Ollscoil na hÉireann, Corcaigh
National University of Ireland, Cork

Department of Psychiatry and Neurobehavioural Science
Head of Dept. Timothy G. Dinan

The Neurobiological Effects of Naturally-Derived Polyphenols and Phospholipids in Cellular & Animal Models of Stress

Thesis presented by
Francisco Donoso

under the supervision of
Prof. John F. Cryan
Prof. Catherine Stanton
Prof. Timothy G. Dinan

for the degree of Doctor of Philosophy
December, 2019

Table of contents

Declaration

This thesis submitted is my own work and has not been submitted for any other degree, either at University College Cork or elsewhere.

Author Contributions

All of the work conducted in this thesis was performed independently by the author with the following exceptions.

Chapter 2

Dr. Valerie Ramírez helped with the experimental design of polyphenol screening in cortical cells.

Chapter 2

Dr. Anna Golubeva and Dr. Gerry Moloney assisted with the RPPA analysis.

Chapter 3

Mr. Patrick Fitzgerald performed the maternal separation and behavioural assessment along with the author.

Chapter 3

Dr. Sian Egerton performed the gut microbiota 16S rRNA sequencing and the short-chain fatty acid determination.

Chapter 3

Dr. Fiona Fouhy and Mr. Thomaz Bastiaanssen carried out the statistical analysis on the gut microbiota data.

Chapter 4

Dr. Marina Schverer performed the immunocytochemistry analysis along with the author.

Chapter 5

Dr. Marina Schverer performed the social defeat procedure and the behavioural assessment along with the author.

Acknowledgments

First of all, I wish to give my gratitude and thanks to my family for all the support and sacrifice that you made on my behalf to get me this far. In particular, I want to thank my father Francisco, my mother Ximena and my sister Gabriela for their encouragement to leave Chile in order to find new opportunities for the development of my career. Thanks for following my scientific work with genuine interest. Thanks for believing in me. I love you all.

I would like to thank Loreto, my partner, who has walked by my side all this time. You have been an important pillar in my life, and undoubtedly your company and love were essential for the completion of my PhD. I hope to be an invaluable support for your own goals, as you have been for mine.

Also, I would like to thank Dr. Gerard Moloney, Mr. Patrick Fitzgerald, Dr. Anna Golubeva, Dr. Harriet Schellekens, Ms. Tara Foley, Dr. Kieran Rea and Dr. Kenneth O'Riordan for their technical support and advice. Your expertise and knowledge made my PhD path an interesting research full of novelty and bright ideas.

To all the people from our laboratory, many friends and colleagues that made of my PhD an enjoyable journey. Special thanks to Dr. Martin Codagnone, Dr. Brunno Rocha Levone, Dr. Matteo Pusceddu, Dr. Marcel van de Wouw, Dr. Marcus Boehme, Mr. James Collins, Ms. Francisca Villalobos, Mr. Thomaz Bastiaanssen, Dr. Sofia Cussotto, Ms. Serena Boscaini, Dr. Christine Fulling, Mr. Simon Spichak, Ms. Katherine Guzzetta, Dr. Minal Jaggar, Mr. Nathaniel Ritz, Ms. Caoimhe Lynch, Dr. Marina Schverer and Dr. Ana Paula Ventura, and many others.

In particular, I want to thank Dr. Gerard Clarke for his support and warm welcome to the laboratory and for his help in my PhD registration and progress, as well as for the kind assistance given during my first days in Cork.

Finally, I wish to give my gratitude to my supervisors Prof. Timothy G. Dinan, Prof. Catherine Stanton and Prof. John F. Cryan for their guidance and encouragement throughout my PhD. Particularly, I would like to thank John for pushing me beyond

my capacities and making of me a better scientist. Perhaps I was not the best PhD student, for that reason I am very grateful to have had an amazing supervisor.

Publications and presentations

Published Papers During Thesis

1. Donoso F, Ramirez VT, Golubeva AV, Moloney GM, Stanton C, Dinan TG and Cryan JF (2019). Naturally-Derived Polyphenols Protect Against Corticosterone-Induced Changes in Primary Cortical Neurons. *The International Journal of Neuropsychopharmacology.*

Manuscripts Submitted/in preparation

2. Donoso F, Egerton S, Bastiaanssen TF, Fitzgerald P, Gite S, Fouhy F, Ross RP, Stanton C, Dinan TG and Cryan JF. Polyphenols Reverse Early-Life Stress-Induced Changes in the Microbiota-Gut-Brain Axis in the Rat. *Psychoneuroendocrinology*, under review.
3. Egerton S, Donoso F, Fitzgerald P, Gite S, Fouhy F, Whooley J, Dinan TG, Cryan JF, Culloty S, Ross RP, Stanton C. Investigating the Potential of Blue Whiting Fish Oil as a Nutraceutical in an Animal Model of Early Life Stress. *Nutritional Neuroscience*, under review.
4. Donoso F, Schverer M, Rea K, Roy BL, Dinan TG, Cryan JF and Schellekens H. Dietary Phospholipids Exert Neuromodulatory Effects on Neuronal Models. *Nutritional Neuroscience*, manuscript in preparation.
5. Schverer M, Donoso F, Rea K, Roy BL, Dinan TG, Cryan JF and Schellekens H. Neurobehavioral Effects of a Phospholipid-Enriched Diet in a Mouse Model of Chronic Psychosocial Stress. *Behavioural Brain Research*, manuscript in preparation
6. Schverer M, O'Mahony SM, O'Riordan KJ, Donoso F, Roy BL, Stanton C, Dinan TG, Schellekens H and Cryan JF. Fat is the New Smart: Dietary Phospholipids and Cognition Across the Lifespan. *Neuroscience Biobehavioural Reviews*, in press.

Poster conferences

1. Donoso F, Ramirez VT, Stanton C, Dinan TG and Cryan JF. Molecular Mechanisms Underlying the Effects of Naturally-Derived Polyphenols in Protecting Against Corticosterone-induced Cytotoxicity in Primary Cortical Cells: Implications for Stress-Related Disorders. *British Neuroscience Association, Festival of Neuroscience*, Dublin, Ireland. April 2019.
2. Donoso F, Egerton S, Fitzgerald P, Gite S, Stanton C, Dinan TG and Cryan JF. Dietary Intervention with Polyphenols Ameliorate Maternal Separation-Induced Depressive Behaviours. *The European Behavioural Pharmacology Society, Biennial Meeting*, Braga, Portugal. August 2019.
3. Donoso F, Egerton S, Fitzgerald P, Gite S, Stanton C, Dinan TG and Cryan JF. Polyphenols Reverse Anxious and Depressive-like Behaviours in the Maternal Separation Model: A role for the Gut Microbiome? *Society for Neuroscience, 49th Annual Meeting*, Chicago, US. October 2019.
4. Donoso F, Egerton S, Fitzgerald P, Gite S, Stanton C, Dinan TG and Cryan JF. Dietary Intervention with Polyphenols Ameliorate Maternal Separation-Induced Depressive Behaviours. *University College Cork, New Horizons Research Conference*, Cork, Ireland. December 2019.

Abstract

The molecular and cellular basis of stress neurobiology remain an important research question in clinical science. Indeed, stress-related mental disorders, including depression and anxiety, are currently a major public health concern. Thus, improving our knowledge about the pathophysiology of these neuropsychiatric disorders may enable the development of novel strategies for their treatment and prevention. On the other hand, the inefficacy of currently available therapies for various stress-related disorders, and the numerous side effects that accompany these treatments, have strengthened the search for less invasive strategies with fewer negative side effects. In this regard, the emerging and compelling evidence for nutrition as a potential therapeutic avenue for the treatment of mental disorders suggests that changes in diet are a viable strategy for improving mental health and treating stress-related psychiatric disorders. Moreover, there is considerable evidence suggesting that certain natural compounds available in diet have a therapeutic potential to improve mental health and disease.

For instance, naturally occurring phytochemicals, namely polyphenols, are molecular compounds found in different plant sources, such as vegetables and fruits. Also, phospholipids are a class of lipid that comprise a major component of all cell membranes, specially concentrated in lean meat and dairy products. Both polyphenols and phospholipids have demonstrated interesting beneficial effects for human health. However, their therapeutic potential to act prophylactically against the detrimental effects of neuropsychiatric disorders have just begun to be taken seriously. Therefore, in this thesis we have tested the hypothesis that polyphenols and/or phospholipids could improve behavioural and neurobiological outcomes in cellular and animal models of stress.

Further, we provide evidence that polyphenols and phospholipids exert neuroprotective effects against the cytotoxicity produced by corticosterone, the main rodent stress hormone, in cortical neurons. Specifically, we have elucidated the potential mechanisms underlying polyphenol-mediated neuroprotection *in vitro*, and demonstrated that phospholipid exposure positively impacts on neurodevelopmental

processes, such as proliferation and differentiation of cultured neural progenitor cells. In addition, we confirmed the therapeutic potential of a dietary intervention with polyphenols by detecting its capacity to reverse depressive- and anxiety-like behaviours induced in a rat model of early-life stress. Moreover, we demonstrated potential implications to modulate BDNF-dependent recovery, regulation of the HPA axis and the microbiota-gut-brain axis in polyphenol-mediated behavioural improvement.

Taken together, our findings support the therapeutic potential of polyphenols for stress-related mental disorders, and we further provide evidence for the possible mechanisms by which they may exert these effects. On the other hand, our data reveal that the novel neuromodulatory potential of phospholipids *in vitro* does not correlate with their inefficacy in attenuating chronic stress-induced behavioural impairment in mice. Nevertheless, these findings contribute to an exciting and growing body of research suggesting that nutritional interventions may have an important role to play in the treatment of stress-related psychiatric conditions.

Chapter 1

General

Introduction

1.1 The stress response

The fight-or-flight, or stress responses is a physiological reaction that occurs in response to intrinsic or extrinsic forces capable of threat to the physical and psychological equilibrium, or *homeostasis* (Jansen *et al.* 1995). All these forces or *stressors*, including harmful events, extreme pain or anguish, or threat to survival, can trigger a repertoire of complex biochemical and behavioural reactions, also known as *allostasis*, a physiological process aimed to counteract the effects of stressors and re-establish homeostasis (Sterling 2012). Within these adaptational responses to stress are included central and peripheral reactions that lead to the facilitation of neural pathways associated with arousal, alertness, vigilance, cognition, and focused attention with concurrent inhibition of pathways that mediate passive functions such as feeding and reproduction (Chrousos *et al.* 1992). When these adaptive systems are turned on and turned off again efficiently and not too frequently, the body is able to cope effectively with challenges that it might not otherwise survive. However, there are a number of circumstances in which allostatic systems may either be overstimulated or not perform normally, and this condition has been termed *allostatic load*, which can lead to disease over long periods (McEwen *et al.* 1993).

1.2 Neurobiology of stress

The impact of the stress response on brain cellular and behavioural function is dependent of the activation of specific neurocircuitry and physiological cascades. For instance, the stress response leads to the activation of neuronal circuits involved in the efferent sympathetic/adrenomedullary system together with the hypothalamic-pituitary-adrenal (HPA) axis (Dunn *et al.* 2008). Acting in concert, both components coordinate emotional, cognitive, neuroendocrine and immune inputs to determine the magnitude and specificity of behavioural and hormonal responses to stress. Specifically, parvocellular corticotropin releasing factor (CRF) is the principal hypothalamic stimulus to the HPA axis activation, that induces the increase of adrenocorticotropic hormone (ACTH) from the pituitary, resulting in glucocorticoid secretion in the adrenal cortex, which is also critical for the regulation of the HPA axis by negative feedback (Fig. 1.2.1) (Tsigos *et al.* 2002).

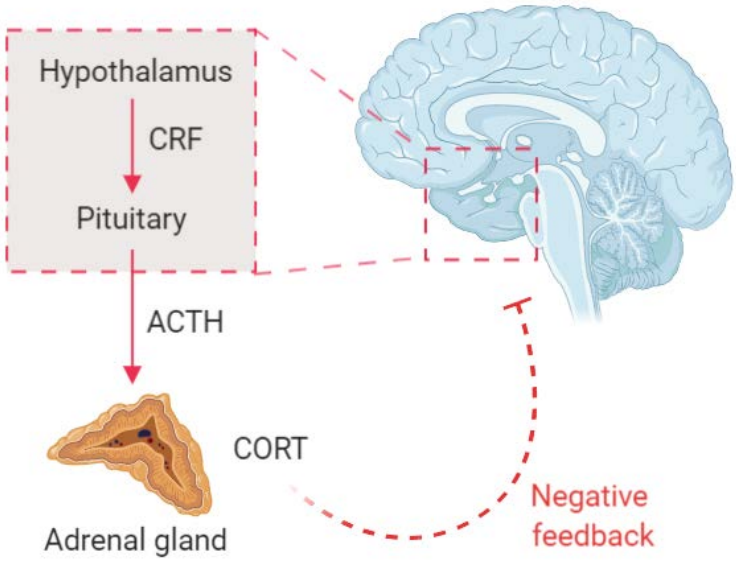

Figure 1.2.1 A simplified schematic representation of the activation of the HPA axis*. During stress the hypothalamus secretes corticotropin releasing factor (CRF), that induces the increase of adrenocorticotropic hormone (ACTH) from the pituitary, resulting in the release of glucocorticoids, such as cortisol (humans) and corticosterone (rodents), which mediate the negative feedback of the HPA axis.*

Glucocorticoids reach every organ by way of the circulation, where their effects are differentiated into two classes of action: modulating actions, which alter the organism's response to the stressor, and preparative actions, which alter the organism's response to a subsequent stressor or aid in adapting to a chronic stressor (Sapolsky *et al.* 2000). The underlying mechanism involves an integrated response, which starts with rapid hormone-induced changes in receptor conformation that lead to slower modulations of gene transcription (de Kloet *et al.* 2005). Specifically, the receptor system of glucocorticoids consists of two related receptor molecules, the low affinity glucocorticoid receptor (GR) and the high affinity mineralocorticoid receptor (MR), which bind the same hormone (primarily cortisol in humans and corticosterone in rodents); however, the GR becomes progressively activated during stress (Kitchener *et al.* 2004).

Glucocorticoid binding provokes conformational changes in the GR that activate multiple functional domains, including nuclear-localization sequences. After translocation into the nucleus, GR associates with specific genomic glucocorticoid response elements (GREs) and nucleates the assembly of transcription regulatory

complexes containing GR, and co-regulatory factors, which together activate or repress the transcription of glucocorticoid-responsive genes (Fig. 1.2.2) (Weikum *et al.* 2017).

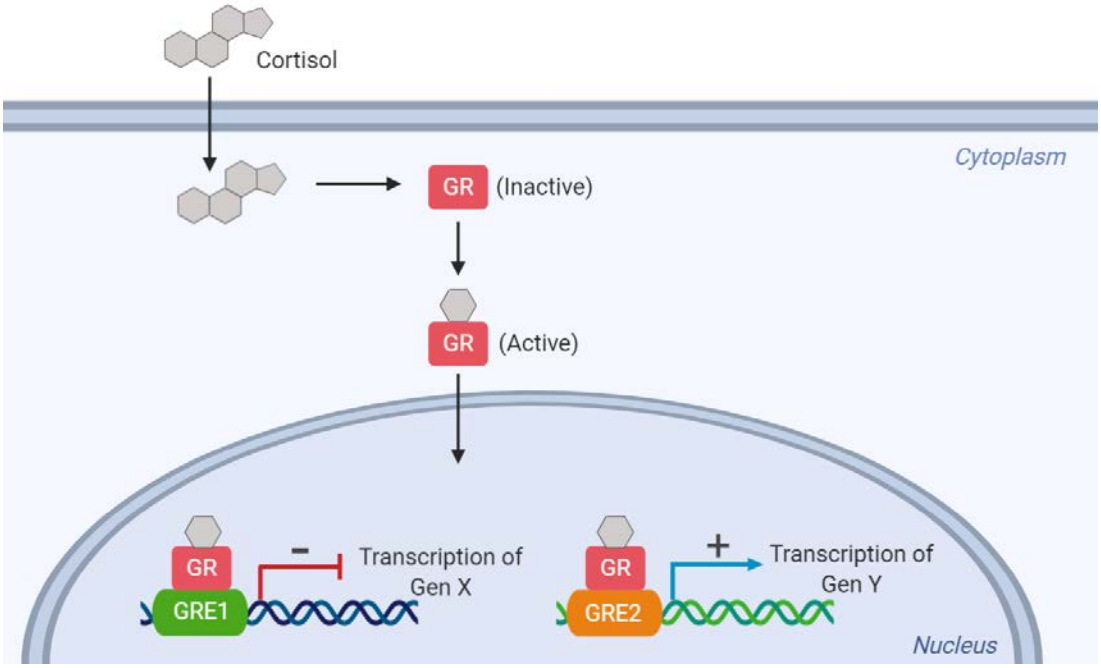

Figure 1.2.2 Glucocorticoid receptor signalling and DNA binding*. Cortisol interacts with the glucocorticoid receptor (GR) in the cytoplasm. Once the GR is activated, it translocates into the nucleus and bind to specific DNA sites named glucocorticoid receptor elements (GREs). GRE1 and GRE2 represent distinct GREs within the genome, Gene X and Gene Y represent the genes under the control of GRE1 and GRE2, respectively.*

Some of the effects produced by activation of the glucocorticoid cascade and catecholamine elevation during stress, include the mobilisation and replenishment of energy stores needed for brain and body function under challenge, and in other contexts have been detected through changes in food intake, dramatic shifts in metabolism, muscle morphology, and increases in blood pressure. These adaptations maintain essential metabolism and body temperature homeostasis (McEwen *et al.* 2003).

At molecular level, some of the genes induced after GR activation and translocation into the nucleus include those associated with the intracellular negative feedback of GR activity (Gjerstad *et al.* 2018). For example, the FK506 binding protein 5 (FKBP5) is a co-chaperone of hsp90 which regulates GR sensitivity. When it is bound to the

receptor complex, cortisol binds with lower affinity and nuclear translocation of the receptor is less efficient. FKBP5 mRNA and protein expression are induced by GR activation via intronic hormone response elements and this provides an ultra-short feedback loop for GR-sensitivity (Binder 2009, Fries *et al.* 2017).

1.3 Stress-related mental disorders

Although it is believed that mild, brief, and controllable states of challenged homeostasis could be perceived as positive stimuli to emotional and intellectual growth and development, if the stress response is inadequate or excessive and prolonged, the cost of reinstating homeostasis might become too high, producing a vulnerable phenotype increasing the risk of developing mental illness (de Kloet *et al.* 2005). Indeed, it is well known that stressful events in early life, or acute and chronic stressors, can exert long-lasting changes later on in brain structure and function (Cryan *et al.* 2013). Accumulating evidence indicates that these adversities are associated with an increased risk for developing stress-related mental disorders such as anxiety and depression (Chapman *et al.* 2004, Heim *et al.* 2012).

Major depressive disorder (MDD), is a serious stress-related mental disorder characterised by at least two weeks of low mood, accompanied by low energy, low self-esteem, loss of interest in usually enjoyable activities, and sometimes includes pain without apparent cause (Smith 2014). On the other hand, anxiety disorders including generalised anxiety disorder (GAD), phobias, panic disorder, obsessive-compulsive disorder and post-traumatic stress disorder, are characterised by strong feelings of apprehension, uneasiness, and an unpleasant state of inner turmoil (Nagata *et al.* 2015). In general, stress and hyper activation of the HPA axis have been demonstrated to mediate, or even promote mental disorders, including anxiety and major depression (Esch *et al.* 2002). In 2015, depressive disorders were estimated to be the third leading cause of disability worldwide. Now, the WHO indicates that depression is a leading cause of disability worldwide and is a major contributor to the overall global burden of disease (Park *et al.* 2019).

1.4 Pathophysiology of stress-related disorders

Stress-related neuropsychiatric disorders, especially depression, do not have a clear aetiology due to their multiple environmental and genetic factors (Saveanu *et al.* 2012). However, there are some promising biomarkers that have been associated with the pathophysiological mechanisms involved in development and onset of these mental disorders (Fig. 1.4.1). For instance, depression, stress and the HPA axis have been associated through several lines of evidence; for example, the core symptoms of depression including low mood, inability to enjoy activities usually considered pleasurable and low energy, are a clear cross-cultural response to stressful events. In addition, the HPA axis is activated in response to stress, leading to a potent release of glucocorticoids into the bloodstream, whereas depression in severe cases is also characterised by over activation of the HPA axis (Pariante 2003). On the other hand, the correlation between early stress events, such as childhood abuse or neglect, and development of anxiety disorders in adulthood, as well as increased hypothalamic CRF neuronal activity strongly support the link between stress, the HPA axis and, anxiety disorders (Arborelius *et al.* 1999).

Studies into the pathophysiology of MDD have confirmed that the neurochemical balance of depressed people is altered, specifically at the main monoamine neurotransmitter level (i.e. dopamine, noradrenaline and serotonin) (Nutt 2008). Accumulated lines of evidence indicate that abnormal concentrations of monoamine neurotransmitters and their metabolites in brain regions that contribute importantly to regulation of mood and motivation, could significantly impact on pathogenesis of depression (Liu *et al.* 2018).

Chronic neuroinflammation has also been associated with the emergence of depressive symptoms (Brites *et al.* 2015). Activation of the peripheral immune system leads to pro-inflammatory cytokine production that eventually reach the CNS stimulating astrocytes and microglia with significant implications for psychiatric disorders (Muller *et al.* 1998). Indeed, human and animal studies revealed increased activation and numbers of microglia in depression and anxiety disorders (Frick *et al.* 2013), and elevated production of cytokines in different animal models of stress (Goshen *et al.*

2007, Liu *et al.* 2013). Taken together, the study of cellular and molecular mechanisms of inflammation in stress-related disorders, offers a new perspective for treatment targeting neuroinflammatory signalling pathways.

1.4.1 The gut microbiota, stress and mental health

Several lines of evidence identify the gut microbiome as a key player in maintaining homeostasis and that a disruption in its composition contributes to various diseases, including mental disorders (Cryan *et al.* 2012, Lucas 2018, Taylor 2019). The human microbiome represents a complex ecosystem that consists of a multitude of microorganisms including bacteria, archaea, yeasts and viruses (Eckburg *et al.* 2005), and there is a growing body of data focused on the impact of the gut microbiome on behaviour supported by the concept of the microbiota-gut-brain axis (Rhee *et al.* 2009, Cryan *et al.* 2019), a complex network formed by the enteric nervous system (ENS), sympathetic and parasympathetic divisions of the autonomic nervous system, neuroendocrine and neuroinmune components of the central nervous system (CNS), and the gut microbiota (Mayer *et al.* 2014).

The brain can influence the gut microbiota indirectly via changes in gastrointestinal motility and secretion, intestinal permeability, or directly, via signalling molecules released into the gastrointestinal lumen from cells in the lamina propria such as enterochromaffin cells, neurons and immune cells. On the other hand, communication from gut microbiota can occur via multiple mechanisms, including receptor-mediated signalling or through direct stimulation of host cells in the lamina propria (Collins *et al.* 2009). For instance, vagal innervation has been identified as a major constitutive communication pathway in probiotic mediated improvements in emotional behaviour and central GABA receptor expression in the mouse (Bravo *et al.* 2011). A probiotic is a mono- or mixed culture of live microorganisms which, applied to animal or man, affect beneficially the host by improving the properties of the indigenous microflora (Jezewska-Frackowiak *et al.* 2018).

Alterations in brain-gut interactions are associated with gut inflammation, chronic abdominal pain syndromes and eating disorders, and that modulation of gut-brain axis

is associated with alterations in the stress response and behaviour (Cryan *et al.* 2012). For instance, studies have demonstrated that chronic stress alters the structure of the intestinal microbiota in rodent models and humans (Bailey *et al.* 2011, Karl *et al.* 2017). In addition, changes in the gut microbiota composition have been associated with depressive- and anxious-like behaviours, as well as with altered serotonin levels in a mouse model of depression (Park *et al.* 2013). In this regard, many studies have reported a restoration of gut microbiota after antidepressant treatments. Taken together, research focused on developing novel therapeutic strategies to treat neuropsychiatric disorders by targeting the gut microbiota is rapidly growing (Long-Smith *et al.* 2019). Indeed, it has recently been proposed that depression is an 'unholy trinity' of stress, inflammation and microbiota (Cruz-Pereira *et al.* 2020).

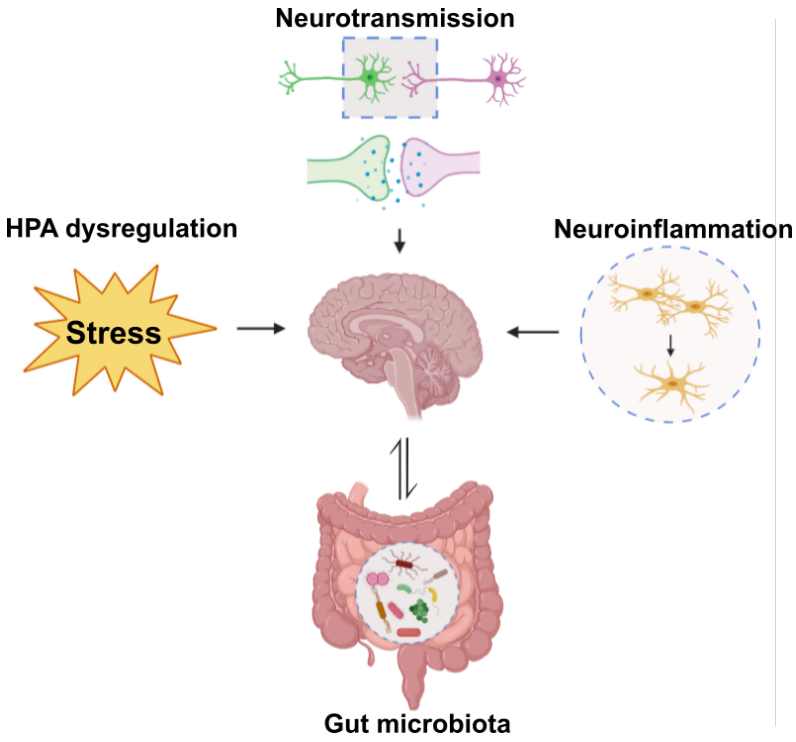

Figure 1.4.1 Physiological components involved in stress-related disorders pathology. *HPA dysregulation, altered neurochemical balance, chronic neuroinflammation and disrupted microbiota-gut-brain axis, are some components associated with the pathophysiological mechanisms of stress-related mental disorders.*

1.5 *In vitro* models of stress-related conditions

In vitro, the Latin for *in glass*, is referred to those scientific studies performed with cells, microorganisms, and biomolecules outside of their usual biological context. Indeed, *in vitro* models for drug discovery and research have gained great interest because of the limitations of animal models in terms of feasibility and ethical concerns. Therefore, by reducing the animal usage for drug research, *in vitro* models can serve as platforms for more controlled screening of compounds with potential pharmacological effects (Elliott *et al.* 2011). In addition, other advantages of *in vitro* experimentation include an expedited study of biological mechanisms of action, such as activation/downregulation of specific signalling pathways as it is easier to measure the impact of a single experimental variable (e.g. a drug) on a simple and well controlled system, and allows testing of a large number of different combinations of experimental parameters (Bajpai *et al.* 2002).

Different *in vitro* models have been developed to understand diverse physiological conditions and cellular processes including cell differentiation and proliferation, metabolism, epigenetics, apoptosis, etc., as well as the elucidation of molecular determinants of disease, including cancer, degenerative and cardiovascular diseases (CVD), viral infective mechanisms, and neurobiological disorders (Abid *et al.* 2005, Katt *et al.* 2016, Mathur *et al.* 2016). Here, *in vitro* models have been extremely important in studying brain development, and exploring therapeutic options for disorders associated with alterations of the central nervous system (CNS) structure and function (Shuler *et al.* 2014). Several classes of *in vitro* models of the brain have been described, including immortalised neuronal cell lines and primary cell cultures from CNS tissue (Fig. 1.5.1 A). However, “neurons” include many diverse cell types among which perhaps just one or a few may be ideal for a particular experiment (Hollenbeck *et al.* 2003).

For instance, it has proven particularly challenging to develop *in vitro* approaches to study psychiatric treatment for mood disorders, as they are linked with the alteration of a number of brain regions and produce a complex array of clinical symptoms. However, basic phenotypes may exist at the level of single neurons (Brennand *et al.*

2012). Indeed, it is hypothesised that many psychiatric disorders are amenable for cell-based *in vitro* studies because of their highly heritable component (Bearden *et al.* 2006). For example, using induced pluripotent stem cell-derived (IPSCs) neural tissue, specific *in vitro* models can be generated from patients with a particular mental disorder, including depression and anxiety (Fig. 1.5.1 B) (Soliman *et al.* 2017). (Tatro *et al.* 2009). Similarly, the recent development of 3D brain organoids derived from human pluripotent stem cells (hPSCs) offers a promising approach for investigating the phenotypic underpinnings of these highly polygenic disorders and for understanding the contribution of individual risk variants and complex genetic background to human pathology (Fig. 1.5.1 B) (Quadrato *et al.* 2016).

On the other hand, blocking or reducing the expression of genes potentially involved in depression can offer a useful tool to understand the role of specific proteins in the development of this mental disorder. Indeed, using knock-down or knock-out techniques in cellular models to manipulate genetic expression of potential biomarkers involved in stress-related neuropsychiatric disorders, allows to elucidate novel signalling pathways and molecular targets for therapeutic approaches (MacLaren *et al.* 2011, Shah *et al.* 2016) (Fig. 1.5.1 C).

An alternative approach to study the neurobiological changes produced during stress-related disorders *in vitro*, use cell-based high glucocorticoid exposure models. These models are based on the high glucocorticoid release produced after abnormal HPA activity during chronic stress events (Pariante 2003). It has been demonstrated that exposure to elevated concentrations of corticosterone (main rodent glucocorticoid hormone) induces neurotoxic effects in different neuronal *in vitro* models, including hippocampal neurons (Latt *et al.* 2018), PC12 cell line (Li *et al.* 2014), hypothalamic neurons (Zhang *et al.* 2012), and neurons derived from brain cortex (Pusceddu *et al.* 2016). For this reason, corticosterone-induced neurotoxicity approaches represent an interesting *in vitro* model to understand the detrimental effects of high glucocorticoid exposure at the molecular and morphological level in neurons, and provide an excellent platform to explore novel drugs with potential therapeutic effects against stress-related disorders (Fig. 1.5.1 C).

Finally, *in vitro* models of stress-related conditions are excellent platforms to test novel chemical-based drugs and naturally-derived compounds with neuropharmacological activity and for selecting which ones with potential therapeutic effect (Kelava *et al.* 2016) (Fig. 1.5.1 C). Indeed, pharmacological research is critical to elucidate signalling pathways associated with stress response and altered gene/protein expression profile of patients suffering of stress-related disorders, and eventually to discover agonist or antagonist drugs able to ameliorate this phenotype (Ghanemi 2014). New treatments for neuropsychiatric disorders associated with stress depend of pharmacological research, and cell-based models of disease-related phenotype represent a powerful tool to carry on these investigations.

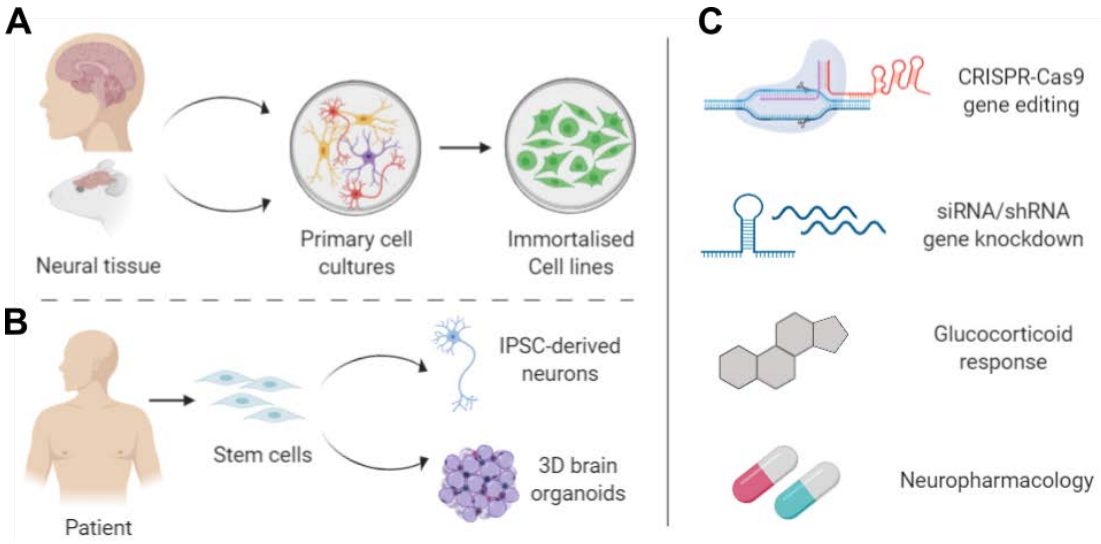

***Figure 1.5.1 Strategies to study stress-related conditions in vitro**. **(A)** Traditional methods to obtain in vitro models of the brain from CNS tissue, including primary cell cultures and immortalised cell lines. (B) Using human pluripotent stem cells from patients with stress-related disorders to generate induced pluripotent stem cell-derived (IPSCs) neuronal cultures and 3D brain organoids. (C) Molecular techniques available to study the neurobiology of stress, such as gene knock-down or knock-out, glucocorticoid exposure and pharmacological screening.*

1.6 Animal models of stress

The necessity to understand the neural processes underpinning behaviour in humans has boosted the field of comparative psychology, which refers to the scientific study of behaviour and cognitive actions of non-human animals (Krupenye *et al.* 2019). Thus, comparative psychology has been crucial for the development of *in vivo* animal

models for evaluating novel treatment approaches in human disease. In addition, the large cost of clinical trials and psychiatric human studies represents a difficult barrier for the development of novel drugs aimed at the central nervous system (CNS) (Frantz 2004).

Indeed, many models and tests for assessing depressive- and anxious-like behaviours in rodents were designed to investigate potential psychiatric drugs such as antidepressants and anxiolytics (Cryan *et al.* 2005). An important number of these animal models of stress-related disorders involve exposure to stressful situations, because it has been demonstrated that trauma or chronic stress are important predisposing factors to depression and anxiety (Kessler 1997). For instance, some chronic stress-based models include chronic unpredictable stress (CUS) and chronic social defeat stress (CSDS) in adult rodents, and maternal separation (MS) and prenatal stress in young rodents (Fig. 1.6.1). CUS is a model that comprises rodent exposure to several different and unpredictable stressors over a number of weeks. It has been reported that CUS induces depressive-like behaviour in adult rodents, specifically anhedonia, a behaviour associated with reduced motivation and inability to experience pleasure (Willner 1997). In contrast, the CSDS procedure exposes the animals to an aggressive and dominant conspecific rodent during a specific period. This psychosocial stress on these animals induces social avoidance behaviours when they are exposed again to an unfamiliar rodent, and exhibit depressive- and anxiety-like behaviours (Finger *et al.* 2011).

On the other hand, the necessity to understand the implications of early-life adversity on emotionality and behaviour, leaded to investigate the role of maternal and environmental influence on the neurobiology of stress in rat pups around 50 years ago (Levine 1967). This work and other later studies (Meaney 2001, Plotsky *et al.* 2005) demonstrated that changes in rodent early postnatal experiences can induce profound long-lasting effects on gene expression, hippocampal architecture and CRF system in adult offspring. Thus, the MS model is based on the negative effects of early life stress on brain function and structure, which are associated with development and onset of depression and anxiety in adulthood (Lupien *et al.* 2009). This procedure involves separation of pups from their mothers for a defined period (usually 3 hours per day

from post-natal day 2 to 12) (O'Mahony *et al.* 2009). Evidence indicates that MS in rodents can produce significant changes in neurochemical balance and depressive- and anxious-like behaviours in adult animals (O'Mahony *et al.* 2011). Finally, the prenatal stress model involves exposure of a pregnant mouse or rat to a stressor during a defined timeline, inducing depressive and anxiety-like behaviours in the litter later in adulthood (Abe *et al.* 2007).

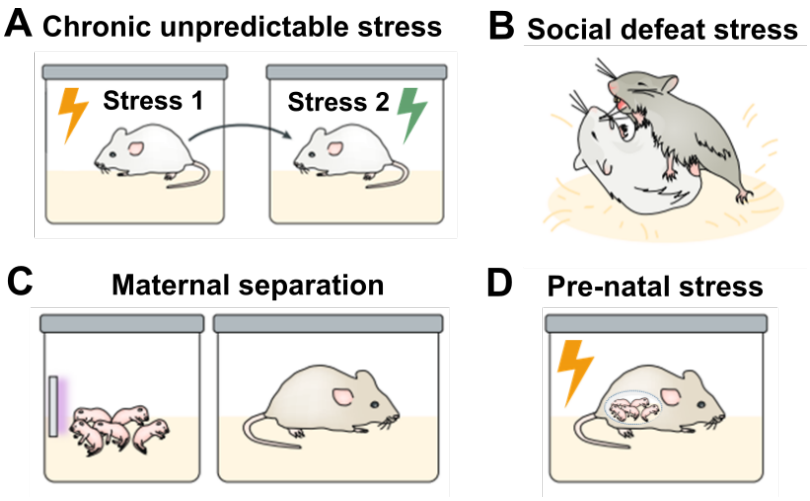

Figure 1.6.1 *Some preclinical approaches to study stress-related conditions in rodent models. Modified from (Gururajan et al. 2019).*

In contrast, other animal models of stress do not involve repeated stress exposure. For instance, preclinical studies have favoured an approach that models HPA hyperactivity by supplementation of corticosterone through injections (Demuyser *et al.* 2016). Indeed, it has been found that HPA axis dysregulation and increased release of peripheral cortisol (corticosterone in rodents) is associated with the neuromolecular and behavioural phenotypes of stress-related disorders (Anacker *et al.* 2011). However, there are some limitations in using corticosterone supplementation, including sex specific effects and hepatic metabolism of exogenous corticosterone (endogenously secreted corticosterone goes directly to systemic circulation) (Gururajan *et al.* 2019).

In order to assess and quantify behaviours associated with depression and anxiety produced by the rodent stress model, several tests have been developed to detect specific symptoms and markers of stress-associated mental disorders (Cryan *et al.*

2007). For example, one of the most reproducible behaviours linked to depression in animals is learned helplessness. This behaviour occurs when the subject, unable to escape, undergoes repeated painful or stressful stimuli that are unavoidable. Eventually, the subject accepts these aversive stimuli without trying to escape or avoid, and is said to have learned that it is helpless. The theory of learned helplessness is associated with the psychopathology of depression and other stress-related mental illnesses in humans (Seligman 1972, Pryce *et al.* 2011).

For instance, the learned helplessness paradigm in rodents can be tested through the forced swim test (FST; see Fig. 1.6.2 A), which is based on the observation that when rats are exposed to water they demonstrate passive immobile behaviour after struggling (swimming/climbing), reflecting behavioural despair (Slattery *et al.* 2012). The tail suspension test (TST; see Fig. 1.6.2 B) is another assessment used to detect behavioural despair related to learned helplessness after stress. Usually, mice are suspended by their tails, in such a way that they cannot escape or hold on to nearby surfaces (Steru *et al.* 1985, Cryan *et al.* 2005). In both tests, immobility is associated with depressive-like behaviours.

Anxiety-like behaviours in rodent models of stress can be assessed using the elevated plus maze test (EPM; see Fig. 1.6.2 C). The EPM consists of a maze composed by two crossed narrow corridors, where two opposite arms are open and the other two opposite arms are closed (open roof, but enclosed by walls). This test is based on rodent's natural aversion to elevated and open spaces (File *et al.* 2004). Similarly, the open field test (OFT; see Fig. 1.6.1 2 D) consists of an arena (circular, square or rectangular), where the animal is placed in the centre. Anxious behaviours are associated with reduced time spent in the centre of the arena, as well as with decreased entries to the centre (Prut *et al.* 2003). Other tests designed to measure anxiety in rodents are based in avoidance of aversive situations, including the elevated zero maze (EZM) and the light/dark box (Fig. 1.6.2 E and F) (Cryan *et al.* 2005).

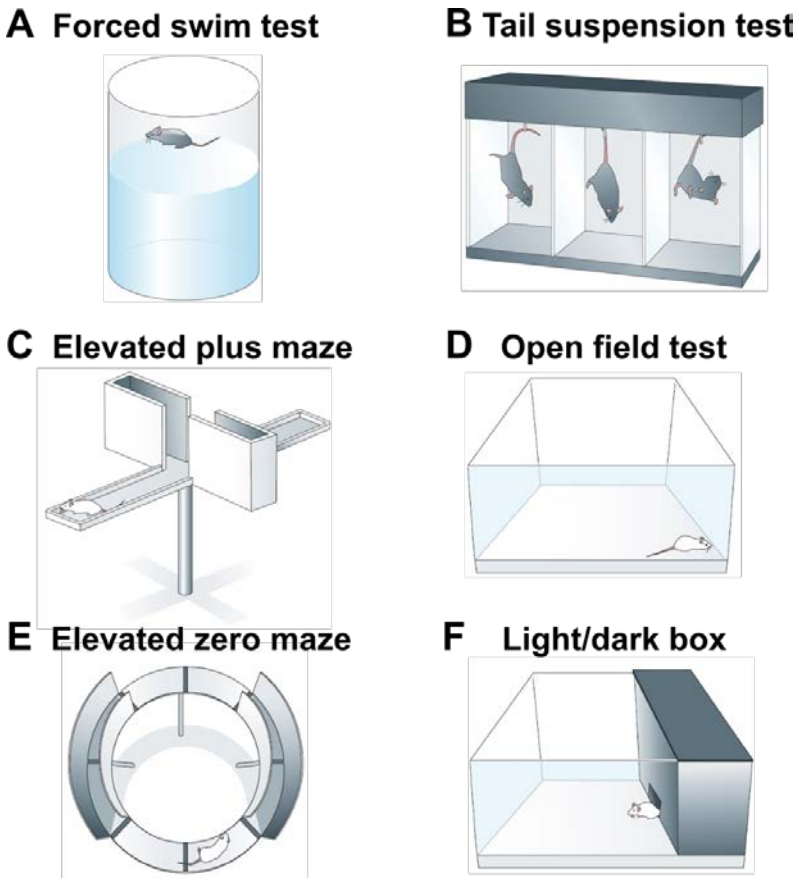

Figure 1.6.2 *Behavioural tests designed to detect anxiety- and depressive-like behaviours in rodent models. Modified from (Cryan et al. 2005).*

Taken together, although several techniques have been developed for understanding underlying mechanisms of novel compound treatments, the final output of the CNS is the behaviour, and should be the basis for conclusions in preclinical studies for drug discovery research.

1.7 Pharmacological treatments for stress-related disorders

Depression and anxiety have significant economic and social consequences worldwide (Greenberg *et al.* 2003), for this reason the search for effective therapeutic strategies to treat these neuropsychiatric disorders has become a major public health priority. Since the monoamine hypothesis of depression was first articulated around 55 years ago (Bunney *et al.* 1965, Schildkraut 1965, Delgado 2000), the initial approaches employed to pharmacologically induce an antidepressant effect involved increasing

the levels of monoaminergic neurotransmitters in the synapse to modulate postsynaptic receptor activity (e.g. serotonin, dopamine, and noradrenaline).

On the other hand, clinical observations have found both direct and indirect evidence of glutamatergic dysfunction in MDD. For example, different studies have reported increased levels of glutamate in plasma of patients diagnosed with MDD (Mauri *et al.* 1998, Kucukibrahimoglu *et al.* 2009); other reports using proton magnetic resonance spectroscopy found reduced metabolic glutamate/glutamine exchange in hippocampus (Block *et al.* 2009) and anterior cingulate cortex (Mirza *et al.* 2004) of people suffering from MDD. In addition, post-mortem analysis of MDD patients revealed changes in expression of N-methyl-D-aspartate (NMDA) receptor subunits. For instance, MDD patients showed altered expression of NR2A, NR2B and NR2C in different brain regions (Beneyto *et al.* 2008, Karolewicz *et al.* 2009, Chandley *et al.* 2014). This evidence has motivated new research focused on antidepressant drugs based on the glutamatergic system and NMDA receptor activity.

In contrast, early research in the field of tranquilizers lead to the discovery of chemical compounds with similar properties of another neurotransmitter, γ-aminobutyric acid (GABA). These drugs showed strong sedative, anticonvulsant, and muscle relaxant properties, with potential use for anxiolytic treatment (Froestl 2011). In this regard, the first successful pharmacological treatment for depression was iproniazid, an antitubercular compound under the trade name Marsilid® in 1958, after clinical observations that reported the side effects of this drug (Loomer *et al.* 1957). At the pharmacological level, Iproniazid was found to block monoamine oxidases (MAO), a family of enzymes that catalyse the oxidative disamination of biogenic amines decreasing monoamine levels in the synapse (Thase 2012). After iproniazid the antidepressant research was focused on the development of new MAO inhibitors (MAOI's), including non-selective MAOI's, and selective MAOI's for MAO-A and MAO-B (Yamada *et al.* 2004).

Although, some MAOI's have proven to be efficacious in the wide spectrum treatment of major depression (Amrein *et al.* 1993), and in some types of anxiety disorders (Yamada *et al.* 2004), there are serious side effects and adverse reactions associated

with MAOI's consumption, including hepatotoxicity, hypertensive crisis (Fiedorowicz *et al.* 2004), and withdrawal syndrome (van Broekhoven *et al.* 2002). For this reason, MAOI's are used in clinical situations in which patients respond poorly to first line antidepressants, and when other drugs have failed; thus, they are considered a last line of treatment.

On the other hand, the study of the first antidepressant also is attributed to Roland Kuhn, a Swiss psychiatrist who examined the therapeutic effects of an unknown compound designated G22355. As a result of the search of antipsychotic drugs for the treatment of schizophrenia, Kuhn noted that G22355 seemed to alleviate depression in the few schizophrenia patients who had prominent depressive symptoms (Brown *et al.* 2015). Eventually, he gave G22355, later named imipramine, to 100 patients suffering depression and his results were published in 1957 (Kuhn 1957). His findings supposed a major breakthrough in the history of psychiatry, that still have an impact on the treatment of depression and on the development of antidepressant drugs (Brown *et al.* 2015).

Since imipramine and other similar drugs (Gillman 2007) discovered later were characterised by a three benzene ring molecular core and their mechanism of action was unknown at the time of discovery, they were classified as tricyclic antidepressants (TCAs) (Hillhouse *et al.* 2015). Now, TCAs are described as having several pharmacological actions, including inhibiting presynaptic noradrenaline and serotonin reuptake transporters, blocking postsynaptic adrenergic α_1 and α_2 receptors, blocking postsynaptic muscarinic receptors, and blocking postsynaptic histamine H_1 receptors (Cusack *et al.* 1994, Owens *et al.* 1997, Vaishnavi *et al.* 2004). Their antidepressant effects are mainly attributed to the inhibition of noradrenaline and serotonin reuptake. On the other hand, because of their non-selective pharmacological targets, TCAs also have negative side effects, including dizziness, memory impairment, and drowsiness (Bet *et al.* 2013).

To date, antidepressant drug discovery research has become more focused on highly selective compounds with specific pharmacological targets. In addition, the evidence has begun to suggest a significant role for serotonin in MDD. During the 1960's, after

a post-mortem study that revealed decreased levels of this neurotransmitter in depressive suicides (Shaw *et al.* 1967), pharmaceutical companies began developing ligands that would selectively inhibit serotonin reuptake, to increase serotonin levels within the synaptic cleft. Thus, the first compound reported as a selective serotonin reuptake inhibitor (SSRI) with potential antidepressant activity was fluoxetine (LY110140) (Wong *et al.* 1974). After FDA approval in 1987, fluoxetine was launched under the trade name Prozac®.

Although, antidepressant drugs based on the monoaminergic hypothesis have had relevant utility in improving mood and normal daily functions in depressed patients, only 60% show considerable improvement after two months of treatment and all patients have to deal with numerous side effects (Al-Harbi 2012). For those patients, these drugs represent a valid alternative for MDD therapy. Indeed, excessive glutamate and subsequent over-stimulation of NMDA receptors leading to excessive Ca^{2+} influx has been implicated in the pathophysiology of many neurodegenerative diseases, including depression and anxiety (Sanacora *et al.* 2012). In addition, there are early findings suggesting that NMDA receptor antagonists possess antidepressant-like activity (Trullas *et al.* 1990), and that chronically administered antidepressants from different classes are able to modulate NMDA receptor function (Nowak *et al.* 1993, Paul *et al.* 1994). Thus, this evidence has raised a lot of interest in NMDA receptor antagonist research for MDD treatment.

The drug that has attracted most attention in this regard is ketamine, a non-selective NMDA receptor antagonist mainly employed as a dissociative anaesthetic, and recently approved by the FDA for treatment of depression at sub-anaesthetic doses (Chang *et al.* 2019). Although it has been reported that ketamine can induce rapid (within 24 hours) and long lasting antidepressant effects (Murrough *et al.* 2013), the mechanism is still unclear. However, accumulated lines of evidence suggest that ketamine-mediated improvements in depressive behaviours might be associated with modulation of synaptogenesis, stimulation of the mammalian target of rapamycin (mTOR) pathway, and increased synaptic proteins (Duman *et al.* 2012, Moda-Sava *et al.* 2019). Unfortunately, similar to other NMDA receptor antagonists, ketamine has demonstrated a tendency to invoke psychotomimetic side effects, including

hallucinations, paranoid delusions, confusion, and learning and memory deficits (Iadarola *et al.* 2015).

In contrast, anxiolytic drugs are chemical compounds designed to alleviate anxiety disorders. GABA is the most abundant inhibitory neurotransmitter in the mammalian brain, because of its capacity to bind to the GABA receptors and subsequently induce neuronal membrane polarisation (Mohler 2006). In this regard, during the 1960's the first drugs discovered that induced anxiolytic effects were benzodiazepines (chemicals with a benzene ring linked to a diazepine ring), which were found to positively modulate $GABA_A$ receptors, (Haefely *et al.* 1975, Masiulis *et al.* 2019). GABA receptors are widely distributed throughout the CNS, and the neurobiological consequences of their activation in different brain regions are not fully understood (Bowery *et al.* 1987, Uzun *et al.* 2010). Indeed, benzodiazepines are associated with adverse reactions, including drowsiness, hangover, and dizziness, as well as with memory impairment/amnesia (Vgontzas *et al.* 1995). On the other hand, pharmacological treatments with SSRIs have displayed positive effects for certain anxiety disorders (Thibaut *et al*. 2017). Moreover, due to their good risk/benefit balance, SSRIs were recommended as first line treatments for anxiety (Wehry *et al.* 2015, Thibaut *et al.* 2017).

Taken together, most of the pharmacological research focused on treatment for stress-related disorders are mainly based on restoring and compensating the neurochemical imbalance observed in these conditions (Fig. 1.7.2). Nevertheless, numerous side effects accompany these treatments, and some medications are ineffective in a proportion of patients, around 20 to 30% for MDD (Olchanski *et al.* 2013, Aust *et al.* 2019). For these reasons, more investigation is urgently required in the field of antidepressant and anxiolytic therapies, and the search for strategies less invasive with fewer negative side effects is needed.

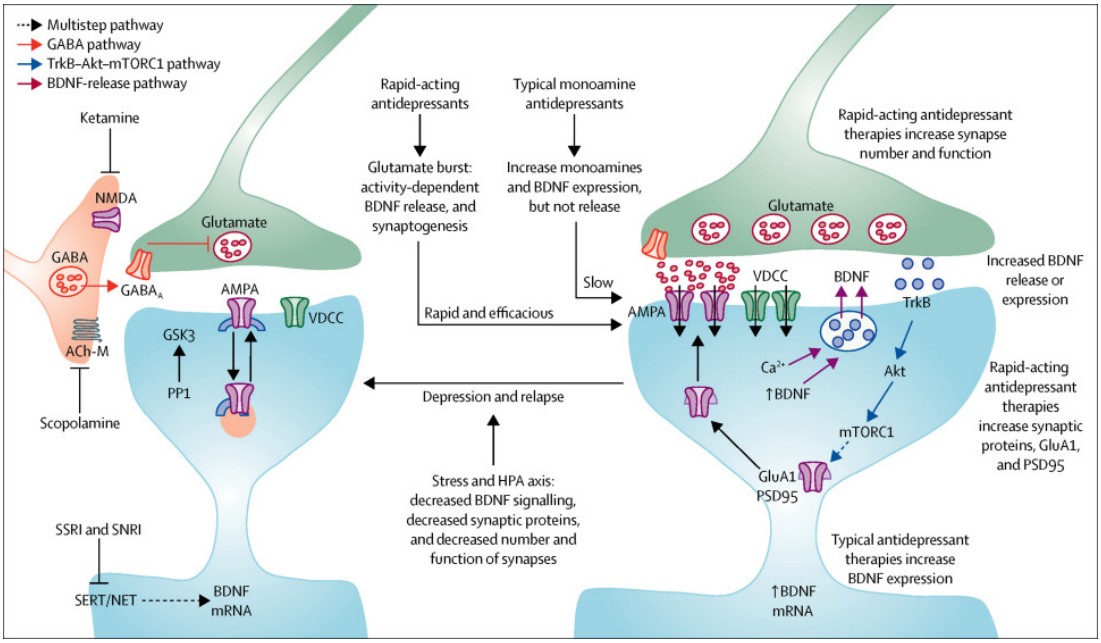

Figure 1.7.2 *Long-term effects of antidepressants on synaptic plasticity. Antidepressant drug actions focused on different cellular pathways associated with neuroplasticity, including mechanisms of neurotransmitter release, postsynaptic Ca^{+2} signalling, trafficking of glutamate AMPA receptor subunits. At molecular level, other pathways have been linked with antidepressant activity, such as BDNF, mTORC1, PSD95, VDCC, and GSK3. AMPA=α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor. BDNF=Brain-derived neurotrophic factor. GSK3=Glycogen synthase kinase-3. mTORC1=mammalian target of rapamycin complex 1. PSD95=Post synaptic density 95. VDCC=voltage dependent calcium channel. Modified from (Duman et al. 2016).*

1.8 The role of nutrition in mental health and disease

It is widely accepted that nutrition and consumption of a balanced diet is an important factor of human health, including metabolism (Oresic 2009), cardiovascular health (Ignarro *et al.* 2007), as well as a protective role against mental illness (Rao *et al.* 2008). Indeed, the relationship between health and nutrition has been recognised by clinicians since Hippocrates, who said "Let food be thy medicine and medicine be thy food."

Evidence in this regard suggests that nutritional deficiencies are associated with a high prevalence of mood symptoms and cognitive dysfunction, which correlate with development and onset of stress-related mental disorders. On the other hand, clinical studies found that depressive symptoms were associated with food insufficiency and nutritional deficiencies (low folate, vitamin, minerals and polyunsaturated fatty acids

intake) in women of childbearing age and elderly populations (Bodnar *et al.* 2005, German *et al.* 2011). In addition, the increased prevalence of mental disorders in developed countries is highly correlated with the consumption of westernised diets (Young 2002), which is characterised by increased intake of red meat, pre-packaged foods, fried foods, high-fat dairy products, refined and processed grains and fruits, and high-sugar drinks (Cordain *et al.* 2005, Yeomans 2017). For instance, a preclinical investigation demonstrated that feeding mice with an energy-dense food (considered as western diet) induced cognitive impairments and increased anxiety-like behaviour in these animals (Andre *et al.* 2014). Similarly, a longitudinal investigation of people aged 60-64 years found that western diet consumption was associated with reduced hippocampal volume (Jacka *et al.* 2015).

The majority of mental disorders are treated with prescription drugs, but many of these drugs cause unwanted side effects and their cost is continuously rising (for more details see section 1.7). For these reasons, nutritional therapies for mental illness have become interesting candidates for alleviating and mitigating stress-related disorder symptoms. In this regard, accumulated work in the field of nutritional psychiatry has proposed different dietary interventions to supplement apparent deficiencies associated with stress-related disorders, including amino acids, polyunsaturated fatty acids (PUFAs), polyphenols, and phospholipids (Sureda *et al.* 2015, Martinez-Cengotitabengoa *et al.* 2017, Smith *et al.* 2018, Adan *et al.* 2019, Boyle *et al.* 2019). Indeed, all of these components have been found enriched in Mediterranean diet, firstly defined because it reflects food patterns typical of countries around the Mediterranean Sea, including Crete, Greece and southern Italy (Willett *et al.* 1995, Gerber *et al.* 2015). In particular, the Mediterranean diet have attracted great attention since it has been associated with low rates of chronic diseases and high adult life expectancy (Martinez-Gonzalez *et al.* 2016, Galbete *et al.* 2018).

Perhaps, one of the most studied nutritional interventions for the treatment of depression is tryptophan supplementation (Shaw *et al.* 2002). Tryptophan is an aromatic amino acid, which is enriched in high-protein sources, including eggs, fish, soybean, cheese, and sesame seed (Markus *et al.* 2008). Tryptophan diet interventions for stress-related disorders, and especially MDD, are based on the monoaminergic

hypothesis of depression (see section 1.7), since tryptophan is an important precursor of serotonin in its synthesis pathway, hence favouring the serotonin production and concentration in the brain (Markus *et al.* 2008).

However, clinical trials in which patients have been administered tryptophan have given conflicting results and reached differing conclusions (Shaw *et al.* 2002). In addition, tryptophan supplementation is known to have side effects. Occasionally, tryptophan administration at higher doses is accompanied with gastrointestinal distress, nausea, dizziness, and tremor. In rare cases, when tryptophan treatment is combined with serotonin drugs (e.g. SSRIs), can result in abnormal serotonin stimulation, which is called "serotonin syndrome". The symptoms of serotonin syndrome include agitation, delirium, coma, mydriasis, sweating, and hyperthermia (Fernstrom 2012).

PUFAs represent another alternative for neuropsychiatric treatment obtained from natural-derived diets. PUFAs are fatty acids constituting a long hydrocarbon chain that possesses two or more carbon-carbon double bonds (Wiktorowska-Owczarek *et al.* 2015). Early studies first linked the beneficial effects of PUFAs with improvements in CVD (Hu *et al.* 2002, Watanabe *et al.* 2017). Later, several studies demonstrated that PUFAs, especially Omega-3 fatty acid derivatives play a significant role in modulation of neurotransmission and cell signal transduction in the CNS (Chalon *et al.* 2001, Alessandri *et al.* 2004). Indeed, abnormalities in fatty acid metabolism and Omega-3 fatty acid deficiencies may play a causal role in development and onset of stress-related conditions, especially in depressive disorders (Lin *et al.* 2017, Messamore *et al.* 2017).

For these reasons, Omega-3 dietary interventions have attracted a lot of interest for the treatment of depression. Particularly, the Omega-3 fatty acids docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) (Fig. 1.8.2), have shown promising results in preclinical studies (Pusceddu *et al.* 2015), as well as in clinical trials treating depressive disorders (Stoll *et al.* 1999, Nemets *et al.* 2006). Although several lines of evidence suggest that Omega-3 PUFAs might have an antidepressant effect, there is

still limited data from clinical studies and in some cases the validity of findings is questionable (Rogers *et al.* 2008).

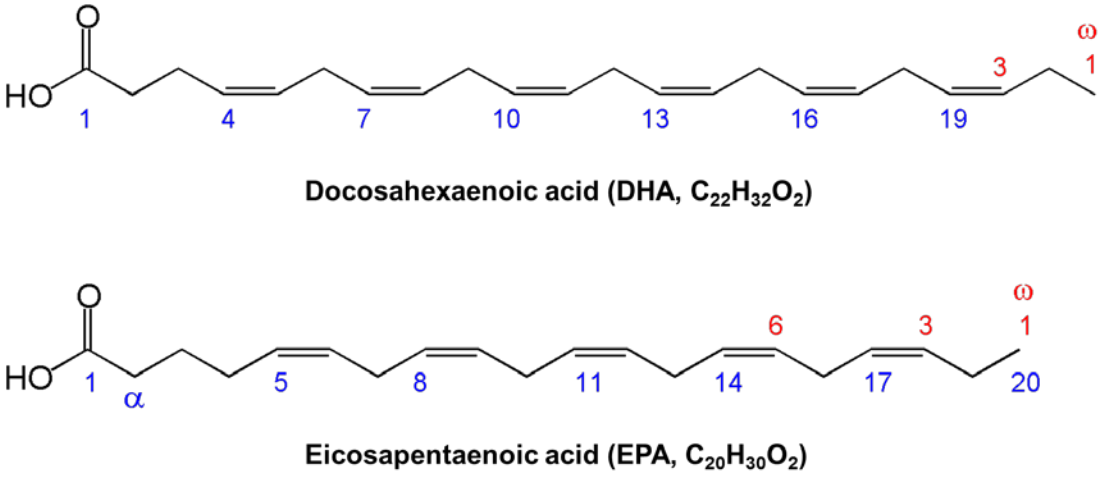

Figure 1.8.2 *Chemical structure of DHA and EPA. DHA=Docosahexaenoic acid. EPA= Eicosapentaenoic acid.*

1.8.1 The impact of nutrition on the microbiota-gut-brain axis

During the last decade, the gut microbiota composition has emerged as a relevant biometric for health status (Claesson *et al.* 2012). In this regard, microbial composition imbalance in the body has been associated with behavioural alteration, including attention deficit disorder and autism (Borre *et al.* 2014, Rosenfeld 2015, Cenit *et al.* 2017), therefore it is considered that positively modulating the microbiota composition might have potential in ameliorating disease. There are numerous environmental and genetic factors able to influence the gastrointestinal microbiota (Spor *et al.* 2011, Blekhman *et al.* 2015), however diet represents the most critical element in terms of changes of microbiota composition during the lifespan (Lankelma *et al.* 2015, Xu *et al.* 2015).

Thus, dietary interventions focused on manipulating gut microbiota composition may have the potential to modulate psychiatric symptoms associated with gut-brain-axis dysfunction. For instance, research using a diet containing meat reported changes to gut microbial diversity, which correlated with improvements in cognition and working memory, and decreased anxiety-like behaviours (Li *et al.* 2009). Specifically, dietary

interventions based on prebiotics (compounds that induce the growth or activity of beneficial bacteria) have attracted a lot of interest in research as modulators of mood in stress-related disorders (Hutkins *et al.* 2016). In this regard, recent preclinical work demonstrated that prebiotics consisting of fructooligosaccharide (FOS) and galactooligosaccharide (GOS) induced antidepressant and anxiolytic effects in chronically stressed mice (Burokas *et al.* 2017).

Dietary interventions designed to treat neuropsychiatric disorders are clearly focused on providing novel and "natural" therapies, which represent valid alternatives for drug resistant patients and those that are afraid or suffering from negative side effects associated with prescription antidepressant and anxiolytic drugs. Therefore, research on dietary interventions for mental disorders is critical to understand the role of nutritional components in mental health and disease. Thus, the purpose of the work presented in this thesis is to further explore the role of naturally-derived compounds, especially polyphenols and phospholipids, which have been gaining increasing interest as potential biomolecules for the development of novel therapies for stress-related disorders. In the following sections the biochemical characteristics and biological implications for mental health of polyphenols and phospholipids will be reviewed in detail.

1.9 Polyphenols

Over the past 20 years, scientists and food manufacturers have become increasingly interested in polyphenols. The key reason for this interest is the recognition of their antioxidant properties, their great abundance in our diet, and their potential role in the prevention and treatment of various diseases associated with oxidative stress, including cancer, CVD, metabolic and neurodegenerative diseases (Manach *et al.* 2004, Pandey *et al.* 2009). Although there are accumulating data from animal and human studies showing that polyphenols are capable of modulating the activity of a wide range of enzymes and cellular receptors (Vauzour *et al.* 2010), polyphenols also have several other specific biological actions that remain poorly understood.

1.9.1 Structure and natural sources of polyphenols

Polyphenols vary both in chemical structure and functional activity, therefore their biological actions strongly depend of their molecular size, specific substituents and solubility. Indeed, their chemical structure is characterized by the presence of at least one aromatic ring with one or more hydroxyl group attached. Polyphenol size range from simple small single phenolic ring compounds to complex, weighty condensed structures; therefore, these compounds have been classified into different groups based on the function, number and distribution of phenolic rings, as well as structural elements present in these rings (Bravo 1998). Although there is no universal classification system of polyphenols, it is widely accepted that they fall into one of two categories: flavonoids and non-flavonoids (Fig. 1.9.1). Flavonoids contain a common structure consisting of two phenolic rings linked by a three carbon atom bridge that form an oxygenated heterocycle. Non-flavonoids include other polyphenolic compounds such as phenolic acids, phenyl alcohols, stilbenes, lignans and chalcones (Motilva *et al.* 2013).

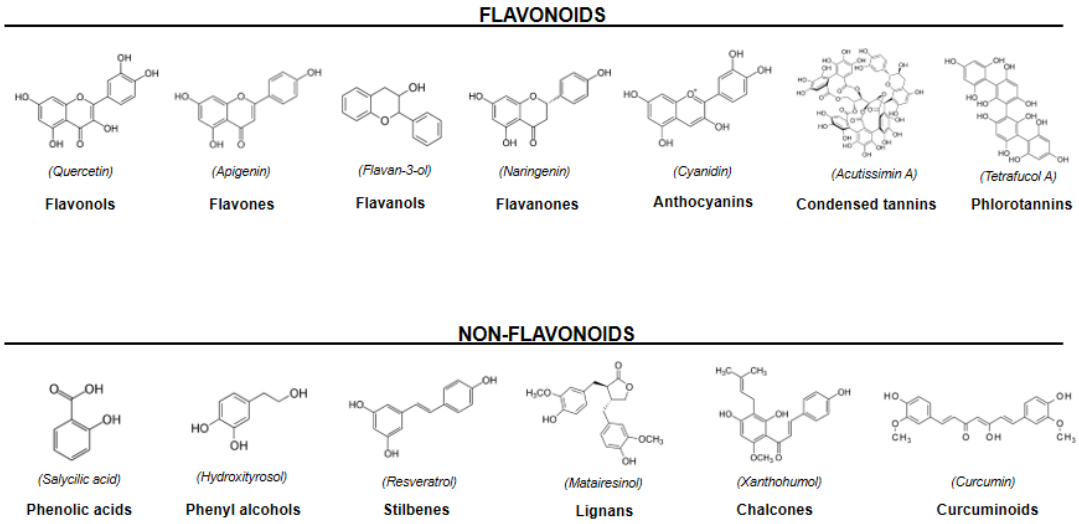

Figure 1.9.1 *Classification of dietary polyphenols, some examples and their molecular structure.*

Polyphenolic compounds are molecules that are mainly found in fruits and vegetables where they act as secondary metabolites which are involved in host defence against pathogens and UV radiation (Haslam 1988). Indeed, major dietary sources of

polyphenols include fruits, vegetables, cereals, tea and wine. Certain polyphenols are found in all plant products (e.g. quercetin), whereas others are specific to particular foods (e.g. flavanones in citrus fruits) (Manach *et al.* 2004). On the other hand, there are many plant products where the polyphenol composition is not fully defined. Furthermore, numerous factors can affect the polyphenol content in certain foods, including ripeness, environmental factors, processing and storage (Manach *et al.* 2004).

1.9.2 Polyphenols as modulators of brain health

It has been recognized for many years that a diet rich in polyphenols can protect against an array of chronic diseases (Vauzour *et al.* 2010), including CVD (Fuhrman *et al.* 2005, Hubbard *et al.* 2006), specific forms of cancer (Zhou *et al.* 2016), and neurodegenerative disorders (Solanki *et al.* 2015). In this regard, evidence supports the neuromodulatory potential of polyphenols via their ability to protect vulnerable neurons and improve neuronal function and regeneration (Spencer 2008). In particular, several *in vitro* studies have confirmed the neuroprotective capacity of polyphenols mainly against cytotoxicity induced by both oxidative stress (Yao *et al.* 2015) and β-amyloid peptide (Aβ) in neuronal-death models (Godoy *et al.* 2016).

There is accumulating data from animal and human studies that support the role of a variety of dietary polyphenols in affecting behaviour and mood through anxiolytic and antidepressant properties (Dias *et al.* 2012) (see Table 1.9.1 to see some examples in pre-clinical studies). For instance, seminal work in this field-tested a polyphenol-rich traditional Chinese herbal medicine, which is used to treat depression, performing neurochemical analyses in chronically stressed rats. Their findings showed that flavonoid-rich treatment induced an increase in neurogenesis and hippocampal Brain-derived Neurotrophic Factor (BDNF) expression, indicating one of the cellular mechanisms underlying the antidepressant action of this Chinese medicine (An *et al.* 2008). Recent studies using resveratrol, a polyphenol found in grape skin and hence red wine, showed that the treatment with this polyphenol ameliorated chronic unpredictable stress-induced depression-like behaviour in rats. This effect was

correlated with a downregulation of the HPA axis hyperactivity and a modulation of the Wnt/catenin pathway (Yang *et al.* 2017).

Table 1.9.1 Evidence of polyphenol-mediated improvements of depressive-like behaviours

Polyphenol	Dose (mg/kg)	Duration	Animal model	Behavioural outcomes	Biochemical outcomes	Reference
Resveratrol	20 – 80 i.p.	5 weeks	CUS in Wistar rats	↑Sucrose preference ↓Immobility FST	↓Corticosterone ↑BDNF, pCREB	(Liu *et al.* 2014)
Resveratrol	15 i.g.	3 weeks	CUS in Sprague Dawley rats	↑Sucrose preference ↓Immobility FST	↓Corticosterone, CRF ↓IL-6, CRP TNF-α ↑BDNF	(Yang *et al.* 2017)
Resveratrol	20 i.p.	2 weeks	Ovariectomy in C57BL/6J	↑Time in centre OFT ↑Open arms time EPM ↓Immobility TST ↓Immobility FST	↔NPC proliferation ↓Microglia activation ↓NF-κB, ↓IL-1β ↑Sirt1	(Liu *et al.* 2019)
Quercetin	20 – 40 p.o.	1 hour	Albino Swiss mice	↑Social interaction ↓Immobility FST	None	(Bhutada *et al.* 2010)
Quercetin	300 p.o.	3 weeks	Wistar rats	↑Open arms time EPM ↑Spatial learning	None	(Priprem *et al.* 2008)
Quercetin	20 i.p.	2 weeks	Restrain-induced chronic stress in Wistar mice	↑Time in light box ↑Open arms time EPM ↓Immobility FST	↑SOD, ↑Catalase activity ↑Brain acetylcholine	(Samad *et al.* 2018)
Naringenin	10 – 50 i.g.	1 hour	ICR mice	↓Immobility TST ↔Immobility FST	None	(Yi *et al.* 2010)
Naringin	50 – 100 i.p.	2 weeks	Doxorubicin injection Wistar rats	↑Time in centre OFT ↓Immobility FST ↑Sucrose preference	↑Antioxidant enzymes	(Kwatra *et al.* 2016)
Curcumin	50 – 200 i.p.	10 days	Wistar Kyoto rats	↓Immobility FST	↑BDNF ↓IL-1β, TNF-α ↓Corticosterone	(Hurley *et al.* 2013)
Curcumin	20 p.o.	3 weeks	Corticosterone -injection	↑Sucrose preference in SPT ↓Immobility in FST	↑BDNF	(Huang *et al.* 2011)
Catechins	5 – 20 p.o.	1 week	ICR mice	↓Immobility in TST ↓Immobility in FST	↓Corticosterone and ACTH	(Zhu *et al.* 2012)

Although the health benefits associated with polyphenol consumption are evident, the mechanisms underlying their neuroprotective effects are unclear. Below, evidence regarding polyphenol-mediated activation of signalling pathways involved in neuroprotection and plasticity is explored.

1.9.3 Neuroprotective mechanisms associated with polyphenols

Several polyphenolic compounds have been identified acting as potent effectors of cellular processes with a capacity to positively modulate neuroprotective mechanisms. Although the molecular mechanism involved in the pathophysiology of neural disease and mental disorders are numerous, a number of studies have suggested an important role for different pathways involved in neuroplasticity and cytoprotective responses that might be linked to polyphenols.

The Nrf2 signalling pathway

For example, the nuclear factor E2-related factor 2 (Nrf2) has been associated with neuroprotective effects of polyphenols (Scapagnini *et al.* 2011), and is probably the most studied pathway associating polyphenolic consequences in animal and human physiology. The Nrf2 transcription factor is a member of the Cap‘n’Collar family of transcription factors, which in normal conditions is inactivated by binding to Keap1, which promotes Nrf2 proteasomal degradation via interactions with a ubiquitin ligase (Sun *et al.* 2007). Several stress signals, including oxidative stress and electrophiles, can disrupt the Nrf2/Keap1 complex and allow Nrf2 to translocate into the nucleus (Fig. 1.9.3.1). There, the activated form of Nrf2 is able to interact with the antioxidant response element (ARE) in the nucleus (Malhotra *et al.* 2010), which positively modulates antioxidant responses, and triggers the simultaneous expression of numerous protective enzymes and scavengers (Scapagnini *et al.* 2011).

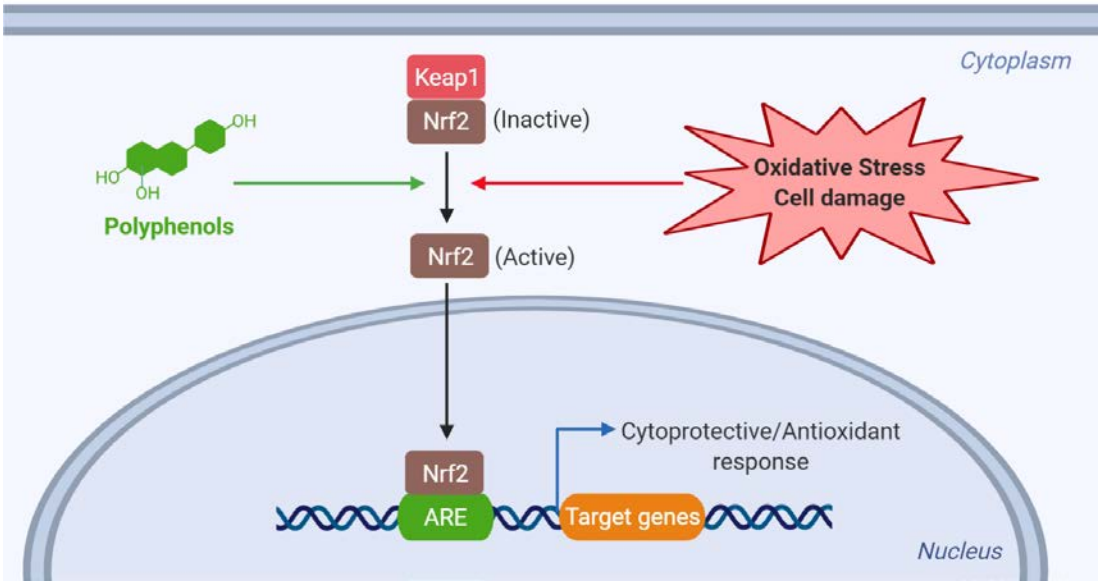

Figure 1.9.3.1 The Nrf2 signalling pathway is activated by oxidative stress or polyphenols. *Intracellular signals like polyphenols or oxidative stress induce the activation of Nrf2, which translocates to the nucleus and interact with antioxidant response elements (ARE), triggering the expression of cytoprotective and antioxidant enzymes.*

The capacity of polyphenols to activate the Nrf2 pathway has been demonstrated in several *in vitro* models (Table 1.9.2). For instance, naringin, a polyphenolic compound mainly found in grapefruit, was shown to protect H9c2 cells (myoblast) against anoxia/ reoxygenation-induced apoptosis *in vitro* via the Nrf2 signalling pathway (Chen *et al.* 2015), as well as to reduce the neurotoxic effects of 6-OHDA in a neuroblastoma cell line (Lou *et al.* 2014).

Table 1.9.2 Evidence of polyphenol-mediated Nrf2 activation *in vitro*

Polyphenol	Cells	Dose	Model	Effect via Nrf2	Reference
Naringin	H9c2 cell line	10 – 40 µg/mL	Anoxia/reoxygenation-induced apoptosis	↓Apoptosis ↑Antioxidant capacity	(Chen *et al.* 2015)
Naringenin	SY5Y cell line	20 – 80 µM	6-OHDA-induced neurotoxicity	↓Neurotoxicity ↑Nrf2 expression ↑ARE activation	(Lou *et al.* 2014)
Resveratrol	Coronary arterial endothelial cells	0.1 – 100 µM	High glucose-induced mitochondrial and cellular oxidative stress	↓Oxidative stress ↑Nrf2 target genes expression	(Ungvari *et al.* 2010)

Resveratrol	Neural stem cells	1 – 20 µM	Oxygen-glucose deprivation/reoxygenation	↑Survival and proliferation ↓Apoptosis ↑SOD activity ↑Nrf2 target genes expression	(Shen *et al.* 2016)
Quercetin	Human hepatocytes	10 – 200 µM	Ethanol-derived oxidative stress	↑HO-1 expression ↓toxicity ↓AST/LDH release	(Yao *et al.* 2007)
Quercetin	Primary cerebellar granule neurons	5 – 100 µM	H_2O_2-induced oxidative stress	↑GSH levels ↑Nrf2 translocation ↑GCLC expression	(Arredondo *et al.* 2010)
Curcumin	MCF-7 cell line	10 – 30 µM	Proliferative conditions	↓Proliferation ↓Fen-1 expression	(Chen *et al.* 2014)
EGCG	Primary vascular endothelial cells	25 – 50 µM	PCB 126-induced inflammation	↑Nrf2-regulated antioxidant enzymes ↓PCB-induced CYP1A1 expression ↓Superoxide	(Han *et al.* 2012)

ARE=Antioxidant Response Elements; AST=Aspartate Transaminase; CYP1A1=Cytochrome P450 1A1; Fen-1=Flap Endonuclease 1; GCLC=Glutamate-Cysteine Ligase Catalytic Subunit; GSH=Glutathione; HO-1=Heme Oxygenase 1; LDH=Lactate Dehydrogenase; PCB=Polychlorinated Biphenyl; SOD=Superoxide Dismutase.

On the other hand, accumulating evidence suggests an important role for Keap1/Nrf2 signalling in mood disorders. Preclinical studies demonstrated that mice subjected to chronic stress have lower protein expression of Keap1 and Nrf2 in the prefrontal cortex (PFC), CA3 and dentate gyrus (DG) of the hippocampus compared with non-stressed animals, which it was correlated with depressive-like behaviours (Yao *et al.* 2016, Zhang *et al.* 2018). Furthermore, Nrf2 knockout mice exhibited depressive-like behaviours accompanied by increased plasma pro-inflammatory cytokines levels and reduced BDNF expression in hippocampal regions (Yao *et al.* 2016). In addition, post-mortem analysis from patients with MDD and bipolar disorder showed decreased expressions of Keap1 and Nrf2 in cortical brain areas (Martin-Hernandez *et al.* 2018, Zhang *et al.* 2018). Taken together, the evidence suggests an important role for the Nrf2 pathway in polyphenol-mediated neuroprotection and mental health.

Polyphenols and BDNF

The role of dietary polyphenols in neuroprotection and brain health has also been associated with the modulation of BDNF expression (Venkatesan *et al.* 2015). BDNF belongs to the neurotrophin family of growth factors, which are implicated in neuronal survival support and providing the growth and differentiation of new neurons and synapses (Huang *et al.* 2001). Indeed, neurotrophin levels, especially BDNF, have been highly associated with the development and treatment of stress-related mental disorders, such as depression and anxiety (Martinowich *et al.* 2007, Brunoni *et al.* 2008).

Studies using different *in vitro* neuronal models have demonstrated the potential of polyphenols in regulating BDNF, and subsequently the neuroprotection associated with these phytochemicals. For instance, tea polyphenols were able to protect neurons through activation of the TrkB/CREB/BDNF pathway against oxidative stress-induced apoptosis (Qi *et al.* 2017). In a different study testing the neuroprotective properties of catechines, a type of polyphenol that belongs to the family of flavonoids, discovered that mitochondrial toxin-induced cytotoxicity was prevented by these catechines by increasing the protein levels of BDNF in primary hippocampal neurons (Nath *et al.* 2012).

The relationship between polyphenol consumption and BDNF expression has been also demonstrated *in vivo*. A clear example was shown in a study where administration of different doses of resveratrol to naïve rats, resulted in dose-dependently elevated mRNA levels of BDNF in hippocampal tissue (Rahvar *et al.* 2011). To demonstrate that this resveratrol-mediated increased expression of BDNF has therapeutic effects in neuropsychiatric disorders, *in vivo* studies using models of chronic stress have been assessed. In this regard, it was shown that the resveratrol antidepressant-like effects in CUS in rats and LPS-induced depressive-like behaviour in mice could be partly mediated by modulation of BDNF expression (Liu *et al.* 2014, Ge *et al.* 2015). Taken together, these data support the role of BDNF in mechanisms associated with neuroprotection by polyphenols.

Polyphenols and neuroinflammation

Brain inflammation, a pathophysiological process characterised by increased microglia and astrocyte activation, increases during aging and is a key feature of neurodegenerative diseases (Sawikr *et al.* 2017). In addition, patients diagnosed with stress-related disorders, such as depression and anxiety, show increased levels of circulating pro-inflammatory cytokines (Brites *et al.* 2015), suggesting a critical role for neuroinflammation in the onset and development of stress-related neuropsychiatric disorders.

In this regard, polyphenols have been reported to exert anti-inflammatory effects in acute and chronic animal models of inflammation (Wang *et al.* 2012, Jhang *et al.* 2015, He *et al.* 2016). In terms of known mechanisms associated with anti-inflammation, polyphenols have been found to regulate immune cells (Shimizu 2017), and to modulate the activity and expression of enzymes involved in arachidonic acid metabolism, including phospholipase A2 and cyclooxygenase (Yahfoufi *et al.* 2018). For instance, further studies have associated the anti-inflammatory capacity of resveratrol with inactivation of the peroxisome proliferator-activated receptor gamma (PPAR-α) (Mohar *et al.* 2012, Barone *et al.* 2019). Curcumin, another non-flavonoid polyphenol, was shown to inhibit the nuclear factor κB (NF- κB) (Chin 2016, Wang *et al.* 2018).

Particularly, polyphenol treatments have displayed promising effects against neuroinflammatory injuries. For example, a study demonstrated that intragastric administration of resveratrol was able to ameliorate neuroinflammation by promoting microglia polarization to the anti-inflammatory phenotype (Yang *et al.* 2017). Quercetin also has demonstrated to have therapeutic potential against neuroinflammation. Oral treatment with quercetin reversed the augmented expression of pro-inflammatory cytokines and the affected haematological parameters found in the rat brain after manganese-induced neuroinflammation (Bahar *et al.* 2017). Similarly, LPS-induced brain inflammation and synaptic dysfunction was attenuated by treatment with quercetin in a mouse model (Khan *et al.* 2018). This evidence highlights the anti-inflammatory capacity of polyphenols as a relevant mechanism of

neuroprotection with important implication for neurodegenerative and stress-related mental disorders.

1.9.4 Impact of polyphenol intake on the microbiota-gut-brain axis

Most of the health benefits of polyphenols are determined by their bioavailability, which is dramatically affected by different biochemical factors, including the type of bioactive compounds, their polarity, molecular mass, source matrix, digestibility by gastrointestinal enzymes, and absorption into the enterocytes (Ozdal *et al.* 2016). In this regard, gut microbiota metabolism plays a critical role in the bioavailability of polyphenol-derived compounds once polyphenols pass the small intestine and encounter the gut microbiota in the colon (Scalbert *et al.* 2000). Polyphenols are extensively conjugated and metabolized in the small intestine and liver during nutrient absorption (Marin *et al.* 2015). Specifically, they are subject to phase I de-glycosylation and phase II metabolism processes, which involve methylation, sulphation, glucuronidation and hydrolysis. All of these metabolic transformations are common to many xenobiotics as a detoxification system and facilitate their elimination by increasing their hydrophilicity (Williamson *et al.* 2017).

On the other hand, polyphenols are capable of modulate the composition of the gut microbial community by inhibiting or stimulating the growth of certain bacteria (Lee *et al.* 2006). Indeed, a number of studies assessing the impact of polyphenols on microbiota composition have been published in recent years (Table 1.9.4.1). For example, a study analysing the effect of isoflavone supplementation on postmenopausal women for two months found an increase in *Bifidobacterium* species in their gut microbiota (Clavel *et al.* 2005). Another study, using rats on a diet intervention with tannins, a class of polyphenolic compounds, detected that *Bacteroides* was significantly increased, while the *Clostridium leptum* cluster was significantly decreased (Smith *et al.* 2005).

Table 1.9.4.1 Evidence of polyphenols modifying gut microbiota composition

Polyphenol	Dose	Duration	Model	Impact on the gut microbiota	Reference
Resveratrol	200 mg/kg p.o.	12 weeks	Kunming mice – High-fat diet	↑*Bacteroides/Firmicutes* ↑*Lactobacillus* ↑*Bifidobacterium* ↓*Enterococcus faecalis*	(Qiao *et al.* 2014)
Resveratrol	1 mg/kg p.o.	25 days	F334 rats – DDS-induced colitis	↑*Lactobacillus* ↑*Bifidobacterium* ↓*Enterococcus*	(Larrosa *et al.* 2009)
Resveratrol+ Quercetin	30+15 mg/kg i.g.	10 weeks	Wistar rats – High-fat diet	↓*Firmicutes/Bacteroides* ↑*Akkermansia* ↑*Ruminococcaceae* ↑*Bacteroidales*	(Zhao *et al.* 2017)
Quercetin	30 mg/kg p.o.	6 weeks	Wistar rats – High-fat sucrose diet	↓*Firmicutes/bacteroides* ↓*Erysipelotrichaceae* ↓*Bacillus* ↓*Euabcterium* ↓*Cylindroides*	(Etxeberria *et al.* 2015)
Quercetin	0.05% w/w p.o.	16 weeks	C57BL/6J mice – High-fat diet	↓*Firmicutes/bacteroides* ↓*Helicobacter* ↔*Lactobacillus* ↔*Parabacteroides* ↓*Desulfovibrio* ↔*Akkermansia*	(Porras *et al.* 2017)
Curcumin	200 mg/kg i.g.	12 weeks	Sprague Dawley rats – High-fat diet	↓Richness ↓α-Diversity ↑*Streptococcus* ↑*Collinsella* ↓*Ruminococcus*	(Feng *et al.* 2017)
Curcumin	100 mg/kg i.g.	12 weeks	Wistar rats – Ovariectomy	↑α-diversity ↑*Bacteroides/Firmicutes* ↓*Helicobacter* ↑*Serratia* ↑*Pseudomonas* ↑*Shewanella*	(Zhang *et al.* 2017)
Condensed tannins	2% w/w p.o.	3 weeks	Wistar Furth rats	↑*Enterobacteriaceae* ↑*Bacteroides* ↑*Prevotella* ↑*Porphyromonas* ↓*Clostridium*	(Smith *et al.* 2004)
EGCG	25 mg/kg p.o.	4 months	C57BL/6J mice – High-fat diet	↓*Firmicutes/Bacteroides* ↓*Lactobacilli* ↑*Clostridium* ↑*Bacteroidetes* ↓*Akkermansia*	(Remely *et al.* 2017)
Tea catechines	0.1% w/w p.o.	7 weeks	db/db mice	↑Richness ↑Evenness ↓*Barnesiella*	(Chen *et al.* 2019)

Thus, the potential of polyphenolic compounds in modifying the gut microbial composition as a strategy to modulate the gut-microbiota-brain axis has attracted great interest in novel treatments for neuropsychiatric disorders.

1.10 Phospholipids

The brain contains an incredible mixture of lipids, which represent important biochemical components of the CNS in terms of neuronal function and structure (Brown *et al.* 2009). Indeed, disturbances in brain lipid balance have been associated with common neurological disorders, including Parkinson's Disease, Alzheimer's Disease, amyotrophic lateral sclerosis, and glioblastomas (Dawson 2015). In particular, phospholipids have attracted great interest due to they represent a major component of all cell membranes (Kent 1995), and their potential link with a number of health benefits in relation to different illnesses, including coronary health disease, inflammation and cancer (Kullenberg *et al.* 2012). However, more research is needed to understand the impact of phospholipid supplementation on brain health, and the implication of potential neuroprotective mechanisms involved in the phospholipid-elicited health benefits.

1.10.1 Structure and natural sources of phospholipids

Phospholipid structure generally consists of a hydrophilic "head" formed by a phosphate group and two hydrophobic "tails" (see Fig. 1.10.1 A), which can be unsaturated fatty acids in the sn-2 position, such as oleic, linoleic or linolenic, arachidonic, or eicosapentanoic acid, while the sn-1 position predominantly carries a saturated fatty acid, such as stearic or palmitic acid (Kullenberg *et al.* 2012). This structure confers their typical amphiphilic characteristics that allow them to form lipid bilayers (Peterson *et al.* 2006) (see Fig. 1.10.1 B).

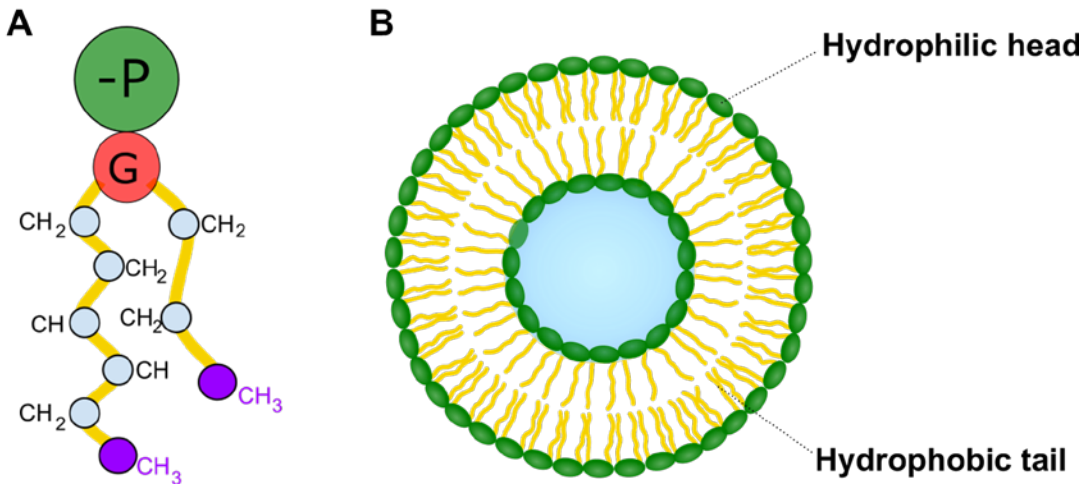

Figure 1.10.1 (A) *Schematic chemical structure of phospholipids and* ***(B)*** *their capacity to form a lipid bilayer. G=Glycerol moiety; –P=Phosphate group.*

Phospholipids differ in not only their hydrophobic tail, but they also show differences in their hydrophilic heads where the phosphate group can be modified with organic molecules such as choline or serine. In this regard, among the most common phospholipids found in food are phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS), phosphatidylinositol (PI), and sphingomyelin (SM) (Cohn *et al.* 2010) (see Fig. 1.10.2).

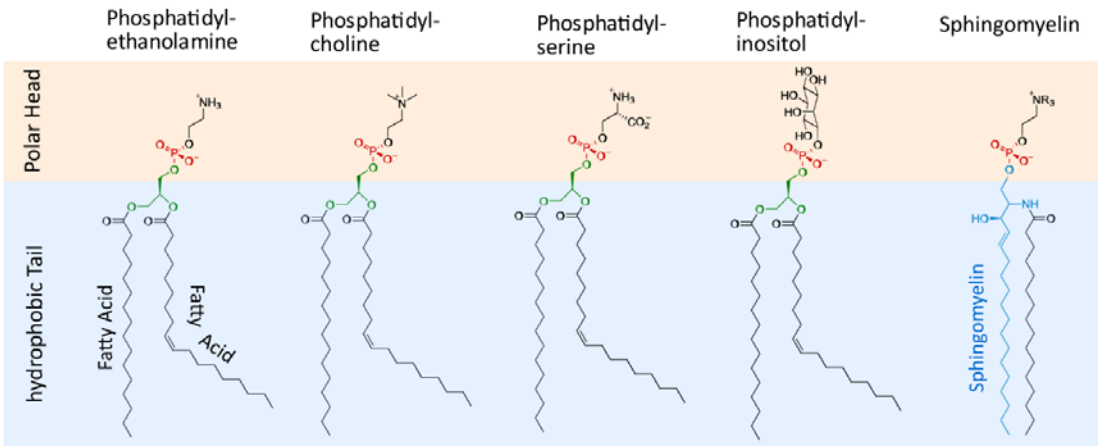

Figure 1.10.2 *Chemical structure of PS, PE, PC, PI and SM.*

Naturally occurring phospholipids can be found in plant and animal food sources, including eggs, organ and lean meats, fish, shellfish, cereal grains, oilseeds and milk

(Cohn *et al.* 2010). Although, among total milk fat, only 0.32 – 1% represent phospholipid compounds, compared to soy lecithin and egg yolk lecithin, milk phospholipids have a more balanced distribution in each subclass (Garcia *et al.* 2012). For this reason, dairy-derived phospholipids have become an interesting target for research in the field of nutritional therapies and functional foods for enhancing cognition (Zheng *et al.* 2019). In particular, the milk fat globule membrane (MFGM) is one unique component, comprised of a tri-layer of polar lipids, glycolipids, and proteins, that may be important for brain development (Moukarzel *et al.* 2018).

1.10.2 Nutritional impact of phospholipids on brain health

Phospholipids are structural components of neural tissues, and they have been found to be critical for neurodevelopment and neuronal function (Svennerholm *et al.* 1972, Burdge *et al.* 1995). In addition, some intracellular phospholipids can be converted into second messengers, participating in key neuronal signalling pathways, such as survival and differentiation (Colombaioni *et al.* 2004, Huang *et al.* 2011). In this context, studies have demonstrated that phospholipid-enriched diets significantly impact behaviour (Kullenberg *et al.* 2012). For example, clinical studies using PS supplementation have demonstrated significant improvements in Age Related Cognitive Decline in terms of cognitive performance (Crook *et al.* 1991). Other clinical study showed that PS supplementation was able to induce an improved mood and reduced levels of cortisol in young adults subjected to acute stress (Benton *et al.* 2001). Similar effects have been detected in preclinical studies. For instance, Mice fed with phospholipid-enriched diet from early life until adolescence exhibited improved working memory performance in adolescence, whereas recognition memory was unchanged (Schipper *et al.* 2016).

On the other hand, fatty acid analysis on the brain of mice subjected to CUS showed altered phospholipid content accompanied with elevated immobility time in the FST. Chronic stress significantly reduced the levels of PI but increased the levels of PC (Faria *et al.* 2014). Also, an *in vitro* study using different types of antidepressant drugs on a neuronal cell line, supports the role of phospholipids in their antidepressant activity. Here, imipramine, paroxetine, and maprotiline increased the levels of

diacylglycerol and PI in neuron-like PC12 cells (Aboukhatwa *et al.* 2010). These and other reports (see Table 1.10.1) suggest potential therapeutic effects of phospholipid supplementation on human cognitive decline and/or chronic stress. Although, the mechanisms underlying the phospholipid-mediated positive effects on behaviour are unclear, there are some candidate signalling pathways potentially implicated in phospholipid activity.

Table 1.10.1 Pre-clinical and clinical evidence of phospholipid-mediated improvements in mental health

phospholipid - based supplement	Dose	Duration	Model	Effects	Reference
Bovine milk-derived phospholipid	2.7 g/day p.o.	6 weeks	Human – Acute psychosocial stress	↑Attention-switching task ↔Cortisol response ↑Mid-stress induction energetic arousal	(Boyle *et al.* 2019)
Phosphatidic acid and PS complex	400 mg/day p.o.	3 weeks	Human – Trier social stress test	↓ACTH, cortisol ↔Heart rate ↓Distress	(Hellhammer *et al.* 2004)
PC-enriched diet	30 mg/day p.o.	1 week	Human – Bipolar disorder patients	↓Mania symptoms	(Cohen *et al.* 1982)
PS	300 mg/day p.o.	1 month	Human – Stressful arithmetic task	↓Stress ↓Cortisol	(Benton *et al.* 2001)
Phospholipid-enriched diet	28% w/w in food p.o.	28 days	C57BL/6 – Males	↑Short-term memory ↔Long-term memory	(Schipper *et al.* 2016)
Lacprodan® MFGM-10	100 mg/kg i.g.	19 days	Sprague Dawley – P2 to P21	↑BDNF, GluR1 ↔MBP ↑Spatial memory	(Brink *et al.* 2018)
Krill PS	100 mg/kg p.o.	30 days	Sprague Dawley – Young males	↑Spatial memory ↑BDNF, IGF	(Park *et al.* 2013)

1.10.3 Phospholipids and neuronal signalling

It is well accepted that the composition of distinct phospholipids in eukaryotic membranes is essential for maintaining cellular homeostasis, while reception and transmission of signals across the plasma membrane has been a function generally attributed to transmembrane proteins (Horejsi *et al.* 2004, Wang *et al.* 2018). However, accumulated evidence support an important contribution of membrane lipids in the process of signal transduction (Sunshine *et al.* 2017). Indeed, alteration of phospholipid composition and biochemistry has been involved in allosteric regulation of G protein-coupled receptor (GPCR) activity (Dawaliby *et al.* 2015), modulation of Toll-like receptors signalling cascade (Erridge *et al.* 2008), and proliferative and

apoptotic pathways (Colombaioni *et al.* 2004, Huang *et al.* 2011). In particular, phospholipid-dependent Protein Kinase C (PKC) activation is one of the most studied signalling pathways associated to phospholipids (Fig. 1.10.3.1). Upon GPCR activation, Phospholipase C (PLC) enzymatically hydrolyses phosphatidylinositol diphosphate (PIP_2), a structural phospholipid component of the cellular membrane, to inositol trisphosphate (IP_3) and diacylglycerol (DAG) (Essen *et al.* 1997). IP_3 is a soluble second messenger molecule able to diffuse through the cytosol, where it binds to its receptor, which is a calcium channel located in the endoplasmic reticulum (ER) (Hanson *et al.* 2004). When IP_3 binds its receptor, calcium is released into the cytosol, thereby activating various calcium regulated intracellular signals, including PKC and muscle contraction mechanisms (Putney *et al.* 2012).

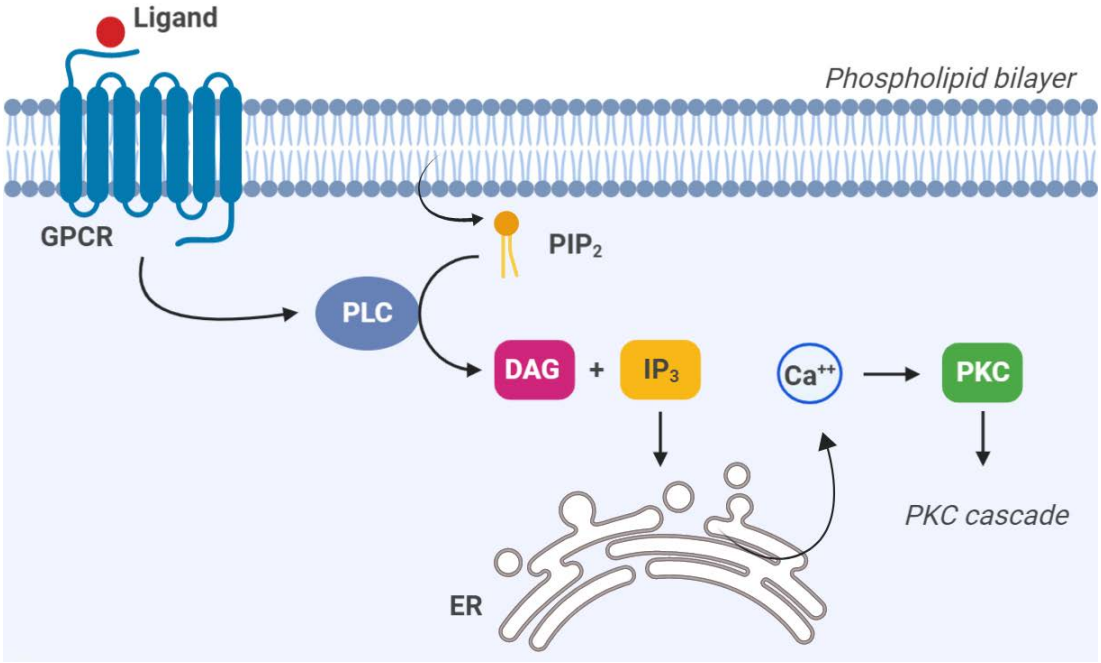

Figure 1.10.3.1 Phospholipid-dependent activation of protein kinase C signalling*. Upon GPCR activation, Phospholipase C (PLC) enzymatically hydrolyses phosphatidylinositol diphosphate (PIP_2), to produce inositol trisphosphate (IP_3) and diacylglycerol (DAG). IP3 induces the release of Ca^{++} from the endoplasmic reticulum (ER), which activates PKC cascade.*

Phospholipids and inflammation

Inflammation is considered a critical component involved in the pathophysiology of stress-related neuropsychiatric disorders (Brites *et al.* 2015) (see section 1.4). Indeed, anti-inflammatory treatments for depression have demonstrated potential in

attenuating depressive symptoms without increased risks of adverse effects (Kohler *et al.* 2014, Miller *et al.* 2015). Interestingly, some of the beneficial effects of phospholipids have been associated to anti/inflammatory processes (Greig *et al.* 2012). Evidence suggests that anti/inflammatory effects of phospholipids involve the activation of peroxisome proliferator-activated receptors (PPARs) (Fig. 1.10.3.2). PPARs are transcription factors, that when bind to peroxisome proliferator hormone response elements (PPREs) in the nucleus, can activate different physiological processes including cellular differentiation, development, metabolism, and inflammation (Komar 2005). Indeed, activation of some PPARs inhibit the production of monocyte inflammatory cytokines (Jiang *et al.* 1998). For instance, a study demonstrated that oxidised phospholipids can activate PPARα in a phospholipase A2-dependent manner (Delerive *et al.* 2000). Taken together, these novel insights have provided interesting ideas into the role of phospholipids in human inflammatory diseases. Moreover, phospholipids are emerging as a class of signalling lipids implicated in neuroinflammatory responses, and being seriously considered as a target for therapeutic treatment of neurological disorders with inflammation component (Kim 2015).

Phospholipids as modulators of Akt

Akt, also known as protein kinase B, is a protein identified as playing a role in the regulation of diverse physiological effects, including cell proliferation, survival and metabolism (Hers *et al.* 2011). Indeed, dysregulation of Akt has been associated with complicated diseases, such as CVD and neurological disorders (Chong *et al.* 2012). For example, interesting insights into treatment for stress-related disorders suggest a role for Akt in their antidepressant actions (Zhang *et al.* 2012, Huang *et al.* 2013). In particular, there is evidence supporting phospholipids as critical modulators of Akt (Huang *et al.* 2011). Since Akt signalling is dependent of lipid membrane balance, dietary phospholipids can induce Akt activation by incorporating into the cellular membrane and altering its composition. On the other hand, phospholipids can act as second messengers in cellular signalling and can activate Akt by interacting directly with it (Huang *et al.* 2011) (Fig. 1.10.3.2).

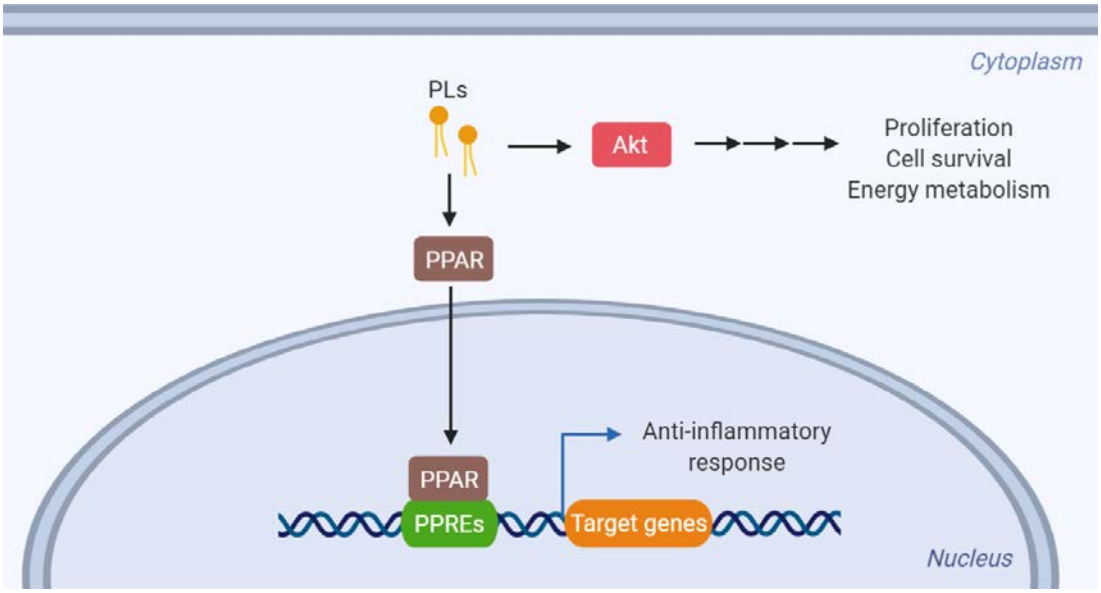

Figure 1.10.3.2 Phospholipids can activate PPAR and Akt signalling pathways*. Phospholipids can interact with Akt in the cytoplasm to induce proliferative- and survival-related pathways. Similarly, phospholipids activate peroxisome proliferator-activated receptors (PPARs), which translocate to the nucleus and bind with specific DNA sites named peroxisome proliferator hormone response elements (PPREs) and induce the expression of genes involved with anti-inflammatory response.*

Although, more research is needed to define the potential implications of phospholipid-Akt interaction as a molecular target for therapeutic strategies for stress-related disorders, the beneficial *in vivo* effects of phospholipids are clearly attracting interest in developing novel dietary treatments for neuropsychiatric mental disorders.

1.10.4 Interaction between dietary phospholipids and the gut microbiota

It is well known that diet can produce dramatic changes in gut microbiota composition, where these alterations are highly associated with health status (see section 1.4.1). However, there is a paucity of information on the effects of dietary phospholipids on microbiota, although this is slowly changing. For instance, a diet based in milk sphingomyelin improved lipid metabolism and altered gut microbiota composition in a mouse model (Norris *et al.* 2016). Specifically, this diet reduced gram-negative bacteria, while relative abundance of *Bifidobacterium* was significantly increased in faecal samples. On the other hand, a dietary intervention study using MFGM supplementation in maternally separated rats showed a significant impact on the

composition of the gut microbiota, alongside with attenuation of some of the long-term effects of early-life stress (O'Mahony *et al.* 2019).

However, much more research is needed to understand the potential of nutritional therapies using phospholipids against stress-related disorders. The purpose of this thesis is to give more insights into the therapeutic potential of dietary phospholipids in stress-related conditions using *in vitro* and animal models.

1.11 Primary hypothesis and aims of thesis

Given that nutritional interventions supported on scientific insights have emerged as valid and less invasive strategies to treat stress-related neuropsychiatric disorders, it is hypothesised that dietary polyphenols and phospholipids will exert positive neurobiological effects during stress-related conditions in cellular and animal models. As such, this PhD thesis is based on the following overarching objectives:

- To identify natural polyphenolics compounds with neuroprotective potential against CORT-induced cytotoxicity in cortical neurons, and to elucidate the molecular components involved in their mechanism.
- To demonstrate that polyphenol-enriched diets improve the depressive phenotype produced by maternal separation, an animal model of early-life stress.
- To determine whether dietary-derived phospholipids are capable of modulating neuronal function *in vitro*, including neuroprotection in cortical cells, and proliferation and differentiation in neural progenitor cells.
- To investigate the potential therapeutic effects of a phospholipid-enriched food against cognitive decline and depressive behaviour caused by a chronic psychosocial stress animal model.

Chapter 2

Naturally-Derived Polyphenols Protect Against Corticosterone-Induced Changes in Primary Cortical Neurons

Francisco Donoso[a, b], Valerie T. Ramírez[a], Anna V. Golubeva[a, c], Gerard Moloney[a, c], Catherine Stanton[a, d], Timothy G. Dinan[a, b] and John F. Cryan[a, c]

[a]APC Microbiome Ireland, University College Cork, Cork, Ireland
[b]Department of Psychiatry, University College Cork, Cork, Ireland
[c]Department of Anatomy & Neuroscience, University College Cork, Cork, Ireland.
[d]Teagasc Food Research Centre, Moorepark, Fermoy, Co. Cork, Ireland.

Published in: *International Journal of Neuropsychopharmacology (2019)*

DOI: 10.1093/ijnp/pyz052

Published online on: 8th December 2019

2.1 Abstract

Polyphenols are phytochemicals that have been associated with therapeutic effects in stress-related disorders. Indeed, studies suggest that polyphenols exert significant neuroprotection against multiple neuronal injuries, including oxidative stress and neuroinflammation, but the mechanisms are unclear. Evidence indicates that polyphenol neuroprotection might be mediated by Nrf2 activation, a nuclear factor associated with antioxidant and cell survival response. On the other hand, in stress-linked disorders, *Fkbp5* is a novel molecular target for treatment because of its capacity to regulate glucocorticoid receptor sensitivity. However, it is not clear the role of *Fkbp5* plays in polyphenol-mediated stress modulation. In this study the neuroprotective effects and mechanisms of the naturally-derived polyphenols xanthohumol and quercetin against cytotoxicity induced by corticosterone (CORT) were investigated in primary cortical cells.

Primary cortical cells containing both neurons and astrocytes were pre-incubated with different concentrations of quercetin and xanthohumol in order to examine the neuroprotective effects of polyphenols on cell viability, morphology and gene expression following CORT insult. Both polyphenols tested prevented the reduction of cell viability and alterations of neuronal/astrocytic numbers due to CORT exposure as assessed by the MTT assay and immunocytochemistry. Basal levels of *Bdnf* mRNA were also decreased after CORT insult, however this was reversed by both polyphenol treatments. Interestingly, the Nrf2 inhibitor blocked xanthohumol, but not quercetin-mediated neuroprotection. In contrast, we found that *Fkbp5* expression was exclusively modulated by quercetin. These results suggest that naturally-derived polyphenols protect cortical cells against CORT-induced cytotoxicity, and enhance cell survival, via modulation of the Nrf2 pathway and expression of *Fkbp5*.

2.2 Introduction

Alterations in hypothalamic-pituitary-adrenal (HPA) axis function have been associated with many neuropsychiatric disorders including depression, anxiety, anorexia nervosa and post-traumatic stress disorder (de Kloet *et al.* 2006, Pariante *et al.* 2008). It is well known that the HPA axis is activated in response to stress leading to increased concentration of glucocorticoids in the circulation. These steroid hormones are critical involved in the homeostatic regulation of metabolism, development and immune function (Sapolsky 2000). Moreover, preclinical studies have shown that chronic exposure to glucocorticoids produces deleterious effects to the structure and functional plasticity of the hippocampus and amygdala of adult rats (Mitra *et al.* 2008, Zhu *et al.* 2013). In addition, stress-induced increase of glucocorticoids has also been associated with impairment in the structure and function of the prefrontal cortex (PFC), a brain region that has been implicated in executive function (Arnsten 2009). Indeed, chronic stress exposure induces loss of dendrites and spines in pyramidal cells of PFC in rodents, which correlates with impaired working memory on the delayed alternation task (Hains *et al.* 2009, Shansky *et al.* 2009). In humans, chronic stress has been shown to weaken PFC functional connectivity and PFC regulation of the amygdala, which correlates with loss of grey matter (Arnsten 2015). Moreover, brain-derived neurotrophic factor (BDNF), which maintains neuronal survival and plasticity, is decreased in chronically stressed animals and patients with major depressive disorder and stress-related disorders (Murakami *et al.* 2005, Brunoni *et al.* 2008). Based on these observations, a drug that can attenuate the effects of chronic exposure to glucocorticoids on brain function may have a therapeutic potential in preventing and treating stress-related disorders.

Polyphenolic compounds are natural phytochemicals found abundantly in plant food sources, and characterised by the presence of multiple hydroxyl structural units on aromatic rings (Vauzour 2012). Several *in vivo* studies have focused on the potential of dietary polyphenols in protecting cognitive function and reducing risk for developing neurodegenerative and neuropsychiatric disorders, including depression and anxiety (Bhutada *et al.* 2010, Hurley *et al.* 2014, Lopresti *et al.* 2014). Indeed, increasing data support the neuromodulatory potential of polyphenols via their ability

to protect vulnerable neurons, improve neuronal function and regeneration (Spencer 2008). In particular, several in vitro studies have confirmed the neuroprotective capacity of polyphenols mainly against cytotoxicity by both oxidative stress and amyloid peptide (Aβ) neuronal insult (Godoy *et al.* 2016).

Polyphenols vary in their chemical structure and functional activity (Vauzour *et al.* 2010). Xanthohumol for example is a prenylated chalcone isolated from the female hop plant, *Humulus lupulus* and shown to possess anti-cancer, anti-inflammatory and neuroprotective effects in vitro (Liu *et al.* 2015, Yao *et al.* 2015). On the other hand, quercetin, a member of the flavonoid family, is one of the most prominent and widely distributed polyphenols in nature, and is found in many fruits, vegetables, leaves and grains (Manach *et al.* 2004). Quercetin has been associated with countless beneficial health effects such as protection against certain types of cancer, CVD and neurodegenerative disorders (Boots *et al.* 2008, Godoy *et al.* 2016).

It is well known that polyphenols can activate the nuclear factor erythroid 2-related factor-2 (Nrf2), a redox-sensitive transcription factor related to cell survival and antioxidant responses (Ungvari *et al.* 2010, Gopinath *et al.* 2012). On the other hand, there is evidence showing that polyphenols are able to modulate the glucocorticoid receptor (GR) function. Indeed, the polyphenolic flavonoid icariin is able to alter the expression of the GR and the FK506 binding protein 5 (FKBP5), which promotes GR stability and reduces GR sensitivity to GC *in vivo* (Wei *et al.* 2016). Actually, modulation of the GR regulatory system is currently an interesting target for treatment for stress-related disorders, therefore more research is needed in this area (O'Leary *et al.* 2013).

Taken together, the purpose of the present study was to explore through an in *vitro* approach new pharmacological strategies to treat stress-related disorders. In particular, we examined whether xanthohumol and quercetin, two naturally-derived polyphenols, protect against corticosterone-induced toxicity in neurons derived from the brain cortex, a key brain area involved in the pathophysiology of stress-related disorders. In addition, we explored the molecular mechanisms potentially implicated in such an effect. Specifically, we examined the interaction between the Nrf2 pathway and the

GR signalling pathway to elucidate the key molecular mechanisms through which polyphenols may protect neuronal integrity and improve stress-related disorders.

2.3 Methods

2.3.1 Chemicals and reagents

Dulbecco's modified Eagle's medium (DMEM), foetal bovine serum (FBS), poly-L-lysine, corticosterone (CORT), trypsin, L-glutamine, penicillin/streptomycin, D-glucose, 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT), dimethyl sulfoxide, Triton™ X-100, RU486, HEPES, NaCl, trigonelline and quercetin were purchased from Sigma. B-27 supplement was obtained from Thermo Fisher Scientific. Xanthohumol (purity 65 – 85%) was provided by Hopsteiner.

2.3.2 Animals

All procedures on live animals were performed under licence from the Government of Ireland Department of Health (B100/3774) in accordance with National and European Union directive 2010/63/EU, with prior ethical approval by University College Cork (AEEC #2012/045). Experiments were conducted in accordance with guidelines established by University College Cork's Animal Welfare Body.

2.3.3 Cell culture

Primary cortical cell cultures, consisting of both neurons and astrocytes, were prepared as described previously (Pusceddu *et al.* 2016) with some modifications. Briefly, PND1 Sprague Dawley male rats were sacrificed, brain removed and cerebral cortices dissected. Tissue was enzymatically dissociated with trypsin (0.25 µg/mL) and then triturated using a glass Pasteur pipette in warmed DMEM-F12 with 10% FBS and 100 µg/mL DNase. Cell suspension was passed through 40 µm strainer and then centrifuged at 1000 rpm for 10 min at room temperature. The pellet was re-suspended in warm culture media (DMEM-F12 supplemented with B-27, 1% FBS, 100 U/mL penicillin, 100 µg/mL streptomycin, L-glutamine 2 mM and D-glucose 55 mM). The suspended cells were cultured at 37 °C with 5% CO2.

2.3.4 Cell treatment

Quercetin and xanthohumol were selected after we detected their positive effects in a preliminary *in vitro* screen testing the potential of several polyphenolic compounds, including resveratrol, naringin and astaxanthin against CORT cytotoxicity in cortical cells (data not shown). Quercetin (0.03, 0.3 and 3 µM) and xanthohumol (0.2, 1 and 5 µM) were added to primary cortical cell cultures at day *in vitro* (DIV) 5, and maintained for 24 hours. The media was replaced with fresh media containing 200 µM CORT at DIV6 and kept for 96 hours (Fig. 2A). The doses of polyphenols were chosen based on previous publications, which showed neuroprotective effects *in vitro* (Yao *et al.* 2015, Godoy *et al.* 2016). To investigate the involvement of Nrf2 signalling in quercetin and xanthohumol-mediated neuroprotective effects, cells were co-treated with a specific Nrf2 inhibitor (5 µM trigonelline) along with quercetin or xanthohumol prior to CORT treatment. The dose of trigonelline is based on previous studies of Nrf2 inhibition in cellular models (Arlt *et al.* 2012, Rizza *et al.* 2015).

2.3.5 Cell viability measurement

Cell viability was determined by MTT assay based on mitochondrial dehydrogenase activity of viable cells as previously described (Mosmann 1983). Briefly, cortical cells were cultured in 24 well plate at a density of 4×10^4 cells per well. After treatment, medium was removed and replaced with fresh culture medium containing MTT (500 µg/mL), and then incubated at 37 °C for 3 hours. Afterwards, 100 µL of dimethyl sulfoxide were added to each well to dissolve the resulting formazan. The absorbance values were measured by spectrophotometry at 570 nm with a microplate reader (BioTek Synergy HT). The results were expressed as a percentage of vehicle control group.

2.3.6 LDH determination

Lactate dehydrogenase (LDH) release is usually quantified as a marker for cell lysis and necrosis (Brito *et al.* 2006). Detection of LDH was measured using the Cytotoxicity Detection Kit LDH (Roche) according to manufacturer instructions. Briefly, cortical cells were cultured in 24 well plate at a density of 4×10^4 cells per well.

After treatment, medium was transferred to a 96 well plate. Mix solution from kit containing both Catalyst and Dye solution was added to the wells and incubated for 30 min at room temperature protected from light. The absorbance values were measured by spectrophotometry at 490 nm with a microplate reader (BioTek Synergy HT).

2.3.7 Immunocytochemistry

Cellular staining for βIII-tubulin and Glial Fibrillary Acidic Protein (GFAP) proteins, molecular markers for neurons and astrocyte respectively, was assessed as through immunofluorescence detection as previously described (Pusceddu *et al.* 2016). Briefly, cortical cells were fixed with ice-cold methanol for 10 min and then blocked overnight in 5% horse serum at 4 °C. Subsequently, the cells were incubated in primary antibody solution (mouse anti-βIII-tubulin 1:300, Promega; rabbit anti-GFAP 1:300, Dako) overnight at 4 °C. The following day, the cells were incubated with the appropriate secondary antibody (Alexa Fluor 594 donkey anti-mouse 1:2000, Thermo Fisher; Alexa Fluor 488 donkey anti-rabbit 1:2000, Thermo Fisher) for 1 hour at room temperature. Finally, cellular counterstaining was assessed with Hoechst 33258 (Sigma) during 5 min. The cells were viewed using an Olympus BX53 upright fluorescence microscope.

2.3.8 Quantitative RT-PCR

Total RNA was isolated from primary cortical cells using the High Pure RNA isolation kit (Roche). Briefly, cells were seeded at density of 1.5×10^6 cells per well in 6-well plates, followed by pre-treatments with quercetin and xanthohumol before CORT insult. Cells were harvested and the lysate transferred into a filter tubes. RNA concentration was assessed using the ND-1000 spectrophotometer. Subsequently, isolated RNA was reverse transcribed into cDNA using the ExiLERATE LNA™ qPCR, cDNA synthesis kit (Exiqon). Following reverse transcription, mRNA expression levels of respective genes were measured using a lightcycler 480 II (Roche). Amplification was performed using ExiLERATE LNA™ qPCR, SYBR® Green master mix kit (Exiqon). Each sample was analysed in triplicate for both target

gene and reference gene (β-actin), and the relative mRNA expressions were calculated using the $2^{-\Delta\Delta Ct}$ method (Livak *et al.* 2001).

Table 2.3.8.1 Real time PCR primers

Target mRNA	Forward primer 5'-3'	Reverse primer 5'-3'	RefSeq
β-actin	TGTCACCAACTGGGACGATA	GGGGTGTTGAAGGTCTCAAA	NM_031144.3
Bdnf	GGACATATCCATGACCAGAAAGAAA	GCAACAAACCACAACATTATCGAG	XM_006234684.3
Creb1	CGTCATCTGCTCCCACTGTA	CCTTCGTTTTTGGGAATCAG	XM_017596652.1
Nqo-1	TCACCACTCTACTTTGCTCCAA	TTTTCTGCTCCTCTTGAACCTC	NM_017000.3
Ho-1	GCCCTGGAAGAGGAGATAGAG	TAGTGCTGTGTGGCTGGTGT	NM_012580.2
Gclc	CAAGGACAAGAACACACCATCT	CAGCACTCAAAGCCATAACAAT	NM_012815.2
Nr3c1	GAAAAGCCATCGTCAAAAGGG	TGGAAGCAGTAGGTAAGGAGA	XM_008772065.2
Gilz	CAGGCCATGGATCTAGTGAA	AGCGTCTTCAGGAGGGTATT	XM_008773446.2
Fkbp5	ACATGCAGGCCGTGATTCAGTA	TTGTCACAGCACTCGACAGCTT	XM_006256224.3
Sgk1	TGAAATAGCCAGTGCCTTGGGT	AAGGTGGACGTTGTCCCATTGT	XM_006227723.1
Foxo3	TGTCAGCAACATGGGCTTGAGT	GGGAAGGTTTGCACTGGCTGAATA	NM_001106395.1
Btg1	TTCAGGCTTCTCCCAAGTGAACTC	CCATTTGCACGTTGGTGCTGTT	NM_017258.1

2.3.9 Reverse-phase protein array (RPPA)

High-throughput RPPA for 249 proteins (antibody list provided in Supplementary Information in Appendix) was performed following the protocol and directions of MD Anderson Center (Houston, Texas 77030, USA), where RPPA was performed. Briefly, cells were seeded at density of 1.5×10^6 cells per well in 6-well plates, followed by pre-treatments with quercetin and xanthohumol before CORT insult. Cells were harvested and then lysed using the lysis buffer suggested by MD Anderson (1% Triton™ X-100, 50 mM HEPES, pH 7.4, 150 mM NaCl, 1.5 mM $MgCl_2$, 1 mM EGTA, 100 mM NaF, 10 mM Na pyrophosphate, 1 mM Na_3VO_4 and 10% Glycerol). Total protein concentration in lysates was determined by performing a BCA assay (Thermo Scientific), and samples were adjusted to a concentration of 1–1.5 μg/μl. Samples were then denatured using the SDS sample buffer recommended by the core facility, boiled for 5 min and then stored at −80 °C before being shipped to MD Anderson on dry ice. Samples were then serially diluted and arrayed onto nitrocellulose-coated slides. Slides were then probed with the core facility's collection of antibodies, and signal was generated using a 3, 3′-diaminobenzidine colorimetric reaction-based system. Results provided by MD Anderson were then analysed using the functional annotation tool DAVID 6.8 in order to detect up- or down-regulation of signalling pathways within the KEGG database.

2.3.10 Statistical analysis

Statistical analysis was performed using the software SPSS 24.0, and the results were presented as mean ± SEM. Data were analysed using one- or two-way analysis of variance (ANOVA) as appropriate. A *p*-value of 0.05 was considered statistically significant.

2.4 Results

2.4.1 CORT-induced changes in cortical cells are mediated by the GR

To investigate the role of the GR in CORT-elicited cytotoxicity in cortical cells, dose and time curve response of CORT were determined by MTT assay. At DIV6 the cells were treated with CORT stimulus for 72 and 96 hours (Fig 1A). As expected, stimulation with CORT caused a significant reduction in cell viability at 96 h (Fig 1B and C). Pre-incubation for 24 hours with the GR antagonist RU486 (50 nM) ameliorated the reduction of cell viability caused by CORT (Fig. 1D).

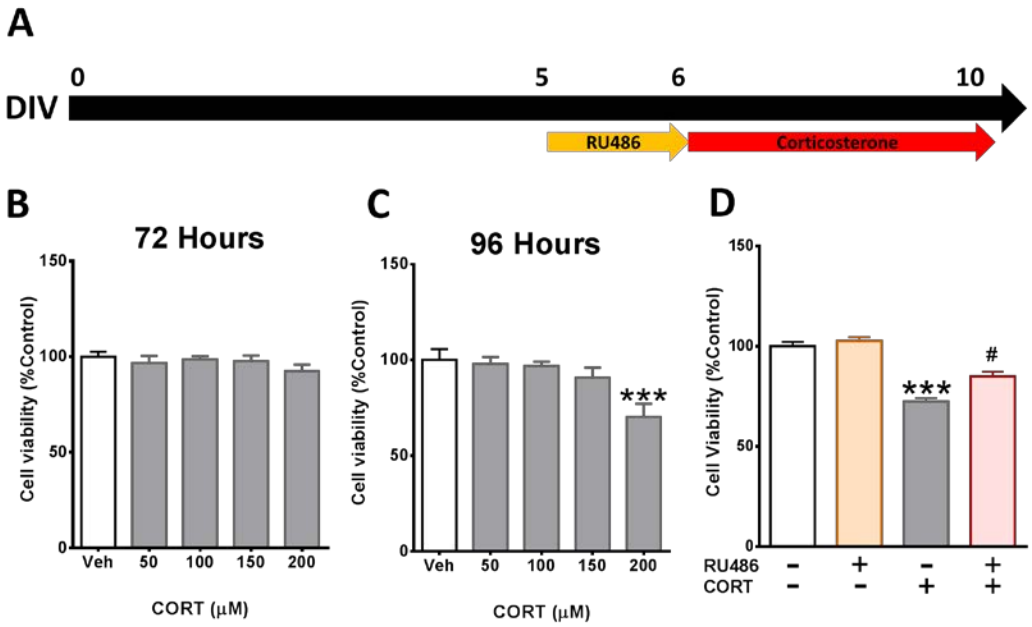

Figure 1 CORT-induced cytotoxicity in cortical cells is mediated by the GR. (A) *Schematic representing the experiment timeline.* ***(B – C)*** *Cortical cells were treated with various concentrations of CORT for 72 and 96 h.* ***(D)*** *Cortical cells were pre-treated with 50 nM of RU486 for 24 h and then with 200 µM CORT for 96 hours. Cell viability was measured by MTT assay. Results are expressed as the mean ± SEM of three independent experiments performed in triplicate (***p < 0.001 versus 'vehicle' groups; #p < 0.05 versus 'CORT' groups).*

2.4.2 Xanthohumol and quercetin prevented CORT-induced cytotoxicity in cortical cells

To determine the protective effects of xanthohumol and quercetin from CORT-induced cytotoxicity, cortical cells were pre-incubated with different concentrations of xanthohumol and quercetin. The reduction of cell viability in cortical cells induced by 96 hours of incubation with 200 µM CORT was significantly prevented by 24-hour pre-treatment with both xanthohumol (1 or 5 µM) and quercetin (3 µM) (Fig. 2 B-C).

2.4.3 Xanthohumol and quercetin prevented CORT-induced alterations in neuron and astrocyte proportion

To further determine the potential protective effects of xanthohumol and quercetin against CORT-induced cytotoxicity, we explored whether these polyphenols prevented the alteration in neuron and astrocyte composition caused by CORT in cortical cells. As illustrated in Fig 2D-H, pre-treatment with both xanthohumol and quercetin prevented the decrease in the number of neurons and the increase in astrocytes induced by treatment with 200 µM CORT.

2.4.4 Xanthohumol and quercetin prevented CORT-induced apoptotic morphological changes in cortical cells

To define the type of cell death triggered by CORT, apoptotic and necrotic features were measured in cortical cells after CORT exposure. Apoptosis is characterised by morphological changes including chromatin condensation and reduction of cell membrane permeability. On the other hand, membrane integrity is completely lost during necrosis (Brito *et al.* 2006). 24-hour pre-incubation with 5 µM xanthohumol and 3 µM quercetin reduced the number of condensed nuclei in cortical cells after 200 µM CORT treatment for 96 hours (Fig. 2I-K). Extracellular LDH activity was not increased after CORT treatment suggesting that plasmatic membrane did not lose integrity, similarly to apoptosis. In contrast, the necrotic/cell lysis inductor Triton™ X-100 produced a significant increase of extracellular LDH activity.

2.4.5 Xanthohumol and quercetin prevented CORT-induced down-regulation of *Bdnf* gene expression in cortical cells

Modulation of BDNF expression is one of the mechanisms which neuroprotective agents exert their effects (Zhao *et al.* 2017). Thus, the effect of xanthohumol and quercetin on CORT-induced reduction in mRNA *Bdnf* levels was investigated in cortical cells. After 96 hours of treatment, 200 µM CORT triggered a considerable reduction of *Bdnf* gene expression in cortical cells as previously reported (Pusceddu *et al.* 2016). However, this *Bdnf* reduction was prevented by a pre-incubation with 5 µM xanthohumol and 3 µM quercetin for 24 hours (Fig. 2N-O). To investigate whether CORT-induced reduction of *Bdnf* expression is mediated by down-regulation of *Creb1*, we first explored whether CORT regulates the *Creb1* mRNA expression in cortical cells (Fig. 2P-Q). Treatment with 200 µM CORT for 96 hours did not induce changes in *Creb1* expression in cortical cells. Moreover, neither 5 µM xanthohumol nor 3 µM quercetin were able to up-regulate *Creb1* gene expression.

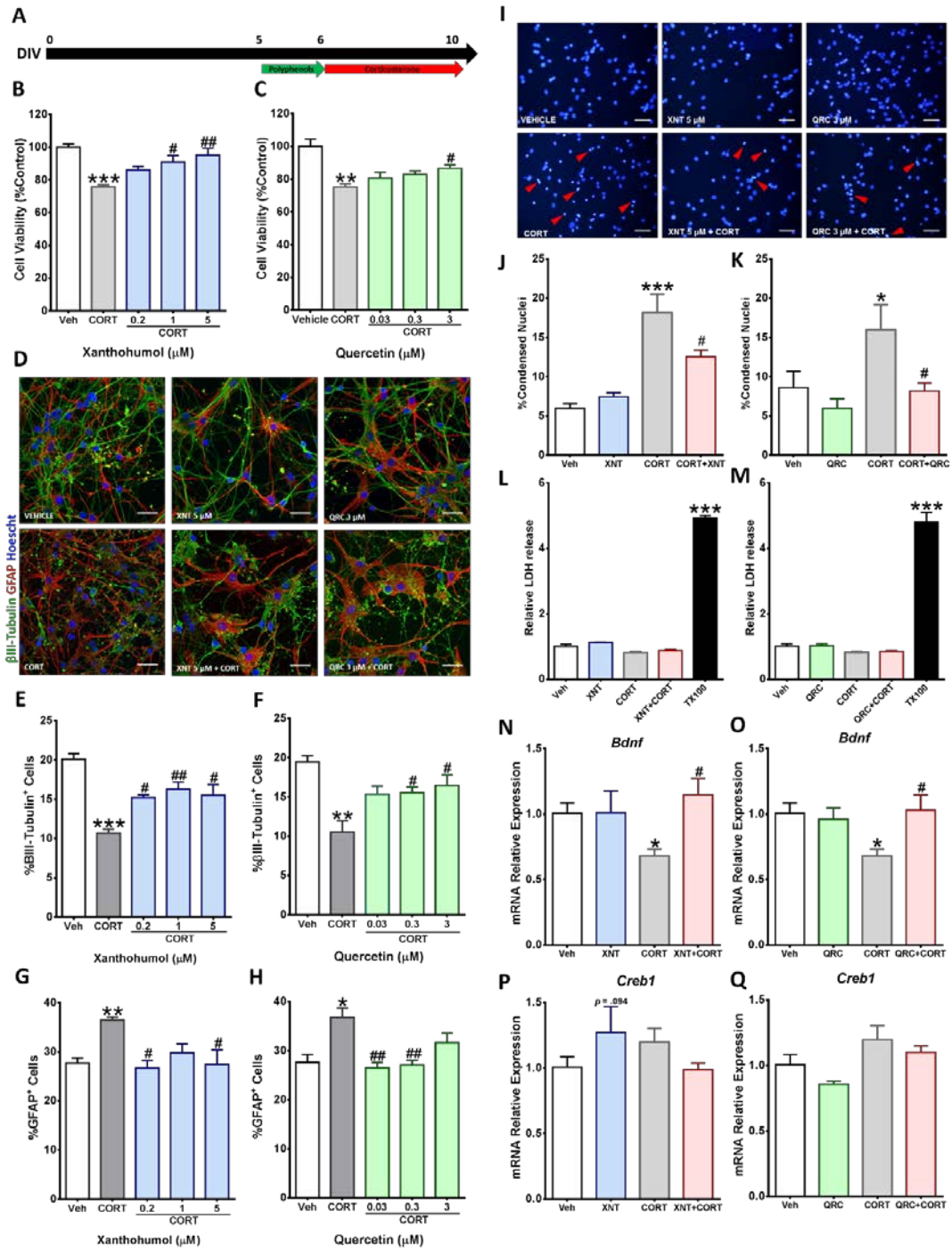

Figure 2 Xanthohumol and quercetin prevented CORT-induced changes in cortical cells. (A) *Schematic representing the experiment timeline.* ***(B – C)*** *Cortical cells were pre-treated with the indicated concentrations of xanthohumol and quercetin for 24 h and then with 200 μM CORT for 96 hours. Cell viability was measured by MTT assay.* ***(D – H)*** *Quantitative analysis of neurons and astrocytes was assessed by immunostaining of βIII-tubulin⁺ and GFAP⁺ cells. Scale bar = 50 μm.* ***(I – K)*** *Cortical cells were pre-treated with 5 μM xanthohumol or 3 μM quercetin for 24 hours, and then exposed 96 hours to 200 μM CORT. Morphological changes of the nuclei were determined by Hoechst 33258 staining. Scale bar = 50 μm. Red arrows indicate representative condensed nuclei.* ***(L – M)***

Lactate dehydrogenase (LDH) activity was measured in cell culture media after polyphenol and CORT treatment. ***(N – Q)*** *The gene expression was quantitatively measured using real time RT-PCR for Bdnf and Creb1 mRNA relative expression. Results are expressed as the mean ± SEM of three independent experiments performed in triplicate (*p < 0.05; **p < 0.01; ***p < 0.001 versus 'vehicle' groups; #p < 0.05; ##p < 0.01 versus 'CORT' groups).*

2.4.6 Inhibiting the Nrf2 pathway attenuated only the protective effect of xanthohumol against CORT-induced cytotoxicity in cortical cells

The activation of Nrf2 pathway has been shown to be involved in the protective mechanisms of polyphenols against CORT inducing its deleterious effects on neuronal models (Freitas *et al.* 2015, Sun *et al.* 2018). Thus we used a pharmacological approach to determine the mediatory role of Nrf2 pathway in the protective effects of xanthohumol and quercetin against CORT-elicited cytotoxicity. We investigated whether trigonelline, an inhibitor of Nrf2 nuclear import (Arlt *et al.* 2012), could abolish the cytoprotective effect of these polyphenols against CORT-dependent reduction in cell viability in cortical cells. At DIV5 the cells received a treatment with both polyphenols and 5 µM trigonelline for 24 hours and subsequently CORT stimulus for 96 hours (Fig. 3A). Trigonelline treatment ameliorated the increase in cell viability induced by treatment with xanthohumol, which suggested that inhibition of Nrf2 pathway blocked the protective effects of xanthohumol against CORT-induced cytotoxicity in cortical cells. In contrast, trigonelline did not affect the protective effects of quercetin against CORT-induced cytotoxicity (Fig. 3B-C).

2.4.7 Xanthohumol induced the expression of Nrf2 target genes in cortical cells

We further investigated whether xanthohumol or quercetin induced the expression of Nrf2-driven cytoprotective/antioxidant genes in cortical cells. At DIV5 the cells received a treatment with either xanthohumol or quercetin along with 5 µM trigonelline for 24 hours (Fig. 3D). After 24-hour treatment with 5 µM xanthohumol, the mRNA expression of Nrf2-activated genes (*Ho-1*, *Nqo-1* and *Gclc*) was significantly increased. Moreover, 5 µM trigonelline co-treatment blocked xanthohumol-induced up-regulation of Nrf2-activated genes, which confirmed the activation of Nrf2-pathway by xanthohumol (Fig. 3E-G). On the other hand, 3 µM

quercetin treatment did not induce expression of Nrf2-driven genes in cortical cells (Fig. 3J-L).

2.4.8 Xanthohumol-induced *Bdnf* gene expression is dependent of Nrf2 activation

The effect of xanthohumol and quercetin on gene expression of *Bdnf* before CORT exposure was studied in cortical cells. Treatment with 5 µM xanthohumol for 24 hours remarkably induced the gene expression of *Bdnf* and *Creb1*. Interestingly, 5 µM trigonelline prevented the increase in *Bdnf* and *Creb1* gene expression induced by treatment with xanthohumol (Fig. 3H-I). In contrast, 3 µM quercetin treatment for 24 hours did not induce gene expression of *Bdnf* and *Creb1* (Fig. 3M-N). These data indicate that xanthohumol-induced *Creb1* and *Bdnf* gene expression may be mediated by the Nrf2-pathway.

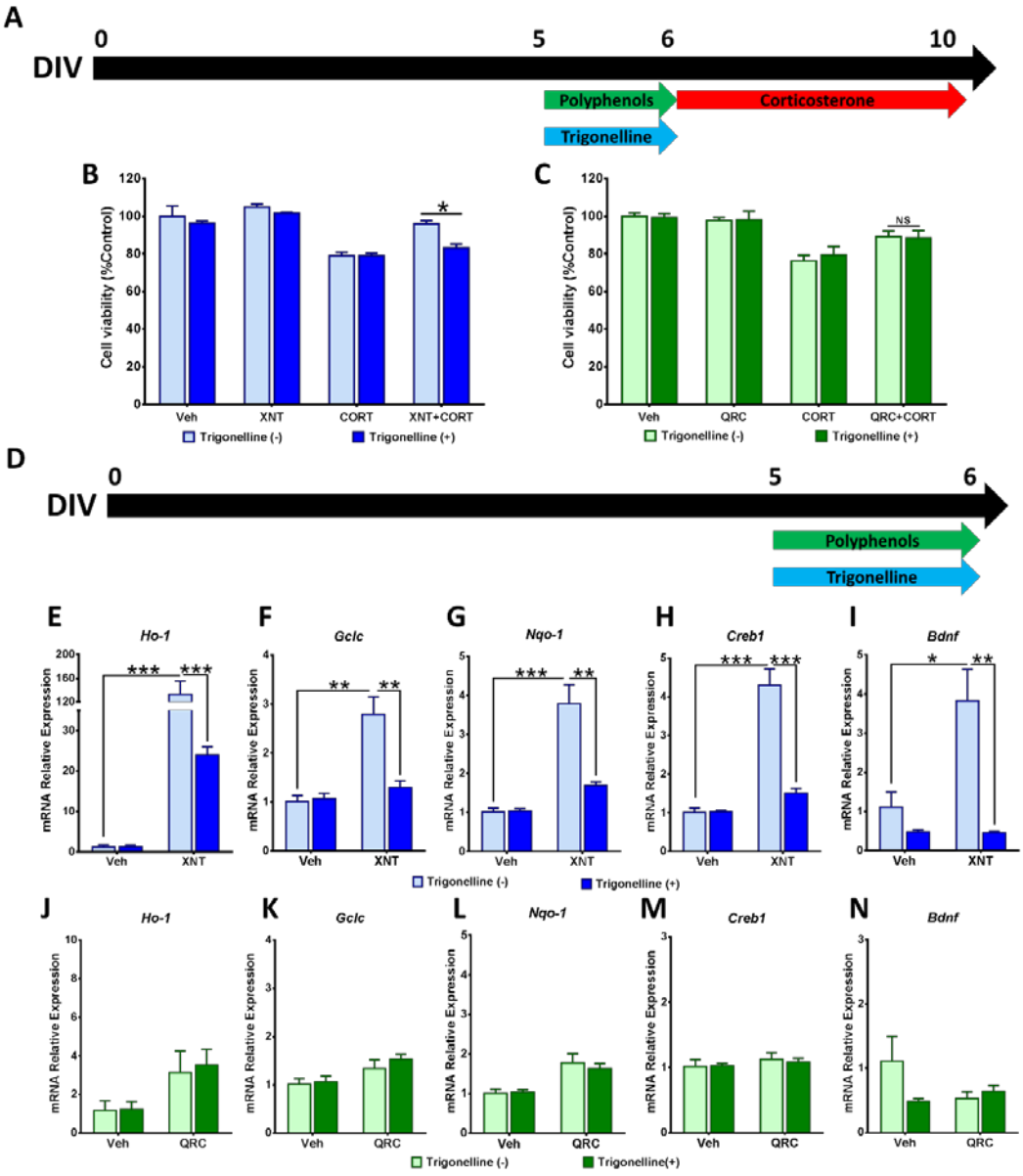

Figure 3 Xanthohumol neuroprotection is mediated by the Nrf2 pathway. (A – C) *Cortical cells were co-treated with 5 µM trigonelline and polyphenols (5 µM xanthohumol or 3 µM quercetin) for 24 hours before 96-hour exposure to 200 µM CORT. Cell viability was detected by MTT assay.* ***(D)*** *Cortical cells were co-treated with 5 µM trigonelline and 5 µM xanthohumol or 3 µM quercetin for 24 hours. Relative expression of Nrf2-activated genes (Ho-1, Nqo-1 and Gclc), Creb1 and Bdnf induced by* ***(E – I)*** *xanthohumol and* ***(J – N)*** *quercetin was determined through real time RT-PCR. Data were expressed as the mean ± SEM of three independent experiments performed in triplicate (***p < 0.001; **p < 0.01; ***p < 0.001). Note: Ho-1 heme oxygenase, Nqo-1 NAD(P)H dehydrogenase (quinone 1), Gclc glutamate-cysteine ligase catalytic subunit.*

2.4.9 Quercetin improved *Fkbp5* gene expression after CORT stimulus in cortical cells

Given that the Nrf2 pathway might not be involved in quercetin neuroprotection, we examined the GR signalling. The effect of xanthohumol and quercetin on GR-regulated genes expression after CORT treatment was studied in cortical cells. Although, we did not find changes in *Btg1*, *N3c1* and *Sgk1* mRNA levels after CORT exposure, the gene expression of *Fkbp5*, *Foxo3* and *Gilz* was significantly increased (Fig. 4). All of these genes have been associated with modulation and regulation of the GR signalling pathway (27030168, 16110340, 27288728). Moreover, *Fkbp5* expression was increased by pre-incubation with 3 µM quercetin followed CORT treatment for 96 h compared to CORT group alone (Fig. 4H). Pre-treatment with 5 µM xanthohumol did not affect the gene expression of any GR-regulated gene (Fig. 4A-F).

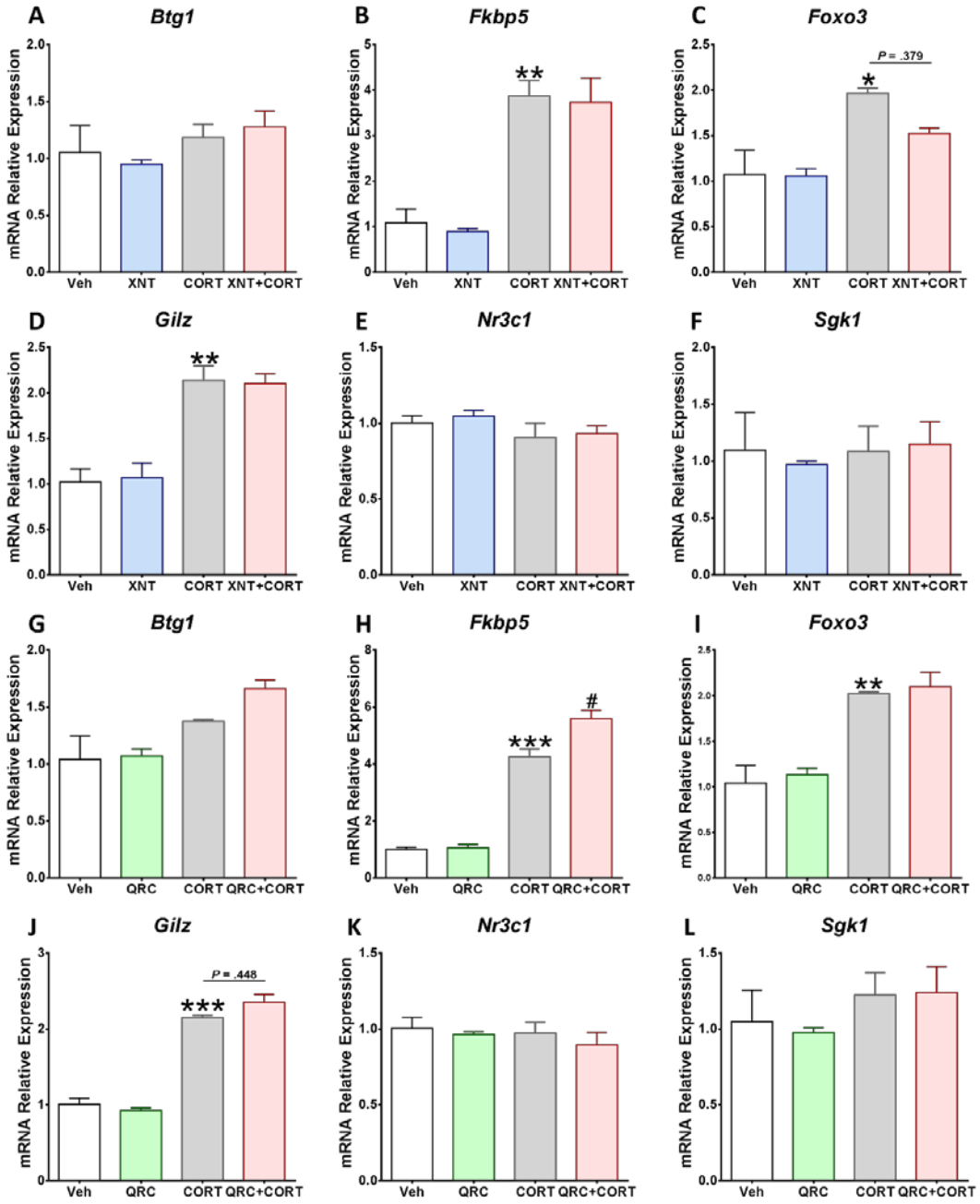

Figure 4 Quercetin enhanced the expression of Fkbp5 after CORT treatment in cortical cells. *After 24-hour exposure to* ***(A – F)*** *5 µM xanthohumol or* ***(G – L)*** *3 µM quercetin, cortical cells were treated during 96 hours with 200 µM CORT. The gene expression of Btg1, Fkbp5, Foxo3, Gilz, Nr3c1 and Sgk1 was quantitatively measured using real time RT-PCR. Results are expressed as the mean ± SEM of three independent experiments performed in triplicate (***p < 0.001; **p < 0.01; *p < 0.05 versus 'vehicle' groups; #p < 0.05 versus 'CORT' groups). Note: Btg1 B-cell translocation gene 1, Fkbp5 FK506 binding protein 5, Foxo3 forkhead box O3, Gilz glucocorticoid-induced leucine zipper, Nr3c1 nuclear receptor subfamily 3 group C member 1, Sgk1 serum and glucocorticoid-regulated kinase 1.*

2.4.10 CORT induced changes in Akt/mTOR/AMPK signalling pathways

In order to investigate the impact of CORT treatment in signalling pathways associated with proliferation, cell growth and apoptosis in cortical cells we studied the activation or downregulation of proteins involved in these physiological processes using the RPPA approach. After treatment with 200 μM CORT for 96 hours we found 46 proteins with altered expression (Fig. 5A), and further analysis revealed that Akt, FOXO, mTOR, AMPK and Neurotrophin signalling pathways were significantly affected (Fig. 5B; KEGG codes: Akt map04151, FoxO map04068, mTOR map04150, AMPK map04152 and Neurotrophin map04722).

2.4.11 Impact of xanthohumol and quercetin on CORT-induced changes in Akt/mTOR, AMPK and Erk1/2 signalling pathways

The effect of xanthohumol and quercetin on CORT-induced alteration in signalling pathways associated with proliferation and apoptosis were next investigated in cortical cells. Pre-incubation with xanthohumol or quercetin did not interfere significantly against CORT-induced changes in Akt, mTOR and Erk1/2 signalling pathways (Fig. 5D, 5F and 5G). Nevertheless, xanthohumol treatment prevented the abnormal increase of pAMPK/AMPK ratio caused after CORT insult (Fig. 5C). Surprisingly, xanthohumol abolished CORT-induced protein overexpression of connexin 43 (Cx43), which is not involved directly with these signalling pathways (Fig. 5H).

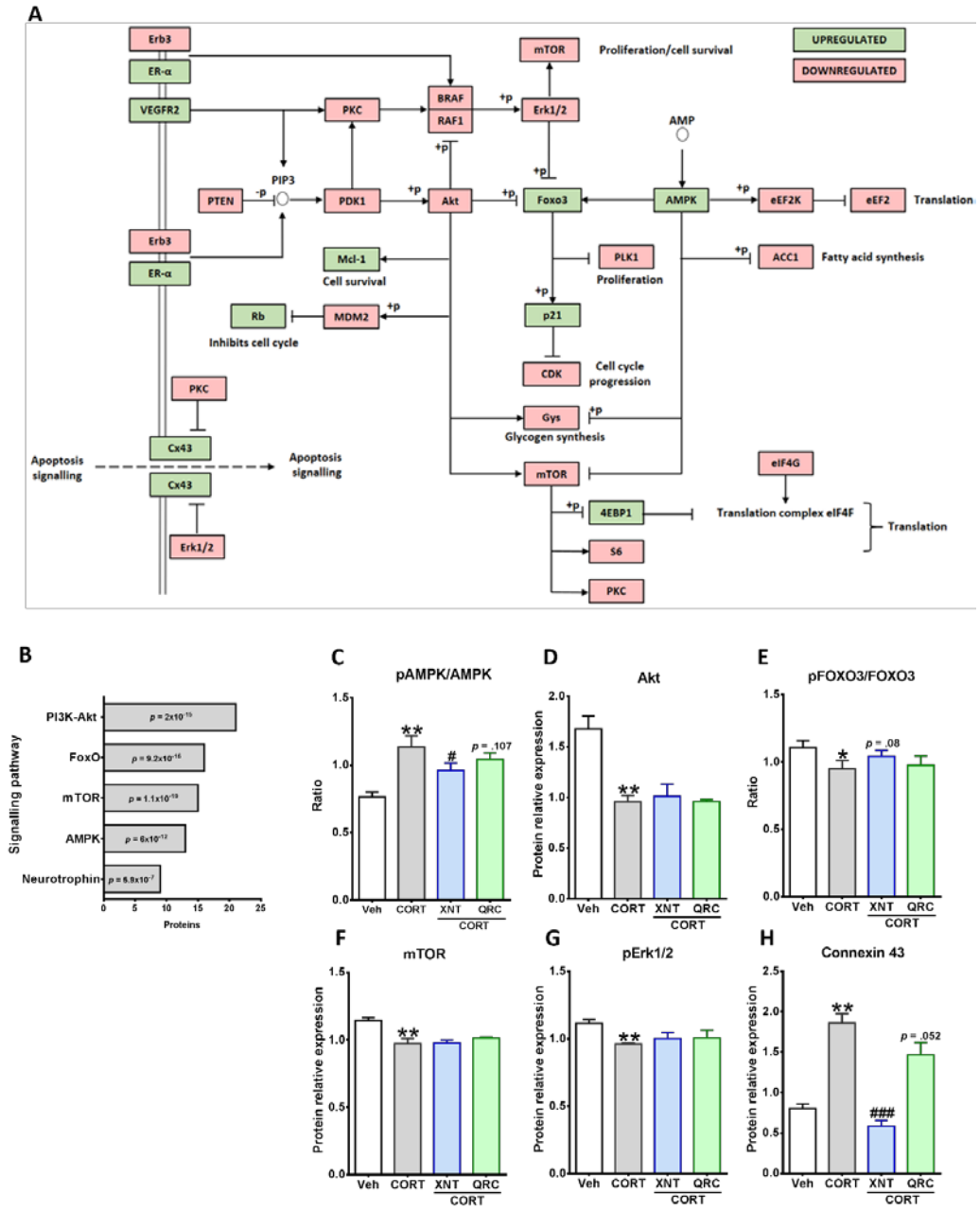

Figure 5 Impact of xanthohumol and quercetin on CORT-induced changes in Akt/mTOR, AMPK and Erk1/2 signalling pathways. (A) *Schematic interaction map of proteins altered by CORT; upregulated expression of proteins is showed in green while downregulated proteins are highlighted in red. Cortical cells were treated with 200 µM CORT for 96 hours. The relative expression of proteins involved in proliferation, cell growth and apoptosis was determined using the RPPA approach.* ***(B)*** *KEEG signalling pathways affected by CORT were detected using the functional annotation tool DAVID 6.8.* ***(C – H)*** *After 24-hour exposure to 5 µM xanthohumol or 3 µM quercetin, cortical cells were treated during 96 hours with 200 µM CORT. The levels of protein expression of AMPK, pAMPK, total Akt, FOXO3, pFOXO3, mTOR, pErk1/2 and connexin 43 were quantitatively measured using RPPA and provided by MD Anderson. Results are expressed as the mean ± SEM of three independent experiments (***p < 0.001; **p < 0.01; *p < 0.05 versus 'vehicle' groups; ###p < 0.001; #p < 0.05 versus 'CORT' groups).*

2.5 Discussion

In the present study, we demonstrated that administration of naturally-derived polyphenols xanthohumol and quercetin significantly protects primary cortical cells against CORT-elicited cytotoxicity, altered neuronal/astrocytic ratio and reduced *Bdnf* expression. In addition, our results establish that trigonelline, an inhibitor of Nrf2, abolishes the neuroprotective effects of xanthohumol, but not quercetin against CORT-induced cell death. Taken together, our findings reveal that although both xanthohumol and quercetin are neuroprotective agents, they recruit different cellular signalling pathways to induce such effects.

Several reports have demonstrated that the glucocorticoid hormone corticosterone exerts toxic effect on neurons (Gao *et al.* 2015, Zhao *et al.* 2018). Additionally, increasing levels of CORT are associated with depressive and anxious-like behaviours in rodent models (Rosa *et al.* 2014, Mendez-David *et al.* 2017). Therefore, preventing the neurotoxic effects of glucocorticoids could represent a novel therapeutic approach for treatment of stress-related disorders. On the other hand, polyphenols have attracted considerable interest due to their multiple health benefits including anti-oxidant, anti-cancer and neuroprotective effects (Vauzour *et al.* 2010). However, the molecular mechanisms underlying polyphenol-mediated neuroprotection in neurons are not fully understood. Thus, the aim of the present study was to investigate whether the naturally-derived polyphenolic compounds xanthohumol and quercetin prevent CORT-induced neurotoxicity in cortical cells.

Mixed cortical cell cultures containing both neurons and astrocytes have been extensively used as a model to study the neurotoxic effects of numerous stimulants *in vitro* (Wie *et al.* 1997, Trackey *et al.* 2001, Huang *et al.* 2009). As expected, our data shows that CORT treatment decreased the cell viability of cortical cells. In addition, further analysis indicated that CORT-exerted cell death is mediated through activation of the GR resulting in the induction of apoptosis. In accordance with our findings, it has been shown before that CORT induces apoptosis via activation of the GR cascade in different neuronal models such as hippocampal neurons (Latt *et al.* 2018), PC12

cells (Li *et al.* 2014), hypothalamic neurons (Zhang *et al.* 2012), and cortical cells (Pusceddu *et al.* 2016).

We next demonstrated that treatment with xanthohumol and quercetin significantly reduced CORT-induced cytotoxicity. In addition, these compounds reduced the alteration of cellular composition in cortical cells produced by CORT. Indeed, our study shows that the reduction in neuronal percentage and increased astrocytic concentration caused by CORT treatment is prevented by these polyphenols. In neural tissue, astrocytes play an important role in regulation of synapse, energy and neurotransmitter balance (Sofroniew *et al.* 2010). Accordingly, astrocytic overgrowth or astrogliosis has been described as a physiological process critical for neural protection and repair (Zhang *et al.* 2007). This is in line with previous studies showing similar effects on astrocytes after CORT insult (Bridges *et al.* 2008, Pusceddu *et al.* 2016).

To further investigate the possible mechanisms underlying the protective effect of xanthohumol and quercetin against CORT-induced cytotoxicity we next examined the BDNF gene expression. BDNF is a neurotrophin involved in neuroplasticity and neuronal survival, which has been associated with neuroprotective effects in *in vitro* and *in vivo* (Almeida *et al.* 2005, Motaghinejad *et al.* 2017). Here we demonstrated that both polyphenols prevent CORT-induced reduction of *Bdnf* mRNA levels. Previous studies have demonstrated that several polyphenols, including xanthohumol and quercetin, induce the activation of Nrf2, which contributes significantly to cytoprotective and antioxidant responses in different cellular models (Tanigawa *et al.* 2007, Ungvari *et al.* 2010, Gopinath *et al.* 2012, Yao *et al.* 2015). We found that trigonelline, an inhibitor of Nrf2, significantly reversed the protective effect of xanthohumol but not quercetin. Moreover, blocking Nrf2 activation abolished the xanthohumol-mediated increase of *Bdnf* mRNA levels confirming previous studies that also highlight the crosstalk between BDNF and Nrf2 signalling (Mendez-David *et al.* 2015, Bouvier *et al.* 2017, Ishii *et al.* 2018).

Surprisingly, quercetin treatment did not induce *Bdnf* gene expression, suggesting that quercetin prevented CORT-induced depletion of BDNF in cortical cells through an

alternative mechanism. Interestingly, there is some evidence indicating that quercetin is able to activate Nrf2 signalling in other *in vitro* studies (Tanigawa *et al.* 2007, Granado-Serrano *et al.* 2012). However, the reason as to why quercetin fails to activate Nrf2 in cortical cells remains unknown.

To elucidate the mechanisms underlying the neuroprotection of quercetin, we examined whether quercetin attenuates activation of the GR cascade following CORT stimulation. We hypothesised that downregulation of the GR signalling pathway could decrease CORT-induced cytotoxicity and eventually reverse BDNF depletion in cortical cells. GR blockade strategies have been proposed to be a novel therapeutic approach to prevent the negative effects of chronic stress (Oomen *et al.* 2007, Revsin *et al.* 2009). In this regard, analysis of FK506 binding protein 51 (FKBP5) a protein which regulates GR sensitivity, is considered an interesting target for the prevention and treatment of stress-related psychiatric disorders (Guidotti *et al.* 2013). When FKBP5 is bound to the receptor complex, glucocorticoids bind with lower affinity and the nuclear translocation of the receptor is less efficient (Binder 2009). Our data demonstrates that quercetin treatment significantly increased expression of *Fkbp5* after CORT stimuli in cortical cells, suggesting that the GR negative feedback system could be activated, thus preventing BDNF depletion.

BDNF-mediated neuroprotective mechanisms are associated with the activation of different molecular pathways related to proliferation and cell growth processes such as Akt, mTOR and Erk1/2 (Han *et al.* 2000, Chen *et al.* 2013). Therefore, we analysed components of these signalling pathways in cortical cells upon CORT stimulation. In this study, we established that CORT treatment significantly influence the proliferative pathways Akt and mTOR, as well as metabolic signalling pathways that possibly trigger apoptosis, such as AMPK and FOXO3. Indeed, downregulation of Akt and mTOR is associated with decreased proliferation in neuronal models (Peltier *et al.* 2007, Bhaskar *et al.* 2009), while activation of AMPK/FOXO3 pathways indirectly lead to apoptotic signalling (Davila *et al.* 2012). Our results demonstrate that xanthohumol treatment reduced AMPK phosphorylation after CORT stimulus, indicating down regulation of this pathway. However, treatment with either xanthohumol or quercetin did not prevent CORT-induced changes at protein levels of

Akt, mTOR and Erk1/2. These data suggest that activation of these proliferative pathways are not critical for BDNF-mediated neuroprotection in cortical cells.

Additionally, we have demonstrated that xanthohumol significantly blocked CORT-induced overexpression of Cx43 in cortical cells. The evidence suggests that Cx43 plays a pro-apoptotic role in different cytotoxic stimuli, which has been suggested before (Huang *et al.* 2001, Dong *et al.* 2015, Yahiro *et al.* 2015), although the mechanisms responsible for Cx43-mediated apoptosis are unknown. Since Cx43 is the structural component of gap junctions and it is responsible for the transfer of water-soluble molecules directly from one cell to another without passing through the membrane, the enhancement of cytotoxic effects on Cx43-transfected cells may be due to increased transfer of drugs or pro-apoptotic molecules from one cell to another (Kameritsch *et al.* 2013).

In conclusion, our present work confirmed that naturally-derived polyphenols xanthohumol and quercetin attenuated CORT-exerted neurotoxicity in primary cortical cultures. We further found that blocking the Nrf2 pathway could abolish the protective effect of xanthohumol, while quercetin neuroprotection may be mediated by FKBP5 modulation. Our data reveal that xanthohumol and quercetin differentially mediate BDNF restoration upon CORT treatment in cortical cells, indicating the possible role of this neurotrophin in the neuroprotective mechanisms of these polyphenols. In addition, our findings provide a novel insight into the mechanisms underlying polyphenol-mediated neuroprotective effect *in vitro* and potential health benefits for stress-related disorders.

Chapter 3

Polyphenols Reverse Early-Life Stress-Induced Changes in the Microbiota-Gut-Brain Axis in the Rat

Francisco Donoso[a, b], Sian Egerton[a, d, e], Thomaz Bastiaanssen[a, c], Patrick Fitzgerald[a, c], Snehal Gite[d], Fiona Fouhy[d], R. Paul Ross[a], Catherine Stanton[a, d], Timothy G. Dinan[a, b] and John F. Cryan[a, c]

[a]APC Microbiome Ireland, University College Cork, Cork, Ireland
[b]Department of Psychiatry, University College Cork, Cork, Ireland
[c]Department of Anatomy & Neuroscience, University College Cork, Cork, Ireland.
[d]Teagasc Food Research Centre, Moorepark, Fermoy, Co. Cork, Ireland.
[e]School of Biological, Earth and Environmental Science, University College, Cork, Ireland.

Submitted to: *Psychoneuroendocrinology*

Under review

3.1 Abstract

There is a growing emphasis on the role of the microbiota-gut-brain axis as modulator of host behaviour and as therapeutic target for neuropsychiatric disorders. In addition, accumulating evidence suggests that early-life stress can exert long-lasting changes on the brain and microbiota, and this early adversity is associated with increased risk for developing depression in later life. The maternal separation (MS) model in rats is a robust paradigm to study the effects of early-life stress on the microbiota-gut-brain axis. Recently, we have shown that polyphenols, naturally occurring compounds associated with several health benefits, have anti-stress effects in *in vitro* models. In this study, we assess the therapeutic potential of a variety of both flavonoid and non-flavonoid polyphenols in reversing the impact of MS on behaviour and the microbiota-gut-brain axis.

Rats underwent a dietary intervention with the naturally-derived polyphenols xanthohumol and quercetin, as well as with a phlorotannin extract for 8 weeks. Treatment with polyphenols prevented the depressive and anxiety-like behaviours induced by MS, where xanthohumol effects were correlated with rescue of BDNF plasma levels. In addition, MS resulted in altered brain levels of 5-hydroxyindoleacetic acid (5-HIAA) and dopamine, accompanied by abnormal elevation of plasma corticosterone. Although polyphenols did not reverse neurotransmitter imbalance, xanthohumol normalised corticosterone levels in MS rats. Finally, we explored the impact of MS and polyphenolic diets on the gut microbiota. We observed profound changes in microbial composition and diversity produced by MS condition and by xanthohumol treatment. Moreover, functional prediction analysis revealed that MS results in altered enrichment of pathways associated with microbiota-brain interactions that are significantly reversed by xanthohumol treatment. These results suggest that naturally-derived polyphenols exert antidepressant-like effects in MS rats, which mechanisms could be potentially mediated by HPA regulation, BDNF levels rescue and modulation of the microbiota-gut-brain axis.

3.2 Introduction

Stress-related psychiatric disorders including depression and anxiety are currently a major public health concern. In 2015, depressive disorders were estimated to be the third leading cause of disability worldwide. Now, the WHO indicates that depression is a leading cause of disability worldwide and is a major contributor to the overall global burden of disease (Johnston *et al.* 2019, Park *et al.* 2019). On the other hand, major depressive disorder is thought to result from the complex interplay of multiple inherited genetic factors and subsequent exposure to a wide range of environmental variables throughout life (aan het Rot *et al.* 2009); therefore, the search for adequate treatments is a great challenge as no established mechanisms have yet been determined (Berton *et al.* 2006). Based on these observations and considering that depression has an inconsistent response to treatment, the development of new antidepressant strategies is increasingly being considered as a critical focus of research.

It is well known that stressful events in early life can exert long-lasting changes in brain structure and function later on (Cryan *et al.* 2013) and accumulating evidence indicates that this early life adversity is associated with an increased risk for developing depression (Chapman *et al.* 2004, Heim *et al.* 2012). For instance, inadequate maternal care has been linked to developmental, emotional and social deficits in humans (Field 1998). In rodents, the maternal separation (MS) model is a well-described paradigm used to investigate the biological and behavioural consequences of early life stress (O'Mahony *et al.* 2011, Nishi *et al.* 2014, Rincel *et al.* 2019). For this reason, the MS model has been used for various psychiatric conditions, especially depression (Vetulani 2013). For example, evidence supports that MS in rats is capable of inducing a depressive phenotype comparable to adult humans including hyperactivity of the HPA axis (Meaney *et al.* 1996) and increasing pro-inflammatory cytokines in plasma (Wieck *et al.* 2013).

The microbiota-gut-brain axis describes the complex bidirectional communication system that exists between the central nervous system (CNS) and enteric microbiota; involving endocrine, immune and neural pathways (Rhee *et al.* 2009, Foster *et al.* 2017, Cryan *et al.* 2019). Accumulating research has focused on the impact of the

microbiota on CNS function and stress perception, and its consequences for behaviour (Cryan *et al.* 2012). Indeed, top down activation of the CNS can influence gut neuromotor and secretory function, immunity and microbiota composition during stress (De Palma *et al.* 2014). In this regard, early-life stress models such as MS have long-term impact on the gut microbiota, which correlate with increased HPA axis activity and behaviour (Bailey *et al.* 1999, O'Mahony *et al.* 2009). Moreover, the MS model is sensitive to reversal treatments that target the gut microbiota (Gareau *et al.* 2007, Fukui *et al.* 2018, Cowan *et al.* 2019, McVey Neufeld *et al.* 2019, O'Mahony *et al.* 2019).

The emerging and compelling evidence for nutrition as a crucial factor in the high prevalence and incidence of mental disorders suggests that changes in diet are a viable strategy for improving mental health and treatment of psychiatric disorders including anxiety and depression (Jacka *et al.* 2014, Lai *et al.* 2014, Adan *et al.* 2019). For instance, dietary polyphenols are a group of naturally occurring phytochemicals which are present in high amounts in fruits and vegetables and are characterised by the presence of multiple hydroxyl groups on aromatic rings (Vauzour 2012). Several studies have focused on the potential of polyphenolic compounds in protecting cognitive function and reducing risk for developing neurodegenerative disorders (Spencer 2008). In particular, some pre-clinical studies have confirmed the antidepressant capacity of polyphenols in different animal models (Anjaneyulu *et al.* 2003, Kulkarni *et al.* 2008, Yi *et al.* 2008). Moreover, dietary polyphenols are capable of modulating the composition of the gut microbial community by inhibiting or stimulating the growth of certain bacteria (Lee *et al.* 2006). Hence, there is increasing interest in using polyphenols to target the microbiota-gut-brain axis to treat mental disorders (Filosa *et al.* 2018, Matarazzo *et al.* 2018).

Polyphenolic compounds are characterised as having different functional activity depending on their chemical structure (Manach *et al.* 2004, Vauzour *et al.* 2010). For instance, phlorotannins are a type of polyphenolic tannin found in marine brown algae, and have been shown to possess anti-oxidant activity, as well as beneficial effects for different diseases such as cancer, cardiovascular problems and diabetes (Kim *et al.* 2011). Other polyphenols can only be isolated from specific sources. Xanthohumol,

for example, is described as a prenylated chalcone, a principal component from the female hop plant, *Humulus lupulus* (Stevens *et al.* 2004). Some health benefits associated with xanthohumol intake include anti-inflammatory and neuroprotective effects (Liu *et al.* 2015). In contrast, some members of the flavonoid family like quercetin are widely distributed in nature (Manach *et al.* 2004). Quercetin is one of the most studied polyphenols and has been demonstrated to confer protection against certain types of cancer, cardiovascular and neurodegenerative disorders (Boots *et al.* 2008).

Recently, we showed that across a wide number of polyphenols, xanthohumol and quercetin were able to reverse the impact of corticosterone exposure in primary cortical neurons (Donoso *et al.* 2019). Moreover, although the antidepressant effects of several polyphenols have been studied in different preclinical studies (Xu *et al.* 2005, Bhutada *et al.* 2010, Liu *et al.* 2014), their therapeutic effects have not yet been examined in models of early life stress yet, nor the mechanisms underlying the polyphenol-mediated alleviation of mood. Therefore, the purpose of this study was to explore the antidepressant effects of different naturally-derived polyphenols, including phlorotannins, xanthohumol and quercetin in the MS model in rats. In addition, the consequences of MS and polyphenol diet intervention on plasma BDNF levels and monoamine neurotransmitter concentration in brainstem, and the potential implication for the HPA axis and the gut microbiome, were explored.

3.3 Methods

3.3.1 Animals

All experimental procedures involving animals were approved by the Ethics Committee of University College Cork. Pregnant Sprague Dawley dams weighing 250–300 g were bred in-house in the Biological Services Unit facility, University College Cork. The pups were housed with their mothers in plastic cages (15 × 22 × 9 cm) in a temperature and humidity controlled room on a 12-h light, 12-h dark cycle (lights on from 7.00–19.00 h). Food and water were available ad libitum.

3.3.2 Drugs

Quercetin (Q4951) was purchased from Sigma. Xanthohumol (A-4-2014) was provided by Hopsteiner, GmbH (Mainburg, Germany). Phlorotannin-rich extract from *Fucus vesiculosus* (Gite *et al.* 2019) was obtained from National University of Ireland, Galway (Galway, Ireland). All diets were prepared by ssniff Spezialdiäten (Ferdinand-Gabriel-Weg, Germany). The resulting chows were isoenergetic and had the same proportion of macronutrients (carbohydrates, proteins and lipids).

3.3.3 Maternal separation procedure

MS was performed as previously described (O'Mahony *et al.* 2009, Pusceddu *et al.* 2015). Briefly, pups were separated from their mother as a whole litter and placed into plastic cages maintained at 30 – 33 °C in a separate room to prevent communication through ultrasonic vocalisation (Hofer *et al.* 1994). Following the 3-hour separation, pups were returned to their original home cage with their mother. This procedure was repeated each day (9.00am–12.00pm) from post-natal day (PND) 2 through PND12. NS-Control rats consisted of non-handled pups, left untouched by the experimenter, and with their respective mothers. After postnatal day 12, pups were left undisturbed except for routine cage cleaning every two days. At weaning, male rats were group-housed (2 – 4) in large cages.

3.3.4 Treatments

The rats were randomly assigned into nine different experimental groups, of which five were relevant to this study. Dose of polyphenols are expressed as a percentage in food (% m/m). [1] NS-Control diet (n = 12); [2] MS-Control diet (n = 12); [3] MS-Phlorotannins 0.03% (n = 10); [4] MS-Xanthohumol 0.015% (n = 10); [5] MS-Quercetin 0.03% (n = 10). Dietary intervention of polyphenols, delivered ad libitum in food, began once the animals were eight weeks old and continued for eight weeks. The concentrations for the polyphenols tested were calculated based on doses previously reported in animal models and considered the average daily food intake and body weight of Sprague Dawley rats aged between 9 and 16 weeks (Laaksonen *et al.* 2013). Estimated doses are as follows; quercetin 20 mg/kg/day (Haleagrahara *et al.* 2009); xanthohumol 10 mg/kg/day (Ceremuga *et al.* 2013); phlorotannins 20 mg/kg/day (Ahn *et al.* 2017). In the interest of reduction in the 3 Rs the control groups used here were also used to assess the impact of a number of other interventions that were run at the same time (Egerton *et al*. unpublished).

3.3.5 Elevated plus maze

The elevated plus maze (EPM) is one of the most commonly used rodent tests for assessing anxiety and was performed as previously described (Cryan *et al.* 2004, Pusceddu *et al.* 2015). Briefly, the maze consisted of two open arms (51 × 10 cm; 5 lux) and two enclosed arms (51 × 10 × 41 cm) that all extended from a common central platform (10 × 10 cm). The apparatus was elevated 55 cm above the floor on a central pedestal. Animals were habituated to the testing room for 30 min prior experiment. At week 12, animals were placed in the centre of the maze facing an open arm to begin. Animal behaviour was recorded for 5 min. Frequency of open and closed arms entries were scored, as well as percentage time spent in each arm.

3.3.6 Open field test

The open field test (OFT) is commonly used as a mechanism to assess anxiolytic effects of compounds (Seibenhener *et al.* 2015). Briefly, at week 13 rats were placed in the centre of a white open field arena (60 × 40 cm; 60 lux) and observed for 10 min.

Animals were habituated to the test room for 30 min prior to the experiment. All trials were conducted between 9.00am and 1.00pm. The arena was cleaned with 70% ethanol to avoid smell cues between each trial. At the end of each trial, animals were returned to their cages. Distance moved, velocity, percentage of time spent in inner zone, and frequency of inner zone entries were analysed and recorded using a tracking system (Ethovision XT 13, Noldus).

3.3.7 Forced swim test

The forced swim test (FST) is the most widely used model for predicting antidepressant activity in rodents, and increased immobility in this test is generally considered to reflect a state of behavioural despair (Porsolt *et al.* 1978). Briefly, at week 15 a modified rat FST protocol (Slattery *et al.* 2012) was used to determine the antidepressant effects of polyphenols in rats. On day one, rats were placed individually in glass cylinders (H: 45 cm; D: 20 cm) filled with water to a depth of 30 cm at 24±1 °C for a 15 min pre-test period. The rats were removed from the water, dried and placed in their home cage. The cylinders of water were changed between each trial. 24 hours after the pre-test, the rats were again placed in the swim apparatus for 5 min and behaviours were monitored from above with a video camera for subsequent analysis. Behaviours rated include immobility, climbing and swimming (scoring of behaviours was blind to the experimental conditions). The 5-min session was scored using a time-sampling technique, whereby the predominant behaviour in each 5-s period of the 300-s trial was recorded. Climbing behaviour consisted of upward-directed movements of the forepaws along the side of the cylinder. Swimming behaviour was defined as movement (usually horizontal) throughout the cylinder. The rat was considered to be immobile when the only activity observed was that which was required by the rat to keep its nose above water.

3.3.8 Plasma corticosterone determination

Blood sample collection was performed as previously described (Pusceddu *et al.* 2015). Briefly, blood samples were collected on day one of FST via a tail-tip incision at five different time points: immediately before (baseline), 30 min, 60 min, 90 min and 120 min after the test was started. Approximately 200 µl of blood was collected

in a tube containing 10 µL of EDTA 0.1 M to avoid coagulation. Blood plasma was obtained by centrifugation at 3500 × *g* at 4 °C for 15 min. Corticosterone levels were measured using the Corticosterone EIA kit (Enzo) according to the manufacturer instructions, and absorbance signal was detected with a conventional plate reader (Synergy HT, Biotek).

3.3.9 Plasma BDNF measurement

Immediately after sacrifice, trunk blood was collected in EDTA Vacutainer tubes. Blood plasma was obtained by centrifugation at 3500 × *g* at 4 °C for 15 min. Protein levels of brain-derived neurotrophic factor (BDNF) were determined using an electrochemiluminescence multiplex system (MSD, Gaithersburg, MD, USA) according to the manufacturer's protocol. BDNF levels were determined and analysed using the MSD QuickPlex SQ 120 Instrument.

3.3.10 Brain monoamines concentration

The monoamine neurotransmitters noradrenaline (NA), serotonin (5-HT), dopamine (DA) and their metabolites 5-HIAA and 3,4-dihydroxyphenylacetic acid (DOPAC) were determined in the brainstem using high-performance liquid chromatography (HPLC) with electrochemical detection as described previously (Clarke *et al.* 2012, Pusceddu *et al.* 2015). Briefly, samples were homogenised in mobile phase (consisting in 0.1 M citric acid, 0.1 M sodium dihydrogen phosphate monohydrate, 5.6 mM 1-octanesulfonic acid, 0.01 mM EDTA, 11.1% (v/v) methanol, and 0.1 µg/mL of N-Methyl 5-HT as internal standard and adjusted to pH 2.8). Then samples were centrifuged 14000 *g* for 15 min at 4 °C, and 20 µL of this supernatant was injected onto the HPLC system (consisting in a CBM-20A system controller, a EC3000 Recipe amperometric detector, a LC-20AD XR pump, a CTO-20A column oven at 30 ^{0}C, a SIL-20AC XR autosampler, and a Prominence DGU-20A3 degasser). A reverse-phase column (Kinetex 2.6u C18 100A 100 mm X 4.6 mm, Phenomenex) was employed in the separation using a flow rate of 0.9 mL/min. Each neurotransmitter was identified through their characteristic retention times and their concentration was determined using the ratios of peak heights of analyte versus internal standard provided by the

LabSolutions software (Shimadzu). Results were expressed as nanograms of neurotransmitter per grams of fresh tissue.

3.3.11 Gut microbiota 16S rRNA sequencing

Microbial DNA was isolated from frozen faecal samples using the QIAGEN QIAamp Fast DNA Stool Mini Kit (Qiagen) according to the manufacturer's directions. DNA concentration and quality was determined using the NanoDrop® ND-1000 UV-Vis Spectrophotometer (Thermo Fisher Scientific). The V3-V4 variable region of the 16S rRNA gene was amplified from the DNA extracts using the Illumina 16S metagenomic sequencing library protocol, and PCR reactions were performed with the KAPA HiFi HotStart PCR Kit (KAPA Biosystems). PCR products were cleaned using AMPure XP magnetic bead-based purification (Beckman Coulter Life Sciences). This was followed by indexing PCR which attached Nextera XT barcodes and Illumina sequencing adapters to the 5′ overhangs and another round of AMPure XP clean-up. Finally, samples were sequenced on the MiSeq™ System (Illumina®), using a 2 x 250bp cycle kit, following standard Illumina sequencing protocols.

3.3.12 Short chain fatty acid determination

The short chain fatty acids (SCFAs) acetate, propionate, butyrate, and valerate, as well as the total branched chain fatty acids (BCFAs) were measured in caecal content using gas chromatography flame ionisation detection (GC-FID) as previously reported (van de Wouw *et al.* 2018). Briefly, samples were vortexed with Milli-Q water (1:10 w/v), left to stand for 10 min at room temperature, and then centrifuged at 14000 *g* for 5 min. The supernatant was filtered (0.2 µm) before transfer to a GC glass vial, and 2-ethylbutyric acid (Sigma) was added as internal standard. SCFA concentrations were measured using a Varian CP-3800 GC flame-ionization system, fitted with a Zebron ZB-FFAP column (30 m × 0.32 mm × 0.25 µm; Phenomenex) and a flame ionisation detector with a CP-8400 auto-sampler. Helium was used as the carrier gas at a flow rate of 1.3 ml/min. The initial oven temperature was set at 100 °C for 0.5 min, raised to 180 °C at 8 °C/min and held for 1 min, then increased to 200 °C at 20 °C/min, and finally held at 200 °C for 5 min. The temperature of the injector and the detector were set at 240 °C and 250 °C respectively. A standard curve made from a standard mix of

acetic acid, propionic acid, n-butyric acid and iso-butyric acid (Sigma) at seven concentrations. Peaks were integrated by using the Varian Star Chromatography Workstation version 6.0 software. Standards were included in each run to maintain calibration.

3.3.13 Statistical analysis

Statistical analysis was performed using the software SPSS 24.0, and the results were presented as mean ± SEM. MS-control group and NS-control group were compared using independent T-test to assess the MS effect. All MS groups were analysed using one-way analysis of variance (ANOVA) followed by Dunnett's post hoc test, where NS-control group was considered as control for the test. A *p*-value of 0.05 was considered statistically significant. FLASH was used to assemble paired-end reads. Further processing of paired-end reads including quality filtering based on a quality score of >25 and removal of mismatched barcodes and sequences below length thresholds was completed using QIIME (version 1.9.0). Denoizing, chimera detection and clustering into operational taxonomic unit (OTU) grouping were performed using USEARCH v7 (64-bit). OTUs were aligned using PyNAST, and taxonomy was assigned using BLAST against the SILVA SSURef database release 123. Statistical microbiome analysis was carried out in R (version 3.6.1) with Rstudio (version 1.2.1335). OTUs unknown on a genus level were excluded, as well as OTUs available in two or fewer samples. The ALDEx2 library (Fernandes *et al.* 2014) was used to compute the centred log-ratio transformed values of the remaining taxa. For principal component analysis, a pairwise implementation of the adonis() PERMANOVA function in the vegan library (Oksanen *et al.* 2017) followed by the Bonferroni-Holm correction was used to test for difference in β-diversity in terms of Aitchison distance. Differential abundance was assessed using a pairwise implementation of the aldex.test() function, followed by Benjamini-Hochberg correction. In these cases, a *q*-value < 0.1 was considered significant. α-diversity was computed using the iNEXT library (Hsieh *et al.* 2016).

3.3.14 Functional prediction of Gut-Brain modules

The Piphillin webservice (Iwai *et al.* 2016) was used to infer the functional metagenome per sample in terms of KEGG orthologues. Next, these KEGG orthologues were processed using the omixer library in R (Darzi *et al.* 2016) in order to calculate abundance of gut-brain modules (GBMs) (Valles-Colomer *et al.* 2019) and gut-metabolic modules (GMMs) in these samples. Then, the same implementation from ALDEx2 was used to assess differential abundance. Scripts are publicly available on GitHub: https://github.com/thomazbastiaanssen/Tjazi doi: 10.5281/zenodo.1480804.

3.4 Results

3.4.1 Quercetin and phlorotannins reversed MS-induced depressive-like behaviours

To investigate the therapeutic effects of the dietary interventions with polyphenols from MS-induced behavioural despair, animals were subjected to a battery of behavioural tests to examine depressive- and anxiety-like behaviours. Firstly, animals did not differ in terms of body weight across the different experimental groups throughout the duration of the treatment (Fig. 1B and C). In FST, analysis yielded a significant effect of MS compared to NS-control group on the time spent immobile (t_{22} = -2.349; p = .028) and swimming (t_{22} = 2.611; p = .016) (Fig. 2A). MS animals exhibited improved depressive-like behaviours with xanthohumol; moreover, quercetin (p = .05) and phlorotannins (p = .002) significantly decreased immobility time in the FST ($F_{3,36}$ = 4.425; main effect p = .010). In addition, treatment with phlorotannins increased (p = .008) swimming time compared to the MS-control group ($F_{3,36}$ = 2.984; main effect p = .044) (Fig. 2A).

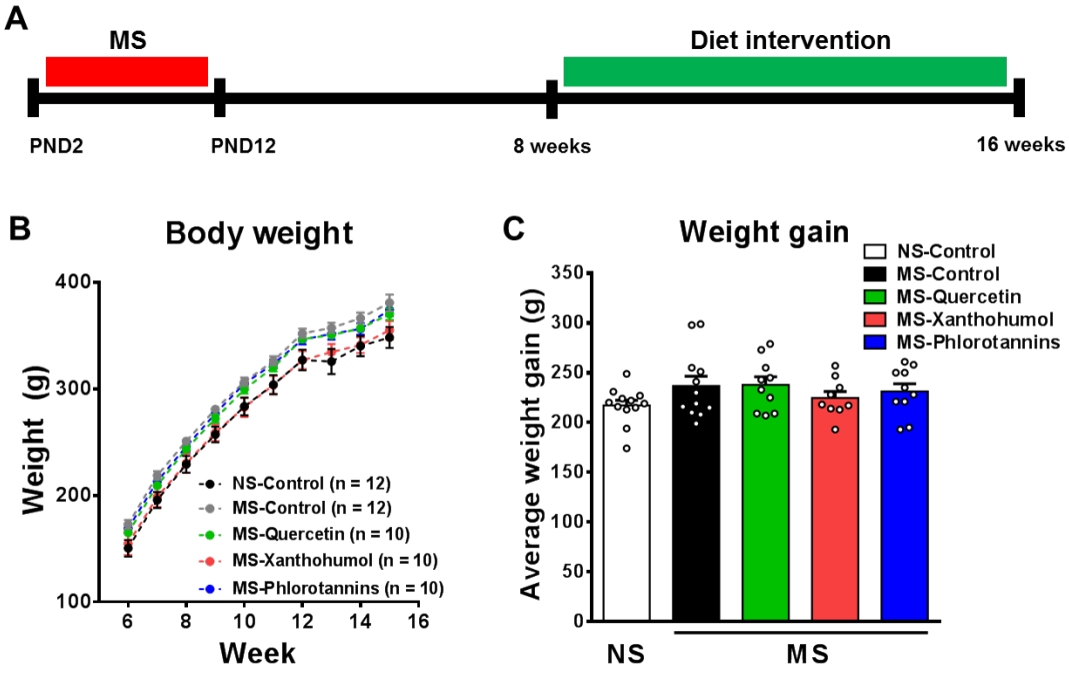

Figure 1 Diet intervention with polyphenols did not affect body weight. ***(A)*** *Schematic representing the experimental timeline.* ***(B)*** *Body weight was measured weekly from 6-week old until the end of the*

diet intervention. ***(C)*** *The weight gain was calculated as the difference between the first body weight record (6 weeks) and the last measurement (15 weeks).*

3.4.2 Polyphenols exerted potential anxiolytic effects in MS animals

MS-induced anxiety-like behaviour in the OFT by significantly reducing the time spent in the centre (t_{21} = 2.156; p = .025), as well as in the number of entries in the centre of the arena (t_{21} = 2.855; p = .009) (Fig.2D and E). Administration of quercetin in MS rats resulted in an apparent increase in the number of entries in the centre of the arena compared to the MS-control group ($F_{3,37}$ = 2.405; main effect p = .083), which was associated with a potential anxiolytic effect (Fig. 2E). Interestingly, treatment with phlorotannins seems to ameliorate MS-induced anxiogenic effects in both, time in centre ($F_{3,37}$ = 2.297; main effect p = .094) and in entries into the centre. However, no differences were found between NS-control and MS animals during the EPM (Fig. 2F and G).

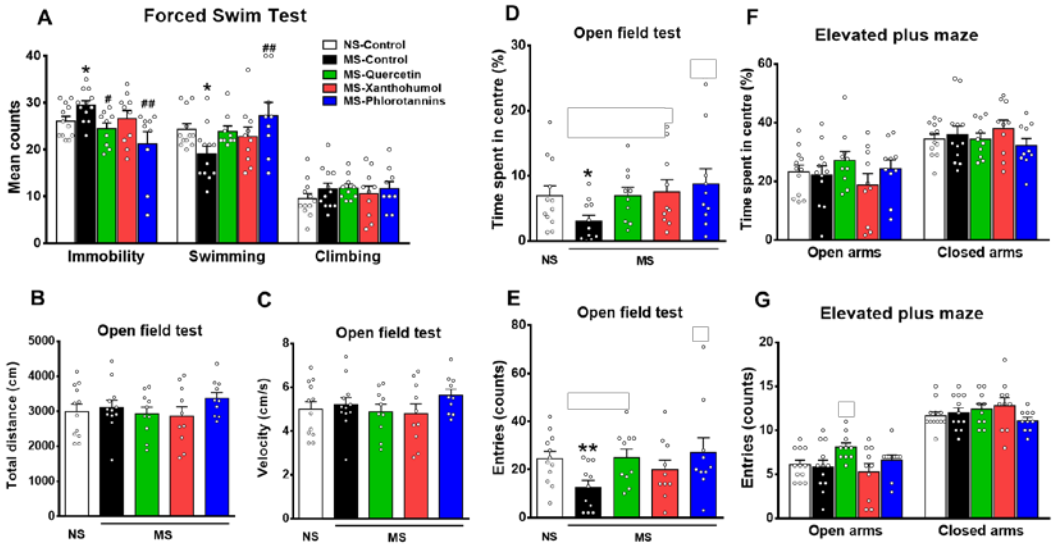

Figure 2 Treatment with polyphenols induced antidepressant- and potential anxiolytic-like effects in MS rats. ***(A)*** *MS-induced increased immobility in the FST is prevented through treatment with quercetin and phlorotannins, while reduced swimming time caused by MS is reversed only by phlorotannins treatment.* ***(B – C)*** *Polyphenolic diets nor MS produced changes in locomotor activity.* ***(D – E)*** *Phlorotannins treatment produced a significant increase in the time spent in centre and number of centre entries when is compared to MS-control group in the OFT.* ***(F – G)*** *However, MS animals did not show anxiety-like behaviours in the EPM. Results are expressed as the mean ± SEM (*p < 0.05; **p < 0.01 versus 'vehicle' groups; #p < 0.05; ##p < 0.01 versus 'CORT' groups).*

3.4.3 Xanthohumol prevented exacerbated corticosterone production in MS rats after acute stress

To determine the role of the HPA axis in MS-induced depressive- and anxiety-like behaviours, the concentration of corticosterone in plasma was measured at different time points after an acute stress (Fig. 3A). Indeed, the corticosterone production was close to being statistically increased in MS-control relative to the NS-control group as revealed by the area under the curve (AUC) of corticosterone response (t_{20} = -1.949; p = .065) (Fig. 3B). Interestingly, dietary intervention with xanthohumol in MS animals induced a significant reduction (p = .010) in corticosterone AUC compared to the MS-control group ($F_{3,34}$ = 3.827; main effect p = .018) (Fig. 3B). In addition, all polyphenolic treatments induced lower baseline levels of plasma corticosterone compared to MS-control group ($F_{3,36}$ = 3.979; xanthohumol p = .006; phlorotannins p = .011), while quercetin showed a trend towards significance (p = .080).

3.4.4 MS produced significant reduction in plasma BDNF

BDNF is a critical modulator of neuroplasticity and survival, abundant in the brain and periphery, including serum and plasma (Lee *et al.* 2010). Preclinical and clinical studies have demonstrated that chronic stress and depressive status reduces BDNF expression (Russo-Neustadt *et al.* 2001, Gonul *et al.* 2005). Indeed, MS rats showed lower levels of plasma BDNF compared to NS animals (t_{19} = 2.672; p = .015). Although not significant, this effect was partially ameliorated by xanthohumol treatment ($F_{3,36}$ = 1.748; main effect p = .175) (Fig. 3D).

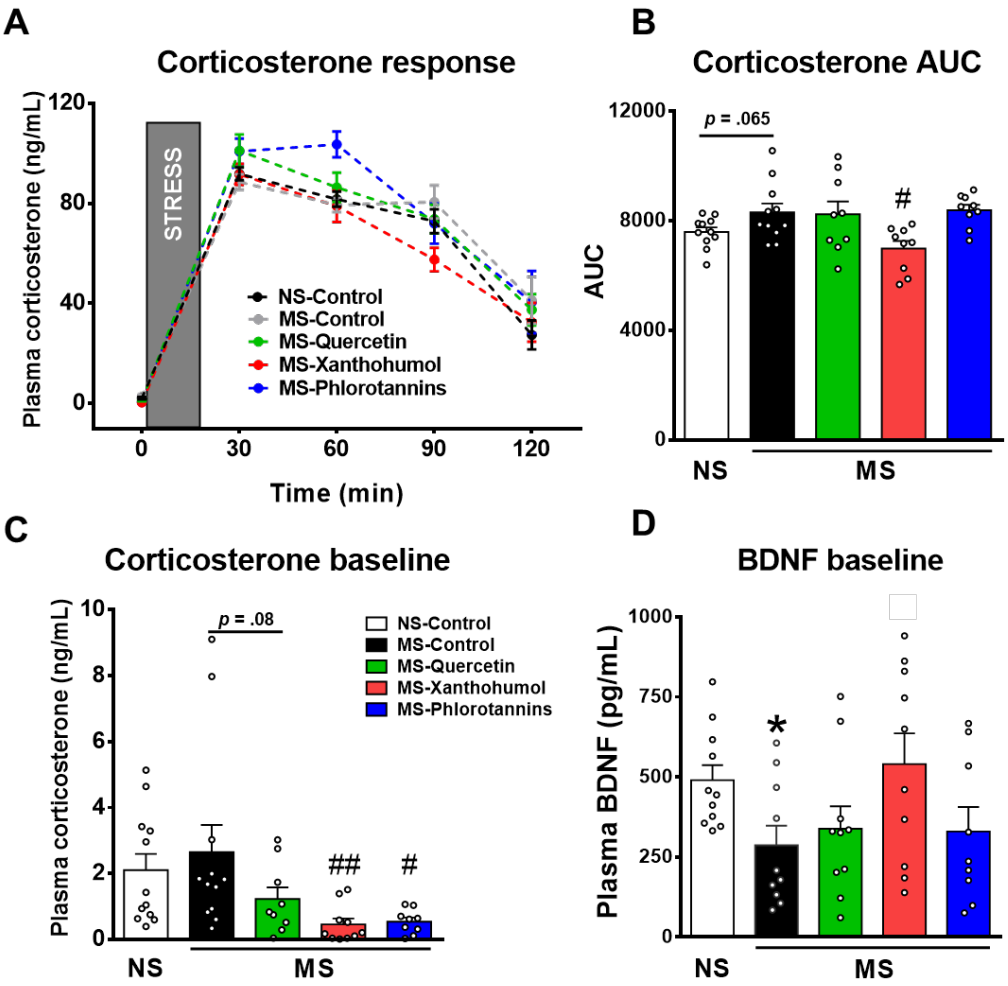

Figure 3 Xanthohumol treatment prevented corticosterone elevation and BDNF reduction in MS rats. (A) *Corticosterone levels in plasma rise after rats are exposed to an acute stress.* ***(B)*** *MS-induced increase in corticosterone release is abolished by treatment with xanthohumol.* ***(C)*** *Baseline levels of corticosterone of rats treated with xanthohumol and phlorotannins are significantly lower compared to MS animals.* ***(D)*** *Rats treated with xanthohumol displayed higher levels of plasma BDNF compared to the MS-control group. Plasma corticosterone was determined using ELISA, and BDNF determination was performed with MSD system. Results are expressed as the mean ± SEM (*p < 0.05 versus 'vehicle' groups; #p < 0.05; ##p < 0.01 versus 'CORT' groups).*

3.4.5 MS induced decreased levels of DA and 5-HIAA in brainstem

To further determine the effects of early life stress on neurochemistry, and its potential implication on the antidepressant and anxiolytic effects of polyphenols, monoamine neurotransmitter concentration was measured in the brainstem. MS produced a significant reduction of DA and 5-HIAA levels ($t_{20} = 6.121$; $p = .000$ and $t_{22} = 3.934$; $p = .001$ respectively) (Fig. 4B and D), reduced 5-HT turnover ($t_{21} = 3.519$; $p = .002$)

(Fig. 4C), and increased DA turnover (t_{22} = -2.153; p = .047) (Fig. 4F). In contrast, treatment with phlorotannins showed a slight increase in the levels of NA (p = 0.177), and 5-HT (p = .066) compared to the MS-control group, although not significant effects were found (Fig. 4A and G).

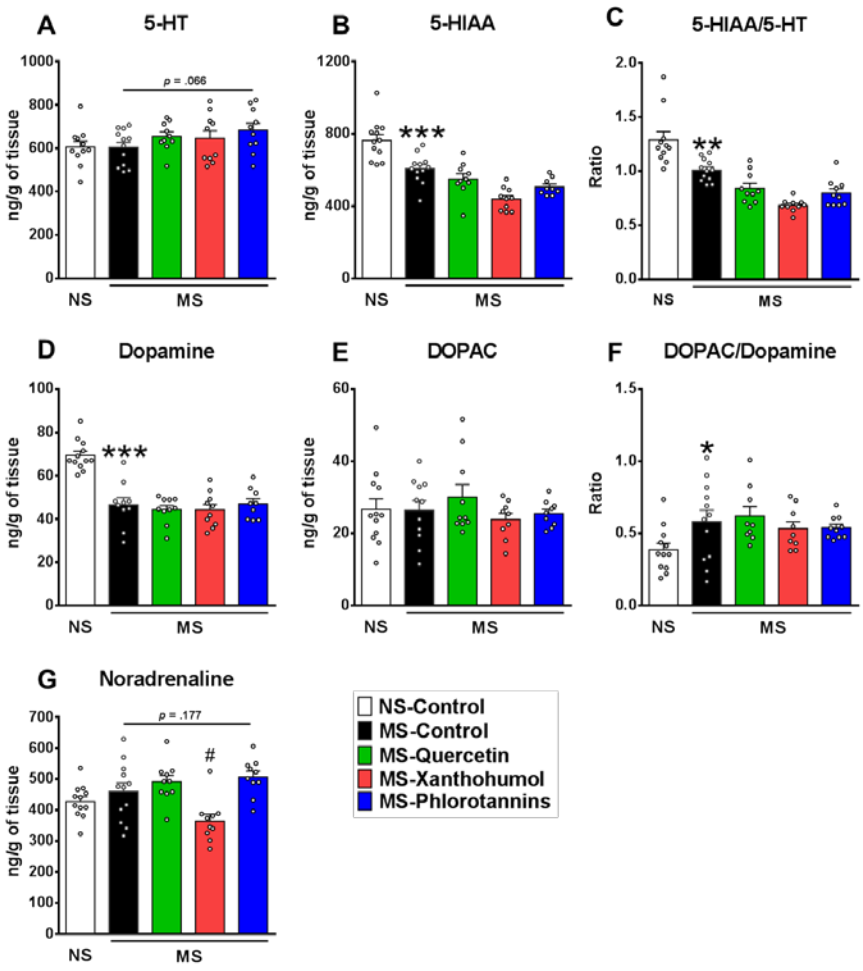

Figure 4 Polyphenols did not prevent MS-induced reductions in brainstem dopamine and 5-HIAA. Monoamine neurotransmitters were measured in the brainstem via HPLC*. MS animals show depleted concentrations of dopamine and 5-HIAA compared to NS-control rats. Phlorotannins diet intervention exerted an increase of noradrenaline and 5-HT MS animals, although not significant. Results are expressed as the mean ± SEM (*p < 0.05; **p < 0.01; ***p < 0.001 versus 'vehicle' groups; #p < 0.05 versus 'CORT' groups).*

3.4.6 MS and dietary treatments induced changes in gut bacterial diversity

To define whether the experimental treatments also altered gut microbiota diversity and bacterial abundance, α- and β-diversity analyses were performed. Although no differences in richness were found using the Chao1 α-diversity metric (Fig. 5A), Shannon entropy and Simpson index both indicate that MS rats treated with phlorotannins showed reduced diversity within this group compared to the MS-control experimental group (p = .072 and p < .05, respectively) (Fig. 5B and C). In other words, while the total estimated amount of OTUs did not differ, the microbial ecosystem of animals treated with phlorotannins were distributed less evenly. On the other hand, principal component analysis (PCA) to measure the diversity among groups, indicated that MS and NS control groups were significantly different from each other ($F_{4,47}$ = 2.012; p = .046) (Fig. 5D). In addition, treatments with quercetin, xanthohumol and phlorotannins also produced significant changes in β-diversity in terms of Aitchison distance compared to MS-control group ($F_{4,47}$ = 2.012; p = .004; p = .045; p = .046, respectively) (Fig. 5D).

3.4.7 Changes in the gut microbiota composition correlated with MS status and polyphenolic diets

Alteration of the gut microbiota composition has been associated with different mental disorders, including major depression and other stress-related psychiatric disorders (Cryan *et al.* 2012). Thus, we examined the differences in the gut microbiota composition of maternally separated rats. Significant differences in terms of the relative abundance between MS-control and NS-control animals were found in 5 specific bacteria based on effect size (*Streptococcus; Ruminococcus; Parabacteroides; Rothia; Christensenellaceae*; q < .1) (Fig. 5E). On the other hand, dietary interventions with quercetin and xanthohumol in MS rats induced significant changes in the abundance of other bacteria genera when compared to the MS-control group. Specifically, quercetin produced a significant increase of *Enterorhabdus* (q < .1), while xanthohumol exerted changes in the abundance of *Asteroplasma, Lachnospiraceae*, and *Coprococcus* (q < .1) (Fig. 5E).

3.4.8 Treatment with phlorotannins and xanthohumol restore MS-induced changes in bacteria associated with microbiota-gut-brain pathways

To investigate the implications of MS-induced changes in gut microbiota composition on metabolic pathways associated with the microbiota-gut-brain axis, we performed a functional prediction based in previously described GBMs (Valles-Colomer *et al.* 2019). MS significantly changed the abundance of bacteria linked to 8 GBMs in terms of effect size, including tryptophan degradation, quinolinic acid metabolism, nitric oxide metabolism, and p-cresol synthesis compared to NS-control group ($q < .1$) (Fig. 5F). Intriguingly, although quercetin did not alter any relevant bacteria, xanthohumol and phlorotannins treatment restored most of the changes produced by MS in these bacteria.

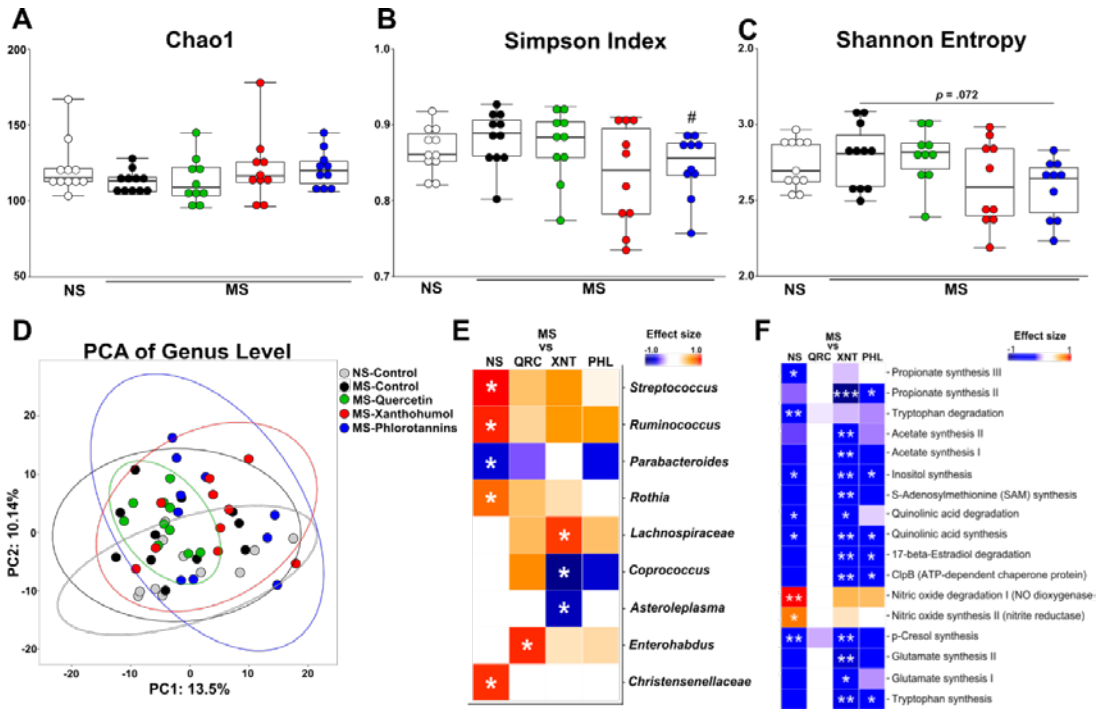

Figure 5 MS and polyphenolic diets induced significant changes in gut microbiota composition and diversity. (A – C) *Chao1, Simpson index and Shannon entropy were used as estimators of bacterial α-diversity.* ***(D)*** *Principal component analysis of genus level was performed to estimate the β-diversity between experimental groups.* ***(E)*** *Bacterial abundances were significantly altered in MS rats in terms of effect size ($q < 0.1$). In contrast, xanthohumol and quercetin changed other bacteria compared to MS animals.* ***(F)*** *Functional prediction of GBMs was utilised to detect potential microbiota-gut-brain pathways affected by MS or dietary treatments. Colours represent effect size, only microbiome features found to be significantly different in at least one comparison are shown (*$q < .1$; **$q < .05$; ***$q < .01$ vs MS-control group).*

3.4.8 Xanthohumol prevented MS-induced reduction of intestinal SCFAs

To determine whether the observed changes in the gastrointestinal microbiota composition and diversity correlate with alteration in SCFA production, the levels of acetate, propionate, butyrate, valerate were determined in caecal content. Interestingly, maternal separation induced a significant reduction of acetate (t_{22} = 2.409; p = .025), propionate (t_{22} = 2.988; p = .01), isobutyrate (t_{21} = 3.354; p = .006), isovalerate (t_{21} = 2.779; p = .016), total SCFAs (t_{21} = 2.228; p = .037), and total BCFAs (t_{21} = 3.181; p = .008). In contrast, phlorotannin treatment significantly reversed the MS-induced propionate reduction ($F_{3,38}$ = 4.646; p = .022), and exerted positive effects on isobutyrate, valerate and total levels of BCFAs.

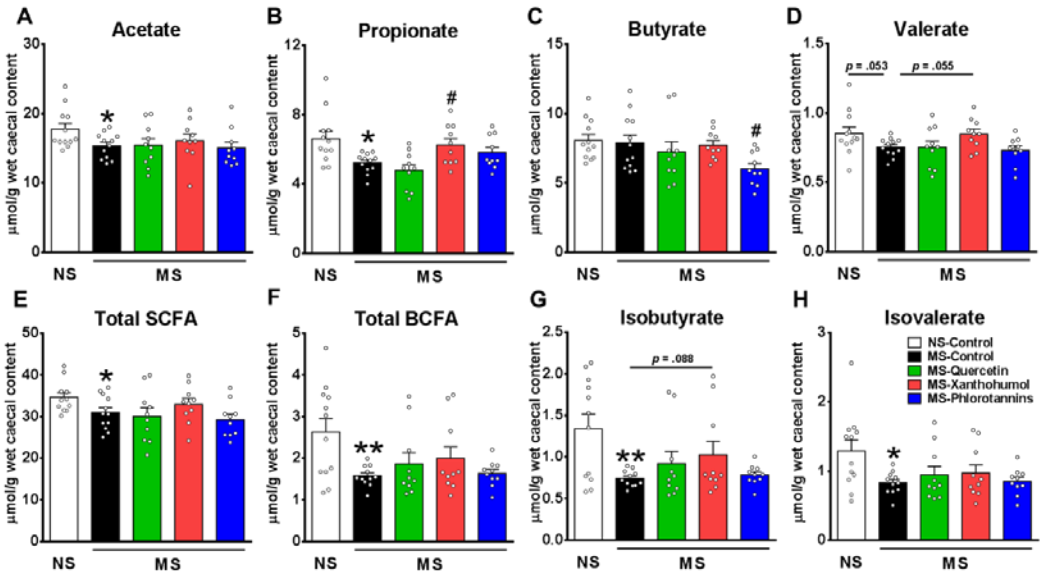

***Figure 6 MS rats exhibited lower levels of gut microbiota-derived metabolites (A – H)**. MS induced significant reduction of gut microbiota-derived metabolites including acetate, propionate, isobutyrate and isovalerate, as well as decreased total short-chain fatty acids (SCFA) and branched-chain fatty acids (BCFA). Xanthohumol treatment ameliorated MS-induced propionate decrease and tends to improve isobutyrate and valerate levels. Fatty acid determination was performed through HPLC in caecal content. Results are expressed as the mean ± SEM (*p < 0.05; **p < 0.01 versus 'vehicle' groups; #p < 0.05 versus 'CORT' groups).*

3.5 Discussion

There has been increasing attention given to the potential of nutritional approaches to ameliorate the effects of stress (Lakhan *et al.* 2008, Marzola *et al.* 2013, Rechenberg *et al.* 2013). In the present study, we tested different naturally-derived polyphenols as potential therapeutic strategies for depression and anxiety associated with early life trauma. Indeed, the polyphenols quercetin, xanthohumol and phlorotannins exert varying degrees of antidepressant and anxiolytic -like responses in rats subjected to MS. Moreover, dietary interventions also modified gut microbial composition and diversity, suggesting that their therapeutic effects could be associated with the microbiome-gut-brain axis.

The MS rat is an excellent model to study the negative effects of early life stress on brain function and structure, which are associated with the development of depression and anxiety (O'Mahony *et al.* 2011, Vetulani 2013). MS in rats induce a robust depressive-like phenotype in adult animals, including changes in gut microbiota, dysregulation of the HPA axis, and an imbalance in neurotransmitter levels (Daniels *et al.* 2004, O'Mahony *et al.* 2009, Liao *et al.* 2019). Furthermore, we demonstrated that all polyphenolic treatments tend to reverse these depressive-like behaviours. In particular, the phlorotannin-enriched diet produced a significant improvement in immobility and swimming behaviour in the FST compared to the MS-control group.

Regarding anxiety, quercetin administration exerted a potential anxiolytic effect in MS animals, resulting in an increase in the number of entries into the open arms of the EPM. Similarly, quercetin- and xanthohumol-enriched diets tend to induce anxiolytic effects in the OFT, while phlorotannin treatment revealed also a potential improvement. Although the concept of a potential therapeutic effect of polyphenols in animal models of stress is not completely new (Anjaneyulu *et al.* 2003, Hurley *et al.* 2014, Aubry *et al.* 2019).

We further investigated the role of the HPA axis in the therapeutic effects of polyphenol administration. Accumulated lines of evidence indicate that depressive or chronically stressed patients have an over activated HPA axis (Pariante *et al.* 2008, Keller *et al.* 2017). Similarly, animals subjected to chronic stress possess a

dysregulated HPA axis and increased baseline levels of glucocorticoids (O'Mahony *et al.* 2011, Uschold-Schmidt *et al.* 2012). Indeed, we demonstrated that the dietary intervention with xanthohumol significantly reduced the exacerbated production of corticosterone in MS animals.

Next, we observed that treatment with xanthohumol shows therapeutic potential in preventing the MS-induced reduction in plasma BDNF, although this effect was found to be not statistically significant. BDNF has strongly been implicated in antidepressant activity and plasma BDNF has been shown to reflect aspects of that centrally and to be a biomarker of antidepressant activity (Sen *et al.* 2008, Lee *et al.* 2010). Although the possible pathways involved in BDNF rescue must be further investigated, it is tempting to speculate that the positive effects of xanthohumol on behaviour could be partly mediated by normalising BDNF expression.

The relationship between stress and the gut microbiota is gaining a lot of attention (Foster *et al.* 2017). Additionally, we have demonstrated that MS is able to induce strong changes to the gut microbiota in terms of composition and diversity which is in line with previous reports (O'Mahony *et al.* 2009, Moussaoui *et al.* 2017). Although, we did not detect changes at the α-diversity level, analysis of β-diversity revealed that MS groups treated with polyphenols differ from the MS control group. We followed this up by assessing differential abundance of bacterial genera between the treatment groups. Notably, only xanthohumol and quercetin treatments produced significant changes in some bacterial genera in MS rats, suggesting that some polyphenol-enriched diets have the potential to modify bacterial composition in the gastrointestinal system. Several studies have demonstrated the capacity of polyphenolic intake to shape the gut microbiota (Etxeberria *et al.* 2013, Ozdal *et al.* 2016). The fact that all types of polyphenol intake was found to alter β-diversity compared to MS control, but only xanthohumol and quercetin yielded differences in the abundances of specific genera may suggest the polyphenols induce a general shift in the microbial composition, which may be indicative of a change in functionality in the microbiome.

Therefore, we performed a functional prediction of the gut metagenome and used this to infer the abundance of GBMs, metabolic modules that are involved in the

microbiota-gut-brain axis (Valles-Colomer *et al.* 2019). Indeed, the analysis predicted that MS is able to increase the abundance of GBMs associated with the modulation of several pathways altered in depression and other neuropsychiatric disorders, including metabolism of tryptophan (Curzon *et al.* 1970, Oxenkrug 2010), inositol (Coupland *et al.* 2005), p-cresol (Persico *et al.* 2013), quinolinic acid (Steiner *et al.* 2011), nitric oxide (Dhir *et al.* 2011), and glutamate (Sanacora *et al.* 2012, Murrough *et al.* 2017). Interestingly, treatment with xanthohumol and phlorotannins reversed these predicted MS-induced changes, suggesting that restoration of these GBMs may partially explain their positive effects in behaviour. An important limitation due to the nature of 16S sequencing is that functional analysis can only be inferential. Future metabolomics-based studies should address this experimentally.

In addition, our data revealed that MS rats exhibited decreased production of SCFAs compared with the NS-control group. We detected a significant reduction of acetate, propionate, isobutyrate, and isovalerate. The production of SCFAs is highly associated to certain bacterial populations in the gut, and there is common agreement surrounding the impact of SCFAs on human metabolism and health (Morrison *et al.* 2016). Indeed, it is widely accepted that SCFAs play a critical role in gut-microbiota-brain communication, and consequences for mental health and behaviour (Stilling *et al.* 2016, Dalile *et al.* 2019). A preclinical study showed that a depression-associated microbiota makeup can impact SCFA production (Kelly *et al.* 2016), and that SCFAs can reverse the enduring effects of stress in a mouse model (van de Wouw *et al.* 2018). In our study, we demonstrated that treatment with xanthohumol specifically prevented the reduction of propionate in MS rats. Since the xanthohumol diet intervention induced acute changes in bacterial composition of the MS gut microbiota, we presume that the changes observed in propionate levels could be a product of improved microbial metabolism.

In conclusion, our present work confirmed that the naturally derived polyphenols xanthohumol, quercetin and phlorotannins display therapeutic potential in the alleviation of depressive- and anxiety-like behaviours in the rat MS model. We further found that treatment with xanthohumol prevented exacerbated production of corticosterone after acute stress in MS animals, and reversed MS-induced plasma

BDNF depletion. In addition, our data revealed that MS-induced behavioural despair correlated with significant changes in bacterial composition and diversity, alteration of predicted microbiota-gut-brain pathways, and reduced SCFA production. Although all polyphenols caused changes in diversity, only xanthohumol induced significant changes in several bacterial taxa and prevented the reduction of propionate in MS rats. Taken together, our findings present evidence of the therapeutic properties of polyphenols and provide a novel insight into the potential mechanisms underlying their antidepressant effect.

Chapter 4

Dietary Phospholipids Exert Neuromodulatory Effects on Neuronal Models

Francisco Donoso[a, b], Marina Schverer[a], Kieran Rea[a], Bernard L. Royd, Timothy G. Dinan[a, b], Harriët Schellekens[a, c] and John F. Cryan[a, c]

[a]APC Microbiome Ireland, University College Cork, Cork, Ireland
[b]Department of Psychiatry, University College Cork, Cork, Ireland
[c]Department of Anatomy & Neuroscience, University College Cork, Cork, Ireland.
[d]Cremo SA, Fribourg, Switzerland.

To be submitted to: *Nutritional Neuroscience*

Manuscript in preparation

4.1 Abstract

Nutrition is a crucial component for maintenance of brain function and mental health. Accumulating evidence suggests that certain molecular compounds derived from diet can exert neuroprotective effects against environmental factors and chronic stress, and moreover improve important neuronal processes, such as plasticity and hippocampal neurogenesis. Dietary phospholipids are naturally occurring amphipathic molecules with promising potential to promote brain health. However, it is unclear whether phospholipids are able to modulate neuronal function directly. In this study, we investigate the neuroprotective effects against corticosterone (CORT)-induced cytotoxicity in primary cultured cortical neurons. In addition, we examine the capacity of phospholipids to modulate proliferation and differentiation of hippocampal neural progenitor cells (NPCs).

We show that certain phospholipids can reverse CORT-induced cytotoxicity and neuronal depletion in cortical cells. On the other hand, phospholipid treatment was unable to prevent the decrease of *Bdnf* expression produced by CORT. Interestingly, only phosphatidylserine was able to increase hippocampal NPCs neurospheres. Finally, phosphatidylethanolamine elicited a significant increase in astrocytic differentiation in hippocampal NPCs. These results suggest that phospholipids protect cortical cells against CORT-induced cytotoxicity, and improve proliferation and astrocytic differentiation in hippocampal NPCs.

4.2 Introduction

It has long been known that neuronal function can affect cognitive processes and emotions, and impairments in neuronal signalling directly impact on mental health (Wohleb 2016, Birkel 2017). The relative abundance of specific nutrients from dietary sources can positively modulate neuronal function and can offer protective effects against environmental and physiological factors (Gómez-Pinilla 2008). Indeed, dietary nutritional components of diet have been demonstrated to be crucial in several brain processes including brain development, neuroplasticity and neurogenesis (Georgieff 2007). In particular, evidence suggest that optimal nutrition is important for the maintenance of adult hippocampal neurogenesis (An *et al.* 2008, Dyall *et al.* 2010, Heberden 2016, Poulose *et al.* 2017), the process where new neurons are produced from neural progenitor cells (NPCs) in the hippocampus (Eriksson *et al.* 1998). The process of neurogenesis is most pronounced during embryonic development, but there is evidence that it persists into adulthood with relevant implications for hippocampal-dependent cognitive function throughout (Kohman *et al.* 2013). However, it is noticeable that although accumulating evidence support the fact that adult human hippocampal neurogenesis exists, in the last few years contradicting reports (Boldrini *et al.* 2018, Sorrells *et al.* 2018) show that the very existence of this neuronal process can still be a subject for debate (Lee *et al.* 2018).

On the other hand, the emerging and compelling evidence for nutrition as a crucial factor in the high prevalence and incidence of mental disorders suggests that changes in diet are a viable strategy for improving mental health and treatment of stress-related psychiatric disorders including anxiety and depression (Sanchez-Villegas *et al.* 2013, Jacka *et al.* 2014, Opie *et al.* 2015, Adan *et al.* 2019). Stress-related mental disorders are associated with alterations in the hypothalamic-pituitary-adrenal (HPA) axis, which can be activated in response to stress leading to a release of glucocorticoids into the circulation (Sapolsky *et al.* 2000). Although these steroid hormones are critically involved in the homeostatic regulation of metabolism, chronic exposure in the central nervous system to glucocorticoids has been associated to deleterious effects on neuronal structure and function (Mitra *et al.* 2008).

Although, all nutrients are important for neuronal function, some appear to have a prime role in terms of neuronal growth and development as well as neuroprotection during vulnerable stages (Calabrese *et al.* 2008, Keunen *et al.* 2014). Based on these observations, the identification of key nutrients and nutritional components that can boost and protect mental health from neuropsychiatric disorders by modulating neuronal processes may represent novel therapeutic strategies against stress-related physiological and environmental factors such as aging and chronic stress.

In this regard, dietary-available phospholipids are a specific type of lipids consisting of a hydrophilic head formed by a phosphate group and two hydrophobic tails (Kullenberg *et al.* 2012). Naturally occurring phospholipids can be found in plant and animal food sources, including eggs, organ and lean meats, fish, shellfish, cereal grains, oilseeds and milk (Cohn *et al.* 2010). Phospholipids are considered to be important components of nutrition, since studies have demonstrated that phospholipid-enriched diets significantly impact behaviour (Kullenberg *et al.* 2012). Indeed, phospholipid supplementation have showed significant improvements in aged patients in terms of cognitive performance (Crook *et al.* 1991), and attenuated the negative effects of stress in young adult subjects (Benton *et al.* 2001). However, is not clear whether the positive effects of phospholipids on behaviour are linked with direct modulation of neuronal function or indirect systemic responses.

Currently, there is a lack of studies looking at phospholipid potential on mental health and disease, and more research is needed to understand their therapeutic effects. The purpose of the present study was to explore through an *in vitro* approach the implications of phospholipids in neuromodulatory processes. We first performed a screening of nine phospholipids to assess their neuroprotective capacity against CORT-induced cytotoxicity in neurons derived from the brain cortex, a key brain area involved in the pathophysiology of stress-related disorders. In addition, we tested the role of phospholipids on proliferation and neural differentiation using hippocampal NPCs formed neurospheres, considering the implications of this brain area in neurodevelopment and neurogenesis. Hippocampal NPCs forming neurospheres are a valuable tool for isolating, and understanding the biology of adult CNS stem cells and neurogenesis (Reynolds *et al.* 2005). Considering that PS, the phospholipid tested in

this study with positive effects on neuroprotection, proliferation and differentiation, crosses the blood-brain barrier (BBB) and it is efficiently absorbed by neurons (Glade *et al.* 2015), it shows potential implications for future pre-clinical and clinical interventions. To date the capacity of other phospholipids to cross the BBB has not been confirmed yet. However, since the chemical and physical properties of the BBB allow the transport of a number of molecules with specific features (Fong 2015), including drugs and chemicals with zwitterionic structure, it is plausible that most of the phospholipids tested in our work are capable of cross it. Together, findings from this study provide new insights into the potential neuromodulatory effects of phospholipids and add important direction to future dietary advice on optimal nutrition for cognition and mental health.

4.3 Methods

4.3.1 Chemicals and reagents

Dulbecco's modified Eagle's medium (DMEM), penicillin/streptomycin, D-glucose, foetal bovine serum (FBS), L-glutamine, Trypsin, DNAase I, corticosterone (CORT), poly-L-lisyne, 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT), fibroblast growth factor (FGF), epidermal growth factor (EGF), dimethyl sulfoxide, phosphatidylcholine (PC; from egg yolk), lysophosphatidylcholine (LPC; from egg yolk), phosphatidylserine (PS; from bovine brain), phosphatidylethanolamine (PE; from egg yolk), phosphatidylinositol (PI; from soybean), phosphatidylglycerol (PG; from egg yolk), phosphatidic acid (PA; from egg yolk), sphingomyelin (SM; from egg yolk) and cardiolipin (CL; from bovine heart) were purchased from Sigma. B-27 supplement was obtained from Thermo Fisher Scientific.

4.3.2 Animals

All procedures on live animals were performed under licence from the Government of Ireland Department of Health (B100/3774) in accordance with National and European Union directive 2010/63/EU, with prior ethical approval by University College Cork (AEEC #2012/045). Experiments were conducted in accordance with guidelines established by University College Cork's Animal Welfare Body.

4.3.3 Primary culturing of post-natal day 1 rat cortical neurons

Mixed neuron and astrocyte cultures were prepared as described previously (Pusceddu *et al.* 2016). Briefly, post-natal day (PND) 1 Sprague Dawley male rats were sacrificed and cerebral cortices dissected. Cortical tissue was dissociated into a cellular suspension using trypsin (0.25%; 37 °C) and physical trituration with a glass Pasteur pipette in warmed DMEM-F12 with 10% FBS and 100 µg/mL DNase I. Cell suspension was passed through 40 µm strainer and then centrifuged at 1000 rpm for 10 min at room temperature. Cells were re-suspended in warmed culture media (DMEM-F12 supplemented with B-27, 1% FBS, 100 U/mL penicillin, 100 µg/mL

streptomycin, L-glutamine 2 mM and D-glucose 55 mM), and then cultured at 37 °C with 5% CO2.

4.3.4 Isolation and culturing of rat hippocampal neural progenitor cells

Hippocampal cultures containing neural stem cells (NSC) and neural progenitor cells (NPC) were obtained as described previously (O'Leime *et al.* 2018). Briefly, hippocampal tissue was dissected from embryonic day (E) 18 rats, and then gently triturated with a glass Pasteur pipette in warmed proliferative media (DMEM-F12 supplemented with 10 ng/mL EGF and 10 ng/mL FGF, B-27, 100 U/mL penicillin, 100 µg/mL streptomycin, 2 mM L-glutamine and 33 mM D-glucose). For differentiation studies, untreated NPCs were dissociated to a single cell suspension after proliferation for 7 days *in vitro* (DIV), and seeded at a density of 4×10^4 cells/well in poly-L-lysine-coated 13 mm glass coverslip in 24-well tissue culture plates using differentiation media (DMEM-F12 supplemented with B-27, 1% FBS, 100 U/mL penicillin, 100 µg/mL streptomycin, 2 mM L-glutamine and 33 mM D-glucose), and then cultured at 37 °C with 5% CO2.

4.3.5 Cell treatment

Phospholipids (0.05 – 30 µg/mL; depending of each phospholipid solubility) were added to cortical cells at day *in vitro* (DIV) 5 and maintained for 24 hours. Then the media was replaced with fresh media containing 200 µM CORT at DIV6 and kept for 96 hours (Fig. 1A). For NPC at proliferative condition, phospholipids (0.05 – 30 µg/mL) were added at DIV0 and maintained until DIV7 (Fig. 1B). For differentiation studies, untreated NPCs were treated with phospholipids (0.05 – 30 µg/mL) for 5 days (Fig. 1C). The doses of phospholipids were chosen based in previous publications, which reported functional activity *in vitro* (Arakawa *et al.* 1991, Kalvodova *et al.* 2005, Treede *et al.* 2007).

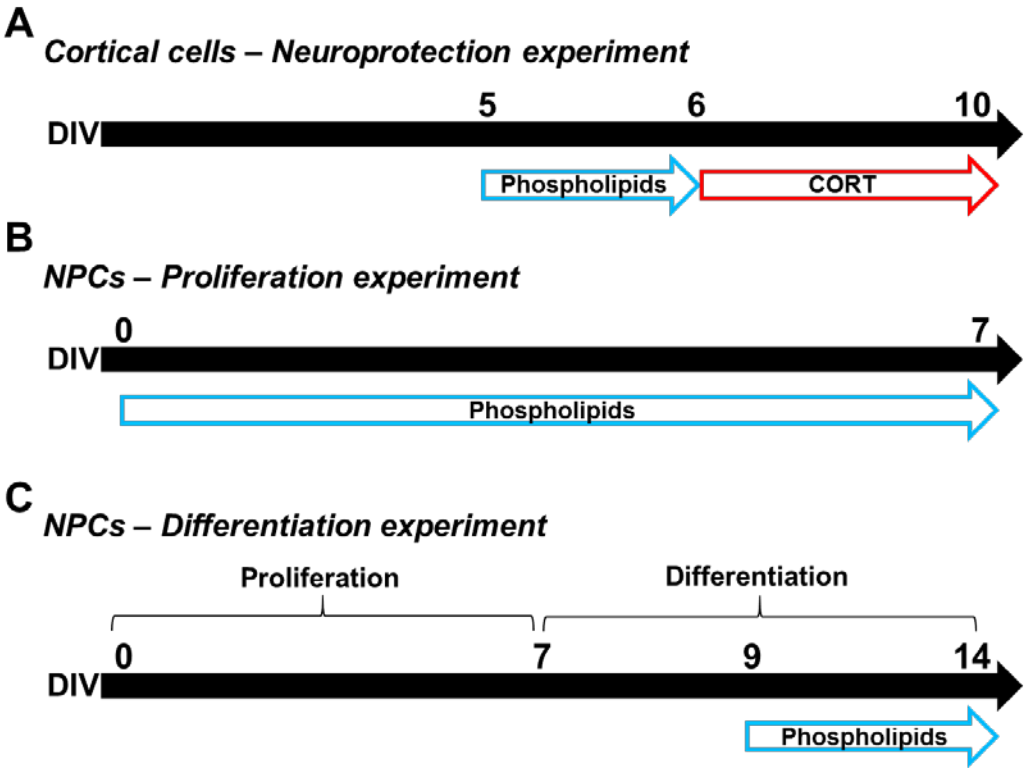

Figure 1 Schematic representing the in vitro experiments performed. ***(A)*** *Neuroprotective experiment. Cortical cells were incubated with various concentrations of phospholipids for 24 hours from DIV5 to DIV6, then the media was replaced with 200 µM CORT and kept until DIV10.* ***(B)*** *Proliferation experiment. NPCs were exposed to phospholipids and allowed to form neurospheres under proliferative conditions from DIV0 to DIV7.* ***(C)*** *Differentiation experiment. NPCs-forming neurospheres were incubated in proliferative conditions from DIV0 to DIV7 without any stimulus. At DIV7 neurospheres were disaggregated and incubated with differentiation media. Phospholipids were added to the media from DIV9 to DIV14.*

4.3.6 Cell viability measurement

Cell viability was determined by the MTT assay which is based on mitochondrial dehydrogenase activity of viable cells as previously described (Mosmann 1983). Briefly, cortical cells or NPCs were cultured at 4×10^4 cells per well in 24 well-plates in triplicate. After treatment, medium was removed and replaced with fresh culture medium containing MTT (500 µg/mL), and then incubated at 37 °C for 3 hours. Then, in order to dissolve the formazan produced after MTT reaction 100 µL of dimethyl sulfoxide were added to each well. The absorbance values were measured by spectrophotometry at 570 nm with a microplate reader (BioTek Synergy HT). The

results were expressed as a percentage of vehicle control group in three independent experiments

4.3.7 Immunocytochemistry

Cellular staining for neuronal (βIII-tubulin) and astrocytic (GFAP) markers, was assessed as through immunofluorescence detection as previously described (Pusceddu *et al.* 2016). Briefly, cortical cells and NPCs were fixed with ice-cold methanol for 10 min and then blocked overnight in 5% horse serum at 4 °C. Then the cells were incubated overnight at 4 °C in primary antibody solution (mouse anti-βIII-tubulin 1:300, Promega; rabbit anti-GFAP 1:300, Dako). The following day, cells are washed and incubated with secondary antibody solution (Alexa Fluor 594 donkey anti-mouse 1:2000, Thermo Fisher; Alexa Fluor 488 donkey anti-rabbit 1:2000, Thermo Fisher) for 1 hour at room temperature. Nuclear staining was performed with Hoechst 33258 (Sigma) for 5 min. Cells then were counted in 5 fields of view for each coverslip and analysed using an Olympus BX53 upright fluorescence microscope. The experiment was performed in duplicate and repeated in three different experiments.

4.3.8 Analysis of NPC neurosphere growth

NPCs were cultured in 6-well plates at 4×10^5 cells per well under proliferative conditions. Selected doses of phospholipids were added at DIV0. Neurospheres were viewed under an inverted Olympus IX70 microscope at DIV2, DIV4 and DIV7 using bright field imaging. At least five images per treatment condition were captured (five different fields of view from one well; 5-8 neurospheres analysed per field of view. Neurospheres were randomly chosen each day), and the neurosphere diameter was quantified using ImageJ 1.51j8 software. Each experiment was repeated 3 times. At DIV0 NPCs received selected doses of phospholipids for 7 days, and micrographs were taken at DIV2, DIV4 and DIV7. Since the number of experimental conditions was elevated, phospholipids were distributed randomly in two experiments performed in different days. In the first experiment, PS, PG, PA, and CL were tested (Fig. 5A); while PC, PI, PE, and SM were examined in the second experiment (Fig. 5C).

4.3.9 Quantitative RT-PCR

Total RNA from primary cortical cells was isolated using the High Pure RNA isolation kit (Roche). Briefly, cells were seeded at density of 1.5×10^6 cells per well in 6-well plates, followed by pre-treatments with phospholipids and CORT insult. Then, cells were homogenised in lysis buffer and transferred into the filter tubes provided in the kit. Following procedure was performed according to manufacturer instructions. RNA concentration was determined using the ND-1000 spectrophotometer, and reverse transcription was assessed using the ExiLERATE LNA™ qPCR, cDNA synthesis kit (Exiqon). Subsequently, PCR reaction was performed using the ExiLERATE LNA™ qPCR, SYBR® Green master mix kit (Exiqon) in a lightcycler 480 II (Roche). Each sample was analysed in triplicate for both target gene and reference gene (β-actin), and the relative mRNA expressions were calculated using the $2^{-\Delta\Delta Ct}$ method (Livak *et al.* 2001).

4.3.10 Statistical analysis

Statistical analysis was performed using the software SPSS 24.0, and the results were presented as mean ± SEM. Data from the neuroprotection experiment was analysed using T-test to compare 'Vehicle' vs 'CORT', then the effect of 'phospholipids+CORT' groups were compared to 'CORT' using one-way ANOVA followed by Dunnett's test. For proliferation studies, the data were analysed using two-way ANOVA followed by Tukey (HSD) post-test. For the differentiation experiment data, 'phospholipids' groups were compared to 'Vehicle' using one-way ANOVA followed by Dunnett's test. A *p*-value of 0.05 was considered statistically significant.

4.4 Results

4.4.1 Phospholipids attenuate CORT-induced cytotoxicity in cortical cells

To test the potential capacity of phospholipids to protect cortical cells from CORT-elicited neurotoxicity, cortical cells were first incubated for 24 hours with different concentrations of each phospholipid to examine potential cytotoxicity (data not shown). None of the phospholipids tested in this study reduced cell viability in the range of concentration examined. Subsequently, cortical cells were pre-incubated for 24 hours with phospholipids at different concentrations, and then exposed to CORT for 96 hours. A protective effect of phospholipid exposure was observed, specifically PS 0.5 – 7.5 µg/mL (main effect $F_{6,14} = 10.863$, $p < .001$), PE 0.75 – 1.50 µg/mL (main effect $F_{6,14} = 11.240$, $p < .001$), PG 0.05 – 0.75 µg/mL (main effect $F_{6,14} = 17.787$, $p < .001$) and PA 0.5 – 15.0 µg/mL (main effect $F_{6,14} = 19.728$, $p < .001$) were able to significantly prevent the cytotoxicity caused by 200 µM CORT exposure (Fig. 2).

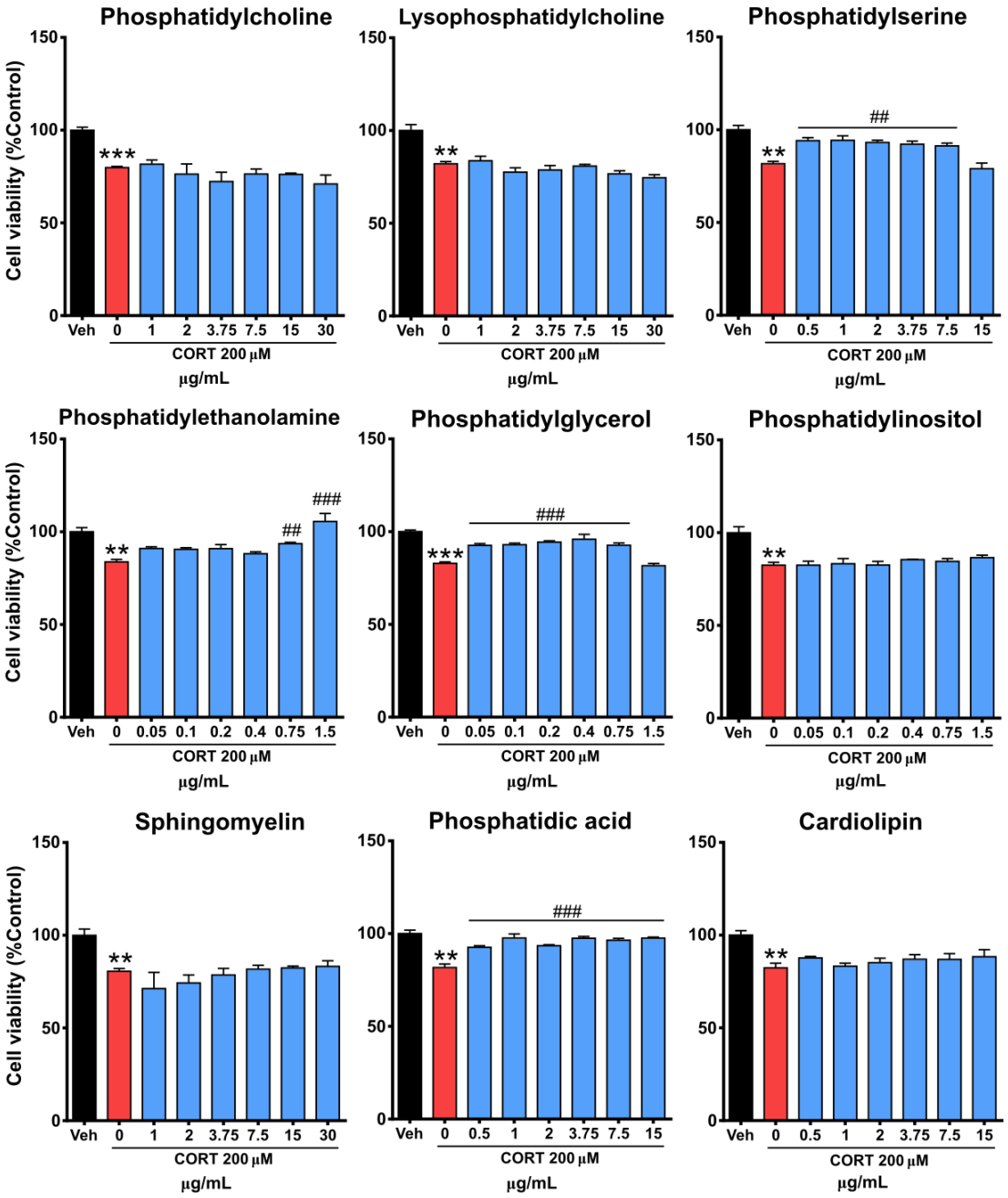

Figure 2 Phospholipids protect against CORT-induced cytotoxicity in cortical cells. *Cortical cells were pre-treated with the indicated concentrations of phospholipids for 24 hours and then with 200 µM CORT for 96 hours. Cell viability was measured by MTT assay. Results are expressed as the mean ± SEM of three independent experiments performed in triplicate (**p < 0.01; ***p < 0.001 versus 'vehicle' groups; ##p < 0.01; ###p < 0.001 versus 'CORT' groups).*

4.4.2 Phospholipids inhibit CORT-induced alterations in neurons but not astrocytes

To further determine the protective effects of PS (4 µg/mL), PE (1.5 µg/mL), PG (0.4 µg/mL) and PA (1 µg/mL) against CORT-induced cytotoxicity in cortical cells, we studied whether the negative impact of CORT on neuronal and astrocytic quantity and morphology (Pusceddu *et al.* 2016) could be ameliorated by pre-treatment with these phospholipids. Indeed, 96-hour exposure with CORT induced a significant reduction in the number of βIII-tubulin$^+$ cells (neurons), from approximately 27% to 18%; while the number of GFAP$^+$ cells (astrocytes) was significantly increased from 34% to 43%. Following a 24-hour pre-treatment with PS, PE and PG ameliorated the negative effects on neuronal integrity (main effect $F_{4,10} = 4.470$, $p = .025$), as measured by number of βIII-tubulin$^+$ cells, after CORT insult (Fig. 3A). In addition, these phospholipids significantly abolished the CORT-induced reduction of βIII-tubulin$^+$ cells (Fig. 3B). However, no phospholipid treatment was able to reverse the CORT-induced increase in GFAP$^+$ cells in cortical cells (Fig. 3C), suggesting this a secondary event following neuronal injury.

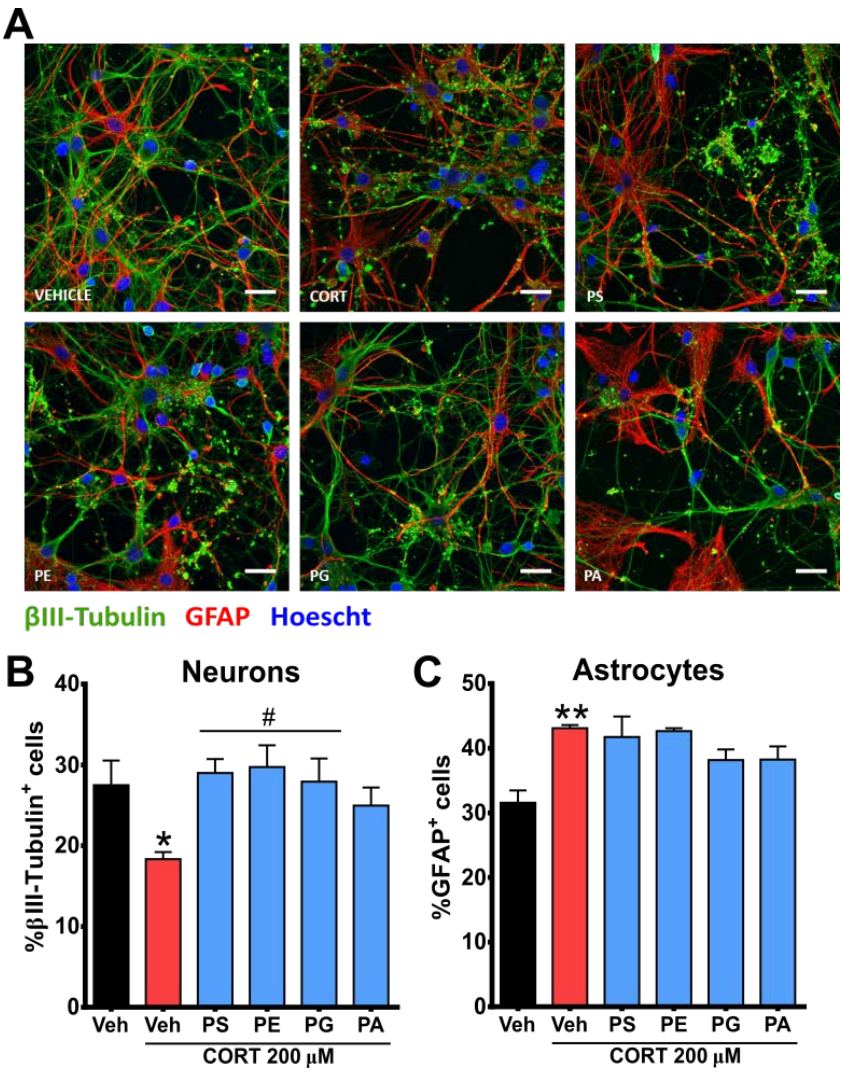

Figure 3 Phospholipids attenuated CORT-induced changes in neuron but not in astrocyte proportion. (A – C) *Cortical cells were pre-treated for 24 hours with phospholipids that showed positive effects in cell viability, including PS (4 µg/mL), PE (1.5 µg/mL), PG (0.4 µg/mL) and PA (1 µg/mL), and then exposed to 200 µM CORT for 96 hours. Quantitative analysis of neurons and astrocytes was assessed by immunostaining of βIII-tubulin+ and GFAP+ cells. Scale bar = 50 µm. (*p < 0.05; **p < 0.01 versus 'vehicle' groups; #p < 0.05 versus 'CORT' groups).*

4.4.3 No effect of phospholipids on CORT-induced reduction of *Bdnf* expression in cortical cells

Since BDNF expression is highly associated with neuronal survival, it is one of the mechanisms used by some neuroprotective agents (Zhao *et al.* 2017). Thus, we hypothesised that the phospholipid mediated protective effect against CORT-induced neuronal injury would occur through this mechanism. Therefore, we explored the effects of PS, PE, PG and PA on CORT-induced decreased of *Bdnf* mRNA expression

in cortical cells. In addition, we measured the mRNA levels of *Creb1*, an important nuclear factor involved in the expression of BDNF (Lee *et al.* 2009). Following a 24-hour treatment with PS, PE, PG and PA did not induce changes in the expression of *Creb1* (Fig. 4A), and its mRNA levels were unaffected after CORT exposure (Fig. 4B), suggesting that neither phospholipids or CORT are capable of modulate the upstream BDNF signalling pathway in terms of *Creb1* expression in cortical cells. Although, 24-hour treatment with phospholipids did not produce significant changes in *Bdnf* expression (Fig. 4C), 200 µM CORT insult significantly altered the expression of *Bdnf* by reducing its mRNA levels around a 60%. Accordingly, we already have demonstrated the capacity of long CORT exposure to negatively modulate *Bdnf* expression in cortical cells (Pusceddu *et al.* 2016) (Fig. 4D). However, phospholipid treatments were not able to ameliorate the *Bdnf* mRNA depletion produced by CORT insult, suggesting that their neuroprotective capacity might be mediated by a different mechanism (Fig. 4D).

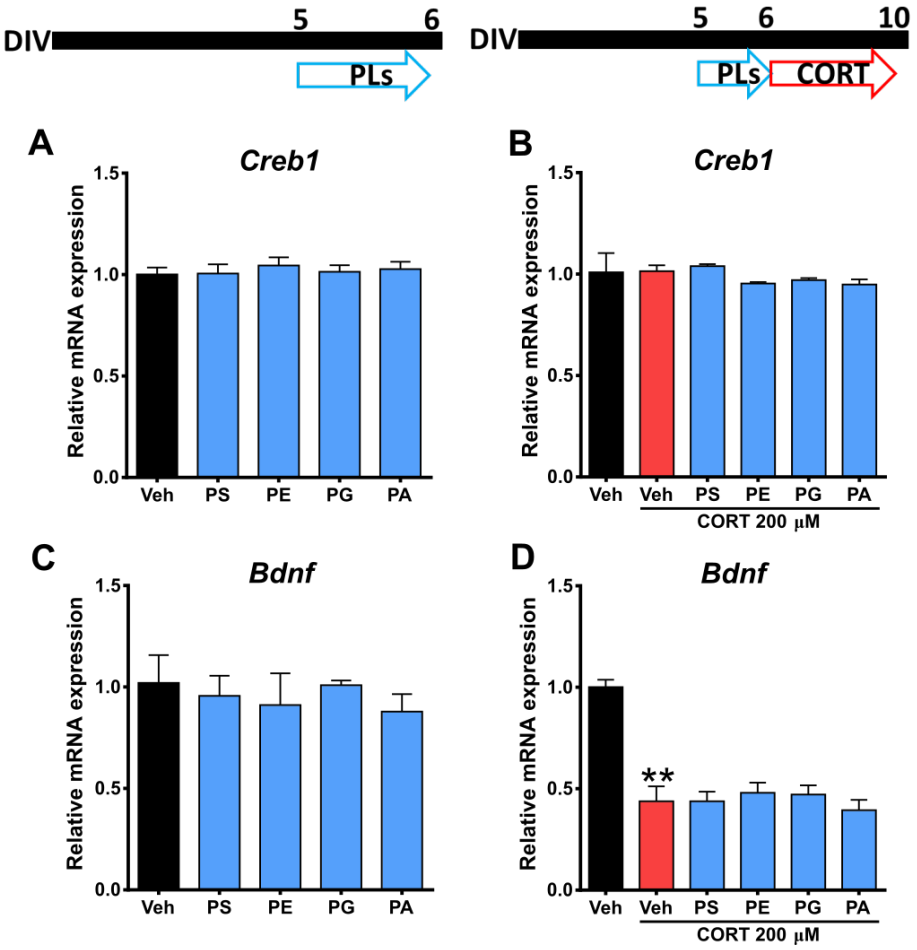

Figure 4 Neuroprotective effects of phospholipids are not associated to BDNF expression*. Total RNA was isolated under two different conditions;* ***(A & C)*** *Cortical cells were incubated with phospholipids for 24 hours, then BDNF and Creb1 expression was analysed.* ***(B & D)*** *Cortical cells were pre-incubated with phospholipids and then exposed to 200 µM CORT for 96 hours. The gene expression was quantitatively measured using real time RT-PCR. Results are expressed as the mean ± SEM of three independent experiments performed in triplicate. (**p < 0.01 versus 'vehicle' groups).*

4.4.4 Phosphatidylserine induces proliferation of neural progenitor cells

Next, we investigated whether phospholipids can modulate proliferation in NPCs by inducing changes in the size of neurospheres. Analysis of untreated NPC neurosphere diameter revealed an increase over 7 days of around 100 – 120%, which it was considered to be strictly dependent of culture conditions. On the other hand, treatment with PG (0.05 µg/mL), PA (4 µg/mL), CL (1 µg/mL), PC (4 µg/mL), PE (0.75 µg/mL), PI (1.5 µg/mL), and SM (30 µg/mL) did not alter the size of NPC neurospheres after a 7-day exposure (Fig. 5B and D). Doses of phospholipids were based in the maximum concentration that is not cytotoxic for neurosphere cultures after 7 DIV (data not shown). Notably, treatment with PS (4 µg/mL) induced a significant increase (p = .014) of the neurosphere diameter at DIV7 by a 14% more compared to the control group (treatment effect $F_{4,10}$ = 4.628, p = .023), suggesting a positive proliferative effect on NPCs (Fig. 5B). LPC was excluded of this experiment since it induced cytotoxic effects on NPC in all doses tested (data not shown).

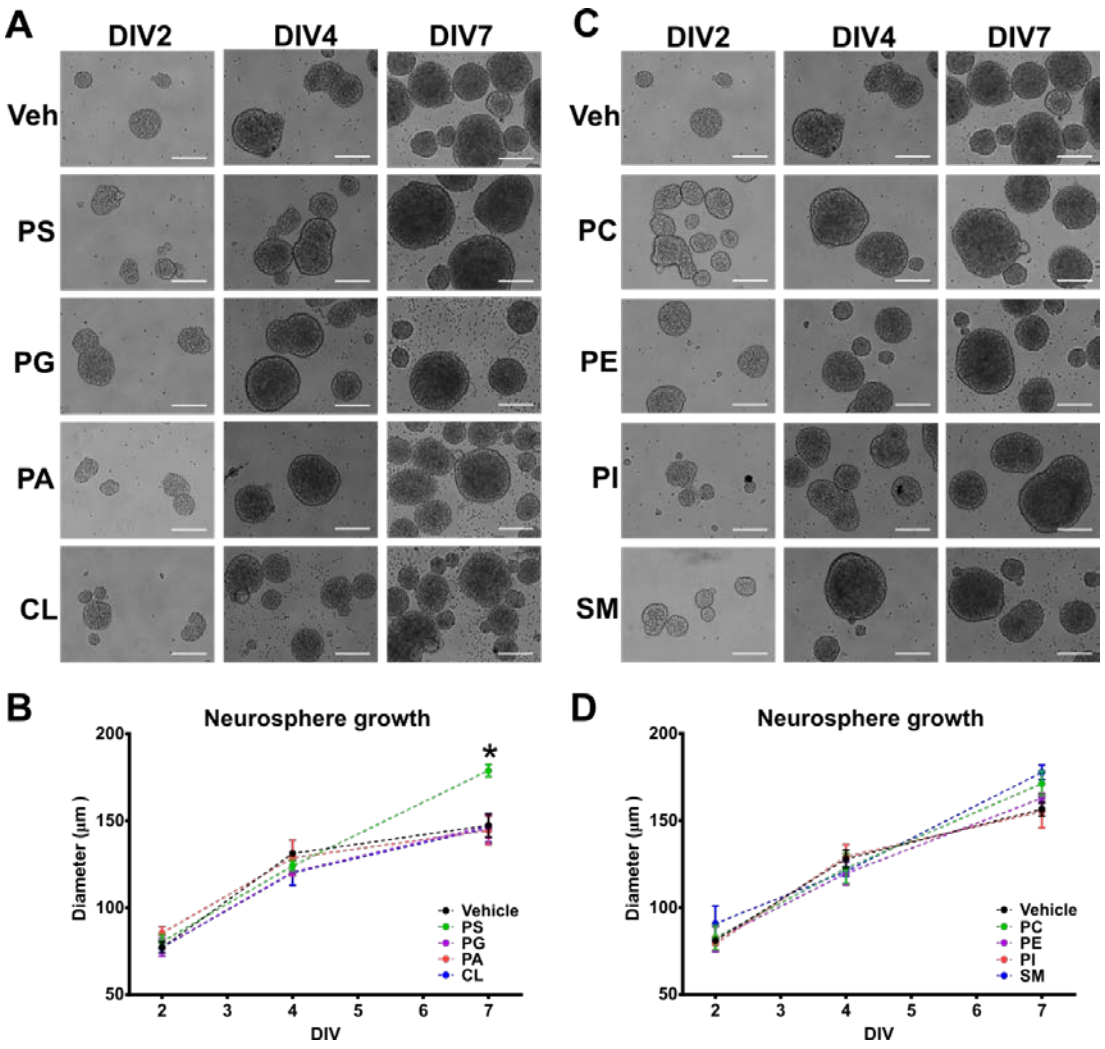

Figure 5 PS induced increase growth size in NPCs. *Selected doses of phospholipids were incubated with NPCs cultures from DIV0 to DIV7. Two independent experiments were performed due to the high number of treatments;* ***(A – B)*** *experiment 1, including PS (4 µg/mL), PG (0.05 µg/mL), PA (4 µg/mL) and CL (1 µg/mL);* ***(C – D)*** *experiment 2, including PC (4 µg/mL), PE (0.75 µg/mL), PI (1.5 µg/mL) and SM (30 µg/mL). Quantitative analysis of NPC growth size was assessed by measuring neurosphere diameter. Scale bar = 100 µm. (*p < 0.05 vs Vehicle group at DIV7).*

4.4.5 Phospholipids induce astrocytic differentiation in neural progenitor cells

To explore the role of phospholipids on neural differentiation, we examined the number of astrocytes and neurons in NPCs after treatment with PC, PS, PG, PE, PI, PA, SM and CL (Fig. 6A). After 5 day-treatment with these phospholipids, no changes were detected in the proportion of βIII-tubulin^{+} cells (Fig. 6B); however, PI tends to

increase the overall number of βIII-tubulin$^+$ cells from approximately 7% to 11%, but this increase failed to reach statistical significance (p = .292). In contrast, PE (p = .035), PS (p = .061) and PG (p = .052) increased the number of GFAP$^+$ cells from approximately 29% to 45% respect to the vehicle group (Fig. 6C), suggesting positive astrocytic differentiation (main effect $F_{8,18}$ = 3.967, p = .007). However, only PE demonstrated to be able to significantly increase the GFAP$^+$ cell number.

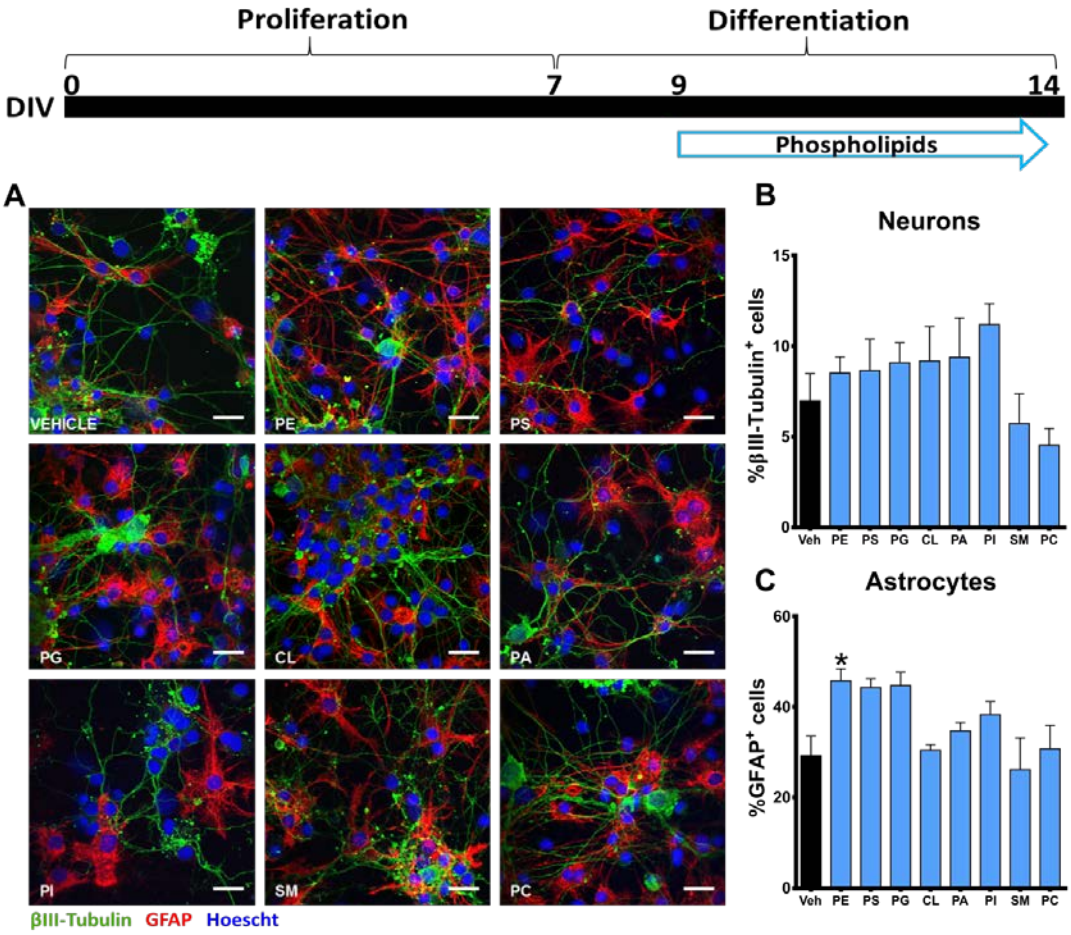

Figure 6 Phospholipids increased astrocyte numbers in differentiated NPCs. (A) *NPCs were incubated with selected doses of phospholipids in differentiation conditions for 5 days.* ***(B – C)*** *Quantitative analysis of neurons and astrocytes was assessed by immunostaining of βIII-tubulin$^+$ and GFAP$^+$ cells. Scale bar = 50 µm. (*p < 0.05 versus 'vehicle' groups).*

4.5 Discussion

Dietary available phospholipids are nutritional lipids with recognised potential to protect neuronal functioning linked with cognition and behaviour, from the negative effects of aging and stress (Crook *et al.* 1991, Benton *et al.* 2001, Kullenberg *et al.* 2012, Boyle *et al.* 2019). In the present study, we demonstrated that natural occurring and dietary phospholipids can significantly modify neuromodulatory processes *in vitro*. In particular, we showed that PS, PE, PG and PA protected cortical cells against CORT-elicited cytotoxicity and altered neuronal/astrocytic ratio. In addition, we demonstrated that PS facilitates neurosphere self-renewal, while PE improved astrocytic differentiation in hippocampal NPCs. Thus, our findings suggest a promising protective and beneficial effect of phospholipids in neuronal function *in vitro*.

Elevated concentration of CORT in rodent models have been associated with depressive- and anxiety-like behaviours (Rosa *et al.* 2014, Mendez-David *et al.* 2017). Similarly, accumulating lines of evidence indicate that depressive or chronically stressed individuals have an over activated HPA axis (Pariante *et al.* 2008, Keller *et al.* 2017). Indeed, several *in vitro* studies showed that chronic exposure to CORT exerts toxic effects on neurons (Gao *et al.* 2015, Pusceddu *et al.* 2016, Zhao *et al.* 2018). Since, phospholipid-enriched diets have demonstrated therapeutic effects against human stress-related conditions (Hellhammer *et al.* 2004, Boyle *et al.* 2019), demonstrating that phospholipids can prevent the neurotoxic effects of glucocorticoids *in vitro*, it could provide novel insights into the mechanism underpinning such effect *in vivo*. Our data show that CORT treatment decreased the cell viability of primary cultured cortical cells. Next, we demonstrated that treatment with PS, PE, PG and PA significantly reduced this CORT-elicited cytotoxicity. However, we did not find a protective effect on CORT-induced cytotoxicity when using the phospholipids PC, LPC, PI, SM, and CL. The reason why other phospholipids tested did not exhibit this neuroprotective effect could be associated to structural differences between these molecules, as similarly occurs in other physiological contexts. For instance, it has been demonstrated that anti-inflammatory properties of phospholipids strongly depend of their structure (Furnkranz *et al.* 2004, Lordan *et al.* 2017). Indeed, although most of

the phospholipids tested shared similar chemical structure in the hydrophobic fatty acid tail, their hydrophilic phosphate head differs from each other. For example, PC is linked with choline, a molecule with demonstrated neuropharmacological potential (Kusuda *et al.* 2019). In contrast, PS has a serine attached to its carbon chain, an amino acid with proven capacity to modulate neuronal function (Billard 2008). Thus, the neuroprotective capacity of PS, PE, PG and PA might be related to their chemical structure in the hydrophilic head. However, future studies are needed to demonstrated this structure-dependent function associated to phospholipids.

In addition, we confirmed the neuroprotective potential of these compounds by preventing the reduction of neuronal percentage in cortical cells caused by CORT treatment. However, none of these phospholipids were able to prevent the CORT-induced increase of astrocytes suggesting a phenotype-specific effect favouring neuronal viability. Accordingly, astrocytic overgrowth or astrogliosis is described as a critical process for neural protection and repair (Zhang *et al.* 2007), and it has been detected in similar studies using CORT high exposure in neural tissue (Bridges *et al.* 2008, Pusceddu *et al.* 2016). Thus, it is likely that the increase in astrocytes is a direct consequence to compensate CORT-induced neuronal injury and not an effect product of exposure to phospholipids.

To further investigate the potential mechanism underpinning the neuroprotective effect of PS, PE, PG and PA against CORT-elicited cytotoxicity we explored the implication of BDNF gene expression. BDNF is a member of the neurotrophin family of proteins involved in neuroplasticity and neuronal survival (Brunoni *et al.* 2008), which also has been associated with neuroprotective effects *in vitro* (Almeida *et al.* 2005). In addition, patients suffering from stress-related disorders display decreased levels of BDNF, and its expression has strongly been implicated in antidepressant activity (Sen *et al.* 2008, Lee *et al.* 2010). We demonstrated that CORT induced a significant reduction of *Bdnf* mRNA levels, however none of the phospholipids were capable of preventing this negative consequence, suggesting that *Bdnf* rescue is not crucial for phospholipid-mediated neuroprotection. In contrast, we did not find differences in *Creb1* mRNA levels after neither CORT or phospholipid treatment. As CREB1 is a key nuclear factor involved in the expression of BDNF (Murphy *et al.*

2013), this result suggests that CORT-induced reduction expression of *Bdnf* mRNA is not dependent of *Creb1* expression.

Neurogenesis is an important brain process highly associated with mental health and cognition (Eisch *et al.* 2008). In order to investigate the effects of phospholipids on neuromodulatory processes associated with neurogenesis and neurodevelopment, we used hippocampal NPC cultures to evaluate proliferation and differentiation *in vitro*. Providing new evidence highlighting a role of phospholipids in this neuronal function would directly support the nutritional impact of phospholipids on brain health and function. In this study, we found increased size of neurosphere diameter after treatment with PS. Neurosphere growth is a common measure of cell self-renewal and proliferation (Molofsky *et al.* 2003). Indeed, an increased in the neurosphere size is associated with proliferative activity of NPCs (O'Leime *et al.* 2018). Interestingly, PC, LPC, PI, PG, PA, PE, SM, and CL did not produce comparable effects to PS, suggesting a specific activity for this compound, which mechanism needs to be further investigated.

In addition, our data revealed that PE, PS and PG have a significant impact on NPC astrocytic differentiation. In this regard, increased astrocyte differentiation has been associated with maintenance of adult neurons in terms of neurite growth and synaptic formation (Yasui *et al.* 2017). For example, high levels of synaptic glutamate can cause over-activation of neurons leading to excitotoxicity. However, astrocytes have specific Na^+ dependent transporters able to remove rapidly extracellular glutamate from the synaptic cleft, improving neuronal survival (Dong *et al.* 2009). Astrogenesis, the process where new astrocytes are produced, is mainly initiated by the activation of JAK-STAT, the canonical pathway regulating astrocyte gene expression (Bonni *et al.* 1997). Nevertheless, the mechanism of phospholipid-mediated astrocytic differentiation in NPCs and the role of JAK-STAT activation herein remains to be investigated.

In conclusion, our present work confirmed that phospholipids modulated important neuronal functions linked to neuroprotection and neurodevelopment *in vitro*. In our knowledge, this is the first study screening a wide range of individual phospholipids

in neuronal *in vitro* models. Indeed, we demonstrated that PS, PE, PG and PA protected cortical neurons against CORT-induced neurotoxicity. In addition, we found that PS produced an increase in NPC neurosphere size, which is associated with active neural proliferation. Finally, we detected that astrocytic differentiation on NPCs was improved after treatment with PE. Our findings support that fact that a diet rich in phospholipids may have potential beneficial effects for brain health (Kullenberg *et al.* 2012, Boyle *et al.* 2019). Although the signalling pathways involved in these phospholipid-mediated neuromodulatory effects, and their capacity to cross the blood-brain-barrier need to be further explored, we provide novel insights into the potential role of dietary available phospholipids on mental health.

Chapter 5

Neurobehavioral Effects of a Phospholipid-Enriched Diet in a Mouse Model of Chronic Psychosocial Stress

Marina Shverer[a], Francisco Donoso[a, b], Kieran Rea[a], Bernard L. Roy[d], Timothy G. Dinan[a, b], John F. Cryan[a, c] and Harriët Schellekens[a, c]

[a]APC Microbiome Ireland, University College Cork, Cork, Ireland
[b]Department of Psychiatry, University College Cork, Cork, Ireland
[c]Department of Anatomy & Neuroscience, University College Cork, Cork, Ireland.
[d]Cremo SA, Fribourg, Switzerland.

To be submitted to: *Behavioural Brain Research*

Manuscript in preparation

5.1 Abstract

Chronic stress is considered a critical risk factor associated with the development of various concerning neuropsychiatric disorders, including depression and anxiety. For this reason, there is growing interest in the search of novel therapeutic strategies to prevent and alleviate the neurobehavioural consequences of stress-related mental disorders. In particular, nutritional approaches have become an interesting alternative due to their non-invasive nature, and few side effects. Phospholipids are a type of dietary lipids present in all cellular membranes, which have been demonstrated to modulate brain function. However, their therapeutic effects against chronic psychosocial stress-induced neurobehavioural changes have not been explored. In this study, we investigated the effects of a phospholipid-enriched dietary intervention in a mouse model of chronic psychosocial stress, specifically in sociability, anxiety- and depressive-like behaviours, as well as the implications of the HPA axis in stress regulation.

After 6 weeks of repeated psychosocial stress (PS), mice developed an increased anxiety-like phenotype and higher HPA axis activity, while no changes in social and depressive-like behaviours were detected. Although phospholipid treatment did not improve dysregulation of the HPA axis, it exerted a potential anxiolytic effect in PS animals. These results provide interesting insights into the effects of dietary phospholipids on behaviour and their therapeutic potential against chronic stress.

5.2 Introduction

Stress-related neuropsychiatric disorders, including depression, anxiety, anorexia nervosa, and post-traumatic stress disorder are considered a challenge to medicine and public health worldwide (deVries *et al.* 2003, Singla *et al.* 2018). Indeed, several lines of evidence suggest that chronic stress events are an important risk factor for developing depression in humans (Hammen 2005, de Kloet *et al.* 2016). Nowadays, one of the most common ways to be exposed to chronic stress is in environments of social context, which can lead to adverse and uncomfortable events or even negative experiences. Thus, these stressors are considered to be part of a specific type of stress, termed psychosocial stress (Siegrist 2008, Lu *et al.* 2016). In rodents, the chronic social defeat stress (CSDS) model is a well described translational paradigm to understand the neurobehavioral consequences of psychosocial stress in humans (Golden *et al.* 2011). Although different protocols of CSDS in rodents have been developed in different laboratories (Sgoifo *et al.* 2002, Finger *et al.* 2011, Golden *et al.* 2011, Vasconcelos *et al.* 2015), similar phenotypic outcomes are obtained after these procedures. Indeed, evidence suggests that CSDS can induce a robust depressive-like phenotype marked by anhedonia, anxiety and social avoidance behaviour in mice (Rygula *et al.* 2006, Macedo *et al.* 2018).

Increasing evidence is demonstrating a direct relationship between diet, stress susceptibility and mental health (Adan *et al.* 2019). Brain health is dependent on the availability of appropriate nutrients, including lipids, amino acids, vitamins and minerals from the diet, which contribute to brain structure, composition and neuronal function (Gómez-Pinilla 2008, Jones 2016, Moore *et al.* 2018). Thus food quality and intake directly impact on brain function, which suggest that health, mood and cognitive performance can be modified by diet (Wahl *et al.* 2016, Meeusen *et al.* 2018). Indeed, poor dietary habits have been shown to be critical in the prevalence of mental disorders (Jacka *et al.* 2014, Lai *et al.* 2014, Dawson *et al.* 2016). The search for new strategies to improve mental health and treatment of neuropsychiatric disorders has directed its attention to the identification of nutritional factors, and their mechanism of action (Adan *et al.* 2019). In this regard, functional foods and dietary components are being considered as potential therapeutic supplements for alleviating the behavioural

symptoms of affective disorders (Owen *et al.* 2017), and their capacity to interact with other systems involved in the pathophysiology of stress-related mental illnesses, such as the hypothalamic-pituitary-adrenal (HPA) axis (Yau *et al.* 2013), the immune system (Klasing 2007), and the microbiota-gut-brain axis (Robertson *et al.* 2017).

Specific dietary lipids known as phospholipids, are biomolecules characterised to have a hydrophilic head formed by a phosphate group and two hydrophobic tails (Kullenberg *et al.* 2012). In addition, phospholipids are primary components of all animal and plant cellular membranes. For this reason, dietary phospholipids can be detected at significant concentration in different food sources, including eggs, organ and lean meats, fish, shellfish, cereal grains, oilseeds and milk (Cohn *et al.* 2010). Particularly, much interest has emerged for dairy-derived phospholipids as crucial nutrients for brain health, since they show a unique composition and a more balanced distribution in each phospholipid subclass compared to other food sources (Garcia *et al.* 2012)(Schverer *et al.* in press). In addition, milk and other dairy products are consumed globally and are considered crucial components for human health, since these foods contain various nutritional compounds, such as vitamins and lipids (Hill *et al.* 2015). For example, a supplementation with bovine milk-derived phospholipids improved cognitive performance in young human adults subjected to acute PS (Boyle *et al.* 2019). However, the implications for a nutritional intervention with dairy-derived phospholipids on prolonged chronic PS and its neurobehavioral consequences have not been investigated previously.

Taken together, the purpose of the present study was to explore the therapeutic potential of a phospholipid nutritional treatment against the detrimental effects on behaviour produced by PS using the mice CSDS model. In particular, we studied the effects of a phospholipid-based product derived from buttermilk on sociability, depressive- and anxiety-like behaviours, and HPA axis response in mice subjected to PS.

5.3 Methods

5.3.1 Animals

All experimental procedures involving animals were approved by the Ethics Committee of University College Cork (AEEC #2018/015). Male C57BL/6J mice (Envigo, 8 weeks old at the beginning of the study) were used. Animals were kept single-house in a temperature and humidity controlled room on a 12-h light, 12-h dark cycle (lights on from 7.00–19.00 h). Food and water were available ad libitum.

5.3.2 Diets

Phospholipid-enriched buttermilk was provided by Cremo. All diets were prepared by ssniff Spezialdiäten (Ferdinand-Gabriel-Weg, Germany). The phospholipid-enriched diet contained 360 mg of phospholipids per 100 g of chow.

5.3.3 Treatments

On arrival, animals were single housed and put under specific diets. Mice were randomly assigned into 4 different groups. [1] non-stressed (NS)-control (n = 10); [2] NS-phospholipid (PL) (n = 10); [3] Stressed (PS)-control (n = 10); [4] PS-PL (n = 10). The concentration of the phospholipid-enriched food was calculated based in previous reports (Kanno *et al.* 2014, Kivity *et al.* 2017) in order to obtain an approximated consumption of 450 mg/kg/day of phospholipids.

5.3.4 Social defeat/overcrowding procedure

The PS stress was carried out for 6 weeks in total as previously described with some modifications (Finger *et al.* 2011, van de Wouw *et al.* 2018). Firstly, 45 male CD1 mice (Envigo, 8-12 weeks old) were tested for aggression for three consecutive days using other CD1 intruder mice in their home cage. The 38 CD1 mice with the shortest attack latencies were selected for the social defeat procedure, while the others were used in the social interaction test. The C57BL/6J mice undergoing the social defeat procedure were pseudo-randomly assigned to a different CD1 aggressor mouse. During the procedure, test mice were gently placed into the CD1 aggressor home cage,

and permitted to interact until the first attack inducing a defeat posture on the test mouse (Beitia *et al.* 2005). Mice were then separated for 2 h by a perforated Plexiglass divider allowing for auditory, olfactory and visual, but not physical contact. The divider was subsequently removed, after which another social defeat took place, and test mice were transferred back to their home cage. For overcrowding sessions, stress mice of one diet group (n = 5) were placed all in one half of a standard holding cage divided by a Plexiglas divider for 24 or 48 hours.

5.3.5 Social interaction test

Social avoidance to the CD1 aggressor mice was performed as previously described (Tsankova *et al.* 2006, Burokas *et al.* 2017). Briefly, after 1-hour habituation in the test room, the social interaction is assessed in two sessions: First, the test mouse is placed in the centre of an open arena (42 × 30 × 24 cm; 5 lux) containing an empty wired mesh cage (9.5 × 7.5 × 7.0 cm), and allowed to explored for 2.5 min. Then, the test mouse is returned to its home cage. Second, 1 min after the first session ended, the same test mouse is placed again in the centre of the arena and allowed to explore for another 2.5 min, but now with an unfamiliar CD1 mouse into the wired mesh cage. Finally, both mice are returned to their home cage, and the open arena and wired mesh cage are cleaned with 70% ethanol. All the sessions were videotaped, and the time spent in the interaction zone was analysed later using a tracking system (Ethovision XT 13, Noldus).

5.3.6 Three-chamber sociability test

To evaluate social and novelty preference in mice, the three-chamber sociability test (3CT) was used. This test was performed as previously described (Burokas *et al.* 2017, van de Wouw *et al.* 2018). Briefly, the procedure consisted of three sessions of 10 min, including habituation, social preference, and social recognition. During habituation, the mouse is placed in the centre of the apparatus (a three-chambered box with small circular openings allowing for access to all chambers; 60 lux), where it is allowed to explore the apparatus with two empty wire cup-like cages in the outer chambers. Secondly, in the social preference session, one of the cages contains a mouse (male conspecific and age-matched), while the other contains an object (rubber

duck). Finally, during the last session of social recognition, the first cage contains the same mouse used in the social preference session (familiar mouse), whereas the object in the second cage is replaced with a novel mouse (male conspecific and age-matched). In between each test mice, the apparatus was cleaned while the location of object and conspecific mice was randomised. All the sessions were videotaped, and the time spent in each chamber was analysed later using a tracking system (Ethovision XT 13, Noldus).

5.3.7 Elevated plus maze

The elevated plus maze (EPM) is one of the most commonly used rodent tests for assessing anxiety and was performed as previously described (Burokas *et al.* 2017). Briefly, the apparatus consisted of two open arms (50 × 5 cm; red light) and two enclosed arms (50 × 5 × 15 cm). After 1-hour habituation to the test room, animals were placed in the centre of the maze facing an open arm to begin. Behaviour was recorded for 5 min. Frequency of open and closed arms entries were scored, as well as percentage time spent in each arm using a tracking system (Ethovision XT 13, Noldus).

5.3.8 Open field test

The open field test (OFT) was used to assess locomotor activity and anxiety-like behaviour, which was conducted as previously described (Burokas *et al.* 2017). Briefly, animals were first habituated to the room for 1 hour. Mice were then placed in the centre of an open field arena (45 × 45 cm; 60 lux) and allowed to explore it for 10 min. The arena was cleaned between mice test with 70% ethanol, and finally the animals were returned to their home cage. Percentage of time spent in inner zone, and frequency of inner zone entries were analysed using a tracking system (Ethovision XT 13, Noldus).

5.3.9 Forced swim test

The forced swim test (FST) is the most widely used model for predicting antidepressant activity in rodents, and increased immobility in this test is generally considered to reflect a state of behavioural despair (Porsolt *et al.* 1978). Briefly, mice were individually placed in a glass cylinder (H: 45 cm; D: 20 cm) filled with water to a depth of 15 cm at 24 ± 1 °C for a 6 min. The mice were removed from the water, dried and placed in their home cage. The water was changed between each trial. The behaviour was monitored from above with a video camera and only the last 4 min were analysed.

5.3.10 Plasma corticosterone determination

Blood sample collection was performed as previously described (van de Wouw *et al.* 2018). Briefly, blood samples were collected on FST day via a tail-tip incision at four different time points: immediately before (baseline), 15 min, 45 min, and 90 min after the test was started. Approximately 40 µl of whole blood was taken per time point using an EDTA-containing capillary, deposited in an Eppendorf and centrifuged for 15 min at 10000 *g* at 4°C. Plasma corticosterone levels were measured using the Corticosterone EIA kit (Enzo) according to the manufacturer instructions, and absorbance signal was detected with a conventional plate reader (Synergy HT, Biotek).

5.3.11 Statistical analysis

Statistical analysis was performed using the software SPSS 24.0, and the results were presented as mean ± SEM. Overall effect of stress condition and diet were assessed using two-way analysis of variance (ANOVA), while multiple comparisons were performed with the Tukey HSD *post hoc* test. A *p*-value of 0.05 was considered statistically significant.

5.4 Results

5.4.1 Phospholipid-enriched diet increases body weight in NS mice

To investigate the therapeutic effect of a nutritional intervention with phospholipids against chronic PS, NS and PS animals received diets from 8-weeks old until week 16 (Fig. 1A). Analysis of body weight (Fig. 1B and C) revealed a significant overall effect of chronic stress ($F_{1,33}$ = 23.760; p < .001) and diet ($F_{1,33}$ = 52.424; p < .001) in total weight gain. In particular, the phospholipid-enriched diet produced a significant increase in weight gain in NS mice and PS mice ($F_{3,33}$ = 26.489; p = .001 and p < .001 respectively) when they were compared to control-diet group. No significant differences were detected in weekly food intake and phospholipid intake between groups (Fig. 1D and E).

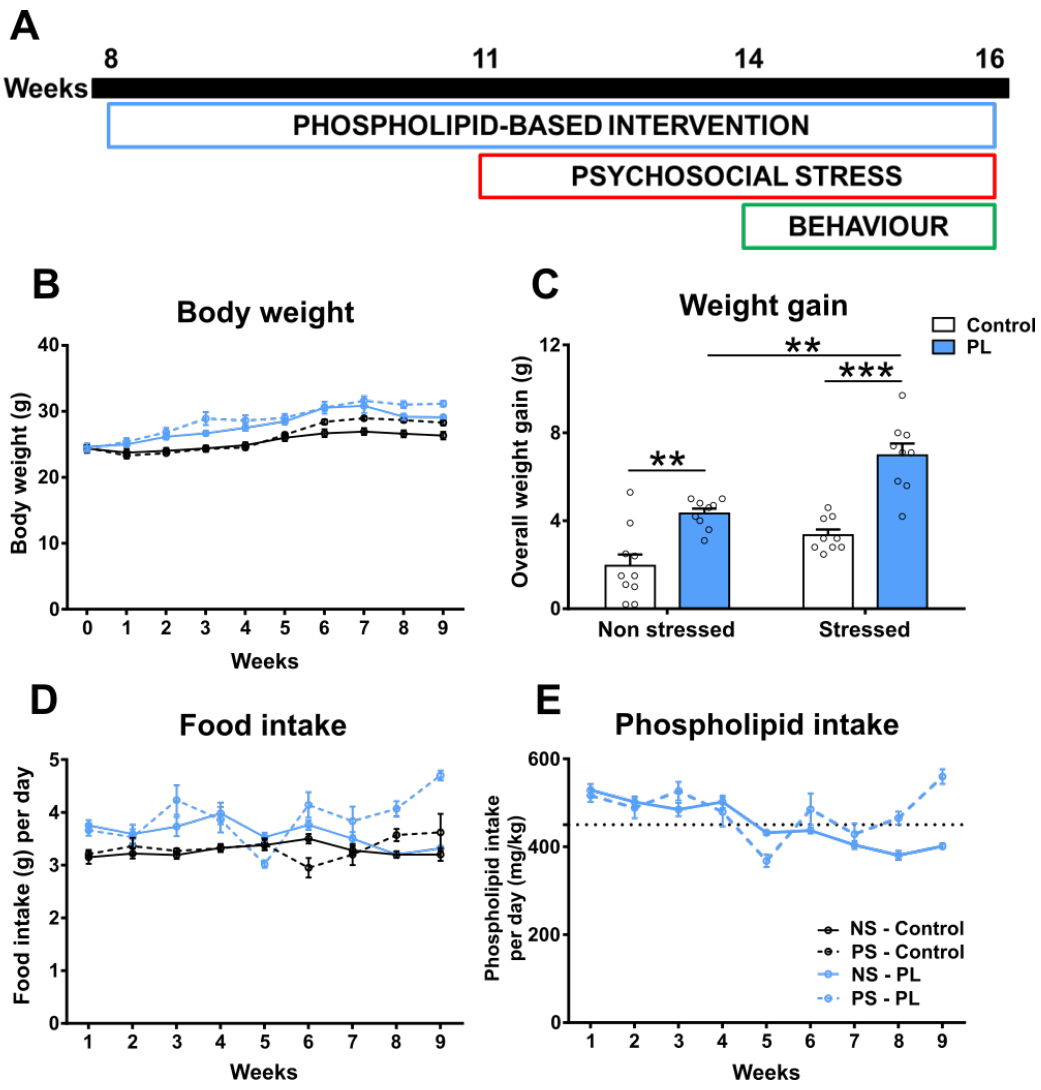

Figure 1 Phospholipid intake monitoring and effect on body weight. (A) *Timeline representing the experimental procedure and dietary intervention.* ***(B – C)*** *Animals were weighed in a weekly basis using a regular scale (± 0.01 g). Weight gain was calculated as the difference between final and initial weight.* ***(D – E)*** *Chow was weighted weekly, and phospholipid consumption was estimated considering total phospholipid concentration in food (360 mg/100 g chow). Results are expressed as the mean ± SEM (**p < 0.01; ***p < 0.001).*

5.4.2 Phospholipid-enriched diet did not alleviate psychosocial stress-induced anxiety-like behaviours

To explore the anxiolytic capacity of a phospholipid-enriched diet, mice were subjected to a battery of behavioural tests to assess anxiety-like behaviours (Fig. 2). In the EPM test, an overall significant anxiogenic effect of chronic stress in terms of time spent in open arms ($F_{1,35}$ = 11.988; p = .001), and entries in open arms ($F_{1,34}$ = 36.567; p = .000) (Fig. 2A and B) was detected. Interestingly, the phospholipid-enriched diet showed a subtle improvement in anxiety-like behaviour in the PS group in terms of time spent and entries in the centre compared to the PS-control diet. However, although this effect was found to be not statistically significant ($F_{3,35}$ = 5.172; p = .317), the stressed animals treated with phospholipids are no longer different that the NS control in terms of time ($F_{3,35}$ = 5.172; p = .397) (Fig. 2A and B). In the OFT, PS displayed an anxiogenic effect on time spent in centre ($F_{1,35}$ = 3.300; p = .078) and entries in the centre of the arena ($F_{1,35}$ = 8.651; p = .006) (Fig. 2C and D).

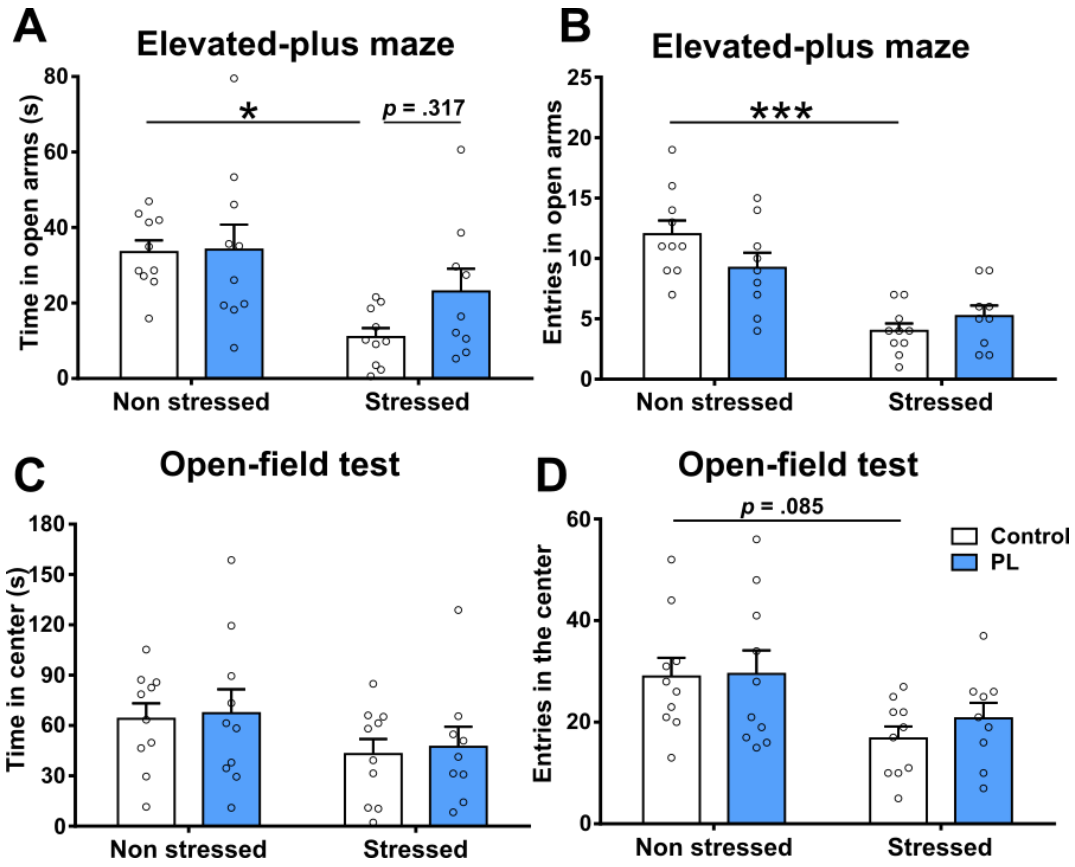

Figure 2 Psychosocial stress-induced anxiety like behaviour was not reversed by phospholipid treatment. (A – B) *PS mice exhibited increased anxiety-like behaviour in the EPM as reflected by the reduced time spent and entries in the open arms.* ***(C – D)*** *Similarly, animals subjected to PS displayed increased anxiety-like behaviour in the OFT. Results are expressed as the mean ± SEM (*p < 0.0; ***p < 0.001).*

5.4.3 Phospholipid-enriched diet had no effect on social behaviour

To study the effects of chronic PS on social behaviour, animals were evaluated in the SIT and 3CT (Fig. 3A and D). PS mice group performed similarly to the NS control group in terms of interaction ratio and time of social avoidance during the SIT (Fig. 3B and C), as well as no difference was found in social preference and novelty as revealed in the 3CT (3E and F). In addition, the phospholipid-enriched diet did not impact on social behaviour in either PS and NS groups.

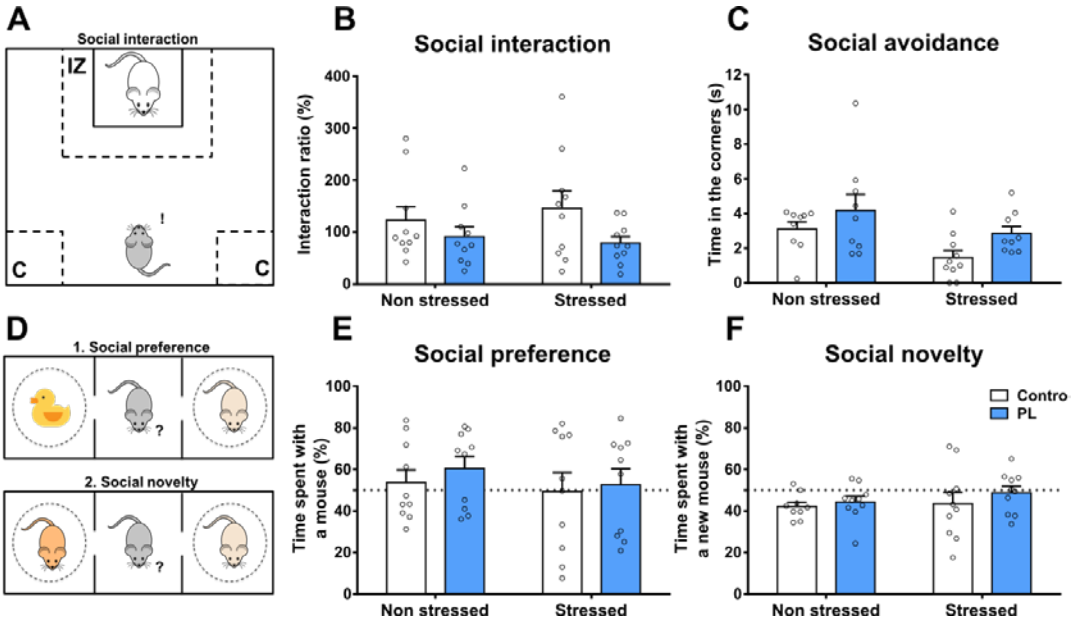

Figure 3 No effects of psychosocial stress nor phospholipid supplementation on social behaviour. ***(A)*** *Schematic representing SIT setup; IZ = Interaction zone; C = Corner.* ***(B – C)*** *PS did not induce social avoidance in mice during the SIT.* ***(D)*** *Schematic representing 3CT setup.* ***(E – F)*** *Social preference and social novelty were not altered by PS or phospholipid supplementation in mice after the 3CT. Results are expressed as the mean ± SEM.*

5.4.4 Chronic psychosocial stress induced high corticosterone production but did not affect depressive-like behaviours

To further examine the depressive-like phenotype in mice subjected to CSDS and the effects of a phospholipid-enriched diet, we measured the immobility time in the FST. Although there was a certain trend toward significance in terms of a PS overall effect in a two-way-ANOVA analysis ($F_{1,33}$ = 3.288; p = .079), was not detected a clear difference between the NS-control and PS-control groups ($F_{3,33}$ = 1.476; p = .237) (Fig. 4A). In addition, to determine the role of the HPA axis in the mouse PS phenotype, the concentration of corticosterone in plasma was measured at different time points after an acute stress. Baseline levels of plasma corticosterone were found to be significantly higher in PS animals compared to NS under the control diet ($F_{3,25}$ = 5.167; p = .019) (Fig. 4B). Moreover, an acute stress produced an exacerbated production of corticosterone in PS animals at 15 and 45 min after ($F_{3,25}$ = 14.478; p < .001; $F_{3,23}$ = 5.639; p = .005) (Fig. 4C). Similarly, the AUC of the corticosterone response over 90 min after acute stress was significantly increased in the PS-control group compared to the NS-control group ($F_{3,20}$ = 4.347; p = .032) (Fig. 4D).

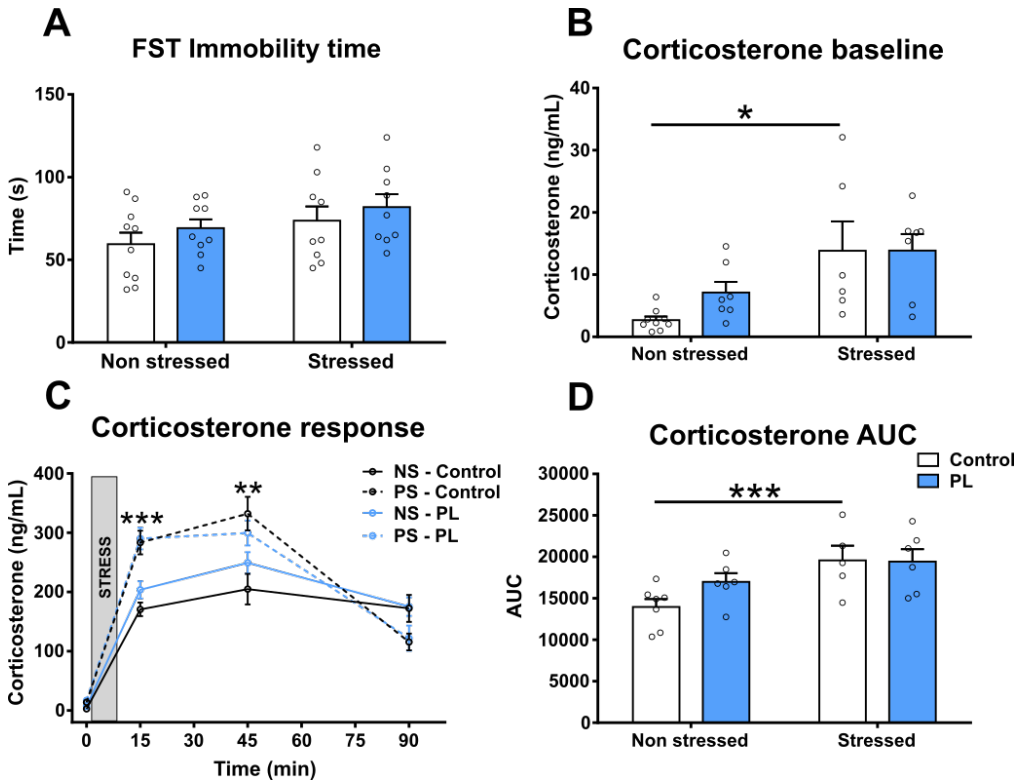

Figure 4 Chronically stressed mice displayed abnormal elevation of plasma corticosterone levels after acute stress. ***(A)*** *No behavioural changes were detected in the FST between NS and PS animals. The FST was used as acute stressor to measure the corticosterone response, which consisted in 6 min swimming in 24 ± 1 °C water.* ***(B)*** *Baseline levels of corticosterone of PS mice are significantly higher compared to NS animals.* ***(C)*** *Corticosterone levels in plasma rise after acute stress. PS-control animals displayed higher peaks of corticosterone concentration at 15 and 45 min compared to NS-control group.* ***(D)*** *Corticosterone release over 90 min after acute stress is significantly higher in PS groups. Plasma corticosterone was determined using ELISA. Results are expressed as the mean ± SEM (*p < .05; **p < .01; ***p < .001, 'NS-control' vs 'PS-control')*

5.5 Discussion

Since there is increasing interest in nutritional strategies to ameliorate the effects of chronic stress (Lakhan *et al.* 2008, Marzola *et al.* 2013, Rechenberg *et al.* 2013), we investigated the therapeutic potential of a phospholipid-enriched dietary intervention against the negative neurobehavioral effects produced in a mouse model of PS. In particular, we evaluated behaviours associated with anxiety, depression, and sociability, as well as the effects on the HPA axis.

The mouse chronic social defeat stress is an interesting model to investigate the neurobehavioral implications of psychosocial stress (Rygula *et al.* 2005, Golden *et al.* 2011, Gururajan *et al.* 2019). Indeed, PS in mice is known to induce significant changes in behaviour associated with depression and anxiety (Venzala *et al.* 2012, Burokas *et al.* 2017). Accordingly, we found that PS mice presented anxiety-like behaviours in the EPM and the OFT. Although not significant, the phospholipid-enriched diet ameliorated these PS-induced anxiety-like behaviours. Similarly, a previous study that used a phospholipid supplementation for 8 weeks failed to restore normal anxiety behaviour in a mouse model of omega 3 deficiency (Carrie *et al.* 2000). Interestingly, the duration of the phospholipid supplementation was the same as in our study, it is tempting to speculate that increased periods of phospholipid treatment would produce significant effects on anxiety-like behaviour.

Next, we examined the consequences of PS on social behaviour using the SIT and the 3CT. We did not detect either social avoidance in the SIT or altered social preference in the 3CT in mice subjected to PS. The SIT is widely used to validate a stressed phenotype in rodents subjected to repeated social defeat, where it is expected to find aversive social behaviour in the test mouse (Golden *et al.* 2011). However, the fact that we found significant increased anxiety- behaviours in other tests and an over-regulated stress neuroendocrine response, confirms a clear stressed phenotype in the PS group compared to NS mice. In this regard, the effect of the phospholipid-enriched diet in both SIT and 3CT was not significant, suggesting that phospholipid supplementation do not impact social behaviour. Further investigation is required to

study the effect of phospholipid dietary interventions in conditions where social behaviour would be altered.

In contrast, the FST revealed a non-significant increase in immobility behaviour in animals subjected to PS, suggesting that the stress paradigm did not result in an increase in depression-related behaviour. Previous studies have reported significant increase of immobility time in the FST after repeated CSDS (Finger *et al.* 2011, Brachman *et al.* 2016, Burokas *et al.* 2017). However, in accordance with a previous report (Slattery *et al.* 2012), this is not the first time where the CSDS procedure fails to induce a depressive-like phenotype in the FST. In this regard, the phospholipid supplementation did not exert antidepressant-like effect in the FST in either NS or PS animals. Perhaps, inducing a strong depressive-like phenotype in a different mouse model would help to understand the preventive capacity of phospholipids against a stress-related mental phenotype.

The CSDS induced a profound anxiety-like phenotype, and increased activity of the HPA axis, as reported previously (Reader *et al.* 2015, Burokas *et al.* 2017). Although the phospholipid treatment showed subtle improvements against PS-elicited anxiety-like behaviour (no statistically significant), it was not able to prevent the exacerbated stress physiological response. Indeed, dysfunctional HPA axis response has been implicated in the pathophysiology of anxiety disorders (Faravelli *et al.* 2012), and pharmacological treatments that have alleviated anxiety-like behaviours, are accompanied by reduction of abnormal HPA axis activation (Sartori *et al.* 2012, Wang *et al.* 2018).

Some aspects of behaviour were not examined in PS mice, where the impact of phospholipids would be interesting to investigate. For instance, it is well known that chronic stress is able to induce cognitive impairment, including disruption of attention, memory and executive functions (Iosifescu 2012, Fjell *et al.* 2014). Indeed, mice have shown altered working memory after prolonged CSDS (Bergamini *et al.* 2016, Burokas *et al.* 2017). On the other hand, clinical and pre-clinical evidence suggest a potential therapeutic effect of dietary phospholipids against chronic stress-induced cognitive impairment (Kanno *et al.* 2014, Boyle *et al.* 2019, O'Mahony *et al.* 2019).

For this reason, further investigation into the protective role of dietary phospholipids against PS-induced cognitive impairment is required.

In conclusion, our present work demonstrates that a phospholipid-enriched dietary intervention shows potential in the alleviation of the anxiety-like phenotype produced in a mouse model of chronic PS. In addition, these data demonstrate that PS and phospholipid supplementation do not impact social and depressive-like behaviours in the mouse. Therefore, whether phospholipids have the potential to alleviate social and cognitive deficits remains unclear. Future studies focusing on animal models with impaired social and cognitive behaviour might be useful to improve our knowledge about phospholipid nutritional impact on mouse behaviour. Nevertheless, future studies are required to investigate alternative phospholipid dose and treatment duration in order to enhance its therapeutic effect against chronic PS.

Chapter 6

General

Discussion

6.1 Overview and summary

Work presented in this thesis has demonstrated that nutritional interventions using dietary-derived polyphenols and phospholipids have therapeutic potential in treating stress-related neuropsychiatric disorders. Firstly, it was shown that the polyphenolic compounds quercetin and xanthohumol protected cortical neurons against corticosterone (CORT)-elicited cytotoxicity *in vitro*. Moreover, treatment with both polyphenols abolished the BDNF reduction produced by CORT, and restored altered neuronal/astrocytic ratio. In addition, the molecular mechanism involved in their neuroprotective effect was explored. We detected an activation of the Nrf2 pathway after xanthohumol treatment, while quercetin was observed modulating glucocorticoid receptor sensitivity by up-regulating FKBP5.

Secondly, the rat maternal separation model was utilised as a paradigm for early-life stress to study the therapeutic potential of polyphenol-enriched diets. maternal separation is known to induce a robust depressive phenotype, including behavioural despair, dysregulation of the HPA axis, an imbalance of monoaminergic neurotransmission, and an alteration of gut microbiota composition. Indeed, nutritional intervention with quercetin, xanthohumol or phlorotannins prevented maternal separation-induced depressive- and anxiety-like behaviours. Treatment with xanthohumol decreased the exacerbated release of CORT in maternally separated animals, and induced significant changes in gut microbiota composition and diversity.

In contrast, we observed a deficiency of monoamine neurotransmitters in the brainstem of maternally separated animals; however, polyphenolic treatments were unable to prevent this deficiency, suggesting that restoration of the neurochemical balance in brainstem is not required for polyphenol-mediated alleviation of depressive- and anxiety-like behaviours in the maternal separation model.

Thirdly, we also investigated the neuromodulatory impact of dietary phospholipids on cellular models of cytotoxicity and neurodevelopment. Using the same approach utilised previously, the neuroprotective capacity of phospholipids was tested against CORT-induced cytotoxicity in cortical neurons. Indeed, we found that certain phospholipids (PS, PE, PG and PA; see Chapter 4) prevented the cytotoxic effects of

CORT as measured by an MTT assay, and ameliorated the CORT-induced neuronal reduction as determined with immunocytochemistry. However, these phospholipids did not affect the CORT-induced reduction of BDNF mRNA levels. In addition, it was demonstrated that PS positively regulated the proliferation in hippocampal NPCs by increasing the size of neurospheres (free-floating clusters of NPCs). On the other hand, it was observed that only PE induced significant astrocytic differentiation in NPCs.

Finally, the behavioural effects of a phospholipid-enriched food were investigated in a mouse model of psychosocial stress. After repeated bouts of social defeat, mice developed an anxiety- and depressive-like phenotype. To examine the potential therapeutic properties of phospholipids, behaviours associated with sociability, anxiety and depression were analysed. Phospholipid supplementation was demonstrated to have anxiolytic potential against chronic stress as assessed in behavioural tests linked to anxiety-like phenotype. However, this treatment did not attenuate the chronic stress-induced depressive-like behaviour observed in mice, and it could not restore normal HPA axis activation after an acute stress.

The studies outlined in this thesis advance our understanding of the role of nutrition, and more precisely, the impact of dietary polyphenols and phospholipids as future therapeutic strategies against stress-related neuropsychiatric disorders.

6.2 Polyphenols and phospholipids as modulators of neuronal function *in vitro*: A cellular convergence for nutritional anti-stress interventions?

In chapters 2 and 4, we investigated the neuroprotective capacity of polyphenols and phospholipids against CORT-induced neurotoxicity in cells derived from brain cortex. CORT is the main stress hormone in rodents, and increasing levels of CORT have been associated with depressive- and anxiety-like behaviours in these animals (Rosa *et al.* 2014, Mendez-David *et al.* 2017). For this reason, long-term exposure of CORT to neurons represents an interesting *in vitro* approach to examine the cellular and molecular implications of chronic stress in neuronal biology (Gao *et al.* 2015, Zhao *et al.* 2018). Indeed, we found that CORT produced significant neurotoxicity in cortical cells. Using this approach, we demonstrated for the first time that treatment with

polyphenolic compounds, xanthohumol and quercetin (chapter 2), and phospholipids, in particular PS, PE, PG and PA (chapter 4), were able to prevent CORT-induced neurotoxicity in primary cortical cells.

Notably, although we found that both treatments were capable of protecting cortical cells against CORT insult, their mechanisms of neuroprotection did not converge. Indeed, our data indicate that while polyphenols reversed the CORT-induced reduction in BDNF levels, phospholipids did not require restoration of BDNF to protect cortical cells. Other studies have suggested neuronal pro-survival mechanisms independent of BDNF (Fujita *et al.* 2002, Choudhury *et al.* 2016). This divergence could also be associated with the fact that polyphenols attenuate CORT-induced astrogliosis, or abnormal increase of astrocytes, in cortical cultures, and phospholipids only prevented CORT-induced reduction of neurons but not attenuate astrocytic increase. Although, in this thesis the signalling pathways linked to polyphenol-mediated neuroprotection were investigated in chapter 2, future work is needed to elucidate the mechanisms involved in the neuroprotective mechanism of phospholipids.

Nevertheless, the clinical implications of this anti-stress convergence of polyphenols and phospholipids are highly promising, since both types of compounds display different mechanism which can be suitable for the treatment of different phenotypes of stress-related neuropsychiatric disorders. Indeed, mental disorders associated with chronic stress, including depression and anxiety, represent a clinical challenge due to the complexity of their aetiology (Biaggi *et al.* 2016, Menard *et al.* 2016, Read *et al.* 2017). Moreover, considering that both polyphenols and phospholipids have biochemical features that allow them to cross the blood-brain-barrier (Fong 2015, Glade *et al.* 2015, Figueira *et al.* 2017), their neuroprotective capacity *in vitro* represent an attractive and plausible strategy to design nutritional therapies to treat stress-related disorders. Thus, in chapter 3 and 5 we aimed to investigate the therapeutic potential of polyphenols and phospholipids in animal models of stress.

6.3 Polyphenols as nutritional strategies for stress-related disorders: Promises and challenges

It is well known that dietary habits have a profound impact on mental health, and for this reason there is increasing interest in discovering novel nutritional approaches for the prevention and treatment of stress-related neuropsychiatric disorders (Jacka *et al.* 2014, Adan *et al.* 2019). Moreover, there is evidence that early-life stress events are an important risk factor for the development of depression and anxiety (Lupien *et al.* 2009, O'Mahony *et al.* 2011). Therefore, in chapter 3 we decided to investigate the therapeutic potential of naturally-derived polyphenols used against the detrimental effects of a rat maternal separation model of early life stress.

This model is an excellent way to study the negative effects of early-life stress, since it has been demonstrated to induce a robust depressive phenotype in adult animals, including changes in gut microbiota composition, dysregulated the HPA axis, and alteration of neurotransmitter levels in the brain (Daniels *et al.* 2004, O'Mahony *et al.* 2009, Liao *et al.* 2019). In this study, we confirmed that maternally separated rats displayed a depressive- and anxiety-like phenotype, which is in line with previous studies (Prut *et al.* 2003, Aisa *et al.* 2007, Desbonnet *et al.* 2010).

In chapter 2 we detected a promising anti-stress capability of polyphenols *in vitro*. However, the exploration of the multisystemic implications of dietary interventions with polyphenolic-enriched foods is necessary to support their therapeutic potential. Therefore, to test the antidepressant and anxiolytic effects of polyphenols, we performed a dietary intervention with 3 different compounds, including xanthohumol, quercetin and phlorotannins. Interestingly, quercetin and phlorotannins attenuated depressive-like behaviours as shown in the FST, suggesting that these polyphenols have potential antidepressant properties. Similarly, intervention with all polyphenolic compounds produced potential anxiolytic effects in maternally separated animals.

In addition, our data confirm that the exacerbated production of CORT in plasma present in maternally separated rats is significantly reversed by treatment with xanthohumol, suggesting a re-normalisation of the HPA axis. These results would correlate with the apparent rescue of early life stress-induced BDNF reduction in

plasma –as long as this consequence would be found statistically significant– suggesting that the improvement of the stress-related phenotype caused by polyphenols may be mediated by BDNF restoration. However, future studies should address the role of BDNF in the beneficial effects of polyphenols, by determining the levels of this neurotrophic factor not only in plasma but also in brain regions at transcriptomic and proteomic level. In this way, the findings of this preclinical investigation would match with the *in vitro* results presented in chapter 2, giving novel insights into the potential mechanism involved in polyphenol-mediated neuroprotection in cortical cells against stress.

On the other hand, more questions about the mechanisms involved in the protective capacity of polyphenols *in vivo* remain unanswered. For instance, we demonstrated in chapter 2 that polyphenol-driven restoration of BDNF in cortical neurons exposed to CORT may be mediated by activation of the Nrf2 pathway (key nuclear factor involved in antioxidant and cytoprotective cellular response) and modulation of the glucocorticoid receptor sensitivity (Kansanen *et al.* 2013, Mendez-David *et al.* 2015). We did not examine the therapeutic implications of activation of these signalling pathways and BDNF restoration produced polyphenols in chapter 3, therefore whether polyphenols used similar mechanisms observed *in vitro* remains unclear.

In this regard, the role of the Nrf2 signalling in mood disorders has been strongly considered as target for the treatment of depression (Hashimoto 2018). Similarly, FKBP5 appears to modulate connectivity deficits in stress related disorders, representing another potential target for the development of therapeutic approaches (O'Leary *et al.* 2013, de Castro-Catala *et al.* 2017). Future studies should evaluate the implications of these signalling pathways in the antidepressant and anxiolytic effects of polyphenols in animal models of early life stress. Transcriptomic and proteomic analysis of Nrf2 and FKBP5 expression in relevant brain areas sensitive to stress, such as prefrontal cortex and hippocampus (Kim *et al.* 2015, Czeh *et al.* 2018), may help to elucidate and understand the mechanisms underlying polyphenol-mediated improvements of depressive-like behaviour caused after early life stress exposure.

Taken together, in chapter 3 we demonstrated that certain polyphenols were able to alleviate depressive- and anxiety-like behaviours. These data correlate with the findings observed in chapter 2, where xanthohumol protected cortical neurons from CORT-induced neurotoxicity and BDNF downregulation, tempting speculation that similar anti-stress mechanism are being activated during xanthohumol treatment *in vitro* and *in vivo*. However, in order to support therapeutic alternatives based in dietary interventions with polyphenols, further investigation must address the potential pharmacological mechanisms of polyphenols in the brain, and their interactions with other physiological systems such as immunity, metabolism and the microbiota-gut-brain axis.

6.4 Phospholipids in models of stress: Highlights and hurdles

Previously, we showed in chapter 4 that phospholipid treatment *in vitro* improved important neuronal function associated with neurodevelopment, including proliferation and differentiation of neural progenitor cells, as well as neuroprotection under stress conditions. Indeed, increased hippocampal neural progenitor cell proliferation was highly associated with neurogenesis (Pino *et al.* 2017), the process where new-born neurons are produced (Cameron *et al.* 2015). Although there is still controversy and debate around the concept of adult hippocampal neurogenesis (Lee *et al.* 2018), a relationship between anti-depression and neurogenesis has been observed. Indeed, hippocampal neurogenesis appears to be reduced in stress-related conditions, and some antidepressant treatments are able to improve neurogenesis in pre-clinical models (Mahar *et al.* 2014, Krzak *et al.* 2017, Sun *et al.* 2017, Micheli *et al.* 2018).

Therefore, given the promising effects of phospholipids against stress observed *in vitro*, in chapter 5 we hypothesised that a diet enriched in phospholipids may ameliorate the detrimental effects on behaviour in a mouse model of psychosocial stress. However, we did not detect significant improvements in the anxiety-like phenotype of psychosocially stressed animals after supplementation with phospholipids, which is contrary to what we observed in chapters 2 and 3. The reason may be linked to cofounding factors involving metabolic changes. Indeed, in chapter

5 we found that a phospholipid-enriched diet significantly increased the body weight of mice. Accumulating evidence suggests that high fat diet-induced obesity facilitates stress-related behaviours in rats (de Noronha *et al.* 2017). Moreover, clinical studies have associated obesity and anxiety symptoms with unregulated HPA axis (Rajan *et al.* 2017, Amiri *et al.* 2019). Therefore, the therapeutic potential of dietary phospholipids in stress-related disorders could be abolished by this increased body weight and obesity phenotype caused by phospholipid intake.

Despite the promising effects of phospholipids observed *in vitro*, these dietary compounds seem to interact with metabolic pathways producing significant an increase in body weight and potential aggravation of the stress-related phenotype in a mouse model of psychosocial stress. Future work is needed to harness the potential of phospholipids *in vivo* without negatively impacting host metabolism. Perhaps, phospholipid supplementation could be tested in stress-related disorders that are accompanied by symptoms of cachexia or sarcopenia, syndromes associated with weight loss due to inappropriate nutrition (Evans *et al.* 2008). Thus, animal models of cachexia and sarcopenia (Ishida *et al.* 2017) tested in a chronic stress paradigm could help to elucidate the potential therapeutic role of dietary phospholipids in both mental health and metabolism.

6.5 The microbiota-gut-brain axis as target for nutritional treatments against stress-related disorders

Given the increasing evidence suggesting a bi-directional communication between the CNS and the gut microbiota (Cryan *et al.* 2019), the relationship with stress (and stress-related mental disorders) has gained a lot of attention. Indeed, novel insights into the regulation of the stress response by the microbiome (Foster *et al.* 2017) highlight its potential implication for the development of new strategies to treat neuropsychiatric disorders associated with chronic stress. For these reasons, and considering the therapeutic potential of polyphenolic dietary interventions observed in chapter 3, we hypothesised whether polyphenol-mediated improvement of depressive- and anxiety-like behaviours were associated with changes in the gut microbiota.

In our work outlined in chapter 3 we confirmed that our model of early-life stress was able to significantly impact composition and diversity of the gut microbiota, which is in line with previous reports (O'Mahony *et al.* 2009, Moussaoui *et al.* 2017). Specifically, although we did not detect significant changes at the α-diversity level comparing the maternal separation and non-separation control groups, we found that phlorotannin treatment induced a decrease in the Simpson index of diversity. In contrast, analysis of β-diversity revealed that maternal separation control group is significantly different from the non-separation control group, suggesting that early-life stress alone is sufficient to induce long-lasting changes in the gut microbiota. Moreover, all groups of maternally separated animals treated with polyphenols differ from the maternal separation control group. We followed this up by assessing differential abundance of bacterial genera between treatment groups. Notably, only xanthohumol and quercetin treatments produced significant changes in some bacterial genera in maternally separated rats, suggesting that some polyphenol-enriched diets have the potential to modify bacterial composition in the gastrointestinal system. Several studies have demonstrated the capacity of polyphenolic intake to shape the gut microbiota (Etxeberria *et al.* 2015, Ozdal *et al.* 2016). The fact that all types of polyphenol intake were found to alter β-diversity compared to the maternal separation control, but only xanthohumol and quercetin yielded differences in the abundances of specific genera, which may suggest that polyphenols induce a general shift in the microbial composition, that may be indicative of a change in functionality in the microbiome.

Therefore, we performed a functional prediction of the gut metagenome and used this to infer the abundance of gut-brain modules (GBMs), metabolic modules that are involved in the microbiota-gut-brain axis (Valles-Colomer *et al.* 2019). Indeed, the analysis predicted that maternal separation is able to increase the abundance of GBMs associated with the modulation of several pathways altered in depression and other neuropsychiatric disorders, including metabolism of tryptophan (Curzon *et al.* 1970, Oxenkrug 2010), inositol (Coupland *et al.* 2005), p-cresol (Persico *et al.* 2013), quinolinic acid (Steiner *et al.* 2011), nitric oxide (Dhir *et al.* 2011), and glutamate (Sanacora *et al.* 2012, Murrough *et al.* 2017). Interestingly, treatment with xanthohumol and phlorotannins reversed these predicted maternal separation-induced

changes, suggesting that restoration of these GBMs may partially explain their positive effects in behaviour. An important limitation due to the nature of 16S sequencing is that functional analysis can only be inferential. Future metabolomics-based studies should address this experimentally.

In addition, our data revealed that maternally separated rats exhibited decreased production of gut microbial metabolites compared with the non-separation group. In particular, we detected a significant reduction of acetate, propionate, isobutyrate, and isovalerate in rats subjected to maternal separation. The production of SCFAs is highly associated with certain bacterial populations in the gut, and there is common agreement surrounding the impact of SCFAs on human metabolism and health (Morrison *et al.* 2016). Indeed, it is widely accepted that SCFAs play a critical role in gut-microbiota-brain communication, and consequences for mental health and behaviour (Stilling *et al.* 2016, Dalile *et al.* 2019). In chapter 3, we demonstrated that treatment with xanthohumol specifically prevented the reduction of propionate in maternally separated rats. Since the xanthohumol diet intervention induced acute changes in bacterial composition of the gut microbiota of maternally separated rats, we hypothesise that the changes observed in propionate levels could be a product of improved gut microbial metabolism.

Taken together, the work outlined in this chapter confirms that dietary interventions based in polyphenols have the capacity to modulate the gut microbiota in terms of composition and diversity, which demonstrates their potential to target the microbiota-gut-brain axis. Indeed, we detected potential Gut-brain pathways altered in maternally separated animals that were restored by treatment with xanthohumol and quercetin as inferred in the functional prediction analysis. Moreover, we detected that maternal separation-induced deficiency of SCFA production was reversed by xanthohumol, suggesting a potential mechanism underlying polyphenol-mediated modulation of the microbiota-gut-brain axis.

6.5.1 Do we require the microbiome to exert effects of nutritional interventions?

The therapeutic potential of dietary polyphenols against stress-related disorders observed in chapter 3 was discussed in section 6.3. Considering the findings from a cellular model of stress in chapter 2, we speculated that the mechanisms underlying mental health benefits of polyphenols might be mediated locally in the brain considering their capacity to cross the blood-brain-barrier and activate anti-stress intracellular pathways. Further, in chapter 3 we explored the implications of the microbiota-gut-brain axis in polyphenol-mediated improvement of depressive-like phenotype in rats subjected to early life stress. Indeed, we demonstrated that polyphenol supplementation induced profound changes in gut microbiota composition and diversity. Moreover, polyphenols reversed the abundance of bacteria associated with metabolic pathways related to gut-brain communication (see section 6.5). Therefore, these findings raise an interesting question about the mechanisms involved in polyphenol mental health benefits; do we require the microbiome to exert beneficial effects of nutritional intervention with polyphenols?

To answer this, future investigations should use models of depleted microbiota such as germ-free and antibiotic treated animals. For instance, germ-free animal models represent an interesting approach to assess the influence of the gut microbiota on brain and behaviour (Luczynski *et al.* 2016, Hoban *et al.* 2018). Also models of antibiotic-induced microbiota depletion in animals allow one to study the implications of a depleted microbiota on brain and behaviour across different stages of life (Desbonnet *et al.* 2015). Thus, looking at the behavioural responses of animals with depleted microbiota under conditions of chronic stress and dietary supplementation with polyphenols may help to elucidate the role of the microbiome in this effect.

6.6 Future directions into polyphenols and phospholipids dietary interventions

In view of the evidence highlighted by this thesis, naturally-derived polyphenols have shown protective effects against stress in cellular and animal models. Thereby, this finding raises the possibility that polyphenol supplementation may represent a

potential strategy to reduce the risk of development of psychopathologies linked to stress-related mental disorders. However, further studies are required to elucidate the mechanisms underlying polyphenol-mediated improvements in behavior and physiology in models of early-life stress. On the other hand, the work outlined in chapter 4 and 5 show that the neuromodulatory potential exerted by phospholipids *in vitro* did not translate to an animal model, with the inefficacy to reverse the anxiety-like phenotype and dysregulated HPA axis produced in a mouse model of chronic psychosocial stress. Thus, future pre-clinical investigations should address alternative phospholipid dose and treatment duration in order to enhance its therapeutic effect against chronic psychosocial stress.

Additional research is required to understand the potential mechanisms involved in the antidepressant and anxiolytic actions of polyphenols; our results obtained from *in vitro* assays in chapter 2 highlight a possible interaction with the Nrf2 and the FKBP5/GR signalling pathways, which may be considered as an explanation for our behavioural findings. Moreover, we have demonstrated that treatment with xanthohumol is capable of preventing CORT-induced downregulation of *Bdnf* expression in cortical cells (Chapter 2). Intriguingly, we observed a similar effect in our *in vivo* study, where dietary intervention with xanthohumol seems to rescue BDNF levels in plasma of animals subjected to early-life stress (Chapter 3). BDNF has strongly been implicated in antidepressant activity, and plasma BDNF has been shown to reflect aspects of that centrally and to be a biomarker of antidepressant effect (Sen *et al.* 2008, Lee *et al.* 2010). Although the possible pathways involved in BDNF rescue must be further investigated, it is tempting to speculate that the positive effects of xanthohumol on behaviour could be partly mediated by normalising BDNF expression. In addition, we have observed profound changes in gut microbiota composition and diversity in rats treated with xanthohumol and phlorotannins, which correlate with predicted restoration of metabolic pathways associated with gut-microbiota communication (Chapter 3). Further studies are needed to experimentally evaluate the role of the microbiota-gut-brain axis in the therapeutic effect of dietary polyphenols against early-life stress induced changes in behaviour.

We also found that phospholipid supplementation was not capable of preventing the anxiety-like phenotype produced in a mouse chronic psychosocial stress paradigm (Chapter 5). Many questions are thus raised: were the source, dose and duration of the treatment appropriate for this dietary intervention? In addition, the novel neuromodulatory effects exerted by phospholipids in neuronal *in vitro* models in terms of NPC proliferation and differentiation (Chapter 4), suggest that the implications of phospholipid nutritional impact on behavior may be crucial in early-life stages, where neurodevelopmental processes are still critical. Future investigation should address the impact of phospholipid supplementation in early-life, such as maternal and post-weaning dietary interventions, and their protective role against stress in different life stages.

Some aspects of behaviour were not examined in mice subjected to chronic psychosocial stress, where the impact of dietary phospholipids would be interesting to investigate. For instance, it is well known that chronic stress is able to induce cognitive impairment, including disruption of attention, memory and executive functions (Iosifescu 2012, Fjell *et al.* 2014). In this regard, clinical and pre-clinical evidence suggest a potential therapeutic effect of dietary phospholipids against chronic stress-induced cognitive impairment (Kanno *et al.* 2014, Boyle *et al.* 2019, O'Mahony *et al.* 2019). However, the therapeutic potential of dietary phospholipids, and more specifically those derived from dairy products, has not been investigated in animal models of psychosocial stress. Thus, further investigation into the protective role of dietary phospholipids against psychosocial stress-induced cognitive deficits is needed.

Finally, considering the role of dietary polyphenols in attenuating depressive- and anxiety-like phenotype in rats, the implications for interventions using these natural compounds for the treatment of stress-related disorders in humans, especially for those associated with early-life trauma, have evident therapeutic potential. Previously, we highlighted the need to find novel approaches for the prevention and attenuation of symptoms linked with stress-related neuropsychiatric disorders such as depression, since available strategies are not effective in all cases, and usually are accompanied by negative side effect. Therefore, clinical trials should investigate the effects of

polyphenol-enriched diets as alternative strategies for the treatment of patients diagnosed with depression and anxiety.

6.7 Conclusions

In this thesis, we have conducted research which has advanced our understanding about the neurobiological effects of naturally-derived polyphenols and phospholipids in cellular and animal models of stress. Indeed, this piece of work involves important implications for polyphenols and phospholipids as therapeutic strategies able to treat and reduce the risk of development of stress-related neuropsychiatric disorders. To our knowledge, this work shows for the first time the beneficial effects of polyphenols against the behavioural and physiological changes produced in a rat model of early-life stress. Further, this thesis emphasises the *in vitro* implications of polyphenols in neuronal mechanisms associated with stress response, survival and plasticity, which could have potential role in polyphenol-mediated improvements in mental health. Moreover, we showed novel insights into the interaction between polyphenols and the gut microbiota, highlighting important implications for the microbiota-gut-brain axis as therapeutic target against stress-related disorders. Nevertheless, in order to build on this work, further investigation is needed to address this experimentally.

On the other hand, although not significant, we demonstrated that treatment with a phospholipid-enriched diet provisionally shows subtle improvements in the anxiety-like phenotype produced in a mouse psychosocial stress paradigm. However, the modulatory effects of phospholipids in neurodevelopmental processes detected *in vitro*, undoubtedly open up for further investigation focused on the nutritional impact of phospholipids at different life stages where brain development could be affected. Indeed, alternative timelines and behavioural outcomes should be considered in future studies for a complete elucidation of the neurobehavioural effects of phospholipids in animal models of stress.

Taken together, our findings demonstrate that naturally-derived polyphenols may represent a potential strategy for the prevention and treatment of stress-related mental disorders. In contrast, more research is required for a better understanding of the therapeutic potential of phospholipid supplementations in stress-related mental

disorders. Thus, this thesis represents a detailed and well-characterised portfolio of pre-clinical studies focused on the neurobiological effects of polyphenols and phospholipids in cellular and animal models of stress, representing an appropriate source of knowledge crucial for human clinical studies investigating novel approaches against stress-related neuropsychiatric disorders.

Appendix

Chapter 2: Supplementary Information

Table S1. List and detail of antibodies for RPPA used by MD Anderson Center

Antibody Name	Origin*	Gene Name	Validation Status*	Slide Id
14-3-3-beta	R	YWHAB	V	GBL1144582
14-3-3-zeta	R	YWHAZ	V	GBL1144583
4E-BP1	R	EIF4EBP1	V	GBL1144348
4E-BP1_pS65	R	EIF4EBP1	V	GBL1144349
53BP1	R	TP53BP1	V	GBL1144437
A-Raf	R	ARAF	V	GBL1144487
ACC1	R	ACACA/ ACACB	C	GBL1144351
ACC_pS79	R	ACACA/ACACB	V	GBL1144350
Akt	R	AKT1/2/3	V	GBL1144660
Akt_pS473	R	AKT1/2/3	V	GBL1144374
Akt_pT308	R	AKT1/2/3	V	GBL1144474
AMPK-a2_pS345	R	PRKAA2	V	GBL1144504
AMPKa	R	PRKAA1/2	C	GBL1144352
AMPKa_pT172	R	PRKAA1/2	C	GBL1144353
AR	R	AR	V	GBL1144565
ARID1A	R	ARID1A	C	GBL1144515
Atg3	R	ATG3	V	GBL1144523
Atg7	R	ATG7	V	GBL1144524
ATM	R	ATM	V	GBL1144506
ATM_pS1981	R	ATM	V	GBL1144507
ATR_pS428	R	ATR	C	GBL1144546
Aurora-B	R	AURKB	V	GBL1144512
Axl	R	AXL	V	GBL1144497
b-Actin	R	ACTB	C	GBL1144480
b-Catenin	R	CTNNB1	V	GBL1144357
b-Catenin_pT41_S45	R	CTNNB1	V	GBL1144481
B-Raf	R	BRAF	C	GBL1144559
B-Raf_pS445	R	BRAF	V	GBL1144361
B7-H4	R	VTCN1	C	GBL1144541
Bad_pS112	R	BAD	V	GBL1144354
Bak	R	BAK1	C	GBL1144355
Bax	R	BAX	V	GBL1144356
Bcl-xL	R	BCL2L1	V	GBL1144358
Beclin	R	BECN1	C	GBL1144591
Bid	R	BID	C	GBL1144359
Bim	R	BCL2L11	V	GBL1144360
BRD4	R	BRD4	V	GBL1144519
c-Abl	R	ABL1	V	GBL1144518

c-IAP2	R	BIRC3	C	GBL1144525
c-Jun_pS73	R	JUN	V	GBL1144367
c-Kit	R	KIT	V	GBL1144670
c-Met_pY1234_Y1235	R	MET	V	GBL1144416
c-Myc	R	MYC	C	GBL1144585
C-Raf	R	RAF1	C	GBL1144485
C-Raf_pS338	R	RAF1	V	GBL1144369
Caspase-3	R	CASP3	C	GBL1144362
Caspase-7-cleaved-	R	CASP7	C	GBL1144363
Caveolin-1	R	CAV1	V	GBL1144364
CD134	R	TNFRSF4	V	GBL1144561
CD20	R	MS4A1	C	GBL1144341
CD4	R	CD4	V	GBL1144563
cdc25C	R	CDC25C	V	GBL1144555
cdc2_pY15	R	CDK1	C	GBL1144545
Cdc6	R	CDC6	V	GBL1144654
CDK1_pT14	R	CDK1/2/3	C	GBL1144534
CDT1	R	CDT1	V	GBL1144655
Chk1_pS296	R	CHEK1	V	GBL1144503
Chk2_pT68	R	CHEK2	C	GBL1144366
Claudin-7	R	CLDN7	V	GBL1144566
COG3	R	COG3	V	GBL1144533
Collagen-VI	R	COL6A1	V	GBL1144577
Connexin-43	R	GJA1	C	GBL1144520
Cox-IV	R	COX4I1	V	GBL1144343
Cox2	R	PTGS2	C	GBL1144488
Creb	R	CREB1	C	GBL1144370
Cyclin-B1	R	CCNB1	V	GBL1144371
Cyclin-D1	R	CCND1	C	GBL1144578
D-a-Tubulin	R	TUBA4A/TUBA3C	V	GBL1144510
DJ1	R	PARK7	V	GBL1144430
DM-Histone-H3	R	HIST1H3A	V	GBL1144568
DUSP4	R	DUSP4	V	GBL1144513
E-Cadherin	R	CDH1	V	GBL1144673
eEF2	R	EEF2	C	GBL1144447
eEF2K	R	EEF2K	V	GBL1144448
EGFR	R	EGFR	V	GBL1144674
EGFR_pY1173	R	EGFR	V	GBL1144372
eIF4E	R	EIF4E	V	GBL1144415
eIF4E_pS209	R	EIF4E	V	GBL1144554
eIF4G	R	EIF4G1	C	GBL1144663
Elk1_pS383	R	ELK1	C	GBL1144373
ER	R	ESR1	V	GBL1144579

ER-a_pS118	R	ESR1	V	GBL1144375
ERCC5	R	ERCC5	C	GBL1144505
Ets-1	R	ETS1	V	GBL1144586
FAK	R	PTK2	C	GBL1144376
FAK_pY397	R	PTK2	V	GBL1144491
FASN	R	FASN	V	GBL1144475
Fibronectin	R	FN1	V	GBL1144377
FOXM1	R	FOXM1	V	GBL1144344
FOXO3	R	FOXO3	V	GBL1144567
FoxO3a_pS318_S321	R	FOXO3	C	GBL1144378
G6PD	R	G6PD	V	GBL1144544
Gab2	R	GAB2	V	GBL1144435
GATA6	R	GATA6	V	GBL1144666
GCLM	R	GCLM	C	GBL1144542
GCN5L2	R	KAT2A	V	GBL1144495
Glutamate-D1-2	R	GLUD1	V	GBL1144575
Glutaminase	R	GLS	C	GBL1144517
Granzyme-B	R	GZMB	V	GBL1144549
GSK-3a-b_pS21_S9	R	GSK3A/GSK3B	V	GBL1144379
Gys	R	GYS1	V	GBL1144444
Gys_pS641	R	GYS1	V	GBL1144445
HER2_pY1248	R	ERBB2	C	GBL1144452
HER3	R	ERBB3	V	GBL1144584
HER3_pY1289	R	ERBB3	C	GBL1144417
Heregulin	R	NRG1	V	GBL1144429
HES1	R	HES1	V	GBL1144521
Hexokinase-II	R	HK2	V	GBL1144570
Histone-H3	R	HIST3H3	V	GBL1144494
HSP27_pS82	R	HSBP1	V	GBL1144380
HSP70	R	HSPA1A	C	GBL1144381
IGF1R_pY1135_Y1136	R	IGF1R/INSR	V	GBL1144489
IGFBP2	R	IGFBP2	V	GBL1144382
IGFRb	R	IGF1R	C	GBL1144383
INPP4b	R	INPP4B	V	GBL1144451
IR-b	R	INSR	C	GBL1144522
IRF-1	R	IRF1	C	GBL1144587
IRS1	R	IRS1	V	GBL1144423
Jagged1	R	JAG1	V	GBL1144514
Jak2	R	JAK2	V	GBL1144479
JNK2	R	MAPK9	C	GBL1144384
JNK_pT183_Y185	R	MAPK8	V	GBL1144428
LC3A-B	R	MAP1LC3A/B	C	GBL1144526
Lck	R	LCK	V	GBL1144385

LDHA	R	LDHA	C	GBL1144436
LRP6_pS1490	R	LRP6	V	GBL1144508
MAPK_pT202_Y204	R	MAPK1/MAPK3	V	GBL1144386
Mcl-1	R	MCL1	V	GBL1144490
MCT4	R	SLC16A3	V	GBL1144532
MDM2_pS166	R	MDM2	V	GBL1144478
MEK1	R	MAP2K1	V	GBL1144387
MEK1_p_S217-S221	R	MAP2K1/MAP2K1	V	GBL1144453
MERIT40_pS29	R	BABAM1	V	GBL1144556
Merlin	R	NF2	C	GBL1144446
MIF	R	MIF	C	GBL1144588
MMP2	R	MMP2	V	GBL1144388
Mnk1	R	MKNK1	V	GBL1144438
MSH6	R	MSH6	C	GBL1144449
MSI2	R	MSI2	C	GBL1144539
mTOR	R	MTOR	V	GBL1144389
mTOR_pS2448	R	MTOR	C	GBL1144390
MYH11	R	MYH11	C	GBL1144576
Myosin-IIa_pS1943	R	MYH9	V	GBL1144477
Myt1	R	PKMYT1	C	GBL1144548
N-Cadherin	R	CDH2	V	GBL1144672
NAPSIN-A	R	NAPSA	C	GBL1144498
NDRG1_pT346	R	NDRG1	V	GBL1144679
NF-kB-p65_pS536	R	RELA	C	GBL1144392
Notch1	R	NOTCH1	V	GBL1144450
Notch3	R	NOTCH3	C	GBL1144580
Oct-4	R	POU5F1	C	GBL1144537
P-Cadherin	R	CDH3	C	GBL1144399
p16INK4a	R	CDKN2A	V	GBL1144492
p21	R	CDKN1A	C	GBL1144573
p27-Kip1	R	CDKN1B	V	GBL1144431
p27_pT198	R	CDKN1B	V	GBL1144427
p38-MAPK	R	MAPK14/11/12	V	GBL1144393
p38_pT180_Y182	R	MAPK11/12/13/14	V	GBL1144394
p44-42-MAPK	R	MAPK1/MAPK3	V	GBL1144662
p53	R	TP53	C	GBL1144395
p70-S6K1	R	RPS6KB1	V	GBL1144396
p70-S6K_pT389	R	RPS6KB1	V	GBL1144397
p90RSK_pT573	R	RPS6K	C	GBL1144482
PAICS	R	PAICS	C	GBL1144500
PAK1	R	PAK1	V	GBL1144550
PAK4	R	PAK4	V	GBL1144511
PAR	R	PAR	C	GBL1144509

PARP	R	PARP1	V	GBL1144564
Paxillin	R	PXN	C	GBL1144398
PD-L1	R	CD274	C	GBL1144540
Pdcd4	R	PDCD4	C	GBL1144425
PDGFR-b	R	PDGFRB	V	GBL1144346
PDHK1	R	PDHK1	C	GBL1144527
PDK1	R	PDPK1	V	GBL1144400
PDK1_pS241	R	PDPK1	V	GBL1144401
PEA-15	R	PEA15	V	GBL1144439
PEA-15_pS116	R	PEA15	V	GBL1144440
PI3K-p110-a	R	PIK3CA	C	GBL1144424
PI3K-p85	R	PIK3R1	V	GBL1144402
PKA-a	R	PRKAR1A	V	GBL1144536
PKC-b-II_pS660	R	PRKCA/B/D/E/H/Q	V	GBL1144470
PKC-delta_pS664	R	PRKCD	V	GBL1144434
PKCa	R	PRKCA	V	GBL1144476
PKM2	R	PKM	C	GBL1144441
PLC-gamma2_pY759	R	PLCG2	C	GBL1144442
PLK1	R	PLK1	C	GBL1144419
PMS2	R	PMS2	V	GBL1144493
PR	R	PGR	V	GBL1144665
PRAS40_pT246	R	AKT1S1	V	GBL1144418
PREX1	R	PREX1	V	GBL1144486
PTEN	R	PTEN	V	GBL1144404
Rab11	R	RAB11A/B	C	GBL1144342
Rab25	R	RAB25	V	GBL1144473
Rad50	R	RAD50	V	GBL1144516
Rad51	R	RAD51	C	GBL1144590
Raptor	R	RPTOR	V	GBL1144467
RBM15	R	RBM15	V	GBL1144471
Rb_pS807_S811	R	RB1	V	GBL1144403
Rictor	R	RICTOR	C	GBL1144468
Rictor_pT1135	R	RICTOR	V	GBL1144469
RIP	R	RIP	C	GBL1144528
RPA32_pS4_S8	R	RPA2	C	GBL1144589
RSK	R	RPS6KA1/2/3	C	GBL1144420
S6_pS235_S236	R	RPS6	V	GBL1144405
S6_pS240_S244	R	RPS6	V	GBL1144406
SDHA	R	SDHA	V	GBL1144502
Shc_pY317	R	SHC1	V	GBL1144443
SHP-2_pY542	R	PTPN11	C	GBL1144483
SHP2	R	PTPN11	V	GBL1144652
SLC1A5	R	SLC1A5	C	GBL1144499

Slfn11	G	SLFN11	C	GBL1144340
Smad1	R	SMAD1	V	GBL1144433
Smad3	R	SMAD3	V	GBL1144422
SOD2	R	SOD2	V	GBL1144569
Sox2	R	SOX2	V	GBL1144538
Src_pY416	R	SRC	V	GBL1144407
Src_pY527	R	SRC	V	GBL1144408
Stat3	R	STAT3	C	GBL1144484
Stat3_pY705	R	STAT3	V	GBL1144562
Stat5a	R	STAT5A	V	GBL1144409
Stathmin-1	R	STMN1	V	GBL1144414
STING	R	TMEM173	V	GBL1144560
TAZ	R	WWTR1	V	GBL1144553
TFAM	R	TFAM	V	GBL1144501
TFRC	R	TFRC	V	GBL1144472
TIGAR	R	TIGAR	V	GBL1144661
TRIM25	R	TRIM25	C	GBL1144543
TSC1	R	TSC1	C	GBL1144664
TTF1	R	NKX2-1	V	GBL1144659
Tuberin	R	TSC2	V	GBL1144410
Tuberin_pT1462	R	TSC2	V	GBL1144411
TUFM	R	TUFM	V	GBL1144552
Tyro3	R	TYRO3	V	GBL1144454
U-Histone-H2B	R	HIST1H2BB	C	GBL1144535
UBAC1	R	UBAC1	V	GBL1144496
ULK1_pS757	R	ULK1	C	GBL1144529
VASP	R	VASP	V	GBL1144412
VEGFR-2	R	KDR	V	GBL1144413
Wee1	R	WEE1	C	GBL1144547
Wee1_pS642	R	WEE1	C	GBL1144558
WIPI1	R	WIPI1	C	GBL1144530
WIPI2	R	WIPI2	C	GBL1144531
XBP-1	G	XBP1	C	GBL1144339
XPF	R	ERCC4	C	GBL1144557
XRCC1	R	XRCC1	C	GBL1144432
YAP	R	YAP1	C	GBL1144581
YAP_pS127	R	YAP1	V	GBL1144421
YB1_pS102	R	YBX1	V	GBL1144426
ZAP-70	R	ZAP70	C	GBL1144551

*V = Validated antibody; C = Validation in progress; R = Rabbit antibody; G = Goat antibody

References

aan het Rot, M., *et al.* (2009). "Neurobiological mechanisms in major depressive disorder." CMAJ **180**(3): 305-313.

Abe, H., *et al.* (2007). "Prenatal psychological stress causes higher emotionality, depression-like behavior, and elevated activity in the hypothalamo-pituitary-adrenal axis." Neurosci Res **59**(2): 145-151.

Abid, K., *et al.* (2005). "An in vitro model of hepatitis C virus genotype 3a-associated triglycerides accumulation." J Hepatol **42**(5): 744-751.

Aboukhatwa, M. A., *et al.* (2010). "Antidepressant stimulation of CDP-diacylglycerol synthesis does not require monoamine reuptake inhibition." BMC Neurosci **11**: 10.

Adan, R. A. H., *et al.* (2019). "Nutritional psychiatry: Towards improving mental health by what you eat." Eur Neuropsychopharmacol.

Ahn, H. S., *et al.* (2017). "Cardioprotective Effects of a Phlorotannin Extract Against Doxorubicin-Induced Cardiotoxicity in a Rat Model." J Med Food **20**(10): 944-950.

Aisa, B., *et al.* (2007). "Cognitive impairment associated to HPA axis hyperactivity after maternal separation in rats." Psychoneuroendocrinology **32**(3): 256-266.

Al-Harbi, K. S. (2012). "Treatment-resistant depression: therapeutic trends, challenges, and future directions." Patient Prefer Adherence **6**: 369-388.

Alessandri, J. M., *et al.* (2004). "Polyunsaturated fatty acids in the central nervous system: evolution of concepts and nutritional implications throughout life." Reprod Nutr Dev **44**(6): 509-538.

Almeida, R. D., *et al.* (2005). "Neuroprotection by BDNF against glutamate-induced apoptotic cell death is mediated by ERK and PI3-kinase pathways." Cell Death Differ **12**(10): 1329-1343.

Amiri, S., *et al.* (2019). "Obesity and anxiety symptoms: a systematic review and meta-analysis." Neuropsychiatr **33**(2): 72-89.

Amrein, R., *et al.* (1993). "RIMA--a new concept in the treatment of depression with moclobemide." Int Clin Psychopharmacol **7**(3-4): 123-132.

An, L., *et al.* (2008). "The total flavonoids extracted from Xiaobuxin-Tang up-regulate the decreased hippocampal neurogenesis and neurotrophic molecules expression in chronically stressed rats." Prog Neuropsychopharmacol Biol Psychiatry **32**(6): 1484-1490.

Anacker, C., *et al.* (2011). "The glucocorticoid receptor: pivot of depression and of antidepressant treatment?" Psychoneuroendocrinology **36**(3): 415-425.

Andre, C., *et al.* (2014). "Diet-induced obesity progressively alters cognition, anxiety-like behavior and lipopolysaccharide-induced depressive-like behavior: focus on brain indoleamine 2,3-dioxygenase activation." Brain Behav Immun **41**: 10-21.

Anjaneyulu, M., *et al.* (2003). "Antidepressant activity of quercetin, a bioflavonoid, in streptozotocin-induced diabetic mice." J Med Food **6**(4): 391-395.

Arakawa, Y., *et al.* (1991). "Neurite-promoting activities of phosphatidylinositol and other lipids on fetal rat septal neurons in culture." J Neurochem **56**(6): 1864-1872.

Arborelius, L., *et al.* (1999). "The role of corticotropin-releasing factor in depression and anxiety disorders." J Endocrinol **160**(1): 1-12.

Arlt, A., *et al.* (2012). "Inhibition of the Nrf2 transcription factor by the alkaloid trigonelline renders pancreatic cancer cells more susceptible to apoptosis through decreased proteasomal gene expression and proteasome activity." Oncogene **32**: 4825.

Arnsten, A. F. (2009). "Stress signalling pathways that impair prefrontal cortex structure and function." Nat Rev Neurosci **10**(6): 410-422.

Arnsten, A. F. (2015). "Stress weakens prefrontal networks: molecular insults to higher cognition." Nat Neurosci **18**(10): 1376-1385.

Arredondo, F., *et al.* (2010). "After cellular internalization, quercetin causes Nrf2 nuclear translocation, increases glutathione levels, and prevents neuronal death against an oxidative insult." Free Radic Biol Med **49**(5): 738-747.

Aubry, A. V., *et al.* (2019). "A diet enriched with curcumin promotes resilience to chronic social defeat stress." Neuropsychopharmacology **44**(4): 733-742.

Aust, S., *et al.* (2019). "Anxiety during ketamine infusions is associated with negative treatment responses in major depressive disorder." Eur Neuropsychopharmacol **29**(4): 529-538.

Bahar, E., *et al.* (2017). "Quercetin Attenuates Manganese-Induced Neuroinflammation by Alleviating Oxidative Stress through Regulation of Apoptosis, iNOS/NF-kappaB and HO-1/Nrf2 Pathways." Int J Mol Sci **18**(9).

Bailey, M. T., *et al.* (1999). "Maternal separation disrupts the integrity of the intestinal microflora in infant rhesus monkeys." Dev Psychobiol **35**(2): 146-155.

Bailey, M. T., *et al.* (2011). "Exposure to a social stressor alters the structure of the intestinal microbiota: implications for stressor-induced immunomodulation." Brain Behav Immun **25**(3): 397-407.

Bajpai, M., *et al.* (2002). "In vitro studies in drug discovery and development: an analysis of study objectives and application of good laboratory practices (GLP)." Drug Metab Rev **34**(4): 679-689.

Barone, R., *et al.* (2019). "Nuclear Peroxisome Proliferator-Activated Receptors (PPARs) as Therapeutic Targets of Resveratrol for Autism Spectrum Disorder." Int J Mol Sci **20**(8).

Bearden, C. E., *et al.* (2006). "Endophenotypes for psychiatric disorders: ready for primetime?" Trends Genet **22**(6): 306-313.

Beitia, G., *et al.* (2005). "Time-dependent behavioral, neurochemical, and immune consequences of repeated experiences of social defeat stress in male mice and the ameliorative effects of fluoxetine." Brain Behav Immun **19**(6): 530-539.

Beneyto, M., *et al.* (2008). "Lamina-specific abnormalities of NMDA receptor-associated postsynaptic protein transcripts in the prefrontal cortex in schizophrenia and bipolar disorder." Neuropsychopharmacology **33**(9): 2175-2186.

Benton, D., *et al.* (2001). "The influence of phosphatidylserine supplementation on mood and heart rate when faced with an acute stressor." Nutr Neurosci **4**(3): 169-178.

Bergamini, G., *et al.* (2016). "Mouse psychosocial stress reduces motivation and cognitive function in operant reward tests: A model for reward pathology with effects of agomelatine." Eur Neuropsychopharmacol **26**(9): 1448-1464.

Berton, O., *et al.* (2006). "New approaches to antidepressant drug discovery: beyond monoamines." Nature Reviews Neuroscience **7**: 137.

Bet, P. M., *et al.* (2013). "Side effects of antidepressants during long-term use in a naturalistic setting." Eur Neuropsychopharmacol **23**(11): 1443-1451.

Bhaskar, K., *et al.* (2009). "The PI3K-Akt-mTOR pathway regulates Abeta oligomer induced neuronal cell cycle events." Mol Neurodegener **4**: 14.

Bhutada, P., *et al.* (2010). "Reversal by quercetin of corticotrophin releasing factor induced anxiety- and depression-like effect in mice." Prog Neuropsychopharmacol Biol Psychiatry **34**(6): 955-960.

Biaggi, A., *et al.* (2016). "Identifying the women at risk of antenatal anxiety and depression: A systematic review." J Affect Disord **191**: 62-77.

Billard, J. M. (2008). "D-serine signalling as a prominent determinant of neuronal-glial dialogue in the healthy and diseased brain." J Cell Mol Med **12**(5B): 1872-1884.

Binder, E. B. (2009). "The role of FKBP5, a co-chaperone of the glucocorticoid receptor in the pathogenesis and therapy of affective and anxiety disorders." Psychoneuroendocrinology **34 Suppl 1**: S186-195.

Birkel, L. (2017). "Decreased use of spatial pattern separation in contemporary lifestyles may contribute to hippocampal atrophy and diminish mental health." Med Hypotheses **107**: 55-63.

Blekhman, R., *et al.* (2015). "Host genetic variation impacts microbiome composition across human body sites." Genome Biol **16**: 191.

Block, W., *et al.* (2009). "Proton MR spectroscopy of the hippocampus at 3 T in patients with unipolar major depressive disorder: correlates and predictors of treatment response." Int J Neuropsychopharmacol **12**(3): 415-422.

Bodnar, L. M., *et al.* (2005). "Nutrition and depression: implications for improving mental health among childbearing-aged women." Biol Psychiatry **58**(9): 679-685.

Boldrini, M., *et al.* (2018). "Human Hippocampal Neurogenesis Persists throughout Aging." Cell Stem Cell **22**(4): 589-599 e585.

Bonni, A., *et al.* (1997). "Regulation of gliogenesis in the central nervous system by the JAK-STAT signaling pathway." Science **278**(5337): 477-483.

Boots, A. W., *et al.* (2008). "Health effects of quercetin: from antioxidant to nutraceutical." Eur J Pharmacol **585**(2-3): 325-337.

Borre, Y. E., *et al.* (2014). "The impact of microbiota on brain and behavior: mechanisms & therapeutic potential." Adv Exp Med Biol **817**: 373-403.

Bouvier, E., *et al.* (2017). "Nrf2-dependent persistent oxidative stress results in stress-induced vulnerability to depression." Mol Psychiatry **22**(12): 1701-1713.

Bowery, N. G., *et al.* (1987). "GABAA and GABAB receptor site distribution in the rat central nervous system." Neuroscience **20**(2): 365-383.

Boyle, N. B., *et al.* (2019). "Effects of milk-based phospholipids on cognitive performance and subjective responses to psychosocial stress: A randomized, double-blind, placebo-controlled trial in high-perfectionist men." Nutrition **57**: 183-193.

Brachman, R. A., *et al.* (2016). "Ketamine as a Prophylactic Against Stress-Induced Depressive-like Behavior." Biol Psychiatry **79**(9): 776-786.

Bravo, J. A., *et al.* (2011). "Ingestion of Lactobacillus strain regulates emotional behavior and central GABA receptor expression in a mouse via the vagus nerve." Proc Natl Acad Sci U S A **108**(38): 16050-16055.

Bravo, L. (1998). "Polyphenols: chemistry, dietary sources, metabolism, and nutritional significance." Nutr Rev **56**(11): 317-333.

Brennand, K. J., *et al.* (2012). "Modeling psychiatric disorders at the cellular and network levels." Mol Psychiatry **17**(12): 1239-1253.

Bridges, N., *et al.* (2008). "The effects of chronic corticosterone on hippocampal astrocyte numbers: a comparison of male and female Wistar rats." Acta Neurobiol Exp (Wars) **68**(2): 131-138.

Brink, L. R., *et al.* (2018). "The role of milk fat globule membranes in behavior and cognitive function using a suckling rat pup supplementation model." J Nutr Biochem **58**: 131-137.

Brites, D., *et al.* (2015). "Neuroinflammation and Depression: Microglia Activation, Extracellular Microvesicles and microRNA Dysregulation." Front Cell Neurosci **9**: 476.

Brito, P. M., *et al.* (2006). "Resveratrol affords protection against peroxynitrite-mediated endothelial cell death: A role for intracellular glutathione." Chem Biol Interact **164**(3): 157-166.

Brown, H. A., *et al.* (2009). "Working towards an exegesis for lipids in biology." Nature Chemical Biology **5**: 602.

Brown, W. A., *et al.* (2015). "The clinical discovery of imipramine." Am J Psychiatry **172**(5): 426-429.

Brunoni, A. R., *et al.* (2008). "A systematic review and meta-analysis of clinical studies on major depression and BDNF levels: implications for the role of neuroplasticity in depression." Int J Neuropsychopharmacol **11**(8): 1169-1180.

Bunney, W. E., Jr., *et al.* (1965). "Norepinephrine in depressive reactions. A review." Arch Gen Psychiatry **13**(6): 483-494.

Burdge, G. C., *et al.* (1995). "Phospholipid molecular species composition of developing fetal guinea pig brain." Lipids **30**(8): 719-724.

Burokas, A., *et al.* (2017). "Targeting the Microbiota-Gut-Brain Axis: Prebiotics Have Anxiolytic and Antidepressant-like Effects and Reverse the Impact of Chronic Stress in Mice." Biol Psychiatry **82**(7): 472-487.

Calabrese, V., *et al.* (2008). "Cellular stress response: a novel target for chemoprevention and nutritional neuroprotection in aging, neurodegenerative disorders and longevity." Neurochem Res **33**(12): 2444-2471.

Cameron, H. A., *et al.* (2015). "Adult neurogenesis: beyond learning and memory." Annu Rev Psychol **66**: 53-81.

Carrie, I., *et al.* (2000). "Phospholipid supplementation reverses behavioral and biochemical alterations induced by n-3 polyunsaturated fatty acid deficiency in mice." J Lipid Res **41**(3): 473-480.

Cenit, M. C., *et al.* (2017). "Gut microbiota and attention deficit hyperactivity disorder: new perspectives for a challenging condition." Eur Child Adolesc Psychiatry **26**(9): 1081-1092.

Ceremuga, T. E., *et al.* (2013). "Investigation of the anxiolytic effects of xanthohumol, a component of humulus lupulus (Hops), in the male Sprague-Dawley rat." AANA J **81**(3): 193-198.

Chalon, S., *et al.* (2001). "Polyunsaturated fatty acids and cerebral function: focus on monoaminergic neurotransmission." Lipids **36**(9): 937-944.

Chandley, M. J., *et al.* (2014). "Elevated gene expression of glutamate receptors in noradrenergic neurons from the locus coeruleus in major depression." Int J Neuropsychopharmacol **17**(10): 1569-1578.

Chang, L., *et al.* (2019). "Comparison of antidepressant and side effects in mice after intranasal administration of (R,S)-ketamine, (R)-ketamine, and (S)-ketamine." Pharmacol Biochem Behav **181**: 53-59.

Chapman, D. P., *et al.* (2004). "Adverse childhood experiences and the risk of depressive disorders in adulthood." J Affect Disord **82**(2): 217-225.

Chen, A., *et al.* (2013). "Neuroprotective effect of brain-derived neurotrophic factor mediated by autophagy through the PI3K/Akt/mTOR pathway." Mol Med Rep **8**(4): 1011-1016.

Chen, B., *et al.* (2014). "Curcumin inhibits proliferation of breast cancer cells through Nrf2-mediated down-regulation of Fen1 expression." J Steroid Biochem Mol Biol **143**: 11-18.

Chen, R. C., *et al.* (2015). "Naringin protects against anoxia/reoxygenation-induced apoptosis in H9c2 cells via the Nrf2 signaling pathway." Food Funct **6**(4): 1331-1344.

Chen, T., *et al.* (2019). "Green Tea Polyphenols Modify the Gut Microbiome in db/db Mice as Co-Abundance Groups Correlating with the Blood Glucose Lowering Effect." Mol Nutr Food Res **63**(8): e1801064.

Chin, K. Y. (2016). "The spice for joint inflammation: anti-inflammatory role of curcumin in treating osteoarthritis." Drug Des Devel Ther **10**: 3029-3042.

Chong, Z. Z., *et al.* (2012). "A Critical Kinase Cascade in Neurological Disorders: PI 3-K, Akt, and mTOR." Future Neurol **7**(6): 733-748.

Choudhury, A., *et al.* (2016). "Administration of N-acetylserotonin and melatonin alleviate chronic ketamine-induced behavioural phenotype accompanying BDNF-independent and dependent converging cytoprotective mechanisms in the hippocampus." Behav Brain Res **297**: 204-212.

Chrousos, G. P., *et al.* (1992). "The concepts of stress and stress system disorders. Overview of physical and behavioral homeostasis." JAMA **267**(9): 1244-1252.

Claesson, M. J., *et al.* (2012). "Gut microbiota composition correlates with diet and health in the elderly." Nature **488**: 178.

Clarke, G., *et al.* (2012). "The microbiome-gut-brain axis during early life regulates the hippocampal serotonergic system in a sex-dependent manner." Molecular Psychiatry **18**: 666.

Clavel, T., *et al.* (2005). "Isoflavones and functional foods alter the dominant intestinal microbiota in postmenopausal women." J Nutr **135**(12): 2786-2792.

Cohen, B. M., *et al.* (1982). "Lecithin in the treatment of mania: double-blind, placebo-controlled trials." Am J Psychiatry **139**(9): 1162-1164.

Cohn, J. S., *et al.* (2010). "Dietary phospholipids and intestinal cholesterol absorption." Nutrients **2**(2): 116-127.

Collins, S. M., *et al.* (2009). "The relationship between intestinal microbiota and the central nervous system in normal gastrointestinal function and disease." Gastroenterology **136**(6): 2003-2014.

Colombaioni, L., *et al.* (2004). "Sphingolipid metabolites in neural signalling and function." Brain Res Brain Res Rev **46**(3): 328-355.

Cordain, L., *et al.* (2005). "Origins and evolution of the Western diet: health implications for the 21st century." Am J Clin Nutr **81**(2): 341-354.

Coupland, N. J., *et al.* (2005). "Decreased prefrontal Myo-inositol in major depressive disorder." Biol Psychiatry **57**(12): 1526-1534.

Cowan, C. S. M., *et al.* (2019). "Early-life stress, microbiota, and brain development: probiotics reverse the effects of maternal separation on neural circuits underpinning fear expression and extinction in infant rats." Dev Cogn Neurosci **37**: 100627.

Crook, T. H., *et al.* (1991). "Effects of phosphatidylserine in age-associated memory impairment." Neurology **41**(5): 644-649.

Cruz-Pereira, J. S., *et al.* (2020). "Depression's Unholy Trinity: Dysregulated Stress, Immunity, and the Microbiome." Annu Rev Psychol **71**: 49-78.

Cryan, J. F., *et al.* (2012). "Mind-altering microorganisms: the impact of the gut microbiota on brain and behaviour." Nat Rev Neurosci **13**(10): 701-712.

Cryan, J. F., *et al.* (2013). "Unraveling the longstanding scars of early neurodevelopmental stress." Biol Psychiatry **74**(11): 788-789.

Cryan, J. F., *et al.* (2005). "The ascent of mouse: advances in modelling human depression and anxiety." Nat Rev Drug Discov **4**(9): 775-790.

Cryan, J. F., *et al.* (2004). "Behavioral characterization of the novel GABAB receptor-positive modulator GS39783 (N,N'-dicyclopentyl-2-methylsulfanyl-5-nitro-pyrimidine-4,6-diamine): anxiolytic-like activity without side effects associated with baclofen or benzodiazepines." J Pharmacol Exp Ther **310**(3): 952-963.

Cryan, J. F., *et al.* (2019). "The Microbiota-Gut-Brain Axis." Physiol Rev **99**(4): 1877-2013.

Cryan, J. F., *et al.* (2007). "Animal models of mood disorders: Recent developments." Curr Opin Psychiatry **20**(1): 1-7.

Curzon, G., *et al.* (1970). "Tryptophan metabolism in depression." J Neurol Neurosurg Psychiatry **33**(5): 698-704.

Cusack, B., *et al.* (1994). "Binding of antidepressants to human brain receptors: focus on newer generation compounds." Psychopharmacology (Berl) **114**(4): 559-565.

Czeh, B., *et al.* (2018). "Long-Term Stress Disrupts the Structural and Functional Integrity of GABAergic Neuronal Networks in the Medial Prefrontal Cortex of Rats." Front Cell Neurosci **12**: 148.

Dalile, B., *et al.* (2019). "The role of short-chain fatty acids in microbiota–gut–brain communication." Nature Reviews Gastroenterology & Hepatology **16**(8): 461-478.

Daniels, W. M., *et al.* (2004). "Maternal separation in rats leads to anxiety-like behavior and a blunted ACTH response and altered neurotransmitter levels in response to a subsequent stressor." Metab Brain Dis **19**(1-2): 3-14.

Darzi, Y., *et al.* (2016). "Towards biome-specific analysis of meta-omics data." ISME J **10**(5): 1025-1028.

Davila, D., *et al.* (2012). "Two-step activation of FOXO3 by AMPK generates a coherent feed-forward loop determining excitotoxic cell fate." Cell Death Differ **19**(10): 1677-1688.

Dawaliby, R., *et al.* (2015). "Allosteric regulation of G protein–coupled receptor activity by phospholipids." Nature Chemical Biology **12**: 35.

Dawson, G. (2015). "Measuring brain lipids." Biochim Biophys Acta **1851**(8): 1026-1039.

Dawson, S. L., *et al.* (2016). "The Importance of Diet and Gut Health to the Treatment and Prevention of Mental Disorders." Int Rev Neurobiol **131**: 325-346.

de Castro-Catala, M., *et al.* (2017). "Interaction between FKBP5 gene and childhood trauma on psychosis, depression and anxiety symptoms in a non-clinical sample." Psychoneuroendocrinology **85**: 200-209.

de Kloet, C. S., *et al.* (2006). "Assessment of HPA-axis function in posttraumatic stress disorder: pharmacological and non-pharmacological challenge tests, a review." J Psychiatr Res **40**(6): 550-567.

de Kloet, E. R., *et al.* (2005). "Stress and the brain: from adaptation to disease." Nat Rev Neurosci **6**(6): 463-475.

de Kloet, E. R., *et al.* (2016). "Stress and Depression: a Crucial Role of the Mineralocorticoid Receptor." J Neuroendocrinol **28**(8).

de Noronha, S. R., *et al.* (2017). "High fat diet induced-obesity facilitates anxiety-like behaviors due to GABAergic impairment within the dorsomedial hypothalamus in rats." Behav Brain Res **316**: 38-46.

De Palma, G., *et al.* (2014). "The microbiota-gut-brain axis in gastrointestinal disorders: stressed bugs, stressed brain or both?" J Physiol **592**(14): 2989-2997.

Delerive, P., *et al.* (2000). "Oxidized phospholipids activate PPARalpha in a phospholipase A2-dependent manner." FEBS Lett **471**(1): 34-38.

Delgado, P. L. (2000). "Depression: the case for a monoamine deficiency." J Clin Psychiatry **61 Suppl 6**: 7-11.

Demuyser, T., *et al.* (2016). "In-depth behavioral characterization of the corticosterone mouse model and the critical involvement of housing conditions." Physiol Behav **156**: 199-207.

Desbonnet, L., *et al.* (2015). "Gut microbiota depletion from early adolescence in mice: Implications for brain and behaviour." Brain Behav Immun **48**: 165-173.

Desbonnet, L., *et al.* (2010). "Effects of the probiotic Bifidobacterium infantis in the maternal separation model of depression." Neuroscience **170**(4): 1179-1188.

deVries, M. W., *et al.* (2003). "Stress, work and mental health: a global perspective." Acta Neuropsychiatr **15**(1): 44-53.

Dhir, A., *et al.* (2011). "Nitric oxide and major depression." Nitric Oxide **24**(3): 125-131.

Dias, G. P., *et al.* (2012). "The role of dietary polyphenols on adult hippocampal neurogenesis: molecular mechanisms and behavioural effects on depression and anxiety." Oxid Med Cell Longev **2012**: 541971.

Dong, L., *et al.* (2015). "Connexin 43 mediates PFOS-induced apoptosis in astrocytes." Chemosphere **132**: 8-16.

Dong, X. X., *et al.* (2009). "Molecular mechanisms of excitotoxicity and their relevance to pathogenesis of neurodegenerative diseases." Acta Pharmacol Sin **30**(4): 379-387.

Donoso, F., *et al.* (2019). "Naturally Derived Polyphenols Protect Against Corticosterone-Induced Changes in Primary Cortical Neurons." Int J Neuropsychopharmacol.

Duman, R. S., *et al.* (2016). "Synaptic plasticity and depression: new insights from stress and rapid-acting antidepressants." Nature Medicine **22**: 238.

Duman, R. S., *et al.* (2012). "Signaling pathways underlying the rapid antidepressant actions of ketamine." Neuropharmacology **62**(1): 35-41.

Dunn, A. J., *et al.* (2008). "The role of corticotropin-releasing factor and noradrenaline in stress-related responses, and the inter-relationships between the two systems." Eur J Pharmacol **583**(2-3): 186-193.

Dyall, S. C., *et al.* (2010). "Omega-3 fatty acids reverse age-related decreases in nuclear receptors and increase neurogenesis in old rats." J Neurosci Res **88**(10): 2091-2102.

Eckburg, P. B., *et al.* (2005). "Diversity of the human intestinal microbial flora." Science **308**(5728): 1635-1638.

Eisch, A. J., *et al.* (2008). "Adult neurogenesis, mental health, and mental illness: hope or hype?" J Neurosci **28**(46): 11785-11791.

Elliott, N. T., *et al.* (2011). "A review of three-dimensional in vitro tissue models for drug discovery and transport studies." J Pharm Sci **100**(1): 59-74.

Eriksson, P. S., *et al.* (1998). "Neurogenesis in the adult human hippocampus." Nature Medicine **4**(11): 1313-1317.

Erridge, C., *et al.* (2008). "Oxidized phospholipid inhibition of toll-like receptor (TLR) signaling is restricted to TLR2 and TLR4: roles for CD14, LPS-binding protein, and MD2 as targets for specificity of inhibition." J Biol Chem **283**(36): 24748-24759.

Esch, T., *et al.* (2002). "The role of stress in neurodegenerative diseases and mental disorders." Neuro Endocrinol Lett **23**(3): 199-208.

Essen, L. O., *et al.* (1997). "Structural mapping of the catalytic mechanism for a mammalian phosphoinositide-specific phospholipase C." Biochemistry **36**(7): 1704-1718.

Etxeberria, U., *et al.* (2015). "Reshaping faecal gut microbiota composition by the intake of trans-resveratrol and quercetin in high-fat sucrose diet-fed rats." J Nutr Biochem **26**(6): 651-660.

Etxeberria, U., *et al.* (2013). "Impact of polyphenols and polyphenol-rich dietary sources on gut microbiota composition." J Agric Food Chem **61**(40): 9517-9533.

Evans, W. J., *et al.* (2008). "Cachexia: a new definition." Clin Nutr **27**(6): 793-799.

Faravelli, C., *et al.* (2012). "The role of life events and HPA axis in anxiety disorders: a review." Curr Pharm Des **18**(35): 5663-5674.

Faria, R., *et al.* (2014). "Alterations in phospholipidomic profile in the brain of mouse model of depression induced by chronic unpredictable stress." Neuroscience **273**: 1-11.

Feng, W., *et al.* (2017). "Modulation of gut microbiota contributes to curcumin-mediated attenuation of hepatic steatosis in rats." Biochim Biophys Acta Gen Subj **1861**(7): 1801-1812.

Fernandes, A. D., *et al.* (2014). "Unifying the analysis of high-throughput sequencing datasets: characterizing RNA-seq, 16S rRNA gene sequencing and selective growth experiments by compositional data analysis." Microbiome **2**(1): 15.

Fernstrom, J. D. (2012). "Effects and side effects associated with the non-nutritional use of tryptophan by humans." J Nutr **142**(12): 2236S-2244S.

Fiedorowicz, J. G., *et al.* (2004). "The role of monoamine oxidase inhibitors in current psychiatric practice." J Psychiatr Pract **10**(4): 239-248.

Field, T. (1998). "Maternal depression effects on infants and early interventions." Prev Med **27**(2): 200-203.

Figueira, I., *et al.* (2017). "Polyphenols journey through blood-brain barrier towards neuronal protection." Sci Rep **7**(1): 11456.

File, S. E., *et al.* (2004). "Animal tests of anxiety." Curr Protoc Neurosci **Chapter 8**: Unit 8 3.

Filosa, S., *et al.* (2018). "Polyphenols-gut microbiota interplay and brain neuromodulation." Neural Regen Res **13**(12): 2055-2059.

Finger, B. C., *et al.* (2011). "High-fat diet selectively protects against the effects of chronic social stress in the mouse." Neuroscience **192**: 351-360.

Fjell, A. M., *et al.* (2014). "What is normal in normal aging? Effects of aging, amyloid and Alzheimer's disease on the cerebral cortex and the hippocampus." Prog Neurobiol **117**: 20-40.

Fong, C. W. (2015). "Permeability of the Blood-Brain Barrier: Molecular Mechanism of Transport of Drugs and Physiologically Important Compounds." J Membr Biol **248**(4): 651-669.

Foster, J. A., *et al.* (2017). "Stress & the gut-brain axis: Regulation by the microbiome." Neurobiol Stress **7**: 124-136.

Frantz, S. (2004). "Therapeutic area influences drug development costs." Nat Rev Drug Discov **3**(6): 466-467.

Freitas, A. E., *et al.* (2015). "Agmatine induces Nrf2 and protects against corticosterone effects in hippocampal neuronal cell line." Mol Neurobiol **51**(3): 1504-1519.

Frick, L. R., *et al.* (2013). "Microglial dysregulation in psychiatric disease." Clin Dev Immunol **2013**: 608654.

Fries, G. R., *et al.* (2017). "The FKBP51 Glucocorticoid Receptor Co-Chaperone: Regulation, Function, and Implications in Health and Disease." Int J Mol Sci **18**(12).

Froestl, W. (2011). "An historical perspective on GABAergic drugs." Future Med Chem **3**(2): 163-175.

Fuhrman, B., *et al.* (2005). "Grape powder polyphenols attenuate atherosclerosis development in apolipoprotein E deficient (E0) mice and reduce macrophage atherogenicity." J Nutr **135**(4): 722-728.

Fujita, R., *et al.* (2002). "The cognition-enhancer nefiracetam is protective in BDNF-independent neuronal cell death under the serum-free condition." Neurochem Int **40**(2): 139-143.

Fukui, H., *et al.* (2018). "Effect of probiotic Bifidobacterium bifidum G9-1 on the relationship between gut microbiota profile and stress sensitivity in maternally separated rats." Sci Rep **8**(1): 12384.

Furnkranz, A., *et al.* (2004). "Regulation of inflammatory responses by oxidized phospholipids: structure-function relationships." Curr Pharm Des **10**(8): 915-921.

Galbete, C., *et al.* (2018). "Evaluating Mediterranean diet and risk of chronic disease in cohort studies: an umbrella review of meta-analyses." Eur J Epidemiol **33**(10): 909-931.

Gao, S., *et al.* (2015). "H2S protects PC12 cells against toxicity of corticosterone by modulation of BDNF-TrkB pathway." Acta Biochim Biophys Sin (Shanghai) **47**(11): 915-924.

Garcia, C., *et al.* (2012). "Phospholipid fingerprints of milk from different mammalians determined by 31P NMR: towards specific interest in human health." Food Chem **135**(3): 1777-1783.

Gareau, M. G., *et al.* (2007). "Probiotic treatment of rat pups normalises corticosterone release and ameliorates colonic dysfunction induced by maternal separation." Gut **56**(11): 1522-1528.

Ge, L., *et al.* (2015). "Resveratrol abrogates lipopolysaccharide-induced depressive-like behavior, neuroinflammatory response, and CREB/BDNF signaling in mice." Eur J Pharmacol **768**: 49-57.

Georgieff, M. K. (2007). "Nutrition and the developing brain: nutrient priorities and measurement." Am J Clin Nutr **85**(2): 614S-620S.

Gerber, M., *et al.* (2015). "The Mediterranean diet: health, science and society." Br J Nutr **113 Suppl 2**: S4-10.

German, L., *et al.* (2011). "Depressive symptoms are associated with food insufficiency and nutritional deficiencies in poor community-dwelling elderly people." J Nutr Health Aging **15**(1): 3-8.

Ghanemi, A. (2014). "Psychiatric neural networks and neuropharmacology: Selected advances and novel implications." Saudi Pharm J **22**(2): 95-100.

Gillman, P. K. (2007). "Tricyclic antidepressant pharmacology and therapeutic drug interactions updated." Br J Pharmacol **151**(6): 737-748.

Gite, S., *et al.* (2019). "Nutraceuticals to promote neuronal plasticity in response to corticosterone-induced stress in human neuroblastoma cells." Nutr Neurosci **22**(8): 551-568.

Gjerstad, J. K., *et al.* (2018). "Role of glucocorticoid negative feedback in the regulation of HPA axis pulsatility." Stress **21**(5): 403-416.

Glade, M. J., *et al.* (2015). "Phosphatidylserine and the human brain." Nutrition **31**(6): 781-786.

Godoy, J. A., *et al.* (2016). "Quercetin Exerts Differential Neuroprotective Effects Against H2O2 and Abeta Aggregates in Hippocampal Neurons: the Role of Mitochondria." Mol Neurobiol.

Golden, S. A., *et al.* (2011). "A standardized protocol for repeated social defeat stress in mice." Nature Protocols **6**(8): 1183-1191.

Gómez-Pinilla, F. (2008). "Brain foods: the effects of nutrients on brain function." Nature Reviews Neuroscience **9**: 568.

Gonul, A. S., *et al.* (2005). "Effect of treatment on serum brain-derived neurotrophic factor levels in depressed patients." Eur Arch Psychiatry Clin Neurosci **255**(6): 381-386.

Gopinath, K., *et al.* (2012). "Naringin modulates oxidative stress and inflammation in 3-nitropropionic acid-induced neurodegeneration through the activation of nuclear factor-erythroid 2-related factor-2 signalling pathway." Neuroscience **227**: 134-143.

Goshen, I., *et al.* (2007). "Brain interleukin-1 mediates chronic stress-induced depression in mice via adrenocortical activation and hippocampal neurogenesis suppression." Molecular Psychiatry **13**: 717.

Granado-Serrano, A. B., *et al.* (2012). "Quercetin modulates Nrf2 and glutathione-related defenses in HepG2 cells: Involvement of p38." Chem Biol Interact **195**(2): 154-164.

Greenberg, P. E., *et al.* (2003). "The economic burden of depression in the United States: how did it change between 1990 and 2000?" J Clin Psychiatry **64**(12): 1465-1475.

Greig, F. H., *et al.* (2012). "Physiological effects of oxidized phospholipids and their cellular signaling mechanisms in inflammation." Free Radic Biol Med **52**(2): 266-280.

Guidotti, G., *et al.* (2013). "Glucocorticoid receptor and FKBP5 expression is altered following exposure to chronic stress: modulation by antidepressant treatment." Neuropsychopharmacology **38**(4): 616-627.

Gururajan, A., *et al.* (2019). "The future of rodent models in depression research." Nat Rev Neurosci.

Gururajan, A., *et al.* (2019). "The future of rodent models in depression research." Nature Reviews Neuroscience.

Haefely, W., *et al.* (1975). "Possible involvement of GABA in the central actions of benzodiazepines." Adv Biochem Psychopharmacol(14): 131-151.

Hains, A. B., *et al.* (2009). "Inhibition of protein kinase C signaling protects prefrontal cortex dendritic spines and cognition from the effects of chronic stress." Proc Natl Acad Sci U S A **106**(42): 17957-17962.

Haleagrahara, N., *et al.* (2009). "Flavonoid quercetin protects against swimming stress-induced changes in oxidative biomarkers in the hypothalamus of rats." Eur J Pharmacol **621**(1-3): 46-52.

Hammen, C. (2005). "Stress and depression." Annu Rev Clin Psychol **1**: 293-319.

Han, B. H., *et al.* (2000). "BDNF protects the neonatal brain from hypoxic-ischemic injury in vivo via the ERK pathway." J Neurosci **20**(15): 5775-5781.

Han, S. G., *et al.* (2012). "EGCG protects endothelial cells against PCB 126-induced inflammation through inhibition of AhR and induction of Nrf2-regulated genes." Toxicol Appl Pharmacol **261**(2): 181-188.

Hanson, C. J., *et al.* (2004). "Cell signalling: IP3 receptors channel calcium into cell death." Curr Biol **14**(21): R933-935.

Hashimoto, K. (2018). "Essential Role of Keap1-Nrf2 Signaling in Mood Disorders: Overview and Future Perspective." Front Pharmacol **9**: 1182.

Haslam, E. (1988). "Plant polyphenols (syn. vegetable tannins) and chemical defense-A reappraisal." J Chem Ecol **14**(10): 1789-1805.

He, B., *et al.* (2016). "Homoplantaginin Inhibits Palmitic Acid-induced Endothelial Cells Inflammation by Suppressing TLR4 and NLRP3 Inflammasome." J Cardiovasc Pharmacol **67**(1): 93-101.

Heberden, C. (2016). "Modulating adult neurogenesis through dietary interventions." Nutr Res Rev **29**(2): 163-171.

Heim, C., *et al.* (2012). "Current research trends in early life stress and depression: review of human studies on sensitive periods, gene-environment interactions, and epigenetics." Exp Neurol **233**(1): 102-111.

Hellhammer, J., *et al.* (2004). "Effects of soy lecithin phosphatidic acid and phosphatidylserine complex (PAS) on the endocrine and psychological responses to mental stress." Stress **7**(2): 119-126.

Hers, I., *et al.* (2011). "Akt signalling in health and disease." Cell Signal **23**(10): 1515-1527.

Hill, D. R., *et al.* (2015). "Clinical applications of bioactive milk components." Nutr Rev **73**(7): 463-476.

Hillhouse, T. M., *et al.* (2015). "A brief history of the development of antidepressant drugs: from monoamines to glutamate." Exp Clin Psychopharmacol **23**(1): 1-21.

Hoban, A. E., *et al.* (2018). "The microbiome regulates amygdala-dependent fear recall." Mol Psychiatry **23**(5): 1134-1144.

Hofer, M. A., *et al.* (1994). "Potentiation of isolation-induced vocalization by brief exposure of rat pups to maternal cues." Dev Psychobiol **27**(8): 503-517.

Hollenbeck, P. J., *et al.* (2003). "Comparing the properties of neuronal culture systems: a shopping guide for the cell biologist." Methods Cell Biol **71**: 1-16.

Horejsi, V., *et al.* (2004). "Transmembrane adaptor proteins: organizers of immunoreceptor signalling." Nat Rev Immunol **4**(8): 603-616.

Hsieh, T., *et al.* (2016). "iNEXT: an R package for rarefaction and extrapolation of species diversity (H ill numbers)." Methods in Ecology and Evolution **7**(12): 1451-1456.

Hu, F. B., *et al.* (2002). "Optimal diets for prevention of coronary heart disease." JAMA **288**(20): 2569-2578.

Huang, B. X., *et al.* (2011). "Phosphatidylserine is a critical modulator for Akt activation." J Cell Biol **192**(6): 979-992.

Huang, E. J., *et al.* (2001). "Neurotrophins: roles in neuronal development and function." Annu Rev Neurosci **24**: 677-736.

Huang, R. P., *et al.* (2001). "Connexin 43 (cx43) enhances chemotherapy-induced apoptosis in human glioblastoma cells." Int J Cancer **92**(1): 130-138.

Huang, W., *et al.* (2013). "Fluoxetine upregulates phosphorylated-AKT and phosphorylated-ERK1/2 proteins in neural stem cells: evidence for a crosstalk between AKT and ERK1/2 pathways." J Mol Neurosci **49**(2): 244-249.

Huang, Y. N., *et al.* (2009). "Methamphetamine induces heme oxygenase-1 expression in cortical neurons and glia to prevent its toxicity." Toxicol Appl Pharmacol **240**(3): 315-326.

Huang, Z., *et al.* (2011). "Curcumin reverses corticosterone-induced depressive-like behavior and decrease in brain BDNF levels in rats." Neurosci Lett **493**(3): 145-148.

Hubbard, G. P., *et al.* (2006). "Ingestion of onion soup high in quercetin inhibits platelet aggregation and essential components of the collagen-stimulated platelet activation pathway in man: a pilot study." Br J Nutr **96**(3): 482-488.

Hurley, L. L., *et al.* (2014). "Antidepressant effects of resveratrol in an animal model of depression." Behav Brain Res **268**: 1-7.

Hurley, L. L., *et al.* (2013). "Antidepressant-like effects of curcumin in WKY rat model of depression is associated with an increase in hippocampal BDNF." Behav Brain Res **239**: 27-30.

Hutkins, R. W., *et al.* (2016). "Prebiotics: why definitions matter." Curr Opin Biotechnol **37**: 1-7.

Iadarola, N. D., *et al.* (2015). "Ketamine and other N-methyl-D-aspartate receptor antagonists in the treatment of depression: a perspective review." Ther Adv Chronic Dis **6**(3): 97-114.

Ignarro, L. J., *et al.* (2007). "Nutrition, physical activity, and cardiovascular disease: an update." Cardiovasc Res **73**(2): 326-340.

Iosifescu, D. V. (2012). "The relation between mood, cognition and psychosocial functioning in psychiatric disorders." Eur Neuropsychopharmacol **22 Suppl 3**: S499-504.

Ishida, J., *et al.* (2017). "Animal models of cachexia and sarcopenia in chronic illness: Cardiac function, body composition changes and therapeutic results." Int J Cardiol **238**: 12-18.

Ishii, T., *et al.* (2018). "When and how does brain-derived neurotrophic factor activate Nrf2 in astrocytes and neurons?" Neural Regen Res **13**(5): 803-804.

Iwai, S., *et al.* (2016). "Piphillin: Improved Prediction of Metagenomic Content by Direct Inference from Human Microbiomes." PLoS One **11**(11): e0166104.

Jacka, F. N., *et al.* (2015). "Western diet is associated with a smaller hippocampus: a longitudinal investigation." BMC Med **13**: 215.

Jacka, F. N., *et al.* (2014). "Food policies for physical and mental health." BMC Psychiatry **14**: 132.

Jansen, A. S., *et al.* (1995). "Central command neurons of the sympathetic nervous system: basis of the fight-or-flight response." Science **270**(5236): 644-646.

Jezewska-Frackowiak, J., *et al.* (2018). "The promises and risks of probiotic Bacillus species." Acta Biochim Pol **65**(4): 509-519.

Jhang, J. J., *et al.* (2015). "Protective Effects of Catechin against Monosodium Urate-Induced Inflammation through the Modulation of NLRP3 Inflammasome Activation." J Agric Food Chem **63**(33): 7343-7352.

Jiang, C., *et al.* (1998). "PPAR-γ agonists inhibit production of monocyte inflammatory cytokines." Nature **391**(6662): 82-86.

Johnston, K. M., *et al.* (2019). "The burden of treatment-resistant depression: A systematic review of the economic and quality of life literature." J Affect Disord **242**: 195-210.

Jones, B. C. (2016). "Nutrition for Brain Health and Cognitive Performance." Nutr Neurosci **19**(7): 327.

Kalvodova, L., *et al.* (2005). "Lipids as modulators of proteolytic activity of BACE: involvement of cholesterol, glycosphingolipids, and anionic phospholipids in vitro." J Biol Chem **280**(44): 36815-36823.

Kameritsch, P., *et al.* (2013). "Gap junctional communication promotes apoptosis in a connexin-type-dependent manner." Cell Death &Amp; Disease **4**: e584.

Kanno, T., *et al.* (2014). "DL-/PO-phosphatidylcholine restores restraint stress-induced depression-related behaviors and spatial memory impairment." Behav Pharmacol **25**(5-6): 575-581.

Kansanen, E., *et al.* (2013). "The Keap1-Nrf2 pathway: Mechanisms of activation and dysregulation in cancer." Redox Biol **1**: 45-49.

Karl, J. P., *et al.* (2017). "Changes in intestinal microbiota composition and metabolism coincide with increased intestinal permeability in young adults under prolonged physiological stress." Am J Physiol Gastrointest Liver Physiol **312**(6): G559-G571.

Karolewicz, B., *et al.* (2009). "Elevated levels of NR2A and PSD-95 in the lateral amygdala in depression." Int J Neuropsychopharmacol **12**(2): 143-153.

Katt, M. E., *et al.* (2016). "In Vitro Tumor Models: Advantages, Disadvantages, Variables, and Selecting the Right Platform." Front Bioeng Biotechnol **4**: 12.

Kelava, I., *et al.* (2016). "Dishing out mini-brains: Current progress and future prospects in brain organoid research." Dev Biol **420**(2): 199-209.

Keller, J., *et al.* (2017). "HPA axis in major depression: cortisol, clinical symptomatology and genetic variation predict cognition." Mol Psychiatry **22**(4): 527-536.

Kelly, J. R., *et al.* (2016). "Transferring the blues: Depression-associated gut microbiota induces neurobehavioural changes in the rat." J Psychiatr Res **82**: 109-118.

Kent, C. (1995). "Eukaryotic phospholipid biosynthesis." Annu Rev Biochem **64**: 315-343.

Kessler, R. C. (1997). "The effects of stressful life events on depression." Annu Rev Psychol **48**: 191-214.

Keunen, K., *et al.* (2014). "Impact of nutrition on brain development and its neuroprotective implications following preterm birth." Pediatric Research **77**: 148.

Khan, A., *et al.* (2018). "Neuroprotective Effect of Quercetin Against the Detrimental Effects of LPS in the Adult Mouse Brain." Front Pharmacol **9**: 1383.

Kim, E. J., *et al.* (2015). "Stress effects on the hippocampus: a critical review." Learn Mem **22**(9): 411-416.

Kim, H.-Y. (2015). "A neuroinflammation emerging target." Nature Chemical Biology **11**: 99.

Kim, S. K., *et al.* (2011). "Medicinal effects of phlorotannins from marine brown algae." Adv Food Nutr Res **64**: 97-109.

Kitchener, P., *et al.* (2004). "Differences between brain structures in nuclear translocation and DNA binding of the glucocorticoid receptor during stress and the circadian cycle." Eur J Neurosci **19**(7): 1837-1846.

Kivity, S., *et al.* (2017). "Phospholipid supplementation can attenuate vaccine-induced depressive-like behavior in mice." Immunol Res **65**(1): 99-105.

Klasing, K. C. (2007). "Nutrition and the immune system." Br Poult Sci **48**(5): 525-537.

Kohler, O., *et al.* (2014). "Effect of anti-inflammatory treatment on depression, depressive symptoms, and adverse effects: a systematic review and meta-analysis of randomized clinical trials." JAMA Psychiatry **71**(12): 1381-1391.

Kohman, R. A., *et al.* (2013). "Neurogenesis, inflammation and behavior." Brain Behav Immun **27**(1): 22-32.

Komar, C. M. (2005). "Peroxisome proliferator-activated receptors (PPARs) and ovarian function--implications for regulating steroidogenesis, differentiation, and tissue remodeling." Reprod Biol Endocrinol **3**: 41.

Krupenye, C., *et al.* (2019). "Theory of mind in animals: Current and future directions." Wiley Interdiscip Rev Cogn Sci: e1503.

Krzak, A. M., *et al.* (2017). "Does neurogenesis relate to depression and do antidepressants affect neurogenesis?" Psychiatr Danub **29**(Suppl 3): 241-246.

Kucukibrahimoglu, E., *et al.* (2009). "The change in plasma GABA, glutamine and glutamate levels in fluoxetine- or S-citalopram-treated female patients with major depression." Eur J Clin Pharmacol **65**(6): 571-577.

Kuhn, R. (1957). "[Treatment of depressive states with an iminodibenzyl derivative (G 22355)]." Schweiz Med Wochenschr **87**(35-36): 1135-1140.

Kulkarni, S. K., *et al.* (2008). "Antidepressant activity of curcumin: involvement of serotonin and dopamine system." Psychopharmacology (Berl) **201**(3): 435-442.

Kullenberg, D., *et al.* (2012). "Health effects of dietary phospholipids." Lipids Health Dis **11**: 3.

Kusuda, R., *et al.* (2019). "Choline attenuates inflammatory hyperalgesia activating nitric oxide/cGMP/ATP-sensitive potassium channels pathway." Brain Res: 146567.

Kwatra, M., *et al.* (2016). "Naringin and Sertraline Ameliorate Doxorubicin-Induced Behavioral Deficits Through Modulation of Serotonin Level and Mitochondrial Complexes Protection Pathway in Rat Hippocampus." Neurochem Res **41**(9): 2352-2366.

Laaksonen, K. S., *et al.* (2013). "Food and water intake, growth, and adiposity of Sprague-Dawley rats with diet board for 24 months." Lab Anim **47**(4): 245-256.

Lai, J. S., *et al.* (2014). "A systematic review and meta-analysis of dietary patterns and depression in community-dwelling adults." Am J Clin Nutr **99**(1): 181-197.

Lakhan, S. E., *et al.* (2008). "Nutritional therapies for mental disorders." Nutrition Journal **7**(1): 2.

Lankelma, J. M., *et al.* (2015). "The gut microbiota in internal medicine: implications for health and disease." Neth J Med **73**(2): 61-68.

Larrosa, M., *et al.* (2009). "Effect of a low dose of dietary resveratrol on colon microbiota, inflammation and tissue damage in a DSS-induced colitis rat model." J Agric Food Chem **57**(6): 2211-2220.

Latt, H. M., *et al.* (2018). "Oxytocin Inhibits Corticosterone-induced Apoptosis in Primary Hippocampal Neurons." Neuroscience **379**: 383-389.

Lee, B., *et al.* (2009). "The CREB/CRE transcriptional pathway: protection against oxidative stress-mediated neuronal cell death." J Neurochem **108**(5): 1251-1265.

Lee, B. H., *et al.* (2010). "The roles of BDNF in the pathophysiology of major depression and in antidepressant treatment." Psychiatry Investig **7**(4): 231-235.

Lee, H., *et al.* (2018). "Adult Human Hippocampal Neurogenesis: Controversy and Evidence." Trends Mol Med **24**(6): 521-522.

Lee, H. C., *et al.* (2006). "Effect of tea phenolics and their aromatic fecal bacterial metabolites on intestinal microbiota." Res Microbiol **157**(9): 876-884.

Levine, S. (1967). "Maternal and environmental influences on the adrenocortical response to stress in weanling rats." Science **156**(3772): 258-260.

Li, W., *et al.* (2009). "Memory and learning behavior in mice is temporally associated with diet-induced alterations in gut bacteria." Physiol Behav **96**(4-5): 557-567.

Li, Z. Y., *et al.* (2014). "Saikosaponin D acts against corticosterone-induced apoptosis via regulation of mitochondrial GR translocation and a GR-dependent pathway." Prog Neuropsychopharmacol Biol Psychiatry **53**: 80-89.

Liao, J. F., *et al.* (2019). "Lactobacillus paracasei PS23 reduced early-life stress abnormalities in maternal separation mouse model." Benef Microbes **10**(4): 425-436.

Lin, P. Y., *et al.* (2017). "Polyunsaturated Fatty Acids in Perinatal Depression: A Systematic Review and Meta-analysis." Biol Psychiatry **82**(8): 560-569.

Liu, D., *et al.* (2014). "Resveratrol reverses the effects of chronic unpredictable mild stress on behavior, serum corticosterone levels and BDNF expression in rats." Behav Brain Res **264**: 9-16.

Liu, M., *et al.* (2015). "Pharmacological profile of xanthohumol, a prenylated flavonoid from hops (Humulus lupulus)." Molecules **20**(1): 754-779.

Liu, T., *et al.* (2019). "Resveratrol ameliorates estrogen deficiency-induced depression- and anxiety-like behaviors and hippocampal inflammation in mice." Psychopharmacology (Berl) **236**(4): 1385-1399.

Liu, W., *et al.* (2013). "Swimming exercise ameliorates depression-like behavior in chronically stressed rats: relevant to proinflammatory cytokines and IDO activation." Behav Brain Res **242**: 110-116.

Liu, Y., *et al.* (2018). "Emotional Roles of Mono-Aminergic Neurotransmitters in Major Depressive Disorder and Anxiety Disorders." Front Psychol **9**: 2201.

Livak, K. J., *et al.* (2001). "Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method." Methods **25**(4): 402-408.

Long-Smith, C., *et al.* (2019). "Microbiota-Gut-Brain Axis: New Therapeutic Opportunities." Annu Rev Pharmacol Toxicol.

Loomer, H. P., *et al.* (1957). "A clinical and pharmacodynamic evaluation of iproniazid as a psychic energizer." Psychiatr Res Rep Am Psychiatr Assoc **8**: 129-141.

Lopresti, A. L., *et al.* (2014). "Curcumin for the treatment of major depression: a randomised, double-blind, placebo controlled study." J Affect Disord **167**: 368-375.

Lordan, R., *et al.* (2017). "Phospholipids of Animal and Marine Origin: Structure, Function, and Anti-Inflammatory Properties." Molecules **22**(11).

Lou, H., *et al.* (2014). "Naringenin protects against 6-OHDA-induced neurotoxicity via activation of the Nrf2/ARE signaling pathway." Neuropharmacology **79**: 380-388.

Lu, Q., *et al.* (2016). "[Relationship between chronic psychosocial stress and BMI among adolescents]." Zhonghua Liu Xing Bing Xue Za Zhi **37**(1): 40-44.

Lucas, G. (2018). "Gut thinking: the gut microbiome and mental health beyond the head." Microb Ecol Health Dis **29**(2): 1548250.

Luczynski, P., *et al.* (2016). "Growing up in a Bubble: Using Germ-Free Animals to Assess the Influence of the Gut Microbiota on Brain and Behavior." Int J Neuropsychopharmacol **19**(8).

Lupien, S. J., *et al.* (2009). "Effects of stress throughout the lifespan on the brain, behaviour and cognition." Nat Rev Neurosci **10**(6): 434-445.

Macedo, G. C., *et al.* (2018). "Consequences of continuous social defeat stress on anxiety- and depressive-like behaviors and ethanol reward in mice." Horm Behav **97**: 154-161.

MacLaren, E. J., *et al.* (2011). "Knockdown of mental disorder susceptibility genes disrupts neuronal network physiology in vitro." Mol Cell Neurosci **47**(2): 93-99.

Mahar, I., *et al.* (2014). "Stress, serotonin, and hippocampal neurogenesis in relation to depression and antidepressant effects." Neurosci Biobehav Rev **38**: 173-192.

Malhotra, D., *et al.* (2010). "Global mapping of binding sites for Nrf2 identifies novel targets in cell survival response through ChIP-Seq profiling and network analysis." Nucleic Acids Res **38**(17): 5718-5734.

Manach, C., *et al.* (2004). "Polyphenols: food sources and bioavailability." Am J Clin Nutr **79**(5): 727-747.

Marin, L., *et al.* (2015). "Bioavailability of dietary polyphenols and gut microbiota metabolism: antimicrobial properties." Biomed Res Int **2015**: 905215.

Markus, C. R., *et al.* (2008). "Effect of different tryptophan sources on amino acids availability to the brain and mood in healthy volunteers." Psychopharmacology (Berl) **201**(1): 107-114.

Martin-Hernandez, D., *et al.* (2018). "Intracellular inflammatory and antioxidant pathways in postmortem frontal cortex of subjects with major depression: effect of antidepressants." J Neuroinflammation **15**(1): 251.

Martinez-Cengotitabengoa, M., *et al.* (2017). "Nutritional supplements in depressive disorders." Actas Esp Psiquiatr **45**(Supplement): 8-15.

Martinez-Gonzalez, M. A., *et al.* (2016). "Mediterranean diet and life expectancy; beyond olive oil, fruits, and vegetables." Curr Opin Clin Nutr Metab Care **19**(6): 401-407.

Martinowich, K., *et al.* (2007). "New insights into BDNF function in depression and anxiety." Nat Neurosci **10**(9): 1089-1093.

Marzola, E., *et al.* (2013). "Nutritional rehabilitation in anorexia nervosa: review of the literature and implications for treatment." BMC Psychiatry **13**(1): 290.

Masiulis, S., *et al.* (2019). "GABAA receptor signalling mechanisms revealed by structural pharmacology." Nature **565**(7740): 454-459.

Matarazzo, I., *et al.* (2018). "Psychobiome Feeding Mind: Polyphenolics in Depression and Anxiety." Curr Top Med Chem **18**(24): 2108-2115.

Mathur, A., *et al.* (2016). "In vitro cardiac tissue models: Current status and future prospects." Adv Drug Deliv Rev **96**: 203-213.

Mauri, M. C., *et al.* (1998). "Plasma and platelet amino acid concentrations in patients affected by major depression and under fluvoxamine treatment." Neuropsychobiology **37**(3): 124-129.

Mayer, E. A., *et al.* (2014). "Gut microbes and the brain: paradigm shift in neuroscience." J Neurosci **34**(46): 15490-15496.

McEwen, B. S., *et al.* (1993). "Stress and the individual. Mechanisms leading to disease." Arch Intern Med **153**(18): 2093-2101.

McEwen, B. S., *et al.* (2003). "The concept of allostasis in biology and biomedicine." Horm Behav **43**(1): 2-15.

McVey Neufeld, K. A., *et al.* (2019). "Neurobehavioural effects of Lactobacillus rhamnosus GG alone and in combination with prebiotics polydextrose and galactooligosaccharide in male rats exposed to early-life stress." Nutr Neurosci **22**(6): 425-434.

Meaney, M. J. (2001). "Maternal care, gene expression, and the transmission of individual differences in stress reactivity across generations." Annu Rev Neurosci **24**: 1161-1192.

Meaney, M. J., *et al.* (1996). "Early environmental regulation of forebrain glucocorticoid receptor gene expression: implications for adrenocortical responses to stress." Dev Neurosci **18**(1-2): 49-72.

Meeusen, R., *et al.* (2018). "Nutritional Supplements and the Brain." International journal of sport nutrition and exercise metabolism **28**(2): 200-211.

Menard, C., *et al.* (2016). "Pathogenesis of depression: Insights from human and rodent studies." Neuroscience **321**: 138-162.

Mendez-David, I., *et al.* (2017). "Differential Peripheral Proteomic Biosignature of Fluoxetine Response in a Mouse Model of Anxiety/Depression." Front Cell Neurosci **11**: 237.

Mendez-David, I., *et al.* (2015). "Nrf2-signaling and BDNF: A new target for the antidepressant-like activity of chronic fluoxetine treatment in a mouse model of anxiety/depression." Neurosci Lett **597**: 121-126.

Messamore, E., *et al.* (2017). "Polyunsaturated fatty acids and recurrent mood disorders: Phenomenology, mechanisms, and clinical application." Prog Lipid Res **66**: 1-13.

Micheli, L., *et al.* (2018). "Depression and adult neurogenesis: Positive effects of the antidepressant fluoxetine and of physical exercise." Brain Res Bull **143**: 181-193.

Miller, A. H., *et al.* (2015). "The role of inflammation in depression: from evolutionary imperative to modern treatment target." Nature Reviews Immunology **16**: 22.

Mirza, Y., *et al.* (2004). "Reduced anterior cingulate cortex glutamatergic concentrations in childhood major depression." J Am Acad Child Adolesc Psychiatry **43**(3): 341-348.

Mitra, R., *et al.* (2008). "Acute corticosterone treatment is sufficient to induce anxiety and amygdaloid dendritic hypertrophy." Proc Natl Acad Sci U S A **105**(14): 5573-5578.

Moda-Sava, R. N., *et al.* (2019). "Sustained rescue of prefrontal circuit dysfunction by antidepressant-induced spine formation." Science **364**(6436).

Mohar, D. S., *et al.* (2012). "The Sirtuin System: The Holy Grail of Resveratrol?" J Clin Exp Cardiolog **3**(11).

Mohler, H. (2006). "GABAA receptors in central nervous system disease: anxiety, epilepsy, and insomnia." J Recept Signal Transduct Res **26**(5-6): 731-740.

Molofsky, A. V., *et al.* (2003). "Bmi-1 dependence distinguishes neural stem cell self-renewal from progenitor proliferation." Nature **425**(6961): 962-967.

Moore, K., *et al.* (2018). "Diet, nutrition and the ageing brain: current evidence and new directions." Proc Nutr Soc **77**(2): 152-163.

Morrison, D. J., *et al.* (2016). "Formation of short chain fatty acids by the gut microbiota and their impact on human metabolism." Gut Microbes **7**(3): 189-200.

Mosmann, T. (1983). "Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays." J Immunol Methods **65**(1-2): 55-63.

Motaghinejad, M., *et al.* (2017). "Curcumin confers neuroprotection against alcohol-induced hippocampal neurodegeneration via CREB-BDNF pathway in rats." Biomed Pharmacother **87**: 721-740.

Motilva, M. J., *et al.* (2013). "Analysis of food polyphenols by ultra high-performance liquid chromatography coupled to mass spectrometry: an overview." J Chromatogr A **1292**: 66-82.

Moukarzel, S., *et al.* (2018). "Milk Fat Globule Membrane Supplementation in Formula-fed Rat Pups Improves Reflex Development and May Alter Brain Lipid Composition." Scientific Reports **8**(1): 15277.

Moussaoui, N., *et al.* (2017). "Chronic Early-life Stress in Rat Pups Alters Basal Corticosterone, Intestinal Permeability, and Fecal Microbiota at Weaning: Influence of Sex." J Neurogastroenterol Motil **23**(1): 135-143.

Muller, N., *et al.* (1998). "Psychoneuroimmunology and the cytokine action in the CNS: implications for psychiatric disorders." Prog Neuropsychopharmacol Biol Psychiatry **22**(1): 1-33.

Murakami, S., *et al.* (2005). "Chronic stress, as well as acute stress, reduces BDNF mRNA expression in the rat hippocampus but less robustly." Neurosci Res **53**(2): 129-139.

Murphy, G. M., Jr., *et al.* (2013). "BDNF and CREB1 genetic variants interact to affect antidepressant treatment outcomes in geriatric depression." Pharmacogenet Genomics **23**(6): 301-313.

Murrough, J. W., *et al.* (2017). "Targeting glutamate signalling in depression: progress and prospects." Nature Reviews Drug Discovery **16**: 472.

Murrough, J. W., *et al.* (2013). "Rapid and longer-term antidepressant effects of repeated ketamine infusions in treatment-resistant major depression." Biol Psychiatry **74**(4): 250-256.

Nagata, T., *et al.* (2015). "Generalized social anxiety disorder: A still-neglected anxiety disorder 3 decades since Liebowitz's review." Psychiatry Clin Neurosci **69**(12): 724-740.

Nath, S., *et al.* (2012). "Catechins protect neurons against mitochondrial toxins and HIV proteins via activation of the BDNF pathway." J Neurovirol **18**(6): 445-455.

Nemets, H., *et al.* (2006). "Omega-3 treatment of childhood depression: a controlled, double-blind pilot study." Am J Psychiatry **163**(6): 1098-1100.

Nishi, M., *et al.* (2014). "Effects of early life adverse experiences on the brain: implications from maternal separation models in rodents." Front Neurosci **8**: 166.

Norris, G. H., *et al.* (2016). "Milk sphingomyelin improves lipid metabolism and alters gut microbiota in high fat diet-fed mice." J Nutr Biochem **30**: 93-101.

Nowak, G., *et al.* (1993). "Adaptive changes in the N-methyl-D-aspartate receptor complex after chronic treatment with imipramine and 1-aminocyclopropanecarboxylic acid." J Pharmacol Exp Ther **265**(3): 1380-1386.

Nutt, D. J. (2008). "Relationship of neurotransmitters to the symptoms of major depressive disorder." J Clin Psychiatry **69 Suppl E1**: 4-7.

O'Leary, J. C., 3rd, *et al.* (2013). "The role of FKBP5 in mood disorders: action of FKBP5 on steroid hormone receptors leads to questions about its evolutionary importance." CNS Neurol Disord Drug Targets **12**(8): 1157-1162.

O'Leary, O. F., *et al.* (2013). "Towards translational rodent models of depression." Cell Tissue Res **354**(1): 141-153.

O'Leime, C. S., *et al.* (2018). "TLX is an intrinsic regulator of the negative effects of IL-1beta on proliferating hippocampal neural progenitor cells." FASEB J **32**(2): 613-624.

O'Mahony, S. M., *et al.* (2011). "Maternal separation as a model of brain-gut axis dysfunction." Psychopharmacology (Berl) **214**(1): 71-88.

O'Mahony, S. M., *et al.* (2009). "Early life stress alters behavior, immunity, and microbiota in rats: implications for irritable bowel syndrome and psychiatric illnesses." Biol Psychiatry **65**(3): 263-267.

O'Mahony, S. M., *et al.* (2019). "The enduring effects of early-life stress on the microbiota-gut-brain axis are buffered by dietary supplementation with milk fat globule membrane and a prebiotic blend." Eur J Neurosci.

Oksanen, J., *et al.* (2017). "Package 'vegan'."

Olchanski, N., *et al.* (2013). "The economic burden of treatment-resistant depression." Clin Ther **35**(4): 512-522.

Oomen, C. A., *et al.* (2007). "Brief treatment with the glucocorticoid receptor antagonist mifepristone normalizes the reduction in neurogenesis after chronic stress." Eur J Neurosci **26**(12): 3395-3401.

Opie, R. S., *et al.* (2015). "The impact of whole-of-diet interventions on depression and anxiety: a systematic review of randomised controlled trials." Public Health Nutr **18**(11): 2074-2093.

Oresic, M. (2009). "Metabolomics, a novel tool for studies of nutrition, metabolism and lipid dysfunction." Nutr Metab Cardiovasc Dis **19**(11): 816-824.

Owen, L., *et al.* (2017). "The role of diet and nutrition on mental health and wellbeing." Proc Nutr Soc **76**(4): 425-426.

Owens, M. J., *et al.* (1997). "Neurotransmitter receptor and transporter binding profile of antidepressants and their metabolites." J Pharmacol Exp Ther **283**(3): 1305-1322.

Oxenkrug, G. F. (2010). "Tryptophan kynurenine metabolism as a common mediator of genetic and environmental impacts in major depressive disorder: the serotonin hypothesis revisited 40 years later." Isr J Psychiatry Relat Sci **47**(1): 56-63.

Ozdal, T., *et al.* (2016). "The Reciprocal Interactions between Polyphenols and Gut Microbiota and Effects on Bioaccessibility." Nutrients **8**(2): 78.

Pandey, K. B., *et al.* (2009). "Plant polyphenols as dietary antioxidants in human health and disease." Oxid Med Cell Longev **2**(5): 270-278.

Pariante, C. M. (2003). "Depression, stress and the adrenal axis." J Neuroendocrinol **15**(8): 811-812.

Pariante, C. M., *et al.* (2008). "The HPA axis in major depression: classical theories and new developments." Trends Neurosci **31**(9): 464-468.

Park, A. J., *et al.* (2013). "Altered colonic function and microbiota profile in a mouse model of chronic depression." Neurogastroenterol Motil **25**(9): 733-e575.

Park, H. J., *et al.* (2013). "Enhanced learning and memory of normal young rats by repeated oral administration of Krill Phosphatidylserine." Nutr Neurosci **16**(2): 47-53.

Park, L. T., *et al.* (2019). "Depression in the Primary Care Setting." N Engl J Med **380**(6): 559-568.

Paul, I. A., *et al.* (1994). "Adaptation of the N-methyl-D-aspartate receptor complex following chronic antidepressant treatments." J Pharmacol Exp Ther **269**(1): 95-102.

Peltier, J., *et al.* (2007). "PI3K/Akt and CREB regulate adult neural hippocampal progenitor proliferation and differentiation." Dev Neurobiol **67**(10): 1348-1361.

Persico, A. M., *et al.* (2013). "Urinary p-cresol in autism spectrum disorder." Neurotoxicol Teratol **36**: 82-90.

Peterson, B. L., *et al.* (2006). "A review of chromatographic methods for the assessment of phospholipids in biological samples." Biomed Chromatogr **20**(3): 227-243.

Pino, A., *et al.* (2017). "New neurons in adult brain: distribution, molecular mechanisms and therapies." Biochem Pharmacol **141**: 4-22.

Plotsky, P. M., *et al.* (2005). "Long-term consequences of neonatal rearing on central corticotropin-releasing factor systems in adult male rat offspring." Neuropsychopharmacology **30**(12): 2192-2204.

Porras, D., *et al.* (2017). "Protective effect of quercetin on high-fat diet-induced non-alcoholic fatty liver disease in mice is mediated by modulating intestinal microbiota imbalance and related gut-liver axis activation." Free Radic Biol Med **102**: 188-202.

Porsolt, R. D., *et al.* (1978). "Behavioural despair in rats: a new model sensitive to antidepressant treatments." Eur J Pharmacol **47**(4): 379-391.

Poulose, S. M., *et al.* (2017). "Nutritional Factors Affecting Adult Neurogenesis and Cognitive Function." Adv Nutr **8**(6): 804-811.

Priprem, A., *et al.* (2008). "Anxiety and cognitive effects of quercetin liposomes in rats." Nanomedicine **4**(1): 70-78.

Prut, L., *et al.* (2003). "The open field as a paradigm to measure the effects of drugs on anxiety-like behaviors: a review." Eur J Pharmacol **463**(1-3): 3-33.

Pryce, C. R., *et al.* (2011). "Helplessness: a systematic translational review of theory and evidence for its relevance to understanding and treating depression." Pharmacol Ther **132**(3): 242-267.

Pusceddu, M. M., *et al.* (2015). "n-3 PUFAs have beneficial effects on anxiety and cognition in female rats: Effects of early life stress." Psychoneuroendocrinology **58**: 79-90.

Pusceddu, M. M., *et al.* (2016). "The Omega-3 Polyunsaturated Fatty Acid Docosahexaenoic Acid (DHA) Reverses Corticosterone-Induced Changes in Cortical Neurons." Int J Neuropsychopharmacol **19**(6).

Putney, J. W., *et al.* (2012). "Phospholipase C signaling and calcium influx." Adv Biol Regul **52**(1): 152-164.

Qi, G., *et al.* (2017). "Neuroprotective action of tea polyphenols on oxidative stress-induced apoptosis through the activation of the TrkB/CREB/BDNF pathway and Keap1/Nrf2 signaling pathway in SH-SY5Y cells and mice brain." Food Funct **8**(12): 4421-4432.

Qiao, Y., *et al.* (2014). "Effects of resveratrol on gut microbiota and fat storage in a mouse model with high-fat-induced obesity." Food Funct **5**(6): 1241-1249.

Quadrato, G., *et al.* (2016). "The promises and challenges of human brain organoids as models of neuropsychiatric disease." Nature Medicine **22**: 1220.

Rahvar, M., *et al.* (2011). "Effect of oral resveratrol on the BDNF gene expression in the hippocampus of the rat brain." Neurochem Res **36**(5): 761-765.

Rajan, T. M., *et al.* (2017). "Psychiatric disorders and obesity: A review of association studies." J Postgrad Med **63**(3): 182-190.

Rao, T. S., *et al.* (2008). "Understanding nutrition, depression and mental illnesses." Indian J Psychiatry **50**(2): 77-82.

Read, J. R., *et al.* (2017). "Multimorbidity and depression: A systematic review and meta-analysis." J Affect Disord **221**: 36-46.

Reader, B. F., *et al.* (2015). "Peripheral and central effects of repeated social defeat stress: monocyte trafficking, microglial activation, and anxiety." Neuroscience **289**: 429-442.

Rechenberg, K., *et al.* (2013). "Nutritional interventions in depression and perinatal depression." Yale J Biol Med **86**(2): 127-137.

Remely, M., *et al.* (2017). "EGCG Prevents High Fat Diet-Induced Changes in Gut Microbiota, Decreases of DNA Strand Breaks, and Changes in Expression and DNA Methylation of Dnmt1 and MLH1 in C57BL/6J Male Mice." Oxid Med Cell Longev **2017**: 3079148.

Revsin, Y., *et al.* (2009). "Glucocorticoid receptor blockade normalizes hippocampal alterations and cognitive impairment in streptozotocin-induced type 1 diabetes mice." Neuropsychopharmacology **34**(3): 747-758.

Reynolds, B. A., *et al.* (2005). "Neural stem cells and neurospheres—re-evaluating the relationship." Nature Methods **2**(5): 333-336.

Rhee, S. H., *et al.* (2009). "Principles and clinical implications of the brain-gut-enteric microbiota axis." Nat Rev Gastroenterol Hepatol **6**(5): 306-314.

Rincel, M., *et al.* (2019). "Maternal separation in rodents: a journey from gut to brain and nutritional perspectives." Proc Nutr Soc: 1-20.

Rizza, S., *et al.* (2015). "S-Nitrosoglutathione Reductase Plays Opposite Roles in SH-SY5Y Models of Parkinson's Disease and Amyotrophic Lateral Sclerosis." Mediators Inflamm **2015**: 536238.

Robertson, R. C., *et al.* (2017). "Omega-3 polyunsaturated fatty acids critically regulate behaviour and gut microbiota development in adolescence and adulthood." Brain Behav Immun **59**: 21-37.

Rogers, P. J., *et al.* (2008). "No effect of n-3 long-chain polyunsaturated fatty acid (EPA and DHA) supplementation on depressed mood and cognitive function: a randomised controlled trial." Br J Nutr **99**(2): 421-431.

Rosa, P. B., *et al.* (2014). "Folic acid prevents depressive-like behavior induced by chronic corticosterone treatment in mice." Pharmacol Biochem Behav **127**: 1-6.

Rosenfeld, C. S. (2015). "Microbiome Disturbances and Autism Spectrum Disorders." Drug Metab Dispos **43**(10): 1557-1571.

Russo-Neustadt, A., *et al.* (2001). "Physical activity-antidepressant treatment combination: impact on brain-derived neurotrophic factor and behavior in an animal model." Behav Brain Res **120**(1): 87-95.

Rygula, R., *et al.* (2006). "Effects of fluoxetine on behavioral deficits evoked by chronic social stress in rats." Behav Brain Res **174**(1): 188-192.

Rygula, R., *et al.* (2005). "Anhedonia and motivational deficits in rats: impact of chronic social stress." Behav Brain Res **162**(1): 127-134.

Samad, N., *et al.* (2018). "Quercetin protects against stress-induced anxiety- and depression-like behavior and improves memory in male mice." Physiol Res **67**(5): 795-808.

Sanacora, G., *et al.* (2012). "Towards a glutamate hypothesis of depression: an emerging frontier of neuropsychopharmacology for mood disorders." Neuropharmacology **62**(1): 63-77.

Sanchez-Villegas, A., *et al.* (2013). "Diet, a new target to prevent depression?" BMC Medicine **11**(1): 3.

Sapolsky, R. M. (2000). "Glucocorticoids and hippocampal atrophy in neuropsychiatric disorders." Arch Gen Psychiatry **57**(10): 925-935.

Sapolsky, R. M., *et al.* (2000). "How do glucocorticoids influence stress responses? Integrating permissive, suppressive, stimulatory, and preparative actions." Endocr Rev **21**(1): 55-89.

Sartori, S. B., *et al.* (2012). "Magnesium deficiency induces anxiety and HPA axis dysregulation: modulation by therapeutic drug treatment." Neuropharmacology **62**(1): 304-312.

Saveanu, R. V., *et al.* (2012). "Etiology of depression: genetic and environmental factors." Psychiatr Clin North Am **35**(1): 51-71.

Sawikr, Y., *et al.* (2017). "Neuroinflammation in Alzheimer's Disease: The Preventive and Therapeutic Potential of Polyphenolic Nutraceuticals." Adv Protein Chem Struct Biol **108**: 33-57.

Scalbert, A., *et al.* (2000). "Dietary intake and bioavailability of polyphenols." J Nutr **130**(8S Suppl): 2073S-2085S.

Scapagnini, G., *et al.* (2011). "Modulation of Nrf2/ARE pathway by food polyphenols: a nutritional neuroprotective strategy for cognitive and neurodegenerative disorders." Mol Neurobiol **44**(2): 192-201.

Schildkraut, J. J. (1965). "The catecholamine hypothesis of affective disorders: a review of supporting evidence." Am J Psychiatry **122**(5): 509-522.

Schipper, L., *et al.* (2016). "A Postnatal Diet Containing Phospholipids, Processed to Yield Large, Phospholipid-Coated Lipid Droplets, Affects Specific Cognitive Behaviors in Healthy Male Mice." J Nutr **146**(6): 1155-1161.

Seibenhener, M. L., *et al.* (2015). "Use of the Open Field Maze to measure locomotor and anxiety-like behavior in mice." J Vis Exp(96): e52434.

Seligman, M. E. (1972). "Learned helplessness." Annu Rev Med **23**: 407-412.

Sen, S., *et al.* (2008). "Serum brain-derived neurotrophic factor, depression, and antidepressant medications: meta-analyses and implications." Biol Psychiatry **64**(6): 527-532.

Sgoifo, A., *et al.* (2002). "Intermittent exposure to social defeat and open-field test in rats: acute and long-term effects on ECG, body temperature and physical activity." Stress **5**(1): 23-35.

Shah, R. R., *et al.* (2016). "Efficient and versatile CRISPR engineering of human neurons in culture to model neurological disorders." Wellcome Open Res **1**: 13.

Shansky, R. M., *et al.* (2009). "Stress-induced dendritic remodeling in the prefrontal cortex is circuit specific." Cereb Cortex **19**(10): 2479-2484.

Shaw, D. M., *et al.* (1967). "5-Hydroxytryptamine in the hind-brain of depressive suicides." Br J Psychiatry **113**(505): 1407-1411.

Shaw, K., *et al.* (2002). "Tryptophan and 5-hydroxytryptophan for depression." Cochrane Database Syst Rev(1): CD003198.

Shen, C., *et al.* (2016). "Resveratrol pretreatment attenuates injury and promotes proliferation of neural stem cells following oxygen-glucose deprivation/reoxygenation by upregulating the expression of Nrf2, HO-1 and NQO1 in vitro." Mol Med Rep **14**(4): 3646-3654.

Shimizu, M. (2017). "Multifunctions of dietary polyphenols in the regulation of intestinal inflammation." J Food Drug Anal **25**(1): 93-99.

Shuler, M. L., *et al.* (2014). "Toward in vitro models of brain structure and function." Proc Natl Acad Sci U S A **111**(38): 13682-13683.

Siegrist, J. (2008). "Chronic psychosocial stress at work and risk of depression: evidence from prospective studies." Eur Arch Psychiatry Clin Neurosci **258 Suppl 5**: 115-119.

Singla, D. R., *et al.* (2018). "Scaling up psychological treatments for common mental disorders: a call to action." World Psychiatry **17**(2): 226-227.

Slattery, D. A., *et al.* (2012). "Using the rat forced swim test to assess antidepressant-like activity in rodents." Nat Protoc **7**(6): 1009-1014.

Slattery, D. A., *et al.* (2012). "Behavioural consequences of two chronic psychosocial stress paradigms: anxiety without depression." Psychoneuroendocrinology **37**(5): 702-714.

Smith, A. H., *et al.* (2004). "Effect of condensed tannins on bacterial diversity and metabolic activity in the rat gastrointestinal tract." Appl Environ Microbiol **70**(2): 1104-1115.

Smith, A. H., *et al.* (2005). "Bacterial mechanisms to overcome inhibitory effects of dietary tannins." Microb Ecol **50**(2): 197-205.

Smith, D. J., *et al.* (2018). "Adjunctive low-dose docosahexaenoic acid (DHA) for major depression: An open-label pilot trial." Nutr Neurosci **21**(3): 224-228.

Smith, K. (2014). "Mental health: a world of depression." Nature **515**(7526): 181.

Sofroniew, M. V., *et al.* (2010). "Astrocytes: biology and pathology." Acta Neuropathol **119**(1): 7-35.

Solanki, I., *et al.* (2015). "Flavonoid-based therapies in the early management of neurodegenerative diseases." Adv Nutr **6**(1): 64-72.

Soliman, M. A., *et al.* (2017). "Pluripotent stem cells in neuropsychiatric disorders." Mol Psychiatry **22**(9): 1241-1249.

Sorrells, S. F., *et al.* (2018). "Human hippocampal neurogenesis drops sharply in children to undetectable levels in adults." Nature **555**(7696): 377-381.

Spencer, J. P. (2008). "Food for thought: the role of dietary flavonoids in enhancing human memory, learning and neuro-cognitive performance." Proc Nutr Soc **67**(2): 238-252.

Spor, A., *et al.* (2011). "Unravelling the effects of the environment and host genotype on the gut microbiome." Nature Reviews Microbiology **9**(4): 279-290.

Steiner, J., *et al.* (2011). "Severe depression is associated with increased microglial quinolinic acid in subregions of the anterior cingulate gyrus: evidence for an immune-modulated glutamatergic neurotransmission?" J Neuroinflammation **8**: 94.

Sterling, P. (2012). "Allostasis: a model of predictive regulation." Physiol Behav **106**(1): 5-15.

Steru, L., *et al.* (1985). "The tail suspension test: a new method for screening antidepressants in mice." Psychopharmacology (Berl) **85**(3): 367-370.

Stevens, J. F., *et al.* (2004). "Xanthohumol and related prenylflavonoids from hops and beer: to your good health!" Phytochemistry **65**(10): 1317-1330.

Stilling, R. M., *et al.* (2016). "The neuropharmacology of butyrate: The bread and butter of the microbiota-gut-brain axis?" Neurochem Int **99**: 110-132.

Stoll, A. L., *et al.* (1999). "Omega 3 fatty acids in bipolar disorder: a preliminary double-blind, placebo-controlled trial." Arch Gen Psychiatry **56**(5): 407-412.

Sun, Q., *et al.* (2018). "Nrf2 Signaling Pathway Mediates the Antioxidative Effects of Taurine Against Corticosterone-Induced Cell Death in HUMAN SK-N-SH Cells." Neurochem Res **43**(2): 276-286.

Sun, Y., *et al.* (2017). "Neuroplasticity and behavioral effects of fluoxetine after experimental stroke." Restor Neurol Neurosci **35**(5): 457-468.

Sun, Z., *et al.* (2007). "Keap1 controls postinduction repression of the Nrf2-mediated antioxidant response by escorting nuclear export of Nrf2." Mol Cell Biol **27**(18): 6334-6349.

Sunshine, H., *et al.* (2017). "Membrane lipids and cell signaling." Curr Opin Lipidol **28**(5): 408-413.

Sureda, A., *et al.* (2015). "Polyphenols and depression: from chemistry to medicine." Curr Pharm Biotechnol **16**(3): 259-264.

Svennerholm, L., *et al.* (1972). "The distribution of lipids in the human nervous system. II. Lipid composition of human fetal and infant brain." Brain Res **47**(2): 457-468.

Tanigawa, S., *et al.* (2007). "Action of Nrf2 and Keap1 in ARE-mediated NQO1 expression by quercetin." Free Radic Biol Med **42**(11): 1690-1703.

Tatro, E. T., *et al.* (2009). "Modulation of glucocorticoid receptor nuclear translocation in neurons by immunophilins FKBP51 and FKBP52: implications for major depressive disorder." Brain Res **1286**: 1-12.

Taylor, V. H. (2019). "The microbiome and mental health: Hope or hype?" J Psychiatry Neurosci **44**(4): 219-222.

Thase, M. E. (2012). "The role of monoamine oxidase inhibitors in depression treatment guidelines." J Clin Psychiatry **73 Suppl 1**: 10-16.

Thibaut, F. (2017). “Anxiety disorders: a review of current literature” Dialogues Clin Neurosci **19**(2): 87-88.

Trackey, J. L., *et al.* (2001). "SIN-1-induced cytotoxicity in mixed cortical cell culture: peroxynitrite-dependent and -independent induction of excitotoxic cell death." J Neurochem **79**(2): 445-455.

Treede, I., *et al.* (2007). "Anti-inflammatory effects of phosphatidylcholine." J Biol Chem **282**(37): 27155-27164.

Trullas, R., *et al.* (1990). "Functional antagonists at the NMDA receptor complex exhibit antidepressant actions." Eur J Pharmacol **185**(1): 1-10.

Tsankova, N. M., *et al.* (2006). "Sustained hippocampal chromatin regulation in a mouse model of depression and antidepressant action." Nature Neuroscience **9**(4): 519-525.

Tsigos, C., *et al.* (2002). "Hypothalamic-pituitary-adrenal axis, neuroendocrine factors and stress." J Psychosom Res **53**(4): 865-871.

Ungvari, Z., *et al.* (2010). "Resveratrol confers endothelial protection via activation of the antioxidant transcription factor Nrf2." Am J Physiol Heart Circ Physiol **299**(1): H18-24.

Uschold-Schmidt, N., *et al.* (2012). "Chronic psychosocial stress results in sensitization of the HPA axis to acute heterotypic stressors despite a reduction of adrenal in vitro ACTH responsiveness." Psychoneuroendocrinology **37**(10): 1676-1687.

Uzun, S., *et al.* (2010). "Side effects of treatment with benzodiazepines." Psychiatr Danub **22**(1): 90-93.

Vaishnavi, S. N., *et al.* (2004). "Milnacipran: a comparative analysis of human monoamine uptake and transporter binding affinity." Biol Psychiatry **55**(3): 320-322.

Valles-Colomer, M., *et al.* (2019). "The neuroactive potential of the human gut microbiota in quality of life and depression." Nature Microbiology: 1.

van Broekhoven, F., *et al.* (2002). "Dependence potential of antidepressants compared to benzodiazepines." Prog Neuropsychopharmacol Biol Psychiatry **26**(5): 939-943.

van de Wouw, M., *et al.* (2018). "Short-chain fatty acids: microbial metabolites that alleviate stress-induced brain-gut axis alterations." J Physiol **596**(20): 4923-4944.

Vasconcelos, M., *et al.* (2015). "Social defeat protocol and relevant biomarkers, implications for stress response physiology, drug abuse, mood disorders and individual stress vulnerability: a systematic review of the last decade." Trends Psychiatry Psychother **37**(2): 51-66.

Vauzour, D. (2012). "Dietary polyphenols as modulators of brain functions: biological actions and molecular mechanisms underpinning their beneficial effects." Oxid Med Cell Longev **2012**: 914273.

Vauzour, D., *et al.* (2010). "Polyphenols and human health: prevention of disease and mechanisms of action." Nutrients **2**(11): 1106-1131.

Venkatesan, R., *et al.* (2015). "Phytochemicals that regulate neurodegenerative disease by targeting neurotrophins: a comprehensive review." Biomed Res Int **2015**: 814068.

Venzala, E., *et al.* (2012). "Chronic social defeat stress model: behavioral features, antidepressant action, and interaction with biological risk factors." Psychopharmacology (Berl) **224**(2): 313-325.

Vetulani, J. (2013). "Early maternal separation: a rodent model of depression and a prevailing human condition." Pharmacol Rep **65**(6): 1451-1461.

Vgontzas, A. N., *et al.* (1995). "Benzodiazepine side effects: role of pharmacokinetics and pharmacodynamics." Pharmacology **51**(4): 205-223.

Wahl, D., *et al.* (2016). "Nutritional strategies to optimise cognitive function in the aging brain." Ageing Res Rev **31**: 80-92.

Wang, C., *et al.* (2012). "Quercetin and allopurinol ameliorate kidney injury in STZ-treated rats with regulation of renal NLRP3 inflammasome activation and lipid accumulation." PLoS One **7**(6): e38285.

Wang, S., *et al.* (2018). "Agarwood Essential Oil Ameliorates Restrain Stress-Induced Anxiety and Depression by Inhibiting HPA Axis Hyperactivity." Int J Mol Sci **19**(11).

Wang, Y., *et al.* (2018). "Role of protein dynamics in transmembrane receptor signalling." Curr Opin Struct Biol **48**: 74-82.

Wang, Y., *et al.* (2018). "Curcumin as a therapeutic agent for blocking NF-kappaB activation in ulcerative colitis." Immunopharmacol Immunotoxicol **40**(6): 476-482.

Watanabe, Y., *et al.* (2017). "Omega-3 polyunsaturated fatty acids for cardiovascular diseases: present, past and future." Expert Rev Clin Pharmacol **10**(8): 865-873.

Wehry, A. M., *et al.* (2015). “Assessment and treatment of anxiety disorders in children and adolescents.” Curr Psychiatry Rep **17**(7): 52.

Wei, K., *et al.* (2016). "Icariin alters the expression of glucocorticoid receptor, FKBP5 and SGK1 in rat brains following exposure to chronic mild stress." Int J Mol Med **38**(1): 337-344.

Weikum, E. R., *et al.* (2017). "Glucocorticoid receptor control of transcription: precision and plasticity via allostery." Nat Rev Mol Cell Biol **18**(3): 159-174.

Wie, M. B., *et al.* (1997). "Eugenol protects neuronal cells from excitotoxic and oxidative injury in primary cortical cultures." Neurosci Lett **225**(2): 93-96.

Wieck, A., *et al.* (2013). "Evidence for a neuroinflammatory mechanism in delayed effects of early life adversity in rats: relationship to cortical NMDA receptor expression." Brain Behav Immun **28**: 218-226.

Wiktorowska-Owczarek, A., *et al.* (2015). "PUFAs: Structures, Metabolism and Functions." Adv Clin Exp Med **24**(6): 931-941.

Willett, W. C., *et al.* (1995). "Mediterranean diet pyramid: a cultural model for healthy eating." Am J Clin Nutr **61**(6 Suppl): 1402S-1406S.

Williamson, G., *et al.* (2017). "Role of the small intestine, colon and microbiota in determining the metabolic fate of polyphenols." Biochem Pharmacol **139**: 24-39.

Willner, P. (1997). "Validity, reliability and utility of the chronic mild stress model of depression: a 10-year review and evaluation." Psychopharmacology (Berl) **134**(4): 319-329.

Wohleb, E. S. (2016). "Neuron-Microglia Interactions in Mental Health Disorders: "For Better, and For Worse"." Front Immunol **7**: 544.

Wong, D. T., *et al.* (1974). "A selective inhibitor of serotonin uptake: Lilly 110140, 3-(p-trifluoromethylphenoxy)-N-methyl-3-phenylpropylamine." Life Sci **15**(3): 471-479.

Xu, Y., *et al.* (2005). "Antidepressant effects of curcumin in the forced swim test and olfactory bulbectomy models of depression in rats." Pharmacol Biochem Behav **82**(1): 200-206.

Xu, Z., *et al.* (2015). "Dietary effects on human gut microbiome diversity." Br J Nutr **113 Suppl**: S1-5.

Yahfoufi, N., *et al.* (2018). "The Immunomodulatory and Anti-Inflammatory Role of Polyphenols." Nutrients **10**(11).

Yahiro, K., *et al.* (2015). "Helicobacter pylori VacA induces apoptosis by accumulation of connexin 43 in autophagic vesicles via a Rac1/ERK-dependent pathway." Cell Death Discov **1**: 15035.

Yamada, M., *et al.* (2004). "Clinical pharmacology of MAO inhibitors: safety and future." Neurotoxicology **25**(1-2): 215-221.

Yang, X., *et al.* (2017). "Resveratrol regulates microglia M1/M2 polarization via PGC-1alpha in conditions of neuroinflammatory injury." Brain Behav Immun **64**: 162-172.

Yang, X. H., *et al.* (2017). "Resveratrol ameliorates chronic unpredictable mild stress-induced depression-like behavior: involvement of the HPA axis, inflammatory markers, BDNF, and Wnt/beta-catenin pathway in rats." Neuropsychiatr Dis Treat **13**: 2727-2736.

Yao, J., *et al.* (2015). "Xanthohumol, a polyphenol chalcone present in hops, activating Nrf2 enzymes to confer protection against oxidative damage in PC12 cells." J Agric Food Chem **63**(5): 1521-1531.

Yao, P., *et al.* (2007). "Quercetin protects human hepatocytes from ethanol-derived oxidative stress by inducing heme oxygenase-1 via the MAPK/Nrf2 pathways." J Hepatol **47**(2): 253-261.

Yao, W., *et al.* (2016). "Role of Keap1-Nrf2 signaling in depression and dietary intake of glucoraphanin confers stress resilience in mice." Sci Rep **6**: 30659.

Yasui, T., *et al.* (2017). "Hypoxia Epigenetically Confers Astrocytic Differentiation Potential on Human Pluripotent Cell-Derived Neural Precursor Cells." Stem Cell Reports **8**(6): 1743-1756.

Yau, Y. H., *et al.* (2013). "Stress and eating behaviors." Minerva Endocrinol **38**(3): 255-267.

Yeomans, M. R. (2017). "Adverse effects of consuming high fat-sugar diets on cognition: implications for understanding obesity." Proc Nutr Soc **76**(4): 455-465.

Yi, L. T., *et al.* (2010). "Involvement of monoaminergic system in the antidepressant-like effect of the flavonoid naringenin in mice." Prog Neuropsychopharmacol Biol Psychiatry **34**(7): 1223-1228.

Yi, L. T., *et al.* (2008). "Antidepressant-like behavioral and neurochemical effects of the citrus-associated chemical apigenin." Life Sci **82**(13-14): 741-751.

Young, S. N. (2002). "Clinical nutrition: 3. The fuzzy boundary between nutrition and psychopharmacology." CMAJ **166**(2): 205-209.

Zhang, H., *et al.* (2012). "Icariin inhibits corticosterone-induced apoptosis in hypothalamic neurons via the PI3-K/Akt signaling pathway." Mol Med Rep **6**(5): 967-972.

Zhang, J. C., *et al.* (2018). "Keap1-Nrf2 signaling pathway confers resilience versus susceptibility to inescapable electric stress." Eur Arch Psychiatry Clin Neurosci **268**(8): 865-870.

Zhang, L., *et al.* (2007). "Caffeic acid attenuates neuronal damage, astrogliosis and glial scar formation in mouse brain with cryoinjury." Life Sci **80**(6): 530-537.

Zhang, Z., *et al.* (2017). "Effect of Curcumin on the Diversity of Gut Microbiota in Ovariectomized Rats." Nutrients **9**(10).

Zhao, H., *et al.* (2017). "Molecular mechanisms of brain-derived neurotrophic factor in neuro-protection: Recent developments." Brain Res **1665**: 1-21.

Zhao, L., *et al.* (2017). "A combination of quercetin and resveratrol reduces obesity in high-fat diet-fed rats by modulation of gut microbiota." Food Funct **8**(12): 4644-4656.

Zhao, X., *et al.* (2018). "Epigallocatechin-3-gallate confers protection against corticosterone-induced neuron injuries via restoring extracellular signal-regulated kinase 1/2 and phosphatidylinositol-3 kinase/protein kinase B signaling pathways." PLoS One **13**(1): e0192083.

Zheng, L., *et al.* (2019). "Dietary Polar Lipids and Cognitive Development: A Narrative Review." Adv Nutr.

Zhou, Y., *et al.* (2016). "Natural Polyphenols for Prevention and Treatment of Cancer." Nutrients **8**(8).

Zhu, H., *et al.* (2013). "High-dose glucocorticoid aggravates TBI-associated corticosteroid insufficiency by inducing hypothalamic neuronal apoptosis." Brain Res **1541**: 69-80.

Zhu, W. L., *et al.* (2012). "Green tea polyphenols produce antidepressant-like effects in adult mice." Pharmacol Res **65**(1): 74-80.