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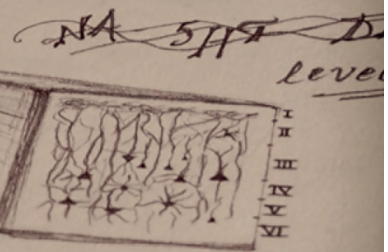
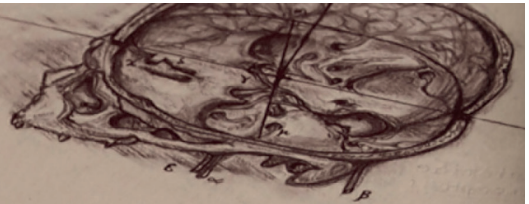
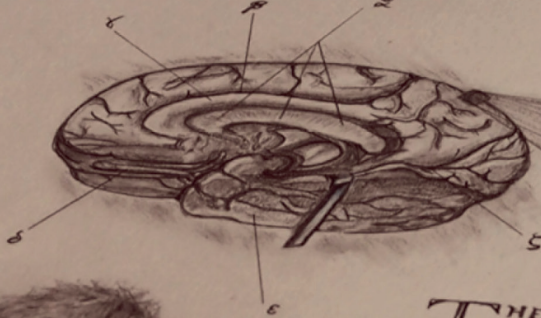
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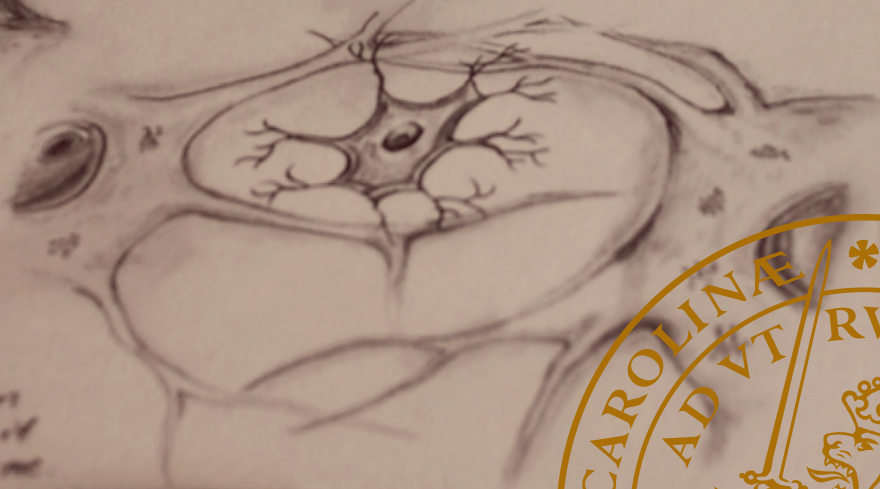
Inflammatory Mechanisms of Depression and Suicidal Behavior

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chemokines
cytokines; pg/ml
- TFWNOG7!



THE inflammatory response is mediated by the activated microglia, the resident immune cells of the CNS.





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Inflammatory Mechanisms of Depression and Suicidal Behavior

Inflammatory Mechanisms of Depression and Suicidal Behavior

Filip Ventorp



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DOCTORAL DISSERTATION

By due permission of the Faculty of Medicine, Lund University, Sweden.
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Faculty opponent
Professor Gregers Wegener
Aarhus University

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Inflammatory Mechanisms of Depression and Suicidal Behavior

Filip Ventorp



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*I dedicate this thesis to my family; you have made me stronger,
better and more fulfilled than I could have ever imagined!*

*... and without whom, this thesis would have been completed two
years earlier!*

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Abbreviations

5-HIAA = 5-Hydroxyindoleacetic Acid

5-HT = 5-hydroxytryptamine

AMPA = α -Amino-3-Hydroxy-5-Methyl-4-Isoxazolepropionic Acid

ANCOVA = Analysis of Covariance

ANOVA = Analysis of Variance

BA = Brodmann Area

BBB = Blood-Brain Barrier

BMI = Body Mass Index

BSA = Brief Scale of Anxiety

CD = Cluster of Differentiation

CMS = Chronic Mild Stress

CPRS = Comprehensive Psychopathological Rating Scale

CRP = C-reactive Protein

CSF = Cerebrospinal Fluid

DOPAC = 3,4-Dihydroxyphenylacetic Acid

DSM = Diagnostic and Statistical Manual of Mental Disorders

ECM = Extracellular Matrix

EPM = Elevated Plus Maze

FST = Forced Swim Test

GLP = Glucagon-Like Peptide

GWAS = Genome-Wide Association Study

HA = Hyaluronic Acid

HVA = Homovanillic Acid

ICD-10 = 10th revision of the International Statistical Classification of Diseases and Related Health Problems

IFN = Interferon

IL = Interleukin
IP = Interferon γ -induced Protein
KO = Knock-Out
LPS = Lipopolysaccharide
LSD = Lysergic Acid Diethylamide
MADRS = Montgomery-Åsberg Depression Rating Scale
MAO = Monoamine Oxidase
MCP = Monocyte Chemoattractant Protein
MDD = Major Depressive Disorder
MIP = Macrophage Inflammatory Protein
MMP = Matrix Metalloproteinase
mRNA = Messenger Ribonucleic Acid
NA = Not Available
NOS = Not Otherwise Specified
NS = Not Significant
NSAID = Nonsteroidal Anti-inflammatory Drug
PFC = Prefrontal Cortex
PI = Propidium Iodide
RANTES = Regulated on Activation, Normal T cell Expressed and Secreted
sCD44 = Soluble CD44
SD = Standard Deviation
sIL = Soluble Interleukin
SIS = Suicide Intent Scale
SSRI = Selective Serotonin Reuptake Inhibitor
SUAS = Suicide Assessment Scale
TARC = Thymus and Activation Regulated Chemokine
TNF = Tumor Necrosis Factor
WHO = The World Health Organization
WT = Wild type

Abstract

During recent decades, increasing evidence has emerged showing that inflammatory mechanisms interact with neurotransmitters and neurocircuits to influence the risk for depression and suicidal behavior. In this thesis, mechanisms beyond conventional pro-inflammatory cytokines and acute-phase proteins are investigated, i.e. chemokines and adhesion molecules such as CD44 and hyaluronic acid. Furthermore, the effect of the conventional anti-depressant fluoxetine and the putative anti-depressant and neuroprotective peptide exendin-4 on inflammation and depression-like behavior in an animal model of endotoxemia was examined.

Six different chemokines were measured in cerebrospinal fluid of suicide attempters and the results were compared to those of healthy controls. Low levels of chemokines were found in the suicide attempters.

Mice lacking the CD44 cell adhesion molecule display increased anxiety after exposure to chronic mild stress. The increased anxiety was associated with decreased cortical serotonin turnover. Patients that recently made a suicide attempt have increased levels of the CD44 ligand hyaluronic acid in the cerebrospinal fluid, and the levels were associated with increased blood-brain barrier permeability.

Exendin-4 prevents LPS-induced behavioral despair by attenuating immobility in the forced swim test in rats. This behavioral effect occurred independently of inflammatory changes in the central nervous system or periphery, but was associated with changed dopamine turnover in the striatum.

Fluoxetine reduced TNF- α production in endotoxin-stimulated BV-2 cells, but did not reduce IL-1- β and TNF- α mRNA expression in the brain of endotoxin-treated rats.

To conclude, immunoproteins that are involved in cell adhesion and cell migration, i.e. chemokines, CD44 and hyaluronic acid, may have important roles in the brain's capacity to manage stress. Dysregulation of these mechanisms could be implicated in suicidal behavior and could potentially be considered as a novel target for intervention in stress-related mental disorders such as depression and suicidality. Fluoxetine and exendin-4 did not affect neuroinflammation in endotoxin treated rats.

Foreword

What can we expect of a biological theory about depression and suicidality?

Trying to understand and carrying out research of depression is intriguing and challenging. The WHO predicts that depression will be the world's leading cause of disease burden by 2030¹, yet depression might just be part and parcel of the human condition. We tend to think that every medical condition has an etiology: a pathogen, a lesion or something else detectable that precedes a pathological process. In the case of depression, we know little for certain about the etiology and pathological processes in biological terms, perhaps because the research field has not advanced sufficiently, or perhaps depression is not best explained in molecular terms. In theory, we can explain the motions of planets in terms of the string theory, but a more suitable explanation uses Newtonian physics (for most applications). Along the same line of thought, I believe molecular biology can explain all aspects of life but, at the same time, it is not suitable for explaining such a complex entity as depression. It is not appropriate to understand the essence of depression in terms of genes, arrangement of molecules or cells, but perhaps the correct unit of analysis is activity of neuronal circuits.

There is no known way to, with absolute certainty, induce a depression in human or in animals using a drug or surgical intervention. There are numerous drugs that cause depression as a side effect, but this only applies to a small fraction of the users. It has also been difficult to pinpoint a specific area in the brain responsible for depressed mood. Research of other human behaviors and cognitive functions, such as fear, the reward system and specific memory functions, has been more successful in locating them to specific brain areas. In the case of depression, over 50 structures have been implied as being involved². It seems that the etiology for one patient could be different from another patient and should be explained by multiple factors, including biological, psychological, and environmental factors. Depression differs from other multifactorial disorders or diseases; whichever biological, psychological or environmental factor contributes to the development of depression, that factor does not seem to be essential. There are always one or more cases of major depression where the particular factor does not appear to be important. It seems not to be a common denominator (besides the feeling of depressed mood). If we want to find a common denominator we probably must define depression more specifically.

This does not mean I believe biological research cannot tell us anything about depression. Although we will never be able to explain depression with one simple

molecular biological mechanism, we might still be able to find out more about some of the different factors contributing to depression. However, there should be no expectations about developing a theory to explain all cases of depression. This thesis concerns the inflammation hypothesis of depression and suicidal behavior, but it will certainly not explain all aspects of depression or suicidality. Nevertheless, it may help to explain one factor, a possible target for treatment, in a subgroup of patients with depressed mood or suicide thoughts.

Aims of the thesis

The main objective of this thesis was to investigate the role of the immune system and inflammation in depression and suicidality. The specific aims were:

1. To investigate the roles of immunoproteins in depression and suicidality. The immune system and inflammation are complex biological responses. A lot of work has been put into investigating the conventional pro-inflammatory cytokines and acute-phase proteins such as CRP. Cell adhesion and cell migration are two inflammatory mechanisms dependent on immunoproteins (e.g. chemokines) and adhesion molecules (e.g. CD44 and HA). One aim of the thesis is to investigate the levels of chemokines in the CSF of suicide attempters to find a potential mechanism contributing to suicidal behavior (Study I).

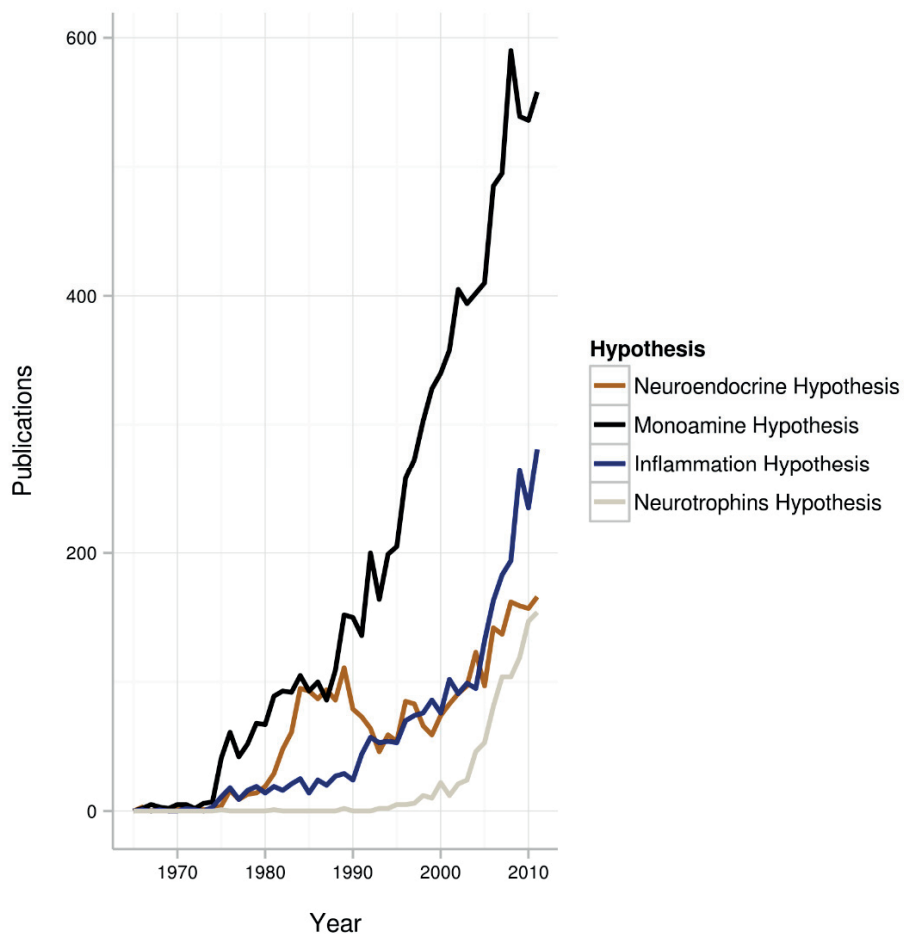
It has been hypothesized that suicidality is associated with low expression of the adhesion molecule CD44 in the brain. The principal ligand of CD44 is the extracellular matrix protein, hyaluronic acid (HA). One aim is to investigate the relevance of CD44-HA signaling in resilience to distress, to find potential mechanisms, and to determine whether the absence of CD44 expression is linked to specific comorbid psychiatric symptoms, such as despair, anxiety and anhedonia (Study III).

2. To find treatment strategies. Drugs with an anti-inflammatory mechanism of action are expected to be beneficial for symptoms of depression and suicidality. One aim of this thesis is to investigate how the conventional anti-depressant fluoxetine (Study IV) and the putative anti-depressant and neuroprotective peptide exendin-4 (Study II) affects inflammation and depressive-like behavior in an animal model of endotoxemia.

Introduction

Inflammation is the cause of various diseases and disorders, and has attracted increasing attention in the field of psychiatry in recent decades. Inflammation has been proposed as a hypothesis of depression and the historical background is presented below, together with a description of MDD and suicidality. The monoamine hypothesis of depression is also included because of its present and historical significance, and since both the monoamine system and inflammatory processes are known to interact.

There is currently no specific definition of the inflammation hypothesis of depression. Some research has focused on peripheral inflammation, and other research considers inflammation in the CNS. Both peripheral and centrally produced immunoproteins, such as cytokines, have been proposed as being important in the development of depression. Since our research group has focused on central inflammation and biological measurements of CSF, two cytokine-producing cell types in the CNS, microglia and astrocytes, are also introduced.



Trends in depression research. The y-axis represents word and phrase usage trends in the Medline database of biomedical abstracts and titles using the MLTrends tool³. The results are normalized to the total number of publications each year.

Major Depressive Disorder

MDD is diagnosed clinically when certain symptoms occur in a combination defined in classification manuals such as DSM-5 and ICD-10. According to DSM-5, a MDD involves some of the following criteria: depressed mood most of the day, nearly every day; markedly diminished interest or pleasure in activities (at least one of the symptoms must be either one of these two criteria); significant weight loss or gain; insomnia or hypersomnia; psychomotor agitation or retardation; fatigue; feelings of worthlessness and guilt; diminished ability to think or concentrate; and recurrent thoughts of death. A patient is diagnosed with MDD if five of these nine symptoms are apparent⁴.

The prevalence of major depression differs globally and from study to study; the variance could be explained by the prevalence period, gender, year of study, depression subtype, survey instrument, age and region⁵. Studies estimate the point prevalence of major depression in Sweden to 4-6%^{6,7}, which is close to an estimated global point prevalence of 4.4–5.0%⁵. According to the WHO, 5% of men and 9% of women will experience a depressive disorder in a given year⁸. The greater occurrence among females is probably not because of genetic and biological factors but rather because females are more exposed to other risk factors, such as adverse experiences in childhood and sociocultural roles with related adverse experiences⁹.

In an American study, the mean age of onset of a MDD was 26¹⁰. The median length of a depressive episode has been estimated at three months¹¹, but other studies report longer durations. Since the duration of a depressive episode can be very long, and because those affected by depressive episodes are of working age¹², the social costs are high. In Europe, an estimated cost of MDD in 2004 was EUR 118 billion, corresponding to 1% of Europe's gross domestic production¹³.

MDD can be inherited to a certain extent. In national Swedish twin studies, genetic factors have been estimated to account for 35-50% of the variability of major depression^{14,15}. However, the genetic factors are not related to single genes, but rather multiple genes with multiple complex gene interactions¹⁶.

Suicidal Behavior

Depression has many side effects, but the one most feared is suicidality. Retrospective studies report that 80% of suicides are associated with depression^{17,18}, but suicidal behavior can be the outcome of other conditions too, such as substance abuse or impulsivity.

There are no universally accepted definitions for suicide and suicidal behavior. The following terminology is recommended in S.K. Goldsmith et al. (Eds.), *Reducing Suicide: A National Imperative*. The definitions are based on the system initiated at the National Institute of Mental Health Center for the Studies of Suicidal Prevention meeting in 1972-1973¹⁹, but have been refined through subsequent research:

Suicide: Fatal self-inflicted destructive act with explicit or inferred intent to die.

Suicidality: All suicide-related behaviors and thoughts, including completing or attempting suicide, suicidal ideation or communications.

Suicide attempt: A non-fatal, self-inflicted destructive act with explicit or inferred intent to die.

Suicidal ideation: Thoughts of harming or killing oneself.

Suicidal communications: Direct or indirect expressions of suicidal ideation or of intent to harm or kill oneself, expressed verbally or through writing, artwork, or other means.

In a meta-analysis, it was concluded that practically all mental disorders have an increased risk of suicide²⁰. The same study showed that, in a population with MDD, the suicide risk is around 20 times higher than in the normal population, but with variation between studies of 0 to 200 times. Like MDD, genetic factors account for 43 to 45% of the variance in suicidal thoughts and behaviors^{21,22}.

The estimated global burden of suicide is just under a million deaths per year²³. According to NASP (National Centre for Suicide Research and Prevention of Mental Ill-Health), around 9000 suicide attempts are made in Sweden each year and around 1500 die by suicide. More men make fatal suicide attempts than women in all countries with the exception of China²⁴. In developed countries, the rate is 2-4 times higher in men than women²³, but females have higher rates of suicidal ideation and behavior than males²⁵. Lifetime risk of completed suicide among people with mood disorders is probably between 5 and 6%²⁶. Psychiatric disorders are present in about 90% of people who kill themselves and, in developed countries, such disorders contribute to 47-74% of the population risk of suicide²⁷.

Since 10% of people who commit suicide do not have any diagnosed psychiatric illness, and 95% of people who have some form of psychiatric illness do not commit

suicide, it has been suggested that suicidal behavior is a distinct psychiatric phenotype, and that suicidal patients have a certain predisposition that is different from other psychiatric illnesses²⁸.

Hypotheses

The Monoamine Hypothesis of Depression

The monoamine hypothesis is based on the idea that depression is caused by a hypoactivity of the monoamine system, i.e. serotonin, dopamine, and noradrenalin in the brain. The hypothesis exists in different versions depending on which monoamine is thought to play a central role, and is sometimes called the catecholamine hypothesis (noradrenalin, dopamine) or the serotonin hypothesis. Today, the most commonly prescribed antidepressant drugs work specifically on the serotonergic system²⁹.

Serotonin was discovered in 1948 as the vasoconstrictor in serum when blood clots³⁰. Soon after, serotonin was also shown to be abundant in the enterochromaffin cells of the gastric and intestinal mucosa, where its release makes the gut contract around the food³¹. About 95% of the serotonin in the body is found in the gastrointestinal tract³². Later, serotonin was isolated from several other different organs, including the brain. At the time, research was conducted on the action of the hallucinogen LSD. The main effects of LSD were well known and it was discovered that LSD blocks the peripheral serotonin receptors.

In 1954, Woolley and Shaw suggested that serotonin plays important roles in mental processes and that the suppression of its action results in mental disorders (e.g. schizophrenia)³³. However, it was not until 1965 that monoamines were explicitly suggested to play a role in mood disorders, when Joseph Jacob Schildkraut integrated the biochemical evidence into a compelling theory of depression in his much cited article 'The catecholamine hypothesis of affective disorders: a review of supporting evidence'³⁴. The two main lines of evidence were that monoamine reducing drugs produce depression (i.e. reserpine) and drugs that increase monoamines reduce depression (i.e. MAO inhibitors).

A few years earlier, the antihypertensive drug reserpine began to be used on psychiatric patients, since it was found to reduce anxiety, obsessive compulsive drives and excessive inhibition³⁵. Reserpine was later replaced, partly because of increasing concerns that it caused depression in some patients³⁶. At the same time, ipronazid, a drug against tuberculosis, was shown to have additional therapeutic effects, such as stimulating appetite, reversing apathy and increasing the patient's

sense of well-being³⁷. It was later reported that ipronazid was a potent inhibitor of MAO³⁸ (the enzyme that breaks down serotonin and other catecholamines), and that reserpine caused depletion of serotonin³⁹ and catecholamines⁴⁰ by blocking the vesicular monoamine transporter. This increased catabolism of monoamines by MAO.

The monoamine hypothesis started to take shape some years before Joseph Schildkraut published his paper. Five years earlier, Everett and Thomas had suggested that brain monoamines were in some way connected to mood⁴¹ and, soon after, Jacobsen offered a similar hypothesis involving the depressogenic action of reserpine and the possible correction of the dysfunction by MAO inhibitors³⁷. However, the general psychiatric community was largely unaware of both these proposals.

This changed in 1965 when Schildkraut published his article in a leading psychiatric journal (*American Journal of Psychiatry*). Schildkraut suggested that depression itself was related to catecholaminergic neurotransmission (i.e. adrenaline, noradrenaline and dopamine), particularly reduced noradrenergic neurotransmission. Two years later, Alec Coppen linked serotonin deficiency rather than catecholaminergic deficiency to depressive illnesses⁴², and the specific behavioral effects of serotonin were further characterized by frog experiments in a study by Lapin and Oxenkrug et. al.^{43,44}.

The Monoamine Hypothesis – Suicidality

At the same time that Schildkraut published his article about the monoamine hypothesis, the concept of suicide as a neurobiological entity started to emerge⁴⁵. The monoamine hypothesis was soon applied to suicidality and, since then, the serotonin system has been the most widely investigated neuromodulatory system in studies of suicide attempters and completers⁴⁵. The first study was published in 1968, when Bourne et al. measured serotonin, 5-HIAA (serotonin metabolite) and noradrenalin in the hindbrain of suicide completers and control objects, and showed lower levels of 5-HIAA in suicide completers⁴⁶. The idea of a dysfunction in serotonin transmission leading to suicide is also supported by a study showing that increased 5-HIAA levels in CSF is correlated with depression and suicide⁴⁷; serotonin (5-HT) was measured in CSF some years later. Besides measuring levels of serotonin and serotonin metabolites in the brain and in CSF, studies of postmortem brain tissue from suicides show changes in serotonin receptor binding and serotonin transporter⁴⁸⁻⁵⁰.

Although psychiatric disorders are present in most suicides, and antidepressant medications alleviate depression and other psychiatric disorders, meta-analyses have generally not detected benefits of antidepressants for suicide or suicide

attempts⁵¹. However, in Sweden, high prescription rates of antidepressants correlate with decreasing suicides rates⁵². Paradoxically, there is some evidence of pro-suicidal effects of anti-depressants in children and young adults^{28,53}.

“However, even in 1965, it was clearly recognized that abnormalities in catecholamine metabolism alone could not conceivably account for all of the diverse clinical and biological phenomena in all types of affective disorders. Thus, in my review, I stressed that this hypothesis was ‘at best a reductionistic oversimplification of a very complex biological state’ that undoubtedly involved many other biochemical abnormalities (including alterations in the metabolism of indoleamines and other neurotransmitters, ionic changes, and endocrine disturbances), as well as physiological and psychological factors.” Schildkraut, 1981.

The Inflammation Hypothesis of Depression

Humans are constantly exposed to bacterial and viral pathogens, and low-grade infections are reported to account for up to 35% of all general practitioner consultations in the United Kingdom⁵⁴. Low-grade systemic inflammation is characterized by increased levels of cytokines, and one early version of the inflammation hypothesis of depression states that depression is caused directly or indirectly by cytokines.

Cytokines are a group of hormone-like molecules facilitating communication between immune cells and between immunocytes and other peripheral cells, such as fibroblast and endothelial cells. The hypothesis was conceptualized in 1991 by RS Smith in an article called ‘The macrophage hypothesis of depression’, where he proposed excessive secretion of the macrophage cytokines IL-1, IFN- α and TNF as the cause of depression⁵⁵. Today, there are other similar hypotheses, such as the ‘Inflammatory & neurodegenerative hypothesis of depression’⁵⁶ or ‘Inflammation and kynurenine pathway hypothesis of depression’; the common denominator is the involvement of inflammatory processes, and the focus is most often on cytokines.

The inflammation hypothesis of depression was the result of some early key discoveries. One was the realization that sickness behavior was not a secondary effect to sickness-induced debilitation, but rather an adaptive response to help fight viral and bacterial infections⁵⁷. Later, it was discovered that proteins secreted by the immune system, i.e. cytokines, were responsible for causing fever and the sickness behavior⁵⁸. Sickness behavior involves symptoms such as loss of energy or fatigue, loss of interest in usual activities, poor appetite and weight loss and sleep changes⁵⁹. These are symptoms that also could be present during a depressive episode and are part of the diagnostic criteria for the disorder⁴.

Another discovery was the neuropsychiatric adverse effects of recombinant IFN- α therapy against certain cancers and virus infections, such as hepatitis C. After a few weeks of treatment, 16-58% of the patients developed a depression according to the criteria of DSM-IV (without the exclusion criteria of substance-induced depression) or as elevated scores on depression rating scales⁶⁰. This can be prevented by prophylactic treatment of SSRIs⁶¹.

The macrophage hypothesis of depression did not attract much attention initially and only a few researchers took notice. One of these was Michael Maes; he was investigating whether depressed patients are more vulnerable to infections and cancers because of a less active immune system. He looked at the immune cells from depressed patients but instead found the opposite; the cells were more active than normal, and released more cytokines⁶²⁻⁶⁴. Maes published several review articles^{65,66} (one together with RS Smith⁶⁷) that described research data obtained in depressed patients, and proposed that depression should be considered an

inflammatory disease. Maes' results were confirmed by some but contradicted by others and at the time were generally considered to be controversial. Nevertheless, research studies connecting an immune response with depression continued to be published.

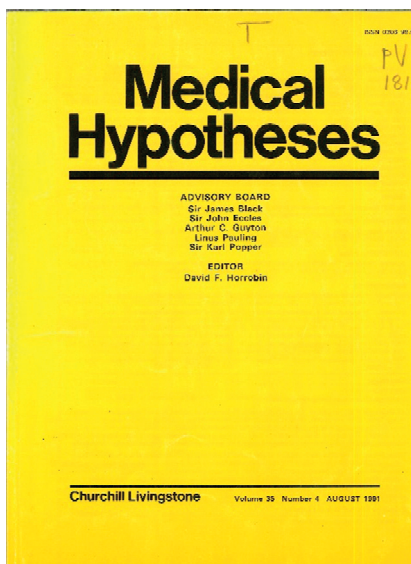
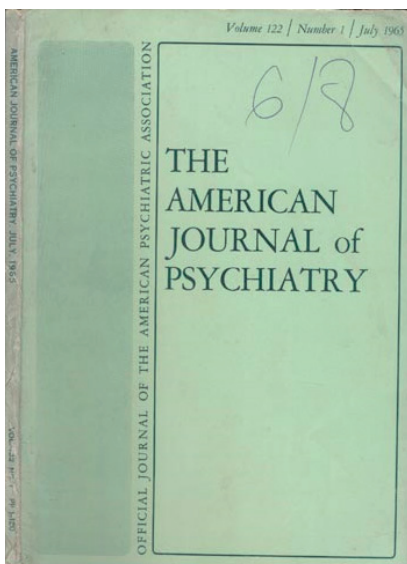
In 1996, an animal model was applied to the inflammation hypothesis of depression. Raz Yirmiya et al. injected rats with the bacterial endotoxin LPS to induce a strong systemic inflammatory response that produced behavioral effects resembling the characteristics of depression. LPS treatment reduced the rats' preference for consumption of saccharin solution and suppressed sexual behavior, indicating an inability to experience pleasure (anhedonia)⁶⁸. This model was pharmacologically validated with chronic treatment (5 weeks) of imipramine (a tricyclic antidepressant) and later fluoxetine (SSRI), which stopped the suppressive effects of LPS on saccharin preference^{68,69}. The induced depression-like behavior was later shown to be independent of the LPS-induced sickness behavior⁷⁰.

Around the same time, Robert Dantzer started to investigate whether sickness behavior was comparable with depression. Dantzer conducted studies assessing the mechanism by which cytokines (i.e. IL-1) induced behavioral changes in the brain. If cytokines play a causative role in the pathophysiology of depression, then antidepressants should interfere with the actions of cytokines. Dantzer and his coworkers injected rats repeatedly with the atypical antidepressant tianeptine before treating them with IL-1 or LPS. In contrast to typical SSRIs, tianeptine is a selective serotonin reuptake enhancer (SSRE). The antidepressant sharply reduced the sickness behavior induced by IL-1 and LPS treatment^{71,72}. The same year, Andrew Miller published the first study showing that pretreatment with paroxetine (SSRI) is an effective strategy for minimizing depression induced by IFN- α therapy in humans⁷³. Yirmiya, Dantzer and Miller's studies provided pharmacological support for the cytokine hypothesis, and the hypothesis attracted progressively greater attention after that.

Around two decades after the publication of the macrophage hypothesis of depression, Maes' discoveries during the 1990s were, to some extent, proved. Three meta-analyses confirmed increased levels of cytokines in major depression, especially the cytokine IL-6⁷⁴⁻⁷⁶. If inflammatory cytokines contribute to depressive symptoms in major depression, then these levels should return to baseline after successful treatment and remission. A meta-analysis of the effects of antidepressant medication on serum levels of cytokines found that antidepressant treatment reduced the levels of IL-1 β and possibly those of IL-6⁷⁷. Depressed patients have also been shown to have higher levels of IL-1 β in the CSF⁷⁸. Today, many researchers hope that targeting inflammatory processes with anti-inflammatory drugs could be a future treatment strategy of MDD^{79,80}.

The Inflammation Hypothesis of Depression - Suicidality

Studies on inflammation and specifically suicidality are rare. One of the first studies looked at soluble IL-2 receptors in the plasma and found higher levels in suicide attempters compared to controls⁸¹. Post-mortem examination of brain from suicide victims showed microgliosis in patients with a psychiatric diagnosis but not in controls that died of other causes⁸². Another group of studies demonstrated a higher transcript level of IL-4 and IL-13 in the orbitofrontal cortical area of suicide victims⁸³. Suicide attempters have also been reported to have higher levels of IL-6 in CSF and higher levels of IL-6 and TNF- α levels in the plasma compared to healthy controls^{84,85}. Some reports have suggested a possible link between suicide and allergic reactions that may alter the function of the orbital prefrontal cortex⁸⁶.



The original cover of *The American Journal of Psychiatry* (122, 509-522 [1965]). The journal ranks second among 135 journals in the category 'Psychiatry'. The article 'The catecholamine hypothesis of affective disorders: a review of supporting evidence' has been cited at least 2309 times, probably many more.

The original cover of *Medical Hypotheses* (351, 298-306 [1991]). The journal fosters diversity and debate in medical science by the absence of peer review filtering. It has published landmarks such as the hypothesis that schizophrenia may be caused by wearing heeled shoes⁸⁷ and that masturbation is a treatment for nasal congestion⁸⁸. The most widely cited article from the journal, 'The macrophage theory of depression' by R. S. Smith has been cited at least 389 times.

Glial Cells in the Central Nervous System

In the brain, neurons transmit electrical signals in the form of action potentials. All other cells are termed glia. Examples of glia are microglia, astrocytes, oligodendrocytes and Schwann cells⁸⁹. In the brain, the glia/neuronal ratio is virtually one-to-one, but the ratio differs substantially between different brain structures⁹⁰. Cytokines in the CNS are predominantly produced by brain-resident microglia and astrocytes⁹¹⁻⁹³.

Microglia

Microglia constitute about 10% of the glial cells⁹⁴. They are of mesodermal origin and enter the brain during early development. These invading cells have amoeboid morphology but transform into branched, ramified microglia known as resting microglia. Microglia can also derive from monocytes that travel from the bone marrow to the brain, where they settle and further differentiate into microglia⁹⁵. After a pathological event, these cells undergo a transformation and again acquire amoeboid morphology and the capacity to migrate, proliferate and phagocytose⁹⁶.

Microglia are immune cells that respond to infiltrating pathogens and injury, and could potentially cause tissue damage through dysregulated inflammation⁹⁷. Microglial-derived cytokines regulate innate defense mechanisms, initiate immune responses, and recruit leukocytes to the CNS, but microglia also receive cytokine signals from astrocytes, neurons, the endothelium and leukocyte infiltrates⁹⁸. Microglia spend most of their time in a non-inflammatory state and play an important role in the development and homeostasis of the CNS^{99,100}. For example, they clear debris and apoptotic cells¹⁰¹, produce trophic factors¹⁰², and play key roles in the regulation of neuronal synapses, learning, memory, neurogenesis and neuroplasticity in the CNS^{103,104}. Both acute stressors and chronic stress can induce microglial activation and change microglial number, morphology and function in animal models of depression¹⁰⁵.

Astrocytes

Astrocytes are divided into two classes: protoplasmic astrocytes found in the grey matter and fibrillary astrocytes in the white matter. Astrocytes can be as diverse as neurons¹⁰⁶, and human astrocytes are distinctly bigger and more complex than those of infra-primate mammals, and have been suggested to contribute to the unique human processing capability¹⁰⁷. Traditionally, astrocytes have been viewed as cells that facilitate the proper functioning of neurons by maintaining ionic balance in the

extracellular space, controlling blood flow, and providing neurons with energy and substrates for neurotransmission and formation and maintenance of the BBB^{89,108}.

Emerging data suggests that astrocytes also have an active role in brain function and information processing⁸⁹. They are involved in the formation of synapses and in modulating synaptic functions through bidirectional communication with neurons¹⁰⁶. As well as microglia, astrocytes are activated in response to injury such as trauma or neurodegenerative disease and result in reactive gliosis¹⁰⁹. Astrocytes can produce a wide variety of cytokines and are, for example, the major source of inducible IL-6 in the CNS⁹², so they play an important role in regulating the CNS immune and inflammatory responses¹¹⁰. The expression of astrocytic cytokines is not limited to disease/trauma states. Astrocytes constitutively release the cytokine TNF- α which influences synaptic strength via rapid effects on the trafficking and expression of AMPA receptors and can therefore modulate plasticity functions, such as long-term potentiation and homeostatic synaptic scaling^{111,112}. Fewer and less active astrocytes have been observed in depressed patients and are therefore suggested to be one factor contributing to depression^{113,114}.

Materials and Methods

Participants

Suicide Attempters

All patients gave informed consent to participate and the studies was approved by the Lund University Medical Ethics Committee. A suicide attempt was defined according to Beck et al. as “those situations in which a person performs a life-threatening behavior with the intent of jeopardizing his life or to give the appearance of such an intent”¹¹⁵. Patients were enrolled between 1987 and 2001 (study I and III) and between 2009 and 2013 (study III). After admission to the Lund University Hospital medical emergency room or intensive care unit, the patients were asked to participate in a research program. Soon after the suicide attempt the patient was evaluated by a psychiatrist by means of the Suicidal Intent Scale. Within a few days they were transferred to a psychiatric ward where, in conjunction with blood collection and lumbar puncture, psychiatrists assessed their current psychiatric symptoms and suicidality using two rating scales (CPRS and SUAS). Patients recruited between 2009 and 2013 used self-report version of SUAS. Axis I diagnoses of the patients were set according to the DSM-III-R (1987-2001) and DSM-IV (2009-2013). In conjunction with the lumbar puncture, all patients also underwent a thorough physical examination and a complete medical history was taken.

Controls

Healthy controls subjects with no previous or ongoing psychiatric condition were recruited through the neuropsychiatric and psychiatric clinics at the Swedish university hospitals in Malmö/Lund and Linköping (study I and III), and from PrecisionMed Inc., San Diego, CA, USA (study I). They were thoroughly checked for psychiatric comorbidity (Axis I and II disorders) by an evaluating psychiatrist and with the structures Clinical Interview for DSM-IV.

Table I. Demographic data of study participants.

| Demographic Data | Study I | | Study III | |
|--------------------------------------|--------------------|------------------|--------------------|------------------|
| | Suicide Attempters | Healthy Controls | Suicide Attempters | Healthy Controls |
| Total Number | 137 | 43 | 94 | 45 |
| Age, mean \pm SD | 38.2 \pm 13.3 | 38.8 \pm 22.3 | 36.0 \pm 12.0 | 30.6 \pm 12.3 |
| Gender, men/women | 67/70 | 36/7 | 47/47 | 37/8 |
| BMI, mean \pm SD | 24.0 \pm 4.1 | 24.0 \pm 3.0 | 23.6 \pm 3.7 | 23.8 \pm 3.2 |

Table II. Somatic diagnoses of study participants.

| Somatic diagnosis | Study I | | Study III | |
|---------------------------------|--------------------|------------------|--------------------|------------------|
| | Suicide Attempters | Healthy Controls | Suicide Attempters | Healthy Controls |
| Anemia | 1 | NA | 1 | NA |
| Asthma | 2 | NA | 0 | NA |
| Cataract | 1 | NA | 0 | NA |
| Chronic Headache | 0 | NA | 1 | NA |
| Diabetes | 3 | NA | 1 | NA |
| Epilepsy | 1 | NA | 0 | NA |
| Gastritis | 3 | NA | 3 | NA |
| Goiter | 1 | NA | 0 | NA |
| Hearing Impairment | 0 | NA | 1 | NA |
| Hepatic Steatosis | 1 | NA | 0 | NA |
| Hypothyroidism | 1 | NA | 0 | NA |
| Irritable Bowel Syndrome | 0 | NA | 1 | NA |
| Migraine | 8 | NA | 2 | NA |
| Peptic ulcers | 2 | NA | 0 | NA |
| Osteoarthritis | 1 | NA | 0 | NA |
| Sciatica | 0 | NA | 1 | NA |
| Spinal disc herniation | 1 | NA | 0 | NA |

Table III. Axis 1 diagnoses of study participants.

| Axis I Diagnoses | Study I | | Study III | |
|--|--------------------|------------------|--------------------|------------------|
| | Suicide Attempters | Healthy Controls | Suicide Attempters | Healthy Controls |
| MDD | 36 | NA | 27 | NA |
| Adjustment disorder | 29 | NA | 17 | NA |
| Dysthymia | 19 | NA | 9 | NA |
| Depression NOS | 15 | NA | 12 | NA |
| Substance dependence/abuse | 11 | NA | 6 | NA |
| Psychotic Disorder | 6 | NA | 5 | NA |
| Bipolar I Disorder, Depressive Episode | 5 | NA | 0 | NA |
| Eating Disorder | 5 | NA | 0 | NA |
| Anxiety Disorder | 4 | NA | 3 | NA |
| Other | 0 | NA | 5 | NA |
| No Axis I diagnosis | 7 | NA | 0 | NA |

Table IV. Somatic medications of study participants.

| Somatic medications | Study I | | Study III | |
|---------------------------|--------------------|------------------|--------------------|------------------|
| | Suicide Attempters | Healthy Controls | Suicide Attempters | Healthy Controls |
| Anabolic steroids | 1 | NA | 2 | 0 |
| Analgesic | 3 | NA | 0 | 1 |
| Antibiotics | 4 | NA | 0 | 0 |
| Anti-epileptic | 1 | NA | 0 | 0 |
| Anti-histamine | 0 | NA | 2 | 1 |
| β -blocker | 1 | NA | 0 | 0 |
| Contraception | 0 | NA | 3 | 5 |
| Estrogen | 1 | NA | 0 | 0 |
| Glucocorticoid | 0 | NA | 0 | 1 |
| Insulin | 1 | NA | 0 | 0 |
| Naturopathic drug | 0 | NA | 1 | 8 |
| NSAID | 1 | NA | 2 | 1 |
| Sedatives | 2 | NA | 0 | 0 |
| Stomach ulcer medications | 3 | NA | 0 | 0 |

Psychometrics

SIS

The Suicide Intent Scale consists of 15 items and assesses both circumstantial evidence and the person's subjective feelings of the intent of a specific suicide attempt. SIS is the most prevalent rating scale used by researchers to measure suicidal intent in adults^{115,116}.

SUAS

The Suicide Assessment Scale assesses suicidality; it includes 20 questions about symptoms relevant to suicidality, desire to live/die, suicidal thoughts and suicidal plans.

CPRS

The Comprehensive Psychopathological Rating Scale was constructed to measure change in the full spectrum of psychopathology over time, particular in response to treatment. Therefore, this scale was constructed with items likely to be change with treatment and avoided variables such as character trait and variables likely to be influenced by sociocultural differences¹¹⁷. To avoid ambiguity of many commonly used psychiatric terms and inconsistency between scale steps, each item has an explicit description and scale steps definitions. The CPRS is designed as a pool of items from which shorter scales can be selected for special purposes.

MADRS

The Montgomery–Åsberg Depression Rating Scale is a depression scale designed (similar to CPRS) to be sensitive to change over time to better evaluate treatments response. It was designed experimentally by identifying the 17 most commonly occurring symptoms (or items) from CPRS in patients diagnosed with a primary depressive illness. Then the ten items which showed the largest changes with treatment and the highest correlation to overall change were used to construct the MADRS scale¹¹⁸. Hence, it is a scale that is derived from the CPRS scale.

BSA

The Brief Scale of Anxiety is derived from the CPRS scale and comprises 10 items. The items were selected by examining the CPRS scores in patients with generalized anxiety disorder or panic disorder¹¹⁹. The scale can also separate between somatic (inner tension) and psychological (worrying) components of anxiety.

Extended Psychosis Subscale

The extended Psychosis subscale consists of CPRS items covering the core symptoms of schizophrenia (7 items) but also other important psychotic symptoms (7 items).

Procedures

Lumbar punctures were performed after a washout period of 14.7 ± 7.7 (mean \pm SD) (study I) and 14.22 ± 6.25 (study III) days from admission to the psychiatric research ward. Occasional doses of benzodiazepines were allowed during the wash-out period. Blood samples were drawn before the spinal tap and checked for the presence of psychotropic drugs. Patients with presence of psychotropic drugs in plasma or with ongoing medication besides benzodiazepines were excluded. The tap was performed in the morning between 8 and 9 a.m. after one night of fasting and bed rest. CSF was taken between L4 and L5 and was immediately centrifuged, aliquoted and stored at -80°C .

Animals

Rats (study II and IV)

All procedures were performed in accordance with national laws and were approved by the Regional Animal Ethics Committee in Malmö/Lund, Sweden. Male Wistar rats (II: Harland, Sweden; IV: Charles River, Italy) weighing around 200 g were used. The animals were allowed to acclimatize for at least five days before treatments with exendin-4 (Sigma, Germany), exendin-4 vehicle (0.1 % bovine serum albumin in saline), LPS (Sigma), LPS vehicle (saline), fluoxetine (Sigma, Germany) or fluoxetine vehicle (1/3 dimethyl sulfoxide and 2/3 saline).

Study II

The rats were given one daily exendin-4 (0.5 µg/mL/kg), or vehicle intraperitoneal (i.p.) injections for five days. On days three and four, the animals also received LPS (1 mg/mL/kg) or saline injection (i.p.) one hour after the exendin-4/vehicle injection. The treatment groups were as follows: Vehicle + Saline (Control), Vehicle + LPS (LPS), Exendin-4 + Saline (Ex4) and Exendin-4 + LPS (LPS+Ex4) (n = 12 animals/group).

Study IV

First injection of fluoxetine (10 mg/kg) was given one day before LPS treatment (mg/kg). Second injection was given one hour before LPS treatment. The animals were sacrificed 24 hours after LPS injection. The 32 rats were divided in 4 treatment groups with 8 animals in each group. First group received vehicle and saline injections, second group fluoxetine and saline, third group vehicle and LPS and fourth group fluoxetine and LPS. All injections were given i.p. at a volume of 1 mL/kg.

CD44KO mice (study III)

CD44 is expressed in various isoforms on numerous cell types and tissues during embryogenesis and in the mature organism. CD44 isoforms are encoded by a single gene containing 19 or 20 exons, 12 of which can be differentially spliced. The CD44KO mice do not express any of the known isoforms since these mice were created by targeting exons encoding the invariant N-terminus region of the molecule. The mice were originally back-crossed into C57Bl/6J mice but the CD44KO mice used in study III has been further back-crossed for at least seven generations onto a DBA/1 background. Heterozygous and homozygous mice were born in Mendelian ratio, survived, grew normally, and were fertile. Animals remained healthy when maintained under specific pathogen-free conditions. No gross deficits in neurological function or behavior were apparent.

All experiments were conducted with the approval from the Tel Aviv University ethical committee. WT littermate mice, derived from common line, were used as controls. After weaning (3-4 weeks old), male mice were divided into separate cages in groups of 3-5 per cage (same experimental group in each cage). The experimental groups consisted of the following: WT (n = 12), CD44KO (n = 19), WT+CMS (n = 12) and CD44KO+CMS (n = 16). At age 5 weeks, mice either underwent CMS for 4 weeks or remained in their standard living conditions.

Animal Procedures

In study II and IV, rats were anesthetized with pentobarbital sodium (60 mg/kg). CSF were collected with transcutaneous cisterna magna puncture and stored at -80 °C. Whole blood was drawn by cardiac puncture, kept at room temperature for 30 minutes and then on ice for 1 hour. Serum was collected following centrifugation (10 min; 1300 g; 4°C) and stored at -80. Brains were removed and brain tissue dissected to specific anatomical sites including the prefrontal cortex, hippocampus and striatum. In study IV, perfusion was carried out with Hank balanced saline solution without Ca^{+2} and Mg^{+2} (HBSS w/o).

In study III, to minimize stress at the time of sacrifice, mice were decapitated; immediately thereafter whole blood and brain tissue were collected as in study II and IV.

Animal Models

LPS Model (study II & IV)

LPS are found in the outer membrane of Gram-negative bacteria and elicit strong immune responses in animals. LPS injections induce sickness behavior that peaks during the first six hours but gradually declines and disappears after 24 hours^{79,120}. At this time, the animals display depression-like behavior⁶⁸. Furthermore, previous studies have shown that serum levels of cytokines, i.e. IL-1 β and IL-6, peak at the same time as the sickness behavior appears, whereas the IL-1 β levels in CSF and IL-1 β mRNA levels in brain tissue peak at the same time as depression-like behavior appears¹²¹.

In study II, to assess if exendin-4 could alleviate inflammation-induced depression-like behavior; rats were given two daily intra-peritoneal LPS injection (1 mg/mL). In study IV, rats were given one LPS injection to evaluate the anti-inflammatory potential of fluoxetine in CNS.

CMS Model (study III)

In the chronic stress model, rodents are continuously exposed to a variety of mild stressors. This is causing behavioral changes that parallel symptoms of depression, especially anhedonic behavior as measured with the sucrose preference test; but also other characteristic changes such as decreased in sexual and aggressive behavior,

self-care and disrupted sleep patterns. This model has also been pharmacologically validated with chronic treatment with all classes of clinically-effective antidepressant drugs¹²².

In study III, CD44KO mice were exposed to a CMS paradigm for four weeks. Two different kinds of mild stressors were applied each day. The CMS protocol was based on Schweizer et al.¹²³ and is detailed in the table below. One week after CMS, mice underwent the behavioral testing.

Table V. Detailed table of the stressors employed over the CMS period.

| Week 1 & 3 | Sunday | Monday | Tuesday | Wednesday | Thursday | Friday | Saturday |
|---------------------------------|---------------|---------------|----------------|------------------|-----------------|---------------|-----------------|
| Water deprivation | 16:00 → | end 08:00 | | | | | |
| Food deprivation | | | | | | | |
| Continuous illumination | | | | | 16:00 → | end 08:00 | |
| Wetting the sawdust | | | 09:00-12:00 | | | 13:00 → | → |
| Empty water bottle | | 08:00-09:00 | | | | | |
| White noise (80 - 90 dB) | | | | 09:00-12:00 | | | |
| Light flicker | 11:00-16:00 | | 14:00-16:00 | | | | |
| Tilted cage | | 11:00-14:00 | | | | | |
| Rat sawdust | | | | | 10:00-16:00 | | |
| Sucrose preference | | | | 16:00 → | end 08:00 | | |

| Week 2 & 4 | Sunday | Monday | Tuesday | Wednesday | Thursday | Friday | Saturday |
|--------------------------|-------------|-------------|-------------|-------------|-------------|-------------|----------|
| Water deprivation | | | | | | | |
| Food deprivation | 16:00 → | 08:00 | | | | | |
| Continuous illumination | | | 16:00 → | end 08:00 | | | |
| Wetting the sawdust | end 08:00 | | | | 09:00-12:00 | | |
| Empty water bottle | 12:00-16:00 | | | | | | |
| White noise (80 - 90 dB) | | 09:00-12:00 | | | 12:00-16:00 | | |
| Light flicker | | | 12:00-16:00 | | | 10:00-12:00 | |
| Tilted cage | | 15:00-16:00 | | 09:00-11:00 | | | |
| Rat sawdust | 09:00-12:00 | | | | | | |
| Sucrose preference | | | | 16:00 → | end 08:00 | | |

Animal Behavior

Unconditional Behavior (study II)

Several behavior tests could be used to examine whether the animals have an intact locomotor capacity. The purpose of unconditional behavior tests was to measure inflammation-induced lethargic behavior to rule out its confounding effects on the subsequent behavior tests.

Rats were individually placed into a novel cage and assessed blindly for every five-second periods over 5 consecutive minutes (in total 60 periods). Each period a rat sat motionless (stillness), or sniffing, rearing, running or walking, grooming, or chewing was measured. The periods of stillness were used as a measure of lethargic behavior.

Open Field (study III & IV)

In study III, to test motor function of CD44KO mice before and after CMS; open-field test was conducted. Mice were introduced to a 50 cm² arena and videotaped for 60 minutes. The videos were analyzed using the EthoVision tracking system (Noldus, Wageningen, The Netherlands) tracking either the center of the animal. The total distance moved in the open field arena was used as measure of motor function.

Open field was also conducted in study IV to measure inflammation-induced lethargic behavior. Horizontal and vertical movements were recorded as infrared beam breaks using equipment and Photo-beam Activity System software (PAS, version 2.0.7.101) from San Diego Instruments.

Forced Swim Test (study II)

The depression-like behavior despair was measured with the FST. The FST is one of the most widely used tools in depression research and used to screen for new anti-depressant drugs by the industry¹²⁴. In study II, the rats were placed in a Plexiglas cylinder (height: 21 cm, diameter: 46 cm) filled with water at 23 ± 1 °C and videotaped for seven minutes. The last four minutes were blindly analyzed, for every 5 s period. The predominant behavior during each period was recorded as swimming, climbing or floating. Behavior was classified as immobility when rats

remained floating motionless in the water making only movements necessary to keep their head above water. Swimming was defined as horizontal movements throughout the cylinder and climbing was defined as vertical movements against the wall.

Sucrose Preference Test (study III)

In study III, sucrose preference was evaluated through the 4 weeks of CMS after 2 training sessions in the week prior to CMS. In each session, the regular water bottle was removed and substituted by two bottles, one containing 40 ml of water and another containing 1 % sucrose solution. The animals were allowed overnight access to the tubes. Thereafter, tubes were removed and the amount of liquid in each tube was measured. Measurement in which a tube was found empty on the following day and the sawdust beneath was wet - were considered as missing values and excluded from calculations. Normally, mice prefer to drink the sucrose solution. Anhedonic behavior was evaluated by calculating the percentage of sucrose solution preference over the total solution intake. No preference to either solution would therefore result in 50 % sucrose solution intake.

Cell Cultures (study II & IV)

Adult primary parenchymal Microglia (study II)

One way to isolate adult primary microglia from rodents is to use enzymatic digestion together with gradient centrifugation as a method to separate microglia from the other CNS cell types and at the same time remove myelin and debris. From studies using chimeric mice, it is known that it is possible to subgroup microglia based on the amount of CD45 molecules on the cell membrane^{125,126}. Resident parenchymal microglia are CD45^{low}, whereas blood derived leukocytes are CD45^{high}.

Rats (weighing around 200 g) were anesthetized with pentobarbital and perfused (cardiac perfusion) with HBSS w/o (Life Technologies, Sweden). Cerebellum and hindbrain were removed and the rest of the brain was cut into small pieces followed by centrifugation (300 g; 2 min; RT) in HBSS w/o. The pellet was then enzymatically homogenized with a pre-heated enzyme mix (37° C) containing papain (Neural Tissue Dissociation Kit, Miltenyi Biotec, Fisher Scientific, Sweden). After 5 min incubation at 37° C, the tissue was dissociated mechanically by pipetting up and down using a serological pipette. This was followed by an addition

of a second enzyme mix containing DNase (Neural Tissue Dissociation Kit, Miltenyi Biotec, Fisher Scientific, Sweden). The tissue was dissociated further with two fire-polished Pasteur pipettes of decreasing diameter and incubated in water bath for a maximum time of 30 minutes with enzymes. The suspension was then filtered through a 70 μ m cell strainer to get a single cell suspension, and centrifuged (300 g; 8 min; RT) in regular HBSS. To get rid of myelin and debris, the pellet was re-suspended in 8 mL 20 % isotonic Percoll (Sigma Aldrich, Stockholm Sweden) with 2 mL HBSS on top of the Percoll layer. Following centrifugation (400 g; 25 min; 22° C) the pellet was collected and washed by centrifugation (300 g; 7 min; 4° C). The cells were then re-suspended in sorting buffer containing HBSS, EDTA (Sigma Aldrich, Sweden), HEPES, 5% FBS (Sigma Aldrich, Sweden).

FACS (study II)

Antibodies targeting CD11b/c (OX-42, FITC conjugated, BD #554861) and CD45 (OX-1, R-Phycoerythrin and Alexa Fluor 750 conjugated, AbD Serotec #MCA43P750) were added to the single cell suspensions (after Percoll gradient centrifugation) which were then incubated for 15 minutes at +4° C. The cells were washed to remove excess antibodies (300 g; 5 min; 4° C) and the pellet was resuspended in sorting buffer. Parenchymal microglia were isolated by cell sorting using FACS (BD FACS Aria III) and based on their characteristic expression of CD11b/c and CD45 (CD11b/c + CD45^{low}, see figure below)¹²⁶.

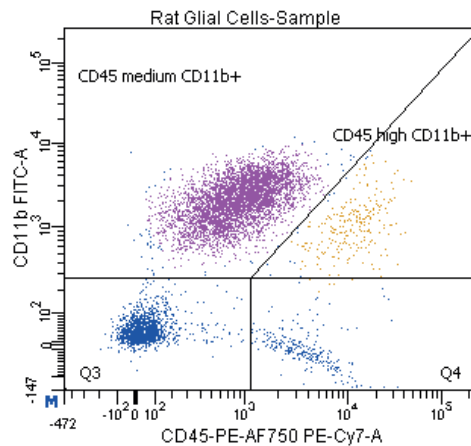


Figure I. Gating of the cell sorting with CD11b/c+CD45low/medium (parenchymal microglia) in Q1, CD11b/c+CD45high in Q2 (macrophages, perivascular microglia), CD11b/c-CD45low in Q3 and CD11b/c-CD45high in Q4.

Cell Cultures (study II)

The acquired cells were re-suspended in culture media (DMEM + high glucose + HEPES (Sigma Aldrich, Sweden), 10 % FBS) and distributed into a Poly-L-Lysine (PLL) (Sigma Aldrich, Sweden) coated 96-well plate (75 000 cells/well). Cells were treated with 2 μ M (8.34 μ g/mL) and 12 μ M (50.21 μ g/mL) exendin-4. After 1 hour of incubation with exendin-4, 2 μ g/mL LPS (Sigma Aldrich, Sweden) was added. After 24 hours of incubation, cell culture media were collected, centrifuged (400 g; 4 min; 4° C), aliquoted and stored in -80 °C. This protocol was repeated at three different occasions.

Cell Viability (study II)

Six hours after the treatment, the percentage of surviving cells was measured with a high content screening platform (CellomicsTM, ArrayScan VTI HCS Reader, Thermo Scientific). The cells nuclei were stained with H342 (Sigma Aldrich, Sweden) (1.25 μ g/mL) and PI (Sigma Aldrich, Sweden) (0.5 %). The percentages of PI positive cells were measured according to a pre-set protocol (background was set to non-stained cells).

BV-2 Cells (study IV)

BV-2 cells were cultivated in Dulbecco's modified eagle medium (Life Technologies) with 10 % fetal bovine serum (Life Technologies). Penicillin/streptomycin broad spectrum antibiotics (Life Technologies), were added to the medium during the growth but not the treatment of cells. Cultures were maintained in 75 cm² culture cell flasks (Nunc, Roskilde, Denmark) in incubator (Sanyo, Osaka, Japan) set at 37°C with 5 % CO₂ and passaged 1/10 at 80 - 90 % confluences. Culture medium was changed every other day. During the experiments, 0.025x10⁶ cells per well, were cultured in 96-well microplates in culture medium (DMEM without phenol). All cells except controls were treated with 1 μ g/mL LPS (Sigma-Aldrich, Stockholm, Sweden). Cells were also treated with anti-depressants or NSAID according to table below. Concentration at 1X represents the plasma concentration of the drug in a chronic (> 2 w) treatment of the anti-depressant in humans. Cells were incubated for 24 h and then medium from wells were collected and stored at -80 °C.

Table VI. Drug concentrations used in study IV.

| | 0.1X | 1X | 5X | 10X | 500X | 100X | Plasma Conc. Ref. |
|-------------------|-----------|------------|-------------|-------------|------------|-----------|-----------------------------------|
| Fluoxetine | 60 ng/mL | 0.60 µg/mL | 3 µg/mL | 6 µg/mL | 30 µg/mL | 60 µg/mL | Bolo et al. ¹²⁷ |
| Sertraline | 19 ng/mL | 0.19 µg/mL | 0.95 µg/mL | 1.9 µg/mL | 9.5 µg/mL | 19 µg/mL | Patel et al. ¹²⁸ |
| Citalopram | 30 ng/mL | 0.3 µg/mL | 1.5 µg/mL | 3 µg/mL | 15 µg/mL | 60 µg/mL | Fredricson et al. ¹²⁹ |
| Paroxetine | 5.5 ng/mL | 55 ng/mL | 0.275 µg/mL | 1.375 µg/mL | 2.75 µg/mL | 5.5 µg/mL | Raptopoulos et al. ¹³⁰ |
| Diclofenac | 0.2 µg/mL | 2 µg/mL | 10 µg/mL | 20 µg/mL | 100 µg/mL | 200 µg/mL | Fowler et al. ¹³¹ |

Cell viability (study IV)

Cellular viability was examined with an XTT assay (Cell proliferation kit II XTT), Roche, Basel, Switzerland). XTT assay is based on the cleavage of tetrazolium salt XTT which in presence of an electron coupling reagent produces formazan salt detectable by a spectrophotometer. This conversion happens only in metabolically viable cells. 50 μ l XTT labeling mixture (XTT labeling reagent and 1:50 electron coupling reagent) was added to 100 μ L of medium and incubated at 37° C for 30 min before absorbance was measured with a spectrophotometer. Microscopy examination was conducted to investigate the morphology of cells.

Protein Measurements

All protein analyses were performed by using enzyme-linked immunosorbent assay (ELISA) or electrochemiluminescence assays. All the electrochemiluminescence assays were analyzed on a SECTOR Imager 6000 (Meso Scale Discovery, Gaithersburg, MD, and the results were analyzed with DISCOVERY WORKBENCH software (Meso Scale Discovery). All assays were performed following manufacturer's protocols except for the chemokine assay (study I) in which 10 % bovine serum albumin (Fisher Scientific, Gothenburg, Sweden) was used in the blocking step and after adding the samples, the plates were incubated overnight at +4 °C.

Samples were assayed in duplicates and the mean from the duplicates was used for statistical analysis. Detection limits and CV-values are depicted in the table below. In study I, all samples below detection limit were assigned the same value, corresponding to the detection limit.

Table VII. Proteins Measurements.

| | Kit Manufacturer | Sample Matrix | Sample Dilution | Detection limit | % above detection limit | Mean CV-values | Study |
|---------------|------------------|---|-----------------|-----------------|-------------------------|----------------|-----------|
| Eotaxin-1 | MSD | Human CSF | 1 | 1.9 pg/ml | 100 % | 8.34 % | Study I |
| IP-10 | MSD | Human CSF | 1 | 2.8 pg/ml | 100 % | 4.56 % | Study I |
| MIP-1 β | MSD | Human CSF | 1 | 10.6 pg/ml | 93.33 % | 16.72 % | Study I |
| MCP-1 | MSD | Human CSF | 1 | 2.2 pg/ml | 99.99 % | 4.42 % | Study I |
| MCP-4 | MSD | Human CSF | 1 | 2.8 pg/ml | 76.11 % | 21.75 % | Study I |
| TARC | MSD | Human CSF | 1 | 2.77 pg/ml | 64.44 % | 52.33 % | Study I |
| IL-1 β | MSD | Rat CSF | 1 | 8.72 pg/mL | 100 % (LPS treatment) | 27.97 % | Study II |
| TNF- α | MSD | Rat CSF | 1 | 1.27 pg/mL | 100 % (LPS treatment) | 44.17 % | Study II |
| IL-1 β | MSD | Rat Serum | 1 | 9.11 pg/mL | 100 % | 16.51 % | Study II |
| IL-1 β | MSD | Adult Rat Primary Parenchymal Microglia | 1 | 24.5 pg/mL | 100 % (LPS treatment) | 19.89 % | Study II |
| TNF- α | MSD | Adult Rat Primary Parenchymal Microglia | 1 | 8.16 pg/mL | 100 % | 4.92 % | Study II |
| HA | R&D Systems | Human CSF | 10 | 370 pg/mL | 100 % | 2.36 % | Study III |
| sCD44 | eBioscience | Human CSF | 10 | 20 pg/mL | 100 % | 5.72 % | Study III |
| MMP9 | MSD | Human CSF | 1 | 12 pg/mL | 100 % | 10.78 % | Study III |
| HA | R&D Systems | Rat CSF | 10 | 370 pg/mL | 100 % | 5.03 % | Study IV |
| TNF- α | MSD | BV-2 Cell Culture | 1 | 11.4 pg/mL | 100 % | 7.06 % | Study IV |
| IL-6 | MSD | BV-2 Cell Culture | 1 | NA | NA | NA | Study IV |
| IL-10 | MSD | BV-2 Cell Culture | 1 | NA | NA | NA | Study IV |

qPCR (study II and IV)

The cytokine mRNA expression in rodent brain tissue was evaluated with RT-qPCR (relative quantification). Brain tissue from striatum, prefrontal cortex and hippocampus was homogenized with Trizol reagent followed by RNA isolation with RNeasy mini kit according to the manufacturer's instructions with the addition of a DNA digestion step (DNase) (Qiagen, Germany). Total RNA was transcribed to cDNA using Super-Script III (Invitrogen, Sweden) following manufacturer's instructions.

In study II and study IV, the expression of IL-1 β and TNF- α in prefrontal cortex and hippocampus (study II) were analyzed with RT qPCR on a C1000 thermal cycler with CFX 96 real-time system (Bio-Rad, Sweden). Maxima SYBR Green qPCR Mix was used as dye (Fermentas, Sweden) and β -actin and Hprt1 were used as the control genes. The PCR program had an initial hot start for 5 min at 95 °C followed by 40 cycles with a denaturing step of 15 s, a 30 s annealing step at 55 °C and an extension step of 30 s at 72 °C. For the melt curve, samples were initially heated to 95 °C for 1 min and then cooled for 1 min at 55 °C followed by 10 s increments of 0.5 °C with the temperature increasing from 55 to 95 °C. All samples were run in triplicates and data were analyzed using the Pfaffl method¹³².

In striatum (study II), the PCR reactions were carried out with TaqMan Universal MasterMix (ABI, Carlsbad, CA). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an endogenous control (Rn99999916_s1, ABI, Carlsbad, CA). Analysis of relative gene expression was carried out using the $2^{-\Delta\Delta CT}$ method¹³³.

Table VIII. qPCR.

| Target Gene | Tissue | Controls Genes | Dye | Study |
|---------------|---------------------------------------|----------------|------------|---------|
| TNF- α | Rat Prefrontal Cortex and hippocampus | B-Actin, Hprt1 | SYBR Green | II & IV |
| IL-1 β | Rat Prefrontal Cortex and hippocampus | B-Actin, Hprt1 | SYBR Green | II & IV |
| IL-1 β | Rat Striatum | GAPDH | TaqMan | II |
| TNF- α | Rat Striatum | GAPDH | TaqMan | II |

HPLC (study II & III)

The levels of serotonin and dopamine and their respective metabolites; 5-HIAA, and DOPAC and HVA, in the prefrontal cortex and striatum were analyzed using HPLC with electrochemical detection. Brain tissue was homogenized using an ultrasonic tissue homogenizer (Biologics, Gainesville, VA) in an antioxidant solution (0.4 N perchlorate, 1.34 mM ethylenediaminetetraacetic acid and 0.53 mM sodium metabisulfite). The suspension was spun down (14,000g, 20 min, 4°) and supernatants collected. A small amount was used for protein determination (BCA Protein Reagent Kit; Pierce, Rockford, IL). The mobile phase consisted of a 10% methanol solution in distilled water containing 21 g/L (0.1 M) citric acid, 10.65 g/L (0.075 M) Na₂HPO₄, 176 mg/L (0.8 mM) heptanesulfonic acid, and 36 mg/L (0.097 mM) EDTA, pH 4.1. Samples were separated on a Microsorb MV C-18 column (5 µm, 4.6 x 250 mm², Varian, Walnut Creek, CA). The monoamines and metabolites were simultaneously examined using a 12-channel coulometric array detector (CoulArray 5200; ESA, Chelmsford, MA) attached to a Waters (Milford, MA) 2695 Solvent Delivery System, under the following conditions: flow rate, 1 ml/min; detection potentials, 50, 175, 350, 400, and 525 mV; and scrubbing potential, 650 mV.

Statistical Analysis

The statistical analyses were undertaken using IBM SPSS Statistics (IBM Corporation, NY, USA). In general, parametric tests were used to evaluate group differences in mean values of analytes. α -level of significance was set at $p = 0.05$.

In study I, some of the chemokines correlated (Pearson's correlations) with gender, age and BMI. Hence, a linear regression model with the appropriate confounders as independent variables (and the chemokine as dependent) were used. If the chemokines were not normally distributed (displayed skewness above 2), they were transformed to normal distribution using the decadic logarithm. One-way ANOVA with Dunnett's post-hoc tests were used to examine chemokine levels between five diagnostic groups (major depressive disorder, dysthymia, adjustment disorder, substance dependence/abuse, depression NOS) and healthy controls. Since individual items on CPRS do not consist of interval scale data, Spearman's Rho was used for correlation analysis of chemokine levels and individual items on CPRS and subscales.

In study II, the effects of the drug treatments (LPS and exendin-4) on animal behavior and on levels of analytes were analyzed with two-way ANOVA. In case

of interaction effects these were further characterized with simple effects analysis. Cell culture data was analyzed with one-way ANOVA. CSF cytokine levels were analyzed with Student's T-test. Spearman's rho was used for correlation analysis.

In study III, all animal behavior tests, cytokines and HA levels in mice serum and the levels of monoamines and metabolites in mice brains were analyzed with two-way ANOVA and one-way ANOVA with post hoc Bonferroni (Four comparisons: WT to CD44KO; WT+CMS to CD44KO+CMS; WT to WT+CMS; CD44KO to CD44KO+CMS). There was a significant relationship between age and CSF sCD44 or HA. In agreement with previous study, there was a trend of gender effects of HA¹³⁴. There was no relationship between any analyte to BMI. Consequently, ANCOVA (GLM) was used to compare means between healthy controls and suicide attempters controlling for age and gender when applicable. The variables were checked of the assumptions of normality; homogeneity of variance and homogeneity of regression slopes and log transformed variables were used if needed. Correlations were done using Spearman's rank correlation.

In study IV, weight differences, before and after second injection of fluoxetine (or vehicle), were analyzed with Independent Student T-test. Weight differences before and after LPS or saline injection, open field test and prefrontal cortex cytokine expression results were analyzed with two-way ANOVA. Fixed factors were fluoxetine/vehicle and LPS/saline. Interaction effects were further characterized with simple effects analysis.

Study I

Study I: The Chemokine Study

Shorena Janelidze, Filip Ventorp, Sophie Erhardt, Oskar Hansson, Lennart Minthon, John Flax, Martin Samuelsson, Lil Träskman-Bendz and Lena Brundin

Highlights

- The levels of Eotaxin-1, MIP-1 β , MCP-1 and MCP-4 were significantly lower in the CSF of suicide attempters compared to healthy controls.
- The low levels of chemokines seem to be primary in patients diagnosed with MDD or depression NOS.

Background Information

In Lund from 1985 to 2001, CSF samples were collected from patients who recently attempted suicide. The principal investigator was prof. Träskman-Bendz and the samples were primarily collected for studying associations between monoamine metabolites and other biomarkers, with observable characteristics, psychological measurements and traits. It was a large initiative and the biobank consists of CSF from around 142 individuals and has resulted in several publications¹³⁵⁻¹⁴⁰.

When the inflammation hypothesis of depression started to emerge, Nässberger and Träskman-Bendz measured sIL-2R in plasma from suicide patients and found that the median sIL-2R concentrations were far above of the ones of healthy controls⁸¹. An attempt to measure sIL-2R in the CSF was done but the concentrations were below detection limit. Fifteen years later, the inflammation hypothesis had momentum and Lena Brundin was collaborating with Träskman-Bendz in projects with the CSF samples. Brundin and Träskman-Bendz started to yet again measure immunoproteins, and at this point, more sensitive assays were available. It was discovered that suicide attempters have higher levels of IL-6 compared to healthy controls⁸⁴. At this time, studies linking inflammation and suicidality were quite scarce and it was decided to continue investigating the role of inflammation in suicidality to further characterize the putative immunological processes in these suicide attempters. It was decided to investigate the levels of chemokines.

Chemokines are a family of cytokines originally identified as factors controlling the chemotaxis of leukocytes under physiological and pathological conditions and are divided into four subgroups (CXC, CC, C and CX₃C) based on the location of cysteine residues in their N-terminal region¹⁴¹. It was later established that chemokines are also expressed in the cells of the CNS, including astrocytes, microglia and neurons¹⁴². Chemokines and chemokine receptors have been implicated in a number of neurological diseases but the role of chemokines in psychiatric disorders is not well investigated.

Our primary hypothesis was that chemokines would be elevated in the patients, in line with the studies showing increased inflammation in the CSF of depressed and suicidal patients. Levels of six chemokines - namely eotaxin-1 (CCL11), interferon γ -inducible protein-10 (IP-10; CXCL10), macrophage inflammatory protein-1 β (MIP-1 β ; CCL4), monocyte chemoattractant protein-1 (MCP-1; CCL2), monocyte chemoattractant protein-4 (MCP-4; CCL13), thymus and activation-regulated chemokine (TARC; CCL17) – were examined in the CSF of a total of 180 patients and healthy controls.

Results and Comments

Levels of chemokines are lower in suicide attempters.

We found that the levels of eotaxin-1, MIP-1 β , MCP-1 and MCP-4 were significantly lower in suicide attempters (n = 137) than in the healthy controls (n = 43) ($p < .05$, Fig. 1). There was a similar trend for IP-10 but these differences were not statistically significant ($p = .052$). The results were reasonably robust and consistent since significance was found using different statistical tests, including non-parametric tests, and since almost all of the chemokines were lower in suicide attempters. Since the CSF of the healthy controls was collected a few years later than that of the suicide attempters, the lower levels of chemokines in suicide attempters could be the results of a longer storage time in the freezer. However, we did not find any significant statistical correlation between storage-time and chemokine levels within the suicide attempter cohort, where we previously have reported higher levels of IL-6⁸⁴.

Chemokine studies in psychiatric patients are scarce but this was not the first study to report low levels of chemokines. Whereas these six chemokines never have been investigated in CSF previously, another chemokine called IL-8, has been confirmed to be lower in the CSF of suicide attempters in more recent reports^{143,144}. Furthermore, Lehto et al. found lower levels of MCP-1, MIP-1 β and IL-8 in the blood of depressed patients compared to controls¹⁴⁵. In contrast, two earlier studies had shown increased blood levels of MCP-1 and eotaxin-1 in depressed patients^{146,147}. Another study found that levels of the chemokines MCP-1 and RANTES were reduced, and eotaxin-1 increased, in the serum of patients with depression and suicidal ideation as compared to those without suicidal ideation¹⁴⁸. Nevertheless, from the current study, we can conclude that there is no increase in chemokines occurring in parallel with the previously observed increase in cytokines. Hence, the lower levels of chemokines in suicide attempters need to be explained in other terms than acute inflammation. Interestingly, chemokines have been associated with other roles in the brain besides neuroinflammation. For example, chemokines regulates synaptic activity by controlling the glutamate release from astrocytes, they play a role in the developmental organization of the brain by regulating the migration of neuronal progenitors, and influence the viability of neurons¹⁴⁹. This is compatible with several studies showing altered brain structure in depressed and suicidal patients^{113,114}.

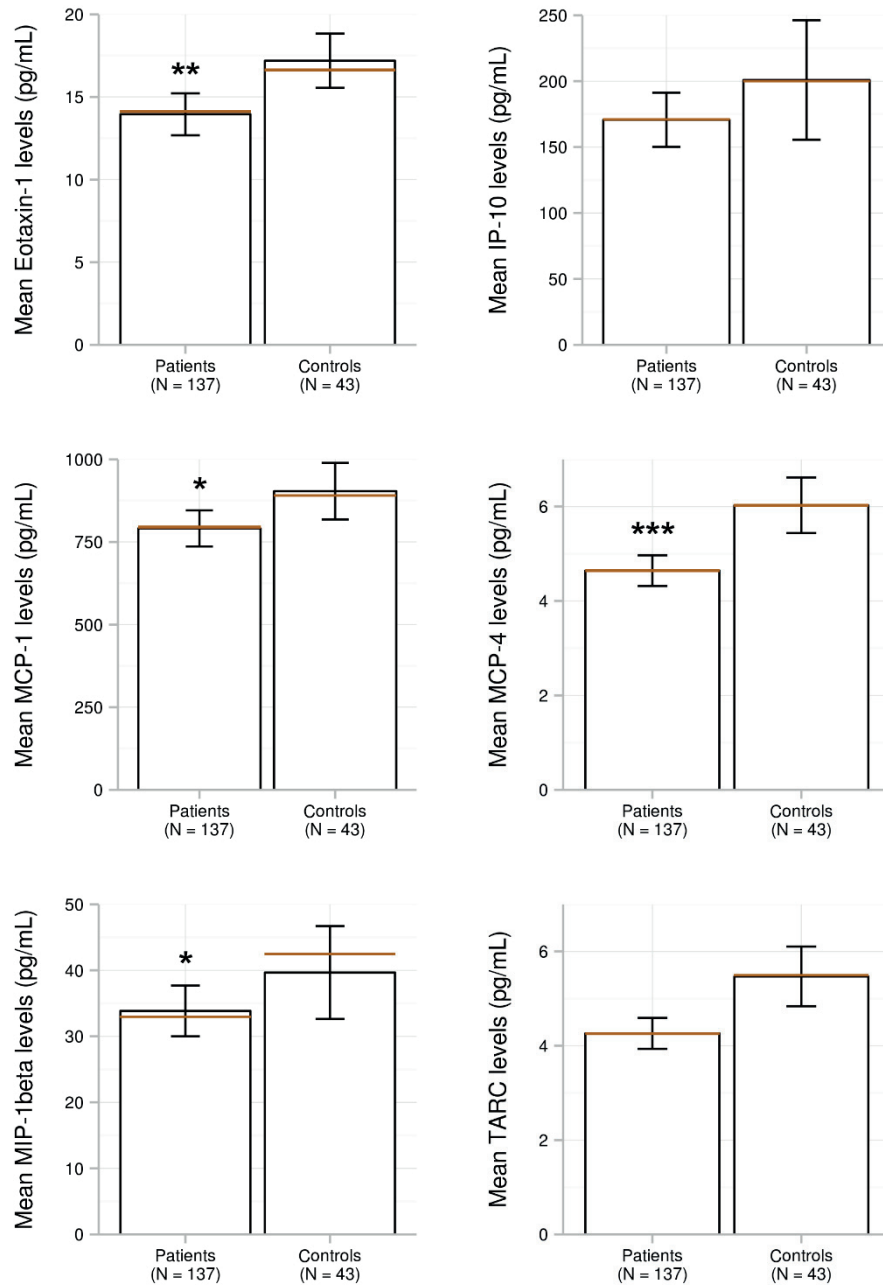


Figure 1. The levels of chemokines in CSF of suicide attempters and healthy controls. Eotaxin-1, IP-10, MCP-1 and TARC were log-transformed for statistical analysis (ANCOVA, adjusting for the effect of age [eotaxin-1, IP-10, MIP-1 β and MCP-1] and gender [eotaxin-1, MIP-1 β , MCP-1 and TARC]. The bronze lines represent the adjusted mean chemokine levels (age and gender when appropriate). * $p < .05$, ** $p < .01$, *** $p < .001$. Error bars: 95 % CI.

Suicide attempters with a Depression NOS (Not Otherwise Specified) diagnosis had the lowest levels of chemokines in CSF.

Patients were subdivided based on their main diagnostic group whereafter chemokine levels were compared between the five largest groups: major depressive disorder, dysthymia, adjustment disorder, substance abuse, depression NOS and healthy controls. We found that patients with a diagnosis of depression NOS had significantly lower levels of IP-10, MIP-1 β , MCP-1, MCP-4 and TARC than healthy controls ($p < .05$, Fig. 2). For Eotaxin-1, MCP-4 and TARC, patients with MDD also had significantly lower levels than controls. MCP-4 levels were also lower in patients with substance abuse. Hence, the low levels of chemokines seem to be primary in patients diagnosed with MDD or "subthreshold depression" (i.e. depression NOS). It is possible, that the results are rather linked to depressive symptoms than to suicide/suicidal behavior per se.

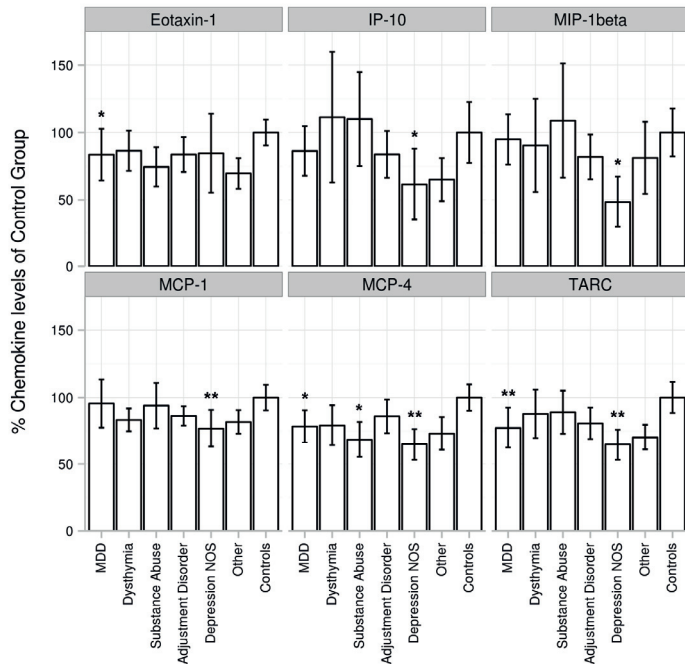


Figure 2. CSF chemokines and psychiatric diagnoses. Patients were divided based on their main diagnostic group (Axis 1 diagnosis) and the chemokine levels were compared between the five largest groups: major depressive disorder (n = 41), dysthymia (n = 19), adjustment disorder (n = 29), substance abuse (n = 11), depression NOS (n = 15) and healthy controls (n = 43). The group “Others” included patients with anxiety, bipolar disorder, Psychotic disorder and patients without any diagnosis (n = 22). Statistical analysis was done by one-way ANOVAs with Dunnett’s post-hoc tests with healthy controls as control category. The levels were transformed in the figure for display purposes only. * p < .05, ** p < .01. Error bars: 95 % CI.

Study II

Study II: The Exendin-4 Study

Filip Ventorp, Cecilie Bay-Richter, Analise Sauro, Shorena Janelidze, Victor Sjödahl-Matsson, Jack Lipton, Ulrika Nordström, Patrik Brundin and Lena Brundin

Highlights

- Exendin-4 prevents LPS-induced behavioral despair as seen as decreased immobility in the FST.
- Exendin-4 effects are not mediated through modulation of neuroinflammation.
- In LPS treated rats, increased dopamine turnover is associated with immobility in the FST.

Background Information

In the early 1980s, scientist discovered several peptide hormones that play a role in digestive and metabolic processes. An endocrinologist named Dr. John Eng was investigating a dried saliva sample from the Gila monster lizard (*Heloderma Suspectum*), and found a peptide with glucose-lowering effects. The peptide was named exendin-4. This peptide shares properties with the endogenous GLP-1; a gut hormone that plays an important role in regulating glucose levels in humans. Both exendin-4 and GLP-1 enhance the body's ability to release insulin in response to elevated levels of glucose, thereby reducing the likelihood that glucose levels will be too high or too low¹⁵⁰. This makes the peptide a potential therapeutic agent for type-2 diabetes and in 2005 the first exendin-4 based treatment was approved by the Food and Drug Administration in the United States.

Exendin-4 passes the BBB¹⁵¹ and the GLP-1 receptors are widely expressed in the brain, with high levels in the hypothalamus and brainstem¹⁵². GLP-1 receptors in the brain are known to regulate appetite and promote satiety¹⁵³. Interestingly, exendin-4 also increases neurogenesis in rodent subventricular zone and dentate gyrus of the hippocampus^{154,155}. In addition, it exhibits neuroprotective properties as it preserves synaptic plasticity in the hippocampus in animal models of Alzheimer's disease (AD)¹⁵⁶⁻¹⁵⁸ and protects dopaminergic neurons and restores dopamine levels in the brain in rodent models of Parkinson's disease (PD)^{159,160}. Today, exendin-4 is tested in clinical treatment trials for both AD and PD¹⁶¹.

Since exendin-4 also protects neurons in LPS-induced models of PD¹⁵⁹ and AD¹⁶² (possibly by reducing inflammatory activity), and since it decreases immobility in the FST in rats¹⁵⁵; we got interested in investigating the putative anti-inflammatory effects of exendin-4 further. We hypothesized that it would have anti-depressive effects in an inflammation-induced model of depression, mediated by its anti-inflammatory properties.



Although the Gila monster is venomous, it represents little threat to humans. The lizard is very sluggish, slow of movement and non-aggressive. The last known report of a death from Gila monster bite occurred in 1930; the victim was very drunk and poked it with a stick.

Results and comments

LPS-injections induce depression-like behavior after 24 h which is prevented by exendin-4 injections.

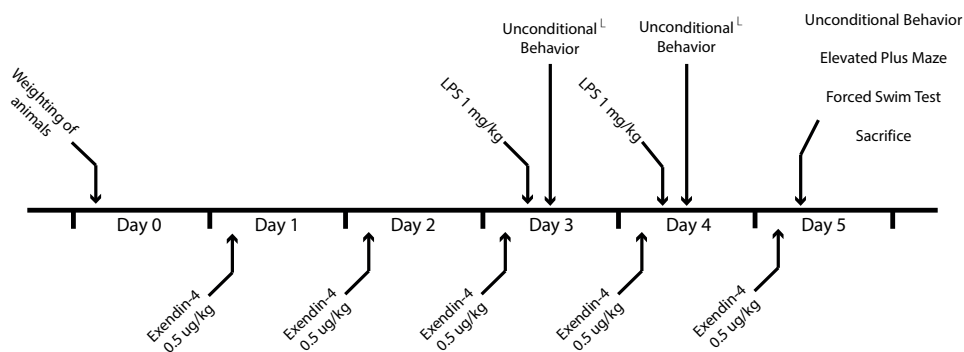


Figure 3. Schematic illustration of the study design.

LPS had the strongest effect on immobility (behavioral despair) out of the three measures in the FST. There was a significant interaction between the LPS and exendin-4 treatments ($p < .05$). LPS treated animals showed increased immobility (14.25 ± 10.85 events, mean \pm SD) when compared to vehicle treated rats (3.09 ± 3.89 , $p < .01$), whereas this effect was not present in the exendin-4 treated rats (6.33 ± 6.34), Fig 4. Exendin-4 has decreased immobility in rodents in earlier studies but never before shown in an inflammation-induced model of depression^{155,159}.

Since exendin-4 decreased immobility, it could be interpreted as exendin-4 has anti-depressant effects. However, the statistical power was relatively weak; some rats did not respond to the LPS and one got hurt by the injections, so they had to be excluded. Hence, the results need to be validated in a larger sample.

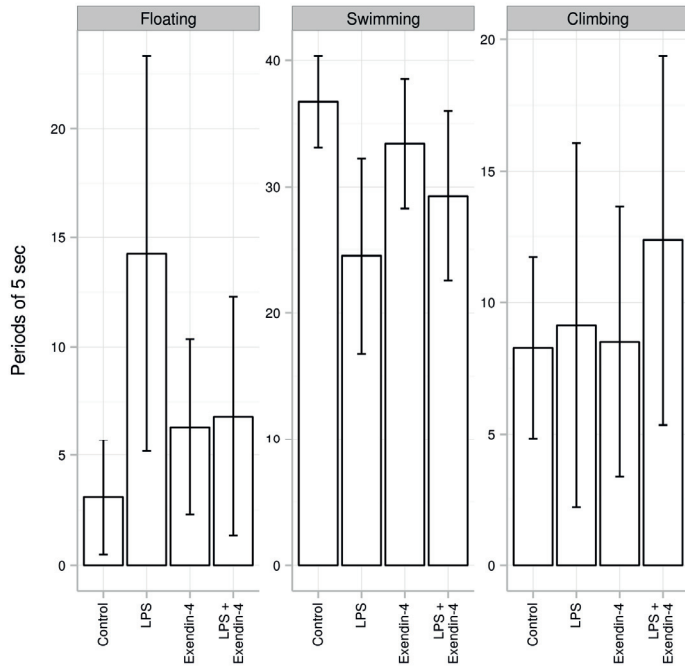


Figure 4. The results of the FST. Rats were treated with LPS (n = 8), Exendin-4 (n = 12), LPS+Exendin-4 (n = 11) or left untreated (Control, n = 11). **(Floating)** Periods of 5 sec the rats spent immobile. There was a significant LPS x Exendin-4 interaction (two-way ANOVA, $p < .05$). LPS significantly increased the time spent immobile (induced behavioral despair) in animals without exendin-4 treatment (simple effect analysis, $p < .01$) but this effect was not present in the Exendin-4 treated rats. **(Swimming)** The number of periods the rats spent swimming (vertical movements). LPS treatment decreased the counted periods of swimming, (two-way ANOVA, $p < .01$) **(Climbing)** The number of counts the rats spent climbing (horizontal movements). There was no effect of either LPS or exendin-4 on climbing behavior (NS). Error bars: 95 % CI.

LPS-injections increase the protein levels of TNF- α and IL-1 β in CSF and IL-1 β mRNA expression in brain tissue. But no significant anti-inflammatory effects of exendin-4.

We hypothesized that the anti-depressant effects of exendin-4 would be mediated by anti-inflammatory properties. In the CSF, TNF- α and IL-1 β protein levels were only measurable in LPS treated rats (below detection limit in non-LPS treated rats). Furthermore, LPS treatment increased the expression of IL-1 β mRNA in all three brain regions analyzed, striatum ($p < .001$), prefrontal cortex ($p < .01$), and hippocampus ($p < .001$). In contrast, there was no effect of LPS on TNF- α mRNA levels in any of the three regions. Exendin-4 treatments did neither affect the CSF levels of the cytokines nor the expression of IL-1 β in the brain, Fig. 5.

Microglia primary cells were isolated from adult rats. Incubation with Exendin-4 did not affect cytokine production from cells cultured in LPS. No significant difference in IL-1 β or TNF- α levels was observed following a 24-hour incubation, Fig. 6. There were no significant differences in cell death (% PI positive cells) between groups after 6-hour incubation confirming that differences in cell viability/numbers did not influence the results.

In conclusion, the LPS injections induced sickness behavior and the LPS-treated rats displayed increased IL-1 β mRNA expression in the prefrontal cortex, striatum and hippocampus 24 hours after the last LPS injection as has been reported in previous studies^{68,79,121}. At this time point, LPS treated rats displayed increased immobility if they were not treated with exendin-4. But we could not find any evidence that the effect of exendin-4 on immobility was mediated through modulation of neuroinflammation as assessed in this model.

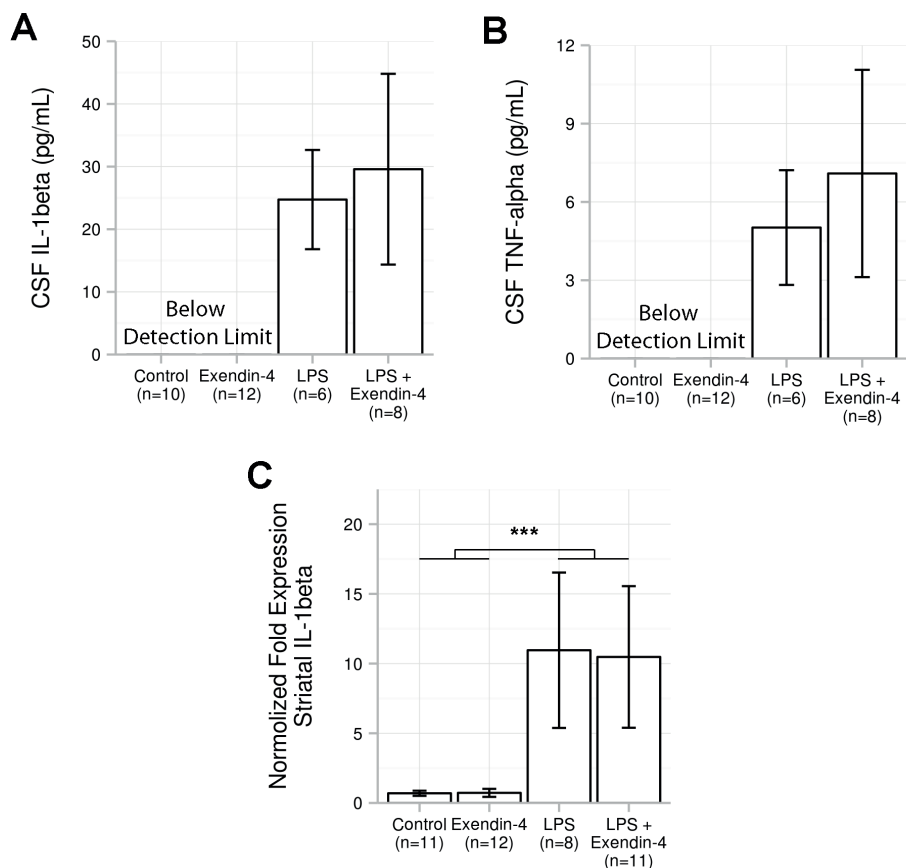


Figure 5. The levels of cytokines in CSF (pg/mL). The LPS-treated rats produced measurable levels of IL-1 β (**A**) and TNF- α (**B**), but exendin-4 treatment had no effect on cytokine levels (Student's t-tests, NS). The levels of cytokines in Non-LPS treated rats were below detection limit. (**C**) The mRNA expression of IL-1 β in the striatum. The IL-1 β expression was higher in LPS treated rats but was not affected by exendin-4 treatment (two-way ANOVA, $p < .001$). *** $p < .001$. Error bars: 95 % CI.

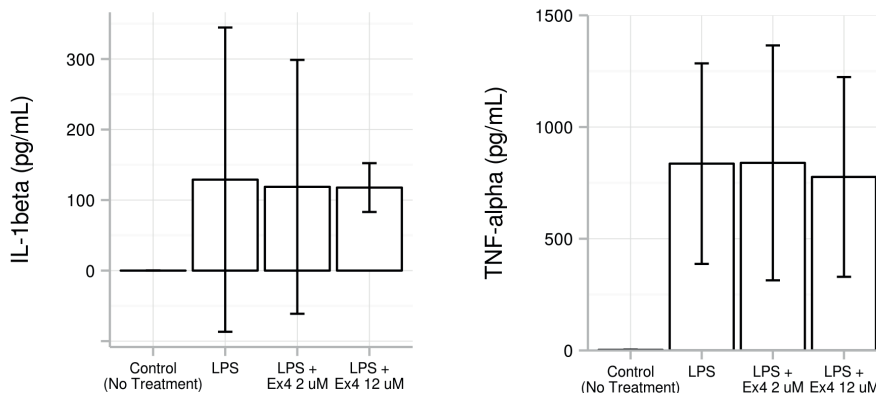


Figure 6. The levels of cytokines in supernatants from adult primary microglia cell cultures ($n = 3$). No significant difference in the levels of IL-1 β and TNF- α could be observed between the LPS treated groups, (Student's t-tests, NS). Error bars: 95 % CI.

LPS-injections increase the levels of HVA in the striatum and striatal dopamine turnover is associated with immobility in the FST.

At Lund University, a research group working with Parkinson's disease, had some preliminary results showing exendin-4 injections to increase the levels of dopamine in the striatum of rats. At the same time, a study showed that exendin-4 treatment potentiates the dopamine release effects of levodopa (L-DOPA)¹⁶³. Since LPS injections in rodents are known to increase the turnover (metabolite/monoamine ratio) of serotonin, dopamine and noradrenaline in multiple structures in the brain^{164,165} and since these changes in turnovers have been suggested to be the cause of the sickness behavior¹⁶⁶, we hypothesized that the effect of exendin-4 was mediated by preventing the LPS modulating effects on brain monoamine levels.

Indeed, the levels of the dopamine metabolite HVA were increased in striatum of LPS treated animals ($p < .05$, Fig. 7A). There was a trend of increased levels of dopamine and a lower DOPAC/dopamine ratio in striatum in exendin-4 treated animals ($p = .059$ and $p = .100$, respectively). Furthermore, the DOPAC/dopamine ratio in the striatum of the LPS-treated animals (with and without exendin-4) was significantly associated with both floating (Spearman's $\rho = 0.6$, $p < .05$, Fig 7B) and climbing ($\rho = 0.7$, $p < .01$). Hence, a low dopamine turnover resulted in less depression-like behavior. The monoamines were however measured in whole tissue and therefore it is not possible to differentiate between intra- and extracellular concentration of dopamine. The exact effects of exendin-4 on dopamine activity should be investigated in future studies.

Exendin-4 did not have any effect on the serotonin levels in any of the regions.

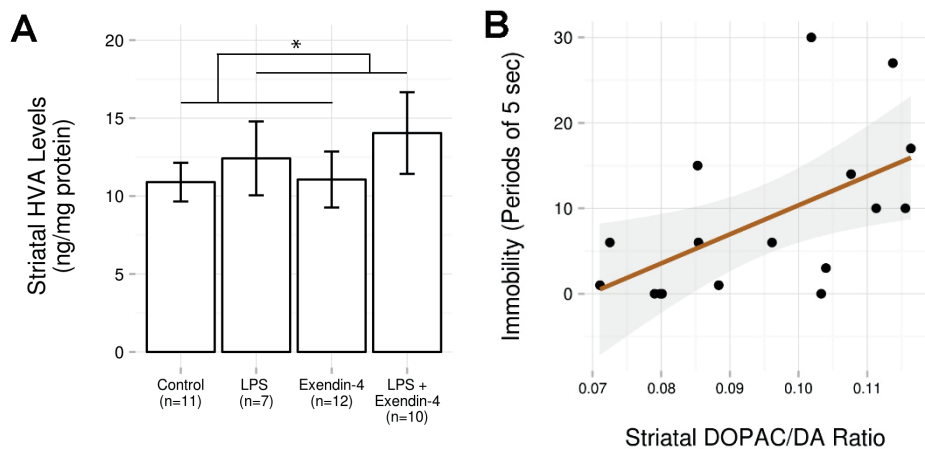


Figure 7. (A) Levels of HVA in the striatum. The LPS treated rats had higher levels of HVA (two-way ANOVA, $p < .05$). **(B)** Association between dopamine turnover in striatum and immobility (Spearman's $\rho = .59$, $p < .05$, $n = 17$) in the forced swim test. Line represents the regression line and the shaded area is the 95 % CI around the line. Error bars: 95 % CI.

Study III

Study III: The CD44 Study

Filip Ventorp, Ran Barzilay, Hadar Segal, Israel Aharoni, Rea Globus, Abraham Weizman, David Naor, Sophie Erhardt, Martin Samuelsson, Jack Lipton, Lil Träskman-Bendz, Shorena Janelidze, Daniel Offen and Lena Brundin

Highlights

- CD44 deficiency in mice increases vulnerability to stress-induced anxiety.
- The stress-induced anxiety is associated with changes in serotonin turnover.
- Suicide attempters have higher levels of HA in CSF and the levels correlate with BBB permeability.

Background Information

Depression and suicidal behavior are to some extent influenced by genes which have been established by several twin-studies and adoption studies. Yet, numerous candidate genes studies have yielded inconsistent findings. For example, a recent meta-analysis of genome-wide association studies for MDD was unable to identify robust and replicable findings¹⁶. Still, genetic studies might point to more generalized processes.

Our interest in the CD44-molecule started with a small GWAS study of suicide victims with and without depression¹⁶⁷. The study identified the CD44 gene as a candidate gene and the gene was also reported to be expressed less in two brain areas of relevance to suicide (BA24, BA9). CD44 is a cell surface adhesion molecule found on most mammalian cells¹⁶⁸ including neurons¹⁶⁹, astrocytes¹⁷⁰ and microglia¹⁷¹. A lot is known about CD44 and tumor metastasis¹⁷² but its role in physiological conditions is less understood. The CD44 gene transcripts undergo complex alternative splicing and there is theoretically over 1000 possible splice-variants resulting in many functionally distinct isoforms¹⁷³. However, The CD44 isoform expressed in the nervous system is predominantly the standard form. CD44 functions as a ligand-binding receptor by interacting with the ECM, especially HA¹⁷⁴. Binding of ligands to the CD44 has been associated with multiple signaling cascades but the most defined cascade includes CD44-HA-dependent activation of RhoGTPase/Rac1 and PKN γ (i.e. cytoskeletal rearrangement)¹⁷⁵. Various immune and neurodevelopmental functions have been reported to be dependent on CD44 such as homing of cells¹⁷⁶ and neuronal axon guidance. CD44 also anchors proteolytic enzymes, such as MMP9, close to the cell surface to retain proteolytic activity to the pericellular region, for example when leukocytes infiltrate tissues¹⁷⁷. MMPs can also shed CD44 from the cell surface into the ECM but the function of the soluble CD44 (sCD44) is not known¹⁷⁸.

During a congress, I met Ran Barzilay from Tel Aviv, whose colleagues worked with the CD44KO mice and rheumatoid arthritis, and we discussed the above mentioned GWAS study. We decided to initiate a collaboration and to investigate if the CD44 gene could be a predisposing factor to suicidal behavior by lowering the ability to cope with stress. Several studies have investigated the role of CD44 in inflammation using CD44KO mice¹⁷⁹⁻¹⁸¹, but no studies have looked at the CD44KO mice in terms of behavior and cognitive functions. Therefore, as a joint venture between Brundin and Offen's research groups, it was decided to expose CD44KO mice to CMS and assess the mice with several behavioral tests related to psychiatry; and at the same time look at CD44 ligands in the CSF of suicide attempters.

Results and Comments

CMS Treatment causes anhedonic behavior in mice and CD44 deficiency increases vulnerability to stress-induced anxiety.

The CMS treatment increased anhedonia, seen as decreased sucrose consumption in the sucrose preference test, as reported in many previous studies¹²². The sucrose preference of WT mice was 66.6 ± 12.4 % (mean \pm SD) whereas in the CD44KO+CMS mice, the preference for sucrose was extinguished (47.7 ± 18.3 %), Fig. 8A.

Of all the behavioral tests, the EPM test showed the most interesting outcomes. Both WT and CD44KO mice who underwent CMS displayed increased anxiety-like behavior, as observed by decreased duration of time spent in the open arm, but this was further exaggerated in the CD44KO mice (13.5 ± 7.5 sec), compared to the WT+CMS mice (28.7 ± 12.2 sec), ($p < .01$, Fig 8B.).

The EPM results support the hypothesis that CD44KO mice have lower ability to cope with stress. The EPM results were replicated in an additional experiment by R Barzilay¹⁸², where the mice were not exposed to other behavioral tests to exclude potential interaction effects.

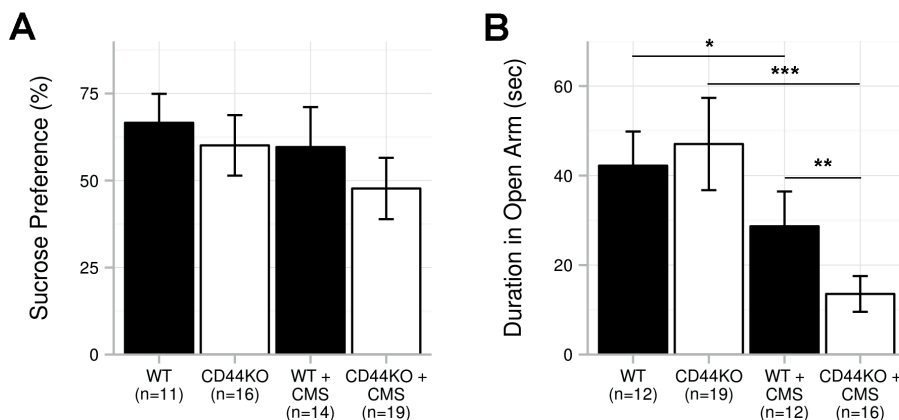


Figure 8. The effects of CD44KO and CMS on behavior. **(A)** Sucrose preference test. Results represent the intake of sucrose containing liquid out of the total liquid intake measured over 4 weekly measurements conducted in each cage. There was a main effect of both genotype ($p < .05$) and CMS ($p < .05$) but no significant genotype \times CMS interaction. **(B)** The result of the EPM test. There was a significant genotype \times CMS interaction ($p < .05$). Specifically, both WT+CMS and CD44KO+CMS mice showed significantly more anxiety compared to WT and CD44KO, respectively (Student T test with Bonferroni correction, $p < .05$ and $p < .001$). However, CD44KO+CMS and spent significantly less time in the open arm compared to WT+CMS mice ($p < .01$). * $p < .05$, ** $p < .01$, *** $p < .001$. Error bars: 95 % CI.

Serotonin turnover in PFC is associated with anxiety-like behavior.

Aberrant monoaminergic turnover has previously been described following various forms of acute and chronic stress in rodents¹⁸³⁻¹⁸⁶. In our study, the CMS exposed animals had a lower turnover of 5-HT (5-HIAA/5-HT ratio) in the prefrontal cortex compared to WT, Fig. 9. Lower 5-HT turnover was even more pronounced in CD44KO+CMS mice (0.93 ± 0.51 ratio, mean \pm SD, $n = 12$) than in WT+CMS mice (0.44 ± 0.05 , $n = 11$). Furthermore, we found a positive correlation between the 5-HT turnover and the time spent in the open arm of EPM (Spearman's $\rho = 0.33$, $p = .025$). Hence, we speculate that in the CD44KO+CMS group, the decreased cortical 5-HT turnover accounts for the increased anxiety in the EPM.

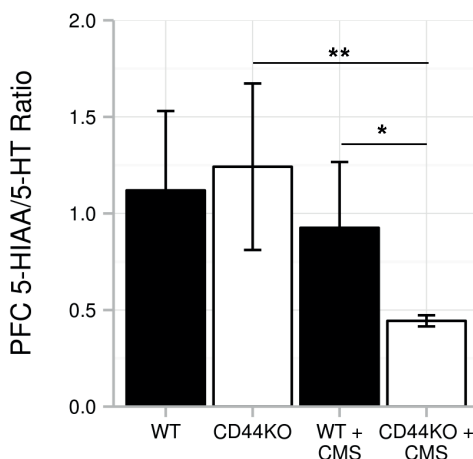


Figure 9. The ratio of 5-HIAA/5-HT (5-HT turnover) in prefrontal cortex in mice. There was an effect of CMS (two-way ANOVA, $p < .01$). There was a significant difference between WT+CMS and CD44KO+CMS (Student T test with Bonferroni correction, $p < 0.05$). * $p < .05$, ** $p < .01$. Error bars: 95 % CI.

Suicide attempters have higher levels of HA and MMP9 in CSF compared to healthy controls. Higher levels of HA are associated with increased BBB permeability.

It is technically difficult to estimate the amount of membrane-bound CD44 receptors in brains of humans and at the same time collect CSF. However, it is possible to measure shedded soluble CD44 and CD44 ligands such as HA and MMPs. Increased HA levels in CSF have previously been reported in patients with CNS tumors¹⁸⁷, meningitis¹⁸⁷, spinal disorders¹⁸⁸, cerebral ischemia¹⁸⁹ and dementia^{134,190} but have never been investigated in psychiatric patients. We found that the levels of HA were significantly higher (49.6 % higher, adjusted for the effect of age and gender) in the suicide attempters ($n = 94$) compared to the healthy controls ($n = 45$), Fig. 10A ($p < .01$). Suicide attempters also had significantly higher levels of sCD44, 39.69 ± 19.72 pg/mL compared to 33.49 ± 11.94 pg/mL (mean \pm SD), but this

significance disappeared after adjusting for age. There was a positive correlation between sCD44 and HA in both suicide attempters and healthy controls, ($\rho = 0.32$, $p < .001$).

The CD44 molecule has been implicated in many important cell functions such as cell adhesion¹⁹¹, cell migration¹⁹², regulation of inflammation¹⁹³, neuronal axon guidance¹⁷⁶ and in BBB permeability. A recent study of CD44KO mice reported increased BBB permeability in the experimental autoimmune encephalomyelitis model, suggesting a role of CD44-HA interaction in maintaining BBB integrity¹⁸¹. Another study reported increased histamine-induced BBB permeability in CD44KO mice compared to WT mice¹⁹⁴. In the suicide attempters, we found a correlation between CSF HA levels and BBB permeability measured as CSF/serum albumin ratio, $\rho = 0.41$, $p < .001$, Fig. 11. (The BBB permeability was measured in a previous articles by L Bayard-Burfield and L Träskman-Bendz¹⁹⁵)

The endothelial glycocalyx contributes to the BBB function and HA is important for the function of the glycocalyx¹⁹⁶. It has been suggested that CSF HA levels increase when the glycocalyx of the BBB is degraded¹⁹⁰. MMPs are involved in both shedding of CD44 molecules and in modulation of the glycocalyx^{178,197,198}. We found increased levels of MMP9 in suicide attempters compared to the healthy controls, Fig. 10B. (135.87 ± 107.47 pg/mL compared to 94.43 ± 74.76) ($p < .01$). Also, the levels of MMP9 correlated with the levels of sCD44, Spearman's $\rho = 0.26$, $p < .01$, $n = 116$.

Loss of and breaching of the BBB has been suggested to contribute to psychiatric disorders^{195,199,200} and CD44-HA interactions might have an important role in this aspect.

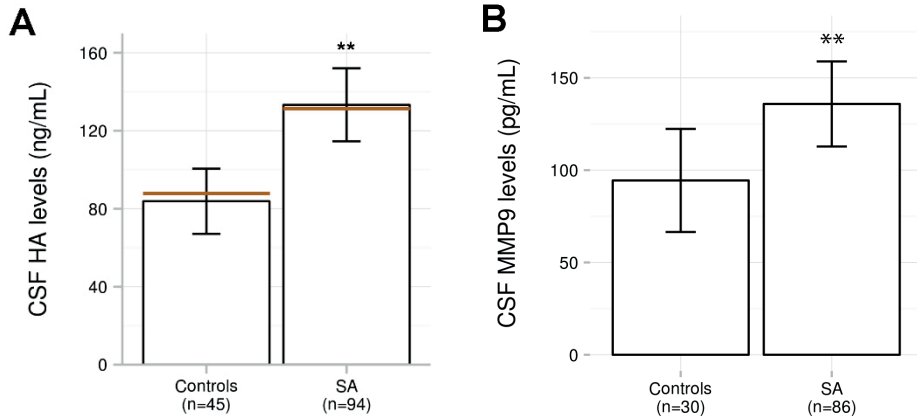


Figure 10. CSF HA (A), and MMP9 (B) levels of suicide attempters and healthy controls. There were higher levels of HA in CSF of suicide attempters compared to healthy controls after adjusting for age and gender (ANCOVA, $p < .01$). The levels of MMP9 was higher in suicide attempters compared to healthy controls, Mann-Whitney U test, $p < .01$. The bronze lines represent the adjusted mean (ANCOVA) levels (age and gender when appropriate). ** $p < .01$. Error bars: 95 % CI (raw uncorrected data).

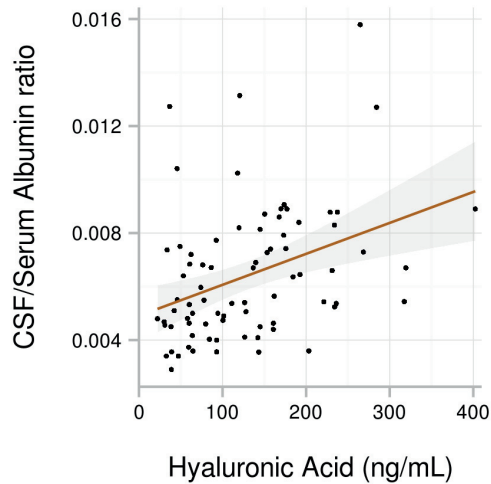


Figure 11. Association between CSF hyaluronic acid levels and the CSF/serum albumin ratio as a measure of BBB integrity. CSF/serum albumin ratio correlated with the HA levels (Pearson's $r = 0.38$, $p = .001$). The shaded area around the regression line is the 95 % CI around the line.

Study IV

Study IV: The Fluoxetine Study

Filip Ventorp, Shorena Janelidze, Simon Ventorp, Oskar Sporre and Lena Brundin

Highlights

- Fluoxetine decreases TNF- α production in LPS stimulated BV-2 cells.
- Fluoxetine did not decrease IL-1 β and TNF- α mRNA expression in brain of LPS treated rats.
- LPS-induced increase of HA in CSF was absent in fluoxetine treated rats.

Background Information

MDD can successfully be treated by medications which increase concentrations of monoamines in the synaptic cleft. Interestingly, several *in vitro* and *in vivo* studies have reported antidepressants to have anti-inflammatory properties such as lowering blood levels of cytokines²⁰¹. We were interested in the potential of antidepressants to exert anti-inflammatory effects on microglial cells. We decided to make experiments using a murine microglia cell line called BV-2 cells. We activated the cells with LPS, treated them with several antidepressants and measured cytokines. We got encouraging results, especially with fluoxetine, a selective serotonin reuptake inhibitor. But at the time we got the results, another research group published a very similar study²⁰². As an alternative, we decided to add an *in vivo* and *ex vivo* experiment with fluoxetine. Fluoxetine has been shown to exert peripheral anti-inflammatory effects in animal models of septic shock and allergic asthma²⁰³ but also in the CNS; for example, fluoxetine prevents MPTP- and LPS-induced degeneration of nigral dopaminergic neurons in an animal model of Parkinson's disease^{204,205}.

We decided to treat rats with LPS to induce a strong inflammatory response and to treat a group of rats with fluoxetine to try to alleviate the inflammation. We also collected CSF. In the CD44 study, suicide attempters have markedly increased levels of HA in the CSF. In the same study, and in a study with Alzheimer's disease and Lewy-Body dementia patients¹³⁴, HA correlates with BBB permeability as measured by CSF albumin/serum albumin ratio^{134,206}. A previous study on the same cohort of suicide attempters, showed increased BBB permeability in suicide attempters compared to controls¹⁹⁵. Since fluoxetine has been shown to prevent disruption of the blood-spinal cord barrier after spinal cord injury²⁰⁷; we decided to measure HA in the CSF and investigate the effects of fluoxetine on HA levels.

Results and Comments

Fluoxetine lowers the levels of TNF- α released from BV-2 cells treated with LPS.

Fluoxetine significantly lowered the concentrations of TNF- α in the cell culture medium at a dose five times higher than reported plasma concentrations after chronic fluoxetine treatment ($p < .05$), Fig. 12A. There was a trend of the same effect of sertraline (SSRI) and diclofenac (NSAID, control substance) but it did not reach significance ($p < .1$). The lowering effect of fluoxetine on cytokines was specific to TNF- α as it did not affect the levels of IL-6 and IL-10, Fig. 12B. Similar results were also reported in the study by Tylan *et al* that preceded ours²⁰². However, they did not investigate any effects of fluoxetine on other cytokines besides TNF- α .

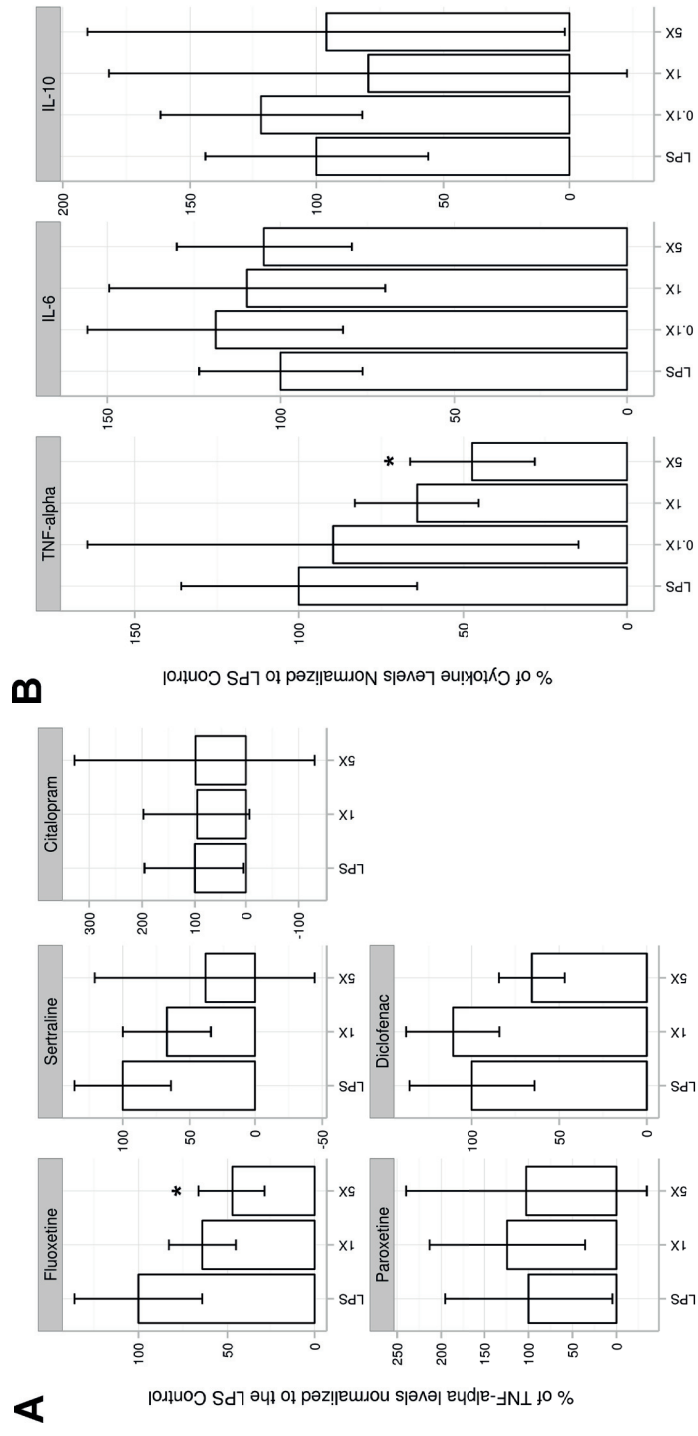


Figure 12. BV-2 cells were treated with LPS (1 µg/mL) together with either an SSRI or control (diclofenac, NSAID). Drug concentrations were calculated based on reported levels of SSRI in serum after acute and chronic treatment (1X). Cell culture medium was collected after 24 hours and cytokines were measured with electrochemiluminescence assays. **(A)** The relative concentration of TNF-α after treatments with LPS, SSRI and diclofenac. Only fluoxetine significantly lowered the concentrations of TNF-α. **(B)** The cytokine lowering effect of fluoxetine was specific to TNF-α i.e. fluoxetine did not affect the levels of IL-6 and IL-10. * $p < .05$ (One-way ANOVA, Dunnett's post hoc test). Error bars: 95 % CI.

LPS injections increase brain mRNA IL-1 β expression. No effects of fluoxetine.

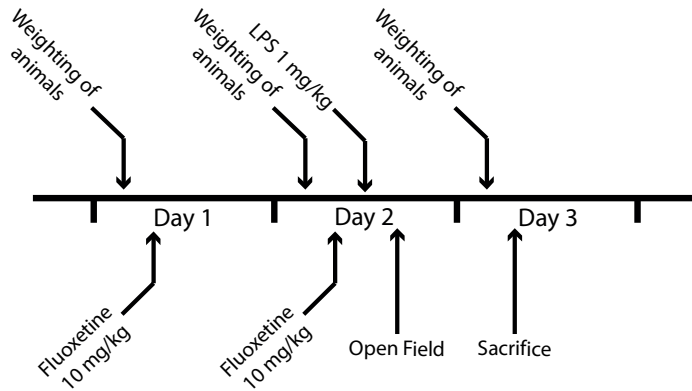


Figure 13. Schematic illustration of the study design.

24 hours after the LPS-injection, the expression of IL-1 β mRNA in prefrontal cortex was increased in LPS treated animals ($p < .001$), Fig. 14. There was no significant effect of fluoxetine on IL-1 β expression. The expression of TNF- α at this time point was unaffected by both LPS and fluoxetine treatment.

Considering that the cytokine lowering effect of fluoxetine was specific to TNF- α in the BV-2 cells, and since we could not measure any effects of fluoxetine on the IL-1 β mRNA expression in the brain in the *in vivo* study, it cannot be concluded that fluoxetine is an anti-inflammatory drug in the CNS.

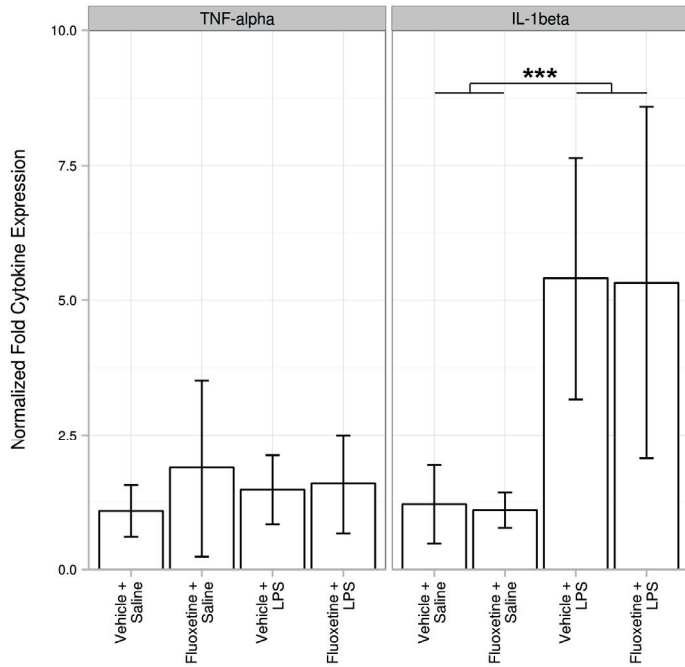


Figure 14. Prefrontal cortex TNF- α and IL-1 β expression in LPS, saline, fluoxetine and vehicle treated rats (n = 8 in each group). Fluoxetine treatment compared to vehicle had no effect on cytokine expressions. LPS treatment increased expression of IL-1 β compared to saline treatment. *** Indicate $p < .001$ (two-way ANOVA, main effects). Error bars: 95 % CI.

Fluoxetine prevents LPS-induced increase of HA levels in CSF.

There was a strong interaction effect of LPS and fluoxetine treatment on the levels of HA in CSF after LPS treatment ($p < .001$). LPS injected animals had higher levels of HA in the CSF compared to saline treated animals, 148.44 ± 33.07 ng/mL (mean \pm SD) compared to 61.85 ± 8.39 . Fluoxetine treatment blocked the LPS-induced increase in HA levels: 51.49 ± 14.13 ($p < .05$), Fig. 15.

Since LPS treatment increased the levels of HA in the CSF of rats; the increased levels of HA we have observed in the suicide attempters (CD44 study) might interpreted to be the result of inflammatory processes. In fact, a previous study has suggested inflammation to be the cause of increased levels of HA in CSF in patients with spinal disorders¹⁸⁸.

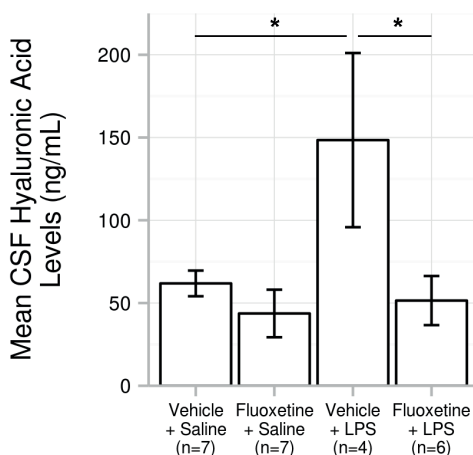


Figure 15. The levels of HA (ng/mL) in the CSF of rats. P-value indicate Student's T-test with Bonferroni correction for group-wise comparisons. * Indicate $p < .050$. Error bars: 95 % CI.

General Discussion

Are depression and suicidality inflammatory disorders?

It is known that inflammation can trigger the onset of depression in a group of patients²⁰⁸. Depression and suicidality are multifactorial syndromes and, in some cases, inflammation may be the final trigger tilting the balance towards illness. At the same time, depression or suicidality are not considered to be inflammatory disorders to the same extent as for example rheumatoid arthritis or hepatitis, simply because inflammation does not seem to be an essential factor. This is apparent when we see reports of increased immunoproteins in plasma and CSF of depressed and suicidal patients. Not all depressed patients have higher cytokine levels or other signs of inflammation, and many patients with elevated cytokine levels do not have MDD.

Many studies reporting inflammation in depressed patients measure several immunoproteins, but the type of immunoprotein varies between studies⁷⁴. The elevations are generally modest, and increased levels of one proinflammatory cytokine (in isolation from other inflammatory signs) are sometimes interpreted as “inflammation” without more in-depth analysis. Instead of connecting aberrant levels of immunoproteins with inflammation as a response to harmful stimuli, a possible approach to understand the observations in psychiatric patients could be to examine the functions of the immune system when not acutely confronted by pathogens, damaged cells, or other irritants.

In the brain, some of the inflammatory pathways and immune cells are involved in everyday maintenance, such as cleaning up cell debris and controlling synapse formation²⁰⁹. Cytokines are pleiotropic – they have different roles depending on context and levels and have different effects on different target cells. For example, TNF- α can induce fever and mediate acute inflammation, but in the brain, it is important for memory formation¹⁰⁷. IL-6 is produced by the muscles during exercise, and is probably important for regulating glucose metabolism and cell proliferation, differentiation, survival, and apoptosis²¹⁰. In the brain, IL-6 has been reported to be both neurotoxic and neuroprotective, depending on the concentration and locales²¹¹. The chemokine receptors are phylogenetically older than the specialized vertebrate immune system²¹² and have been suggested to be involved in processes relating to CNS development, modulating neural transmission, and to have neuroprotective properties²¹³. CD44-HA signaling is important for regulating the immune response, but is also implicated in neuronal axon guidance, developmental fate of glial progenitors^{214,215} and in BBB permeability¹⁸¹. Consequently, changes in inflammatory pathways do not necessarily imply

inflammation the way we commonly use the word, hence depression and suicidality should not simply be reduced to “inflammatory disorders”. Nevertheless, inflammatory mechanisms may be part of the complex pathophysiology of depression and suicidality.

Is inflammation linked to depression or suicidality?

Some of the arguments of the inflammation hypothesis of depression are only valid for depression, for example the resemblance of depression to sickness behavior and that IFN- α treatment frequently causes depression. The studies that make up this thesis use material from suicide attempters but use animal models of depression. Is this an inconsistency? Suicidal behavior is suggested to be a distinct psychiatric phenotype, so biological suicide research is separated from other psychiatric disorders, e.g. research into depression and anxiety. The main rationale is that not all people who share risk factors for suicide commit suicide. For example, why do some people who suffer from major depression commit suicide while others never consider it? People with depression who commit suicide may be biologically different from those who do not.

It is not possible to model suicidality in animals directly but it is possible to model aspects of it such as hopelessness, aggression, impulsivity and most importantly depression. Also, many of the different factors contributing to depression may also contribute to suicidal behavior, and the two behavioral phenotypes may be biologically similar. In fact, recurrent thoughts of death and suicidal behavior is one of the criteria in diagnosing a MDD. Many depressed patients have suicidal ideations but many other factors influence whether depressed patients commit suicide. Indeed, findings of suicide research are very like findings of depression research; most patients with suicidal behavior also have depression symptoms. For example, retrospective studies report that 50-80% of suicides are associated with depression^{17,18}.

Secondly, various inflammation hypotheses are also applied to other psychiatric disorders such as psychosis²¹⁶, obsessive-compulsive disorder²¹⁷ and anorexia nervosa²¹⁸. Inflammation or dysregulated inflammatory pathways may be factors contributing to a variety of psychiatric disorders and not linked to a specific set of symptoms, in the same way that smoking is a factor contributing to different diseases with different pathogenesis (not only lung cancer). The outcome (depression, suicidality or other psychiatric disorders) depends on other biological, psychological and environmental factors. This does not exclude the possibility that some inflammatory mechanisms might be linked to distinct symptoms; for example, we hypothesize that CD44 expression is more directly linked to vulnerability to developing anxiety specifically.

What is the clinical prospect of the inflammation hypothesis of depression?

It is natural to think anti-inflammatory treatment could show some positive effects in psychiatric patients with ongoing inflammation or dysregulated inflammatory pathways²¹⁹. On the other hand, inflammatory processes could be a contributing factor but not a successful treatment target. Smoking causes lung cancer but, when lung cancer is manifested, giving up smoking will not cure the cancer. However, if the onset of psychiatric illnesses is not linked to inflammatory processes, anti-inflammatory treatments could still have some positive effects, since inflammatory pathways interact so extensively with other systems in the brain.

Several clinical studies have investigated the effect of NSAID as an add-on treatment to conventional anti-depressants (e.g. fluoxetine and sertraline)²¹⁹⁻²²². In a double-blind study, Müller et al. used celecoxib as a six-week add-on to reboxetine treatment (n = 20) and the group showed significantly greater improvement compared to the reboxetine-alone group²²⁰. The kynurenine to tryptophan ratio (thought to be a measure of pro-inflammatory activity) predicted the response to celecoxib²¹⁹. However, there was little outcome difference between the groups. One study also reported significantly lower levels of IL-6 in the serum of the celecoxib group, and the levels of IL-6 predicted the anti-depressant response in both the placebo and celecoxib group²²². In the most recent study (n = 66), cimicoxib, when added to standard sertraline therapy, showed beneficial effects in a subgroup of 23 patients with severe depression²¹⁹. New biopharmaceutical drugs have also been examined. The TNF- α antagonist infliximab showed no generalized efficacy in treatment-resistant depression but was reported to improve depressive symptoms in patients with high baseline of inflammatory biomarkers²²³.

Even though large clinical trials are lacking, results in these pilot studies are rather promising. They also indicate that it is only in the subgroup of psychiatric patients who show signs of low-grade inflammation that an anti-inflammatory treatment might be beneficial. Therefore, when finding candidates for an anti-inflammatory treatment, it is important to distinguish between depressive patients with an ongoing inflammation and depressive patients without inflammation.

Main contributions of this thesis, future directions and concluding remarks

The results presented in this thesis show that dysregulation or absence of different inflammatory mechanisms might be involved in etiology of depression and suicidality. Some of the results contradicted our primary hypothesis, i.e. that increased inflammation causes the symptomatology. For example, we measured lower levels instead of increased levels of chemokines in the CSF of suicide attempters. Instead of being signs of inflammation, this could be the result of an inability of the brain to make appropriate adaptive responses to environmental stressful stimuli (stress sometimes precede suicidal behavior), but prospective studies are needed to confirm such a hypothesis. Many studies have examined the physiological roles of chemokines in the brain, such as in intercellular communication, cell proliferation and synaptic activity²²⁴. Consequently, it is difficult to link our results to a specific mechanism, thereby explaining its role on behavior, without further experiments. Hopefully, such studies will be made in the future

This thesis also examines inflammatory pathways that are important for cell adhesion and cell migration, i.e. CD44 and HA. Cell adhesion and migration have not been studied much in relation to depression and suicidality. However, the neuronal cell adhesion molecule (NCAM) has been linked with serotonergic and dopaminergic neurotransmission in rodents. NCAMKO mice show increased anxiety-like behavior and functional alterations in 5-HT receptors²²⁵ and recently it has been demonstrated that stress-induced changes in dopaminergic circuitry are mediated through NCAM²²⁶. We report that CD44KO mice develop more anxiety after exposure to CMS, and the increased anxiety was associated with changes in serotonin (5-HT) turnover in prefrontal cortex. Consequently, the absence of cell adhesion molecules in the brain might mediate a vulnerability to environmental stressors and it would be interesting to further investigate if cell adhesion molecules are linked to depression and suicidality *per se* or more directly to anxiety. As for chemokines, it is difficult to pinpoint a specific mechanism to explain how cell adhesion molecules might affect such complex concepts as behavior and monoamine transmission. We hypothesized that it might be associated with dysregulation of inflammation, BBB-permeability and neuroplasticity, but we did not perform any conclusive experiments to address the matter. Hopefully it will spur other studies looking at cell adhesions molecules as potential markers of vulnerability to stress.

Elevated levels of the CD44 ligand HA in the CSF of suicide attempters was associated with increased BBB-permeability, which may be another factor lowering resilience to stressors. These results further highlight the importance of CD44-HA interactions in suicidal behavior, but could also be interpreted as evidence of low-

grade inflammation in the brain of suicide attempters, since low-molecular weight HA (LMW-HA) is known to increase during inflammation²²⁷. This is supported by the finding that rats that experience a strong peripheral inflammatory response after LPS treatment also have increased levels of HA in the CSF. However, HA would not be a very sensitive or specific marker for suicidal behavior or depression, but LMW-HA has potential as a marker for low-grade inflammation in the brain or of increased BBB-permeability. Consequently, it could be a good marker of factors that might indirectly increase the risk of developing depression and suicidal behavior.

To conclude, immunoproteins that are involved in cell adhesion and cell migration, i.e. chemokines, CD44 and HA, might have important roles in the brain's capacity to manage stress. Dysregulation of these mechanisms may be implicated in suicidal behavior and could potentially be considered as a novel target for intervention in stress-related mental disorders such as depression and suicidality. Cell adhesion and migration are important inflammatory mechanisms during pathological conditions, but finding a single and specific mechanism explaining the behavioral changes could be found by investigating these immunoproteins roles in physiological conditions such as maintaining a trophic and functional environment in the CNS.

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