EVALUATION OF ALTERATION IN THE EXPRESSION OF REACTIVE ASTROCYTE GENES AND DIET-RELATED GENES DUE TO STRESS AND INULIN PREBIOTIC TREATMENTS IN AGED MICE

FINAL YEAR PROJECT OF BIOCHEMISTRY AND MOLECULAR BIOLOGY

Júlia Borràs Bisa

Education: Double degree in Biotechnology, and Biochemistry and Molecular Biology (2016 – 2021)

University: Rovira i Virgili, Tarragona

Academic supervisor: Dr Manuel Suárez Recio, Biochemistry and Biotechnology Department, manuel.suarez@urv.cat

In collaboration with: APC Microbiome Ireland

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This project is based on the results obtained in the Extracurricular Internship carried out in APC Microbiome Ireland under the professional supervision of Gerard Moloney and Joana da Cruz Pereira, and the academic supervision of Manuel Suárez Recio. Due to pandemic situation, this internship lasted just two months, although my first intention was to be there for four months. Therefore, we had to think a Plan B and change the main objective of the project.

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ABSTRACT

Neuroinflammation, triggered by factors such as aging and social defeat stress (SDS), is a common characteristic of important neurodegenerative age-associated disorders, which are becoming one of the major health threats of the actual society. Astrocytes, which are a type of glial cell, respond to neuroinflammation through reactive astrogliosis, a process in which astrocytes exhibit heterogeneity at multiple levels and the expression of some genes is altered. Preliminary data from this study showed that, in a social cognition task, aged mice exposed to stress could not discriminate between a familiar and a novel mouse, but mice exposed to stress and fed with inulin-supplemented diet, rescued the ability to discriminate them. Given that inulin acts as a prebiotic - therefore shaping the gut microbiome – this fibre can potentially alter social behaviour by acting on the gut microbiome-brain axis. The current project focused on the evaluation of the impact of stress on reactive astrogliosis-related genes and a diet-related gene expression, and the potential of inulin to modulate gene expression alterations caused by stress in male C57BL/6 aged mice. Concretely, the expression of glial fibrillary acidic protein (*Gfap*), signal transducer and activator of transcription 3 (*Stat3*), serine peptidase inhibitor clade A member 3N (*Serpina3n*), complement component 3 (*C3*), complement component 4b (*C4b*), C-X-C motif chemokine ligand 10 (*Cxcl10*) and aryl hydrocarbon receptor (*Ahr*) in three different brain regions, amygdala, prefrontal cortex (PFC) and hippocampus, was analysed after SDS exposure and inulin-supplementation. To do so, RNA extraction of amygdala, PFC and hippocampus from control and treated mice, cDNA synthesis and relative quantification through qPCR were performed. Results suggested that just in some specific cases gene expression was altered by SDS and inulin-supplemented chow diet. In general, the gene expression of individuals was highly variable, and it made difficult to draw strong conclusions. Furthermore, we suggest that aging, which is an evident neuroinflammatory factor, could previously had altered gene expression in the brain of aged mice, thus masking SDS effect. Overall, although more studies need to be conducted, this research provides more information in the knowledge of neuroinflammation and gut-microbiome-brain axis.

Key words: gene expression, inulin, neuroinflammation, reactive astrogliosis, social defeat stress

ABBREVIATIONS

3Ch	Three-chamber test
ACTB	Beta-actin
AHR	Aryl hydrocarbon receptor
BBB	Blood-brain-barrier
C3	Complement component 3
C4B	Complement component 4b
CCK	Cholecystokinin
CNS	Central nervous system
CXCL10	C-X-C motif chemokine ligand 10
FOS	Fructooligosaccharide
GALT	Gastrointestinal associated lymphoid tissue
GF	Germ-free
GFAP	Glial fibrillary acidic protein
GIT	Gastrointestinal tract
GLP-1	Glucagon-like peptide 1
GM	Gut microbiota
GOS	Galactooligosaccharide
IL	Interleukin
LPS	Lipopolysaccharide
MLN	Mesenteric lymph node
NSC	Neural stem cell
PFC	Prefrontal cortex
PP	Peyer's patch
PYY	Peptide YY
qPCR	Quantitative polymerase chain reaction
RG	Radial glial
SCFA	Short chain fatty acid
SDS	Social defeat stress
SERPINA3N	Serine peptidase inhibitor clade A member 3N
STAT3	Signal transducer and activator of transcription 3

1. INTRODUCTION

1.1. NEUROINFLAMMATORY DISORDERS

Neuroinflammation, which is defined as an inflammatory response centralized in the brain and spinal cord, is a common characteristic of the pathogenic mechanisms involved in important neurodegenerative age-associated disorders, as multiple sclerosis, Parkinson's and Alzheimer's diseases (Gambino *et al.* 2019). These disorders usually are related to dementia, characterized by a deterioration in memory, thinking, behaviour and the ability to perform usual activities (World Health Organization, 2020).

In 2010, it was estimated that 35.6 million people worldwide were diagnosed with dementia and this number is expected to nearly double every 20 years (Bettens *et al.* 2010). Hence, neuroinflammatory disorders are becoming one of the major threats to the society health and many efforts are focused on this area of research.

From a molecular point of view, neuroinflammation is mediated by the production of cytokines, chemokines, secondary messengers, and reactive oxygen species. There are different degrees of neuroinflammatory response, which depends on the context, duration and primary stimulus or insult. Inflammatory mediators are produced by central nervous system (CNS) cells, specifically, microglia and astrocytes, which play an essential role in the neuroinflammatory process (DiSabato *et al.* 2016).

1.2. CENTRAL NERVOUS SYSTEM AND ASTROCYTES

1.2.1. Central nervous system

The CNS is the part of the nervous system formed by the brain and spinal cord. Its responsibilities are receiving, processing, and responding to sensory information (Thau *et al.* 1943).

The brain is composed of billions of neurons that form numerous connections and synapses. It is divided into three parts: cerebrum, cerebellum, and brain stem. The cerebrum is formed by two hemispheres, left and right, which are continuously communicating (Jawabri and Sharma 2019). The most commonly studied brain regions are described below and represented in **Figure 1**:

- ***Cerebral cortex***. It is the outermost layer of the brain, and it is composed of grey matter. There are four lobes in the cortex: frontal, parietal, occipital, and temporal, and each lobe has different function (Jawabri and Sharma 2019).

- ***Prefrontal cortex (PFC).*** It is located on the forward-facing part of the frontal lobe. PFC functions are inhibiting inappropriate behaviour, providing continuity in the thought process, and keeping the mind focused on goals. PFC is connected to other cortex lobes, amygdala and thalamus, and it is involved in emotional brain (Ackerman 1992).
- ***Thalamus.*** It is located above the brain stem, in the centre of the brain, and divided into two masses. It sorts information from four of the senses (sight, hearing, taste, and touch) and relays it to the cerebral cortex. Some other sensations, such as pain, temperature, and pressure, are also related to thalamus (Ackerman 1992; Mandal and Robertson n.d.)
- ***Hypothalamus.*** It is one of the smallest parts of the brain (2 % of the total volume of the brain) and connects the CNS to the endocrine system through the pituitary gland (Lechan and Toni 2000). It is often related to complex functions of the organism, as vegetative system control, homeostasis, thermoregulation, and emotional behaviour (Swaab *et al.* 2018).
- ***Cerebellum.*** It is located at the back of the brain and consists of grey matter (on the surface of the cerebellum) and white matter (under the cerebellar cortex). Cerebellum regulates and coordinates voluntary movements. Besides, it coordinates balance and posture, motor learning and speech. Finally, it is also involved in cognitive function (Han and Seladi-Schulman 2021).
- ***Amygdala.*** It is a paired structure, and it is located in the medial temporal lobe, in front of the hippocampus. Amygdala is related to emotion and memory control. Besides, it detects threats and activates appropriate fear-related behaviours. Recent studies also relate amygdala to positive emotions elicited by appetite stimuli (Salzman n.d.).
- ***Hippocampus.*** It is a dense pack of neurons that curves into a S-sharped structure and is embedded deep into temporal lobe. It has a major role in learning and memory (Anand and Dhikav 2012).

The limbic system is an assembly of linked structures in the brain, which are involved in the behaviour and the emotional responses that are necessaries for animal's survival: feeding, reproduction, fight responses, etc. Some structures related to limbic system are amygdala, hippocampus, and prefrontal cortex (The limbic system 2019).

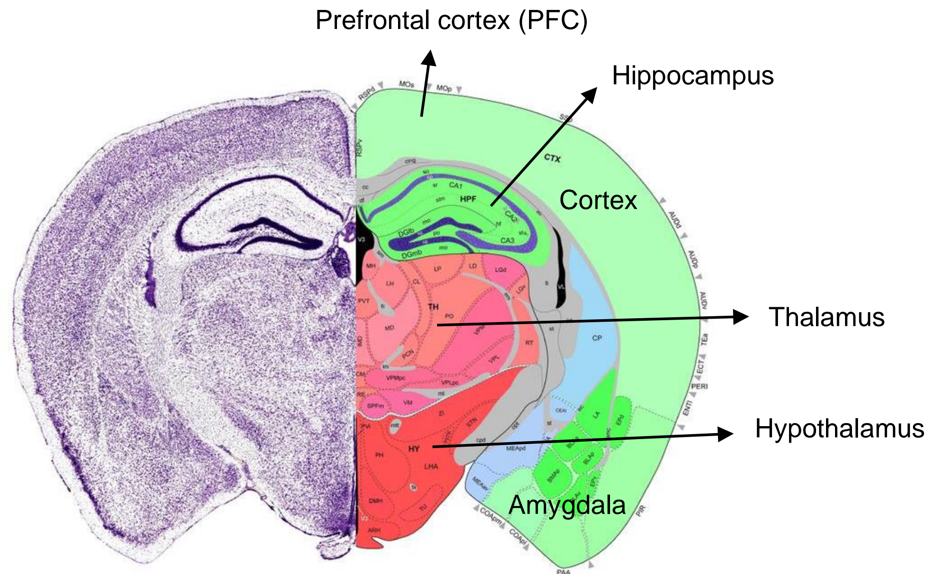

Figure 1 | Coronal section of mouse brain. Extracted and adapted from the Allen Institute for Brain Science (2021).

1.2.2. Definition and characteristics of astrocytes

Astrocytes, in conjunction with oligodendrocytes, microglia, ependymal cells, Schwann cells and satellite cells define the group of glial cells. The first four types of glial cells are located in the brain and spinal cord and the latter two are found in the peripheral nervous system (Carlson 2018). Although historically glial cells were defined as a glue that held the CNS together and were thought of solely as support cells for neurons, they participate in some crucial functions for the organism. Their functions include regulation of myelination in the CNS, contribution to the blood-brain barrier, removal, recycling and storage of transmitter substances, storage of glycogen, among others (Maynard and Downes 2019). For 50 years, it has been thought that glia:neuron ratio was 10:1 but the new counting method using an isotropic fractionator has demonstrated this ratio is less than 1:1 (von Bartheld *et al.* 2016).

Astrocytes are the most common glial cell type, named for its characteristic star-like shape due to processes extending radially from the cell body (Ramachandran 2002). Their nuclei is approximately the same size as many neuronal nuclei but is larger than oligodendrocyte nuclei (Treuting *et al.* 2018).

The morphology and location of astrocytes classify them into two main subpopulations: the fibrous and protoplasmic astrocytes in the white and grey matter, respectively (Chaboub and Deneen 2013). Protoplasmic astrocytes have many finely branching processes, which envelop synapses and whose endfeet cover blood vessels. They function in part by regulating the extracellular concentration of various molecules as well

as proper neuronal synaptic function. Fibrous astrocytes express glial fibrillary acidic protein (GFAP) and have long, thin, unbranched processes whose endfeet envelop nodes of Ranvier, short segments where the axon is naked. Their exact function remains elusive (Wang and Bordey 2008; Chaboub and Deneen 2013).

The generation of mature astrocytes is the culmination of series of differentiation steps that are composed of intermediate precursor population. Neuroepithelial cells, which are the neural stem cells (NSCs), form radial glial (RG) cells in the ventricular zone (Chandrasekaran *et al.* 2016). They are the earliest cells to be derived from the lateral wall of the neural tube. In the neonatal subventricular zone, RG cells can generate intermediate progenitors cells, which behave as neural progenitors for both neurons and glial cells during forebrain development (Noctor *et al.* 2001; Chandrasekaran *et al.* 2016). However, brain cells are not developed at the same time: neurons are generated first, followed by astrocytes, and finally, oligodendrocytes (Wang *et al.* 2016). In embryonic development, RG cells transition from neurogenesis to gliogenesis in a process called the gliogenic switch, and gliogenesis continues during the neonatal and postnatal period (Wang and Bordey 2008; Molofsky and Deneen 2015). The gliogenic switch is governed by extracellular and intracellular mechanisms, such as the fibroblast growth factor (Dinh Duong *et al.* 2019) or the epigenetic regulator Polycomb group proteins (Hirabayashi *et al.* 2009).

Astrocytes are implicated in many important functions within the brain, some of which are cited below. ***(1) Formation and maintenance of the blood-brain-barrier (BBB).*** BBB is a highly specialized brain microvascular structure made up of endothelial cells coupled with astrocytes that protects the brain from toxic substrates in the blood and maintains ion gradient (Wang and Bordey 2008). The main functional component of the BBB are the endothelial tight junctions (Sofroniew and Vinters 2010), and astrocytes use their endfeet to make physical contact with endothelial cells and secrete diffusible molecules that regulate formation of tight junctions (Chaboub and Deneen 2013). ***(2) Metabolism.*** Astrocytes uptake glutamate, the major neurotransmitter in nervous system, through glutamate transporters GLAST or GLT1. Glutamate is turned into glutamine with the help of glutamine synthetase, and then it is transported back to neurons (Chaboub and Deneen 2013). Astrocytic glutamate uptake also induces glycolysis, resulting in the production and shuttling of lactate from astrocytes to neurons for oxidative metabolism (Stobart and Anderson 2013). ***(3) Communication between astrocytes and neurons.*** Astrocytes exhibit regulated increases in intracellular calcium (Ca^{2+}) concentration as a way of astrocyte-neuron intercellular communication. Ca^{2+} elevation can be triggered by transmitters released during neuronal activity and elicit the

release from astrocytes of neuroactive molecules such as glutamate into the extracellular space (Halassa *et al.* 2007; Sofroniew and Vinters 2010). ***(4) Synaptogenesis and neurotransmission.*** Billions of neurons connect to form functional networks via synapses and astrocytes participate in this process because they increase the number of synaptic structures within the neural circuits. (Chandrasekaran *et al.* 2016). ***(5) Ion balance.*** The implication of astrocytes in ion homeostasis was one of the firsts functions described. Activation of neurons results in the accumulation of K^+ extracellularly and astrocytes take up excess amount of K^+ and dilute it (Holthoff and Witte 2000).

*1.2.3. **Astrocytes throughout lifespan***

Astrocytes change with aging, and this is manifested by different parameters. First, as Salas *et al.* (2020) reviewed, astrocytes respond to brain aging by changing their morphology, increasing their volume and complexity, and these alterations seem to occur in a region-specific manner. Second, although for years it was believed that the number of astrocytes increased with brain aging, some studies revealed that there were no differences between the cell number of adult (5 months-old) and old (21 months-old) mice brain (Grosche *et al.* 2013). Moreover, a recent transcriptomic study suggested that the astrocyte number practically does not change with brain aging (Ximerakis *et al.* 2019). Third, cultures of old astrocytes produced significantly more superoxide radical, lipoperoxidation and protein oxidation and damage than cultures of adult astrocytes (García-Matas *et al.* 2008). Finally, with aging, astrocytes become less capable of doing their functions. For instance, aging downregulates expression of genes related to excitotoxicity and it leads to a deficiency in glutamate and potassium homeostasis (Limbad *et al.* 2020). Besides, astrocytes lose their ability to provide functional support to neurons and myelination, which is highly active in young mice and greatly inhibited in aged mice (Wang *et al.* 2020). Another important function of astrocytes is the formation and maintenance of BBB, so perivascular astrocyte senescence leads to altered BBB function in old age (Ferrer 2017). These functional alterations in aged glial cells contribute to the deterioration of the neural tissue in older individuals. And since hypothalamic glial cells are involved in systemic homeostasis, their modifications with aging may contribute to the deterioration of other body tissues (Chowen and Garcia-Segura 2021).

1.2.4. Astrocyte reactivity

Astrocytes respond to all forms of injury and disease in the CNS through a process called reactive astrogliosis. It is not a simple all-or-none phenomenon as it can vary from subtle to deep changes. Reactive astrocytes exhibit heterogeneity at multiple levels: cell morphology, gene expression, cell signalling and cell function, among others (Anderson *et al.* 2014). Reactive astrocytes are characterized by an increased expression of GFAP, so changes in GFAP protein amounts and the level of *GFAP* gene expression are widely used to quantify astrocyte reactivity although they are variable depending on the region of the brain (Salas *et al.* 2020). For instance, in rat cerebral cortex, staining for GFAP was lower than in rat cerebral hippocampus (Kimelberg 2004). Therefore, it is necessary to find better markers of astrocyte reactivity and recent transcriptomic studies have contributed to the discovery of other reactive astrocyte genes more specific and with higher expression (Liddelow and Barres 2017). For instance, complement component 3 (C3) and complement component 4b (C4B) proteins play a key role in the complement system and the genes that codifies for them (*C3* and *C4B*) are upregulated in reactive astrocytes. C-X-C motif chemokine ligand 10 (CXCL10) protein binds to the receptor CXCR3, and this union triggers an influx of inflammatory leukocytes into the CNS. The gene that codifies for CXCL10, *CXCL10*, is also upregulated in reactive astrocytes. Besides, the expression of serine peptidase inhibitor clade A member 3N (*SERPINA3N*) gene, that codifies for a peptidase inhibitor called SERPINA3N, is induced by inflammation. Finally, signal transducer and activator of transcription 3 (STAT3) is a transcription factor encoded by *STAT3* gene, whose expression is upregulated in reactive astrogliosis (Herrmann *et al.* 2008; Zamanian *et al.* 2012; Liddelow *et al.* 2017; Clarke *et al.* 2018).

There are two different types of reactive astrocytes, A1 and A2, induced by neuroinflammation and ischemia, respectively. Based on the upregulated genes by these two types of astrocytes, it is suggested that A1 neuroinflammatory reactive astrocytes might have harmful functions, meanwhile A2 ischemic reactive astrocytes might have helpful functions (Liddelow and Barres 2017).

In aging, the brain becomes more vulnerable to injury and some studies showed that astrocytes from aged brains have a reactive phenotype typical of neuroinflammatory A1-like reactive astrocytes (Clarke *et al.* 2018). Besides, it is well studied that exposure to stress induces alteration in immune system and inflammatory responses in brain and periphery (Grippo and Scotti 2013). Therefore, both aging and stress are factors that trigger neuroinflammation.

1.3. GUT-BRAIN AXIS

The gut-brain axis is the term for the bidirectional communication system between the CNS and the gastrointestinal tract, where serotonin is the key neurotransmitter (Chassaing and Gewirtz 2018).

Much of the earlier gut-brain axis work is focused on the gastrointestinal function since it plays an essential role in the regulation of gastrointestinal tract (GIT) motility, secretion, digestion, absorption, regulation of appetite, energy balance and metabolism (Washabau and Day 2012). Nevertheless, recent research has taken an increasing focus on cognitive and psychological effects of gut-brain communication. A bad network between these two body regions leads to gut inflammation disorders, altered responses to acute and chronic stress and altered behaviour (Cryan *et al.* 2019).

1.3.1. Microbiome-gut-brain axis

There is clear evidence of the importance of microbiota in this bidirectional communication between the GIT and the brain. There are many pathways of communication between the gut microbiota (GM) and the brain, some of which are represented in **Figure 2** and are described below. ***(1) Immune mediators,*** as pro-inflammatory cytokines (IL-6 and IL-1β), are stimulated when lipopolysaccharide (LPS) from gram-negative bacteria cell walls contacts toll-like receptors, expressed by immune cells. Then, the inflammatory response signals the brain directly via the circumventricular organs or via the vagus nerve (Sherwin *et al.* 2016). ***(2) Vagus nerve*** is the 10th cranial nerve, the fastest route to connect the gut and the brain and it is composed of 80 % afferent and 20 % efferent fibres (Cryan *et al.* 2019). ***(3) Short chain fatty acids (SCFAs)***, specifically, acetate, butyrate and propionate, are generated by microbial fermentation of host dietary-resistant starch and nonstarch polysaccharides, and they are the major signalling molecules mediating microbiota and host communication (Martin *et al.* 2018). SCFAs can bind to G-protein coupled receptors and influence brain physiology and behaviour (Sherwin *et al.* 2016). ***(4) Enteroendocrine cells*** are situated in the intestine and can produce signalling molecules, such as glucagon-like peptide 1 (GLP-1), peptide YY (PYY) and cholecystokinin (CCK), which reduce appetite. Bacterial commensals are capable of regulating enteroendocrine signalling, for example, it was suggested *Escherichia coli* proteins are involved in host satiety, as increased PYY and suppressed acutely food intake (Collins *et al.* 2012).

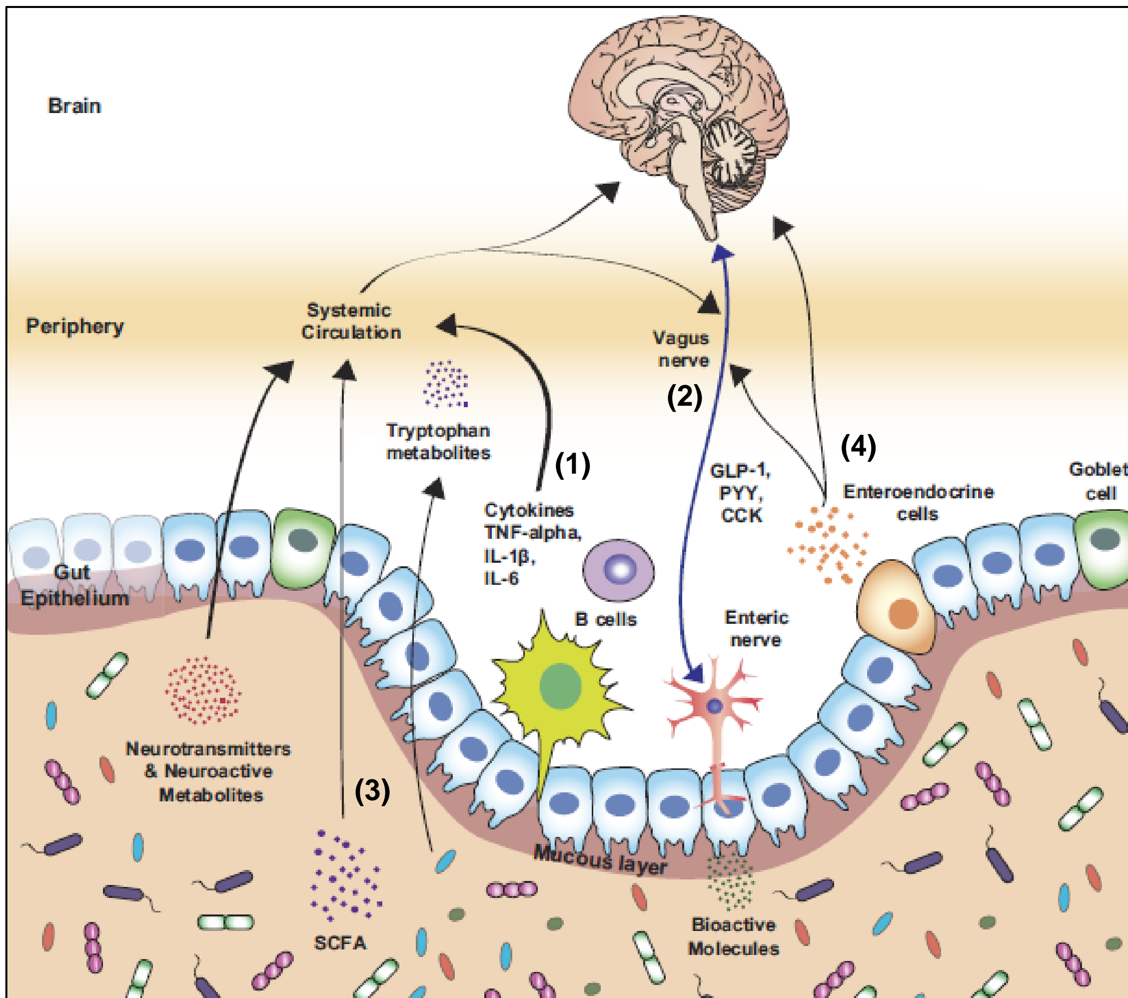

Figure 2 | Communication mechanisms between the gut microbiota (GM) and the brain, including **(1)** immune response, **(2)** vagus nerve, **(3)** short chain fatty acids (SCFAs) and **(4)** enteroendocrine cells. CCK, cholecystokinin; GLP-1, glucagon-like peptide 1; IL, interleukin; PYY, peptide YY; TNF, tumor necrosis factors. Adapted from Cryan *et al.* (2019).

1.3.2. Possible involvement of astrocytes in gut-brain communication

Astrocytic activation can be affected by various factors within or outside the CNS, such as gut microbiota-mediated metabolites, which are recognized by aryl hydrocarbon receptors (AHR) in astrocytes (Ma *et al.* 2019). An upregulation of AHRs triggers an anti-inflammatory phenotype (Rothhammer *et al.* 2016).

The effect of GM on astrocytes depends on the bacterial species. For instance, as Ma *et al.* (2019) reviewed, some ampicillin-sensitive microbes catalysed dietary tryptophan to AHR agonist, leading to protection of neurons from inflammation. However, *Porphyromonas gingivalis*, a common species in inflammatory diseases, stimulated astrocytes and induced neuroinflammatory responses.

Therefore, it is demonstrated that astrocytes take part in gut-brain communication but after GM activity, the astrocytes' response is variable.

1.4. GUT MICROBIOME

The human microbiome has emerged as an area of utmost interest. Over the past two decades, microbiome research has accelerated and is revealing the huge impact that the microbiota has on our daily lives. It is a critical determinant of human health and disease and a key regulator of host physiology and metabolism (Cryan *et al.* 2019).

Human microbiota includes all the microorganisms that live in and on us, including bacteria, viruses, fungi, and protozoa, but the bacterial population is currently the most well characterized. The GM is comprised of all the bacteria residing in the GIT, mainly of the phyla *Firmicutes, Bacteroidetes, Actinobacteria* and *Proteobacteria* (Belizário and Napolitano 2015). While bacteria colonize oral and nasal cavity, placenta, vagina, eyes, lungs, skin and GIT, the vast majority of bacteria reside within the GIT and predominantly anaerobic species dwell in the colon (10^{10}-10^{12} CFU/mL) (Corrigan *et al.* 2018).

Although it is suggested that bacteria outnumber human cells by a ratio of at least 10:1, recent studies describe a ratio of 1.3 bacterial cells for every 1 human cell (Sender *et al.* 2016). However, these estimates do not take into consideration fungi, viruses, and phage present in various body environments.

1.4.1. Microbiome throughout lifespan

Microbiota co-evolves with the organism from before birth to old age and is not static throughout the lifespan. Specifically, it changes at both extremes of life, in early life and aging, where it is different from the typical adult microbiota (Cryan *et al.* 2019). For a long time, placenta was believed to be a sterile environment, but recent studies show that it has a unique bacterial community, composed of non-pathogenic commensal microbiota from the *Proteobacteria, Bacteroidetes, Firmicutes, Tenericutes* and *Fusobacteria* phyla (Aagaard *et al.* 2014). It changes depending on the birth method (Dominguez-Bello *et al.* 2010) and the way of feeding, as the contribution of maternal microbes to human gut colonisation continues after birth via microbes in breast milk (Collado *et al.* 2016). Palmer *et al.* (2007) studied the development of the human infant microbiota and reported that in the first 6 months of life, there is a remarkable diversity of community profiles. However, these communities converge toward a genetic adult-like profile, and they become more similar to one another. A study conducted with adolescents established that their GM is richer in *Bifidobacterium* and *Clostridium* compared to adult GM (Agans *et al.* 2011). The adult pattern of microbiota is characterized by a predominance of *Bacteroides* and *Firmicutes*, followed by a common presence of *Verrucomicrobia*, and very low existence of *Proteobacteria* and aerobic Gram-negative bacteria (Palmer *et al.* 2007). Elderly people (> 65 years) have a different GM composition compared to adults

and it can be associated with several reasons, such as a reduced mobility and changes in diet (Cryan *et al.* 2019). As Rondanelli *et al.* (2015) reviewed, elderly population is subjected to a reduction in the diversity of the microbiota, which is significantly variable between individuals. In particular, the proportion of the *Bacteroidetes* and *Firmicutes* ranged from 3 % to 92 % and from 7 % to 94 %, respectively (Claesson *et al.* 2011). Vaiserman *et al.* (2017) delved into different studies which concluded that *Bifidobacteria* spp., *Enterobacter* spp., *Klebstella* spp. and *Faecalibacterium prausnitzii* decrease with aging and *Bacteroides* spp., *Clostridium* spp., *Providencia* spp., *Proteust* spp. and facultative anaerobes increase with aging. Besides, several studies reveal that *Firmicutes*/*Bacteroidetes* ratio decreases with aging although there are opposing suggestions about how *Firmicutes* and *Bacteroidetes* change through lifespan. The GM of individuals living for over 100 years is characterized by an enrichment in *Proteobacteria* (Vaiserman *et al.* 2017). A schematic representation of major age-associated changes in GM is given in **Figure 3.**

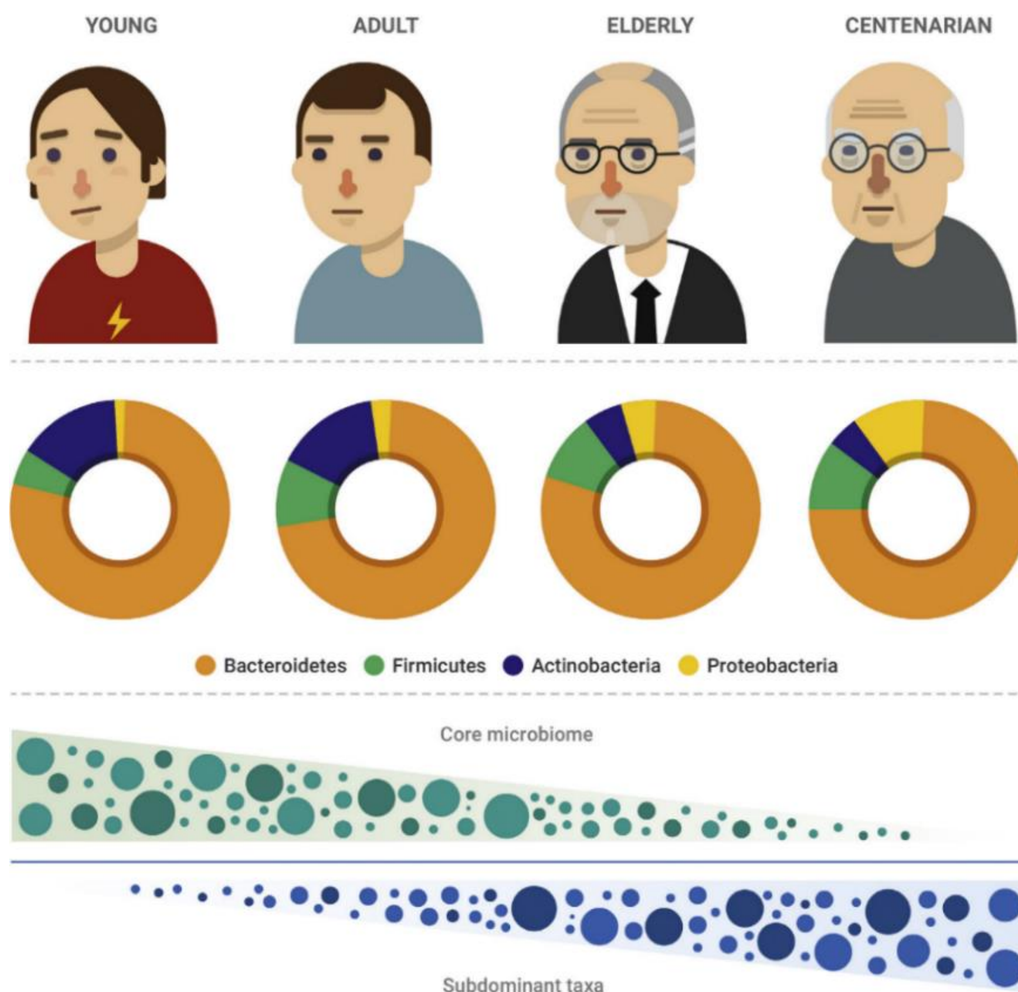

Figure 3 | Age-associated changes in human intestinal microbiota composition. Extracted from Vaiserman *et al.* (2017).

1.4.2. Gut microbiome and gut immunology

Gastrointestinal tract mainly has two functions: ***(1) digestion and nutrients uptake*** and ***(2) maintaining immune homeostasis***. Gastrointestinal associated lymphoid tissue (GALT) comprises the Peyer's patches (PPs) and the mesenteric lymph nodes (MLNs), which are aggregations of lymphoid follicles. PPs are usually located in the small intestine, mainly in the distal ileum, where microbiota is more abundant and diverse (Mason *et al.* 2008). The GM regulates not only the local intestinal immune system but also takes part in systemic immune responses (Wu and Wu 2012).

Germ-free (GF) animals have never been exposed to microorganisms and they are used in studies to reveal the function and importance of microbiota. As Chassaing and Gewirtz (2018) reviewed, GF mice exhibit deficiencies in the development of GALT since they presented fewer and smaller PPs and MLNs than specific pathogen-free animals and exhibited impaired development of isolation lymphoid follicles. Beside, germ-free mice have enlarged cecum and are susceptible to opportunistic pathogens (Mason *et al.* 2008).

1.4.3. Factors influencing the human gut microbiome

All microorganisms live in a dynamic equilibrium, which can be altered leading to a state of dysbiosis. Dysbiosis is often defined as an imbalance in the gut microbial community associated with disease (Chassaing and Gewirtz 2018). Some of the factors that can affect the development and homeostasis of the GM are described below. ***(1) Delivery mode*** largely shapes the bacterial colonization in the neonate, although the development of microbiota starts in the utero (Corrigan *et al.* 2018). Babies born vaginally have more diverse gut microbial communities similar to their mother's vaginal microbiota, while microbiomes of babies delivered by Caesarean section are similar to their mother's skin microbiota (Dominguez-Bello *et al.* 2010). ***(2) Ethnicity and geographical location*** also affect the diversity and composition of GM. ***(3) Stress*** is defined as an organism's total response to environmental demands or pressures (exercise, critical illness…), and stress mediators. GIT and the GM are sensitive to stress and some studies show that enteric bacteria respond to the release of norepinephrine by the host. It may shift the microbial colonization patterns on the mucosal surface and alter the susceptibility of the host to infection (Lyte *et al.* 2011). ***(4) Antibiotics*** are a common therapy for a variety of ailments and they target not only pathogenic microorganisms but also the host-associated microbial communities in the gut (Cresci and Bawden 2015). Among other effects, antibiotics disturb intestinal mucosa homeostasis, which leads to a decrease of resistance against *Clostridium difficile*. The pathological overgrowth of *C. difficile* is

related to antibiotic-associated diarrhoea, one common side effect after antibiotic administration (Gu *et al.* 2016). Antibiotic treatment also causes microbiota diversity decrease, and even though most of the microbiota returns to normal levels, some members are lost indefinitely (Löfmark *et al.* 2006). Besides, there is increasing evidence that antibiotics administered early in life have a profound effect on the gut microbiome that can result in later development of obesity, asthma, inflammatory bowel disease, etc. (Gilbert *et al.* 2018). ***(5) Diet composition*** is a crucial factor to determine the host microbiota. In the initial phase of life, infants can be fed with breast milk, which contains 600 different species of bacteria and some prebiotics, or formula milk (Cresci and Bawden 2015). In adult phase, gut microbiota varies according to the diet, specially, to the consumption of dietary fibre from fruits, vegetables and other plants (Cresci and Bawden 2015).

1.4.4. Inulin

Among components of the diet that influence the gut microbiome, fibre is one of the most important. Based on The Institute of Medicine (US) definition, dietary fibres include nondigestible carbohydrates and lignin, intrinsic and intact in plants. Fibres are not digested by human enzymes, and when they reach the colon, they are fermented by commensal microbiota (Dahl and Stewart 2015). These compounds are considered prebiotics because they stimulate the growth and/or activities of beneficial bacteria and provide health benefits to the human body (Roberfroid *et al.* 2010). In this regard, inulin is a soluble fibre that consists in repetitive fructosyl units linked by β(2,1) bonds. It is naturally found in more than 36,000 species of plants, including vegetables such as wheat, garlic, onion, leek, chicory roots, artichoke, asparagus and banana (Shoaib *et al.* 2016; Bărboi *et al.* 2020). Previous authors described some beneficial effects of inulin for the human health. For instance, as Le Bastard *et al.* (2020) reviewed, in several studies, inulin supplementation increased *Bifidobacterium*, *Anaerostipes, Faecalibacterium*, and *Lactobacillus* relative abundance, and decreased *Bacteroidetes* relative abundance. Moreover, inulin supplementation obtained by extraction from chicory root in the diet of 174 healthy young adults significantly increased total SCFAs concentrations by 12 %, which are required for optimal health (Baxter *et al.* 2019). Furthermore, in distal colitis induced by dextran sodium sulphate in rats, dietary inulin intake prevented inflammation, increased *Lactobacillus* and, overall, reduced colitis symptoms (Videla *et al.* 2001). And, among this large variety of inulin effects, it has also been involved in a long-term protective effect against food allergies in the offspring when pregnant and lactating mice are exposed to inulin/galactooligosaccharides (GOSs) prebiotics mixture (Bouchaud *et al.* 2016).

Going back to the concept of brain-gut-axis, in both human and mice, it has been observed that changes in the microbial composition lead to changes in the behaviour of the host (Desbonnet *et al.* 2014; Leclercq *et al.* 2017). For instance, male mice fed with two prebiotics (short-chain GOSs and long-chain FOSs) showed reduced anxiety-like and improved social behaviour in adulthood (Szklany *et al.* 2020). Similarly, in preliminary results from this study performed by the Cryan lab from APC microbiome (data not shown), beneficial effects of inulin were observed. Specifically, although aged mice exposed to stress could not discriminate between a familiar and a novel mouse, aged mice exposed to stress and fed with a diet supplemented with inulin rescued their ability to discriminate between a familiar and a novel mouse. Therefore, inulin administration increased mice preference for a novel mouse versus a familiar mouse, altering their behaviour.

2. HYPOTHESIS

As previously described, the gut-brain axis plays an essential role in the regulation of gastrointestinal function, regulation of appetite, energy balance and cognitive and psychological function.

It is well established that prebiotics such as inulin stimulate the growth and/or activities of beneficial bacteria and, by the gut-brain axis, it is expected that the administration of prebiotics may alter the state of the brain. Specifically, it is expected that inulin supplementation could recover the normal state of astrocytes and, therefore, normal expression levels of reactive astrocytes-related genes, which could be previously altered by some stimulus such as stress. In this sense, aged and stressed mice that in Three-Chamber (3Ch) test performed by APC Microbiome group (data not shown) could not discriminate between familiar and novel mouse, although it is still not confirmed, must have reactive astrogliosis.

Considering all this, **the hypothesis of this work is that stress alters the expression of some reactive astrocytes- and diet-related genes in aged male mice and that inulin supplementation can modify this alteration.**

3. OBJECTIVES

To confirm the hypothesis, the principal aim of this project is **to evaluate the role of stress and inulin supplementation in the alteration of the expression of reactive astrocytes- and diet-related genes.** To achieve this goal, a few specific objectives have been proposed:

- To determine the impact of stress on reactive astrocytes-related genes (*Gfap, Stat3, Serpina3n, C3, C4b* and *Cxcl10*) expression and a diet-related gene (*Ahr*) expression in samples from aged male mice.
- To quantify the levels of expression of some reactive astrocytes-related genes and a diet-related gene from aged male mice subjected to inulin supplementation.
- To evaluate the potential of inulin to modulate gene expression alterations caused by stress.
- To compare the expression of these seven genes in three different regions of the brain related to limbic system: amygdala, PFC, and hippocampus.

4. MATERIALS AND METHODS

4.1. ANIMAL EXPERIMENT DESIGN

The objective of this project is to evaluate the effect of stress in the relative expression of some reactive astrocytes-related genes and a diet-related gene in aged male mice and the ability of inulin supplementation to modify gene expression alterations caused by stress. Hence, animals were subjected to two factors: stress treatment and diet. All the animal experimental procedure was performed by the group of Microbiota-Gut-Brain Axis from APC Microbiome before my arrival to Ireland, and all the experiments were conducted in accordance with European Directive 86/609/EEC, Recommendation 2007/526/65/EC, and approved by the Animal Experimentation Ethics Committee of University College Cork. **Figure 4** shows a scheme of the experimental design followed in this project. The study was performed with 10 males C57BL/6 mice (Charles River, Kent, United Kingdom) aged 19-month-old per group, which are considered old mice and are used as models of aging. These mice were housed in groups of 2-3 at arrival and housed in the animal facility for 5 months. During this period, mice were kept under a 12 h light/dark cycle at a temperature of 21 ± 1 ºC and fed *ad libitum* with a standard chow diet (2018 Teklad global 18% protein, Envigo, United Kingdom). After this period, mice were divided into four groups (n = 10 per group) according to the treatment received: **a)** non-stress treatment and chow diet, **b)** non-stress treatment and chow diet enriched with 10 % of fructooligosaccharide (FOS) - inulin (mixture of 92 ± 2 % of inulin and 8 ± 2 % of FOS, Orafti® Synergy1, Beneo, Tienen, Belgium), **c)** stress treatment and chow diet, and **d)** stress treatment and chow diet enriched with 10 % of FOS - inulin.

For the social defeat stress procedure, adult male CD1 mice were used as aggressors (Envigo, United Kingdom). Prior to the defeat sessions, a CD1 mouse was exposed to another CD1 mouse until the first attack. Mice with the shortest attack latencies were selected as aggressors to be used in subsequent social defeats. For each defeat session, experimental mice were exposed to a different aggressor CD1 mouse each day over the 6 days. The session involved a single initial exposure of the test mouse to the aggressive CD1 in the aggressor home-cage until the first attack with expression of submissive posturing or until 5 min had passed. The latency to attack or display a submissive posture was recorded. The mice were then separated by a perforated Plexiglas® wall that allowed non-physical contact for 2 h. Then, the separator was removed, and, after another defeat, mice were returned to their home-cage.

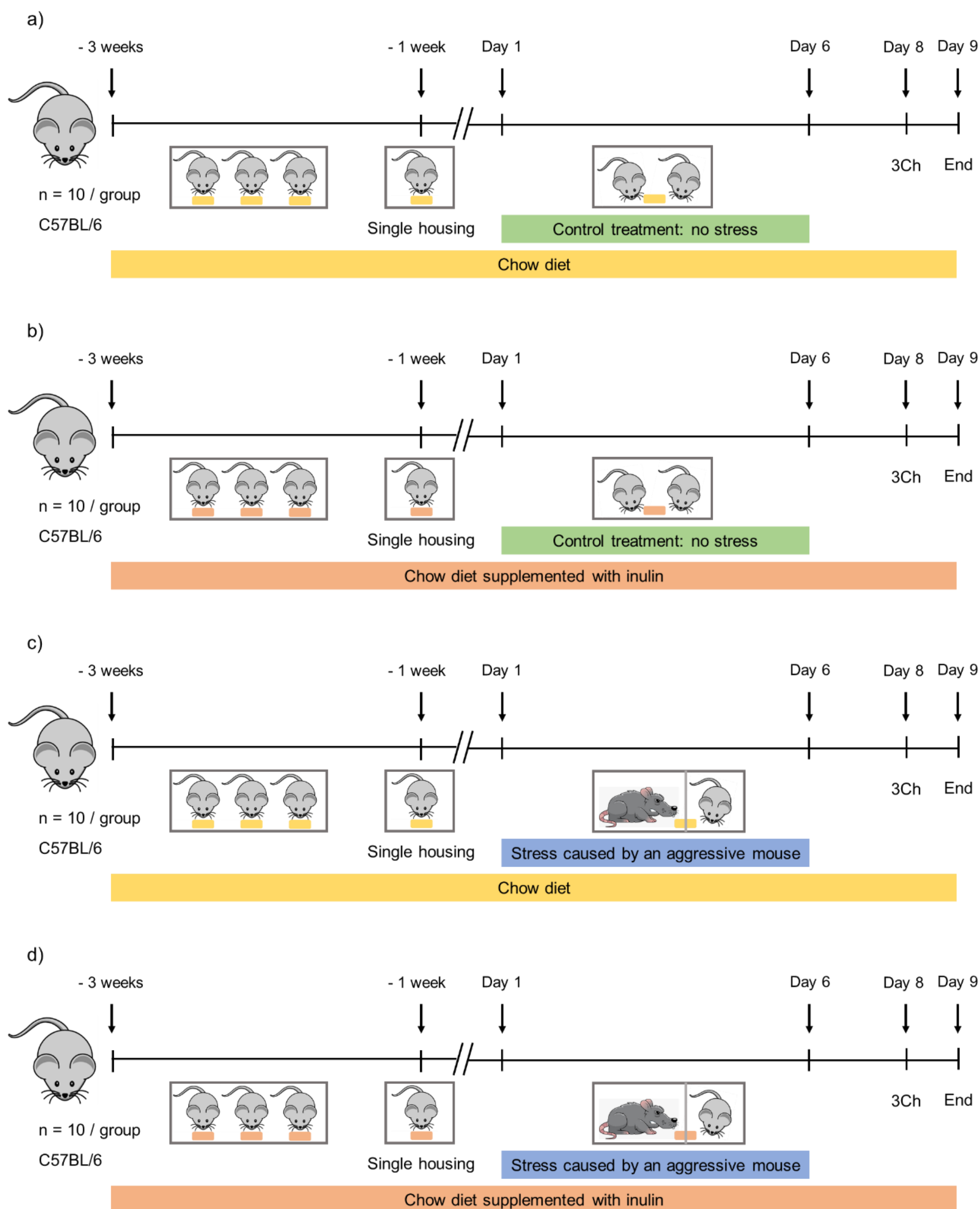

Figure 4 | Experimental timeline of steps performed to evaluate the effect of stress and inulin-supplemented diet on aging male C57BL/6 mice (n = 10 per group). **a)** Group 1: control mice, which were subjected to chow diet and non-stress exposure, **b)** Group 2: mice subjected to inulin-supplemented diet and non-stress exposure, **c)** Group 3: mice subjected to chow diet and social defeat stress (SDS) caused by an aggressor CD1 mouse, and **d)** Group 4: mice subjected to inulin-supplemented diet and SDS. Three weeks before the stress treatment (-3 weeks), groups 2 and 4 started a diet supplemented with inulin. A week before the stress treatment (-1 week), all the mice were single housed. At day 1, mice from groups 3 and 4 were introduced in the aggressor's home-cage until the first defeat or until 5 min had passed. The mice were then separated by a perforated wall for 1 hour and then returned to the home-cage. SDS lasted 6 days. At day 8, they performed the Three-Chamber (3Ch) test. Finally, at day 9, all of them were sacrificed.

Finally, four different groups of mice were obtained and tested in 3Ch, schematically represented in **Figure 5**. The 3Ch test assesses social behaviour and deficits in social interactions in experimental mice. This test measures the sociability, which is the propensity to spend time with another mouse versus time alone in an identical but empty chamber (or with an object), and social novelty, which is the propensity to spend time in a chamber with a novel mouse rather than a familiar mouse. Stimulus mouse in the two sessions, sociability, and social novelty, were placed under a wire cage. To quantify the sociability and social novelty of the experimental mouse, the time it spent in each chamber and the time it sniffed at each wire cage were measured (Three-Chamber social test n.d.). The following day, mice were killed by decapitation.

The main objective of 3Ch test was analysing the role of inulin in the behaviour of aging and stressed mice because repeated social defeat stress (SDS) had been shown to promote neuroinflammation and behaviour changes, such as reduced social behaviour (Wohleb *et al.* 2011; Tripathi *et al.* 2021). However, how inulin affects had not previously been studied.

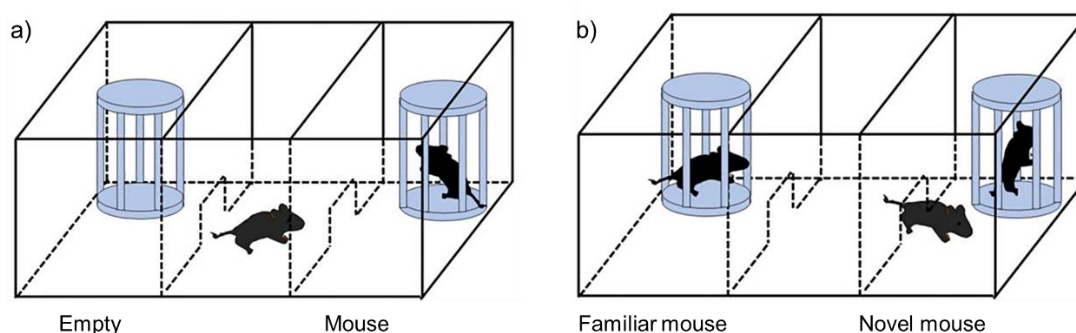

Figure 5 | Schematic representation of Three-Chamber (3Ch) test. **a)** Experimental design for 3Ch test to measure sociability. **b)** Experimental design for 3Ch test to measure social novelty. In both cases, mouse can freely move between the three chambers. All conspecific mice are placed under a wire cage. Adapted from Lim *et al.* (2017).

From 3Ch test performed by APC Microbiome group prior to my arrival (data not shown) it was deduced that stress did not impact social interaction in aged mice. Regarding social novelty test, aged mice spent more time in the novel mouse chamber rather than in the familiar mouse chamber, meanwhile aged mice exposed to stress could not discriminate between a familiar and a novel mouse. As it was previously described (in 1.4.4.), those animals exposed to stress and fed with an inulin-supplemented chow diet rescued their ability to discriminate. Therefore, we wanted to link the astrocyte activity in social behaviour- and emotions-related brain regions to the behaviour outcomes previously obtained.

4.2. SAMPLE PROCESSING

Immediately upon sacrifice, their brains were dissected. In this process, amygdala, PFC, and hippocampus were dissected, snap frozen in dry ice, and stored at -80 ºC.

These three regions were chosen because all are related to limbic system, which is involved in the behaviour and the emotional responses. In this sense, Tripathi *et al.* (2021) recently reviewed that chronic SDS enhances microglia activation in amygdala, PFC and hippocampus and activated microglia induces reactive astrocytes. Thus, these regions were the most appropriated for our work. The objective was to link the gene expression to the social behaviour previously analysed in 3Ch test.

4.3. PRIMERS

Most of the genes analysed in the current work (*Gfap, Stat3, Serpina3n, C3, C4b* and *Cxcl10*) are related to astrocyte reactivity and in previous studies they were upregulated in neuroinflammatory situations, as aging, stress, LPS injection, among others. Besides, one of the analysed genes (*Ahr*) codifies for a receptor and precursors of its ligands can come from the diet.

To compare gene expression, as it is described later, a quantitative polymerase chain reaction (qPCR) was performed using PrimeTime® Std qPCR Assay 20X (Integrated DNA Technologies, Coralville, Iowa, United States of America) that contained the probe, primer forward and primer reverse for each gene. Sequences of them are detailed in **Table 1**.

Table 1 | Sequence of the probe, primer forward and primer reverse of the analysed genes. Fw, forward; Rv, reverse.

Gene	Contains	Sequence
Actβ	Probe	5'-/56-FAM/CTGGCCTCA/ZEN/CTGTCCACCTTCC/3IABkFQ/-3'
	Primer Fw	5'-GACTCATCGTACTCCTGCTTG-3'
	Primer Rv	5'-GATTACTGCTCTGGCTCCTAG-3'
Gfap	Probe	5'-/56-FAM/CAACCTCCA/ZEN/GATCCGAGAAACCAGC/3IABkFQ/-3'
	Primer Fw	5'-AACCGCATCACCATTCCTG-3'
	Primer Rv	5'-GCATCTCCACAGTCTTTACCA-3'
Stat3	Probe	5'-/56-FAM/CGTGCCAAT/ZEN/TGTGATGCCTCCTTG/3IABkFQ/-3'
	Primer Fw	5'-GTTCAAGCACCTGACCCTTAG-3'
	Primer Rv	5'-AGTCTCGAAGGTGATCAGGT-3'

Table 1 Continuation | Sequence of the probe, primer forward and primer reverse of the analysed genes. Fw, forward; Rv, reverse.

Gene	Contains	Sequence
Serpina3n	Probe	5'-/56-FAM/CTTTAAAGC/ZEN/CAAATGGAAGGTGCCCT/3IABkFQ/-3'
	Primer Fw	5'-GACATTGATGGTGCTGGTGA-3'
	Primer Rv	5'-CGCGTAGAACTCAGACTTGAAC-3'
C3	Probe	5'-/56-FAM/AGGGTCCCA/ZEN/GCTACTAGTGCTACTG/3IABkFQ/-3'
	Primer Fw	5'-CCTTCCACCTTTTTCCTTCACT-3'
	Primer Rv	5'-CTCCAGCCGTAGGACATTG-3'
C4b	Probe	5'-/56-FAM/CTGGCCTGG/ZEN/GTGTTCAGCTTCT/3IABkFQ/-3'
	Primer Fw	5'-GTTGATCAGAAGGGAGCAGAC-3'
	Primer Rv	5'-GGCTTCTGCAGGGATGAG-3'
Cxcl10	Probe	5'-/56-FAM/AGACCATCA/ZEN/AGAATTTAATGAAAGCGTTTAGCC/3IABkFQ/-3'
	Primer Fw	5'-ACGTGTTGAGATCATTGCCA-3'
	Primer Rv	5'-AGTTAAGGAGCCCTTTTAGACC-3'
Ahr	Probe	5'-/56-FAM/AGCCATTCA/ZEN/GCGCCTGTAACAAGA/3IABkFQ/-3'
	Primer Fw	5'-GTGTAGAGCACAAATCAGAGACT-3'
	Primer Rv	5'-GAGGAAGCATAGAAGACCAAGG-3'

4.4. RNA EXTRACTION OF TISSUE

RNA extraction of the samples was performed using the *mir*Vana™ miRNA Isolation Kit (Thermo Fisher Scientific, Waltham, Massachusetts, United States of America) and it consisted in a physicochemical extraction.

Briefly, 300 µL of Lysis/Binding Buffer were added to amygdala frozen tissue, and 500 µL of the same reagent were added to PFC and hippocampus frozen tissue. Up and down pipetting was useful to rupture tissue and mix it with the buffer. Then, 1/10 volume of miRNA Homogenate Additive was added to the tissue lysate, which corresponds to 30 µL in amygdala samples and 50 µL in PFC and hippocampus samples and mixed well by vortexing. The solution was incubated for 10 min in ice. After this time, a volume of Acid-Phenol:Chloroform equal to the lysate volume previously added (300 µL or 500 µL according to the sample) was added to the solution and vortexed for 30-60 seconds. Samples were centrifuged for 5 min at 10,000 x g at room temperature to separate the aqueous and organic phases. The aqueous phase was recovered, quantified, and transferred to a fresh tube. Next, 1.25 volumes of room temperature 100 % ethanol were

added to the aqueous phase and mixed. For each sample, a Filter Cartridge was placed into one of the Collection Tubes and up to 700 µL of the lysate/ethanol mixture were pipetted onto the Filter Cartridge. If there were more than 700 µL, the leftover solution was stored at -20 ºC in the freezer. Collection Tubes with a Filter Cartridge containing lysate/ethanol were centrifuged for 30 sec at 10,000 x g at room temperature. Next, the flow-through was discarded, 700 µL of miRNA Wash Solution 1 was added to the same Filter Cartridge and it was centrifuged for 30 sec at 10,000 x g at room temperature. Flow-through was discarded and 500 µL of Wash Solution 2/3 was applied to the same Filter Cartridge. It was centrifuged at the same conditions that previous step and a second 500 µL aliquot of Wash Solution 2/3 was added repeating the procedure. After discarding the flow-through from the last wash, a centrifuge of 1 min was performed to remove residual fluid from the filter. Following that, 100 µL of pre-heated (95 ºC) Elution Solution was added to the Filter Cartridge previously transferred to a fresh Collection Tube. Finally, tubes were spinned for 45 sec at maximum speed to recover the RNA.

Before storing it at -20 ºC, RNA concentration and quality were determined using NanoDrop™ ND-1000 spectrophotometer (Thermo Fisher Scientific).

4.5. SYNTHESIS OF cDNA

To analyse genetic expression, it is necessary to obtain double strain complementary DNA (cDNA) from RNA by reverse transcription or retrotranscription. It was performed using High Capacity cDNA Reverse Transcription Kit (Applied Biosystem™ by Thermo Fisher Scientific, Warrington, United Kingdom).

Firstly, RNA samples were diluted with UltraPure™ Distilled Water DNase/RNase Free (Invitrogen™ by Life Technologies, Warrington, United Kingdom) in a MicroAmp™ Optical 96-Well Reaction Plate (Applied Biosystem™ by Thermo Fisher Scientific, Warrington, United Kingdom) to get 150 ng of RNA in a total volume of 20.0 µL in amygdala samples, 300 ng of RNA in a total volume of 20.0 µL in PFC samples and 400 ng of RNA in a total volume of 20.0 µL in hippocampus samples. These amounts were adjusted considering RNA concentration in each tube. Next, master mix was prepared by mixing 2.0 µL of 10X RT Buffer, 0.8 µL of 25X dNTP Mix (100 mM), 2.0 µL of 10X RT Random Primers, 1.0 µL MultiScribe™ Reverse Transcriptase and 4.2 µL of Nuclease-free H_2O per sample. Then, 10.0 µL of the master mix and 10.0 µL of the diluted RNA were distributed in each well of a 96-well plate. The plate was sealed and centrifuged to spin down the contents and to eliminate air bubbles. Finally, the plate was loaded into the thermal cycle, and it started running (programme described in **Figure 6**).

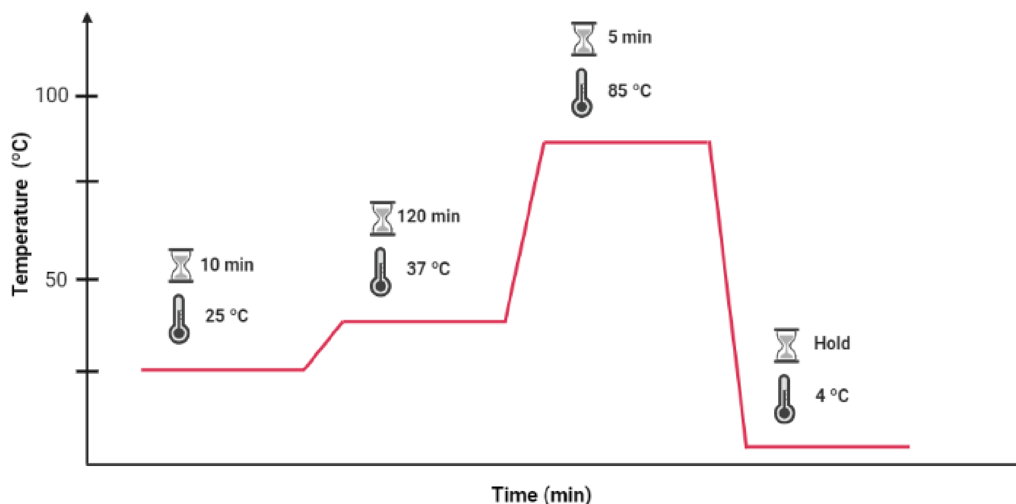

Figure 6 | Incubation conditions (time and temperature) to get cDNA.

After running this programme, RNA concentration was 3.75 ng/µL in amygdala samples, 7.50 ng/µL in PFC samples and 10.00 ng/µL in hippocampus samples. Besides, a 1.5 dilution was performed in PFC and hippocampus samples, getting final RNA concentrations of 5.00 ng/µL and 6.67 ng/µL, respectively. RNA from amygdala was not diluted because the concentration was lower than the other ones.

4.6. RELATIVE QUANTIFICATION BY qPCR

qPCR, also called real-time PCR, is used to amplify, and quantify a target gene after each cycle. Quantification is based on the fluorescence emitted by a fluorochrome, as TaqMan probes. The number of amplified products in each cycle is proportional to the emitted fluorescence increase. In this analysis, the results are expressed in cycle threshold (Ct), that is, how many cycles of qPCR has to be performed to make the amplifying curve cross the threshold line. Ct value is used as a relative measure of target gene concentration in each sample, and it is inversely proportional to this concentration. The lower Ct values (below 19) denote high concentrations of target gene and the higher Ct values (beyond 38) denote low concentrations of target gene (Real-Time PCR: understanding Ct n.d.).

qPCR performed on thermocycler LightCycler® 480 II (Roche) was used to assess relative expression of the samples of interest. For each gene, a master mix consisting of 5.0 µL of TaqMan™ Universal Master Mix II, no UNG (Applied Biosystems by Thermo Fisher Scientific, Vilnius, Lithuania), 1.0 µL of PrimeTime® XL qPCR Assay 20X (Integrated DNA Technologies, Coralville, Iowa, United States of America) and 2.0 µL of UltraPure™ Distilled Water DNase/RNase Free (Invitrogen™ by Life Technologies, Warrington, United Kingdom) per well was prepared and distributed (8.0 µL/well). Next, 2.0 µL of sample were added to each well. All the experiments included a non-template

control (NTC) and beta-actin (*Actb*) as a reference gene. Two technical replicates were performed per each biological replicate. Thermal conditions applied are described in **Figure 7**.

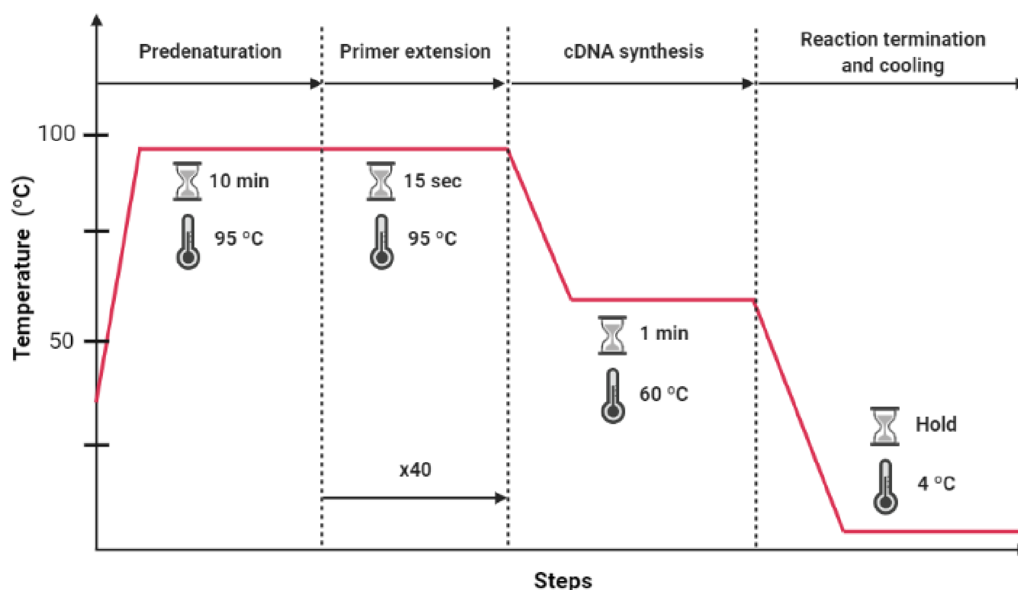

Figure 7 | Incubation conditions applied during qPCR analysis.

Finally, $2^{-\Delta\Delta Ct}$ method, also known as Livak method, was used to calculate relative expression of *Gfap, Stat3, Serpina3n, Ahr, C3, C4b and Cxcl10* genes (formula 1). First, we calculated the average value of Ct from the two technical replicates per each biological replicate. Then, for each sample we calculated ΔCt as Ct of gen of interest (GOI) – Ct of reference gene, and ΔΔCt as ΔCt of the sample – ΔCt of the calibrator (average of GOI Ct – average of reference gene Ct).

$$\text{Relative expression} = 2^{-\Delta\Delta Ct} \quad (1)$$

4.7. STATISTICAL ANALYSIS

Data were statistically analysed with IBM SPSS Statistics and graphs were represented using GraphPad's Prism 5. First, the normality of the data distribution was evaluated by Shapiro-Wilk test. Second, when data were normally distributed, parametric data were analysed by analysis of variance (ANOVA). If F-test was statistically significant, the Student's test (two means) or the Tukey's HSD test (more than two means) were performed for means comparison, using a significant level of $p < 0.05$. When data were not normal distributed (nonparametric), Mann-Whitney test (two means) or the Kruskal-Wallis test (more than two means) were performed for means comparison, using a significant level of $p < 0.05$.

5. RESULTS AND DISCUSSION

5.1. QUANTIFICATION OF RNA

After performing RNA extraction, RNA quality and concentration was measured by NanoDrop™ ND-1000 spectrophotometer (Thermo Fisher Scientific). The average concentration was around 30 ng/µL in amygdala samples, around 90 ng/µL in PFC samples, and around 60 ng/µL in hippocampus samples. Regarding RNA quality, we focused on 260/280 and 260/230 ratios. The first one is a DNA and RNA purity index, and it is considered that the sample is pure when this value is around 1.8 and 2.0, respectively. If this ratio is lower, it may indicate presence of protein, phenol, or others. 260/230 ratio is used as a secondary nucleic acid purity index. Expected values are between 2.0 and 2.2, and the ratio could be lower if there are contaminants that absorb at 230 nm (NanoDrop 2007). In this study, ratios were around 1.5-2.0, correct and appropriate to continue analysing gene expression of interest genes.

5.2. EXPRESSION OF ASTROCYTES- AND DIET-RELATED GENES

The main goal of this study consisted in evaluating the role of stress and inulin supplementation in the alteration of the expression of some reactive astrocytes- and a diet-related genes. To achieve this objective, the expression of reactive astrocytes-related genes (*Gfap, Stat3, Serpina3n, C3, C4b* and *Cxcl10*) and a diet-related gene (*Ahr*) were analysed in three parts of the brain, which have different characteristics and functions. *Actb* was used as a reference gene to normalize the expression of target genes. It was selected since it is one of the most commonly used reference genes and in previous studies it has been demonstrated that *Actb* is stably expressed in different areas of the mouse brain (olfactory bulb, cerebellum, cortex, hypothalamus, hippocampus, brainstem, and striatum) (Gilsbach *et al.* 2006).

Regarding gene expression analyses, authors describe that it is necessary to analyse gene expression in different tissues to get a better comprehension of all the processes. In fact, Clarke *et al.* (2018) profiled astrocytes from three functionally distinct brain regions, hippocampus, striatum and cortex, and observed that hippocampus and striatum, which are vulnerable regions to cognitive decline, suffer more dramatic transcriptional changes than cortex. Likewise, Boisvert *et al.* (2018) compared mRNA from astrocytes from visual cortex, motor cortex, hypothalamus and cerebellum and reported that different brain regions had distinct gene expression profiles, which changed with age. Hence, we obtained different gene expression according to the brain region analysed, as it is described in previous studies.

5.2.1. Glial fibrillary acidic protein (Gfap) expression

As described above, one of the specific objectives of this project is to determine the impact of stress on gene expression. In the amygdala of those animals fed with a chow diet, *Gfap* expression was lower in the samples from animals subjected to stress than in samples from control mice (**Figure 8a**). In PFC the results were similar, as the only difference in *Gfap* expression was between animals fed with inulin-based diet subjected to control and stress treatment. Those exposed to stress treatment had a lower expression than those exposed to control treatment (**Figure 8b**). Finally, in hippocampus, there were no significant differences between groups (**Figure 8c**).

Gfap is well known as a reactive astrocytic marker (Zamanian *et al.* 2012; Salas *et al.* 2020) although reactive astrocytes are characterized by altered expression of hundreds of genes, not only *Gfap*. In our study, in amygdala and PFC from male aged mice brain, stress caused a decrease of *Gfap* expression although we anticipated an increase of *Gfap* expression since stress and aging are neuroinflammatory factors (Grippo and Scotti 2013). Neuroinflammation induces reactive astrocytes type A1, which lose the ability to help neuronal survival, synapsis and phagocytosis, among others (Liddelow *et al.* 2017). A1 astrocytes are characterized by an altered gene expression profile and, as Clarke *et al.* (2018) studied, normal aging induced A1-like astrocyte reactivity. In a previous study that compared aging astrocyte transcriptome from visual cortex, motor cortex, hypothalamus, and cerebellum of the mouse brain, *Gfap* was upregulated in the 4 brain regions with aging (Boisvert *et al.* 2018). In an independent study, it was also suggested that oxidative and inflammatory phenotypes, which are common in aged rats, precede the increase of *Gfap* expression (Bellaver *et al.* 2017). Despite we expected that stress would have caused an increase of *Gfap* expression, there are not previous studies about how stress affects the expression of this gene.

Next, the second and third objectives were to quantify the level of expression of some genes from animals fed with a chow diet supplemented with inulin, and to evaluate the potential of inulin to modulate gene expression alterations caused by stress. Inulin treatment did not affect *Gfap* expression, as comparing the expression in samples of animals fed with chow and inulin-based diet, none of them had significant differences.

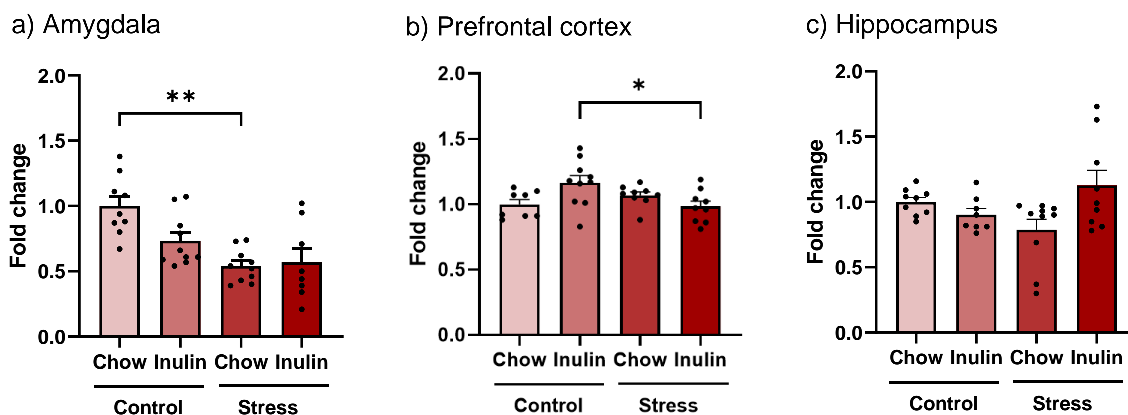

Figure 8 | Fold change representation of glial fibrillary acidic protein (*Gfap*) expression of each group normalized to control group (Control-Chow). **a)** Amygdala, **b)** Prefrontal cortex and **c)** Hippocampus samples. *Gfap* expression was relative to reference gene (*Actb*) expression. Each column represents the mean of biological replicates (8-10 replicates) and vertical bars indicate the standard error of the mean. Asterisks (*) indicate significant differences between groups according to Tukey's HSD test for parametric data or Kruskal-Wallis test for nonparametric data (* $p < 0.05$, ** $p < 0.01$)

5.2.2. Signal transducer and activator of transcription 3 (Stat3) expression

Regarding stress effect on *Stat3* expression, in amygdala of mice tested, it was constant in all the groups, so stress did not affect (**Figure 9a**). In PFC, the expression of individuals from each group was very variable and there were not significant differences (**Figure 9b**). Finally, in hippocampus there were no stress effects because the expression was the same among control and stressed groups (**Figure 9c**).

Stat3 codifies for the transcription factor STAT3, which is activated in different types of cells after the release of cytokines implicated in injury response. Some of these cytokines (such as interleukin-6, leukaemia inhibitory factor and epidermal growth factor) are involved in the outbreak of reactive astrogliosis (Herrmann *et al.* 2008). In a previous transcriptomic study of reactive astrocytes, over 1000 genes of a vast spectrum of biological processes were induced at least two-fold in comparison to quiescent astrocytes. *Stat3* was increased two-fold in reactive astrocytes induced by ischemia, which causes cell death, and by systemic LPS injection, which causes neuroinflammation (Zamanian *et al.* 2012). After ischemic stroke, some pathways in which *Stat3* took part were induced: acute phase response signalling; IL-6 signalling; role of macrophages, fibroblasts, and endothelial cells in rheumatoid arthritis; hepatocyte growth factor signalling; IL-10 signalling; and oncostatin M signalling (Zamanian *et al.* 2012).

Stat3 was described as one of the genes that were more upregulated in A2 reactive astrocytes (reactivity induced by ischemia). Specifically, the upregulation was significant

in hippocampal and striatal astrocytes, but not in cortical astrocytes (Clarke *et al.* 2018). It is suggested that STAT3 is necessary for the induction of reactive astrogliosis, thus, normal aging could induce astrocyte reactivity in a region-specific manner. In this case, *Stat3* expressed in astrocytes from our experimental mice might have been altered for the aging factor, previous than stress factor. Hence, no alteration in *Stat3* expression caused by stress should be expected.

Regarding inulin supplementation, in hippocampus, there was a clear diet effect as in both control and stressed groups, there was a decrease in *Stat3* expression in samples from animals fed with inulin-supplemented diet, compared to samples from animals fed with a chow diet (**Figure 9c**). Similarly, in a recent study, male mice were fed with a microbiota-accessible-carbohydrates-supplemented diet, such as inulin, and this treatment prevented neuroinflammation and cognitive decline. Precisely, in hippocampus, microbiota-accessible carbohydrates supressed the activation of neuroglia and inflammation caused by high-fat and fibre deficient diet (Shi *et al.* 2020). Moreover, it is well studied that human hippocampal volume is associated to the diet: if it is predominated by healthy ingredients, hippocampus is bigger than if it is based on unhealthy ingredients (Jacka *et al.* 2015; Akbaraly *et al.* 2018). Therefore, hippocampus seems to be sensitive to the diet and this is what can be confirmed from our results.

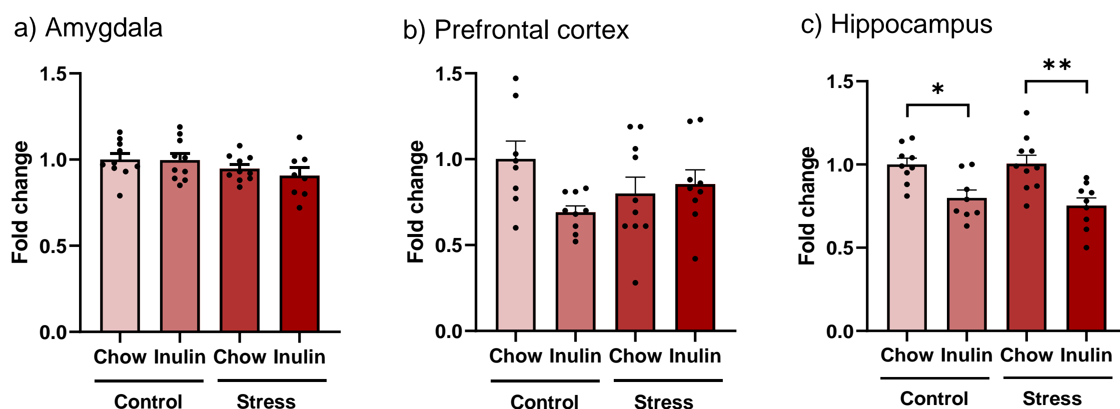

Figure 9 | Fold change representation of signal transducer and activator of transcription 3 (*Stat3*) expression of each group normalized to control group (Control-Chow). **a)** Amygdala, **b)** Prefrontal cortex and **c)** Hippocampus samples. *Stat3* expression was relative to reference gene (*Actb*) expression. Each column represents the mean of biological replicates (8-10 replicates) and vertical bars indicate the standard error of the mean. Asterisks (*) indicate significant differences between groups according to Tukey's HSD test for parametric data or Kruskal-Wallis test for nonparametric data (* $p < 0.05$, ** $p < 0.01$)

5.2.3. Serine peptidase inhibitor clade A member 3N (Serpina3n) expression

In none of the three regions of the brain evaluated *Serpina3n* expression had significant differences between the four tested groups (**Figures 10a-10c**). The expression of individuals of some groups was highly variable and significant stress effect could not be described.

Serpina3n gene codifies for serine peptidase inhibitor clade A member 3N (SERPINA3N), which is an acute phase protein increased in the blood during the inflammatory process (Dimberg *et al.* 2011). For instance, in a previous study, trimethyltin chloride was used to cause neuroinflammation, what is related to an upregulation of *Serpina3n*. Melatonin acted as an anti-inflammatory agent and caused a decrease of *Serpina3n* expression. However, the overexpression of *Serpina3n* in the mouse hippocampus eliminate the protective effect of melatonin (Xi *et al.* 2019).

In regard to *Serpina3n* expression, it was previously described as reactive astrocyte-associated gene (Clarke *et al.* 2018) and is considered a member of a core of aging-upregulated genes because it was upregulated in 4 different regions of the brain (Boisvert *et al.* 2018). Nevertheless, none of them were amygdala, PFC, or hippocampus so it cannot directly be compared to our results. In a previous study, aged astrocytes responded to LPS-induced neuroinflammation with an increase of some genes, among which, *Serpina3n*. Remarkably, the upregulation of *Serpina3n* was dramatically higher in aged than in younger mice (Clarke *et al.* 2018). Therefore, if the increase of *Serpina3n* expression was higher in front of two inflammation triggering factors, aging and LPS, than in front of just LPS factor, aged mice submitted to stress were expected to have a higher expression of *Serpina3n* than aged not stressed mice. However, the mean expression of group 1 (control and chow) and group 3 (stress and chow) shows that stress caused a nonsignificant decrease in the expression (in amygdala and hippocampus), or it did not have any effect (in PFC). In this sense, since there is evidence of aging altering *Serpina3n* expression, stress effects could be potentially masked by the aging effect.

In relation to the supposed ability of inulin supplementation to modify altered gene expression levels, in amygdala and PFC (**Figure 10a** and **Figure 10b**), *Serpina3n* expression was the same independently of the diet. In contrast, in hippocampus (**Figure 10c**), inulin supplementation non-significantly increased *Serpina3n* expression, which was decreased in a non-significant way by stress treatment. Nevertheless, there are no previous studies describing the effect of inulin modifying alterations in gene expression caused by stress, and further research is needed.

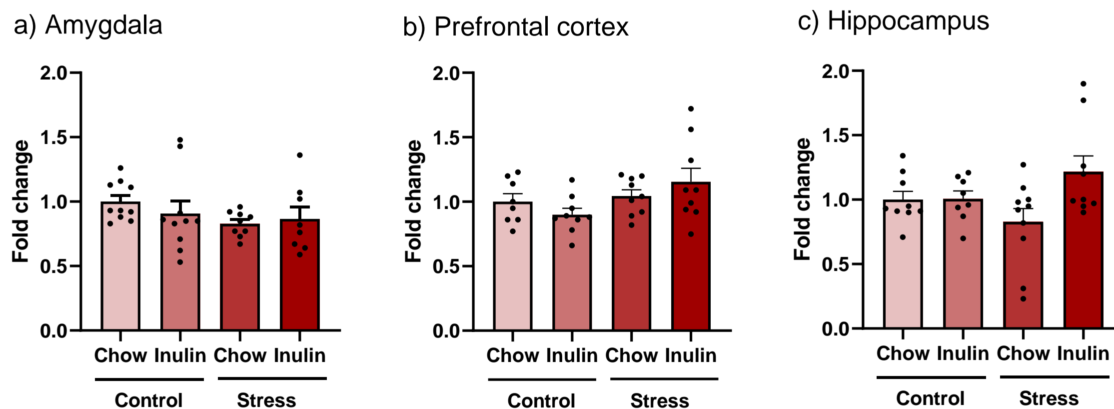

Figure 10 | Fold change representation of serine peptidase inhibitor clade A member 3N (*Serpina3n*) expression of each group normalized to control group (Control-Chow). **a)** Amygdala, **b)** Prefrontal cortex and **c)** Hippocampus samples. *Serpina3n* expression was relative to reference gene (*Actb*) expression. Each column represents the mean of biological replicates (8-10 replicates) and vertical bars indicate the standard error of the mean.

5.2.4. Complement component 3 (C3) expression

C3 was a problematic gene because after qPCR analysis, Ct values were very high (around 38-40), and as described before, it denotes low concentrations of target gene (Real-Time PCR: understanding Ct n.d.). Therefore, it is important to keep in mind that the results from this gene can be imprecise, and it can justify the big variability between individuals from the same group (**Figures 11a-11c**).

C3 is a complement component indispensable for triggering the biochemistry cascade as a part of the immune system response. Some CNS pathologies are followed by an excessive immune reaction and current efforts are aimed at blocking the activation of C3 (Jung *et al.* 2016). In the same way as previous analysed genes, *C3* is identified as a reactive astrocyte-associated gene, and during aging, it was significantly upregulated in hippocampal and striatal astrocytes (Clarke *et al.* 2018). Some authors studied its activation in front of stress and saw that C3 mRNA levels increased in PFC from mice exposed to chronic and unpredictable stress (Crider *et al.* 2018), which had similar neuroinflammatory effects than repeated SDS, the kind of stress to which mice were subjected in our study. In addition, recent studies described that C3 mediates neuroinflammation and behaviour deficits through modulation of IFNβ, which is induced by chronic stress (Tripathi *et al.* 2021). All things considered, we expected a high *C3* expression since it is a gene upregulated in aging- and stress-induced reactive astrocytes. However, as Ct values from qPCR analysis of this gene were too high and variable, we cannot compare the expression obtained to the results from other studies. It would be necessary to repeat *C3* analysis with different primers because they can be

the weak spot, as samples were the same than in the other genes' analysis, in which we got normal Ct values.

Referring to inulin supplementation effect, in PFC (**Figure 11b**), where stress treatment did not cause any difference in the *C3* expression, animals fed with chow diet supplemented with inulin had a lower *C3* expression. However, there are not previous evidence about inulin effect in *C3* expression to compare to our results.

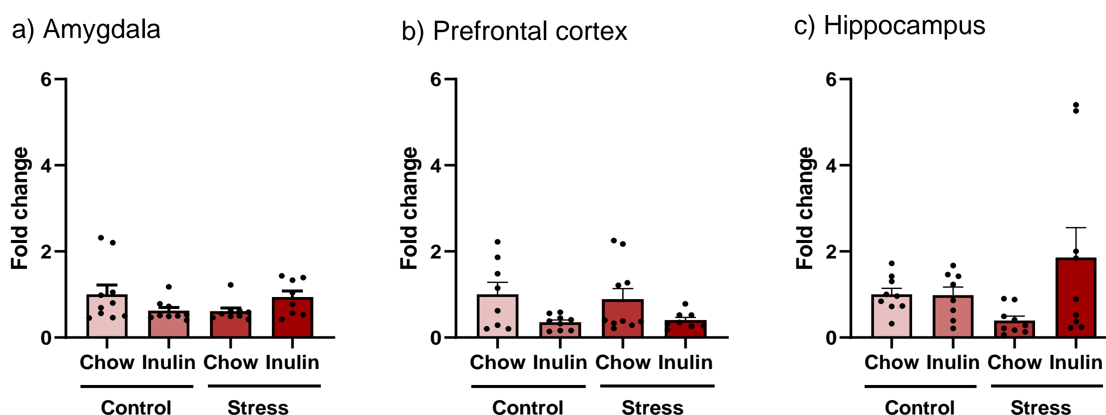

Figure 11 | Fold change representation of complement component 3 (*C3*) expression of each group normalized to control group (Control-Chow). **a)** Amygdala, **b)** Prefrontal cortex and **c)** Hippocampus samples. *C3* expression was relative to reference gene (*Actb*) expression. Each column represents the mean of biological replicates (8-10 replicates) and vertical bars indicate the standard error of the mean.

5.2.5. Complement component 4b (C4b) expression

In relation to stress effect on *C4b* expression, it was non-existent between groups in amygdala samples (**Figure 12a**). However, in PFC there was a non-significant stress effect, as the first two groups (control treatment) had a lower expression than the second two groups (stress treatment) (**Figure 12b**). Finally, in the hippocampus, the *C4b* expression in animals fed chow was the same independently of stress treatment (**Figure 12c**).

As previously described, stress induces neuroinflammation (Grippo and Scotti 2013), and this phenotype is involved in the conversion of quiescent astrocytes to A1 reactive astrocytes. Therefore, it was expected to get an upregulation of genes associated to astrocyte reactivity, as those involved in the complement pathway, *C3* and *C4b.* This is what can be observed in PFC samples (**Figure 12b**), since *C4b* expression in not stressed groups was lower than in stressed groups, although not significant differences were observed.

The effect of inulin supplementation in amygdala and PFC (**Figure 12a** and **Figure 12b**) was lacking. However, in hippocampus (**Figure 12c**), *C4b* expression was significantly higher in the fourth group (treated with stress and inulin) versus the rest of the groups. The gut-brain communication is well studied, so it is not surprising that diet alterations can cause changes in the expression of astrocyte-related genes. Some authors studied how components of diet, such as astaxanthin or cashew-derived protein with high fibre, improve Alzheimer's disease and cerebral ischemia, respectively (Wattanathorn *et al.* 2017; Chen *et al.* 2021), both related to neuroinflammatory phenotypes. In the same way, the removal of some ingredients can improve inflammatory responses in hippocampus (Mazzoli *et al.* 2021). Based on previous studies, we expected a reduced *C4b* expression in stressed animals treated with inulin, but it did not occur. We suggest that mechanisms related to stress and diet response interacted and caused an increased *C4b* expression. Many details about gut-brain communication still need to be elucidated.

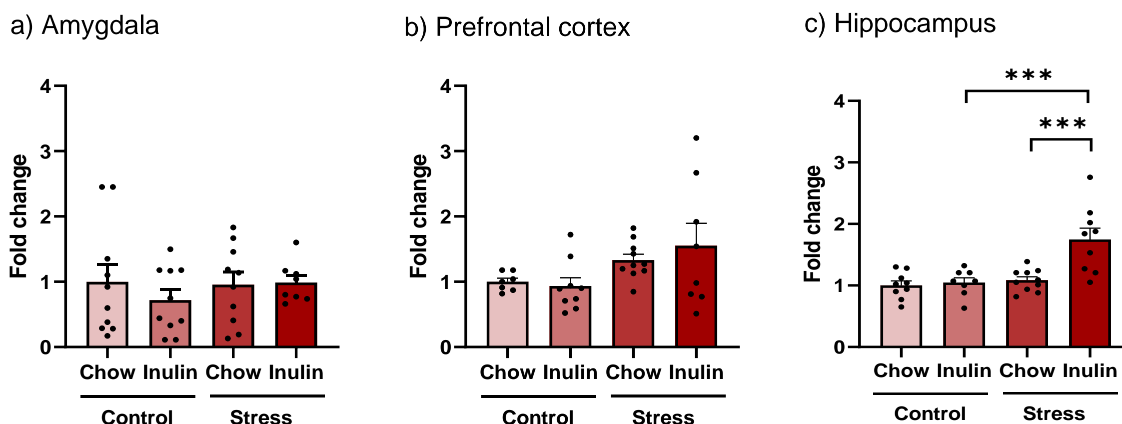

Figure 12 | Fold change representation of complement component 4b (*C4b*) expression of each group normalized to control group (Control-Chow). **a)** Amygdala, **b)** Prefrontal cortex and **c)** Hippocampus samples. *C4b* expression was relative to reference gene (*Actb*) expression. Each column represents the mean of biological replicates (8-10 replicates) and vertical bars indicate the standard error of the mean. Asterisks (*) indicate significant differences between groups according to Tukey's HSD test for parametric data or Kruskal-Wallis test for nonparametric data (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

5.2.6. C-X-C motif chemokine ligand 10 (Cxcl10) expression

To determine the first specific objective of the current work, gene expression in each brain region must be analysed. Stress treatment caused a significant increase of *Cxcl10* expression in the amygdala of those animals fed with inulin-based diet although the expression of this gene in this area was very variable among individuals from each group (**Figure 13a**). In PFC, the expression of all individual samples was very similar inter and intra groups except for two individuals from the first group, which had the highest expression (**Figure 13b**). Finally, in hippocampus of animals fed with chow diet, there

was a significant difference between animals subjected to a control treatment and animals subjected to a stress treatment. In this case, stress decreased *Cxcl10* expression (**Figure 13c**).

During neuroinflammation, chemokines attract inflammatory leukocytes into the CNS and, depending on the context they can have positive or negative effects. CXCL10 is the most studied chemokine for its role in mediating the influx of leukocytes into the CNS in some important diseases (Michlmayr and McKimmie 2014). According to Clarke *et al.* (2018), *Cxcl10* is one of those genes previously identified as a reactive astrocyte-associated gene, and they confirmed through RNAseq analysis that it was significantly upregulated in hippocampal and striatal astrocytes during aging. Besides, they also evaluated gene expression changes in response to LPS treatment using *in situ* hybridization and observed an increase in the number of *Cxcl10*. In our study we only obtained this result in amygdala, comparing the expression between animals fed with an inulin-supplemented diet subjected to control and stress treatment. In the rest of the brain regions, we did not get significantly higher expression in groups treated with stress than in groups not treated with stress. Similarly, in a study performed in choroid plexus, the barrier between the blood and the cerebrospinal fluid, Baruch *et al.* (2014) demonstrated that the expression of type II IFN dependent genes, such as *Cxcl10*, was decreased in aging. Hence, these results suggest the same that we deduced from our results: in the presence of a neuroinflammatory factor, gene expression is not always the same, as it depends on the brain region.

To elucidate inulin supplementation effect on *Cxcl10* expression, attention should be focused on hippocampus (**Figure 13c**). In this region, stress caused a decrease in the gene expression and inulin-supplemented chow diet increased *Cxcl10* expression to levels like control. In this case, again, alteration of the expression was not predictable based on the previous evidence. Therefore, further studies are required for a better comprehension.

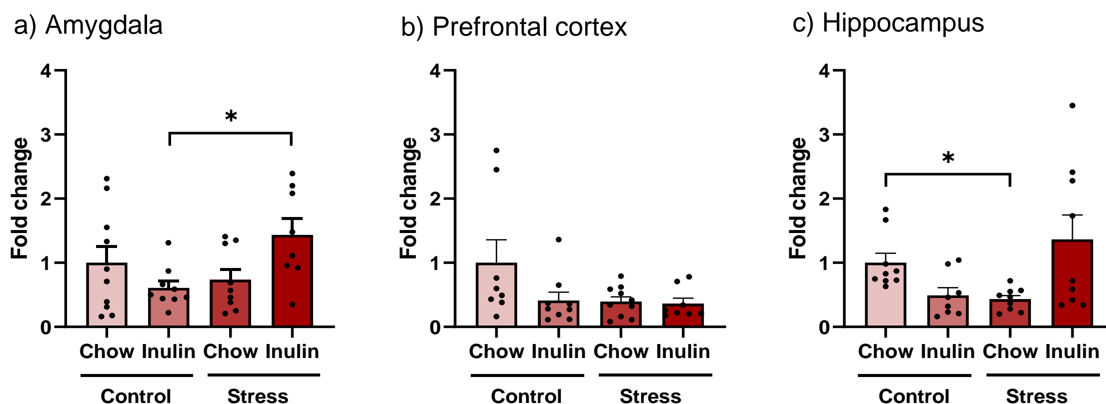

Figure 13 | Fold change representation of C-X-C motif chemokine ligand 10 (*Cxcl10*) expression of each group normalized to control group (Control-Chow). **a)** Amygdala, **b)** Prefrontal cortex and **c)** Hippocampus samples. *Cxcl10* expression was relative to reference gene (*Actb*) expression. Each column represents the mean of biological replicates (8-10 replicates) and vertical bars indicate the standard error of the mean. Asterisks (*) indicate significant differences between groups according to Tukey's HSD test for parametric data or Kruskal-Wallis test for nonparametric data (* $p < 0.05$).

5.2.7. Aryl hydrocarbon receptors (Ahr) expression

The impact of stress in *Ahr* expression in the different brain regions (**Figures 14a-14c**) was non-existent because it was practically the same in control and stressed groups.

The aryl hydrocarbon receptor (AHR) is a transcription factor activated by ligands, which can be heme-derived molecules, arachidonic acid metabolites, tryptophan (Trp) metabolites, among others (Quintana and Sherr 2013). Trp is an essential amino acid provided by dairy products, meats, fishes, cereals and some fruits. It can be metabolized by the host or by the GM, which can convert Trp to tryptamine and indole. These metabolites are used as a precursors for the synthesis of AHR agonists (Rothhammer *et al.* 2016; Gasaly *et al.* 2021).

AHR is involved in a molecular pathway through which described factors modulate the immune response in health and disease (Quintana and Sherr 2013). To study how *Ahr* expression in astrocytes regulate the inflammation in the CNS, Rothhammer *et al.* (2016) deleted *Ahr* in astrocytes and saw that experimental autoimmune encephalomyelitis scores were worse in AHR^- mice than in normal mice. Besides, inflammation and neurodegeneration markers expression were increased in microglia from AHR^- mice. Hence, these results suggested that the connection between AHR agonists, some generated from dietary Trp metabolized by GM, and AHR has anti-inflammatory effects.

Regarding diet role in *Ahr* expression, inulin-supplemented chow diet had an evident effect in PFC (**Figure 14b**), especially in those animals submitted to stress stimulus. The

expression in PFC from animals fed with inulin-supplemented diet was significantly lower than the expression in PFC from animals fed with chow diet.

Considering all these results, we suggest that inulin is metabolized by GM and resultant metabolites are AHR agonists precursors. Therefore, as diet treatment reduces neuroinflammation, it may reflect in a lower expression of *Ahr*. However, further studies are needed to confirm this point.

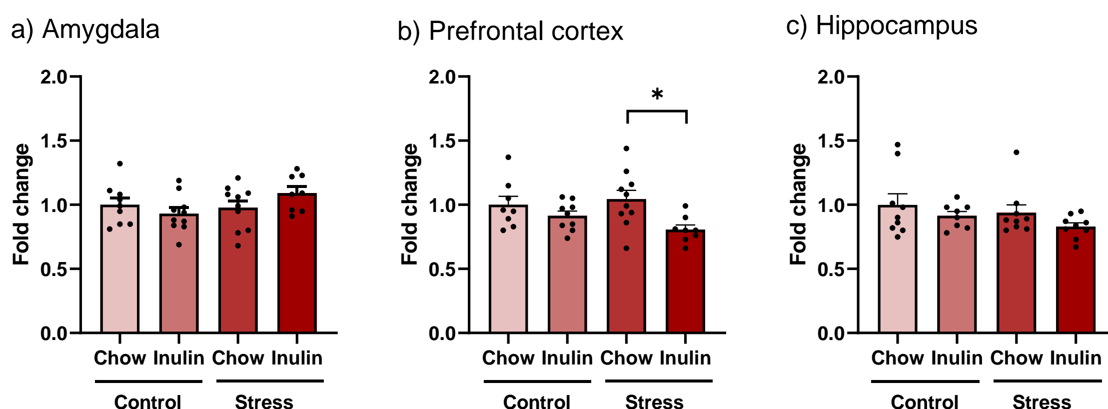

Figure 14 | Fold change representation of aryl hydrocarbon receptors (*Ahr*) expression of each group normalized to control group (Control-Chow). **a)** Amygdala, **b)** Prefrontal cortex and **c)** Hippocampus samples. *Ahr* expression was relative to reference gene (*Actb*) expression. Each column represents the mean of biological replicates (8-10 replicates) and vertical bars indicate the standard error of the mean. Asterisks (*) indicate significant differences between groups according to Tukey's HSD test for parametric data or Kruskal-Wallis test for nonparametric data (* $p < 0.05$).

From a general point of view, the gene expression in amygdala was very similar between the four analysed groups. It could be due to the fact that amygdala is smaller than the other brain regions and in the RNA extraction process we obtained very low concentrations. In PFC and hippocampus, we did not get much more significant differences between groups, but we can see a tendency to increase or decrease the expression of almost all the genes. It could be interesting to compare expression in amygdala to results from other studies, to check if stress and diet do not affect this region. However, we have not found specific evidence from the amygdala in other studies. Besides, drawing conclusions about the differences in gene expression in PFC and hippocampus is difficult because we could not see a clear stress treatment effect nor ability of inulin supplementation to modify gene expression alterations caused by stress. We must keep in mind that we are analysing the gene expression in tissues, which are composed by more than 5-6 different types of cells, and it is hard to separate cell specific gene expression in whole tissues. We should consider repeating the experiment using primary astrocyte cultures or single-cell flow cytometry. In this way, we would probably

get less variability between animals, and therefore, clearer differences between groups. We should also contemplate the option of using more than one reference gene, as some authors suggested (Vandesompele *et al.* 2001), to increase the precise normalization of gene expression.

There are many previous studies focused on the role of aging in neuroinflammation, and they describe the increase of reactive astrocytes-related genes with aging. All our groups were composed of aged mice, and we analysed the role of stress in these aging groups. In this sense, it could be possible that aging factor had already altered *Gfap, Stat3, Serpina3n, C3, C4b, Cxcl10* and *Ahr* expression, and stress effect was hidden by neuroinflammatory aging effect, which is clearly demonstrated. In those cases where a stress or diet effect was reported, it could be due to the expression of this gene in this specific brain region is highly modulated by these factors. It is also worth noting that in some cases inulin supplementation modulated the level of expression of some genes that were not altered by stress. Therefore, it can affect gene expression independently of the stress effect.

6. CONCLUSIONS

This study focused on the analysis of reactive astrocyte-related genes (*Gfap, Stat3, Serpina3n, C3, C4b* and *Cxcl10*) and a diet-related gene (*Ahr*) expression in three different brain regions (amygdala, PFC, and hippocampus). We have compared the expression of four groups composed of male aged mice, which were subjected to stress (control or social defeat stress caused by an aggressive mouse) and diet (chow or inulin-supplemented chow) treatments. The following conclusions have been reached:

Objective 1:

- In general, expression of the genes in most of the brain regions was not significantly affected by stress exposure. However, in this condition, some genes, such as *Gfap, Serpina3n, C4b* and *Cxcl10*, had their expression altered in some specific regions.
- Aging could previously have caused neuroinflammation, reactive astrogliosis, and alterations in gene expression, thus masking the effect of stress.

Objective 2:

- In general, inulin prebiotic supplementation did not affect the expression of analysed genes, except for *Stat3* and *Ahr*, in which inulin caused a reduction of their expression. Besides, *Cxcl10* expression was also altered by inulin supplementation, which increased it.

Objective 3:

- The potential of inulin to modulate gene expression alterations caused by stress could not be evaluated because as indicated in objective 1, in general, stress did not cause significant alterations.

Objective 4:

- Gene expression of the selected genes is different depending on the region evaluated (amygdala, PFC or hippocampus) thus confirming the suitability of evaluating different regions.

7. FUTURE PERSPECTIVES

To continue this study, we could follow different avenues. Firstly, we could analyse gene expression of other parts of the brain also related to neuroinflammation, such as choroid plexus and meninges. Besides, as we just used one side of the brain, we could send the remaining sample for transcriptomics or proteomics, to see what the profile is and directly focus on those genes related to altered transcripts. Finally, we also could work with a transgenic mouse line that interferes with astrocytes, to see the response in aging and describe the role of astrocytes in aging.

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