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The Glutamatergic System and Pain: Influence of Stress, Oestrous Cycle and Gut Microbiota

Thesis presented by

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for the degree of

Doctor of Philosophy

Under the supervision of

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Department of Anatomy and Neuroscience

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January 2019





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Declaration

This is to certify that the work I am submitting is my own and has not been submitted for another degree, either at University College Cork or elsewhere. All external references and sources are clearly acknowledged and identified within the contents. I have read and understood the regulations of University College Cork concerning plagiarism.



Jahangir Sajjad

Author Contributions

The author conducted all work under the supervision of Dr. Siobhain O'Mahony and Professor John Cryan in this thesis except the following:

- **Chapter 2**. Anna Golubeva and Valeria Felice helped in setting up aspartate assay. Tara Foley and Anna Golubeva assisted in experiments.
- **Chapter 3**. Rachel Moloney performed colorectal distension. Tara Foley assisted in aspartate assay experiments.
- **Chapter 4.** Tara Foley assisted in aspartate assay experiments. Amy Murphy and James Keane performed the microbiota analysis. Fiona Fouhy performed bioinformatics.
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List of Publications and Presentations

Published Manuscripts

Sex-dependent activity of the spinal excitatory amino acid transporter: Role of estrous cycle
J. Sajjad, V. Felice, A. Golubeva, M. Corten, J. F. Cryan and S. M. O' Mahony.
Journal: Neuroscience. 2016 Oct 1;333:311-9, PMID: 27471194

Estrous cycle influences excitatory amino acid transport and visceral pain sensitivity in the rat: effects of early-life stress
R. D. Moloney, J. Sajjad (Joint 1st author), T. Foley, T. G. Dinan, J. F. Cryan and S. M. O'Mahony
Journal: Biol Sex Differ. 2016 Jul 14;7:33, PMID: 27429736

Oral Presentations

Early-life stress and the oestrogen interact to influence both visceral pain sensitivity and glutamate Metabolism
Registrar Prize meeting, Irish Institute of Clinical Neuroscience, Dublin, November 2015

Riluzole reverses the inhibitory effects of oestrogen on spinal excitatory amino acid transporters in rats
Pharmacology 2014 meeting, British Pharmacology Society, London (International)

Sex hormones modulate glutamate reuptake by spinal excitatory amino acid transporters in rat spinal Cord
Young Neuroscientists Symposium, September 2014, Neuroscience Ireland, Dublin (National)
Registrar's Prize in Clinical Neuroscience, November 2014, Irish Institute of Clinical Neuroscience, Dublin (National)

Abstract

Women have a higher incidence of various visceral and neuropathic chronic pain conditions. Most of the animal studies investigating pain mechanisms show oestrogen to be pronociceptive. However, similar studies in healthy humans are equivocal. The predisposition of chronic pain conditions in female patients with a history of stress emphasises the role of chronic stress in the pathogenesis of these conditions. Since glutamatergic system plays an import role in the transmission of pain-related information in the central nervous system (CNS), its impaired modulation may be a key phenomenon responsible for the pathogenesis of pain conditions and associated gender differences. The microbiota-gut-brain axis is shown to be capable of influencing the CNS function in health and various diseases, but as yet it is to be determined whether it has any role in the modulation of pain-related central nervous mechanisms.

Extensive research is being carried out to understand the mechanisms through which sex hormones and chronic stress may influence the pain neurotransmission resulting in sex differences in pain. However, these mechanisms are not fully understood yet. As microbiota-gut-brain axis has been shown to influence a number of CNS disorders, and there is emerging evidence of its role in pain modulation in preclinical studies, we specifically concentrated on gut microbiota and its associated mechanisms influencing pain sensitivity. Our objectives were to explore the interplay between sex, stress, glutamatergic system, gut microbiota, and their effect on pain responses. We performed experiments both in animals and healthy humans.

We were able to demonstrate that in rats, synaptic glutamate metabolism in the spinal cord and anterior cingulate cortex of brain varied significantly depending upon the phase of

the oestrous cycle. Endogenous oestrogen exerted its effects through oestrogen receptor-α and was also shown to regulate glutamate receptor subunits expression. By utilising animal models of visceral hypersensitivity and stress (early-life stress & depression) in Wistar-Kyoto (WKY) and maternally separated rats, we could demonstrate that stress significantly altered pain responses through modifying the glutamatergic system and such effect was oestrous phase specific. In WKY rats, short-chain fatty acid producing gut microbiota abundance had a positive correlation with synaptic glutamate transporter function in a sex-specific manner. Finally, in healthy humans, we demonstrated a positive correlation between the abundance of butyrate producing bacteria in the bowel and pain sensitivity. Furthermore, hormonal contraceptive use was associated with increased lipopolysaccharide-binding protein levels.

Taken together, our results show that oestrogen plays a vital role in glutamatergic neurotransmission and pain responses. Aberrant changes in the pain-related sensory mechanisms may develop by factors such as stress and altered gut microbiota. The emerging relationship between sex hormones, neuroendocrine system, and the gut microbiota in relation to pain modulation supports further investigations in large scale studies. Trials are needed to establish if interventions to correct gut microbiota imbalance can have analgesic effects.

Chapter 1

Introduction

1.1 Pain

Pain is an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage (Treede, 2018). Depending on the cause, pain can be divided into two types: nociceptive and neuropathic pain (Spahr *et al.*, 2017). Table 1.1 (adapted from Dworkin *et al.*) details pain types and associated characteristics (Dworkin *et al.*, 2003). Nociceptive pain occurs in response to mechanical, chemical or thermal stimulation and disappears when the stimulus is eliminated. It is a protective phenomenon which results in a number of processes including localisation of the painful stimulus and withdrawal, and the subsequent emotional, psychophysiological, and behavioural reactions. Neuropathic pain occurs due to abnormalities in the somatosensory system caused by imbalances between excitatory and inhibitory signalling, alterations in ion channels and changes in the central pain modulation (Colloca *et al.*, 2017). Depending upon the body region involved, nociceptive pain can be further divided into two types: “somatic” and “visceral” (Cervero, 2009). While somatic pain is usually described as a “sharp,” “burning” or “prickling” sensation, it can be “dull” or “aching” when originating from the deep tissues. The stimuli for the somatic pain may originate from the skin, connective tissues, muscles, bones, and joints, and generally result in localised pain (Carver and Foley, 2003). Visceral pain, on the other hand, refers to pain that arises from stimuli to the internal organs or the tissues that support them. Visceral pain is usually cramping in nature and poorly localised.

Table 1.1. Types of pain, area of origin and related conditions and diseases are enlisted (Dworkin *et al.*, 2003)

Type	Subtype	Body area	Related conditions and diseases
Nociceptive	Somatic-superficial	Skin Cornea Teeth	Superficial injury
	Somatic-deep	Muscles Joints Bones and cartilage	Trauma / fractures Compartment syndrome Arthritis Bone cancer and metastasis
	Visceral	Gastrointestinal tract Genitourinary system Heart Lungs	Irritable Bowel Syndrome Inflammatory bowel disease Pancreatitis appendicitis endometriosis Noncardiac Chest pain Cardiac Angina Nonulcer dyspepsia Painful bladder syndrome Ureteric colic Cholelithiasis
Neuropathic	Peripheral		Acute and chronic inflammatory demyelinating polyradiculoneuropathy Alcoholic polyneuropathy Chemotherapy-induced polyneuropathy Complex regional pain syndrome Entrapment neuropathies (e.g., carpal tunnel syndrome) HIV sensory neuropathy Iatrogenic neuralgias (e.g., postmastectomy pain or postthoracotomy pain) Idiopathic sensory neuropathy Nerve compression or infiltration by tumour Nutritional deficiency-related neuropathies Painful diabetic neuropathy Phantom limb pain Postherpetic neuralgia Postradiation plexopathy Radiculopathy (cervical, thoracic, or lumbosacral) Toxic exposure-related neuropathies Tic douloureux (trigeminal neuralgia) Posttraumatic neuralgias
	Central		Compressive myelopathy with spinal stenosis HIV myelopathy Multiple sclerosis–related pain Parkinson's disease–related pain Postischaemic myelopathy Postradiation myelopathy Poststroke pain Posttraumatic spinal cord injury pain Syringomyelia

1.1.1 Visceral Pain

The pain resulting from the stimulation of receptors in the internal body organs or associated tissue, e.g., heart, stomach, intestine, urinary bladder, is defined as visceral pain (Cervero and Laird, 1999; Sikandar and Dickenson, 2012). Hollow body organs, e.g., the urinary bladder, ureter, and gut show more visceral pain responses than the parenchymous viscera such as liver and pancreas (Giamberardino, 1999; Robinson and Gebhart, 2008). In parenchymous body organs, such as the liver, diseases affecting these are commonly not associated with pain, rather the symptoms arise from the altered organ functions (Robinson and Gebhart, 2008). The mechanical, chemical and ischaemic stimuli are the typical triggers known to induce visceral pain (Ness and Gebhart, 1988, 1990; Al-Chaer and Traub, 2002). Mechanical stimuli include spasm or distension of hollow viscus walls, for example, ureteric/biliary colic due to stones and intestinal obstruction pain (Giamberardino, 2013). Chemical stimuli include inflammation (Bercik *et al.*, 2004), whereas ischaemic stimulus can result from the blockade of supplying artery, e.g., ischaemic cardiac pain, and mesenteric ischaemia (Stoney and Reilly, 1983; Sengupta, 2009; Walker, 2009).

Visceral pain is often felt at some distant cutaneous regions sharing the same dermatomal origin as the internal organ affected (Giamberardino and Vecchiet, 1995; Laird *et al.*, 2001). The most common examples of this are a cardiac pain being referred to the left shoulder, or pain from the large bowel being referred to the umbilical region.

1.1.2 Somatic Pain

Somatic pain can be “superficial somatic” or “deep somatic.” The superficial somatic pain originates from the skin and other superficial areas. Examples of injuries that induce superficial somatic pain include skin wounds and burns. Deep somatic pain is initiated by stimulation of nociceptors in ligaments, tendons, bones, blood vessels, fasciae, and muscles.

Examples of deep somatic pain include ligamentous sprains, bone fractures, and cancerous bone pains (Carver and Foley, 2003; Mantyh, 2014).

Somatic pain is a key component of the body's normal defence mechanisms, protecting the body from the potentially hostile surrounding environment by initiating behavioural and reflex avoidance responses (Willer, 1977; Woolf, 2010). The absence of these responses in patients with peripheral neuropathies often results in tissue damage and even permanent deformities, as seen in diabetic feet (Allan *et al.*, 2016). Congenital insensitivity to pain is a rare genetic condition characterised by inability to feel pain in response to any noxious stimulus (Schon *et al.*, 2018). Patients suffering from this condition can easily develop significant trauma to their bodies such as skin cuts, burns, bone fractures and joint deformities (Zhang *et al.*, 2016). Somatic pain is, therefore, a very important adaptive component of the nervous system, which should only be temporarily suppressed during surgical procedures and for pain relief procedures, e.g., nerve root injections for brachialgia or sciatica. Clinical pain also includes exaggerated pain response to noxious stimuli (hyperalgesia) (Lee *et al.*, 2011) and pain produced by stimuli which generally would not elicit pain (allodynia) (Lolignier *et al.*, 2015).

1.1.3 Pain Pathways

1.1.3.1 Nociceptors

Nociceptors are the neuronal apparatus responsible for the detection of noxious stimuli. Some nociceptors are thinly myelinated A-fibres, but the majority are unmyelinated C fibres (Wooten *et al.*, 2014). Somatic nociceptors can be classified as cutaneous, muscle and joint nociceptors depending upon their location in the body. Cutaneous nociceptors are mostly C-fibre mechano-heat (CMHs), A-fibre mechano-heat (AMHs) and high threshold mechanoreceptors (HTMs). The C fibres are also called polymodal nociceptors and can be

mechano-heat (C-MH), mechano-chemical (C-MC) or mechano-heat-chemical (C-MHC) (Campbell and Meyer, 1983; Martin *et al.*, 1987; Dubin and Patapoutian, 2010; Wooten *et al.*, 2014). Table 1.2 (derived from Dubin, *et al*.) summarises fibre characteristics (Dubin and Patapoutian, 2010).

As their names indicate, AMHs and CMHs respond to noxious mechanical and heat stimuli, whereas HTMs respond only to intense noxious mechanical stimuli but not to heat stimuli. The sensory nerve endings in the skin are mostly associated with specialised structures, e.g., Merkel Cells, Ruffini endings and Pacinian corpuscles (Garcia-Mesa *et al.*, 2017). These structures play a role in the sensation of touch, pressure, and vibration. Lack of these unit on the viscera limits the visceral sensation to distension, inflammation and ischaemia only. Muscles have nociceptive afferent myelinated (group III) and unmyelinated (group IV) fibres with a relatively lower excitatory threshold (Abrahams, 1986; McCord and Kaufman, 2010).

The visceral afferent nociceptive information is carried out almost exclusively through thinly myelinated Aδ and unmyelinated C fibres (Sengupta and Gebhart, 1994; Robinson and Gebhart, 2008). Common forms of noxious cutaneous stimulation, such as pricking, cutting, do not generally elicit pain response when applied to body viscera. Moreover, different visceral organs may have specific nociceptors that respond to a particular stimulus. For example, the heart has unmyelinated fibres which are individually responsive to chemicals released by the ischaemic myocardium (Casati *et al.*, 1979; Baker *et al.*, 1980; Fu *et al.*, 2010; Goodman-Keiser *et al.*, 2010). In the gut, there are tension receptors located on the muscular wall which respond to distension, contraction, and constriction of the gut (Leek, 1977; Clarke and Davison, 1978; Lu *et al.*, 2010; Humenick *et al.*, 2015).

Table 1.2. Characteristics of unmyelinated C and myelinated A-fibres (Dubin and Patapoutian, 2010)

Unmyelinated C-Fibre	Myelinated A-Fibre
Small Diameter, conduction velocity 0.4-1.4 m/s	Conduction velocity 5-30 m/s
Polymodal nociceptors C-mechano-heat, C-mechano-chemical, C-mechano-heat-chemical	A-mechano-heat, A-heat, A-mechanical
Broadly distributed with less precise localisation	Highly sensitive
Group IV are small unmyelinated C fibres with conduction velocity <2.5 m/s and respond to strong pressure	Group III are small myelinated A-fibres with conduction velocity 2.5-20 m/s and respond to pressure and pain

1.1.3.2 Afferents Neurons

The primary nociceptive afferents innervating the viscera reach the central nervous system (CNS) mainly via sympathetic nerves, but some afferent fibres also project to CNS via parasympathetic nerves, including the pelvic nerve and the vagus nerve (Grundy, 2002; Almeida *et al.*, 2004; Berthoud *et al.*, 2004; Anand *et al.*, 2007; Vermeulen *et al.*, 2014). The vagus nerve innervates the gastrointestinal tract (GIT) from the oesophagus to the proximal third of the transverse colon. The remainder of the large bowel and rectum are supplied by pelvic nerves (Bennett and Stockley, 1975; Mei, 1983; Wingate, 1985; Vermeulen *et al.*, 2014). Somatic afferent neurones enter the spinal cord at the corresponding spinal segments through the spinal nerves. The cell bodies of these first order neurons are located in the dorsal root ganglia of the spinal cord (Figure 1.1). These neurons then synapse with the second-order neurons in the dorsal horn and lateral grey matter of the spinal cord, where the pain signals travel further in the ascending sensory pathways (Armett *et al.*, 1961; Scheibel and Scheibel, 1968; Handwerker *et al.*, 1975; Moreno-Lopez *et al.*, 2013).

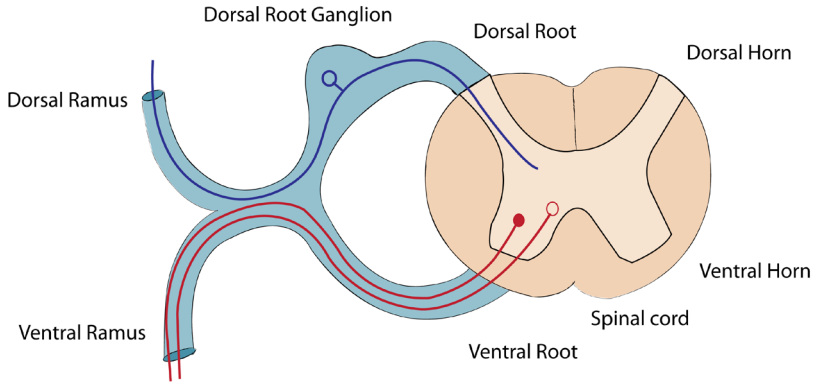


Figure 1.1. A schematic showing spinal cord cross section along with a spinal nerve.

1.1.3.3 Ascending Sensory Pathways

The second-order neurons, with cell bodies lying in the dorsal horn, ascend mainly in the anterolateral system containing spino-thalamic and spino-reticular tracts. Other tracts include spino-mesencephalic, spino-tectal and spino-hypothalamic fibres (Purves *et al.*, 2001c). The visceral afferents terminate in laminae I, II, V–VII and X of the dorsal horn in the spinal cord (Purves *et al.*, 2001a), as described in Figure 1.2. Laminae I and V form parts of the anterior and lateral spinothalamic tracts, whereas laminae VII and X constitute part of the dorsal column pathway (Rustioni *et al.*, 1979; Bennett *et al.*, 1983).

The two major tracts of the spinal cord known to transmit the visceral nociception are the spinothalamic tracts and the dorsal column. The nerve cells in the anterior and lateral spinothalamic tracts make synaptic connections in different locations along their pathway, e.g., within the mesencephalic reticular formation and the periaqueductal grey via spino-reticular tract, in the tectum via spino-tectal tract, and in the intralaminar thalamus via spinothalamic tract (Jones *et al.*, 2006; Sharma *et al.*, 2009). Based on this knowledge, procedures such as anterior cordotomy and commissural myelotomy have been reported to be performed in the early 20th century to relieve visceral pain (Spiller and Martin, 1912). However, these techniques have been abandoned due to associated serious adverse events.

The dorsal column of the spinal cord is generally known to be involved in the transmission of proprioception, vibration, and fine touch. However, its role in neurotransmission of visceral pain has also been established (Willis *et al.*, 1999; Palecek, 2004). A surgical lesion of the postsynaptic dorsal column has been shown to dramatically reduce the electrical responses of the ventral posterolateral nucleus (VPL) of the thalamus to colorectal distension in rats (Al-Chaer *et al.*, 1996b, a). With the evolution of techniques in surgical lesions in dorsal columns, a recent, more accurate technique 'punctate midline myelotomy' has been described for intractable visceral cancer pain (Nauta *et al.*, 2000). This procedure has been found to be very efficient and is associated with low morbidity and surgical mortality (Hong and Andren-Sandberg, 2007).

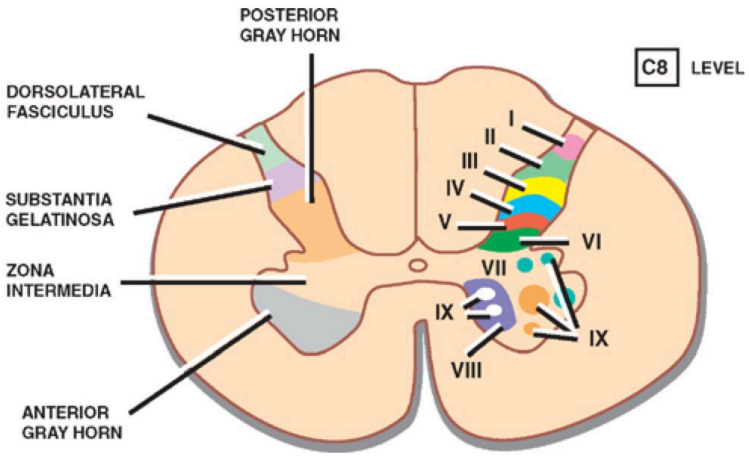


Figure 1.2. A cross section of spinal cord showing laminae of Rexed in grey matter. The visceral afferents terminate in laminae I, II, V–VII and X of the dorsal horn in the spinal cord (C.D. Alberstone, 2009).

1.1.3.4 Pain Processing in the Brain

The cell bodies of the third order neurons of nociception pathways are located in the ventral-posterior-lateral, the ventral-posterior-inferior and the intralaminar nuclei of the thalamus (Steeds, 2009). The fibres from ventral-posterior-lateral nucleus course through the posterior limb of the internal capsule and terminate in the primary somatosensory cortex (also known as postcentral gyrus) through the corona radiata. The primary somatosensory cortex

is located in the postcentral gyrus of the parietal lobe (Figure 1.3). The ventral-posterior-inferior nucleus of the thalamus project mainly to secondary somatosensory cortex located on the superior aspect of the lateral fissure. The intralaminar nuclei project fibres to the striatum (caudate and putamen), primary and secondary somatosensory cortex, cingulate cortex, and prefrontal cortex (Eto *et al.*, 2011; Fuchs *et al.*, 2014; Seminowicz and Moayedi, 2017).

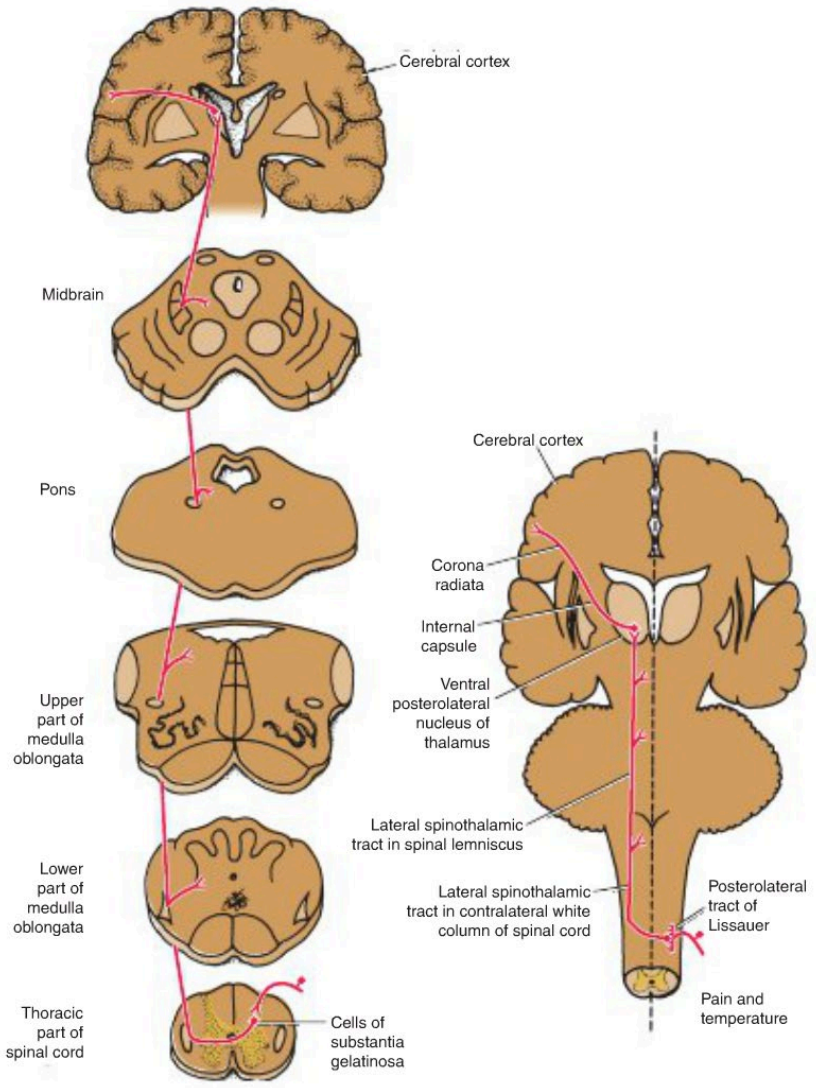


Figure 1.3. A schematic showing fibres carrying pain signals, travelling through the spinothalamic tracts to the postcentral gyrus of the brain cortex (Snell, 2010).

Clinical imaging studies such as electroencephalography (EEG), magnetic resonance imaging (MRI), and positron emission tomography have shown that nociception is not only

processed at the primary and secondary somatosensory cortices but also in the anterior cingulate cortex, insular cortex, and the supplementary motor cortex, thalamus and cerebellum (Apkarian *et al.*, 2005). Figure 1.4 depicts these connections. In the context of nociception, these brain structures such as the primary and secondary somatosensory, the cingulate and the insular cortices are often referred to as "pain matrix", i.e., a network of cortical areas through which pain is generated from nociception (Melzack, 1999; Tracey and Mantyh, 2007). The somatosensory cortices process information about sensory features of pain such as location and duration (Kenshalo *et al.*, 1988; Greenspan *et al.*, 1999; Garland, 2012). The anterior cingulate cortex (ACC) and anterior insular cortex are connected with the limbic cortex, which plays a role in the emotional aspect of pain (Bush *et al.*, 2000). The ACC, which is thought to play a dominant role in cognitive and emotional processing, is also known to be involved in descending modulation of pain (Tracey and Mantyh, 2007). In posttraumatic stress disorder, there is decreased activation of ACC seen in functional imaging, which may indicate impairment of emotion regulation in this condition (Etkin and Wager, 2007). A similar effect of reduced activation of ACC is seen in depressed patients in response to emotional stimuli (Fitzgerald *et al.*, 2008). Depressive disorders are often associated with chronic pain. Central neuronal plasticity may underlie these both conditions. A study showed that degree of depression was associated with the magnitude of activation of brain areas involved in the affective dimension of pain (Giesecke *et al.*, 2005). Patients with lesions in ACC and insula show altered emotional responses to pain and based on this, surgical cingulotomy can be helpful in patients with cancer pain refractory to medical management (Viswanathan *et al.*, 2013; Agarwal *et al.*, 2016).

Certain areas of the brain also play an important role in descending modulation of pain transmission which can either result in inhibition or facilitation (Ren and Dubner, 2002). These

brain regions include the frontal lobe, ACC, insula, amygdala, hypothalamus, PAG, nucleus cuneiformis, and rostral ventromedial medulla (RVM) (Ossipov *et al.*, 2014). This pain modulatory system, which works through the attentional modulation of pain, regulates the nociceptive processing mainly at the dorsal horn and is mediated largely through the PAG and RVM (Reynolds, 1969; Yaksh and Rudy, 1978; Gebhart, 2004; D'Mello and Dickenson, 2008). PAG has been shown to have increased activity in people who were distracted compared to those who were attentive to pain stimulation (Tracey *et al.*, 2002). The former group also had low pain score rating. During distraction, the cingulo-frontal cortex exerts influences on the PAG and posterior thalamus to gate the pain modulation (Valet *et al.*, 2004).

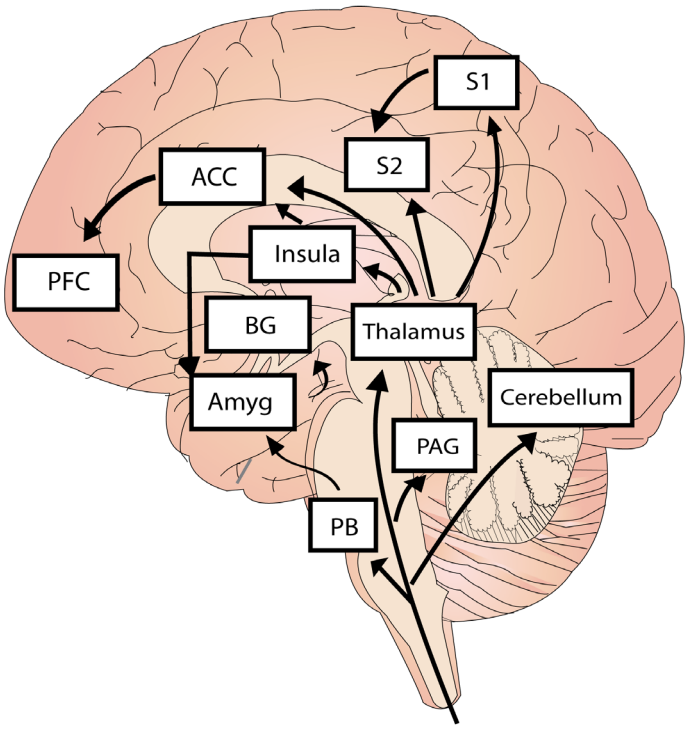


Figure 1.4. Afferent pain pathways to multiple brain regions. Nociceptive information from the thalamus is projected to the insula, anterior cingulate cortex (ACC), primary somatosensory cortex (S1) and secondary somatosensory cortex (S2), whereas information from the amygdala (AMY) is projected to the basal ganglia (BG). Information is also relayed into PAG, periaqueductal grey; PB, parabrachial nucleus; PFC, prefrontal cortex (Bushnell *et al.*, 2013).

1.1.4 Neurotransmitter Signalling in the Pain Pathways

Some of the neurotransmitters and receptors involved in pain processing include; glutamatergic, GABAergic, opioid, nicotinic, cannabinoid, and glucocorticoid neurotransmitter-receptors systems (Otsuka and Yanagisawa, 1990; Argoff, 2011). Table 1.3 (adapted from Steeds *et al.*) lists the neurotransmitters involved in the transmission of pain (Steeds, 2009). Among these, glutamate is the chief excitatory and gamma amino butyric acid (GABA) is a major inhibitory neurotransmitter involved in the pain processing.

Table 1.3. List of neurotransmitters involved in pain neurotransmission (Steeds, 2009)

Class	**Neurotransmitter**	**Comments**
Amines	Noradrenaline Serotonin	Involved with modulation of descending pain pathways
Endogenous opioid peptides	Enkephalins B-Endorphins	Widespread in CNS, particularly along pain pathways. Inhibitory action on opioid receptors
Non-opioid peptides	Substance P	Widespread especially in dorsal root ganglion (DRG) of C fibres. Associated with inflammation
	Gelanin	Widespread. Involved with antinociception
	Cholecystokinin	Present in DRG, dorsal horn, and spinal tracts. May be involved with visceral pain
Excitatory amino acids	Glutamate	Act on N-methyl-D-aspartate (NMDA) and non-NMDA receptors. Involved in development, memory, and neuronal plasticity
Inhibitory amino acids	GABA Glycine	Regulate behaviour associated with non-noxious stimuli
Others	Cannabinoids	Receptors in the brain and spinal cord and on primary afferent neurons. Involved in antinociception
	Nitric Oxide	In sensory neurons and dorsal horn. Involved in peripheral and central sensitisation. Linked with NMDA activity

1.1.4.1 Glutamatergic System

The study of the expression of various components of the glutamatergic system and functions of regulators of synaptic glutamate concentration is vital to explore multiple factors affecting nociception. The role of glutamatergic pathway in nociceptive processes has been associated with different types of pain such as neuropathic and visceral pain (Jensen and

Baron, 2003; Osikowicz *et al.*, 2013; Zunhammer *et al.*, 2016). Upregulation of glutamate clearance at synapses has been shown to attenuate the neuropathic pain and visceral pain (Gosselin *et al.*, 2010; Chen *et al.*, 2012; Moon *et al.*, 2014; Nicholson *et al.*, 2014b). Therefore, the drugs that can downregulate glutamatergic neurotransmission appear to be an attractive target for the future therapy of various pain syndromes. Glutamate is a major excitatory neurotransmitter in the CNS that participates in rapid signal transmission across the synapses. Glutamate receptors are found throughout the brain and spinal cord, both in neurons and glial cells. Glutamate concentration in the neuronal synapses is regulated by a number of mechanisms. Abnormal regulation of glutamate metabolism can lead to deleterious effects, for example; excessive concentration of extracellular glutamate can lead to postsynaptic excitotoxicity and cell death (Choi, 1994; Doble, 1996; Heath and Shaw, 2002).

Glutamine is the precursor of glutamate found in presynaptic neurons (Hamberger *et al.*, 1979; Holten and Gundersen, 2008). Glutamate, once formed, is transported and stored in synaptic vesicles in the presynaptic neuron by vesicular glutamate transporters (VGLUTs). During signal transmission, glutamate is released into the synaptic cleft, which, in turn, causes activation of ionotropic glutamate receptors, also called N-methyl-D-aspartate (NMDA) receptors. Activation of these receptors results in neurotransmission across the synapse. The concentration and activity of glutamate are controlled by sodium-dependent excitatory amino acid transporters (EAATs), through which an influx of glutamate occurs into glial cell end feet wrapped around the synaptic cleft (Rothman *et al.*, 2003). This movement is sodium-dependent and involves an influx of sodium and glutamate, and efflux of potassium molecule (Zerangue and Kavanaugh, 1996; Rose *et al.*, 2009). Inside glial cells, the glutamate is converted into glutamine and transported back in to the presynaptic neuron, where it is stored

again in synaptic vesicles in the form of glutamate by the action of glutaminase (Thanki *et al.*, 1983). Figure 1.5 summarises glutamate metabolism.

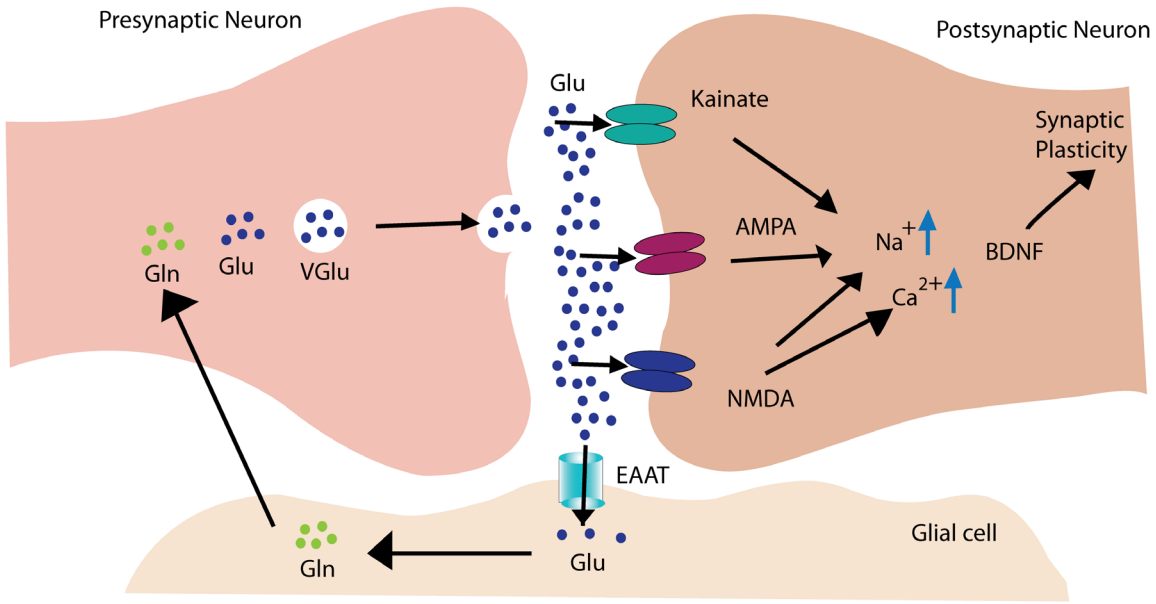


Figure 1.5. Glutamatergic neurotransmission and glutamate metabolism across the synaptic cleft. Glutamate (Glu) is released into synapse by the presynaptic neuron. The Glu in the synapse is cleared by EAAT into the glial cell where it is converted into glutamine (Gln). Glutamine is transported into presynaptic neuron where after conversion into glutamate is stored in vesicular glutamate transporters (VGLUTs).

1.1.4.1.1 Glutamate Transporters

To maintain a normal synaptic transmission and to avoid excitotoxicity, the regulation of glutamate metabolism is critical. EAATs regulate the concentration of glutamate in the synaptic clefts by rapidly transporting glutamate into the cells. Five EAA transporters have been identified; EAAT1 to EAAT5. L-glutamate/L-aspartate transporter (EAAT1), glutamate transporter-1 (EAAT2), and excitatory amino acid carrier-1 (EAAT3) were first identified in 1992 (Kanai and Hediger, 1992; Pines *et al.*, 1992; Storck *et al.*, 1992). EAAT1 and EAAT2 are primarily found on glial cells, while EAAT3 is a neuronal membrane transporter (Eulenburg and Gomeza, 2010). EAAT4 and EAAT5 were later identified in the cerebellum and retina,

respectively (Fairman *et al.*, 1995; Arriza *et al.*, 1997). Primarily astrocytes, but also microglia and oligodendrocytes are involved in the regulation of extracellular glutamate (Olive, 2009).

The majority of the EAA transporters in the glial cells are associated with synapses (Minelli *et al.*, 2001) and therefore have a vital role in regularisation of neurotransmission and prevention of excitotoxicity. Interestingly, in certain pathophysiologic states, such as ischaemia, the high extracellular K^+ may lead to reverse transport of glutamate out of glial cells into the extracellular space (Szatkowski *et al.*, 1990; Rossi *et al.*, 2000). This mechanism may have a causative role in brain cell death following ischaemic brain stroke (Grewer *et al.*, 2008). Another morbid pathological condition associated with EAAT2 dysfunction is amyotrophic lateral sclerosis (Rosenblum and Trotti, 2017). In the spinal cord, EAAT1 and EAAT2 are the mediators of glutamate clearance at synapses, and their expression has an important role in visceral hypersensitivity[1] (Tao *et al.*, 2005; Yan *et al.*, 2009; Bradesi, 2010). Moreover, transgenic mice overexpressing EAAT2 demonstrated twofold glutamate uptake across these transporters and a significant reduction in visceromotor response (VMR) to colorectal distension[2] (Lin *et al.*, 2009b). It has been shown in rats that intrathecal administration of spinal glutamate transporter antagonist results in increased sensitivity to colorectal distension (Gosselin *et al.*, 2010). On the other hand, maternal separation (an animal model of early-life distress) leading to visceral hypersensitivity, resulted in a selective reduction in spinal EAAT1 expression (Gosselin *et al.*, 2010). Therefore, there is a need to study these transporters extensively to achieve specific therapeutic targets in pain syndromes.

1. Visceral hypersensitivity is defined as a heightened sensation in response to a physiological stimulus to viscera.
2. The abdominal musculature contractions in response to experimental colorectal distension is termed as visceromotor response.

1.1.4.1.2 Glutamate Receptors

There are two main types of glutamate receptors- ionotropic and metabotropic (Kew and Kemp, 2005; Pleasure, 2008). As their names indicate, ionotropic receptors are voltage sensitive, whereas metabotropic receptors are ligand sensitive. Ionotropic receptors are fast acting, which upon activation leads to an immediate influx of sodium resulting in postsynaptic cell membrane depolarisation. Ionotropic receptors are named after their respective ligands as *N*MDA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA), and kainic acid as shown in Figure 1.5.

Metabotropic receptors are slow acting receptors and act via gene expression and protein synthesis via G-protein coupling effect (Niswender and Conn, 2010). The metabotropic receptors, summarised in table 1.4, are grouped into three categories (Kew and Kemp, 2005).

Table 1.4. Glutamate receptors and their properties

Receptors	Protein Subunits	Properties
	Inotropic Glutamate Receptors	
NMDAR	NR1, NR2A, NR2B, NR2C, NR2D, NR3A, and NR3B	$Ca^{2+,}$ Na^{+}
AMPAR	$GluR_1$, $GluR_2$, $GluR_3$, and $GluR_4$	Na^{+}, (Ca^{2+})
Kainate Receptors	$GluR_5$, $GluR_6$, $GluR_7$, KA1, and KA2	
	Metabotropic Receptors	
Group 1	$mGluR_1$ and $mGluR_5$	G_q / G_{11} → ↑PLC
Group 2	$mGluR_2$ and $mGluR_3$	
Group 3	$mGluR_4$, $mGluR_6$, $mGluR_7$, and $mGluR_8$	G_i / G_0 → ↓AC

Glutamate activates sodium and calcium dependent ionotropic receptors and stimulates metabotropic receptors coupled via G proteins (G_q, G_{11}, G_i, G_0) to activation of phospholipase C (PLC) and inhibition of adenylate cyclase (AC) activity (Kew and Kemp, 2005).

Group I metabotropic receptors are located on postsynaptic neurons, group II on both presynaptic and postsynaptic neurons, and group III receptors are situated mainly on presynaptic neurons. The presynaptic location of group II and III indicate their inhibitory effects on glutamate transmission (Lesage and Steckler, 2010). Dysfunction of group II and III

have been shown to be implicated in nervous system disorders such as anxiety, fear, stress, schizophrenia, Alzheimer's and Parkinson's disease (Swanson *et al.*, 2005).

N-methyl-D-aspartate receptors (NMDARs) are expressed by all DRG neurons (Marvizon *et al.*, 2002), and it has been shown that direct stimulation of peripheral afferent nerve terminal fields with NMDAR agonists causes pain responses in both human and animal models (Cairns *et al.*, 2003; Cairns *et al.*, 2006). Stimulation of NMDARs in peripheral nerves and central nerve endings in spinal cord results in the release of pronociceptive neuropeptides, such as substance P, calcitonin gene-related peptide, and brain-derived nerve growth factor (BDNF) (McRoberts *et al.*, 2001; Zhao *et al.*, 2006a).

1.1.4.1.3 Measurement of Extracellular Glutamate

Extracellular glutamate can be measured by both in-vivo and ex-vivo techniques. Table 1.5 summarises these techniques.

A) In-vivo Measurement of Extracellular Glutamate

i- Microdialysis

Microdialysis has been used to analyse the extracellular concentration of a number of electrolytes, neurotransmitters, hormones, and drugs from various regions of the brain and spinal cord (Chefer *et al.*, 2009). Microdialysis probes are constructed utilising fused silica inlet, and outlet tubes joined together with a semi-permeable dialysis membrane with around 2 mm active length (Baker *et al.*, 2003). The in-vivo microdialysis probe is inserted into the target brain region after drilling small holes into the skull of animals using a stereotactic frame to identify the specific brain. There are various modifications, but for glutamate analysis, the most common approach is the no-net-flux method. In this method, after the insertion of the active probe into the desired tissue, increasingly concentrated exogenous glutamate solution is passed through the inlet probe at regular intervals. The final glutamate concentrations are

measured from the outlet probe. The point where there is no change in the concentration of both inlet and outlet solutions, i.e., no-net-flux, is inferred to the extracellular glutamate concentration of the tissue assessed (Jacobson *et al.*, 1985; Lerma *et al.*, 1986; Miele *et al.*, 1996; Galvan *et al.*, 2003; Melendez *et al.*, 2005; Miller *et al.*, 2008; Berglind *et al.*, 2009).

Table 1.5. lists some studies utilising various technique to measure glutamate in animal pain models

Technique	Pain model	Species, CNS region, awake/anaesthesia	Reference
Microdialysis	Plantar incision	Rat, dorsal horn, awake	(Zahn *et al.*, 2002)
	Pinching or saline injection in hind paw	Rat, periaquiductal grey, awake	(Rouge-Pont *et al.*, 1998; Silva *et al.*, 2000)
	Spinal capsaicin	Rat, intrathecal, awake	(Malmberg *et al.*, 1995)
	Phorbol ester into the dorsal horn	Rat, intrathecal, awake	(Palecek *et al.*, 1999)
	Mustard oil joint into temporo-mandibular joint	Rat, spinal trigeminal nucleus, barbiturate anaesthesia	(Bereiter and Benetti, 1996; Bereiter *et al.*, 2002)
	Formalin/capsaicin injection into hindpaw	Mouse, spinal cord dorsal horn,	(Watanabe *et al.*, 2012)
	Spinal cord injury	Rat, the ventro-postero-lateral nucleus of the thalamus, awake	(Ghanbari *et al.*, 2014)
Microsensors	Tail pinch	Rats, prefrontal cortex, awake	(Rutherford *et al.*, 2007)
	Electrical stimulation of hind limb	Rats, dorsal brainstem, anaesthetised	(Onifer *et al.*, 2012)
Electrophysiological measurements	Peripheral nerve injury	Mice, anterior cingulate cortex	(Xu *et al.*, 2008; Cao *et al.*, 2009b)
	Hindpaw injection of Complete Freund's adjuvant	Rats, anterior cingulate cortex	(Zhao *et al.*, 2006b)

ii- Microsensors

Microsensors are prepared by coating 300-500 µM long carbon fibre electrodes with a hydrogel containing glutamate oxidase and peroxidase (Oldenziel and Westerink, 2005).

Microsensors utilise the principle of a one-step or two-step redox reaction (Qin *et al.*, 2008). A one-step redox reaction involves the action of glutamate oxidase, coated onto the sensors, which produces H_2O_2 detected by electrodes. A more recent form of these microsensors utilises two-step redox reaction, where H_2O_2 produced is further reduced by a second enzyme peroxidase (Oldenziel *et al.*, 2006). The resultant current generated by this reaction is calibrated to determine the concentration of extracellular glutamate, which can range from 1-10 µM in normal brain tissue and can reach 100 µM upon activation (Baker *et al.*, 2002).

B) In-vitro Analysis of Extracellular Glutamate

i- Electrophysiological Measurements in Brain Slices

Cavelier and Attwell originally described this method (Cavelier and Attwell, 2005), whereas some modifications have been published in the literature (Herman and Jahr, 2007; Meur *et al.*, 2007). Briefly, brain slices are dissected in an oxygenated buffer solution containing sodium kynurenate (to block glutamate receptors), at 4°C and a pH of 7.4.

The cells are whole-cell voltage-clamped with a pipette solution containing QX-314 (to suppress voltage-gated sodium currents). The external solution, as for brain slicing but without sodium kynurenate, is superfused at 25°C. Tetrodotoxin, picrotoxin, strychnine, and glycine are used in the bathing fluid to block action potentials, block $GABA_A$ and glycine receptors, and to fully activate the glycine-binding site on NMDA receptors. The slices need to be pretreated with bafilomycin to deplete glutamate from synaptic vesicles, so slices are incubated in a solution (without sodium kynurenate) to which bafilomycin is added. Control slices are incubated for the same period in a solution containing sodium kynurenate. Excitatory postsynaptic tonic NMDA-receptor currents are evoked by placing a concentric stimulating electrode at a standard location, mediated by extracellular glutamate. Compared

to in-vivo techniques, these methods give extracellular glutamate concentrations to be very low, i.e., 23 to 89 nM (Cavelier and Attwell, 2005; Herman and Jahr, 2007).

ii- Glutamate Uptake Assay by Radiolabelling

Glial glutamate uptake by glutamate transporter can be measured by radiolabelling the glutamate, a technique described by Frizzo *et al.* (Frizzo *et al.*, 2002; Thomazi *et al.*, 2004). During the procedure, animals are decapitated, their brains immediately removed and humidified with Hank's balanced salt solution (HBSS), at pH 7.2. Brain tissue is dissected into Petri dishes with HBSS and slices (0.4 mm) obtained using a McIIwain tissue chopper. Slices are transferred to 24-well culture plates: one plate maintained at 35 °C and the other on the ice. The slices from the first plate are washed once with 1 mL of 35 °C HBSS and the second with 1 mL of 4 °C sodium-free HBSS for the analysis of non-specific uptake. Slices are pre-incubated at 35 °C for 30 min, followed by the addition of Ci mL^{-1} L- [^{3}H] glutamate, and 100 M (final concentration) glutamate. Incubation is stopped after 3, 5 or 7 min for striatum, hippocampus, and cortex, respectively, with two ice-cold washes of l mL HBSS, immediately followed by the addition of 0.5N NaOH, which are then kept overnight.

To measure sodium-independent uptake, the same protocol described above is used, though with differences in the temperature and the medium used. Sodium-independent uptake is determined on ice (4 °C), using *N*-methyl-d-glucamine instead of sodium chloride. The results are subtracted from the total uptake to obtain the specific sodium-dependent uptake. The radioactivity is measured using a liquid scintillation counter. The final radioactivity is normalised to protein measured from the same tissue to get the total glutamate uptake. Since EAATs transport both glutamate and aspartate, either of these compounds can be used to assess the EAAT function (Ryan *et al.*, 2009).

1.1.4.2 GABAergic System

γ-aminobutyric acid (GABA) is widely distributed throughout the CNS. GABA receptor agonists, as well as the inhibitors of its metabolism, have been shown to display antinociceptive activity (Sands *et al.*, 2003; Jasmin *et al.*, 2004; Hama and Borsook, 2005; Enna and McCarson, 2006). There are two distinct types of GABA receptors: ionotropic $GABA_A$ and metabotropic $GABA_B$ receptors.

GABAergic neurons, as well as $GABA_A$ and $GABA_B$ receptors, are located in the areas of the CNS related to the neurotransmission of pain signals. In the brain, the activation of $GABA_A$ receptors in the thalamus induces an antinociceptive response (Reyes-Vazquez *et al.*, 1986; Umorin *et al.*, 2016). Furthermore, there are GABAergic projections from the substantia nigra to the PAG and dorsal medullary raphe nucleus that regulate the behavioural and responses to pain (Kirouac *et al.*, 2004; Omelchenko and Sesack, 2010; Dieb *et al.*, 2016). There is data indicating that $GABA_A$ receptors are located on inhibitory neurons projecting from the rostral ventral medulla to the dorsal horn (Gilbert and Franklin, 2001; Kato *et al.*, 2006). In the spinal cord, GABA receptors are present on the laminae I and II of the dorsal horn. Activation of presynaptic $GABA_B$ receptors on substance P or glutamate containing neurons tends to enhance the pain thresholds by inhibiting the release of these transmitters (Malcangio and Bowery, 1994). Thus, stimulation of $GABA_B$ receptors located presynaptically on the descending inhibitory serotonergic or noradrenergic terminals may lower the pain threshold by diminishing the release of transmitter from these cells (Yang *et al.*, 2002). Likewise, direct $GABA_A$ or $GABA_B$ receptor-mediated inhibition of opioid-containing neurons tends to facilitate pain transmission by reducing the release of this endogenous analgesic (Mahmoudi and Zarrindast, 2002).

1.2 Anatomical Sex Differences in Pain Pathways

Animal models have shown females to be more sensitive to visceral pain stimuli such as colorectal distension and urinary bladder distension (Holdcroft *et al.*, 2000; Ness *et al.*, 2001; Ji *et al.*, 2006; Ball *et al.*, 2010; Ji *et al.*, 2012). In contrast to this, some studies have shown no gender differences in visceral pain (Wang *et al.*, 2009; Larauche *et al.*, 2012).

Sex differences have been investigated in various aspects of pain, including physiological and pathophysiological characteristics, behaviours to pain and responses to different interventions (Fillingim *et al.*, 2009; Bartley and Fillingim, 2013). The cause of most of these sex differences is multifactorial, but there are anatomical and molecular differences present in the CNS in both sexes, accountable for these differences in pain associated characteristics.

1.2.1 Sex Difference Along Nociceptive Afferents Signalling

The lumbosacral spinal cord is an important area in the gut-related visceral pain and sciatica associated neuropathic pain pathways. Mills *et al.* have demonstrated that male rats have more DRG neurons as compared to females in the lumbar spine (Mills and Sengelaub, 1993). They found that this distribution was testosterone dependent as no difference was found in day 18 female and male embryos, whereas this difference emerged at the age of 10 days postnatal. This hypothesis of testosterone effect was endorsed by the finding that perinatal treatment with testosterone in females abolished this difference (Mills and Sengelaub, 1993). However, this difference is related to perineal musculature activity rather than visceral pain.

The hormonal modulation of DRGs was further suggested by the presence of oestrogen receptors in DRGs in 1994 for the first time (Sohrabji *et al.*, 1994). It was also noted that cyclical changes in the expression of oestrogen receptors in DRGs occurred in the

different phases of the oestrous cycle in female rats. A few years later the distribution of both oestrogen receptor alpha (ERα) and oestrogen receptor beta (ERβ) in this region was described (Taleghany *et al.*, 1999). The expression of mRNA of ERβ in almost all neurons in DRG, in both male and female rats was seen. In contrast, ERα mRNA along with ERα protein was mainly found in small-sized DRG neurons. These were found to be concentrated in DRGs in those levels related to visceral organs (lumbosacral spinal segments L6-S1) than those related to hind limbs (lumbar segments L4-L5). Moreover, DRG neurons were shown to be enriched with ERα mRNA at lumbar segments L1-L2 in males when compared to females. Later, it was established that approximately 17% of the L6-S1 DRG neurons contain ER-α, 23% contain ER-β and 5% express immunoreactivity for both subtypes of the ER suggesting that oestrogen may produce different effects in different neurons depending on their ER receptor expression (Papka and Storey-Workley, 2002).

It has yet to be established whether these gender differences in afferents have any role in visceral pain modulation. The electrophysiological studies of colonic afferents have shown no differences in responses to colorectal distension in intact and ovariectomised female rats, suggesting oestrogen may modulate visceral pain pathways distal to the primary afferents (Ji *et al.*, 2011).

1.2.2 Spinal Mechanisms Involved in Sex Differences

1.2.2.1 Dorsal Horn Neurons

Different segments of the spinal cord may be involved in the transmission of visceral pain depending on its origin. For example, lumbosacral and thoracolumbar dorsal horn neurons have been shown to be involved in the transmission of the pain resulting from CRD (Ness and Gebhart, 1987, 1988). It has been shown that the activation of afferents to cervical

segments 1, 2, 6 & 7 led to inhibition of evoked responses to visceral stimulation of lumbosacral neurons (Qin *et al.*, 1999).

1.2.2.2 Glial Excitatory Amino Acid Transporters

To our knowledge, sex-specific comparisons of mRNA expression of these transporters have not been studied previously. Further studies are needed to elucidate the function of these transporters in males and females. This can be achieved by glutamate uptake assays on rat spinal cord slices and by comparing the results of males with females in various phases of the oestrous cycle. The factors affecting the glutamate transport, such as sex hormones, can also be elucidated with this technique.

1.2.2.3 Glutamate Receptors

In the CNS, NMDARs activity is modulated by oestrogen. It has been shown that NMDARs showed more activity in DRGs in female rats as compared to males, and this effect was further potentiated in females by exogenous oestrogen (McRoberts *et al.*, 2007). Double immunofluorescent labelling studies have shown co-localisation of oestrogen receptor alpha (ER-α) and NR1 (a subunit of NMDAR) in the dorsal horn of the lumbosacral cord, suggesting a direct modulation of glutamate receptor activity by oestrogen through ER-α (Tang *et al.*, 2008). Further studies to evaluate the expression of oestrogen receptors and glutamate receptors in the lumbosacral cord in females across their oestrous cycle may add more knowledge to effects of endogenous oestrogen on these receptors' expression.

1.2.2.4 Other Modulators

Toll-like receptors (TLRs) are a group of pathogen-recognition receptors activated by various bacterial and viral components. Their activation results in the transcription of inflammatory cytokines, such as interleukins and tumour necrosis factor α. In the gut, TLRs are among the key mediators of the host defence system, being responsible for maintenance

of mucosal and commensal homeostasis. Patients with irritable bowel syndrome show greater expression of TLR4 and TLR5 in their colonic mucosa and increased blood levels of TLR-agonist-induced cytokines as compared to healthy individuals (Dinan *et al.*, 2006; Liebregts *et al.*, 2007; Scully *et al.*, 2010; Brint *et al.*, 2011; McKernan *et al.*, 2011).

From the family of TLRs, TLR4 has an important role in both somatic and visceral pain. Various animal models have shown increased lumbar spinal mRNA expression of TLR4 in peripheral nerve lesions (DeLeo *et al.*, 2004; Raghavendra *et al.*, 2004). TLR4-knockout mice have been shown to develop fewer pain responses and decreased expression of inflammatory cytokines (Tanga *et al.*, 2004). Moreover, administration of TLR4 antagonist administration to mice has been demonstrated to relieve neuropathic pain (Bettoni *et al.*, 2008). Our lab has demonstrated reduced visceral nociceptive responses in TLR4 deficient mice. It has also been demonstrated that selective antagonism of TLR4 resulted in reduced visceral hypersensitivity in wild- type mice and reversed visceral hypersensitivity in stressed mice (Tramullas *et al.*, 2014).

Although the role of TLRs in chronic pain has been increasingly emphasised, only a few studies have been carried out to see gender differences in their involvement. Sorge *et al.* have demonstrated a significant sex difference in the involvement of spinal TLR4 in the mediation of chronic pain through the administration of spinal intrathecal lipopolysaccharide (LPS-agonist of TLR4), where allodynia in females was observed less as compared to males (Sorge *et al.*, 2011). This difference was considered to be testosterone dependent. The TLR4 knockout female mice did not show a reduction in allodynia following lumbar nerve lesion as compared to males. These findings suggest pain processing at the level of the spinal cord in females is independent of the TLR4 pathway (Sorge *et al.*, 2011; Stokes *et al.*, 2013).

1.2.3 Role of Supraspinal Mechanisms in Sex Differences in Pain Signalling

1.2.3.1 Brain-derived Neurotrophic Factor

BDNF is a growth factor regulating neuronal growth and differentiation. Its actions are mediated by a high-affinity receptor called tropomyosin receptor kinase B. BDNF and its receptor's expression has been noted in all the key areas in pain pathways such as DRGs, spinal cord, thalamus, periaqueductal grey, hypothalamus, parabrachial nucleus, amygdala and brain cortex (Conner *et al.*, 1997; Yan *et al.*, 1997a; Yan *et al.*, 1997b; Zhou *et al.*, 2004; Salio *et al.*, 2005; Bruns and Miller, 2007). The role of BDNF at these anatomical sites, as a pain modulator, has also been established (Merighi *et al.*, 2008).

Li *et al*., have shown the effects of BDNF in males and females differentially. It facilitates the visceral pain in females but shows the opposite effect in male rats. After the intraperitoneal acetic acid (AA) injection (an animal model of visceral pain), females showed more pain behaviours than males. Ovariectomy attenuated this higher sensitivity to AA-induced pain response in females. Pre-treatment with anti-BDNF antibody significantly exacerbated the pain response in males but attenuated it in females. While exogenous BDNF administration did not alter AA injection-induced pain response in females, BDNF pre-treatment attenuated the pain response in males but exacerbated it in females with ovariectomy (Li *et al.*, 2010). This effect can be partly explained by the findings of higher whole blood BDNF concentration in females (Trajkovska *et al.*, 2007) and which displays a hormonal change-related rhythmic variation (Piccinni *et al.*, 2008). The ability of oestrogen to regulate BDNF transcription and expression has also been demonstrated (Allen and McCarson, 2005).

1.2.3.2 Spino-parabrachial-amygdala Pathway and Cortex

The parabrachial nucleus (PBn) in the brainstem has long been recognised as an important relay centre in visceral nociceptive sensory processing (Cechetto *et al.*, 1985;

Bernard *et al.*, 1994; Bester *et al.*, 1997). Dorsal horn cell fibres project into the PBn, which in turn project in to the amygdala. This spino-parabrachial–amygdala pathway is thought to constitute an essential circuit for the affective-motivational component of visceral pain (Gauriau and Bernard, 2002). Murphy *et al.* found no sex differences in the anatomical organisation of spino-PBn neurons, but they discovered that noxious CRD activated spino-PBn neurons more in males compared to females. Moreover, morphine attenuated this activation significantly in males compared to females (Murphy *et al.*, 2009). This suggests that spino-PBn pathway contributes to the sex difference in visceral pain by activating an opioidergic inhibitory circuit in males. Furthermore, the amygdala has been shown to be affected by the sex hormones (Myers *et al.*, 2011). Oestrogen and progesterone implants, when placed in the amygdala in ovariectomised rats, resulted in heightened responses to CRD but not to mechanical pain, suggesting that an amygdala-dependent mechanism may have a role in the exacerbation of visceral symptomatology in females. Wang *et al.* measured functional activation of brains in male and female rats exposed to colorectal distension. They demonstrated greater changes in limbic (amygdala & hypothalamus) and paralimbic region (ventral striatum, nucleus accumbens, raphe) in females but greater cortical activation in male rats. In males, this was consistent with greater cortical inhibition of the limbic region (Wang *et al.*, 2009). Increased activation of the prefrontal cortex and limbic/paralimbic changes in females suggest their role in affective responses during visceral pain. A clinical gender study utilising functional MRI of the brain during pain showed that women demonstrated greater activation in the cingulate cortex, insula, premotor cortex, and cerebellum and stronger deactivation in the caudate. In comparison, men showed increased activity in the supplementary motor area. In other words, during pain, women significantly activate brain

areas associated with the affective and motivation components of pain (Kano *et al.*, 2013; Calvo-Perxas *et al.*, 2016; Kisler *et al.*, 2016).

In summary, there are anatomical and physiological gender differences present along the whole visceral pain pathways responsible for differential incidence and prevalence of visceral pain syndrome. However, most of the studies described above have not taken the phase of the oestrous/menstrual cycle into account when comparing females with males. As there is growing evidence of the role of sex hormones on various neurotransmission-related mechanisms, it is imperative to assess the factors modulating pain pathways in the context of the oestrous/menstrual cycle.

1.3 Hormonal Modulation of Pain Depicted in Preclinical & Clinical Pain Models

Numerous preclinical and clinical studies have shown that gonadal hormonal variations can lead to altered pain responses.

1.3.1 Effect of Sex Hormones on Pain in Preclinical Visceral Pain Models

During the oestrous cycle, systemic levels of oestrogen change in females depending upon the phase of the cycle. Oestrous cycle in female rats, which has been divided into four distinct phases (metestrus, diestrus, proestrus, and oestrus) lasts for four days on average. These phases can be identified by the relative populations of different vaginal epithelial cells under the microscope after vaginal smearing (Byers *et al.*, 2012). The oestrogen level is highest during proestrus, and then it continues to decline in oestrus through to diestrus (Kalra and Kalra, 1974; Santmyire *et al.*, 2010).

The role of gonadal hormones in visceral nociception has been investigated, with most studies indicating that oestrogen is pronociceptive (Ji *et al.*, 2003; Lu *et al.*, 2007; Ji *et al.*, 2008; Tang *et al.*, 2008; Fan *et al.*, 2009a; Lu *et al.*, 2009; Chaloner and Greenwood-Van Meerveld, 2013), a few have concluded no effect (Robbins *et al.*, 2010), and some of the earlier studies

demonstrating antinociceptive effects on visceral pain (Giamberardino *et al.*, 1997; Bradshaw, 1999; Bradshaw and Berkley, 2002).

Female rats have been shown to demonstrate higher visceromotor responses to colorectal distension in proestrus as compared to other phases (Ji *et al.*, 2008; Peng *et al.*, 2008), although there are reports of no effect of the oestrous cycle on visceral pain (Cason *et al.*, 2003). Furthermore, a study revealed more ureteral pain sensitivity in metestrus and diestrus (Giamberardino *et al.*, 1997). These discrepancies may result from varying factors including strain differences, the accuracy of oestrous phase determination, study design, duration of pain stimulus and sample size. Ovariectomy of female rats resulted in decreased pain responses as compared to intact females, whereas replacement of oestrogen in these ovariectomised animals led to the restoration of increased visceromotor responses to colorectal distension (Ji *et al.*, 2003; Tang *et al.*, 2008). However, co-administration of progesterone blunted this effect of exogenous oestrogen in ovariectomised rats (Fan *et al.*, 2009a). Interestingly, direct activation of oestrogen receptor alpha (ER-α) had pronociceptive effects (Ji *et al.*, 2011), whereas ER-β activation resulted in lower visceromotor responses to colorectal distension (Cao *et al.*, 2012) suggesting that oestrogen exerts it pronociceptive effects via ER-α.

As there are a number of reports elucidating the effects of hormones on visceral pain responses, only a few reports are available in the literature detailing the direct implications of these sex hormones on neurotransmission in CNS. Further studies are needed to examine how pain modulatory systems such as the excitatory glutamatergic system are moderated by the effects of changing levels of gonadal hormones during the oestrous cycle, and how factors such as early-life stress and depression may impact on this hormonal modulation.

1.3.2 Hormonal Modulation of Pain in Clinical Studies

Women have cyclical variations of sex hormones across the menstrual cycle, which on average lasts around 28 days. The menstrual cycle can be divided into four phases depending upon the development of ovarian follicle and corpus luteum: early follicular (low-oestrogen, low progesterone), late follicular (high-oestrogen, low progesterone), early luteal (low-oestrogen, low progesterone) and late luteal (low-oestrogen, intermediate progesterone).

Cephalic pain disorders, e.g., migrainous headaches and temporomandibular joint disorder, are worsened in early follicular and late luteal phases of the menstrual cycle when the blood levels of oestrogen are low (Johannes *et al.*, 1995; Dzoljic *et al.*, 2002; LeResche *et al.*, 2003; Mattsson, 2003). A similar variation of non-cephalic pain disorders symptomatology has been reported, e.g., exacerbation of irritable bowel syndrome (IBS)-related abdominal pain and rheumatoid joint pain in early follicular phase (Latman, 1983; Houghton *et al.*, 2002). Moreover, the chronic neck pain-related disability index was found to be low in the late follicular stage where the systemic levels of oestrogen were high (Balter *et al.*, 2013). It is interesting to note that the disorders mentioned above are more prevalent in females, endogenous oestrogen seems to be antinociceptive in the studies mentioned above. In contrast, some reports in literature demonstrated no effect of menstrual cycle stage on pain sensitivity (Klatzkin *et al.*, 2010; Banz *et al.*, 2012; Balter *et al.*, 2013).

Similar to observational experiments of menstrual cycle-related hormonal changes and their impact on pain sensitivity, the effect of exogenous ovarian hormones (hormone replacement therapy in menopausal women & hormonal contraception) on pain sensitivity has been assessed on healthy individuals and those with existing pain syndromes. Some reports have shown exogenous oestrogen to increase experimentally induced pain sensitivity, e.g., cold pressor pain and facial pain (Fillingim and Edwards, 2001; Bragdon *et al.*, 2002;

Drobek *et al.*, 2002; Kowalczyk *et al.*, 2006). However, other studies have failed to demonstrate such differences (Dao *et al.*, 1998; Isselee *et al.*, 2001; Nekora-Azak *et al.*, 2008). These inconsistencies may arise from different methodologies used in different studies, insufficient sample size and inaccuracy of determination of menstrual cycle stage. In a review of 13 experimental studies, only four confirmed the cycle stage with hormone assays (Sherman and LeResche, 2006). Another possible reason for such inconsistency may be a lack of consideration of newly emerging factors affecting neuronal mechanisms, such as birth delivery mode, gut microbiota and gut permeability, and early-life abuse.

We have summated relevant anatomical and physiological differences along the pain pathways in CNS, accountable for the gender differences in pain. Sex hormones seem to play a major role in the modulation of visceral pain as demonstrated by various preclinical and clinical studies. The clinical studies which fail to show the hormonal modulation do not necessarily point toward the inference of lack of such effect, as there is a possibility that the presence of two opposing factors may nullify each other's effect. For example, the effect of low-oestrogen may have been revoked due to the presence of stress and depression, which co-occurs with chronic pain in subjects during the clinical experiments (Sheng *et al.*, 2017). Studies of gender differences are paramount as these aim toward appropriate pain management across the groups. Further studies are needed targeting the excitatory glutamatergic system among both sexes, and factors affecting its functioning are required to be explored.

1.4 Role of Stress in Hormonal Modulation of Pain

1.4.1 Hypothalamic-Pituitary-Adrenal (HPA) Axis and Stress

Acute stress activates HPA axis, sympathetic nervous system, and sympathoadrenal system (Ulrich-Lai and Herman, 2009). Corticotropin-releasing hormone (CRF), the chief regulator of the HPA axis, is synthesised and released from the periventricular nucleus (PVN) of the hypothalamus (Bonfiglio *et al.*, 2011). Vasopressin is also synthesised in PVN (Ohbuchi *et al.*, 2015). After release, CRF reaches anterior pituitary gland through hypophysial portal vessels and binds to CRF type 1 receptors (Hauger *et al.*, 2006). Through cyclic adenosine monophosphate (cAMP) pathway, CRF induces the release of adrenocorticotropic hormone (ACTH) into the circulation (Lewis *et al.*, 2016). Circulating ACTH binds to melanocortin type-2 receptor in the adrenal cortex and stimulates glucocorticoid synthesis (Clark *et al.*, 2016). Glucocorticoid receptors are widely distributed throughout the brain and peripheral tissues. The glucocorticoids, e.g., cortisol in humans and corticosterone in rodents regulate a number of physiological processes in the body including metabolism, cardiovascular, immune, and behavioural processes (Strelzyk *et al.*, 2012). Cortisol is released in a diurnal pattern with systemic levels reaching a peak in the morning to facilitate arousal and steadily declining thereafter. Throughout the day, cortisol maintains blood glucose and suppresses nonvital organ systems to provide energy to an actively functioning brain and neuromuscular system (Edwards *et al.*, 2001). Cortisol is also a potent anti-inflammatory hormone; it prevents inflammation and its associated widespread tissue damage (Yeager *et al.*, 2011).

Activation of the HPA axis is a tightly controlled mechanism involving neuronal and endocrine systems. The endocrine regulation is maintained through negative feedback by circulating glucocorticoids via fast non-genomic and delayed genomic mechanisms. The neuronal regulation is maintained through a number of projections to PVN arising from the

brainstem, lamina terminalis, hypothalamic nuclei, and limbic system (Ulrich-Lai and Herman, 2009). Excessive and repetitive activation of HPA axis, as seen in chronic stress and depression, may attribute to the dysfunction of normal HPA axis (Hannibal and Bishop, 2014).

The repetitive, excessive activation of the HPA axis, as observed in chronic stress states can result in cortisol dysfunction (Stephens and Wand, 2012). Cortisol dysfunction can manifest as depletion of cortisol, insufficient free (unbound) cortisol, impaired cortisol secretion or CRH function, glucocorticoid receptor (GR) resistance or downregulation, or hypersensitivity of the negative feedback system (Hannibal and Bishop, 2014). In the presence of GR resistance, cortisol may enhance its affinity with mineralocorticoid receptors (MR) which may initiate pro-inflammatory effects (Sorrells *et al.*, 2009). Furthermore, binding of cortisol of MR downregulates the negative feedback mechanism resulting in higher CRH levels (Fries *et al.*, 2005). Higher CRH levels activate inflammatory cells, increase noradrenaline release, and upregulate glutamate and its receptors in the amygdala to condition a fear-based stress response (McEwen and Kalia, 2010). In humans, stress-induced inflammation has been associated with many pain disorders such as fibromyalgia, chronic pelvic pain, temporomandibular joint dysfunction, chronic low back pain, sciatica, and rheumatoid arthritis (Quartana *et al.*, 2010; Tak and Rosmalen, 2010; Tak *et al.*, 2011).

Acute pain is likely to elicit cortisol secretion and is commonly associated with hypercortisolism. Repeated cortisol secretion following maladaptive responses to acute pain is likely to perpetuate hypocortisolism and chronic pain (Tennant, 2013). As cortisol is a potent anti-inflammatory hormone, its dysfunction can shift the balance toward a pro-inflammatory state. The resultant elevated levels of inflammatory cytokines may sensitise nociceptors, manifesting as an increase in pain sensitivity. HPA axis dysfunction is commonly implicated in

chronic pain disorders such as fibromyalgia, chronic pelvic pain and temporomandibular joint disorder (Eller-Smith *et al.*, 2018).

1.4.2 Sex Differences and Effect of Sex Hormones on HPA Axis

Animal studies have shown that glucocorticosteroid levels are higher in females compared to males (Heinsbroek *et al.*, 1991; Yoshimura *et al.*, 2003). However, similar studies in humans are inconsistent. Some studies founds men to have higher HPA activation responses to physiological stress (Seeman *et al.*, 2001; Stroud *et al.*, 2002; Traustadottir *et al.*, 2003), while other found no such differences (Kirschbaum *et al.*, 1992; Buske-Kirschbaum *et al.*, 2003; Kudielka *et al.*, 2004). Larger cortisol response to acute stress has been noted in women with major depression in some studies (Peeters *et al.*, 2003), while other groups failed to observe such findings (Young *et al.*, 2004). These inconsistencies can be partly because of the fact that most of these studies focus only on the acute stress in otherwise healthy individuals but from heterogenous populations. Moreover, the newly emerging factors such as gut microbiota and their related diseases, when considered, may change the inclusion criteria of participants and comparison groups completely.

In male rats, testosterone has been shown to inhibit stress-induced ACTH release, and peripherally, testosterone may interfere with cortisol metabolism in the liver and the adipose tissue (Viau and Meaney, 1996; Barat *et al.*, 2007). In rodents, basal and stimulated ACTH and corticosterone levels tend to be greater in females than in males. Ovariectomy has been shown to attenuate the response of HPA axis in rodents, whereas oestrogen replacement has reversed this attenuation (Lesniewska *et al.*, 1990b; Norman *et al.*, 1992). Similarly, gonadectomy increased ACTH responses in male rats, which were reversed by testosterone replacement (Viau and Meaney, 1996). Animal studies suggest that locally activated androgen receptor may facilitate negative feedback regulation of glucocorticoid synthesis by

upregulating GRs in the pituitary gland (Miyamoto *et al.*, 2007). Interestingly, it has been shown that although men had significantly higher ACTH and cortisol concentrations than women, the hormone response to CRH was conversely markedly higher in women than in men (Vicennati *et al.*, 2006). Moreover, short-term oestrogen treatment seems to enhance HPA response to psychosocial stress in healthy men (Kirschbaum *et al.*, 1996).

A number of animal experiments have suggested oestrogen to have a causative role in enhanced HPA responsiveness, which may be due in part to impairment of glucocorticoid negative feedback. As mentioned previously, female rats have higher peak diurnal plasma corticosterone as compared to males. Potential mechanisms of action include competition between ER-α and GR to regulate transcription at an AP-1 site and reduction of GR binding in the hippocampus, hypothalamus, and anterior pituitary (Weiser and Handa, 2009). Oestrogen may also modulate PVN neurosecretory function directly. For example, oestrogen supplementation has shown a greater induction of c-fos mRNA in PVN neurons in response to stress (Ochedalski *et al.*, 2007). Other studies have elaborated oestrogen to regulate PVN neuropeptides and upregulated stress-induced CRH mRNA (Liu *et al.*, 2012). Expression of ER-β in preautonomic neurons in PVN makes it unlikely to influence the HPA modulation (Kudwa *et al.*, 2014). Oestrogen seems to impair the glucocorticoid induced negative feedback on HPA axis via oestrogen receptor alpha in the hypothalamus (Weiser and Handa, 2009; Liu *et al.*, 2012). The predominance of chronic pain states, e.g., fibromyalgia, chronic fatigue syndrome, chronic pelvic pain, temporomandibular joint dysfunction, chronic low back pain in women and simultaneous higher incidence of chronic stress in these women, suggests an interplay between cortisol failure and oestrogen. This effect may not be apparent in a normally functioning HPA axis in healthy women.

1.5 Mechanism of Action of Oestrogen in Nociceptive Modulation

1.5.1 General Actions

Oestrogen is one of the steroid hormones and is generated from cholesterol (Berg *et al.*, 2002). Cholesterol is converted into pregnenolone which is a precursor of all the steroid hormones. The androstenedione and testosterone, derived from pregnenolone, are converted into estrone and oestradiol by aromatisation by the P450 aromatase monooxygenase enzyme (Becker, 2007). The secretion of oestrogens is modulated by the gonadotropin-releasing hormone (GnRH) secreted by the hypothalamus (Marques *et al.*, 2018). GnRH, in turn, induces the secretion of follicle stimulating hormone and luteinising hormone. These hormones act on the steroid producing cells, and oestrogens are generated (Ghayee and Auchus, 2007). The central nervous system has the ability to synthesise oestrogen from cholesterol due to the presence of all required enzymes within CNS (Hojo *et al.*, 2008). Moreover, testosterone and androstenedione have the capability of freely entering the brain; therefore can provide a source of oestrogen synthesis in the brain (Banks, 2012).

Oestrogen receptors when in a free state, are usually located in the cell cytoplasm and are attached to heat-shock proteins (Fliss *et al.*, 2000). Under the effect of oestrogen, the heat-shock proteins are dissociated from oestrogen receptors, and motor proteins such as dynein get attached to the oestrogen-oestrogen receptor complex, which allows the transport into the nucleus (Belmont, 2010). The oestrogen binds to specific sequences of DNA in the nucleus called oestrogen-response elements (ERE) leading to gene transcription, and the particular proteins are generated (Klinge, 2001). After transcription is complete, the oestrogen receptors attach back to heat-shock proteins and become inactive. Oestrogen receptors can also alter the activity of other transcription factors without binding to EREs and may affect the production of collagenase and insulin-like growth factors for example (Gunter *et al.*, 2008).

Being a phosphoprotein, oestrogen receptors can also be activated by phosphorylation in the absence of oestrogen through the mitogen-activated protein kinase pathway (Lannigan, 2003).

Oestrogens have the capability of acting rapidly via transcription-independent mechanisms (Vasudevan and Pfaff, 2008). These pathways are mediated by cell membrane-bound oestrogen receptors. G-protein coupled receptor GPR30 may also be activated by oestrogens. Oestrogens can also affect several intracellular pathways including mitochondrial activated protein kinase pathway, protein kinase C pathway, calcium channel modulation, and activation of transcription factors.

1.5.2 Rapid Dendritic Spine Formation

Oestrogen can induce rapid dendritic spine formation in the ACC. The formation of the dendritic spine, their number and morphology are very important in the cell to cell communication. Oestrogen can induce synaptic density which is linked with enhanced learned memory. Thus, oestrogen can rapidly and persistently potentiate NMDA transmission, increased pERK and pCREB in the hippocampus and rACC (Cao *et al.*, 2009a), remodelling of dendritic spines (Shansky and Morrison, 2009), and pain-related aversion (Cao *et al.*, 2009b). Functional MRI studies in humans have shown increased activation of ACC and cerebellum during low-oestrogen phases of menstrual cycle suggesting that the affective component of pain may be enhanced during the low-oestrogen phase in healthy women (Sundström Poromaa and Gingnell, 2014).

1.5.3 Action Through Descending Inhibitory Pathways

The excitability of pain processing neurons is influenced by several descending pathways that reach the medulla oblongata and dorsal horn of the spinal cord. Examples

include opioid, noradrenergic, and serotonergic systems, of which, the endogenous opioid is the most potent descending pain modulation pathway (Purves *et al.*, 2001b). Endogenous opioids are manufactured in the CNS, pituitary region, and adrenal glands. Major examples of opioids include enkephalin and dynorphin. Enkephalins such as met-enkephalin and leu-enkephalin are widely expressed in the brain and are concentrated in the superficial lamina of the spinal dorsal horn (Fukushima *et al.*, 2011). The regional concentration of dynorphin is similar to that of enkephalins; it can be both pronociceptive and antinociceptive (Gintzler and Liu, 2012). The opioids act primarily via G-protein coupled opioid receptors inducing hyperpolarisation through potassium conductance and voltage-gated calcium channels. The three well described subtypes include δ, μ, and κ receptors; μ being the most potent (Traynor, 2012).

The enkephalinergic neurons in lamina II exert an inhibitory effect on nociceptive signals (Todd, 2010). They are activated by ascending afferent fibres as well as descending fibres from pain modulating regions of the brain. Co-expression of ERα by most of the enkephalinergic neurons in the dorsal horn indicated a robust mechanistic role between two systems (Amandusson *et al.*, 1996).

The opioid system plays a pivotal role in pregnancy-induced analgesia, which is primarily mediated through spinal dynorphin activity (Gupta *et al.*, 2001). Spinal dynorphin and enkephalin pathways get activated simultaneously, but as pregnancy progresses, dynorphin remains concentrated in lumbar regions receiving pelvic inputs. Enkephalins, however, gradually increase in concentration throughout the spinal cord as pregnancy advances (Gupta and Gintzler, 2003). Exogenous oestrogen treatment in ovariectomised rats has shown increased expression of enkephalin gene and subsequent higher concentration of enkephalins in the CNS (Eckersell *et al.*, 1998).

Oestrogen can also modulate the expression and function of opioid receptors in brain regions along the pain pathways. For example, in the parabrachial nucleus, μ-opioid receptor expression fluctuates along the oestrous cycle being lowest in the proestrus phase (Murphy *et al.*, 2009). A similar trend has been seen in periaqueductal grey (Loyd *et al.*, 2008). In healthy women, Mu-opioid receptor activation in the brain in response to painful stimuli is oestrogen-dependent (Lawson *et al.*, 2010). Women in the low-oestrogen state have shown a lower capacity to activate the opioid system in comparison to men and in turn demonstrate higher pain responses (Smith *et al.*, 2006).

1.6 Gut Microbiota and Pain

1.6.1 Gut Microbiota

The term human gut microbiota refers to all non-pathogenic commensal microorganisms residing in our digestive tract which are involved in vital bodily functions such as digestion, absorption of nutrients and modulation the immune system (Wu and Wu, 2012; Aziz *et al.*, 2013; Purchiaroni *et al.*, 2013; El Aidy *et al.*, 2014; Moloney *et al.*, 2016; Foster *et al.*, 2017). The overall collection of the genome of gut microbiota is termed as gut microbiome (Qin *et al.*, 2010). The gastrointestinal tract in the newborn infant is virtually sterile although some studies are highlighting the possibility of prenatal colonisation (Jimenez *et al.*, 2008). Initial gastrointestinal microbiota is largely determined by delivery mode (Dominguez-Bello *et al.*, 2010; Mueller *et al.*, 2015) and this microbiota profile can remain in residence from months to years (Jakobsson *et al.*, 2014) and has the capacity to influence health outcomes (Negele *et al.*, 2004; Parfrey and Knight, 2012). The initial composition of gut microbiota in infants is similar to that of the mother and their surroundings (Mueller *et al.*, 2015; Rodríguez *et al.*, 2015), but it tends to change dramatically over the first two years of life (Palmer *et al.*, 2007). Following this dynamic period, it tends to develop a profile that will mostly habitate

the gut for life (Rodriguez *et al.*, 2015). However, a number of factors in prenatal and early postnatal life can alter the microbiota composition and the adult life. These factors include maternal diet, use of drugs including antipsychotics, antiepileptics, and antibiotics, birth through caesarean section, formula feeding, and early-life stress (O'Mahony *et al.*, 2014; Cong *et al.*, 2015; Mueller *et al.*, 2015; Codagnone *et al.*, 2018).

In adults, the gut microbiota is mainly composed of phyla Firmicutes and Bacteroidetes, representing 79.4% and 16.9% of the microbiota respectively, followed by Actinobacteria (2.5%) and Proteobacteria (1%) (Tap *et al.*, 2009; Arumugam *et al.*, 2011). While gut microbiota tends to be relatively stable over time, it does change with age (O'Toole and Jeffery, 2015), disease (Larsen *et al.*, 2010) and antibiotic usage (Jernberg *et al.*, 2010; Modi *et al.*, 2014; Mikkelsen *et al.*, 2015). A shift in the microbiota has been observed toward a Bacteroidetes predominated population in older individuals compared to younger individuals (O'Toole and Jeffery, 2015). Metagenomic studies have further grouped the gut bacteria into three distinct enterotypes depending upon the composition. The enterotype-1 is enriched with Bacteroides along with Parabacteroides. The enterotype-2 mainly comprises of *Prevotella* and *Desulfovibrio*, whereas, *Ruminococcus* is a major representative of enterotype-3 (Arumugam *et al.*, 2011). This concept that each human being has a characteristic enterotype similar to blood grouping has become debatable due to recent reports of microbiota composition dependency on long-term diet preferences (Wu *et al.*, 2011; Claesson *et al.*, 2012).

In the last few years, the utilisation of new techniques such as gene sequencing for the characterisation of gut microbiota in relation to health and disease has advanced our knowledge to a new level (Fraher *et al.*, 2012). The composition of gut microbiota is found to be altered in various gut-related disorders, e.g. inflammatory bowel disease (Sartor and

Mazmanian, 2012; Matsuoka and Kanai, 2015), IBS (Distrutti *et al.*, 2016; Moloney *et al.*, 2016), and colorectal cancer (Louis *et al.*, 2014; Gagnière *et al.*, 2016). Altered gut microbiota is also found to be implicated in certain psychiatric disorders including depression (Naseribafrouei *et al.*, 2014; Lach *et al.*, 2018), autism (Mayer *et al.*, 2014a), and Alzheimer's disease (Hill *et al.*, 2014; Sarkar *et al.*, 2018). Recent preclinical and clinical studies have shown an association of altered gut microbiota with a number of CNS disorders including Parkinson's disease (Felice *et al.*, 2016; Dinan and Cryan, 2017a), ischaemic stroke, amyotrophic lateral sclerosis (Fang, 2016) and multiple sclerosis (Winek *et al.*, 2016; Sherwin *et al.*, 2018).

The gut microbiota can be altered by oral administration of prebiotics, probiotics, antibiotics. A more advanced method of modulating gut bacteria involves faecal microbiota transplant (FMT). Prebiotics are defined as a selectively fermented ingredients that allow specific changes, both in the composition and/or activity in the gastrointestinal microflora that confer benefits upon host wellbeing and health (Gibson *et al.*, 2004). The oligosaccharides, such as fructans, galactans, and lactulose are examples of prebiotics. The probiotics, on the other hand, are live microorganisms that, when administered in adequate amounts, confer a health benefit on the host (Sanders, 2008). The examples of probiotics include *Lactobacillus Casei* and *Bifidobacterium Bifidum.*

1.6.2. Communication Between Gut Microbiota and Central Nervous System

Bidirectional communication between the brain and gut microbiota, and recognition that microbiota influences various signalling pathways in the brain have led to the concept of microbiota-gut-brain-axis (Cryan and Dinan, 2012; Forsythe *et al.*, 2012; Montiel-Castro *et al.*, 2013a). Gut microbiota and the brain communicate through three distinct pathways, 1) neuronal (vagus nerve and enteric nervous system);2) endocrine (HPA axis), and 3) immune (bacterial metabolites, cytokines) systems (Cryan and Dinan, 2012; Montiel-Castro *et al.*,

2013b; Dinan and Cryan, 2016). This relationship (as shown in Figure 1.6) is evident in psychologically stressful situations where increased cortisol levels can enhance cytokine secretion through inflammatory cells, but can also alter gut microbial composition (O'Mahony *et al.*, 2011; Moloney *et al.*, 2016) and gut barrier function (Vanuytsel *et al.*, 2014). On the other hand, gut bacteria have the capability of producing cytokines and metabolites, and to modulate the normal immune status of the body, which may have direct implications for the development of many nervous system disorders.

The production and metabolism of certain neurotransmitters, short-chain fatty acids, and tryptophan by microbiota are associated with the modulation of some physiological functions of the brain such as behaviour and cognition (Cryan and Dinan, 2012; Dinan *et al.*, 2015; Jenkins *et al.*, 2016). Among various bacterial products, lipopolysaccharide (LPS) has been emphasised to be associated with the development of some neurological disorders mentioned earlier. LPS is a constituent of the cell membrane of Gram-negative bacteria and is secreted to the surrounding environment as part of the normal physiological activity of these bacteria (Alexander and Rietschel, 2001). LPS has been found to increase the permeability of not only the gut mucosa (Anitha *et al.*, 2012) but also the blood-brain barrier (Jangula and Murphy, 2013). There are studies of microglial activation in the brain by LPS (Qin *et al.*, 2007). Therefore, LPS seems to have a pro-inflammatory effect on the brain through increased transport of bacterial products and direct activation of microglia.

Gut bacteria can also stimulate vagal nerve endings to its anxiogenic, anxiolytic, anti-inflammatory and immunomodulatory effects on the brain depending on the nature of the stimulus (Bravo *et al.*, 2011; Forsythe *et al.*, 2012). The recognition of these communication pathways and associated implications has resulted in the increase in research exploring the

role of gut microbiota in the development and progression of neurological disorders (Mayer *et al.*, 2014b).

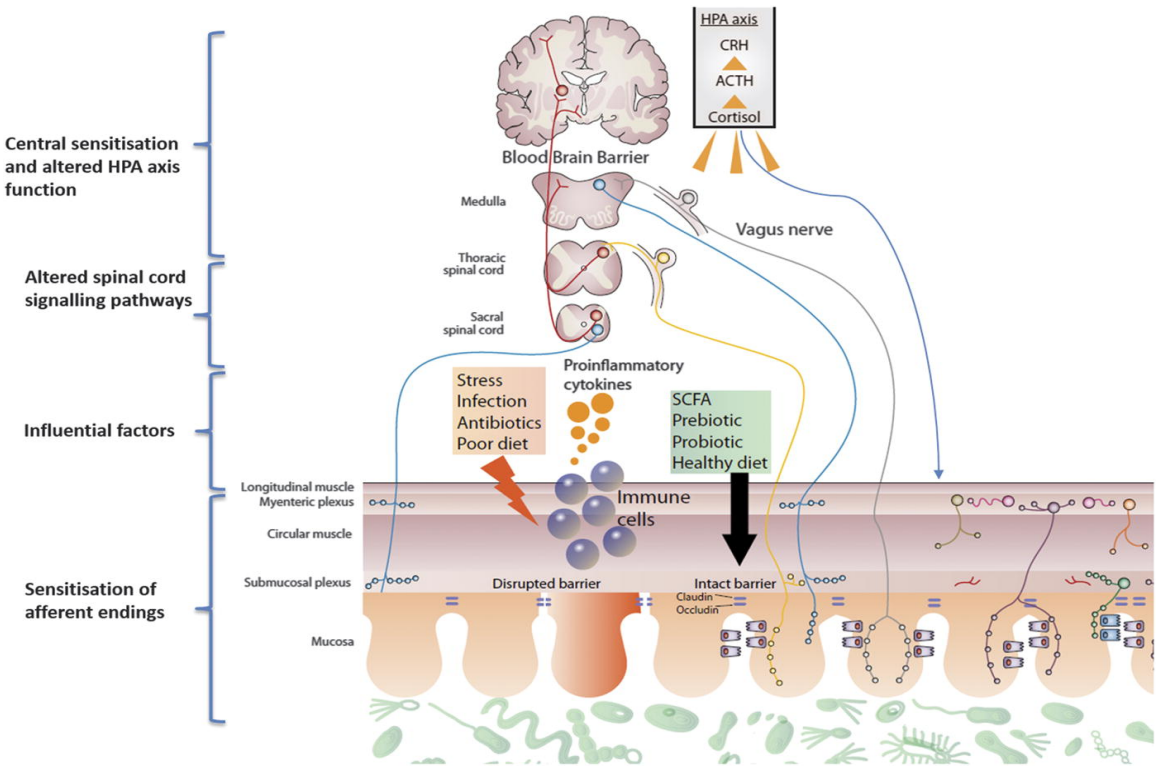


Figure 1.6. Populations of neurons, whose cell bodies either reside within (intrinsic) or outside (extrinsic) the GI wall. The gut microbiota are capable of impacting at both local and extraintestinal levels to influence visceral sensation. Factors affecting gut microbiota include stress, infection, antibiotics, and diet. These may induce an imbalance in the microbiota which can ultimately result in peripheral or central sensitisation. In turn, neuroplasticity contributes to abnormal sensation and results in the development of discomfort and pain. Reversal of the bacterial imbalance is possible through the consumption of prebiotics or probiotics and the introduction of healthy diet habits which have been associated with a reduction in visceral pain particularly related to the gut.
Key: (1) extrinsic neurons-red: second- and third order neurons of the anterolateral tract; blue: autonomic nervous system neuron; grey: vagal afferent neuron; yellow: spinal afferent neuron and (2) intrinsic neurons-green: secretomotor neuron; purple: intrinsic primary afferent; orange: motor neuron; pink: interneuron. GI, gastrointestinal; SCFA, short-chain fatty acid (O' Mahony *et al.*, 2017).

1.6.3 Role of Gut Microbiota in Pain

1.6.3.1 Preclinical Studies on Microbiota and Pain

The presence of the gut microbiota is very important for the regulation of normal excitability of the myenteric complex in the enteric nervous system, as shown in a comparative study of germ-free and control mice (McVey Neufeld *et al.*, 2013). Some preclinical studies have shown the links between disruption of normal gut microbiota (dysbiosis) with changes in the visceral pain perception. For example, Aguilera *et al.* demonstrated that an antibiotic-

induced a colonic dysbiosis (increments in *Bacteroides* spp, *Clostridium coccoides* and *Lactobacillus* spp and reduction in *Bifidobacterium* spp) in mice was associated with increased levels of secretory-IgA, and TLR 4 and 7. This resulted in attenuation in visceral pain-related responses to intraperitoneal AA or intracolonic capsaicin (Aguilera *et al.*, 2015). Similarly, early-life stress-induced dysbiosis resulted in increased visceral pain responses in the stressed group of adult rats (O'Mahony *et al.*, 2009; O'Mahony *et al.*, 2014; Luczynski *et al.*, 2017).

The administration of probiotics in animals has shown to attenuate the visceral pain responses. For example, administration of probiotic *Lactobacillus rhamnosus* reduced the visceromotor response to colorectal distension in adult rats who had neonatal inflammation-induced visceral hypersensitivity (Kannampalli *et al.*, 2014). Similarly, the administration of probiotic *Bifidobacterium infantis* reduced CRD-induced pain behaviours in both adult Sprague-Dawley and Wistar-Kyoto rat strains (McKernan *et al.*, 2010). In another set of experiments, the increased visceral hypersensitivity, secondary to antibiotic-induced dysbiosis, normalised following the administration of *Lactobacillus paracasei* to these animals (Verdú *et al.*, 2006). Miquel *et al.* were able to demonstrate the antinociceptive effects of *Faecalibacterium prausnitzii* in IBS like models in rodents (Miquel *et al.*, 2016). These studies have shown the association between the visceral hypersensitivity and the gut microbiota composition. However, more extensive research is required in this field to explore the causative mechanisms responsible for this relationship.

1.6.3.2 Clinical Studies on Microbiota and Pain

IBS is a classic example of a functional gastrointestinal disorder associated with visceral pain in humans. Patients typically develop recurring abdominal pains related to the bowel movements and altered bowel habits without the presence of an identified underlying organic disease (Schmulson and Drossman, 2017). Although the aetiology of IBS is multifactorial, the

changes in the gut microbiota have been increasingly observed in both adult and paediatric patients.

In a study evaluating the relative abundance of microbiota in infants with or without colic, *Proteobacteria* were significantly increased in infants with colic when compared to control infants (de Weerth *et al.*, 2013b). In contrast, *Bifidobacteria* and *Lactobacilli* were significantly reduced in infants with colic shown in the same study. In another study on preterm babies, the administration of probiotic *Lactobacillus rhamnosus* alleviated the colic symptoms (Partty *et al.*, 2013). Furthermore, the use of *Lactobacillus rhamnosus* moderately increases treatment success in children with abdominal pain-related functional gastrointestinal disorders, particularly IBS (Horvath *et al.*, 2011).

Adult IBS patients, with predominate diarrhoea, have been shown to have significant populations of *Enterobacteriaceae*, and lower levels of *Fecalibacterium* genera compared to healthy individuals (Carroll *et al.*, 2012). IBS patients demonstrate an abnormal IL-10/IL-12 ratio, indicative of a pro-inflammatory state. This ratio may be normalised with probiotic *B. infantis* resulting in a reduction in the abdominal pain and discomfort (O'Mahony *et al.*, 2005). Probiotic *Lacbacillus acidophilus* induces colonic mu-opioid receptor expression and downstream signalling suggesting a possible mechanism by which probiotics may modulate pain sensation in humans (Ringel-Kulka *et al.*, 2014). The gut microbiota or probiotics may act directly through bacterial metabolites on the pain receptors in the gut wall, or indirectly by influencing intestinal epithelial barrier, the mucosal and systemic immune mechanisms (Theodorou *et al.*, 2014). The observed increased thickness of the somatosensory cortex in female IBS patients may suggest an underlying interplay of the microbiota-gut-brain axis and the sex hormones (Jiang *et al.*, 2013).

The studies mentioned above suggest a plausible link between the gut microbial composition and symptomatology of visceral pain. However, a lot of research is needed to study further the bidirectional interplay between the gut and the brain in relation to pain. The influence of other factors affecting visceral pain, such as stress, dietary factors, sex hormones needs to be studied extensively in the future preclinical and clinical research. Table 1.6 (modified from (O' Mahony *et al.*, 2017)) enlists the preclinical and clinical studies signifying the role of gut microbiota in pain modulation. The possible mechanisms by which gut microbiota may influence the nociceptive neurotransmission directly include regulation of neurotransmitter synthesis in CNS (Desbonnet *et al.*, 2010; Lyte, 2013, 2014; Dinan and Cryan, 2017c), alterations in various receptors such as adrenergic receptor, cholecystokinin B receptor, opioid and cannabinoid receptors (Rousseaux *et al.*, 2007; O'Mahony *et al.*, 2014), excitability of sensory neurons (McVey Neufeld *et al.*, 2013).

Table 1.6. List of preclinical and clinical studies elucidating role of gut microbiota on pain disorders (O' Mahony *et al.*, 2017)

Preclinical and clinical studies of pain disorders and gut microbiota		
Pain model/disorder	**Gut microbiota involvement**	**Reference**
Preclinical studies		
Chemotherapy-induced mechanical hyperalgesia	Oxaliplatin-induced mechanical hyperalgesia was reduced in germ-free mice and in mice pretreated with antibiotics. Restoring the microbiota of germ-free mice abrogated this protection	(Shen *et al.*, 2017)
Antibiotic cocktail in adulthood	A reduction in visceral pain to acetic acid and intracolonic capsaicin	(Aguilera *et al.*, 2015)
Antibiotic cocktail in adulthood	Increased visceral pain to colorectal distension in mice; reversed by *L. paracasei*	(Verdú *et al.*, 2006)
Antibiotic vancomycin and cocktail in early-life	Increased visceral pain seen during colorectal distension in rats	(O'Mahony *et al.*, 2014)
Maternal separation	Early-life stress leads to a reduced diversity of faecal microbiota in rats Administration of *Faecalibacterium prausnitzii* reduces pain following maternal separation	(O'Mahony *et al.*, 2009; Miquel *et al.*, 2016)
Wistar-Kyoto	Administration of *Bifidobacterium infantis* reduced pain to colorectal distension in rats	(McKernan *et al.*, 2010)
Zymosan inflammation-induced visceral pain	Administration of the probiotic *Lactobacillus GG* reduced the pain response to the inflammatory stimulus	(Kannampalli *et al.*, 2014)
Gastric distension–induced visceral pain	Administration of *Lactobacillus reuteri* reduced jejunal spinal nerve firing evoked by gastric distension or capsaicin	(Perez-Burgos *et al.*, 2015)
Clinical studies		
Irritable bowel syndrome		
Microbiota changes from control	GI infection leads to persistent IBS symptoms	(Thabane *et al.*, 2007)
	Patients with IBS show a reduced bacterial diversity as well as changes to the Firmicutes to Bacteroidetes ratio	(Jeffery *et al.*, 2012)
	Increased numbers of *Dorea, Ruminococcus*, and *Clostridium* spp; decreased numbers of *Bifidobacterium* and *Faecalibacterium* spp; a correlation is seen between the changes in the above microbial groups and IBS symptom scores	(Rajilic-Stojanovic *et al.*, 2011; Jalanka-Tuovinen *et al.*, 2014)
	IBS was associated with an enrichment in bacteria related to *Ruminococcus* which correlates with symptoms	(Malinen *et al.*, 2010; Rajilic-Stojanovic *et al.*, 2011; Carroll *et al.*, 2012)
	Diarrhoea-predominant IBS patients have significantly higher levels of Enterobacteriaceae and lower levels of *Fecalibacterium* genera. This is associated with higher beta-diversity	(Ringel-Kulka *et al.*, 2016)

	IBS patients show significantly higher numbers of *Veillonella* and *Lactobacillus*	(Pimentel *et al.*, 2011; Hungin *et al.*, 2013)
Manipulating the microbiota	Antibiotic rifaximin reduces pain Probiotics can reduce abdominal pain in IBS. More specifically, *Bifidobacterium infantis*-treated patients experienced a reduction in for abdominal pain	(O'Mahony *et al.*, 2005; Hungin *et al.*, 2013; Gulden *et al.*, 2015; Curro *et al.*, 2017)
Paediatric irritable bowel syndrome		
Microbiota changes from control	*Ruminococcus*-like microbe was associated with IBS symptoms in this paediatric population. Visceral pain also correlated with an increased bacterial taxa from the genus *Alistipes*	(Saulnier *et al.*, 2011)
Manipulating the microbiota	Prebiotics and synbiotics effective in paediatric IBS A mixture of *Bifidobacterium infantis* M-63, *breve* M-16V and *longum* BB536 improved abdominal pain in children	(Saulnier *et al.*, 2011)
Functional dyspepsia	GI infection leads to functional dyspepsia	(Simren *et al.*, 2013)
Functional abdominal pain	*L*. *acidophilus* was seen to reduce functional abdominal pain in adults	(Ringel-Kulka *et al.*, 2014)
Colonic diverticulosis	Depletion of *Clostridium* cluster IV. *Clostridium* cluster IX, *Fusobacterium*, and *Lactobacillaceae* were reduced in symptomatic vs asymptomatic patients with colonic diverticulosis	(Barnes and Yeh, 2015)
Infantile colic		
Microbiota changes from control	Increased pathogenic bacteria and reduced *Lactobacilli*, *bifidobacteria* were seen in this colic group	(Savino *et al.*, 2004; de Weerth *et al.*, 2013a)
Manipulating the microbiota	Prebiotic (PDX/GOS) and probiotics (*L. actobacillus reuteri* and *L. rhamnosus*) reduced colics	(Savino *et al.*, 2011; Partty *et al.*, 2013)
Interstitial cystitis	Reduced gut levels *of E. sinensis, C. aerofaciens, F. prausnitzii, O. splanchnicus*, and *L. longoviformis* are seen in patients with interstitial cystitis	(Braundmeier-Fleming *et al.*, 2016)
Osteoarthritis and joint pain	A positive correlation seen between knee osteoarthritic pain scores and abundance of *streptococcus* species	(Boer *et al.*, 2017)

1.7 Experimental Models of Pain

1.7.1 Animal Models of Pain

Literature is populated with several validated experimental pain models utilising animals. These pain models are designed considering pain stimulus type and resulting pain behaviours. Similarly, some models are developed that mimic pain resulting from some pathological conditions. Table 1.7 lists various animal models of pain utilised in the preclinical studies (Gregory *et al.*, 2013).

Table 1.7. Animal models of pain

Type of Pain Stimulus			Pain Model	Reference
Outcome Measure of Pain Behaviour	Reflexive Pain Tests	Thermal	Tail flick test	(Dewey *et al.*, 1970)
			Hot-plate test	(Woolfe and Macdonald, 1944)
			Hargreaves test	(Hargreaves *et al.*, 1988)
		Mechanical	Paw withdrawal with Von-frey filament	(Dixon, 1980; Sluka, 1997; Tverskoy *et al.*, 1998)
			Pressure application	(Randall and Selitto, 1957; Sufka, 1994; Schafers *et al.*, 2003; Luis-Delgado *et al.*, 2006; Barton *et al.*, 2007)
	Non-reflexive Pain tests	Spontaneous pain behaviour	Paw elevation/licking (injection of formaldehyde, capsaicin, mustard oil)	(Dubuisson and Dennis, 1977; Cortright *et al.*, 2008)
		Avoidance of evoked stimuli	Thermal escape test	(Mauderli *et al.*, 2000)
			Conditioned place avoidance	(Johansen *et al.*, 2001)
			Place Escape avoidance paradigm	(LaBuda and Fuchs, 2000; Moqrich *et al.*, 2005)
Animal Models of Disease		Inflammatory pain	Capsaicin injection	(Tominaga *et al.*, 1998; Sluka, 2002)
			Carrageenan injection	(Radhakrishnan *et al.*, 2003; Radhakrishnan *et al.*, 2004; Pratt *et al.*, 2013)
			Complete Freund's adjuvant	(Ren *et al.*, 1992; Parvathy and Masocha, 2013)
			Cortical/thalamic injection of the excitotoxic agent	(Oliveras and Montagne-Clavel, 1994, 1996; LaBuda *et al.*, 2000)
			Spinal cord injury	(Vierck *et al.*, 1971; Levitt and Heybach, 1981;

Neuropathic pain		Yezierski and Park, 1993; Yezierski *et al.*, 1998)
	Peripheral nerve injury Ligating/transacting/injecting a peripheral nerve	(Kim and Chung, 1992; Ringkamp *et al.*, 1999; Decosterd and Woolf, 2000)
Neoplastic pain models	Xenograft injection of cancer cell Orofacial region Metastasis induced bone pain	(Wacnik *et al.*, 2005; Nagamine *et al.*, 2006; Cata *et al.*, 2008; Hidaka *et al.*, 2011)
Arthritis pain models	Rheumatoid arthritis-injection of collage type-II antibodies Osteoarthritis-ligamentous injury	(Christianson *et al.*, 2010; Longo *et al.*, 2012; Vincent *et al.*, 2012; Sluka *et al.*, 2013)
Muscle Pain models	Myositis- injecting irritants	(Hoheisel and Mense, 1989; Hoheisel *et al.*, 1993; Radhakrishnan *et al.*, 2003; Uhelski and Fuchs, 2009)
Postoperative pain	Hindpaw/calf surgical incision	(Brennan *et al.*, 1996; Pogatzki *et al.*, 2002)
Visceral pain	Writhing test-Intraperitoneal injection of acetic acid	(Reichert *et al.*, 2001; Lin *et al.*, 2009b; Li *et al.*, 2010)
	Colorectal distension	(Ness and Gebhart, 1987; Lin *et al.*, 2009b; Larauche *et al.*, 2012; O'Mahony *et al.*, 2012)
	Cystitis-bladder injury with irritants	(Lanteri-Minet *et al.*, 1995; Bon *et al.*, 1998; Malykhina *et al.*, 2013; Stemler *et al.*, 2013)

The models are categorised as physiological (reflexive and non-reflexive) and models of disease (Gregory *et al.*, 2013).

1.7.2 Human Models of Experimental Pain

Human pain models help to evaluate the mechanism of action and effect of analgesics and bridge the gap between basic studies and clinical therapeutics. Table 1.8 enlists various types of human models of experimental pain (extracted from a review by Olesen *et al.* (Olesen *et al.*, 2012)).

Table 1.8. Experimental human models of pain

Area Assessed	**Type of Stimuli**		**Reference**
Skin	Mechanical	Touch	(Curatolo *et al.*, 2000; Bars *et al.*, 2001)
		Pinprick	(Curatolo *et al.*, 2000; Bars *et al.*, 2001)
		Pressure	(Curatolo *et al.*, 2000; Kinser *et al.*, 2009)
	Electrical	Neurophysiological electrical stimulation	(Koppert *et al.*, 2001; Hegarty and Shorten, 2012)
	Thermal	Cold	(Fowler *et al.*, 1988)
		Contact heat	(Roberts *et al.*, 2008)
		Laser	(Frahm *et al.*, 2010)
	Evoking Hyperalgesia	Capsaicin	(Scanlon *et al.*, 2006)
		Nerve growth factor	(Rukwied *et al.*, 2010)
		Glutamate	(Gazerani *et al.*, 2006)
		Burns injury with ultraviolet radiation	(Bishop *et al.*, 2009)
		Freeze Lesion	(Kilo *et al.*, 1994)
		Mustard Oil	(Koltzenburg *et al.*, 1992)
		Menthol	(Binder *et al.*, 2011)
		Acid Phosphate Buffer	(Steen *et al.*, 1995)
		Sodium Lauryl Sulfate	(Petersen *et al.*, 2010)
		Electrical Stimulation	(Koppert *et al.*, 2001)
Muscle and Bone	Mechanical	Muscle Pressure Algometry	(Polianskis *et al.*, 2001; Graven-Nielsen and Arendt-Nielsen, 2003)
		Mechanical pressure on bone (tibia, mastoid process etc.)	(Andresen *et al.*, 2011; Finocchietti *et al.*, 2012)
	Electrical	Needle electrodes	(Schulte *et al.*, 2003)
	Thermal	Intramuscular saline injections	(Graven-Nielsen *et al.*, 2002)
	Evoking Hyperalgesia	Ischaemic stimulation with a tourniquet	(Graven-Nielsen and Arendt-Nielsen, 2003)
		Exercise induced muscle pain	(Graven-Nielsen, 2006)
		Chemically induced hyperalgesia	(Graven-Nielsen and Arendt-Nielsen, 2003; Mork *et al.*, 2003)
Viscera	Mechanical	Bowel distension with a barostat	(van der Schaar *et al.*, 1999; Staahl *et al.*, 2006; Arendt-Nielsen *et al.*, 2009; Olesen *et al.*, 2010a)
	Electrical	Depolarisation of afferents by electrical current	(Drewes *et al.*, 2003; Brock *et al.*, 2009)
	Temperature	Luminal heat stimulation	(Staahl *et al.*, 2006; Arendt-Nielsen *et al.*, 2009; Olesen *et al.*, 2010a)
	Evoking Hyperalgesia	Chemicals such as acid, capsaicin, and glycerol	(Drewes *et al.*, 2003; Brock *et al.*, 2009; Olesen *et al.*, 2009; Olesen *et al.*, 2010b)

Examples of mechanical, electrical, and thermal pain and evoked hyperalgesia applied to somatic and visceral areas are listed (Olesen *et al.*, 2012).

1.8 Aims of the Thesis

We designed our experiments to evaluate the sex differences in glutamatergic signalling. We explored specific factors affecting glutamatergic system and pain, such as cyclical sex hormone variation in females, early-life stress, gut microbiota and exogenous oestrogen.

Aim 1: Is the Spinal Glutamatergic System Modulated by Sex Hormones?

Most pain-related studies investigating sex differences in animals have not investigated the effects of physiological variations of sex hormones. We aimed to look at the function of synaptic glutamate regulators under different hormonal, physiological states in animals (Chapter 2). We investigated mechanisms responsible for the effects of hormones on glutamate metabolism. We carried out ex-vivo studies on rat spinal cord to achieve our goals. Our hypothesis was that oestrogen will have an inhibitory action on glutamate transporters.

Aim 2: Is the Hormonal Modulation of the Glutamatergic System Affected by Stress?

Early-life stress is a known risk factor for visceral hypersensitivity in adult life. We assessed the glutamatergic turnover in animal models of early-life stress and visceral hypersensitivity with an aim to establish the interplay between stress, hormones and the neurotransmission in pain states (Chapter 3 & 4). We performed colorectal distension in rats exposed to early-life stress and animal models of visceral hypersensitivity and studied glutamatergic system in both the spinal cord and the brain. We hypothesised that animal stress models will show a comparatively high visceral pain sensitivity, and it will be associated with glutamate transporter function inhibition.

Aim 3: Is There a Role of Gut Microbiota on Glutamatergic Signalling and Pain Sensitivity?

The role of the microbiota-gut-brain axis in CNS disorders has an emerging recognition. We aimed to establish if gut microbiota had any role in glutamatergic signalling and pain

sensitivity. We correlated synaptic glutamate transporter function with the relative abundance of gut microbiota in WKY rats (Chapter 4). We assessed pain sensitivity in humans and correlated it with their gut microbiota (Chapter 5). Our hypothesis was that the abundance of short-chain fatty acid producing gut bacteria will be more in the subjects demonstrating higher pain thresholds.

Chapter 2

Sex-Dependent Activity of The Spinal Excitatory Amino Acid Transporter: Role of Oestrous Cycle

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2.1 Abstract

Background: Females are more likely to experience visceral pain than males, yet mechanisms underlying this sex bias are not fully elucidated. Moreover, pain sensitivity can change throughout the menstrual cycle. Alterations in the glutamatergic system have been implicated in several pain disorders; however, whether these are sex-dependent is unclear. Thus, we aimed to investigate sex differences in the spinal cord glutamate uptake and how it varies across the oestrous cycle. *Methods*: The activity of the glutamate transporters, excitatory amino acid transporters (EAATs) was assessed using an *ex-vivo* aspartate radioactive uptake assay in the lumbosacral spinal cord in Sprague-Dawley male and female rats. The gene expression of EAATs, glutamate receptor subunits NR1 and NR2B and the oestrogen receptors ERα & ERβ in the spinal cord were also analysed. *Results:* EAAT activity was lower in females, particularly during the oestrus phase, and this was the only cycle stage that was responsive to the pharmacological effects of the EAATs activator riluzole. Interestingly, EAAT1 mRNA expression was lower in high-oestrogen and high-ERα states compared to diestrus in females. Conclusion: We conclude that the spinal EAAT activity in females is different to that in males, and varies across the oestrous cycle. Furthermore, the expression levels of oestrogen receptors also showed a cycle-dependent pattern that may affect EAATs function and expression.

2.2 Introduction

In general, women are more sensitive to pain than men, and some painful disorders such as IBS (Chang and Heitkemper, 2002; Mogil and Bailey, 2010), chronic pelvic pain (Scialli, 1999), temporomandibular disorder (Cairns, 2010), fibromyalgia (Yunus, 2002), biliary colic (Palsson and Sandblom, 2015), esophagitis (Krarup *et al.*, 2013), and rheumatoid arthritis (Brennan and Silman, 1995) are more prevalent in women. Similar associations have been reported in rodents, where female rats showed exaggerated visceromotor responses to colorectal distension (Holdcroft *et al.*, 2000; Ji *et al.*, 2006; Ji *et al.*, 2012; Winston *et al.*, 2014; Guo *et al.*, 2015) as well as other types of pain (Mogil and Bailey, 2010). On the other hand, there are some reports of increased visceral and somatic hypersensitivity in male rats exposed to early-life stress as compared to female rats (Prusator and Greenwood-Van Meerveld, 2015). Significant gender differences in incidence, symptomatology, and therapeutic outcome in various pain disorders indicate the necessity for gender-tailored treatment approaches. However, to achieve this, it is necessary to have a comprehensive understanding of the molecular mechanisms responsible for these pain-related gender differences.

IBS is the most common functional gastrointestinal disorder with symptoms of altered bowel habit and visceral hypersensitivity (Kennedy *et al.*, 2014; Hyland *et al.*, 2015). The majority of women with IBS who seek health care are of reproductive age. Population surveys have reported that the prevalence of IBS declines after the age of 40 years (Hungin, 2003); suggesting a role of female sex hormones in the aetiology of IBS. Studies on gender differences in visceral pain are increasingly being carried out, yet few of these have evaluated the impact of menstrual cycle on pain perception (Stening, 2007; Heitkemper and Chang, 2009a). Moreover, there is a paucity of information on the potential mechanisms underlying sex differences in visceral pain. Of these, plasma levels of cytokines have been shown to vary

depending on the cycle stage (O'Brien *et al.*, 2007). Furthermore, there is growing evidence of the involvement of gonadal hormones (e.g. oestrogen) in visceral and somatic pain sensitivity; however, conflicting results have been reported. Some studies have shown ovarian hormones (e.g. oestrogen) to be antinociceptive (Bradshaw, 1999; Sanoja, 2005; Heitkemper and Chang, 2009a), whereas others indicate that these hormones may be pronociceptive (Ji *et al.*, 2003; Lu *et al.*, 2009; Chaloner and Greenwood-Van Meerveld, 2013). Noteworthy, visceromotor response to colorectal distension was shown to be higher in proestrus (high-oestrogen state) than in metestrus/diestrus (low-oestrogen states) (Ji *et al.*, 2008; Peng *et al.*, 2008). A need, therefore, arises to assess the underlying mechanisms that are implicated in visceral pain with regard to the changing levels of gonadal hormones across the female cycle.

During pain signal transmission, glutamate is released into the synaptic cleft, which, in turn, causes activation of NMDA receptors and hence neurotransmission across the synapse occurs. The synaptic concentration and resultant activity of glutamate are controlled by EAATs which transport glutamate from the synaptic cleft into the glial cells. In the spinal cord, EAAT1 and EAAT2 are the most efficient mediators of glutamate clearance at synaptic cleft (Danbolt, 2001; Holmseth *et al.*, 2012), and their gene expression has an important role in visceral sensitivity (Lin *et al.*, 2009b; Gosselin *et al.*, 2010). It has been shown that intrathecal administration of a glutamate transporter antagonist results in increased sensitivity to colorectal distension in rats (Gosselin *et al.*, 2010). It has also been demonstrated that transgenic mice overexpressing EAAT2 demonstrated a twofold increase in the glutamate uptake across these transporters and a significant reduction in the visceromotor response to colorectal distension (Lin *et al.*, 2009b). Furthermore, maternally separated rats have shown an increase in visceral sensitivity associated with a selective reduction in spinal EAAT1

expression (Gosselin *et al.*, 2010). However, most of the preclinical studies assessing the role of EAATs in pain have been carried out in male animals.

Oestrogen levels fluctuate during the phases of the menstrual cycle (humans) and oestrous cycle (rodents) (Marcondes *et al.*, 2001; Stricker *et al.*, 2006) and were shown to alter the activity of glutamate receptors (Tang *et al.*, 2008). Moreover, NMDARs showed higher activity in DRGs in female rats as compared to males, and this effect was further potentiated in females by exogenous oestrogen (McRoberts *et al.*, 2007). Co-localisation of oestrogen receptor alpha (ER-α) and NR1 (a subunit of NMDAR) in the dorsal horn of the lumbosacral spinal cord suggests a direct modulation of glutamate receptor activity by oestrogen through ER-α (Tang *et al.*, 2008). Furthermore, it has been shown that the threshold for visceral pain is lower in high-oestrogen proestrus phase (Ji *et al.*, 2008).

Thus, we aimed to investigate if the activity of spinal glutamate transporters/or efficiency of glutamate reuptake differs between sexes, across the oestrous cycle and in response to the EAATs activator riluzole. We further sought to determine if subunits of the NMDA and oestrogen receptors are differentially expressed in males and females, as well as throughout different phases of the oestrous cycle.

2.3 Materials and Methods

2.3.1 Animals

Adult male and female Sprague-Dawley rats weighing 250–300 g (Harlan, UK) were housed in a local animal facility with food (2018 Teklad Global 18% Protein Rodent, Envigo) and water ad libitum, on a 12:12-hour dark–light cycle (lights on at 7:00 AM) with the temperature at 20°C ± 1°C. Animals were group housed by 4 to 5 per cage in plastic cages with sawdust bedding, shredded paper, and a cardboard roll. They were allowed to habituate in the new environment for a week before the commencement of experiments. Female rats underwent vaginal smearing on the day of each experiment to assess their oestrous cycle phase (Byers *et al.*, 2012). One cohort of 10 males and 30 female rats was used for the aspartate uptake studies. Another cohort of 10 males and 29 females was used for gene expression analysis of glutamate receptors subunit and oestrogen receptors in the spinal cord. All experiments were in full accordance with the European Community Council Directive (2010/63/EU) and approved by Animal Experimentation Ethics Committee of University College Cork.

2.3.2 Vaginal Smearing

Females were vaginally lavaged with saline, and cells were immediately viewed under the microscope at round 08:00 am on the day of the experiment. The stage of oestrous cycle was determined as previously described (Ji *et al.*, 2008). Briefly, the metestrus was characterised by the appearance of small leukocytes mixed with round nucleated epithelial cells, whereas, diestrus smear had fewer leukocytes along with cornified epithelial cells. The smears from proestrus rats had a predominance of round nucleated cells of uniform size, with a complete absence of leukocytes. Finally, in oestrus, nonnucleated cornified epithelial cells were predominant. Since metestrus only lasts for a short period (5–6 h) and the plasma

oestrogen concentration in metestrus does not differ largely from that in diestrus, data from these two groups of rats were pooled. Animals were lavaged immediately before euthanasia, to determine oestrous stage accurately for all tissue collected.

2.3.3 Aspartate Uptake

Aspartate uptake on brain slices has been previously described (Thomazi *et al.*, 2004); here we optimised this technique to be used on spinal cord slices.

2.3.4 Reagents

Aspartic Acid, D- [2,3-^{3}H] (specific gravity 12.9 Ci/mmol) was purchased from Perkin-Elmer, USA. RIPA buffer and Peirce BCA protein assay kit were purchased from Fisher Scientific Ireland. (3*S*)-3-[[3-[[4-(Trifluoromethyl) benzoyl] amino] phenyl] methoxy]-L-aspartic acid (TFB-TBOA), 2-amino-6-trifluoromethoxybenzothiazole (Riluzole) and 17-β-estradiol-3-benzoate were purchased from Tocris UK. All other reagents were purchased from Sigma-Aldrich. HBSS was prepared in-house containing (in mM): 137 NaCl; 0.63 Na_2HPO_4; 4.17 $NaHCO_3$; 5.36 KCl; 0.44 KH_2PO_4; 1.26 $CaCl_2$; 0.41 $MgSO_4$; 0.49 $MgCl_2$ and 1.11 glucose, pH 7.2. In sodium-free HBSS, NaCl was replaced by 137 mM N-methyl-D-glucamine.

2.3.5 Spinal Cord Slice Preparation

Animals were euthanised by decapitation immediately after vaginal smearing. The spinal cord was removed by hydraulic pressure into HBSS filled Petri dishes, and 0.4 mm thick slices were obtained from the lumbosacral region using a McIlwain tissue chopper. These slices were separated by fine dissection under a microscope and transferred into either (1ml) sodium-containing HBSS (labelled as Na^+) or (1ml) sodium-free HBSS (labelled as Na^-) on 24 well cell culture plates. Na^+ plates were maintained at 35 °C and Na^- on the ice. The slices from Na^+ plates were washed once with 1 mL of 35 °C sodium-containing HBSS and Na^- plate with

1 mL of 4 °C sodium-free HBSS to assess sodium-dependent and independent uptake respectively.

2.3.6 Total Uptake

Spinal cord slices were pre-incubated at 35 °C and on ice in Na^+ and Na^- HBSS respectively for 30 min. Then a solution containing 1µL 0.66 µCi/mL aspartic acid D- [2,3-^{3}H], 15 µL of cold 100 µM D-Aspartate and 84 µL H_2O was added. After 3 minutes of incubation, slices were washed twice with 1 mL of the corresponding ice-cold HBSS. Samples were then transferred into 1.5 mL tubes containing volume RIPA buffer and then were mechanically homogenised with pestles. To improve the efficiency of lysis, tissue homogenate was incubated for further 15 minutes at 4 °C. Tissue lysate was cleared by centrifugation at 14000g for 10 min at 4 °C and used for the radioactivity analysis. Radioactivity was measured using a Perkin-Elmer scintillation counter. Data obtained from Na^- samples were subtracted from those of the Na^+ samples to specifically estimate sodium-dependent uptake mediated by EAAT 1 & 2 transporters. Uptake procedure was performed in triplicate. Protein was measured using Peirce BCA protein assay kit. Aspartate uptake in each slice was presented as scintillation per minute (CPM) per mg of protein.

2.3.7 Effect of Compounds on Aspartate Uptake

To assess the influence of various drugs on spinal cord aspartate uptake, slices of the spinal cord were pre-incubated with these drugs for 30 minutes before addition of tritium labelled aspartate. Drug concentrations used were as follows: TFB-TBOA 200 ηM in 0.00008% DMSO (Jiang *et al.*, 2012), Riluzole 10 µM in 0.0025% DMSO (Gosselin *et al.*, 2010) and 17-β-estradiol-3-benzoate 100 ηM in 0.000025% DMSO (a dose-response experiment was performed, data not shown). DMSO was used as a vehicle control.

2.3.8 Quantitative Real-time PCR (RT-qPCR)

Lumbosacral spinal cord samples were collected from a separate cohort of female and male animals (n=39). Samples were snap frozen and stored at −80°C until the analysis. Total RNA was isolated using a *mir*Vana miRNA isolation kit (Life Technologies). RNA concentration was quantified using the NanoDrop spectrophotometer. 10 ng/mL of RNA was reverse transcribed to cDNA using the Applied Biosystems High Capacity cDNA kit (Applied Biosystems, Warrington, UK). RT-qPCR was carried out using TaqMan probes designed by Applied Biosystems on the ABI7300 Real-Time PCR machine (Applied Biosystems, Warrington, UK) according to the manufacturer's protocol. The following primers were used: Rn00665089_m1 for EAAT1, Rn00691548_m1 for EAAT2, Rn01436038_m1 for NR1, Rn00680474_m1 for NR2B, Rn01640372_m1 for ESR1, Rn00562610_m1 for ESR2, Rn01485494_m1 for β-Actin as an endogenous control.

RT-qPCR was carried out experimental samples were run in triplicate with 1µL cDNA per reaction. Cycle threshold (Ct) values were calculated; averaged Ct values of each triplicate were used in further analysis. The Ct value for the target gene in each sample was normalised to its endogenous control Ct value and transformed to relative gene expression value using $2^{-\Delta\Delta Ct}$ equation (Livak and Schmittgen, 2001). The gene expression levels in female groups were presented as a fold change vs the male group.

2.3.9 Statistical Analysis

All data was normally distributed according to Gaussian distribution analysis. An unpaired two-tailed student's *t*-test, a one-way ANOVA or two-way ANOVA with Tukey's post-hoc analysis was utilised. Values of $P < 0.05$ were considered statistically significant.

2.4 Results

2.4.1 Oestrogen and TFB-TBOA Reduce Spinal Cord Aspartate Uptake in Male Rats

To validate the aspartate uptake technique and ensure we are able to detect alterations to the EAATs activity in the spinal cord, spinal cord slices were pre-incubated with TFB-TBOA which is a high-affinity selective blocker of the glial glutamate transporters EAAT1 and EAAT2. There are various analogues available, but we chose TFB-TBOA because of its high potency, high selectivity for EAATs and is not neurotoxic (Bozzo and Chatton, 2010). Administration of TFB-TBOA significantly reduced the aspartate uptake, thus validating the technique ($p < 0.01$, Figure 2.1A).

We next sought to determine the effect of female gonadal hormone oestrogen on the aspartate uptake in spinal cord tissue in males. We wanted to explore if exogenous oestrogen was directly able to modify the function of EAATs. When spinal cord slices from male rats were pre-incubated with female sex hormone oestrogen (17-β-estradiol-3-benzoate), aspartate uptake was significantly reduced ($p < 0.01$, $F_{(2, 27)} = 7.14$, Figure 2.1B). The vehicle, DMSO, did not affect the aspartate uptake.

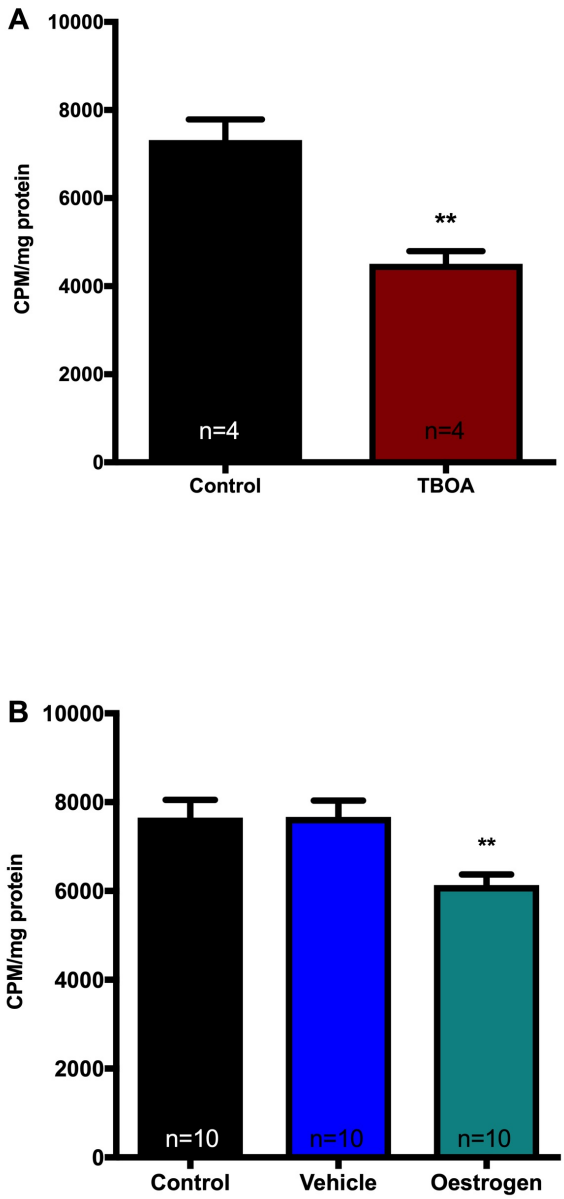


Figure 2.1. Effect of TBOA and oestrogen on EAAT function in male rat spinal cord. (A) EAAT blocker TFB-TBOA decreased aspartate reuptake in spinal cord slices obtained from male rats, thus proving the validity of the technique applied, n=4, ** $p < 0.01$. (B) 17-β-estradiol-3-benzoate decreased the uptake of aspartate in male rats. Vehicle solution of 0.000025% DMSO did not have an effect on aspartate uptake. Data are presented as mean ± standard error of the mean (SEM), n=10, **$p < 0.01$ *vs.* control and vehicle.

2.4.2 Reduced Aspartate Uptake in Females Relates to Oestrous Cycle Stage

Based on the findings that oestrogen had a significant effect on aspartate uptake in male rats, we investigated EAATs function across the female oestrous cycle which is characterised by different oestrogen systemic levels. Here we show that oestrous cycle had a significant effect on the spinal aspartate uptake ($p < 0.0001$, $F_{(3,24)}=10.91$, Figure 2.2). Post-

hoc analysis showed that males and diestrus females did not have any differences in aspartate uptake. However, EAATs activity was significantly lower in high-oestrogen states, i.e., proestrus and oestrus females as compared to both diestrus female rats and males ($p < 0.05$ and $p < 0.0001$ respectively).

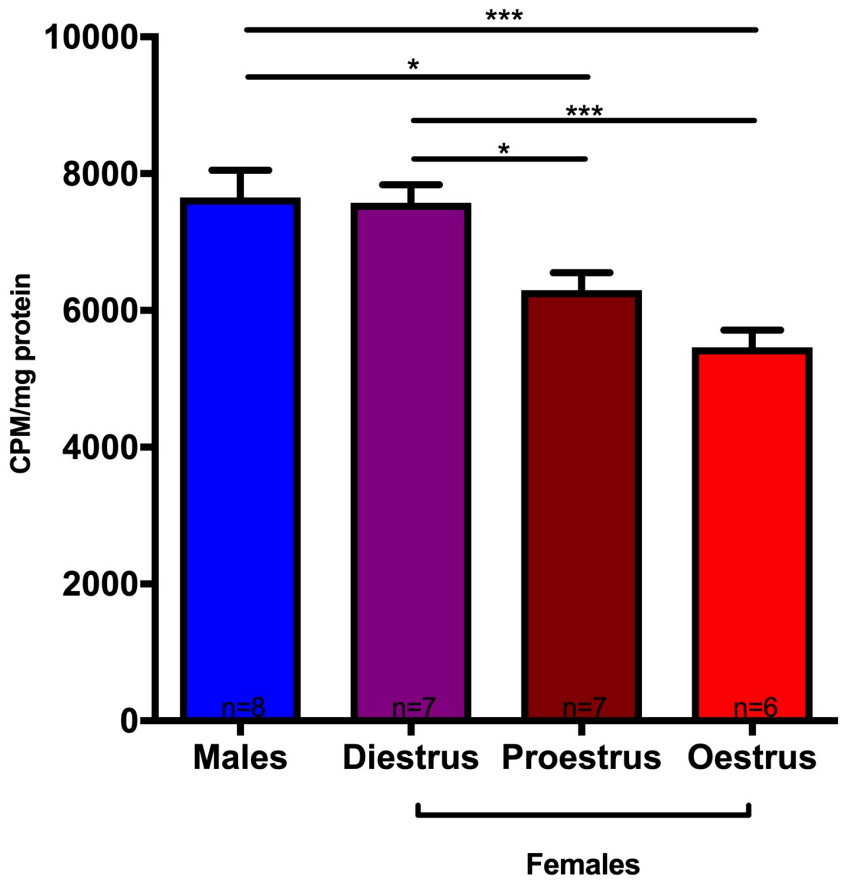


Figure 2.2. Aspartate uptake in the spinal cord of males and females across oestrous cycle. In oestrus and proestrus states, the aspartate uptake is significantly low compared to diestrus females and male rats. Values represent mean ± SEM, n=6-8. *$p < 0.05$ vs proestrus, ***$p < 0.001$ vs. oestrus.

2.4.3 Pharmacological Enhancement of EAATs Reverses the Inhibitory Effect of Endogenous Oestrogen on Aspartate Uptake

Here we assessed the effect of riluzole on spinal EAATs function across different stages of oestrous cycle in female rats. A two-way ANOVA revealed a significant interaction between oestrous phase and riluzole treatment ($p < 0.0001$, $F_{(2,34)}=17.63$, Figure 2.3). Post-hoc analysis showed that it significantly enhanced the EAATs function in oestrus phase only, thus possibly

reversing the inhibitory effect of endogenous oestrogen ($p < 0.0001$). Notably, riluzole had no effect on aspartate uptake in diestrus and proestrus phases of the oestrous cycle.

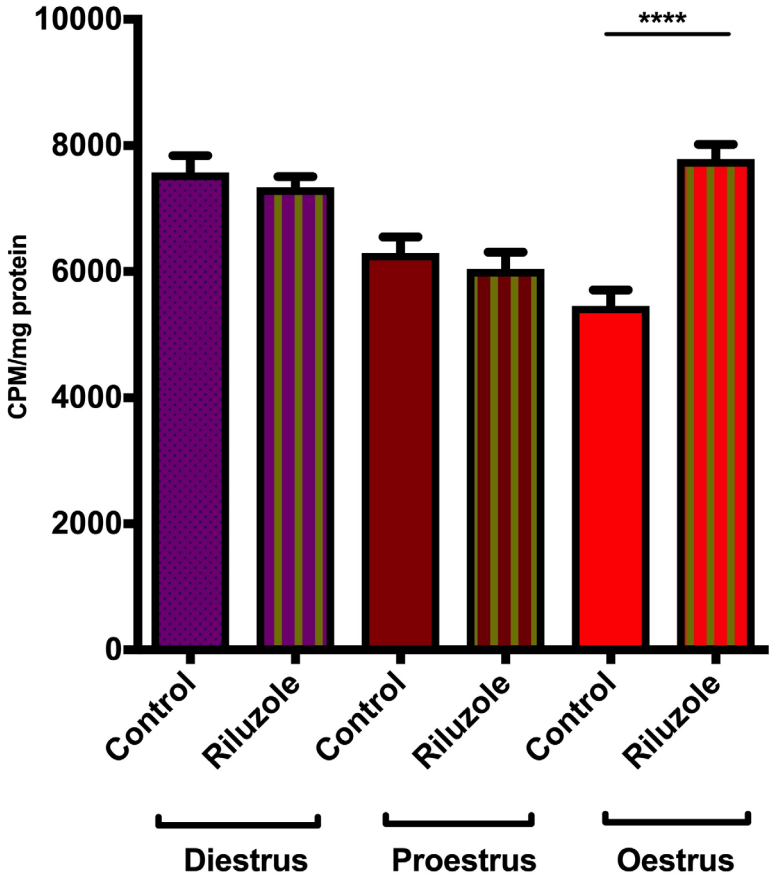


Figure 2.3. Effect of riluzole on aspartate uptake in the spinal cord across the oestrous cycle. Riluzole significantly enhanced the aspartate uptake in oestrus phase, but no effect was seen in other stages of oestrous cycle. Values represent mean ± SEM, n=7, ***$p < 0.0001$ vs. oestrus group.

2.4.4 Diestrus Female Rats Have Increased mRNA Expression of EAAT1 in Lumbosacral Spinal Cord

The EAAT1 mRNA expression levels showed significant variation across the oestrous cycle (one-way ANOVA, $p < 0.05$, $F_{(3,29)} = 4.3$). Post-hoc analysis revealed increased expression of EAAT1 in diestrus as compared to proestrus females ($p < 0.05$, Figure. 2.4A). There was no significant difference in the expression of EAAT2 across the groups (Figure. 2.4B)

2.4.5 NMDA-Receptor Subunit-1 Gene Expression Is Reduced in Proestrus

We compared the expression of glutamate receptor (NMDAR) subunits 1 and 2b genes in the spinal cord of males and females in the different phases of the oestrous cycle. Expression of subunit-1 was significantly lower in proestrus compared to diestrus and oestrus females (post-hoc analysis, $p < 0.05$, and $p < 0.001$ respectively) t (one-way ANOVA showed $p < 0.01$, $F_{(3, 35)} = 5.4$, Figure 2.4C). Expression of the NMDAR 2b subunit was not different across all groups (Figure 2.4D).

2.4.6 Expression of ERα and ERβ change throughout the oestrous cycle

As aspartate uptake appeared to be lower in high-oestrogen states, we evaluated differential expression of oestrogen receptor genes, ERα, and ERβ, across the same phases. A one-way ANOVA showed a significant effect of oestrous cycle on ERα ($p < 0.0001$, $F_{(3,35)} = 13.67$, Figure 2.4E). Post-hoc comparisons showed significantly higher expression of ERα in oestrus females as compared to males, diestrus and proestrus females ($p < 0.0001$).

Expression of ERβ also varied significantly across oestrous cycle (one-way ANOVA, $p < 0.01$, $F_{(3,35)} = 5$, Figure 2.4F). However, its expression was higher in diestrus as compared to proestrus (post-hoc analysis, $p < 0.001$).

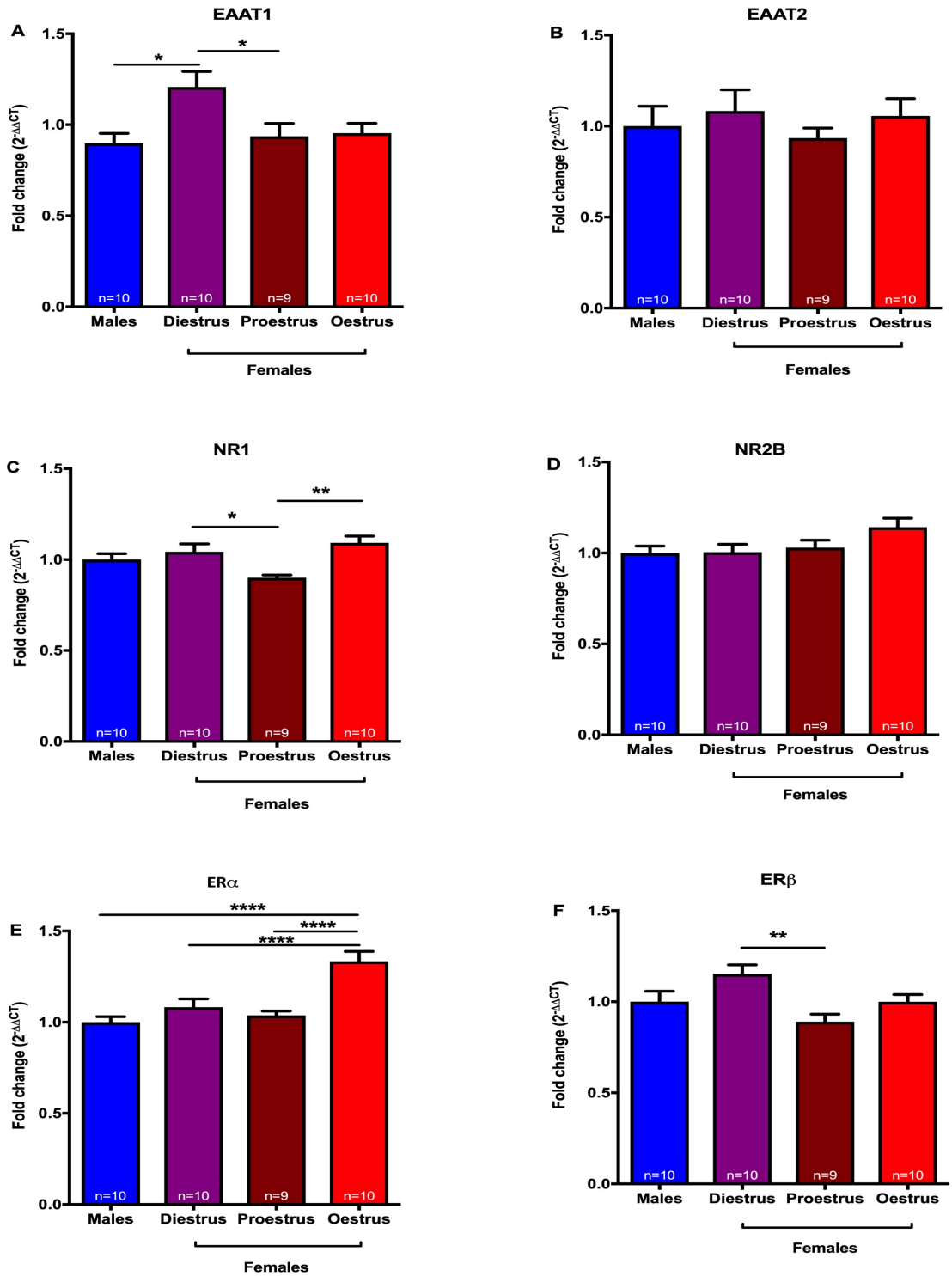


Figure 2.4. mRNA expression of EAAT1 & 2, NR1, NR2B, and oestrogen receptors in males and females. (A) EAAT1 gene mRNA expression in the spinal cord is higher in the diestrus phase. * $p < 0.05$ vs. diestrus (B) mRNA expression of EAAT2 gene does not change across the oestrous cycle (C) mRNA expression of the NMDA-receptor subunit-1 gene was changed across the oestrous cycle. *$p < 0.05$, **$p < 0.01$ vs proestrus. (D) mRNA expression of NMDA-receptor subunit-2B gene in the spinal cord of male and female rats was not different between sexes or changed during the oestrous cycle. (E) mRNA expression of ERα gene in the spinal cord was different between the sexes and between stages of the oestrous cycle. ****$p < 0.00001$, ***$p < 0.0001$ vs oestrus. (F) mRNA expression of ERβ gene was different in stages of the oestrous cycle. **$p < 0.01$ (diestrus vs. proestrus). Values represent mean ±SEM, n=9-10.

2.5 Discussion

In this study, we have demonstrated that EAAT activity in the lumbosacral spinal cord differs between male and female rats. The activity of these transporters is decreased in spinal cord slices from male rats when exogenous oestrogen is applied. Moreover, uptake via EAATs varies across the oestrous cycle in female rats gradually decreasing from the diestrus to the oestrus phase. This indicates that the gonadal hormone oestrogen is capable of influencing the activity of these transporters and can be artificially manipulated. This provides insights into sex differences in visceral pain as the lumbosacral level of the spinal cord receives sensory signals from the gastrointestinal tract. Alterations in the expression of these transporters in this part of the spinal cord have been previously associated with visceral pain in an animal model of IBS (Gosselin *et al.*, 2010), a disorder that is more prevalent in females. Hence, we proposed to assess in healthy rats if a difference in the activity of these transporters existed between the sexes that may help explain differences in pain that are contributed to by gonadal hormones.

Interestingly, we observed an increase in the RNA expression levels of the EAAT1 transporter in the diestrus phase compared to males and the other phases. This may indicate that a compensatory higher expression of the transporter is possible in low-oestrogen states that allow maintenance of same EAAT activity as male rats, while in proestrus this expression is decreased and may be associated with the exaggerated decrease in activity compared to male rats and the diestrus phase. The functional consequences of this may be seen as higher synaptic glutamate levels during high-oestrogen states and result in increased pain signalling.

No difference was noted between males and females or between the different stages with regard to the expression of EAAT2 mRNA, indicating that the sex and cyclical difference seen in the uptake of aspartate were related to the expression of EAAT1 solely.

Here we also demonstrate that riluzole, which enhances EAAT function increases uptake of aspartate during the oestrus phase in female rats only. This indicates that EAAT1 function in female rats may only be susceptible to manipulation during this stage of the cycle. As riluzole has been shown to have a therapeutic effect in chronic pain states (Gosselin *et al.*, 2010; Nicholson *et al.*, 2014a), our study highlights the need to take fluctuating female hormones into account in pain management with riluzole or other EAAT enhancers. Our results correlate with other preclinical *in-vivo* studies, which have shown that ovariectomy in rats (resulting in oestrogen deficiency) significantly decreased the magnitude of visceromotor response to colorectal distension, and this effect was reversed by administration of oestrogen to these animals (Ji *et al.*, 2003).

In the spinal cord, EAAT1 constitutes 40% of all high- affinity EAATs, and it is abundantly present in lumbar dorsal horn (Queen *et al.*, 2007). In physiological conditions, EAATs remove glutamate from the synaptic cleft by co-transporting glutamate and sodium into adjacent glial cells. The inefficient function of EAATs can lead to high extracellular glutamate concentration causing neuronal damage (Kanai *et al.*, 2013). In the spinal cord, glutamate transporters have been shown to be downregulated following chronic sciatic nerve injury (Sung *et al.*, 2003; Yan *et al.*, 2009; Ramos *et al.*, 2010). Drugs inhibiting EAATs such as TBOA have shown the pronociceptive effect by increasing synaptic glutamate and hence neuronal excitability (Liaw *et al.*, 2005).

Interestingly in pathological chronic pain states, these compounds have shown opposite i.e. antinociceptive effect (Minami *et al.*, 2001; Yang *et al.*, 2015). Tao *et al.* have

proposed various possible causes of this phenomena including sustained higher extracellular glutamate leading to neurotoxicity; glutamate conversion to inhibitory molecule gamma amino butyric acid (GABA); presynaptic glutamate trans- porter blockade leading to depletion of presynaptic gluta- mate and postsynaptic glutamate receptor desensitisation (Tao *et al.*, 2005). The exogenous oestrogen decreased the EAATs activity in the male spinal cord at 100nM concentration in our study. This concentration of oestrogen is higher than the physiological one but was used to allow the penetration into the spinal cord tissue in our ex-vivo experiments. In females, the proestrus phase exhibited less active EAAT state most likely under the effect of high endogenous oestrogen. This effect proved to be sustainable and persisted in oestrus phase where oestrogen usually decreases its circulatory levels. Mitrovic *et al.* demonstrated that the exogenous progesterone showed no effect on glial glutamate uptake in male rats, alone or in combination with oestrogen (Mitrovic *et al.*, 1999). In our study, the glutamate uptake was high in metestrus and diestrus, implying that progesterone does not reduce the uptake in this case.

Remarkably, the oestrogen receptor ERα gene expression was upregulated in the oestrus phase suggesting an enhanced hormone signalling. Furthermore, studies have shown that selective activation of spinal ERα mimics the pronociceptive effects of 17b-estradiol on visceral sensitivity (Ji *et al.*, 2011). Oestrogen generates its effects via the nuclear receptors: oestrogen receptors alpha (ERα) and beta (ERβ), which upon their activation instigate transcriptional changes for target genes modulating cellular function (Dechering *et al.*, 2000). Oestrogen also has non-genomic effects through cellular membrane oestrogen receptors by initiating activation of intracellular signalling pathways depending upon their location e.g. mitogen-activated protein kinase/extracellular signal-regulated kinases (MAPK/ERK) in the spinal cord (Bjornstrom and Sjoberg, 2005). Oestrogen potentially exerts its nociceptive

effects on visceral pain pathways through ERα leading to activation of ERK pathway (Ji *et al.*, 2011). Our study showed increased expression of spinal ERα mRNA in the oestrus phase which may be linked to the decreasing levels of oestrogen. We also noted an increase in ERβ in the diestrus phase which is related to decreased oestrogen and visceral pain. Moreover, ERβ ligands have an antinociceptive effect in rats during colorectal distension (Cao *et al.*, 2012). Future work is needed to focus on whether this is causally related to the changes in glutamate transporter per se and downstream pain states. This may include the use of selective ERα and ERβ inhibitors while assessing the glutamate uptake.

Interestingly, oestrogen has been shown to upregulate NMDA receptors (NDMARs), which are heterotetramers composed of two N1 and two N2 or three subunits. NMDA-receptor activity is subject to changes in receptor abundance, distribution, phosphorylation status, and subunit composition and may be altered by ischaemia, inflammation, ageing, or sex hormones (Tang *et al.*, 2008; Ji *et al.*, 2015). Oestrogen also increases receptor phosphorylation of the NR1 subunit contributing to an increase in NMDA-receptor activity in a model of visceral pain (Tang *et al.*, 2008). This may result in a negative feedback mechanism and thus decreased expression of the NR1 subunit mRNA, which is possibly the case in our data as there was a selective reduction of NR1 mRNA in proestrus. This effect was not present in oestrus phase of the oestrous cycle most likely due to the decreasing concentration of systemic oestrogen levels in the oestrus phase. We noted no change in the NR2B subunit between the sexes or between cycle stages. This conflicts with other studies possibly due to our use of healthy rats and Ji *et al.*, using a model of inflammatory visceral pain (Ji *et al.*, 2012).

One possible pathway summarising the overall action involves NMDA-receptor activation – Ca^{2+} influx – protein kinase A (PKA) activation – ERK phosphorylation and subsequent synaptic plasticity (Thomas and Huganir, 2004). A few studies have also shown

modulation of synaptic glutamate transporters in the brain by oestrogen, although both enhancement (Keller *et al.*, 1997; Liang *et al.*, 2002) and inhibition (Sato *et al.*, 2003) of glutamate uptake have been reported.

These findings suggest high-affinity glutamate transporters as potential therapeutic targets for chronic pain states that tend to fluctuate over the female cycle. Pharmacological strategies to upregulate EAAT function e.g. riluzole; ceftriaxone and valproate can potentially alleviate pain in certain chronic pain conditions. However, a human trial failed to show the efficacy of riluzole in chronic neuropathic pain (Galer *et al.*, 2000). Interestingly there have been some reports of its efficacy in visceral pain states such as IBS (Mishra *et al.*, 2014). Our data would suggest that care is needed as to when its efficacy may be most potent across the menstrual cycle in females.

2.6 Conclusions

Here we show that the spinal glutamate transporter activity is oestrogen state-dependent which can potentially affect the efficiency of excitatory glutamate neurotransmission. Oestrogen potentially exerts its effects through ERα which may lead to altered expression of EAATs and glutamate receptor subunits. While more research is needed in this area this study sheds some light on gender specific pain therapies.

Chapter 3

Oestrous Cycle Influences Excitatory Amino Acid Transport and Visceral Pain Sensitivity in the Rat: Effects of Early-Life Stress

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3.1 Abstract

Background: Early-life stress (ELS) is a recognised risk factor for chronic pain disorders, and females appear to be more sensitive to the negative effects of stress. Moreover, oestrous cycle-related fluctuations in oestrogen levels have been linked with alternating pain sensitivity. Aberrant central circuitry involving both the ACC and the lumbosacral spinal cord has also been implicated in the modulation of visceral pain in clinical and preclinical studies. Here we further investigate changes in visceral pain sensitivity and central glutamatergic systems in rats with respect to oestrous cycle and ELS.

Methods: We investigated visceral sensitivity in adult female Sprague-Dawley rats, which had undergone maternal separation (MS) in early-life or remained non-separated (NS), by performing colorectal distension (CRD). We also assessed excitatory amino acid uptake through EAATs in the lumbosacral spinal cord and ACC.

Results: NS animals in proestrus and oestrus exhibited reduced EAAT uptake and decreased threshold to CRD. Moreover, total pain behaviours were increased in these stages. MS rats exhibited lower pain thresholds and higher total pain behaviours to CRD across all stages of the oestrous cycle. Interestingly, cortical EAAT function in MS rats was inhibited in the low-oestrogen state- an effect completely opposite to that seen in NS rats.

Conclusion: This data confirms that oestrous cycle and ELS are significant factors in visceral sensitivity and fluctuations in EAAT function may be a perpetuating factor mediating central sensitisation.

Keywords: Visceral pain, colorectal distension, ELS, excitatory amino acid transporter, glutamatergic system, aspartate uptake.

3.2 Introduction

Chronic pain syndromes such as IBS, fibromyalgia, migraine and interstitial cystitis display a striking female preponderance with females presenting at the clinic up to 10 times more often than their male counterparts (Unruh, 1996; Mogil, 2012). Moreover, females report more intense pain, of longer duration and more frequently. With these high prevalence rates, a growing body of evidence suggests that gonadal hormones play a significant role in pain processing (Iacovides *et al.*, 2015). In line with this, recent evidence suggests the possible interaction of gonadal hormones with the brain-gut axis and downstream pain processing (Heitkemper and Jarrett, 2001; Ji *et al.*, 2003; Myers *et al.*, 2011). Indeed, gonadal hormone binding sites are widely distributed in areas of the CNS involved in pain processing. Sex steroids also have receptors located throughout the entire intestine, with both oestrogen and progesterone exhibiting direct effects on visceral organs (Arendt-Nielsen *et al.*, 2004). Furthermore, oestrogen has been implicated in the possible modulation of visceral pain perception (Ji *et al.*, 2003; Chaloner and Greenwood-Van Meerveld, 2013). However, both clinical studies and animal models have revealed conflicting evidence on the role of the female hormonal cycle on pain perception (Fillingim and Ness, 2000; Heitkemper and Chang, 2009b). Numerous studies in the rat have shown a decreased threshold and greater sensitivity in the proestrus phase, however, others have reported no difference between oestrous stages (Sapsed-Byrne *et al.*, 1996; Ji *et al.*, 2003, 2006; Ji *et al.*, 2008). These discrepancies may be due in part to strain differences and the inaccuracy of oestrous cycle determination.

Oestrogen receptors have also been shown to interact with other key neurotransmitter systems such as the glutamatergic system, which itself is critical to pain processing mechanisms (Zhang *et al.*, 2012). In particular, the process of central sensitisation, implicates a critical role of excessive glutamatergic signalling leading to the development of

plasticity, thus maintaining the chronic pain state (David-Pereira *et al.*, 2015; Falnikar *et al.*, 2015). The glial EAATs, in particular, EAAT 1 and EAAT 2 are crucial in the maintenance of homeostasis within the glutamatergic synapse. However, their expression has been shown to be altered in chronic pain models (Gosselin *et al.*, 2010; Moloney *et al.*, 2015a). Intrathecal administration of EAAT blockers in naïve animals results in spontaneous somatic pain, as well as hyperalgesia and allodynia, implying that a continuous spinal glutamate uptake has a key basal antinociceptive action (Aanonsen and Wilcox, 1987; Brambilla *et al.*, 1996; Kontinen and Meert, 2002). Moreover, EAAT 2 has also been shown to play a vital role in numerous models of visceral pain (Bradesi *et al.*, 2011) with an increased expression showing antinociceptive effects (Lin *et al.*, 2009a). Moreover, pharmacological activation of these transporters has also demonstrated therapeutic potential (Gosselin *et al.*, 2010).

Many chronic pain disorders are also included in categories of stress-induced disorders as a significant proportion of patients attribute the onset and exacerbation of their symptoms to an early-life stressful period or chronic stress throughout life. Indeed, it is now becoming more evident that females appear more sensitive to the negative effects of stress (Bangasser *et al.*, 2013). ELS during childhood is related to increased risk to develop depression, anxiety and chronic pain in adulthood (Raphael and Widom, 2011; Burke *et al.*, 2017). Maternal separation in the early days of life in animals is a widely used model of ELS and has been used to elucidate the underlying mechanisms of depression as well as chronic pain disorders (O'Mahony *et al.*, 2009; O'Mahony *et al.*, 2011; Moloney *et al.*, 2015b). The model is based on the evidence that adverse environmental alterations during early-life can cause long-lasting effects into adulthood, e.g., increased visceral pain sensitivity (O'Mahony *et al.*, 2011). In the present study, we aimed to assess whether maternal separation and the oestrous cycle alter visceral sensitivity and if changes in phenotype were associated with changes in excitatory

amino acid transport via glial uptake in the lumbosacral spinal cord and the ACC, two critical regions within the CNS, known to play a role in visceral pain processing (Lieberman and Eisenberger, 2015; Moloney *et al.*, 2015b).

3.3 Material and Methods

3.3.1 Animals

Adult male and female Sprague-Dawley rats (250-300g) (Harlan, UK) were used as breeding partners to generate offspring in this study. Upon arrival, animals were housed according to sex, four to five animals per cage in plastic cages and were maintained in a temperature-controlled room (20±1 °C) with a 12-hour light/dark cycle (7:00 am to 7:00 pm). The animals were allowed one week to acclimatise to the animal facility in University College Cork after arrival. Breeding pairs were housed together until confirmation of pregnancy. Females were then group housed throughout gestation until gestation day 19 after which time they were housed singly and allowed to give birth. Two cohorts of animals were used in the current study; (1) the first cohort was used for behavioural analysis of visceral sensitivity. (2) The second cohort was used for naïve sample collection to assess EAAT function. Group sizes were 9-10 animals and was based on previous experiments. All experiments were conducted in accordance with the European Directive 2010/63/EU and approved by Animal Experimentation Ethics Committee of University College of Cork.

3.3.2 Maternal Separation

Maternal Separation was performed from postnatal day 2 (PND 2) to PND 12 inclusive as previously described (Neumann *et al.*, 2005; O'Mahony *et al.*, 2009). Briefly, litters were randomly assigned to either the NS group or the maternally separated (MS) group. NS animals were left undisturbed except for routine husbandry practices. MS pups were separated daily from their mothers in a separate room and placed in a clean cage with fresh bedding, placed on top of heated pads (30°C–33°C), for 3 hours from 9:00 am until 12:00 pm after which time animals were placed back in their mother's home cage. Following the separation period (PND2-12) animals were left undisturbed except for weekly cage cleaning. Offspring were

weaned and sexed at PND 21. Animals were allowed to mature to adulthood (8 weeks), and all female animals were used for the remainder of the study.

3.3.3 Vaginal Smears

Females were vaginally lavaged daily with saline for at least two consecutive oestrous cycles, and cells were immediately viewed under a microscope prior to behavioural assessment. Those rats that were regularly cycling were used in this study. The stage of oestrous cycle was determined as previously described (Ji *et al.*, 2008). Since metestrus only lasts for a short period (5-6 hours) and the plasma oestrogen concentration in metestrus do not differ from that in diestrus, data from these two groups of rats were pooled. For cohort 1, the behavioural study, animals were lavaged immediately before balloon insertion into the colorectum, to assess oestrous stage during visceral pain measurement. For cohort 2, tissue collection only, the animals underwent vaginal smears for cycle phase estimation immediately before decapitated for tissue collection. Toward the end of each study there was occasions where the lavaged rats were not in the correct phase (we aimed for 9-10 per group), and then we waited a day or two until we lavaged again to check the stage and if it fit into the groups that required more numbers.

3.3.4 Colorectal Distension

CRD was performed as previously described (O'Mahony *et al.*, 2010; O'Mahony *et al.*, 2012). Briefly, animals were fasted overnight and anaesthetised with isoflurane (3-5% in oxygen) followed by insertion of a 6 cm latex balloon into the colorectal cavity, 1 cm from the anus. The animals were allowed to recover for 10 minutes before CRD commenced (9:00 am to 12:00 pm), in unrestrained freely moving animals. The paradigm used was an ascending phasic distension from 0 mmHg to 80 mmHg over 8 minutes. The parameters of interest were (1) the threshold pressure (mmHg) that evokes the first visually identifiable visceral pain

behaviour and (2) the total number of pain behaviours over the distension period. Postures defined as visceral pain behaviours were abdominal retractions and/or abdominal withdrawal reflex. The experimental groups were randomised, and behavioural testing was performed by an experimenter blinded to treatment groups to eliminate any bias. All animals underwent CRD only once.

3.3.5 Sample Preparation for Aspartate Transport Assay

The animals were euthanised by decapitation immediately after vaginal smearing. Their spinal cords were removed by hydraulic pressure into HBSS filled Petri dishes, and 0.4 mm thick slices were obtained from lumbosacral spinal cord using a McIlwain tissue chopper. Similarly, brain tissue was removed from the skull and sectioned using a vibratome to obtain ACC sections. These slices were separated by fine dissection under a microscope and transferred to 24-well culture plates filled with both sodium-containing HBSS (labelled as Na^+) and sodium-free HBSS (labelled as Na^-) separately. The Na^+ plate was maintained at 35 °C and Na^- on the ice. The slices from Na^+ plate were washed once with 1 mL of 35 °C HBSS and Na^- plate with 1 mL of 4 °C sodium-free HBSS to assess sodium-dependent and independent uptake respectively.

3.3.6 Aspartate Transport Assay

Since EAATs show high-affinity to both glutamate and aspartate, we used aspartate. This protocol has been previously described in brain slices (Thomazi *et al.*, 2004); we optimised this technique to be used on spinal cord slices for the first time. Aspartic Acid, D-[2, 3-^{3}H] (specific activity 12.9 Ci/mmol) was purchased from Perkin-Elmer, USA. RIPA buffer and Pierce BCA protein assay kit were purchased from Fisher Scientific Ireland. All other reagents were purchased from Sigma-Aldrich. HBSS was prepared containing (in mM): 137 NaCl; 0.63

Na_2HPO_4; 4.17 $NaHCO_3$; 5.36 KCl; 0.44 KH_2PO_4; 1.26 $CaCl_2$; 0.41 $MgSO_4$; 0.49 $MgCl_2$ and 1.11 glucose, in pH 7.2. In sodium-free HBSS, NaCl was replaced by 137 mM N-methyl-D-glucamine.

Spinal cord and ACC slices were pre-incubated at 35 °C and ice in Na^+ and Na^- HBSS respectively for 30 min. Then a solution containing 1µL 0.66 µCi/mL aspartic acid D- [2,3-^{3}H], 15 µL of cold 100 µM D-Aspartate and 84 µL H_2O was added. After 3 and 7 minutes of incubation of spinal cord and ACC respectively, the slices were washed twice with 1 ml of corresponding ice-cold HBSS. Tissue was transferred into 1.5 ml tubes containing RIPA buffer and was mechanically dissociated with pestles. This mixture was homogenised for 15 minutes at 4 °C and residue was removed. Radioactivity was measured in terms of counts per minute (CPM) using a liquid scintillation counter and results for Na^- samples were subtracted from those of Na^+ samples to achieve sodium-dependent uptake-hallmark of EAAT 1 & 2 function. Uptake procedure was performed in triplicate. Protein was measured using Peirce BCA protein assay kit. Final scintillation result for each slice was divided by respective protein value to achieve aspartate uptake in terms of CPM/mg-indicative of spinal and cortical EAAT's function.

3.3.7 Statistical Analysis

All data was normally distributed according to Gaussian distribution analysis. Data are expressed as mean ± SEM. Two-Way-ANOVA and Tukey post-hoc test were used in all cases. $P < 0.05$ were considered statistically significant. The sample size (n=9/10) was based on previous studies showing that it was sufficient to observe statistically significant results.

3.4 Results

3.4.1 Early-life Stress and Oestrous Cycle-dependent Variations in Visceral Sensitivity

A two-way ANOVA analysis of threshold sensitivity revealed a significant effect of stress ($F_{(1, 50)} = 11.42$, $p < 0.01$), oestrous cycle ($F_{(2, 50)} = 6.847$, $p < 0.01$) and an interaction effect of stress x oestrous cycle ($F_{(2, 50)} = 5.561$, $p < 0.01$, Figure 3.1A, n=9/10 per group). Overall MS rats displayed a lower threshold compared to NS rats. Post-hoc analysis revealed significant differences between groups with NS animals in both the proestrus ($p < 0.0001$) and oestrus ($p < 0.01$) phases exhibiting decreased threshold values compared to NS metestrus/diestrus animals. In addition, a difference was noted between MS rats and NS rats in metestrus/diestrus ($p < 0.0001$). There were no between-group differences observed in MS animals.

A two-way ANOVA analysis of total pain behaviours also revealed a significant effect of stress ($F_{(1, 50)} = 8.657$, $p < 0.01$) and a significant effect of oestrous cycle ($F_{(2, 50)} = 3.571$, $p < 0.05$) but no interaction effect of oestrous cycle x stress ($F_{(2, 50)} = 1.305$, $p > 0.05$, Figure 3.1B, n=9/10 per group). Overall MS rats showed a higher number of pain behaviours, with post-hoc analysis revealing significantly increased total pain behaviours in the NS animals in the proestrus ($p < 0.01$) phase compared to NS animals in the metestrus/diestrus phase. There were no between-group differences observed in MS animals.

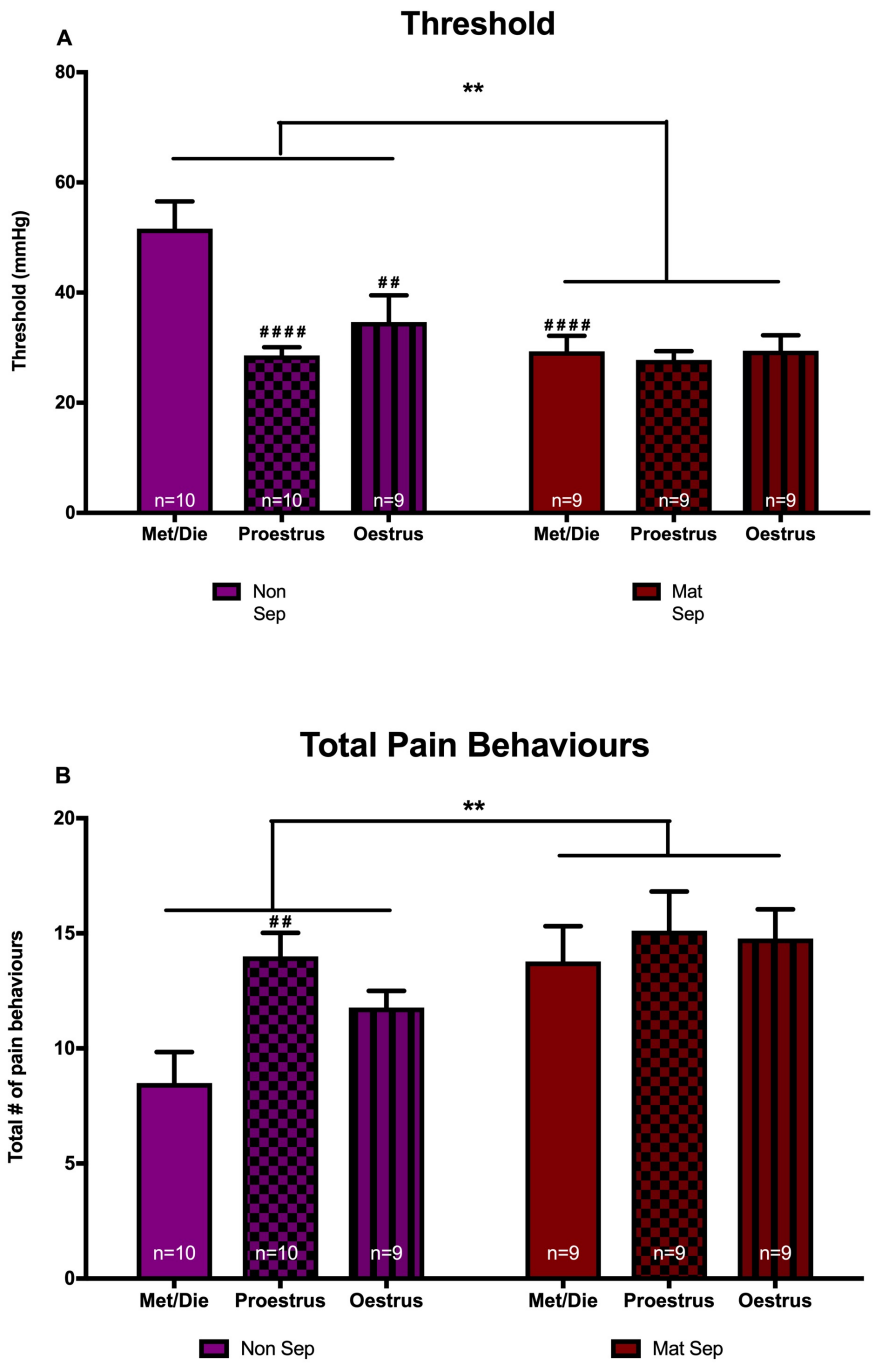


Figure 3.1. Early-life stress and oestrous cycle-dependent variations in visceral sensitivity. MS animals exhibit visceral hypersensitivity with a significantly lower threshold of visceral distension required for identifiable abdominal contraction [A] and increased total pain behaviours [B] compared to NS (**$p < 0.01$ non-sep vs mat-sep, $^{\#\#}p < 0.01$, $^{\#\#\#\#}p < 0.0001$ vs. met/die non-sep, n=9-10/group).

3.4.2 Early-life Stress and Oestrous Cycle-dependent Variations in Central EAAT Activity.

3.4.2.1 Lumbosacral Spinal Cord

Two-way ANOVA analysis of EAAT function within the lumbosacral spinal cord revealed a significant effect of the oestrous cycle ($F_{(2, 53)} = 4.349$, $p < 0.05$, Figure 3.2, n=10 per group) but no significant effect of stress or interaction effect. Post-hoc analysis revealed NS animals in the oestrus phase ($p < 0.01$) and the proestrus phase ($p < 0.05$) to have reduced EAAT function compared to NS animals in the metestrus/diestrus phase.

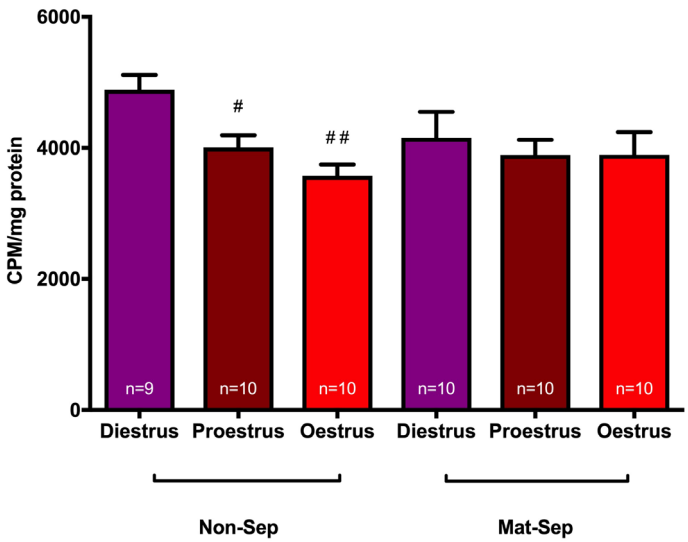


Figure 3.2. Early-life stress and oestrous cycle-dependent variations in spinal EAAT function. Oestrous cycle plays a role in EAAT function in the lumbosacral spinal cord with both the oestrus and proestrus phases of the cycle inducing significant reductions in EAAT function ($^{\#}p < 0.05$, $^{\#\#}p < 0.01$ vs. met/die non-sep, n=9-10/group).

3.4.2.2 Anterior Cingulate Cortex

A significant interaction of stress x oestrous cycle was observed for EAAT function within the ACC ($F_{(2, 51)} = 27.00$, $p < 0.0001$, Figure 3.3, n=10 per group) with post-hoc analysis revealing significant decreases in both the proestrus ($p < 0.0001$) and oestrus ($p < 0.0001$) phases in NS animals. However, cortical EAAT function in MS rats was inhibited in low-oestrogen state, i.e., diestrus, rather than high-oestrogen states- an effect completely opposite to that seen in NS rats. Moreover, a student t-test direct comparison between both

groups showed that aspartate uptake was significantly low in MS diestrus rats compared to NS diestrus rats ($p < 0.0001$).

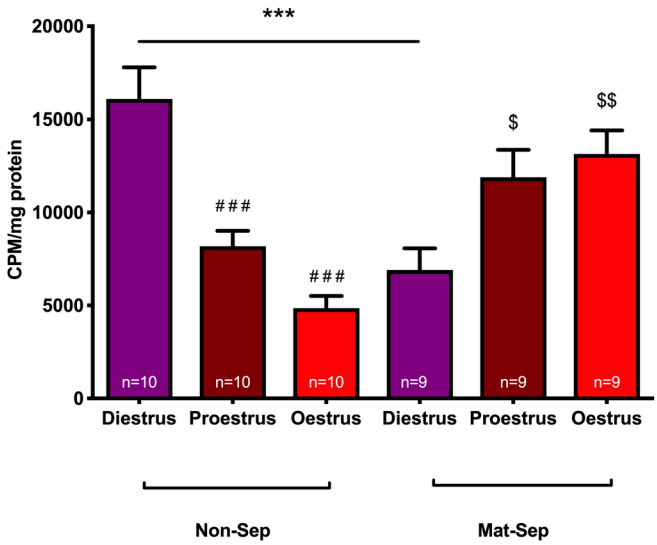


Figure 3.3. Early-life stress and oestrous cycle-dependent variations in central EAAT function. Oestrous cycle and ELS play a role in EAAT function in the ACC with both the oestrus and proestrus phases of the cycle inducing significant reductions in EAAT function in NS rats ($^{####}p < 0.0001$ vs met/die non-sep, n=10/group) and oestrus and proestrus phases showing increased EAAT function in MS rats ($^{\$}p < 0.05$, $^{\$\$}p < 0.01$ vs. met/die mat-sep, n=10/group). Diestrus phase aspartate uptake was significantly low in MS group compared to NS group ($^{***}p < 0.0001$, n=9-10).

3.5 Discussion

Here we show that ELS and the oestrous cycle play significant roles in visceral sensation. MS animals displayed similar visceral pain responses at all stages of the cycle while in NS controls, fluctuations in visceral sensitivity occurred across the cycle. We noted an early-life stress-induced increase in visceral sensitivity in the combined metestrus and diestrus phases but not in the proestrus and oestrus phases.

Furthermore, excitatory amino acid transport, both within the lumbosacral spinal cord and the ACC, were also altered in response to stress and oestrous cycle, thus indicating that aberrant excitatory transport may in part lead to fluctuations in visceral sensitivity.

Functional gastrointestinal disorders, characterised by visceral pain have been more commonly reported in females, in particular, premenopausal women, with increasing focus on the effect of hormonal cycles on pain processing (Mayer *et al.*, 2004). Female IBS patients report increased visceral pain during menses suggesting enhanced visceral sensitivity during the perimenstrual period (Heitkemper and Chang, 2009b). The hormonal cycle in the rodent is much shorter than that in humans with the cycle completed in just 4-5 days. Progressing through the stages may occur over the course of just a few hours, making it difficult to investigate the role of the oestrous cycle in nociceptive assays due to the inconsistent methodology. For this reason, many studies disregard the cycle (Kamp *et al.*, 2003; Tammpere *et al.*, 2005; Arvidsson *et al.*, 2006), however, it is increasingly more appreciated that this is a significant confound and makes the interpretation of data difficult. Indeed, the oestrous stage has previously been shown to alter the responses in somatic nociceptive assays, in particular, the tail flick test (Martinez-Gomez *et al.*, 1994; Kayser *et al.*, 1996; Fillingim and Ness, 2000). This current study found significant changes in visceral sensitivity across the stages of the oestrous cycle in controls; with heightened visceral pain response in the proestrus and

oestrous phases. This is in agreement with previous reports (Sapsed-Byrne *et al.*, 1996; Ji *et al.*, 2008), however, it is pertinent to note that other studies have shown findings on the contrary, with evidence showing no changes in visceral pain throughout the cycle (Ji *et al.*, 2006). These discrepancies seen in animal models may be due in part to species and strain differences but also the impact of stress. Although not widely acknowledged in the literature, the methodology used to assess oestrous stage, i.e., vaginal lavage, can be stressful to animals and may result in changes in the cycle. Thus, it is important to acclimate animals to the vaginal smearing process itself as was performed in this study.

The most potent and prevalent endogenous oestrogen, oestradiol, is at its highest level during the proestrus phase, which also coincides with the highest level of visceral pain sensitivity, highlighting the potential role of the gonadal hormones in visceral pain. Moreover, its contribution to visceral hypersensitivity has been investigated in animal models through ovariectomy and hormone replacement studies (Ji *et al.*, 2003; Myers *et al.*, 2011). These studies reported reduced visceral sensitivity in ovariectomised females compared with intact females. Oestrogen replacement was reported to increase visceral sensitivity back to levels of that seen in the intact females (Ji *et al.*, 2003). Furthermore, the implantation of micropellets containing oestradiol and progesterone into the amygdala increased visceral pain behaviours in ovariectomised rats, with no differences seen when placed in neighbouring brain regions (Myers *et al.*, 2011). Interestingly no changes were observed in somatic pain thresholds. These findings suggest that female sex steroids do indeed play a significant role in visceral nociception.

Stress and altered HPA axis function are significant risk factors for the development of visceral pain. Thus, it is interesting to note that hormonal fluctuations have also been shown in stress responsivity and altered neurobiology of the HPA axis. Females have been found to

have higher corticotropin-releasing hormone, adrenocorticotropic hormone, and corticosterone levels during proestrus, the phase of the cycle in which oestradiol levels are higher than during other phases of the oestrous cycle (Buckingham *et al.*, 1978; Bohler *et al.*, 1990; Atkinson and Waddell, 1997). Furthermore, plasma adrenocorticotrophic hormone and corticosterone levels in response to stress are higher during proestrus than during other phases of the cycle (Pollard *et al.*, 1975; Viau and Meaney, 1991; Carey *et al.*, 1995). Moreover, neurotransmitter systems implicated in the control of HPA function (Kaneko and Hiroshige, 1978; Plotsky *et al.*, 1989) show variations related to the oestrous cycle and are sensitive to gonadal steroid levels (Rance *et al.*, 1981a; Rance *et al.*, 1981b; Biegon and McEwen, 1982). Indeed, here we specifically focus on the glutamatergic system and the role of glial glutamate transport in visceral nociception.

Here we show that the activity of glutamate transporters in the lumbosacral spinal cord and ACC are significantly decreased during the phases of the oestrous cycle when visceral sensitivity is higher. The EAATs are responsible for removing excess glutamate from the synaptic cleft thereby reducing the availability of glutamate and preventing central sensitisation. Studies from our group have shown that expression of these transporters is reduced in animal models of heightened sensitivity to colorectal distension including the maternal separation model (Gosselin *et al.*, 2010) as well the CBA/J strain of mouse (Moloney *et al.*, 2015a).

Water avoidance stress has also been shown to alter expression levels of these transporters in the spinal cord which was positively correlated with pain behaviours (Bradesi *et al.*, 2011; Lin *et al.*, 2011). Taken together, it is plausible that glutamate uptake enhancers may prove to be a novel therapeutic strategy for the treatment of visceral hypersensitivity (Gosselin *et al.* 2010). Indeed, recent studies show downregulation of spinal EAAT transporters

in various models of chronic pain (Mao *et al.*, 2002; Sung *et al.*, 2003; Weng *et al.*, 2005). Moreover, the importance of glial glutamate uptake is further highlighted by reports where EAAT overexpression reduces pain sensitivity (Lin *et al.*, 2009a; Lin *et al.*, 2011; Yang *et al.*, 2011). Previous studies have shown higher glutamate concentration in ACC in animals exposed to ELS (Mathew *et al.*, 2003).

Overall, in MS rats, stress increased visceral sensitivity in metestrus/diestrus phases but had no effect in proestrus and oestrus compared to NS rats. The reason for this is not clear, but it is interesting to note that glutamate uptake in the spinal cord paralleled this. However, uptake in the ACC was opposite to the that of the NS controls. This may be compensatory as uptake was higher in the proestrus and oestrus in the MS animals, which may indicate that higher activity of these transporters is necessary to maintain behaviours at the same level. Moreover, it appears that visceral sensitivity in MS rats is linked to changes in EAAT function in the ACC, while both the spinal and ACC EAAT function was related to pain behaviours in the NS group. Both ACC and spinal cord EAAT function are reduced in the higher oestrogen state, i.e., proestrus and ensuing oestrus, paralleling that of the pain behaviours. Other studies have indicated that oestrogen can affect the expression of the EAATs as well as L-glutamate uptake activity in cultured midbrain astrocytes (Pawlak *et al.*, 2005). Oestrogen exerts if effects through either membrane-bound or nuclear receptors and stimulation of PI3-kinase coupled to nitric oxide production may be involved in the inhibitory regulation of glutamate transporter activity by oestrogen. The presence of nuclear oestrogen receptors in astrocytes has been demonstrated in-vitro and in-vivo (Garcia-Ovejero *et al.*, 2002). Moreover, it should be appreciated that the expression of oestrogen receptors can change (Garcia-Ovejero *et al.*, 2002) and hence influence the level of impact of oestrogen changes also.

Our data further support an important role of glutamate transport at both the level of the spinal cord but also higher brain centres in the pathophysiology of visceral pain.

3.6 Conclusion

The findings presented here highlight the important contribution of female sex hormones to the control, processing, and manifestation of visceral pain. Indeed, our studies suggest a complex link between steroid signalling, stress, and glutamatergic neurotransmission. Taken together these findings have added to the accumulating literature implicating the role of sex hormones and glutamate in visceral pain.

Chapter 4

Spinal and Cortical Aspartate Uptake is Associated with Gut Microbiota in Wistar-Kyoto Rats- An Animal Model of Visceral Hypersensitivity

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4.1 Abstract

Background: Chronic stress, pain, and depression are interrelated. Oestrous cycle influences glutamatergic signalling in the central nervous system (CNS) through the involvement of hypothalamic-pituitary-adrenal (HPA) axis. Repeated stimulation of HPA axis in chronic stress may lead to important structural and functional changes in the CNS affecting neurotransmission leading to depressive characteristics. There is evolving evidence that microbiota-gut-brain axis is involved in the pathogenesis of certain CNS disorders and it may have a role in altered pain signalling in CNS. Here we further investigated changes in central glutamatergic system in Wistar-Kyoto (WKY) rats (an animal model of depression) with respect to oestrous cycle. We also evaluated if caecal microbiota had any correlation with changes in central glutamatergic system.

Methods: We assessed the functional activity of the EAATs in the lumbosacral spinal cord and ACC in WKY rats. Additionally, the caecal microbiota composition of these animals was investigated.

Results: EAAT function was similar in males compared to females in all phases of the oestrous cycle at the level of lumbosacral spinal cord. In the ACC, the glutamate transporter uptake was higher in diestrus females compared to males, proestrus and oestrus females. In males, aspartate uptake in the ACC was found to correlate positively with *Bacteroides*. Aspartate uptake in the spinal cord was found to correlate positively with relative abundances of the *Lachnospiraceae NK4A136* in males. Further positive associations with aspartate uptake in the spinal cord were also observed for *Alistipes* and *Bifidobacteruim* during oestrus in females, as well as for the *Eubacterium coprostanoligenes* during proestrus. *Clostridium sensu stricto1* was also found to be negatively associated with aspartate uptake in the ACC in males and diestrus females.

Discussion: This data suggests that EAAT function is not regulated by oestrogen in the lumbosacral spinal cord. In the ACC, glial transporter function changed across oestrous cycle, indicating that glutamate metabolism in areas of the brain processing affective components of pain is dependent on oestrous phase. Short-chain fatty acid (SCFA) producing gut bacteria seem to have positive correlation with aspartate uptake in males and oestrous state specific females.

4.2 Introduction

Chronic stress has been linked with the aetiogenesis of a number of disorders including chronic pain syndromes, autoimmune disorders, hypertension and depression (O'Malley *et al.*, 2011). In fact, the patients with chronic pain states such as irritable bowel syndrome have elevated baseline levels of norepinephrine in their blood indicating the relationship of stress and the pain disorders (Posserud *et al.*, 2004). Chronic stress can lead to abnormal changes in the CNS, which may, in turn, dysregulate the body response to stress resulting in increased pain sensitivity. Activation of the HPA axis plays an important role in the stress responses through the secretion of cortisol from adrenal glands. Repeated stimulation of the HPA axis over an extended period of time has been shown to be implicated in the development of depression and IBS (Dinan *et al.*, 2008; Thomson and Craighead, 2008).

The rodent adult females have higher basal and stress-state levels of ACTH and corticosterone as compared to males (Lesniewska *et al.*, 1990a). Moreover, there is a growing evidence toward the role of gonadal hormones in the regulation of HPA axis. For example, female rats have higher blood CRH, ACTH, and corticosterone levels during high-oestrogen state, i.e., proestrus compared to diestrus and oestrus (McCormick *et al.*, 2002). Fluctuations in the circulating levels of oestrogen, during the menstrual cycle, can also lead to variation in the symptomatology of chronic pain syndromes through the activation of glutamatergic receptors (Heitkemper and Chang, 2009b; Mermelstein, 2009). Moreover, gonadal hormones have been shown to affect visceral sensitivity in animal models significantly; oestrogen being pronociceptive in rodents (Ji *et al.*, 2003; Myers *et al.*, 2011).

The WKY rat strain demonstrates hyperresponsiveness to stress (Pare, 1993; De La Garza and Mahoney, 2004) and elevated basal and stress-state ACTH and corticosterone levels compared to other strains (Rittenhouse *et al.*, 2002). Moreover, these animals showed a

decrease in expression of the astrocytic marker glial fibrillary acidic protein (GFAP)(Gosselin *et al.*, 2009) and increased pain induced activation in the prefrontal cortex (infralimbic, prelimbic and ACC) when compared to Sprague-Dawley rats (Gibney *et al.*, 2010). Changes in GFAP expression have been shown to modify glial glutamate metabolism through direct effects on EAAT's activity (Sullivan *et al.*, 2007), which is also implicated in major depression (Choudary *et al.*, 2005). Previously, WKY rats have been shown to be more sensitive to painful stimuli in tests such as colorectal distension and tactile allodynia, compared to Sprague-Dawley (SD) rats in both male and female sexes (SM *et al.*, 2013; Ackerman *et al.*, 2015). However, there is no comparative study available in the literature comparing the pain responses in two sexes of WKY rats, as previously done on SD rats. In female WKY rats, the responses to painful stimuli across the oestrous cycle have currently not been demonstrated.

Multiple studies now imply that the visceral pain can by influenced by the gut microbiota (Table 1.6). In rodent studies, probiotics have been shown to decrease stress-induced or antibiotic-induced visceral pain (Verdu *et al.*, 2006; Ait-Belgnaoui *et al.*, 2009; McKernan *et al.*, 2010). Some probiotics have been shown to alleviate abdominal pain in the humans (Curro *et al.*, 2017). Interestingly, visceral hypersensitivity can be transferred via the microbiota of IBS patients to animals previously lacking microbes (Crouzet *et al.*, 2013). However, the mechanisms underlying the effects of the microbiota on visceral pain perception remain to be explained.

There is a paucity of data examining whether glutamate metabolism is altered in the key pain processing central nervous areas, in relation to the physiological alterations of circulating gonadal hormones; and if present how do these changes correlate with depression and gut microbiota. Thus, here we aim to investigate the changes in glial aspartate uptake in the spinal cord and ACC, in response to intrinsically alternating blood oestrogen levels during

various oestrous cycle stages in WKY rats, an animal model of depression. We also studied if there was a correlation between aspartate uptake and faecal microbiota.

4.3 Material and Methods

4.3.1 Animals

Adult WKY rats (weighing 125-150 g females and 150-175 g males) were housed in a local animal facility with food and water ad libitum, on a 12:12-hour dark–light cycle (lights on at 7:00 AM) with the temperature at 20°C ± 1°C. Animals were group housed by 4 to 5 per cage in plastic cages with sawdust bedding, shredded paper, and a cardboard roll. They were allowed to habituate in the new environment for a week before the commencement of experiments. Female rats underwent vaginal smearing on the day of each experiment to assess their oestrous cycle phase (Byers *et al.*, 2012). One cohort of 10 males and 30 female rats was used for the aspartate uptake studies. All experiments were in full accordance with the European Community Council Directive (2010/63/EU) and approved by Animal Experimentation Ethics Committee of University College Cork.

4.3.2 Vaginal Smears

Females were vaginally lavaged with saline, and cells were viewed under a microscope. The stage of oestrous cycle was determined as previously described (Ji *et al.*, 2008). Since metestrus only lasts for a short period (5-6 hours) and the plasma oestrogen concentration in metestrus do not differ from that in diestrus, data from these two groups of rats were pooled. Animals were lavaged immediately before euthanasia, to determine oestrous stage accurately for all tissue collected.

4.3.3 Sample Preparation for Aspartate Transport Assay

The animals were euthanised by decapitation immediately after vaginal smearing. Spinal cords were removed by hydraulic pressure into HBSS filled Petri dishes and 0.4 mm thick, slices were obtained from lumbosacral spinal cord using a McIlwain tissue chopper.

Brain tissue was removed from the skull and sectioned using a vibratome to obtain ACC sections. These slices were separated by fine dissection under a microscope and transferred to 24-well culture plates filled with both sodium-containing HBSS (labelled as Na^+) and sodium-free HBSS (labelled as Na^-) separately. The Na^+ plate was maintained at 35 °C and Na^- plate was kept on ice. The slices from the Na^+ plate were washed once with 1 mL of 35 °C HBSS and slices from Na^- plate were washed with 1 mL of 4 °C sodium-free HBSS to assess sodium-dependent and independent uptake, respectively.

4.3.4 Aspartate Transport Assay

Since EAATs show high-affinity to both glutamate and aspartate, we chose aspartate for uptake study as it is widely available. This protocol has been previously described in brain slices (Thomazi *et al.*, 2004). We have optimised this technique to be used on spinal cord slices for the first time. Aspartic Acid, D-[2, 3-^{3}H] (specific activity 12.9 Ci/mmol) was purchased from Perkin-Elmer, USA. RIPA buffer and Pierce BCA protein assay kit were purchased from Fisher Scientific Ireland. All other reagents were purchased from Sigma-Aldrich. HBSS was prepared containing (in mM): 137 NaCl; 0.63 Na_2HPO_4; 4.17 $NaHCO_3$; 5.36 KCl; 0.44 KH_2PO_4; 1.26 $CaCl_2$; 0.41 $MgSO_4$; 0.49 $MgCl_2$ and 1.11 glucose, in pH 7.2. In sodium-free HBSS, NaCl was replaced by 137 mM N-methyl-D-glucamine.

Spinal cord and ACC slices were pre-incubated at 35 °C and ice in Na^+ and Na^- HBSS respectively for 30 min. Following this, a solution containing 1µL 0.66 µCi/mL aspartic acid D-[2,3-^{3}H], 15 µL of cold 100 µM D-Aspartate and 84 µL H_2O was added. After 3 and 7 minutes of incubation of spinal cord and ACC respectively, the slices were washed twice with 1 ml of corresponding ice-cold HBSS. Tissue was transferred into 1.5 mL tubes containing RIPA buffer and was mechanically dissociated with pestles. This mixture was homogenised for 15 minutes at 4 °C, and the residue was removed. Radioactivity was measured in terms of scintillations

per minute (CPM) using a liquid scintillation counter and the results for Na^- samples were subtracted from those of Na^+ samples to achieve sodium-dependent uptake-hallmark of EAAT 1 & 2 function. Uptake procedure was performed in triplicate. Protein was measured using Peirce BCA protein assay kit. Final scintillation result for each slice was divided by respective protein value to achieve aspartate uptake in terms of CPM/mg which is indicative of spinal and cortical EAAT's function.

4.3.5 Caecal Microbiota Analysis

In order to acquire caecal microbiota data, caecal DNA was extracted, followed by Illumina MiSeq sequencing and bioinformatics analysis.

A 0.25 g aliquot of the faecal sample was added to 1 mL lysis buffer (500 mM NaCl, 50 mM Tris-HCl, pH 8.0, 50 mM EDTA, and 4% sodium dodecyl sulfate) together with 0.4g of zirconia beads. The samples were homogenised for 3 minutes using the Biospec Minibeadbeater at maximum speed. The homogenised samples were then heated to 70°C for 15 minutes and then centrifuged at 16,000 xg for 5 minutes. The supernatant was then removed, fresh lysis buffer added and the beadbeating, heating and centrifugation steps repeated. The nucleic acids were then precipitated using 10 M ammonium acetate, followed by addition of isopropanol. The nucleic acids were then pelleted, washed and resuspended in 1X TE buffer. Subsequently, removal of RNA, protein, and purification of the DNA was completed using components of the QIAGEN QIAamp DNA Stool Mini kit along with the wash buffers and elution buffer, and DNA was stored at -20 °C.

The V3-V4 variable region of the 16S rRNA gene was amplified from the DNA extracts using the 16S metagenomic sequencing library protocol (Illumina). The DNA was amplified with primers specific to the V3-V4 region of the 16S rRNA gene which also incorporates the Illumina overhang adaptor (Forward primer 5'

TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG; reverse primer 5' GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC). Each PCR reaction contained DNA template, 5 µL forward primer (1 µM), 5 µL reverse primer (1 µM), 12.5 µL 2X Kapa HiFi Hotstart ready mix (Anachem, Dublin, Ireland), PCR grade water to a final volume of 25 µL. PCR amplification was carried out as follows: heated lid 110°, 95 °C x 3 minutes, 25 cycles of 95 °C x 30 s, 55 °C x 30 s, 72 °C x 30 s, then 72 °C x 5 mins and held at 4 °C. PCR products were visualised using gel electrophoresis (1X TAE buffer, 1.5 % agarose, 100 V). Successful PCR products were cleaned using AMPure XP magnetic bead based purification (Labplan, Dublin, Ireland). A second PCR reaction was completed on the purified DNA (5 µl) to index each of the samples, allowing samples to be pooled for sequencing on the one flow cell and subsequently demultiplexed for analysis. Two indexing primers (Illumina Nextera XT indexing primers, Illumina, Sweden) were used per sample. Each PCR reaction contained 5 µL index 1 primer (N7xx), 5 µL index 2 primer (S5xx), 25 µL 2x Kapa HiFi Hot Start Ready mix, 10 µL PCR grade water. PCRs were completed as described above, but only 8 amplification cycles were completed instead of 25. PCR products were visualised using gel electrophoresis and subsequently cleaned (as described above). Samples were quantified using the Qubit™ 3.0 Fluorometer (Biosciences, Dublin, Ireland) along with the high sensitivity DNA quantification assay kit. Samples were then pooled in an equimolar fashion. The sample pool was prepared following Illumina guidelines. Samples were sequenced on the MiSeq sequencing platform (Clinical Microbiomics, Denmark), using a 2 x 250 cycle kit, following standard Illumina sequencing protocols.

250 base pair paired-end reads were assembled using FLASH (FLASH: fast length adjustment of short reads to improve genome assemblies). 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. Singleton removal (to ensure a minimum of 2 copies to be counted), denoising, chimera detection and clustering into operational taxonomic units (OTUs) (97% identity) were performed using USEARCH v7 (64-bit) (Edgar, 2010). OTUs were aligned using PyNAST (PyNAST: python nearest alignment space termination; a flexible tool for aligning sequences to a template alignment) and taxonomy was assigned using BLAST against the SILVA SSURef database release v123. Data were not rarefied/normalised for further analysis. The richness and α-diversity indices were generated in QIIME Version 1.9.0 (Caporaso *et al.*, 2010).

4.3.7 Statistical Analysis

Aspartate assay data were normally distributed according to Gaussian distribution analysis. Data are expressed as mean ± SEM. One-way-ANOVA and Tukey post-hoc test were used in all cases. $P < 0.05$ was considered statistically significant. The sample size (n=10) was based on previous studies showing that it was sufficient to observe statistically significant results. Spearman rank correlation coefficients were calculated for determination of the existence of significant associations between aspartate uptake and microbial relative abundances in the gut. *P*-values were corrected for multiple comparisons as described by Benjamini and Hochberg (1995). Canonical Correspondence Analysis (CCA) was carried out using the vegan package in R (Oksanen *et al.*, 2018).

4.4 Results

4.4.1 Sex and Oestrous Cycle Have no Effect on the Spinal Glial Aspartate Uptake

The aspartate uptake in the lumbosacral spinal cord of male WKY rats was similar to that of females. Moreover, there was no statistically significant change noticed in spinal aspartate uptake across the oestrous cycle stages (Figure 4.1).

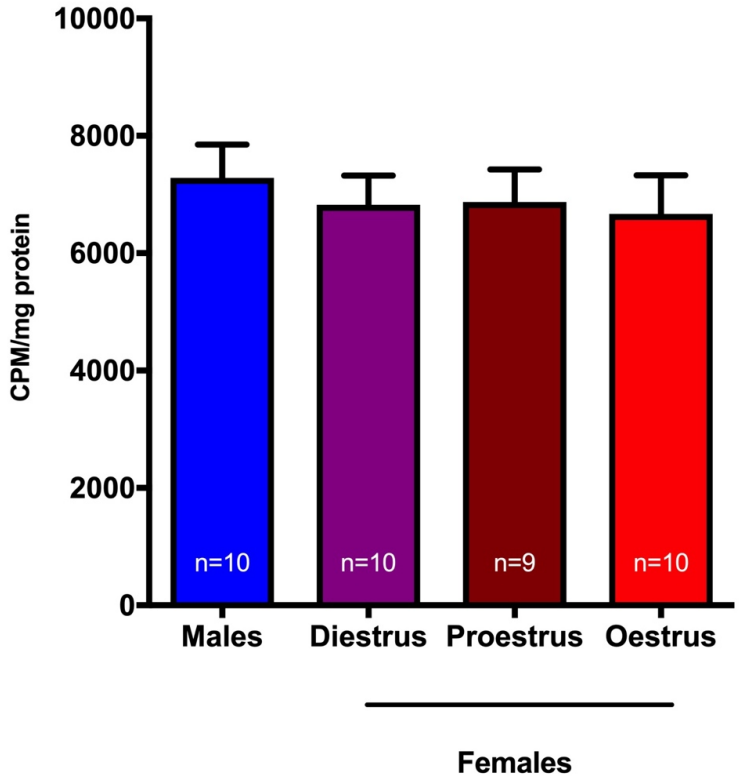


Figure 4.1. Aspartate uptake in lumbosacral spinal cord. Uptake was similar in males and females across all oestrous stages (n=9-10/group).

4.4.2 Aspartate Uptake in ACC Relates to Oestrous Cycle

Here we show that the oestrous cycle had a significant effect on the cortical aspartate uptake ($p < 0.01$, $F_{(3,34)} = 6.4$, Figure 4.2). Post-hoc analysis revealed that EAAT activity was considerably lower in the high-oestrogen state, i.e., proestrus and the following oestrus state as compared to diestrus female rats ($p < 0.01$ and $p < 0.05$ respectively).

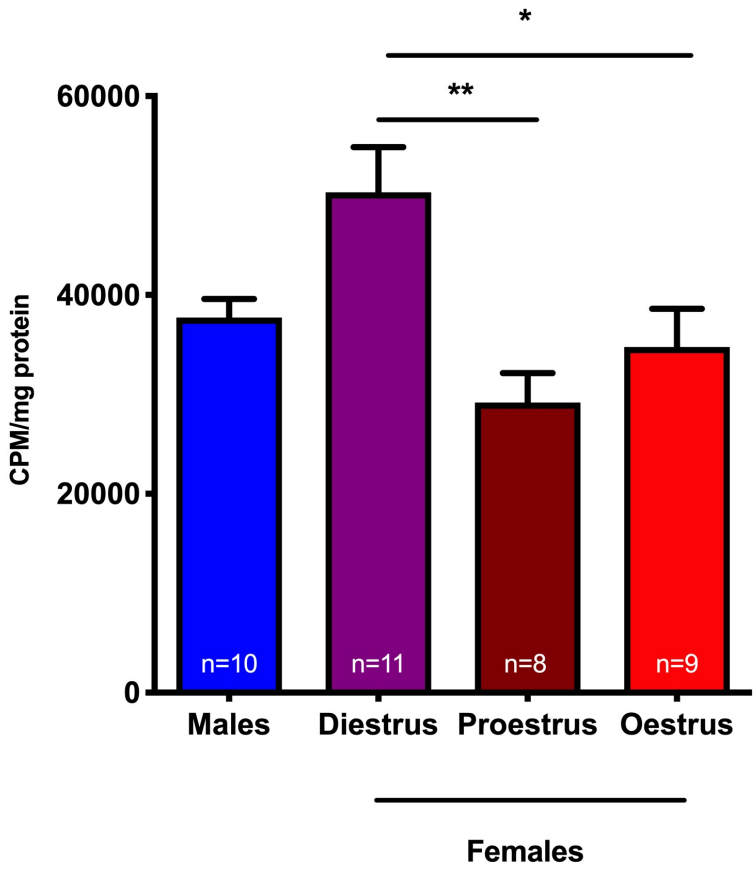


Figure 4.2. Aspartate uptake at ACC. Aspartate uptake in females shows significant oestrogen-dependent changes in EAAT activity (one-way ANOVA, $p < 0.01$, $F_{(3,34)} = 6.4$, n=9-10/group). Uptake was higher in diestrus compared to proestrus females (**$p < 0.01$, Tukey's post-hoc) and oestrus females (*$p < 0.05$, Tukey's post-hoc).

4.4.3 Associations between Bacterial Relative Abundances in the Gut and Aspartate Uptake in the ACC and Spinal Cord.

Changes in relative abundances of the most prominent bacterial taxa are illustrated as a function of increasing levels of aspartate uptake in the ACC and spinal cord between samples at the genus level in females during different stages of the oestrous cycle as well as in males (Figure 4.3A). CCA revealed that the effect of aspartate uptake in the ACC on bacterial dispersion was orthogonal to the effect exerted by aspartate uptake in the spinal cord (Figure 4.3B). However, neither aspartate uptake in the ACC ($p = 0.912$) nor that in the spinal cord ($p = 0.586$) were found to significantly influence the gradient of bacterial dispersion between samples with a clear majority of taxa observed to cluster together at the centre of the plot. Sample groups also failed to form distinct clusters indicating that differences in the

relationships between bacterial abundances of the gut microbiota and aspartate uptake in the ACC and spinal cord are not evident according to either gender or oestrous cycle stage. Despite this, significant associations were observed between aspartate uptake and the relative abundances of individual taxa at the genus level (Figure 4.3C). Aspartate uptake in the ACC was found to correlate positively with *Bacteroides* (q = 0.0003; Spearman's rho 0.967) but negatively with *Clostridium sensu stricto1* (q = 0.0215; Spearman's rho -0.816) in males. *Clostridium sensu stricto1* was also found to be negatively associated with aspartate uptake in the ACC (q = 0.0091; Spearman's rho -0.905) during diestrus in females, as was *Alloprevotella* (q = 0.0145; Spearman's rho -0.881). Aspartate uptake in the spinal cord was found to correlate positively with relative abundances of the *Lachnospiraceae NK4A136 group* (q = 0.0427; Spearman's rho 0.766) in males. Further positive associations with aspartate uptake in the spinal cord were also observed for *Alistipes* (q = 0.0344; Spearman's rho 0.783) and *Bifidobacteruim* (q = 0.0427; Spearman's rho 0.766) during oestrus in females, as well as for the *Eubacterium coprostanoligenes group* (q = 0.0333; Spearman's rho 0.943) during proestrus.

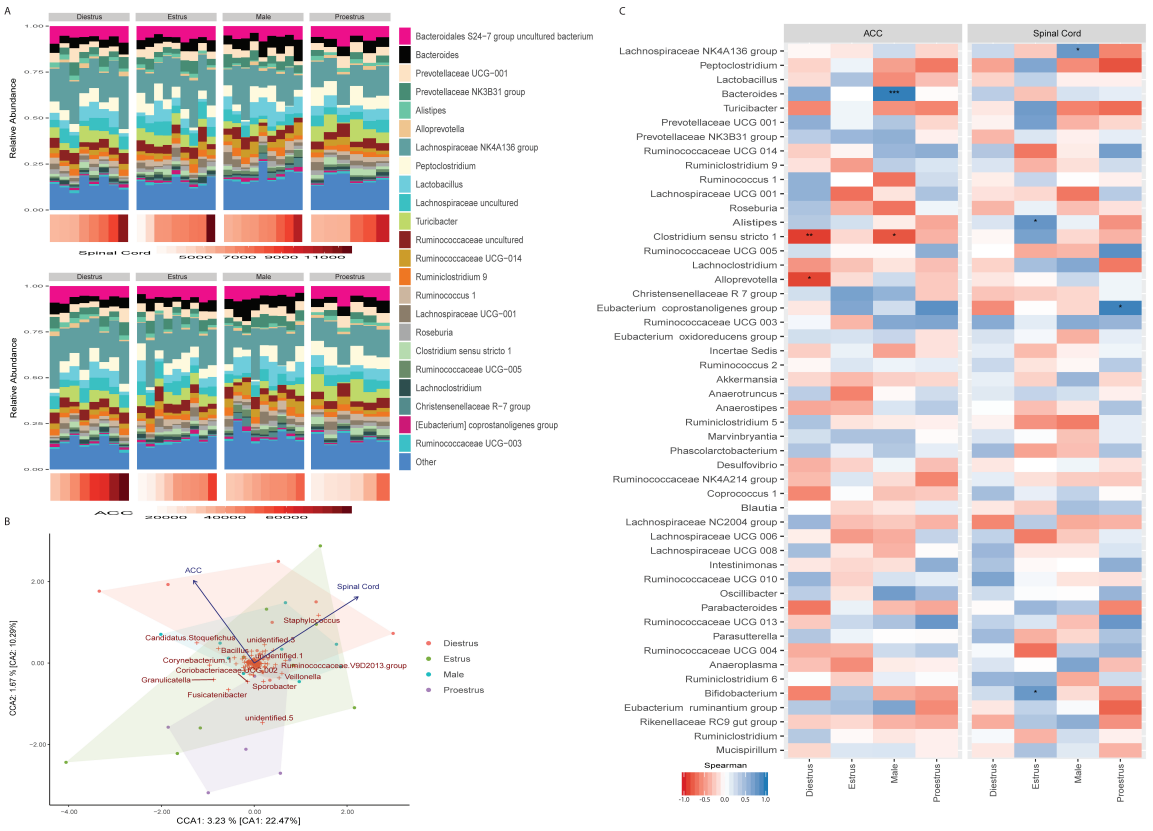


Figure 4.3. Associations between bacterial relative abundances and aspartate uptake in the ACC and Spinal Cord. A) The Influence of increasing aspartate uptake, in the ACC and spinal cord respectively, on sample relative abundances at the genus level, both in males and across the oestrous cycles in females. B) CCA of microbiota composition regressed on measures of aspartate uptake in both the ACC and spinal cord. Filled circles represent samples while red crosses represent bacterial taxa (genus level) with the top 5% of taxa which best fit the axes being indicated by name. The ACC and Spinal Cord vectors each point to the direction of taxa which exhibit the strongest association with the variable while their magnitude indicate the strength of the variable in explaining the bacterial dispersion observed. The Canonical variates explain 14.4% and 16.3% of the total explainable variance in the first and second axis respectively. C) Heat map of the 50 most abundant bacterial taxa (genus level) correlated against aspartate uptake in the ACC and spinal cord using Spearman's correlation (* $q < 0.05$; ** $q < 0.01$; *** $q < 0.001$; q = False Discovery Rate adjusted *p*-value (q-value) after Benjamini-Hochberg multiple testing corrections).

4.5 Discussion

Here we demonstrate that the circulating oestrogen regulates the function of sodium-dependent glutamate transporters in female WKY rats. Cortical EAAT activity was upregulated in the diestrus stage of the oestrous cycle when compared with males, proestrus females and oestrus females. However, there was no such change in glutamate metabolism seen at the level of lumbosacral spinal cord. In males, aspartate uptake in the ACC and spinal cord was found to correlate positively with *Bacteroides* and *Lachnospiraceae NK4A13,6,* respectively. In females, positive associations with aspartate uptake in the spinal cord were observed for *Alistipes* and *Bifidobacteruim* during oestrus, as well as for the *Eubacterium coprostanoligenes* during proestrus.

The lumbosacral spinal cord is an important part of the CNS in relation to processing of pain signals from most of the abdominal viscera. Afferents from the viscera play little role in the hormonal modulation of pain responses (Ji *et al.*, 2012). A decreased expression of EAAT in the spinal cord of females WKY rats exposed to chronic stress has been shown to be associated with exaggerated pain responses (Ackerman *et al.*, 2015). In our study, there was no change observed in the function of lumbosacral spinal EAATs, between the sexes and across oestrous cycle stages. This may suggest that in this animal strain, the hormonal state-dependent function of EAATs may have more of a role in pain processing along the higher ascending pain pathways rather than lumbosacral cord.

The ACC is an area of the brain that interconnects other key areas of the brain involved in cognition and behaviour such as frontal cortex, the thalamus, and the amygdala (Vogt, 2005; Shackman *et al.*, 2011). Clinical studies have demonstrated activation of ACC using imaging techniques in response to painful stimuli, suggesting its role in the pain processing (Fomberstein *et al.*, 2013). The rostral part of ACC is particularly involved in the processing of

affective components of pain (Johansen *et al.*, 2001) and increased expression of glutamate receptors in ACC has been observed in rats who are hypersensitive to visceral pain (Fan *et al.*, 2009b). As chronic pain and depression are closely related, various studies have investigated and elucidated the role of ACC in the development of depression (Pizzagalli, 2011). ACC volume reduction changes on MRI have been reported in depressed patients (Drevets *et al.*, 1998), and some studies have shown ablative procedures such as anterior cingulotomy to be effective in drug-resistant depressed patients (Shields *et al.*, 2008). The reduction in the volume of ACC in depressed patients is believed to be caused by a decrease in glial density rather than neuronal tissue (Drevets *et al.*, 1998; Rajkowska and Miguel-Hidalgo, 2007). Since glial cells participate in the uptake and recycling of glutamate, reduction in glial density seems to be accountable for the reported decreased glutamate levels in depressed patients (Taylor *et al.*, 2009). In an animal model of depression, i.e., WKY rats, the glutamate receptors have been reported as either less functional or less in number (Lei *et al.*, 2009). A decrease in functional connectivity of ACC has also been noted in other behavioural CNS disorders, such as autism (Zhou *et al.*, 2016). Gut microbiota seems to influence the structure and functioning of ACC significantly. In germ-free (GF) mice, the volumes of ACC was seen to be reduced along with dendritic changes (Luczynski *et al.*, 2017). Interestingly, feeding mice with *Bacteroides Fragilis* reversed the autism like behaviour in these mice (Li *et al.*, 2017a). Our study showed that *Bacteroides* had a positive correlation with ACC glial aspartate uptake in males. Interestingly, this effect was sex-specific. Moreover, *Lachnospiraceae had* a positive correlation with lumbar spinal glial aspartate uptake in males. *Lachnospiraceae* and *Alistipes* abundance has been found to be reduced in animal models of major depressive disorder (Jiang *et al.*, 2015; Li *et al.*, 2017b). Intriguingly in our data, *Bifidobacterium* correlated positively with aspartate uptake in oestrus female rats. Probiotic *Bifidobacterium longum* has been

shown in animal models to reduce depression symptoms (Pinto-Sanchez *et al.*, 2017). In summary, SCFA-producing bacteria, i.e. *Bacteroides*, *Lachnospiraceae*, and *Bifidobacterium* had a positive correlation on ACC and spinal cord in a sex-dependent manner.

Oestrogen has a significant influence on the glutamate metabolism through the downregulation of EAATs via the nitric oxide pathway (Sato *et al.*, 2003). The high-oestrogen state, i.e., proestrus showed a marked decreased activity of EAATs in ACC in our study, compared to diestrus and oestrus. SCFAs have been shown to enhance cellular oestrogen sensitivity through mitogen-activated protein kinase pathway (Jansen *et al.*, 2004) and through this pathway, SCFAs-producing gut bacteria may have shown positive correlations with aspartate uptake in WKY rats that exhibit phenotypic features of depression with an altered ACC structure and function.

Our data suggests that excitatory glial transport in the ACC is dependent on the oestrous cycle phase of the WKY female rats and gut microbiota may be an important modulating factor. As ACC is related to affective aspects of pain, our study suggests future preclinical and clinical trials evaluating pain associated behavioural traits in both sexes and across the oestrous/menstrual cycle in females, with an emphasis on gut microbiota analysis.

Chapter 5

Gender Differences in the Role of Gut Microbiota in Pain Sensitivity

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5.1 Abstract

Relative to men, women present with some chronic pain conditions more commonly. Consistent differences exist between healthy men and women in terms of pain perception. The underlying mechanisms of these differences are incompletely understood and could inform the development of effective treatments for pain. The gut microbiota can influence nervous system functioning, including sensory signalling. We hypothesised that the gut microbiota and components of the gut-brain axis may influence electrical pain thresholds. Further, we hypothesised that gender, menstrual cycle, and hormonal contraceptive use may account for inter-gender differences in pain perception.

Non-obese males (n=15) and females (n=16), of which nine were using hormonal contraceptives, were recruited. Male subjects were invited once, whereas females were invited three times across the menstrual cycle, based on self-reported early follicular (EF), late follicular (LF) and mid-luteal (ML) phase. On test days, electrical stimulation on the right ankle was performed. Cortisol levels were measured in morning saliva samples, levels of lipopolysaccharide-binding protein (LBP), soluble CD14 (sCD14) (only EF/ML), TNF-α, IL-8, and IFN-γ were measured in plasma and faecal microbiota-, and short-chain fatty acid (SCFA) composition was determined in the stools.

We observed that, in women but not in men, abundance of genus *Anaerostipes* (linked to butyrate formation) correlated positively with electrical pain sensation threshold (PST); a trend was also observed toward positive correlation between prevalence of genus *Anaerostipes* and pain tolerance threshold (PTT) (False discovery rate (FDR) <0.1), in women but not in men. Interestingly, PTT/PST was significantly lower in women than men, but not PST and PTT. Further, hormonal contraceptive use was associated with increased LBP levels (LF & ML phase), but not sCD14 and inflammation.

Differential butyrate formation and absorption in healthy men and women may account for inter-gender differences in electrical pain perception. Further research is needed to investigate the potential of PTT/PST as a measure of inter-individual electrical pain tolerance differences, and to examine whether hormonal contraceptives increase LBP levels independently of gastrointestinal permeability.

5.2 Introduction

Chronic pain is a major healthcare challenge as it confers a substantial burden on individuals, employers, health care systems and society in general (Breivik *et al.*, 2013). Understanding the mechanisms which regulate pain sensitivity is important for the development of new effective treatments.

Several chronic pain conditions are more prevalent among women than men. In general, laboratory tests demonstrate that women exhibit greater pain sensitivity compared with men (Mogil, 2012). Differences in pain perception (tolerance & threshold), between adult men and women, are greatest for electrical stimulation and pressure, whereas inter-gender differences in heat pain threshold are highly variable when assessing differences between men and women (Riley *et al.*, 1998). Interestingly, in a meta-analysis including only healthy children, a greater number of studies demonstrated no significant inter-gender differences in pain perception; however, for those of age > 12 years, girls reported greater pain intensity in response to the cold pressor test compared to boys (Boerner *et al.*, 2014). This suggests that the onset of puberty and the associated hormonal changes may contribute to pain gender bias in teenagers and possibly adults (Boerner *et al.*, 2014).

Within each of our gastrointestinal tracts, a unique combination of different communities of organisms exists (O'Mahony, 2017). Microbes actively participate in shaping and maintaining our physiology-almost as an extra organ (Clarke *et al.*, 2014). Furthermore, it is now accepted that important relationships exist between the gut microbiota, mood disorders, bowel disorders and neurodevelopmental disorders (e.g., autism) (Dinan and Cryan, 2017b). Interactions between the gut microbiota and the brain are mediated through the immune system, the neuroendocrine system (e.g., tryptophan metabolism) and neural

pathways (vagus nerve) (Dinan and Cryan, 2017b). The nervous system is a master regulator of host function and, by signalling within this system, bacteria influence complex physiological processes. Gut bacteria are active producers of metabolites such as SCFAs, enabling the host nervous system to sample the chemical signature of gut bacterial communities. SCFAs such as acetate, propionate, and butyrate can act on intestinal epithelial cells through G protein-coupled receptors 41, 43 and 109A, and also can directly or indirectly influence peripheral organs (Canfora *et al.*, 2015; Morris *et al.*, 2016). For example, at the intestinal level, dysbiosis and subsequent low levels of the important epithelial cell fuel, butyrate, can adversely affect intestinal barrier integrity and drive inflammatory diseases (Canfora *et al.*, 2015; Morris *et al.*, 2016).

Microbial composition or activity of the gut can be modified by diet, antibiotics, host genetics, and stress (O'Mahony *et al.*, 2017). Altered gut microbiota is also associated with changes to the intestinal permeability, low-grade inflammation and metabolic dysfunction (Cani *et al.*, 2012). Microorganisms or components of microorganisms are able to activate macrophages and increase levels of pro-inflammatory cytokines, such as TNF-α. Administration of lipopolysaccharide (LPS), a cell wall component of Gram-negative bacteria increases several cytokines (TNF-α, interleukins (IL) 6, 8 and 10), cortisol and lowers pressure pain threshold (Wegner *et al.*, 2014) and electrical pain threshold (de Goeij *et al.*, 2013). Hence, increased systemic levels of bacterial components may initiate a reduction of pain thresholds and contribute to chronic pain through activation of inflammatory and stress pathways.

The microbiota and its manipulation have been associated with several types of pain including visceral pain (O'Mahony *et al.*, 2014), inflammatory pain (Amaral *et al.*, 2008),

migraine (van Hemert *et al.*, 2014), interstitial cystitis (Braundmeier-Fleming *et al.*, 2016), as well as autoimmune-related pain in rheumatoid arthritis (McLean *et al.*, 2015). We have shown preclinically that manipulating the microbiota with antibiotics increases visceral pain (O'Mahony *et al.*, 2014) and leads to changes in signalling pathways in the spinal cord (O'Mahony *et al.*, 2014). Furthermore, we have shown that germ-free mice show a reduced pain tolerance to colorectal distension than conventionally colonised mice (Luczynski *et al.*, 2017). Sex differences are noted in the composition of the gut microbiota with clear effects of gonadectomy and hormone replacement on gut bacteria (Markle *et al.*, 2013; Org *et al.*, 2016). Whether the gut microbiota is involved in the aetiology of somatic pain disorders or gating somatic pain sensitivity and pain tolerance in healthy individuals is understudied. Sex-specific associations have been shown for pain symptoms in myalgic encephalomyelitis/chronic fatigue syndrome (ME/CFS) patients (Wallis *et al.*, 2016). In male ME/CFS patients, *Eubacterium* was negatively correlated to pain symptoms, whereas *Lactobacillus* and *Streptococcus* were positively correlated to pain symptoms (Wallis *et al.*, 2016). In contrast, *Streptococcus* was negatively correlated to pain symptoms in female ME/CFS patients (Wallis *et al.*, 2016).

Whether specific microbiota taxa are related to pain thresholds at the visceral or somatic level in chronic pain patients, or pain disorder-free individuals remains to be investigated. Here, we examined gut microbiota abundance, cortisol awakening response, inflammatory factors and those associated with gut permeability in association with gender differences in electrical pain sensitivity in healthy adult volunteers.

5.3 Materials & methods

5.3.1 Study Population

Students and employees of University College Cork were recruited and screened through a survey. The following inclusion criteria were used: 18 -35 years of age, regular menstrual cycle (28±4 days), no hormonal treatment during the past three months, and body mass index (BMI) <30 kg/m^2. All subjects were screened through an online survey. Exclusion criteria were: current / recent (3 months) use of psychotropic drugs, beta-blockers or analgesic medication (24 h before measurements), neurological disease, diabetes mellitus and gastrointestinal disease (e.g., inflammatory bowel disease, inflammatory bowel syndrome). 15 non-obese men and 16 non-obese women were included. Eight women used oral contraceptives, and one woman used a Nuova ring (etonogestrel / ethinyl oestradiol vaginal ring). Each was asked not to engage in strenuous physical activities, sleep regular hours and refrain from alcohol the day before the experiment. Smoking was not allowed for a period of 2 h before the test session. This pilot study was approved by the local ethical committee of University College Cork (UCC) (CREC: ECM 3 granted on the 7th July 2015).

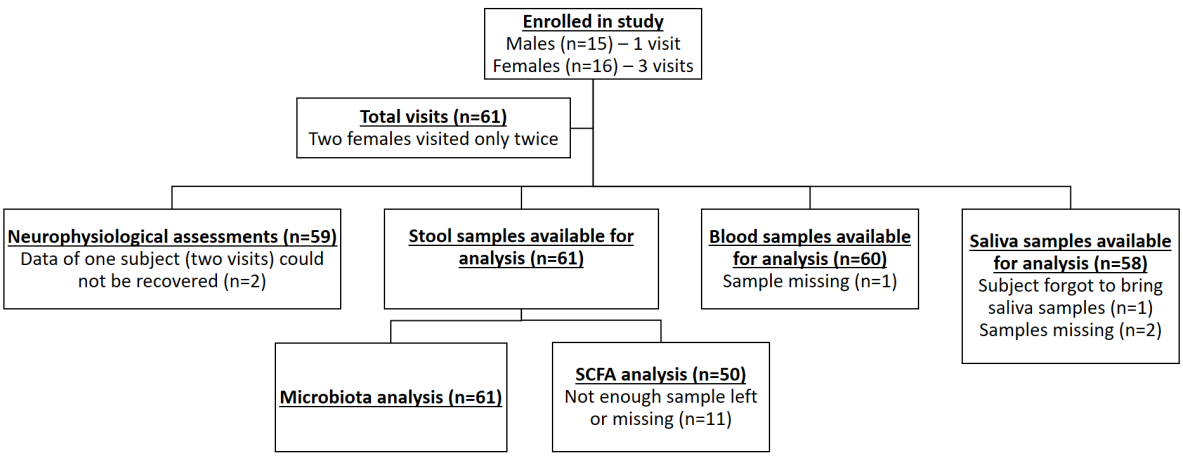


Figure 5.1. Flowchart availability raw data and samples. stool, blood and saliva samples and neurophysiological assessments available for analysis.

Of the recruited population, two females only visited twice (Figure 5.1). Raw data/samples of 59, 61, 50, 60 and 58 subjects were available for the analyses of neurophysiological assessments, faecal microbiota, faecal SCFAs, blood plasma markers, and salivary cortisol, respectively (Figure 5.1). Age, smoker's status, native English language rate were evaluated through (online) questionnaires (Table 5.1).

Table 5.1. Age, smoker's status and native English language performance of the study population

Characteristic	*Male (n=15)*	*Female HC (n=9)*	*Female nHC (n=7)*
Age	23.5 ±1.1	22.8 ±1.3	26.1 ±1.8
Smoker	3 (20%)	1 (11%)	1 (14%)
Native English speaker	13 (87%)	6 (67%)	2 (29%)

Data is presented as mean ± SEM. Abbreviations: HC = hormonal contraceptive users, nHC = non-hormonal contraceptive users

5.3.2 Beck's Depression Inventory

The Beck's depression inventory (Beck *et al.*, 1961) was completed by 21 subjects of whom 19 answered all questions. Only fully completed questionnaires were used for further analyses.

5.3.3 Hospital Visits and Sample Collection

Male subjects were invited to attend at Cork University Hospital once, whereas females were invited to attend three times across a single menstrual cycle. A menstrual cycle diary was provided to record the menstruation onset of the current menstrual cycle. Self-reported menstrual cycle days 2-7, 9-13, and 18-23 were defined as early follicular phase (EF), late follicular (LF) phase, and mid-luteal (ML) phase, respectively. During these visits, subjects brought saliva and stool samples, which were subsequently stored at -80 °C. Furthermore, an electrical stimulation test was performed, and blood samples were collected.

5.3.4 Saliva Sample Collection

Subjects were asked to collect saliva samples upon awakening and 30, 45 and 60 minutes after awakening in a *salivette* through chewing on a cotton swab for around 1.5 minutes and transferring the bud from the mouth directly in the tube, without using hands/fingers. Next, they were instructed to place *salivettes* directly into a refrigerator, or into a plastic container with an ice pack prior to refrigerator placement. Additional notes included instructions not to brush teeth throughout the collection process, drink or eat anything prior to sample 1, between samples 2, 3 and 4, or 15 minutes prior to sample 2.

5.3.5 Stool Sample Collection

Subjects were asked to collect stool samples as fresh as possible, ideally produced on the morning of the visit. If morning collection was not possible, they were asked to collect a sample from the evening/night before and store it in a cool place. Samples were collected in a plastic container with an anaerobic gas producing sachet (Thermo Fisher Scientific, Massachusetts, USA) Subjects were asked to store samples in a cool area prior to attendance at the Hospital.

5.3.6 Neurophysiological Assessments

Quantitative sensory testing was performed by a trained clinician. The pain perception threshold to transcutaneous constant current electrical stimulation was assessed on subjects lying supine in a warm, quiet environment using Neuropack-S neuro-diagnostic stimulator with disposable surface electrodes (Natas Medical Instruments Inc, California, USA). Pain perception and pain tolerance thresholds were recorded during electrical stimulation; manual 5 mA current increase (up to maximum 100 mA) using a standardised technique at the right ankle just below the medial malleolus. Stimuli were set at 1 Hz, duration 200 µs. If two

threshold values differed by > 10% between runs, testing was repeated until three consecutive thresholds were recorded, each within 10% of the others. Data for one subject could not be recovered, so data of 30 subjects instead of 31 were used for further analysis.

5.3.7 Blood Sample Collection

Venous blood was collected in EDTA tubes by a single venepuncture from antecubital fossa after completion of the neurophysiology assessment. Blood samples were centrifuged, plasma was separated and stored at -80 °C.

5.3.8 Faecal Microbiota Analysis

In order to acquire faecal microbiota data, faecal DNA was extracted, followed by Illumina MiSeq sequencing and bioinformatics analysis.

A 0.25 g aliquot of the faecal sample was added to 1 mL lysis buffer (500 mM NaCl, 50 mM Tris-HCl, pH 8.0, 50 mM EDTA, and 4% sodium dodecyl sulfate) together with 0.4g of zirconia beads. The samples were homogenised for 3 minutes using the Biospec Minibeadbeater at maximum speed. The homogenised samples were then heated to 70°C for 15 minutes and then centrifuged at 16,000 xg for 5 minutes. The supernatant was then removed, fresh lysis buffer added and the beadbeating, heating and centrifugation steps repeated. The nucleic acids were then precipitated using 10 M ammonium acetate, followed by addition of isopropanol. The nucleic acids were then pelleted, washed and resuspended in 1X TE buffer. Subsequently, removal of RNA, protein, and purification of the DNA was completed using components of the QIAGEN QIAamp DNA Stool Mini kit along with the wash buffers and elution buffer, and DNA was stored at -20 °C.

The V3-V4 variable region of the 16S rRNA gene was amplified from the DNA extracts using the 16S metagenomic sequencing library protocol (Illumina). The DNA was amplified with primers specific to the V3-V4 region of the 16S rRNA gene which also incorporates the

Illumina overhang adaptor (Forward primer 5' TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG; reverse primer 5' GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC). Each PCR reaction contained DNA template, 5 µL forward primer (1 µM), 5 µL reverse primer (1 µM), 12.5 µL 2X Kapa HiFi Hotstart ready mix (Anachem, Dublin, Ireland), PCR grade water to a final volume of 25 µl. PCR amplification was carried out as follows: heated lid 110°, 95 °C x 3 minutes, 25 cycles of 95 °C x 30 s, 55 °C x 30 s, 72 °C x 30 s, then 72 °C x 5 mins and held at 4 °C. PCR products were visualised using gel electrophoresis (1X TAE buffer, 1.5 % agarose, 100 V). Successful PCR products were cleaned using AMPure XP magnetic bead based purification (Labplan, Dublin, Ireland). A second PCR reaction was completed on the purified DNA (5 µl) to index each of the samples, allowing samples to be pooled for sequencing on the one flow cell and subsequently demultiplexed for analysis. Two indexing primers (Illumina Nextera XT indexing primers, Illumina, Sweden) were used per sample. Each PCR reaction contained 5 µL index 1 primer (N7xx), 5 µL index 2 primer (S5xx), 25 µL 2x Kapa HiFi Hot Start Ready mix, 10 µL PCR grade water. PCRs were completed as described above, but only 8 amplification cycles were completed instead of 25. PCR products were visualised using gel electrophoresis and subsequently cleaned (as described above). Samples were quantified using the Qubit™ 3.0 Fluorometer (Biosciences, Dublin, Ireland) along with the high sensitivity DNA quantification assay kit. Samples were then pooled in an equimolar fashion. The sample pool was prepared following Illumina guidelines. Samples were sequenced on the MiSeq sequencing platform (Clinical Microbiomics, Denmark), using a 2 x 250 cycle kit, following standard Illumina sequencing protocols.

250 base pair paired-end reads were assembled using FLASH (FLASH: fast length adjustment of short reads to improve genome assemblies). 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. Singleton removal (to ensure a minimum of 2 copies to be counted), denoising, chimera detection and clustering into OTUs (97% identity) were performed using USEARCH v7 (64-bit) (Edgar, 2010). OTUs were aligned using PyNAST (PyNAST: python nearest alignment space termination; a flexible tool for aligning sequences to a template alignment) and taxonomy was assigned using BLAST against the SILVA SSURef database release v123. Data were not rarefied/normalised for further analysis. Richness and α-diversity indices were generated in QIIME Version 1.9.0 (Caporaso *et al.*, 2010).

5.3.9 Faecal Short-chain Fatty Acid Analysis

Faecal supernatant and standards for SCFA analysis were prepared (protocol S1). In short, faecal samples were mixed with 5 mL milliQ/gram, acidified to pH ±2-3, spun and filtered (0.2 μm). The concentration of SCFA was determined by gas chromatography flame ionisation detection (GC-FID) using a Varian 3500 GC system, fitted with a ZB-FFAP column and a flame ionisation detector. Helium was supplied as the carrier gas at an initial flow rate of 1.3 mL/min. The initial oven temperature was 100 °C and was maintained for 0.5 min, and then raised to 180 °C at 8 °C/min and held for 1.0 min, before being increased to 200 °C at 20 °C/min, and finally held at 200 °C for 5.0 min. The temperatures of the detector and the injection port were set at 250 °C and 240 °C, respectively. Samples were run in duplicate, and the injected sample volume was 0.5 μL. Blanks were measured in between different samples to ensure there was no potential carryover from previous samples. Peaks were integrated using Varian Star Chromatography Workstation version 6.0. Subsequently, peaks were quantified, and concentrations of acetate, propionate, isobutyrate, n-butyrate, isovalerate and valerate were calculated. Isobutyrate, isovalerate and valerate were excluded from

further analysis since some samples had concentrations < 0.1 mM (lowest standard) for these SCFA. Acetate concentrations greater than 10 mM (greatest standard) were not excluded. Duplicate samples with a %C.V. > 15% for acetate were repeated. One repeated duplicate had a %C.V. of 16% for acetate and was included. %C.V.s of all duplicates were < 15% and <1 0% for propionate and n-butyrate, respectively. A correction was made for the solution (milliQ and 5M HCl) that was added to the faeces and moles short-chain fatty acid/gram wet faeces were calculated.

5.3.10 Biochemical Analyses of Proteins in Plasma

After thawing, plasma samples were used directly for analysis, or they were distributed in aliquots and frozen again. All plasma aliquots were spun at 1000 G for 10 min prior to analysis to remove excessive (gelatinous) masses. Duplicate oestradiol level measurements were performed in pretreated EDTA plasma samples (dilution factor, 1.6) using an oestradiol parameter assay kit (ELISA, R&D systems, Minneapolis, USA) according to manufacturer's instructions. To determine oestradiol concentrations, a four-parameter logistic curve was generated from the standard dilution series (MyAssays Ltd). Values of one subject fell outside the standard curve and were excluded. Duplicates with calculated concentration intraplate coefficient variation (CV) > 15% were repeated.

Duplicate LBP and soluble cluster of differentiation 14 (sCD14) level measurements were performed in EDTA plasma samples (dilution factor 400, 300 respectively) using commercially available ELISAs for free human LBP (Enzo Life Sciences, New York, USA) and soluble CD14 (R&D systems, Minneapolis, USA). All procedures were carried out according to manufacturer's instructions, except for the fact that a standard GEN5 pathlength correction (absorbance 977 nm, 900 nm) was included as an additional control (BioTek Synergy HT,

Vermont, USA). To determine LBP and sCD14 concentrations, a four-parameter logistic curve was generated from the standard dilution series (MyAssays Ltd). Duplicates of LBP with calculated concentration intraplate CV > 15% were repeated. Calculated concentration intraplate CV of duplicates of sCD14 were ≤10%. Plasma levels of pro-inflammatory cytokines interferon gamma (IFN-γ), IL-1β, IL-6, IL-8, and TNF-α were determined using a V-PLEX MULTI-SPOT assay system (Mesoscale Discovery, Maryland, USA) according to manufacturer's instructions. EDTA plasma samples (dilution factor, 1 or 2) were analysed in duplicate or triplicate. IL-1β and IL-6 data were excluded from further analysis since part of the sample concentrations were below standard curve range or detection range. Duplicates with calculated concentration intraplate CV > 10% for IFN-γ or TNF-α were repeated. Calculated concentration intraplate CV of included duplicates/triplicates were ≤ 10%, ≤ 10% and ≤ 15% for IFN-γ, IL-8, and TNF-α, respectively.

5.3.11 Cortisol Awakening Response

Saliva samples were used directly for cortisol analysis after thawing. All samples were spun at 1000 G for 10 min prior to analysis to remove excessive (gelatinous) masses. Duplicate cortisol level measurements were performed in saliva samples (dilution factor, 2 or 3) using a cortisol ELISA kit (Enzo Life Sciences, New York, USA). All procedures were done according to manufacturer's instructions, except for the fact that a standard GEN5 pathlength correction (absorbance 977 nm, 900 nm) was included as an additional control (BioTek Synergy HT, Vermont, USA) and a standard concentration of 0 pg/mL was included. A logistic curve was generated from the standard dilution series (GEN5), and concentrations were calculated. For validity testing purposes, the concentration of several samples was measured on three different plates. Values of the last plate were used for further analyses, except for 1 duplicate for which practical mistakes were noted. If sample values fell outside the standard curve

range, these were repeated in a lower or higher dilution (dilution factor, 1 or 5). If at least one value of an undiluted measurement was below the standard curve range, or a sample was not available, this resulted in exclusion of the total cortisol awakening response. If calculated intraplate CV for samples was higher than 20%, these were repeated. Area under the curve (AUC) of the cortisol awakening response was calculated by the following formula:

$$AUC=30*([cort]^{t=0min}+[cort]^{t=30min})/2+15*([cort]^{t=30min}+[cort]^{t=45min})/2+15*([cort]^{t=45min}+[cort]^{t=60min})/2$$

Since cortisol awakening response data appeared to be highly vulnerable for variation, Grubbs' tests were performed for the seven groups (males, females using contraceptives (3 phases) and normally menstruating females (3 phases)) to define outliers. If a Grubbs' value was higher than the critical value (α=0.05, two-sided) for 1. cortisol concentration of one of the four timepoints, 2. cortisol %increase over the first 30 minutes or 3. AUC, this resulted in the exclusion of the total cortisol awakening response. For validity determination purposes, cortisol concentration of samples was determined on 3 different plates as described in the previous section. Samples with a calculated intraplate C.V. > 20% or sample analyses of which practical mistakes were noted were excluded from further analysis. Interplate C.V. was calculated for 33 samples.

5.3.12 Statistical Analyses

Kruskal-Wallis and Friedman tests with Dunn's post-hoc test were performed to determine differences between groups or within groups using GraphPad Prism 5. Comparisons with a *p*-value ≤ 0.05 were considered as statistically significant different. For statistical analysis of microbiota relative abundance, Kruskal-Wallis tests were performed using R-studio and Friedman tests were performed using SPSS version 21. FDR-adjusted p-values were calculated for all taxa (phylum, family, genus) that were observed for a certain comparison.

For FDR adjustments, only data of a specific group comparison was taken into account. For example, FDR adjustments were made for Kruskal-Wallis tests that were run for seven groups ([1]males, female contraceptive users ([2]EF, [3]LF, [4]ML), and normally menstruating females ([5]EF, [6]LF, [7]ML), thereby not taking into account comparisons of another Kruskal-Wallis test run for four groups ([1]males, total females ([2]EF, [3]LF, [4]ML). An FDR-adjusted p-value < 0.1 was considered acceptable for Dunn's post-hoc testing using GraphPad Prism 5. For the Dunn's post-hoc test, comparisons with *p*-values ≤ 0.05 were considered as statistically significant different.

Spearman's rank correlations of bacterial genera and other parameters (data of faecal SCFAs, intestinal integrity markers, inflammatory markers, oestradiol, and cortisol) were performed in R-Studio. Two fungal families were removed from the data set. FDR-adjusted p-values were calculated for all correlations that yielded a correlation coefficient. Correlation coefficients and FDR-adjusted p-values were calculated separately for "males only" and "females only."

Correlations between all parameters (except the microbiota) were performed in the same way as the genera correlations described above.

5.4 Results

5.4.1 Age-matched, Non-obese Normally Menstruating Females Score Higher for Beck's Perceived Stress Scale Than Men.

We demonstrated that Beck's depression inventory score was significantly greater in normally menstruating females compared to males, whereas a "trend" toward a greater Beck's score was observed for female contraceptive users compared to males (Supplementary Figure 1). However, it should be noted that for our study the mean score of female responders (8.9) can be linked to a relatively stable mood, since scores >20 indicate moderate+ depression and scores lower than 10 represent 'normal' mood fluctuations.

5.4.2 Oestradiol Status

To confirm oestradiol differences between males and females and differences across the menstrual cycles, total oestradiol levels were measured in plasma. Contrary to expectations, no significant differences were observed between males and females (Supplementary Figure 2). R&D reports in their oestradiol manual a mean 31.7% difference between reference values of (apparently healthy) males and females (EDTA plasma samples). In this study, we observed a non-significant 26.7% difference between female and male samples. Further, we identified a considerable overlap in oestradiol plasma concentrations between males and females (consistent with the reference data provided by R&D). Our data did not demonstrate significant differences across the phases of the menstrual cycle (Supplementary Figure 2). Our specific interest was to include a phase with high levels of oestradiol levels.

5.4.3 Electrocutaneous Pain Assessment

Across the menstrual cycle, we did not observe significant PST and PTT differences in females using hormonal contraceptives and non-users (Figure 5.2). There was no significant difference in PST and PTT between males and females (Supplementary Figure 3).

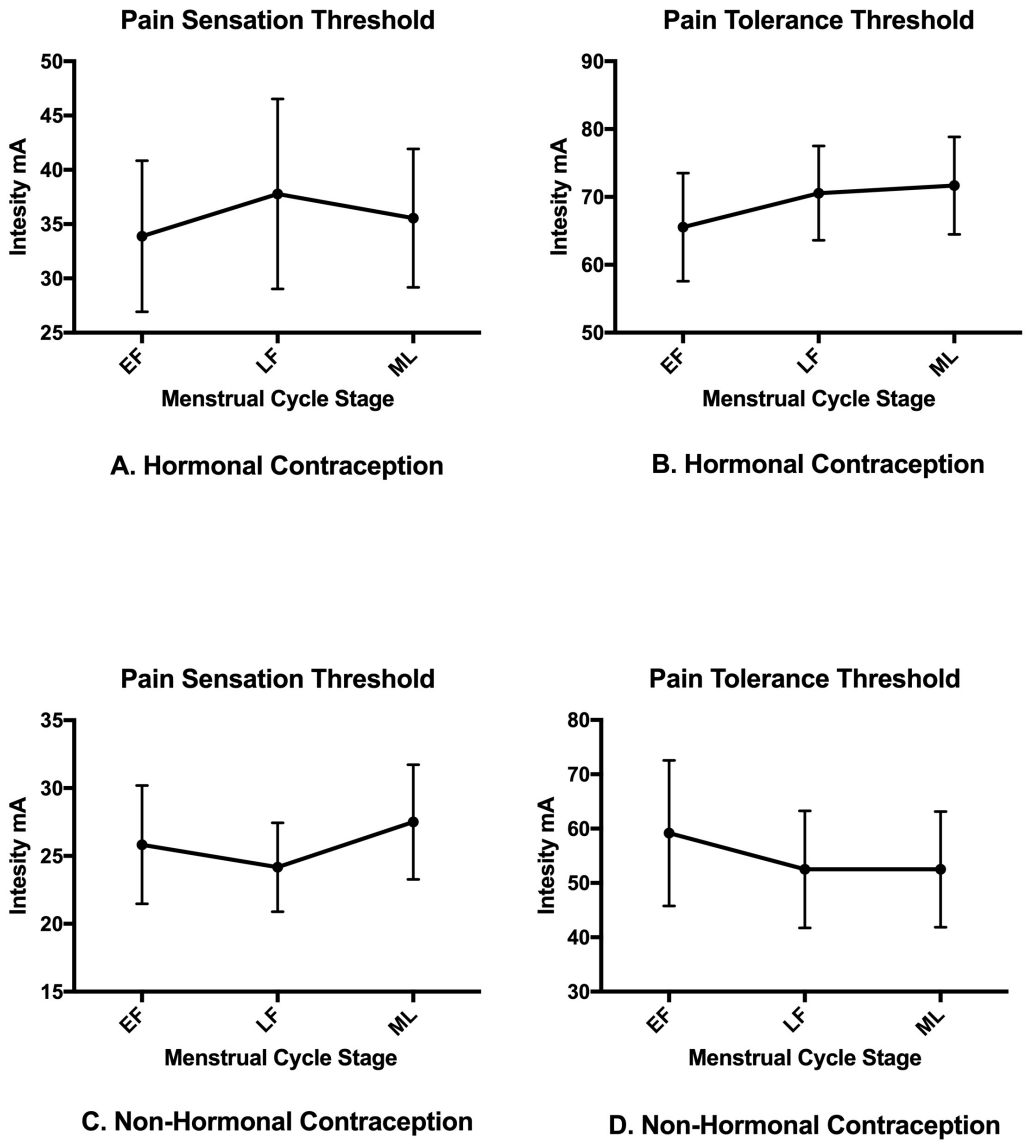


Figure 5.2. Pain tolerance thresholds & pain sensation thresholds did not vary significantly along menstrual cycle. There was no significant difference seen in PTT and PST along menstrual cycle stages in both hormonal and non-hormonal contraception female groups. Similarly, no differences were seen between males and females (data not shown).
Abbreviations: EF = early follicular phase, LF = late follicular phase, ML = mid-luteal phase.

When we investigated the ratio of PTT/PST, we demonstrated significantly lower PTT/PST ratios in females in all phases of the menstrual cycle (EF, ML: $p \leq 0.01$, LF: $p \leq 0.05$) compared to males (Figure 5.3A). In categorising women as contraceptive users or non-users, significantly lesser PTT/PST ratios were demonstrated only for the EF phase of contraceptive users ($p \leq 0.05$) and the ML phase of non-users ($p \leq 0.05$) (Figure 5.3B).

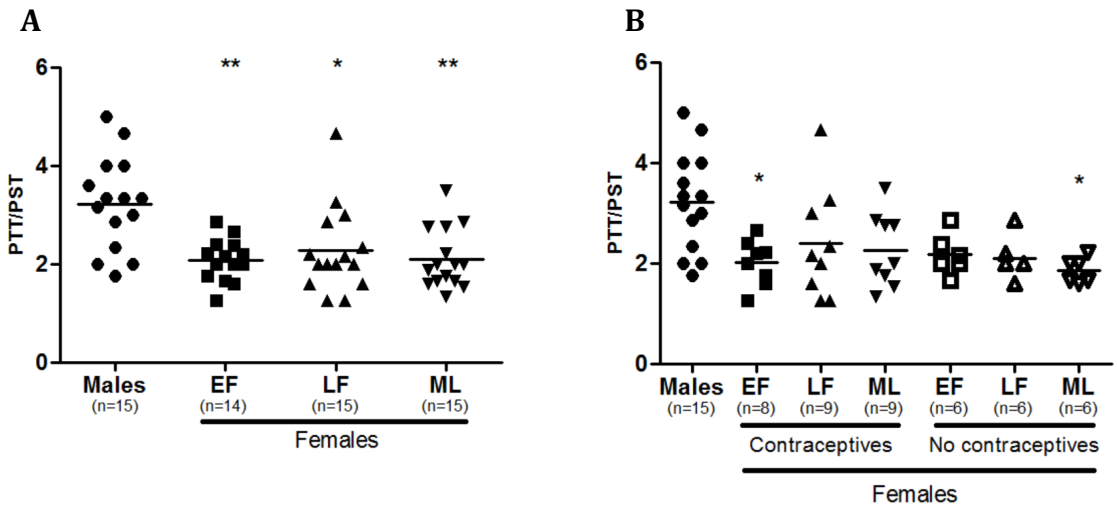


Figure 5.3. The ratio electrocutaneous pain tolerance threshold/pain sensation threshold is lower in women than men. Ratio PTT/PST was determined in males, female contraceptive users and normally menstruating females. Kruskal-Wallis test and Friedman test with Dunn's post-hoc test were performed to assess differences between selected groups, and differences across the menstrual cycle. $^{**}p \leq 0.01$, $^{*}p \leq 0.05$
Abbreviations: EF = early follicular phase, LF = late follicular phase, ML = mid-luteal phase.

5.4.4 Microbiota Composition

To evaluate whether gender, menstrual cycle or hormonal contraceptive use was associated with microbiota composition, richness/α-diversity indices and relative abundance of microbiota were determined. The number of reads and OTUs per sample were variable, whereas the joining efficiency/quality was high (in general over 90%) (Table 5.2).

Table 5.2. Quality parameters microbiota data

	Mean ± SEM	Lowest value	Highest value
Number of reads	67,199 ± 3,424	15,734	146,997
Joining efficiency	94.03 ± 0.25	86.40	97.35
Number of merged paired-ends reads	49,675 ± 2,547	12,899	110,628
Number of OTUs	43,718 ± 2,272	12,274	97,637

Number of reads, joining efficiency, number of merged paired-ends reads and number of operational taxonomical units (n=61).

5.4.4.1 Alpha diversity

We investigated potential differences in Chao1, number of observed species, Shannon's diversity, Simpson's diversity, and Phylogenetic diversity. No differences were observed for these α-diversity indices between men and women (contraceptive use y/n) and across the menstrual cycle (Table 5.3).

Table 5.3. Gender, menstrual cycle phase, and hormonal contraceptive use did not influence microbiota richness and diversity indices

	Males	Females HC			Females nHC		
	(n=15)	EF (n=8)	LF (n=9)	ML (n=9)	EF (n=7)	LF (n=6)	ML (n=7)
Chao1	311.9 ±22.69	318.9 ±37.88	328.1 ±38.18	314.2 ±36.29	338.6 ±32.86	319.4 ±38.27	358.3 ±47.30
Observed species	262.0 ±19.77	269.5 ±34.75	271.0 ±37.13	262.7 ±35.58	291.3 ±27.33	282.3 ±37.94	309.9 ±45.74
Shannon's diversity	5.392 ±0.136	5.237 ±0.286	5.361 ±0.210	5.229 ±0.267	5.653 ±0.104	5.623 ±0.111	5.893 ±0.183
Simpson's diversity	0.947 ±0.005	0.942 ±0.011	0.953 ±0.006	0.944 ±0.009	0.963 ±0.003	0.961 ±0.003	0.967 ±0.003
Phylogenetic diversity	19.12 ±1.236	20.04 ±2.184	19.78 ±2.317	19.44 ±2.324	21.61 ±1.820	21.05 ±2.489	22.36 ±3.024

Data is presented as a mean ± standard error of the mean (SEM). Kruskal-Wallis test and Friedman test with Dunn's post-hoc test were performed to assess differences between selected groups, and differences across the menstrual cycle. Abbreviations: HC = hormonal contraceptive users, nHC = non-hormonal contraceptive users, BMI = body mass index, EF = early follicular phase, LF = late follicular phase, ML = mid-luteal phase.

5.4.4.2 Relative abundance of gut microbiota

When evaluating differences in relative abundance group distributions for phylum (Figure 5.4), family (Figure 5.5), and genus, significant differences were observed for five taxa when comparing males and females (3 phases) (Table 5.4). For relative abundance group distribution comparisons of males and females (contraceptive use y/n, 3 phases), significant differences were observed for another 14 taxa (Supplementary Table 1). Across the menstrual cycle of all females, significant differences were shown for 14 taxa (Supplementary Table 2). Across the menstrual cycle of contraceptive users and normally menstruating females, significant differences were demonstrated for 10 and 5 taxa, respectively (Supplementary Tables 3 & 4). However, only for genus *Erysipelatoclostridium* a FDR lower than 10% was demonstrated; hormonal contraceptive use was associated with an increased relative abundance of *Erysipelatoclostridium*, at least for the late follicular phase (Supplementary Table 1, Figure 5.6).

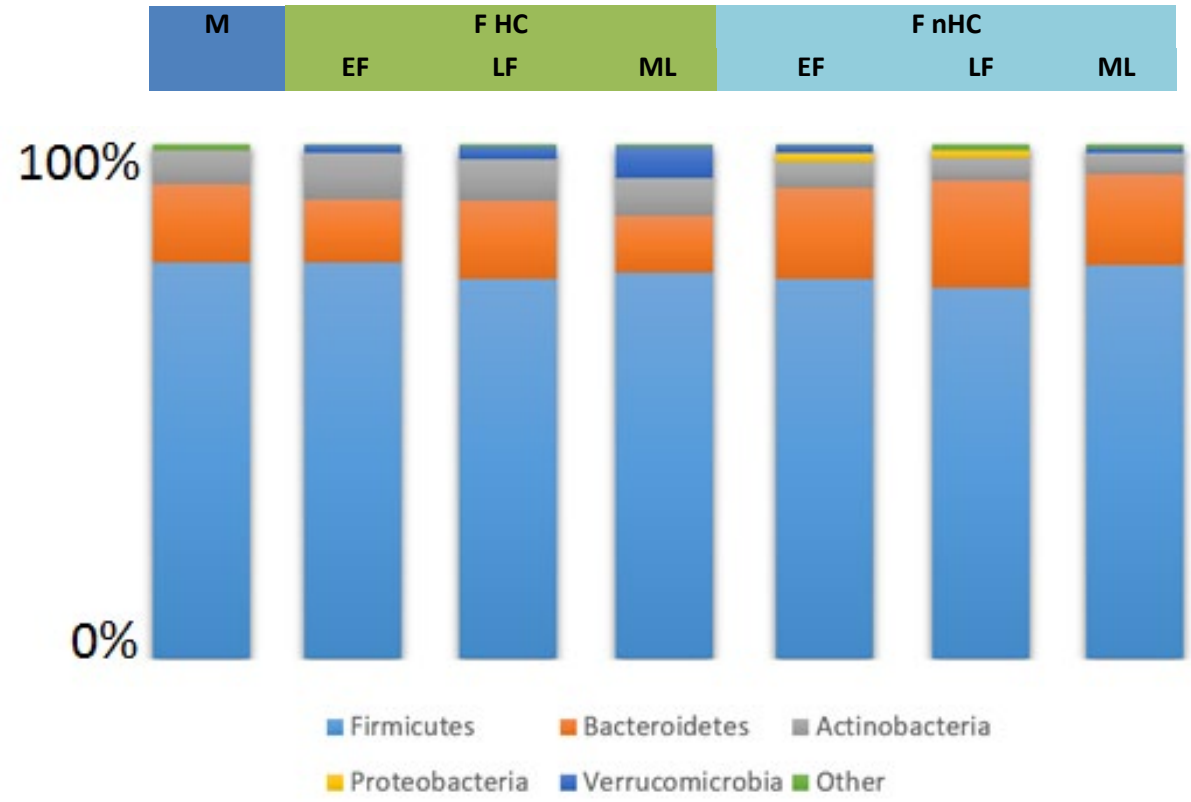


Figure 5.4. Gender, menstrual cycle, and hormonal contraceptive use did not influence phylum relative abundance. Only phyla with a relative abundance ≥1% are visualised as independent taxa. Other phyla are listed under 'other'.
Abbreviations: HC= hormonal contraceptive users, nHC = non-hormonal contraceptive users, EF = early follicular phase, LF = late follicular phase, ML = mid-luteal phase.

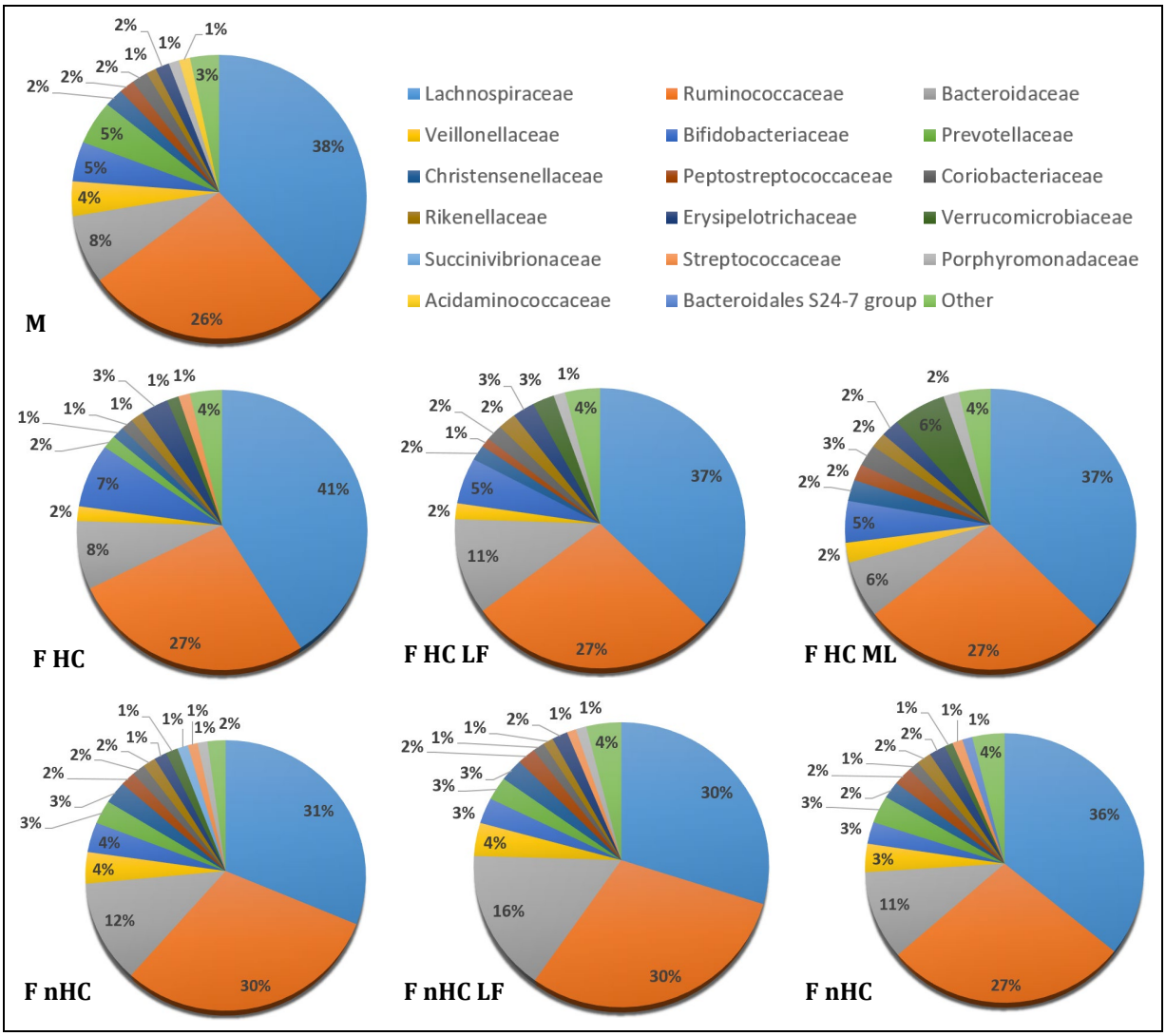


Figure 5.5. Gender, menstrual cycle, and hormonal contraceptive use did not influence family relative abundance. Only families with a relative abundance ≥1% are visualised as independent taxa. Other families are listed under 'other.'

Abbreviations: HC= hormonal contraceptive users, nHC = non-hormonal contraceptive users, EF = early follicular phase, LF = late follicular phase, ML = mid-luteal phase.

Table 5.4. Kruskal-Wallis tests for all phyla, families, and genera comparing males and total females

Taxa	p-value	FDR-adjusted p-value
Bacteria_Actinobacteria_Coriobacteriia_Coriobacteriales_Coriobacteriaceae_Slackia	0.002866224	0.854134703
Bacteria_Firmicutes_Erysipelotrichia_Erysipelotrichales_Erysipelotrichaceae_Solobacterium	0.023224437	3.460441134
Bacteria_Saccharibacteria	0.029649725	2.945206024
Bacteria_Saccharibacteria_uncultured.bacterium	0.029649725	2.208904518
Bacteria_Saccharibacteria_uncultured.bacterium	0.029649725	1.767123615

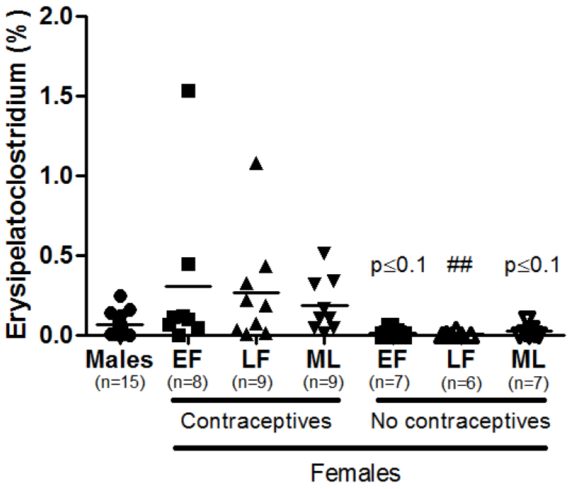


Figure 5.6. Hormonal contraceptive use is associated with an increased relative abundance of the Erysipelatoclostridium. Relative abundance of Erysipelatoclostridium in faecal contents of males, and females (using hormonal contraceptives y/n) across the menstrual cycle. Kruskal-Wallis test and Friedman test with Dunn's post-hoc test were performed to assess differences between selected groups., $p \leq 0.1$ & #: compared to hormonal contraceptive users (same phase). ##$p \leq 0.01$.
Abbreviations: EF = early follicular phase, LF = late follicular phase, ML = mid-luteal phase.

5.4.5 Short-chain Fatty Acid Content

We observed no association of these factors with faecal acetate, propionate and n-butyrate levels (Figure 5.7 & Table 5.5). This is in line with our finding that gender, menstrual cycle, and hormonal contraceptive use did not influence microbiota composition, except for the relative abundance of *Erysipelatoclostridium* that was associated with hormonal contraceptive use.

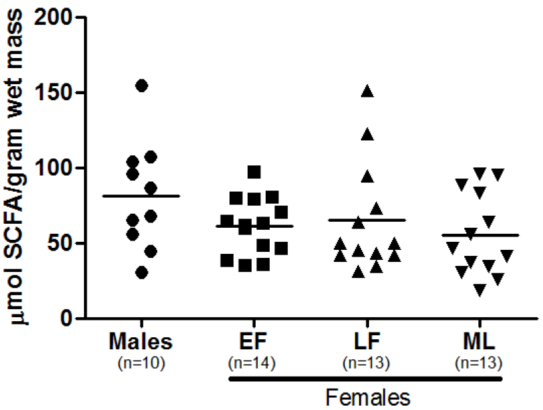


Figure 5.7. Gender, menstrual cycle, and hormonal contraceptive use did not influence SCFA levels in faecal wet mass. SCFA content (sum of acetate, propionate and n-butyrate) in the faecal wet mass of males, and females across the menstrual cycle. Kruskal-Wallis test and Friedman test with Dunn's post-hoc test were performed to assess differences between selected groups.

Additionally, to our knowledge, no other studies have found differences in faecal SCFA levels between "healthy" males and females, across the menstrual cycle or differences associated with hormonal contraceptive use.

Table 5.5. Gender, menstrual cycle, and hormonal contraceptive use did not influence acetate, propionate and n-butyrate levels in faecal wet mass

	Males	**Females HC**			**Females nHC**		
	(n=10)	EF (n=7)	LF (n=8)	ML (n=6)	EF (n=7)	LF (n=5)	ML (n=7)
Acetate (µmol/gram wet mass)	51.29 ±7.247	36.99 ±4.650	35.18 ±5.689	34.11 ±7.236	43.46 ±4.471	52.00 ±12.08	38.75 ±7.137
Propionate (µmol/gram wet mass)	15.38 ±2.692	10.17 ±1.069	10.12 ±1.972	8.678 ±2.024	13.03 ±2.353	14.51 ±5.004	9.925 ±1.973
n-Butyrate (µmol/gram wet mass)	14.65 ±2.557	7.955 ±1.288	10.16 ±3.111	7.511 ±2.570	11.60 ±1.763	13.90 ±3.300	10.87 ±1.983

Data is presented as mean ±SEM SCFA content in faeces (wet mass) of males, and females (using hormonal contraceptives y/n) across the menstrual cycle. Kruskal-Wallis test and Friedman test with Dunn's post-hoc test were performed to assess differences between selected groups. Abbreviations: EF = early follicular phase, LF = late follicular phase, ML = mid-luteal phase.

5.4.6 Hormonal Contraceptive Use Is Associated with Increased Levels of LBP, But Not Soluble CD14 and Inflammation

Levels of indirect intestinal integrity marker LBP were determined in plasma. LBP levels of females using hormonal contraceptives were greater compared to those of males ($p \leq 0.01$) and female non-users (LF: $p \leq 0.05$, ML: $p \leq 0.01$), except for the EF phase (Figure 5.8). Since it normally takes ±1-3 days to initiate a period after ingestion of the last pill of the contraceptive pill strip, and phases are based on self-reported menstrual cycle start date, we speculate that the two contraceptive pill users with high LBP levels during the EF already started ingesting

contraceptive pills of their new strip, which thereby may have caused the high LBP levels compared to the other five contraceptive pill users with relatively low LBP levels.

Another indirect intestinal integrity marker, sCD14, were determined in the EF phase and ML phase of contraceptive users and non-users. For this marker, no differences were observed between the groups and phases (Figure 5.9).

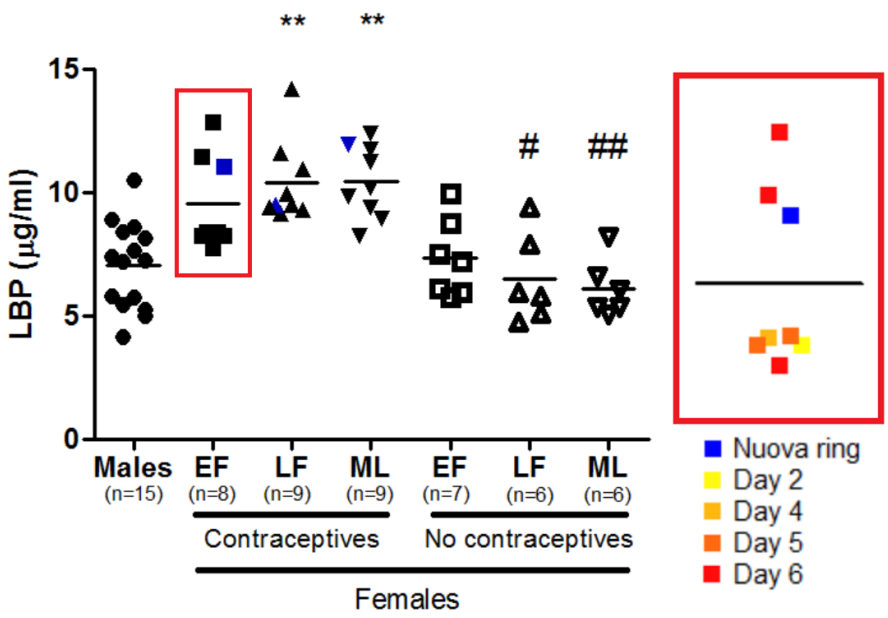


Figure 5.8. Hormonal contraceptive use is associated with increased systemic LBP levels. Plasma LBP concentrations of males, and females (using hormonal contraceptives y/n) across the menstrual cycle. Kruskal-Wallis test and Friedman test with Dunn's post-hoc test were performed to assess differences between selected groups. *compared to males, #compared to hormonal contraceptive users (same phase). *$p \leq 0.05$, **$p \leq 0.01$

Abbreviations: EF = early follicular phase, LF = late follicular phase, ML = mid-luteal phase, LBP = lipopolysaccharide-binding protein.

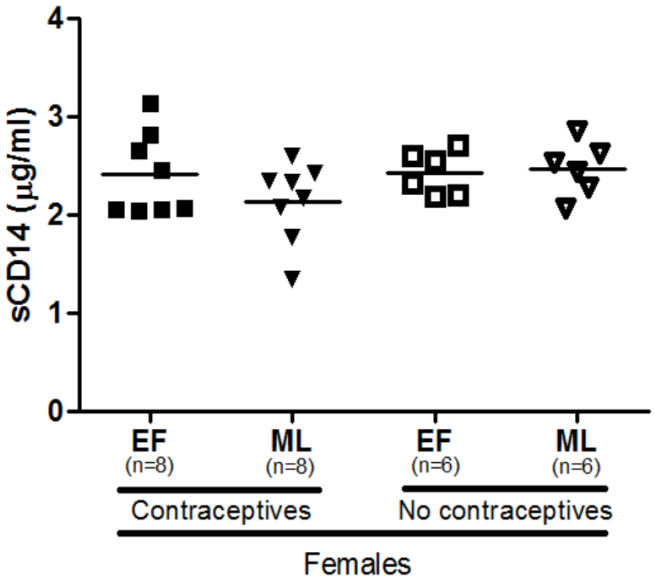


Figure 5.9. Hormonal contraceptive use does not affect systemic sCD14 levels. No differences in sCD14 were observed between the early follicular phase and the mid-luteal phase, and between contraceptive users and normally menstruating females plasma sCD14 concentrations of females (using hormonal contraceptives y/n) in the EF and ML phase. Kruskal-Wallis test and Friedman test with Dunn's post-hoc test were performed to assess differences between selected groups. Abbreviations: EF = early follicular phase, ML = mid-luteal phase, sCD14 = soluble cluster of differentiation 14.

To investigate whether gender, menstrual cycle or hormonal contraceptive use influenced the inflammatory state, plasma levels of TNF-α, IFN-γ and IL-8 were determined. In this study, we did not observe any differences in plasma TNF-α, IL-8 or IFN-γ between males and females, across the menstrual cycle or an association with hormonal contraceptive use (Figure 5.10A-C). However, in the EF phase of hormonal contraceptive users, we found a slight, but significant increase in IL-8 compared to the LF and ML phase, that may not be biologically relevant (Figure 5.10D).

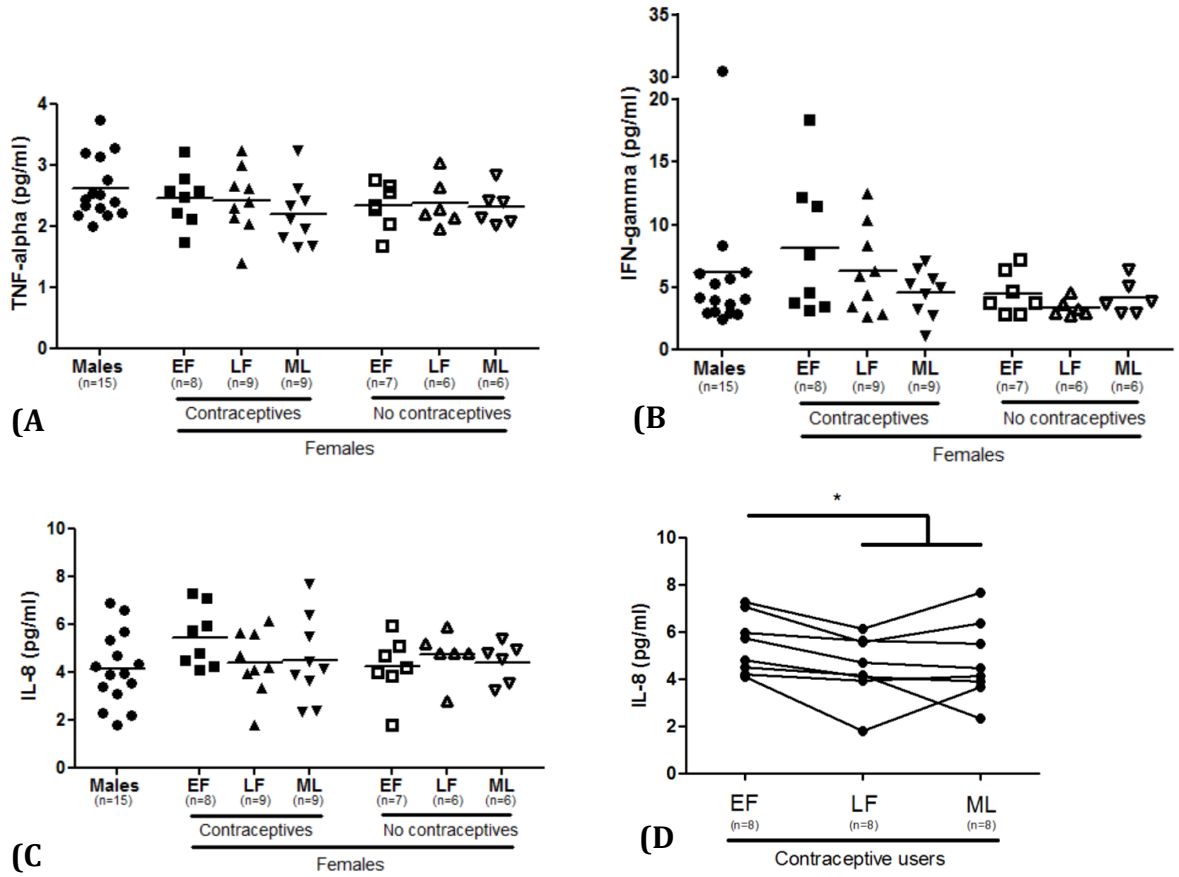


Figure 5.10. IL-8 levels were higher in the early follicular phase compared to the late follicular phase and mid-luteal phase in hormonal contraceptive users. Plasma TNF-α **(A)**, IFN-γ **(B)** and IL-8 **(C & D)** concentrations of males, and females (using hormonal contraceptives y/n) during the EF, LF, ML phase. Kruskal-Wallis test and Friedman test with Dunn's post-hoc test were performed to assess differences between selected groups. *p≤0.05.

Abbreviations: EF = early follicular phase, ML = mid-luteal phase, TNF-α = tumour necrosis factor α, IFN-γ = interferon γ, IL-8 = interleukin 8.

5.4.7 Gender, Menstrual Cycle, and Hormonal Contraceptive Use Did Not Influence Cortisol Awakening Response

When looking at the CAR profile of all included subjects and timepoints (n=51), we observed a significant increase of salivary cortisol levels 30 minutes after awakening compared to levels at awakening (Figure 5.11). At 60 minutes after awakening, a significant decrease in cortisol levels was demonstrated compared to the peak (30 minutes) (Figure 5.11). This profile was not observed in the separate groups, except for the early follicular phase of females using hormonal contraceptives (Table 5.6). In addition, no significant differences were observed between groups/menstrual cycle phases for 1) cortisol levels at a specific timepoint,

2) cortisol increase during the first 30 minutes after awakening and 3) cortisol output during the first hour after awakening (AUC) (Table 5.6). Of special interest, for the late follicular phase of normally menstruating females, a mean negative cortisol awakening response was observed (Table 5.6). We also observed relatively high variation in mean cortisol levels at awakening and cortisol increase across the menstrual cycle (measurements on three different days), especially in females using hormonal contraceptives.

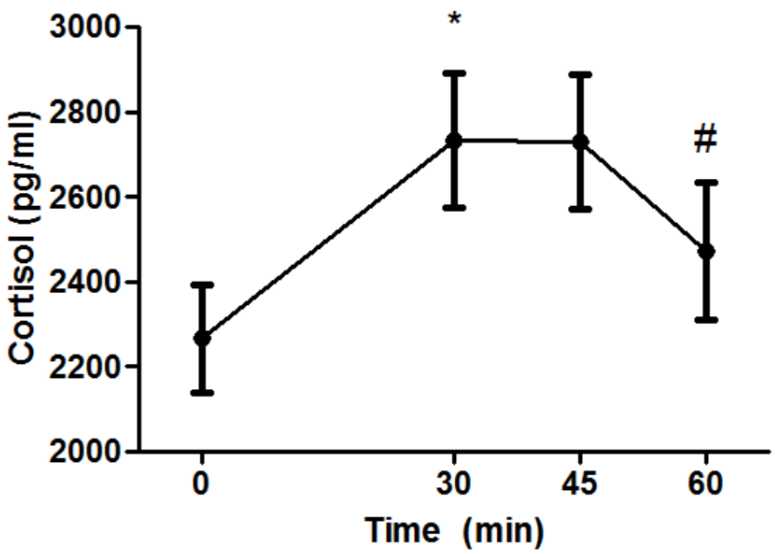


Figure 5.11. Mean cortisol levels during the awakening response of the study population. Salivary cortisol levels upon awakening (t=0 min) and 30, 45 and 60 minutes after awakening. 51 cortisol awakening responses were included. Friedman test with Dunn's post-hoc test was performed to assess differences between selected timepoints. *compared to t=0min, #compared to t=30min, *$p \leq 0.05$.

Table 5.6. Gender, menstrual cycle, and hormonal contraceptive use did not influence cortisol awakening response

Cortisol level (pg/ml)	**Males**	**Females HC**			**Females nHC**		
	(n=10)	EF (n=7)	LF (n=8)	ML (n=8)	EF (n=7)	LF (n=4)	ML (n=7)
Awakening	1851 ±274	2005 ±317	2344 ±311	2968 ±455	2006 ±328	2441 ±373	2025 ±221
Awakening +30min	2243 ±474	2999* ±488	3083 ±435	2815 ±327	2944 ±281	2152 ±146	2617 ±293
Awakening +45min	2470 ±188	2577 ±465	3021 ±536	3018 ±523	2804 ±297	2040 ±355	2599 ±334
Awakening +60min	2137 ±353	2317# ±460	2891 ±534	2698 ±506	2676 ±339	1853 ±353	2411 ±278
Cortisol increase 0-30min (%)	32.6 ±19.5	48.8 ±10.9	32.2 ±12.0	0.5 ±9.7	89.5 ±49.2	-2.4 ±20.9	41.0 ±25.7
The AUC (arbitrary unit * 1000)	131 ±15	154 ±26	172 ±25	173 ±24	158 ±13	130 ±6	146 ±10
Negative response (%)	30	0	13	50	14	75	29

Salivary cortisol levels upon awakening, 30, 45 and 60 minutes after awakening, %salivary cortisol increase during the first 30 minutes after awakening, cortisol awakening response AUC and %negative cortisol awakening responses. Friedman tests with Dunn's post-hoc test were performed to assess differences between selected timepoints within a group. Kruskal-Wallis and Friedman tests with Dunn's post-hoc test were performed to assess differences between selected groups *compared to t=0min, #compared to t=30min, *p≤0.05. Abbreviations-HC = hormonal contraceptive users, nHC = non-hormonal contraceptive users, EF = early follicular phase, LF = late follicular phase, ML = mid-luteal phase.

Another factor that we further investigated was the interplate variation of the cortisol assays used since cortisol analyses were determined over several different plates (only 96 spots per plate available). Mean %CV was 12.8 for 33 included samples that were run on 3 different plates. This is in line with the high %CV (13.4, 7.8) that the manufacturer reports for samples with cortisol levels of 451pg/ml and 969pg/ml, respectively. Strikingly, the 12.8% variation was an average, and observed interplate variation ranged from 3.9% to 33.0%. Taken together, relatively low sample size, high variability of the CAR, high variation of the cortisol assay, and potential non-compliance have a complicated demonstration of differences in HPA axis functioning between the groups.

5.4.8 Relative Abundance of Specific Genera Correlate Positively with Propionate, SCD14, TNF-α and Pain Sensation Threshold

Our secondary outcomes included investigating if associations existed between the gut microbiota, SCFA composition, indirect intestinal integrity markers, inflammation, HPA axis functioning and electrical pain thresholds in men and women.

Here, we focused specifically on the relative abundance of genera that correlated with other parameters in men or women. We did not find any significant (FDR-corrected) correlations for men only (Supplementary Table 5 & Supplementary Figure 4). This is likely caused by the low sample size for men (n=15). For women (n=46 visits), we found several significant correlations between genus abundance and the other parameters (Supplementary Table 6 & Supplementary Figure 4). After filtering out low abundant/low prevalent genera (since these are probably not/less relevant), five remained (Figure 5.11).

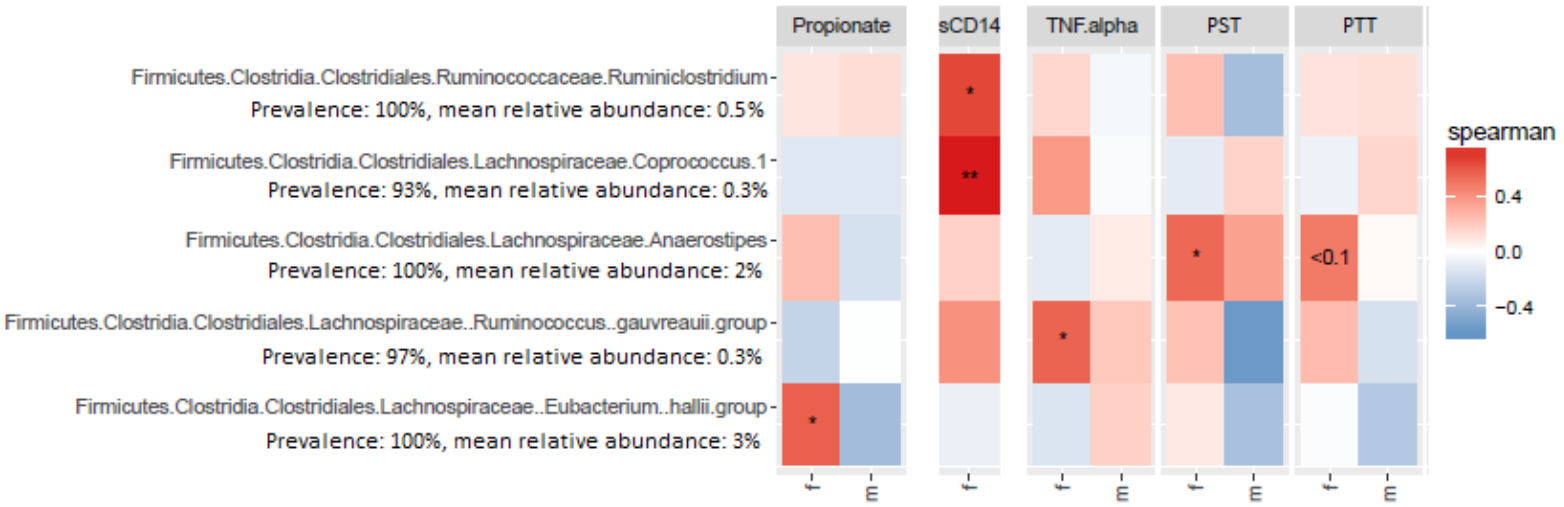
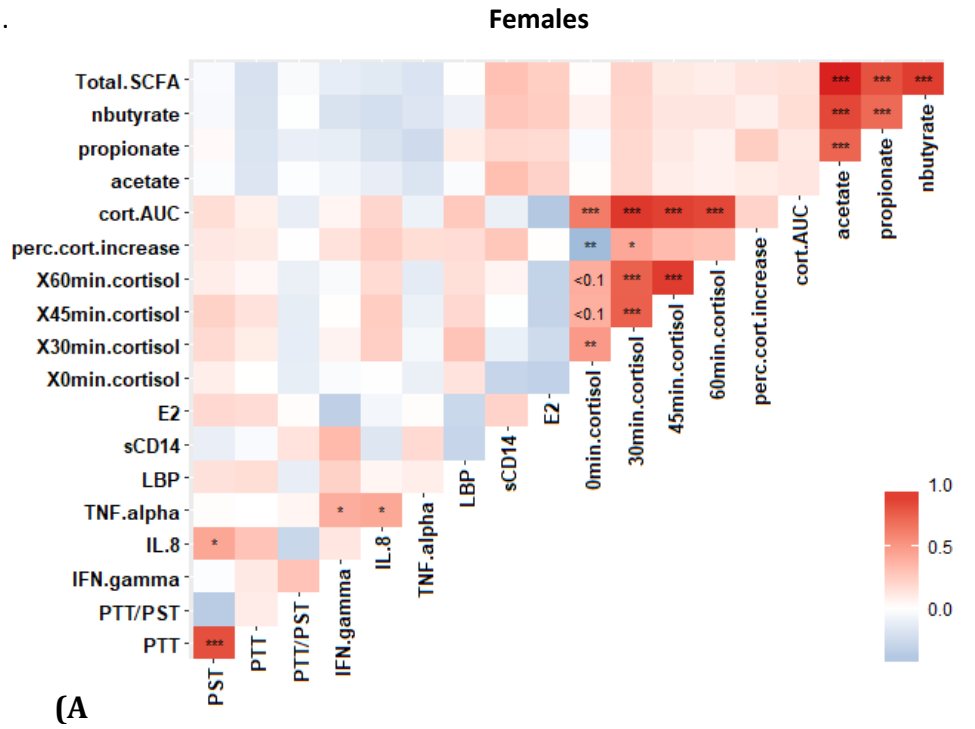


Figure 5.11. Overview of high prevalent/abundant genera that correlated with investigated parameters
Spearman correlation coefficients expressed in colours, positive (red), negative (blue). *FDR-corrected p-value <0.05, **FDR-corrected p-value <0.01 FDR were calculated separately for men and women.
Abbreviations: f=females, m=males, sCD14= soluble CD14, PST=pain sensation threshold, PTT=pain tolerance threshold

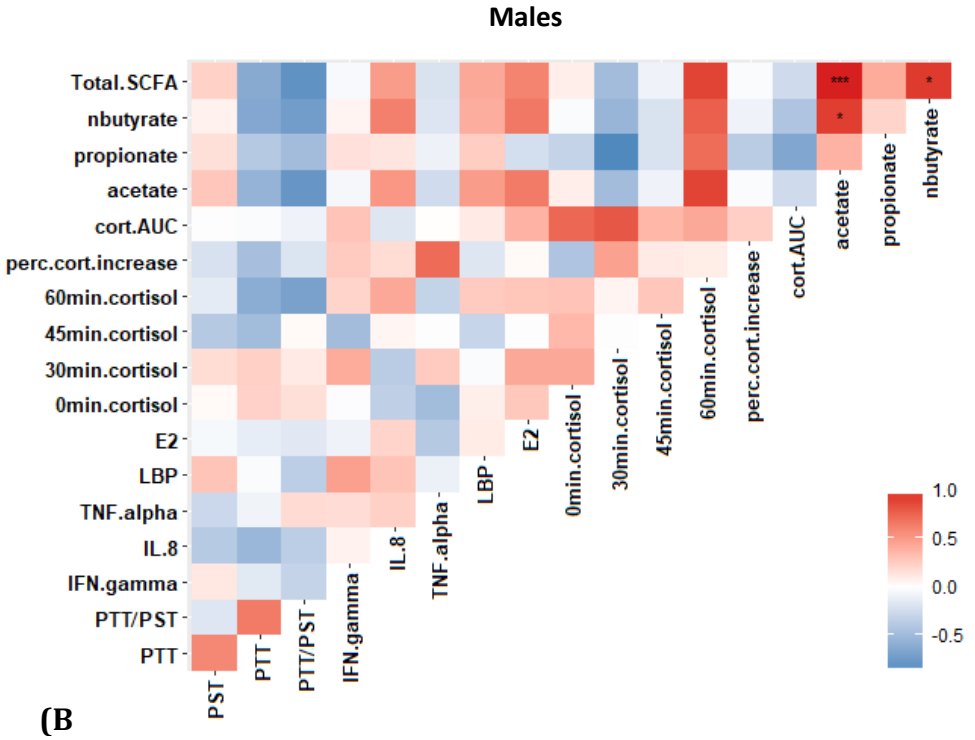
We did not find a significant correlation between *Anaerostipes* and faecal butyrate. Interestingly, we demonstrated a positive correlation between genus *Anaerostipes* and PST in females (correlation coefficient: 0.55, FDR-corrected *p*-value: 0.03). For PTT a trend for a

significant correlation with *Anaerostipes* was observed (correlation coefficient 0.49, FDR-corrected p-value <0.1). In males, the correlation coefficients for correlations between *Anaerostipes* and PST and *Anaerostipes* and PTT were respectively, 0.35 and 0.02. Other genera that correlated with some of our parameters were *Ruminiclostridium*, *Coprococcus 1*, Rumminococcus Gauvreauii and *Eubacterium hallii*; the first two correlated positively to plasma sCD14, Rumminococcus Gauvreauii with TNF-α and *Eubacterium hallii* with propionate (Figure 5.11 & Supplementary Table 6). Next, we investigated potential associations between SCFAs data, cortisol awakening response data, oestradiol, LBP, sCD14 and inflammatory markers data, and pain data for males and females apart (Figure 5.12A, B & Supplementary Tables 7 & 8).

.



(A



(B

Figure 5.12. Correlation plots of females (A) and males (B) Spearman correlation coefficients expressed in colors, positive (red), negative (blue). *FDR-corrected p-value <0.05, **FDR-corrected *p*-value <0.01 FDR were calculated separately for men and women.
Abbreviations: sCD14= soluble CD14, PST=pain sensation threshold, PTT=pain tolerance threshold

5.5 Discussion

In this study, we identified compositional alteration of the gut metagenome that may be related to altered pain sensitivity. Interestingly, we found a positive correlation between butyrate producing bacteria and pain sensitivity. We observed that in female participants only, an abundance of genus *Anaerostipes* correlated positively with PST. Although PTT and PST were not any different individually between the sexes, the ratio of PTT to PST was significantly low in women compared to men. Additionally, hormonal contraceptive use was associated with increased LBP levels.

Most of the pain models on humans measure either pain threshold or pain tolerance as a measure of experimental pain responses (Lund *et al.*, 2005; Hegarty and Shorten, 2012; Nakashima *et al.*, 2015). Although reliable, there is a presence of interpersonal variability within a group, mostly because the measurements are very subjective, purely relying on subject self-reporting. The neurophysiological pain stimulation has some advantages over the other forms such as thermal or mechanical pain. The electrical stimulations can be pre-programmed, and measurement are not influenced by the observer's bias. Moreover, if stimulations are applied over a motor nerve such as tibial nerve, the movement of corresponding muscle on stimulation may correlate directly with PST. A major shortcoming of electrical stimulations is that it bypasses the pain receptors and activates the nerve fibres directly and therefore, it does not cause a specific nociceptor activation (Reddy *et al.*, 2012). In our study, there was no significant difference observed in PST or PTT between males and females. In addition, the menstrual cycle did not seem to have any effect on the pain responses. This can be partly due to a smaller number of participants in non-hormonal contraception group, or due to the fact that our method of menstrual cycle phase determination was based on self-reporting. However, one interesting observation was that

the ratio of PTT to PST was significantly low in women, which signifies decreased tolerance to repeated painful stimuli in women. The significance of this ratio has not been studied previously, therefore, leads to a future direction of further research work taking into account the PTT/PST ratio for pain assessments.

In our experiments, women with a higher abundance of butyrate producing gut bacteria had high pain tolerance. The production of SCFAs, e.g. butyrate, propionate in the gut is dependent upon the diet, gut transit time and the composition of the colonic microbial flora. Butyrate beneficially affects inflammatory processes such as cytokine release and myeloperoxidase activity (Ogawa *et al.*, 2003). It also supports mucosal barrier function, hence, decreases colonic epithelial permeability (Yan and Ajuwon, 2017). Despite all these benefits, the role of butyrate in visceral pain is controversial. Butyrate administration has been shown to induce colonic hypersensitivity in Sprague-Dawley rats (Bourdu *et al.*, 2005). In contrast, an animal study on neuropathic pain found butyrate administration to be very beneficial (Kukkar *et al.*, 2014). A human study observed intraluminal injections of butyrate into the bowel to decrease visceral pain perception in healthy subjects (Vanhoutvin *et al.*, 2009). The known mechanisms through which butyrate may modulate pain include serotonergic modulation, activation of transient receptor potential vanilloid 1 (TRPV1) receptor, and desensitisation of sensory neurons expressing TRPV1 receptors (Fukumoto *et al.*, 2003; Kannampalli *et al.*, 2011). SCFAs have the ability to inhibit histone deacetylases, which may be responsible for their behavioural effect (Mátis *et al.*, 2013). Our somatic pain study showing a positive correlation between pain sensitivity and butyrate producing *Anaerostipes* abundance*,* supports the finding of this human visceral pain study mentioned. Interestingly, we observed relevant findings in women only. Since no microbiota intervention

was performed on our participants, an interaction between the neuroendocrine system and gut microbiota may be accountable for the findings we observed.

Recent reviews suggest that gut microbiota may influence neuroendocrine function through direct production of neuroendocrine metabolites and indirectly as a modulator of inflammatory and immune responses (Cussotto *et al.*, 2018). Gut microbiota have the ability to produce different neurotransmitters such as dopamine, noradrenaline, and GABA (Roshchina, 2016). LPS, a product of Gram-negative bacteria, has the ability to cross intestinal barrier and can activate TLR4 and HPA axis in CNS (Maes *et al.*, 2008). The gut microbiota mediate the production of immune mediators such as TNF-α, IL-1β, and IL-6 which in turn, reach the brain and stimulate the HPA axis (Schirmer *et al.*, 2016). Moreover, the gut microbiota is able to directly influence the production of glucocorticoid hormones (Mukherji *et al.*, 2013). Sudo *et al*., have shown that germ-free (GF) mice exhibit altered HPA axis function, with elevated levels of plasma ACTH and corticosterone when compared to specific-pathogen free (SPF) mice. GF mice also had reduced BDNF expression levels in the cortex and hippocampus when compared to SPF mice (Sudo *et al.*, 2004). Several other studies have confirmed that GF animals have high levels of ACTH and corticosterone following a stressful stimulus (Clarke *et al.*, 2013; Crumeyrolle-Arias *et al.*, 2014). SCFA-producing gut microbiota can directly influence oestrogen function by modulating its cellular sensitivity through mitogen-activated protein kinase pathway (Jansen *et al.*, 2004).

Since HPA axis is closely related to sex hormones, these studies suggest a plausible relationship between HPA, sex hormones, and gut microbiota leading to differential pain responses in men and women. Further research on a larger scale is, however, required to completely understand these mechanisms and to develop therapeutic interventions.

Chapter 6

General Discussion

6.1 Overview and Summary

The goal of the thesis is to explore the factors responsible for sex differences in pain. The particular focus was to see the effect of endogenous cyclical changes in oestrogen levels, stress and gut microbiota on EAAT functioning and pain sensation. To achieve this goal, we optimised an aspartate assay utilising radiolabelling on spinal cord and ACC. This assay has previously been used on brain slices but was optimised to be used in the spinal cord of rats for the first time. The validity of aspartate assay was confirmed with the use of an EAAT function enhancer and a blocker. To ascertain the effect of stress, well established animal models of early-life stress (maternally separated rats) and depression (Wistar-Kyoto rats) were utilised. In an attempt to translate the results of animal experiments onto humans, a multifactorial experimental pain study was performed on healthy men and women during different menstrual cycles phases. In the human study, our particular focus was to see if the difference in gut microbiota can influence the pain sensation.

Our first aim was to determine whether the EAAT function in the lumbosacral spinal cord changed in different stages of the oestrous cycle, where a particular oestrous phase would indicate a specific sex hormonal status. We carried out ex-vivo studies on rat spinal cord to achieve our goals (Chapter 2). We were able to demonstrate that the function of EAAT was dependent upon the oestrous phase. Hight oestrogen states, i.e., proestrus and oestrus were associated with downregulated EAAT function. Exogenous oestrogen showed a similar effect on male rat spinal cord glutamate metabolism.

Furthermore, with the utilisation of the PCR, we were able to demonstrate that there was a higher expression of oestrogen receptor ER-α during high-oestrogen states, and so was the expression of glutamate receptors subunit NR1. These findings suggest a positive

modulatory effect of oestrogen on glutamatergic signalling such that the endogenous oestrogen would enhance glutamate receptors expression and decrease glutamate uptake; thus, promoting a higher synaptic glutamate concentration and activity. These results denote an association of glutamate uptake and oestrogen levels, which in the context of pain sensitivity would suggest that in high-oestrogen states, the rats would be more sensitive to pain.

Based on our first aim and its results, our second aim was to look at the synaptic glutamatergic uptake in different oestrogen states and correlate it directly with the pain sensitivity in stress states (Chapter 2 & 3). We achieved this by performing colorectal distension (an established method to assess visceral pain) on maternally separated rats (an established model of earl life stress (ELS)). On a second cohort of the same rat model of ELS, we performed aspartate uptake assay and made a direct correlation between stress, hormones and the neurotransmission in pain states. Since anterior cingulate cortex (ACC) plays an important role in affective pain responses, we performed aspartate assay in both spinal cord and ACC. We were able to show that the visceral pain sensitivity corresponded with the EAAT function, as pain responses were considerably high in proestrus and oestrus phases. Furthermore, excitatory amino acid transport, both within the lumbosacral spinal cord and the ACC, were also altered in response to stress and oestrous cycle, thus indicating that aberrant excitatory transport may in part lead to fluctuations in visceral sensitivity.

After determining the effects of ELS on glutamatergic metabolism and visceral sensitivity, our goal was to see the if a similar effect can be confirmed on another animal stress model. We achieved this goal by utilising Wistar-Kyoto (WKY) rats which are an accepted model of depression and visceral hypersensitivity. We determined that the circulating oestrogen regulates the function of sodium-dependent glutamate transporters in female WKY

rats. Cortical EAAT activity was upregulated in the diestrus stage of the oestrous cycle as compared to males and females in proestrus and oestrus. However, there was no such change observed in glutamate metabolism seen at the level of the lumbosacral spinal cord. The aspartate uptake correlated positively with SCFA-producing gut bacteria in sex and hormonal state-dependent manner. These results highlight how chronic stress and early-life stress can lead to increased pain sensitivity and change the glutamatergic signalling in varying oestrogen environment. As chronic stress leads to dysfunction of normal HPA axis, the interaction of stress in these animals with oestrogen and gut microbiota may be accountable for these altered pain responses and glutamate metabolism through the altered HPA axis.

Finally, we performed neurophysiological electrical stimulation at the ankle in healthy humans to evaluate pain responses. We aimed to explore the sex differences in pain sensitivity in humans and various factors influencing it (Chapter 5). As microbiota-gut-brain axis has been shown to influence a number of CNS disorders, and there is emerging evidence of its role in pain processing in preclinical studies, we particularly focused on gut microbiota and its associated mechanisms influencing pain sensitivity. We achieved this goal by recruiting healthy non-obese young adults of both sexes. We utilised electrical stimulation along the tibial nerve and assessed pain sensory thresholds (PST) and pain tolerance thresholds (PTT) in men and women across their menstrual cycle. We could not see any significant difference in either PST or PTT between men and women, and among women in their different phases of the menstrual cycle. However, the PTT/PST ratio, indicating decrease tolerance to repeated painful stimuli, was significantly low in women in comparison to men. Interestingly, we noted a positive correlation between *Anaerostipes* and pain sensitivity in women. Use of hormonal contraception seemed to be associated with higher lipopolysaccharide-binding protein (LBP), a marker of gut mucosal wall integrity.

6.2 Can Preclinical Experimental Findings of Hormonal Modulation of Pain be Translated to Humans?

It is very important to determine the translatability of findings of animal studies to humans. In the context of oestrogen, the distribution patterns of ER mRNA are similar in the brains of most of the mammals (Heldring *et al.*, 2007; Vanderhorst *et al.*, 2009). Furthermore, ER-α distribution patterns in the brain stem and spinal cord of primates are identical to that of rodents. There is also molecular similarity seen in the ERs between species (Vanderhorst *et al.*, 2009).

On the other hand, the physiological outcome of ER activation varies, probably because the binding characteristics of transcription factors may vary considerably (Odom *et al.*, 2007). Direct comparison between rodent oestrous cycle and the human menstrual cycle is also not straightforward. The human hormonal cycle is a lot more complex process comprising of distinct ovarian and uterine cycles correlating with each other. The length of the human menstrual cycle is very variable among different individuals and can be affected by a number of modifiable factors (Liu *et al.*, 2004). Similarly, the experiment setup on rodents can easily dysregulate the oestrous cycle. Although the ovariectomy can significantly reduce the oestrogen levels in rodents, the extragonadal production of oestrogen and release from storage in fatty areas continue to occur and may affect the pain-related sensory neurotransmission (Alagwu and Nneli, 2005). Ovariectomy affects the interactions of oestrogen with the HPA axis, and an altered HPA axis may further confound the experimental results. Notably, the absence of the direct cortical interoceptive representation and the direct medial motivational pathway in subprimates implies that they cannot experience feelings from the body in the same way that primates do, particularly pain (Craig, 2003).

Although the experimental animal data may not translate entirely to humans, various animal models used today are providing vital information in the understanding of the pathogenesis of a number of diseases. Since rodents' genome is very similar to the human genome, genetically engineered mice/rats for a particular disease provide a valid resource for research on pathogenesis and therapy of that disease (Vandamme, 2014). The Royal College of Surgeons rat is the first known animal with inherited retinal degeneration (Adachi *et al.*, 2016). The Zucker rat was bred to be a genetic model for research on obesity and hypertension (Buckley and Rasmussen, 2012). Genetically engineered knockout rat disease models for Parkinson's disease (Dave *et al.*, 2014), Alzheimer's disease (Do Carmo and Cuello, 2013), hypertension (Moreno *et al.*, 2011), and diabetes (Plum *et al.*, 2005) are commercially available and are extensively being used in preclinical research. It is not possible to create such models in humans, and trials to treat patients with these diseases require rigorous ethical approval where the success of similar experiments on animals needs to be sought beforehand.

We utilised maternally separated Sprague-Dawley rats and WKY rats; although not genetically engineered, both are validated models of ELS and depression, respectively, based on their phenotypic characteristics. Both rat models are commonly used to study visceral hypersensitivity. To investigate visceral pain and hypersensitivity, we used colorectal distension, which is an established model of gut-related visceral pain. We were able to investigate the interplay of endogenous oestrogens, glutamatergic metabolism, and stress, which provides us with very important information regarding the pathogenesis of visceral hypersensitivity in stress.

6.3 Are EAATs the Only Modulators of Glutamatergic Transmission?

The glutamatergic system can be regulated at a synapse at multiple levels including presynaptic glutamate release, glial glutamate uptake from the synapse, and through the

expression of glutamate receptors on the postsynaptic neurons. The modulation by glial uptake and glutamate receptor expression has been elaborated in earlier segments of the thesis (Chapter 1 and 2). The presynaptic glutamate release is regulated by voltage-gated K^+ (Kv) channels. A subtype Kv3.4 is robustly expressed at DRG neurons (Ritter *et al.*, 2015a), and its dysfunction has been shown to be implicated in the pathophysiology of neuropathic pain (Ritter *et al.*, 2015b). These findings suggest that this ion channel might play a significant role as a regulator of nociceptive synaptic activity at the level of the first synapse in the pain pathway. However, until recently, it was unclear whether the modulation of these receptors affects the actual neurotransmission along the synapse. With the help of immunofluorescence, measurement of excitatory postsynaptic currents and application of Kv3.4 enhancers, it has been demonstrated that these channels significantly prolong the action potential, slow the maximum rate of repolarisation in small diameter DRG neurons, and potentiate monosynaptic excitatory postsynaptic currents in dorsal horn laminae I and II through a presynaptic mechanism (Muqeem *et al.*, 2018). These results strongly suggest that presynaptic Kv3.4 channels are one of the major regulators of nociceptive synaptic transmission in the spinal cord. More pain-related research including hormonal modulation of these particular channels is needed to establish their role in preclinical and clinical pain modulation.

6.4 Can EAATs Be a Therapeutic Target for Clinical Pain?

As we have detailed the importance of the role of EAATs in pain transmission (Chapter 2,3, and 4), it is important to see what evidence is available regarding the effectiveness of their enhancers as analgesics. Riluzole (used in the treatment of amyotrophic lateral sclerosis), ceftriaxone (a broad-spectrum antibiotic), and valproate (an antiepileptic) are known enhancers of the EAATs.

6.4.1 Riluzole As an Analgesic

Riluzole has been shown to be effective in alleviating neuropathic pain and improving functional recovery in cervical spondylotic myelopathy rodent model (Moon *et al.*, 2014). Riluzole can reverse visceral hypersensitivity induced by ELS in rodents (Sung *et al.*, 2003; Gosselin *et al.*, 2010). In rodent studies of neuropathic pain involving spinal nerve avulsion, riluzole, when administered, was able to prevent the onset of evoked pain hypersensitivity but was unable to reverse the pre-established hypersensitivity (Chew *et al.*, 2014). Some studies have shown its analgesic properties in rodent neuropathic models only when injected intraperitoneally compared to intrathecal injections, indicating a peripherally mediated antinociceptive mechanism (Hama and Sagen, 2011). However, central co-administration of riluzole and minocycline seems to reduce morphine induced drug tolerance in rats (Habibi-Asl *et al.*, 2009). Although preclinical studies suggest a promising effect of riluzole as an analgesic, only a few human studies have attempted to examine its potential an effective clinical analgesic. Hammer *et al.*, studied the effect of riluzole on a human model of inflammatory pain induced by thermal injury on healthy humans and found no benefit of riluzole on pain scoring (Hammer *et al.*, 1999). Another group studied the effect of riluzole at different doses on humans with existing peripheral neuropathic pain. They were not able to see any efficacy of riluzole on neuropathic pain (Galer *et al.*, 2000). In contrast, a group found riluzole to be effective as an add on to standard IBS treatment regimen, in relieving IBS related visceral pain (Mishra *et al.*, 2014).

6.4.2 Ceftriaxone as an Analgesic

Chronic treatment with ceftriaxone dampened visceral pain responses in mice in inflammatory colitis, colorectal and bladder distension models (Lin *et al.*, 2009a; Yang *et al.*, 2011). Chronic administration of ceftriaxone in rodents also proved to be effective in the

prevention of morphine tolerance (Yan *et al.*, 2009; Ramos *et al.*, 2010). Ceftriaxone is also capable of both potentiating analgesic effect of nicotine and diminishing development of tolerance to nicotine analgesia (Schroeder *et al.*, 2011). Ceftriaxone has been shown to reduce both thermal hyperalgesia and tactile allodynia (Yan *et al.*, 2009; Hu *et al.*, 2010; Ramos *et al.*, 2010) in a rodent model of neuropathic pain. In the cervical nerve root injury-based model of radicular pain, both thermal hyperalgesia and mechanical allodynia were attenuated by chronic treatment with ceftriaxone (Nicholson *et al.*, 2014a). Another rodent pain study found ceftriaxone to exert its antinociceptive effect in both somatic and visceral pain (Stepanovic-Petrovic *et al.*, 2014). Similar to that of riluzole, the clinical data for the effectiveness of ceftriaxone as an analgesic in humans is scarce, although it is a widely used antibiotic. A single dose preoperative dose of ceftriaxone before ulnar and radial nerve decompression, significantly reduced postoperative pain scoring compared to normal saline and cefazoline (Macaluso *et al.*, 2013).

Although preclinical studies show us the promising analgesic effects of these EAATs' modulators, the scarcity of relevant human data limits their clinical use. The lack of clinical data is partly due to ethical hurdles for clinical trials, research costs particularly for riluzole, and availability and usage of already licenced analgesics. Since we have described the role of EAAT and glutamate metabolism in the pathogenesis of a number of chronic pain disorder, it is paramount to establish human studies and trials utilising EAAT enhancers. The recruitment should include both healthy individuals and patients with existing chronic pain disorders, taking into consideration sex, menstrual cycle phases, use of contraceptives, normal births and deliveries through C-section, gut microbiota profiles and presence of stress conditions.

6.5 Can Interventions Make Gut Microbiota a Potential Therapeutic Target in the Future?

Recent research supporting a role for the microbiota in maintaining healthy brain function suggests targeting gut microbiota as a therapeutic potential in CNS disorders (Forsythe *et al.*, 2012). There is growing evidence to support the use of antibiotics, prebiotics, and probiotics as a treatment option in IBS (Didari *et al.*, 2015; Quigley, 2015). The use of probiotics has shown symptomatic pain relief in IBS patients (Brenner *et al.*, 2009; Whorwell, 2009; Moayyedi *et al.*, 2010). Other possible microbiota interventions include dietary modification and faecal microbiota transplant (FMT) from healthy individuals to patients (Gupta *et al.*, 2016).

B. infantis treatment, a probiotic for example, was shown to reverse behavioural deficits, restore basal noradrenaline concentrations in the brainstem of adult rats subjected to the early-life stress of maternal separation, an animal model of brain-gut axis dysfunction (Desbonnet *et al.*, 2010; O'Mahony *et al.*, 2011). A preclinical study using the same probiotic found that CRD-induced visceral pain behaviours were significantly reduced in the viscerally hypersensitive Wistar-Kyoto rat strain (McKernan *et al.*, 2010). *L. paracasei* administration has also shown efficacy in reducing visceral hypersensitivity in a mouse model of IBS (Verdu *et al.*, 2006). Table 1.6 in Chapter 1 outlines similar studies in the context of pain modulation.

Understanding the role of gut microbiota in the bidirectional interaction between the gut and the brain is a novel and appealing research field. Microbiota-gut-brain-axis is being studied in relation to a number of CNS related disorders such as autism (Strati *et al.*, 2017), Parkinsonism (Perez-Pardo *et al.*, 2017), and Alzheimer's disease (Jiang *et al.*, 2017) to name a few. Currently, microbiota involvement and the mechanism of action involved in pain modulation are not yet clearly established. To be able to target the gut microbiota to alleviate painful symptoms requires a lot more preclinical and clinical research focusing on the interactions between gut microbiota, sex, pre-existing stress in both health individuals and

patients with existing pain disorders. Our experimental model (Chapter 5), although involved a small number of subjects, showed exciting results of correlation of *Firmicutes* with pain sensitivity. These results set a future direction toward human/clinical studies at a larger scale that should include interventions targeting gut microbiota with dietary modifications, prebiotics, probiotics, antibiotics, and FMT.

6.6 Future Directions

My thesis investigated the modulation of EAAT function and pain sensitivity by oestrous cycle and demonstrated the effects of stress and gut microbiota on the modulation of pain. Our results add significant information to existing knowledge of nociceptive processes; however, more research is required to understand the pathogenesis of visceral pain disorders fully. We suggest following future research themes bases on our results.

We are able to demonstrate EAAT function alterations in different phases of the oestrous cycle, and riluzole reversed the oestrus phases induced inhibition of EAAT function. Although visceral pain responses correlated to EAAT function, it is not known whether alteration in visceral pain responses was due to altered synaptic glutamate transmission under the influence of EAATs or the effect of endogenous oestrogen on other mechanisms responsible for pain modulation described earlier. We, therefore, suggest ex-vivo electrophysiological studies on male and female rodent spinal cord and brain slices and investigate whether the synaptic evoked potential change and correspond to the EAAT function. The resultant correlation between oestrous cycle, EAAT function, and synaptic action potential may confirm EAATs as therapeutic targets. The effect of EAAT enhancers on neurotransmission may also be investigated by pre-treating the CNS slices with these drugs.

We suggest that all animal experiments examining nociception in males and females must consider the oestrous phase of the female rodent being assessed. We performed colorectal

distension on maternally separated rats across their oestrous cycle. There is room to perform this nociceptive test on WKY rats in males and females to reproduce the results. Likewise, pain models for neuropathic pain can be studied in the similar settings. Moreover, microbiota analysis can be performed and correlated with pain model results. Interventions such as the use of prebiotics, probiotics, and antibiotics can be performed to see their effect. We were able to see a positive correlation of SCFAs-producing gut bacteria on EAAT functioning in WKY rats, further correlation with pain responses may be beneficial to further understanding of microbiota-gut-brain axis in pain modulation.

Our human study showed exciting results as the relative abundance of *Anaerostipes* correlated positively with pain sensitivity. We were not able to see any significant effect of menstrual cycle status on experimental pain sensitivity; however, some trends were noted. Our sample size was relatively small. We suggest our human study experimental setup to be performed on a larger scale. This setup can also be performed on patients with existing pain disorders and by having a larger number of participants, a better understanding of the interaction between nociception, sex, stress, and microbiota-gut-axis can be elucidated.

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Supplementary Data

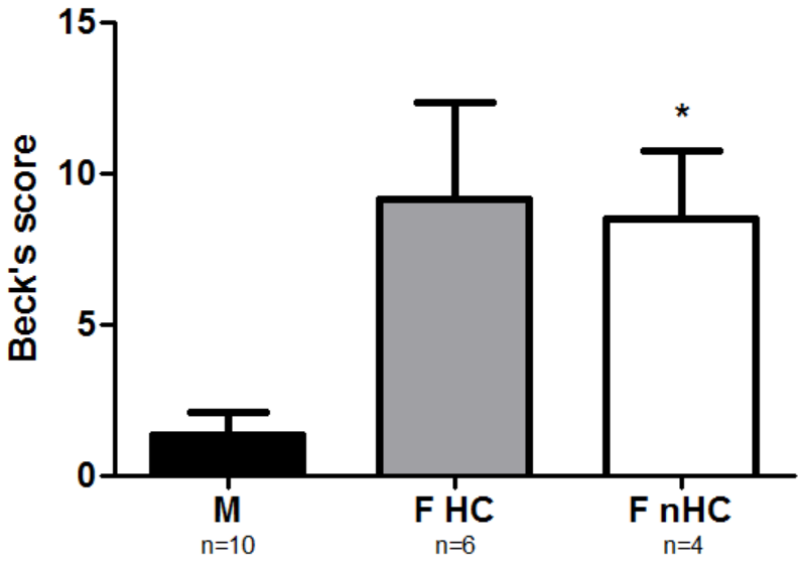


Figure S1. Beck's depression inventory score was higher in normally menstruating females than males. Scores of the Beck's depression inventory that was filled in by the study participants. Kruskal-Wallis test with Dunn's post-hoc test was performed to assess differences between selected groups. *p≤0.05
Abbreviations: M = males, F HC = females using hormonal contraceptives, F nHC = females not using hormonal contraceptives

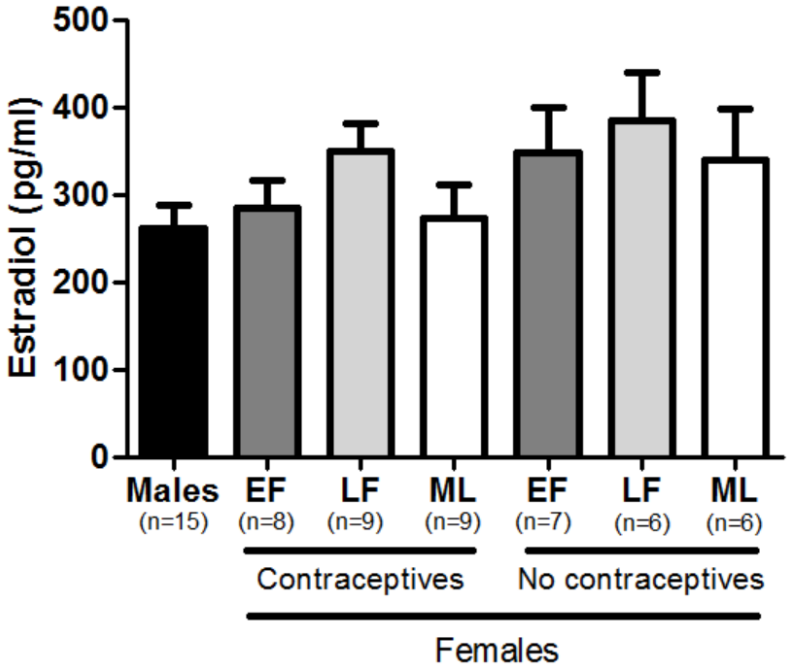


Figure S2. Gender, menstrual cycle and hormonal contraceptive use did not influence plasma oestradiol levels. Plasma oestradiol concentrations of males, and females (using hormonal contraceptives y/n) across the menstrual cycle. Kruskal-Wallis test and Friedman test with Dunn's post-hoc test were performed to assess differences between selected groups.
Abbreviations: EF = early follicular phase, LF = late follicular phase, ML = mid-luteal phase

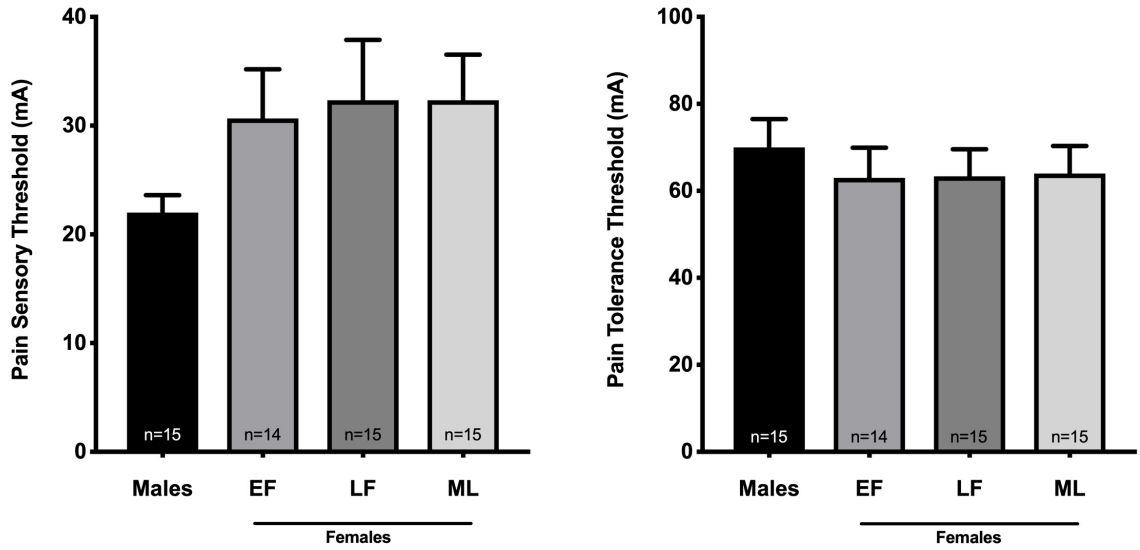


Figure S3. Pain tolerance thresholds & pain sensation thresholds did not vary significantly along menstrual cycle and between both sexes. There was no significant difference seen in PTT and PST along menstrual cycle stages in both hormonal and non-hormonal contraception female groups combined. Similarly, no differences were seen between males and females

Abbreviations: EF = early follicular phase, LF = late follicular phase, ML = mid-luteal phase.

Table S1. Kruskal-Wallis tests for all phyla, families and genera comparing males, females using contraceptives (three phases), and normally menstruating females (three phases)

Taxa	p-value	FDR-adjusted p-value
Bacteria_Firmicutes_Erysipelotrichia_Erysipelotrichales_Erysipelotrichaceae_Erysipelatoclostridium	0.000184811	**0.055073793**
Bacteria_Actinobacteria_Coriobacteriia_Coriobacteriales_Coriobacteriaceae_Slackia	0.002042006	0.304258923
Bacteria_Tenericutes_Mollicutes_Mollicutes.RF9_uncultured.bacterium	0.011961159	1.188141827
Bacteria_Tenericutes_Mollicutes_Mollicutes.RF9_uncultured.bacterium	0.011961159	0.89110637
Bacteria_Firmicutes_Clostridia_Clostridiales_Lachnospiraceae_Lachnospiraceae.UCG.001	0.01461519	0.871065329
Bacteria_Firmicutes_Clostridia_Clostridiales_Ruminococcaceae_Candidatus.Soleaferrea	0.0157854	0.784008217
Bacteria_Firmicutes_Clostridia_Clostridiales_Family.XI_Peptoniphilus	0.016332206	0.695285339
Bacteria_Firmicutes_Erysipelotrichia_Erysipelotrichales_Erysipelotrichaceae_Dielma	0.018019838	0.671238954
Bacteria_Firmicutes_Bacilli_Lactobacillales_Lactobacillaceae_Pediococcus	0.020295746	0.67201469
Bacteria_Proteobacteria	0.025986384	0.774394228
Bacteria_Firmicutes_Clostridia_Clostridiales_Eubacteriaceae	0.031915405	0.864617348
Bacteria_Firmicutes_Clostridia_Clostridiales_Lachnospiraceae_Lachnospiraceae.NK4A136.group	0.043469868	1.079501722
Bacteria_Actinobacteria_Coriobacteriia_Coriobacteriales_Coriobacteriaceae_Eggerthella	0.044918523	1.02967076
Bacteria_Firmicutes_Clostridia_Clostridiales_Lachnospiraceae_Lachnospiraceae.UCG.004	0.045605592	0.97074761

Table S2. Friedman tests to compare potential phylum, family and genera differences across the menstrual cycle in all females

Taxa	p-value	FDR-adjusted p-value
Bacteria_Firmicutes_Clostridia_Clostridiales_Lachnospiraceae_Coprococcus 3	0.010	2.92
Bacteria_Firmicutes_Clostridia_Clostridiales_Lachnospiraceae	0.011	1.606
Bacteria_Firmicutes_Clostridia_Clostridiales_Ruminococcaceae_Ruminococcaceae UCG-005	0.012	1.168
Bacteria_Firmicutes_Bacilli_Lactobacillales_Streptococcaceae_Streptococcus	0.017	1.241
Bacteria_Firmicutes_Clostridia_Clostridiales_Ruminococcaceae_Ruminococcus 2	0.020	1.168
Bacteria_Bacteroidetes_Bacteroidia_Bacteroidales_Bacteroidales S24-7 group_uncultured bacterium	0.027	1.314
Bacteria_Firmicutes_Clostridia_Clostridiales_Lachnospiraceae_[Eubacterium] oxidoreducens group	0.030	1.251429
Bacteria_Firmicutes_Clostridia_Clostridiales_Ruminococcaceae_Faecalibacterium	0.030	1.095
Bacteria_Firmicutes_Clostridia_Clostridiales_Ruminococcaceae_Ruminococcaceae UCG-011	0.036	1.168
Bacteria_Actinobacteria_Coriobacteriia_Coriobacteriales_Coriobacteriaceae_Enterorhabdus	0.038	1.1096
Bacteria_Firmicutes_Clostridia_Clostridiales_Family XIII_Family XIII UCG-001	0.041	1.088364
Bacteria_Firmicutes_Clostridia_Clostridiales_Lachnospiraceae_uncultured	0.046	1.119333
Bacteria_Firmicutes	0.046	1.033231
Bacteria_Firmicutes_Clostridia_Clostridiales_Ruminococcaceae_Ruminococcaceae UCG-010	0.049	1.022

Table S3. Friedman tests to compare potential phylum, family and genera differences across the menstrual cycle in female contraceptive users

Taxa	p-value	FDR-adjusted p-value
Bacteria_Firmicutes_Clostridia_Clostridiales_Lachnospiraceae_uncultured	0.002	0.542
Bacteria_Firmicutes_Clostridia_Clostridiales_Peptococcaceae	0.006	0.813
Bacteria_Firmicutes_Bacilli_Lactobacillales_Streptococcaceae	0.011	0.993667
Bacteria_Firmicutes_Clostridia_Clostridiales_Peptococcaceae_uncultured	0.015	1.01625
Bacteria_Firmicutes_Clostridia_Clostridiales_Ruminococcaceae_Ruminiclostridium 5	0.021	1.1382
Bacteria_Firmicutes_Clostridia_Clostridiales_Ruminococcaceae_Ruminococcaceae UCG-005	0.025	1.129167
Bacteria_Firmicutes_Clostridia_Clostridiales_Lachnospiraceae_Dorea	0.034	1.316286
Bacteria_Firmicutes	0.034	1.15175
Bacteria_Firmicutes_Clostridia_Clostridiales_Ruminococcaceae_Ruminococcaceae UCG-011	0.038	1.144222
Bacteria_Firmicutes_Bacilli_Lactobacillales_Streptococcaceae_Streptococcus	0.044	1.1924

Table S4. Friedman tests to compare potential phylum, family and genera differences across the menstrual cycle in normally menstruating females

Taxa	p-value	FDR-adjusted p-value
Bacteria_Firmicutes_Clostridia_Clostridiales_Lachnospiraceae_[Ruminococcus] gauvreauii group	0.016	4.368
Bacteria_Firmicutes_Clostridia_Clostridiales_Ruminococcaceae_Faecalibacterium	0.016	2.184
Bacteria_Firmicutes_Clostridia_Clostridiales_Ruminococcaceae_Ruminococcus 2	0.018	1.638
Archaea_Euryarchaeota_Methanobacteria_Methanobacteriales_Methanobacteriaceae	0.050	3.4125
Archaea_Euryarchaeota	0.050	2.73

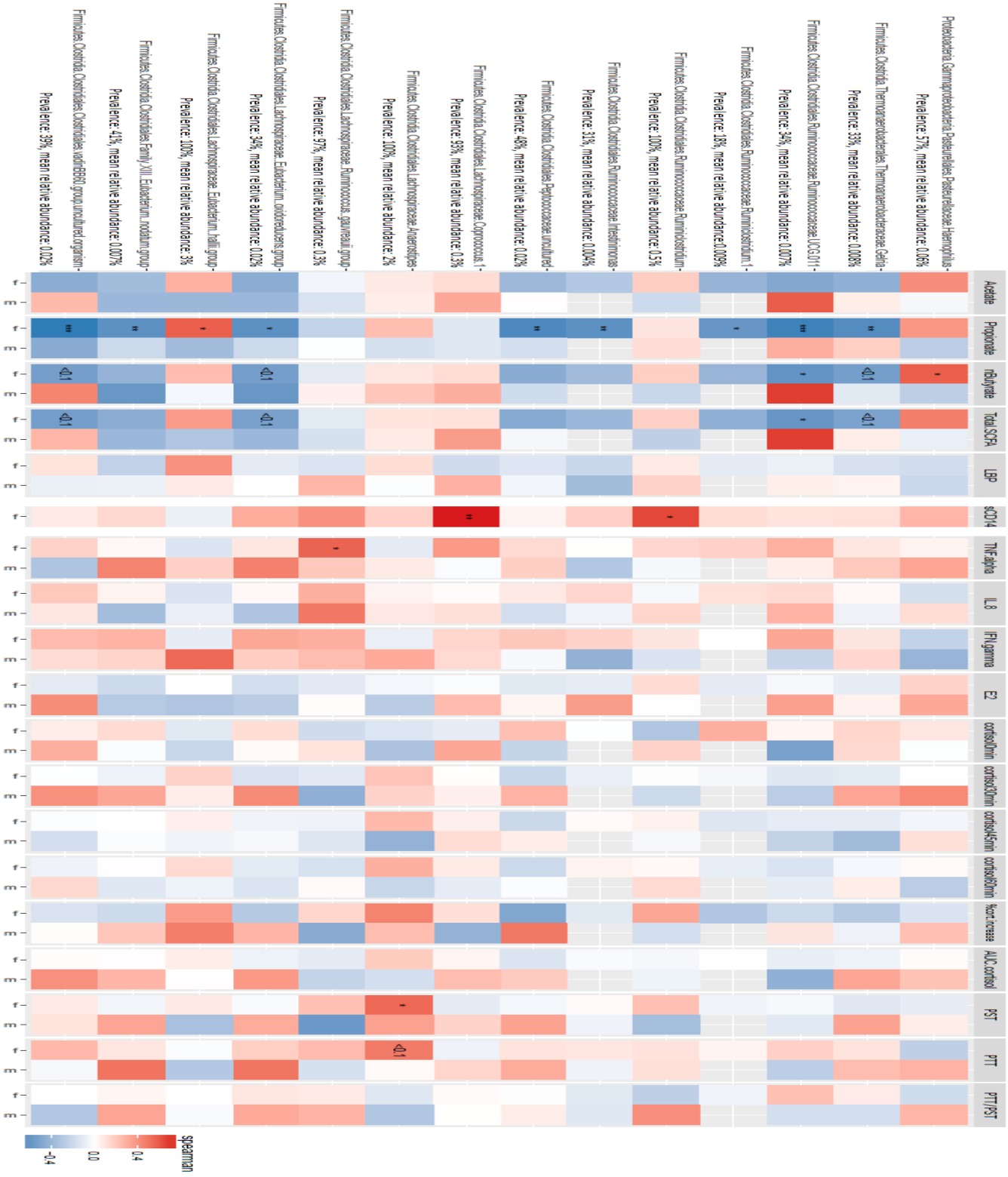


Figure S4. Overview of genera that correlated significantly with investigated parameters for males and females apart. Spearman correlation coefficients expressed in colours, positive (red), negative (blue). *FDR-corrected p-value <0.05, **FDR-corrected p-value <0.01**,** ***FDR-corrected p-value <0.001, FDR were calculated separately for men and women.

Table S5. Spearman correlation coefficient overview of genera correlated to investigated parameters (males)

Genus	Parameter	Correlation coefficient	p-value	FDR-adjusted p-value
Firmicutes.Negativicutes.Selenomonadales.Acidaminococcaceae.Phascolarctobacterium	Propionate	0.912946	0.000226	0.728491
Firmicutes.Clostridia.Clostridiales.Lachnospiraceae.Lachnospiraceae.NK4A136.group	E2	0.807143	0.000435	0.701605
Firmicutes.Clostridia.Clostridiales.Lachnospiraceae.Tyzzerella.4	E2	-0.78021	0.0006	0.645137
Firmicutes.Clostridia.Clostridiales.Lachnospiraceae.Fusicatenibacter	TNF.alpha	0.760714	0.00151	1.217414
Firmicutes.Erysipelotrichia.Erysipelotrichales.Erysipelotrichaceae.Turicibacter	TNF.alpha	0.757143	0.001635	1.054277
Firmicutes.Clostridia.Clostridiales.Lachnospiraceae.Lachnospiraceae.NK4A136.group	Acetate	0.866667	0.002681	1.440814
Firmicutes.Clostridia.Clostridiales.Ruminococcaceae.Ruminiclostridium.9	Total.SCFA	0.866667	0.002681	1.234983
Firmicutes.Clostridia.Clostridiales.Ruminococcaceae.Ruminococcaceae.UCG.014	LBP	-0.69534	0.004001	1.612534
Proteobacteria.Deltaproteobacteria.Desulfovibrionales.Desulfovibrionaceae.Desulfovibrio	cortisol60min	0.812897	0.004249	1.522157
Firmicutes.Clostridia.Clostridiales.Lachnospiraceae.Lachnospiraceae.NK4A136.group	Total.SCFA	0.842424	0.004459	1.437591
Firmicutes.Clostridia.Clostridiales.Ruminococcaceae.Ruminiclostridium.9	Acetate	0.842424	0.004459	1.306901
Fusobacteria.Fusobacteriia.Fusobacteriales.Fusobacteriaceae.Fusobacterium	E2	-0.68417	0.004906	1.318054
Firmicutes.Clostridia.Clostridiales.Lachnospiraceae.Lachnospiraceae.UCG.005	cortisol30min	0.830303	0.005557	1.378088
Firmicutes.Clostridia.Clostridiales.Ruminococcaceae.Ruminiclostridium.6	E2	0.673816	0.005882	1.354608
Firmicutes.Clostridia.Clostridiales.Family.XIII..Eubacterium..brachy.group	Perc.cortisol.increase	0.795205	0.005961	1.281262
Proteobacteria.Deltaproteobacteria.Desulfovibrionales.Desulfovibrionaceae.Desulfovibrio	Total.SCFA	0.795205	0.005961	1.201183
Firmicutes.Negativicutes.Selenomonadales.Veillonellaceae.Veillonella	PST	0.663611	0.006989	1.32536
Firmicutes.Clostridia.Clostridiales.Ruminococcaceae.Acetanaerobacterium	cortisol45min	0.785334	0.007105	1.272531
Actinobacteria.Actinobacteria.Actinomycetales.Actinomycetaceae.Actinomyces	E2	-0.66131	0.00726	1.231869
Firmicutes.Negativicutes.Selenomonadales.Veillonellaceae.Veillonella	PTT	0.656452	0.007858	1.266645
Firmicutes.Clostridia.Clostridiales.Family.XIII..Eubacterium..brachy.group	PTT	-0.65506	0.008036	1.233778
Proteobacteria.Gammaproteobacteria.Enterobacteriales.Enterobacteriaceae.Enterobacter	IFN.gamma	-0.65487	0.00806	1.181166
Firmicutes.Clostridia.Clostridiales.Lachnospiraceae.Lachnospiraceae.UCG.005	AUC.cortisol	0.806061	0.008236	1.154412
Firmicutes.Erysipelotrichia.Erysipelotrichales.Erysipelotrichaceae.Holdemanella	cortisol60min	0.769345	0.009274	1.245837
Proteobacteria.Deltaproteobacteria.Desulfovibrionales.Desulfovibrionaceae.Desulfovibrio	Acetate	0.769345	0.009274	1.196004
Firmicutes.Negativicutes.Selenomonadales.Acidaminococcaceae.Acidaminococcus	Propionate	0.768037	0.00947	1.174298
Firmicutes.Clostridia.Clostridiales.Ruminococcaceae.Candidatus.Soleaferrea	IFN.gamma	0.657143	0.009527	1.137646

Table S6. Spearman correlation coefficient overview of genera correlated to investigated parameters (females)

Genus	Parameter	Correlation coefficient	p-value	FDR-adjusted p-value
Firmicutes.Clostridia.Clostridiales.Clostridiales.vadinBB60.group.uncultured.organism	Propionate	-0.74957	2.61E-08	**1.05E-04**
Firmicutes.Clostridia.Clostridiales.Ruminococcaceae.Ruminococcaceae.UCG.011	Propionate	-0.71527	2.14E-07	**4.32E-04**
Firmicutes.Clostridia.Clostridiales.Peptococcaceae.uncultured	Propionate	-0.67505	1.77E-06	**2.38E-03**
Firmicutes.Clostridia.Clostridiales.Lachnospiraceae.Coprococcus.1	sCD14	0.756912	3.14E-06	**3.17E-03**
Firmicutes.Clostridia.Thermoanaerobacterales.Thermoanaerobacteraceae.Gelria	Propionate	-0.65338	4.86E-06	**3.93E-03**
Firmicutes.Clostridia.Clostridiales.Lachnospiraceae..Eubacterium..oxidoreducens.group	Propionate	-0.62659	1.52E-05	**1.02E-02**
Firmicutes.Clostridia.Clostridiales.Family.XIII..Eubacterium..nodatum.group	Propionate	-0.62637	1.54E-05	**8.89E-03**
Firmicutes.Clostridia.Clostridiales.Ruminococcaceae.Intestinimonas	Propionate	-0.62424	1.67E-05	**8.44E-03**
Firmicutes.Clostridia.Clostridiales.Ruminococcaceae.Ruminococcaceae.UCG.011	n-Butyrate	-0.60581	3.44E-05	**1.54E-02**
Firmicutes.Clostridia.Clostridiales.Ruminococcaceae.Ruminiclostridium.1	Propionate	-0.59324	5.48E-05	**2.21E-02**
Firmicutes.Clostridia.Clostridiales.Ruminococcaceae.Ruminococcaceae.UCG.011	Total.SCFA	-0.59039	6.07E-05	**2.23E-02**
Firmicutes.Clostridia.Clostridiales.Lachnospiraceae..Ruminococcus..gauvreauii.group	TNF.alpha	0.567852	6.41E-05	**2.16E-02**
Proteobacteria.Gammaproteobacteria.Pasteurellales.Pasteurellaceae.Haemophilus	n-Butyrate	0.583449	7.77E-05	**2.42E-02**
Firmicutes.Clostridia.Clostridiales.Lachnospiraceae..Eubacterium..hallii.group	Propionate	0.584053	0.000103	**2.97E-02**
Firmicutes.Clostridia.Clostridiales.Lachnospiraceae.Anaerostipes	PST	0.551763	0.000103	**2.79E-02**
Firmicutes.Clostridia.Clostridiales.Ruminococcaceae.Ruminiclostridium	sCD14	0.660189	0.000132	**3.33E-02**
Proteobacteria.Betaproteobacteria.Burkholderiales.Alcaligenaceae.Parasutterella	Propionate	-0.5503	0.000234	5.57E-02
Firmicutes.Clostridia.Clostridiales.Clostridiales.vadinBB60.group.uncultured.organism	Total.SCFA	-0.54884	0.000245	5.51E-02
Firmicutes.Clostridia.Clostridiales.Clostridiales.vadinBB60.group.uncultured.organism	n-Butyrate	-0.54342	0.00029	6.18E-02
Firmicutes.Clostridia.Clostridiales.Lachnospiraceae..Eubacterium..oxidoreducens.group	n-Butyrate	-0.54157	0.000307	6.21E-02
Firmicutes.Clostridia.Thermoanaerobacterales.Thermoanaerobacteraceae.Gelria	n-Butyrate	-0.54094	0.000313	6.03E-02
Proteobacteria.Gammaproteobacteria.Enterobacteriales.Enterobacteriaceae.Escherichia.Shigella	TNF.alpha	0.507276	0.000376	6.90E-02
Cyanobacteria.Melainabacteria.Gastranaerophilales.uncultured.bacterium.	Propionate	-0.532	0.000411	7.22E-02
Tenericutes.Mollicutes.Mollicutes.RF9.uncultured.bacterium.	PTT	-0.50923	0.000415	6.99E-02
Firmicutes.Clostridia.Clostridiales.Lachnospiraceae..Eubacterium..oxidoreducens.group	Total.SCFA	-0.52884	0.000451	7.29E-02
Firmicutes.Clostridia.Clostridiales.Lachnospiraceae..Eubacterium..ventriosum.group	LBP	-0.49433	0.000558	8.67E-02
Proteobacteria.Betaproteobacteria.Burkholderiales.Oxalobacteraceae.Herbaspirillum	Propionate	-0.52099	0.000567	8.49E-02
Bacteroidetes.Bacteroidia.Bacteroidales.Porphyromonadaceae.uncultured	AUC.cortisol	0.516551	0.000648	9.35E-02
Firmicutes.Clostridia.Thermoanaerobacterales.Thermoanaerobacteraceae.Gelria	Total.SCFA	-0.51539	0.000666	9.28E-02
Firmicutes.Clostridia.Clostridiales.Lachnospiraceae.Howardella	PTT	0.490648	0.000721	9.72E-02
Firmicutes.Clostridia.Clostridiales.Lachnospiraceae.Anaerostipes	PTT	0.489528	0.000745	9.71E-02
Bacteroidetes.Bacteroidia.Bacteroidales.Porphyromonadaceae.Parabacteroides	LBP	-0.48239	0.000791	9.99E-02

Table S7. Spearman correlation coefficients of investigated parameters, excluding microbiota (males)

Parameter 1	Parameter 2	Correlation coefficient	Raw p-value	FDR-corrected p-value
Total.SCFA	acetate	0.987879	9.31E-08	1.42E-05
Total.SCFA	n-butyrate	0.915152	0.000204	0.015642
n-butyrate	acetate	0.90303	0.000344	0.017524
Total.SCFA	PTT/PST	-0.81099	0.004414	0.168843
cort.AUC	30min.cortisol	0.806061	0.004862	0.148779
acetate	PTT/PST	-0.7805	0.007717	0.196796
PTT/PST	PTT	0.648479	0.008923	0.195034
cort.AUC	0min.cortisol	0.733333	0.015801	0.302186
n-butyrate	PTT/PST	-0.73172	0.016152	0.274577
perc.cort.increase	TNF.alpha	0.721212	0.018573	0.284169
propionate	30min.cortisol	-0.88571	0.018845	0.262123
60min.cortisol	acetate	0.885714	0.018845	0.24028
60min.cortisol	Total.SCFA	0.885714	0.018845	0.221797
PST	PTT	0.587558	0.021265	0.232399
PTT/PST	60min.cortisol	-0.69513	0.025647	0.261595
E2	n-butyrate	0.660606	0.037588	0.359439
PTT	IL.8	-0.53085	0.041745	0.375705
PTT	n-butyrate	-0.65036	0.041749	0.354865
E2	acetate	0.648485	0.04254	0.34256
PTT	Total.SCFA	-0.62581	0.05294	0.404991

Table S8. Spearman correlation coefficients of investigated parameters, excluding microbiota (females)

Parameter 1	Parameter 2	Correlation coefficient	Raw p-value	FDR-corrected p-value
cort.AUC	30min.cortisol	0.92561	0	0
Total.SCFA	acetate	0.982176	0	0
60min.cortisol	45min.cortisol	0.909233	2.22E-16	1.27E-14
Total.SCFA	n-butyrate	0.90788	6.66E-16	2.85E-14
cort.AUC	45min.cortisol	0.890767	6.22E-15	2.13E-13
cort.AUC	60min.cortisol	0.870557	1.41E-13	4.02E-12
n-butyrate	acetate	0.872233	2.30E-13	5.62E-12
PTT	PST	0.840712	9.33E-13	1.99E-11
Total.SCFA	propionate	0.830019	3.54E-11	6.74E-10
45min.cortisol	30min.cortisol	0.771603	3.52E-09	6.02E-08
60min.cortisol	30min.cortisol	0.762544	6.88E-09	1.07E-07
propionate	acetate	0.749531	2.62E-08	3.73E-07
n-butyrate	propionate	0.726642	1.11E-07	1.45E-06
cort.AUC	0min.cortisol	0.645296	5.24E-06	6.4E-05
30min.cortisol	0min.cortisol	0.502787	0.000807	0.009205
perc.cort.increase	0min.cortisol	-0.49791	0.000924	0.00988
IL.8	PST	0.44003	0.0028	0.02816
TNF.alpha	IL.8	0.433597	0.002925	0.027789
perc.cort.increase	30min.cortisol	0.433972	0.004586	0.04127
TNF.alpha	IFN.gamma	0.405138	0.005767	0.049307
60min.cortisol	0min.cortisol	0.405401	0.00855	0.069625
45min.cortisol	0min.cortisol	0.39216	0.011216	0.087182