Decreasing Pro-Inflammatory Signalling as an Antidepressant Strategy in Pre-Clinical Models of Major Depressive Disorder

by

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I certify that this thesis does not incorporate without acknowledgement any material previously submitted for a degree or diploma at any university; and to the best of my knowledge and belief it does not contain any material previously published or written by another person except where due reference is made in the text.

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Signed

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I am grateful to all the people that made this possible.

To my parents, whose moral and economical support made this possible.

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TABLE OF CONTENT

Summary

Mounting evidence suggests an involvement of neuroinflammation in major depressive disorder (MDD). Psychological stress activates pro-inflammatory signalling by means of Nod-like receptors family pyrin domain containing 3 (NLRP3) inflammasome assembly and processing of pro-inflammatory cytokines, which exacerbate depressive-like symptoms.

To test the antidepressant efficacy of inhibiting pro-inflammatory signalling during stress exposure we used caspase 1 (*Casp1*) knockout $^{(-/-)}$ mice and mice simultaneously lacking *Casp1*, inducible nitric oxide synthase (*Nos2*) and interferon gamma receptor (*Ifngr*) genes in pre-clinical MDD paradigms. We assessed: a) baseline behaviour, b) the behavioural response to chronic stress, c) the levels of the circulating adrenocorticotropic hormone (ACTH) and corticosterone (CORT) following stress, in (*Casp1, Ifngr, Nos2*)$^{-/-}$ triple knockout mice, and d) gut microbiome composition in response to chronic stress and pharmacological CASP1 inhibition with minocycline.

We found that *Casp1*$^{-/-}$ mice and (*Casp1, Ifngr, Nos2*)$^{-/-}$ mice display decreased anxiety- and depressive-like behaviour. These two mouse strains also have increased locomotor activity and *Casp1*$^{-/-}$ mice has increased locomotor skills. Similarly, CASP1 inhibition with minocycline ameliorated stress-induced depressive-like behaviour in wild-type (wt) mice and shifted gut microbiota composition compared to stress treatment alone. We observed shifts in gut microbiome composition following stress with increased representation of bacterial species conducive to a pro-inflammatory environment, which were decreased following pharmacological CASP1 inhibition. Moreover, we observed that CASP1 inhibition affects the response to chronic stress by preventing the exacerbation of depressive- and anxiety-like behaviours. Lastly, we found that (*Casp1, Ifngr, Nos2*)$^{-/-}$mice display a decreased exacerbation of anhedonic-like behaviour after stress compared to wt mice. Interestingly, no differences were found in the level of circulating CORT and ACTH between (*Casp1, Ifngr, Nos2*)$^{-/-}$ and wt mice.

Together, these results point towards antidepressant-like effects of pro-inflammatory signalling inhibition in MDD, and suggest that at least some of these effects are mediated by changes in gut microbiome composition. Based on our findings and corroborated by the increasing evidence connecting psychological stress, immune activation, gut dysbiosis and co-morbid illnesses, we formulate the “microbiome-inflammasome” hypothesis of major depression and co-morbid systemic illnesses. This hypothesis speculates that NLRP3-

orchestrated processes represent a key node in a) stress-induced gut dysbiosis, b) dysbiosis-induced inflammatory dysregulation which lead to depressive symptoms, c) dysbiosis-induced dysregulation of neurotransmitter production, which exacerbates depressive symptoms, d) dysbiosis-induced immune activation, which leads to the development of co-morbid systemic conditions, and e) immune dysregulation resulting from systemic conditions leading to MDD and dysbiosis.

Future studies should aim at streamlining diagnostic strategies to identify MDD patients with dysregulated immune profiles, who could benefit from anti-inflammatory therapies. Finally, the clinical safety and efficacy of direct inhibition of pro-inflammatory pathways as well as their indirect inhibition by means of psychobiotics administration, faecal microbiota transplantation and diet should be further investigated in MDD patients. This could lead to the identification of novel, more efficacious, personalized therapeutic strategies in MDD.

Chapter 1 - Introduction and thesis overview

Major depressive disorder (MDD)

MDD is a mental illness that affects the way a person feels, thinks, and behaves by causing long-lasting feelings of sadness and loss of interest in activities that were previously enjoyed.[1] According to the 5th edition of the Diagnostic and Statistical Manual of Mental Disorders (DSM-5), patients diagnosed with MDD present an impaired ability to work, study or cope with daily activities accompanied by symptoms such as depressed mood, anhedonia, feelings of guilt or low self-worth, disturbed appetite, dysregulation of sleep patterns, fatigue, psychomotor alterations and poor concentration.[1] Global estimates suggest that about 350 million individuals of all ages suffer from MDD, making this condition one of the leading causes of disability worldwide.[2,3] In Australia, epidemiological estimates suggest that 1 in 5 women and 1 in 8 men will experience a depressive episode during their lives, with some three million people experiencing depression at any given time.[4] Further, suicide associated with MDD represents the second leading cause of death in individuals aged 15-29 worldwide (with an estimated 800,000 individuals who take their lives every year) and represents the first leading cause of death in individuals aged 15-24 in Australia.[2-4]

Treatment modalities in MDD

Several treatment approaches are available for MDD. Treatment selection for MDD patients is usually shaped around the symptom profile that a patient presents at treatment onset. The main modalities of MDD treatment are a) pharmacotherapy (such as treatment with monoamine reuptake inhibitors), b) psychological treatment (such as cognitive behavioural therapy), and c) physical treatment (such as transcranial magnetic stimulation and electroconvulsive therapy).[5] In mild depression, psychotherapy is advocated, since the outcomes of pharmacological treatments are considered poor.[6] In moderate to severe depression, often the first line of therapy revolves around pharmacotherapy, sometimes in combination with psychotherapy.[7] In treatment-resistant depression (when a patient does not achieve remission following two or more trials of antidepressant drugs[8]), cognitive behavioural therapy as an adjunctive therapy to antidepressants has been confirmed to be effective in a multicentre clinical trial.[9] Similarly, the efficacy of electroconvulsive therapy has been established in treatment-resistant depression,[10,11] while the efficacy of transcranial magnetic stimulation is still being debated.[12,13]

Pharmacotherapy in MDD

Antidepressant medications represent the core of MDD pharmacotherapy. Commercially available antidepressant drugs act via increasing the levels of available neurotransmitters at the synapses through disparate mechanisms. Several classes of antidepressants are available such as a) selective-serotonin-reuptake inhibitors (SSRIs) and b) serotonin-norepinephrine-reuptake inhibitors (SNRIs), both of which inhibit the reuptake of neurotransmitters at the synaptic cleft and therefore increase the extracellular concentration of such neurotransmitters,[14,15] c) monoamine oxidase inhibitors (MAOIs), which prevent the enzymatic degradation of monoamines[16] and d) tricyclic antidepressants (TCAs), which inhibit the synaptic reuptake of serotonin (5HT) and norepinephrine (NE).[17] Meta analyses of randomized controlled trials report antidepressant medications to be 20-30% more effective than placebo, presenting higher response rates in patients with greater symptom severity.[18,19] If a patient only responds partially after the first treatment trial: a) higher doses of the same antidepressant might be prescribed, b) the treatment can be switched from one antidepressant to another, c) a non-antidepressant drug can be added to the regimen (augmentation), or d) a second antidepressant can be added to the regimen (combination).[20] These approaches should ultimately increase the likelihood of full remission (the absence of depressive symptoms and the return to a one's normal self).

The serendipitous discovery of the "mood elevating" effects of the MAOI iproniazid (used in the treatment of tuberculosis) and those of the TCA Imipramine (used as an antihistaminic) led these drugs to represent the first class of antidepressants at the beginning of the 1950s.[21,22] The mood enhancing effects of iproniazid and imipramine, together with their inferred monoamine-enhancing effects and the mood depressing effects of drugs that depleted catecholamines led to the formulation of the monoamine hypothesis of depression. This theory suggests that low levels of monoamines such as 5HT and NE are responsible for the symptoms observed in MDD.[23-26] However, this theory, which has represented a pivotal point in shaping MDD pharmacotherapy, has since been considered controversial by some. This is because the monoamine hypothesis of depression fails to address some major issues, such as the delayed onset of action of antidepressant drugs, the fact that only a subset of depressed patients achieve remission following antidepressant treatment and the fact that reuptake inhibitors are only slightly better than placebo in double blind, placebo controlled studies.[26-28] Further, given the heterogeneity of the disease and the inter-individual variability of MDD symptoms, pharmacological treatments present high rate of failure and patients might need to trial several

antidepressant drugs to determine which is effective. Given the poor understanding of the molecular underpinnings of MDD, the low success rate of existing therapies (only 1 in 3 MDD patients achieves full remission after the first line of treatment) and the high incidence of side effects, the need emerges for a better understanding of the molecular causes of this disease. Such an understanding could lead to the development of more efficacious and personalised pharmacotherapies**.**

Selective-serotonin-reuptake inhibitors

The most common class of antidepressant drug prescribed to adults diagnosed with MDD is SSRIs. These medications (such as citalopram, escitalopram, fluoxetine, sertraline, paroxetine, viladozone and fluvoxamine) selectively act by interfering with the reuptake of 5HT mediated by the 5HT transporter after 5HT is released from the axon terminals and bind to 5HT receptors in the synaptic cleft.[14] Given this mechanism of action, the mood-enhancing effects of SSRIs are attributed to an increase in extracellular 5HT available at the synaptic cleft. If a patient fails to respond (or responds partially) to the initial doses of SSRIs, they are usually prescribed higher doses of SSRIs or add-on therapies, given that the recommended goal in clinical MDD is remission, which is having minimal or no depressive symptoms at all.[29] One major drawback of SSRIs therapy is the side effects profile which, although an improvement on older classes of antidepressant medications such as TCA and MAOIs, is still considered quite concerning given the current prevalence of prescription.[30] The most common side effects are related to the serotonergic system and are dose-dependent. They include but are not limited to gastrointestinal disturbances, weight gain, sexual dysfunction, headaches, anxiety, agitation and insomnia.[30] Patients who fail to respond to SSRIs are often prescribed SNRIs.

Serotonin-norepinephrine-reuptake inhibitors

SNRIs (such as duloxetine, venlafaxine, bupropion, desvenlafaxine, and levomilnacipran) were developed more recently than SSRIs and are often prescribed to patients who do not respond to SSRIs. The mechanism of action of SNRIs is the inhibition of the synaptic reuptake of 5HT and NE, which result in an increased extracellular availability of those neurotransmitters.[15] Some studies suggested that SNRIs might be more effective than SSRI in achieving MDD remission. However, there seems to be a high inter-individual response variability to these medications, possibly because of pharmacogenomics differences amongst the population.[31] The most common side effects experienced by patients prescribed SNRIs are nausea, drowsiness, headaches, appetite changes and diminished libido.[32,33] Importantly, *in-utero* exposure to the SNRI venlafaxine has been

shown to increase cardiac anomalies in the offspring, suggesting that this drug should not be prescribed during pregnancy.[34] Finally, discontinuation syndrome has been reported to be worse for SNRIs than SSRIs.[32]

Tryciclic antidepressant and monoamine oxidase inhibitors

TCAs (such as imipramine, desipramine, clomipramine, amoxapine, amitriptyline and nortriptyline) are one of the earliest classes of antidepressants discovered. Although TCAs are effective in ameliorating depressive symptoms, they also (unlike SSRIs and SNRIs) interact with histaminic, muscarinic and alpha 1 postsynaptic receptors, actions considered to underlie at least some of their most substantial side effects, such as constipation, dry mouth, drowsiness, liquid retention, weight gain, and sexual dysfunction.[35,36] Therefore, they have largely been replaced by other classes of antidepressants with fewer side effects. Like SNRIs, TCAs act by inhibiting the reuptake of 5HT and NE and therefore increase the bioavailability of these neurotransmitters at the synapses.[17] While the clinical efficacy of TCAs can be compared to that of SSRIs, the dropout rates are higher for TCAs, possibly because of the greater side effects that they present.[17] Accordingly, TCAs are often only used in treatment resistant depression, when other lines of pharmacotherapy have proved ineffective.

MAOIs (such as tranylcypromine, rasagiline, selegiline, and phenelzine) are compounds that hinder the degradation of monoamines by the endogenous MAO family of enzymes. Such compounds act by decreasing the degradation rate of neurotransmitters, which increases their bioavailability.[16] MAOIs were the first class of antidepressants to be serendipitously discovered in the 1950's when iproniazid (used for the treatment of tuberculosis) showed mood enhancing effects.[37] Much like TCAs, MAOIs are now only prescribed for treatment-resistant patients if other lines of pharmacotherapy (such as SSRIs and SNRIs) have failed.

Ketamine

Over the past two decades the sedative drug ketamine, which acts as a glutamate receptor antagonist, has gained increasing attention for its rapid and sustained antidepressant effects.[38] In fact intravenous administration of low-dose ketamine (0.5mg/kg) has been shown to promptly reduce depressive symptoms, hopelessness and suicidality in MDD patients.[39] These fast-onset antidepressant effects represent a striking contrast when compared to other classes of approved antidepressant drugs which have a delayed onset of several days up to several weeks.[40] Low doses of ketamine seem to be

well-tolerated although a minority of patients present short-term mild-to-moderate dissociative symptoms and increased heart rate and blood pressure, which resolve within 4 hours of administration.[39] It has been suggested that the acute antidepressant effects of ketamine might be mediated by its neuroplasticity-enhancing properties.[38] However, no study has so far investigated the safety of long-term ketamine administration nor the physical, psychiatric or neurological side effects thereof. Therefore, particular care should be taken until more evidence is available. Future larger size double blind, placebo-controlled studies should address these aspects to guide informed policy decisions.

The potential of anti-inflammatory medications in the treatment of MDD

Recently, the potential of non-steroidal anti-inflammatory drugs has come to the forefront of MDD pharmacotherapy. In fact, studies have reported increased activation of pro-inflammatory pathways in at least a subset of patients diagnosed with MDD.[41-43] This suggests that targeting pro-inflammatory mediators could prove useful as a stand-alone or adjunctive therapy for those MDD patients that present altered inflammatory profiles. Unsurprisingly, it has been reported that only MDD patients with increased levels of immune mediators benefit from anti-inflammatory therapies, while others patients do not.[42,44]

While a body of work exists describing the dysregulation of immune processes in MDD, there is a paucity of translational studies investigating whether targeting pro-inflammatory mediators could represent a valid therapeutic approach. Therefore, in the first part of this investigation (study 1), we tested the effects of genetic ablation or pharmacological inhibition of the pro-inflammatory mediator CASP1. In the second part of this investigation (study 2), we investigated the antidepressant-like effects of simultaneously deleting the genes *Casp1*, *Nos2* and *Ifngr* in a pre-clinical model of MDD.

Disease hypotheses

Based on the available pre-clinical, clinical, molecular and behavioural evidence accumulated over the past several decades, multiple hypotheses have been developed in order to address the pathophysiology of MDD. Five main hypotheses have been formulated: a) the monoamine-serotonin hypothesis,[45,46] b) the glutamate hypothesis,[47,48] c) the corticosteroid receptor hypothesis,[49,50] d) the brain derived neurotrophic factor (BDNF) hypothesis,[51,52] and e) the macrophage (or cytokine) hypothesis.[53-59] However, despite the attempts of researchers to shed light on the dysfunctions of specific biological systems that lead to MDD, this disorder seems to arise from a complex interplay of

genetics and environmental factors: these theories are therefore not necessarily mutually exclusive.[60,61] In this work, we have focussed on the macrophage (or cytokine) hypothesis of MDD, and our results corroborate this hypothesis. Given the available evidence to date, corroborated by the findings of our study, we have formulated a novel hypothesis of depression, the "microbiome-inflammasome" hypothesis of major depression and co-morbid systemic dysfunctions (see chapter 6). This hypothesis takes into account the multidirectional communication pathways linking the brain, the immune system and the gut microbiome in responding to psychological stress. When those pathways become dysregulated, they can increase the risk of developing MDD and co-morbid systemic illnesses.

Innate and acquired immunity

The work presented here has been shaped around the increasing pre-clinical and clinical evidence suggesting an involvement of neuroinflammatory pathways in the response to psychological stress[62,63] and in the development of MDD[58,64] and co-morbid systemic illnesses.[46,65-70] Generally, the immune system is considered to have 2 "arms", the innate (non-specific) and the adaptive (acquired) arms. Innate immunity indicates non-specific mechanisms, which represent the first line of defense against pathogens and come into play immediately or within a few hours of an antigen being recognized by the body. Macrophages and neutrophils are part of the innate arm of the immune system and are essential for the control of bacterial infections. Activated macrophages secrete cytokines (small proteins released by immune cells which affect the communication and interaction between cells[71]) such as interleukins and interferons, and chemokines (cytokines with chemotactic properties[72]) such as intercellular adhesion molecules and macrophage inflammatory proteins. These molecules create an inflammatory state with the final aim of fighting the invading pathogen and returning the system to homeostasis.[73]

Stimulation of the innate immune response subsequently triggers the activation of the adaptive immune response, which has a delayed activation compared to the former (4-7 days) and it is characterized by lymphocyte-mediated processes. Lymphocytes can be classified into B cells, which secrete antibodies, and T cells [either 'helper' (Th), which secrete cytokines, attract macrophages and activate B cells, or 'cytotoxic', which actively kill the invading pathogens]. The adaptive response begins with the ingestion of a pathogen by an immature dendritic cell within the inflamed tissue. Subsequently, the cell digests the pathogen and undergoes specific changes becoming a mature antigen-presenting cell. Subsequently, antigen-presenting cells travel to a nearby lymph node to

present the antigens to T lymphocytes which in turn become activated and fight the invading pathogen.[73]

Psychoneuroimmune interactions and the cytokine hypothesis of depression

Psychoneuroimmunology is the study of the reciprocal interactions between behavioural traits and the immune system, which are mediated by the nervous and endocrine systems.[273] In MDD, increasing evidence suggests that the communication networks existing between the nervous, immune and endocrine systems lie at the crossroads of psychosocial stress, onset of depressive symptomatology and antidepressant response.[274] Numerous studies suggest anti-inflammatory and endocrine-modulating effects of antidepressants, antidepressant effects of anti-inflammatory medications and differential responses to antidepressants driven by polymorphisms in inflammation-related genes.[196,275-277] With regard to the immune players of such communication, cytokines have gained increasing attention over the past 20 years. Cytokines are pleiotropic signalling molecules with immunomodulatory function that are expressed constitutively and on-demand in the periphery as well as in the CNS, and have been associated in at least a subset of patients with onset, course and severity of neuropsychiatric disorders, as well as with the response to therapeutic drugs.[42,58,278-285]

Exposure to psychological stressors primes the immune system towards the creation of a pro-inflammatory environment in the brain, a phenomena called *sterile inflammation* which prepares the CNS and the body to trigger a potential full-blown immune response.[286,287] Two main pro-inflammatory gene expression programs delineate such response: 1) the first involves the expression of genes (such as IL1B, IL6 and TNF) that result in the activation of transcription factors entailing NFKB1 and activator protein-1; 2) the second is characterized by the induction of transcription factors such as IFN regulatory factors (IRFs) by type I and II IFNs.[288] While on one hand these programs are essential for the response to the stressor and for the restoration of homeostasis, on the other hand they require high amounts of energy and have the potential for collateral damage. In fact, repeated or chronic exposure to stress results in a sustained inflammatory milieu in the brain which can be deleterious and lead to the development of MDD and co-morbid systemic illnesses.[193,289]

These lines of evidence have led to the formulation of the “cytokine hypothesis” (or “macrophage hypothesis”) of depression, which suggests that cytokines and an out-of-balance brain-immune communication represent key factors underpinning the

pathophysiology of MDD.[53,74,206,290,291] The cytokine hypothesis is supported by mounting evidence, such as: a) illnesses characterized by chronic inflammatory responses (i.e. type-1 diabetes and systemic lupus erythematous) are associated with increased depression rates,[211,213] b) administration of pro-inflammatory cytokines as a therapeutic strategy (i.e. IFNA administration in cancer and hepatitis-C) induces a dose-response depressive symptomatology entailing depressed mood, malaise and anorexia, as well as some of the underlying molecular features of MDD, such as decreased monoamines levels,[67,292-295] c) administration of pro-inflammatory cytokines in humans and animals induces sickness or depressive-like behaviour. The latter represents a physiological adjustment to the activation of the immune system, encompassing a behavioural repertoire adopted following a raise in the levels of pro-inflammatory cytokines. The symptoms include low motivation, fatigue, malaise, loss of interest in social activities, inability to seek and experience pleasure, exaggerated pain responses, lack of concentration and sleep pattern alterations, manifestations that closely resembles the clinical symptomatology of MDD.[54,243,249] Lastly, polymorphisms in inflammation-related genes have been associated with increased susceptibility to MDD and with differences in antidepressant response.[59] These lines of evidence suggest that neuroinflammatory pathways are involved in the onset of depressive symptoms as well as in the response to antidepressant treatment, and they provide fertile ground in which to investigate novel diagnostic and therapeutic opportunities in the field of neuro-immuno-psychiatry.[54,56-59,64,193,196,280,291,296,297]

Major depression and dysregulated inflammatory pathways

Psychoneuroimmunology research has highlighted that at least a subgroup of MDD patients presents a systemic low-grade chronic inflammatory profile underlined by increased T-cell, monocytic, microglial and astrocytic activation.[64,193,296,298,299] This is underlined by increased expression of Th1 pro-inflammatory cytokines such as IL1, IL2, IL6, TNF and IFNG, and in decreased expression of Th2-related anti-inflammatory cytokines, such as IL4 and IL10 as well as in decreased expression of regulatory T cells.[41,80,82,83,290,300-303] Such skewed inflammatory balance triggers multiple dysfunctions in the body, such as changes in metabolic processes, neurotransmitter systems, gut microbiome composition and decreased neurogenesis leading to hippocampal atrophy, processes relevant to MDD.[133,296,304] Accordingly, volumetric decreases are observed in the hippocampus and in other forebrain regions in MDD patients and can be reversed by antidepressant treatment; these findings support the neurotrophic hypothesis of depression. The latter suggests that MDD is underlined by decrements in neurotrophic

factors and neurogenesis, potentially as a result of increased systemic inflammation, which leads to atrophy of specific brain areas.[167,305-307] In fact, pro-inflammatory cytokines and increased glucocorticoids production down-regulate neurotrophins (such as BDNF and nerve-growth factor) and neurogenesis of human hippocampal progenitor cells during and following stress, while antidepressants reverse such decreases in humans and in pre-clinical models of depression.[308,309]

Minocycline for the treatment of MDD

Minocycline is a second-generation tetracycline which in an earlier case report was described to have antidepressant effects on a bipolar disorder patient.[90] Subsequently, minocycline was shown to ameliorate disease progression in a mouse model of Huntington disease via decreasing pro-inflammatory signalling through inhibition of CASP1, CASP3 and NOS2.[91] Given its reported immunomodulatory properties it was hypothesized that minocycline might have antidepressant effects depending on its anti-inflammatory properties. Indeed, pre-clinical studies showed that minocycline has antidepressant-like effects in the forced swim test, while synergizing with sub-threshold doses of antidepressants and glutamate antagonists.[92,93] Further studies in rodent models of chronic stress suggested that the antidepressant-like effects of minocycline might be mediated by its negative modulatory effects on microglial activation.[94] Other studies have suggested that minocycline might interact with the glutamatergic and/or noradrenergic systems; therefore, it cannot be excluded that such mechanisms may also be involved in its antidepressant effects.[95-97] A recent clinical study has investigated the effects of minocycline as an adjunctive treatment in MDD patients. Although no differences were found in the Montgomery-Asberg depression scores (the primary outcome measure in that study), minocycline-treated patients reported improved quality of life and improved social and occupational functioning (secondary outcome measures).[98] These seemingly contradicting results suggest that larger sample sizes might have been needed to observe statistically significant changes in depression scores. Another clinical trial has investigated the effects of minocycline in the treatment of bipolar disorder. In that study, the authors observed a reduction in the severity of depressive symptoms as well as improvements in cognitive functions.[99] Other clinical studies have investigated minocycline as a stand-alone or adjunctive treatment in psychotic depression and schizophrenia, yielding promising results.[100-103]

In study 1, we treated wt mice with minocycline to assess if such treatment could prevent the exacerbation of anxiety- and depressive-like behaviour following exposure to a chronic

stress regimen. Moreover, we sought to investigate the effects of minocycline administration during stress exposure on gut microbiome composition, a mechanism that we hypothesized to be involved in its antidepressant-like effects.

The hypothalamic-pituitary-adrenal (HPA) axis

The three main response systems activated in response to stressful stimuli are the sympathetic nervous system (SNS), the hypothalamic-pituitary-adrenal (HPA) axis and the locus coeruleus-norepinephrine (LC-NE) system. After stress is perceived by the amygdala, the hypothalamus activates the SNS, which triggers the production of epinephrine and NE in the LC (in the brain) and the adrenal medulla (in the blood). Upon release, these neurotransmitters upregulate pro-inflammatory signalling in virtually every organ. Subsequently the HPA axis is activated and corticotropin releasing hormone (CRH) and arginine-vasopressin (AVP) are secreted in the hypothalamus. These hormones cause the release of ACTH from the pituitary gland, which in turn stimulates the release of glucocorticoids from the adrenal glands. Finally, glucocorticoids interact with the glucocorticoid receptor (NR3C1) and the mineralocorticoid receptor (NR3C2) in multiple tissues to activate intracellular signalling cascades which lead to anti-inflammatory gene regulation.[104,105]

Interestingly, NR3C1 is highly expressed in the hippocampus, highlighting the role of this brain region in the stress response.[106] Glucocorticoid receptors are also present within individual components of the HPA axis, where they sustain an inhibitory feedback loop on CRH and AVP in the hypothalamus and on ACTH secretion from the pituitary.[104,106] HPA axis-produced glucocorticoids regulate many bodily functions such as stress-related responses, metabolism, immunity and brain function. Specifically, in the brain, glucocorticoids are involved in the regulation of neuronal survival, neurogenesis, memory formation and emotion regulation.[104]

Given its role as a key player in the stress response and as a stress mediator, it is unsurprising that the HPA axis presents abnormalities in MDD. In fact, a subset of MDD patients have a) increased levels of circulating cortisol,[107] b) decreased levels of glucocorticoid receptors,[108] and c) possible increased activity and size of the pituitary and adrenal glands.[109] These lines of evidence highlight an over-activity of the HPA axis accompanied by impaired inhibitory feedback and glucocorticoid resistance.[110] Therefore, the HPA axis seems one of the key systems dysregulated in MDD, and therapeutic targeting of this stress-response system could represent a valid therapeutic strategy in

MDD.[108,110,111] At the same time, it has been hypothesized that hyperactivity of the HPA axis mediated by genetics and early life experiences could be a causative factor rather than a consequence in MDD.[112] In fact, while glucocorticoids are essential in homeostasis and stress responses, they can also lead to dysfunctions in many bodily systems.[104] Of central importance to this work, it was recently shown that increased CASP1 activity is responsible for cleaving NR3C1, thus facilitating glucocorticoid resistance, which could at least partially contribute to the decreased level of glucocorticoid receptors and increased glucocorticoid resistance observed in MDD patients with heightened inflammatory profiles.[111,113] Taken together, the available evidence suggests that regulating HPA axis activity could represent a valuable therapeutic tool in MDD.

The microbiome-gut-brain (MGB) axis

Recently, the role of the gut microbiome in behaviour, its interconnectedness with brain processes, and its potential involvement in the pathophysiology of MDD have come to the forefront in psychiatry.[114-116] The term microbiome refers to all bacteria, bacterial genomes and bacterial metabolites and byproducts present in a specific habitat at any given point.[117] Its phylogenetic composition is determined by both selective pressure form the host and microbial competition.[118]

The microbiome-gut-brain (MGB) axis consists of a communication network that controls and integrates gut and brain function, and that seems to be a central modulator of health and disease.[119] More specifically, there seems to exist a bidirectional communication between the gut and the brain, which occurs through multiple intertwined pathways, mediated by the vagus nerve,[120] the immune system,[121,122] and the bacterial metabolome (the ensemble of bacterial metabolic by-products and end products).[123,124]

The contribution of the gut microbiome in affecting host behaviour is suggested by the high comorbidity rates between psychiatric illnesses and gastroinstestinal ailments.[114,125] For example, irritable bowel syndrome (IBS) patients present high rates of mood disorders, and antidepressant drugs represent one of the most common pharmaceutical approaches in IBS.[126,127] Moreover, at least some irritable bowel disease patients undergoing faecal microbiota transplantation (FMT) report improved mood following treatment, suggesting that such procedure might prove useful in MDD treatment.[128]

Recently, there has been increasing research trying to determine whether the gut microbiome plays a causal role in MDD onset, course and remission. Several studies have investigated the phylogenetic composition of the gut microbiota of MDD patients compared

to healthy controls. Although the available studies to date present variability between cohorts, it seems that gut microbiome composition is altered in MDD patients compared to controls, and that those changes might be sex-dependent.[129]

In a pioneer study, Naseribafrouei and colleagues found that microbiota composition can predict whether an individual is currently depressed, suggesting that microbiome screening could be helpful in the diagnostic process.[130] In that study, the levels of *Lachnospiracea* and *Bacteroidetes* were decreased in MDD patients, while *Alistipes and Oscillibacter* were increased. Interestingly, low levels of *Bacteroidetes* have been associated with chronic low-grade inflammation and obesity, suggesting that the low levels observed in MDD patients might be involved in the high levels of MDD-obesity co-morbidity.[43,131,132]

A study in a Chinese cohort found increased Proteobacteria and *Bacteroidetes* (driven by increased *Parabacteroides* and *Alistipes* abundance) levels in MDD patients, while the abundance of Firmicutes, *Lachnospiraceae* and *Faecalibacterium,* was reduced.[133] Some Proteobacteria have been shown to trigger depressive symptoms secondary to NLRP3 inflammasome activation. Therefore their increased abundance in MDD patients might be involved with the immune dysregulations observed in MDD.[134,135] *Bacteroidetes* convert tryptophan to indole; therefore, their increased levels in MDD patients could be connected to the serotonergic deficiencies observed in MDD.[136] *Lachnospiraceae* levels were also decreased in MDD cohort, and this genus is a key producer of the anti-inflammatory short-chain fatty acids (SCFAs), which are involved in intestinal barrier integrity.[137-140]

Another Chinese cohort displayed alterations in Bacteroidetes (which were decreased in MDD), Actinobacteria (which were increased in MDD) and Firmicutes (some increased, others decreased in MDD).[123] In that study, the author performed FMT from MDD patients to germ-free mice, which was sufficient to instate depressive-like behaviour in the receiving mice.[123] This result suggests that behaviours associated with specific enterotypes might be transmissible via the gut microbiota. Another study reported decreased bacterial diversity and increased inflammatory markers in MDD patients.[141] Following human-to-mouse FMT, increased kineurine levels and increased plasma kyneurine/tryptophan ratio (underlying features of MDD which contribute to serotonergic imbalance) were observed in the mice receiving faecal material from MDD patients.[141]

Those studies suggest that MDD is associated with altered gut microbiome composition, and that the latter may play a causal role in MDD onset. However, some of these studies present contrasting findings, possibly because gut microbiome composition is affected by

ethnicity, diet, and medication status. Therefore, there is a need for studies on larger cohorts that take such variables into consideration.

Supporting a role for the microbiome in shaping behaviour, specific bacterial strains termed “psychobiotics” have been shown to elicit positive effects on mood, if ingested in the right amount.[142] Patients dosed with psychobiotics have reported decreased depression and anxiety scores and increased overall quality of life.[143] Moreover, biochemical studies have reported that psychobiotics decrease the levels of pro-inflammatory markers,[144] while affecting region-specific patterns of brain activity.[145,146]

Together, the available evidence to date, suggests that the gut microbiome is dysregulated in MDD and might play a causal role in MDD onset. Therefore, a clearer understanding of the contribution of the gut microbiome in MDD seems crucial, and could lead to the development of novel therapeutic strategies aiming at modulating gut microbiome composition via dietary interventions, psychobiotic supplementation and FMT.

Mouse strains used

Casp1 knockout mouse model

Given the increasing evidence of an involvement of neuroinflammatory pathways and more specifically of the NLRP3 inflammasome and IL1B in the response to psychological stressors and MDD onset, in the first study we used *Casp1*$^{-/-}$ mice to investigate the behavioural and biochemical effects of lacking *Casp1* on innate behaviour and on the response to chronic restraint stress (CRS). *Casp1*$^{-/-}$ mice have been shown to be overtly normal despite having very low levels of IL1A and undetectable levels of IL1B.[147] Interestingly, they have similar pro-inflammatory and behavioural responses to systemic lipopolysaccharide (LPS) administration compared to wt mice but they are resistant to the exacerbation of depressive-like behaviour and to the spike of pro-inflammatory cytokines in response to intracerebroventricular LPS administration.[148] Moreover, they display increased survival and decreased inflammation-induced brain transcription in response to the administration of lethal endotoxin doses compared to wt mice.[147,149] Furthermore, mice with impaired IL1 signalling are resistant to the exacerbation of depressive-like behaviour and to the decrease in neurogenesis elicited by stress exposure.[150,151]

Given their decreased inflammatory profiles and the fact that impairment of IL1B signalling decreases the deleterious effects of stress, we hypothesized that *Casp1*$^{-/-}$ mice might have decreased depressive-and anxiety-like behaviour and be resistant to the exacerbation of

anxiety-and depressive-like behaviour following chronic stress exposure. Of note, following the generation of this transgenic mouse model, further investigations unveiled that this is a double knockout mouse model. These mice in fact lack the *Casp1* and *Casp11* genes, because *Casp1* and *Casp11* are neighbouring on the genome and too close to segregate by recombination.[147,152,153] The results obtained involving this model in study 1 should therefore be interpreted in light of this.

(Casp1, Ifngr, Nos2)$^{-/-}$ triple knockout mouse model

In the second study we aimed to determine if the simultaneous ablation of multiple pro-inflammatory cytokines would affect baseline behaviour and the behavioural response to a chronic stressor, as well as affect the circulating levels of the stress hormones ACTH and CORT following stress exposure. To do so, we used mice lacking CASP1, NOS2 and INFGR [(*Casp1, Ifngr, Nos2*)$^{-/-}$] and investigated their behavioural phenotype at baseline and following chronic stress exposure. Studies investigating mouse models with genetic deletion of each of these cytokines have been reported, but to the best of our knowledge this is the first study to investigate the effects of multiple pro-inflammatory gene deletion on behaviour and on the response to chronic stress.

Ifng$^{-/-}$ mice have been previously reported not to display developmental defects but to present increased susceptibility to bacterial and viral infections.[154,155] Moreover, *Ifng*$^{-/-}$ mice present decreased depressive- and anxiety-like behaviour and heightened emotionality. These behaviours are coupled with increased noradrenergic and serotonergic activity, increased baseline CORT levels, decreased hippocampal neurogenesis and decreased nerve growth factor in the prefrontal cortex.[156,157] Furthermore, *Ifng*$^{-/-}$ mice have been shown not to be resistant to chronic stress but to present altered changes in monoamines, CORT and cytokine levels in response to stress compared to wt.[157]

Similarly, genetic deficiency and pharmacological inhibition of NOS2 has been shown to decrease depressive-like behaviour, suggesting that NOS2-produced nitric oxide is involved in the modulation of depressive-like behaviour in mice.[158] Therefore, given the observed antidepressant-like phenotypes of the single KO mouse models, we hypothesized that the simultaneous deletion of *Casp1, Ifngr and Nos2* might have an additive effect and result in greater antidepressant-like effects compared to the individual KOs.

Pre-clinical paradigms of MDD used

While it is accepted that certain features of MDD are purely human, such as suicidality, guilt and sadness, other aspects of the depressive symptomatology such as anhedonia, despair, and sleep and appetite alterations can be reproduced in laboratory animals, and some of these symptoms can be ameliorated with clinically used antidepressant drugs. Pre-clinical research on MDD relies on “animal models of depression”, research tools used to study MDD and antidepressant action by simulating MDD physiopathology and symptomatology. These paradigms usually involve chronic stress procedures (as opposed to acute stress paradigms) that exacerbate anxiety- and depressive-like behaviours.[159] Such procedures mimic at least some of the underlying pathophysiological changes of human MDD, such as a) neuroendocrine, b) immune, c) autonomic and cardiovascular and d) central neurotransmitter alterations.[160] Usually, the changes triggered in chronically stressed animals include: a) behavioural despair, b) anxiety-like behaviour, c) anhedonic-like behaviour, d) changes in appetite or metabolism, e) neuroanatomical changes and f) alterations of the sleep cycle.[160,161] Generally, to be considered a valid pre-clinical MDD model, a paradigm should possess good face validity (have phenomenological and pathophysiological similarities to MDD), construct validity (have similar aetiology to MDD), and predictive validity (being responsive to common treatments used in MDD).[162,163]

One of the challenges to meet in modelling MDD in animals is to produce long-lasting depressive-like states that resemble MDD symptomatology. Pre-clinical MDD research relies on procedures that involve presenting the animals with stressors known to simulate MDD risk factors, such as the exposure to repeated predictable, unpredictable and uncontrollable stressors. These procedures are coupled to tests to quantify depressive- and anxiety-like behaviours which assess the efficacy of the stress paradigm.[159] Chronic stress models rely on physical, psychosocial or early life stressors. The most used paradigms in MDD research are a) CRS, involving placing the mice in restrainers which limit their ability to move freely, b) chronic unpredictable mild stress (CUMS), in which the mice are presented with a series of mild stressors in a randomized and unpredictable fashion, c) early maternal separation stress, involving separating the newborn mice from the dam for a set amount of time at a specific postnatal stage, and d) social defeat stress, involving introducing an intruder mouse in the cage of the resident mouse for a set amount of time over prolonged periods of time.[164-166] While chronic stress models have good face validity, meaning that they closely resemble MDD phenomenology and pathophysiology, they have the drawback of being low-throughput. In fact, implementing chronic stress

paradigms requires greater sample size and more physical space than acute stress paradigms, resulting in higher costs. Moreover, the genetic and epigenetic inter-individual biological variability involved in stress susceptibility and resilience phenotypes needs to be taken into account when designing experiment. Therefore, often relatively large sample size is needed to detect statistically significant differences amongst the groups studied.[161,167]

To induce depressive-like symptoms we used CRS in study 1, which involved *Casp1*$^{-/-}$ and minocycline-treated mice (chapter 4), and CUMS in the study 2, which involved (*Casp1*, *Ifngr, Nos2*)$^{-/-}$ mice (chapter 5). CRS is a paradigm in which rodents are immobilized in restrainers, devices which physically limit their ability to move. CRS is considered a paradigm to model MDD since it increases behavioural despair and anxiety-like behaviour, symptoms which resemble core features of clinical depression.[168,169] This paradigm has good face validity since it increases anxiety- and depressive-like behaviours and results in morphological changes in brain areas involved in fear and anxiety responses in humans.[169-171] Moreover, CRS: a) increases the level of circulating stress hormones, b) downregulates NR3C1expression, and c) attenuates the glutamate-induced release of brain-derived neurotrophic factor in the prefrontal cortex, one of the brain regions relevant to depression.[168] Furthermore, CRS impairs hippocampal neurogenesis in mice,[172] a phenomenon which is considered to mirror the clinical course of depression.[173] Nevertheless, CRS is considered to have good predictive validity, since antidepressant drugs prevent and reverse the exacerbation of CRS-induced depressive-like behaviour.[174] In study 1, we applied CRS for 4-6h day for 21 days.

In study 1, when CRS was performed, we did not observe a decrease in sucrose preference in the sucrose preference test following stress, a test designed to assess the extent of anhedonic-like behaviour (the inability to seek or experience pleasure, see "behavioural experiments" section below). Anhedonia is considered one of the main dimensions of MDD, and it is considered fairly important to reproduce this symptom when modelling MDD in pre-clinical models.[175] We therefore decided to use the CUMS paradigm for study 2, because this paradigm has been shown to decrease sucrose preference more reliably than CRS. The CUMS is a paradigm that has been used to model depressive-like states in rodents for over 20 years.[176,177] The strength of the CUMS paradigm lies in its: a) face validity, since CUMS elicits depressive-like symptoms and the neuroplasticity losses associated with stress exposure in humans, b) predictive validity, since most antidepressant drugs reverse CUMS-induced behaviours, and c) construct validity, since

the behavioural and biological response to CUMS in animals resemble those responses in humans.[163,164] This paradigm consists of chronically exposing the mice to multiple mild social and environmental stressors, which are randomly scheduled, and applied every day for a set period of time (between 4 to 10 weeks).[164,176,178-180] Examples of such stressors include cage tilting, intruder stress, light cycle reversal, fasting, removal and/or soiling of bedding. Usually the stressors schedule is randomized (or semi-randomized) to maximise the unpredictability of the regimen and to prevent habituation, one of the drawbacks of the exposure to homotypic chronic stress paradigms.[181]

Therefore, in study 2 we applied CUMS for 28 days, presenting mice to various randomly scheduled, low intensity social and environmental stressors, applied each day during the light phase of the light cycle (except for the light cycle reversal stress, applied during the weekend). Depending on the duration of the stressor, one (if it lasted more than two hours) or two (if it lasted two hours or less) stressors were applied each day. The schedule was randomized weekly to maximise the degree of unpredictability and to avoid habituation, which is one of the drawbacks in modelling depression in rodents.[164] The stressors were: a) two hours restraint in polypropylene restrainers on an open bench, b) eight hours removal of bedding and nesting material, c) eight hours of soiled bedding, obtained by adding 200 mL of autoclaved water to 100 g of bedding, d) eight hours of 45º cage tilting obtained by introducing a Plexiglas “tilter” inside the cage to allow the cage to be returned to the individually ventilated cage rack, e) two hours of predator stress, obtained by introducing in the cage a 5 mL test tube modified with ten 2 mm holes containing two fresh rat faecal pellets, f) five minutes forced swim test, performed once at the beginning and once at the end of the stress period (representing both a stressor and a behavioural test), g) sixteen hours of overnight fasting in clean cages, h) two hours of social stress, consisting in pair housing two mice from different litters in a neutral cage, i) two hours of light cycle disruption during the light phase, and j) forty-eight hours of light cycle reversal over the weekends. Mice were tested weekly in the sucrose preference test to monitor changes in their preference for a sucrose solution, considered an index of anhedonic-like behaviour.[179] This parameter, together with the floating time in the forced swim test, was used to assess the effectiveness of the CUMS procedure and to determine when anhedonic and depressive-like behaviour had been induced.

Diagnostics and therapeutic implications

The diagnostic process in MDD lacks biomarkers to assist with the identification of potentially affected individuals.[182] Currently, MDD diagnosis relies on a combination of

interviews, self-reported questionnaires and checklists based on the DSM-5.[183] However, the validity and objectivity of this routine assessment system is strongly debated, since it seems to hinder the development of personalized therapeutic plans.[184-186] As highlighted by researchers and psychiatrists, this is a major shortcoming for the credibility of psychiatry. The search for reliable biomarkers, together with cognitive, imaging and genetic measures in the diagnosis of MDD is therefore extremely important.[187-189]

Given the multilayered contribution of biological mediators and bodily systems in MDD pathophysiology, a panel aiming at profiling a number of diagnostic and predictive biomarkers from a biological sample (such as plasma or saliva) could be more viable than a single-biomarker approach in MDD diagnosis.[182,190-194] Ideally, a panel of diagnostic and prognostic biomarkers should provide in-depth information about the inflammatory profile of the patient. This could help identify individuals that might benefit from anti-inflammatory approaches as a stand-alone therapy or in combination with standard antidepressant treatment. Further, clarifying the correlation between subtypes of MDD and inflammatory profiles could help in identifying MDD subtypes via biological testing. This could lead to enhanced diagnostic power and to the fine-tailoring of individualized treatments.[187]

If some MDD patients could benefit from anti-inflammatory medications as an antidepressant therapy, it is vital to identify such patients via a sound diagnostic screening able to predict a positive therapeutic outcome. It has been previously suggested that cytokine profiles could be useful in diagnosing MDD and to predict response patterns to specific antidepressants. However, the fact that the levels of these cytokines are increased in other diseases makes them less-than-unequivocal candidate biomarkers in MDD. However, these molecules could still be quantified as part of the diagnostic process. Preliminary studies yielded contrasting results. In a small-scale study investigating cytokine profiles in young women diagnosed with different MDD subtypes, the authors found no differences in cytokine levels between healthy controls and MDD patients, and cytokine levels were increased following antidepressant treatment.[195] However, meta-analyses suggest that the levels of IL1, IL6 and C-reactive protein (CRP) decrease in response to antidepressant treatment.[196,197] These inconsistent findings suggest that the understanding of cytokine trends in MDD course and remission is still far from complete, and that larger scale studies are needed to delineate such profiles and concretize their translational value.

Some researchers have suggested that IL6 could represent a valid marker for MDD, since its levels are relatively uniformly increased in MDD and are relatively stable over extended periods of time. Therefore, the one-off measurement of levels of this cytokine could represent a useful diagnostic marker in MDD, which could be added to the psychological assessment of depressive symptoms.[198] Similarly, high levels of positive acute phase proteins, such as CRP and high-sensitivity CRP seem to be one of the most reproducible findings in MDD.[76,199,200]

However, some researchers have emphasized that immune processes are dysregulated only in a subset of MDD patients, suggesting that only patients that present an immune activation could benefit from anti-inflammatory therapies.[42,201] [42,75,202-208] A recent clinical study proved this concept. The authors administered for 12 weeks either the TNF inhibitor *infliximab* or placebo to treatment-resistant depression patients.[44] The authors hypothesized that high circulating level of high-sensitivity CRP and TNF before treatment might be predictive of effective treatment response to *infliximab*.[44] The results showed that only patients with heightened inflammatory profiles before treatment benefitted from the anti-inflammatory therapy, while patients with low pre-treatment inflammatory biomarkers receiving *infliximab* seemed to do worse than placebo-treated controls.[44] Therefore, it seems that a preliminary screening to identify patients that could benefit from anti-inflammatory therapies might be needed. To the best of our knowledge, no studies to date have investigated the potential of screening the baseline levels of inflammatory mediators, either to predict treatment-response patterns or to predict MDD subtypes. Addressing this research question would involve the preliminary screening of inflammatory markers in patients that are diagnosed with depression to determine if any specific combination or patterns of immune dysregulation matches classically diagnosed depression subtypes. Following screening, depending on their inflammatory profile, these patients could be assigned different types of pharmacotherapy (i.e. antidepressant drugs as a primary treatment and anti-inflammatory add-on medications or only one of the two) to determine the efficacy of anti-inflammatory drugs in treating depressive symptoms. Finally, the levels of inflammatory mediators could be measured following intervention to assess the effects of treatment and to determine if the immune profile is “normalized”.

When designing therapies that target immune processes, it is extremely important to consider safety and the inter-individual differences in genetic and epigenetic architecture of immune processes. Beyond the obvious importance of keeping therapeutic approaches safe, it is necessary to take into account the fact that each patient has a unique

immunological profile and that the latter could present one or several dysregulated inflammatory pathways before treatment. This raises the concept of personalized medicine; the latter entails pharmaco-genomics and pharmaco-epigenomics, the study of the intra-individual genetic and epigenetic variability that plays a role in shaping biochemical and clinical profiles, as well as in triggering different responses to therapeutic drugs. Such an approach could help developing tailored antidepressant treatments with immunomodulatory properties.

Studies aiming at investigating the efficacy of the therapeutic approaches outlined above are needed. Ideally, randomized controlled trials should be designed, aimed at investigating: a) the clinical safety of targeting specific immune mediators as a stand-alone or adjunctive therapy in the treatment of MDD, b) the neurochemical and neurobehavioural changes resulting from such therapies, and c) the gut microbiome changes brought on by such therapies. This could lead to improved therapeutic and diagnostic potential and to individually tailored therapies for MDD treatment.

Chapter 2 – Neuroimmunomodulation in Major Depressive Disorder: Focus on CASP1, NOS2 and IFNGR

Abstract

MDD is one of the leading causes of disability worldwide, and its incidence is expected to increase. Despite tremendous efforts to understand its underlying biological mechanisms, MDD pathophysiology remains elusive and pharmacotherapy outcomes are still far from ideal. Low-grade chronic inflammation seems to play a key role in mediating the interface between psychological stress, depressive symptomatology and MDD onset. Here, we review the available pre-clinical and clinical evidence of an involvement of pro-inflammatory pathways in the pathogenesis, treatment and remission of MDD. We focus on CASP1, NOS2 and IFNG, three inflammatory systems dysregulated in MDD. Treatment strategies aiming at targeting such pathways alone or in combination with classical therapies could prove valuable in MDD. Further studies are needed to assess the safety and efficacy of immune modulation in MDD and other psychiatric disorders with neuroinflammatory components.

Introduction

MDD is a psychiatric disorder with significant morbidity, mortality, disability and economic burden worldwide.[3,209] In addition to the psychosocial and psychophysical dysfunctions associated with MDD, several conditions are often co-morbid, including but not limited to obesity, type-2 diabetes, heart conditions, autoimmune diseases, neurodegenerative disorders and cancer.[210-214] Multiple hypotheses have been formulated attempting to describe the elusive pathophysiology of MDD, including the monoamine hypothesis, the neurotrophic hypothesis, the glutamate hypothesis and the cytokine (or macrophage) hypothesis.[48,68-70,215] However, no single hypothesis seems to fully explain the onset, course and remission of the disease. To complicate matters further, currently approved antidepressant drugs present numerous side effects and are effective only in a subset of patients.[29,216,217] Therefore the quest for a better understanding of the molecular underpinnings of this disease represents an essential step in the identification of novel therapeutic strategies that could target the causal biological mechanisms of MDD.

Emerging evidence suggests that dysregulated neuro-immune pathways could underlie depressive symptomatology in at least a subset of patients diagnosed with MDD.[54,59,64,193,209,218,219] Three crucial inter-linked networks seem to influence the bidirectional communication between the brain and the immune system, namely a) increased oxidative stress, driven by NO overproduction, b) chronic inflammation, driven by CASP1 and NLRP3 inflammasome over activation and c) Central Nervous System (CNS) Th1 lymphocyte infiltration, driven by INFG. NO was recently shown to be necessary for IFNG-mediated suppression of IL1B processing, highlighting the interconnectedness of innate and adaptive immune responses, as well as the therapeutic potential of interfering with inflammatory processes in MDD.[220] The possible involvement of these three networks in MDD is briefly summarized here and will be described in detail throughout this review.

Reactive oxygen species (ROS) are normally produced during cell metabolism and in physiological processes, and are largely quenched and neutralized by endogenous antioxidant elements.[221] However, stress-induced excess of oxidative products can overwhelm the antioxidant capacity, eliciting oxidative stress and causing protein, lipids and/or DNA damage.[222] Preclinical and clinical studies suggest that chronic stress, which appears to be a risk factor in developing MDD, is associated with increased ROS

production.[223-230] One of the free radicals often produced during psychological stress is NO, mainly by action of NOS2.[231]

Inflammatory factors play key roles in tissue repair and in orchestrating the first line of defense to neutralize invading pathogens such as virus, bacteria and protozoa.[232,233] However, pathological activation of both the innate and the adaptive inflammatory cascades caused by stress, metabolic imbalances, autoimmune diseases, and other insults can alter brain function and increase the likelihood of developing MDD and co-morbid systemic illnesses.[65,85,234] One of the inflammatory elements that plays a prominent role in the activation of the innate inflammatory cascade is CASP1, a protein that in the NLRP3 inflammasome renders the mature forms of IL1B and IL18, chief pro-inflammatory factors that modulate brain-immune interactions.[235,236]

It has been shown that reactive T cells are capable of infiltrating the brain in response to antigens derived from the CNS, where they produce pro-inflammatory cytokines.[237] At the same time, there is evidence suggesting that a Th1 immunophenotype prevails in MDD, which leads to the instauration of a chronic, low-grade inflammatory profile.[80,82] Moreover, the key Th1-pro-inflammatory cytokine INFG, involved in both innate and adaptive immunity is a powerful inducer of indoleamine 2,3-dioxygenase 1 (IDO1).[238,239] IDO1-induced tryptophan catabolism increases kyneurine and quinolinic acid, leading to hyposerotonergia and hyperglutamatergia, which are involved in MDD.[68] In this review, we will summarize pre-clinical, clinical and genetic evidence supporting the involvement of innate and adaptive neuroimmune and oxidative pathways in the pathophysiological processes underlying depressive symptomatology. To this end, we will focus on CASP1 (involved in chronic inflammation), NOS2 (involved in oxidative stress), and IFNG (involved in hyposerotonergia and hyperglutamatergia).

Bidirectional communication between the brain and the immune system

Although the CNS is considered to have its “own immune system”, independent from the peripheral immune system, it is accepted that the two constantly communicate and cooperate, that the CNS is involved in regulating immunity, and that immune responses in the periphery lead to changes in the CNS resulting in changes in behaviour.[240,241] To date, pre-clinical and clinical studies have widely described a role for cytokine signalling in the CNS that results in neurochemical, neuroendocrine and behavioural changes.[54,242-244]

Upregulation of macrophage-produced pro-inflammatory cytokines [such as IL1, IL6, TNF) and IFNG, by psychosocial, physical or bacterial stress, leads to a number of endocrine

and neurochemical responses, such as activation of the SNS and of the HPA axis (see chapter 1 for details). Activation of the SNS leads to an activation of the HPA axis which finally stimulates the release of glucocorticoids. This leads to the transcriptional upregulation of anti-inflammatory genes and suppression of HPA axis activity to avoid potential deleterious side effects.[104,105,246-248]

The combination of these events causes an inflammation-mediated transcriptional upregulation in the brain, which results in microglial activation and in the adoption of sickness or depressive-like behaviour (discussed in details below).[54,243,249] Depending on the temporal and qualitative cytokine profile (i.e. acute or chronic activation and cytokine milieu), microglial activation can lead to either neuroprotection or neurodegeneration, suggesting how delicate the equilibrium between restoring homeostasis and tipping into neurotoxicity can be.[250] Altogether, these events produce alterations in several major neurotransmitter systems, such as decreases of available 5HT and DA, two of the most relevant neurotransmitters involved in MDD pathogenesis.[251,252]

Glucocorticoids exert a number of functions in the host response to stressors, with the final goal of restoring homeostasis.[253] However, when the system fails to return to a homeostatic state, the HPA axis can become hyperactive (one of the consistent findings in MDD): a phenomenon underlined by increased cortisol levels, blunted ACTH response to CRH, glucocorticoid resistance, impairment in gluco- and mineral-corticoid signalling, and enlargement of the pituitary and adrenal glands.[254-258] Cytokines are considered to be involved in this impaired feedback mechanisms, through derangement of nuclear factor kappa B subunit 1 (NFKB1), and signal transducers and activators of transcription, all of which inhibit NR function.[110] Aside from decreasing corticosteroids expression, cytokines also block corticosteroids translocation from the cytoplasm to the nucleus and disrupt corticosteroids-DNA binding via nuclear protein-protein interactions.[258] Interestingly, greater extent of such effects has been described in men with high anger and hostility indexes.[259,260] Antidepressant drugs seem to normalize the HPA axis abnormalities observed in MDD and to enhance the expression and function of corticosteroids.[108,261]

Peripheral cytokines can cross the blood-brain barrier (BBB) in a number of ways: a) via CNS lymphatic vessels, b) via active transport and leaky or compromised BBB, c) via crossing at circumventricular organs and d) by binding to receptors found in the blood vessels that course through the brain.[262-265] Moreover, cytokines can affect brain function indirectly, through vagal nerve activation or by binding to cell-surface proteins found in

brain endothelial cells, a process that results in the production of second messengers that in turn diffuse into the brain.[262,264,266,267]

Nonetheless, cytokines are produced *de-novo* in the brain in response to stress exposure.[268-270] Increased concentrations of brain cytokines trigger the activation of microglial cells, immune cells inhabiting the brain parenchyma, which represent the chief innate immune cells in the brain.[241,271] Interestingly, the brain regions presenting the highest concentrations of pro-inflammatory cytokines are the prefrontal cortex, the hypothalamus and the hippocampus, areas involved in the regulation of cognitive functions, mood, and response to antidepressant treatments.[87,272]

Studies investigating the involvement of pro-inflammatory cytokines in MDD have led to the formulation of the cytokine hypothesis of depression, suggesting that cytokine inmbalances lead to MDD pathogenesis (see chapter 1 for details).[53,74,206,290,291]

Cytokine signalling and nitrosative stress

Of central significance for this review, oxidative stress plays a role in the pathophysiology of MDD and of other serious medical conditions.[310,311] In fact, cytokine-induced microglial activation following stressor exposure leads to the prompt up-regulation of ROS via the induction of NOS2, an event that leads to overall increased oxidative stress, which in turn activates a positive feedback loop (co-activation state) that results in the release of more pro-inflammatory cytokines.[298] Oxidative stress is characterized by the increased generation and activity of free ROS, such as NO, which contributes to protein and DNA damage, and can lead to tissue damage and irreversible changes in brain function, events that can flow into neurodegeneration and cognitive impairments.[312] Such stress-induced oxidative processes are gaining increasing attention in psychiatry, since an expanding body of evidence suggests oxidative and nitrosative stress involvement in MDD pathogenesis.[64,230,298,313-315]

The involvement of oxidative and nitrosative stress in the etiology of depression is confirmed by increased circulating and urine oxidative stress markers (such as NO, arachidonic acid, malondialdehyde and 8-hydroxy-2-deoxyguanosine) and nitrosative stress markers (such as immunoglobulin M –IgM- antibodies directed against phosphatidylitol and nitro-bovine serum albumin) in MDD patients, together with decreased levels of antioxidant molecules (such as vitamins C and E).[316-320] Interestingly, the concentration of oxidative stress markers seems to correlate with the severity and

chronicity of depression, as well as with the efficacy of antidepressant treatment.[230,298,313,315,317,321,322] Accordingly, some antioxidant compounds seem to have antidepressant properties, and antidepressant drugs (such as paroxetine) partially reverse oxidative damage by enhancing the protective antioxidant status following stress.[314,323-325]

Of crucial importance for this work, the NO system is being widely investigated in MDD research, because NO levels are increased in depressed patients and in pre-clinical models of chronic stress, and NO inhibition leads to antidepressant-like effects in clinical settings and in pre-clinical behavioural paradigms relevant to clinical depression (these topics are discussed in detail in the NOS2 section).[227,320,326-328] The increased levels of oxidative and nitrosative stress molecules can easily damage neurons, since the latter use high amounts of oxygen due to their high energy needs, and are built with high levels of polyunsaturated fatty acids, molecules particularly vulnerable to attack by free radicals.[329] Nonetheless, the brain presents lower concentrations of antioxidant compounds compared to other organs, making it more susceptible to damage by free radicals.[316] Unsurprisingly, some areas (i.e. the subfields *Cornu Ammonis* –CA- 1 and CA4) of the hippocampus (a brain region involved in mood regulation and adult neurogenesis) are some of the brain areas most sensitive to oxidative damage.[64]

The role of CASP1 in MDD

As mentioned above, exposure to psychological stress results in a physiological reaction called “sterile inflammation”, initiated by the recognition of endogenous danger signals, termed damage-associated molecular patterns (DAMPs), by glial cells, macrophages and olygodendrocytes.[63,287,330] DAMPS are nuclear, cytosolic, mitochondrial or extracellular molecules which are normally hidden from the immune system but upon activation are exposed and released in the extracellular space where they become noticeable to and stimulate an activation of the immune system.[287,331] In line with this understanding, increased levels of DAMPs have been found in rodent blood and hippocampus following stress exposure.[268,332]

Once DAMPs are released in the extracellular space, they function as alarm signals which alert immune cells through pattern recognition receptors, such as toll-like receptors (TLR), nucleotide-binding oligomerization domain-like receptors, RIG-I-like receptors or absent In Melanoma 2-like receptor, in order to get ready for a potential full-blown immune response.[63,333,334] It has been hypothesized that such processes could represent an adaptive characteristic of the acute stress response; for example, if an animal were

running away from a predator and were wounded during the chase, it might have better chances of surviving if its immune system were primed and ready to respond.[335] Another theory, one that places this mechanism in a modern context, suggests that such stress responses are activated when an individual is exposed to social evaluation, rejection, isolation, exclusion or conflict, possibly due to the potentially physically harmful significance of such social situations throughout history.[336]

Taken together, DAMP activation and release induce the activation of downstream signalling cascades such as the transcription factors NFKB1 and IRFs, events that lead to the transcriptional upregulation of a number of immune response genes, such as IL1B, IL6, TNF and many more. These changes result in the creation of a pro-inflammatory milieu in the brain and periphery and in the activation of the afferent nerves, which in turn leads to *de-novo* production of pro-inflammatory cytokines in the brain and culminates with the onset of depressive-like behaviour.[54,243,249]

Moreover, DAMP activation results in the assembly of cytosolic multi-molecular signalling complexes called inflammasomes.[79,334] A peculiar role in DAMPs signalling is played by the NLRP3 inflammasome, an entity that consists of the NLRP3 protein, the adaptor apoptosis-associated speck-like protein containing a CARD (ASC), and the cysteine-protease CASP1.[235] Upon TLR-mediated DAMPs signalling to immune cells, the inflammasome platform is assembled, and the inactive procaspase 1 zymogen is proteolitically cleaved into the enzymatically active heterodimer.[337,338] In turn, activated CASP1 cleaves pro-IL1B and pro-IL18 into their mature, releasable, bioactive isoforms.[235,339] Increased circulating levels of IL1B activate the HPA axis, which increases glucocorticoids production.[247] Interestingly, glucocorticoids increase NLRP3 transcriptional and translational levels, thus priming the NLRP3 inflammasome to readily respond to subsequent stimuli such as alarmins or danger-associated molecules.[340]

Significantly for this review, CASP1 has been reported to be involved in the development of depressive-like behaviour in pre-clinical models of stress and to be increased in MDD patients, leading to the formulation of the “inflammasome hypothesis” of depression.[86,235,341-343] This hypothesis suggests that the inflammasome is a key mediator between the stress response, the exacerbation of depressive symptomatology, the response to antidepressant drugs and the onset of co-morbid illnesses.[339,342] Accordingly, studies have found that CASP1 and NLRP3 transcripts and their protein products are increased in peripheral blood mononuclear cells from MDD patients compared to healthy

controls, suggesting that increased NLRP3 inflammasome activity is relevant to MDD, while antidepressant treatment can decrease such hyperactivity.[78] In addition, IL1B and IL18 are increased in MDD patients, and their circulating levels seem to correlate with the severity of depression.[78] Correspondingly, antidepressant treatment decreases IL1B levels.[196]

Casp1 deficient mice have previously been shown to have the same behavioural and pro-inflammatory response to systemic LPS administration to wt mice but to be resistant to the development of depressive-like behaviour and to the increase of pro-inflammatory cytokines following intracerebroventricular administration of LPS.[148] Moreover, *Casp1*$^{-/-}$ mice were shown to be resistant to lethal doses of LPS, and have decreased levels of inflammation-induced brain and systemic transcription in a pre-clinical model of systemic inflammatory response syndrome.[147,149,344] Furthermore, *Casp1*$^{-/-}$ mice are resilient to pre-clinical models of intestinal colitis, a phenotype underlined by reduced clinical and histological scores, as well as decreased levels of circulating pro-inflammatory cytokines.[345]

Interleukin 1B

Upon inflammasome-mediated IL1B release, IL1B binds to the interleukin-1 receptor (IL1R1), resulting in the activation of many transcription factors [such as NFKB1, c-jun N-terminal kinase, AP-1 and p38 mitogen-activated protein kinase (MAPK)], which results in the expression of many acute-phase inflammation genes, such as NOS2, IL6 and cyclooxygenase type 2.[338,346] Recently, it was suggested that activation of the NLRP3 inflammasome mediates IL1B orchestrated inflammation (that results in depressive-like behaviour) in the prefrontal cortex following chronic stress, and that fluoxetine is able to reverse such changes.[62,86]

Accordingly, mice lacking the IL1 receptor are resistant to developing depressive-like behaviour following chronic stress exposure while being protected against the decrease in neurogenesis observed in wt mice following chronic stress.[150,151] *Il1b* deficient mice have been shown to respond normally to the peripheral administration of LPS but to have impairments in developing a localized acute phase response at the site of tissue damage.[347]

Interleukin 1A

IL1A shares many features with IL1B and it is an equally potent pro-inflammatory cytokine. Similarly to IL1B, it is produced as a 31 kilodalton precursor which can be cleaved into

smaller isoforms and binds to the IL1R1, triggering identical intracellular signalling cascades to IL1B.[348] However, IL1A also presents many differences to IL1B. For example, unlikely the IL1B precursor which is not active, both the pro-IL1A and the cleaved IL1A are active ligands of the IL1R1.[349] Moreover, while IL1B is a released protein, IL1A can be secreted or act as a membrane-bound cytokine, although the factors that control such translocation have not been fully elucidated yet.[348,350] During apoptosis, IL1A is sequestered in the nucleus to avoid its release, while during necrotic activity it is secreted and acts as an alarmin.[348] Furthermore, while IL1B is cleaved by CASP1 in the NLRP3 inflammasome, IL1A is cleaved via calcium-dependent cysteine proteases of the calpain family.[351] Finally, while IL1B is produced on-demand only in immune cells in response to stressful stimuli, IL1A is constitutively expressed in a variety of cell types (such as endothelial cells) but can also be produced by immune cells in response to inflammatory insults.[352] Such expression occurs rapidly in response to cytokine exposure, oxidative stress, and other stimuli such as lipid overload and hormonal stimuli.[346,348,353-355] Interestingly, it has been reported that IL1A-mediated activation of p38-MAPK inhibits NR3C1 function, suggesting that at least part of the mechanism conferring glucocorticoid resistance in MDD could be associated with an excessive production of IL1A.[356] To the best of our knowledge, no studies have investigated the extent of anxiety- and depressive-like behaviour in *Il1a*$^{-/-}$ mice compared to wt mice. However, transgenic *Il1a*$^{-/-}$ mice were shown to have similar ischemic brain damage to wt (and so did *Il1b*$^{-/-}$ mice), while mice lacking both IL1A and IL1B were shown to have drastically reduced infarct size, suggesting a compensatory mechanism within the IL1 system.[357] Another important function of IL1A is its involvement in atherogenesis and its role in mediating fatty acid-induced vascular inflammation.[358] Finally, middle aged (12 months) *Il1a*$^{-/-}$ mice were shown to have increased litter size and pregnancy rates compared to wt mice, probably due to reduced gonadal apoptotic activity.[359]

Interleukin 18

IL18 is considered a prototypical Th1 cytokine for its ability to stimulate IFNG activity, and it is expressed in macrophages and dendritic cells.[360] Unlike pro-IL1B, pro-IL18 is constitutively expressed and substantially pooled inside cells, and inflammatory stimulations don't have a big impact on its transcription.[360,361] However, circulating IL18 levels increase during psychological stress and in response to HPA axis activation.[362] Circulating IL18 binds to the IL18 receptor (IL18R) in T-cells, B-cells and natural killer cells. This activates p38-MAPK, c-Jun N-terminal kinase and NFKB1 cascades which

potentiate antimicrobial and antiviral immunity.[361,363] Although IL18 is known for its ability to promote both Th1- and Th2-related inflammatory responses, its predominant role in enhancing Th1 activity makes this cytokine a candidate therapeutic target in a number of Th1-related inflammatory and autoimmune diseases, including MDD.[360]

In line with its role as a stress-related molecule, IL18 is increased in MDD patients and in patients diagnosed with panic disorder.[364] Pre-clinical studies have shown that *Il18*$^{-/-}$ mice have decreased production of IFNG, impaired natural killer cell activity and abnormal Th1 responses.[365] Moreover, *Il18*$^{-/-}$ mice display decreased depressive- and anxiety-like behaviour, as well as gene expression changes across various brain regions and in particular decreased vasopressinergic and oxytocinergic neurotransmission within the amygdala.[366,367] Other studies have shown that immobilization stress in mice induced pro-IL18 via ACTH and a superoxide-activated CASP1 pathway.[368] Given that IL6 is not induced in response to stress in *Il18*$^{-/-}$ mice, it seems that IL18 mediates stress-induced IL6 upregulation.[368] Other studies found that IL18 is involved in stress-induced microglial activation in rodents while contributing to dopaminergic degeneration.[369,370] Finally, *Il18*$^{-/-}$ mice have been shown to be hyperphagic and prone to both obesity and insulin resistance.[371]

Interleukin 33

IL33 is another member of the IL1 family, that performs alarmin and nuclear transcription factor roles, and is considered to trigger predominantly Th2-related immune responses, such as the production of IL4, IL5 and IL13 and anti-inflammatory gene expression.[372] Like other members of the IL1 family, IL33 can be beneficial or detrimental, depending on its spatio-temporal expression. Constitutively, IL33 is expressed in quiescent endothelial and epithelial cells as well as in microglial cells, astrocytes, fibroblasts and keratinocytes.[373,374] In these cells, IL33 is localized in the nucleus, where it modulates gene expression via a) acting as a transcription factor, b) regulating chromatin structure, c) sequestering NFKB1 and therefore curbing pro-inflammatory signalling.[375-377] IL33 is constitutively expressed and localized in the cytoplasm. However, if a barrier is breached and IL33 is released from destroyed cells, it acts as an alarmin upon binding the IL33 receptor (ST2).[378] The signalling cascade in response to ST2 activation results in the transcriptional modulation of hundreds of genes with a pattern that resembles that of IL1R1 activation.[379]

Two single nucleotide polymorphisms in the *IL33* gene (rs11792633 and rs7044343) have been found to moderate the correlation between history of childhood abuse and recurrent

depression in a women cohort.[374] In the same study, the authors found that patients with a history of recurrent depression had greater peripheral levels of IL33 and IL1B.[374] Finally, the authors reported increased IL33 expression in the paraventricular nucleus of the hypothalamus and in the prefrontal cortex of rats exposed to an acute stressor, suggesting that stress increases IL33 expression in those brain regions.[374] Accordingly, circulating IL33 levels are increased in bipolar disorder patients.[380]

The combination of the inflammatory events triggered by psychosocial stress has the final goal of dealing with the stressor and of restoring homeostasis in the system by activating neuroprotective mechanisms. However, if these events do not subside upon termination of the stimulus, or if the stimulus is repeated over time, they can transform into maladaptive events and lead to pathological states. Such states can flow into neurodegeneration and depressive symptomatology, while increasing the potential for neurodegeneration-related co-morbid illnesses.[58] Finally, high levels of IL1B caused by stress exposure can upregulate the enzyme IDO1, which leads to decreased levels of available tryptophan, resulting in overall decreased brain 5HT.[381]

The role of NOS2 in MDD

NO is a small intercellular and intracellular signalling molecule with a very short half-life (3-6 s) that freely diffuses across cell membranes and plays important physiological roles in the brain through modulating different pathways such as neurogenesis, neurotransmission, synaptic plasticity, learning, and pain perception.[382] NO also plays a crucial role in the regulation of emotional and cognitive processes, suggesting that it could be involved in the aetiology of MDD and anxiety disorders through its participation in neurotransmission, neuromodulation and synaptic plasticity.[383] Three isoforms of the NOS enzyme produce NO: NOS2*,* neuronal (NOS1) and endothelial (NOS3), all of which have specific spatio-temporal patterns of regulation. In this review, we will focus on the inducible isoform because it is considered to be the most relevant to MDD pathophysiology.

Over the past two decades, several lines of evidence have brought NO and specifically the NOS2 isoform to the forefront in psychiatry: a) the levels of NO and its metabolites are increased in MDD patients and patients who attempt suicide compared to healthy controls,[328,384,385] b) transcription of the *NOS2* gene is increased in the peripheral blood of patients with recurrent depressive disorder,[386] c) a polymorphism (-1026C/A) in the *NOS2* promoter is associated with the risk of recurrent depressive disorder,[387] d) IgM levels against NO adducts are elevated in MDD patients, suggesting that the protein damage

created by NO results in the formation of immunogenic peptides, that in turn results in an autoimmune-like response,[388,389] e) the SSRI paroxetine is a NOS2 inhibitor,[390,391] f) adjuvant NOS2 inhibition enhances the efficacy of serotonergic antidepressants,[326] g) NOS2 is increased in the hippocampus and cerebral cortex in mice following stress as a response of increased NFKB1 activity, and NOS2 inhibition results in antidepressant-like effects in rodents,[158,228,392] h) NOS2 inhibition in rodents results in antidepressant-like effects,[158] i) NO-mediated pathways are involved in the development of post-traumatic stress disorder in animal models.[393]

Nitric oxide is synthesized from L-arginine and molecular oxygen, and this process requires a number of co-factors to take place.[394] The architecture of the *Nos2* promoter region suggests that this gene has a tight and complex pattern of transcriptional control since it is rich in positive and negative regulatory regions, and it is responsive to many transcription factors, pro- and anti-inflammatory cytokines as well as bacterial degradation by-products.[395] NOS2 is calcium-independent, is expressed in macrophages and microglia, consists of both soluble and membrane bound-NOS and is synthesized on-demand.[396] In fact, whereas there is no detectable physiological NOS2 expression in the brain, a profound neuronal, glial and vascular transcriptional up-regulation of the *NOS2* gene can be observed in response to traumatic events such as ischemia and systemic inflammation, most likely through activation of the *NOS2* promoter by inflammation-related molecules, such as cytokines, IRF1and NFKB1.[229,344,395,397,398] Following induction, NOS2 produces NO continuously until the proteasome degradation pathway inactivates the enzyme.[399] Interestingly, in the brain, glial NOS1-generated NO negatively regulates *NOS2* expression through suppression of *NFKB1* gene transcription.[400]

Several studies have targeted the NO system in pre-clinical MDD research, yielding promising results. For example, NO seems to decrease the production of NE, to decrease the levels of nitrate and nitrite in the hippocampus and cerebral cortex, and to decrease 5HT turnover in the frontal cortex.[394,401,402] Moreover NO has an inhibitory effect on dopamine transporters; therefore, it indirectly increases the availability of inter-synaptic dopamine.[403] Finally, the relevance of the L-arginine-nitric oxide-cyclic guanosine monophosphate pathway to MDD has recently emerged. In fact, several molecules such as bupropion (a norepinephrine-dopamine reuptake inhibitor), venlafaxine (a SNRI), mementine (an NMDDA receptor antagonist) and berberine (a plant alkaloid), all of which produce antidepressant-like effects, modulate this signalling pathway.[404] Taken together,

these findings suggest that NO modulation could represent a useful approach in the treatment of MDD.

The role of interferon-gamma in MDD

IFNG is a pleiotropic soluble cytokine which orchestrates several distinct cellular programs via transcriptional and translational control over a large set of genes. It is also the sole member of the type II IFNs.[405,406] IFNG is produced by a number of immune cells such as lymphocytes, cytotoxic lymphocytes, B cells and antigen-presenting cells.[407,408] The IFNGR is expressed on almost all cell types and its activation triggers the janus kinase 1 and 2 signal transducer and activator of transcription 1 pathway, as well as additional pathways, such as the extracellular-signal-regulated-kinase 1/2.[409,410] Activation of the IFNGR results in the transcription of genes with IFNG-stimulated response elements (ISREs) within their promoter region until signal transducer and activator of transcription 1 dissociates following complete dephosphorylation within 1-2 hours.[411,412] The genes transcribed in response to IFNGR activation are at least 200, together with many micro RNAs and long non-coding RNAs[413] (for a database of IFN-regulated genes see [414]). At the same time, after IFNGR stimulation, the secondary transcription factors IRF1, IRF2 and interferon consensus sequence binding protein are upregulated. This in turn results in the transcriptional induction of a subset of inflammatory-related genes such as *NOS2* (stimulated by IRF1) and guanylate-binding protein. Finally, IFNG can activate and be activated by CASP1, highlighting the interconnectedness of these 2 inflammatory pathways.[415-418] The ensemble of these processes highlights the multilayered complexity of events that arise following IFNGR activation.[412,419]

Studies have shown that *ex-vivo* peripheral blood mononuclear cells from MDD patients display increased IFNG and neopterin production upon stimulation, as well as decreased tryptophan bioavailability.[205] Nevertheless, IFNG transcriptional levels (together with those of TNF) in patients with multiple sclerosis correlate with the severity of the depressive symptomatology during flare-ups.[420] At the same time, most categories of antidepressants (tricyclic, SSRI, SNRI and reversible inhibitors of monoamine oxidase A) suppress the IFNG/IL10 ratio through suppressing IFNG and stimulating IL10 *in-vivo* and *ex-vivo.*[421,422] These findings suggest that MDD patients have increased systemic IFNG and neopterin production by activated T cells and macrophages. This could be responsible for an upregulation of the enzyme IDO1 (since the latter presents 2 ISREs at the promoter region that lead to maximum promoter activity) and consequent tryptophan depletion through upregulation of the kyneurine/ tryptophan pathway, events that result in a decrease of

available 5HT levels and in an increase of the toxic metabolite kyneurine.[66,205,423,424] Accordingly, a polymorphism (CA repeat, rs3138557) in the *IFNG* gene correlates with lower levels of serum tryptophan and 5-hydroxindolacetic acid (the main metabolite of 5HT) and higher levels of kyneurine, suggesting that carriers of the CA allele have decreased 5HT levels and are therefore more susceptible to developing MDD.[425] Similarly, the presence of the high producer T allele +874(T/A) polymorphism (rs2430561) has been associated with increased IDO1 activity.[426] Interestingly, IFNG signalling promotes the creation of a pro-inflammatory milieu, and drives Th1 development;[427,428] therefore, increased production and signalling of IFNG during early life and traumatic events could be one of the drivers of the Th1/Th2 shift towards Th1 in MDD and the onset of depressive symptomatology during childhood or later in life.[83]

Animal studies have shown that *Ifng*$^{-/-}$ mice do not show developmental defects but present compromised immune responses and increased susceptibility to infections.[155] With regard to their behavioural phenotypes, *Ifng*$^{-/-}$ mice display decreased anxiety- and depressive-like behaviour as well as heightened emotionality in several pre-clinical behavioural paradigms.[156,157,429] These behaviours are underlined by a) increased serotonergic and noradrenergic activity (i.e. greater metabolite accumulation) in the central amygdaloid nucleus, together with b) increased baseline plasma corticosterone, c) decreased neurogenesis in the hippocampus, and d) decreased levels of nerve-growth factor in the prefrontal cortex, suggesting that IFNG modulates anxiety and depressive states and is involved in CNS plasticity.[156,157] On the other hand, while IFNG deficiency was shown not to confer resistance to a chronic stress regimen in mice, it was shown to attenuate monoamine, corticoid and cytokine alterations in response to stressors.[157] Given this evidence for an involvement of IFNG in pathways relevant to depressive symptoms and depressive-like behaviour, targeting IFNG and/or its receptor could hold potential in the quest for novel therapeutic strategies in MDD treatment.

Chapter 3 - Hypotheses and aims

Rationale of the study

Given the emerging role of inflammatory mediators and of the gut microbiome in the aetiology and treatment of MDD, we ought to investigate if inhibiting pro-inflammatory mediators would result in antidepressant-like effects in pre-clinical models of MDD and affect gut microbiome composition. In the first study (study 1, chapter 4) we investigated whether genetic deficiency and pharmacological inhibition of CASP1 affect behaviour, chronic stress response and gut microbiome composition. In the second study (study 2, chapter 5), we tested the effects of simultaneously deleting the genes a) *Nos2*, b) *Casp1* and c) *Ifngr* on innate anxiety- and depressive-like behaviours, on those behaviours following chronic stress and on the levels of the circulating stress-related hormones ACTH and CORT.

CASP1 is a cysteine protease activated by a variety of physical and physiological stressors in the NLRP3 inflammasome.[78,235,430] Its activation leads to the cleavage and release of the bioactive forms of IL1B, IL18 and IL33.[343] CASP1 transcriptional and protein levels are increased in the blood of MDD patients, and decrease following antidepressant treatment.[78,342,430] These findings suggest a role for CASP1 in MDD pathogenesis and remission, potentially through its involvement in neuroinflammation and neurodegeneration.[78,342,430] Given that stress induces pro-inflammatory signalling and that *Casp1*$^{-/-}$ mice display decreased inflammation-induced brain transcription in response to endotoxic shock compared to wt mice, inhibiting CASP1 might result in a protective effect against psychological stress.[149] Accordingly, CASP1 inhibition with minocycline (a second class tetracycline with anti-inflammatory effects) has been shown to have antidepressant and neuroprotective outcomes, highlighting the role of the NLRP3 inflammasome-IL1 system in the psychological stress response and neuroinflammation.[93,94,431] Similarly, IL1 blockade has been shown to prevent the anhedonic and anti-neurogenic effects of stress in the mouse brain.[151]

NOS2 is one of the four isoforms of the enzyme NOS, a family of enzymes that produce the molecular compound and free radical NO, which is involved in a number of processes, such as the response to psychological stress, neurogenesis, neurotransmission, learning, immune system modulation, blood vessel function and pain perception.[396] Exposure to stressful stimuli has been shown to increase *Nos2* expression in the rat brain via NFKB1 activation,[227,228] and the production of NO is increased in MDD patients, while

polymorphisms in the *NOS2* genes associate with major depression.[328,385,387] Correspondingly, inhibition of NOS2 in rodents induces antidepressant-like effects.[158,432] Finally, NO modulates several neurotransmitter systems, making it an appealing candidate target in the treatment of MDD and other psychiatric disorders.[382]

IFNG is a soluble cytokine and a fundamental mediator of innate and adaptive immunity.[407] This cytokine plays a role in Th1-mediated cell responses, Th1 and T-reg cell differentiation, macrophage activation and immunoediting.[416] Its circulating levels have been shown to correlate with stress perception and anxiety responses in students before an examination,[83] while *ex-vivo* peripheral blood mononuclear cells from MDD patients produce more IFNG upon stimulation compared to healthy controls.[205] Accordingly, many antidepressant drugs suppress IFNG production.[81,433] To test our hypotheses we used mice with genetic deletion or pharmacological inhibition of CASP1, and mice with simultaneous genetic deletion of *Casp1*, *Nos2* and *Ifngr*.

Aims and hypotheses

Overarching Hypothesis

Our overarching hypothesis was that the genetic deletion or pharmacological inhibition of pro-inflammatory mediators in mice would decrease anxiety- and depressive-like behaviours. Moreover, that this would have a protective effect on the stress response resulting in a decreased exacerbation of anxiety-and depressive-like behaviours following exposure to chronic stress regimens. Furthermore, that such inhibition would result in decreased levels of circulating ACTH and CORT after chronic stress compared to wt mice. Finally, that chronic stress and CASP1 inhibition with minocycline treatment would affect gut microbiome composition.

Aim 1

Our first aim was to determine if genetic deficiency and pharmacological inhibition of CASP1 with minocycline affect behaviour in mice. Moreover, to determine if decreasing CASP1 activity via genetic deletion or minocycline treatment could prevent the exacerbation of depressive- and anxiety-like behaviours following exposure to a chronic stress regimen. Furthermore, to determine if stress and pharmacological inhibition of CASP1 and their combination during stress exposure affect gut microbiome composition in mice.

Hypothesis 1

We hypothesized that genetic deficiency of *Casp1* would affect baseline behaviour and prevent the exacerbation of anxiety- and depressive-like behaviours following chronic stress. Moreover, we hypothesized that CASP1 pharmacological inhibition would prevent the exacerbation of anxiety- and depressive-like behaviours following chronic stress while affecting gut microbiome composition. Furthermore, we hypothesized that chronic stress exposure alone would affect gut microbiome composition.

Aim 2

Our second aim was to determine if the simultaneous genetic ablation of *Casp1*, *Nos2* and *Ifngr* affects anxiety- and depressive-like behaviours at baseline and anxiety- and depressive-like behaviours following chronic stress. Moreover, to determine if the contemporaneous deficiency of *Casp1*, *Nos2* and *Ifngr* affects the levels of the stress-related hormones ACTH and CORT following chronic stress exposure.

Hypothesis 2

Our second hypothesis was that the contemporaneous genetic ablation of *Casp1*, *Nos2* and *Ifngr* would affect anxiety- and depressive-like baseline behaviours, while preventing the exacerbation of anxiety- and depressive-like behaviours following chronic stress. Moreover, that the contemporaneous genetic deficiency of *Casp1*, *Nos2* and *Ifngr* would decrease the circulating levels of the stress hormones ACTH and CORT following chronic stress exposure.

Methods

In order to test our hypotheses, we used wt C57BL/6J mice and mice with genetic deletion of either *Casp1* or mice with simultaneous deletion of *Casp1*, *Nos2* and *Ifngr* [(*Casp1*, *Ifngr, Nos2*)$^{-/-}$]. We chose to use these mice given the available literature on the stress resilience phenotypes of *Il1b*$^{-/-}$, *Nos2*$^{-/-}$ and *Ifngr*$^{-/-}$ mice (see chapter 2 for details).

Statistical analyses

In order to detect statistical differences between the groups here studied we used a general linear model for repeated measures (repeated measures ANOVA with mixed design) using the software Statistical Package for the Social Sciences version 23.0 for windows (SPSS, Chicago, Illinois, USA),[434] given that the same behavioural tests were repeated before and after stress exposure. We investigated whether there was a main effect of genotype (between subjects factor), a main effect of stress (within subjects factor) and/or a stress*genotype interaction. The significance threshold was set at $P<0.05$. In

order to validate the assumption of sphericity of the variances (a condition in which the variances of the differences between all within-subject conditions are equal) we used Mauchly's sphericity test. We reported estimates of effect size as partial eta squared (η^2_p). If stress*genotype or stress*treatment interactions were statistically significant, they were unpacked to determine the major contributing factors (i.e. stress or genotype/treatment) for the differences observed.[434] Enzyme-linked immunosorbent assays (ELISAs) results were compared by using 2-tailed unpaired t-test with a confidence level of 95%. Assistance with the statistical analysis was provided by a statistical consultant employed by Flinders University (Mr. Pawel Skuza).

Comparison of gut microbiome composition between groups (beta-diversity) was carried out using Bray Curtis similarity matrices in PRIMER (v6, PRIMER-E Ltd, Plymouth, UK). Matrices were created from the abundance of sample-normalized, square-root transformed, relative OTUs (operational taxonomic units). Changes at the community level were assessed using one-way permutational multivariate analyses of variance (PERMANOVA) tests with 9,999 random permutations and the significance threshold was set at $P<0.01$. The contribution of individual taxa to between-group variation was assessed by similarity percentages (SIMPER) analysis, as previously reported.[435] If specific bacterial taxa were identified as contributing to changes in microbiome composition, the variation in their relative abundance was further assessed through Mann-Whitney U tests between groups. Differences of median relative abundance between groups were assessed using Hodges-Lehmann estimator. Statistical analyses of gut microbiome experiments were carried out and results interpreted by Dr. Lex Leong, Dr. Jocelyn Choo, and A/Prof. Geraint B. Rogers, affiliated with the Infection and Immunity Theme, South Australian Health and Medical Research Institute, Adelaide, SA, Australia, and the Department of Infectious Disease, Flinders University School of Medicine and Flinders Medical Centre, Adelaide, SA, Australia.

Power analyses

The required sample size to detect statistically significant differences in our studies was determined with results obtained in preliminary pilot experiments.

Study 1

In the experimental design phase for study 1, power calculation was performed based on the effect size seen in a pilot study investigating the effects of CASP1 deficiency on floating time in the forced swim test (our primary outcome measure). Cohen's *d* for that

study was 0.78, meaning that a sample size of n>45 (we used n=47) would result in 80% power to detect an antidepressant-like effect at $P \leq 0.05$.

Study 2

In the experimental design phase for study 2, power analysis was based on the effect size previously observed in a preliminary study investigating the effects of *Casp1, Nos2 and Ifngr* genetic deficiency on total floating time in the forced swim test. Cohen's *d* for that study was 0.84, meaning that a sample size of n=34 (we used n=36) would result in 80% power to detect an antidepressant-like phenotype at $P \leq 0.05$.

Behavioural experiments

In order to assess behaviour at baseline and following chronic stress exposure, we performed widely adopted behavioural tests to assess anxiety- and depressive-like behaviours. Specifications for each behavioural test can be found in the material and methods section of chapter 4 and 5.

Forced swim test

The forced swim test (or Porsolt swim test) is a test used to assess behavioural despair (also called depressive-like or hopelessness behaviour) in rodents. This test is based on the notion that when rodents are forced to swim in a cylinder filled with water from which they cannot escape, they will rapidly realize that they are not able to exit the cylinder and will become immobile, only making minimal movement to float with their head above the surface of the water. In the forced swim test, the total amount of time spent immobile (inactive or floating behaviour) within a specific timeframe (usually 5 minutes) is considered an index of depressive-like or hopelessness behaviour; the higher the immobility time, the higher the behavioural despair or depressive-like phenotype. A range of antidepressant treatments has been shown to decrease floating in the forced swim test by increasing active behaviour. The latter is usually classified as either swimming (when the rodent swims in the cylinder moving both its hind and back legs) or climbing (when the rodent makes vigorous movements to attempt escaping, with its body almost parallel to the walls of the container in which the test is performed).[436-439]

Sucrose preference test

The sucrose preference test is considered a measure of anhedonic-like (the inability of seeking or pleasurable stimuli and reward) behaviour. In this test, mice are given the choice of drinking either plain water or a sugary solution (usually containing varying concentrations of sucrose). Under normal conditions, the mice display preference for the

sweet solution over standard water, while in times of increased stress the mice drink the same amount from either bottles or prefer to drink standard water. Usually mice are first habituated to the novel drink by being presented with two identical bottles containing the sweet solution. Then, one of the bottles is replaced with normal water and the preference for the sweet solution is calculated as the percentage of the sweet solution drunk over the total volume of liquid drunk over a set amount of time (usually 1, 12 or 24 hours). To avoid preference for one side, the position of the bottles can be switched at regular intervals during the test.[176,177]

Marble Burying test

The marble-burying test is used to assess novelty-induced anxiety and anxiolytic activity. In this test, rodents are individually placed in a novel arena with regular bedding in which colorful glass marbles have been introduced. Digging is one of the innate rodent behaviours, and in times of increased stress or in novel environments rodents display increased digging behaviour. As a result of this behaviour, the marbles are indirectly buried and the number of marble buried gives an index of digging considered predictive of anxiety-like behaviour. However, some scientists have argued that anxiety-like measures in the marble burying test do not correlate with anxiety measures in other tests. Some have argued that this test is more representative of repetitive behaviour (and hence more similar to a test for the quantification of obsessive-compulsive behaviour).[440,441] This test was only performed in study 1 (chapter 4).

Rotarod

The rotarod is a test performed to assess locomotor coordination, balance and motor skill learning. This test is performed by placing the animal on a rotating (at constant or accelerating speed) drum. The average latency to fall from the apparatus over several trials (3 per day over 4 days in this study) is used for the analysis.[442] This test was performed only in study 1 (chapter 4).

Open field test

The open field test is used to assess exploratory and locomotor activity in rodents. Animals are individually placed in the corner of a brightly lit arena from which escape is prevented by walls and their locomotor activity is recorded for a set amount of time (usually 5 to 60 minutes). This test was initially designed to measure the emotionality elicited by the exposure to a novel, brightly lit arena by counting the number of defecations and urinations.[443] This test can also assess anxiety-like behaviour as measured by the time

spent in a central subsection of the arena (to which rodents display an innate avoidance) or the ratio between the distance travelled in the centre of the arena over the total distance travelled.[443,444]

Elevated plus maze test

The elevated plus maze is a maze which comprise two open and two closed arms and it is used to quantify anxiety-like behaviour in rodents. This test relies on the innate avoidance that rodents have for open spaces, in which they feel more vulnerable due to the increased likelihood of being vulnerable to predators. Usually the total time spent in the open arms and/or the ratio between the time spent in the open arms over the time spent in the closed arms are used as indexes of anxiety-like behaviour, since anxiolytic treatments decrease these parameters.[444,445]

Novelty suppressed feeding

The novelty suppressed feeding is a test based on a phenomenon called *hyponeophagia*, which is the reduction in the amount of feeding in response to a novel environment. In this test, rodents are fasted for a period of time (usually overnight/16 hours) and then placed in a novel arena with a single pellet of food in the middle of the arena. The animals face the choice between hiding in one of the corners or approach and consume the food in the centre of the arena while experiencing avoidance deriving from the novel environment. Higher latency to eat is connected to higher avoidance and anxiety-like behaviour.[446] This test was only performed in study 1.

Gut microbiota assessment

Following the experiment in which we investigated the behavioural effects of concomitant pharmacological inhibition of CASP1 with minocycline and exposure to CRS, we decided to investigate the gut microbiota composition of these mice to assess the effects of a) chronic stress, b) pharmacological CASP1 inhibition with minocycline and c) concomitant treatment with minocycline and CRS. In order to determine gut microbiota composition, we used paired-end 16S rRNA analysis, a sequencing technique used to distinguish bacterial families and species based on the unique signatures of bacterial 16S rRNA. This technique relies on the amplification and sequencing of a region (300 base pairs in our study) of bacterial genes coding for the 16S rRNA, which is a part of ribosomes and is involved in the translation process. This technique is widely used to identify bacterial populations, given that 16S rRNA is highly conserved amongst bacterial families and therefore allows for the use of universal primers for amplification. Moreover, since it is one

of the most characterized techniques to identify microorganisms, databases are available that allow for the identification of the sequencing results. Furthermore, this technique allows for the identification of both cultured and uncultured microbial families. Finally, this approach is cheaper than other sequencing approaches such as shotgun sequencing.[447,448] This analysis was performed in study 1 (chapter) as a core service by the David R. Gunn Genomics Facility, South Australian Health and Medical Research Institute.

ELISAs

To investigate if the levels of the stress hormones ACTH and CORT were different amongst groups in study 2 we used ELISAs. ELISA is a molecular technique used to determine the presence of a specific substance such as a peptide, a protein, a hormone or an antibody in a biological sample, such as blood, urine or cell culture supernatant.[449] The "direct" version of this assay (like the CORT assay used in this study) relies on the specificity of monoclonal antibodies to exclusively bind to a specific antigen. After the antigen is immobilized, the detection antibody is added and it forms a complex with the antigen. Finally, adding a substrate produces a visible signal quantifiable with a spectrophotometer which indicates the quantity of antigen in the sample.[450] The ELISA kit used to determine the levels of ACTH in this study is a variation of the standard ELISA procedure. In fact, this assay relies on the principle of competitive inhibition. In this type of assay, the concentration of the antigen of interest is measured by observing the interference of the expected signal output. The more antigen in the sample that needs to be analysed, the higher the reduction in intensity. We chose ELISA assays to determine the levels of the proteins of interest because this approach allows for the determination of the amount of a mature protein.

Outcome measures

Primary outcome measures

Our primary outcome measure was to assess depressive-like behaviour in the forced swim test at baseline and following exposure to a chronic stress regimen.

Secondary outcome measures

Secondary outcome measures included anhedonic-like behaviour, anxiety-like behaviour, locomotor activity (study 1 and 2), respirometry and gut microbiome composition (study 1 only), and circulating levels of the stress-related hormones ACTH and CORT (study 2 only).

Chapter 4 - Inflammasome signalling affects anxiety- and depressive-like behaviour and gut microbiome composition

Published paper, "Molecular Psychiatry", (D.O.I. 10.1038/mp.2016.46.)

In this paper, generation and genotyping of *Casp1*$^{-/-}$ transgenic mice was performed as a service from the Australian National University core facility. The behavioural experiments involving *Casp1*$^{-/-}$ mice were performed by the candidate when the candidate was working as a research assistant and volunteer in the Translational Psychiatry research group at the John Curtin School of Medical Research, Australian National University. The behavioural experiments involving CASP1 pharmacological inhibition with minocycline were performed by the candidate at the South Australian Health and Medical Research Institute. The statistical analyses performed on the data involving *Casp1*$^{-/-}$ and minocycline treated mice were performed by the candidate. Assistance in the statistical analysis was provided by a statistical consultant employed by Flinders University (Mr. Pawel Skuza). The gut microbiota composition study, using paired-end 16S RNA analysis, was performed as a core service by the David R. Gunn Genomics Facility, South Australian Health and Medical Research Institute and the statistical analysis for that experiment and interpretation were performed by Dr. Lex Leong, Dr. Jocelyn Choo, and A/Prof Geraint B. Rogers.

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Abstract

The inflammasome is hypothesized to be a key mediator of the response to physiological and psychological stressors, and its dysregulation may be implicated in MDD. Inflammasome activation causes the maturation of CASP1 and activation of IL1B and IL18, two pro-inflammatory cytokines involved in neuroimmunomodulation, neuroinflammation, and neurodegeneration. In this study, C57BL/6 mice with genetic deficiency or pharmacological inhibition of CASP1 were screened for anxiety- and depressive-like behaviours, and locomotion at baseline and after chronic stress. We found that genetic deficiency of *Casp1* decreased depressive- and anxiety-like behaviours, and conversely increased locomotor activity and skills. We also showed that *Casp1* deficiency prevented the exacerbation of depressive-like behaviour. Furthermore, pharmacological CASP1 antagonism prevented stress-induced increase in depressive-like behaviour. Chronic stress or pharmacological inhibition of CASP1 affected faecal microbiome composition and were both associated with a dysbiotic state. Analysis of individual bacterial taxon relative abundance provided evidence of both synergistic and antagonistic effects of chronic restraint and CASP1 inhibition. Our results suggest that CASP1 inhibition has a protective effect in modulating the relationship between stress and microbiome composition, which supports the notion of a microbiome-gut-inflammasome-brain (MGIB) axis, in which the gut microbiome, via inflammasome signalling, modulates inflammatory pathways that will alter brain function and affect depressive- and anxiety-like behaviours. Our data suggest a novel opportunity for translation into MDD treatment, if future studies demonstrate that the MGIB axis represents a feasible therapeutic target in the treatment of psychiatric disorders.

Keywords: caspase 1/interleukin 1 converting enzyme, interleukin 1 beta (IL1B), interleukin 18 (IL18), inflammasome, chronic restraint stress, major depressive disorder, minocycline, gut microbiome.

Introduction

Increasing evidence suggests an involvement of neuroinflammatory pathways in the etio-pathophysiology of MDD, and antidepressant response.[58,64] Depressive symptoms are underlined by increased levels of pro-inflammatory cytokines (i.e., IL1B, IL6), decreased levels of anti-inflammatory cytokines, (i.e. IL4 and IL10), and are associated with polymorphisms in inflammation-related genes.[59,202,203] IL1 receptor type-I and its ligands are expressed in brain areas relevant to stress response[451-453] and IL1B signalling is fundamental in mediating the deleterious neurobehavioural and neuroendocrine responses to stress and adaptation.[150,454] Chronic stress or IL1B administration triggers depressive-like behaviour.[455]

A variety of stressors activate the inflammasome through the NLRP3 or P2X purinoceptor 7 receptors, resulting in CASP1 maturation, which processes and releases bioactive IL1B and IL18.[235,343] CASP1 and NLRP3 mRNA are increased in blood cells of depressed patients,[78] suggesting that the inflammasome is a key mediator by which physical and psychological stressors contribute to the development of depression, leading to the "inflammasome hypothesis" of depression.[342] If that proves to be correct, CASP1-inhibiting compounds may have antidepressant effects. Minocycline is a semisynthetic tetracycline antibiotic that inhibits CASP1 and CASP3 transcription and has anti-apoptotic, anti-inflammatory, and neuroprotective properties as well as acute antidepressant-like effects.[91,92,95,456-459]

Casp1$^{-/-}$ mice are overtly normal, despite having undetectable IL1B and low IL1A levels.[147] They have decreased systemic inflammatory response and increased survival to lethal endotoxin doses when compared to wt mice.[147,149] This is underlined by reduced inflammation-induced brain transcription, decreased inflammasome assembly, and consequently decreased circulating IL1B and IL18.[147,149]

The MGB axis is a complex multi-organ bidirectional signalling system between the microbiome and the brain that plays a fundamental role in host physiology, homeostasis, development and metabolism.[460] Growing evidence shows reproducible and consistent effects of microbial states on mouse behaviour, supporting a role for the microbiome in modulating behaviour.[145,461,462] Differences in anxiety-related behaviours are commonly reported in mice with altered gut microbiomes, implicating the role of gut microbiome in stress and depression.[463,464] *Casp1*$^{-/-}$ mice display depressive-like behaviour and anorexia after peripheral but not central LPS administration and differ in gut microbiome

composition compared to wt mice.[148,465,466] Therefore, our primary and secondary hypotheses were, respectively, a) that decreased CASP1 activity would result in decreased depressive-like behaviour and b) that CASP1 inhibition and CRS would result in changes in the gut microbiome. The null hypothesis was that there would be no difference in these parameters between *Casp1*$^{-/-}$, wt and minocycline-treated mice.

Materials and methods

Procedures were approved by the Animal Ethics Committees of the Australian National University and the South Australian Health and Medical Research and are in accordance with the Australian Code for the Care and Use of Animals for Scientific Purposes (8th edition, 2013). Male mice (C57BL/6J background, wt n=81; *Casp1*$^{-/-}$ n=20) aged 60-90 days were obtained from the Australian Phenomics (Canberra, Australia) or the Bioresources Facilities (Adelaide, Australia). Genetic *Casp1* deficiency was confirmed by genotyping in experimental mice (Supplementary Fig. 4.6). Littermates were group housed (Green Line IVC Sealsafe PLUS mouse, Tecniplast, Varese, Italy) in a temperature (22°C ±1°C) and light (12h cycles, lights on at 7:00 am) specific pathogen free room with water and food *ad libitum*. Animals were assigned and randomized as described in the supplementary materials and methods. The investigators were not blinded to group assignment. Behavioural phenotyping was performed between 9:00 am to 4:00 pm. Animals were given 30 min habituation to the behavioural testing room. Tests were performed from the least to the most invasive to minimize the influence of prior test history (in order: rotarod, elevated plus maze, marble burying test, open field test, sucrose preference test, novelty suppressed feeding, forced swim test. See supplementary methods for details).[467] Following CRS this order was reversed for a bell-shaped stress exposure (Supplementary Fig. 4.7).

CRS

After baseline behavioural testing, animals were submitted to restraint stress for 21 days. Every day, mice were placed in a horizontal resting position inside a well-ventilated (12 holes, 0.5mm diameter) 50mL falcon tube at 10:00 am and after 4-6 hours they were unrestrained.

Pharmacological CASP1 inhibition with minocycline

Wt mice were treated with either saline [0.2mL, intraperitoneally (i.p.), n=27] or minocycline (LKT laboratories, St. Paul, MN, USA) [5mg/kg/d in 10mL/kg saline, (i.p.), n=27]. Treatment lasted for the same duration of the restraint procedure (21 days).

Respirometry

Minocycline- or saline-treated restrained animals were individually housed in the Promethion Metabolic Monitoring System (Sable Systems International, Las Vegas, NV) for 48 hours to assess the effects of minocycline on exploratory behaviour, food intake, energy expenditure, volume of oxygen inhaled and of carbon dioxide exhaled at baseline and after chronic restraint.

16S rRNA analysis

Please see supplementary materials and methods for a detailed explanation of the methods used for the 16S rRNA analysis. Briefly, faecal pellets were collected with autoclaved toothpicks, placed in 1.5mL tubes, snap-frozen on dry ice and stored at -80°C. Following DNA extraction, faecal microbiome profiling was performed by paired-end 16S rRNA gene amplicon sequencing, based on the Illumina MiSeq platform to a depth of approximately 40,000 reads per sample. Sequence data processing was performed as previously described.[468]

Statistical analysis

Power analysis was performed based on the effect size seen in a previous pilot study for the effects of *Casp1* deficiency on total floating time in the forced swim test (our primary outcome measure). Cohen's *d* for that study was 0.78, meaning that a sample size of n=47 would result in over 80% power to detect an antidepressant-like effect at $P \leq 0.05$. Statistical analyses were performed using the Statistical Package for the Social Sciences version 23.0 for Windows (SPSS, Chicago, Illinois, USA) using a general linear model for repeated measures. The effects of genotype, stress, treatment and their interaction were explored and the significance set at $P<0.05$. Sphericity of the variances of the groups was assessed with the Mauchly's sphericity test. If the assumption of sphericity was violated, the Greenhouse-Geisser correction was generated. Effect size was reported as partial eta-squared (η^2_p). Significant stress*genotype or stress*treatment interaction was unpacked as described previously.[434] Comparison of microbiome composition between groups (beta-diversity) was performed using Bray Curtis similarity matrices in PRIMER (v6, PRIMER-E Ltd, Plymouth, UK). Matrices were generated from sample-normalized, square-root transformed, relative OTU (Operational Taxonomic Units) abundance. Community level changes were assessed for significance using one-way permutational multivariate analyses of variance (PERMANOVA) tests with 9,999 random permutations and at a significance threshold of $P<0.01$. The contribution of individual taxa to between-group variation was assessed by similarity percentages (SIMPER) analysis, as previously

reported.[435] Where specific bacterial taxa were identified as contributing to change in microbiome composition, variation in their relative abundance was further assessed through Mann-Whitney U tests between groups. Differences of median relative abundance between groups were assessed using Hodges-Lehmann estimator.

Results

Our primary outcome measure was the assessment of depressive-like behaviour in the forced swim test. Secondary outcome measures included anxiety-like behaviour, changes in the sucrose preference test, locomotor activity, gut microbiome and respirometry. Analyses and results of behavioural tests are available in supplementary tables 4.1, 4.2 and 4.3.

***Casp1* deficiency decreases depressive and anxiety-like behaviours**

Our results showed that *Casp1* deficiency decreased depressive- and anxiety-like behaviours. In the forced swim test, the total floating time was lower in *Casp1*$^{-/-}$ compared to wt mice ($F_{1,45}$=117.04, P<0.0001) (Fig. 4.1a and supplementary table 4.1). Additionally, swimming and climbing behaviours were higher in *Casp1*$^{-/-}$ mice compared to wt (respectively $F_{1,45}$=117.10, P<0.0001, and $F_{1,45}$=38.69, P<0.0001). Anxiety-like behaviour had a significant main effect of genotype in 4 tests: (1) elevated plus maze, (2) novelty suppressed feeding, (3) marble burying, and (4) open field tests. We found a significant main effect of genotype in the elevated plus maze open to closed arms time ratio ($F_{1,45}$=4.16, P=0.047) (Fig. 4.1b), suggesting an anxiolytic phenotype in *Casp1*$^{-/-}$ mice. Accordingly, in the novelty suppressed feeding, *Casp1*$^{-/-}$ mice showed decreased latency to eat in a novel environment following fasting ($F_{1,43}$=32.17, P<0.0001) (Fig. 4.1c). In the marble burying test, which is considered predictive of anxiolytic compounds,[469] we observed a decreased number of marbles buried by *Casp1*$^{-/-}$ mice ($F_{1,45}$=11.55, P=0.001) (Fig. 4.1d). Moreover, *Casp1*$^{-/-}$ mice displayed a decreased number of faecal boli during the open field test ($F_{1,45}$=4.72, P=0.035) (Fig. 4.1e), while no differences were observed for the time spent in the center area of the arena, another measure of anxiety-related behaviour ($F_{1,45}$=0.05, P=0.826). In the sucrose preference test, *Casp1*$^{-/-}$ mice displayed an increased preference for a 1% sucrose solution ($F_{1,33}$=5.52, P=0.025) (supplementary table 4.1), suggesting greater hedonic-like behaviour.

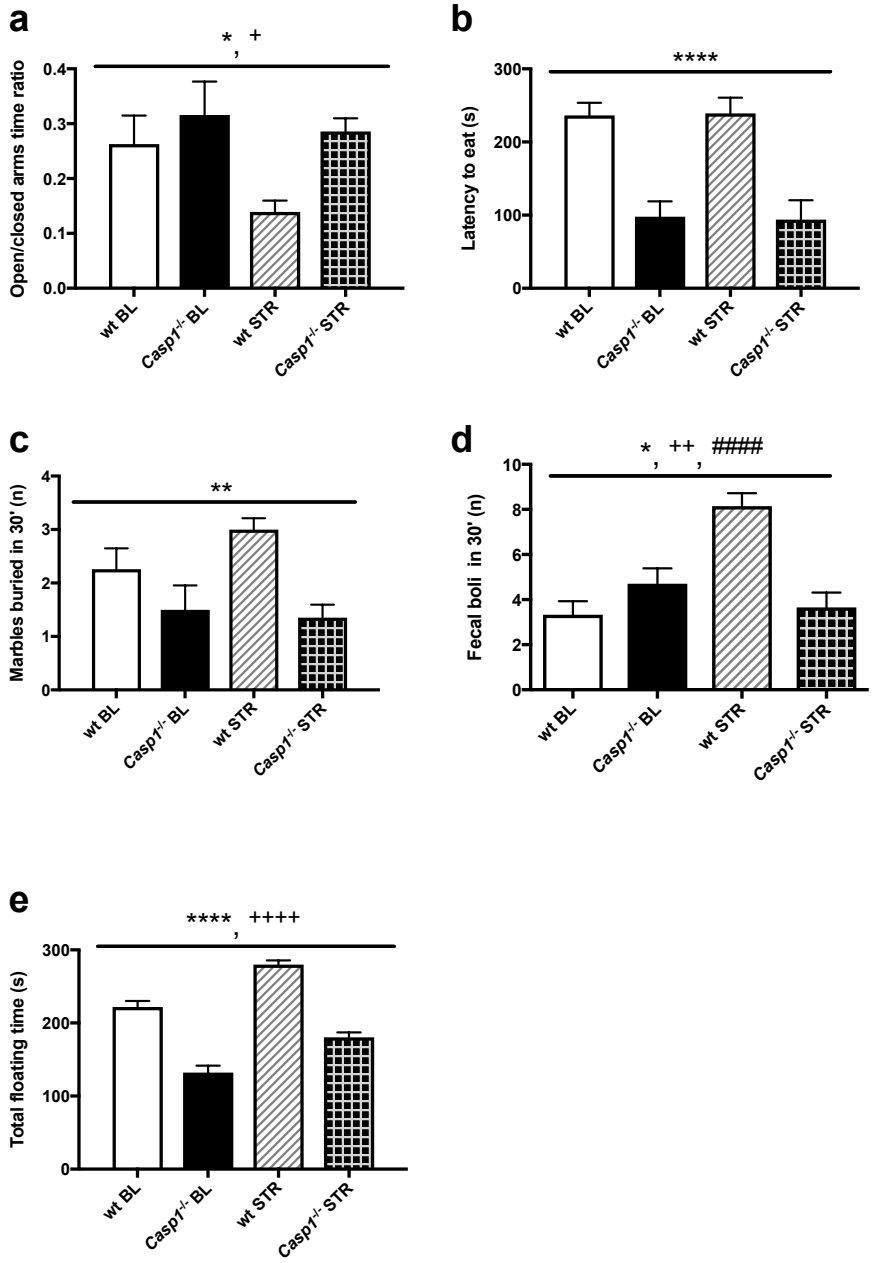

Figure 4.1. *Casp1* deficiency decreases anxiety-like and depressive-like behaviour and affects CRS response.

(**a**) *Casp1*$^{-/-}$ mice displayed decreased floating time in the forced swim test in comparison to wt) mice and (**b**) displayed decreased anxiety-like behaviour as measured by the open/closed arms time ratio in the elevated plus maze. (**c**) In the novelty suppressed feeding test, *Casp1*$^{-/-}$ mice showed significantly decreased latency to feed following 16 hours of fasting but not water deprivation. (**d**) Moreover, *Casp1* deficiency resulted in less marbles buried in the marble-burying test. (**e**) In the open field test, we observed a decreased number of faecal boli as a result of *Casp1* deficiency, as well as a different response to chronic restraint stress. Data are presented as means ± s.e.m. Genotype effect * =P<0.05; ** =P<0.01; *** =P<0.001; **** =P<0.0001; stress effect + =P<0.05; ++ =P<0.01; +++ =P<0.001; ++++ =P<0.0001; genotype*stress effect # =P<0.05; ## =P<0.01; ### =P<0.001; ####=P<0.0001. wt = wild-type; BL = baseline; STR= after CRS paradigm. (wt n=27; *Casp1*$^{-/-}$ n=20).

Casp1 deficiency affects CRS response

Our results suggest that *Casp1*$^{-/-}$ mice had an attenuated response to chronic stress. We found a significant (genotype*stress) interaction for swimming and climbing time in the forced swim test (respectively $F_{1,45}$=7.02, *P*=0.011, and $F_{1,45}$=8.60, *P*=0.005). Wt mice showed a greater decrease in swimming time (70%, $F_{1,45}$=45.48, *P*<0.0001) than *Casp1*$^{-/-}$ mice (14%, $F_{1,45}$=5.33, *P*=0.026) following stress. Accordingly, wt animals displayed a greater reduction in climbing time (91%, $F_{1,45}$=33.33, *P*<0.0001) compared to *Casp1*$^{-/-}$ mice (64%, $F_{1,45}$=78.13, *P*<0.0001) following restraint (Fig. 4.1a). We found a significant (genotype*stress) interaction for body weight changes ($F_{1,45}$=6.06, *P*=0.018), which decreased in wt mice following restraint ($F_{1,45}$=14.24, *P*<0.0001, average delta body weight= -1.3 g) but remained unchanged in *Casp1*$^{-/-}$ mice ($F_{1,45}$=0, *P*=1, average delta body weight= 0 g). Furthermore, we found a significant (genotype*stress) interaction in the number of defecations in the open field test ($F_{1,45}$=30.93, *P*<0.0001) (Fig 4.1d); *Casp1*$^{-/-}$ mice did not show an increase in this parameter following restraint ($F_{1,45}$=1.73, *P*=0.196) while wt mice did ($F_{1,45}$=48.98, *P*<0.0001).

Casp1 deficiency increases locomotion and locomotor skills

We found that *Casp1* deficiency increases locomotor activity in the open field test ($F_{1,45}$=10.54, *P*=0.002) (Fig. 4.2a). Moreover, *Casp1*$^{-/-}$ mice acquired skills more quickly than wt mice to perform in the accelerating rotarod test ($F_{1,45}$=15.35, *P*<0.0001) (Fig. 4.2b and supplementary table 4.2).

CRS increases anxiety-like and depressive-like behaviours

CRS (4h/day for 21 days) increased the floating time in the forced swimming test ($F_{1,45}$=66.92, *P*<0.0001) (Fig. 4.1a) while decreasing swimming ($F_{1,45}$=37.80, *P*<0.0001) and climbing behaviour ($F_{1,45}$=109.52, *P*<0.0001). It also increased anxiety-like behaviour in the elevated plus maze test, decreasing the time spent in the open arms ($F_{1,45}$=5.65, *P*=0.022) and the open to closed arms time ratio ($F_{1,45}$=4.55, *P*=0.038) (Fig. 4.1b), as well as in the open field test, increasing the number of defecations ($F_{1,45}$=12.74, *P*=0.001) (Fig. 4.1e). Furthermore, restraint decreased body weight gain ($F_{1,45}$=6.06, *P*=0.018) and food intake ($F_{1,43}$=5.75, *P*=0.021). Nevertheless, restrained mice showed an increase in ratio quotient (RQ, $F_{1,28}$=4.79, *P*=0.037). Following restraint, no changes were observed in the sucrose preference test ($F_{1,33}$=0.05, *P*=0.817) (supplementary table 4.1) or in locomotor activity in the open field test ($F_{1,45}$=3.64, *P*=0.063) (Fig. 4.2a).

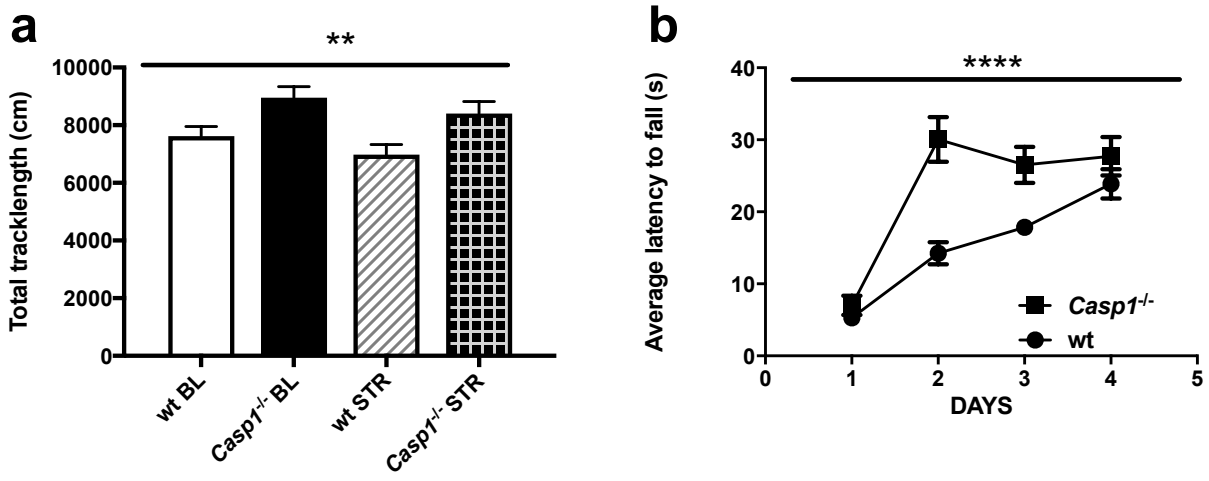

Figure 4.2. *Casp1* deficiency increases spontaneous locomotion and locomotory skills.

(**a**) *Casp1*$^{-/-}$ mice had increased locomotor activity in the open field test when compared to wt mice and (**b**) acquired more quickly the skills required to perform the rotarod test. Data are means ± s.e.m. Genotype effect * =*P*<0.05; ** =*P*<0.01; *** =*P*<0.001; **** =*P*<0.0001; stress effect + =*P*<0.05; ++ =*P*<0.01; +++ =*P*<0.001; ++++ =*P*<0.0001; genotype*stress effect # =*P*<0.05; ## =*P*<0.01; ### =*P*<0.001; ####=*P*<0.0001. wt = wild-type; BL = baseline; STR= after CRS paradigm. (wt n=27; *Casp1*$^{-/-}$ n=20).

Minocycline treatment affects stress response and metabolic parameters

We found a significant (treatment*stress) interaction in the floating time in the forced swim test ($F_{1,28}$=6.67, *P*=0.015) (Fig. 4.3a and supplementary table 4.3). Saline- and minocycline-treated animals displayed similar floating times at baseline ($F_{1,28}$=2.35, *P*=0.137); however, minocycline-treated mice were less immobile than saline-treated mice following restraint ($F_{1,28}$=5.25, *P*=0.030). No differences were observed between restrained mice receiving saline or minocycline in terms of locomotion, food intake, energy expenditure, body mass and volume of oxygen inhaled (not shown). We found a significant effect of treatment and stress on the volume of carbon dioxide exhaled (respectively $F_{1,28}$=5.64, *P*=0.025 and $F_{1,28}$=8.13, *P*=0.008) (Fig. 4.3b).

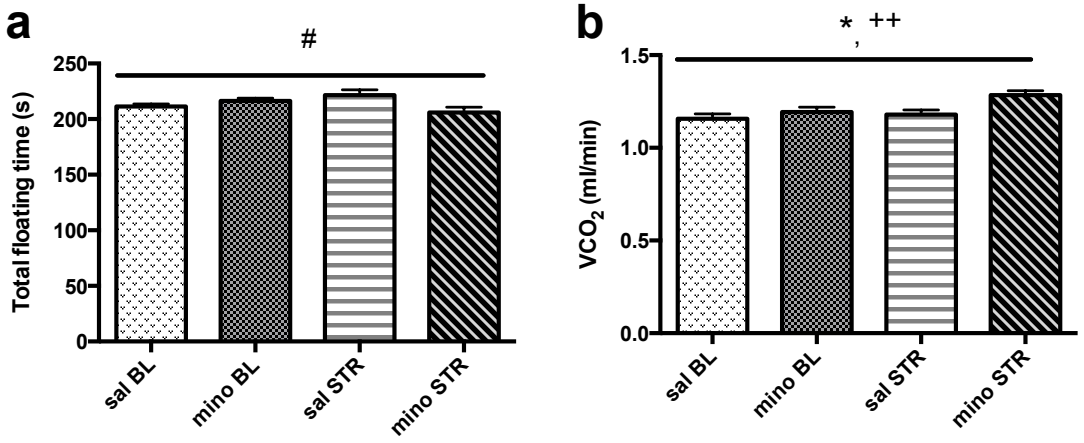

Figure 4.3. CASP1 antagonism affects CRS response.

(**a**) Minocycline treatment (mino) in wt animals during CRS (STR) prevented stress-induced increased floating time in the forced swim test. (**b**) Respirometry measurement for volume of CO_2 exhaled revealed a significant effect of stress as well as treatment. Data are means ± s.e.m. Treatment effect: * =*P*<0.05; ** =*P*<0.01; *** =*P*<0.001; **** =*P*<0.0001; stress effect: + =*P*<0.05; ++ =*P*<0.01; +++ =*P*<0.001; ++++ =*P*<0.0001; treatment*stress effect: # =*P*<0.05; ## =*P*<0.01; ### =*P*<0.001; ####=*P*<0.0001. (sal n=15, mino n=15).

CRS affects the gut microbiome

CRS (4-6h per day for 21 days) affected the gut microbiome compared to non-stressed animals (PERMANOVA *P*=0.0027, *t*=2.3492). Dysbiosis (an alteration of the relative abundance of bacterial taxa) was associated with a non-significant trend towards an increased ratio of Firmicutes to Bacteroidetes (Fig. 4.4a). In particular, restrained animals had significantly lower relative abundances of the genera *Allobaculum* (difference in median relative abundance -7.8%, *P*<0.0001 Mann Whitney U test), *Bifidobacterium* (-4.6%, *P*=0.0002), *Turicibacter* (-3.4%, *P*<0.0007), *Clostridium* (-0.7%, *P*<0.0001), and the family S24-7 (-5.8%, *P*=0.0021); and high relative abundances of the family Lachnospiraceae (+0.3%, *P*=0.0244). Variance in the relative abundance of these taxa accounted for >40% of intergroup variance.

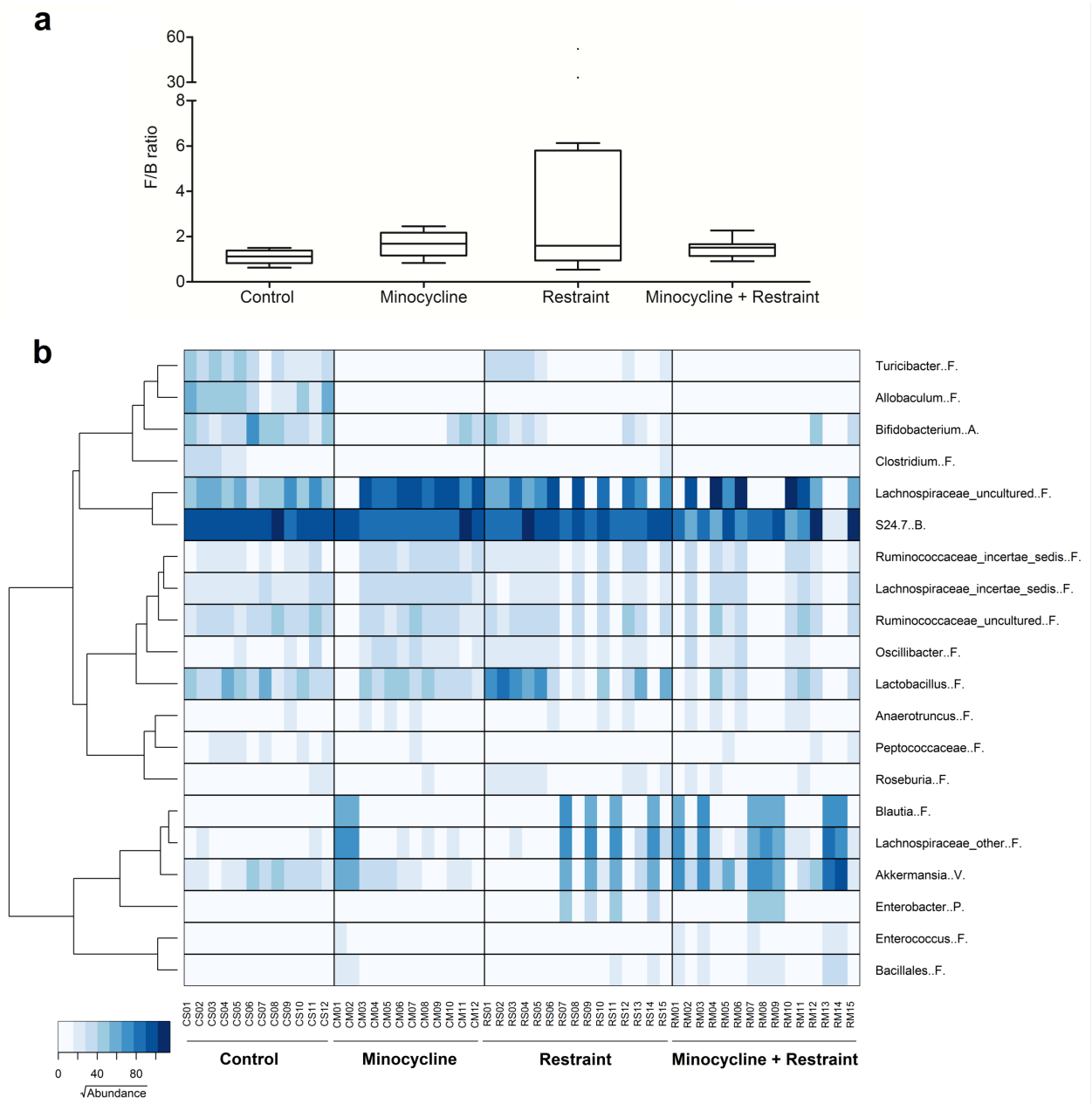

Figure 4.4. Minocycline treatment and CRS affect the gut microbiome and CRS changes the gut Firmicutes-Bacteroidetes ratio.

(**a**) Box and whiskers plot displayed the analysis of the differences of the main composition of the microbiome (Firmicutes to Bacteroidetes). Upper and lower quartiles defined the box with median midline, and the whiskers were assessed using Tukey's method. (**b**) Microbiome distribution at species level of taxon contributing to 97.5% of sample variations. Heatmap shows square root-transformed read counts for the 20 taxa determined by SIMPER analysis. The dendrogram shows the clustering of genera based on Ward's hierarchical clustering method. Phyla are abbreviated as follows: Actinobacteria (A), Bacteroidetes (B), Firmicutes (F), Proteobacteria (P) and Verrocomicrobia (V). (control n=12; minocycline n=12; restraint n=15; restraint + minocycline n=15)

Minocycline affects the gut microbiome

Minocycline treatment (5mg/kg/day/21 days) affected microbiome composition compared to saline-treated controls (PERMANOVA P=0.0001, t=3.0947) (Fig. 4.4b). In particular, minocycline-treated animals had lower relative abundances of the genera *Allobaculum* (difference in median relative abundance -7.8%, P<0.0001 Mann Whitney U test), *Bifidobacterium* (-5.8%, P<0.001), *Turicibacter* (-4.2%, P<0.0001), *Clostridium* (-0.7%, P<0.0001), and the family S24-7 (-7.4%, P=0.003); and significantly high relative abundances of the family Lachnospiraceae (+25.3%, P=0.005), and Ruminococcaceae incertae sedis (+2.4%, P=0.024). Variance in the relative abundance of these taxa accounted for >67% of intergroup variance.

Effect of CRS on the gut microbiome when combined with minocycline

Combining chronic restraint with minocycline treatment resulted in a microbiome composition that was different to that in non-restrained saline-treated controls (PERMANOVA P=0.0002, t=3.4593) (Fig. 4.4b). When assessed globally, the differences in composition between chronic restraint, minocycline-treated animals and animals that received each treatment alone, were not significant (given a PERMANOVA threshold of P<0.01). However, when assessed at the level of individual taxa, there was evidence of individual, synergistic, and antagonistic effects of restraint and minocycline. For example, significant reductions in the relative abundance of both *Turicibacter* and *Bifidobacterium* were observed in both restrained and minocycline-treated animals (Fig. 4.5a-b). In contrast, a positive additive effect of the two treatments was observed for other taxa, including *Akkermansia*, *Blautia*, and members of Lachnospiraceae (Fig. 4.5c-e). Evidence of an antagonistic effect between minocycline and restraint was observed for *Lactobacillus* and *Anaerovorax*, with increased abundance observed in restrained animals, but a reduction in relative abundance when minocycline was also administered (Fig. 4.5f-g).

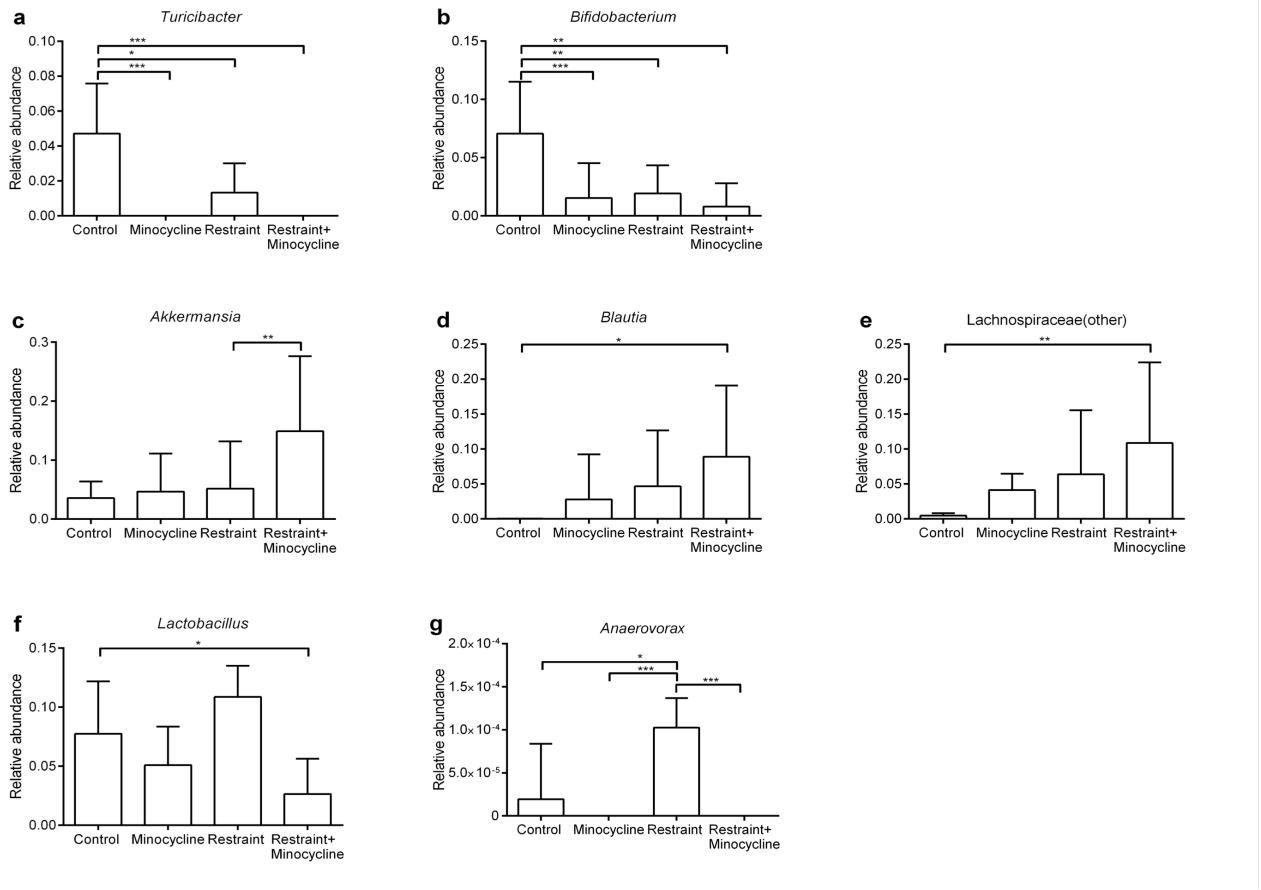

Figure 4.5. The effect of minocycline treatment, CRS and their combination assessed at the level of individual taxa.

Individual minocycline effect on the (**a**) *Turicibacter* and (**b**) *Bifidobacterium* populations; Synergistic effect of minocycline and CRS on the (**c**) *Akkermansia*, (**d**) *Blautia*, and (**e**) Lachnospiraceae populations; and antagonistic effect of minocycline and CRS on the (**f**) *Lactobacillus* and (**g**) *Anaerovorax* populations. Significant difference between treatment groups are represented with asterisks: * =P<0.05, **=P<0.01, ***=P<0.001. (control n=12; minocycline n=12; restraint n=15; restraint + minocycline n=15).

Discussion

CASP1 is a cysteine protease that cleaves pro-IL1B and pro-IL18 into their mature isoforms in the NLRP3 inflammasome in response to stressful stimuli such as psychosocial and microbial stress, ATP, toxins and particulate matter.[235,470] Since *Casp1*$^{-/-}$ mice lack CASP1 mRNA and its mature protein product, they have decreased inflammasome bioactivity and inflammasome-driven IL1B and IL18 production, and could be helpful in identifying the role of CASP1 in behaviour, either innate or following stress-induced inflammasome activation.[147] Our data highlights a role for CASP1 in both the modulation of innate behaviour and in the response to chronic stress, since CASP1 modulation decreased baseline anxiety- and depressive-like behaviours and the exacerbation of depressive-like behaviours following chronic restraint stress. Our results

are in line with studies reporting that modulation of IL1B-mediated pathways could potentially attenuate the behavioural and molecular effects of stress-induced inflammation.[151,471] Our findings strengthen the role for CASP1 as a potential target for therapies aiming at modulating inflammasome-mediated pathways in psychiatric disorders.

Minocycline exerts anti-inflammatory and neuroprotective effects in animal models of neurodegenerative disorders, neurotoxicity and brain injury, in addition to presenting acute antidepressant-like effects in the forced swim test by increasing climbing, potentially through interaction with glutamatergic and/or noradrenergic systems.[92,95-97] These antidepressant-like effects might also be related to the protection of serotonergic and dopaminergic circuitries.[93,472] Consistent with this literature, we found that minocycline prevented the exacerbation of depressive-like behaviour in the forced swim test following chronic restraint stress. Given this finding, we suggest that minocycline may be valuable in the treatment of MDD and other psychiatric disorders. Indeed, a proof-of-concept trial investigating minocycline augmentation for MDD reported improved global impression, functioning and quality of life even though the primary outcome measure (Montgomery–Asberg Depression Rating Scale) was not affected.[98] Similarly, two clinical trials investigating minocycline as a stand-alone or adjuvant treatment in psychotic depression and schizophrenia yielded promising results.[100-103]

CRS significantly altered gut microbiome composition. Changes included a substantial, although not statistically significant, increase in the ratio of Firmicutes to Bacteroidetes. Similar changes have been described in IBS patients and in animal models of hypertension,[473,474] two conditions associated with chronic low-grade inflammation.[475,476] Further, significant changes were observed in microbiome composition at the genus level. For example, levels of *Bifidobacterium*, a genus associated with the suppression of inflammation through inhibition of the NFKB1 pathway,[477] were significantly reduced in animals undergoing restraint. This supports the notion that NFKB1 is increased in response to stress and is a critical mediator of stress-induced depressive-like behaviour and stress-impaired neurogenesis.[478] The genus *Allobaculum* was absent in restrained animals, despite representing a substantial component of the microbiome in control animals. *Allobaculum* consists of mucin-degrading bacteria, whose relative abundance is inversely correlated with dietary-induced inflammation markers, including leptin and IL22.[479,480] Conversely, chronic restraint led to an increase in the relative abundance of *Lactobacillus*. Bacteria from this genus are implicated in inflammasome activation through stimulation of CASP1-dependent IL1B production by macrophages,[481] and the abundance

shift of this genus in response to stress could be responsible for the increased IL1B levels observed in depression and in animal models of stress.[64,300,482]

Minocycline treatment also significantly altered microbiome composition. Dysbioses induced by minocycline and restraint separately were not significantly different when assessed on a microbiome-wide level: a finding that may stem from the dual role of minocycline as antibiotic and inhibitor of CASP1. However, when restrained mice were treated with minocycline, evidence of both synergistic and antagonistic effects on microbiome composition was observed. For example, *Akkermansia*, *Blautia*, and an uncultured member of the Lachnospiraceae family, were significantly increased in mice undergoing concomitant restraint and minocycline treatment, despite non-significant increases with either treatment in isolation. This effect is notable given that *Akkermansia* attenuates inflammation in adipose tissue through induction of Foxp3 T-reg cells, and suppression of IL6 and IL1B.[483-485] Moreover, a similar increase in *Akkermansia* was reported in a study in which minocycline rebalanced the gut microbiome in a rat model of hypertension.[473] Lachnospiraceae is one of the most abundant families of Firmicutes and is associated with beneficial production of SCFAs from complex polysaccharides.[486] An increase in Lachnospiraceae relative abundance in minocycline-restraint animals is consistent with changes in the gut microbiome of *Casp1*$^{-/-}$ mice.[466]

A significant effect of restraint and minocycline was also observed for *Lactobacillus,* which was reduced in animals receiving both treatments, but not in animals receiving either treatment in isolation. Previous inflammasome studies have reported a significant reduction in *Lactobacillus* in *Casp1* and *Nlrp6* deficient mice compared to wt.[466,487] Consistent with this, relative abundance of *Lactobacillus* in minocycline-treated mice trended downwards, while in restrained mice not receiving minocycline it trended upwards. The genus Anaerovorax was also significantly increased in chronic-restraint mice, but was absent in mice receiving minocycline, whether undergoing chronic restraint or not. Relatively little is known about the role of this genus in host physiology, and given its low abundance in the microbiome, this finding should be interpreted with caution.

This study has several limitations. In this study, we chose minocycline as a CASP1 inhibiting compound to further explore the relationship between the microbiome and host behaviour.[461,462] Minocycline is an antibiotic, and would therefore probably affect the gut microbiome regardless of CASP1 inhibition.[488] Since minocycline has antidepressant effects, its influence on the gut microbiome could be at least partially responsible for such

effects.[93,458,472] Further work is required to elucidate the exact mechanisms of gut microbiome-host interactions, including the analysis of SCFAs production. The dysbioses we observed are likely to translate into complex metabolomic shifts; a metabolomic profiling approach to investigate this is beyond the scope of the work presented here. It could be argued that the withdrawal of food and water during the restraint period might impact the microbiome.[489] However, where food is withheld for a short period, substrates for microbial fermentation will continue to transit the gastrointestinal tract for some time, and a resultant substantial microbiome shift is unlikely. Mice were submitted twice to a battery of behavioural tests; exposure to one test could impact subsequent test performance.[467,490] However, we tried to minimize bias by performing the tests from the least to the most invasive and by giving the animals recovery time in between tests to minimize the influence of previous tests.[467,490] Our CRS paradigm did not decrease sucrose preference, which replicates published findings.[491] While this test is considered a model of clinical anhedonia, it has highly variable outcomes, even within the same facility.[177] Finally, it was reported that *Casp1*$^{-/-}$ mice generated using strain 129 embryonic stem cells are *Casp1*, *Casp11* double knockouts, since *Casp1* and *Casp11* are neighbouring on the genome and are too close to segregate by recombination.[147,152,153] The findings in this study should therefore be interpreted as the result of lacking both *Casp1* and *Casp11* rather than *Casp1* alone.

In summary, our findings suggest that CASP1 inhibition has a protective effect on the stress response by modulating the interface between stress and microbiome composition. This supports the concept of a microbiome-gut-inflammasome-brain (MGIB) axis, in which the gut microbiome modulates inflammatory pathways, via the inflammasome signalling platform, that will alter brain function and affect depressive- and anxiety-like behaviours. Reduction of inflammasome bioactivity may represent a feasible therapeutic strategy in the treatment of MDD and other neuropsychiatric disorders with inflammatory components, through modulation of the gut microbiome. Future studies should address the tolerability, safety and long-term effects of inflammasome modulation as a therapeutic strategy, and the effects of its discontinuation.

ACKNOWLEDGMENTS

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Adelaide, Australia). We acknowledge the support of Neil Dear, Sian Dear and their team at SAHMRI Research and Biomedical Services in the conduct of the animal experiments.

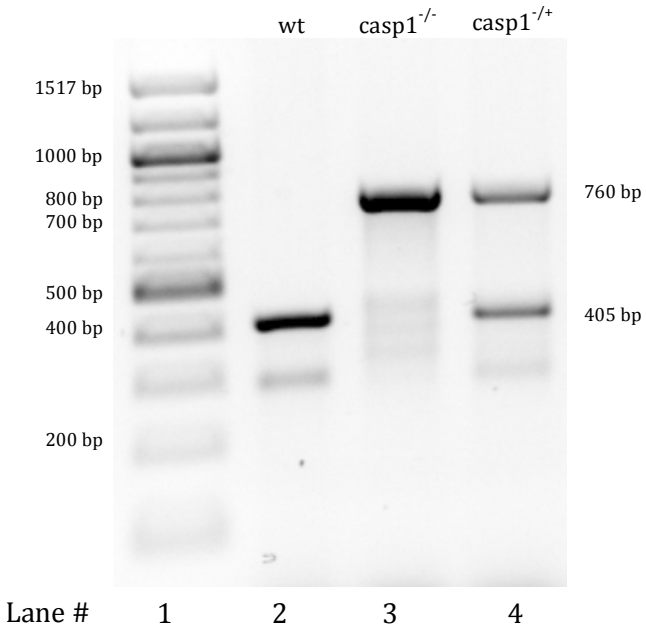
CONFLICT OF INTEREST

The authors declare no conflict of interest.

Supplementary figures

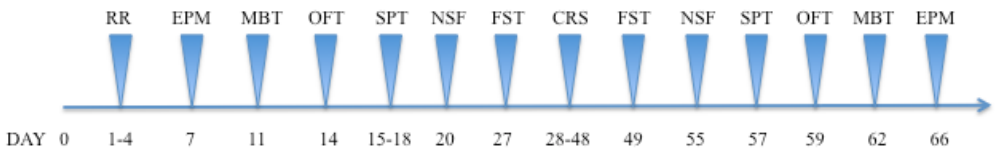
Supplementary figure 4.6. Confirmation of *Casp1* genetic deficiency.

Casp1 genetic deficiency was confirmed by endpoint PCR with primers designed to amplify either the *Casp1* gene (for wt and heterozygous *Casp1*$^{-/+}$ mice) or the *neo* cassette (for homozygous *Casp1*$^{-/-}$ and heterozygous *Casp1*$^{-/+}$ mice) used to inactivate the *Casp1* gene. Wt mice display one band at 405 bp (second lane), *Casp1*$^{-/-}$ mice displays one band at 760 bp (third lane) and *Casp*$^{-/+}$ mice display two bands, one at 405 bp and one at 760 bp (fourth lane).



Supplementary figure 4.7. Timeline of behavioural experiments.

RR= rotarod test; EPM= elevated plus maze test; MBT= marble burying test; OFT= open field test; SPT= sucrose preference test; NSF= novelty suppressed feeding test; FST= forced swim test; CRS= chronic restraint stress.



Supplementary methods

Experiment 1: Baseline and post chronic restraint-stress behaviour of Casp1 knockout vs. wt mice. Wt (n=27) and *Casp1*$^{-/-}$ (n=20) mice were tested in the rotarod, elevated plus maze, marble burying test, open field test, sucrose preference test, novelty suppressed feeding and forced swim test. Subsequently, they were subject to CRS for 21 days (4-6 hours/day), and the behavioural tests were performed in the reverse order, to obtain a bell-shaped stress exposure (see supplementary Fig. 4.7 for a timeline of the tests). The number of experimental animals was decided based on prior pilot studies carried out to determine the adequate size effect.

Experiment 2: Baseline and post chronic restraint-stress behaviour and metabolic parameters of wt mice with concomitant minocycline treatment and resulting changes in the gut microbiome. Fifty-four wt mice were randomly (by drawing cage numbers from a hat) assigned to one of four groups: 1) Restraint + Minocycline n=15, 2) Restraint + Saline n=15, 3) Minocycline n=12, 4) Saline n=12. Fifteen minocycline-treated wt mice and 15 saline-treated wt mice as control were tested in the forced swim test to assess depressive-like behaviour and subject to respirometry assessment in metabolic cages (Sable Systems International, Las Vegas, NV) at baseline. Subsequently, they underwent CRS (21 days, 4-6 hours/day) and concomitant daily treatment with minocycline [5mg/kg/d in 10mL/kg saline, (i.p.)], or saline [0.2mL, intraperitoneally (i.p.)]. After the restraint procedure concluded, the pharmacological treatment was interrupted and the mice were tested in the forced swim test to assess the effects of chronic minocycline treatment on the exacerbation of depressive-like symptoms. Moreover, the mice were subject to respirometry assessment to quantify metabolic changes in response to concomitant restraint and minocycline treatment. An additional 12 minocycline- and 12 saline-treated mice were used as controls. These mice were tested at baseline in the forced swim test and subject to respirometry, then treated for 21 days with either minocycline or saline and left undisturbed in their home cage (without undergoing restraint). Subsequently, they underwent forced swim test and metabolic monitoring to assess the behavioural and metabolic effects of each treatment alone. Faecal pellets were collected from all mice at the end of the experimental procedures and used to assess the composition of faecal microbial communities in response to CRS and pharmacological minocycline treatment.

Rotarod test

Mice were tested for four days on the rotarod apparatus (Harvard Bioscience, Holliston, MA, USA) to assess locomotor coordination, balance and motor skill learning.[442] Animals

were given 3 trials per day with 5 minutes rest in their home cage between trials. The test was performed by placing one mouse at the time on a rotating drum and measuring the latency to fall from the apparatus. The apparatus was set on the accelerating mode (4 to 40 rpm in 120 seconds). The average latency to fall from the apparatus for each mouse over three trials was used for the statistical analysis.

Elevated plus-maze test

Animals were placed in the central square (10cm x 10cm) of a plus-shaped maze with two open and two closed arms (30cm long, 10cm wide, 20cm walls, 1m above the ground). The time spent in each of the arms was detected by a camera coupled to the Biobserve viewer II software (Biobserve, St. Augustin, Germany). An entry in an arm required the animal to enter that arm with all four paws. The total time spent in the open arms and the ratio open/closed arms time were used as anxiety measures, because anxiolytic drugs decrease such parameters.[444,445]

Marble burying test

Sixteen marbles were placed 4cm apart in a cage containing 5cm of regular bedding to quantify digging and burrowing behaviours. Animals were placed in a corner facing the center. After 30 minutes the number of marbles completely buried was recorded and used as a measure of anxiety, because anxiolytic drugs decrease this measure.[440]

Open field test

Animals were placed in the center of a novel, brightly lit arena (50cm x 50cm x 50cm) and their locomotor activity was recorded for 30 minutes by a camera coupled to the Biobserve Viewer II software (Biobserve, St. Augustin, Germany). Total distance (locomotor activity) and the time spent in the center of the arena (center time) were used to quantify locomotor activity and anxiety-like behaviour. The number of defecations was recorded and used as an index of emotionality.[443,444]

Sucrose preference test

Mice were individually housed and given 2 identical drinking bottles containing a 1% sucrose solution in standard drinking water for 24h in order to familiarize them to the novel drink. In the following day, one of the bottles was replaced with a bottle containing standard drinking water and mice were given a free choice to drink from either bottle for 48 hours (training). On the fourth day (test day), the amount of liquid drunk from each bottle was recorded and sucrose preference was calculated as the percentage of the volume of sucrose drunk over the total volume of fluid drunk.[176,177]

Novelty suppressed feeding

Animals were fasted for 16 hours in clean cages prior to this test. Subsequently they were individually placed in a corner of a brightly lit arena (60cm x 40cm x 40cm) containing 5cm of regular bedding and one single pellet of regular chow in the center. The latency to eat (the time it took the animal to stand next to the food on its rear paws, hold the pellet with its front paws and bite it) was recorded with a stopwatch. Animals that didn't eat within fifteen minutes were excluded from the analysis.[446]

Forced swim test

In order to quantify depressive-like behaviour, mice were individually placed in an open, clear Plexiglas cylindrical container (40cm tall, 20cm diameter) containing 20cm of water at 23°C ± 1°C. A camera coupled with the FST High-throughput Forced Swim Test Analysis software (Biobserve, St. Augustin, Germany) was used for the detection of active (climbing and swimming) and inactive (floating) behaviour twice a second for 5 minutes.[438,439]

16S rRNA analysis

Faecal pellets were collected with autoclaved toothpicks, placed in 1.5mL tubes, snap-frozen on dry ice and stored at -80°C. DNA extraction was performed using PowerSoil DNA Isolation Kit (MO BIO Laboratories, Inc., Carlsbad, CA, USA) optimized for the Biomek 4000 Automation Workstation (Beckman Coulter Inc., Lane Cove, NSW, Australia). Faecal microbiome profiling was performed by paired-end 16S rRNA gene amplicon sequencing, based on the Illumina MiSeq platform. The V4 hypervariable region of the bacterial 16S rRNA gene was amplified from faecal DNA extracts using modified universal bacterial primer pairs 515F (5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGGTGCCAGCMGCCGCGGTAA-3') and 806R (5'-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGGACTACHVGGGTWTCTAAT-3'), with Illumina adapter overhang sequences. Barcoded Amplicons were generated, cleaned, indexed and sequenced according to the Illumina MiSeq 16S Metagenomic Sequencing Library Preparation protocol (http://support.illumina.com/downloads/16s_metagenomic_sequencing_library_preparation.html) with modifications. Briefly, an initial PCR reaction contained at least 12.5 ng DNA, 5 µL of forward primer (1 µM), 5 µL of reverse primer (1 µM) and 12.5 µL of 2x KAPA HiFi Hotstart ReadyMix (KAPA Biosystems, Wilmington, MA, USA) in a total volume of 25 µL. The PCR reaction was performed on a Veriti 96-well Thermal Cycler (Life Technologies, Scoresby, Australia) using the following program: 95°C for 3 min, followed by 25 cycles of

95°C for 30 sec, 55°C for 30 sec and 72°C for 30 sec and a final extension step at 72°C for 5 min. Amplicons were indexed for multiplexing using the dual-index approach of the Nextera XT Index Kit (Illumina, San Diego, CA, USA) and cleaned using Agencourt AMPure XP (Beckman Coulter). Library preparation QC involved Qubit dsDNA High Sensitivity assay for quantitation and Bioanalyzer using Agilent High Sensitivity DNA Kit (Agilent Technologies, Santa Clara, CA, USA) for amplicon assessment. The final library was paired-end sequenced at 2 x 300 bp using a MiSeq Reagent Kit v3 on the Illumina MiSeq platform. Sequencing was performed at the David R Gunn Genomics Facility, South Australian Health and Medical Research Institute. Sequencing was performed to a depth of approximately 40,000 reads per sample using the Illumina MiSeq platform. Sequence data processing was performed as described previously.[468]

Supplementary tables

Supplementary table 4.1. Statistical report of *Casp1*$^{-/-}$ vs. wt mice behavioural results.

Values in columns 3-6 are means ± s.e.m. BL=baseline; STR=stress; df=degrees of freedom; G=genotype effect; S=stress effect; GxS=genotype x stress interaction; η^2_p=partial eta squared; *=P<0.05; **=P<0.01; ***=P<0.001; ****=P<0.0001.

TEST	MEAUSRE	wt BL (n=27)	casp1-/- BL (n=20)	wt STR (n=27)	casp1-/- STR (n=20)	MAUCHLY'S W	df	F-TEST	*P*-VALUE	Partial Eta Squared
Elevated plus maze	Open/closed arms time (ratio)	0.263 ± 0.052	0.316 ± 0.061	0.139 ± 0.021	0.286 ± 0.024	1.000	1,45	G F=4.16	G *P*=0.047 *	G η2p=0.085
						1.000	1,45	S F=4.55	S *P*=0.038 *	S η2p=0.092
						1.000	1,45	GxS F=1.703	GxS *P*=0.199	GxS η2p=0.036
Elevated plus maze	Open arms time (s)	51.089 ± 6.088	54.525 ± 7.073	32.67 ± 3.064	52.225 ± 3.56	1.000	1,45	G F=3.71	G *P*=0.060	G η2p=0.076
						1.000	1,45	S F=5.65	S *P*=0.022 *	S η2p=0.112
						1.000	1,45	GxS F=3.42	GxS *P*=0.071	GxS η2p=0.071
Novelty suppressed feeding	Latency to eat (s)	236.407 ± 17.131	97.889 ± 20.980	239.074 ± 21.505	93.944 ± 26.338	1.000	1,43	G F=32.17	G *P*<0.0001 ****	G η2p=0.428
						1.000	1,43	S F=0.01	S *P*=0.972	S η2p=0.000
						1.000	1,43	GxS F=0.034	GxS *P*=0.854	GxS η2p=0.001
Novelty suppressed feeding	Food intake (g in 5')	0.159 ± 0.06	0.181 ± 0.08	0.143 ± 0.06	0.163 ± 0.008	1.000	1,43	G F=9.34	G *P*=0.004 **	G η2p=0.178
						1.000	1,43	S F=5.75	S *P*=0.021*	S η2p=0.118
						1.000	1,43	GxS F=0.02	GXS *P*=0.896	GxS η2p=0.000
Marble burying test	Marbles buried (n)	2.259 ± 0.391	1.500 ± 0.455	3 ± 0.212	1.35 ± 0.246	1.000	1,45	G F=11.55	G *P*=0.001 **	G η2p=0.204
						1.000	1,45	S F=0.81	S *P*=0.372	S η2p=0.018
						1.000	1,45	GxS F=1.85	GXS *P*=0.181	GxS η2p=0.039
Open field test	Defefecations (n)	3.333 ± 0.592	4.7 ± 0.688	8.148 ± 0.572	3.65 ± 0.665	1.000	1,45	G F=4.72	G *P*=0.035 *	G η2p=0.095
						1.000	1,45	S F=12.74	S *P*=0.001 **	S η2p=0.221
						1.000	1,45	GxS F=30.93	GXS *P*<0.0001****	GxS η2p=0.407
Open field test	Centre time (s)	112.07 ± 10.871	117.885 ± 12.631	108.304 ± 13.956	110.07 ± 16.215	1.000	1,45	G F=0.05	G *P*=0.826	G η2p=0.001
						1.000	1,45	S F=0.45	S *P*=0.504	S η2p=0.010
						1.000	1,45	GxS F=0.06	GXS *P*=0.815	GxS η2p=0.001
Open field test	Locomotor activity (cm)	7621.707 ± 331.197	8952.625 ± 384.816	6976.396 ± 356.673	8405.457 ± 414.417	1.000	1,45	G F=10.54	G *P*=0.002 **	G η2p=0.190
						1.000	1,45	S F=3.64	S *P*=0.063	S η2p=0.075
						1.000	1,45	GxS F=0.03	GXS *P*=0.876	GxS η2p=0.001
Sucrose preference test	Sucrose preference (%)	76.722 ± 1.609	89.136 ± 1.656	79.834 ± 4.967	84.199 ± 5.111	1.000	1,33	G F=5.52	G *P*=0.025 *	G η2p=0.143
						1.000	1,33	S F=0.05	S *P*=0.817	S η2p=0.002
						1.000	1,33	GxS F=1.06	GXS *P*=0.311	GxS η2p=0.031
Forced swim test	Floating (s)	221.978 ± 8.184	132.069 ± 9.509	279.863 ± 5.838	180.325 ± 6.784	1.000	1,45	G F=117.04	G *P*<0.0001 ****	G η2p=0.722
						1.000	1,45	S F=66.92	S *P*<0.0001 ****	S η2p=0.598
						1.000	1,45	GxS F=0.55	GXS *P*=0.462	GxS η2p=0.012
Forced swim test	Swimming (s)	62.864 ± 6.197	129.652 ± 7.200	18.826 ± 5.431	112.139 ± 6.310	1.000	1,45	G F=117.10	G *P*<0.0001 ****	G η2p=0.722
						1.000	1,45	S F=37.80	S *P*<0.0001 ****	S η2p=0.457
						1.000	1,45	GxS F=7.02	GXS *P*=0.011 *	GxS η2p=0.135
Forced swim test	Climbing (s)	15.168 ± 2.667	38.278 ± 3.099	1.31 ± 1.634	13.625 ± 1.898	1.000	1,45	G F=38.69	G *P*<0.0001 ****	G η2p=0.462
						1.000	1,45	S F=109.52	S *P*<0.0001 ****	S η2p=0.709
						1.000	1,45	GxS F=8.60	GXS *P*=0.005 **	GxS η2p=0.161
Body weight	Mass (g)	29.568 ± 0.42	32.863 ± 0.488	28.27 ± 0.363	32.863 ± 0.421	1.000	1,45	G F=53.22	G *P*<0.0001 ****	G η2p=0.542
						1.000	1,45	S F=6.06	S *P*=0.018 *	S η2p=0.119
						1.000	1,45	GxS F=6.06	GXS *P*=0.018 *	GxS η2p=0.119

Supplementary table 4.2. Statistical report of *Casp1*$^{-/-}$ vs. wt mice rotarod tests results.

Values in columns 4-5 are means ± s.e.m. (in seconds). df=degrees of freedom; G=genotype effect; η^2_p=partial eta squared; *=P<0.05; **=P<0.01; ***=P<0.001; ****=P<0.0001.

TEST	MEASURE	DAY	wt (n=27)	casp1-/- (n=20)	MAUCHLY'S W	df	F-TEST	*P*-VALUE	Partial Eta Squared
Rotarod	Latency to fall (s)	1	4.877 ± 1.060	7.000 ± 1.231	0.777 (*P*=0.051)	1,45	G F=15.35	*G P*<0.0001 ****	G η2p=0.254
		2	14.383 ± 2.134	30.050 ± 2.479					
		3	17.889 ± 1.681	26.500 ± 1.953					
		4	24.346 ± 2.201	27.700 ± 2.558					

Supplementary table 4.3. Statistical report of minocycline- (mino) vs. saline-treated (sal) wt mice tests results.

Values in columns 3-6 are means ± s.e.m.. BL=baseline; STR=stress; df=degrees of freedom; T=treatment effect; S=stress effect; TxS=treatment x stress interaction; η^2_p=partial eta squared; *=P<0.05; **=P<0.01; ***=P<0.001; ****=P<0.0001.

TEST	MEAUSRE	wt sal BL (n=15)	wt mino BL (n=15)	wt sal STR (n=15)	wt mino STR (n=15)	MAUCHLY'S W	df	F-TEST	*P*-VALUE	Partial Eta Squared
Forced swim test	Floating (s)	211.305 ± 2.376	216.453 ± 2.376	221.512 ± 4.801	205.956 ± 4.801	1.000	1,28	T F=2.14	T *P*=0.154	T η2p=0.071
						1.000	1,28	S F=0.01	S *P*=0.971	S η2p=0.000
						1.000	1,28	TxS F=6.68	TxS *P*=0.015 *	TxS η2p=0.193
Respirometry	VCO2 (ml/min)	1.157 ± 0.026	1.193 ± 0.026	1.18 ± 0.024	1.284 ± 0.024	1.000	1,28	T F=5.64	T *P*=0.025 *	T η2p=0.168
						1.000	1,28	S F=8.13	S *P*=0.008 **	S η2p=0.225
						1.000	1,28	TxS F=2.89	TxS *P*=0.100	TxS η2p=0.093
Respirometry	RQ (ratio)	0.825 ± 0.011	0.816 ± 0.011	0.831 ± 0.008	0.850 ± 0.008	1.000	1,28	T F=0.22	T *P*=0.644	T η2p=0.008
						1.000	1,28	S F=4.79	S *P*=0.037 *	S η2p=0.146
						1.000	1,28	TxS F=2.35	TxS *P*=0.136	TxS η2p=0.078

Chapter 5 - Mice lacking Casp1, Ifngr and Nos2 genes exhibit decreased depressive- and anxiety-like behaviour and altered stress responsiveness

Abstract

Converging evidence suggests an involvement of pro-inflammatory pathways in a subset of MDD patients. Pre-clinical and clinical studies suggest that decreasing pro-inflammatory signalling might prove useful in the treatment of MDD. In this investigation, we used mice with simultaneous genetic deficiency of *Casp1*, *Ifngr* and *Nos2* (*Casp1, Ifngr, Nos2*)$^{-/-}$ to assess depressive- and anxiety-like phenotypes at baseline and following 4 weeks of CUMS exposure. Moreover, we assessed the levels of ACTH and CORT following stress. We found that (*Casp1, Ifngr, Nos2*)$^{-/-}$ mice display decreased depressive- and anxiety-like behaviour and increased hedonic-like behaviour while displaying increased locomotor activity. Moreover, we found that *Casp1, Ifngr, Nos2* deficiency prevented the exacerbation of anhedonic-like behaviour mice following stress exposure. Furthermore, (*Casp1, Ifngr, Nos2*)$^{-/-}$ mice showed a heightened emotional state following chronic stress. Finally, the plasma levels of ACTH and CORT were not different in (*Casp1, Ifngr, Nos2*)$^{-/-}$ mice compared to wt mice following stress. Together, our results suggest that simultaneously targeting multiple pro-inflammatory pathways could represent a valuable approach in MDD therapies. Randomized controlled trials should investigate the safety of such approach in clinical settings and its effectiveness as an antidepressant strategy. If these studies prove successful, inhibiting pro-inflammatory signalling in MDD patients with dysregulated inflammatory pathways could represent a novel stand-alone or adjuvant therapy in MDD.

Keywords:

Major depressive disorder, MDD, inflammation, NLRP3, CASP1, nitric oxide synthase, interferon gamma, interferon gamma-receptor, chronic unpredictable mild stress, antidepressant.

Introduction

Increasing evidence suggests an involvement of neuroinflammatory pathways in the development, treatment and remission of MDD.[58,64] Clinical depressive symptoms are underlined by a serotonergic deficiency and a glutamatergic overproduction, potentially due to a chronic and systemic low-grade cell-mediated inflammatory response.[64,68] This long-lasting immune activation seems to be characterized, in at least a subset of patients, by increased levels of Th1-related cytokines [mostly pro-inflammatory, such as IL1, IL2, IL12 and IFNG] and TNF and decreased levels of Th2-related cytokines (mostly anti-inflammatory, such as IL4, IL10, and IL13).[68] Dysregulation of three major inflammatory systems seem to exist in MDD patients: a) increased oxidative stress by means of NO overproduction driven by NOS2, b) low-grade chronic pro-inflammatory status driven by CASP1 overproduction and NLRP3 inflammasome over activation and c) Th1 lymphocyte infiltration in the CNS driven by INFG (please see chapter 2 for further details).

In order to assess the effects of simultaneously decreasing the levels of these three pro-inflammatory mediators, we generated a triple knockout mouse model lacking *Casp1*, *Ifngr* and *Nos2*, to assess the effects of lowered Th1 activity on: a) baseline behaviour, b) the response to CUMS, and c) HPA axis response to stress as measured by the levels of circulating ACTH and CORT.

Materials and methods

All procedures were approved by the South Australian Health and Medical Research Institute Ethics committee and are in accordance with the Australian Code for the Care and Use of Animals for Scientific Purposes (8th edition, 2013). All efforts were made to minimize animal suffering. Male C57BL/6J mice (wt, n=16) aged 60 days were obtained from the South Australian Health and Medical Research Institute Bioresources Facility (Adelaide, Australia). Age-matched (*Casp1, Ifngr, Nos2*)$^{-/-}$ mice (n=20) with C57/BL6J background were generated by backcrossing mice with single gene deletion and selecting mice that were homozygous for each of the required genes (see supplementary Fig. 5.6 for an example of endpoint PCR genotyping). After seven days acclimatization, mice were single housed in transparent Plexiglas cages (Green Line IVC Sealsafe PLUS mouse, Tecniplast, Varese, Italy) in a temperature (21°C ±1°C), humidity (50%) and light (12 hour cycles, lights on at 7:00 am) controlled room with water and food *ad libitum*. Behavioural testing was performed in the light phase of the light cycle between 9:00 am to 4:00 pm. At the endpoint of the experiment, mice were euthanized by cervical dislocation and blood

was collected by cardiac puncture in ethylenediaminetetraacetic acid coated tubes. For a timeline of the experimental procedures, see Fig. 5.1.

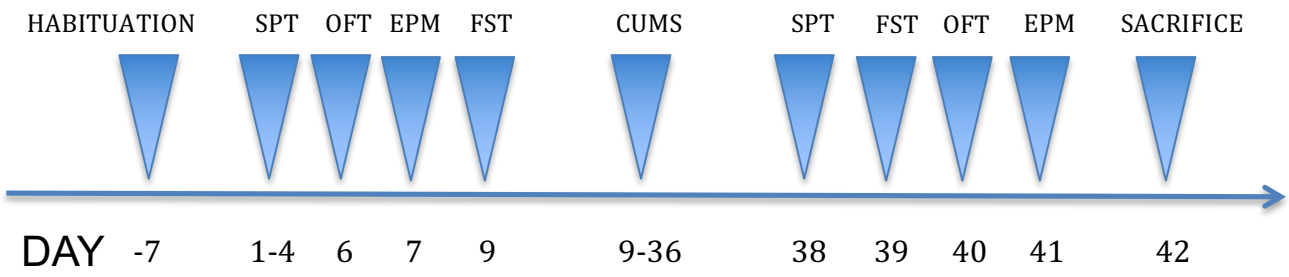


Figure 5.1. Timeline of behavioural experiments performed in study 2.

SPT= sucrose preference test; OFT= open field test; EPM= elevated plus maze test; FST= forced swim test; CUMS= chronic unpredictable mild stress.

CUMS

The CUMS procedure used in this study is a variation of the procedure previously described as a naturalistic model of depression in rodents (please see chapter 1 for details).[176]

Day #	Stress Day #	Stress 1/Procedure	Stress 2
1		Sucrose preference test	
2		Sucrose preference test	
3		Sucrose preference test	
4		Sucrose preference test (test)	
5		Move to individual cages	
6		Open field test	
7		Elevated plus maze	
8		**START CUMS**	
9	1	Forced swim test	2h Restraint
10	2	Cage Tilting	
11	3	Social stress	Overnight Fast
12	4	Wet Bedding	
13	5	Predator stress	2h Restraint
14	6	Light cycle reversal	
15	7	Light cycle reversal	
16	8	Sucrose preference test	
17	9	2h Restraint	Predator stress
18	10	No Bedding	
19	11	2h Restraint	Overnight fast
20	12	Cage Tilting	
21	13	Light cycle reversal	
22	14	Light cycle reversal	
23	15	Wet Bedding	
24	16	Sucrose preference test	Sucrose preference test
25	17	2h light cycle disruption	
26	18	Social stress	Overnight (16h) fast
27	19	Cage Tilting	
28	20	Light cycle reversal	
29	21	Light cycle reversal	
30	22	No bedding	
31	23	Social stress	2h Restraint
32	24	Sucrose preference test	Sucrose preference test
33	25	2h light cycle disruption	Overnight Fast
34	26	Wet bedding	
35	27	Light cycle reversal	
36	28	Light cycle reversal	
37		**END CUMS**	
38		Sucrose preference test	
39		Forced swim test	
40		Open field test	
41		Elevated plus maze	
42		**SACRIFICE**	

Table 5.1. Calendar of the CUMS protocol used in study 2.

Behavioural testing

Mice were submitted to the open field test, elevated plus maze, forced swim test and sucrose preference test. Mice were given at least half an hour habituation to the test room prior to each test. All tests were videorecorded by a camera coupled to Ethovision XT 10 computer software (Noldus, Wageningen, Holland) for behaviour recognition and scoring (please see chapter 4 for details).

ACTH and CORT measurement

ACTH was measured in plasma by using a competitive inhibition ELISA kit following manufacturer's instruction (Cloud-Clone Corp., Wuhan, Hubei, China). Circulating CORT was measured by using a competitive immunoassay ELISA kit following manufacturer's directions (Enzo Life Sciences, Farmingdale, New York, USA).

Statistical analysis

Power analysis was performed based on the effect size seen in a previous pilot study investigating the effects of simultaneous *Casp1, Nos2 and Ifngr* deficiency on total floating time in the forced swim test (our primary outcome measure). Cohen's *d* for that study was 0.84, meaning that a sample size of n=36 would result in over 80% power to detect an antidepressant-like effect at $P \leq 0.05$. Statistical analyses of the behavioural tests were performed using the Statistical Package for the Social Sciences version 23.0 for windows (SPSS, Chicago, Illinois) using a general linear model for repeated measures (repeated measures ANOVA). The effects of genotype, stress, treatment and their interaction were explored and the significance set at $P \leq 0.05$. Sphericity of the variances of the groups was assessed with Mauchly's sphericity test. Effect size was reported as partial eta-squared (η^2_p). If the stress*genotype interaction was significant, it was unpacked as described previously.[434] Statistical analyses of the ELISA results were performed by two-tailed unpaired t-test.

Results

Our primary outcome measure was depressive-like behaviour in the forced swim test. Secondary outcome measures included anhedonic- and anxiety-like behaviours, locomotor activity and the levels of circulating ACTH and CORT following stress exposure. Results and analyses of the behavioural tests are available in supplementary table 5.2. Mice that didn't display >65% sucrose preference (an accepted criterion to characterize

anhedonic-like behaviour) at baseline were considered anhedonic at baseline and therefore excluded from all subsequent tests and analyses.

Casp1, Nos2 and Ifngr deficiency decreases depressive-like and anxiety-like behaviour

Our results suggest that simultaneous *Casp1*, *Nos2* and *Ifngr* genetic deficiency decrease depressive- and anxiety-like behaviour. In fact, the total floating time in the forced swim test was lower in (*Casp1, Ifngr, Nos2*)$^{-/-}$ mice compared to wt mice ($F_{1,34}$=14.618, *P*=0.001) (Fig. 5.2a and supplementary table 5.2). At the same time, swimming and climbing behaviours were more prevalent in (*Casp1, Ifngr, Nos2*)$^{-/-}$ mice compared to wt mice (respectively $F_{1,34}$=25.256, *P*=0.001 Fig. 5.2b and $F_{1,34}$=5.929, *P*=0.020, Fig. 5.2c). Similarly, preference for a 1% sucrose solution in the sucrose preference test was increased in the (*Casp1, Ifngr, Nos2*)$^{-/-}$ genotype compared to wt mice ($F_{1,34}$=23.331, *P*<0.001) (Fig. 5.2d) suggesting greater reward seeking and hedonic behaviours in (*Casp1, Ifngr, Nos2*)$^{-/-}$ mice compared to wt mice. Furthermore, (*Casp1, Ifngr, Nos2*)$^{-/-}$ mice spent more time in the open arms of the elevated plus maze compared to wt mice ($F_{1, 34}$=15.480, *P*<0.001) (Fig. 5.3a) suggesting an anxiolytic phenotype. Contrastingly, no differences were observed in the total time spent in the centre of the open field arena compared to wt mice ($F_{1, 34}$=0.200, *P*=0.658) (Fig. 5.3b) or in the ratio centre/total distance in the open field test ($F_{1,34}$=3.330, P=0.077*)*, another measure of anxiety-like behaviour. Unexpectedly, we observed an increased number of faecal boli ($F_{1, 34}$=4.128, *P*=0.050) (Fig. 5.3c) in the open field test as a result of *Casp1, Ifngr* and *Nos2* deficiency.

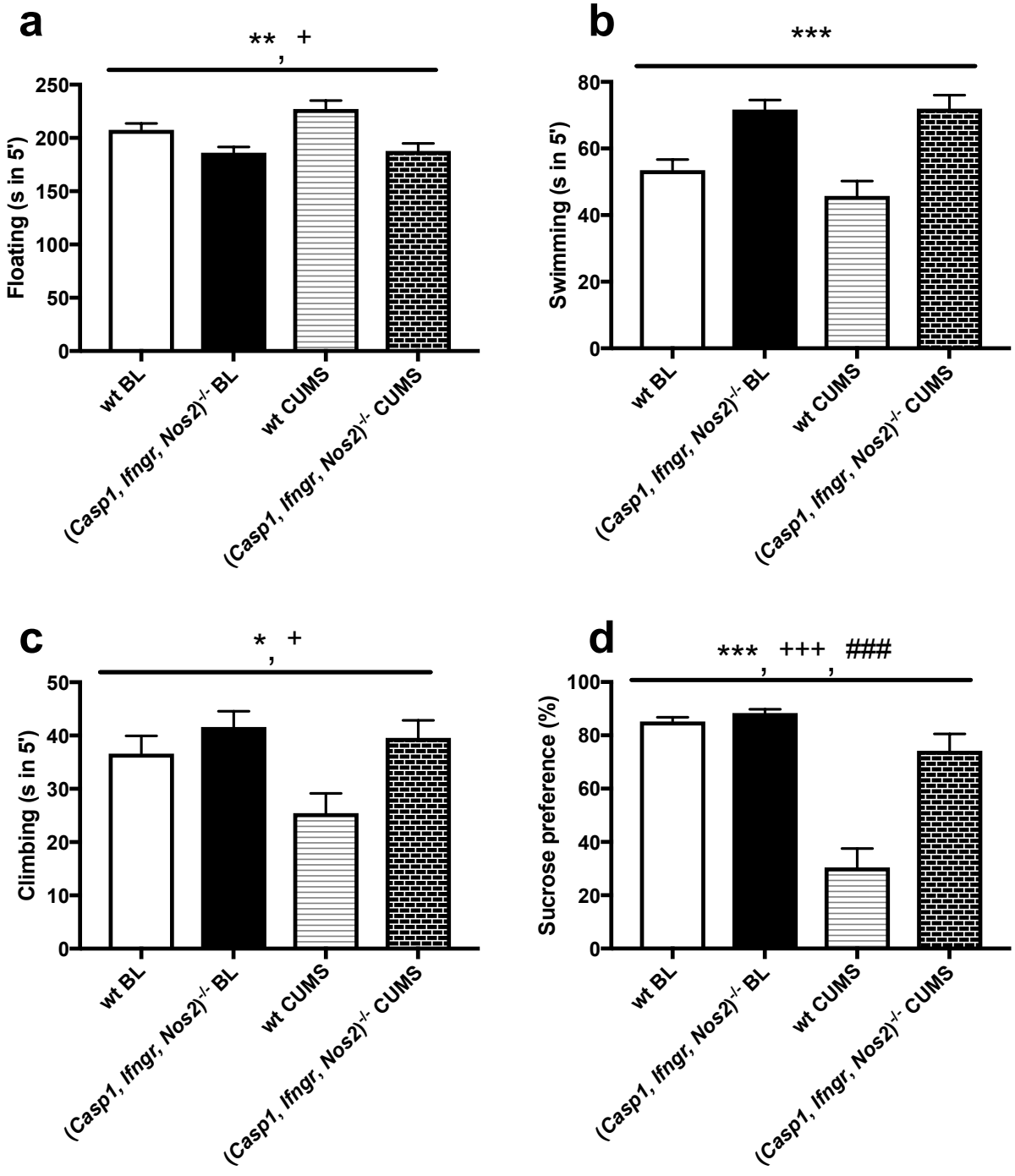


Figure 5.2. *Casp1*, *Nos2* and *Ifngr* deficiency (*Casp1, Ifngr, Nos2*)$^{-/-}$ decreases depressive-like behaviour.

(**a**) (*Casp1, Ifngr, Nos2*)$^{-/-}$ mice displayed decreased floating time in the forced swim test in comparison to wt mice while displaying (**b**) increased swimming and (**c**) climbing behaviours. Moreover, (**d**) (*Casp1, Ifngr, Nos2*)$^{-/-}$ mice displayed increased preference for a 1% sucrose solution. Data are presented as means ± s.e.m. Genotype effect * =P<0.05; ** =P<0.01; *** =P<0.001; stress effect + =P<0.05; ++ =P<0.01; +++ =P<0.001; genotype*stress effect # =P<0.05; ## =P<0.01; ### =P<0.001. wt = wild-type; BL = baseline; CUMS= after chronic unpredictable mild stress paradigm. (wt n=16; (*Casp1, Ifngr, Nos2*)$^{-/-}$ n=20).

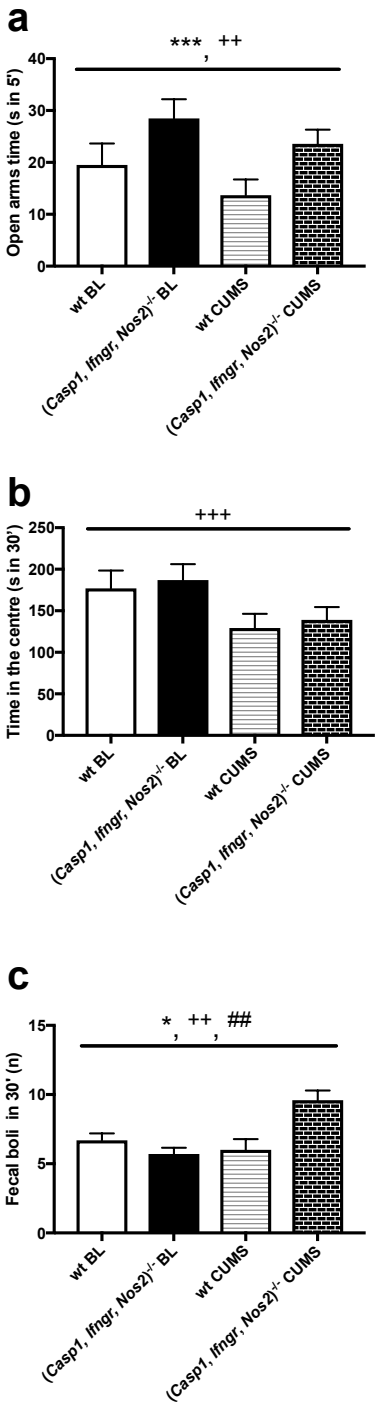


Figure 5.3. *Casp1*, *Nos2* and *Ifngr* deficiency (*Casp1, Ifngr, Nos2*)$^{-/-}$ decreases anxiety-like behaviour.

(**a**) (*Casp1, Ifngr, Nos2*)$^{-/-}$ mice displayed increased time spent in the open arms of the elevated plus maze but (**b**) similar time in the centre area of the open field test. The (*Casp1, Ifngr, Nos2*)$^{-/-}$ genotype had a main effect on (**c**) the number of defecations during the open field test, which was driven by the increased number of faecal boli in (*Casp1, Ifngr, Nos2*)$^{-/-}$ mice following CUMS, while this parameter remained unchanged in wt mice. Data are presented as means ± s.e.m. Genotype effect * =P<0.05; ** =P<0.01; *** =P<0.001; stress effect + =P<0.05; ++ =P<0.01; +++ =P<0.001; genotype*stress effect # =P<0.05; ## =P<0.01; ### =P<0.001; wt = wild-type; BL = baseline; CUMS= after chronic unpredictable mild stress paradigm. (wt n=16; (*Casp1, Ifngr, Nos2*)$^{-/-}$ n=20).

Casp1, Nos2 and Ifngr deficiency affects the response to chronic stress in a mouse model of CUMS.

Our results suggest that (*Casp1, Ifngr, Nos2*)$^{-/-}$ mice had an attenuated response to chronic stress. Following 28 days of CUMS, the preference for a 1% sucrose solution in the sucrose preference test varied as a function of genotype ($F_{1,34}$=17.485, *P*<0.001) (Fig. 5.2d). Wt mice showed a ~74% decrease in sucrose preference compared to baseline ($F_{1,34}$=57.25 *P*<0.001), reaching an average after-stress sucrose preference of 30.3%; this is below the anhedonic threshold which is usually set at 65% of preference.[177,493] Conversely, (*Casp1, Ifngr, Nos2*)$^{-/-}$ mice did not meet the anhedonic threshold, even though they showed a decrease of ~16% ($F_{1,34}$=4.78, *P*=0.036) compared to baseline, reaching an after-stress sucrose preference of 74.2%. This result suggests that (*Casp1, Ifngr, Nos2*)$^{-/-}$ mice are resistant to chronic stress-induced anhedonia, an endophenotype considered a predictor of resilience to stress-induced decrease in neurogenesis.

In the open field test, we found a significant stress*genotype interaction for the measure of total distance travelled ($F_{1,34}$=11.091, *P*=0.002) (Fig. 5.5a). This result was driven by the 17.5% decrease in locomotor activity in (*Casp1, Ifngr, Nos2*)$^{-/-}$ mice following stress ($F_{1,34}$=24.68, *P*<0.001), while the distance travelled by wt mice was unchanged ($F_{1,34}$=0, *P*=0.981) compared to baseline. Similarly, we found a significant stress*genotype interaction for the average moving velocity in the open field test ($F_{1,34}$=11.154, *P*=0.002) (Fig. 5.5b). In fact, while the average velocity recorded for wt mice was unchanged ($F_{1,34}$=0, *P*=0.979) following CUMS, (*Casp1, Ifngr, Nos2*)$^{-/-}$ mice displayed a statistically significant 17.6% reduction ($F_{1,34}$=24.80, *P*<0.001).

Interestingly, we found a significant stress*genotype interaction for the number of faecal boli in the open field test ($F_{1,34}$=14.285, *P*<0.001) (Fig. 5.3c). In fact, while this measure was not different between genotypes at baseline ($F_{1,34}$=2.11, *P*=0.155), and it was unchanged in wt mice following chronic stress ($F_{1,34}$=0.58, *P*=0.453), (*Casp1, Ifngr, Nos2*)$^{-/-}$ mice showed a 68.4% increase ($F_{1,34}$=23.23, *P*<0.001).

Casp1, Nos2 and Ifngr deficiency does not affect ACTH and CORT plasma levels following stress

Following 28 days of CUMS, we found that *Casp1, Nos2* and *Ifngr* deficiency does not affect the plasma levels of ACTH ($t_{2, 25}$=0.1465, *P*=0.8847) (Fig. 5.4a) or CORT ($t_{2, 28}$=0.3851, *P*=0.7031) (Fig. 5.4b).

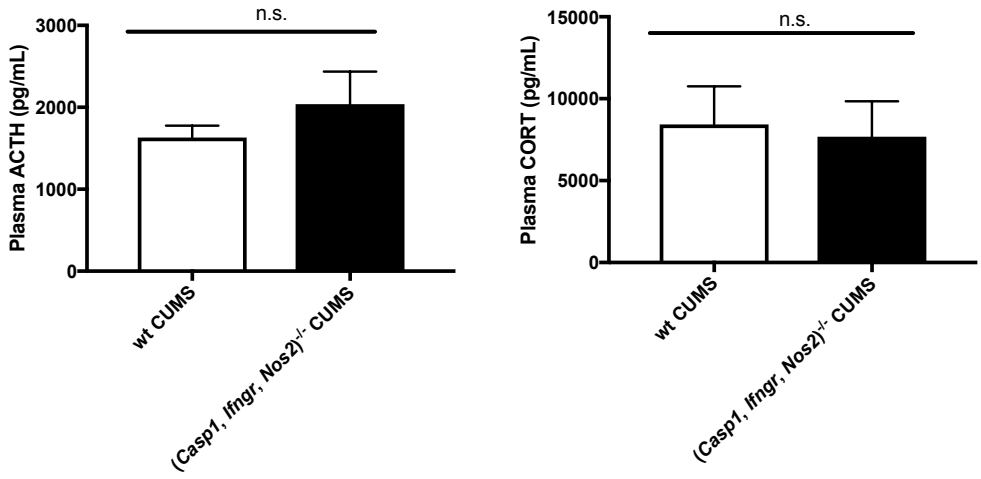


Figure 5.4. *Casp1*, *Nos2* and *Ifngr* deficiency (*Casp1, Ifngr, Nos2*)$^{-/-}$ does not affect the levels of ACTH and CORT following CUMS.

(**a**) (*Casp1, Ifngr, Nos2*)$^{-/-}$ mice displayed similar levels of ACTH and (**b**) CORT compared to wt mice following 28 days of CUMS. Data are presented as means ± s.e.m. n.s.= not significant. [ACTH wt n=14, *(Casp1, Ifngr, Nos2)*$^{-/-}$ n=13; CORT wt n=12, *(Casp1, Ifngr, Nos2)*$^{-/-}$ n=18].

CUMS affects anxiety- and depressive-like behaviour.

In line with the literature, CUMS exposure for 28 days exacerbated depressive-like behaviour, as measured by increased floating ($F_{1,34}$=4.299, *P*=0.046) (Fig. 5.2a) and decreased climbing behaviours ($F_{1,34}$=6.545, *P*=0.015) (Fig. 5.2c) in the forced swim test. Moreover, CUMS induced anhedonia, as measured by the decreased preference for a 1% sucrose solution in the sucrose preference test ($F_{1,34}$=50.384, *P*<0.001) (Fig. 5.2d). Furthermore, CUMS increased anxiety-like behaviour, as measured by the decreased time spent in the open arms of the elevated plus maze ($F_{1,34}$=10.423, *P*=0.003) (Fig. 5.3a) and the decreased time spent in the centre section of the open field test arena ($F_{1,34}$=12.583, *P*<0.001) (Fig. 5.3b). No differences were observed in the central distance/ total distance ratio in the open field test, another measure of anxiety-like behaviour ($F_{1,34}$=2.442, *P*=0.127).

Casp1, Nos2 and Ifngr deficiency increases locomotor activity

We found that *Casp1*, *Nos2* and *Ifngr* deficiency increases locomotor activity in the open field test ($F_{1,34}$=58.883, *P*<0.001) (Fig. 5.5a). Accordingly, the average moving velocity was increased in (*Casp1, Ifngr, Nos2*)$^{-/-}$ mice ($F_{1,34}$=58.777, *P*<0.001) (Fig. 5.5b). Similarly, in the open field test, the number of centre visits but not the total time spent in the centre was increased in (*Casp1, Ifngr, Nos2*)$^{-/-}$ mice (respectively $F_{1, 34}$=35.424, *P*<0.001 and $F_{1,34}$=0.200, *P*=0.658) (Fig. 5.3b). Furthermore, in the elevated plus maze, (*Casp1, Ifngr,*

Nos2)$^{-/-}$ mice displayed increased number of entries in any of the arms ($F_{1,34}$=20.348, *P*<0.001) (Fig. 5.5c), irrespective of whether they were open or closed. These results suggest a hyperlocomotive state as a result of *Casp1*, *Nos2* and *Ifngr* deficiency.

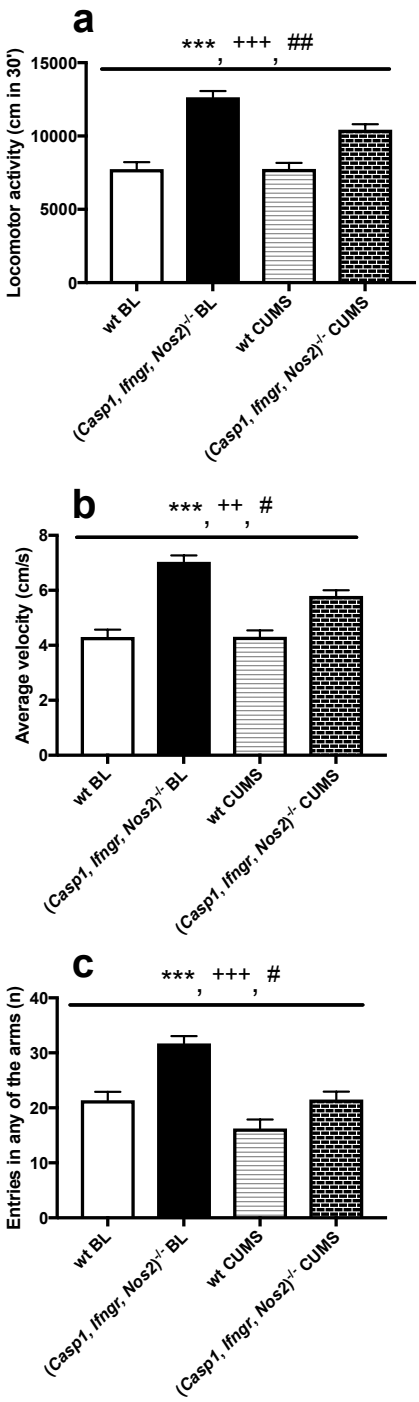


Figure 5.5. *Casp1*, *Nos2* and *Ifngr* deficiency (*Casp1, Ifngr, Nos2*)$^{-/-}$ increases locomotor activity.

(**a**) (*Casp1, Ifngr, Nos2*)$^{-/-}$ mice displayed increased locomotor activity and (**b**) average moving velocity in the open field test. Moreover, (**c**) (*Casp1, Ifngr, Nos2*)$^{-/-}$ genotype had a main effect on the total number of entries in any of the arms of the elevated plus maze, irrespective of them being open or closed. All those parameters showed a stress*genotype interaction, with a common trend of being decreased as a result of *Casp1, Ifngr* and *Nos2* deficiency following CUMS. Data are presented as means ± s.e.m. Genotype effect * =P<0.05; ** =P<0.01; *** =P<0.001; stress effect + =P<0.05; ++ =P<0.01; +++ =P<0.001; genotype*stress effect # =P<0.05; ## =P<0.01; ### =P<0.001. wt = wild-type mice; BL = baseline. (wt n=16; (*Casp1, Ifngr, Nos2*)$^{-/-}$ n=20).

Discussion

Increasing evidence suggests a dysregulation of inflammatory pathways underpinning MDD pathophysiology. In this study we aimed at determining if lacking CASP1, NOS2 and IFNGR affects depression- and anxiety-like behaviours in mice both at baseline and following exposure to chronic stress. Moreover, we aimed at investigating if the simultaneous genetic deletion of *Casp1*, *Nos2* and *Ifngr* affects the HPA response to stress, as measured by the levels of ACTH and CORT following stress exposure. To test our hypotheses, we used (*Casp1, Ifngr, Nos2*)$^{-/-}$ mice, which were generated by cross breeding single knockout mice lacking each of those proteins. We tested these mice in paradigms to assess depressive- and anxiety-like behaviours (forced swim test, sucrose preference test and elevated plus maze test) and locomotor activity (open field test). Subsequently, we exposed these mice to CUMS and repeated the behavioural tests to determine whether the genetically modified mice displayed a different response to stress exposure. Finally, we investigated the post-stress levels of circulating ACTH and CORT to understand if (*Casp1, Ifngr, Nos2*)$^{-/-}$ mice display different levels of such mediators.

We found that (*Casp1, Ifngr, Nos2*)$^{-/-}$ mice display decreased depressive-like behaviour at baseline, as measured by decreased floating time in the forced swim test, and increased hedonic-like behaviour, as measured by increased preference for a 1% sucrose solution in the sucrose preference test. Moreover, (*Casp1, Ifngr, Nos2*)$^{-/-}$ mice display decreased levels of anxiety-like behaviour in the elevated plus maze, as measured by the higher amount of time spent in the open arms of the maze compared to wt mice. At the same time, we observed that (*Casp1, Ifngr, Nos2*)$^{-/-}$ mice display increased locomotor activity and greater moving velocity compared to wt mice in the open field test and a higher number of visits in any of the arms of the elevated plus maze, irrespective of whether they were open or closed. These results suggest a heightened locomotor state. Although decreased floating time is considered an index of antidepressant activity, it has also been suggested that higher locomotor activity could be a confounding factor in this test. In fact, increased locomotor activity can lead to a decrease in total floating time which is not connected with antidepressant activity but which is a side effect of the higher locomotor activity. Similar increases in locomotor activity have been observed in genetically modified mice with impaired DA transporter function, which results in increased extracellular DA levels, and in mice dosed with psychostimulants such as amphetamines, which increase the levels of DA at the synapses.[494-496] This suggests that (*Casp1, Ifngr, Nos2*)$^{-/-}$ mice

might have increased levels of available DA. Moreover, given that the glutamatergic system is involved in the regulation of locomotor activity, it cannot be excluded that (*Casp1, Ifngr, Nos2*)$^{-/-}$ mice might present differences in this neurotransmitter system.[497,498] Accordingly, mice lacking the glial glutamate and aspartate transporter, which have increased forebrain glutamatergic signalling, display hyperactivity during novel exposure to the open field test.[499] Similarly, increased locomotor activity has been found in mice treated with oestrogen, a phenomenon mediated by the oestrogen receptor alpha.[500]

After 28 days of CUMS exposure, we observed a different response to stress in *(Casp1, Ifngr, Nos2)*$^{-/-}$ mice. In line with the existing literature, CUMS increased floating and decreased swimming and struggling time.[180,491] However, while CUMS decreased sucrose preference in both wt and (*Casp1, Ifngr, Nos2*)$^{-/-}$ genotypes, the decrease observed in wt mice was greater and fell below the anhedonic threshold of 65% preference, while the decrease in (*Casp1, Ifngr, Nos2*)$^{-/-}$ mice did not, suggesting that (*Casp1, Ifngr, Nos2*)$^{-/-}$ mice might be protected from developing anhedonic-like behaviour following stress. Accordingly, the hippocampal inhibition of NOS2 by means of aminoguanidine was previously shown to prevent the decrease in sucrose preference following CUMS in rats.[501] Moreover, it was previously shown that *Ifng* deficiency affects basal emotionality while blunting some of the neurochemical, HPA axis and cytokine alterations associated with exposure to chronic stressors.[157] Similarly, it was shown that NOS2 inhibition in mice induces acute antidepressant-like effects while preventing the exacerbation of depressive-like behaviour following unpredictable chronic stress.[158] Nevertheless, we previously showed that *Casp1*$^{-/-}$ mice have decreased anxiety and depressive-like behaviour at baseline and decreased exacerbation of those behaviours following chronic stress.[122] It could be that simultaneously deleting *Casp1, Ifngr* and *Nos2* results in a complex neuro-behavioural phenotype which decreases anxiety-and depressive-like behaviours at baseline while preventing the exacerbation of anhedonic- but not depressive- or anxiety-like behaviours following chronic stress exposure.

Interestingly, we found that (*Casp1, Ifngr, Nos2*)$^{-/-}$ but not wt mice display a greater number of faecal pellets excreted during the open field test following exposure to unpredictable chronic mild stress. Similar effects have been reported in germ-free mice exposed to maternal separation stress and in rats exposed to water avoidance stress.[502,503] This increase has been hypothesized to associate with stress-induced colonic hypermotility and hyperalgesia.[502,503] The fact that (*Casp1, Ifngr, Nos2*)$^{-/-}$ mice only displayed increased defecation following chronic stress but not as a basal behaviour, and

the fact that these mice do not present differences in markers of HPA axis activity following chronic stress, might suggest that such effects are related to stress response systems other than the HPA axis, such as the LC-NE system or the SNS. Accordingly, the changes in faecal pellet output mediated by stress are an accepted measure of modulation of distal colonic motility by the autonomic nervous system, and this measure is increased in rodent models of stress susceptibility (Lewis and Fisher rats).[504] However, it cannot be excluded that the changes in faecal output might be mediated by changes in gut bacteria composition, which might differ in (*Casp1, Ifngr, Nos2*)$^{-/-}$ compared to wt mice as a result of their gene deletions. Further studies should investigate gut microbiome composition of these mice, which could also be a causative factor of the behavioural differences observed here. Interestingly, in another study we found that wt but not *Casp1*$^{-/-}$ mice display increased number of defecations in the open field test following chronic restraint stress, and we hypothesized that this might be a result of the resilience to stress displayed by *Casp1*$^{-/-}$ mice given that highly emotional states increase defecation.[122] This apparently contrasting result might be due to the different stress paradigm used in the present study or to the different gut bacteria composition of these two strains. Moreover, given that (*Casp1, Ifngr, Nos2*)$^{-/-}$ mice display an increased number of defecations following stress as compared to baseline while this parameter is unchanged for wt mice, it could be hypothesized that chronic stress exacerbates such anxiety-like behaviour in a greater fashion in (*Casp1, Ifngr, Nos2*)$^{-/-}$ compared to wt mice.

Future directions

The transgenic mouse model used in this study displays increased locomotor activity compared to wt mice. Pre-clinical studies have identified several systems to modulate spontaneous locomotor activity such as the dopaminergic system, the glutamatergic system and the *Casp1* system.[122,494-500] Therefore, it could be that (*Casp1, Ifngr, Nos2*)$^{-/-}$ mice have increased levels of movement-related neurotransmitters, such as DA or glutamate. In order to test this hypothesis, further studies should investigate the levels of monoamines in brain regions relevant to MDD (such as the prefrontal cortex and the hippocampus) in (*Casp1, Ifngr, Nos2*)$^{-/-}$ compared to wt mice. This could be achieved via high-pressure liquid chromatography (HPLC) analyses of post-mortem brain homogenates or via microdialisis in living rodents. Indirect assessments could include *post-mortem* immunohistochemical analysis of brain areas relevant to MDD to determine the levels of markers of neurotransmission activity, such as tyrosine hydroxylase for DA or of N-acetylaspartate and N-acetylaspartylglutamate for glutamate.[505,506] If (*Casp1, Ifngr, Nos2*)$^{-/-}$

mice indeed have increased levels of such neurotransmitters they might prove useful as a model of resilience to dopaminergic or glutamatergic neurodegeneration in diseases such as Parkinson's and Alzheimer's disease (respectively PD and AD). In fact, in those diseases, the main neurodegenerative features include inflammation-mediated loss of dopaminergic neurons in areas involved with movement and locomotor activity, such as the substantia nigra and the striatum.[507] Accordingly, it was previously shown the CASP1 and CASP3 inhibitor minocycline prevents nigrostriatal dopaminergic neurodegeneration in a mouse model of PD.[457] Moreover, oxidative stress seems to correlate with neurodegeneration, although the causal effects have not been fully elucidated yet.[508] If (*Casp1, Ifngr, Nos2*)$^{-/-}$ mice have altered DA levels, they could also be useful as a pre-clinical model of attention-deficit hyperactivity disorder (ADHD). Abnormal functioning of the DA transporter and receptor related to genetic variability have been described in ADHD patients, suggesting that abnormal DA system physiology is involved in the pathogenesis of this disorder.[509,510]

This study should be considered in light of some limitations. The mouse model used in this study is a transgenic model that simultaneously lacks three proteins. Therefore, it is difficult to ascertain which differences in behaviour are amenable to which protein. This model was generated given the evidence that each of the *Casp1*, *Nos2* and *Ifngr* single KOs display altered response to stress exposure or antidepressant-like phenotypes; therefore, we hypothesized that by combining these models, the effects might be additive and result in a greater antidepressant-like phenotype. In order to bypass this limitation, it might prove valuable to generate a combination of double KOs to ascertain if any two of these KOs in combination have greater antidepressant-like behaviour than the respective individual KOs.

In conclusion, in this study we have investigated the effects of simultaneous *Casp1*, *Nos2* and *Ifngr* gene deletion in a CUMS model of MDD. We found that (*Casp1, Ifngr, Nos2*)$^{-/-}$ mice show decreased levels of depressive- and anxiety-like behaviour while displaying increased locomotor activity and moving velocity. At the same time, following chronic stress exposure, these mice present an attenuated exacerbation of anhedonic-like behaviour compared to wt mice. Interestingly, following stress exposure, plasma levels of ACTH and CORT were not different between (*Casp1, Ifngr, Nos2*)$^{-/-}$ and wt mice. Moreover, (*Casp1, Ifngr, Nos2*)$^{-/-}$ mice displayed increased defecation following chronic stress, suggesting that they might present differences in gut motility and/or gut microbiome composition.[502,503] Future studies could investigate the behaviour and stress responses of

mice lacking a combination of 2 of the 3 proteins investigated here (i.e. (*Casp1, Nos2*)$^{-/-}$), in order to shed light on the potential mechanisms of their resilience to developing anhedonic-like behaviour following stress. Further investigations should elucidate the molecular mechanisms underlining the increased locomotor activity of (*Casp1, Ifngr, Nos2*)$^{-/-}$ mice, which could prove valuable in the study of motor-related neurodegenerative conditions, such as PD and AD, and in the study of conditions in which the DA system is altered, such as ADHD. The gut microbiome composition of (*Casp1, Ifngr, Nos2*)$^{-/-}$ mice could be investigated, which could explain at least some of the stress-induced behavioural differences observed in this study. Finally, pre-clinical and clinical investigations aiming at determining the safety and efficacy of inhibiting pro-inflammatory signalling in MDD should be designed to determine the translational value of such approach.

ACKNOWLEDGEMENTS

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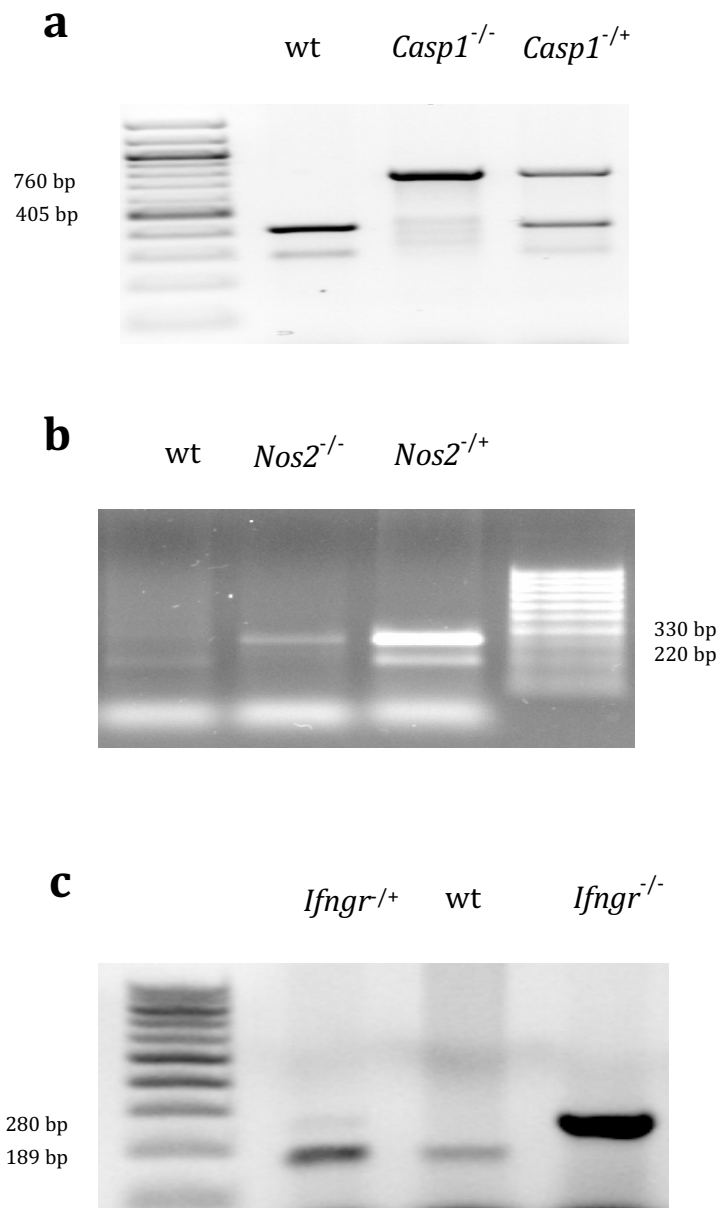
CONFLICT OF INTEREST

The authors declare no conflict of interest.

Supplementary figures



Supplementary figure 5.6. Confirmation of *Casp1*, *Nos2* and *Ifngr* genetic deficiency.

Casp1, *Nos2* and *Ifngr* genetic deficiency was confirmed by endpoint PCR. (**a**) Electrophoresis gel to confirm *Casp1* deficiency. Wt mice display one band at 405 bp (lane 2), *Casp1*$^{-/-}$ mice display one band at 760 bp (lane 3) and *Casp1*$^{-/+}$ display two bands, one at 405 bp and one at 760 bp (lane 4). (**b**) Electrophoresis gel to confirm *Nos2* deficiency. Wt mice display one band at 220 bp (lane 2), *Nos2*$^{-/-}$ mice display one band at 330 bp (lane 3) and *Nos2*$^{-/+}$ mice display two bands, one at 220 bp and one at 330 bp. (**c**) Electrophoresis gel to confirm *Ifngr* deficiency. Wt mice display one band at 189 bp (lane 3), *Ifngr*$^{-/-}$ mice display one band at 280 bp (lane 4) and *Ifngr*$^{-/+}$ mice display two bands, one at 189 bp and one at 280 bp (lane 2).

Supplementary tables

Supplementary table 5.2. Statistical report of (*Casp1*, *Ifngr*, *Nos2*)$^{-/-}$ vs. wt mice behavioural results.

Values in columns 3-6 are means ± s.e.m.. BL=baseline; CUMS=stress; df=degrees of freedom; G=genotype effect; S=stress effect; GxS=genotype x stress interaction; η^2_p=partial eta squared; *=*P*<0.05; **= *P* <0.01; ***= *P* <0.001.

TEST	MEAUSRE	wt BL (n=16)	KO BL (n=20)	wt CUMS (n=16)	KO CUMS (n=20)	MAUCHLY'S W	df	F-TEST	*P*-VALUE	Partial Eta Squared
Forced swim test	Floating (s)	207.623 ± 6.097	186.102 ± 5.453	227.147 ± 7.895	187.790 ± 7.061	1.000	1,34	G F=14.618 S F=4.299 GxS F=3.040	G *P*=0.001 ** S *P*=0.046 * GxS *P*=0.090	G η2p=0.301 S η2p=0.112 GxS η2p=0.082
Forced swim test	Swimming (s)	53.533 ± 3.199	71.720 ± 2.862	45.748 ± 4.510	71.976 ± 4.034	1.000	1,34	G F=25.256 S F=1.774 GxS F=2.023	G *P*<0.001 *** S *P*=0.192 GxS *P*=0.164	G η2p=0.426 S η2p=0.050 GxS η2p=0.056
Forced swim test	Climbing (s)	36.618 ± 3.325	41.590 ± 2.974	25.458 ± 3.685	39.572 ± 3.272	1.000	1,34	G F=5.929 S F=6.545 GxS F=3.150	G *P*=0.020 * S *P*=0.015 * GxS *P*=0.085	G η2p=0.148 S η2p=0.161 GxS η2p=0.085
Sucrose preference test	Sucrose preference (%)	85.243 ± 1.540	88.390 ± 1.377	30.457 ± 7.074	74.224 ± 6.327	1.000	1,34	G F=23.331 S F=50.384 GxS F=17.485	G *P*<0.001 *** S *P*<0.001 *** GxS *P*<0.001 ***	G η2p=0.960 S η2p=0.597 GxS η2p=0.340
Open field test	Locomotor activity (cm)	7744.286 ± 473.444	12649.357 ± 423.461	7756.406 ± 414.117	10430.527 ± 370.397	1.000	1,34	G F=58.883 S F=10.852 GxS F=11.091	G *P*<0.001 *** S *P*<0.002 *** GxS *P*=0.002 **	G η2p=0.634 S η2p=0.242 GxS η2p=0.246
Open field test	Average velocity (cm/s)	4.306 ± 0.264	7.034 ± 0.236	4.313 ± 0.230	5.798 ± 0.206	1.000	1,34	G F=58.777 S F=10.892 GxS F= 11.154	G *P*<0.001 *** S *P*<0.002 ** GxS *P*=0.001 **	G η2p=0.634 S η2p=0.243 GxS η2p=0.247
Open field test	Defecations (n)	6.688 ± 0.507	5.700 ± 0.453	6.000 ± 0.782	9.600 ± 0.700	1.000	1,34	G F=4.128 S F=7.005 GxS F=14.285	G *P*= 0.050 * S *P*= 0.012 ** GxS *P*= 0.001 **	G η2p=0.108 S η2p=0.171 GxS η2p=0.296
Open field test	Centre visits (n)	79.500 ± 6.883	139.650 ± 6.157	70.313 ± 7.989	106.650 ± 7.146	1.000	1,34	G F=35.424 S F=12.942 GxS F=4.123	G *P*<0.001 *** S *P*=0.001 ** GxS *P*=0.050 *	G η2p=0.946 S η2p=0.108 GxS η2p=0.108
Open field test	Centre time (s)	176.995 ± 21.473	187.032 ± 19.206	129.172 ± 17.253	139.128 ± 15.431	1.000	1,34	G F=0.200 S F=12.583 GxS F=0.00	G *P*=0.658 S *P*<0.001 *** GxS *P*=0.998	G η2p=0.006 S η2p=0.270 GxS η2p=0.000
Open field test	Centre/total distance	0.153 ± 0.013	0.179 ± 0.012	0.137 ± 0.013	0.166 ± 0.012	1.000	1,34	G F=3.330 S F=2.442 GxS F=0.030	G *P*=0.077 S *P=0.127* GxS *P*=0.864	G η2p=0.930 S η2p=0.067 GxS η2p=0.067
Elevated plus maze	Open arms time (s)	19.505 ± 4.144	28.492 ± 3.706	13.675 ± 3.045	23.576 ± 2.724	1.000	1,34	G F=15.480 S F=10.423 GxS F=1.999	G *P*<0.001 *** S *P*=0.003 ** GxS *P*=0.166	G η2p=0.969 S η2p=0.235 GxS η2p=0.056
Elevated plus maze	Entries in any arm (n)	21.375 ± 1.527	31.700 ± 1.366	16.250 ± 1.637	21.500 ± 1.464	1.000	1,34	G F=20.348 S F=38.389 GxS F=4.210	G *P*<0.001 *** S *P*<0.001 *** GxS *P*=0.048 *	G η2p=0.374 S η2p=0.530 GxS η2p=0.110
Elevated plus maze	Open arms latency (s)	20.940 ± 7.309	5.208 ± 6.537	53.140 ± 18.663	36.240 ± 16.692	1.000	1,34	G F=1.464 S F=5.564 GxS F=0.002	G *P*=0.235 S *P*=0.024 * GxS *P*=0.966	GxS η2p=0.217 GxS η2p=0.141 GxS η2p=0.000
Elevated plus maze	Open/closed arms time ratio	0.091 ± 0.030	0.233 ± 0.027	0.058 ± 0.015	0.105 ± 0.013	1.000	1,34	G F=17.820 S F=13.019 GxS F=4.511	G *P*<0.001 *** S *P*=0.001 ** GxS *P*=0.041 *	G η2p=0.778 S η2p=0.117 GxS η2p=0.117
Elevated plus maze	Head directed to open arms (s)	30.255 ± 2.984	41.452 ± 2.669	21.230 ± 1.754	21.164 ± 1.569	1.000	1,34	G F=5.135 S F=45.423 GxS F=6.679	G *P*<0.030 * S *P*<0.001 *** GxS *P*=0.014 *	G η2p=0.131 S η2p=0.571 GxS η2p=0.164

Chapter 6 - The microbiome-inflammasome hypothesis of major depression

Manuscript formatted for invited submission to "Bioessays", Hypothesis section in response to the paper published in chapter 4.

The hypotheses in this manuscript were conceived by the candidate. The candidate has ideated and written this manuscript which was subsequently edited by the supervisor, the co-supervisor and a third contributing author.

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Abstract

We propose the “microbiota-inflammasome” hypothesis of depression and co-morbid systemic dysfunctions. Our model is corroborated by an emergent body of literature that supports a role for the multidirectional communication between the gut microbiota, the NLRP3 inflammasome, and the brain, in psychological stress response, leading to the development of MDD and co-morbid systemic illnesses. The microbiota-inflammasome theory of depression is supported by the following lines of evidence: a) increased NLRP3 signalling activates the HPA axis and increases pro-inflammatory signalling affecting depression- and anxiety-related behaviours and gut microbiota composition, b) changes in intestinal structural integrity resulting from stress and/or gut dysbiosis (the alteration of normal gut microbiota composition and expansion of pathogenic bacteria) increase bacterial translocation in physiologically sterile compartments, fuelling enteric, systemic and central pro-inflammatory signalling mediated at least partially by NLRP3 inflammasome signalling, c) stress-induced changes in gut microbiome composition alter the availability of neurotransmitters and neuroactive compounds, at least partially through activation of inflammatory cascades affecting behaviour, d) increased NLRP3 signalling sustained by stress, gut dysbiosis, and leaky gut, increases the likelihood of developing co-morbid NLRP3-mediated systemic conditions, e) NLRP3-driven systemic dysregulation increases the likelihood of developing MDD and gut dysbiosis. If this hypothesis proves to be true, further safety and efficacy translational studies of NLRP3 inflammasome inhibition by means of pharmacological antagonism, faecal microbiota transplantation, psychobiotics supplementation, or dietary change, could give rise to novel therapeutic strategies in the treatment of MDD and co-morbid systemic illnesses.

Keywords: NLRP3, inflammasome, caspase 1, gut microbiota, dysbiosis, stress response, co-morbid conditions, danger associated molecular patterns, Toll-like receptors, probiotics, psychobiotics.

Introduction

The NLRP3 inflammasome is an innate intracellular immune sensor, capable of detecting stress, danger, or damage. Once activated, it triggers intracellular cascades that return the system to homeostasis.[79] One of the main avenues through which NLRP3 exerts its effects is via activating IL1B-mediated cascades.[77] Since IL1B signalling is a crucial node in gut-immune-brain communication, understanding NLRP3 function and dysfunction has become an essential milestone in understanding health and disease across a range of contexts.[79,511-513] Moreover, given the presence of NLRP3 inflammasomes in many immune cell types (including microglial cells, monocytes, granulocytes, epithelial cells and T and B cells), inflammasome-mediated processes are highly relevant to the fields of immunology, psychiatry, and nutrition.[79,511] Inflammasome-mediated loss of homeostasis has been implicated in the pathogenesis, progression and treatment response of MDD, IBD, cancer, diabetes, obesity, as well as neurodegenerative and autoimmune diseases.[511,514-519]

Based on the concept of a MGB axis (please see chapter 1), we have introduced the concept of a microbiota-inflammasome-gut-brain axis (MGIB) The MGIB axis is a bidirectional communication system, interfacing psychological stress, immune system function and gut microbiome composition.[122] Exposure to psychological stress increases NLRP3 inflammasome signalling, resulting in the activation of IL1- and TNF-mediated pathways.[84,451-453] The extent to which NLRP3 is activated in response to external triggers appears to be determined, to a large extent, by the composition and function of the gut microbiome, an entity that is independently linked to the risk of MDD and co-morbidities.[62,114,122,123,134,135,519-521]

In this paper we focus on the MGIB axis, specifically on the NLRP3-mediated pathways that link psychosocial stress, the gut microbiota, and exacerbation of depressive symptomatology.[78,122,270,342,430,514,516,521] We hypothesise a mechanism by which stress exposure, via influencing the gut microbiome, affects NLRP3- and IL1-driven pathways to influence brain function. These processes lead to depressive- and anxiety-like behaviour that increase the risk of MDD and associated co-morbidities (Fig. 6.1). We further hypothesise that, through NLRP3-mediated pathways, stress results in alteration of the composition of the gut microbiota, further compounding pathogenic processes that lead to MDD.

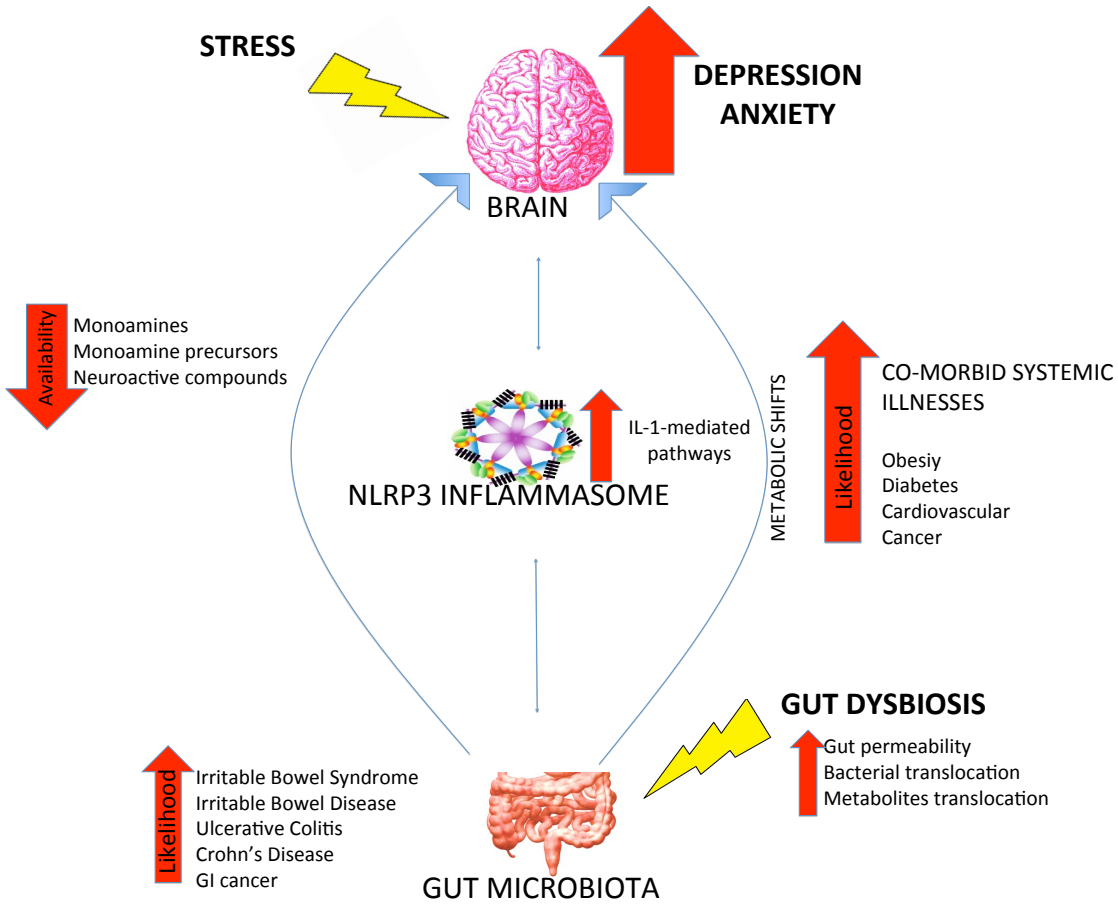

Figure 6.1. The microbiome-inflammasome theory of depression and co-morbid systemic illnesses.

Psychological stress exposure increases NLRP3 signalling, which triggers anxiety and depressive behaviours. At the same time, stress-evoked gut microbiome changes mediated by the NLRP3 inflammasome and IL1-activated pathways alter the bioavailability of monoamine precursors and neuroactive compounds produced by the gut microbiome, which results in the exacerbation of depressive symptomatology. On the other hand, changes in intestinal structural integrity (i.e. leaky gut) result in the translocation of bacteria and their by-products in physiologically sterile bodily compartments, fuelling pro-inflammatory signalling which increase anxiety and depressive behaviours. At the same time, gut dysbiosis alters the levels of microbiome-produced monoamine, neuroactive compounds and other metabolites, which affect anxiety and depressive behaviours. Finally, increased NLRP3 activity fuelled by stress, dysbiotic states and leaky gut increases the likelihood of co-morbid NLRP3-mediated systemic illnesses such as neurodegenerative and autoimmune diseases, IBD, obesity, diabetes and cancer.

The microbiota-inflammasome theory of depression and co-morbid systemic illnesses

Converging evidence linking NLRP3 inflammasome-mediated processes in stress response, MDD development, and systemic illnesses, has led to the formulation of the "inflammasome hypothesis of depression".[342] In this hypothesis, psychological stress activates the NLRP3 inflammasome and therefore IL1B release. If protracted, increased systemic inflammation puts patients at an increased risk of depressive symptomatology and co-morbid illnesses, and an increased likelihood of developing MDD.[342]

Although supported by clinical and pre-clinical evidence, the inflammasome theory of depression does not take into account the involvement of the gut microbiota in the dysregulation of NLRP3-mediated enteric, central, and systemic inflammatory processes.[114,122,123,130,133,134,137,141,466,522-524] We suggest that stress exposure increases NLRP3 signalling, which in turn increases anxiety and depressive behaviours and alters gut microbiota composition. At the same time, stress-induced changes in the gut microbiota alter the bioavailability of monoamine precursors and neuroactive compounds, resulting in exacerbation of depressive symptomatology. We suggest that gut dysbiosis, by increasing systemic and central NLRP3-mediated pro-inflammatory signalling, contribute both to depressive symptoms and to the risk of NLRP3-related co-morbid illnesses (Fig. 6.1).[511,514-519]

In summary, the proposed microbiota-inflammasome hypothesis of depression is based on the following notions: a) psychosocial stress increases NLRP3 inflammasome signalling, resulting in increased HPA axis activation and increased systemic and central pro-inflammatory signalling, b) stress- and/or dysbiosis-mediated changes in gut barrier function (i.e. the development of "leaky gut") result in increased bacterial and bacterial by-products translocation to otherwise sterile enteric compartments, fuelling pro-inflammatory signalling, c) inflammation-mediated shifts in gut microbiota composition resulting from stress alter levels of microbiota-produced neurotransmitter precursors and neuroactive compounds, and d) increased NLRP3 activity fuelled by chronic stress, dysbiosis, and leaky gut, increases the likelihood of increased anxiety and depressive behaviour, and comorbid NLRP3-mediated systemic conditions.[511,514-519]

The NLRP3 inflammasome mediates the psychological stress response

Psychosocial stress triggers *sterile inflammation*, an inflammatory process that is initiated by the extracellular release of DAMPs.[84] DAMPs activate a number of immune cell types throughout the body, priming the body and the brain for a potential full immune response following damage resulting from confrontation with peers or predators.[63] When DAMPS are released, they activate the immune system through pattern recognition receptors (PRRs).[79] The cytokines released in response activate the HPA axis, which by increasing glucocorticoid release, down-regulates immune responses to return to homeostasis.[525]

However, when exposure to a stressor is repeated or prolonged, NLRP3 activity does not reach resolution.[62] This failure to resolve can lead to a state of systemic low-grade chronic inflammation that results in the exacerbation of depressive symptoms (depressive-like behaviour in rodents).[78,430] Interestingly, it was shown recently that increased CASP1 activity is responsible for cleaving glucocorticoid receptors and increasing glucocorticoid resistance, a process which could contribute to the decreased level of glucocorticoid receptors and resistance to glucocorticoids observed in MDD patients.[111,113,525] Ultimately, depressive symptoms are compounded by HPA axis dysfunction, functional and structural brain changes (such as monoaminergic deficiency and hippocampal atrophy) and gut dysbiosis. In turn, these changes further fuel systemic inflammation exacerbating anxiety and depressive symptoms and increase the likelihood of co-morbid systemic illnesses.[85,114,342]

The NLRP3 inflammasome is gaining increasing attention in MDD research for its involvement in stress responses, gut dysbiosis, and pro-inflammatory pathways, which incite depressive symptoms and precipitate co-morbid conditions.[62,516,526] NLRP3 mRNA and protein are increased in peripheral blood mononuclear cells in MDD patients, together with CASP1 and IL1B levels, and normalized by antidepressant treatment.[78] Moreover, NLRP3 signalling is involved in the development of stress-induced depressive-like behaviour in pre-clinical models of depression.[62,122]

The NLRP3 inflammasome regulates intestinal homeostasis

The NLRP3 inflammasome plays a key role in maintaining intestinal homeostasis and in mediating the communication between the gut microbiome, the immune system, and the brain.[523] NLRP3-produced IL1 and IL18 play dichotomous roles in gut function and dysfunction. In fact, while IL1 is a pro-inflammatory cytokine, which at high levels can become detrimental to gut function, IL18 plays a protective role in maintaining intestinal

integrity and promoting epithelium repair.[338,527,528] Accordingly, mice lacking NLRP3, CASP1 or the adaptor protein apoptosis-associated speck-like protein containing a CARD (ASC), display increased mortality to experimental colitis. This is characterized by a loss of epithelial integrity, bacterial dispersion, increased leukocyte infiltration, and upregulated colonic chemokine production.[520,524,528] Together, these observations suggest that NLRP3 activity is necessary to combat intestinal stress and maintain gut homeostasis.[520,524,528] Other groups have, however, suggested entero-protective effects of NLRP3 inflammasome inhibition in experimental colitis.[529] These apparently contrasting findings suggest that the fine-tuning of NLRP3 activity is essential for gut homeostasis, and that NLRP3 modulation (but not total inhibition) might prove valuable in treating conditions connected to altered inflammatory profiles and dysbiotic states, including MDD.[114]

Genetic evidence further supports the role of NLRP3 inflammasome in chronic inflammatory conditions. On one hand, NLRP3 hyperfunctional mutations increase IL1B processing, leading to cryopyrin-associated periodic syndromes.[530] On the other, hypofunctional NLRP3 phenotypes correlate with Crohn's disease in a sex-dependent manner.[531] Given the importance of NLRP3 activity regulation, it represents a potential therapeutic target for modulating gut homeostasis and could have clinical benefits in treating the dysbiotic features that may accompany a subset MDD cases and co-morbid systemic dysfunctions.[114,123,130]

The NLRP3 inflammasome mediates the cross-talk between the gut microbiota and the immune system

While the gut microbiota has a substantial influence on the development and regulation of the immune system, host immunity also helps to shape the composition of the gut microbiota.[532,533] The majority of immune pathways that are involved in regulation of microbiota are activated through NLRs, cytoplasmic sensors of cellular and tissue stress. Mice lacking *Nlrp3*, *Nlrp6*, or other inflammasome components, exhibit an "inflammasome-mediated dysbiosis", characterized by *Prevotellacea* and TM7 over-representation and increased expression of pro-inflammatory cytokines, increased experimental colitis severity and an autoimmune-like response.[487,534] *Nlrp6*$^{-/-}$ mice susceptibility to dysbiosis seems to relate to a defect in goblet cell-mediated intestinal mucus production, suggesting that a similar mechanism might be driving the *Nlrp3*$^{-/-}$-mediated dysbiosis.[535] Similarly, *Nod1*$^{-/-}$ and *Nod2*$^{-/-}$ mice exhibit altered gut microbiota profiles, characterized by under-representation of *Bacteroidetes vulgatus*, increased enteric interferon-gamma production and altered MYD88 signalling.[536-538] Other innate immunity-driven mechanisms seem to be

involved in fuelling gut dysbiosis, such as the dysregulated production of antimicrobial peptides by Paneth cells in intestinal crypts, which can lead to shifts in microbiota composition and colonization of physiologically sterile inner mucus layers.[539-541] Taken together, these findings suggest that NLRP3 inflammasome bioactivity is crucial to maintenance of gut homeostasis.

The NLRP3 inflammasome mediates metabolites-brain interactions

Interactions between commensal microbiota and the host have developed over millennia and have a beneficial influence on many aspects of physiology.[542] Disruption of these interactions can precipitate enteric, autoimmune, metabolic and psychiatric disorders.[114,122,130,145] The principal pathways of microbiota-gut-brain interaction are the host-microbe metabolic axes, multidirectional cross-talk networks linking bacterial metabolites, and host cellular pathways.[124 63] Such cross-talk acts principally through two distinct pathways: 1) vagus nerve activation by microbiota-produced gene expression by-products and metabolites, which directly affect brain function and indirectly regulate immune system balances,[543] and 2) direct interaction of the microbiota and their metabolites with components of the immune system, such as the NLRP3 inflammasome, which influence pro-inflammatory pathways and affect brain function and host behaviour.[122,544] Through such pathways, commensal bacteria can make a substantial beneficial contribution to the regulation of host immunity. For example, many gut commensals, such as spore-forming *Clostridia*, influence intestinal T-reg cell induction through the production of SCFAs. In turn, T-regs mediate anti-inflammatory and immunoregulatory processes.[522,545-547] This is of particular relevance in MDD, where a shift towards Th1 response has been described in at least a subset of patients.[80,82,83,548] Some commensals (such as *Helicobater hepaticus*) have also been shown to stimulate pro-inflammatory Th1 and Th17 responses in immunocompromised but not in wt mice, suggesting that extra attention is needed in shaping therapies in immunocompromised patients.[549]

The "depression gut microbiota"

The gut microbiome is essential to human health and its disruption is implicated in diverse pathologies. Indeed, the emerging literature on gut-brain interactions and the association of gastrointestinal diseases (such as IBD) with psychiatric conditions, highlight the contribution of the gut microbiota in psychopathology.[125,550] It has been suggested that clinical MDD is associated with altered gut microbiology, and the majority of the studies that have investigated the "depression microbiota" have reported compositional

differences between depressed patients and healthy controls. Typically, alterations in overall microbiota structure, rather than in the abundance of individual species, have been described (for a summary of studies reporting bacterial changes in MDD patients, see table 6.1).

Increased in MDD	Decreased in MDD	Reference
Actinomycineae Coriobacterineae Coriobacterineae Lactobacillaceae Streptococcaceae Clostridiales *Eubacteriaceae* *Lachnospiraceae* *Ruminococcaceae* *Erysipelotrichacea*	Bacteroidaceae Lachnospiraceae Acidaminococcaceae Veillonellaceae *Sutterellaceae*	Zheng et al., 2016
Thermoanaerobacteriaceae	Prevotellaceae	Kelly et al., 2016
	Bifidobacterium *Lactobacillus*	Aizawa et al., 2016
Enterobacteriaceae *Alistipes* Acidaminococcaceae Fusobacteriaceae Porphyromonadaceae Rikenellacea	Bacteroidaceae Erysipelotrichaceae Lachnospiraceae Prevotellaceae Ruminococcacea Veillonellaceae	Jiang et al., 2015

Table 6.1. Summary of studies reporting increased and decreased levels of bacterial families in MDD patients.

For example, Jiang and colleagues compared the gut microbiota composition in a Chinese population amongst active depressed patients (n=29), depressed patients responding to treatment (n=17) and healthy controls (n=30), reporting greater bacterial diversity in the active MDD patients.[133]

The authors also reported increases in the relative abundance of discrete phylogenetic groups. For example, Proteobacteria were increased in patients with active MDD, while the relative abundance of Firmicutes was reduced. *Proteus mirabilis* (a member of the Proteobacteria phylum) has been shown to trigger monocyte-induced NLRP3 activation and IL1B production through the release of the virulence molecule hemolysin.[134] Bacterial components from other Proteobacteria, such as the structural LPS produced by

Pseudomonas species, have been shown to trigger depressive symptoms via TLR4-mediated NLRP3 inflammasome activation and production of LPS-reactive immunoglobulins.[135] Notably, levels of these pro-inflammatory markers are increased in MDD patients.[54] The authors also reported the relative abundance of *Bacteroidetes* to be significantly higher in the active depression group (mostly the result of increased *Parabacteroides* and *Alistipes* abundance). Since these bacteria are able to convert tryptophan to indole, they might influence tryptophan availability and potentially disrupt the enteric serotonergic balance and be involved in the serotonergic unbalances observed in MDD.[136] Interestingly, the abundance of the genus *Alistipes* is increased in MDD, chronic fatigue syndrome, pediatric IBS and in rodent stress models, and appears to correlate with inflammation.[551,552] Moreover, a diabetogenic effect of this genus has been hypothesized, which could contribute to the MDD-diabetes comorbidity. Similarly, *Lachnospiraceae*, a key producer of the SCFA butyrate, which helps to maintain intestinal barrier integrity, was decreased in this cohort. Such changes might contribute, in part, to the increased susceptibility of MDD patients to leaky gut and gastrointestinal pathology.[137-140] The genus *Faecalibacterium*, is a well-recognised bacterial contributor to the suppression of inflammation, and its lower abundance in MDD patients is consistent with the heightened inflammatory state in MDD.[553] *Oscillibacter* was also increased in active MDD patients.[130] This genus produces the SCFA valeric acid, which resembles gamma-aminobutyric acid (GABA) and binds GABAA receptor, involved in gastrointestinal functions.[554]

Zheng and colleagues investigated faecal microbiota composition in a second Chinese population of MDD patients (n=58) and demographically matched healthy controls (n=63). The authors reported alterations of taxa belonging to the phyla Bacteroidetes (decreased in MDD), Actinobacteria (increased in MDD) and Firmicutes (some members increased in MDD, others decreased).[123] The authors went on to transfer faecal microbiota from MDD patients and healthy controls to germ free mice, and reported that mice receiving the "depression microbiota" displayed increased anxiety-like and depressive-like behaviour. These findings suggest that the depressive phenotype is transmissible via the gut microbiome.[123]

Kelly and colleagues also investigated the relationship between the gut microbiome and MDD using an Irish cohort of MDD patients (n=34) and gender, age and ethnicity matched healthy controls (n=33). In contrast to the findings of Jiang and colleagues, the authors reported decreased bacterial diversity in the MDD group compared to healthy controls, which were associated with an increase in inflammatory markers (IL6, IL8, TNF, and

CRP). Using a similar approach to Zheng and colleagues, the authors demonstrated that the transfer of gut microbiota from MDD patients to antibiotic-treated rats resulted in a recapitulation phenotype. Specifically, rats that received depression-associated microbiota displayed anhedonic-like and anxiety-like behavior, and MDD-like biological traits, including increased plasma kineurine levels and an increased plasma kyneurine/tryptophan ratio.[141]

Naseribafrouei and colleagues reported a correlative study of faecal microbiota composition in Norwegian MDD patients (n=37) and healthy controls (n=18) and found that the complete microbiota correlated with depression with high predictive accuracy independent of medication status.[130] *Lachnospiracea* and *Bacteroidetes* were under-represented in the gut microbiome of MDD patients. Again, levels of *Alistipes and Oscillibacter* were increased in MDD. Low levels of *Bacteroidetes* are associated with chronic low-grade inflammation and obesity, suggesting that such a decrease could bridge chronic low-grade inflammation, depression and obesity.[43,131,132]

By demonstrating that altered gut microbiology is not only associated with depression, but that it has a causal contribution to the development of depressive-like behaviour, these studies provide exciting insight into pathological mechanisms, and opportunities for the improvement of therapies. However, it is important to note the variability in the findings reported. Larger studies that take into consideration factors such as diet and pharmacological treatment are now required to corroborate these early investigations, and define MDD-associated microbiome characteristics more clearly.

MDD is associated with altered metabolic pathways

The shifts in gut microbiota composition observed in MDD patients result in peripheral and central disturbances of metabolic pathways. These include a) altered amino acid profiles, which could be responsible for altered neurotransmitter signatures in MDD and altered low- and very low- density lipoproteins, b) lipid metabolism-related molecules, which could be responsible for dysregulated lipid balances and high co-morbidity between MDD, metabolic syndrome and obesity and c) altered levels of energy metabolism-related molecules, which could be responsible for the energy deficiencies observed in MDD.[555-560]

Interestingly, following the instillation of gut microbiota from MDD patients into rodent models, altered behaviour can be observed resembling that of the human donors, suggesting that behaviours associated with specific gut microbiota may be transmissible.[123] This intriguing observation raises the question of whether transferring the

microbiota from healthy, non-depressed individuals could provide clinical benefit in MDD. Strikingly, peripheral microbiota-driven metabolic changes are also transmissible and are reflected centrally in the recipient's hippocampus.[123] Further studies should investigate whether profiling the peripheral metabolome might have diagnostic value in humans.

Inflammasome signalling modulation affects host behaviour and gut microbiota composition

The involvement of the MGB axis in the pathophysiology of MDD is increasingly recognized.[116,123,130,133,141] However, the molecular mechanisms underpinning such communication remain poorly understood. Recently, we reported that genetic deletion or CASP1 pharmacological inhibition, and therefore decreased NLRP3 inflammasome signalling, attenuates anxiety- and depressive-like behaviours, while preventing the exacerbation of stress-induced depressive-like behaviour in mice.[122] In that study, we characterized gut microbiota changes stemming from chronic stress and pharmacological inhibition of NLRP3 inflammasome bioactivity with minocycline. Minocycline administration was found to result in gut microbiota shifts similar to those observed in *Casp1*$^{-/-}$ mice.[122,466] Stressed mice showed subtle shifts in the Firmicutes/Bacteroidetes ratio, which are thought to correlate to chronic low-grade inflammation in other diseases.[65] *Bifidobacterium*, a genus associated with inflammatory pathways suppression via nuclear factor NF-kB inhibition, was decreased in stressed mice,[477] while *Lactobacillus*, a genus involved in activation of the inflammasome through CASP1-mediated IL1B production by macrophages, was increased.[122] Interestingly, the levels of *Akkermansia* were increased in mice undergoing chronic stress concomitantly receiving the CASP1 inhibitor minocycline. This effect is relevant since *Akkermansia* decreases inflammatory signalling in adipocytes through T-reg cells induction, which in turn results in the suppression of IL1B and IL6, and might be involved in minocycline's immunosuppressive effects.[483,484] Similarly, Lachnospiraceae, a family associated to the production of the anti-inflammatory SCFA, was increased in stressed mice receiving minocycline.[122] These findings highlight the far-reaching effects of stress exposure on the gut, and support the microbiome-inflammasome theory of depression proposed here.

The microbiome-gut-inflammasome-brain axis

Our findings, and those of other researchers, suggest that by modulating NLRP3 inflammasome signalling, it is possible to attenuate stress-induced neuroinflammation and prevent stress-induced gut dysbiosis.[122,149,341,342,430,526] We introduced the notion of a microbiome-gut-inflammasome-brain axis, where the gut microbiome affects anxiety and

depression-related behaviours at least in part via activating inflammasome-mediated pathways.[122] While these changes in brain function are connected to shifts in microbiome profiles, they are actually driven by the host's metabolic pathways.[123] Such alterations of metabolic profiles are reflected in the host's hippocampus, a brain region relevant to MDD.[123] Given these findings, we suggest that the MGIB axis is crucial for MDD onset and progression.

Translational implications of the microbiome-inflammasome hypothesis of depression and co-morbid systemic illnesses and future directions

The MGIB axis could represent a valuable therapeutic target for MDD treatment via modulation of NLRP3 inflammasome bioactivity to potentially decrease inflammation and depressive symptoms. For example, it has been reported that fluoxetine, a common antidepressant, inhibits NLRP3 inflammasome activation.[526] An alternative approach to such a pharmaceutical approach might be to manipulate the gut microbiome, to reduce NLRP3 inflammasome bioactivity and increase the availability of monoamine precursors and neuroactive compounds (for example, through faecal microbiome transfer, psychobiotic supplementation, or dietary measures).[128,142,561] These approaches are supported by pre-clinical evidence that NLRP3 inhibition and gut microbiome modulation decreases neuroinflammation via preventing microglial activation while attenuating the loss of hippocampal neurogenesis, important hallmarks of MDD progression.[62,562]

Modulation of NLRP3 inflammasome activity via adjunct anti-inflammatory therapies seems to hold potential in MDD and co-morbid systemic illnesses, although clinical investigations remain limited.[276,563,564] [107] The indirect modulation of inflammasome signalling through manipulation of the gut microbiome also has therapeutic potential.[128,142,561] Demonstration of the efficacy of these strategies under clinical trial conditions could lead to a paradigm shift in the 60 year-old MDD pharmacotherapy.[23,565]

NLRP3 modulation in MDD

Pre-clinical and clinical evidence points to a role for the NLRP3 inflammasome in MDD onset, antidepressant response and remission.[78,526] A proof-of-concept placebo-controlled trial investigating the effects of minocycline augmentation for MDD reported improvements in several outcome measures, including global impression, functioning, and quality of life.[98] However, the study's primary outcome measure, the Montgomery–Asberg Depression Rating Scale, was unaffected.[98] Other clinical trials have investigated the efficacy of anti-inflammatory augmentation in MDD, finding that such approach increases the efficacy and

decrease the latency of antidepressant effects onset.[276,563,564] Similarly, genetic deletion or pharmacological inhibition of inflammasome assembly abrogates LPS- and stress-induced depressive-like behaviour.[62,122,341] These changes are mediated by decreased inflammasome and pro-inflammatory cytokine activity.[62,122,341] Such a reduction in neuroinflammation suggests that humans might benefit from similar therapies. A clinical trial investigating the effects of TNF inhibition in depression treatment found that only patients with dysregulated inflammatory profiles benefit from such therapy,[42] raising the possibility that only depressed patients with increased NLRP3 baseline activity might benefit from NLRP3-directed therapies. However, when designing therapies targeting NLRP3, it is important to take into consideration the effects that such will have on physiological immune responses, whose functionality needs to be preserved.

Faecal microbiota transplantation in MDD

Although, to date, no clinical trials have investigated the efficacy of FMT with depressive symptomatology as a primary outcome measure, anecdotal and indirect evidence of mood enhancing and anti-inflammatory effects of such treatment are available from patients undergoing such transplantation procedures for the treatment for IBD, *Clostridium difficile* infection and Crohn's disease.[128] Accordingly, pre-clinical studies have shown the transmissibility of gut microbiota-driven behaviours.[123,141] Though no study has investigated the reverse approach (i.e. FMT from healthy donors to depressed recipients), the transmissibility of microbiota-associated behaviours suggests that FMT might prove valuable in treating MDD.[123,141] When designing FMT-related therapies, it is fundamental to maximize the safety of the procedure (i.e. screening for pathogens) while taking into account potential short-term adverse effects (i.e. abdominal discomfort and fever) and long-term adverse effects (i.e. onset of latent infections and increased risk for other microbiota-related conditions).[566] Double-blind, placebo controlled clinical trials are needed to assess the safety and efficacy of FMT in MDD and the resulting changes in gut microbiota composition and biochemical parameters in the recipients.

Psychobiotics supplementation in MDD

Probiotics are bacteria that yield beneficial health outcomes, while prebiotics are compounds that ferment in the gut producing changes in gut bacteria composition and/or function.[567] Psychobiotics are probiotics and prebiotics that positively affect mental health and can ameliorate psychiatric symptoms by: a) competitive exclusion of pathogens, b) modulation of pro- and anti-inflammatory pathways, c) communication with the CNS via the vagus nerve, d) changes in neurotransmitters levels.[142,568-572] Clinical trials involving

psychobiotics administration are sparse but on the rise, and are yielding promising results (table 6.2).

Bacterial Strain	Condition	Probiotic Treatment	Mood effects	Biological effects	Reference
***Bifidobacterium longum* NCC3001**	Irritable bowel syndrome	42 days	Decreased depression scores Increased quality of life Amelioration in general physical health Amelioration in problems with work or other daily activities	No difference in serum inflammatory markers No changes in gut microbiome composition *Bifidobacterium longum* detected in 80% of treated patients at end of treatment Reduced amygdala and frontal and temporal cortices engagement in response to fearful stimuli (correlated to IBS symptoms relief) Heightened occipital regions engagement in response to fearful stimuli	Pinto-Sanchez, 2017
Lactobacillus helveticus* *Bifidobacterium longum	Low mood	56 days	None found	High levels of baseline vitamin D predicted better treatment response	Romijn et al, 2017
Lactobacillus acidophilus* *Bifidobacterium bifidum* *Streptoccocus thermophiles	SSRI treatment resistant depression	56 days	Decreased depression scores Improved quality of life	None tested Hypothesized intestinal anti-inflammatory effects	Bambling et al, 2017
***Lactobacillus gasseri* CP2305 (inactivated)**	Stressed students	84 days	Improved sleep quality Decreased stress levels	Improved sleep electroencephalogram Prevented increases in basal cortisol levels Prevented increases in expression of stress-responsive microRNA miR-144 (females only) Normalized bowel habits	Nishida et al, 2017
Lactobacillus acidophilus* *Lactobacillus casei* *Bifidobacterium bifidum	Major Depression Disorder	56 days	Decreased depression scores	Decreased insulin levels Decreased hsCRP levels Increased glutathione levels	Akkasheh et al, 2016
***Bifidobacterium longum* 1714**	Healthy subjects	28 days	Decreased daily stress levels Decreased anxiety response to a stressor	Improved visuospatial memory Decreased cortisol response to a stressor Enhanced prefrontal cortex activity	Allen et al, 2016
***Bifidobacterium bifidum* W23** ***Bifidobacterium lactis* W52** ***Lactobacillus acidophilus* W37** ***Lactobacillus brevis* W63** ***Lactobacillus casei* W56** ***Lactobacillus salivarius* W24** ***Lactococcus lactis* (W19 and W58)**	Healthy subjects	28 days	Reduced rumination Reduced aggressive thoughts	None investigated	Steenbergen et al, 2015
***Lactobacillus acidophilus* LA5 and *Bifidobacterium lactis* BB12 (GROUP 1)** ***Lactobacillus casei*, *Lactobacillus acidophilus*, *Lactobacillus rhamnosus*, *Lactobacillus bulgaricus*, *Bifidobacterium breve*, *Bifidobacterium longum*, *Streptoccocus thermophilus* (GROUP 2)**	Healthy subjects	42 days	Decreased anxiety scores Decreased depression scores	Improved general health	Mohammadi et al, 2015
***Bifidobacterium animalis subsp lactis* I-2494** ***Lactobacillus bulgaricus* I-1632 and I-1519** ***Lactococcus lactis subsp lactis* I-1631**	Healthy subjects	28 days	None investigated	Altered activity of interoceptive and somatosensory regions Decreased activity of mid insula cortex and primary somatosensory cortex Decreased activity of frontal, prefrontal, and temporal cortices, parahippocampal gyrus, and the periaqueductal gray	Tillisch et al, 2013
***Lactobacillus helveticus* R0052** ***Bifidobacterium longum* R0175**	Healthy subjects	30 days	Decreased scores of somatisation, depression and anger–hostility	None investigated	Messaoudi et al, 2011
***Lactobacillus casei* Shirota**	Healthy subjects	20 days	Patients more depressed at baseline reported improved mood	None investigated	Benton et al, 2007

Table 6.2. Summary of clinical studies reporting biological and/or mood outcomes following psychobiotics supplementation.

Spore-forming bacteria: an example of mood-enhancing bacteria

Interestingly, spore-forming bacteria present in the gut are involved in regulating host's 5HT biosynthesis.[573] Although it is accepted that peripherally produced 5HT cannot cross the BBB, spore-forming bacteria might improve mood by increasing faecal α-tocopherol (a

form of vitamin E) and tyramine (a catecholamine-releasing trace amine) levels.[573] Alpha-tocopherol, which is decreased in MDD, is beneficial in several diseases and ameliorates depressive-like behaviour in pre-clinical models, while tyramine is a neurotransmitter-like molecule with antidepressant and anxiolytic effects.[574-576] Spore-forming bacteria might, therefore, have monoamine-independent mood-enhancing effects elicited by increased production of beneficial metabolites and neuroactive compounds.

In a pilot study with SSRIs resistant patients, 8 week supplementation with *Lactobacillus acidophilus*, *Bifidobacterium bifidum*, *Streptoccocus thermophiles and* magnesium orорate resulted in a reduction in depression scores and improved overall quality of life.[143] Similarly, in a randomized controlled trial (RCT), the administration of *Lactobacillus acidophilus*, *Lactobacillus casei*, and *Bifidobacterium bifidum* led to a decrease in depression scores, and levels of insulin and high sensitivity CRP, while glutathione levels increased.[144] Accordingly, another RCT investigating the effects of 6 weeks of supplementation with *Bifidobacterium longum* in IBS patients reported decreased depression scores and increased quality of life.[577] Imaging analysis identified attenuated amygdala and temporal activation in response to fearful stimuli, which correlated with IBS symptoms amelioration.[577] Moreover, a cocktail of *Bifidobacterium animalis* subsp. *lactis*, *Streptococcus thermophiles*, *Lactobacillus bulgaricus*, and *Lactococcus lactis* subsp. *lactis* decreased neural activity in negative emotion- and sensation-processing brain areas in healthy women.[145] These results suggest that the introduction of commensal bacteria to the gut can have a profound influence on region-specific brain activity. The identification of such effects represents an important step in the application of psychobiotic supplementation for conditions that alter brain activity, such as MDD and other psychiatric and neurodegenerative disorders.[577,578] Accordingly, *Bifidobacterium longum* administration in healthy volunteers increased prefrontal cortex activity and decreased daily stress levels, while attenuating cortisol and anxiety responses to a stressor.[146]

An important challenge in psychobiotics research is the identification of bacterial strains that provide consistent efficacy. For example, in one study, healthy volunteers dosed for 30 days with *Lactobacillus helveticus* and *Bifidobacterium longum* displayed decreased anxiety and cortisol levels while in another study, 21 days of treatment did not affect psychological symptoms.[570,579] Another study investigating the efficacy of a combination of *Lactobacillus helveticus* and *Bifidobacterium longum* in patients presenting low mood found no significant differences in mood scores across treatment groups.[580] Two other studies reported that *Lactobacillus casei* Shirota improved mood in healthy subjects with

low baseline mood score, and decreased anxiety scores in CFS patients.[581,582] Interestingly, gut *Lactobacillus* and *Bifidobacteria* representation was increased in the CFS cohort following supplementation, suggesting that effective psychobiotic treatment might have a "ripple effect" (the effects on overall gut balances) on gut microbiota composition.[582]

Most recently, the use of a "parapsychobiotic" (an inactivated psychobiotic) containing *Lactobacillus gasseri*, was shown to prevent the rise in stress-responsive micro RNAs and cortisol, while improving sleep quality and bowel habits in a RCT with chronically stressed students.[583] These findings suggest that the administration of live bacteria might not be essential to achieving beneficial effects.

Psychobiotics supplementation in pre-clinical models of MDD

Accordingly to the promising results obtained in clinical trials, pre-clinical studies have shown beneficial effects of psychobiotics in modulating the stress response, neurotransmitter systems and inflammatory and metabolic pathways (table 6.3).

Bacterial Strain	Host	Condition	Probiotic Treatment	Mood effects	Biological effects	Reference
***Bifidobacterium bifidum* W23** ***Bifidobacterium lactis* W52** ***Lactobacillus acidophilus* W37** ***Lactobacillus brevis* W63** ***Lactobacillus casei* W56** ***Lactobacillus salivarius* W24** ***Lactococcus lactis* W19** ***Lactococcus lactis* W58**	Rat (Sprague-Dawley)	Healthy High-fat diet	35 days	Reduced depressive-like behaviour independent of diet	Skewed cytokine production towards IFNG, IL2 and IL4 and decreased TNF and IL6 Lowered hippocampal transcript levels of factors involved in HPA axis regulation Increased level of indole-3-propionic acid	Abildgaard et al, 2017
***Bifidobacterium breve* 1205**	Mouse (BALB/c)	Acute stress	180 days	Reduced anxiety-like behaviour	Increased spleen weight	Savignac et al, 2014
***Bifidobacterium longum* 1714**	Mouse (BALB/c)	Acute stress	180 days	Reduced depressive-like behaviour Reduced obsessive/compulsive-like behaviour	None found	Savignac et al, 2014
***Lactobacillus rhamnosus* JB-1**	Mouse (BALB/c)	Chronic Stress	28 days	Reduced depressive-like behaviour Reduced anxiety-like behaviour Reduced fear conditioning	Attenuated stress-induced increase of corticosterone Higher levels of GABAB1b receptor in prelimbic and cortical areas Higher levels of GABAB1b receptor in amygdala and dentate gyrus	Bravo et al, 2011
***Lactobacillus helveticus* R0052** ***Bifidobacterium longum* R0175**	Rat (Wistar)	Healthy	14 days	Decreased anxiety-like behaviour	None investigated	Messaoudi et al, 2011
***Bifidobacterium breve* 6330**	Rat (Sprague-Dawley)		42 days	None investigated	Increased hippocampal BDNF expression Decreased BDNF exon IV expression (responsive to stress)	O'Sullivan et al, 2011
***Bifidobacterium infantis* 35642**	Rat (Sprague-Dawley)	Maternal separation stress	45 days	Decreased depressive-like behaviour	Decreased IL10 Decreased noradrenaline in the amygdaloic cortex Decreased 5HIAA in the amygdaloic cortex Increased amygdalar CRF	Desbonnet et al, 2010
***Bifidobacterium infantis* 35642**	Rat (Sprague-Dawley)	Baseline	14 days	None found	Decreased 5HIAA in the frontal cortex Decreased DOPAC in the amygdaloic cortex Increased plasma tryptophan level Increased plasma kyneurenic acid Decreased body weight gain Ex-vivo (peripheral whole blood) attenuation of IFNG, TNFA and IL6	Desbonnet et al, 2009
***Lactobacillus rhamnosus* GR-1**	Primary bovine mammary epithelial cells	*E. Coli*-induced mastitis	Pre-treatment for 24h	N/A	Attenuated NLRP3 activation Attenuated IL1B, IL6, IL8, IL18 and TNFA mRNA Upregulated IL10 mRNA	Wu et al, 2016

Table 6.3. Summary of pre-clinical studies reporting biological and/or behavioural outcomes following psychobiotics supplementation.

Bifidobacterium infantis for 14 days in mice: a) suppressed the production of pro-inflammatory cytokines, b) increased the levels of available tryptophan, c) decreased 5HT degradation by-products, and d) decreased dopamine degradation by-products.[584] Correspondingly, *Bifidobacterium infantis* normalized depressive-like behaviour and noradrenalin levels in a pre-clinical paradigm of depression.[585] Although these studies did not assess NLRP3 or IL1B levels, the immuno- and neuro-regulatory effects achieved might be mediated, at least partially, by reduced inflammasome signalling.[454,586] *Bifidobacterium longum* showed anxiolytic and antidepressant effects in an innately anxious mouse strain, while *Bifidobacterium breve* had anxiolytic effects and decreased body weight gain, suggesting an effect of the former in mood and anxiety and of the latter in anxiety and metabolism.[587] Some studies suggest that psychobiotics might upregulate

neurotrophic pathways. In fact, *Bifidobacterium breve* upregulated BDNF expression in the rat hippocampus, and BDNF is known to modulate neurogenesis.[588,589]

Similarly, *Lactobacillus rhamnosus* for 28 days attenuated stress-induced rise of CORT levels as well as depressive- and anxiety-like behaviours in mice.[590] Interestingly, *L. rhamnosus* triggered vagus nerve-mediated GABAergic upregulation.[590] Since animal models of depression have decreased GABA levels, *L. rhamnosus* could be useful in MDD treatment.[591] Lastly, a recent study reported that a psychobiotic cocktail decreased depressive-like behavior in rats, while skewing cytokine balance and affecting hippocampal gene expression and plasma metabolomics.[592]

The need for biochemical outcome measures in clinical trials

While clinical trials investigating psychobiotic supplementation often have self-reported questionnaires as their primary outcome measure, there is a lack of biochemical outcome measures. No clinical study has so far investigated the effects of psychobiotics on NLRP3 inflammasome expression or bioactivity. Promisingly, one study on rheumatoid arthritis patients found that *Lactobacillus casei* supplementation led to a decrease in IL1, IL6, IL12 and TNF, while increasing IL10 production.[593] Accordingly, pre-clinical studies report decreased levels of inflammatory cytokines and neurotransmitter degradation in psychobiotics-treated rodents, suggesting promising immunomodulatory and monoaminergic outcomes that might be achieved in MDD patients with altered inflammatory profiles.[584,585] The only report of psychobiotics effects on the NLRP3 inflammasome involves a bovine model of mastitis in which *L. rhamnosus* attenuated NLRP3 inflammasome activation as well as CASP1, IL1B, IL18, TNF and IL6 expression, while increasing IL10 due to decreased TLR4 signaling.[594] These effects might be due to the *L. rhamnosus* production of lactate, which attenuate TLR4-mediated NLRP3 signaling.[595] Clinical studies investigating psychobiotics on NLRP3 inflammasome activation and cytokine production as well as neurotransmitter and gut microbiota changes are warranted to assess if similar outcomes can be achieved in humans.

Diet in MDD

One of the most potent influences on gut microbiome composition and function is diet.[596] Depressive symptoms prompt the consumption of foods high in sugar and saturated fats, driving gut dysbiosis and compounding depressive symptoms. However, by stimulating the production of immunomodulatory compounds, such as SCFAs, a diet rich in fibre, for example, should be considered as an adjunct therapy for MDD.[561] Indeed, a study has

shown, for example, that MDD patients who consume fermented milk products have increased levels of *Bifidobacterium*, a genus that is reduced in MDD patients.[597]

Conclusions

The evidence for an involvement of NLRP3-mediated pathways in the cross-talk networks linking the gut microbiota, the immune system and the brain, is compelling. The microbiota-inflammasome theory of depression presented here suggests that NLRP3 signalling triggered by psychological stress and/or gut dysbiosis affects anxiety and depressive-like behaviours resulting in decreased levels of available monoamine precursors and neuroactive compounds while fuelling gut dysbiosis and increasing the risk for NLRP3-driven co-morbid illnesses. Conversely, chronic stress exposure results in immune balances disruption that can trigger dysbiotic states which fuel systemic low-grade inflammation, increasing the susceptibility to co-morbid systemic illnesses. New therapeutic strategies might target the microbiota-gut-inflammasome-brain axis, either through the direct inhibition of NLRP3, or through the modulation of gut microbiota. The latter could involve using psychobiotics, faecal microbiota transplantation, or dietary measures to decrease NLRP3 inflammasome signalling during chronic stress to reduce pro-inflammatory pathways, and decrease risks of neuroinflammation and neurodegeneration. While these approaches offer exciting opportunities for novel therapies, further pre-clinical and clinical research is required if they are to be translated into clinical practice.

Conflict of interest: The authors declare no conflict of interest.

Chapter 7 - Discussion and future directions

Increasing evidence suggests an involvement of inflammatory pathways in the response to stress.[63,287,330,331,333,334] Stressors prolonged or repeated over time can lead to changes in the host ranging from alterations in neurotransmitter systems to gut microbiome shifts to dysregulation of metabolic pathways, which can increase the risk of developing MDD and co-morbid conditions.[133,296,304] Given the evidence of dysregulated inflammatory pathways in MDD, it seems plausible that modulating inflammatory signalling could be valuable in MDD treatment. This thesis therefore investigated whether decreasing stress-responsive pro-inflammatory pathways is beneficial in pre-clinical models of MDD.

We hypothesized that *Casp1* deficiency and pharmacological CASP1 inhibition with minocycline would decrease anxiety- and depressive-like behaviour in mice at baseline and following stress. Moreover, that chronic stress and CASP1 inhibition would affect gut microbiome composition. Nevertheless, that simultaneously deleting the genes coding for CASP1, NOS2 and IFNGR would affect baseline behaviour while preventing the exacerbation of anxiety- and depressive-like behaviour following stress. Finally, we hypothesized that genetic deletion of *Casp1, Ifngr* and *Nos2* would decrease the levels of circulating CORT and ACTH following stress.

To test our hypothesis, we used knockout mice lacking *Casp1* or *Casp1*, *Ifngr* and *Nos2*, and wt mice with and without minocycline treatment. We assessed the baseline levels of a) depressive-like behaviour in the forced swim test, b) anxiety-like behaviour in the elevated plus maze, c) anhedonia in the sucrose preference test and d) locomotor activity and anxiety-like behaviour in the open field test. Subsequently, we exposed these mice to chronic stress paradigms used to trigger depressive-like behaviour and we investigated the effects of genetic deficiency or pharmacological inhibition of such proteins on: a) the stress-induced exacerbation of anxiety- and depressive-like behaviour, b) gut microbiome composition, c) the circulating levels of the stress hormones CORT and ACTH, and d) metabolic parameters.

The results obtained partially support our hypothesis. In fact, *Casp1* and *Casp1*, *Ifngr* and *Nos2* genetic deficiency affect depressive-like behaviour. Both *Casp1*$^{-/-}$ and (*Casp1, Ifngr, Nos2*)$^{-/-}$ mice displayed decreased floating time in the forced swim test, suggesting an antidepressant-like phenotype.[439,493,598] Contrastingly, mice lacking IL1R1 were previously

shown to have no differences in depressive-like behaviour at baseline compared to wt mice.[471] The mouse models used in this study display decreased anxiety-like behaviour at baseline, suggesting that their genotype could be driving such behavioural difference. Accordingly, mice lacking *Il1r* display decreased anxiety-like behaviour.[471] This is consistent with the notion that pro-inflammatory signalling increases anxiety-like behaviour and suggests that *Casp1*$^{-/-}$ and (*Casp1, Ifngr, Nos2*)$^{-/-}$ mice display an anxiolytic phenotype.[444] The fact that the mouse models used display decreased anxiety- and depressive-like behaviour at baseline suggest that CASP1, IFNGR and NOS2 might be involved in the regulation of behaviour. Such effects could be mediated by the influence of CASP1 on HPA axis, which in addition to stress responses also modulates physiological homeostatic processes involved in shaping basal behaviour.[599] Moreover, (*Casp1, Ifngr, Nos2*)$^{-/-}$ had increased preference for a sugary solution as compared to wt mice, suggesting increased reward-seeking and hedonic-like behaviour compared to wt mice.

Following stress exposure, *Casp1*$^{-/-}$ mice had an attenuated response to stress compared wt mice, as measured by the decreased exacerbation of stress-induced anxiety- and depressive-like behaviour. While *Casp1*$^{-/-}$ mice display decreased climbing time in the forced swim test, their swimming time was not affected. Those two behaviours have been respectively correlated with the serotonergic and noradrenergic neurotransmitter systems.[600] Given that swimming time did not decrease in *Casp1*$^{-/-}$ mice following stress, this could suggest that *Casp1* deficiency affects serotonergic pathways and that it might have a protective effect against stress-induced serotonergic neurodegeneration. On the other side, following 28 days of CUMS, (*Casp1, Ifngr, Nos2*)$^{-/-}$ mice displayed an attenuated exacerbation of anhedonic-like behaviour. In fact, wt mice displayed a substantial decrease in sucrose preference (dropping below the “anhedonic” threshold of 65%), while (*Casp1, Ifngr, Nos2*)$^{-/-}$ mice did not. These mice did not have altered CORT and ACTH levels compared to wt mice at the experimental endpoint. This suggests that (*Casp1, Ifngr, Nos2*)$^{-/-}$ mice might have a similar HPA axis response to that of wt mice and produce the same amount of stress hormones in response to stress. However, it is unclear whether there could have been HPA axis differences at earlier timepoints. Given that (*Casp1, Ifngr, Nos2*)$^{-/-}$ mice display an increased locomotor state and their HPA axis responses are not improved compared to wt following CUMS, it cannot be excluded that the observed decreases in floating and increased swimming and struggling times in the forced swim test might be related to their hyperlocomotive state rather than an

antidepressant-like phenotype. Therefore, those results should be interpreted in light of this limitation.

Nevertheless, this does not exclude the possibility that (*Casp1, Ifngr, Nos2*)$^{-/-}$ mice might have differences in stress-related pathways downstream of the HPA axis, or in other stress response systems, such as the LC-NS. This hypothesis could be tested via transcriptional profiling of brain regions relevant to MDD (such as the hippocampus and the prefrontal cortex) via RNA microarray analyses or RNA sequencing (a deeper, more informative and sensitive high-throughput approach).[601] Another approach to further elucidate downstream signalling pathways could involve the investigation of other stress-related proteins downstream of the glucocorticoid production, such as the expression or sensitivity of NR3C1 or specific intracellular signalling pathways activated by NR3C1 receptor activation, such as the NFKB1 and signal transducer and activator of transcription 5A. This kind of investigation could help shed light on the underpinnings of the resilience to developing anhedonic-like behaviour in (*Casp1, Ifngr, Nos2*)$^{-/-}$ mice and determine if indeed this genotype results in an antidepressant-like phenotype or simply to a heightened locomotor state which has a confounding effect on the forced swim test.

It was previously shown that IL1B administration and chronic stress modulate the antineurogenic effects of stress (namely the decreased neuronal proliferation observed in the hippocampus following exposure to chronic stress).[151] Accordingly, IL1 blockade or *Il1r1* deficiency prevents the decrease in neurogenesis and exacerbation of anhedonic behaviour following chronic stress.[151] Therefore, since CASP1 activates IL1B in response to stress, and *Casp1*$^{-/-}$ mice have very low levels of IL1, it cannot be excluded that the stress-resilience phenotypes observed here might be mediated by the protective effects of impaired IL1 signalling on stress-induced decrease in neurogenesis.[151]

Similar hyperlocomotive states have been reported in transgenic mice with increased levels of extracellular DA and in mice treated with psychostimulants such as methamphetamines, which are known to increase the levels of synaptic DA.[494-500] For example, it was previously shown that mice with genetic deletion of the DA transporter (which causes hyperdopaminergia) display increased locomotion, especially when exposed to a novel environment.[495] This hypermobility state can be reversed by modulating the AMPA glutamate receptor, suggesting that the glutamate system might be affected as a result of combined *Casp1*, *Ifngr* and *Nos2* deficiency.[495] This indicates that (*Casp1*)$^{-/-}$ and (*Casp1, Ifngr, Nos2*)$^{-/-}$ mice might have increased DA or glutamate levels

and/or altered architecture of the dopaminergic or glutamatergic neurotransmitter systems, which could explain the stress resilience of these mice. Accordingly, antidepressant drugs can indirectly increase DA levels, and some MDD patients present altered functioning of the dopaminergic system, such as decreased dopamine release, impaired downstream dopamine signalling and changes in receptor number and/or function. (Reviewed by Dunlop and Nemeroff [602]) Further investigations into the mechanisms that underpin these phenotypes could be valuable in AD and PD research and in other diseases accompanied by dopaminergic and glutamatergic neurodegeneration. Indeed, one recently published study suggests that *Casp1*$^{-/-}$ mice might have decreased dopaminergic neuronal death in an experimental model of PD.[603]

The hypothesis that the models used in this study might have altered levels of neurotransmitters could be tested by investigating the levels of central monoamines in brain regions relevant to MDD in (*Casp1, Ifngr, Nos2*)$^{-/-}$ mice at baseline and following stress to determine if they differ from wt mice. HPLC analyses of brain homogenates or microdialisates could be performed, in order to quantify the levels of available monoamines. Indirect approaches include immunohistochemistry analysis of MDD-related brain areas to investigate the levels of markers of neurotransmission, such as tyrosine hydroxylase and DA receptors for DA and N-acetylaspartate and N-acetylaspartylglutamate for glutamate.[505,506] If this hypothesis proves true, (*Casp1*)$^{-/-}$ and (*Casp1, Ifngr, Nos2*)$^{-/-}$ mice might be useful in PD and AD research, given that both these diseases present increased dopaminergic and glutamatergic degeneration, and in ADHD research, a condition in which the DA system is altered.[457,494-496,507,509,510]

While several studies have investigated the acute antidepressant effects of minocycline in rodents, to the best of our knowledge no other study has investigated the antidepressant effects of minocycline in a rodent model of chronic restraint stress.[92,456,459] Our finding that minocycline prevented the exacerbation of depressive-like behaviour following exposure to CRS corroborates the notion that minocycline has antidepressant-like effects and that it might hold potential in MDD treatment. Our results are in line with studies investigating the effects of minocycline as a suppressant of microglia activation in stress models.[94,604] This suggests that the antidepressant-like effects of minocycline might be connected to its inhibitory effects on microglial activation, a crucial mechanism in the exacerbation of depressive symptomatology. Indeed, minocycline was recently reported to improve global impression, functioning and quality of life in a MDD cohort although depression scores were unaffected.[98] Another clinical trial has investigated minocycline in the treatment of

bipolar disorder. The authors observed reduced severity of depressive symptoms and improvements in cognitive functions in minocycline-treated patients.[99] Similarly, clinical trials of minocycline as a stand-alone or augmentation therapy in psychotic depression and schizophrenia have yielded promising results.[100-103]

Our findings, and those of other studies, suggest that the mechanisms of CASP1 inhibition with minocycline might present two levels of antidepressant activity: the first achieved by directly decreasing pro-inflammatory signalling and microglial activation and the second achieved by modulating gut microbiota composition, which indirectly modulates immune processes.[122,605]

Turcibacter was absent in minocycline-treated mice, and in mice concomitantly receiving stress and minocycline. The presence of this genus might be dependent on TNF.[606] This is supported by our finding, given that *Turcibacter* was increased following stress, and stress increases the production of TNF.[41] This genus was absent in minocycline-treated mice, in line with the notion that minocycline attenuates TNF expression.[607] We observed decreased levels of *Allobaculum* in mice receiving minocycline, and similar changes were reported in high fat diet studies. Lachnospiraceae was increased in mice receiving minocycline, and this family produces anti-inflammatory SCFAs, which have anti-inflammatory properties.[486] Therefore, Lachnospiraceae might be involved in the anti-inflammatory effects of minocycline.

Stressed mice receiving minocycline showed a decreased exacerbation of depressive-like behaviour following stress. This is in line with the reported antidepressant-like effects of minocycline.[92,456] These findings also support the notion that minocycline treatment during stress decreases microglia-induced neuroinflammation, given that neuroinflammation is involved in MDD.[94]

The microbiome of mice undergoing stress and receiving minocycline showed shifts in the representation of bacterial families known to affect immune processes. For example, *Akkermansia* was increased, and this genus has immunosuppressive effects via inducing Foxp3 T-reg cells.[483-485] Conversely, the levels of *Lactobacillus* were decreased in restrained mice receiving minocycline, a finding consistent with the decrease observed in inflammasome-deficient mouse models, such as *Casp1* and *Nlrp6* deficient mice.[466,487] *Lactobacillus* levels were decreased in restrained mice receiving minocycline, while they were increased in mice receiving restraint alone.

Minocycline treatment resulted in antidepressant-like effects while modulating gut microbiota composition. However, it seems unlikely that microbiota changes are responsible for its acute antidepressant-like effects.[92,456] Instead, it seems plausible that the acute inhibition of CASP1, CASP3 and NOS2 by minocycline might affect feedback loops that modulate NLRP3 inflammasome activity. Future studies should investigate the possibility that microbiota changes might be involved in the antidepressant effects of minocycline. To ascertain the clinical safety of minocycline, it will prove relevant to assess the microbiome shifts brought up by minocycline administration in humans, since other antibiotics have the potential to instate dysbiotic states and to increase the likelihood of systemic illnesses.

The gut microbiome composition of mice exposed to stress trended towards an increased F/B ratio. Similar changes were observed in IBS patients and were associated with heightened anxiety and depression symptoms.[474] Similarly, increased F/B ratio was reported in rat models of hypertension.[473] Since both these conditions are underlined by low-grade chronic inflammation,[475,476] it cannot be excluded that the F/B shift observed in the gut microbiota of stressed mice might be involved in the establishment of a systemic low-grade pro-inflammatory profile during stress.[85] The latter is thought to be a driving factor in increasing the likelihood of developing MDD and co-morbid systemic illnesses.[65,67,85,290,476]

The levels of *Bifidobacterium* were reduced in restrained mice. This genus inhibits NFKB1 (which triggers depressive-like behavior) and other pro-inflammatory cytokines in response to LPS.[477] *Allobaculum* was not detected in restrained animals and its abundance correlates with inflammatory markers, such as IL22 and leptin.[479,480] *Lactobacillus* was increased in stressed animals. These bacteria are involved in inflammasome activation via stimulating CASP1-mediated IL1B production in macrophages.[481] Therefore, their increase in response to stress could contribute to the exacerbation of depressive-like behaviour.[64,300,482] Together, the changes observed in gut microbiota composition following chronic stress exposure seem to point towards the establishment of a pro-inflammatory environment. Such an environment could represent a bridging mechanism underlying the increased likelihood of developing MDD and inflammation-related co-morbidities following exposure to prolonged psychosocial stressors.[270,342]

The results obtained in this study corroborate the increasing body of knowledge suggesting that MDD is underlined by dysregulated inflammatory pathways and that

targeting such pathways could prove valuable in MDD therapy. Moreover, our results suggest that the gut microbiome plays important roles in behaviour, in stress responses and in the exacerbation of inflammatory processes. The latter can increase the risk for co-morbid systemic illnesses, such as metabolic syndrome, obesity, cardiovascular disease, diabetes and IBD. Although our results clarify some of the links connecting inflammation, stress response and gut microbiota composition, a complete understanding of the translational power of the stress-immune-microbiome axis has yet to fully develop.

Limitations of the study and how they might affect the validity of the findings

It is important to consider this study in light of some limitations. In the first study we used *Casp1*$^{-/-}$ mice to assess their innate depressive- and anxiety-like behaviour and the exacerbation of these behaviours in response to stress. However, it was previously reported that all *Casp1*$^{-/-}$ mice generated using strain 129 embryonic stem cells are in fact *Casp1*, *Casp11* double knockouts, because *Casp1* and *Casp11* are neighbouring on the genome and too close to segregate by recombination.[147,152,153] Therefore the findings of the first study should be interpreted as a result of the simultaneous deletion of *Casp1* and *Casp11* rather than *Casp1* alone.

In the first study, we used minocycline as a pharmacological inhibitory compound of CASP1 activity. However, minocycline also presents some degree of inhibitory activity towards CASP3 and NOS2, and therefore it cannot be excluded that at least some of the effects observed following minocycline treatment (both in behaviour and in gut microbiome composition) could be amenable to the simultaneous inhibition of CASP1, CASP3 and NOS2 rather than to a specific CASP1 inhibition.[488] Moreover, since minocycline is an antibiotic, it is expected that it would affect gut microbiome composition regardless of its anti-inflammatory properties.[488] Yet, since minocycline exerts antidepressant effects as well as affecting the gut microbiome, and since gut microbiome composition is relevant to mood and behaviour, its impact on the gut microbiome could be involved in the mechanism of action underlying its antidepressant-like effects.[93,458,472]

In both studies here presented, mice were submitted to a battery of behavioural tests twice, one at the beginning and one at the end of each study. It is known that exposure to behavioural tests can affect the performance in other behavioural tests, and that exposure to a specific test can affect the outcomes of the same test if repeated.[467,490] We tried to reduce this bias by performing the behavioural tests from the least to the most stressful and by allowing the mice to recover in between tests.[467,490]

Another limitation is that the CRS paradigm used in the first study did not decrease sucrose preference, while the CUMS paradigm used in the second study did. The sucrose preference test is a paradigm able to model clinical anhedonia, one of the core symptoms of MDD. Although our results are in line with other findings, which highlight the difficulty in reproducing the outcome of this test even within the same research facility, this represents a limiting factor in interpreting the effectiveness of the stress paradigm used in the study involving *Casp1*$^{-/-}$ mice.[164,177,180,491]

The results of the behavioural phenotyping of (*Casp1, Ifngr, Nos2*)$^{-/-}$ mice suggest a phenotype characterized by decreased anxiety- and depressive-like behaviour and increased locomotion. Moreover, that these differences are not mediated by altered HPA axis activity, since the levels of CORT and ACTH are similar to those of wt mice. It is true that these mice were generated to determine the pre-clinical therapeutic potential of inhibiting multiple Th1- pathways in response to chronic stress. However, this design has the intrinsic limitation of impeding the attribution of the differences observed to one single protein or pathway. Yet, the effects of singularly inhibiting these mediators have been previously reported, and therefore our findings can be interpreted in light of and compared to such studies. Future studies could investigate the behavioural phenotype of double KO mice lacking 2 of the proteins investigated here at one time [i.e. (*Casp1, Ifngr*)$^{-/-}$].

Finally, (*Casp1, Ifngr, Nos2*)$^{-/-}$ mice lack *Ifngr* and therefore their IFNGR-activated intracellular signalling cascade is impaired. However, it could be that the stress-induced rise of IFNG levels might alter feedback or feedforward loops or compensative mechanisms to counterbalance the high circulating levels of this cytokine. Further studies should investigate these possibilities.

Future directions

Our findings corroborate an increasing wealth of knowledge, which points towards a central and systemic dysregulation of inflammatory mediators in MDD. These changes result in neurological and systemic dysfunctions, as well as shifts in gut microbiome composition, events that increase the likelihood of co-morbid systemic illnesses. Although further studies are required, it seems that pharmacological modulation of dysregulated inflammatory pathways could represent a valid therapeutic strategy in MDD and other psychiatric disorders with inflammatory components.

Casp1$^{-/-}$ mice displayed antidepressant-like behaviour at baseline and following CRS compared to wt mice. These differences could be attributable to a protective effect of

Casp1 deficiency on the decrease in neurogenesis brought up by inflammasome-mediated neuronal apoptosis in response to stress exposure. In order to test this hypothesis, neurogenesis studies aiming at quantifying the rate of newly formed neurons in the hippocampus of *Casp1*$^{-/-}$ mice undergoing chronic stress regimen could be performed. Such studies could involve the immunohistochemical staining of newly formed neurons in the dentate gyrus of the hippocampus, one of the brain areas relevant for both neurogenesis and MDD.

Minocycline treatment caused shifts in gut microbiome composition. Further studies could investigate the effects of minocycline on the gut metabolome, which is likely to present substantial changes in light of the shifts of gut microbiota composition. Such changes could represent an intermediate effector in the antidepressant effects of minocycline.[608] In fact, the metabolome is considered to be a pivotal interface between the gut microbiota and the host via affecting immune processes.[609] Accordingly, recent findings suggest that the gut metabolome reflects the metabolome composition in brain areas relevant to MDD. Moreover, that the gut metabolome plays fundamental roles in shaping immune processes, which are likely to mediate the behavioural outcome of gut microbiota composition.[123]

Both *Casp1*$^{-/-}$ and *(Casp1, Ifngr, Nos2)*$^{-/-}$ mice displayed increased locomotor activity, a phenotype that suggests an effect of their genetic deletions on movement-related brain circuitries. In order to investigate if these models have altered neurotransmitters level, studies could be performed either *in-vivo*, by microdialisis, or on brain tissues collected after euthanasia by HPLC-MS analyses. At the same time, an indirect interrogation of the proportion of dopaminergic and glutamatergic neurons could be performed by immunohistochemical staining of the enzyme TH2, the rate-limiting enzyme for the production of the DA and of the glutamate transporter solute carrier family 17 member 8. If *Casp1*$^{-/-}$ and (*Casp1, Ifngr, Nos2*)$^{-/-}$ mice have increased levels of DA, they could be useful in experimental models of AD and PD, in which dopaminergic neurodegeneration is usually induced by chemical compounds. If these mice show resilience to paradigms of AD or PD progression, that might suggest that inhibition of CASP1, or the simultaneous inhibition of CASP1, NOS2 and IFNGR might prove valuable in the treatment of AD or PD.

(*Casp1, Ifngr, Nos2*)$^{-/-}$ mice displayed resilience to the exacerbation of anhedonic-like behaviour following stress. To better understand this result it might prove valuable to interrogate the molecular mechanisms that underpin the resilience to anhedonia in the

(*Casp1, Ifngr, Nos2*)$^{-/-}$ genotype. This could help understand the potential effects of inhibiting those pathways in humans.

Beyond understanding the mechanisms underlying stress resilience and behavioural changes, it is important to assess if the inhibition of any immune mediator represents a safe therapeutic approach. In fact, such manipulation might impair immune functioning. In other words, it is important to understand if such inhibition would still allow the mounting of an appropriate inflammatory response to immunological challenges to fight the triggering stimulus (i.e. a bacterial, viral or fungal infection). For example, it was previously shown that mice lacking *Ifng* or *Ifngr* have delayed encephalomyelitis virus clearance from the brain and spinal cord and more intense inflammation even though they have decreased levels of IL6 and TNF.[610] Accordingly, mice lacking *Ifngr* have increased mortality following Bacillus Calmette-Guérin infection.[154] Similarly, it was reported that CASP1 is necessary for (immune) cellular recruitment following influenza infection and therefore to induce protective antiviral immunity.[611] These results suggest that extra care is required in designing and trialing novel antidepressant strategies that inhibit immune mediators.

Studies could be performed in pre-clinical models of immune challenges to investigate the safety of inhibiting pro-inflammatory signalling in the search for novel antidepressant approaches. If these studies prove to be safe and successful, individual or combined inhibition of CASP1-, NOS2-, and IFNG-mediated pathways could be trialled in clinical settings. Preliminary studies are essential before such therapies can reach mainstream clinical practice, given that an immunological failure in pre-clinical models would represent a limiting factor (and potentially a dead end) to this kind of approach. In order to test these research questions, pre-clinical studies could investigate the immunological outcomes of inhibiting inflammasome and other pro-inflammatory mediators in the response to infections; moreover, studies in which the exposure to infectious agents is coupled to acute and chronic stress paradigm could be designed to determine the molecular and behavioural outcomes of such treatments in combination. If these studies prove safe and efficacious, the next step could involve randomized controlled trials to assess translatability. Finally, if clinical trials replicate the safety and efficacy of modulating pro-inflammatory mediators directly (i.e. via pharmacological inhibition) or indirectly (i.e. with diet, probiotics supplementation or faecal microbiome transplantation), alone or in combination, such approaches could become part of routine treatments for MDD as a stand-alone or as adjunctive therapies.

Concluding remarks

This study investigated the efficacy of decreasing pro-inflammatory signalling (CASP1, NOS2 and IFNGR) as an antidepressant strategy in pre-clinical models of MDD. We found that such approach decreased depressive- and anxiety-like behaviours at baseline compared, while decreasing the exacerbation of depressive symptoms following chronic stress. Moreover, we found that minocycline treatment affected gut microbiome composition; we hypothesize that such effects might be involved in the antidepressant-like effects of minocycline. Furthermore, we found that stress affected gut microbiome composition, shifting the balance between bacterial species that are connected to immune activation. This suggests that the gut microbiome, together with the NLRP3 inflammasome, is a major bridging axis in mediating the deleterious effects of stress on behaviour and on gut microbiome composition, and that targeting of this axis could hold therapeutic potential in MDD treatment. Finally, plasma ACTH and CORT levels in (*Casp1, Ifngr, Nos2*)$^{-/-}$ mice resembled those of wt mice following stress, suggesting that the observed stress resilience of this mouse strain might be mediated by systems other than the HPA axis.

Our results suggest that a) CASP1 is a regulator of innate behaviour and of locomotor activity, b) CASP1 modulates the development of stress-induced depressive-like behaviour, c) minocycline has antidepressant-like effects in a chronic stress regimen in mice, d) minocycline treatment may exert its antidepressant-like effects at least partially through its influence on gut microbiome composition, e) genetic deletion of *Casp1*, *Nos2* and *Ifngr* decreases anxiety- and depressive-like behaviour and increases locomotor activity at baseline while preventing the stress-induced exacerbation of anhedonic behaviour, f) CASP1, NOS2 and IFNG inhibition could be valuable in the treatment of MDD.

Further clinical trials are warranted and strongly encouraged to determine the safety and efficacy of inhibiting inflammasome bioactivity as well as NOS2- and IFNG-mediated pathways in the treatment of MDD while investigating the effects of such strategies on gut microbiome composition and immune competency. Finally, we suggest that therapeutic strategies aiming at regulating the microbiome-inflammasome-gut-brain axis via inflammasome inhibition or gut microbiome modulation (through diet, psychobiotics supplementation and faecal microbiome transplantation) could prove valuable in the treatment of MDD and other psychiatric conditions with neuroinflammatory components.

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Abbreviations

η^2_p: Partial eta squared

5HIAA: 5-Hydroxyindoleacetic acid

5HT: Serotonin

16S rRNA: 16 Svedberg ribosomal ribonucleic acid

ACTH: Adrenocorticotropic hormone

AD: Alzheimer's disease

AIM2: Absent in melanoma 2

ANOVA: Analysis of variance

ASC: Adaptor protein apoptosis-associated speck-like protein containing a CARD

BBB: Blood-brain barrier

BL: Baseline

CA1: Cornu Ammonis 1

CA4: Cornu Ammonis 4

CASP1: Caspase 1

CASP1: Caspase 1 (human gene)

Casp1: Caspase 1 (mouse gene)

***Casp1*$^{-/-}$**: Caspase 1 knockout mouse

(*Casp1, Ifngr, Nos2*)$^{-/-}$: Caspase 1, interferon gamma receptor, nitric oxide synthase knockout mouse

CASP2: Caspase 2

CASP3: Caspase 3

CCL5: Chemokine (C-C motif) ligand 5

CFS: Chronic fatigue syndrome

CNS: Central nervous system

CORT: Corticosterone

CRH: Corticotropin releasing hormone

CRS: Chronic restraint stress

CUMS: Chronic unpredictable mild stress

DAMP: Damage-associated molecular patterns

DNA: Deoxyribonucleic acid

DOPAC: 3,4-Dihydroxyphenylacetic acid

DSM-V: 5th Edition Diagnostic and Statistical Manual of Mental Disorders

ELISA: Enzyme-linked immunosorbent assay

EPM: Elevated plus maze

FST: Forced swim test

GABA: Gamma-aminobutyric acid

HPA: Hypothalamic-pituitary-adrenal

HPLC-MS: High performance liquid chromatography coupled to mass spectrometry

hsCRP: High-sensitivity C-reactive protein

IBD: irritable bowel disease

IBS: Irritable bowel syndrome

IDO1: Indoleamine 2,3-dioxygenase 1

IFN: Interferon

IFNG: Interferon gamma

IFNGR: Interferon gamma receptor

IFNGR: Interferon gamma receptor (human gene)

Ifngr: Interferon gamma receptor (mouse gene)

***Ifngr*$^{-/-}$**: Interferon gamma receptor knockout mouse

IgA: immunoglobulin A

IgM: Immunoglobulin M

IL1: Interleukin 1

IL1A: Interleukin 1 alpha

Il1a: Interleukin 1 alpha (mouse gene)

***Il1a*$^{-/-}$**: Interleukin 1 alpha knockout mouse

IL1B: Interleukin 1 beta

Il1b: Interleukin 1 beta (mouse gene)

***Il1b*$^{-/-}$**: Interleukin 1 beta knockout mouse

IL1R1: Interleukin 1 receptor

IL2: Interleukin2

IL2: Interleukin 2

IL4: Interleukin 4

IL5: Interleukin 5

IL6: Interleukin 6

IL10: Interleukin 10

IL12: Interleukin12

IL13: Interleukin 13

IL18: Interleukin 18

***Il18*$^{-/-}$:** Interleukin 18 knockout mouse

IL18R: Interleukin 18 receptor

IL22: Interleukin 22

IL33: Interleukin 33

i.p.: Intraperitoneally

IRF: Interferon regulatory factor

IRF1: Interferon regulatory factor 1

IRF2: Interferon regulatory factor 2

ISRE: Interferon gamma stimulated response element

KO: Knockout

LC-NE: Locus coeruleus-norepinephrine

LPS: Lipopolysaccharide

MAOI: Monoamine oxidase inhibitor

MBT: Marble burying test

MDD: Major depressive disorder

MGB: Microbiota-gut-brain

MGIB: Microbiota-gut-inflammasome-brain

MYD88: Myeloid differentiation primary response 88

mRNA: Messenger ribonucleic acid

NE: Norepinephrine

NFKB1: Nuclear factor kappa B subunit 1

NLR: Nod-like receptor

NLRP3: Nod-like receptors family pyrin domain containing 3

Nlrp3: Nod-like receptors family pyrin domain containing 3 (mouse gene)

***Nlrp3*$^{-/-}$**: Nod-like receptors family pyrin domain containing 3 knockout mouse

Nlrp6: Nod-like receptors family pyrin domain containing 6 (mouse gene)

***Nlrp6*$^{-/-}$**: Nod-like receptors family pyrin domain containing 6 knockout mouse

NO: Nitric oxide

***Nod1*$^{-/-}$**: Nucleotide-binding oligomerization domain-containing protein 1

***Nod2*$^{-/-}$**: Nucleotide-binding oligomerization domain-containing protein 2

NOS1: Neuronal nitric oxide synthase

NOS2: Inducible nitric oxide synthase

NOS2: Inducible nitric oxide synthase (human gene)

Nos2: Inducible nitric oxide synthase (mouse gene)

***Nos2*$^{-/-}$**: Inducible nitric oxide synthase knockout mouse

NOS3: Endothelial nitric oxide synthase

NR3C1: Glucocorticoid receptor

NR3C2: Mineralocorticoid receptor

NSF: Novelty suppressed feeding

OFT: Open field test

OTU: Operational taxonomic unit

p38 MAPK: p38 mitogen-activated protein kinase

PCR: Polymerase chain reaction

PD: Parkinson's disease

PERMANOVA: Permutational multivariate analyses of variance

PPR: Pattern recognition receptor

RLR: Rig-like receptor

ROS: Reactive oxygen species

RNA: Ribonucleic acid

RR: Rotarod

SCFA: Short chain fatty acid

SIMPER: Similarity percentages

SNRI: Serotonin-norepinephrine-reuptake inhibitors

SNS: Sympathetic nervous system

SPT: Sucrose preference test

SSRI: Selective-serotonin-reuptake inhibitor

ST2: Interleukin 33 receptor

STAT1:

T-reg: Regulatory T cell

TCA: Tricyclic antidepressant

Th1: T cell-helper 1

Th2: T cell-helper 2

Th17: T cell-helper 17

TLR: Toll-like receptor

TLR4: Toll-like receptor 4

TNF: Tumour necrosis factor

TPH2: Tryptophan hydroxylase 2

wt: Wild-type

Table of figures

Table of tables