THE UNIVERSITY of ADELAIDE

Exploring the Role of Spinal Glia and Toll-Like Receptor 4 in the Development of Endometriosis and Neuroimmune-Associated Pain

A thesis submitted in fulfilment for the degree of

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by

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Table of Contents

Abstract

Endometriosis is an oestrogen-dependent, chronic inflammatory condition in females, characterised by the presence of endometrial-like tissue forming lesions on extra-uterine sites; typically within the pelvis. The most common and debilitating clinical symptom is pain, including dysmenorrhoea, dyspareunia and persistent pelvic pain. However, the severity of reported pain seldom correlates with the extent of endometriosis lesions, and removal of lesions does not always eliminate pain. This disconnection between the peripheral pathology and pain symptoms suggests that central sensitisation processes may occur in women with endometriosis. Accordingly, researchers have thus far investigated neuronal contributions to central sensitisation associated with this condition. However, it is well recognised that adaptations in the reactivity of spinal glial cells (astrocytes and microglia) may also facilitate central sensitisation, by releasing inflammatory mediators that enhance excitatory, and/or reduce inhibitory, neuronal signalling ('neuroimmune communication'). Whether these glial-mediated inflammatory processes contribute to central sensitisation in the context of endometriosis remains to be established. Therefore, the initial aims of this thesis were to develop and optimise a minimally-invasive mouse model of endometriosis (Chapter 3), and to determine whether spinal glial adaptations that may contribute to central sensitisation occur in this model (Chapter 4). Recent studies suggest that inflammatory mediators, released by highly reactive spinal glial cells, may facilitate inflammatory disease processes in the periphery by stimulating neurogenic inflammation. In this case, neuroimmune-mediated central sensitisation may not only contribute to exaggerated pain (peripheral-to-central signalling), but also in sensitising afferent neurons to establish and maintain peripheral inflammatory conditions (central-to-peripheral signalling). Moreover, the central inflammation induced by spinal glia, as well as peripheral inflammation by peritoneal immune cells (such as macrophages), can be

activated by stimulation of the innate immune pattern recognition receptor, Toll-like receptor 4 (TLR4). While preliminary studies have suggested that altered TLR4 signalling may contribute to endometriosis, a role for peripheral and central TLR4 activity in the development of lesions and/or the associated pain has yet to be thoroughly investigated. The subsequent aims of this study were therefore to characterise endometriosis-like lesion development and neuroimmune-associated pain by manipulating TLR4 activity both centrally and peripherally (Chapters 5-7). The studies presented herein demonstrate that: mice with endometriosis-like lesions display changes in spinal glial expression, which correlate spatially with the locations of lesions in the periphery; an overall reduction in TLR4 activity promotes lesion development, alters glial-associated inflammatory signalling and attenuates pain behaviour; enhanced central TLR4 activity may stimulate lesion growth; and enhanced peripheral TLR4 activity facilitates lesion establishment, alters glial-associated inflammatory signalling and heightens pain behaviour. Collectively, these results highlight that central neuroimmune pathways, including those stimulated via TLR4, may be involved in endometriosis-related pain and possibly neurogenically-mediated lesion growth. In addition, both a deficiency and an excess in peripheral TLR4 activity can lead to a greater burden of endometriosis lesions. With future research, tailoring treatments to maintain a fine balance of receptor activity in central and peripheral tissues may prove to be a necessary and viable method to limit lesion development and pain symptoms in endometriosis patients.

Thesis declaration

'I certify that this work contains no material which has been accepted for the award of any other degree or diploma in my name, in any university or tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text. In addition, I certify that no part of this work will, in the future, be used in a submission in my name, for any other degree or diploma in any university or other tertiary institution without the prior approval of the University of Adelaide and where applicable, any partner institution responsible for the joint-award of this degree.'

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'I acknowledge the support I have received for my research throughout the provision of an Australian Government Research Training Program Scholarship.'

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Publications arising from this thesis

Manuscripts

Dodds KN, Beckett EAH, Evans SF, Hutchinson MR. Spinal glial adaptations occur in a minimally-invasive mouse model of endometriosis: Potential implications for lesion etiology and persistent pelvic pain. 2018, *Reprod Sci*, **in press** doi: 10.1177/1933719118773405.

Dodds KN, Beckett EAH, Evans SF, Hutchinson MR. Lesion development is modulated by the natural estrous cycle and mouse strain in a minimally-invasive model of endometriosis. 2017, *Biol Reprod*, **97** (6): 810-21.

Dodds KN, Beckett EAH, Evans SF, Grace PM, Watkins LR, Hutchinson MR. Glial contributions to visceral pain: implications for disease etiology and the female predominance of persistent pain. 2016, *Transl Psychiatry* **6**: e888.

Abstracts

Dodds KN, Beckett EAH, Staikopoulos V, Evans S, Hutchinson MR. Novel mouse model of endometriosis permits identification of spinal glia and nociception characteristics involved in pelvic pain. *J Neurochem*, **134** (1): S267.

Additional studies and co-authored publications

During my PhD candidature, I contributed to several additional primary research papers that are not presented in my thesis. These publications are listed below.

Arkwright JW, Underhill ID, Dodds KN, Brookes SJH, Costa M, Spencer NJ, Dinning PG. A composite fibre optic catheter for monitoring peristaltic transit of an intra-luminal bead. 2016, *J Biophotonics*, **9** (3): 305-10.

Dodds KN, Staikopoulos V, Beckett EAH. Uterine contractility in the non-pregnant mouse: changes during the oestrous cycle and effects of chloride channel blockade. 2015, *Biol Reprod*, **92** (6): 141.

Sia TC, Kuizenga MH, Dodds KN, Wiklendt L, Arkwright JW, Thomas A, Brookes SJ, Spencer NJ, Wattchow DA, Dinning PG, Costa M. Neurally-mediated propagating Discrete Clustered Contractions superimposed on myogenic ripples in ex vivo segments of human ileum. 2015, *Am J Physiol Gastrointest Liver Physiol*, **308** (1): G1-11.

Herweijer G, Kyloh M, Beckett EA, Dodds KN, Spencer NJ. Characterization of primary afferent spinal innervation of mouse uterus. 2014, *Front Neurosci*, **8**: 202.

Research grants, awards and conference presentations

Throughout my PhD candidature, I received several competitive research grants, awards and scholarships in recognition of, and to support, this work. I was also fortunate to attend multiple national and international conferences to present my research findings.

Research grants

From Targets to Novel Drugs Focus Grant (Principal Investigator 2015-16; Bayer Health Care Pharmaceuticals; €25,000).

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Runner up, David Healy Award (2017; World Endometriosis Society).

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Invited presentations

Dodds KN, Beckett EAH, Evans SF, Hutchinson MR. Development of a novel mouse model to investigate potential neuroimmune contributions to endometriosis. (ASCEPT 7th National Symposium on Advances in Gastrointestinal and Urogenital Research, Adelaide, Australia, Oct 2015).

Conference presentations

Dodds KN, Beckett EAH, Evans SF, Hutchinson MR. Endometriosis causes spinal glial adaptations: implications for persistent pelvic pain. (Oral presentation, 13th World Congress on Endometriosis, Vancouver, Canada, May 2017).

Dodds KN, Beckett EAH, Liu J, Evans SF, Hutchinson MR. Toll-like receptor 4 (TLR4) knock-out promotes lesion development in a mouse model of endometriosis. (Poster, Australasian Neuroscience Society, Hobart, Australia, Dec 2016).

Dodds KN, Beckett EAH, Evans SF, Hutchinson MR. Endometriosis-like lesions on peripheral tissues in the mouse induces subtle changes in spinal cord glial reactivity. (Poster, Society for Neuroscience, San Diego CA, USA, Nov 2016).

Dodds KN, Beckett EAH, Staikopoulos V, Evans SF, Hutchinson MR. Novel mouse model of endometriosis permits identification of spinal glia and nociception characteristics involved in pelvic pain. (Oral presentation, Australian Society for Medical Research, Adelaide, Australia, June 2015; Poster, Australasian Neuroscience Society, Cairns, Australia, Aug 2015; Poster – Prize Winner, Centre for Neuroscience Collaborators Day 2015, Adelaide, Australia, Sept 2015).

Thesis explanation

The format of this thesis is as follows: a traditional literature review; a literature review by publication; five primary research papers by publication; a general discussion; references; and an appendix. The second literature review (Chapter 2) and the first two primary research chapters (Chapters 3 & 4) were published in peer-reviewed journals during this PhD candidature. Here, these chapters are presented in their **original** manuscript format, except that the language has been translated into Australian English for consistency throughout the thesis. The formatted PDF (.pdf) versions of these publications are provided in Appendix 1. Literature citations from each chapter have also been collated within the References section. The remaining primary research studies (Chapters 5-7) have also been presented in manuscript style, which may result in some repetition between chapters (particularly in the abbreviations, and the *Introduction* and *Methods* sections). In all primary research chapters, figures within the *Methods* sections are continuous with the text, whereas figures, tables and supplementary materials pertaining to the experimental results are presented, in order of mention, at the end of each *Results* section.

Abbreviations

17βHSD2	17β-hydroxysteroid dehydrogenase 2
AMPA	α-amino-3-hydroxyl-5methyl-4-isoxazolepropionic acid
ANOVA	Analysis of variance
AP1	Activator protein 1
ATP	Adenosine triphosphate
AUC	Area under curve
BDNF	Brain-derived neurotrophic factor
cAMP	Cyclic adenosine monophosphate
CC	Chemokine (C-C motif)
CD11b	Cluster of differentiation 11b
CGRP	Calcitonin gene-related peptide
CI	Confidence interval
CNS	Central nervous system
COX	Cyclooxygenase
CREB	cAMP response element-binding protein
CV	Coefficient of variation
CX3	Chemokine (C-X3-C) motif
CXC	Chemokine (C-X-C) motif
DAB	3,3'-diaminobenzidine
DAMP	Damage-associated molecular pattern
DRG	Dorsal root ganglia
EAAT1	Excitatory amino acid transporter 1
EAAT2	Excitatory amino acid transporter 2
ELISA	Enzyme-linked immunosorbent assay
ER	Oestrogen receptor
ERK	Extracellular signal-regulated kinase

GABA	γ-aminobutyric acid
GFAP	Glial fibrillary acidic protein
GLAST	Glutamate-aspartate transporter
GLT-1	Glutamate transporter 1
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GnRH	Gonadotropin-releasing hormone
GS	Glutamine synthetase
HMGB1	High mobility group box 1
Hsp	Heat-shock protein
Iba-1	Ionised calcium-binding adaptor molecule 1
ICAM-1	Intercellular adhesion molecule 1
IFN	Interferon
IL	Interleukin
iNOS	Inducible nitric oxide synthase
IRF3	Interferon regulatory factor 3
JNK	c-Jun N-terminal kinase
LPS	Lipopolysaccharide
MAMP	Microbial-associated molecular pattern
MAPK	Mitogen-activated protein kinase
MCP-1	Monocyte chemoattractant protein 1
MIF	Macrophage migration inhibitory factor
MMP	Matrix metalloproteinase
mRNA	Messenger ribonucleic acid
MyD88	Myeloid differentiation primary response 88
NFκB	Nuclear factor κ-light-chain-enhancer of activated B cells
NGF	Nerve growth factor
NK	Natural killer
NK1R	Neurokinin-1 receptor

NMDA	N-methyl-D-aspartate
NSAID	Non-steroidal anti-inflammatory drug
P2X	Ionotropic purinoceptor
P2Y	Metabotropic purinoceptor
PAMP	Pathogen-associated molecular pattern
PBMC	Peripheral blood mononuclear cell
PG	Prostaglandin
PKC	Protein kinase C
PR	Progesterone receptor
RANTES	Regulated upon activation normal T-cell expressed and secreted
RIPA	Radio-immunoprecipitation assay
ROI	Region of interest
RT	Room temperature
SD	Standard deviation
TGF	Transforming growth factor
TIR	Toll-interleukin 1 receptor
TIRAP	TIR domain-containing adaptor protein
TLR	Toll-like receptor
TNF	Tumour necrosis factor
TRAM	TRIF-related adaptor molecule
Treg	Regulatory T-cell
TRIF	TIR-domain-containing adaptor-inducing interferon β
TRPV1	Transient receptor potential vanilloid 1
VEGF	Vascular endothelial growth factor

Chapter 1. An introduction to endometriosis

This chapter provides an introduction to endometriosis and its pathogenesis, with particular focus on contributions of the immune system and hormonal regulation. An overview of the current animal models used to study endometriosis, and potential neural mechanisms underlying the associated pain symptoms, will also be presented. In conjunction with the literature on glial contributions to pain reviewed in Chapter 2, this offers the relevant background information used to formulate the aims and hypotheses explored throughout this thesis.

1.1 Definition and epidemiology

Endometriosis is a chronic, oestrogen-dependent, inflammatory condition that is classically defined by the presence of endometrial-like tissue fragments (lesions) outside the uterus. In the general population, the estimated prevalence is 5-10% of women of reproductive age (Giudice & Kao, 2004), but can approach 40-70% in women with pelvic pain, and 30-50% in women with infertility (Meuleman *et al.*, 2009; Janssen *et al.*, 2013). Endometriosis is thus considered one of the commonest benign gynaecological conditions, affecting approximately 176 million females worldwide (Adamson *et al.*, 2010).

Endometriosis lesions are comprised of endometrioid epithelial and stromal cells that display a similar cellular architecture to the eutopic endometrium. They are highly vascularised, innervated, and contain numerous immune cells that contribute to the inflammatory microenvironment, including macrophages, mast cells, T and B lymphocytes, and natural killer (NK) cells (Greaves *et al.*, 2017a). Typically, endometriosis lesions form on structures in the lower pelvic cavity, including the ovaries, parietal peritoneum, uterus, uterosacral ligaments, rectovaginal septum, rectosigmoid colon, bladder, and the pouch of Douglas (Stegmann *et al.*, 2008). One of the perplexing aspects

of endometriosis is that lesion pathology can manifest in a variety of different forms. Macroscopically, lesions can be characterised as early-stage 'red' polypoid lesions (red, red-pink, and clear vesicles); intermediate-stage 'black' typical lesions (black and blue); and late-stage 'white' plaque lesions (white and yellow-brown) (American Society for Reproductive Medicine, 1997). This colour coding is considered to reflect the extent of vascularity (red), fibrosis (white), and bleeding (black) within lesions, which may be found in various multi-coloured combinations (Nisolle & Donnez, 1997; Stegmann *et al.*, 2008). Endometriosis lesions vary widely in size, and may be accompanied by the development of adhesions. These rigid structures often distort and impair the function of affected organs. In addition to morphology, lesions may be classified by their location and depth of invasion, which comprise (I) minimal; (II) mild; (III) moderate; and (IV) severe endometriosis (American Society for Reproductive Medicine, 1997). These stages are assessed on a weighted point system of whether endometriotic tissues infiltrate superficially (<5 mm) or deeply (>5 mm), are associated with adhesions (filmy or dense), cover of the pouch of Douglas, and involve the ovaries.

Pelvic pain is the most common clinical symptom associated with endometriosis and usually begins in adolescence at, or soon after, the onset of menarche (Sinaii *et al.*, 2008). The severity of pain varies considerably between patients and can occur as persistent non-menstrual pain, cyclic menstrual pain (dysmenorrhoea), pain upon defaecation (dyschezia) or pain during sexual intercourse (dyspareunia). Consequently, women with the condition often suffer major social and physical debility, including an average of 11 hours of lost productivity at school or work per week (Nnoaham *et al.*, 2011; Rogers *et al.*, 2013). Endometriosis-related pain is also associated with a high frequency of emergency department presentations (Nicholson *et al.*, 2001; Gao *et al.*, 2006), and additional patient-borne expenses for the care of endometriosis itself and the wide range of comorbid

conditions, such as painful bladder syndrome, migraine, inflammatory bowel disease (IBS), depression and anxiety (Mirkin *et al.*, 2007; Pope *et al.*, 2015). The average annual cost per patient in Canada for medical bills and lost productivity was $5,200 in 2009 (€10,000 in Europe), yielding an extrapolated total national expenditure of $1.8 billion (Levy *et al.*, 2011; Simoens *et al.*, 2012). Endometriosis and its associated pain therefore constitute a substantial burden not only on the quality of life of individual women, but also on their families, communities and the finite resources of national health and welfare services.

Besides pain, endometriosis is strongly associated with sub- or infertility, although in some cases patients may be asymptomatic. While both pelvic pain and infertility are suggestive but non-pathognomonic signs of the condition, there are currently no non-invasive imaging techniques or biomarkers available for diagnosis. To date, the only reliable means of diagnosing endometriosis is the macroscopic visualisation of lesions during exploratory laparoscopy (Dunselman *et al.*, 2014). This factor and a reticence of patients to present with symptoms they may consider embarrassing or 'just part of being a woman' have resulted in an average diagnostic delay of eight years from first experience of symptoms (Hadfield *et al.*, 1996), and has made endometriosis the second commonest indication for surgery in women of premenopausal age (Patel *et al.*, 2018). The true prevalence of endometriosis is therefore likely to be underestimated due to difficulties with correct symptom recognition by women themselves and primary care clinicians, and accurate diagnosis by gynaecologists.

Postsurgical staging of endometriosis can be difficult because lesions are dynamic, and may be found during periods of growth, regression or active remodelling (Wiegerinck *et al.*, 1993; Stegmann *et al.*, 2008). There are no correlations with the frequency of endometriosis and age, and there is also no relationship between age at diagnosis and lesion

severity (Savaris *et al.*, 2014). Patients are therefore unable to be treated based on whether the lesions and symptoms are likely to progress, remain static, or subside. Many clinicians and patients also believe that endometriosis-associated pain is due to the lesions, yet pain symptoms can remain in some women despite complete excision of lesions, and the degree of experienced pain correlates poorly with lesion characteristics (see Chapter 1.5) (Vercellini *et al.*, 1996; Chapron *et al.*, 2005). Combined with a limited understanding of how and why lesions develop, there are no substantive prevention or curative methods for either endometriosis or persistent pelvic pain, although several appropriate interventions, including surgical removal of lesions, can lessen its impact (see Chapter 1.3). Clearly, considerable research efforts are required to address this significant unmet human need.

1.2 Pathogenesis of lesions

The distinct aetiology and pathogenic mechanisms that lead to the development of endometriosis have not yet been fully elucidated. Proposed by Sampson in 1927, the principal theory for the origin of endometriosis is retrograde menstruation, where shed endometrial tissue is flushed back through the fallopian tubes and deposited within the peritoneal cavity during menses (Sampson, 1927). Here, viable epithelial and stromal cells can implant upon ectopic sites, and progress into mature lesions. Several lines of evidence support the contribution of retrograde menstruation to endometriosis, as they imply a higher likelihood of pelvic exposure to menstrual debris. This includes: endometriosis is typically diagnosed in reproductively-aged (menstruating) females, and spontaneously develops only in menstruating species (Eskenazi & Warner, 1997; D'Hooghe *et al.*, 2009); women with early menarche, short or heavy menstrual cycles display an increased risk for developing lesions (Cramer *et al.*, 1986; Darrow *et al.*, 1993); the incidence of endometriosis is increased in women with medical conditions associated with outflow obstruction and increased retrograde menstrual flow (Halme *et al.*, 1984); and suppression

of menstruation (via pregnancy or hormonal therapy) is associated with a reduced risk of lesion development (Missmer *et al.*, 2004; Vercellini *et al.*, 2014). In addition, the distribution of lesions within the pelvis is consistent with an accumulation of refluxed tissue in these locations (Jenkins *et al.*, 1986; Bricou *et al.*, 2008).

However, retrograde menstruation is a phenomenon that occurs in approximately 90% of reproductively mature females (Halme *et al.*, 1984; O *et al.*, 2017), while a much smaller proportion (~10%) develop endometriosis lesions. Other mechanistic hypotheses have therefore been suggested, such as coelomic metaplasia (where mesothelial cells in the peritoneum undergo metaplastic transition into endometrial glandular cells) (Matsuura *et al.*, 1999), neonatal seeding of endometrial stem cells (Brosens & Benagiano, 2013), lymphatic dissemination of endometrial tissues (Jerman & Hey-Cunningham, 2015), and bacterial contamination of refluxed endometrial tissues (Khan *et al.*, 2018). Although retrograde menstruation remains the most accepted and plausible source for endometrial cells within the peritoneum, it is not wholly sufficient to explain lesion pathogenesis. A genetic component to risk is likely as the incidence of endometriosis is greater in women with a positive family history (Moen & Magnus, 1993; Sapkota *et al.*, 2015). Additional factors that may predispose women to the condition have been considered, which act by promoting the survival, adhesion, invasion, growth, and maintenance of lesions. Both peritoneal immune dysfunction and altered sex hormone activity have been implicated as leading contributors to these crucial events (Patel *et al.*, 2018).

1.2.1 Dysregulation of the peritoneal immune response

Immunological perturbations have been observed in endometriosis patients for decades, although were often regarded as secondary side effects and not further evaluated as potential causes or targets for therapeutic intervention. Although the causal relationship between altered immune activity and endometriosis remains contentious, it is becoming

clear that immune dysfunction is critical in the development of endometriosis lesions.

1.2.1.1 Defective immunosurveillance

After translocating to the peritoneal cavity, ectopic endometrial tissues must first evade host immune defences in order to establish as lesions. The initial detection and inflammatory response against 'foreign' endometrial debris requires activation of the innate immune system, and it has been suggested that lesion formation may be enhanced where immune cells are unable to identify and clear endometrial tissues from the peritoneal space. Current mechanisms contributing to this hypothesis reflect both an inherent resistance of refluxed endometrial tissues, and an impaired function of peritoneal immune cells (Christodoulakos *et al.*, 2007; Herington *et al.*, 2011; Riccio *et al.*, 2018). An intrinsic abnormality of eutopic endometrial cells might increase the propensity for lesions to develop through either reduced expression of apoptotic molecules (Meresman *et al.*, 2000; Dmowski *et al.*, 2001) or the release of decoy factors, such as soluble intercellular adhesion molecule (ICAM)-1 (Somigliana *et al.*, 1996; Vigano *et al.*, 1998). This may result in failure of the immune system to recognise the ectopic endometrial tissue (Berbic *et al.*, 2010). Studies on the peritoneal immune system have also revealed that endometriosis patients display a decreased cytotoxic activity of NK and T-cells (Oosterlynck *et al.*, 1991; Guo *et al.*, 2016b); decreased phagocytic activity of macrophages (Chuang *et al.*, 2009); decreased maturation of immune cell populations (Garzetti *et al.*, 1993; Kikuchi *et al.*, 1993); and the potential for increased immune cell apoptosis (Garcia-Velasco *et al.*, 1999; Selam *et al.*, 2002; Sturlese *et al.*, 2011).

1.2.1.2 Inflammatory hyperresponsiveness

Although the capacity of immune cells to detect ectopic endometrial tissues may be compromised in women with endometriosis, these cells retain the ability to produce an

inflammatory response. Studies have consistently reported that there is increased recruitment of activated immune cells in the peritoneum of endometriosis patients (Zeller *et al.*, 1987; Beste *et al.*, 2014), which secrete higher basal and stimulated levels of cytokines, chemokines, growth and angiogenic factors, compared to cells from healthy women (Rana *et al.*, 1996; Kyama *et al.*, 2006; Montagna *et al.*, 2008). Local production of inflammatory mediators is believed to provide an ideal environment for lesion establishment, by inhibiting endometrial cell degradation and promoting tissue repair (Ahn *et al.*, 2015; Izumi *et al.*, 2018).

Inflammatory mediators linked to endometriosis exert their pro-lesion effects by regulating cellular activity, such as gene transcription. In particular, cytokines and chemokines are pleiotropic factors that act on many different target cells, to induce proliferation, differentiation and chemotaxis. Numerous cytokines pertinent to endometriosis have been implicated in these processes, including the common interleukins (IL)-1β, IL-4, IL-6, IL-10, interferon (IFN)-γ and tumour necrosis factor (TNF)-α; as well as IL-8, IL-17, IL-33 and transforming growth factor (TGF)-β; and the chemokines (C-C motif) ligand 2 (CCL2; MCP-1) and CCL5 (RANTES) (Herington *et al.*, 2011; Wu *et al.*, 2015). IL-8 and TNF-α, for example, promote proliferation and attachment of endometriotic cells to mesothelial structures (Zhang *et al.*, 1993; Garcia-Velasco & Arici, 1999; Iwabe *et al.*, 2000). IL-1β and TGF-β can induce the expression of vascular endothelial growth factor (VEGF)-A in endometriotic tissues; a factor imperative for angiogenesis and therefore lesion vascularisation (Taylor *et al.*, 2001; Young *et al.*, 2015). TNF-α, IL-1β and TGF-β may enhance migration and invasion abilities of ectopic endometrial cells, possibly through epithelial-to-mesenchymal transition processes and the upregulated expression of matrix metalloproteinases (MMPs) (Gottschalk *et al.*, 2000; Sillem *et al.*, 2001; Kao *et al.*, 2011; Yang & Yang, 2017). IL-1β has also been implicated in the development of postsurgical

adhesions associated with endometriosis (Stocks *et al.*, 2017). Many of these mediators reciprocally stimulate immune cells to release more cytokines and other inflammatory products, including cyclooxygenase (COX)-2 and prostaglandins. In addition, IL-8, CCL2 and CCL5 can recruit more specialised immune cells, such as T-cells, to the area, resulting in a highly complex inflammatory milieu (Wu *et al.*, 2015).

The peritoneal inflammatory environment is also enhanced by neurogenic mechanisms (Laux-Biehlmann *et al.*, 2015; McKinnon *et al.*, 2015). It is well established that sensory (nociceptive) nerves can initiate or exacerbate peripheral inflammation by the local release of neuropeptides, such as calcitonin gene-related peptide (CGRP) and substance P. This may occur due to stimulation of nerve terminals at the peripheral site ('axonal reflex'), or by antidromic signals originating from the central nervous system (CNS) ('dorsal root reflex'; see Chapter 2.12). Both substance P- and CGRP-expressing neurons are present within endometriosis lesions (Berkley *et al.*, 2004; Tokushige *et al.*, 2006; Tokushige *et al.*, 2010), as well as the receptor for substance P, neurokinin-1 receptor (NK1R) (McKinnon *et al.*, 2013). Upon release, substance P and CGRP directly induce the immune-mediated secretion of cytokines, such as TNF-α and IL-6, and attract more immune cells by altering vascular blood flow and permeability (Tuluc *et al.*, 2009; Raddant & Russo, 2011). Specific to endometriosis, sensory nerves containing substance P and CGRP may be activated by inflammatory factors released from menstrual debris or established lesions, or by mediators secreted from nearby immune cells (Chiu *et al.*, 2012; McKinnon *et al.*, 2015). The ensuing neurogenic inflammation may therefore further enrich the inflammatory environment and, by a positive feedback loop, continue to advance lesion growth.

1.2.1.3 Toll-like receptor 4-mediated immunity

Toll-like receptors (TLRs) are a major family of pattern recognition receptors that are

expressed by cells of the innate immune system. Each TLR consists of three key domains: an extracellular (or extra-endosomal) N-terminal ligand recognition domain, a transmembrane helix, and an intracellular (or intra-endosomal) C-terminal signalling domain (Botos *et al.*, 2011). The N-terminal is composed of leucine-rich repeats that allow TLRs to detect unique sets of conserved molecular patterns expressed by pathogens-, microbes- and/or host damage-associated molecules (PAMPs, MAMPs and DAMPs, respectively) (Bell *et al.*, 2003; Nicotra *et al.*, 2012). Upon ligand stimulation, TLRs must dimerise, which permits the C-terminal domain (the Toll/IL-1 receptor (TIR) domain) to bind and activate the adaptor proteins, TIR domain-containing adaptor protein (TIRAP) and TRIF-related adaptor molecule (TRAM) (Botos *et al.*, 2011). TIRAP and TRAM subsequently recruit myeloid differentiation primary response 88 (MyD88) and TIR-domain-containing adaptor-inducing IFN-β (TRIF)-dependent proteins, respectively, which result in divergent signalling outcomes (Akira & Takeda, 2004). For instance, activation of TIRAP-MyD88 induces the phosphorylation of mitogen-activated protein kinases (MAPKs), with the production of activator protein 1 (AP1) and early-phase nuclear factor κ-light-chain-enhancer of activated B cells (NFκB). This culminates in the upregulated transcription of genes largely relating to proinflammatory cytokines, such as IL-1β and TNF-α (Fitzgerald *et al.*, 2001; Kawai & Akira, 2005). Alternatively, TRAM-TRIF signalling activates IFN regulatory factor 3 (IRF3) and late-phase NFκB, to produce type-1 IFNs (e.g. IFN-β) and anti-inflammatory cytokines (e.g. IL-10, TGF-β) (Fitzgerald *et al.*, 2003; Kawai & Akira, 2005). Collectively, these mediators assist in resolving the immune insult, and may initiate processes of tissue repair (Takeuchi & Akira, 2010).

Typical TLR-mediated immune responses hence serve as defence mechanisms against invading pathogens or to minimise harm. However, where TLR function is inappropriate, resulting in aberrant immune activity, this can potentiate inflammatory processes that are

damaging to the host. For example, basal TLR signalling in the gastrointestinal tract is involved in maintaining commensal microbiota populations and mucosal homeostasis. Receptor dysfunction with either immune hypo- or hyperresponsiveness can disrupt barrier integrity and perpetuate tissue injury, with the consequent development of chronic inflammation and IBS (Cario, 2010; Frosali *et al.*, 2015). Evidence has also been provided that neuroinflammatory processes evoked by abnormal TLR activation can contribute to the development of neurodegenerative diseases, including amyotrophic lateral sclerosis, Parkinson's disease and Alzheimer's disease (Glass *et al.*, 2010; Lehnardt, 2010).

Given that: (1) TLR-mediated recognition of foreign stimuli can trigger inflammatory responses; (2) dysregulation of these immune functions have been heavily implicated in endometriosis lesion pathology; and (3) abnormal TLR activity can contribute to the progression of other chronic inflammatory conditions; a specific role for TLR-mediated immunity in endometriosis is now being explored. Particular attention has been given to the TLR4 subtype and its major exogenous ligand, lipopolysaccharide (LPS; or endotoxin). LPS is a component of the outer cell wall of gram-negative bacteria and thus is considered a PAMP, which can evoke strong TLR4-mediated proinflammatory responses. Notably, TLR4 can also be activated by numerous DAMPs, such as heat-shock protein (Hsp)-70, which are not associated with infection but rather endogenous responses to tissue injury or stress.

In endometriosis, high levels of LPS have been measured in the menstrual and peritoneal fluids from patients compared to controls (Khan *et al.*, 2010), and messenger ribonucleic acid (mRNA) transcript and protein expression of TLR4 is significantly increased in lesions compared to eutopic endometrial tissues (Allhorn *et al.*, 2008; Hayashi *et al.*, 2013). LPS- or Hsp-70-stimulated proliferation of endometriotic cells is also enhanced compared to normal endometrial cells, and dependent on the activity of NFκB, TNF-α and IL-8 (Iba *et*

al., 2004; Khan *et al.*, 2008b; Khan *et al.*, 2010; Khan *et al.*, 2013b). Moreover, peritoneal macrophages from endometriosis patients secrete significantly greater levels of IL-6 and IL-8 in response to LPS or Hsp-70 than lesion-free controls (Khan *et al.*, 2008b; Khan *et al.*, 2013b). Chronic LPS stimulation following the surgical induction of endometriosis in young mice has also been shown to induce an NFκB-dependent increase in endometriosis-like lesion number, size, cytokine expression, and proportion of proliferative cells (Azuma *et al.*, 2017). While still preliminary, these findings indicate a strong potential for TLR4 involvement in the pathogenesis of endometriosis, and warrants further exploration.

1.2.2 Altered sex hormone activity

The roles of ovarian sex hormones, oestrogen and progesterone, have also been considered to facilitate the development of endometriosis. Historically, studies have focussed on implicating high levels of oestrogen, since symptoms of the condition often first present at, or soon after menarche, and improve with the decline in oestrogen during menopause (Goldstein *et al.*, 1980; Oxholm *et al.*, 2007). In addition, the endometrium, a key component of lesions, involutes in the absence of oestrogen (Al-Sabbagh *et al.*, 2012). Hormonal fluctuations across the menstrual cycle are also well known to contribute to symptoms of pelvic pain, as is observed in endometriosis patients (Hassan *et al.*, 2014). Hence, endometriosis is commonly termed an 'oestrogen-dependent' condition.

1.2.2.1 Enhanced sensitivity to oestrogen

The endometrium is a primary target for oestrogen, which is cyclically produced by the ovaries under the hierarchical control of the hypothalamic-pituitary endocrine axis. In the ovaries, androgens are converted to bioactive oestrogens via the action of aromatase P450. Levels of oestrogen escalate during the first half (proliferative phase) of the menstrual cycle, which stimulates endometrial growth and vascularisation. In addition, oestrogen can

activate COX-2, with the subsequent production of prostaglandin E_2 (PGE_2) (Sacco *et al.*, 2012). Prostaglandins contribute to local inflammation and may additionally amplify oestrogen levels by stimulating aromatase P450 (Attar & Bulun, 2006). Therefore, oestrogenic action not only promotes biological effects in the endometrium favourable for lesion development, but can also self-sustain high concentrations in the absence of negative regulation (Kitawaki *et al.*, 2002; Rizner, 2009).

A role for oestrogen in endometriosis is supported by studies revealing that oestrogen receptors and aromatase P450 are localised within lesions (Kitawaki *et al.*, 1997; Matsuzaki *et al.*, 2001). Importantly, aromatase is not expressed in normal endometrial tissues (Kitawaki *et al.*, 1997); indicating that lesions acquire the ability to produce and respond to oestrogen locally, which may lend to a self-perpetuating cycle of growth. While its activity may not be wholly necessary for lesion formation (Burns *et al.*, 2012), administration of exogenous oestrogen in animal models has been shown to significantly augment the incidence, size, adherence, and invasiveness of ectopic endometrial tissues (Cummings & Metcalf, 1995; Fortin *et al.*, 2004; Burns *et al.*, 2012). Recurrence of lesions and pain symptoms that have previously regressed may also occur upon withdrawal of oestrogen suppressing drugs in premenopausal women (Waller & Shaw, 1993), or with oestrogen replacement in postmenopausal women (Matorras *et al.*, 2002) and in animal models of endometriosis (Rajkumar *et al.*, 1990). The mechanisms leading to, and resulting from, altered oestrogen signalling in endometriosis thus continue to be an active area of research.

1.2.2.2 Reduced sensitivity to progesterone

As a complimentary hypothesis, lesions and endometrial tissues from women with endometriosis may be functionally resistant to progesterone (Patel *et al.*, 2017). Progesterone is produced by the corpus luteum in the second half (secretory phase) of the

menstrual cycle, which causes the superficial endometrial layers to undergo extensive remodelling (including decidualisation) in preparation for blastocyst implantation (Brosens *et al.*, 2004). In the absence of conception, corpus lutea degenerate with a parallel decline in progesterone. Due to its relative anti-inflammatory nature, this sets up a controlled activation of resident immune cells, contributing to endometrial breakdown and expulsion (Brosens *et al.*, 2004). It has been postulated that the specific failure of progesterone to act appropriately alters the phenotype of endometrial tissue shed at menstruation; with a prolonged reduction in anti-inflammatory signalling, and highly robust endometrial debris capable of survival under mostly oestrogenic stimulation (Bruner-Tran *et al.*, 2013; Li *et al.*, 2016b).

At a molecular level, these important endometrial functions occur by the coordinated transcription of genes regulated by progesterone receptor (PR) signalling. PR expression is altered in endometriosis lesions (Attia *et al.*, 2000), and the endometrial transcriptome exhibits a delayed transition from the proliferative (high oestrogen) to secretory (high progesterone) phase in endometriosis patients (Burney *et al.*, 2007; Aghajanova & Giudice, 2011). Analyses of secretory phase endometrium from women with endometriosis show persistent expression of genes associated with cellular proliferation (Burney *et al.*, 2007), and reduced expression of progesterone-regulated genes, such as the PR coactivator, Hic5 (Aghajanova *et al.*, 2009), and 17β-hydroxysteroid dehydrogenase 2 (17βHSD2) (Burney *et al.*, 2007). Importantly, 17βHSD2 is an enzyme that plays a key role in converting bioactive oestrogen into its less potent metabolites. In addition, the lack of PR-regulated gene expression leading to decidualisation may contribute to implantation failure observed in infertile endometriosis patients (Kao *et al.*, 2003; Aghajanova *et al.*, 2011). Taken together, these data strongly support a role for disturbed sex hormone pathways, resulting in enhanced oestrogen activity, in the development of endometriosis.

1.3 Current therapeutic strategies to manage endometriosis

Current therapeutic approaches aim to relieve the symptoms of endometriosis through surgical and/or medical interventions, with pelvic pain as the first priority. Many of these options are unsatisfactory, however, because they do not treat the underlying cause(s) of the condition, and only temporarily suppress lesion activity. Importantly, the only available method to remove lesions is by surgical excision or ablation, which usually occurs during the laparoscopy required for diagnosis. This procedure is far from ideal due to the attendant risks, morbidity and financial costs associated with all surgeries. In addition, about 20% of endometriosis patients do not report an improvement in pain symptoms (Abbott *et al.*, 2004) and up to 40% have relapse of the condition within five years of the primary surgery (Abbott *et al.*, 2003; Vercellini *et al.*, 2006). Reoperation occurs in over half of the patient population, and approximately 30% require three of more surgeries that may include hysterectomy or oophorectomy (Cheong *et al.*, 2008). However, some patients with severe recurrent symptoms do not show further visible lesions (Abbott *et al.*, 2003); suggesting that not all postoperative pain is due to persistent endometriosis, and may reflect independent central neural contributions to pain (Stratton & Berkley, 2011). Surgery itself may also induce pain or contribute to its severity in endometriosis patients (Kehlet *et al.*, 2006). Adjuvant medical therapies must therefore be used to reduce the risk of lesion and pain recurrence (Guo, 2009).

Drugs used to alleviate endometriosis-associated pelvic pain have traditionally focussed on hormonal suppression, due to the known influence of high oestrogen levels on lesion growth and dysmenorrhoea. These include progestin-containing oral contraceptives and gonadotropin releasing hormone (GnRH) analogues, which inhibit oestrogen action or its production from the ovaries to low levels comparable with menopause or pregnancy. The induction of a hypo-oestrogenic state causes atrophy of endometrial tissues and

amenorrhoea, which counteract the proliferative and inflammatory activities that normally produce pain symptoms (Stratton & Berkley, 2011). Reduced menstruation, and thus a decreased risk for retrograde menstruation, may also prevent new endometriosis lesions from developing. However, these agents cannot be used for prolonged periods, due to significant side effects associated with systemically low oestrogen (e.g. progressive bone loss and severe vasomotor symptoms) (Rice, 2002). Non-steroidal anti-inflammatory drugs (NSAIDs) are the only inflammation-targeted therapies routinely recommended to treat endometriosis pain, which act by blocking COX-2-mediated production of PGE_2. Again, however, these drugs have numerous adverse effects upon chronic use, and often provide inadequate analgesic relief (Rice, 2002). Pain management therefore usually requires repeated courses and combinations of drug therapies to target multiple pathways (such as tricyclic anti-depressants and nerve block agents), until symptoms subside at menopause.

Due to these limitations, there is an obvious need to identify alternative therapies that provide specific and efficient treatment of endometriosis and its symptoms. For this to occur, it is critical to further understand the complex pathophysiology leading to the development of lesions. In many studies, this important work is facilitated by the use of animal models.

1.4 Animal models of endometriosis

Endometriosis is a condition that only develops spontaneously in humans and some non-human primates, such as the baboon (D'Hooghe *et al.*, 2009). It is a significant challenge to study the pathophysiology in women because there are no reliable, non-invasive, early detection methods available, and therefore patients already demonstrate established lesions at the time of clinical presentation. The variability of presenting symptoms, lack of biomarkers, and heterogeneity in lesions further complicate research, making it almost impossible to control for both the pelvic condition (e.g. normal pelvis, endometriosis, or

other pelvic pathology) and for symptoms (e.g. no symptoms, pelvic pain, and/or infertility). Moreover, lesion progression may only be confirmed and monitored by repeated laparoscopic examinations, raising ethical concerns. A vast number of studies therefore rely on experimental animal models to investigate the *in vivo* formation, consequences and potential treatment of endometriosis, with the principal species being non-human primates, and laboratory rodents (rats and mice).

1.4.1 Non-human primate models

Non-human primates, such as the rhesus monkey, cynomolgus monkey and baboon, are considered to represent the most physiologically relevant animal models of endometriosis. This is primarily due to their human-like qualities, with respect to phylogenetic homology, and reproductive anatomy and physiology (D'Hooghe *et al.*, 2009). The study of endometriosis in non-human primates is also an attractive option due to the occurrence of retrograde menstruation; the presence of minimal-to-severe spontaneous endometriosis (macroscopically and histologically); non-invasive menstrual cycle monitoring via perineal skin analysis; collection of naturally occurring peritoneal fluid; continuous breeding capabilities; and the potential for frequent blood sampling, repeated laparoscopies, and complex experimental surgeries, owing to their relatively large body size (D'Hooghe *et al.*, 2009). Thus far, non-human primates have provided important insights into both the natural progression and pathophysiology of endometriosis, and have acted as preclinical buffers for the translation of new therapeutics between rodents and humans. For example, familial clustering akin to that described in humans (Hadfield *et al.*, 1997; Zondervan *et al.*, 2004), and much of the early work linking environmental toxins (xeno-oestrogens) to endometriosis (Rier *et al.*, 1993; Rier *et al.*, 2001), was discovered through observations in rhesus monkeys. Nevertheless, their uses are limited for a number of reasons, including expensive purchase and agistment costs, the requirement for specialised infrastructure and

training for animal handling, as well as ethical sensitivity (D'Hooghe *et al.*, 2009; Greaves *et al.*, 2017a). Logistically, it may also take a long and unpredictable time to acquire sufficient numbers of animals for research, especially for studies on the advanced stages of lesion development. For these reasons, laboratory rodents are currently the major animal species utilised by researchers for *in vivo* investigations of endometriosis.

1.4.2 Rodent models

Rodents models of endometriosis are advantageous due to the high availability, ease of handling and experimental manipulation, and relatively low cost of animals; thereby offering a more tangible option for the evaluation of endometriosis lesions. One of the key differences between rodents and humans (and non-human primates) is that animals undergo an oestrous rather than a menstrual reproductive cycle. This means that, in the absence of implantation, endometrial tissue is resorbed by infiltrating immune cells as opposed to being shed through the process of menstruation. As a result, rodents do not naturally develop endometriosis and instead rely on the manual transplantation of endometrial-like tissues into ectopic sites, which provides a recapitulation of the lesions observed in humans (Vernon, 1990). These models of induced endometriosis can be classified into two main types: homologous (autologous or syngeneic) and heterologous (xenograft). The generation of homologous models involves the transfer of endometrial tissue within an individual (autologous) or between genetically identical (syngeneic) immunocompetent animals, and has been achieved using both rats and mice. In contrast, heterologous (xenograft) models are established by implanting fragments of human endometrium, menses, or endometriosis lesions into specialised immunodeficient mice. An overview of the common and recently developed rodent models of endometriosis is provided below.

1.4.2.1 Rat models

The most renowned rat model of endometriosis was developed by Vernon and Wilson in 1985, in which full thickness uterine squares were sewn onto the cascading mesenteric arteries of the same animal (Vernon & Wilson, 1985). Hence, it was termed the autologous rat model of surgically-induced endometriosis. Later modifications to this technique involved suturing of uterine tissues not only within the intestinal mesentery, but also to the abdominal wall and ovaries to more closely reflect the distribution of lesions in women (Berkley *et al.*, 2001). The significant benefits of this model are that the endometriosis-like lesions establish in known peritoneal locations, and develop into robust, uniform, cystic-type structures that share common gene expression profiles to human lesions (Flores *et al.*, 2007; Konno *et al.*, 2007; Umezawa *et al.*, 2008). This allows the ectopic endometrial implants to be easily retrieved, and their size and phenotype monitored following various manipulations; providing a suitable method to evaluate treatment options that aim to induce lesion regression (Lenhard *et al.*, 2007). Since rats (and mice) have bicornuate uteri, the endometriotic implants induced using one uterine horn can also be compared against the remaining intact uterus tissues.

The autologous rat model of surgically-induced endometriosis therefore replicates several fundamental aspects of the condition and has been highly valuable for our understanding of the pathogenesis, biological effects, and prospective pharmacotherapies for endometriosis. Indeed, the rat model is still in use by the original authors today, having recently demonstrated a link between stress and the growth of ectopic endometrial cysts (Cuevas *et al.*, 2018). However, this model of endometriosis invariably relies on the surgical placement of full-thickness uterine fragments (endometrium plus myometrium) and thus does not truly simulate lesion formation via the random peritoneal dissemination of shed endometrial tissue. Surgical procedures alone can also induce artificial

inflammatory responses that may confound results (Long *et al.*, 2016). Moreover, the use of rats in research is generally limited by the low number of genetically modified strains that are commercially available, and in this respect mouse models may be favoured.

1.4.2.2 Mouse models of endometriosis

1.4.2.2.1 Homologous (autologous and syngeneic) models

Homologous mouse models are the most widely used animals for studies of endometriosis. Many modifications and improvements have been developed since initial descriptions of the autologous mouse model of surgically-induced endometriosis (Cummings & Metcalf, 1995), which was based on the rat model by Vernon and Wilson (Vernon & Wilson, 1985). Major variations have included suturing of uterine tissues from a donor mouse to peritoneal sites of a recipient mouse (i.e. syngeneic surgically-induced model), and the blind injection of donor endometrial fragments into a recipient via an intraperitoneal syringe or small abdominal incision (i.e. syngeneic injection model). Occasionally, donor mouse tissues have been injected subcutaneously (Cheng *et al.*, 2011; Ferrero *et al.*, 2017), although this method lacks authentic interactions between the endometrial and peritoneal surfaces. All syngeneic mouse models can be used to explore specific contributions of donor and host tissues to the development of endometriosis and, as alluded to above, may utilise genetically engineered animals to enhance this. So far, studies have included mice that express ubiquitous or cell-specific fluorescent proteins to non-invasively track cells and monitor lesion growth (Hirata *et al.*, 2005; Greaves *et al.*, 2014a), as well as conditional or constitutive gene alterations to investigate the roles of particular proteins (Daftary *et al.*, 2013; Guo *et al.*, 2013; Rakhila *et al.*, 2014; Heard *et al.*, 2015; Vallcaneras *et al.*, 2017).

Significant attention has been given to a recent variation in the syngeneic injection model, where donor menstrual-like endometrium was used to inoculate recipient mice (Greaves *et*

al., 2014a). This protocol involves a series of manipulations to circulating levels of oestrogen and progesterone, which transform the endometrium into a phenotype that mimics human menstrual debris (Cousins *et al.*, 2014). The collected endometrial tissue subsequently injected into ovariectomised, oestrogen-supplemented mice therefore provides a closer representation of retrograde menstruation observed in humans. Intriguingly, since this method was published, the spiny mouse (Acomys cahirinus) has been discovered to undergo spontaneous endometrial decidualisation, demonstrating for the first time natural menstruation in a rodent (Bellofiore *et al.*, 2017). It is yet to be determined whether this strain of mouse can spontaneously develop endometriosis or has the ability to be utilised in homologous models with induced endometriosis-like lesions.

The menstruating mouse model of endometriosis highlights one of the substantial drawbacks of many studies using the syngeneic injection technique, which involves the ovariectomisation of mice and manual supplementation with oestrogen and progesterone. This method avoids the natural fluctuations in sex hormones that occur during the oestrous cycle, and can provide uniform, supraphysiological levels of oestrogen that are believed to promote lesion establishment and growth. Therefore, while there are benefits of controlling sex hormones for endometriosis research, in many cases the continuous systemic oestrogen stimulation may not reflect the clinical setting. These models, for example, are impracticable for studies of physiological functions that require intact hormone systems, such as fertility. Mouse models of endometriosis that involve ovariectomy also inadvertently undergo surgery and, as mentioned, this in itself may induce an inflammatory response (Long *et al.*, 2016). Many syngeneic surgically-induced mouse models of endometriosis bypass the hormonal limitation by using gonad-intact animals. Thus, while surgery-induced inflammation may be present in these mice, the cyclic variations in sex hormones allow for mechanistic links between lesions and infertility to be explored (Bilotas

et al., 2015; Cohen *et al.*, 2015).

1.4.2.2.2 Heterologous (xenograft) models

Since laparoscopy is required for the diagnosis of endometriosis, this procedure fortuitously provides clinicians with an opportunity to collect patient tissues and fluids for use in endometriosis research. Heterologous, or xenograft, models are considered to produce 'humanised' endometriosis-like lesions, as they are derived from such tissues taken from endometriosis patients (e.g. endometriosis lesions, menstrual effluent or eutopic endometrium) that are inoculated into immunodeficient mice (Bruner *et al.*, 1997). The use of human specimens via this approach is significant, because it is believed that the endometrium from endometriosis patients is abnormal and this contributes to lesion susceptibility (Carvalho *et al.*, 2011). Virtually all other rodent models, with the exception of the few using menstrual-like endometrium (Greaves *et al.*, 2014a), are generated using healthy uterine tissues. Xenografted mice are used to explore many aspects of endometriosis, although an overwhelming number of studies employ this model to test new therapeutic agents. Comparable to the syngeneic mouse models, several groups have also labelled donor human endometrial tissues with fluorescent proteins prior to xenotransplantation. Various techniques have been successful, including lipophilic dye infusion (Tabibzadeh *et al.*, 1999) or transfection with adenoviral vectors (Fortin *et al.*, 2004). However, many of these fluorescent labels are only transiently stable, and therefore longitudinal studies may be unfeasible.

The major weakness of xenograft models is that immunodeficient mice must be used to prevent rejection of the human endometrial transplants. As such, the full immune cell complement cannot be analysed, which removes one of the significant factors believed to be involved in the pathogenesis of endometriosis. Immunocompromised mice may, however, allow some interactions between the immune system and lesions to be explored,

when select immune cell populations are ablated (Sohngen *et al.*, 2014) or are introduced by adoptive transfer (Bruner-Tran *et al.*, 2010). A further challenge of this model is that the endometriosis biopsies used to induce lesions may be highly variable. For example, human lesions can be obtained from several locations (e.g. ovaries, pouch of Douglas, parietal peritoneal wall); can exhibit different degrees of invasiveness (e.g. superficial, deeply infiltrating nodules); as well as diverse macroscopic appearances (e.g. red, black, white ± adhesions). The heterogeneous nature of donor tissues may therefore limit the reproducibility and comparison of results between studies.

1.4.3 Summary

It is clear that animal models of endometriosis are invaluable for studying the pathophysiological mechanisms underlying lesion formation, as well as the debilitating effects of endometriosis-associated pain and infertility. As with many rodent models of human disease, the extent to which the ('non-physiological') induction of endometriosis-like lesions is truly representative of the condition is debatable, although they provide the best readily available tool for researchers at present. Currently, there is no single animal model optimal for all studies relating to endometriosis. Due to the number of theories on the aetiology and pathogenesis of this disorder, many factors need to be carefully considered when deciding on the appropriate animal model to address each research hypothesis.

It is also evident that most rodent models of endometriosis require surgical procedures; either a ventral midline abdominal incision with uterine tissues sutured to visceral structures, and/or a dorsal flank incision for ovariectomy, typically followed by oestrogen supplementation of supraphysiological levels. However, two of the main biological systems affected by endometriosis (i.e. the nervous and reproductive systems) can be significantly modulated by incisional injury and altered sex hormone activity. While many

studies utilise sham control animals, the relatively larger effects of these somatic modifications might mask subtle but important changes in proteins or behaviours of interest. Therefore, the use of current animal models may be particularly inadequate to explore several mechanisms contributing to endometriosis-associated pain and infertility. For future studies, there is a need for a well-characterised rodent model of endometriosis using animals that are gonad intact, surgery-free, and fully immunocompetent.

1.5 Mechanisms contributing to endometriosis-related pain

Pelvic pain is one of the most common and debilitating symptoms reported by women with endometriosis, and the mechanisms leading to pain in these patients is a major focus of current research. The physiological perception of pain arises from the activation of nociceptive sensory neurons by noxious stimuli, such as tissue injury or inflammation. These peripheral signals are conveyed to the spinal cord via the release of neurotransmitters, then projected to the brain by ascending second-order neurons. Here, the signal is integrated by multiple brain regions involved in somatosensation, emotion and cognition, which leads to the conscious experience of pain (Dubin & Patapoutian, 2010).

Hence, the presence of inflammatory lesions in the peritoneal cavity was originally believed to be the defining cause of pelvic pain associated with endometriosis. However, clinical observations have repeatedly found that the degree of reported pain seldom correlates with lesion severity, and removal of lesions does not reliably eliminate pain (Fauconnier & Chapron, 2005; Vercellini *et al.*, 2007). This disconnection between the peripheral pathology and pain symptoms suggests that sensitisation of nociceptive pathways may alter the pain experienced by endometriosis patients. At present, it is unclear whether the lesions themselves lead to exaggerated pain or if women with endometriosis are inherently hypersensitive to noxious stimuli. Notwithstanding this ambiguity, many molecular changes along the pain neuroaxis have been implicated in endometriosis, which

can amplify the noxious signals from lesions. Factors contributing to both peripheral and central pain sensitisation in endometriosis have been reviewed recently at length (McKinnon *et al.*, 2015; Morotti *et al.*, 2017; Coxon *et al.*, 2018), and the major findings from these discussions are summarised below.

1.5.1 Peripheral adaptations

Peripheral sensitisation is defined as the increased responsiveness and reduced threshold of nociceptive neurons in the periphery to stimulation of their receptive fields (Merskey & Bogduk, 1994). Interactions between the innervation and complex inflammatory environment of lesions has therefore been considered to induce peripheral adaptations that may lead to heightened nociception and the resulting pelvic pain associated with endometriosis.

1.5.1.1 Innervation of endometriosis lesions

Endometriosis lesions develop in close proximity to existing nerve fibres of the peritoneal cavity, and it is thought that compression or local aggravation of these neurons by infiltrating lesions can stimulate nociceptive pathways (Anaf *et al.*, 2000; Mechsner *et al.*, 2009; McKinnon *et al.*, 2012). A direct sensory innervation has also been described within superficial (Tokushige *et al.*, 2006; Mechsner *et al.*, 2009), ovarian (Tokushige *et al.*, 2010) and deeply infiltrating endometriosis lesions (Anaf *et al.*, 2002; Wang *et al.*, 2009), and the density of nerves correlates with the severity of pelvic pain associated with each anatomical site (Anaf *et al.*, 2002; McKinnon *et al.*, 2012). In addition, it is well established that endometriosis lesions demonstrate newly formed nerve endings (Asante & Taylor, 2011), and mediators that support neurogenesis, including the neurotrophins, nerve growth factor (NGF) and brain-derived neurotrophic factor (BDNF), are highly expressed both within lesions (Anaf *et al.*, 2002; Peng *et al.*, 2018) and the peritoneal fluid of endometriosis

patients (Barcena de Arellano *et al.*, 2011; Kajitani *et al.*, 2013).

Neurons innervating endometriosis lesions from both animal models and humans express many of the neurochemical qualities (receptors and neurotransmitters) required for pain signalling. Notably, this includes high expression of the key nociceptive ion channel, transient receptor potential vanilloid 1 (TRPV1), on lesion-associated sensory nerves (Greaves *et al.*, 2014b; Bohonyi *et al.*, 2017), which is associated with an increase in reported pelvic pain (Poli-Neto *et al.*, 2009; Rocha *et al.*, 2011; Liu *et al.*, 2012a). Neuronal receptors for other pain-producing mediators such as purines (Greaves *et al.*, 2014b; Ding *et al.*, 2017), oestrogen (Alvarez *et al.*, 2014), and proinflammatory cytokines (Alvarez & Levine, 2014), have also been described. Sensory nerves within lesions strongly express the neuropeptides, substance P and CGRP, which are implicated in both nociceptive transmission and neurogenic inflammation, and may therefore perpetuate inflammatory pain (Berkley *et al.*, 2004; Tokushige *et al.*, 2006; Tokushige *et al.*, 2010). In addition, the imbalance between sympathetic nerve fibres relative to the high density of sensory input in lesions has also been postulated to assist in maintaining local inflammation (Ferrero *et al.*, 2010; Arnold *et al.*, 2013; Morotti *et al.*, 2017).

1.5.1.2 Pronociceptive inflammatory stimuli

The dynamic inflammatory environment present within lesions and the peritoneal fluid of women with endometriosis can contribute to lesion development and, as indicated above, it is believed that these mediators also facilitate the generation of pain. Peripheral sensitisation by inflammation may occur via direct excitation of lesion-associated nerve terminals, or indirectly by changing the expression or responses of nociceptive ion channels and receptors through activation of second messenger systems (Schaible *et al.*, 2011). Numerous pronociceptive mediators that can induce these effects have been identified in endometriosis patients, including proinflammatory cytokines, chemokines, growth factors

and prostaglandins (Laux-Biehlmann *et al.*, 2015; McKinnon *et al.*, 2015). For example, a general increase in the concentration of proinflammatory cytokines of the peritoneal fluid correlates with the degree of pelvic pain in women with endometriosis (McKinnon *et al.*, 2012; Neziri *et al.*, 2014), and TNF-α activity within endometriosis lesions has been specifically implicated in upregulating the expression of NK1R (McKinnon *et al.*, 2013). Associations between prostaglandins and pain sensitisation are well established (Ma & Quirion, 2008; Schaible *et al.*, 2009), and the overexpression of PGE_2 and $PGF_{2\alpha}$ signalling pathways contributes to hyperalgesia in women and rodent models of endometriosis (Buchweitz *et al.*, 2006; McAllister *et al.*, 2016; Greaves *et al.*, 2017b). Adding complexity to this finding, endometriosis patients with pelvic pain display elevated peritoneal levels of oxidised lipoproteins, which can facilitate the non-enzymatic (i.e. COX-independent) generation of additional prostanoid-like molecules (Ray *et al.*, 2015).

1.5.2 Central adaptations

Continuous nociceptive input from sensitised peripheral neurons can initiate processes of central sensitisation where the excitability of spinal projection neurons, and therefore activity within the brain, also becomes enhanced (Woolf & Salter, 2000). Heightened neurotransmission within the CNS can, however, become independent of peripheral signals, due to long-lasting molecular changes in central nerve fibres (Ji *et al.*, 2003). As a result, the perception of pain may no longer reflect the peripheral noxious stimulus, and can remain even after the initiating insult has resolved. Albeit to a lesser extent than those observed in the periphery, a growing number of studies have investigated changes in central neural activity associated with endometriosis pain, which fall into two broad themes: adaptations in the spinal cord, and those of brain activity.

1.5.2.1 Adaptations in the spinal cord

Evidence indicating that neuronal adaptations in the spinal cord occur due to the presence of endometriosis has mostly been demonstrated by animal studies on pelvic organ cross-sensitisation. This mechanism implies that noxious information from an inflamed pelvic organ is relayed via the spinal cord to an adjacent normal structure, which results in functional changes of the unaffected organ (Malykhina, 2007). The connection between distinct pelvic organs arises from the convergence of sensory neurons from both organs into shared a spinal level. Adaptations consistent with sensitisation can therefore be investigated in the dorsal root ganglia (DRG), spinal dorsal horn and secondary organ (Li *et al.*, 2008; Peng *et al.*, 2010; Lei & Malykhina, 2012; Yoshikawa *et al.*, 2015). Similar to local stimulation of sensory nerve terminals, antidromic signalling from the spinal cord can trigger the release of neuropeptides, such as substance P and CGRP, at the peripheral site, leading to neurogenic inflammation and pain (Pan *et al.*, 2010; Pan & Malykhina, 2014). Identification of these 'viscero-visceral' or 'viscero-somatic' sensitising events suggests an anatomical and physiological basis for the occurrence of referred pain, and may partially explain the range of comorbid pelvic pain disorders commonly reported by endometriosis patients.

In the experimental setting, women with endometriosis-associated pelvic pain have a generalised increase in sensitivity to stimulation of sites close (e.g. colon or abdomen) and remote (e.g. hand or foot) from the pelvis (Bajaj *et al.*, 2003; Laursen *et al.*, 2005; He *et al.*, 2010; Issa *et al.*, 2012). Animals with endometriosis-like lesions also display referred hyperalgesia in the ureter (Lopopolo *et al.*, 2014; Iuvone *et al.*, 2016), colon (Chen *et al.*, 2015; Wang *et al.*, 2015), vagina (Berkley *et al.*, 2001; McAllister *et al.*, 2016) and abdomen (Greaves *et al.*, 2017b). Specifically, vaginal hyperalgesia can persist when endometriosis-like lesions are removed (Berkley *et al.*, 2007) and may be modulated by

fluctuations in the levels of oestrogen (Cason *et al.*, 2003; Berkley *et al.*, 2007; Nagabukuro & Berkley, 2007; Zhang *et al.*, 2008a). In addition, colonic hypersensitivity is associated with increased spinal activation of neuronal ion channels and transcription factors (Chen *et al.*, 2015; Wang *et al.*, 2015). Other DRG or spinal adaptations observed in animal models of endometriosis include the upregulated expression of nociceptive receptors (e.g. TRPV1, prostaglandin receptors), enzymes (e.g. COX-2) and neurotransmitters (e.g. substance P, CGRP) (Greaves *et al.*, 2017b; Hernandez *et al.*, 2017; Lian *et al.*, 2017).

1.5.2.2 Adaptations in brain activity

Central sensitisation in women with endometriosis-associated pain has been investigated by several neuroimaging studies, which have revealed that patients show decreased grey matter volume in key brain regions associated with the processing of noxious stimuli (As-Sanie *et al.*, 2012); elevated levels of excitatory neurotransmitters in the brain (As-Sanie *et al.*, 2016); and increased functional connectivity between brain regions involved in somatosensation (As-Sanie *et al.*, 2016). Animal studies have additionally demonstrated that endometriosis can alter electrical activity and opioid receptor expression in brain regions critical for endogenous pain control (Chadha *et al.*, 2008; Torres-Reveron *et al.*, 2016), and psychological stress can worsen endometriosis-associated hyperalgesia (Hernandez *et al.*, 2017). Moreover, animals with endometriosis-like lesions and pain exhibit an increase in depressive and anxiety-like behaviours, with changes in corresponding brain gene expression and electrophysiology (Li *et al.*, 2018).

1.6 Neuroimmune contributions to central sensitisation and pain – does this play a role in endometriosis?

Within the CNS, interactions between neurons and glial cells, a phenomenon termed ‘neuroimmune communication’, is well recognised to facilitate the development of central

sensitisation and pain (Milligan & Watkins, 2009; Grace *et al.*, 2014). Glia, such as astrocytes and microglia, are populations of immune-like cells that, under ambient conditions, are primarily responsible for maintaining neuronal homeostasis. However, in response to strong or prolonged immune challenges (such as peripheral inflammation), glial cells can shift their reactivity from a regulatory to rapid response state. Importantly, this includes the upregulated production and secretion of proinflammatory cytokines, which can increase the excitability of adjacent CNS neurons (in a manner similar to peripheral sensitisation), leading to central sensitisation and an augmented perception of pain.

While it is clear that neuronal mechanisms of central sensitisation have been considered in the context of endometriosis, a role for adaptations in glial populations is yet to be explored. The mechanisms of central sensitisation that occur due to neuroimmune signalling, and the potential significance of heightened glial reactivity in female-dominant visceral pain conditions (such as endometriosis), have been comprehensively reviewed as part of this PhD study. This discussion is presented henceforth in its peer-reviewed manuscript form.

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ii. permission is granted for the candidate in include the publication in the thesis; and
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Signature		Date	19/03/2018

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Contribution to the Paper	Assisted with manuscript conception, reviewed manuscript and provided expert advice on neuroimmune contributions to persistent pain.		
Signature		Date	20/03/2018

Chapter 2. Glial contributions to visceral pain: implications for disease aetiology and the female predominance of persistent pain

This chapter has been peer-reviewed and formally published as a full review paper [Dodds KN et al. (2016) *Transl Psychiatry*, **6**: e888].

2.1 Abstract

In the central nervous system, bidirectional signalling between glial cells and neurons ('neuroimmune communication') facilitates the development of persistent pain. Spinal glia can contribute to heightened pain states by a prolonged release of neurokine signals that sensitise adjacent centrally-projecting neurons. While many persistent pain conditions are disproportionately common in females, whether specific neuroimmune mechanisms lead to this increased susceptibility remains unclear. This review summarises the major known contributions of glia and neuroimmune interactions in pain, which has been determined principally in male rodents and in the context of somatic pain conditions. It is then postulated that studying neuroimmune interactions involved in pain attributed to visceral diseases common to females may offer a more suitable avenue for investigating unique mechanisms involved in female pain. Further, we discuss the potential for primed spinal glia and subsequent neurogenic inflammation as a contributing factor in the development of peripheral inflammation, therefore representing a predisposing factor for females in developing a high percentage of such persistent pain conditions.

2.2 From 'hysteria' to a molecular understanding of female pain

Historical descriptions of chronic debilitating pain without obvious visible cause were originally restricted to females, and dated back over two thousand years to the era of renowned Greek physician Hippocrates (460-370 BC). Episodes of severe emotional and physical distress in women were diagnosed as 'hysteria', a condition attributed to the movement of the uterus outside of the pelvis (the 'wandering womb') (King, 1998). Towards the end of the nineteenth century, the stigma surrounding female hysteria diminished due to accumulating evidence that men could also suffer from persistent pain, work which was largely pioneered by Sigmund Freud (1856-1939) (Freud & Freud, 2001). Considering pain as sex-independent in this context, along with general medical advances from the mid-twentieth century, has contributed to an immense expansion in our understanding of the mechanisms underlying the development of persistent pain. Notably, this is now known to involve bidirectional signalling between neurons and glia within the central nervous system (CNS).

However, a key discrepancy that remains in the literature is the clear over-representation of females among patients with persistent pain. There is an almost unanimous consensus that women are not only more sensitive in detecting painful stimuli, but are also the predominant sex with the most common painful disorders (Berkley, 1997; Greenspan *et al.*, 2007; Fillingim *et al.*, 2009; Mogil, 2012). This includes, but is not limited to, conditions associated with neuropathic pain, musculoskeletal pain (such as back pain, fibromyalgia, osteoarthritis and complex regional pain syndrome), orofacial pain (including temporomandibular joint pain), abdominal and pelvic pain (such as irritable bowel syndrome, painful bladder syndrome and dyspareunia), and headache/migraine (Fillingim *et al.*, 2009).

Extensive epidemiological, clinical and experimental evidence implicates several

biopsychosocial factors as contributing to the disparity in pain susceptibility across the sexes (Greenspan *et al.*, 2007). Despite this, a dichotomy exists in the pain research field at large, where the vast majority of preclinical studies have characterised pain models using male subjects only (Mogil & Chanda, 2005). Moreover, evidence implicating neuroimmune signalling in the development of persistent pain has primarily been acquired using animal models of neuropathic and somatic inflammatory pain. This has included, but is not restricted to, muscle inflammation, spinal cord injury, peripheral nerve injury, arthritis, bone cancer, and chemotherapy. While many of these pathologies are important for understanding female pain, there is a lack of research into the large number of female-dominant conditions that stem from the viscera. Consequently, the specific biological mechanisms underlying the predisposition of females to persistent pain remain elusive.

It is possible that past research generalising nociceptive mechanisms across the sexes has limited our approach in effectively treating female pain. Is it appropriate to assume that females process pain via identical mechanisms to males? Can we learn from, adapt and update aspects of the ancient Greek philosophy, by regarding female pain as a fundamentally distinct entity? And, to what extent do the sex-specific anatomical and neuroendocrine systems influence the heightened sensitivity of females to persistent pain?

To consider these questions, this review provides a summary of neuroimmune contributions, specifically those provided by astrocytes and microglia, to persistent pain signalling within the spinal cord. The concept that female sex hormones may modulate central neuroimmune signalling is then discussed, and that variations in these processes may have relevance for female-dominant pain conditions, as exemplified by several visceral inflammatory diseases. Additionally, the dorsal root reflex is re-explored as a central driver of peripheral neurogenic inflammation, leading to the hypothesis that sensitised spinal glia might contribute to, and predispose, a subpopulation of females to

persistent inflammatory pain.

2.3 Persistent pain arises from central sensitisation

Pain is a complex, unpleasant sensory and emotional experience that arises in response to, or is described in terms of, tissue damage (Merskey & Bogduk, 1994). Distinct from the well-established protective and adaptive functions of acute pain, pain persisting beyond tissue healing is maladaptive and serves no known physiological function. In contrast to acute pain, the mechanisms involved in the development and maintenance of persistent pain are not fully understood. One potential mechanism that has received detailed investigation is the process of 'central sensitisation', whereby long-lasting molecular changes cause amplification of pain signalling by nociceptive neurons within the CNS. Central sensitisation can include conditions of both hyperalgesia (heightened pain to a previously noxious stimulus), and allodynia (pain caused by a normally innocuous stimulus) (Woolf & Salter, 2000; Campbell & Meyer, 2006). It is now acknowledged that the development of central sensitisation engages not only neuronal, but also glial processes. Hence, the following sections outline the rationale for considering persistent pain to be a 'gliopathy' (Ji *et al.*, 2013), in addition to the previously described 'neuropathy'.

2.4 Glia and the tetrapartite synapse support the maintenance of CNS homeostasis

Glia are a non-neuronal, immune-like cell population that constitute the vast majority of cells within the CNS. They comprise satellite glial cells in the ganglia, and microglia, astrocytes and oligodendrocytes within the spinal cord and brain. The anatomical co-localisation of astrocytes and microglia in the spinal cord, combined with pre- and postsynaptic neurons, forms a key site of interaction termed the 'tetrapartite synapse' (DeLeo *et al.*, 2006; Ren & Dubner, 2015). Each cell within this functional unit reciprocally signals to another, contributing to a 'neuroimmune communication' that allows glia to

respond rapidly to disruptions in neuronal signalling (Milligan & Watkins, 2009; Grace *et al.*, 2014). The reactivity state and control of astrocytes and microglia is therefore critical in maintaining healthy CNS activity.

2.5 Dysregulation of healthy glial activity contributes to the development of persistent pain

Following injury and aberrant nociceptive events, microglia and astrocytes increase their expression and secretion of various proinflammatory cytokines and chemokines (Milligan & Watkins, 2009). The stimulation of glial cells can occur by neurokine products released as a result of tissue injury, or by neurotransmitters released from activated neurons. Many of the proinflammatory responses of glia are important in protecting against challenges that disrupt the homeostatic balance of the CNS, such as during the sickness response — a constellation of adaptive behaviours and physiological responses that promote recovery from illness (Maier & Watkins, 1998). However, under certain conditions glial reactivity is not advantageous and can instead be detrimental to neuronal function, such as during the manifestation of persistent pain.

In response to strong or persistent receptor stimulation, microglia switch from a surveillance state to an active response state, and astrocytes transition from a regulatory to reactive state (Ji *et al.*, 2013). Under these circumstances, the release of proinflammatory mediators by glia can contribute to ongoing nociception, by inducing long-lasting plastic changes of synaptic connectivity that enhances the transmission of ascending nociceptive information. As such, glia and their products are sufficient to create exaggerated pain. This has been shown where intrathecal transfer of highly reactive microglia alone, or injection or induction of their proinflammatory products (such as interleukin (IL)-1β and tumour necrosis factor (TNF)-α) into naïve animals, can induce symptoms of neuropathic pain (Tsuda *et al.*, 2003; Coull *et al.*, 2005; Kawasaki *et al.*, 2008a).

The downstream effects of enhanced glial reactivity are strengthened by the fact that immune mediators, including those released by glia, are substantially more potent in modulating neuronal signalling compared to classical neurotransmitters on a per molecule basis (Ji *et al.*, 2013). Glial proliferation, morphological changes, and increases in protein expression can persist for months after initial injury, even beyond tissue healing (Beggs *et al.*, 2012; Schwaller *et al.*, 2015). Moreover, proinflammatory mediators and glial-derived neurotransmitters can reciprocally stimulate glia in an autocrine and paracrine manner, thereby amplifying a positive feedback loop of unfavourable activity (Shiga *et al.*, 2001; Anderson *et al.*, 2004; Zhang *et al.*, 2014).

2.5.1 How do glia become activated?

Glia function as a product of their microenvironment, and as such the types of receptors they express vary from site to site, and many receptors can be upregulated to make glia more 'tuned' to ongoing stimulation. Within the spinal cord, microglia are sensitive to adenosine triphosphate (ATP) that binds to ionotropic (e.g. P2X4 and P2X7) and metabotropic (e.g. P2Y6 and P2Y12) purinergic receptors (Kobayashi *et al.*, 2006; Inoue, 2008; Morioka *et al.*, 2013; Shieh *et al.*, 2014). Chemokine receptors, such as CX3CR1 (with CX3CL1/fractalkine as ligand) and CCR2 (activated by CCL2/MCP-1), also contribute to the microglial proinflammatory response (Verge *et al.*, 2004; Thacker *et al.*, 2009; Toyomitsu *et al.*, 2012; Hu *et al.*, 2013), as well as receptors for the sensory neuropeptide, calcitonin gene-related peptide (CGRP) (Nieto *et al.*, 2015), and interferons (IFN), such as IFN-γ (Tsuda *et al.*, 2009). Akin to microglia, astrocytes can respond to ATP via the surface expression of P2X7 (Duan *et al.*, 2003; Narcisse *et al.*, 2005) and P2Y1 (Kobayashi *et al.*, 2006; Zeng *et al.*, 2008), and can be stimulated by IFN-γ (Zhang *et al.*, 2013a), CGRP (Reddington *et al.*, 1995; Cady *et al.*, 2011; Hansen *et al.*, 2015), and several mediators released by microglia themselves, including TNF-α and IL-18 (see reviews by

Ji *et al.* (2013) and Hansen and Malcangio (2013)). There is also evidence that astrocytes express tachykinergic NK1Rs (Miyano *et al.*, 2010), with substance P potentiating the IL-1β-mediated induction of IL-1β and prostaglandin E_2 (PGE_2) secretion from spinal cord astrocytes (Palma *et al.*, 1997).

Furthermore, a receptor family expressed by both glial cell types that has gained much recent attention, with regard to pain and immunity, are the Toll-like receptors (TLRs) (Nicotra *et al.*, 2012). TLRs allow glia to sense the presence of pathogen- or microbial-associated molecular products (PAMPs and MAMPs, respectively). Importantly, some receptor subtypes, such as TLR4, can additionally recognise endogenous 'self' warning molecules. Numerous putative ligands have been identified for these so-called damage-associated molecular patterns (DAMPs) in the processing of pain, including high mobility group box 1 protein (HMGB1) (Tong *et al.*, 2010; Ren *et al.*, 2012; Agalave *et al.*, 2014), heat-shock protein (Hsp)-90 (Hutchinson *et al.*, 2009), and fibronectin (Tsuda *et al.*, 2008).

2.5.2 What proinflammatory products do glia release upon activation?

Glial-induced upregulation of proinflammatory signalling is achieved through the induction of gene expression by numerous second messenger-mediated pathways. This includes activation of transcription by phosphorylation of mitogen-activated protein kinases (MAPKs) and nuclear factor-κB (NFκB). Specifically, the MAPKs implicated here are p38 in microglia (Svensson *et al.*, 2003), c-Jun N-terminal kinase (JNK) in astrocytes (Zhuang *et al.*, 2006), and extracellular signal-regulated kinases (ERKs) in both glial cell types (Zhuang *et al.*, 2005; Wang *et al.*, 2011). The proinflammatory products subsequently released from microglia include IL-1β, IL-6, IL-18, TNF-α, PGE_2, nitric oxide and brain-derived neurotrophic factor (BDNF), and IL-1β, IL-6, TNF-α, IFN-γ, CCL2, CXCL1, CXCL21 and matrix metalloproteinase (MMP)-9 from astrocytes (for reviews see Old and Malcangio (2012); Clark *et al.* (2013); Mika *et al.* (2013); Sofroniew (2014)). In addition,

astrocytes can increase their release of gliotransmitters, such as ATP (Werry *et al.*, 2006), glutamate, and D-serine (Mothet *et al.*, 2005).

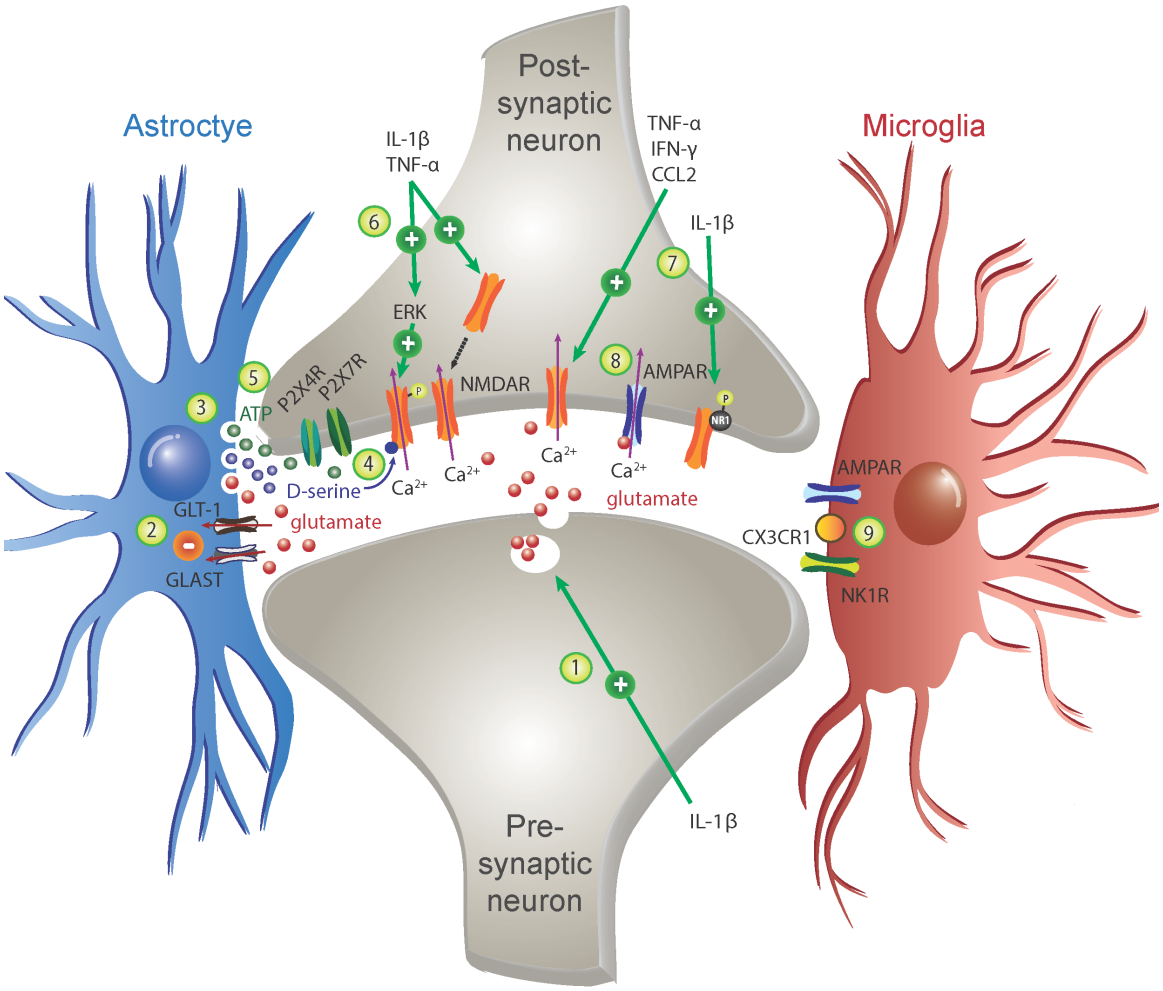
Since the discovery of neuroimmune contributions to pain more than two decades ago (Garrison *et al.*, 1991; Garrison *et al.*, 1994; Meller *et al.*, 1994), knowledge of glial-mediated molecular alterations in central sensitisation has grown exponentially. Overall, their proinflammatory effects enhance excitatory tone and synaptic efficiency, thereby facilitating an exaggerated pain state. The sequelae of mediators released and resultant outcome are now realised to be highly dependent on the type of glial cell that is activated, the degree of its reactivity, and the nature of the stimulus (Ransohoff & Perry, 2009; Kosek *et al.*, 2015). For this reason, we will provide a brief summary of the major known excitatory and inhibitory adaptations, and strongly encourage readers to explore other excellent in-depth reviews (see Milligan and Watkins (2009); Gao and Ji (2010); Hansen and Malcangio (2013); Ji *et al.* (2013); Taves *et al.* (2013); Grace *et al.* (2014)).

2.6 Glia enhance excitatory nociceptive signalling

Glial-derived proinflammatory mediators enhance nociceptive signalling in the spinal cord firstly by facilitating glutamatergic neurotransmission (Fig. 2.1). IL-1β has been shown to increase presynaptic release of glutamate (Yan & Weng, 2013), and IL-1β, TNF-α, CCL2 and IFN-γ increase postsynaptic NMDA and AMPA receptor currents (Vikman *et al.*, 2003; Kawasaki *et al.*, 2008b; Gao *et al.*, 2009; Gruber-Schoffnegger *et al.*, 2013; Liu *et al.*, 2013; Clark *et al.*, 2015). Postsynaptic neurons may further be excited by the release of glutamate from reactive astrocytes (Parpura *et al.*, 1994; Jourdain *et al.*, 2007). TNF-α can increase postsynaptic NMDA and AMPA-mediated activity by trafficking more receptor to the cell surface (Choi *et al.*, 2010), and by increasing subsequent Ca^{2+} conductance through phosphorylation of neuronal ERK (Xu *et al.*, 2010). In addition, IL-1β can induce Src kinase-mediated phosphorylation of the NR1 subunit on NMDA (Viviani

et al., 2003; Zhang *et al.*, 2008b). D-serine, a powerful neuromodulator released by reactive astrocytes, enhances depolarising NMDA cation currents by binding to the NMDA receptor glycine site (Lefevre *et al.*, 2015). There is also a persistent decrease in astrocytic expression of GLAST and GLT-1 (Sung *et al.*, 2003; Xin *et al.*, 2009); loss of function of these glutamate transporters causes an elevation in extracellular glutamate concentrations within the synapse (Liaw *et al.*, 2005; Weng *et al.*, 2006). Thus, the resultant aberrant uptake and/or release of glutamate, as well as the enhanced activity of its postsynaptic receptors, can contribute to excessive nociceptive signalling reaching the brain.

Additionally, increased exocytosis of ATP from reactive astrocytes (Hansen & Malcangio, 2013) can directly stimulate neuronal excitation (Jahr & Jessell, 1983) or induce glutamate release from presynaptic neurons (Nakatsuka & Gu, 2001), an effect which is facilitated by the upregulation of purinoceptors, such as P2X4 (Tsuda *et al.*, 2008; Ulmann *et al.*, 2008), P2X7 (Kobayashi *et al.*, 2011; Ying *et al.*, 2014) and P2Y12 (Kobayashi *et al.*, 2008; Tozaki-Saitoh *et al.*, 2008). Levels of other cytokine and chemokine receptors are also upregulated, including IL-6 induced microglial CX3CR1 (Verge *et al.*, 2004; Lee *et al.*, 2010) that enhances pain via IL-1β (Willemen *et al.*, 2010). Under certain conditions, such as IL-1β stimulation, both glial cell types may increase NK1R expression (Guo *et al.*, 2004). This potentiates the response to substance P (Miyano *et al.*, 2010), in turn facilitating the release of astrocytic ATP (Werry *et al.*, 2006) and proinflammatory cytokines, including TNF-α, IL-6 and PGE_2 (Luber-Narod *et al.*, 1994; Derocq *et al.*, 1996; Palma *et al.*, 1997). Lastly, TNF-α, IL-1β and IL-6 can elicit long-term synaptic plasticity by inducing the phosphorylation of the transcription factor cyclic adenosine monophosphate (cAMP) response element-binding protein (CREB) (Kawasaki *et al.*, 2008b), which may lead to the CREB-mediated transcription of COX-2 and NK1 (Samad *et al.*, 2001; Ji *et al.*, 2002; Ji *et al.*, 2003).


Astroctye
Post-
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NMDAR
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AMPAR
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P2X4R
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ATP
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D-serine
glutamate
glutamate
GLT-1
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GLAST
AMPAR
CX3CR1
9
NK1R
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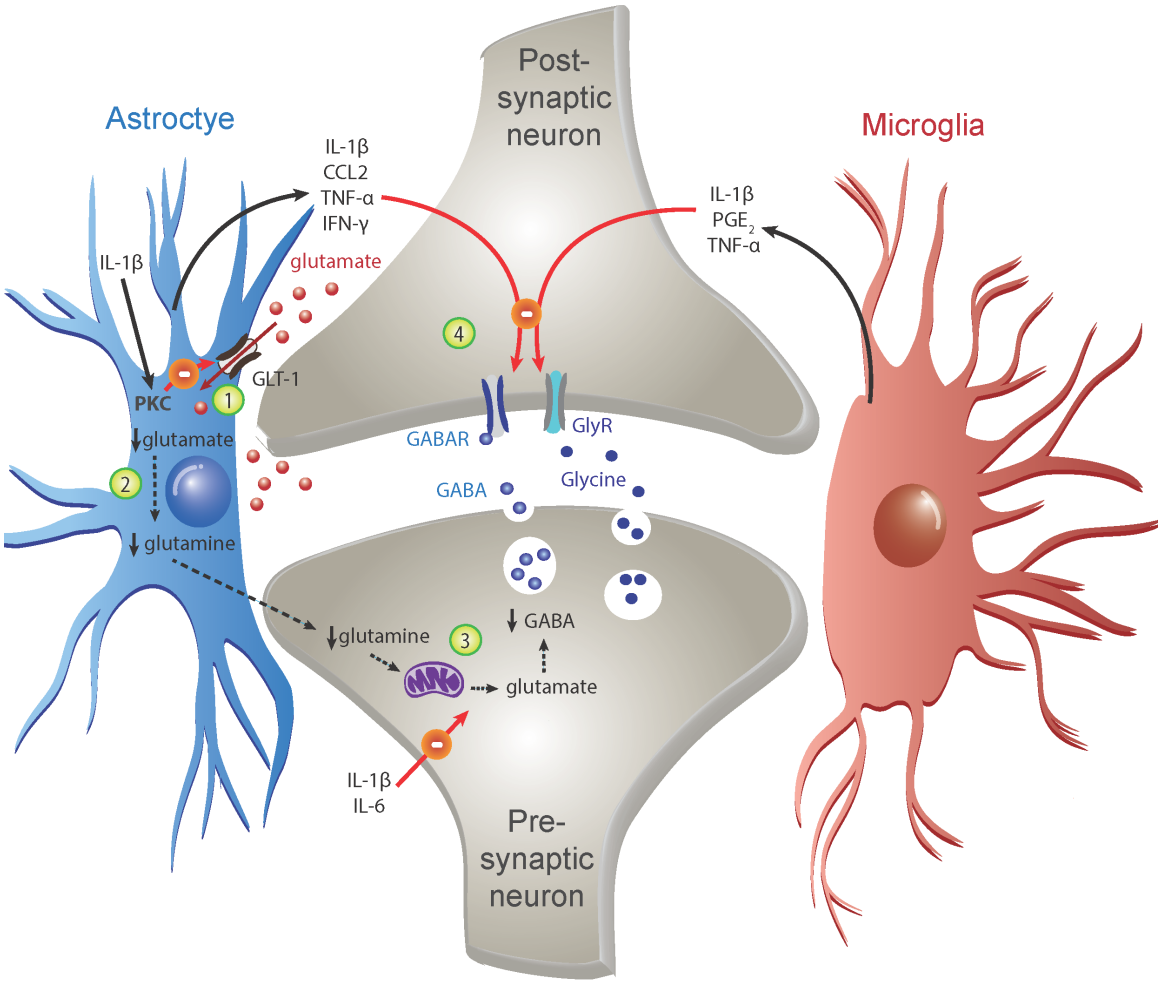
IL-1β
Pre-
synaptic
neuron

Figure 2.1 Schematic representation of the major proinflammatory glial-mediated alterations to excitatory synapses within the spinal dorsal horn that contribute to central sensitisation. Strong or long-term noxious activation of astrocytes and microglia within the spinal dorsal horn can lead to the aberrant synthesis and release of proinflammatory mediators, such as TNF-α and IL-1β. The overarching effect of these neurokine signals in excitatory synapses contributes to central sensitisation and facilitates the transmission of nociceptive signals to the brain. Some of the major known adaptations include: (1) Increased release of the excitatory neurotransmitter, glutamate, from presynaptic nerve terminals. (2) Suppression of astrocytic glutamate reuptake via downregulation of GLT-1 and GLAST activity. (3) Release of the glutamate from astrocytes, which is capable of increasing the excitability of nearby neurons. (4) D-serine, also released from astrocytes, enhances Ca^{2+} influx via binding to glycine sites on NMDA receptors on postsynaptic neurons. (5) Astrocytic release of ATP also increases postsynaptic excitability via activation of ligand-gated purinergic receptors, P2X4 and P2X7. (6) TNF-α and IL-1β increase translocation of NMDA receptors to the postsynaptic membrane and increases their conductance via an ERK dependent pathway. (7) IL-1β, TNF-α, IFN-γ and CCL2 increase NMDA receptor-mediated excitatory signalling; in the case of IL-1β this is thought to involve the phosphorylation of receptor subunits including NR1, 2a and 2b. (8) Proinflammatory cytokines have been linked to increased expression and activation of AMPA receptors at excitatory synapses. (9) Reactive microglia have increased expression of receptors for various neurotransmitters and chemokines (e.g. AMPARs, NK1Rs and CX3CR1), which can induce the further release of proinflammatory cytokines upon stimulation, thereby perpetuating neuronal excitation.

2.7 Glia attenuate the inhibition of nociceptive signalling

Heightened glial activation can also induce disinhibition; that is, a loss of inhibitory signals within the CNS that usually suppress nociceptive transmission, such as GABA and glycine signalling (Fig. 2.2). Activation of microglial TLR4 by LPS in rodent spinal slices induces IL-1β release, which suppresses postsynaptic GABA receptor function through activation of protein kinase C (PKC) (Yan *et al.*, 2015a). IL-1β-induced PKC activation also attenuates astrocytic GLT-1 activity, leading to increased glutamate within the synaptic cleft (Yan *et al.*, 2015a). This not only drives a sustained excitation of postsynaptic neurons, but also a deficiency in the supply of glutamine, which is metabolised from glutamate following its reuptake. Consequently, glutamate-glutamine cycle-dependent GABA synthesis by the presynaptic neuron is attenuated (Jiang *et al.*, 2012). Moreover, TNF-α can prevent action potentials in inhibitory presynaptic neurons (Zhang *et al.*, 2010); IL-1β and IL-6 suppress presynaptic GABA and glycine currents (Kawasaki *et al.*, 2008b); and PGE_2, CCL2, and IFN-γ can attenuate postsynaptic electrical activity mediated by GABA or glycine (Ahmadi *et al.*, 2002; Gosselin *et al.*, 2005; Vikman *et al.*, 2007). Thus, suppression of inhibitory influences within the spinal cord by glial-derived factors may exacerbate pain, by potentiating the transduction of nociceptive information.



<u>Figure 2.2</u> Schematic depicting the major proinflammatory glial-mediated changes to inhibitory synapses within the spinal dorsal horn that facilitate central sensitisation. As mentioned in Figure 1, prolonged stimulation of astrocytes and microglia can lead to the increased synthesis and release of various proinflammatory cytokines and chemokines. Within inhibitory synapses of the spinal cord dorsal horn, the effects of these mediators ultimately lead to a reduction in inhibitory neurotransmission ('disinhibition'), which further facilitates central sensitisation. For example: (1) IL-1β can mediate a decrease in the astrocytic uptake of glutamate, via a PKC-mediated suppression of glutamate transporter GLT-1. (2) The reduced uptake of glutamate via GLT-1 leads to decreased availability of glutamine for GABA synthesis. (3) IL-1β and IL-6 inhibit presynaptic GABA and glycine currents. (4) Lastly, IL-1β, PGE_2, CCL2, TNF-α and IFN-γ decrease GABA and glycine receptor activity; in the case of IL-1β this is thought to be mediated via a PKC-dependent pathway.

2.8 Female sex hormones and neuronal hypotheses underlying the sexual dimorphism of pain

In addition to many pain syndromes having greater prevalence in females than males, other anecdotal evidence suggests that sex steroid hormones can have a direct influence on somatic and visceral persistent pain. In women, for instance, certain painful conditions typically occur during the menstrual years, and symptoms tend to fluctuate with the menstrual cycle (Riley III *et al.*, 1999; Houghton *et al.*, 2002). Symptom severity of several visceral pain conditions, such as irritable bowel syndrome, has been reported to decrease following menopause (Palsson *et al.*, 2003), and increase with hormone replacement therapy in postmenopausal women (Ruigomez *et al.*, 2003). Similarly, nociceptive stimuli in rodent visceral pain models are sensitive to both the changing steroid hormone levels throughout the oestrous cycle (Cason *et al.*, 2003; Ji *et al.*, 2008; Ball *et al.*, 2010), and during hormone supplementation following ovariectomy (Ji *et al.*, 2005; Berkley *et al.*, 2007; Robbins *et al.*, 2010). Thus, it has been suggested that either elevated or fluctuating levels of sex hormones play a key role in exacerbating persistent pain (Traub & Ji, 2013).

However, the mechanisms underlying this modulation remain unclear and, to date, much of the research has focused on sex steroid-mediated alterations in neural activity and/or molecular targets expressed by neurons. For example, antagonism of neuronal NMDA receptors, often coexpressed with estrogen receptor-α (ER-α), can attenuate the visceromotor reflex to colorectal distension with greater potency in untreated ovariectomised rats, compared to those with oestradiol replacement (Ji *et al.*, 2008). Colorectal distension is correlated with an increase in PKA-mediated NMDAR NR1 subunit expression and phosphorylation in ovariectomised, oestrogen-supplemented animals, compared to those not receiving oestrogen (Ji *et al.*, 2008). Furthermore, intrathecal administration of oestrogen or an ER-α-selective agonist can cause an increase

in distension-evoked dorsal horn neuron phosphorylated ERK expression, and reverse the decrease in distension-evoked visceromotor reflex produced by ovariectomised rats (Ji *et al.*, 2011).

2.9 Does female sex hormone modulation of glial reactivity contribute to the female predominance of persistent pain?

Despite our understanding of the tetrapartite synapse in facilitating nociceptive signalling, it is likely that the contribution of glia has not yet received sufficient attention with regards to the female susceptibility to persistent pain. Intriguingly, TLRs - which, as discussed previously, are one receptor family expressed by glia and play an important role in the immunological response to pathogenic stimuli - are well situated to serve as an important molecular target for persistent pain conditions. This is particularly true for hormonally-regulated female pain, as oestrogen appears to influence TLR4-mediated proinflammation and pain in various conditions. For instance, glucuronide metabolites (which typically have a longer half-life than the parent molecule) of oestrogen cause potent activation of TLR4 *in vitro*, correlating with enhanced mechanical allodynia in rats *in vivo* (Lewis *et al.*, 2015). The proinflammatory response to LPS is potentiated by oestrogen in female but not male neonatal microglia (Loram *et al.*, 2012). Moreover, while adult hippocampal microglia from ovariectomised rats in *ex vivo* preparations show a downregulation in LPS-induced inflammation upon oestrogen supplementation, IL-1β messenger ribonucleic acid (mRNA) is potentiated when oestrogen is administered *in vivo* (Loram *et al.*, 2012). Long-term oestrogen exposure in ovariectomised mice promotes the expression of inflammatory mediators by CNS and peritoneal macrophages, in response to LPS activation *in vivo* (Soucy *et al.*, 2005) and *ex vivo* (Calippe *et al.*, 2010), respectively. Intravenous administration of LPS in humans induces a similar decrease in visceral and musculoskeletal pain thresholds, although intriguingly a much more pronounced increase in circulating

levels of plasma TNF-α and IL-6 was evidenced in females compared to males (Wegner *et al.*, 2015). A recent randomised control trial additionally showed that low-dose LPS was perceived to increase pain from suprathreshold noxious thermal stimuli in women only, and impaired conditioned pain modulation, a measure of endogenous pain inhibition (Karshikoff *et al.*, 2015).

Other studies have reported that TLR-mediated responses are important in male but not female pain. Using LPS-induced (in TLR4 mutant mice) (Sorge *et al.*, 2011) and spinal nerve ligation (in *TLR4*-knockout mice) (Stokes *et al.*, 2013) models of pain enhancement, it was reported that mechanical allodynia is TLR4-dependent in males but TLR4-independent in females. Inhibition of spinal p38 MAPK has been effective in attenuating inflammatory and neuropathic pain in male, but not female mice (Taves *et al.*, 2015). It has further been proposed that female pain is independent of microglia in a rodent model of mechanical allodynia, alternatively involving the recruitment of T-cells (Sorge *et al.*, 2015). However, this argument bears further consideration given that males are comparable to females in the generation of autoimmune T-cells, but the phenotype of regulatory T-cells (Treg), which serve to suppress inflammatory processes, may be more aggressive in males (Reddy *et al.*, 2005).

Perhaps these opposing results mirror the highly complex, and well recognised, nature of oestrogen being both a pronociceptive and antinociceptive hormone (see reviews Fillingim and Ness (2000); Aloisi and Bonifazi (2006); Craft (2007); Sanoja and Cervero (2010); Amandusson and Blomqvist (2013)). Regardless, it is evident that the effects of female sex hormones on TLR4-mediated signalling are multifaceted and, given the range of receptors and pathways utilised by glia, highlight the need for research into neuroimmune mechanisms that may be specific to pain in females.

2.10 Somatic versus visceral pain

Persistent pain is a cardinal feature of chronic inflammation of peripheral tissues; thus, our increase in knowledge of neuroimmune signalling has led to investigations of the link between glia and persistent pain associated with inflammation. These data have been primarily acquired using animal models of neuropathic and somatic inflammatory pain, with considerably less attention given to pain arising from the viscera. Whilst there are many commonalities in the processing of somatic and visceral pain, there are also several important clinical distinctions (for reviews see Gebhart and Ness (1991); Giamberardino and Vecchiet (1995); Cervero and Laird (1999)). For instance, pain cannot be evoked from all viscera; visceral pain is diffuse and poorly localised, due to relatively few visceral afferents with extensive receptive fields; visceral pain can often be referred to remote locations, attributable to visceral and somatic afferent pathways converging into shared spinal levels; injury to the viscera does not necessarily cause pain; and intense motor and autonomic reflexes, such as nausea and muscle tension, usually accompany visceral pain. This aside, the fundamental mechanisms leading to the perception of somatic and visceral pain are similar, where enhanced activity from peripheral nociceptors activates ascending central pathways to the brain. Consequently, the involvement of neuroimmune signalling in persistent pain attributed to visceral inflammation has gained interest in the past few years (Lu, 2014).

2.11 Neuroimmune contributions to the female predominance of pain associated with inflammation of the pelvic viscera

The viscera are also where sex divergences in pain processing become particularly intriguing, due to the unique organisation of the reproductive and pelvic anatomy in males and females. It has been estimated that women are at greater risk of developing persistent pain within the pelvis, currently affecting between 15% and 24% of women (Mathias *et*

al., 1996; Grace & Zondervan, 2004) versus 1.8-12% in men (Ejike & Ezeanyika, 2008; Suskind *et al.*, 2013), including pain due to menstruation, intercourse, pregnancy and childbirth, and infection and inflammation via the vagina, cervix and uterus (Berkley, 1997; Latthe *et al.*, 2006; Curran, 2015). Spinal microglia been found to contribute to pain in male animals with chronic prostatitis (Zhang *et al.*, 2013b; Wong *et al.*, 2015). To our knowledge, however, there are currently no comprehensive studies investigating glial contributions to pain associated with visceral diseases that have been restricted to, or with a substantial focus on, females. This alternative scope in research could reveal distinct female pain mechanisms that may be exploited to improve pain management.

Potential neuroimmune contributions to three visceral conditions that have a greater prevalence in, or are exclusive to, females are discussed below: inflammatory bowel disease, painful bladder syndrome, and endometriosis. These pathologies share several features of neuropathic pain and somatic inflammation, such as heightened neural activity, decreased pain thresholds and increased pain behaviour, indicating that central neuroimmune adaptations are probably taking place. This is supported by evidence demonstrating that experimentally-induced inflammatory bowel disease, cystitis or endometriosis can result in the sensitisation of adjacent pelvic organs (e.g. intestines, bladder, and uterus) (Miranda *et al.*, 2011; Chen *et al.*, 2015; Wang *et al.*, 2015; Yoshikawa *et al.*, 2015). A similar phenomenon is observed clinically with the clustering of comorbidities in women with pelvic pain, such as patients with irritable bowel often presenting with viscero-visceral (e.g. bladder or menstrual pain) or viscero-somatic (e.g. pelvic muscle spasm, temporomandibular pain) complaints.

2.11.1 Inflammatory bowel disease (IBD)

IBD comprises ulcerative colitis and Crohn's disease, which both involve colonic inflammation; however, each has distinctive pathologic features (Podolsky, 1991). While

the prevalence of ulcerative colitis in males and females is generally similar, the female-male ratio of Crohn's disease in adults is increased up to approximately 1.2-1.3 times (Bernstein *et al.*, 2006; Kappelman *et al.*, 2007). Studies on glia and IBD have utilised rodent models of di- or trinitrobenzene sulfonic acid-induced colitis, and potential differences between the sexes have not been analysed (Riazi *et al.*, 2008; Huang *et al.*, 2010; Kannampalli *et al.*, 2014; Song *et al.*, 2014). Nonetheless, marked increases in reactivity were described for microglia in the spinal cord and hippocampus (Riazi *et al.*, 2008; Kannampalli *et al.*, 2014), and activated satellite glia in the DRG (Kannampalli *et al.*, 2014). This is associated with an upregulation of TNF-α levels (Riazi *et al.*, 2008; Kannampalli *et al.*, 2014), and closer apposition between satellite glial cells and primary afferent neurons in the DRG (Kannampalli *et al.*, 2014) via enhanced neuron-glia gap junction coupling (Huang *et al.*, 2010). Associated centrally-derived hyperalgesia was assessed by various methods, including increased visceromotor reflex activity (Kannampalli *et al.*, 2014) and abdominal withdrawal reflex (Song *et al.*, 2014), to graded colonic distension. Intracerebroventricular (Riazi *et al.*, 2008), intrathecal or systemic (Kannampalli *et al.*, 2014) minocycline or intrathecal administration of an anti-TNF-α antibody (Song *et al.*, 2014) attenuated the respective pain behaviours examined.

2.11.2 Painful bladder syndrome

Contributions of neuroimmune overactivity to persistent pain have also been suggested in animal models of, and human patients with, painful bladder syndrome. Formally known as interstitial cystitis, painful bladder syndrome affects approximately 3-7% of adult females and 2-4% of males, encompassing a range of bladder disorders that involve persistent pelvic pain or discomfort, non-specific urinary symptoms, and often cystitis (Berry *et al.*, 2011; Suskind *et al.*, 2013; Vella *et al.*, 2015). In a preliminary study using pooled data from male and female cats with spontaneous feline interstitial cystitis, the fluorescent

intensity and number of glial fibrillary acidic protein (GFAP)-immunopositive astrocytes in the S1 spinal cord dorsal horn was increased compared to healthy unaffected cats (Birder *et al.*, 2010). In addition, it has recently been demonstrated that peripheral blood mononuclear cells (PBMCs) from women with painful bladder have an increased proinflammatory response to TLR2 and 4 stimulation *in vitro* (Schrepf *et al.*, 2014). The magnitude of the proinflammatory response also positively correlated with the extent of pelvic and extra-pelvic pain, and the manifestation of comorbid conditions (Schrepf *et al.*, 2015). This observation has great importance, as the TLR responsivity of PBMCs could serve as a neuroimmune biomarker for persistent pain (Kwok *et al.*, 2013), given the functional similarities between TLR signalling of immune cells in the periphery and in the CNS. Thus, the heightened TLR-responsivity of peripheral immune cells in females with painful bladder syndrome may indicate that CNS sensitisation involving neuroimmune modulation may be occurring in parallel, and remains to be explored further.

2.11.3 Endometriosis

Endometriosis is an oestrogen-dependent, chronic inflammatory medical condition in women, defined as the presence of endometrial tissue in extra-uterine locations, and commonly associated with painful pelvic symptoms. It affects an estimated 5-10% of women of reproductive age (Eskenazi & Warner, 1997), and up to 60% of women with persistent pelvic pain (Janssen *et al.*, 2013). Endometriosis-associated pain is thought to solely arise from the presence of lesions, yet pain symptoms attributed to the disease can occur in women with lesions removed (Abbott *et al.*, 2003), and the severity of experienced pain correlates poorly with the degree of lesions (Gruppo Italiano per lo Studio dell'Endometriosi, 2001; Vercellini *et al.*, 2007). Thus, it exemplifies all that is female: from the unique visceral anatomy to the complex hormonal interplay, and the long-standing association with unexplained persistent pain.

Given that the conditions mentioned above affect visceral organs present in both sexes, studying endometriosis (and indeed other female-specific conditions, such as vulvodynia) may provide further insight into subpopulation adaptations of neuroimmune-mediated pain. Neural changes have been studied in detail (Brawn *et al.*, 2014; Morotti *et al.*, 2014), and it has been suggested that pain attributed to endometriosis is likely to involve neuronal processes leading to central sensitisation (Bajaj *et al.*, 2003; Berkley *et al.*, 2005; Berkley *et al.*, 2007; Brawn *et al.*, 2014). However, a potential role for glia has yet to be investigated. Accumulating evidence nevertheless demonstrates that there are alterations in peripheral immune function in endometriosis patients (Olovsson, 2011; Khan *et al.*, 2013a). LPS-stimulated peritoneal macrophages from women with endometriosis secrete significantly higher levels of proinflammatory cytokines (e.g. IL-6 and TNF-α) than non-diseased counterparts, an effect that can be attenuated by pretreatment with a TLR4-neutralising antibody (Khan *et al.*, 2008b). TLR4 mRNA transcript expression is increased up to six-fold in endometriosis lesions compared to eutopic endometrium (Allhorn *et al.*, 2008), and TLR2 and -9 mRNA from peritoneal effusions are upregulated in endometriosis patients compared to healthy controls (Yeo *et al.*, 2013). It remains to be determined if the increased TLR levels are due to an upregulation of the receptors per immune cell, or recruitment of TLR-bearing cells to the diseased area. There is now also solid evidence from multiple lines of investigation that the development and maintenance of endometriosis involves atypical peritoneal macrophage activity (Khan *et al.*, 2008a; Capobianco & Rovere-Querini, 2013).

Collectively, these data suggest that several alterations in neural, immune and neuroimmune functions exist in the female-predominant conditions of IBS, painful bladder and endometriosis. Studies that further investigate visceral disease-associated modifications in neuroimmune signalling are desirable. Such information would further

our knowledge of persistent pain mechanisms, and may also identify a molecular basis of pain susceptibility in the subpopulation of females.

2.12 Does the dorsal root reflex and neurogenic inflammation contribute to the development of visceral inflammatory disease?

Besides painful symptoms, many chronic inflammatory diseases present with visible tissue abnormalities and consequently a vast number of studies focus on characterising and treating these lesions. However, attention has recently shifted to unravelling the complex molecular pathways that instead underlie disease aetiology. This is particularly interesting in the example of endometriosis, which is generally attributed to the movement of menstrual debris through the fallopian tubes into the abdominopelvic cavity during menses (retrograde menstruation) (Sampson, 1927). Whilst it is estimated that approximately 90% of women aged 15-49 years will exhibit retrograde menstruation (Blumenkrantz *et al.*, 1981), only around one in ten will develop endometriosis lesions. Similarly, in many patients the onset of IBD follows a bout of gastroenteritis (Garcia Rodriguez *et al.*, 2006), yet not all individuals with gastroenteritis will develop IBD. Thus it seems other factors affect the likelihood of disease formation in subsets of patients, leaving them susceptible to developing disease compared to their peers.

It is well established that sensitised sensory nerves can initiate or exacerbate inflammatory conditions by the release of neuropeptides from peripheral nerve terminals, such as CGRP and substance P (Foreman, 1987; O'Connor *et al.*, 2004; Xanthos & Sandkuhler, 2014). This results in oedema, immune cell infiltrate, and other sequelae reminiscent of inflammation; hence has been termed neurogenic inflammation (Richardson & Vasko, 2002). The release of such peptides in the periphery is known to occur via two antidromic signalling mechanisms. Initially, there is strong local stimulation of peripheral nerve terminals at the site of disease, known as the ‘axonal reflex’. With increased afferent input,

the central terminals of sensory neurons within the spinal dorsal horn may also be excited, leading to anterograde propagation of action potentials back to the periphery (the 'dorsal root reflex') (Rees *et al.*, 1995; Sluka *et al.*, 1995; Willis, 1999).

Centrally-derived neurogenic inflammation via the dorsal root reflex contributes to pathology in several animal models of peripheral inflammation, mostly involving the skin (Lin *et al.*, 1999; Lin *et al.*, 2000; Weng & Dougherty, 2005; Chen *et al.*, 2006; Lin *et al.*, 2007; Wei *et al.*, 2010) and joints (Rees *et al.*, 1994; Rees *et al.*, 1996; Zhang *et al.*, 2000), but also colitis (Lin *et al.*, 2009). Compared with control animals receiving infused saline, colonic tissues from rats stimulated with intrathecal substance P to the lumbar spine showed increased protein expression of the proinflammatory cytokine, macrophage migration inhibitory factor (MIF), mucosal oedema and lymphocyte infiltration, effects that were attenuated by intrathecal pretreatment with an NK1R antagonist. The efferent propagation of inflammation via central dorsal horn activation has also been supported in humans, by observations that relapses in ulcerative colitis have been associated with electrical stimulation of the spinal cord (Barbara *et al.*, 1999; Kemler *et al.*, 1999; Peck & Wood, 2000).

2.13 Does central glial stimulation and over-activity trigger peripheral neurogenic inflammation of the viscera?

In addition to neuropeptides, it has been suggested that proinflammatory cytokines are able to stimulate dorsal horn afferents to influence the development of peripheral inflammation (Boyle *et al.*, 2006; Fiorentino *et al.*, 2008). It has been reported that spinal IL-1β, associated with reactive astrocytes, can contribute to the induction and maintenance of temporomandibular arthritis and associated pain (Fiorentino *et al.*, 2008). In these experiments, central disruption or inhibition of spinal IL-1 receptor type-1 (a receptor for IL-1β) signalling in mice with established arthritis, resulted in significant attenuation of

joint pathology. Mice without previously established arthritis showed an upregulation of astrocyte reactivity within the dorsal horn following local spinal overexpression of IL-1β, as well as joint changes indicative of the initial stages of arthritic disease. Enhanced CGRP expression was observed in primary sensory fibres of mice with IL-1β-overexpression (peripheral projections, DRG and central projections), which also displayed spontaneous behaviour indicative of pain. It was suggested that bidirectional crosstalk between the CNS and peripheral joints, via spinal IL-1β stimulation of sensory afferents to release CGRP in the periphery, may play a role in the exacerbation of inflammation and pain (Fiorentino *et al.*, 2008). Therefore, heightened spinal glial reactivity and proinflammatory signalling may contribute to ongoing peripheral inflammation, as well as enhancing pain by central sensitisation.

This raises the interesting question as to whether centrally-derived neurogenic inflammation, generated in part by neuroimmune signalling, contributes to the perpetuation of other inflammatory diseases. Indeed, neurogenic inflammatory processes have been implicated in the exacerbation of IBD, cystitis and endometriosis (Jasmin *et al.*, 1998; Wesselmann, 2001; Engel *et al.*, 2011; Origoni *et al.*, 2014). In endometriosis, neurogenic inflammation is thought to create an optimal peritoneal environment for ectopic lesion formation in the visceral tissues (Laux-Biehlmann *et al.*, 2015; McKinnon *et al.*, 2015). In this setting, enhanced afferent signalling in response to accumulating endometrial debris may facilitate lesion development by a positive feedback loop (Fig. 2.3). Further research into the role of glia and the dorsal root reflex in the development of inflammation are recommended.

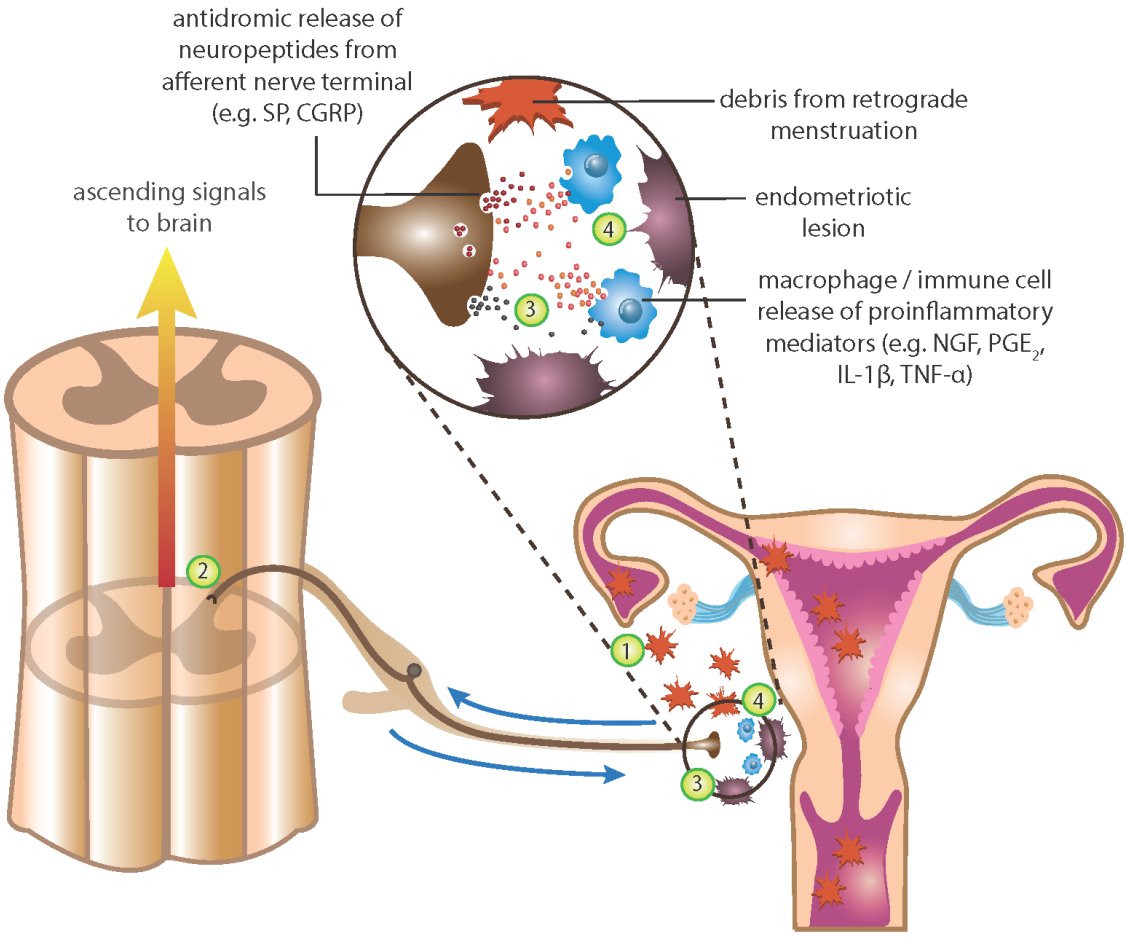


Figure 2.3 Possible involvement of centrally-mediated neurogenic inflammation in the development of visceral inflammatory disease in the periphery: example for endometriosis. (1) During menstruation, endometrial debris passes both per vaginum and in a retrograde fashion through the fallopian tubes to the peritoneal cavity. (2) In certain women, the inflammatory events initiated by ectopic endometrial tissue activate sensory afferents innervating adjacent visceral structures, which transmit the noxious information to the spinal dorsal horn. In addition to exciting ascending neural signals projecting to the brain, afferent neurotransmitter release could potentially also activate spinal astrocytes and microglia, whose proinflammatory products contribute to the development of central sensitisation and exaggerated pain (see Figure 1 and 2 for details). (3) Strong ongoing afferent stimulation associated with regular monthly menstruation and dysmenorrhea, as well as the excitatory environment created by reactive glia, may reciprocally activate the central terminals of sensory nerves. This can then induce the antidromic release of neuropeptides (such as substance P and CGRP) at the peripheral site of disease (the 'dorsal root reflex'). (4) The subsequent induction of neurogenic inflammation, including the release of cytokines (IL-1β and TNF-α), PGE_2 and nerve growth factor (NGF) from local immune cells, may then contribute to an environment that encourages the implantation of endometrial debris onto the peritoneum, and the development of endometriotic lesions (including the associated neovascularisation and sprouted innervation).

2.14 Early life stressors as central glial primers for visceral inflammation

It is now realised that glia have the ability to be 'primed' by prior experience to over-respond to new immune challenges (a 'two-hit hypothesis' (Grace *et al.*, 2014)). This is shown where laparotomy and intraperitoneal injection of LPS each individually cause modest increases in mechanical allodynia. However, allodynia is potentiated up to a three-fold when laparotomy and LPS are administered sequentially, with enhanced pain being associated with heightened microglial reactivity (Hains *et al.*, 2010).

Many studies are currently investigating the impact of early life stressors, such as maternal separation or injury, on long-lasting glial alterations in the adult. Such events can be the 'first hit' that primes glia to over-respond and be detrimental in restoring 'second hit' immune challenges later in life. Visceral hyperalgesia can be enhanced by early adverse events (Rosztoczy *et al.*, 2003; Ness & Randich, 2010; Pierce *et al.*, 2014; Pierce *et al.*, 2015), although associations with glia have thus far been described only for somatic pain. For instance, incisional surgery of the neonatal rat hind paw caused an increase in the intensity of microglial activation and expression within the dorsal horn that persisted into adulthood (Beggs *et al.*, 2012). This was associated with hyperalgesia following incisional surgery as an adult, and was prevented by intrathecal administration of minocycline at the time of adult injury. Thus, this suggests that early adverse life events provoking long-term heightened glial reactivity may lead to greater sensitivity to future harmful stimuli.

Priming of spinal glia may provide an explanation for why some subpopulations, such as females, are predisposed to developing certain painful conditions. If the neuroimmune communication has been primed prior to a persistent pain-triggering insult, then this mechanism may inherently increase disease burden in females (or males) due to the increased release of proinflammatory products, and may also be exacerbated by the activity of sex hormones, such as oestradiol. Early aggravation of spinal glia might therefore

contribute to the development of peripheral inflammation, via the dorsal root reflex or otherwise. Regarding endometriosis, clinical records from female monkeys have indicated that animals exposed to prior adverse life events, such as laparoscopic examination and caesarean section, were associated with an increase in the incidence of developing endometriosis (D'Hooghe *et al.*, 1996; Coe *et al.*, 1998). The initial scenario of gastroenteritis preceding IBD could further represent the 'first hit' of irritation that sensitises the neuroimmune system, later contributing to disease progression. Direct evidence linking early-life glial priming and the incidence of visceral inflammation in adulthood await to be studied.

2.15 Beyond 'hysteria' towards targeted treatment of female pain

Our current understanding of central sensitisation leading to the development of persistent pain involves interactions between neurons and highly reactive glia. Studying alterations in these neuroimmune connections under various conditions provides enormous potential for meaningful new research discoveries and, given the significant female predominance of pain, may contribute to understanding the biological mechanisms that underlie sex differences in pain processes. Using both male and female subjects will be crucial for this future pain research. Exploring painful conditions of the viscera that are most prevalent or specific to each of the sexes, such as IBD, painful bladder syndrome and endometriosis in females and prostatitis in males, may additionally provide clues into the unique anatomical and neuroendocrine influences on pain sensitivity. Indeed, the potential contribution of neuroimmune and neurogenic signalling to inflammation and pain is a novel avenue for gynaecological and urogenital research. While much of this review has focused on female sex hormones and pain, male sex hormones may also play a critical role, where low testosterone levels are an emerging link to persistent pain states in both sexes (Aloisi *et al.*, 2007; White & Robinson, 2015). Thus, prospective studies comparing root causes of sex-

specific pain conditions may have important implications for both future pain prevention and treatment strategies.

As we unravel the molecular pathways involved in enhancing nociceptive transmission, this will provide opportunities for resultant drug discovery. New pharmacotherapies that aim to target glia to modulate their deleterious, proinflammatory contributions to pain are now steadily emerging (Grace *et al.*, 2014; Ji *et al.*, 2014). This is emphasised by recent exciting studies that have for the first time demonstrated an upregulation of central glial cell reactivity in pain patients *in vivo* (Banati *et al.*, 2001; Albrecht *et al.*, 2015; Loggia *et al.*, 2015). While the translation of results from animals to humans has been variable in effectiveness, an issue plaguing the field of pain at large (Mogil *et al.*, 2010; Borsook *et al.*, 2014), it is likely that the future analgesic success of these agents will be highly dependent on the type of injury or disease, the selection of drug and dosing regimen, the route of delivery and the timing of treatment. With continued investigations, the neuroimmune system represents a key target to decrease the burden of persistent pain.

Thesis aims and hypotheses

The introductory chapters (Chapter 1 and 2) of this thesis aimed to highlight that endometriosis is a common condition in females associated with the oestrogen-dependent growth of inflammatory lesions, and significant pelvic pain. It is highly likely that processes of peripheral and central sensitisation contribute to the observed pain symptoms. However, current animal models utilised to study both lesion pathophysiology and pain mechanisms in endometriosis consistently require incisional surgery, sex hormone supplementation, and/or the creation of immunocompromised animals; thereby limiting the potential of future research relating to these themes.

The aim of the first primary research study (Chapter 3) in this thesis was therefore:

- To devise and refine an immunocompetent, minimally-invasive mouse model of endometriosis that allows for studies of central sensitisation and pain behaviour, without the confounding factors of surgery and/or exogenous hormone manipulation.

Based on the well-known roles of oestrogen and inflammation in endometriosis, and the proposed origin of lesions via retrograde menstruation, we hypothesised that:

- Endometriosis-like lesions in the mouse will develop more frequently when induced during periods of naturally high oestrogen;
- C57BL/6 wildtype mice, which exhibit an inherently proinflammatory immune phenotype, will produce a greater number and altered phenotype of lesions compared to BALB/c wildtype mice; and
- Lesion incidence will increase in parallel with graded increase in the volume of injected endometrial tissue.

At present, studies investigating endometriosis-associated pain mechanisms have primarily focused on neuronal adaptations alone. However, it is well established that neuroimmune signalling, mediated by spinal glial cells, can also lead to central sensitisation and pain in other peripheral inflammatory states. This includes several female-dominant, pelvic pain-producing conditions, such as IBS and bladder pain syndrome.

Hence, the aim of the second primary research study (Chapter 4) was:

- To determine whether the expression of spinal glial cells (astrocytes and microglia) are altered secondary to the development of endometriosis-like lesions, using the minimally-invasive mouse model of endometriosis (previously characterised in Chapter 3).

Given that an increase in spinal glial reactivity is typically reported to correlate with heightened central sensitisation and pain, and endometriosis lesions can develop in various peritoneal locations, we hypothesised that:

- Astrocyte and microglial expression will be increased in the thoracolumbar spinal cord of mice with endometriosis-like lesions; and
- Spinal levels showing the greatest alterations in glial reactivity will correspond to the peritoneal locations where lesions have developed.

Further, a growing body of literature suggests that aberrant endometrial and/or peritoneal activity of the innate immune receptor, TLR4, may be involved in the pathogenesis of endometriosis lesions. It is also recognised that TLR4-mediated proinflammatory signalling in the spinal cord, due to its expression by immune-like glial cells, can facilitate the development of central sensitisation and pain.

The third primary research study (Chapter 5) therefore aimed:

- To characterise the development of endometriosis-like lesions, as well as spinal glial adaptations and pain behaviour, in mice with genetic knockout of *TLR4* compared to wildtype controls.

Most studies have implicated exaggerated TLR4-mediated inflammation in the development of endometriosis lesions and in neuroimmune-associated pain. This is due to the well-known effects that inflammatory mediators produce regarding both endometrial growth, and neuronal adaptations that result in nociceptive hyperexcitability, respectively. As genetic knockout of *TLR4* reduces activity in the TLR4 signalling pathway, it was hypothesised that:

- Endometriosis-like lesion development will be attenuated in *TLR4*-knockout mice; and
- Spinal glial expression and pain behaviour, associated with the presence of lesions, will also be reduced in mice with genetic knockout of *TLR4*.

Whilst conventional knockout mice provide an exceptional tool to study protein function, it cannot be tacitly assumed that signalling pathways (and therefore biological processes) will not adapt to the deficient protein during development and maturation. Therefore, additional experiments must be performed to support conclusions drawn from these animals. This may include, for example, experiments using targeted antagonist drugs in wildtype animals.

As an addendum to Chapter 5, the aim of the fourth primary research study (Chapter 6) was therefore:

- To assess which of the endometriosis-associated changes observed in *TLR4*-knockout mice could be reproduced by acute pharmacological blockade of TLR4 in the peripheral tissues (i.e. the peritoneal cavity).

Since the TLR4 antagonist was to be applied only within the periphery (not the CNS), and during the earliest phase of lesion formation, we hypothesised that:

- The incidence of endometriosis-like lesions will mirror the result observed in *TLR4*-knockout mice; and
- Changes in spinal glial reactivity and pain behaviour will occur as expected in wildtype mice, and will not be altered by acute peripheral TLR4 blockade.

Lastly, the current evidence linking aberrant TLR4 activity to the development of endometriosis has largely been collected via *in vitro* experiments; studies investigating this relationship *in vivo* are lacking, and therefore the translational and clinical relevance is uncertain. In addition, it remains to be determined whether prior or pre-existing peritoneal inflammatory responses can alter the development of lesions. On a separate note, reports have intriguingly suggested that glial-mediated central sensitisation may not only affect neuronal projection pathways leading to pain (i.e. an orthodromic peripheral-to-central effect), but also afferent pathways leading to neurogenic inflammation in peripheral tissues (i.e. an antidromic central-to-peripheral effect). This indicates that enhanced spinal glial reactivity may be a *causative factor*, as well as a consequence, of inflammatory conditions in the periphery.

Therefore, the final aims of this thesis, presented in the fifth primary research study (Chapter 7), were:

- To investigate whether acute *in vivo* stimulation of <u>peripheral</u> TLR4 with LPS can alter the development of endometriosis-like lesions, the associated glial adaptations, and pain; and
- To examine lesion development under the influence of enhanced spinal glial reactivity, by acute *in vivo* stimulation of <u>central</u> TLR4 with LPS.

Based on the information presented above, our hypotheses were that:

- The development of endometriosis-like lesions will be enhanced by acute peripheral stimulation of TLR4, leading to further alterations in spinal glial reactivity and amplification of pain behaviour; and
- Lesion development will be enhanced by acute central stimulation of TLR4, and pain behaviour exaggerated due to the direct action of LPS on TLR4-expressing glial cells within the CNS.

Statement of Authorship

Title of Paper	Lesion development is modulated by the natural oestrous cycle and mouse strain in a minimally-invasive model of endometriosis
Publication Status	☑ Published ☐ Accepted for Publication ☐ Submitted for Publication ☐ Unpublished and Unsubmitted w ork w ritten in manuscript style
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Principal Author

Name of Principal Author (Candidate)	Kelsi N. Dodds		
Contribution to the Paper	Designed experiments, performed all experiments, analysed and interpreted data, generated figures, wrote manuscript and acted as corresponding author.		
Overall percentage (%)	85%		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
Signature		Date	28/02/2018

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);
ii. permission is granted for the candidate in include the publication in the thesis; and
iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

Name of Co-Author	Elizabeth A. H. Beckett		
Contribution to the Paper	Supervised development of work, assisted with experimental design, statistical analysis, figure generation and reviewed the manuscript.		
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Contribution to the Paper	Assisted with experimental design, data interpretation and reviewed the manuscript.		
Signature		Date	20/03/2018

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Contribution to the Paper	Supervised development of work, assisted with experimental design, data interpretation and reviewed the manuscript.		
Signature		Date	20/03/2018

Chapter 3. Lesion development is modulated by the natural oestrous cycle and mouse strain in a minimally-invasive model of endometriosis

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3.1 Abstract

Many rodent models of endometriosis are invasive, involving surgery to implant donor endometrial tissue into recipient animals. Moreover, few studies have compared and contrasted lesions between rodent strains and oestrous stages without exogenous hormone manipulation. This is despite extensive data demonstrating that genetic and hormonal factors can influence endometriosis progression. Here, we have refined a minimally-invasive model of endometriosis using naturally cycling mice (donor and recipient matched for cycle phase) to investigate lesion development in two different strains (C57BL/6 and BALB/c), induced in oestrous stages of high and low oestrogen (pro-oestrus or oestrus, respectively), and with varying amounts of donor endometrial tissue (7.5-40 mg), injected intraperitoneally. The overall probability of developing endometriosis-like lesions was higher in pro-oestrus than oestrus, and increased with greater masses of donor tissue. Similarly, the total number of lesions (0-3) increased from 7.5 to 40 mg, and was significantly greater in pro-oestrus C57BL/6 mice but not BALB/cs. The dominant lesion type also differed between mouse strains; C57BL/6 mice were more likely to develop dense-type lesions, whereas BALB/c mice developed a greater proportion of cystic-type. These data further support a role for oestrogen in the development of endometriosis, and that genetic variance can influence the degree and characteristics of lesions. Our minimally-invasive model would be beneficial for studies with outcome measurements particularly sensitive to incisional injury, such as pain, or alterations to sex hormones, including fertility.

3.2 Introduction

Endometriosis is a female-specific chronic inflammatory condition, classically defined as the presence of endometrial-like tissue outside the uterus. Affecting an estimated 10% of women of reproductive age worldwide, endometriosis is highly associated with dysmenorrhea, infertility and persistent pelvic pain (Eskenazi & Warner, 1997). The mechanistic event underpinning the development of endometriosis lesions is attributed to the flow of menstrual debris through the fallopian tubes to the peritoneal cavity during menses (retrograde menstruation), with the subsequent implantation of endometrial cells (Sampson, 1927). However, this hypothesis cannot fully explain the pathogenesis of endometriosis, as approximately 90% of women aged 15-49 years will exhibit retrograde menstruation (Halme *et al.*, 1984; O *et al.*, 2017), yet a much smaller proportion are at risk of developing lesions.

Female rodents do not undergo a menstrual cycle with shedding of the endometrium akin to humans (with the exception of the spiny mouse (Bellofiore *et al.*, 2017)). Rather, an oestrous cycle where the endometrium, in the absence of implantation, is reabsorbed by the activity of infiltrating leucocytes. As such, rodents will not spontaneously develop endometriotic lesions, and animal models to date have required manual implantation of endometrial fragments into the abdominal space. The vast majority of these models require surgery: either a ventral midline abdominal incision with uterine tissues sutured to visceral structures, such as the renowned rat autologous model (Vernon & Wilson, 1985; Berkley *et al.*, 2001); and/or a dorsal flank incision and ovariectomy, typically followed by oestrogen supplementation (Hull *et al.*, 2008; Greaves *et al.*, 2014a) (for reviews of rodent models used for endometriosis research see Pullen *et al.* (2011) and Greaves *et al.* (2017a)).

It is widely acknowledged that long-lasting modulation of somatosensory processing can occur in animals following incisional surgery or similar techniques. This includes changes

in the sensitivity of both peripherally- and centrally-located neurons (Xu & Brennan, 2009, 2010); altered expression of proteins integral to neurotransmission, such as spinal metabotropic glutamate receptors (Dolan *et al.*, 2004); as well as sensitisation of the neuroimmune system, demonstrated by increased reactivity of spinal microglia and heightened somatosensensory activity (Hains *et al.*, 2010). Therefore, while many studies utilise sham controls, subtle changes in proteins or behaviours of interest in models of endometriosis may be masked by a relatively larger effect of the surgery. This limits the use of available models for experiments that are particularly sensitive to tissue injury, such as observations of the nervous system (as mentioned above), and therefore may particularly impact on studies of peripheral and central sensitisation, and pain. These investigations remain imperative, as the severity of pain reported by women seldom correlates with the extent and duration of endometriosis (Gruppo Italiano per lo Studio dell'Endometriosi, 2001), suggesting a more complex aetiology beyond that observed at the peripheral lesion site.

Animal models of endometriosis that involve ovariectomy have the additional complication of being impracticable for studies of physiological functions that require natural fluctuations in sex hormones, such as fertility. It has long been hypothesised that endometriosis lesions thrive under the influence of heightened or prolonged oestrogen stimulation (Kitawaki *et al.*, 2002; Rizner, 2009), since symptoms of the condition often first present at, or soon after menarche, and improve when oestrogen levels fall at menopause; and the endometrium involutes in the absence of oestrogen. Consequently, manipulation of sex hormones in basic scientific work has further implicated a role for oestrogen in lesion development, where exogenous administration of oestradiol in ovariectomised rodents enhances endometrial implant growth (Cummings & Metcalf, 1995; Bruner *et al.*, 1997); is modulated by oestrogen receptor signalling (Burns *et al.*,

2012); and can lead to recurrence of lesions that have previously regressed (Rajkumar *et al.*). Researchers have also recently developed a hormonal protocol that transforms the rodent endometrium into a phenotype that mimics human menstrual debris, which is subsequently collected for use in an animal model (Greaves *et al.*, 2014a). Therefore, while there are many benefits of controlling sex hormones for endometriosis research, in many cases the continuous systemic oestrogen stimulation is of supra-physiological levels and may not necessarily reflect the clinical setting.

In addition to altered sex hormone activity, the variable susceptibility among human females in developing endometriosis lesions is also thought to be influenced by genetic (and environmental/epigenetic) factors (Borghese *et al.*, 2017). Comparing lesion characteristics in rodent models of diverse genetic backgrounds, where the susceptibility of each strain is unknown, may complicate research findings. Determining the behaviour of specific rodent strains may therefore provide further insight into the varied types of endometriosis lesions and their associated symptoms. For example, C57BL/6 and BALB/c are two strains of commonly used wildtype mice that are known to differ in their immune profiles; the former considered Th1-dominant and the latter Th2-dominant in the immune response (Watanabe *et al.*, 2004). This affects the timing, composition and location of cytokine release in response to specific immune insults, and hence how each strain might respond to donor tissue in endometriosis models.

Given endometriosis continues to be an area of significant unmet medical need, and the mechanistic disconnects between clinical presentation of the condition and animal models, we sought to refine and carefully characterise an animal model of endometriosis in mice. In this study, we have utilised a minimally-invasive, gonad intact mouse model of endometriosis which may be compatible with experiments that are sensitive to surgery or incisional injury, and sex hormone manipulation. Further, we have considered the extent

of lesion development in two genetically diverse mouse strains, during oestrous stages of relatively high and low oestrogen concentrations, and with increasing amounts of inoculated endometrial tissue.

3.3 Methods

3.3.1 Animals

Cytological evaluation of vaginal smears from 12 ± 3 week-old, virgin female mice (weighing 20.6 ± 2.0 g) was used to determine current oestrous stage, as described previously (Dodds *et al.*, 2015). C57BL/6NHsd (C57BL/6; n = 43 donors and 43 recipients) or BALB/c (n = 38 donors and 43 recipients) mice in pro-oestrus and oestrus were selected for experimental use, corresponding to the respective oestrous stages of relatively high and low oestrogen (Cason *et al.*, 2003). Animals were obtained from the University of Adelaide Laboratory Animal Services; all experimental procedures were performed in accordance with the National Health and Medical Research Council Australian code for the care and use of animals for scientific purposes (8th edition, 2013) and the University of Adelaide Animal Ethics Guidelines, and were approved by the University of Adelaide Animal Ethics Committee.

3.3.2 Induction of endometriosis

Donor mice were anaesthetised by isoflurane inhalation, sacrificed by cervical dislocation and their uterus removed. Tissues were placed immediately in cold (4°C) phosphate-buffered saline (0.01 M PBS composed of 13.7 mM NaCl, 0.27 mM KCl, 0.15 mM KH_2PO_4 and 0.8 mM Na_2HPO_4; pH 7.4) in a glass Petri dish. Residual connective tissue, fat, the ovaries and cervix were removed, and each horn was opened along the mesometrial border. The uterine horns were then pinned flat to the Sylgard®-coated base (Dow Corning; Michigan, USA) of the glass Petri dish using entomology pins. The endometrium was

removed by sharp dissection, measured for wet weight, and sectioned into 2-3 mm^2 fragments. Once the desired amount of endometrium was collected (7.5, 15, 25 or 40 mg), the fragments were suspended in a syringe with 0.5 ml sterile saline (0.9% NaCl; room temperature (RT)) and passed once through a 21-gauge needle. This was to ensure smooth delivery of content to recipient animals.

Recipient mice of syngeneic strain and identical oestrous stage were then intraperitoneally injected with the donor endometrial fragment suspension (ENDO mice) at the ventral midline between the left inguinal nipples. Control recipient mice were injected with an equal volume of sterile saline only. Following 21 days development, ENDO mice were deeply anaesthetised with isoflurane gas and decapitated, to allow for an unobstructed view of the abdominopelvic cavity. After thorough examination, the number and location of endometriosis-like lesions was recorded. Identified lesions and control tissues for histological comparison from saline-injected mice (endometrium, lymph nodes, gastrointestinal tract, fat) were immediately fixed with cold (4°C) paraformaldehyde fixative (4% in PBS; pH 7.2) for 4-6 hours.

3.3.3 Histological Assessment

Endometriosis-like lesions and control tissues were further fixed in 10% neutral-buffered formalin (Chem-Supply; South Australia, Australia) overnight (4°C), placed in cassettes and submerged into 70% ethanol, then dehydrated in graded alcohols, cleared with xylene, and embedded with warmed liquid paraffin wax. Once solidified, serial sections of 5 μm were cut using a rotary microtome and collected onto albumin-coated slides. Routine hematoxylin and eosin staining was then performed, and the slides scanned using a NanoZoomer (Hamamatsu Photonics; Shizuoka Pref., Japan) and viewed with NanoZoomer Digital Pathology software view.2 (Histalim; Montpellier, France). Lesions were assessed in a blinded fashion for the presence of both glands and stroma, which are

typically prerequisite for a confirmed diagnosis of endometriosis in humans (Clement, 2007). Parameters including inflammatory cell infiltration, blood vessels, connective tissue and the presence of cysts were also recorded. Immunohistochemical staining for F4/80 was performed to further confirm the presence of macrophages within endometriosis-like lesions (for details see Supplementary Fig. 3.1). Only lesions classified as dense- or cystic-type were counted toward the total number of lesions obtained from an ENDO animal (see *Results* section 3.4.1 below). The size of endometriosis-like lesions was defined in this study as the maximum diameter of the cut surface area in histological sections. Note that due to their variable shape, lesions were orientated during embedding so that the greatest dimension would be sectioned longitudinally.

3.3.4 Statistical analysis

In total, 14 experimental groups were analysed: C57BL/6 and BALB/c mice in pro-oestrus injected with 7.5, 15, 25 and 40 mg donor endometrial tissue (eight groups pro-oestrus); and C57BL/6 and BALB/c mice in oestrus injected with 15, 25 and 40 mg endometrium (six groups oestrus).

SPSS Statistics 24 software (IBM; New York, USA) was used for statistical analyses and GraphPad Prism 7 software (GraphPad Software Inc.; California, USA) was used to generate figures. A logistic regression model was used to investigate the association of at least one lesion present (lesion success) with the mouse model variables; a linear regression model and two-way ANOVA with Holm-Šídák multiple comparisons were performed to assess for associations between the total number of lesions, after determining that the assumptions of a linear model were upheld; a logistic generalised estimating equation model was performed to analyse the associations between lesion type (cystic or dense) as well as lesion locations; and a linear mixed-effects model was used to determine differences in lesion sizes.

The independent variables in these statistical models were mouse strain (C57BL/6 or BALB/c), oestrous stage at induction (pro-oestrus or oestrous) and mass of injected tissue (7.5, 15, 25 or 40 mg), which were analysed individually or with strain-stage, stage-mass or mass-strain interactions. From the statistical models, an odds ratio or estimate, 95% confidence interval (CI), comparison *P* value and interaction *P* value (where applicable) were calculated. A *P* value of <0.05 was considered statistically significant. Values are presented as mean ± standard deviation in *Results* section 3.4.1 and mean ± standard error for sections 3.4.2-3.4.5.

3.4 Results

3.4.1 Endometriosis-like lesions develop with distinct characteristics

Endometriosis-like lesions were successfully established in our minimally-invasive mouse model. No interruptions to the oestrous cycle were observed in recipient ENDO mice throughout the 21-day development period, indicating that the ovaries were intact and functioning as normal. The 7.5 mg recipient group were injected with an average of 7.6 ± 0.2 mg donor endometrium in 5.1 ± 0.3 pieces (n = 12); the 15 mg ENDO animals 16.3 ± 1.2 mg in 9.0 ± 1.5 pieces (n = 26); the 25 mg group with 25.0 ± 2.4 mg in 18.5 ± 4.0 pieces (n = 24); and 40 mg ENDO animals 41.1 ± 2.3 mg in 28.8 ± 3.5 pieces (n = 24). No overt effects were observed on the reproductive organs or tissues adjacent to endometriosis-like lesions in ENDO mice, except for occasional areas of erythema surrounding the lesions. Following histological processing and imaging, all endometriosis-like lesions collected from ENDO mice were initially classified into one of three categories based on their characteristics: dense, cystic or necrotic-type lesions.

Macroscopically, dense-type lesions appeared opaque and light pink to deep red in colour with a smooth surface, in some cases speckled with darker red blood vessels, and ranged

from 221 to 4260 μm in length (average lesion length 1219 ± 832.8 μm; $n = 45$) (Fig. 3.1A). On histological inspection it was confirmed that dense lesions contained both epithelial (glandular) and stromal endometrial cells (Fig. 3.1D). These lesions were encapsulated by a sheath of connective tissue, vascularised with single-layer walled small blood vessels, and commonly infiltrated by inflammatory cells, such as macrophages and lymphocytes (identified by their morphological characteristics). Identified macrophages often contained areas of punctate brown staining akin to hemosiderin deposition, as has been observed in other animal models (Bergqvist *et al.*, 1985) and human endometriosis lesions (Moen & Halvorsen, 1992).

Cystic-type lesions were characterised by the presence of a fluid-filled cyst in addition to endometrial glands and stroma. Cystic lesions were again light pink in colour although translucent due to the cysts; in many cases the donor endometrial fragments could be seen inside. The cysts often had a 'bubbled' or multi-lobed appearance (Fig. 3.1B, E). These lesions were commonly surrounded by a capsule of connective tissue that appeared contiguous with adjacent structures. Cystic-type lesions were also vascularised, and displayed inflammatory cell infiltrate within the stroma (Fig. 3.1E-a). Cysts typically contained inflammatory cells (including macrophages and lymphocytes) and red blood cells (Fig. 3.1E-b), as well as a yet-unidentified lightly eosinophilic web-like structure (Fig. 3.1E-c). The presence of macrophages in lesions was further confirmed by distinct F4/80-positive structures within the cyst lumen and stroma (Supplementary Fig. 3.1). Cystic-type lesions were generally larger than dense lesions, ranging from 503 to 3700 μm (average 1990 ± 847.1 μm; $n = 50$).

Necrotic lesions were ubiquitously white in colour with an irregular surface, and small in size (range 564 to 650 μm; average 606.5 ± 35.87 μm; $n = 4$) (Fig. 3.1C). Histologically they were defined by a majority presence of necrosis within a lesion, determined as areas

of dense hematoxylin staining without discernible cellular or tissue structure (Fig. 3.1F). This meant that in many necrotic lesions obvious glands and stroma were not identified. Interestingly, necrotic lesions were only observed in ENDO animals induced with pro-oestrus endometrial tissue. Due to the single time-point of tissue collection in this study, it is unknown whether necrotic lesions were previously fully formed lesions that were degenerating at the time of collection, or if they never formed lesions and were deteriorating donor endometrial tissue left over from the initial ENDO injection. For these reasons, and that glands and stroma were not consistently present, necrotic-type lesions were excluded from further analysis.

3.4.2 Endometriosis lesions develop in diverse peritoneal locations

The location of endometriosis-like lesion development was random across the various ENDO groups; there were no significant associations with oestrous stage, mass of injected tissue, or strain of mouse. Lesions formed in several locations within the peritoneal cavity, typically on the anterior surfaces; likely because of the posture and gravity in quadrupedal animals. Most often lesions tended to form on connective tissue-like structures, such as mesenteric attachments between the stomach and pancreas (47/95 lesions; 49.5% total). It was common to also observe lesions on the anterior abdominal wall, at a level typical of the injection site (18/95 lesions; 18.9% total), as well as within fatty structures such as the gonadal white adipose tissue (24/95 lesions; 25.3% total) (Fig. 3.2). Few lesions (3/95 lesions; 3.1% total) formed in the subcutaneous space, likely due to residual fragments of endometrial tissue embedding at the insertion point upon needle withdrawal. A single lesion also formed on connective tissue anchoring the descending colon to the posterior abdominal wall (1.1% total), and two lesions were found on connective tissue bridging the posterior uterine body with the anterior descending colon/rectum (2.1% total).

3.4.3 Successful development of endometriosis-like lesions is dependent on the quantity of donor endometrium and oestrous stage at induction

Controlling for both mouse strain and stage, the incidence of endometriosis-like lesion development increased as the amount of donor endometrial tissue injected into ENDO mice progressed from 7.5 to 40 mg. Collectively amongst the ENDO groups, the rate of lesion establishment was 33.3% for animals injected with 7.5 mg (n = 4/12); 69.2% for 15 mg animals (n = 18/26); 83.3% for those in the 25 mg group (20/24); and 100% for recipient mice injected with 40 mg endometrium (n = 24/24). Using the 7.5 mg group as reference (14% estimated probability of developing lesions), 15 mg ENDO animals were 16.9 times more likely to develop lesions (73% probability; P = 0.004), 48.5 times more likely with 25 mg (88% probability; P = 0.001), and were all expected to develop lesions in the 40 mg group (100% probability; P <0.001).

Interestingly, endometriosis-like lesions were 1.9 times more likely to establish in pro-oestrus as opposed to oestrus (P = 0.009), when controlled for both strain and group mass. For C57BL/6 ENDO mice, lesions were observed in 33.3% (n = 2/6) of animals injected with 7.5 mg tissue in pro-oestrus, and in all animals with 15 mg and above (100%; 19/19). BALB/c mice in pro-oestrus developed lesions in 33.3% of 7.5 mg animals (n = 2/6), 71.4% of 15 mg animals (n = 4/7), and 100% of animals injected with 25 and 40 mg of donor endometrial tissue (n = 12/12) (Fig. 3.3A). In contrast, lesions developed in only half of 15 mg C57BL/6 and BALB/c ENDO mice in oestrus (50%; n = 3/6 per strain), two-thirds of 25 mg mice (66.7%; n = 4/6 per strain) and all animals at 40 mg (100%; n = 6/6 per strain) (Fig. 3.3B). ENDO animals in oestrus with 7.5 mg donor tissue were not analysed.

No mouse strain effect was observed for the successful development of lesions. The incidence of lesion development was equal in both C57BL/6 and BALB/c mice, except at 15 mg in pro-oestrus where C57BL/6 ENDO mice had a slightly increased rate (by 28.6%)

compared to BALB/cs (100% vs. 71.4% respectively; 95% CI: -0.05, 0.62).

3.4.4 Total number of endometriosis-like lesions developed by ENDO mice is modulated by quantity of donor endometrium, oestrous stage at induction and mouse strain

In addition to the frequency of ENDO animals developing lesions, there was a positive correlation between the number of total endometriosis-like lesions per animal and the mass of donor endometrium. Controlled for strain and stage, animals injected with 7.5 mg tissue developed 0-1 lesions (mean 0.13 ± 0.21); 15 mg animals 0-2 lesions (mean 0.79 ± 0.13); 25 mg-injected animals 0-3 lesions (mean 1.25 ± 0.14); and 40 mg animals 1-3 lesions (mean 1.67 ± 0.14) (all pairwise comparisons $P \leq 0.033$).

In C57BL/6 ENDO mice, a greater total number of lesions per animal were observed in the pro-oestrus groups as opposed to oestrus groups. On average 7.5 mg pro-oestrus mice developed 0.33 ± 0.24 lesions; 15 mg mice developed 1.14 ± 0.22 lesions in pro-oestrus versus 0.50 ± 0.24 lesions in oestrus; the 25 mg pro-oestrus group developed 1.67 ± 0.24 lesions versus 0.67 ± 0.24 lesions in oestrus; and the 40 mg groups developed 1.67 ± 0.24 versus 1.50 ± 0.24 lesions, respectively. Therefore overall, pro-oestrus C57BL/6 mice had a probability of developing 0.60 ± 0.21 more lesions at any given group mass compared to those in oestrus ($P = 0.005$), with the most significant difference at 25 mg (probability of 1.00 ± 0.34 more lesions in pro-oestrus than oestrus; $P = 0.004$) (Fig. 3.4A). This is reiterated by a reduced average mass of endometrium that was required to obtain 0 (7.68 ± 0.11 vs 19.66 ± 2.07 mg; $P = 0.004$), 2 (22.03 ± 3.25 vs 37.97 ± 0.62 mg; $P = 0.017$) and 3 lesions (34.77 ± 5.84 vs >40 mg) in pro-oestrus versus oestrus, respectively (Fig. 3.4B). The pattern for the predicted number of lesions per group mass in pro-oestrus was an increase by 0.81 ± 0.33 lesions from 7.5 mg to 15 mg ($P = 0.014$), which then remained stable at 25 mg (+ 0.52 ± 0.33 lesions from 15 mg to 25 mg; $P = 0.113$) and 40 mg (+ 0.00

± 0.34 lesions from 25 mg to 40 mg; $P = 1.000$). In oestrus, the 15 mg and 25 mg groups developed similar numbers of lesions (+ 0.17 ± 0.34 lesions from 15 mg to 25 mg; $P = 0.627$) until 40 mg, which showed an increase by 0.83 ± 0.34 lesions (from 25 mg; $P = 0.015$).

In contrast, BALB/c ENDO mice developed similar total numbers of lesions per group mass regardless of oestrous stage (Fig. 3.4C). For 7.5 mg animals in pro-oestrus, the average number of lesions was 0.33 ± 0.29; 15 mg mice developed 1.00 ± 0.26 lesions in pro-oestrus versus 0.86 ± 0.28 lesions in oestrus; the 25 mg pro-oestrus group developed 1.50 ± 0.26 lesions versus 1.60 ± 0.23 lesions in oestrus; and the 40mg groups developed 2.33 ± 0.21 versus 1.89 ± 0.24 lesions, respectively. Again, the absence of separation between pro-oestrus and oestrus is shown by similar average quantities of endometrium used to produce 1, 2, and 3 lesions (0 lesions 9.24 ± 2.32 vs 19.72 ± 2.27 mg, respectively; $P = 0.032$) (Fig. 3.4D). The predicted number of lesions per group mass in pro-oestrus did not significantly differ when compared incrementally until 40 mg, with an estimated 0.83 ± 0.33 more lesions than at 25 mg ($P = 0.013$). Although no differences were observed from 7.5 mg to 15 mg and then 15 mg to 25 mg, overall the 25 mg group was likely to develop 1.17 ± 0.39 lesions more than at 7.5 mg ($P = 0.003$) indicating a steady increase in lesion number throughout this range. In oestrus, the predicted number of lesions increased from 15 mg to 25 mg (increase by 0.74 ± 0.36; $P = 0.039$) that remained stable from 25 mg to 40 mg (increase by 0.29 ± 0.34; $P = 0.390$). Between the 15 mg to 40 mg groups lesions were estimated to increase by 1.03 ± 0.37 ($P = 0.005$).

3.4.5 Endometriosis-like lesion characteristics differ between mouse strains

A total of 95 lesions were collected from ENDO animals, 50 of which were cystic (52.6%) and 45 classified as dense-type (47.4%). No differences in the ratio of cystic-to-dense lesions were detected in pro-oestrus versus oestrus ($P = 0.186$), where there were 33

(56.9%) cystic and 25 (43.1%) dense lesions in pro-oestrus, versus 17 (45.9%) cystic and 20 (54.1%) dense lesions in oestrus (Fig. 3.5A). Similar proportions of the lesion types were also observed across the group masses (all pairwise comparisons $P \geq 0.172$). ENDO mice with 7.5 mg donor tissue developed a total of 2 cystic and 2 dense lesions (50% each); 15 mg animals had 12 (57.1%) cystic and 9 (42.9%) dense lesions; 25 mg animals developed 13 (43.3%) cystic and 17 (56.7%) dense lesions; and finally 40 mg ENDO animals developed 23 (57.5%) cystic and 17 (42.5%) dense lesions (Fig. 3.5B). However, BALB/c mice were 2.7 times more likely to develop cystic versus dense lesions compared to C57BL/6 mice ($P = 0.022$). From BALB/c mice a total of 31 (63.3%) cystic and 18 (36.7%) dense lesions were collected, whereas C57BL/6 mice developed 19 (41.3%) cystic and 27 (58.7%) dense lesions (Fig. 3.5C).

Adjusted for mass and strain, the average diameter of endometriosis-like lesions in pro-oestrus was 1.42 ± 0.14 mm and in oestrus 1.32 ± 0.18 mm ($P = 0.573$) (Fig. 3.6A). Lesions from animals receiving 7.5 mg donor endometrium were 0.86 ± 0.44 mm in size; 15 mg animal lesions 1.43 ± 0.19 mm; 25 mg animal lesions 1.33 ± 0.16 mm; and 40 mg animals had lesions 1.86 ± 0.14 mm in size. Statistically, lesions from the 40 mg group were 1.00 ± 0.46 mm larger than those from 7.5 mg animals ($P = 0.033$) and 0.53 ± 0.21 mm larger than 25 mg animals ($P = 0.013$) (Fig. 3.6B). Moreover, C57BL/6 mice developed lesions 1.22 ± 0.16 mm in size and BALB/c mice 1.52 ± 0.60 mm ($P = 0.104$) when controlled for mass and stage (Fig. 3.6C). Post-hoc analysis interestingly revealed that within the 40 mg group there was a significant difference in lesion size between the mouse strains, where those from BALB/c ENDO mice were estimated to be 0.72 ± 0.27 mm larger than in C57BL/6 animals ($P = 0.010$) (Fig. 3.6D). This difference is most likely due to the greater numbers of cystic-type lesions that were developed by BALB/c over C57BL/6 ENDO mice, and that cystic lesions were typically larger than dense lesions.

3.4.6 Figures

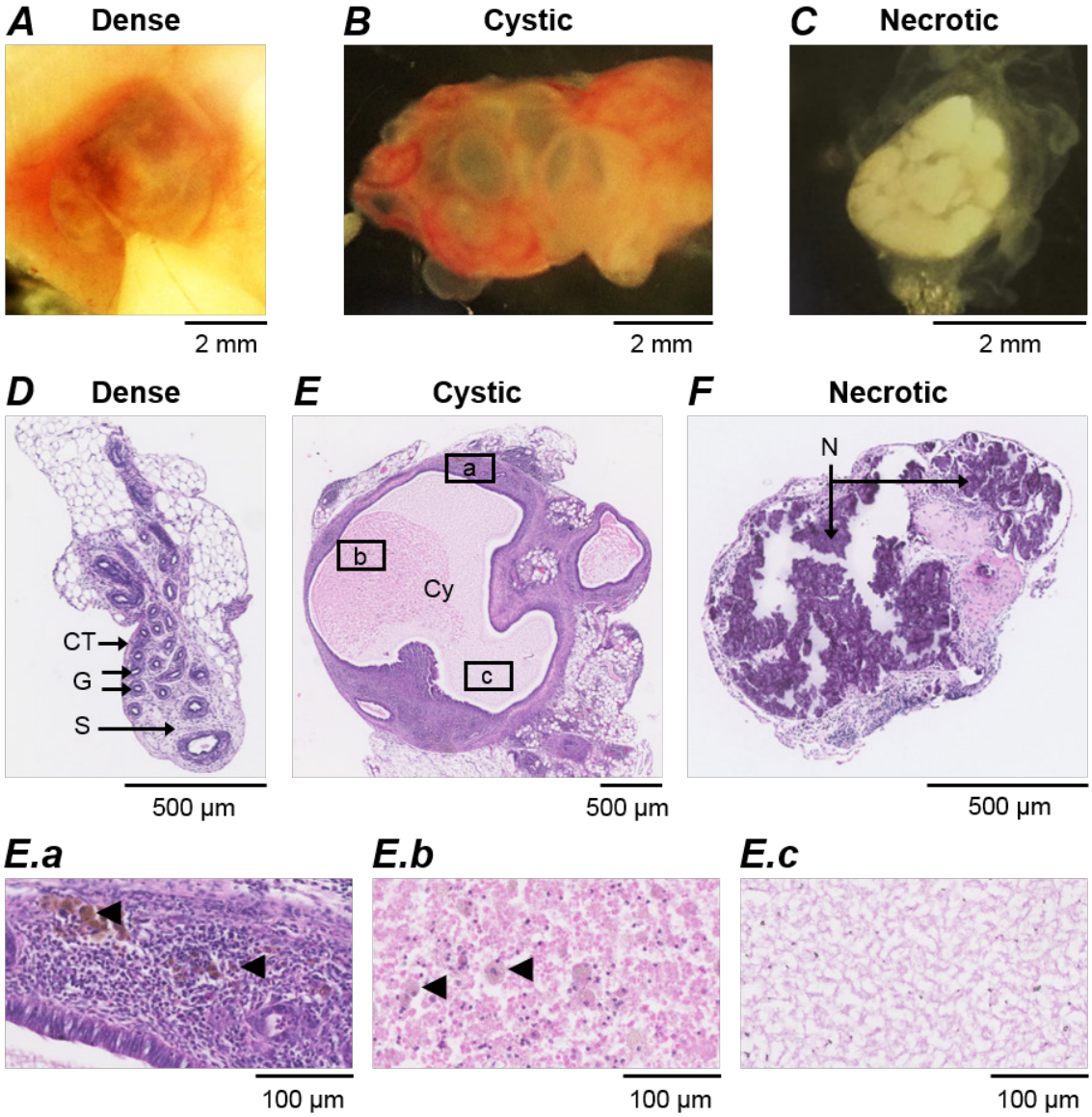
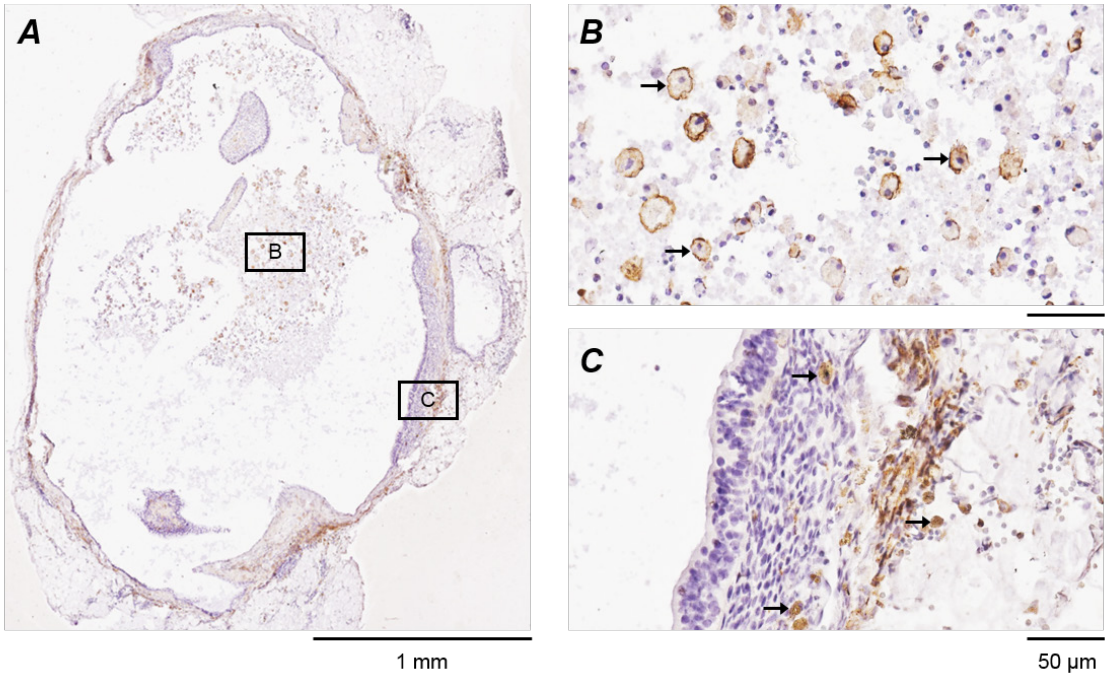


Figure 3.1 Macroscopic and histological characteristics of endometriosis-like lesions developed in the minimally-invasive mouse model of endometriosis (ENDO). (*A*) Macroscopic image of a dense-type lesion embedded within gonadal white adipose tissue. The lesion is opaque and pink-red coloured, and the surface is generally smooth; note connective tissue strand adhering the anterior surface of the lesion to an adjacent region of fat. **(*B*)** Cystic-type lesions had a 'bubbled' appearance and were often translucent. **(*C*)** Necrotic-type lesions were easily identified by their striking white colour and small size. **(*D*)** Histological example of a dense-type lesion, showing the connective tissue capsule (CT), endometrial glands (G) and endometrial stroma (S). **(*E*)** Cystic-type lesions contained one or more fluid-filled cysts (Cy). Within the stroma **(*E.a*, inset)** and cysts **(*E.b*, inset)**, macrophages (arrowheads) often stained brown within their cytoplasm, indicating possible hemosiderin deposition. **(*E.c*, inset)** Cysts also contained a lightly eosinophilic web-like structure with sporadic nuclei of an unidentified cell type. **(*F*)** Necrotic-type lesions showed areas of hematoxylin-rich tissue necrosis (N), with minimal discernible endometrial glands or stroma. Magnification in **(*E.a-c*)** 40x inset.



Supplementary Figure 3.1 Immunohistochemical labelling for the macrophage-specific marker F4/80 in endometriosis-like lesions from ENDO mice. (*A*) Low magnification example of a cystic-type lesion from an ENDO mouse displaying macrophage infiltration within the cyst lumen and throughout the stroma (n = 6). In brief, sections of endometriosis-like lesions were blocked for non-specific staining of tissues, then incubated in 1.25 µg/ml rat anti-mouse F4/80 primary antibody (#14-4801-82; clone BM8; eBiosciences; San Diego, CA), overnight at 4°C. After washing, 1 µg/ml biotinylated rabbit anti-rat secondary IgG (#BA-4001; Vector Laboratories; Burlingame, CA) was applied to sections (40 minutes; RT), followed by Vectastain ABC-HRP reagent (#PK-4000; Vector Laboratories; Burlingame, CA) (30 minutes; RT). The immunocomplex was visualised with precipitation of 3,3'-diaminobenzidine (DAB) (#K3468; Dako; Carpinteria, CA) (15 minutes; RT) and lightly counterstained with hematoxylin. Negative controls were performed by omitting the primary or secondary antibody. **(*B, inset*)** Higher magnification of F4/80-positive macrophages within the cyst of the endometriosis-like lesion (arrows). **(*C, inset*)** F4/80-positive macrophages were also clearly visible at higher magnification within the surrounding stroma of endometriosis-like lesions (arrows). Scale bar in **(*C*)** also applies to **(*B*)**. Magnification in **(*B, C*)** 40x inset.

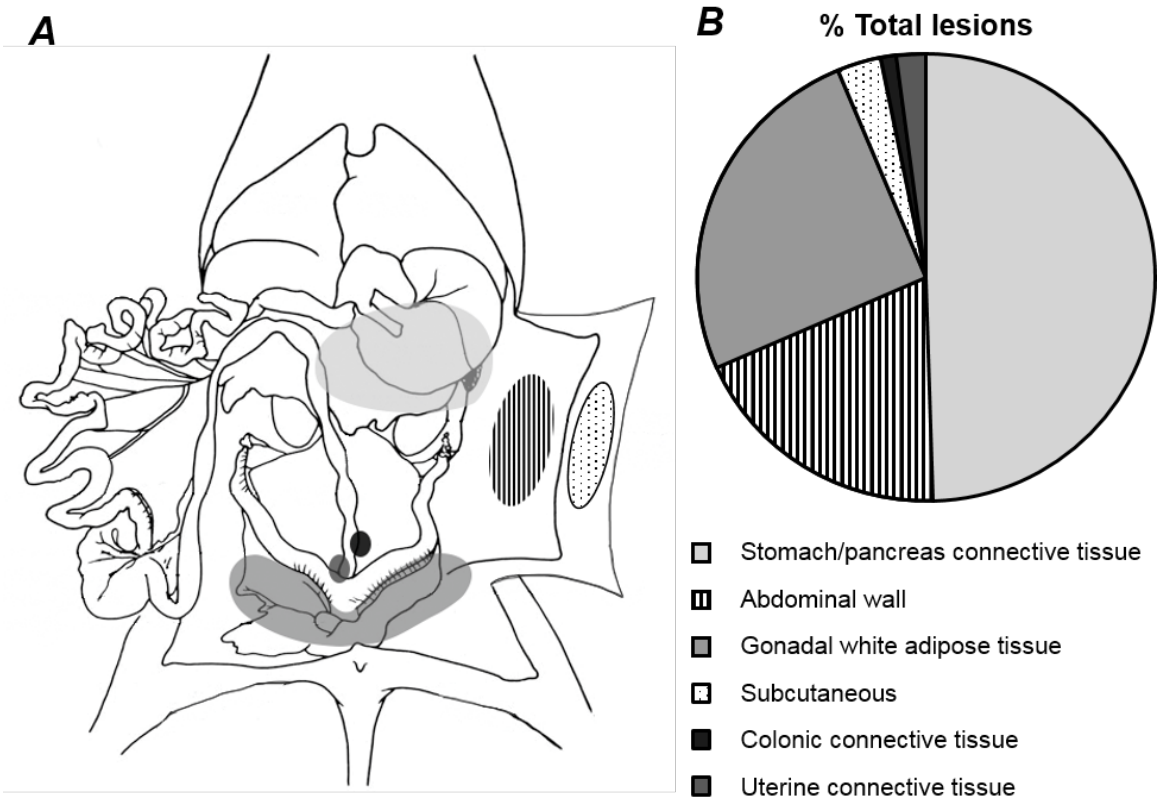


Figure 3.2 Anatomical locations of endometriosis-like lesions established in ENDO mice. (*A*) Schematic diagram of the anterior view of the internal female mouse anatomy, depicting the locations of endometriosis-like lesions established in ENDO mice. Commonly, lesions were found most superiorly on connective tissue attachments near the stomach and pancreas, on the anterior abdominal wall (ipsilateral to the injection site), and inferiorly within the gonadal white adipose tissue. Few lesions formed in the subcutaneous space (also ipsilateral to injection), on connective tissue of the colon, and connective tissue between the colon and uterus. **(*B*)** Summarised data of the proportion of lesions in each location. Outline of mouse anatomy in **(*A*)** adapted with permission from Elsevier and Jackson Laboratories (Cook, 1965).

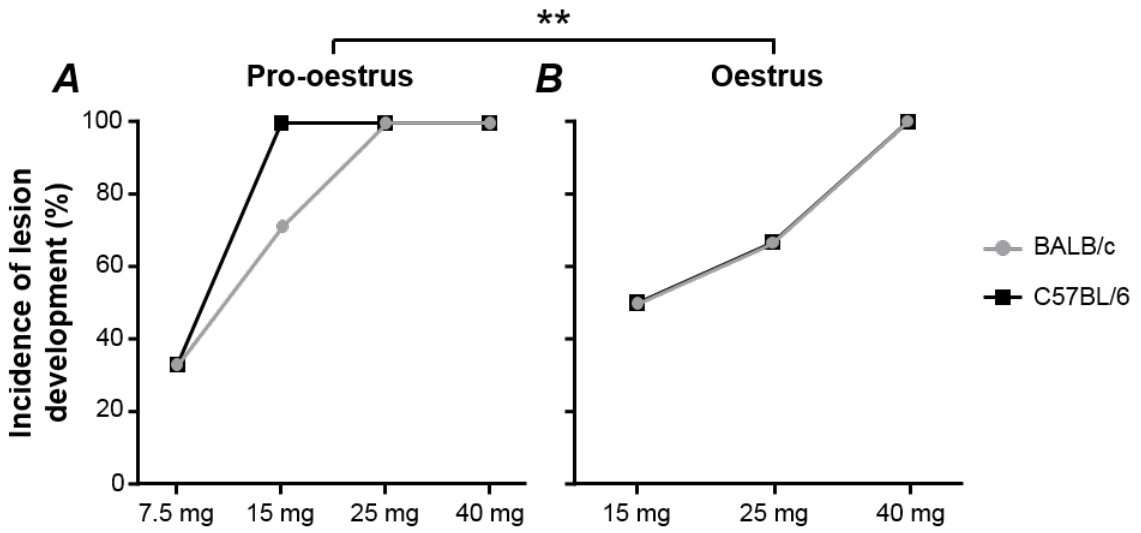
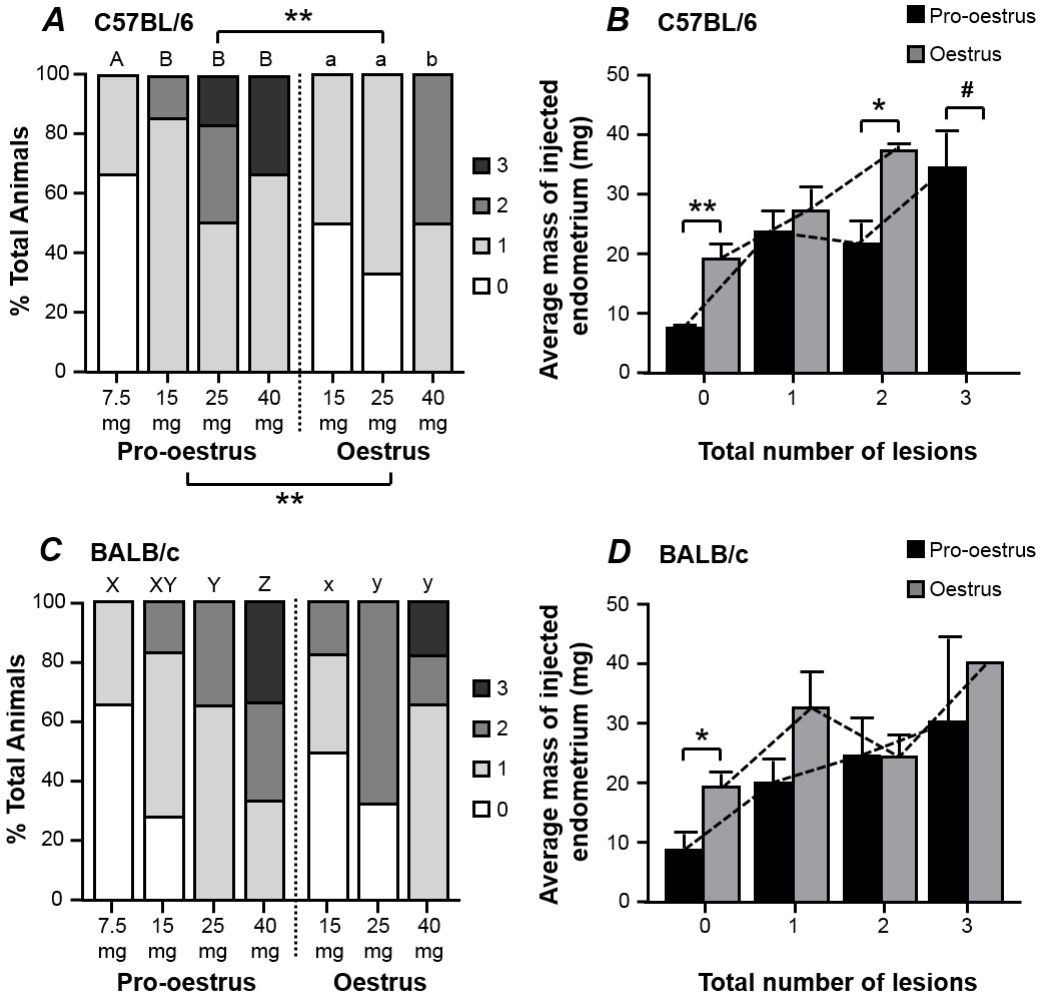


Figure 3.3 Modulation of endometriosis-like lesion prevalence in ENDO mice by the quantity of donor endometrium and oestrous stage at induction. (*A*) Proportion of C57BL/6 and BALB/c ENDO animals in pro-oestrus, grouped by mass of donor endometrial tissue, which developed at least one endometriosis-like lesion three weeks post-injection. **(*B*)** Proportion of animals that successfully established lesions when induced in oestrus. Overall, the probability of lesion development increased as the mass of donor endometrial tissue increased (statistics not shown), and was also greater in pro-oestrus (high oestrogen) than oestrus (low oestrogen). However, there was no effect of mouse strain on lesion establishment. ** denotes $P < 0.01$.



<u>Figure 3.4</u> Total number of endometriosis-like lesions developed by ENDO mice is influenced by mouse strain, oestrous stage, and mass of injected donor endometrial tissue. (*A*) Summarised data showing the proportions of total endometriosis-like lesions (0-3) developed by C57BL/6 mice in pro-oestrus and oestrus, at each mass of injected endometrial tissue. With increasing amounts of donor endometrium, more lesions established and, interestingly, a greater total number of lesions developed in pro-oestrus compared to oestrus (particularly at 25 mg). **(*B*)** This is supported by data displaying the average amount of endometrial tissue required to produce a given number of lesions. Here, the histogram indicates that to generate 0, 2 or 3 lesions pro-oestrus C57BL/6 mice required less donor endometrial tissue compared to those in oestrus. **(*C*)** While the total number of lesions was also greater with increased tissue mass in BALB/c mice, there was no statistical difference between the oestrous stages. **(*D*)** A comparison between tissue volume and lesion number further indicates little effect of oestrous stage influencing lesion number in BALB/c mice (except for 0 lesions). As indicated by the dashed lines linking the average mass of tissue per lesion number, note the similar patterns of lesion development between pro-oestrus C57BL/6 mice in (***B***) and oestrus BALB/c mice in (***D***), and visa versa. Bars not sharing the same superscript within the same panel are statistically different ($P < 0.05$). Uppercase letters are used for analysis within pro-oestrus only, and lowercase letters are used for analysis within oestrus. * denotes $P < 0.05$; ** $P < 0.01$; # no lesions developed for statistical comparison.

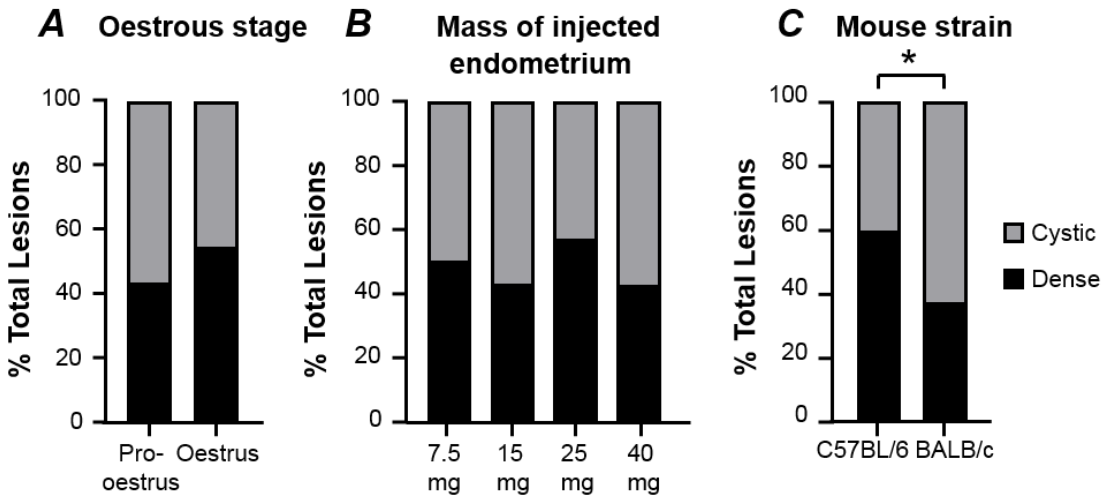


Figure 3.5 Probability of lesion type is altered in C57BL/6 and BALB/c ENDO mice. (***A***) Proportion of lesions characterised as cystic or dense-type were similar in pro-oestrus (n = 58 lesions from 40 animals) and oestrus (n = 37 lesions from 26 animals). **(*B*)** Lesion type did not differ amongst different group masses (7.5 mg n = 4 lesions from 4 animals; 15 mg n = 19 lesions from 18 animals; 25 mg n = 30 lesions from 20 animals; and 40 mg n = 40 lesions from 24 animals). **(*C*)** There was a significant difference in the ratio of cystic-to-dense lesions between the C57BL/6 (n = 46 lesions from 34 animals) and BALB/c mouse strains (n = 49 lesions from 32 animals). BALB/c ENDO mice were 2.7 times more likely to develop cystic-type lesions than C57BL/6s. * denotes P <0.05.

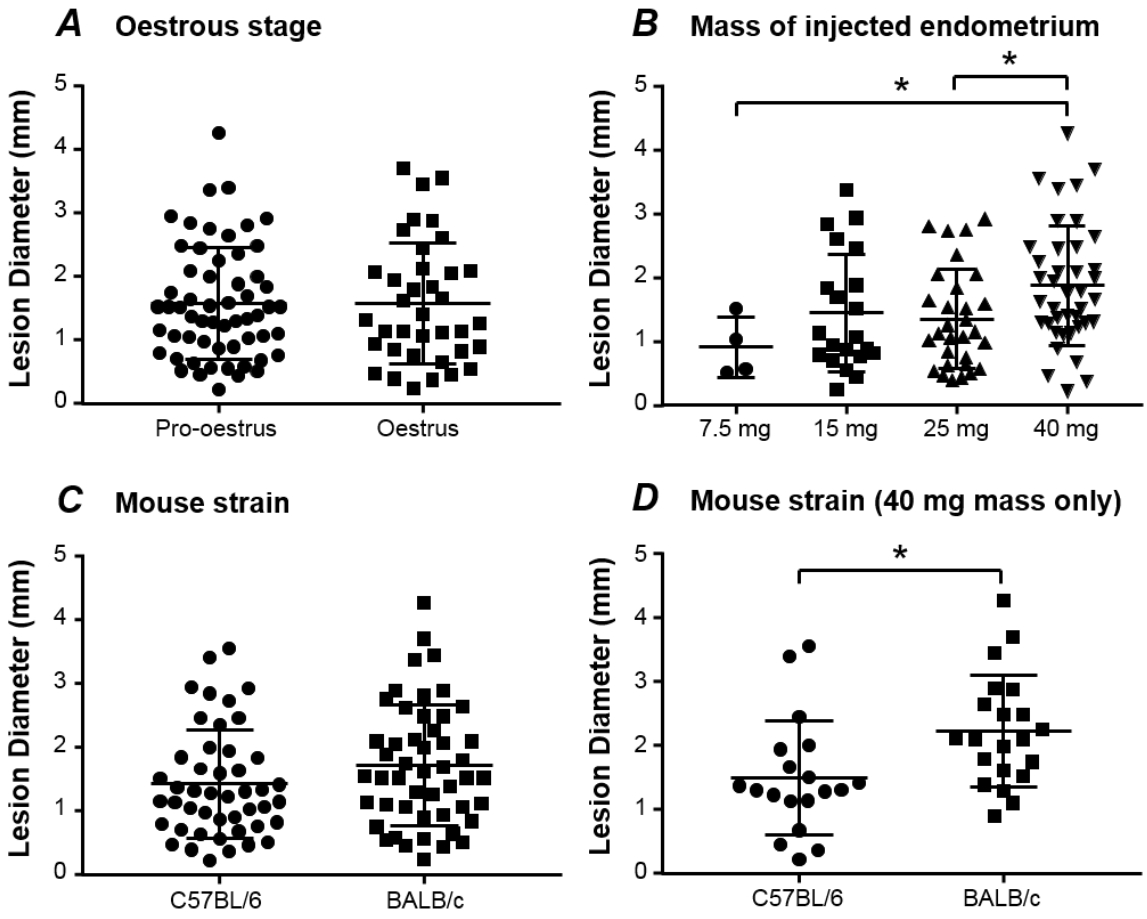


Figure 3.6 Maximal diameter of endometriosis-like lesions varied with mass of injected tissue and mouse strain. (*A*) The average size of lesions was similar regardless of oestrous stage at the time of endometriosis induction. **(*B*)** However, lesions tended to be larger in animals injected with 40 mg donor endometrial tissue compared to the 7.5 mg and 25 mg groups. **(*C*)** While overall there was no difference in the size of lesions between the two mouse strains, at 40 mg only **(*D*)** lesion diameter was greater in BALB/c ENDO mice compared to C57BL/6s. * denotes $P < 0.05$.

3.5 Discussion

In this study, we have carefully characterised the expression of endometriosis-like lesions in a minimally-invasive, hormonally intact mouse model. Akin to the human condition, lesions were phenotypically heterogeneous; the vast majority presenting with well-organised endometrial glands and stroma, with or without cysts. Lesions were also of variable size, and adhered to a number of visceral structures within the peritoneum. We have demonstrated that viable lesions were able to form both without using surgical procedures, or any exogenous sex hormone administration to either donor or recipient animals. In addition, our data show distinct patterns in lesion development dependent on the oestrogen status, mouse strain, and mass of endometrial tissue injected.

3.5.1 Location of endometriosis-like lesion development is indiscriminate

The anatomical locations of lesions within the abdominopelvic cavity of ENDO mice did not form any discernible pattern, using the model permutations investigated in this study. Most lesions adhered on superficial peritoneal locations anywhere from the diaphragm to pelvis, as a result of posture and gravity in quadrupedal mice. This is in opposition to humans, where the majority of lesions are typically distributed deeper within the pelvis due to the influence of a bipedal gait (Chapron *et al.*, 2006).

Regardless, the visceral tissues which lesions adhered to appeared similar to those observed in humans, with the exception that lesions did not form on the surfaces of the visceral organs. Most commonly, lesions established on the peritoneum and connective tissues around the stomach, with occasional lesions associated with the colon and uterus. These structures may resemble connective tissues within the female pelvis where endometriosis is frequent, including the uterosacral ligaments and the rectouterine pouch (Redwine, 1987; Chapron *et al.*, 2003). The affected locations in our ENDO mice have also been described

in models by other laboratories (Burns *et al.*, 2012; Greaves *et al.*, 2014a). Thus, both the phenotypic characteristics and anatomical distribution of lesions in our minimally-invasive mouse model of endometriosis appear to be valid for its use in future investigations.

3.5.2 Prevalence and total number of endometriosis lesions increases with greater endometrial debris

As mentioned, the mechanical event believed to underpin the development of endometriosis is a retrograde menstruation, with the subsequent deposition, attachment and growth of endometrial fragments within the abdominopelvic cavity. In order to replicate this process in rodent models, including the method used in this study, endometrial fragments are administered by intraperitoneal injection. We observed that endometriosis-like lesions were more likely to develop in our ENDO animals as the amount of injected donor endometrium increased. In addition to the success rate, the total number of lesions also increased with greater doses of tissue.

This indicates that in our model we can reasonably predict the success and number of lesions that will develop depending on the amount of donor tissue injected (i.e. dose-dependency). These results correlate with clinical observations, where it is consistently reported that, proportionally, endometriosis patients have earlier age of menarche, shorter menstrual cycle lengths, heavier menstrual effluent, and increased duration of menstrual flow than control counterparts (Vercellini *et al.*, 1997; Matalliotakis *et al.*, 2008); all characteristics that may contribute to large and frequent volumes of retrograde menstruation. Under normal conditions, refluxed endometrial debris is primarily cleared from the peritoneum by cells of the immune system. However, greater volumes of menstrual debris are hypothesised to overwhelm the peritoneal macrophage clearing mechanism, resulting in a permissive environment for developing endometriosis. Hence, one of the current successful therapies for preventing the recurrence of endometriosis is to

suppress endometrial growth and menstruation (usually by hormonal manipulation, such as levonorgestrel (Lockhat *et al.*, 2004)), thereby reducing the volume of menstrual debris for retrograde transport into the abdominopelvic cavity.

We also found that lesions developed from the greatest endometrial mass administered (40 mg) tended to be larger in size compared to those from smaller injections (7.5 and 25 mg). It may be speculated that administration of larger quantities of endometrial fragments challenges the immune clearance system to such an extent that the response mounted is insufficient and thus the lesions that establish consequently grow larger in size. In any case, the dose-response nature of our model may be vital for future studies investigating the effects of interventions on total lesion number, and supports that the quantity of injected endometrial tissue must be considered when comparing results between laboratories. Additionally, this flexible dosing approach allows for exploration of the signalling factors derived from the endometrial tissue that may condition the environment for endometriosis-like lesion development.

3.5.3 Endometriosis lesions are more likely to develop under conditions of naturally high oestrogen

The endometrium is one of the primary targets for sex hormone stimulation, with oestrogen producing many biological effects including hyperplasia, hypertrophy and vascularisation, and therefore favourable for the development of endometriosis lesions. While not wholly necessary for lesion establishment (Burns *et al.*, 2012), administration of exogenous oestrogen has been demonstrated in numerous studies to augment the total number, size, adherence, and invasiveness of lesions and promote tissue integrity (Cummings & Metcalf, 1995; Fortin *et al.*, 2004; Burns *et al.*, 2012).

Here, we are the first to investigate the effect of natural fluctuations in oestrogen in freely

cycling mice on the development of endometriosis, by inducing the condition during oestrous stages of pro-oestrus (pre-ovulation; higher oestrogen) and oestrus (post-ovulation; lower oestrogen). Other laboratories using naturally cycling rodents typically use oestrus (Berkley *et al.*, 2004) or di-oestrus endometrium (McAllister *et al.*, 2012; Peterse *et al.*, 2016) (the latter hormonally comparable to menses in humans) and/or administer exogenous oestrogen at least to the donor animal regardless of oestrous stage (Mariani *et al.*, 2012). Endometriosis-like lesions in this study were achieved at a significantly higher rate during pro-oestrus than in oestrus, irrespective of mouse strain or volume of donor tissue. C57BL/6 mice also developed a greater total number of lesions in pro-oestrus, although no other differences in lesion characteristics such as size, type or location were found to be dependent on oestrous stage.

Thus, our data show that several previous experimental observations of endometriosis can be replicated in rodent models without the use of additional hormones. In addition, we further support a role for oestrogen in enhancing susceptibility to lesion formation. That we did not observe other oestrous-dependent variations in lesion characteristics likely relates to a limitation of our minimally-invasive mouse model, which is that the inoculated donor endometrium was healthy. In addition, the tissue was not menstrual-like (Greaves *et al.*, 2014a) nor at an equivalent oestrous stage to the menstrual phase in humans (Peterse *et al.*, 2016), as our aim was to characterise lesion development using endometrium influenced by natural periods of low and high oestrogen. While we were able to positively identify endometriosis-like lesions generated from such tissue, considerable evidence from studies on human lesions and eutopic menstrual tissue from endometriosis patients has shown to be atypical compared to healthy endometrium.

One of the major purported differences is that the endometrium from women with endometriosis may be functionally resistant to progesterone stimulation (Patel *et al.*, 2017).

The failure of progesterone to act appropriately ultimately affects the phenotype of endometrial tissue shed at menstruation, which displays a prolonged reduction in anti-inflammatory signalling, and highly robust, pro-growth endometrial debris under mostly oestrogenic stimulation (Bruner-Tran *et al.*, 2013). Therefore, while the donor tissue used in our model may not fully mimic the endometrial characteristics observed in endometriosis patients, it does not preclude from using donor tissues in future studies that more accurately represent the pathophysiology of lesion development in humans.

3.5.4 Quantity and characteristics of endometriosis lesions differ between mouse strains of diverse genetic backgrounds

Another novelty of the present study is that we comprehensively compared and contrasted the development of endometriosis-like lesions in two genetically diverse, wildtype inbred mouse strains. Using a surgical model with oestrogen supplementation, only one study to our knowledge has investigated lesion formation in similar mice, which reported no strain differences in their number or histological characteristics in recipients with a maximum 15 mg donor tissue (Somigliana *et al.*, 1999). These experimental conditions would most closely resemble our 15 mg pro-oestrus group. Considering this permutation alone, we would also be unable to discern any variations in lesion number or characteristics between the mouse strains. However, when analysed in combination with greater volumes of endometrium and at different oestrous stages, several critical differences were established.

C57BL/6 mice generated a significantly greater total number of lesions in pro-oestrus than oestrus; an effect that was not observed in BALB/cs. Based on the previous information linking sex hormones and endometriosis, the higher lesion count in pro-oestrus C57BL/6 mice likely also reflects the effects of relatively high oestrogen levels at induction (and therefore a pro-survival, pro-growth, pro-vascularisation microenvironment surrounding the endometrial debris). It was unexpected, and unknown at this stage why BALB/c mice

did not show a similar cycle-sensitivity.

As speculated, mouse strain differences in the development of endometriosis lesions could be attributed to variations in their immune responses to the donor endometrial tissue. In addition to an excess of refluxed menstrual debris, dysregulation of peritoneal macrophage function has also been implicated in the implantation and growth of endometrial lesions (Herington *et al.*, 2011). The specific mechanisms contributing to the evasion of ectopic endometrium from immune clearance are vast, and include decreased cell cytotoxic activity (Guo *et al.*, 2016b); altered levels of proinflammatory cytokines, growth and angiogenic factors (Kyama *et al.*, 2006); decreased recognition of ectopic endometrium (Kuessel *et al.*, 2017); and increased immune cell apoptosis (Sturlese *et al.*, 2011) (for reviews see Christodoulakos *et al.* (2007) and Herington *et al.* (2011)).

Although immune responses of the wildtype mice used in this study may not necessarily have been inappropriate, their different genetic regulation may result in activation of distinct inflammatory cells, signalling cascades and mediators that ultimately determines the fate of the endometrial debris. Previous studies have demonstrated that C57BL/6 and BALB/c mice exhibit strikingly different cytokine signatures to several bacterial infections (Panthel *et al.*, 2003; Jiang *et al.*, 2010); show oestrous cycle-dependent (Krzych *et al.*, 1978) and brain region-specific (Liu *et al.*, 2011) inflammatory responses to an identical stimulus; and are diversely susceptible to experimental autoimmune conditions (Sun *et al.*, 1997), and tumorigenesis (Freeman *et al.*, 2006; Suzuki *et al.*, 2006).

In general, C57BL/6 mice are genetically inclined to mount a Th1/M1 macrophage-dominant immune response, characterised by an IFN-γ-mediated upregulation of multiple proinflammatory mediators such as TNF-α, IL-1β, IL-6 and inducible nitric oxide synthase (iNOS). BALB/c mice alternatively produce Th2/M2 macrophage-dominant responses, an IL-4-driven milieu of anti-inflammatory cytokines, including IL-5, IL-8 and IL-10 (Mills

et al., 2000; Muraille *et al.*, 2014). A previous study showed that adoptive transfer of M1 macrophages into the peritoneum of recipient mice resulted in significantly reduced growth of endometriosis-like lesions, while conversely enhanced growth in the presence of M2 macrophages (Bacci *et al.*, 2009). A similar phenomenon could therefore be associated with the opposing predominant lesion types observed here, where C57BL/6 mice (Th1/M1) developed more lesions with small dense-type characteristics, as opposed to the large cystic-type in BALB/cs (Th2/M2).

Our findings highlight that caution should be taken when translating results between different strains of mice (and between species), and to carefully consider strain prior to the initiation of new experiments. Given that macrophages from peritoneal fluid and ectopic lesions in endometriosis patients express higher levels of M2 markers (Bacci *et al.*, 2009), we suggest that BALB/c mice may be a preferred mouse strain that more closely represents the human condition (and therefore better suited for human xenograft mouse models), although further investigations are necessary. Future studies conducting screening of novel pharmacological interventions should consider conducting tests in multiple strains to avoid unfortunate false-negative results. Additionally, these data point to the need for personalised medicine in the treatment of endometriosis through phenotyping of the lesion types and targeted treatment selection.

3.5.5 Conclusions

As with all rodent models of endometriosis, the absence of natural menstruation means that care must be taken when translating research to humans. Nevertheless, it is hoped that the minor interventions required to successfully develop lesions in this study may be particularly beneficial for researchers investigating endometriosis-induced alterations to the CNS, reproductive processes or any other biological system that is sensitive to surgery or the manipulation of sex hormones. Examples of potential applications include maternal

or early life influences of lesion development, the impact of surgical removal of lesions, contributions to and consequences of pain and central sensitisation, and the effect of lesions on fertility. With such possibilities, this model may be useful for studying new molecular targets and therapeutics, to ultimately provide better treatment outcomes for women often long-suffering with endometriosis.

Statement of Authorship

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Name of Principal Author (Candidate)	Kelsi N. Dodds		
Contribution to the Paper	Designed experiments, performed all experiments, analysed and interpreted data, generated figures, wrote manuscript and acted as corresponding author.		
Overall percentage (%)	85%		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
Signature		Date	30/04/2018

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);

ii. permission is granted for the candidate in include the publication in the thesis; and

iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

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Signature		Date	30/04/2018

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Contribution to the Paper	Helped to interpret data, and to evaluate and edit the manuscript.		
Signature		Date	30/04/2018

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Contribution to the Paper	Supervised development of work, assisted with experimental design, data interpretation, and reviewed the manuscript.		
Signature		Date	30/04/2018

Chapter 4. Spinal glial adaptations occur in a minimally-invasive mouse model of endometriosis: potential implications for lesion aetiology and persistent pelvic pain

This chapter has been peer-reviewed and formally published as a primary research paper [Dodds KN et al. (2018) *Reprod Sci*, **in press** doi: 10.1177/1933719118773405].

4.1 Abstract

Glial adaptations within the central nervous system are well known to modulate central sensitisation and pain. Recently, it has been suggested that activity of glial-related proinflammatory cytokines may potentiate peripheral inflammation, via central neurogenic processes. However, a role for altered glial function has not yet been investigated in the context of endometriosis; a chronic inflammatory condition in women associated with peripheral lesions, often manifesting with persistent pelvic pain. Using a minimally-invasive mouse model of endometriosis, we investigated associations between peripheral endometriosis-like lesions and adaptations in central glial reactivity. Spinal cords (T13-S1) from female C57BL/6 mice with endometriosis-like lesions (ENDO) were imaged via fluorescent immunohistochemistry for the expression of glial fibrillary-associated protein (GFAP; astrocytes) and CD11b (microglia) in the dorsal horn (n = 5). Heightened variability (P = 0.02) as well as an overall increase (P = 0.04) in the mean area of GFAP-immunoreactivity was found in ENDO versus saline-injected control animals. Interestingly, spinal levels showing the greatest alterations in GFAP-immunoreactivity appeared to correlate with the spatial location of lesions within the abdominopelvic cavity. A subtle but significant increase in the mean area of CD11b-immunostaining was also observed in ENDO mice compared to controls (P = 0.02). This is the first study to describe adaptations in non-neuronal, immune-like cells of the central nervous system attributed to the presence of endometriosis-like lesions.

4.2 Introduction

Endometriosis is an oestrogen-dependent, chronic inflammatory condition, defined by the presence of endometrial-like lesions in extra-uterine locations; typically within the pelvis (Johnson *et al.*, 2017). Common symptoms reported by women with endometriosis include dysmenorrhea, pain during sexual intercourse, discomfort upon movements of the bowel and bladder, and persistent pelvic pain (Stratton & Berkley, 2011; Hansen *et al.*, 2014). However, clinical observations (and indeed animal studies (McAllister *et al.*, 2009)) have repeatedly found that the degree of reported pain does not necessarily correlate with the severity of endometriosis lesions, and removal of lesions does not always eliminate pain (Fauconnier & Chapron, 2005). Despite this, current analgesic, anti-inflammatory, surgical and hormonal therapies maintain a primary focus on suppression or excision of lesions.

The disconnection between the peripheral pathology and pain symptoms suggests that central sensitisation processes may influence the extent of pain experienced by endometriosis patients. This is supported by experimental research, which has demonstrated that women with endometriosis-associated persistent pelvic pain have a generalised increase in sensitivity to stimulation of sites close (e.g. abdomen) and remote (e.g. hand or foot) from the pelvis (Bajaj *et al.*, 2003; Laursen *et al.*, 2005; He *et al.*, 2010). Endometriosis patients also show decreased grey-matter volume in several brain regions associated with the processing of noxious stimuli (As-Sanie *et al.*, 2012); elevated levels of excitatory neurotransmitters in the brain (As-Sanie *et al.*, 2016); and increased functional connectivity between brain regions involved in somatosensation (As-Sanie *et al.*, 2016).

Within the central nervous system (CNS), bidirectional signalling between glial cells, such as astrocytes and microglia, and neurons ('neuroimmune communication') is well recognised to facilitate the development of central sensitisation. Glia are a population of immune-like cells that, under ambient conditions, are primarily responsible for maintaining

normal neuronal activity. This is achieved, for example, by the ability of astrocytes to facilitate clearance of the excitatory neurotransmitter, glutamate, from synapses (Liaw *et al.*, 2005). Microglia, classically known as macrophages of the CNS, can alternatively preserve homeostasis by recognising, sequestering and processing antigens and cellular debris (Nimmerjahn *et al.*, 2005). However, in response to strong or prolonged immune challenges (such as peripheral inflammation), adaptations in glial morphology and function can occur, shifting their reactivity from a regulatory to rapid response state (Milligan & Watkins, 2009). Importantly, this includes altered cell-specific protein expression, and upregulated secretion of proinflammatory cytokines, such as interleukin (IL)-1β and tumour necrosis factor (TNF)-α (Ji *et al.*, 2013). Continued aberrant release of these signals contributes to central sensitisation by inducing molecular changes in adjacent neurons that enhance excitatory (e.g. glutamatergic), and decrease inhibitory (e.g. GABAergic), neuronal signalling (Dodds *et al.*, 2016 - Chapter 2). Ultimately, this may lead to augmented transmission of peripheral nociceptive signals that reach the brain, and consequently exaggerated (hyperalgesia and allodynia) and/or persistent pain perception.

Evidence of pain facilitation by the neuroimmune system has been demonstrated in pre-clinical models of both neuropathic pain and somatic inflammation (Old *et al.*, 2015). Recently, the altered reactivity of spinal glial cells has also been established in animal models of visceral inflammatory conditions, including colitis (Kannampalli *et al.*, 2014), pancreatitis (Feng *et al.*, 2010; Qian *et al.*, 2011), and cystitis (Liu *et al.*, 2016a). While neuronal mechanisms of central sensitisation have been considered in the context of endometriosis (Brawn *et al.*, 2014), a potential role for adaptations in glial populations are yet to be explored.

Intriguingly, recent reports have suggested that proinflammatory cytokines, released by highly reactive spinal glia, may promote inflammatory disease processes in the periphery

by stimulating neurogenic inflammatory pathways (Boyle *et al.*, 2006; Boettger *et al.*, 2010; Bressan *et al.*, 2010; Luo *et al.*, 2014). The release of mediators (such as neuropeptides) from afferent neurons that lead to neurogenic inflammation can occur by local stimulation of their peripheral terminals ('axonal reflex'); or by the antidromic propagation of signals from their central terminals within the spinal cord, back to the periphery ('dorsal root reflex') (Willis, 1999). Therefore, it has been hypothesised that neuroimmune-mediated central sensitisation may not only affect projection neurons contributing to persistent pain (peripheral-to-central signalling), but also in sensitising afferent neurons to establish and maintain peripheral inflammatory conditions (central-to-peripheral signalling) (Gong *et al.*, 2010; Majima *et al.*, 2018). In endometriosis, neurogenic inflammation within the peritoneal environment is believed to assist in lesion formation, where stimulation of afferent endings by refluxed endometrial debris creates a positive feedback loop of inflammation favourable for growth (Laux-Biehlmann *et al.*, 2015; McKinnon *et al.*, 2015). However, a possible role for centrally-derived neurogenic inflammation in this process remains to be considered (Dodds *et al.*, 2016 - Chapter 2).

Hence, there exists strong potential for glial adaptations and subsequent neuroimmune-mediated central sensitisation to impact on both the pain symptoms and lesion pathology associated with endometriosis. In this study, we have utilised a minimally-invasive mouse model of endometriosis to assess the expression of glial (astrocytes and microglia) cell-specific protein markers in the spinal dorsal horn, which are indicative of their degree of adaptation and reactivity. Further, we have investigated a relationship between the peripheral location of endometriosis-like lesions, and the distribution of glial expression within specific spinal levels.

4.3 Methods

4.3.1 Animals

Female C57BL/6NHsd mice (C57BL/6; $n = 16$) 8-12 weeks in age, weighing 19.5 ± 1.1 g, were obtained from the University of Adelaide Laboratory Animal Services. Animals underwent daily cervical smear testing to determine their oestrous cycle, as described previously (Dodds *et al.*, 2015). Each was selected for use only when in the pro-oestrus phase of the oestrous cycle (high oestrogen; pre-ovulation), as pro-oestrus conditions at the time of induction have been shown to generate more robust and consistent endometriosis-like lesions than the oestrus phase (low oestrogen; post-ovulation) in C57BL/6 mice (Dodds *et al.*, 2017 - Chapter 3). All animal use was conducted in accordance with the National Health and Medical Research Council Australian code for the care and use of animals for scientific purposes (8th edition, 2013) and the University of Adelaide Animal Ethics Guidelines, and was approved by the University of Adelaide Animal Ethics Committee.

4.3.2 Induction of the minimally-invasive mouse model of endometriosis

To induce endometriosis-like lesions in mice, we employed a method previously developed by our laboratory (Dodds *et al.*, 2017 - Chapter 3). This particular model was favoured as it avoids surgery and exogenous hormone administration, which in themselves can alter glial reactivity (Hains *et al.*, 2010; Loram *et al.*, 2012; Lewis *et al.*, 2015), and therefore could confound results. Donor animals ($n = 5$) were sacrificed by cervical dislocation whilst under deep inhaled isoflurane anaesthesia. The uterus was removed and placed into a sterilised glass Petri dish containing cold (4°C) 0.01 M phosphate-buffered saline (PBS; composed of 13.7 mM NaCl, 0.27 mM KCl, 0.15 mM KH_2PO_4 and 0.8 mM Na_2HPO_4; pH 7.4). The ovaries, cervix and remaining mesometrium were detached leaving only the two

uterine horns. Each horn was opened along the mesometrial border and pinned flat to the Sylgard®-lined (Dow Corning; Michigan, USA) base of the Petri dish using entomology pins. The endometrium (40 mg) was then carefully removed by sharp dissection and further cut into segments of 2-3 mm^2. The resultant endometrial fragments were placed into a 1-ml syringe containing 0.5 ml sterile saline (0.9% NaCl) attached to a 21-gauge needle. To ensure smooth delivery of the donor endometrium, the fragments were plunged through the needle once and re-aspirated, before being intraperitoneally injected into a recipient mouse (ENDO; n = 5) (1 donor: 1 recipient) at the ventral midline between the left inguinal nipples. Control animals were injected with an equal volume of sterile saline alone (n = 6).

Following 21 days of development, all animals were anaesthetised with isoflurane and decapitated. Spinal cords (spanning thoracic to sacral, inclusive), endometriosis-like lesions (where applicable) and control tissues (such as lymph nodes and fat), were carefully removed and immersed in cold (4°C) 4% paraformaldehyde fixative (pH 7.2) overnight. Lesions and control tissues then underwent standard histological processing, sectioning and staining for hematoxylin and eosin, to assess for the presence of epithelial glands and stroma – criteria typically required for a positive diagnosis of endometriosis in humans (Clement, 2007). Confirmed lesions were then measured and classified as dense-, cystic- or necrotic-type based on additional phenotypic characteristics, as described previously (Dodds *et al.*, 2017 - Chapter 3).

4.3.3 Immunohistochemistry

Spinal cord tissues were washed (4 x 15 min; PBS) and cryoprotected in 30% sucrose at 4°C for two nights. Following dissection into regions T13-L1, L2-L3, L4-L5 and L6-S1, the spinal cord segments were submerged into individual plastic moulds containing Tissue-Tek® OCT compound (#IA018; ProSciTech; Queensland, Australia) and frozen by being placed into isopentane cooled by liquid nitrogen. All blocks were then insulated and stored

at -80°C.

Spinal segments were sectioned in duplicate (per antibody label) at 10 µm using a Leica CM1850 cryostat (Leica Biosystems; Nusslock, Germany) at approximately -15°C, and collected onto SuperFrost® glass microscope slides (Menzel-Gläser; Braunschweig, Germany). It was ensured that each section was taken at least 50 µm apart to prevent cell overlap during analysis. After air-drying for a minimum of 30 min, slides were briefly rinsed with PBS to remove residual OCT before undergoing heat-mediated antigen retrieval using sodium citrate buffer (0.01 M with 0.05% Tween 20; pH 6.0). Retrieval buffer was preheated to 65°C using the PT Link™ system (#PT101; Dako; Glostrup, Denmark). Slides were submerged in the buffer and the temperature raised to 97°C for 10 min. After returning to 65°C, slides were removed and cooled with 0.01% Tween 20 at room temperature (RT).

To assess for adaptations in astrocytes, sections were blocked for 1 h at RT in a humid chamber with 10% normal donkey serum/0.01% Triton X-100. Sections were then incubated in Alexa Fluor® 488-conjugated mouse monoclonal anti-glial fibrillary acidic protein (GFAP) antibody (#53-9892-82, clone GA5; RRID: AB_10598515; 1 µg/ml; eBioscience; California, USA) for two nights at 4°C in a dark, humid chamber.

For the assessment of microglial adaptations, sections were blocked for 1 h at RT in a humid chamber with 5% bovine serum albumin/0.03% Triton X-100. Slides were then incubated in rat monoclonal anti-CD11b (#ab64347, clone M1/70; RRID: AB_1140550; 5 µg/ml; Abcam; Cambridge, UK) for two nights at 4°C. After washing (4 x 10 min; PBS), sections were then incubated with donkey anti-rat Alexa Fluor® 568 secondary antibody (#ab175475; RRID: AB_2636887; 4 µg/ml; Abcam; Cambridge, UK) for 1 h at RT in a dark, humid chamber.

All sections were given a final rinse with PBS (4 x 10 min) and mounted with Tris-based Fluoro-Gel medium (#IM030; ProSciTech; Queensland, Australia). Control samples were prepared by omitting either primary or secondary antibodies from the incubation solutions (data not shown).

4.3.4 Image acquisition

Slides were viewed with a Leica TCS SP5 scanning confocal microscope (Leica Microsystems; Wetzlar, Germany) using appropriate excitation wavelengths at 20x magnification with oil immersion. Images were acquired using Leica Application Suite Advanced Fluorescence version 2.6.3 (Leica Microsystems; Mannheim, Germany). Final images are digital composites of 1 µm Z-series scans (approximately 11-16 optical sections through a depth of 10-15 µm). All images per antibody label were taken at the same gain and offset parameters between animals. Each spinal dorsal horn per section was imaged separately; unless otherwise stated, anatomical descriptions of ipsilateral and contralateral regarding the dorsal horn (and location of lesions) are used in reference to the ENDO injection site, which was always the animals' left-hand side.

4.3.5 Image analysis

Semiquantitative analyses were performed on collected images using ImageJ Fiji software (Schindelin *et al.*, 2012). Prior to analysis, the maximized Z-stack of images were converted from Leica image files (.lif) to 8-bit greyscale .tiff images, and signal pixels of positive staining areas/region of interest (ROI) were selected using the *Threshold* function. The ROI was an ellipsoid shape that remained a consistent size for each spinal level between animals. For T13-L1 the area of analysis was 1.42×10^5 µm^2; L2-L3 1.57×10^5 µm^2; L4-L5 1.78×10^5 µm^2; and L6-S1 1.45×10^5 µm^2. On spinal cord images, the ROI was specifically positioned so that the length extended from the most superficial margin of

Rexed's lamina I, through to the deepest margin of lamina IV, and the full medial-lateral grey matter width (Fig. 4.1). Depending on the spinal level, the length of the ROI sometimes also included the superficial portion of lamina V. Importantly, the selected laminae contain a large proportion of visceral afferent terminals, and the expression of neurochemical substances associated with nociceptive signalling, such as calcitonin gene-related peptide (CGRP) and substance P (Sengul & Watson, 2012). The percentage of the ROI occupied by immunofluorescence was calculated by the *Measure* function, which divides the signal pixels in the selected ROI by the total number of pixels in the ROI, multiplied by 100. Duplicate area measurements for each dorsal horn were then averaged to obtain a single percentage area value per dorsal horn, per spinal level, per animal. All images were measured blinded as to animal treatment.

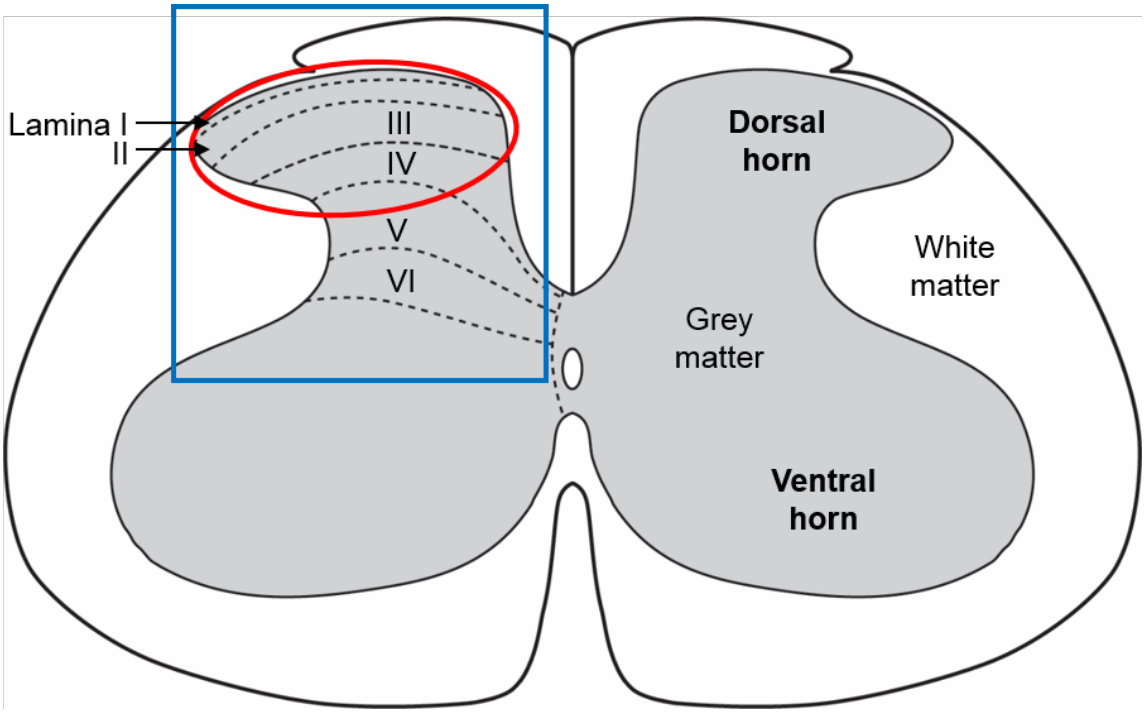


Figure 4.1 Schematic diagram of a transverse section of the mouse lumbar spinal cord. Major regions of white and grey matter, the dorsal and ventral horns, and locations of Rexed's laminae I-VI within the dorsal horn are shown. The red ellipsoid denotes the approximate position of ROIs used for measurements of glial immunoreactivity, and the blue box represents fields-of-view of the 20x images captured for analysis.

4.3.6 Statistical analysis

Microsoft® Excel® 2013 (Microsoft Corporation; Washington, USA) was used to generate coefficient of variation (CV) percentages and F-test scores of variability around the standard deviation (SD) for the area of positive immunolabelling values. A two-way ANOVA with Bonferroni post-hoc correction was performed using GraphPad Prism® 7 software (GraphPad Software Inc.; California, USA) to determine the 'treatment' effect of ENDO animals versus saline controls. GraphPad Prism® 7 was also used to generate area under the curve (AUC) scores for each dorsal horn (ipsilateral T13-S1 and contralateral T13-S1) per animal. For summarised AUC data, statistical comparisons between ENDO animals and saline controls were determined by a student unpaired two-way t-test with Welch correction. Data in the text are expressed as mean ± SD, and *P* values of <0.05 were considered statistically significant.

4.4 Results

4.4.1 Endometriosis-like lesions successfully established in ENDO mice

Endometriosis-like lesions were successfully induced in C57BL/6 ENDO mice during the 21-day development period (Somigliana *et al.*, 1999; Dodds *et al.*, 2017 - Chapter 3). On average, ENDO animals received 40.9 ± 1.4 mg of donor endometrial tissue in 28.2 ± 1.9 pieces. The total number of recovered lesions ranged from 1-4, were 221 to 2450 μm in diameter (average 1205 ± 654 μm) and consisted of 36.3% cystic, 45.5% dense, and 18.2% necrotic-type lesions (Dodds *et al.*, 2017 - Chapter 3) (Table 4.1).

4.4.2 Astrocyte reactivity is increased and highly variable in the spinal dorsal horn of ENDO mice

In saline control animals, the area of GFAP-immunoreactivity in the spinal dorsal horn was

consistent across spinal levels, and between animals (n = 6). The average area occupied by GFAP-positive structures in T13-L1 was 5.1 ± 0.7% (CV 13%); L2-L3 5.3 ± 0.6% (CV 12%); L4-L5 4.8 ± 0.8% (CV 17%); and L6-S1 5.1 ± 1.0% (CV 20%) (minimum value from any segment 3.3%; maximum value 6.7%) (representative images shown in Fig. 4.2A, C, E, G). In contrast, astrocyte reactivity marker expression in ENDO mice was highly variable (n = 5). On average, the area of GFAP-immunoreactivity in T13-L1 was 6.6 ± 1.6% (CV 24%); L2-L3 6.2 ± 1.4% (CV 22%); L4-L5 6.1 ± 2.6% (CV 43%); and L6-S1 5.4 ± 0.9% (CV 17%) (minimum value from any segment 3.5%; maximum value 10.8%) (representative images in Fig. 4.2B, D, F, H). Statistically, the variability of the SD between ENDO and saline-injected animals was significantly increased for T13-L1 (P = 0.009), L2-L3 (P = 0.02) and L4-L5 (P = 0.0007), but not L6-S1 (P = 0.78) (Fig. 4.3A-B).

In addition to altered GFAP variability, a significant overall treatment effect was observed for animals with endometriosis-like lesions compared to controls (P = 0.02), with the most significant increase in GFAP-immunoreactivity at the level of T13-L1 (P = 0.02) (L2-L3 P = 0.59; L4-L5 P = 0.09; L6-S1 P >0.99) (Fig. 4.3A-B). A comparison of summarised AUC values further reiterated that overall, spinal GFAP astrocyte expression was increased in ENDO animals (average AUC 18.3 ± 4.0) compared to saline controls (15.2 ± 2.0) (P = 0.04) and highly variable (P = 0.02) (Fig. 4.3C).

4.4.3 Spinal levels of altered astrocyte reactivity correlate with peripheral locations of endometriosis-like lesions in ENDO mice

Associations between the spinal sites of altered GFAP-immunoreactivity and the peripheral location of lesions were further analysed. Owing to the heterogeneity of lesion development across the ENDO animals, a general overview of observations are described below, followed by case-by-case analyses.

All ENDO animals displayed area percentages of GFAP-immunoreactivity that were altered beyond 2 SD of the mean saline control values for discrete spinal segments (Fig. 4.4). In general, the greatest deviations from control values were observed in T13-L1 and L2-L3 in animals with lesions in the mid-upper region of the abdominopelvic cavity, and in L4-L5 in those with lesions in the mid-lower regions. No change, or only non-significant deviations >1 but <2 SD, from mean values were observed in L6-S1. In addition, the major changes to GFAP-immunoreactivity in ENDO mice largely occurred in the ipsilateral dorsal horn (regarding lesion location from the midline), or across both dorsal horns within the same spinal segment. There did not appear to be any correlations between lesion location or type with the directionality or degree of change in area percentage values. However, the sole animal that showed a significant decrease (>2 SD) in GFAP-immunoreactivity compared to control values (all others increased) was the only animal to develop a lesion on the anterior abdominal wall (somatic involvement versus all other lesions on visceral surfaces).

With a single lesion on the ipsilateral abdominal wall, roughly midway along the length of the abdomen, ENDO animal 1 showed an increase in GFAP-immunoreactivity >2 SD (by 31% from control mean) in the ipsilateral T13-L1. Interestingly, a decrease in area >2 SD (average 30% reduction from control mean) was observed for both dorsal horns at the level of L2-L3 (Fig. 4.4A).

ENDO animal 2 also had one lesion, attached to connective tissue around the stomach and pancreas, biased toward the animal's left-hand side (Fig. 4.4B). In this animal, GFAP-immunoreactivity was increased >3 SD (by 65%) from mean control values in the ipsilateral T13-L1, and increased >2 SD in both the contralateral (by 32%) and ipsilateral (by 25%) dorsal horns in L2-L3.

ENDO animal 3 developed two lesions, also in the region of the stomach/pancreas,

although the larger of the two lesions was located closer to the animal's right-hand side (Fig. 4.4C). As such, the contralateral T13-L1 dorsal horn showed a 66% increase (>3 SD) in GFAP-immunoreactivity, and the ipsilateral T13-L1 a 36% increase (>2 SD). The contralateral L2-L3 also increased by 34% (>2 SD).

ENDO animal 4 had two lesions around the stomach/pancreas (the larger lesion biased to the animal's left-hand side), as well as one lesion within the ipsilateral gonadal white adipose tissue (Fig. 4.4D). Although no change was seen in the T13-L1 dorsal horn (as with ENDO animals 1-3 and 5), an increase >3 SD occurred in the ipsilateral L2-3 (by 51%) and L4-L5 (by 82%) compared to control mean values.

Lastly, ENDO animal 5 had four lesions in total: two at the midline in the region of the stomach/pancreas, and two within the contralateral gonadal white adipose tissue (Fig. 4.4E). A significant increase in the area of GFAP-immunoreactivity >3 SD from control values was observed in both the contralateral (by 72%) and ipsilateral (by 52%) T13-L1 dorsal horn. Increased values also occurred in the ipsilateral L2-L3 by 25% (>2 SD). However, the greatest change occurred in spinal segment L4-L5, with an increase >3 SD in both the contralateral (by 94%) and ipsilateral (by 126%) dorsal horn compared to controls.

4.4.4 Microglial reactivity is subtly increased in the spinal dorsal horn of ENDO mice

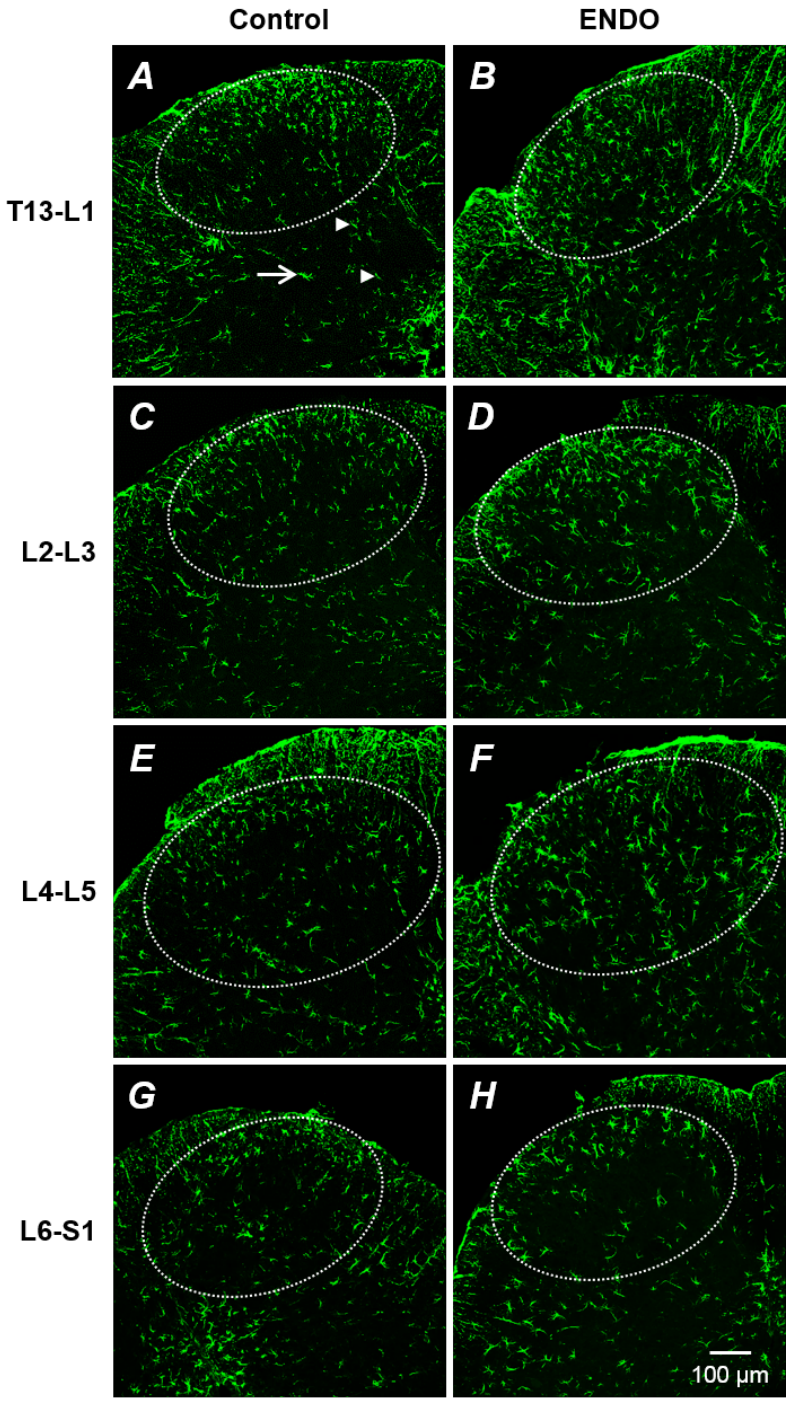
The total area of CD11b-immunoreactivity in the spinal dorsal horn of saline control animals was also consistent both across spinal levels, and between individual animals (n = 6). The average area occupied by CD11b-positive structures in T13-L1 was 4.1 ± 0.6% (CV 14%); L2-L3 4.3 ± 0.7% (CV 16%); L4-L5 4.5 ± 0.5% (CV 12%); and L6-S1 4.5 ± 0.6% (CV 14%) (minimum value from any segment 3.4%; maximum value 5.5%)

(representative images shown in Fig. 4.5A, C, E, G). In ENDO mice (n = 5), the area of CD11b reactivity was 4.9 ± 0.6% (CV 12%) in T13-L1; 4.7 ± 0.7% (CV 14%) for L2-L3; 5.0 ± 0.5% (CV 11%) in L4-L5; and 4.9 ± 0.9% (CV 18% for L6-S1) (minimum value from any segment 2.9%; maximum value 6.1%) (representative images shown in Fig. 4.5B, D, F, H). Statistically, no differences were detected between saline control and ENDO animals regarding variability in CD11b expression per spinal level (all comparisons P ≥0.24). However, there was an overall treatment effect observed for ENDO animals versus saline controls (P = 0.01), with a significant increase in CD11b area observed for T13-L1 (P = 0.02; all other levels P ≥0.33) (Fig. 4.6A-B). Collectively, there was also a subtle but significant increase in the AUC of CD11b reactivity in ENDO (average AUC 14.6 ± 1.5) versus saline animals (13.1 ± 1.3; P = 0.02) (Fig. 4.6C).

4.4.5 Figures and tables

Model parameters				Lesion characteristics		
Animal ID	**Injected tissue mass (mg)**	**Injected tissue pieces**	**No. of lesions**	**Class**	**Diameter (µm)**	**Location**
ENDO 1	40	28	1	Dense	1300	Ab
ENDO 2	39.7	26	1	Cystic	1280	G/P CT
ENDO 3	43.1	31	2	Dense	1510	G/P CT
				Necrotic	650	G/P CT
ENDO 4	40.1	27	3	Cystic	1370	G/P CT
				Dense	679	GWAT
				Dense	221	GWAT
ENDO 5	41.1	29	4	Cystic	2450	G/P CT
				Cystic	2000	G/P CT
				Dense	1230	GWAT
				Necrotic	564	GWAT

Table 4.1 Characteristics of model parameters and identified endometriosis-like lesions in ENDO mice. *AB* = abdominal wall; *G/P CT* = gastric/pancreatic connective tissue; *GWAT* = gonadal white adipose tissue.



<u>Figure 4.2</u> Distribution of GFAP-immunopositive astrocytes in the spinal dorsal horn of control and ENDO mice. (*A-B*) Representative images of the T13-L1 spinal dorsal horn (dashed ellipsoid) in control and ENDO mice, respectively. ENDO animals showed a significant increase in the total area occupied by GFAP-immunoreactivity compared to controls. Characteristic stellate GFAP-immunopositive astrocytes can be identified (arrows) as well as fragments of their processes (arrowheads). ***(C-D)*** Whilst variable, significant increases in the dorsal horn area of GFAP-immunoreactivity were also often observed in L2-L3 of ENDO animals versus controls, ***(E-F)*** as well as in L4-L5. ***(G-H)*** The percentage area of GFAP immunostaining in control and ENDO mice were similar for spinal levels L6-S1. Magnification 20x; n = 24 per spinal level from 6 control animals; n = 20 per spinal level from 5 ENDO animals. Scale bar in (***H***) applies to all panels.

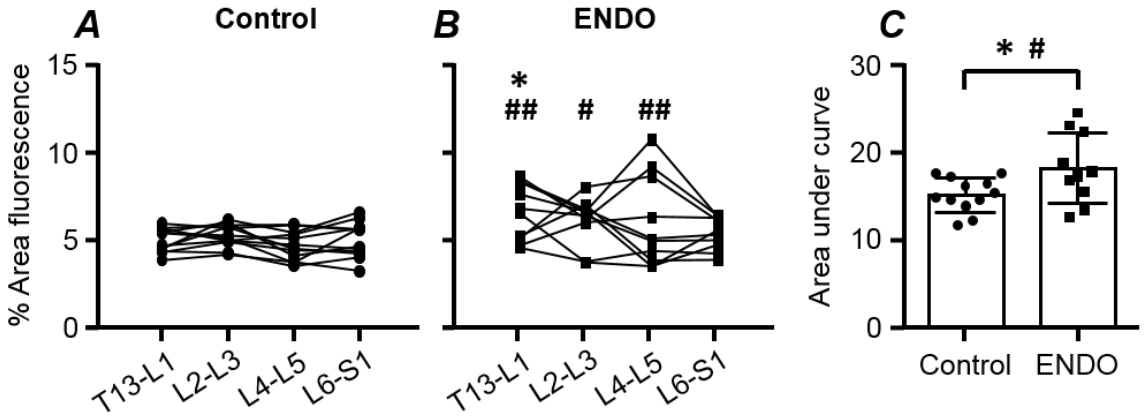
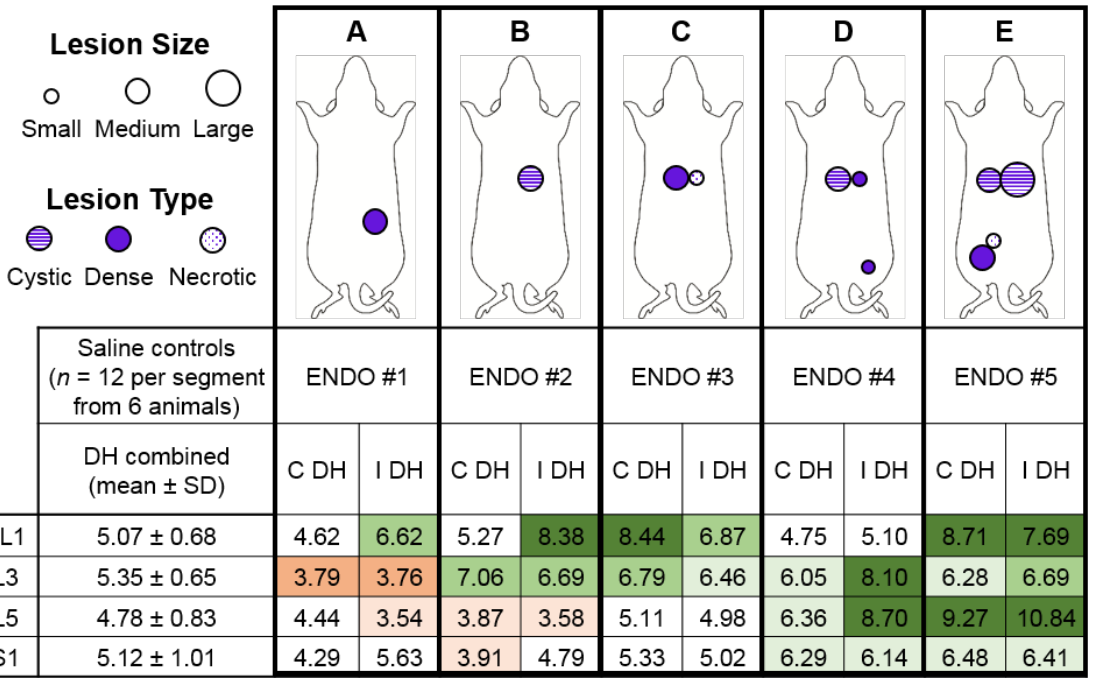


Figure 4.3 Calculated area of GFAP-immunoreactivity (astrocytes) in the spinal dorsal horn of control and ENDO mice. (*A*) Percentage area of each dorsal horn (linked by horizontal bars) occupied by GFAP immunostaining in saline-injected control mice was relatively consistent across spinal levels T13-S1 (n = 12 per segment from 6 animals). **(*B*)** In contrast, a significant increase in variability was observed for levels T13-L5 in animals with endometriosis-like lesions (n = 10 per segment from 5 animals). The mean area of GFAP-immunoreactivity for the T13-L1 dorsal horn in ENDO mice was also significantly increased compared to controls. **(*C*)** Summarised data comparing the area under the curve values for each dorsal horn further indicate an overall mean increase and heightened variability of GFAP immunostaining in ENDO animals versus saline-injected controls. # denotes a significant difference in variability of dorsal horn area P <0.05; ## P <0.01; * denotes a significant increase in the mean dorsal horn area P <0.05. Significance notations in panel **(*B*)** refer to corresponding spinal levels in panel **(*A*)**. Y-axis labels in panel **(*A*)** also apply to panel **(*B*)**.



	Saline controls (n = 12 per segment from 6 animals)	ENDO #1		ENDO #2		ENDO #3		ENDO #4		ENDO #5	
	DH combined (mean ± SD)	C DH	I DH	C DH	I DH	C DH	I DH	C DH	I DH	C DH	I DH
T13-L1	5.07 ± 0.68	4.62	6.62	5.27	8.38	8.44	6.87	4.75	5.10	8.71	7.69
L2-L3	5.35 ± 0.65	3.79	3.76	7.06	6.69	6.79	6.46	6.05	8.10	6.28	6.69
L4-L5	4.78 ± 0.83	4.44	3.54	3.87	3.58	5.11	4.98	6.36	8.70	9.27	10.84
L6-S1	5.12 ± 1.01	4.29	5.63	3.91	4.79	5.33	5.02	6.29	6.14	6.48	6.41

Increase >1 SD, <2 SD >2 SD, <3 SD >3 SD from mean of controls
Decrease >1 SD, <2 SD >2 SD, <3 SD >3 SD from mean of controls

Figure 4.4 Spatial distribution of calculated percentage area values of GFAP-immunoreactivity in the T13-S1 spinal dorsal horn of individual ENDO mice. ***(A-E)*** Schematic diagram of ENDO animals 1-5 showing the approximate location of identified endometriosis-like lesions, with representative circles depicting their size and type. Row values in the table immediately below each ENDO animal correspond to the average percentage area of GFAP-immunoreactivity per dorsal horn spanning T13-S1. Combined contralateral and ipsilateral values from saline-injected control animals (n = 6) are displayed in the far left-hand column. Green color-coding in ENDO animal cells indicates an increase in percentage area of GFAP-immunoreactivity compared to the mean control values for a particular spinal segment, graded in intensity from low change (light; <2 SD) to high change (dark; >3 SD). Orange color-coding indicates a decrease in percentage area of GFAP-immunoreactivity. No colour per cell denotes no change from the mean of control values. *C* = contralateral; *I* = ipsilateral; *DH* = dorsal horn. 'Contralateral' and 'ipsilateral' dorsal horn is used in reference to the ENDO injection site (animal's left-hand side).

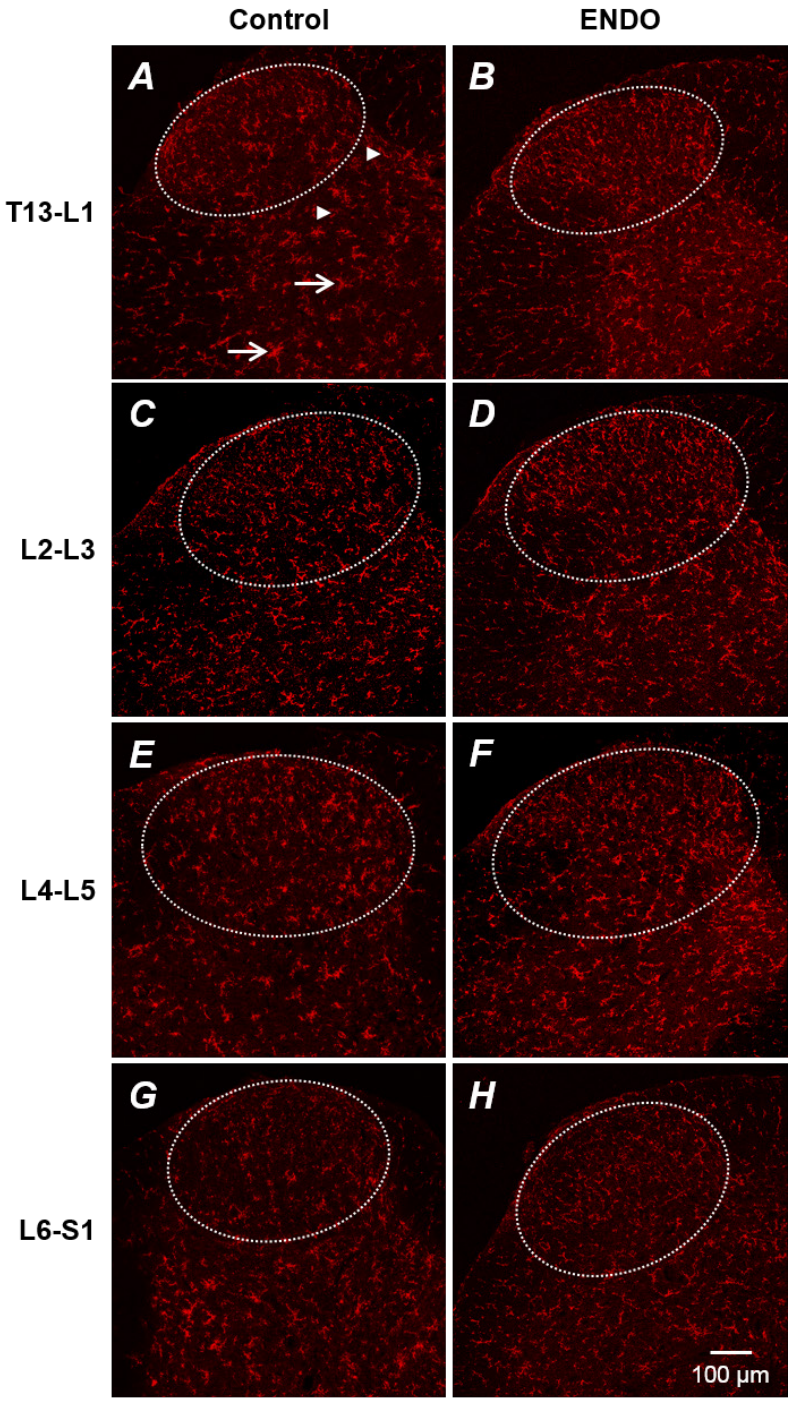


Figure 4.5 Distribution of CD11b-immunopositive microglia in the spinal dorsal horn of control and ENDO mice. (*A-B*) Representative images of the T13-L1 spinal dorsal horn (dashed ellipsoid) in control and ENDO mice, respectively, depicting a subtle increase in total area occupied by CD11b-immunoreactivity in ENDO animals compared to controls. Discrete microglial cells can be identified (arrows) as well as fragments of cell bodies and processes (arrowheads) **(*C-D*)** The percentage area of CD11b immunostaining between control and ENDO animals was generally similar across spinal levels L2-L3, **(*E-F*)** L4-L5, **(*G-H*)** and L6-S1. Magnification 20x; n = 24 per spinal level from 6 control animals; n = 20 per spinal level from 5 ENDO animals. Scale bar in (***H***) applies to all panels.

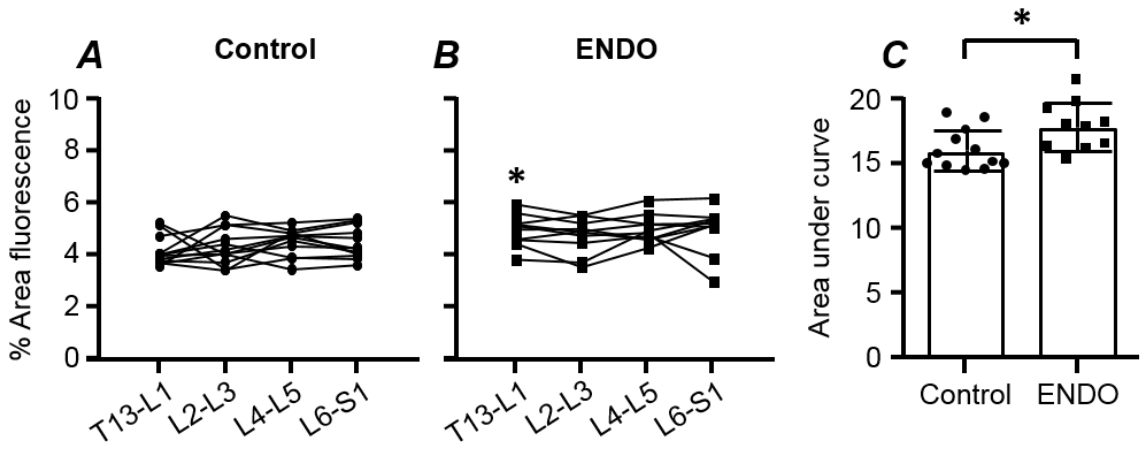


Figure 4.6 Calculated area of CD11b-immunoreactivity (microglia) in the spinal dorsal horn of control and ENDO mice. (*A*) Percentage area of each dorsal horn (linked by horizontal bars) occupied by CD11b immunostaining in saline-injected control mice was relatively consistent across spinal levels T13-S1 (n = 12 per segment from 6 animals). **(*B*)** CD11b-immunoreactivity within the T13-L1 dorsal horn in ENDO mice was increased compared to controls (n = 10 per segment from 5 animals). **(*C*)** Summarised data comparing the area under the curve values for each dorsal horn further indicate an overall mean increase in CD11b immunostaining in ENDO animals versus saline-injected controls. * denotes a significant increase in the mean CD11b-immunopositive area within the dorsal horn P <0.05. Significance notations in panel **(*B*)** refer to corresponding spinal levels in panel **(*A*)**. Y-axis labels in panel **(*A*)** also apply to panel **(*B*).**

4.5 Discussion

To our knowledge, this is the first study to describe central adaptations in astrocytic GFAP and microglial CD11b reactivity markers in mice with peripheral endometriosis-like lesions. These data support the hypothesis that endometriosis-like lesions have potential to cause adverse changes in distant locations, and thus may affect multiple biological systems. To date, the majority of studies investigating the effects of endometriosis lesions on the central nervous system (including central sensitisation) have focused exclusively on changes within neural networks and signalling mechanisms. However, the evidence here suggests that glia, the non-neuronal, immune-like cells of the nervous system, may also be modified in the presence of this condition. Intriguingly, we have also demonstrated an association between the peripheral location of endometriosis-like lesions and the spinal levels that showed robust changes in astrocyte expression.

We acknowledge that analyses in this study were limited to gross glial cell morphology, and further work examining specific markers of activation may elucidate additional population differences between groups. A further limitation of our data is the relatively small sample size of ENDO animals examined. Whilst the potential for statistical error in low-powered studies cannot be overlooked, the changes in spinal glial expression of ENDO mice are likely to be genuine, compared with control values that showed a high degree of consistency between animals and across spinal levels.

Nevertheless, in keeping with many studies describing inflammation-induced changes in spinal glial populations (Ji *et al.*, 2013; Old *et al.*, 2015; Dodds *et al.*, 2016 - Chapter 2), ENDO mice showed an overall increase in astrocytic GFAP-immunoreactivity compared to controls. Under pathological conditions, reports of dysregulated GFAP-immunoreactivity associated with molecular changes to the glutamatergic system are vast. Notably, this includes altered astrocytic protein expression of glutamate transporters,

GLAST (EAAT1) and GLT-1 (EAAT2) (Sung *et al.*, 2003; Xin *et al.*, 2009), and the metabolic enzyme, glutamine synthetase (GS) (Chen *et al.*, 2010). Increased presynaptic release of glutamate (Yan & Weng, 2013; Clark *et al.*, 2015), and greater sensitivity of the glutamate receptors, such as N-methyl-D-aspartate (NMDA) (Zhang *et al.*, 2008b; Choi *et al.*, 2010; Gruber-Schoffnegger *et al.*, 2013), have also been well-reported. As such, glutamate reuptake and breakdown are attenuated, leading to enhanced excitatory stimulation and pain (Weng *et al.*, 2006). The altered GS activity has an additional downstream impact on glutamate-glutamine cycle-dependent synthesis of GABA (Jiang *et al.*, 2012) – one of the major inhibitory neurotransmitters of the CNS.

Although a direct neuroimmune link has yet to be established, alterations in the NR1 subunit of the NMDA receptor has been shown in the brain of rats with experimental endometriosis (Torres-Reveron *et al.*, 2016), and increased levels of excitatory neurotransmitters reported in the CNS of human endometriosis patients (As-Sanie *et al.*, 2016). Vital to our hypothesis of central-to-peripheral signalling in endometriosis, spinal glutamatergic signalling is also an important contributing factor to dorsal root reflexes generated by inflammatory stimuli (Zhang *et al.*, 2000). Thus, a relationship between the altered astrocytic GFAP-immunoreactivity observed here and glutamatergic signalling in ENDO mice, may be a significant factor in the development of central sensitisation associated with endometriosis.

Whilst there was increased GFAP-immunoreactivity in the thoracolumbar spinal cord, a noteworthy decrease was also observed in the mid-lumbar region of one ENDO animal. Interestingly, the endometriosis-like lesion associated with this finding occurred on the anterior abdominal wall; the only lesion with involvement of the somatic nervous system, opposed to all others which were associated with visceral structures. Decreased spinal GFAP-immunoreactivity is uncommon in the literature regarding inflammatory pain,

although has been described as a contributing factor in models of other CNS disorders, such as stress-induced visceral hyperalgesia (Bradesi *et al.*, 2011).

Therefore, the variability in glial expression – regardless of deviations being increased or decreased from control levels – might furthermore be an alternative and important component of central sensitising processes. Statistically, we found that the percentages of spinal GFAP-immunoreactivity in ENDO mice were highly variable across spinal levels, and between animals. It is well acknowledged that glia are a product of their microenvironment, and respond accordingly to differing types and degrees of immune perturbations (Dodds *et al.*, 2016 - Chapter 2). Consequently, the observed variability is likely attributed to the heterogeneous quantities, phenotypes and locations of the endometriosis-like lesions developed by ENDO animals. The ability to determine graded changes in GFAP-immunoreactivity and lesion characteristics will therefore be a key consideration in future studies.

It was unsurprising that comparatively minor changes in spinal microglial CD11b expression occurred in ENDO animals, given that the duration of the model was relatively chronic. It has been shown that spinal microglia are often 'first-responders' to immune insults, whereas astrocytes become more active during the later stages (Tanga *et al.*, 2004; Zhang & De Koninck, 2006). Indeed, it was recently demonstrated that microglial signalling precedes, and is often required, to transform astrocytes from a resting to reactive state (Liddelow *et al.*, 2017). Thus, a more robust change in microglial CD11b expression might have been observed if tissue were examined during the acute lesion induction phase. In addition, other animal models with more severe endometriosis may see further dramatic changes in microglial reactivity, compared to our minimally-invasive model.

That we observed a greater change in astrocyte GFAP reactivity as opposed to microglial CD11b in our endometriosis model may also reflect a sex- or condition-specific

neuroimmune response. To date, the vast majority of neuroimmune studies in pain models, including those on visceral inflammation, have used male animals. This has led to debate over the type and extent of glial cell involvement in females with persistent pain conditions (Mogil & Chanda, 2005; Mogil, 2012). Although the exact mechanisms are yet to be unravelled, there are clear sex-dependent central glial responses to similar peripheral immune challenges, which lead to divergent behavioural outcomes (Stokes *et al.*, 2013; Sorge *et al.*, 2015). It was recently shown, for example, that while astrocytes are involved in neuropathic pain of both sexes, microglia are only involved in the development of male neuropathic and inflammatory pain (Chen *et al.*, 2017). Thus, our female-specific inflammatory model may be a further example of an astrocyte-dominant pain condition.

The general increase in spinal CD11b-immunoreactivity might furthermore indicate a sensitisation or priming of microglia associated with endometriosis-like lesions. This is significant, as it has been shown that first 'hits' of acute inflammation (e.g. laparotomy) can induce long-lasting changes in microglial CD11b-immunoreactivity that further potentiate pain responses to subsequent inflammatory challenges (second 'hit'; e.g. endotoxin exposure); even after resolution of the initial painful event (Hains *et al.*, 2010). A similar concept has been suggested for bladder pain syndrome (Schrepf *et al.*, 2015). We therefore speculate that our single ENDO injection could be a first 'hit' conditioning stimulus, resulting in mild sustained microglial sensitisation. To fully characterise the microglial response to endometriosis-like inflammation, prospective studies may consider analysing 'two-hit' inflammatory paradigms. This may have important implications for the surgical removal of endometriosis lesions, since patients require laparoscopic surgery for diagnosis and can undergo multiple procedures (Saraswat *et al.*, 2018). It may also contribute to a greater risk of lesion recurrence, and/or incidence of chronic postsurgical pain.

Finally, the location of endometriosis-like lesions in ENDO mice appeared to dictate the regions of spinal cord that showed the most dramatic changes in astrocytic GFAP-immunoreactivity. In most studies of both somatic and visceral inflammation, the experimental injuries are usually focal and of equal magnitude between animals, which lends to an expected region of interest and graded glial responses within the spinal cord. Such responses allow for discrete, targeted drug delivery or intervention, even with known neuroanatomical differences between the somatic and visceral sensory systems (Cervero & Laird, 1999). This becomes much more difficult to predict in conditions such as endometriosis, where lesions can form in diffuse locations, upon multiple tissue types, and with various phenotypes; despite ENDO animals being induced under identical experimental conditions. Hence, it was favoured that each animal in this study was analysed individually.

Our observation of glial changes in multiple spinal regions per ENDO animal, owing to the distribution of lesions, may be important in the pathogenesis of pelvic organ cross-sensitisation. Women with pelvic pain disorders including endometriosis frequently suffer from comorbid conditions such as pelvic floor muscle spasm, painful bladder syndrome and irritable bowel syndrome (Mirkin *et al.*, 2007). Such phenomena occur due to the convergence of neural pathways from two or more pelvic organs, where noxious sensory information from one organ can be transmitted to another via a CNS-mediated relay of neurogenic inflammation (Malykhina, 2007). This mechanism is analogous to the dorsal root reflex mentioned earlier, although impacts adjacent pelvic organs in addition to perpetuating the initial insult. Pelvic organ cross-sensitisation is associated with increased spinal NMDA receptor activity (Peng *et al.*, 2008), and has been demonstrated in rats with experimentally-induced endometriosis (Chen *et al.*, 2015; Wang *et al.*, 2015). Thus, the breadth of spinal levels affected in ENDO animals could contribute to cross-sensitisation

of other visceral organs and, in line with our central-to-peripheral hypothesis, may involve a role for inflammatory products generated by highly reactive glia (Majima *et al.*, 2018).

4.5.1 Conclusions

In summary, this is the first study to provide evidence that central glial adaptations occur in association with endometriosis lesions. Future research should now determine the cellular signalling and functional consequences of the observed changes in microglial CD11b and astrocytic GFAP expression. Where accompanying alterations to spinal proinflammatory cytokines occur, the implications for endometriosis patients may be two-fold: that glial-mediated proinflammatory activity may contribute to central sensitisation and the development of altered pain behaviour (spinal cord to brain); and/or central sensitisation that perpetuates the development of lesions via neurogenic inflammation (spinal cord to periphery). This is a fresh directive in the area of endometriosis research, which has predominantly focused on reducing the lesions themselves and manipulating only the neural aspect of pain. These hypotheses may therefore provide scope for prospective therapeutic interventions that target neuroimmune interactions in the CNS as opposed, or in adjuvant to, existing suboptimal anti-inflammatory and analgesic therapies.

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Overall percentage (%)	85%		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
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By signing the Statement of Authorship, each author certifies that:

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Chapter 5. Genetic knockout of the innate immune receptor Toll-like receptor 4 (TLR4) promotes lesion development and alters neuroimmune-associated pain in a mouse model of endometriosis

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5.1 Abstract

Toll-like receptor 4 (TLR4) is an innate immune receptor capable of initiating proinflammatory responses following detection of pathogenic and/or damage-associated stimuli. Aberrant TLR4-mediated signalling can contribute to the progression of peripheral inflammatory conditions, and the development of central sensitisation and pain. We therefore investigated the role of TLR4 in endometriosis; a chronic inflammatory condition in women associated with peripheral lesions and pain. Using a minimally-invasive model, endometriosis-like lesions (ENDO) were induced in female wildtype (WT) and *TLR4*-knockout (-/-) mice. More lesions developed in *TLR4*$^{-/-}$ ENDO mice compared to WT and in a wider range of peritoneal locations. Various lesion characteristics were altered in *TLR4*$^{-/-}$ ENDO animals, including a smaller diameter of dense-type lesions; an increased proportion of necrotic-type lesions; and heightened variability in lesion-associated cytokines. Whilst the degree of change in spinal astrocytic GFAP-immunoreactivity was similar between *TLR4*$^{-/-}$ and WT ENDO mice, spinal expression of inflammatory cytokines were altered between strains, and facial grimace scores were attenuated in *TLR4*$^{-/-}$ ENDO mice. These data suggest that adequate function of peripheral TLR4 assists with limiting the establishment of endometriosis-like lesions. However, central TLR4 activation may contribute to neuroimmune-mediated pain associated with lesion incidence. Further research into the potentially opposing roles of peripheral and central TLR4 in endometriosis may provide useful insight for treating both lesion pathology and pain

symptoms of this condition.

5.2 Introduction

Endometriosis is a chronic inflammatory condition in females where tissue similar to the uterine endometrium forms lesions on extra-uterine sites, typically within the pelvis. While pain is a common and debilitating symptom (including dysmenorrhea, dyspareunia and persistent pelvic pain), the often paradoxical relationship between lesion pathology and the severity of pain remains poorly understood. Furthermore, the mechanisms underlying the aetiology and pathogenesis of endometriosis are yet to be fully elucidated. Whilst it is widely recognised that the ovarian steroid hormones, particularly oestrogen, can modulate its development (Huhtinen *et al.*, 2012; Patel *et al.*, 2017), lesions can continue to establish in ovariectomised animals without oestrogen supplementation (Burns *et al.*, 2012). This indicates that in addition to sex hormones, the foundation and growth of endometriosis likely involves complex interactions between multiple biological systems.

One of the major emerging hypotheses for this contribution is dysregulation of the peritoneal immune response. It is widely believed that endometrial debris is effluxed via retrograde menstruation, providing endometrium-like constituents within the peritoneum that attach, proliferate and mature into established endometriosis lesions (Sampson, 1927). A reduced ability of immune cells, such as macrophages, to recognise and clear ectopic endometrial tissue from the peritoneal space, has therefore been suggested (Christodoulakos *et al.*, 2007; Herington *et al.*, 2011). Many studies have also reported an increase in immune cell secretion of proinflammatory cytokines, growth and angiogenic factors associated with endometriosis that can promote lesion establishment and growth (for review see Ahn *et al.* (2015) Izumi *et al.* (2018)).

Both the recognition and initial inflammatory response against ‘foreign’ endometrial debris

in the peritoneum requires activation of the innate immune system, and recent studies have begun to focus on a key family of pattern recognition receptors that mediate such processes, the Toll-like receptors (TLRs). Each TLR subtype (10 in humans; 13 in mice) is capable of detecting unique sets of conserved molecular epitopes expressed on pathogens and microbes, and endogenous damage-associated signals. Upon stimulation, intracellular transcription pathways are activated, culminating in the rapid production and secretion of proinflammatory mediators (such as interleukin (IL)-1β and tumour necrosis factor (TNF)-α) that assist with eliminating the immune insult. Typical TLR-mediated immune responses to infection or injury hence serve as defence mechanisms against invading pathogens or to minimise harm. However, where TLR function is inappropriate, resulting in aberrant immune activity, this can potentiate inflammatory processes that are harmful to the host (Nicotra *et al.*, 2012).

In endometriosis, particular attention has been given to the TLR4 subtype, its exogenous ligand, lipopolysaccharide (LPS; or endotoxin), and the endogenous stress-related TLR4 ligand, heat shock protein (Hsp)-70. Higher levels of endotoxin have been measured in the menstrual and peritoneal fluids in women with endometriosis compared with controls (Khan *et al.*, 2010), and TLR4 expression is increased in lesions compared to eutopic endometrial tissues (Allhorn *et al.*, 2008; Hayashi *et al.*, 2013). LPS- or Hsp-70-stimulated proliferation of endometriotic cells is enhanced compared to eutopic endometrium; an effect abrogated by treatment with antagonists of TLR4, transcription factor NFκB, TNF-α, and IL-8 (Iba *et al.*, 2004; Khan *et al.*, 2008b; Khan *et al.*, 2010; Khan *et al.*, 2013b). Moreover, peritoneal macrophages from endometriosis patients secrete greater TLR4-mediated levels of IL-6 and IL-8 in response to LPS or Hsp-70 than lesion-free counterparts (Khan *et al.*, 2008b; Khan *et al.*, 2013b). In mice, repeated intraperitoneal injections of LPS has also been shown to induce an NFκB-dependent increase in the number of established

endometriosis-like lesions (Azuma *et al.*, 2017). While still preliminary, it is evident that TLR4-mediated inflammatory cytokine signalling may be a promising mechanistic target in the development of endometriosis.

In addition to the traditional immunological role of TLR4, it is well acknowledged that its inflammatory signalling molecules can act as neuromodulators; capable of modifying the electrophysical properties of neurons, and subsequent neurotransmission ('neuroimmune communication') (Rostene *et al.*, 2007; Dodds *et al.*, 2016 - Chapter 2). In the spinal cord, TLR4 is expressed on the surface of glia (astrocytes and microglia), which are resident immune-like cells of the central nervous system (CNS), primarily responsible for maintaining neuronal homeostasis. Strong or prolonged exposure to TLR4-associated immune mediators, released by highly reactive glia, can stimulate transcriptional and epigenetic processes in adjacent neurons, which alters their expression of neurotransmitters and receptors. Such plastic changes can reinforce or diminish synaptic activity, and ultimately manifests as central sensitisation. Under certain circumstances this modifies behaviour, including pain perception observed (clinically and experimentally) as hyperalgesia (heightened pain to a previously noxious stimulus) and/or allodynia (pain caused by a normally innocuous stimulus) (Dodds *et al.*, 2016 - Chapter 2).

Evidence for glial TLR4-mediated central sensitisation has been presented extensively in various models of neuropathic pain (Tanga *et al.*, 2005; Hutchinson *et al.*, 2008; Sun *et al.*, 2015); cancer- (Liu *et al.*, 2010; Mao-Ying *et al.*, 2012) and chemotherapy-induced pain (Yan *et al.*, 2015b; Wardill *et al.*, 2016); inflammatory pain (Agalave *et al.*, 2014; Su *et al.*, 2018); opioid-induced hyperalgesia (Hutchinson *et al.*, 2010b; Bai *et al.*, 2014); and visceral pain (Tramullas *et al.*, 2014; Yuan *et al.*, 2015). Rodent models of endometriosis also display hyperalgesic behaviour (McAllister *et al.*, 2012; Greaves *et al.*, 2017b; Hernandez *et al.*, 2017), and we have recently demonstrated that a marker for astrocyte

reactivity (glial fibrillary-associated protein; GFAP) is increased in expression and highly variable in the thoracolumbar spinal cord of C57BL/6 mice with endometriosis-like lesions (Dodds *et al.*, 2018 - Chapter 4). A subtle increase in the microglial marker, CD11b, was also observed. However, it remains to be established whether TLR4 contributes to this altered spinal glial reactivity, and therefore neuroimmune-mediated pain associated with lesions in endometriosis.

This study aimed to further characterise a role for TLR4 in the incidence, phenotype and inflammatory profile of endometriosis-like lesions, using wildtype and *TLR4*-knockout (-/-) mutant mice using a minimally-invasive model. In addition, we investigated whether TLR4 contributes to neuroimmune adaptations and pain attributed to endometriosis, by examining spinal glial expression, associated inflammatory cytokine expression, and pain behaviour in this model.

5.3 Methods

5.3.1 Animals

Female BALB/c-wildtype (WT; $n = 48$) and BALB/c-*TLR4*$^{-/-}$ mice (*TLR4*$^{-/-}$; $n = 48$) 8-13 weeks in age, weighing 19.4 ± 1.7 g, were obtained from the University of Adelaide Laboratory Animal Services. Breeding pairs of *TLR4*$^{-/-}$ mice, which had been back-crossed onto a BALB/c background strain for more than 10 generations, were kindly obtained from Professor Paul Foster (University of Newcastle; New South Wales, Australia) and were originally sourced from Professor Shizuo Akira (Osaka University; Osaka, Japan) (Hoshino *et al.*, 1999). All animals underwent daily cervical smear testing to determine their oestrous cycle phase, as described previously (Dodds *et al.*, 2015). Each was selected for use only when in the pro-oestrus phase of the oestrous cycle (high oestrogen; pre-ovulation), as pro-oestrus conditions at the time of induction have been shown to support

robust and consistent endometriosis-like lesions in gonad-intact mice (Dodds *et al.*, 2017 - Chapter 3). All animal use was conducted in accordance with the National Health and Medical Research Council Australian code for the care and use of animals for scientific purposes (8th edition, 2013) and the University of Adelaide Animal Ethics Guidelines, and was approved by the University of Adelaide Animal Ethics Committee.

5.3.2 Minimally-invasive mouse model of endometriosis

5.3.2.1 Model induction

The method for inducing endometriosis in the mouse for this study was designed and validated by our group previously (Dodds *et al.*, 2017 - Chapter 3). Donor animals (n = 18 per strain) were sacrificed by cervical dislocation whilst under deep inhaled isoflurane anaesthesia. The uterus was removed and placed into a sterilised glass Petri dish containing cold (4°C) 0.01 M phosphate-buffered saline (PBS; composed of 13.7 mM NaCl; 0.27 mM KCl; 0.15 mM KH_2PO_4; and 0.8 mM Na_2HPO_4; pH 7.4). Each horn was opened along the mesometrial border and pinned flat to the Sylgard®-lined (Dow Corning; Michigan, USA) base of the Petri dish using entomology pins. The endometrium (40 mg) was then carefully removed by sharp dissection and cut into segments of 2-3 mm^2. Endometrial segments were then aspirated with 0.5 ml sterile saline (0.9% NaCl) into a 1-ml syringe attached to a 21-gauge needle. To ensure smooth delivery of the donor endometrium, the fragments were plunged through the needle once and re-aspirated, before being intraperitoneally injected into a recipient mouse (ENDO; n = 18 per strain) (1 donor: 1 recipient) at the ventral midline between the left inguinal nipples.

In total, four experimental groups of ENDO animals were generated: WT donor to WT recipient (WT ENDO; n = 12); WT donor to *TLR4*$^{-/-}$ recipient (WT>*TLR4*$^{-/-}$ ENDO; n = 6); *TLR4*$^{-/-}$ donor to WT recipient (*TLR4*$^{-/-}$>WT ENDO; n = 6); and *TLR4*$^{-/-}$ donor to *TLR4*$^{-/-}$

recipient (*TLR4*$^{-/-}$ ENDO; *n* = 12). Control animals were injected with an equal volume of sterile saline alone (*n* = 12 per strain).

Following 21 days of development, all animals were deeply anaesthetised with isoflurane gas and decapitated. WT ENDO mice, *TLR4*$^{-/-}$ ENDO mice, and their respective controls were then randomly assigned to one of two groups for tissue processing: the first cohort of animals had tissues fixed for further histological analyses (*n* = 6 per group); whereas the second had tissues fresh frozen for immunoassay (n = 6 per group). Tissues from WT-*TLR4*$^{-/-}$ ENDO cross-injected mice were fixed only for histological assessment of lesions.

5.3.2.2 Assessment of endometriosis-like lesion characteristics

After thorough examination of the peritoneal cavity in all ENDO mice, the number and location of potential endometriosis-like lesions were recorded. Lesions and control tissues (lymph nodes, fat etc.) retrieved from the first cohort of animals were immersed in cold (4°C) 10% neutral-buffered formalin (Chem-Supply; South Australia, Australia) overnight (4°C). All tissues then underwent standard histological processing, sectioning and staining for hematoxylin and eosin to confirm the 'diagnosis' of endometriosis-like lesions, in which endometrial glands and stroma were positively identified (Clement, 2007). The size and classification of confirmed lesions (cystic, dense or necrotic) was subsequently determined, as described previously (Dodds *et al.*, 2017 - Chapter 3).

Endometriosis-like lesions from animals in the second experimental cohort were pinned to the Sylgard®-coated base of a Petri dish containing cold PBS, and photographed with a digital camera (iSight; Apple Inc.; California, USA) mounted on the eyepiece of a stereomicroscope (#SMZ445; Nikon; Tokyo, Japan). Lesions were then measured from the images using ImageJ Fiji software (Schindelin *et al.*, 2012), and classified based on their macroscopic appearances (Dodds *et al.*, 2017 - Chapter 3).

Due to their variable shape, the size of endometriosis-like lesions from both cohorts of animals was defined in this study as the maximum diameter of their greatest longitudinal surface. Only lesions classified as dense- or cystic-type were counted toward the total number of viable lesions obtained from an ENDO animal. Necrotic-type lesions, except where specified, were excluded from analysis due to inconsistencies in meeting the diagnostic criteria (Dodds *et al.*, 2017 - Chapter 3).

5.3.3 Fluorescent immunohistochemistry for visualisation of glial markers

5.3.3.1 Tissue processing

In the first cohort of WT saline, WT ENDO, *TLR4*$^{-/-}$ saline and *TLR4*$^{-/-}$ ENDO animals (*n* = 6 per group), spinal cords (spanning thoracic to sacral, inclusive) were carefully removed and immersed in cold (4°C) 4% paraformaldehyde fixative (pH 7.2) overnight. Tissues were then washed (4 x 15 min; PBS) and cryoprotected in 30% sucrose at 4°C for two nights. Following dissection into regions T13-L1, L2-3, L4-5 and L6-S1, spinal cord segments were submerged into individual plastic moulds containing Tissue-Tek® OCT compound (#IA018; ProSciTech; Queensland, Australia) and frozen by being placed into isopentane cooled with liquid nitrogen. All blocks were then insulated and stored at -80°C.

Spinal segments were sectioned in duplicate (per antibody label) at 10 µm using a Leica CM1850 cryostat (Leica Biosystems; Nusslock, Germany) at -15 ± 0.5°C, and collected onto SuperFrost® glass microscope slides (Menzel-Gläser; Braunschweig, Germany). Each section was taken at least 50 µm apart to prevent cell overlap during analysis. After air-drying for 30 min, slides were rinsed with PBS to remove residual OCT before undergoing heat-mediated antigen retrieval using sodium citrate buffer (0.01 M with 0.05% Tween 20; pH 6.0). Retrieval buffer was preheated to 65°C using the PT Link™ system (#PT101; Dako; Glostrup, Denmark). Slides were submerged in the buffer and the

temperature raised to 97°C for 10 min. After returning to 65°C, slides were removed and cooled with 0.01 % Tween 20 at room temperature (RT).

Sections were then blocked for 1 h at RT in a humid chamber with 10% normal donkey serum/0.01% Triton X-100. To visualise astrocytes, sections were incubated in Alexa Fluor® 488-conjugated mouse monoclonal anti-GFAP antibody (#53-9892-82, clone GA5; RRID: AB_10598515; 1 µg/ml; eBioscience; California, USA) for two nights at 4°C in a dark, humid chamber. For microglial assessment, slides were incubated in rabbit polyclonal anti-ionised calcium-binding adaptor molecule 1 (Iba-1) (#019-19741; RRID: AB_839504; 0.5 µg/ml; WAKO, Osaka, Japan) for two nights at 4°C. After washing (4 x 10 min; PBS), sections were then incubated with donkey anti-rabbit Alexa Fluor® 488 secondary antibody (#ab150073; RRID: AB_2636877; 2 µg/ml; Abcam, Cambridge, UK) for 2 h at RT in a dark, humid chamber. All sections were given a final rinse (4 x 10 min; PBS) and mounted with Tris-based Fluoro-Gel medium (#IM030; ProSciTech; Queensland, Australia). Control samples were prepared by omitting either primary or secondary antibodies from the incubation solutions (data not shown).

5.3.3.2 Image acquisition

Slides were viewed with a Leica TCS SP5 scanning confocal microscope (Leica Microsystems; Wetzlar, Germany) using appropriate excitation wavelengths at 20x magnification with oil immersion. Images were acquired using Leica Application Suite Advanced Fluorescence version 2.6.3 (Leica Microsystems; Mannheim, Germany). Final images are digital composites of 1 µm Z-series scans (approximately 10-16 optical sections through a depth of 13-17 µm). All images per antibody label were taken at the same gain and offset parameters between animals. Each spinal dorsal horn per section was imaged separately.

5.3.3.3 Image analysis

Semiquantitative analyses were performed on collected images using ImageJ Fiji software (Schindelin *et al.*, 2012). Prior to analysis, the maximised Z-stack of images were converted from Leica image files (.lif) to 8-bit greyscale .tiff images. Signal pixels of positive staining areas in the region of interest (ROI) were selected using the *Threshold* function, and the percentage of the ROI occupied by immunofluorescence was calculated. The ROI was an ellipsoid shape that remained a consistent size for each spinal level between animals, and was placed over Rexed's laminae I-IV (Dodds *et al.*, 2018 - Chapter 4). Duplicate area measurements for each dorsal horn were then averaged to obtain a single percentage area value per dorsal horn, per spinal level, per animal. All images were measured blinded as to mouse strain and treatment.

5.3.4 Multiplex enzyme-linked immunosorbent assay (ELISA) for cytokine protein quantification

5.3.4.1 Tissue processing

Proinflammatory cytokine expression was assessed in spinal cord segments T13-L1, L2-L3, L4-L5 and L6-S1 from the second cohort of WT saline, WT ENDO, *TLR4*$^{-/-}$ saline and *TLR4*$^{-/-}$ ENDO animals (n = 6 per group). For statistical analyses, cytokine concentrations from each spinal level were combined for each animal. Endometriosis-like lesions (pooled per animal) from the WT and *TLR4*$^{-/-}$ ENDO mice were also examined. Each sample was weighed and snap frozen with liquid nitrogen, then submerged in a 10 µl/mg.tissue^{-1} volume of radio-immunoprecipitation assay buffer (RIPA; composed of 50 mM HEPES; 150 mM NaCl; 12 mM sodium deoxycholate; 10 mM NaF; 10 mM $Na_4P_2O_7$; 5 mM EDTA; 1% Triton X-100; 0.1% SDS; and 5% ethylene glycol) supplemented with 1% protease inhibitor cocktail (#P8340 Sigma Aldrich; NSW, Australia). Samples were homogenised

in the buffer for 30 s at RT using a pellet pestle attached to its motor (components #Z359963 and #Z359971; Sigma Aldrich; New South Wales, Australia), then centrifuged at 15,000 RPM for 30 min at 4°C. The resulting supernatant was isolated, aliquoted and stored at -80°C. Total protein concentration was quantified using the Pierce™ BCA Protein Assay Kit (#23225; ThermoFisher Scientific; Victoria, Australia) as per the manufacturer's instructions. A working concentration of 2 mg/ml for all samples was used for cytokine analysis.

5.3.4.2 Cytokine quantification

Cytokine concentrations (pg/mL) were measured using MILLIPLEX® MAP Mouse High Sensitivity T-cell Magnetic Bead Panel kit (#MHSTCMAG-70K; Merck Millipore; Darmstadt, Germany), prepared as per manufacturer's instructions. The cytokines analysed were: IL-1β, IL-2, IL-6, IL-10, IL-17A, interferon (IFN)-γ, TNF-α and granulocyte-macrophage colony stimulating factor (GM-CSF). Each 96-well plate included an 8-point standard curve and two quality controls provided by Merck Millipore. All standards, quality controls and samples were loaded onto plates in duplicates. Plates were read using a MAGPIX® Luminex xMAP® platform, and experimental data calibrated against the standard curves of each corresponding cytokine using a cubic spline algorithm calculated by MILLIPLEX® Analyst 5.1 software (Merck Millipore; Darmstadt, Germany).

5.3.4 Facial grimace scoring for assessment of pain behaviour

Pain behaviour was measured in a blinded manner throughout the 21-day development period for all mice in the second experimental cohort. Once daily (between 09:00-10:00 am) from 24 h post-injection, animals were individually weighed and 2-3 images of the face photographed using a digital camera (iSight; Apple Inc.; California, USA). Images were then de-identified, and scores determined using a validated mouse grimace scale

designed to measure spontaneously emitted pain (Langford *et al.*, 2010). Briefly, the scoring method consisted of five distinct criteria: orbital tightening, nose bulge, cheek bulge, ear position and whisker position. Each criterion was scored as 0 = absent, 1 = moderate, and 2 = severe. Total daily scores for each animal were then grouped per week of the experimental time-course.

5.3.5 Statistical analysis

All data were statistically analysed using GraphPad Prism® 7 software (GraphPad Software Inc.; California, USA). A D'Agostino-Pearson omnibus K2 test was initially performed to assess normality. Total number, and proportions of cystic-and dense-type lesions between WT and *TLR4*$^{-/-}$ ENDO mice, were assessed using a one-way ANOVA with Tukey multiple comparisons. Differences in the number of necrotic-type lesions, as well as cytokine concentrations within endometriosis-like lesions retrieved from WT and *TLR4*$^{-/-}$ ENDO mice, were assessed by a two-tailed Mann-Whitney test. A two-way ANOVA with appropriate post-hoc tests were used to determine differences in facial pain scores and spinal glial GFAP and Iba-1 immunolabelling values between WT and *TLR4*$^{-/-}$, saline control and ENDO animals. Area under the curve (AUC) scores for GFAP and Iba-1 were also subsequently generated for each dorsal horn per animal, and compared between control and ENDO mice using a Student unpaired two-tailed *t*-test with Welch correction. For spinal cytokine concentrations, a one-way ANOVA with Bonferroni post-hoc correction or a Kruskal-Wallis test with Dunn multiple comparisons was performed to identify statistical significance between groups. Microsoft® Excel® 2013 (Microsoft Corporation; Washington, USA) was additionally used to generate F-test scores of variability around the standard deviation (SD) for glial immunolabelling values and cytokine concentrations. Unless otherwise specified, data in the text are expressed as mean ± SD, and *P* values of <0.05 were considered statistically significant.

5.4 Results

5.4.1 TLR4$^{-/-}$ ENDO mice develop a greater total number of endometriosis-like lesions

Endometriosis-like lesions successfully established in all ENDO mice. WT ENDO animals developed 1-3 lesions (n = 26 lesions from 12 animals; average 2.2 ± 0.7), with a mass of injected endometrial tissue 42.3 ± 1.7 mg in 29.4 ± 3.1 pieces. In contrast, a significantly greater number of endometriosis-like lesions were retrieved from *TLR4*$^{-/-}$ ENDO mice (P <0.0001), with 2-7 lesions (n = 63 lesions from 12 animals; average 5.3 ± 1.8) developed from 40.6 ± 2.9 mg donor tissue in 26.8 ± 2.5 pieces. The WT and *TLR4*$^{-/-}$ cross-injected ENDO animals both developed an intermediary total number of lesions, where 3.3 ± 0.8 lesions were retrieved from WT>*TLR4*$^{-/-}$ ENDO mice (P = 0.03 vs. *TLR4*$^{-/-}$ ENDO) and 4.5 ±1.8 lesions from *TLR4*$^{-/-}$>WT ENDO mice (P = 0.008 vs. WT ENDO) (Fig. 5.1).

5.4.2 Endometriosis-like lesions from TLR4$^{-/-}$ ENDO mice develop in a wider variety of peritoneal locations

The locations and structures on which endometriosis-like lesions established in WT ENDO mice were similar to those reported for this model previously (Dodds *et al.*, 2017 - Chapter 3, 2018 - Chapter 4). The majority of lesions (17/26; 65.4%) in WT ENDO mice attached to connective tissues associated with the stomach and pancreas; followed by the gonadal white adipose tissue (6/26 lesions; 23.1%); and the abdominal wall (3/26 lesions; 11.5%) (Fig. 5.2A). Most endometriosis-like lesions from *TLR4*$^{-/-}$ ENDO mice were also observed in these locations, with 23/63 lesions (36.5%) associated with gastric/pancreatic connective tissue, 16/63 lesions (25.4%) in gonadal white adipose tissue, and 5/63 (7.9%) on the abdominal wall. The remainder of endometriosis-like lesions from *TLR4*$^{-/-}$ ENDO mice were retrieved from additional peritoneal locations, some of which included attachments

to visceral organs. For instance, 3/63 lesions (4.8%) established on the uterine surface or associated mesometrium; a further 3/63 lesions (4.8%) were found on the liver; and 1/63 lesions (1.6%) developed on the surface of the distal colon. Other lesions were associated with connective tissue attachments, such as those bridging the rectum and uterus (7/63; 11.1%), and the mesentery of the small intestine (4/63 lesions; 6.3%). One lesion (1.6%) was also located within the subcutaneous space (Fig. 5.2B).

5.4.3 Phenotypic profiles of endometriosis-like lesions are altered in TLR4$^{-/-}$ ENDO mice

In both mouse strains, cystic-type lesions were the dominant phenotype developed compared with dense-type lesions ($P \leq 0.0001$). The proportions of cystic- and dense-type lesions were comparable between WT and *TLR4*$^{-/-}$ ENDO animals. Cystic-type lesions accounted for 83.3 ± 25.6% (21/26 lesions) in WT ENDO mice and 74.5 ± 24.8% (43/63 lesions) in *TLR4*$^{-/-}$ ENDO mice ($P = 0.83$). Dense-type lesions accounted for 16.66 ± 25.6% (5/26 lesions) in WT ENDO mice and 25.5 ± 24.8% (20/63 lesions) in *TLR4*$^{-/-}$ ENDO mice ($P = 0.83$) (Fig. 5.3A). Although not considered to be a viable endometriosis-like lesion phenotype in our model, the number of necrotic-type lesions was also noted. While WT ENDO mice had 0-2 necrotic lesions (average 0.2 ± 0.6), *TLR4*$^{-/-}$ ENDO animals developed a significantly greater number of between 0-8 (average 2.2 ± 2.9 necrotic lesions; $P = 0.006$) (Fig. 5.3B).

The average diameter of cystic-type lesions from *TLR4*$^{-/-}$ ENDO mice was 1.9 ± 0.9 mm, which was similar in size to those from WT ENDO mice (1.8 ± 0.8 mm; $P = 0.78$) (Fig. 5.3C). However, the mean diameter of dense-type lesions from *TLR4*$^{-/-}$ mice (0.7 ± 0.3 mm) was significantly smaller than those from WT ENDO counterparts (1.3 ± 0.4 mm; $P = 0.0003$) (Fig. 5.3D). The average diameter of necrotic-type lesions was not measured.

5.4.4 Inflammatory cytokine profiles of endometriosis-like lesions differ between WT and TLR4$^{-/-}$ ENDO mice

Detectable concentrations were found for all cytokine targets in pooled endometriosis-like lesions from WT and *TLR4*$^{-/-}$ ENDO mice. To assist with readability, all data values are specified in Table 5.1. The expression of IL-6 in lesions from *TLR4*$^{-/-}$ ENDO mice was increased in variability ($P = 0.004$) and total concentration compared to WT ENDO mice ($P = 0.008$). Heightened variability in GM-CSF was also observed in *TLR4*$^{-/-}$ ENDO mice compared to WT ENDO ($P = 0.01$), as well as for IL-2 ($P = 0.006$) and IL-10 ($P = 0.01$); although the mean values of these cytokines were not statistically different between groups (all comparisons $P \geq 0.1$). No differences in mean values (all comparisons $P \geq 0.2$) or variability (all comparisons $P \geq 0.1$) were observed between WT and *TLR4*$^{-/-}$ endometriosis-like lesions for IFN-γ, IL-1β, IL-17A or TNF-α (Fig. 5.4).

5.4.5 ENDO animals show heightened variability in spinal astrocytic GFAP-immunoreactivity compared to controls in both WT and TLR4$^{-/-}$ mice

In WT control animals, the area of GFAP-immunoreactivity in the spinal dorsal horn was reasonably consistent across spinal levels, and between animals. The average area occupied by GFAP-immunopositive structures in T13-L1 was 5.4 ± 1.0%; L2-L3 5.4 ± 0.9%; L4-L5 4.8 ± 0.8%; and L6-S1 5.2 ± 0.8% (Fig. 5.5A, C). In contrast, astrocytic GFAP expression in WT ENDO mice was highly variable. On average, the area of GFAP-immunoreactivity in T13-L1 was 5.2 ± 1.3%; L2-L3 6.1 ± 1.6%; L4-L5 5.9 ± 1.8%; and L6-S1 5.0 ± 0.6% (Fig. 5.5B, D). Statistically, the variability between ENDO and control animals was increased for L2-L3 ($P = 0.04$) and L4-L5 ($P = 0.01$), but not T13-L1 ($P = 0.4$) or L6-S1 ($P = 0.2$) (Fig. 5.5C-D). Whilst there was no overall 'treatment' effect observed for WT ENDO ($P = 0.3$), a comparison of the summarised AUC values reiterated that GFAP-immunoreactivity was highly variable in WT ENDO animals (average AUC 17.2 ± 4.1)

compared to controls (15.6 ± 1.8; $P = 0.01$) (Fig. 5.5G).

The area of spinal GFAP-immunoreactivity was similarly consistent in *TLR4*$^{-/-}$ control mice. GFAP-immunopositive structures in T13-L1 occupied an average area of 5.4 ± 0.8%, L2-L3 4.5 ± 0.8%, L4-L5 5.2 ± 0.5% and L6-S1 5.1 ± 0.5% (Fig 5.5E). For *TLR4*$^{-/-}$ ENDO mice, the area of GFAP-immunoreactivity was 5.7 ± 1.6% in T13-L1; 5.8 ± 1.2 % for L2-L3; 6.1 ± 1.2% in L4-L5; and 5.8 ± 0.9% for L6-S1. The variability of GFAP-immunoreactivity in *TLR4*$^{-/-}$ ENDO mice was significantly increased compared to *TLR4*$^{-/-}$ control animals for T13-L1 ($P = 0.03$) and L4-L5 ($P = 0.002$), but not L2-L3 ($P = 0.2$) or L6-S1 ($P = 0.06$). An overall 'treatment' effect was additionally observed in *TLR4*$^{-/-}$ ENDO animals versus controls ($P = 0.02$), with the most significant increase in GFAP-immunoreactivity at the level of L2-L3 ($P = 0.01$) (all other levels $P \geq 0.1$) (Fig. 5.5F). Summarised AUC values further demonstrated that spinal GFAP expression was increased in *TLR4*$^{-/-}$ ENDO animals (average AUC 17.6 ± 3.2) compared to *TLR4*$^{-/-}$ saline controls (15.0 ± 1.1; $P = 0.02$) and highly variable ($P = 0.001$) (Fig. 5.5G).

Area of GFAP-immunoreactivity in *TLR4*$^{-/-}$ control mice did not significantly differ by mean ($P = 0.4$) or variability (all comparisons $P \geq 0.05$) from WT controls. Likewise, spinal GFAP-immunoreactivity in *TLR4*$^{-/-}$ ENDO mice was similar to WT ENDO (mean $P = 0.6$; all variability comparisons $P \geq 0.2$).

5.4.6 Spinal expression of microglial Iba-1 is unchanged between control and ENDO animals of both WT and TLR4$^{-/-}$ mouse strains

For both WT and *TLR4*$^{-/-}$ control animals, Iba-1-immunoreactivity in the spinal dorsal horn was generally consistent. The average area of Iba-1-positive structures from WT control mice in T13-L1 was 4.3 ± 0.7%; L2-L3 4.0 ± 0.5%; L4-L5 4.1 ± 0.7%; and L6-S1 3.8 ± 0.7% (Fig. 5.6A, C). In WT ENDO animals, the area of Iba-1-immunoreactivity was 4.2 ±

0.7% in T13-L1; 4.1 ± 0.6% for L2-L3; 4.4 ± 0.4% in L4-L5; and 4.3 ± 0.5% for L6-S1 (Fig. 5.6B, D). No differences were detected between WT control and ENDO regarding a 'treatment' effect ($P = 0.2$) or variability in Iba-1 expression (all comparisons $P \geq 0.1$) (Fig. 5.6G).

Iba-1-immunopositive structures in T13-L1 from *TLR4*$^{-/-}$ control animals was 4.1 ± 0.7%, L2-L3 3.8 ± 0.5%, L4-L5 4.0 ± 0.4% and L6-S1 4.0 ± 0.6% (Fig 5.6E). In *TLR4*$^{-/-}$ ENDO mice ($n = 6$), the area of Iba-1-immunoreactivity was 4.1 ± 0.7% in T13-L1; 3.9 ± 0.6% for L2-L3; 3.8 ± 0.5% in L4-L5; and 4.2 ± 0.4% for L6-S1 (Fig. 5.6F). Similar to WT animals, no 'treatment' effect was detected between the *TLR4*$^{-/-}$ groups ($P = 0.7$), nor any changes in the variability of Iba-1-immunoreactivity at any spinal level (all comparisons $P \geq 0.2$) (Fig. 5.6G).

Values of Iba-1-immunoreactivity in *TLR4*$^{-/-}$ control mice did also not significantly differ by mean ($P = 0.6$) or variability (all comparisons $P \geq 0.05$) from WT controls; and the area of spinal GFAP-immunoreactivity in *TLR4*$^{-/-}$ ENDO mice was similar to WT ENDO (mean $P = 0.2$; all variability comparisons $P \geq 0.3$).

5.4.7 Spinal inflammatory cytokine profiles differ between WT and TLR4$^{-/-}$ *ENDO mice*

Detectable concentrations were found for all cytokine targets in spinal cords from WT and *TLR4*$^{-/-}$ control and ENDO mice. To assist with readability, all data values are specified in Table 5.2. No statistical differences were detected for any cytokines between WT and *TLR4*$^{-/-}$ control animals (all comparisons $P \geq 0.2$). In addition, no changes were observed in the mean values (all comparisons $P \geq 0.1$) or variability (all comparisons $P \geq 0.2$) of IL-6 or IL-10 between any groups.

In WT ENDO animals, spinal GM-CSF expression was elevated compared to WT controls

(P = 0.0007) and *TLR4*$^{-/-}$ ENDO mice (P = 0.005). Levels between *TLR4*$^{-/-}$ controls and *TLR4*$^{-/-}$ ENDO mice were unchanged (P >0.9). TNF-α expression was also increased in WT ENDO mice compared with WT controls (P = 0.006), but levels in *TLR4*$^{-/-}$ control and *TLR4*$^{-/-}$ ENDO animals were similar (P >0.9) and no changes were observed between ENDO strains (P = 0.07). Spinal IL-2 was reduced in WT ENDO mice compared with WT controls (P = 0.04), and unchanged between *TLR4*$^{-/-}$ animals (P = 0.4) and ENDO strains (P = 0.1). In *TLR4*$^{-/-}$ ENDO animals, IFN-γ was significantly increased from *TLR4*$^{-/-}$ controls (P = 0.001), although no differences were detected between WT animals (P >0.9) or between ENDO strains (P = 0.1). IL-17A expression in *TLR4*$^{-/-}$ ENDO animals were reduced compared to *TLR4*$^{-/-}$ controls (P = 0.04), and unchanged between WT groups and ENDO strains (all comparisons P >0.9) (Fig. 5.7; Table 5.2A).

While no changes were detected in mean values of IL-1β between groups (all comparisons $P \geq 0.1$), there was a decrease in variability between *TLR4*$^{-/-}$ control and *TLR4*$^{-/-}$ ENDO mice (P = 0.04) (all other variability comparisons P >0.2). Variability was increased for GM-CSF between WT control and WT ENDO mice (P <0.0001) as well as WT ENDO and *TLR4*$^{-/-}$ ENDO mice (P = 0.0006) (all other comparisons P >0.3). IL-2 decreased in variability between WT control and WT ENDO mice (P = 0.006) and WT ENDO compared to *TLR4*$^{-/-}$ ENDO mice (P = 0.002) (all other comparisons P >0.5). IFN-γ values were increased in variability between WT control and WT ENDO mice (P = 0.02) as well as *TLR4*$^{-/-}$ control and *TLR4*$^{-/-}$ ENDO mice (P = 0.02) (all other comparisons P >0.7). No changes in variability were observed for TNF-α (all comparisons P >0.1) or IL-17A (all comparisons P >0.4) (Fig. 5.7; Table 5.2B).

5.4.8 Facial pain expression is attenuated in TLR4$^{-/-}$ ENDO mice compared to WT ENDO controls

Facial grimace scores were maintained for all experimental groups throughout the three-

week experimental time course. In both mouse strains, the most common facial grimace features observed for ENDO animals were orbital tightening, and changes in ear and whisker positions. For WT ENDO mice, the median pain score was 1 (range 0-3), which was significantly elevated compared to WT controls (median 0; range 0-1; P <0.0001) (Fig. 5.8A-B). *TLR4*$^{-/-}$ ENDO mice also showed an increase in spontaneous pain behaviour compared to *TLR4*$^{-/-}$ controls, with a median score of 1 (range 0-2) versus 0 (range 0-1; P = 0.001), respectively (Fig 5.8C-D). While no differences in pain criteria were detected between WT and *TLR4*$^{-/-}$ control animals (P = 0.9), scores from *TLR4*$^{-/-}$ ENDO mice were reduced compared to those of WT ENDO mice (P = 0.001) (Fig. 5.8B, D).

5.4.9 Figures and tables

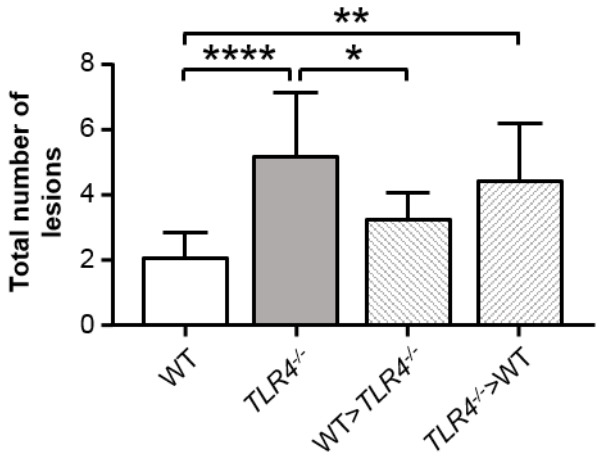

Figure 5.1 Total number of endometriosis-like lesions established in WT, *TLR4*$^{-/-}$, and WT-*TLR4*$^{-/-}$ cross-injected ENDO mice. Summarised data shows a significantly greater number of endometriosis-like lesions retrieved from *TLR4*$^{-/-}$ ENDO mice compared with WT ENDO mice (n = 12 animals per group). Cross-injected ENDO animals (WT>*TLR4*$^{-/-}$ or *TLR4*$^{-/-}$>WT; n = 6 animals per group) developed an intermediate total number of lesions, more than WT alone but less than those with complete *TLR4*-knockout. * denotes P <0.05; ** P <0.01; **** P <0.0001. Data expressed as mean ± SD.

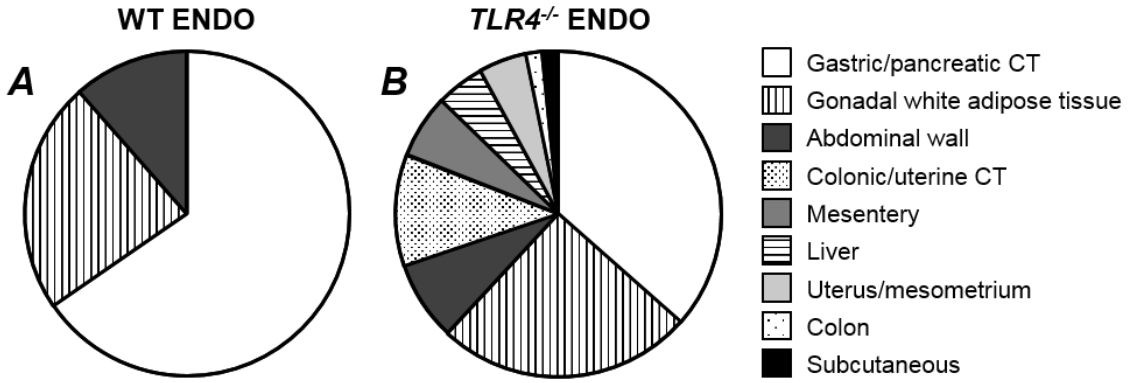

Figure 5.2 Diversity of anatomical locations of endometriosis-like lesions established in WT and *TLR4*$^{-/-}$ ENDO mice. (*A*) Summarised data of the proportions of endometriosis-like lesions found in each peritoneal location of WT ENDO mice (*n* = 12 animals) show that lesions were typically found attached to connective tissues near the stomach/pancreas, within the gonadal white adipose tissue, or on the surface of the anterior abdominal wall. **(*B*)** In contrast, *TLR4*$^{-/-}$ ENDO mice (*n* = 12 animals) developed endometriosis-like lesions in a much greater range of locations, including on the surface of the liver, colon and uterus, as well as attached to their associated connective tissues, and within the mesentery. *CT* = connective tissue.

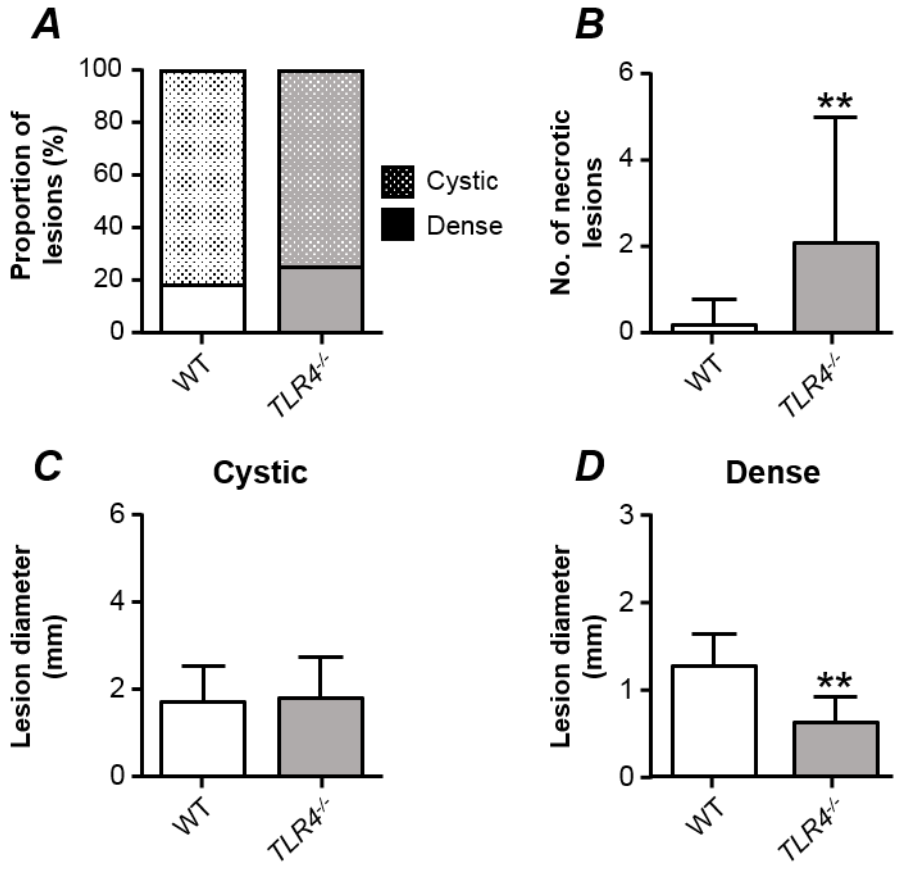

Figure 5.3 Endometriosis-like lesion phenotype profiles in WT and *TLR4*$^{-/-}$ ENDO mice. (*A*) Proportions of cystic and dense lesion types were similar between WT and *TLR4*$^{-/-}$ ENDO mice (n = 12 animals per group). **(*B*)** Although not considered to be viable endometriosis-like lesions, the number of retrieved necrotic-type tissues was significantly greater in *TLR4*$^{-/-}$ ENDO animals compared to WT. **(*C*)** The average size of cystic-type lesions from *TLR4*$^{-/-}$ ENDO mice was not significantly different from those developed by WT ENDO mice. **(*D*)** However, the size of dense-type lesions were significantly reduced in *TLR4*$^{-/-}$ ENDO animals compared to WT. ** denotes P <0.01. Data in ***(B-D)*** expressed as mean ± SD.

Endometriosis-like lesions		
Cytokine (pg/mL)	WT ENDO (mean ± SD)	*TLR4*$^{-/-}$ ENDO (mean ± SD)
GM-CSF	17.9 ± 15.2	39.5 ± 52.6
IL-1β	15.2 ± 9.7	23.6 ± 8.9
IL-2	5.0 ± 2.5	11.2 ± 10.7
IL-6	45.7 ± 24.7	175.5 ± 112.7
IL-10	2.7 ± 2.4	7.5 ± 8.3
IL-17A	0.6 ± 0.4	0.4 ± 0.3
IFN-γ	17.8 ± 22.6	39.2 ± 44.9
TNF-α	10.9 ± 7.9	10.3 ± 7.8

Table 5.1 Cytokine protein concentrations of endometriosis-like lesions developed by WT and *TLR4*$^{-/-}$ ENDO mice. *n* = 6 animals per group.

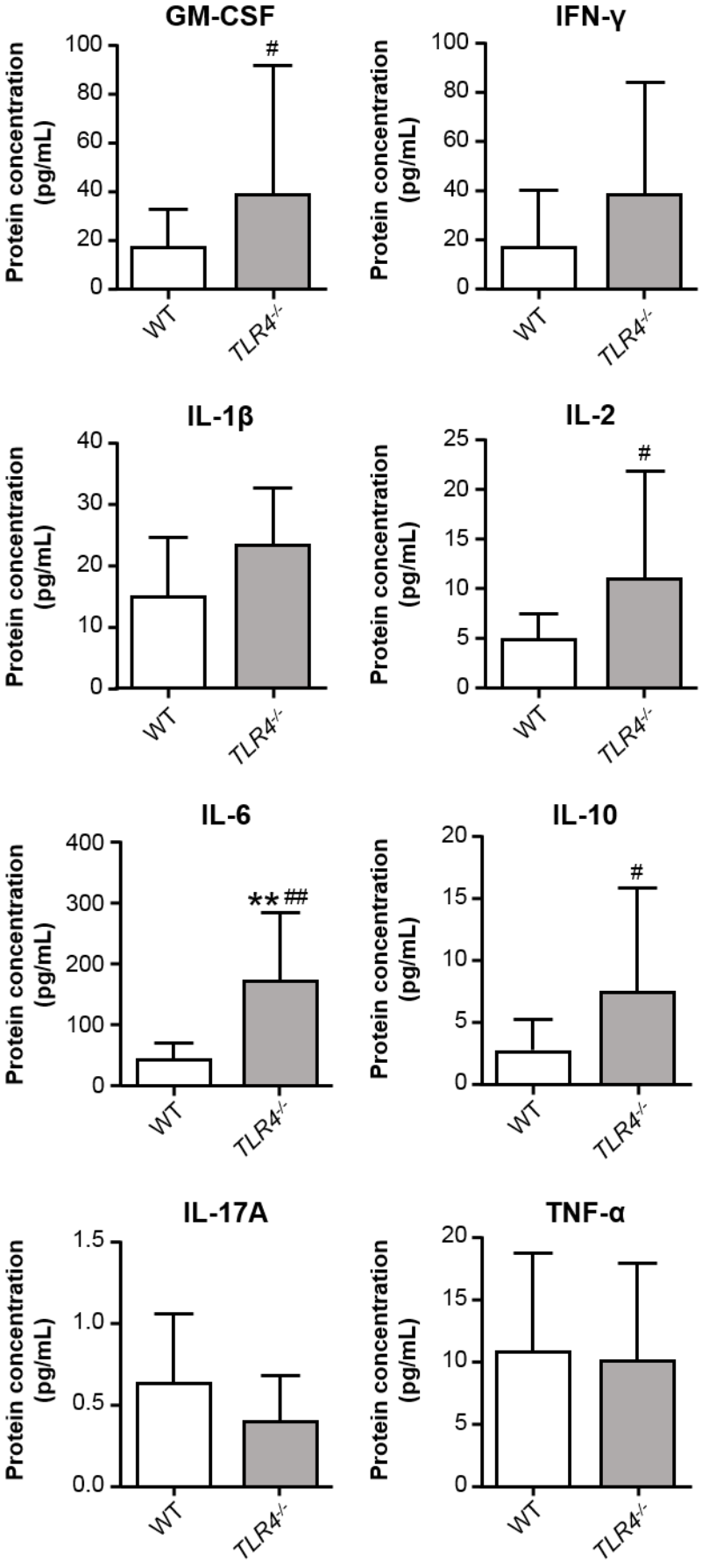
GM-CSF
Protein concentration (pg/mL)
100
80
60
40
20
0
#
WT
TLR4-/-
IFN-γ
Protein concentration (pg/mL)
100
80
60
40
20
0
WT
TLR4-/-
IL-1β
Protein concentration (pg/mL)
40
30
20
10
0
WT
TLR4-/-
IL-2
Protein concentration (pg/mL)
25
20
15
10
5
0
#
WT
TLR4-/-
IL-6
Protein concentration (pg/mL)
400
300
200
100
0
**##
WT
TLR4-/-
IL-10
Protein concentration (pg/mL)
20
15
10
5
0
#
WT
TLR4-/-
IL-17A
Protein concentration (pg/mL)
1.5
1.0
0.5
0.0
WT
TLR4-/-
TNF-α
Protein concentration (pg/mL)
20
15
10
5
0
WT
TLR4-/-

Figure 5.4 Endometriosis-like lesion cytokine profiles in WT and *TLR4*$^{-/-}$ ENDO mice. Endometriosis-like lesions from *TLR4*$^{-/-}$ ENDO mice (n = 6 animals) displayed a significant increase and greater variability in protein levels of IL-6 than those obtained from WT ENDO mice (n = 6 animals). Heightened variability in the expression of GM-CSF, IL-2 and IL-10 was also observed in lesions from *TLR4*$^{-/-}$ ENDO compared to WT ENDO mice. No differences were observed in the lesions between WT ENDO and *TLR4*$^{-/-}$ ENDO mice for concentrations of IFN-γ, IL-1β, IL-17A or TNF-α. * denotes mean comparison P <0.05; # variability comparison P <0.05; ## P <0.01. Data expressed as mean ± SD.

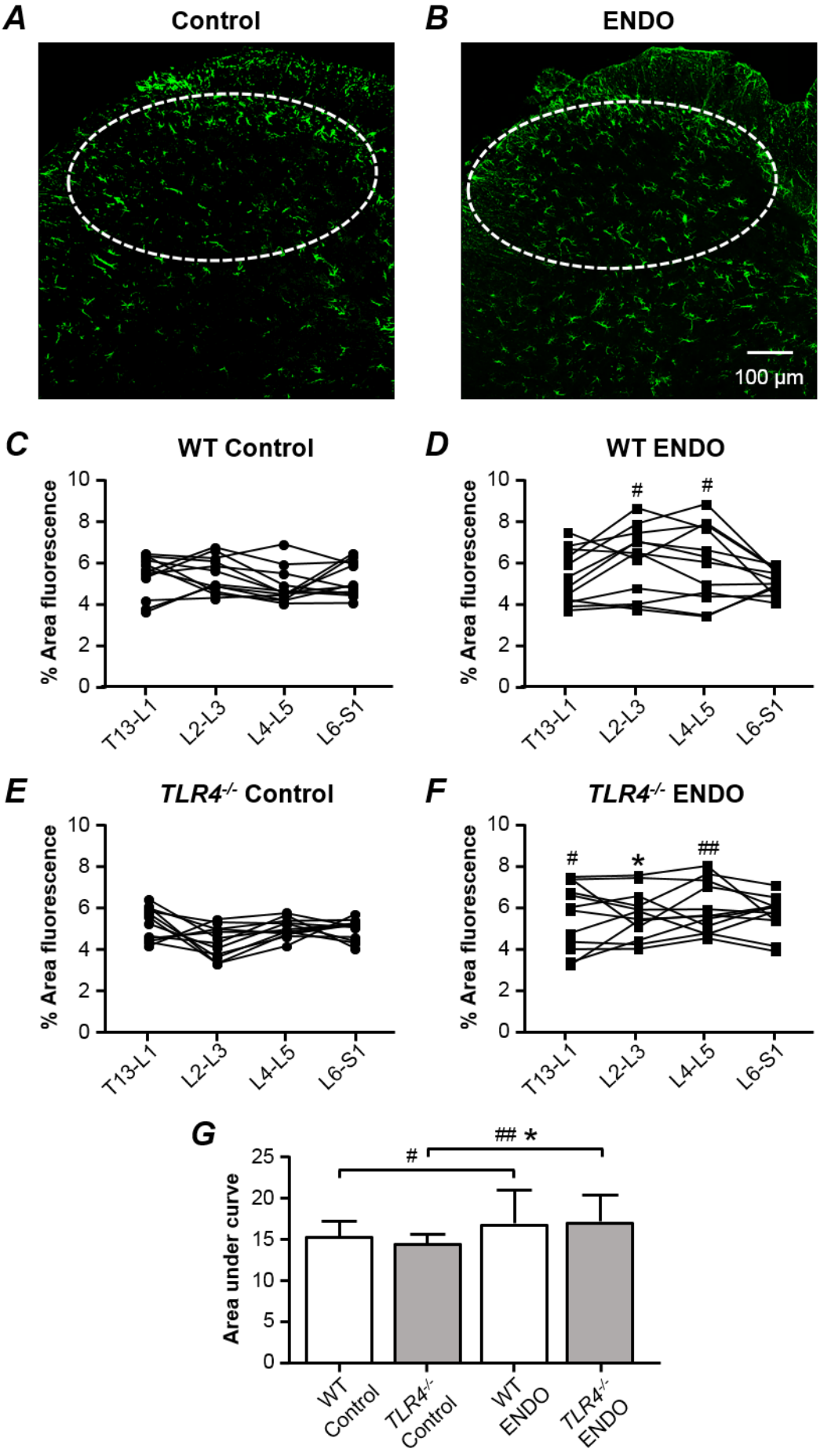
A
Control
B
ENDO
100 µm
C
WT Control
D
WT ENDO
% Area fluorescence
T13-L1
L2-L3
L4-L5
L6-S1
E
TLR4-/- Control
F
TLR4-/- ENDO
G
Area under curve
WT Control
TLR4-/- Control
WT ENDO
TLR4-/- ENDO

Figure 5.5 Distribution and calculated area of GFAP-immunoreactivity (astrocytes) in the T13-S1 spinal dorsal horn of WT and *TLR4*$^{-/-}$ ENDO mice compared to saline-injected controls. (*A-B*) Representative images of astrocytic GFAP-immunoreactivity in the L4-L5 spinal dorsal horn (dashed ellipsoid) from a saline control and ENDO animal, respectively. This ENDO animal showed an increase in GFAP-immunoreactivity compared to a saline-injected control. **(*C*)** Percentage area of each dorsal horn (linked by horizontal bars) occupied by GFAP immunostaining in WT control mice was relatively consistent across spinal levels T13-S1. **(*D*)** In contrast, a significant increase in variability was observed for levels L2-L5 in WT mice with endometriosis-like lesions. **(*E*)** Area of GFAP-immunoreactivity in *TLR4*$^{-/-}$ saline mice was also consistent across spinal levels, and **(*F*)** increased in variability for T13-L1 and L4-L5 in *TLR4*$^{-/-}$ ENDO mice. Calculated GFAP area in L2-L3 for *TLR4*$^{-/-}$ ENDO additionally showed a significant mean increase in GFAP-immunoreactivity compared to *TLR4*$^{-/-}$ saline controls. **(G)** Summarised data comparing AUC values for each dorsal horn further indicate heightened variability of GFAP immunostaining in both strains of ENDO animals versus their respective controls, as well as an overall increase in GFAP-immunoreactivity measured in *TLR4*$^{-/-}$ ENDO mice. Magnification in ***(A-B)*** 20x; scale bar in ***(B)*** also applies to ***(A)***. ***** denotes mean comparison $P < 0.05$; # variability comparison $P < 0.05$; ## $P < 0.01$. Significance notations in panel **(*D*)** refers to corresponding spinal levels in panel **(*C*)**; significance notations in panel **(*F*)** refers to corresponding spinal levels in panel **(*E*)**. All groups $n = 12$ from 6 animals. Data in ***(G)*** expressed as mean ± SD.

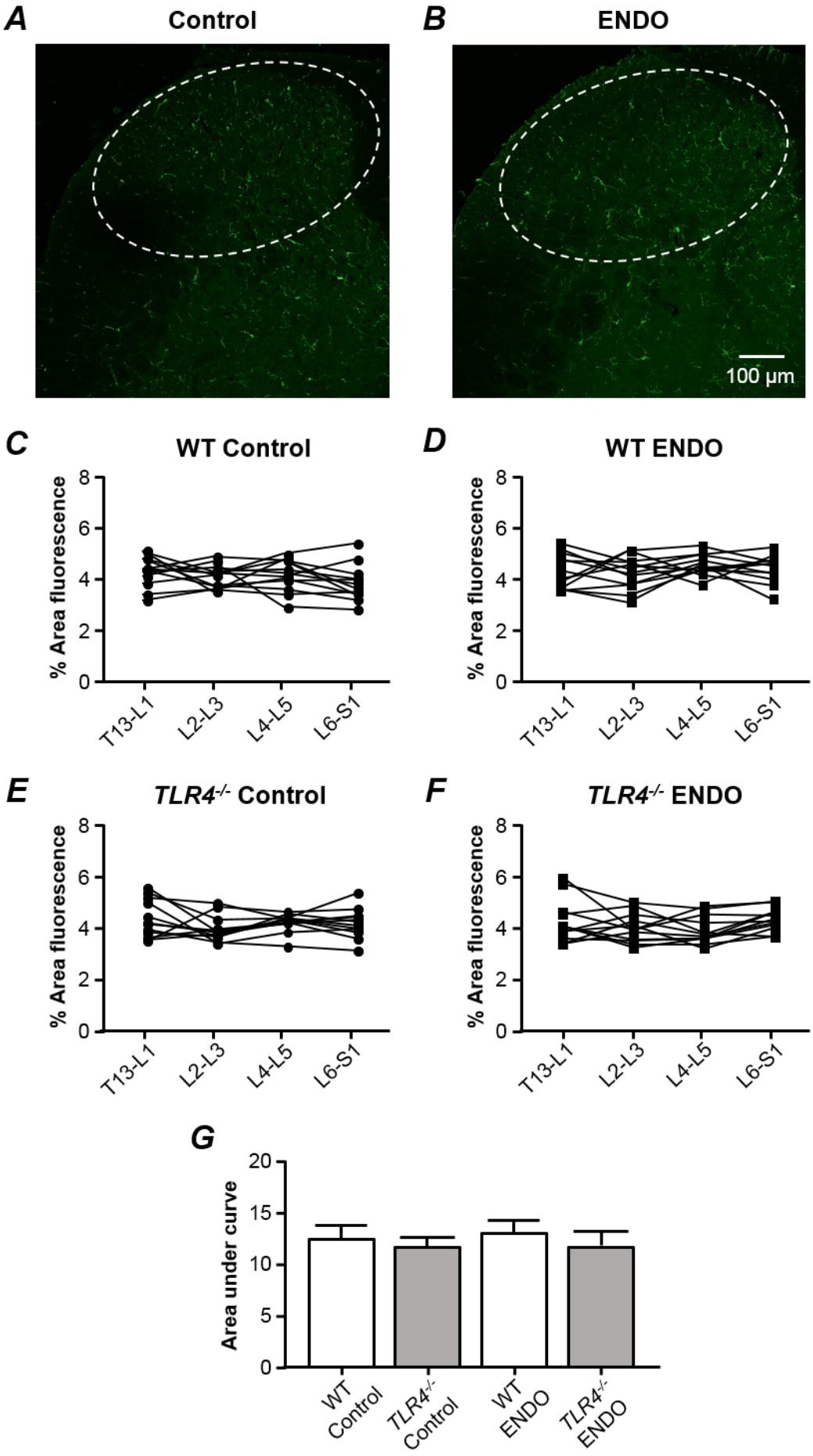
A
Control
B
ENDO
100 µm
C
WT Control
D
WT ENDO
% Area fluorescence
0
2
4
6
8
T13-L1
L2-L3
L4-L5
L6-S1
E
TLR4-/- Control
F
TLR4-/- ENDO
G
Area under curve
0
5
10
15
20
WT Control
TLR4-/- Control
WT ENDO
TLR4-/- ENDO

Figure 5.6 Distribution and calculated area of Iba-1-immunoreactivity (microglia) in the T13-S1 spinal dorsal horn of WT and *TLR4*$^{-/-}$ ENDO mice compared to saline-injected controls. (*A-B*) Representative images of microglial Iba-1-immunoreactivity in the L4-L5 dorsal horn (dashed ellipsoid) from a saline control and ENDO animal, respectively. These examples show a similar degree of Iba-1-immunoreactivity between the two experimental groups. **(*C*)** Percentage area of each dorsal horn (linked by horizontal bars) occupied by Iba-1 immunostaining in WT control mice was reasonably consistent across spinal levels T13-S1. **(*D*)** These values were unchanged for WT ENDO mice. **(*E-F*)** Similarly, the area of Iba-1-immunoreactivity was not statistically different in mean or variability values at any spinal level between *TLR4*$^{-/-}$ saline and ENDO animals. **(*G*)** Summarised AUC data for each dorsal horn further indicate no differences in spinal Iba-1immunostaining between saline and ENDO, or between WT and *TLR4*$^{-/-}$ mice. Magnification in **(*A-B*)** 20x; scale bar in **(*B*)** also applies to **(*A*)**. All groups n = 12 from 6 animals. Data in ***(G)*** expressed as mean ± SD.

A

WT spinal cord		
Cytokine (pg/mL)	Control (mean ± SD)	ENDO (mean ± SD)
GM-CSF	7.2 ± 2.3	11.4 ± 5.8
IL-1β	43.1 ± 11.7	41.3 ± 11.7
IL-2	142.6 ± 34.3	117.5 ± 18.3
IL-6	25.5 ± 9.2	31.3 ± 11.5
IL-10	15.3 ± 7.4	18.4 ± 9.4
IL-17A	3.0 ± 0.8	3.0 ± 0.8
IFN-γ	21.4 ± 17.3	28.7 ± 28.3
TNF-α	2.3 ± 1.0	3.3 ± 1.2

B

TLR4$^{-/-}$ spinal cord		
Cytokine (pg/mL)	Control (mean ± SD)	ENDO (mean ± SD)
GM-CSF	8.0 ± 2.2	7.9 ± 2.7
IL-1β	47.6 ± 14.2	39.2 ± 9.2
IL-2	154.3 ± 32.2	138.1 ± 37.1
IL-6	23.9 ± 8.6	30.4 ± 10.0
IL-10	16.6 ± 6.3	20.9 ± 7.3
IL-17A	3.5 ± 0.9	2.9 ± 0.7
IFN-γ	16.6 ± 16.1	39.4 ± 26.4
TNF-α	2.5 ± 0.9	2.6 ± 0.9

Table 5.2 Cytokine protein concentrations within the spinal cords of WT and *TLR4*$^{-/-}$ control and ENDO mice. (*A*) Values obtained from WT mice (n = 6 animals per group). **(*B*)** Values obtained from *TLR4*$^{-/-}$ mice (n = 6 animals per group).

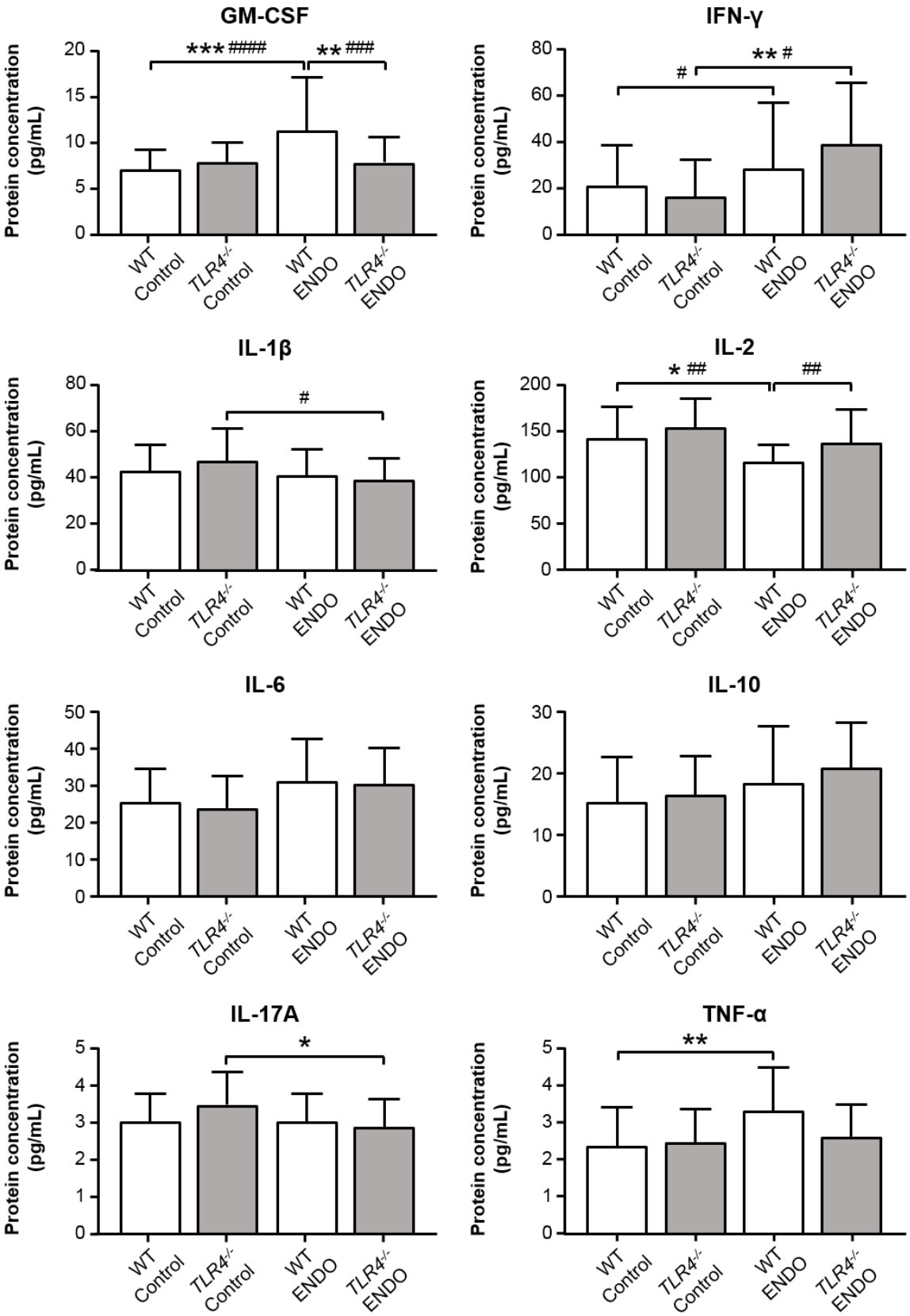
GM-CSF
IFN-γ
IL-1β
IL-2
IL-6
IL-10
IL-17A
TNF-α
Protein concentration (pg/mL)
WT Control
TLR4-/- Control
WT ENDO
TLR4-/- ENDO

Figure 5.7 Inflammatory cytokine expression in the T13-S1 spinal cord of WT and *TLR4*$^{-/-}$ ENDO mice compared to saline-injected controls. Spinal cords from WT ENDO mice displayed an increase in the mean protein levels and variability of GM-CSF compared to WT controls, and *TLR4*$^{-/-}$ ENDO mice. A mean increase in TNF-α was also observed in WT ENDO mice compared to WT controls, and IL-2 was decreased in mean and variability. IFN-γ was increased in variability for both WT and *TLR4*$^{-/-}$ ENDO animals compared to their respective controls, and increased by mean values for *TLR4*$^{-/-}$ ENDO mice only. *TLR4*$^{-/-}$ ENDO mice further displayed a mean decrease in the concentration of IL-17A compared with *TLR4*$^{-/-}$ controls, and decrease in the variability of IL-1β. No differences between groups were observed for spinal cord concentrations of IL-6 or IL-10. * denotes mean comparison $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. # denotes variability comparison $P < 0.05$; ## $P < 0.01$; ### $P < 0.001$; #### $P < 0.0001$. All groups $n = 6$ animals. Data expressed as mean ± SD.

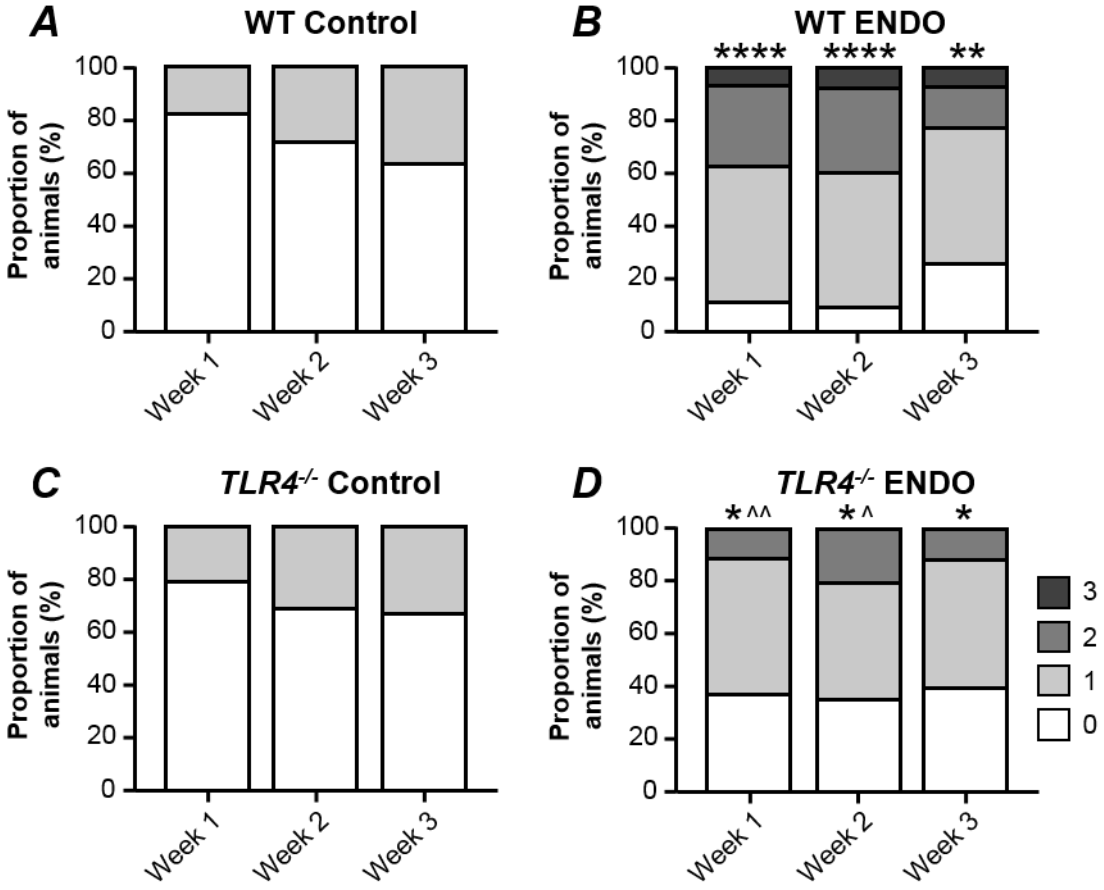

Figure 5.8 Facial pain scores following development of endometriosis-like lesions in WT and *TLR4*$^{-/-}$ ENDO mice compared to saline-injected controls. (*A*) The majority of WT control animals had a grimace score of 0 over the three-week developmental period. **(*B*)** In contrast, WT ENDO mice showed significantly more signs of pain, with a higher proportion of animals scoring ≥1. **(*C*)** Similarly, *TLR4*$^{-/-}$ control animals were mostly pain free, compared to **(*D*)** *TLR4*$^{-/-}$ ENDO animals that scored ≥1. However, facial pain criteria from *TLR4*$^{-/-}$ ENDO mice was significantly lower overall compared to WT ENDO mice. * denotes mean comparison to saline controls $P < 0.05$; ** $P < 0.01$; **** $P < 0.0001$. Asterisk significance notations in panel **(*B*)** refer to corresponding weeks in panel **(*A*)**; those in panel **(*D*)** refer to corresponding weeks in panel **(*C*)**. ^ denotes mean comparison to WT ENDO $P < 0.05$; ^^ $P < 0.01$. Caret significance notations in panel **(*D*)** refer to corresponding weeks in panel **(*B*)**. All groups $n = 6$ animals.

5.5 Discussion

To our knowledge, this is the first study to describe both peripheral and central effects of TLR4 *in vivo* in the development of endometriosis and its associated pain, respectively. Our findings reveal central TLR4-mediated neuroimmune adaptations as a consequence of endometriosis and implicate basal peripheral TLR4 activation as an inhibitor of lesion development. Intriguingly, our research thus demonstrates pivotal but opposing roles for TLR4 in the lesion pathophysiology and pain symptoms of endometriosis.

5.5.1 Peripheral TLR4 activation appears necessary to limit lesion development in endometriosis

A greater number of endometriosis-like lesions were retrieved from TLR4-deficient animals, suggesting that TLR4-mediated signalling pathways play a role in limiting lesion development. WT-*TLR4*$^{-/-}$ cross-injected ENDO animals additionally showed an intermediary growth of lesions, indicating that the immune statuses of ectopic endometrial tissue and the peritoneal environment are both important in lesion generation. This result was somewhat unexpected, as it has been hypothesised, based on *in vitro* and chronic TLR4 activation studies, that enhanced TLR4 activity likely contributes to endometriosis (Iba *et al.*, 2004; Khan *et al.*, 2008b; Khan *et al.*, 2010; Khan *et al.*, 2013b; Azuma *et al.*, 2017).

TLR4 is an innate immune receptor that initiates inflammatory cascades following the recognition of pathogen-, microbial- and/or damage-associated ligands. Under ambient conditions the inflammatory responses are generally successful in resolving an immune insult however, in pathological states, inappropriate TLR-mediated inflammation can contribute to disease progression. It is therefore possible that TLR4 plays a dichotomous role in the development of endometriosis; with its pattern recognition abilities and controlled inflammatory signalling essential for limiting the development of lesions (as

indicated here), while excessive receptor activation and/or exaggerated production of inflammatory mediators (akin to other studies) facilitates lesion establishment. If proven to be accurate, determination of which factor contributes more significantly to clinical outcomes would therefore better direct future TLR4-specific therapeutic research.

Several lines of evidence, as well as our observation that more necrotic lesions were also retrieved from *TLR4*$^{-/-}$ ENDO mice, support the above hypothesis. Although necrotic tissues did not continue to form viable endometriosis-like lesions, their presence likely reflects peritoneal immune cell dysfunction, with reduced ability to recognise and/or clear donor endometrial tissues. In women, the TLR4 A896G (D299G) polymorphism is associated with an increased risk of developing endometriosis (Latha *et al.*, 2011). This genetic variation can lead to decreased recruitment of the TLR4 adaptor proteins, MyD88 and TRIF (Figueroa *et al.*, 2012), resulting in receptor hypoactivity and the failure to mount an appropriate immune response upon stimulation (e.g. by ectopic endometrium). Moreover, C3H/HeJ mice are naturally deficient in TLR4 (Poltorak *et al.*, 1998), and peritoneal macrophages isolated from these animals display reduced TNF-α inflammatory activity when incubated with peritoneal fluid from endometriosis patients (Takeshita *et al.*, 1998). Suppressed proinflammatory responses associated with reduced TLR4 activation may therefore permit implantation of endometrial debris, and the subsequent development of endometriosis lesions.

Cytokine profiles of endometriosis-like lesions from *TLR4*$^{-/-}$ ENDO mice differed from those of WT animals. Despite being unable to identify the source of cytokines, or determine whether the effects were compensatory or maladaptive, their expression may provide clues for the increased lesion burden in *TLR4*$^{-/-}$ animals. Although many classical proinflammatory cytokines associated with TLR4 appeared unchanged (such as IFN-γ, IL-1β and TNF-α), levels of IL-2, IL-6, IL-10 and GM-CSF were altered. Typically, GM-CSF

and IL-2 regulate the activity of macrophages and lymphocytes (such as regulatory T (Treg) cells), respectively (Waldmann, 2006b). Secretion of IL-10 and other mediators by Treg cells can suppress autoimmunity; terminate existing immune responses; and promote the differentiation of alternatively activated (M2/Th2) macrophages (Waldmann, 2006a; Tiemessen *et al.*, 2007). Interestingly, Treg populations are altered in tissues from endometriosis patients (Olkowska-Truchanowicz *et al.*, 2013; Tanaka *et al.*, 2017), and their activity can influence the early establishment of endometriosis-like lesions (Stanic *et al.*, 2014; Tanaka *et al.*, 2017). Endometriosis is also often considered an M2-mediated disorder (Podgaec *et al.*, 2007; Smith *et al.*, 2012), and adoptive transfer of M2 macrophages into the peritoneum of recipient ENDO mice enhances lesion formation (Bacci *et al.*, 2009). The potential role of IL-6 secretion in lesions from *TLR4*$^{-/-}$ mice is less clear, given that IL-6 is often associated with TLR4 stimulation, and has the ability to perform both pro- and anti-inflammatory roles (Greenhill *et al.*, 2011). However, the release of IL-6 from M2 macrophages can promote migration of endometriotic cells, and thus may further contribute to the development of endometriosis (Woo *et al.*, 2017).

The proportion of endometriosis-like lesion phenotypes was similar between groups, and comparable to our previous report on BALB/c (WT) ENDO mice (Dodds *et al.*, 2017 - Chapter 3). Although cystic-type lesions in WT and *TLR4*$^{-/-}$ ENDO mice were of a similar size, diameters of the less prevalent dense-type lesions were reduced in *TLR4*$^{-/-}$ ENDO mice. Exogenous administration of IL-2 has been shown to reduce the size of endometriosis-like lesions, and thus altered levels of IL-2 in lesions from *TLR4*$^{-/-}$ ENDO animals may have contributed to this finding (Quereda *et al.*, 2008). We also observed an increase in attachments of endometriosis-like lesions to visceral organs in *TLR4*$^{-/-}$ ENDO mice. This may indicate that TLR4-mediated inflammatory activity assists in the growth and attachment lesions, or could simply be incidental findings owing to the assumed

reduction of donor tissue clearance in *TLR4*$^{-/-}$ ENDO mice.

5.5.2 Central TLR4 activation may be implicated in neuroimmune-related inflammatory signalling and pain associated with endometriosis

Both WT and *TLR4*$^{-/-}$ ENDO animals displayed a heightened variability in spinal astrocytic GFAP-immunoreactivity compared to control animals. This finding is complementary to our observations in C57BL/6 ENDO mice (Dodds *et al.*, 2018 - Chapter 4), and further supports the notion that endometriosis-like lesions are peripheral inflammatory stimuli that can lead to central adaptations in glial reactivity. *TLR4*$^{-/-}$ ENDO mice also displayed a subtle overall increase in spinal GFAP expression, as might be expected with a greater peripheral input (i.e. more lesions).

Lesion-associated adaptations in spinal glial reactivity were largely similar between WT and *TLR4*$^{-/-}$ groups, yet the inflammatory cytokine output in spinal cords from these animals were markedly different. Pain behaviour also developed to a lesser degree in *TLR4*$^{-/-}$ ENDO mice compared to WT, even with a significantly greater formation of endometriosis-like lesions. Firstly, these findings emphasise the concept that the existence of lesions does not necessarily dictate the degree of pain in endometriosis. Secondly, dissociations between glial reactivity and pain have been documented (Colburn *et al.*; Honore *et al.*, 2000; Ducourneau *et al.*, 2014), and morphological analysis alone may not fully represent the extent of glial-mediated inflammatory signalling (Norden *et al.*, 2016). Therefore, an absence of overt differences in spinal gliosis between WT and *TLR4*$^{-/-}$ groups does not preclude a neuroimmune-mediated pain effect. The divergence in inflammatory mediator composition in WT and *TLR4*$^{-/-}$ ENDO mice may therefore contribute to the differential pain scores observed.

Inflammatory cytokine profiles of spinal cords from WT ENDO mice displayed altered

levels of GM-CSF, TNF-α, IL-2 and IFN-γ. Conversely, *TLR4*$^{-/-}$ ENDO mice show adaptations in IFN-γ, IL-17A and IL-1β. While beyond the scope of this study to discuss potential contributions of each cytokine selected for analysis, all have been shown to facilitate central sensitisation by numerous processes (for review, see Clark *et al.* (2013); Ji *et al.* (2013); Dodds *et al.* (2016 - Chapter 2)). The precise mechanisms leading to this cytokine shift remains to be determined, but our data point towards contributions of both TLR4-dependent (WT) and independent (*TLR4*$^{-/-}$) immune pathways. Sex differences in central pain mechanisms are of significant recent interest: whilst some suggest spinal glial adaptations are essential for pain generation in females (Yang *et al.*, 2015; Liu *et al.*, 2016a; Smeester *et al.*, 2016; Chen *et al.*, 2017), others report development of TLR4-independent, and indeed glial-independent, pain in female but not male animals (Sorge *et al.*, 2011; Sorge *et al.*, 2015; Woller *et al.*, 2016). We cannot discount the possibility that lesions from *TLR4*$^{-/-}$ mice were less noxious than those from WT animals, resulting in the altered cytokine expression and pain behaviour by mechanisms other than spinal TLR4. However, given that glial-mediated TLR4 signalling plays a significant role in many other pathological conditions, the presence or absence of this receptor has likely influenced the combination of lesion-associated cytokines released within the spinal cord; thus contributing to the unique pain responses observed in WT and *TLR4*$^{-/-}$ ENDO animals.

These results suggest that targeting spinal neuroimmune mechanisms may be a viable method to treat endometriosis-related pain. Intrathecally-administered drugs that block the activation of glial cells (Liu *et al.*, 2012b; Kannampalli *et al.*, 2014; Liu *et al.*, 2016a), TLR4 (Yuan *et al.*, 2015; Li *et al.*, 2016a), or neutralise proinflammatory cytokines (Saito *et al.*, 2010), have shown analgesic potential in several inflammatory pain models. Given that existing treatments for endometriosis patients often provide suboptimal pain relief, these neuroimmune-targeted agents may therefore present new opportunities for improved

analgesia as adjuvant drug therapies.

5.5.3 Conclusions

In summary, data from the current study have highlighted that adequate activation of peripheral TLR4 assists in minimising the development of endometriosis-like lesions. Yet in contrast, central TLR4 signalling may facilitate the generation of lesion-associated pain behaviour. This research not only improves our understanding of the underlying mechanisms involved, but also reveals a promising opportunity to intervene in the complex pathophysiology of both lesion development and pain symptoms in endometriosis. It is important to acknowledge that although conventional knockout systems provide a powerful tool for investigation of function, the recruitment of pathways to compensate for a deficiency in TLR4 protein, such as TLR2 activation, may occur. Nevertheless, it is evident that a fine balance of TLR4 signalling in endometriosis is required, and our data point towards a potential therapeutic benefit of inhibiting spinal TLR4, while maintaining receptor-related activity in the periphery. As with all cases of TLR4-targeted treatment options, the effects on both efficacy of lesion burden and potential consequences besides pain (such as fertility (Schjenken *et al.*, 2015)) will be paramount.

Statement of Authorship

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Name of Principal Author (Candidate)	Kelsi N. Dodds		
Contribution to the Paper	Designed experiments, performed all experiments, analysed and interpreted data, generated figures, and wrote the manuscript.		
Overall percentage (%)	85%		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
Signature		Date	30/04/2018

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);
ii. permission is granted for the candidate in include the publication in the thesis; and
iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

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Chapter 6. Acute pharmacological blockade of peripheral Toll-like receptor 4 (TLR4) enhances lesion development in a mouse model of endometriosis

This chapter is an unpublished and unsubmitted primary research study written in manuscript style.

6.1 Abstract

Endometriosis is a chronic inflammatory condition in women associated with abdominopelvic endometrial-like lesions. Primarily, lesions are thought to arise from immune-evaded implantation of endometrial debris effluxed via retrograde menstruation. Toll-like receptor 4 (TLR4) is an innate immune cell receptor capable of detecting and initiating proinflammatory responses against endogenous and exogenous ligands, and total genetic knockout of *TLR4* in mice facilitates endometriosis lesion development. We therefore sought to determine whether this finding could be replicated by acute pharmacological blockade of peripheral TLR4. Endometriosis-like lesions were induced in female BALB/c mice by intraperitoneal injection of syngeneic donor endometrium, four hours following a single intraperitoneal injection of the TLR4 antagonist, (+)-N-phenethylnoroxymorphone (1j-ENDO), or vehicle (Veh-ENDO). Following 21 days of development, the total number of endometriosis-like lesions were significantly increased in 1j-ENDO animals compared to Veh-ENDO. Lesion size and phenotypic characteristics were similar between groups, although lesions established in a greater range of peritoneal locations in 1j-ENDO mice. Central adaptations, including the degree of change in spinal glial immunoreactivity and spontaneous pain behaviour, were unchanged between 1j-ENDO and Veh-ENDO animals. These data further demonstrate the importance of peripheral TLR4 activity in regulating endometriosis lesion development, likely through early recognition and clearance of 'foreign' endometrial debris within the peritoneal cavity.

The subsequent return of TLR4 activity permits lesion growth, associated central neuroimmune adaptations and pain behaviour to progress as standard.

6.2 Introduction

Endometriosis is a common gynaecological, chronic inflammatory condition developed by approximately 5-10% of reproductively-aged women (Giudice & Kao, 2004). It is characterised by lesions within the abdominopelvic cavity, composed of tissue similar to the uterine endometrium. Endometriosis patients commonly present clinically with dysmenorrhoea, dyspareunia and/or persistent pelvic pain (Stratton & Berkley, 2011). Importantly, a further consequence of endometriosis affecting the female reproductive organs is sub- or infertility (Bulletti *et al.*, 2010). A key mechanistic event believed to contribute to the pathogenesis of lesion establishment is the peritoneal deposition of endometrial tissue, via retrograde menstruation (Sampson, 1927). However, reverse flow of menstrual debris occurs in approximately 90% of women, whilst a much smaller proportion go on to develop mature endometriosis lesions (Halme *et al.*, 1984; O *et al.*, 2017). This strongly suggests that other pathobiological factors facilitate this process and, as such, dysfunction in the peritoneal immune response to recognise, sequester and clear the ectopic endometrial tissue has been suggested.

As contributors to first line host defence, Toll-like receptors (TLRs) of the innate immune system are principally involved in the recognition of conserved molecular patterns associated with invading pathogens, microbes and/or endogenous tissue damage (PAMPs, MAMPs and DAMPS, respectively). Upon TLR activation, intracellular signalling pathways are initiated that lead to upregulated synthesis and secretion of proinflammatory mediators (such as interleukin (IL)-1β and tumour necrosis factor (TNF)-α), which assist in removal of the immune insult (Akira & Takeda, 2004). Recent research on the relationship between endometriosis and innate immunity has given particular focus to the

TLR4 subtype, due to its ability to detect bacterial endotoxin (lipopolysaccharide; LPS) (Khan *et al.*, 2010) and host proteins released from tissue stress or injury, such as heat shock protein (Hsp)-70 (Khan *et al.*, 2008b), high mobility group box 1 (HMGB1) protein (Yun *et al.*, 2016), and hyaluron metabolites (Dechaud *et al.*, 2001).

Some have postulated that excessive TLR4 activation and the associated receptor-mediated proinflammatory cytokine release may exacerbate development of endometriosis-like lesions, possibly through a sterile or subclinical inflammatory process (Khan *et al.*, 2013a; Kobayashi *et al.*, 2014; Khan *et al.*, 2018). In contrast, however, we have previously demonstrated that genetic knockout of *TLR4*, and therefore a ubiquitous decrease in receptor expression and activity, promotes lesion development in a minimally-invasive mouse model of endometriosis (Dodds *et al.*, 2018 - Chapter 5). A reduction in lesion-associated spontaneous pain behaviour, likely due to alterations in glial TLR4-mediated central sensitisation, was also observed in the *TLR4*-knockout mice. We therefore hypothesised that TLR4 may play a dichotomous role in the development of endometriosis; with its pattern recognition abilities and controlled inflammation essential for limiting lesion formation, whilst enhanced receptor activation and/or exaggerated inflammatory signalling may alternatively facilitate this process.

Although conventional knockout systems are considered the gold standard for investigation of function, biological processes may adapt to disturbed signalling pathways during development and maturation. Therefore, despite the deficiency and/or mutation in specific proteins, phenotypes (including disease processes and behaviour, such as pain) can remain unaltered due to compensatory recruitment of redundant or parallel pathways (Eisener-Dorman *et al.*, 2009). Caution must therefore be taken when drawing conclusions from rodent knockout systems, such as our *TLR4*-knockout ENDO model (Dodds *et al.*, 2018 - Chapter 5). Moreover, previous investigations on the potential role(s) of TLR4 in

endometriosis have largely included studies either *in vitro*, or by chronic receptor stimulation *in vivo*. It is unknown whether an acute alteration in peripheral TLR4 activity *in vivo* can regulate the development of lesions and associated pain. Hence, we sought to determine which of our findings from *TLR4*-knockout mice could be reproduced, by pharmacologically blocking intraperitoneal TLR4 activity in wildtype animals during the early phase of endometriosis-like lesion development.

6.3 Methods

6.3.1 Animals

Female BALB/c mice (n = 24) 8-14 weeks in age, weighing 19.0 ± 1.4 g, were obtained from the University of Adelaide Laboratory Animal Services. All animals underwent daily cervical smear testing to determine their oestrous cycle, as described previously (Dodds *et al.*, 2015). Each was selected for experimental use only when in pro-oestrus, as this phase at the time of induction has been shown to generate robust and consistent endometriosis-like lesions in gonad-intact BALB/c mice (Dodds *et al.*, 2017 - Chapter 3). All procedures were conducted in accordance with the National Health and Medical Research Council Australian code for the care and use of animals for scientific purposes (8th edition, 2013) and the University of Adelaide Animal Ethics Guidelines, and approved by the University of Adelaide Animal Ethics Committee.

6.3.2 Minimally-invasive mouse model of endometriosis

6.3.2.1 Acute peripheral antagonism of TLR4

Recipient mice received a single administration of drug or vehicle solution prior to the induction of endometriosis-like lesions (ENDO). Using a 30-gauge needle, drug-treated mice received 1 mg/kg of the TLR4 antagonist, (+)-N-phenethylnoroxymorphone (1j)

(Selfridge *et al.*, 2015), via intraperitoneal injection at the ventral midline between the left inguinal nipples (1j-ENDO; n = 6). Control (vehicle-treated) mice (Veh-ENDO; n = 6) alternatively received an equivalent volume of vehicle solution (1% dimethyl sulfoxide (DMSO)).

6.3.2.2 Induction of endometriosis-like lesions

Approximately three hours following 1j or vehicle administration to recipient ENDO animals, donor mice (n = 12) were sacrificed by cervical dislocation whilst under deep inhaled isoflurane anaesthesia. The uterus was removed and placed into a sterilised glass Petri dish containing cold (4°C) 0.01 M phosphate-buffered saline (PBS). Each horn was opened along the mesometrial border and pinned flat to the Sylgard®-lined (Dow Corning; Michigan, USA) base of the Petri dish using entomology pins. The endometrium (40 mg) was then carefully removed by sharp dissection and further cut into segments of 2-3 mm^2. The resultant endometrial segments were suspended in a 1-ml syringe containing 0.5 ml sterile saline (0.9% NaCl; room temperature (RT)) and passed once through a 21-gauge needle, to ensure smooth delivery of content to recipient ENDO animals.

Four hours post-1j or vehicle administration, recipient ENDO mice received the donor endometrial suspension, via a second intraperitoneal injection at the same anatomical site (1 donor: 1 recipient). The timing of drug and ENDO administration was selected as a mid-range point, based on previous evidence demonstrating 1j antagonistic potency of at least 1-24 hours (Selfridge *et al.*, 2015). Following 21 days of development, ENDO animals were deeply anaesthetised with isoflurane gas and decapitated (Fig. 6.1).

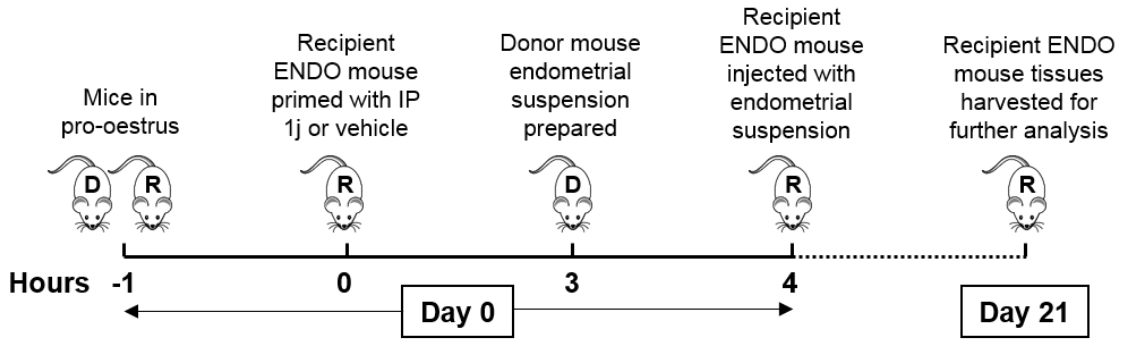

Figure 6.1 Schematic diagram of the experimental timeline for drug priming and induction of endometriosis-like lesions in ENDO mice. Pro-oestrus recipient mice were initially administered the TLR4 antagonist, 1j (1 mg/kg) or vehicle solution via IP injection. Three hours later, endometria from pro-oestrus donor mice were harvested, fragmented and placed into a syringe with sterile saline. At a total of four hours post-drug priming, recipient animals were IP injected with the donor endometrial suspension. Following 21 days of endometriosis-like lesion development, tissues from recipient ENDO mice were collected for further analysis. *D* = donor; *R* = recipient; *IP* = intraperitoneal.

6.3.2.3 Assessment of endometriosis-like lesion characteristics

After thorough examination of the peritoneal cavity, the number and locations of endometriosis-like lesions from ENDO mice were recorded. Identified lesions and control tissues (lymph nodes, fat etc.) were carefully removed and immediately fixed with cold (4°C) 10% neutral-buffered formalin (Chem-Supply; South Australia, Australia) overnight. All tissues then underwent standard histological processing, sectioning and staining for hematoxylin and eosin, to assess for the presence of epithelial glands and stroma – criteria typically required for a positive diagnosis of endometriosis in humans (Clement, 2007). Confirmed lesions were measured (maximum diameter of greatest longitudinal surface) and classified as dense-, cystic- or necrotic-type based on additional phenotypic characteristics, as described previously (Dodds *et al.*, 2017 - Chapter 3). Only lesions considered dense- or cystic-type were counted toward the total number of viable lesions obtained from an ENDO animal. Necrotic-type lesions, except where specified, were excluded from analysis due to inconsistencies in meeting the diagnostic criteria.

6.3.3 Fluorescent immunohistochemistry for visualisation of glial markers

6.3.3.1 Tissue processing

The immunohistochemical protocol for visualising spinal glia in this study has been described in detail previously (Dodds *et al.*, 2018 - Chapter 4). Briefly, spinal cords (spanning thoracic to sacral, inclusive) were carefully removed from ENDO mice and immersed in 4% paraformaldehyde fixative (PFA; pH 7.2) (4°C; overnight). Tissues were then washed and cryoprotected in 30% sucrose (4°C; two nights). Following dissection into regions T13-L1, L2-3, L4-5 and L6-S1, spinal cord segments were submerged into individual plastic moulds containing Tissue-Tek® OCT compound (#IA018; ProSciTech; Queensland, Australia) and snap frozen. Spinal segments were cryostat sectioned (10 µm) in duplicate per antibody label and collected onto SuperFrost® glass microscope slides (Menzel-Gläser; Braunschweig, Germany). After air-drying, slides were briefly rinsed before undergoing heat-mediated antigen retrieval (97°C for 10 min) with sodium citrate buffer (0.01 M with 0.05% Tween 20; pH 6.0) and cooled with 0.01% Tween 20.

To visualise astrocytes, sections were blocked with 10% normal donkey serum/0.01% Triton X-100 (RT; 1 h), and incubated in Alexa Fluor® 488-conjugated mouse monoclonal anti-glial fibrillary acidic protein (GFAP) antibody (#53-9892-82, clone GA5; RRID: AB_10598515; 1 µg/ml; eBioscience; California, USA) (4°C; two nights). For microglial assessment, sections were blocked with 10% normal donkey serum/0.01% Triton X-100 (RT; 2 h), and incubated in rabbit polyclonal anti-ionised calcium-binding adaptor molecule 1 (Iba-1) (#019-19741; RRID: AB_839504; 0.5 µg/ml; WAKO, Osaka, Japan) (4°C; two nights). After washing, sections were then incubated with donkey anti-rabbit Alexa Fluor® 488 secondary antibody (#ab150073; RRID: AB_2636877; 2 µg/ml; Abcam, Cambridge, UK) (RT; 2 h). All sections were given a final rinse and mounted with Tris-based Fluoro-Gel medium (#IM030; ProSciTech; Queensland, Australia).

6.3.3.2 Image acquisition

Slides were viewed with a Leica TCS SP5 scanning confocal microscope (Leica Microsystems; Wetzlar, Germany) using appropriate excitation wavelengths at 20x magnification with oil immersion. Images were acquired using Leica Application Suite Advanced Fluorescence version 2.6.3 (Leica Microsystems; Mannheim, Germany). Final images are digital composites of 1-1.5 µm Z-series scans (approximately 8-14 optical sections through a depth of 10-16 µm). All images per antibody label were taken at the same gain and offset parameters between animals. Each spinal dorsal horn per section was imaged separately.

6.3.3.3 Image analysis

Semiquantitative analyses were performed on collected images using ImageJ Fiji software (Schindelin *et al.*, 2012). All images were measured blinded as to animal treatment. Maximized Z-stack of images were converted from Leica image files (.lif) to 8-bit greyscale .tiff images, and signal pixels of positive staining areas in the region of interest (ROI) were selected. The ROI was an ellipsoid shape that remained a consistent size for each spinal level between animals and was positioned over Rexed's laminae I-IV (Dodds *et al.*, 2018 - Chapter 4). The percentage of immunofluorescence in the ROI was calculated, and the duplicate area measurements for each dorsal horn were averaged to obtain a single percentage area value per dorsal horn, per spinal level, per animal.

6.3.4 Facial grimace scoring for assessment of pain behaviour

Endometriosis-like lesion-induced pain was measured in a blinded manner throughout the 21-day development period for all ENDO mice. Once daily (between 09:00-10:00 am) from 24 hours post-ENDO induction, animals were individually weighed and 2-3 images of the face were photographed using a digital camera (iSight; Apple Inc.; California, USA).

Images were then de-identified, and scores were determined using a validated mouse grimace scale designed to measure spontaneously emitted pain (Langford *et al.*, 2010). Briefly, the scoring method consisted of five distinct criteria: orbital tightening, nose bulge, cheek bulge, ear position and whisker position. Each criterion was scored as 0 = absent, 1 = moderate, and 2 = severe. Total daily scores for each animal were then grouped per week (1-3) of the development period.

6.3.5 Solutions and drugs

PBS (pH 7.4) was composed of 13.7 mM NaCl; 0.27 mM KCl; 0.15 mM KH_2PO_4; and 0.8 mM Na_2HPO_4 dissolved in deionised water. PFA, sodium citrate buffer and all antibody-related solutions were made with PBS as the solvent. 1j compound, kindly obtained from Professor Kenner C. Rice (National Institute on Drug Abuse; Maryland, USA), was dissolved from powder in neat DMSO (#D5879; Sigma Aldrich; New South Wales, Australia) to make stock concentrations of 20 mg/ml, and stored at RT. When required, aliquots were diluted to a final concentration of 200 µg/ml using sterile saline.

6.3.6 Statistical analysis

All data were analysed using GraphPad Prism® 7 software (GraphPad Software Inc.; California, USA). A D'Agostino-Pearson omnibus K2 test was initially performed to assess normality. Total number of viable endometriosis-like lesions retrieved from ENDO mice were assessed by a Student unpaired one-tailed *t*-test, and number of necrotic-type lesions assessed using a one-tailed Mann-Whitney test. The proportions of dense- and cystic-type lesions between Veh-ENDO and 1j-ENDO mice were determined using a one-way ANOVA with Tukey multiple comparisons, and their sizes by a Student two-tailed unpaired *t*-test. A two-way ANOVA with Bonferroni post-hoc analysis was used to determine differences in facial pain scores and spinal glial GFAP and Iba-1

immunolabelling values between Veh-ENDO and 1j-ENDO animals. Area under the curve (AUC) scores for GFAP and Iba-1 were generated for each dorsal horn per animal and compared between groups using a Student unpaired two-tailed *t*-test. Microsoft® Excel® 2013 (Microsoft Corporation; Washington, USA) was additionally used to generate F-test scores of variability around the standard deviation (SD) for glial immunolabelling values. Unless otherwise specified, data in the text are expressed as mean ± SD, and *P* values of <0.05 were considered statistically significant.

6.4 Results

6.4.1 Total number of endometriosis-like lesions is increased in ENDO mice with acute TLR4 blockade

Endometriosis-like lesions were successfully established in all ENDO mice. An average of 3.5 ± 0.8 endometriosis-like lesions (range 2-4; *n* = 21 lesions from 6 animals) were retrieved from Veh-ENDO mice, administered with an average donor endometrial tissue of 41.4 ± 1.0 mg in 26.5 ± 1.9 pieces. In contrast, a significantly greater number of endometriosis-like lesions developed in 1j-ENDO mice (*P* = 0.03), with 5.5 ± 1.8 lesions (range 3-7; *n* = 33 lesions from 6 animals) obtained from donor endometrial injections of 39.9 ± 3.9 mg in 25.3 ± 2.9 pieces (Fig. 6.2A).

Although necrotic-type endometriosis-like lesions do not reliably exhibit clear epithelial glands and stroma (Dodds *et al.*, 2017 - Chapter 3), we previously found this lesion type to be significantly increased in number in *TLR4*-knockout ENDO mice (Dodds *et al.*, 2018 - Chapter 5); hence their incidence was also recorded in the present study. A total of 25 necrotic-type lesions were retrieved from 1j-ENDO mice (average 4.2 ± 3.6 lesions; *n* = 5 of 6 animals). Veh-ENDO mice developed a total of 9 necrotic-type lesions (average 1.5 ± 2.3 lesions; *n* = 3 of 6 animals), which approached, but did not reach, significance from

the 1j-ENDO group ($P = 0.06$) (Fig. 6.2B).

6.4.2 Acute TLR4 inhibition does not alter endometriosis-like lesion phenotype characteristics

In both experimental groups (1j-ENDO and Veh-ENDO), the proportion of cystic-type lesions was higher than dense-type lesions ($P \leq 0.003$). The proportions of cystic and dense-type lesions retrieved from Veh-ENDO mice were comparable to 1j-ENDO animals. Cystic-type lesions comprised 79.2 ± 24.6% (17/21 lesions) in Veh-ENDO mice and 75.8 ± 19.0% (24/33 lesions) in 1j-ENDO ($P = 0.9$). Dense-type lesions accounted for 20.8 ± 24.6% (4/21 lesions) in Veh-IT ENDO animals and 24.2 ± 19.0% (9/33 lesions) in LPS-IT ENDO animals ($P = 0.9$) (Fig. 6.3A).

The average diameter of cystic-type lesions from 1j-ENDO mice was 2.1 ± 1.0 mm, which was similar in size to those from Veh-ENDO mice (2.4 ± 0.9 mm; $P = 0.4$) (Fig. 6.3B). Likewise, the diameter of dense-type lesions from 1j-ENDO mice (1.3 ± 0.3 mm) did not significantly differ to those from Veh-ENDO counterparts (1.3 ± 0.4 mm; $P = 0.8$) (Fig. 6.3C). The average diameter of necrotic-type lesions was not measured.

6.4.3 Peritoneal locations of endometriosis-like lesion establishment have larger diversity in ENDO mice with acute TLR4 blockade

The peritoneal locations of endometriosis-like lesions from Veh-ENDO animals were similar to those observed in this model previously (Dodds *et al.*, 2017 - Chapter 3, 2018 - Chapter 4, 2018 - Chapter 5). The majority (10/21 lesions; 47.6%) were found attached to connective tissues surrounding the stomach and pancreas, followed by within the gonadal white adipose tissue (5/21; 23.8%). An equal number of endometriosis-like lesions established on the surface of the anterior abdominal wall, and on connective tissues associated with the distal colon (2/21 lesions each; 9.5% per location). Atypically, one

lesion was retrieved from the surface of the diaphragm (4.8%) and one was located within the mesentery (4.8%) (Fig. 6.4A).

In 1j-ENDO mice, endometriosis-like lesions were associated with gastric/pancreatic connective tissues (12/33; 36.4%), gonadal white adipose tissue (5/33; 15.2%), attached to the posterior peritoneal wall (6/33 lesions; 18.2%) or anterior abdominal wall (5/33; 15.2%). Two lesions were attached to the connective tissue of the colon and uterus (6.0%), whilst single lesions were found located within the attachment between the rectum and uterus (1/33 lesions; 3.0%), adhered to the diaphragm (3.0%), and within the peri-renal adipose tissue (3.0%) (Fig. 6.4B).

6.4.4 Spinal glial reactivity associated with endometriosis-like lesions in the periphery is unchanged following acute TLR4 inhibition

We have previously shown that astrocytic GFAP- and microglial Iba-1- (or CD11b)-immunoreactivities in the spinal dorsal horn of control mice (no endometriosis-like lesions) occupy a consistent total area across spinal levels, and between individual animals (Dodds *et al.*, 2018 - Chapter 4, 2018 - Chapter 5). Hence comparisons in the current study were between the ENDO drug-treated (1j-ENDO) and vehicle-treated (Veh-ENDO) mice only.

The average area of GFAP-positive structures from Veh-ENDO animals in T13-L1 was 5.4 ± 1.1% (CV 21%); L2-L3 4.9 ± 1.0% (CV 21%); L4-L5 5.4 ± 1.1% (CV 21%); and L6-S1 4.5 ± 0.4% (CV 8%) (Fig. 6.5A). In 1j-ENDO animals, the area of GFAP-immunoreactivity was 6.1 ± 0.9% in T13-L1 (CV 16%); 5.8 ± 1.3% for L2-L3 (CV 22%); 5.4 ± 1.0% in L4-L5 (CV 19%); and 4.4 ± 0.3% for L6-S1 (CV 7%) (Fig. 6.5B). Statistically, no differences were detected between Veh-ENDO and 1j-ENDO mice regarding a 'treatment' effect ($P = 0.1$) or variability in GFAP expression (all spinal level

comparisons $P \geq 0.5$) (AUC mean and variability comparisons $P \geq 0.1$) (Fig. 6.5C).

Iba-1-immunopositive structures in T13-L1 from Veh-ENDO animals was 3.2 ± 0.3% (CV 9%), L2-L3 3.4 ± 0.4% (CV 11%), L4-L5 3.6 ± 0.5% (CV 14%) and L6-S1 3.5 ± 0.5% (CV 13%) (Fig. 6.5D). In 1j-ENDO mice, the area of Iba-1-immunoreactivity was 3.3 ± 0.4% in T13-L1 (CV 11%); 3.4 ± 0.4 % for L2-L3 (CV 11%); 3.6 ± 0.3% in L4-L5 (CV 9 %); and 3.6 ± 0.5% for L6-S1 (CV 13%) (Fig. 6.5E). Similar to Veh-ENDO animals, no 'treatment' effect of ENDO versus saline was detected ($P = 0.3$), nor any changes in the variability of Iba-1-immunoreactivity at any spinal level between the groups (all comparisons $P \geq 0.1$) (AUC mean and variability comparisons $P \geq 0.3$) (Fig. 6.5F).

6.4.5 Spontaneous pain behaviour is unchanged in ENDO mice primed by acute TLR4 blockade

Facial grimace scores were observed for all ENDO mice throughout the three-week experimental time course. As with analysis of spinal glial reactivity, we have previously demonstrated that spontaneous pain behaviour in ENDO animals is significantly heightened compared to control mice (no endometriosis-like lesions) (Dodds *et al.*, 2018 - Chapter 5); therefore, comparisons here were made between ENDO drug-treated (1j-ENDO) and vehicle-treated (Veh-ENDO) mice only. Regardless of experimental group (1j-ENDO or Veh-ENDO), the most common facial grimace features observed were orbital tightening, and changes in ear and whisker positions. For Veh-ENDO mice, the median pain score was 1 across all three weeks (range 0-2 for week 1; 0-3 for weeks 2-3) (Fig. 6.6A). 1j-ENDO animals displayed a median facial grimace score of 2 during week 1, and 1 in weeks 2-3 (range 0-2 across all three weeks). Overall, however, no statistical differences in pain criteria were determined between Veh-ENDO and 1j-ENDO mice ($P = 0.09$) or for any particular week (all comparisons $P > 0.1$) (Fig 6.6B).

6.4.6 Figures

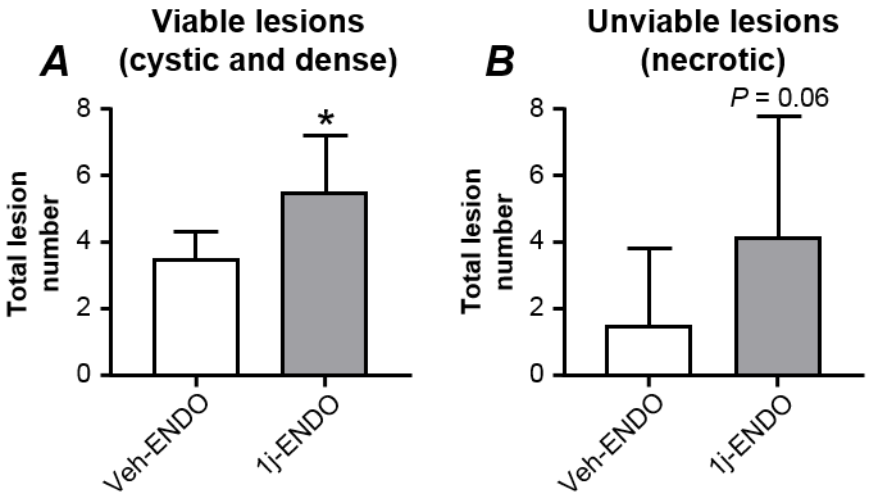

Figure 6.2 Total number of endometriosis-like lesions established in Veh-ENDO and 1j-ENDO mice. (*A*) Summarised data shows a significantly greater number of viable (cystic- and dense-type) endometriosis-like lesions were retrieved from 1j-ENDO mice compared to Veh-ENDO ($n = 6$ animals per group). **(*B*)** Whilst not considered an authentic lesion phenotype, the number of retrieved necrotic-type tissues was also compared. No statistical differences were observed between ENDO groups, although this approached significance for 1j-ENDO mice ($P = 0.06$). * denotes $P < 0.05$. Data expressed as mean ± SD.

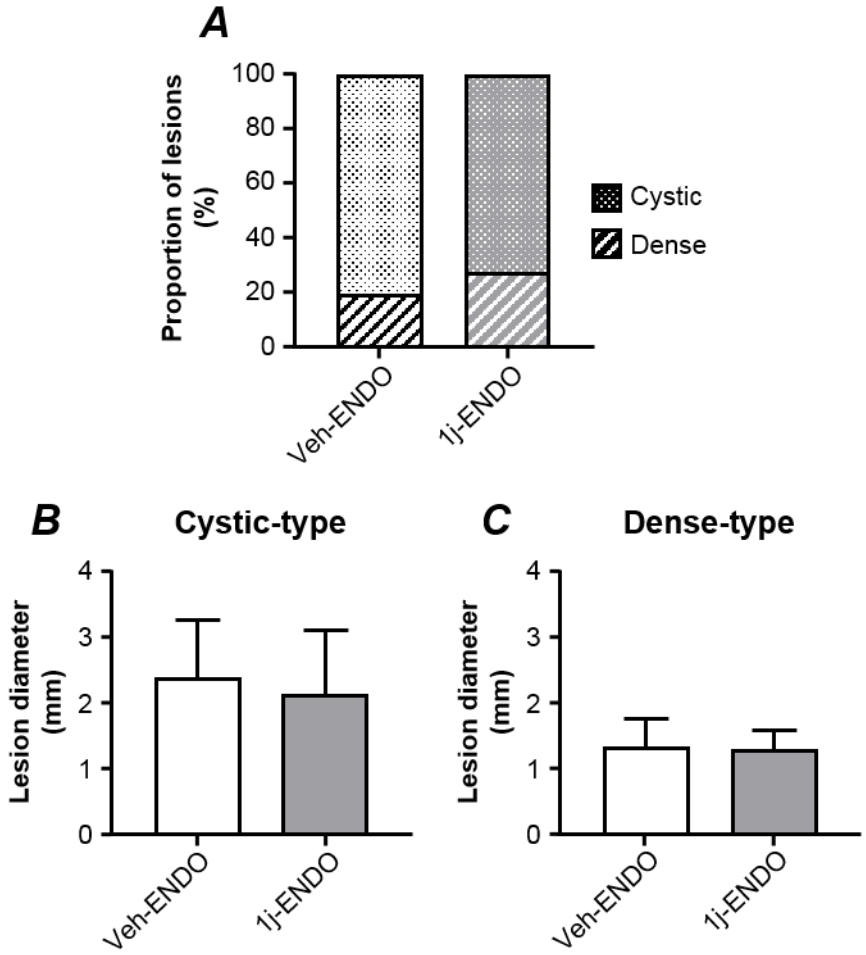

Figure 6.3 Endometriosis-like lesion phenotype profiles in Veh-ENDO and 1j-ENDO mice. (*A*) Proportions of cystic- and dense-type lesion types were similar between Veh-ENDO and 1j-ENDO animals (n = 6 animals per group). **(*B*)** The average diameter of cystic-type lesions from 1j-ENDO mice was not significantly different from those developed by Veh-ENDO mice. **(*C*)** Likewise, the size of dense-type lesions were comparable between Veh- and 1j-ENDO animals. Data in ***(B-C)*** expressed as mean ± SD.

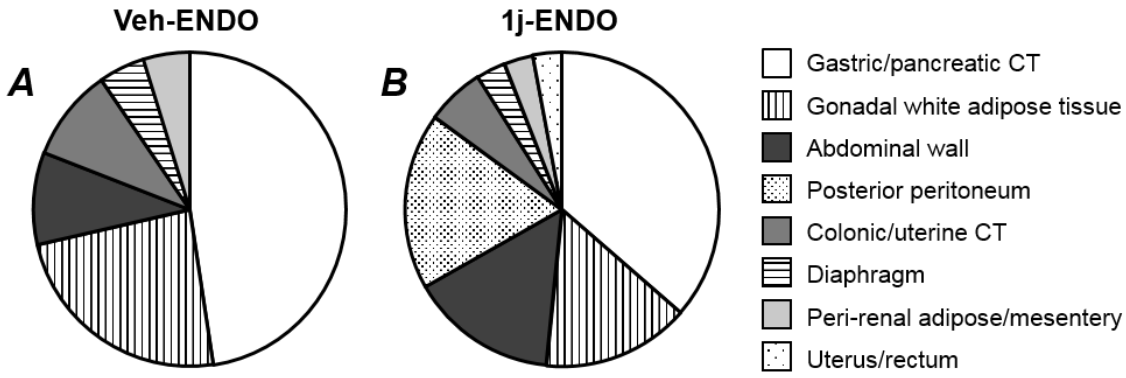

Figure 6.4 Anatomical locations of endometriosis-like lesion establishment in Veh-ENDO and 1j-ENDO mice. (*A*) Summarised data of the proportions of endometriosis-like lesions found in each peritoneal location of Veh-ENDO mice ($n = 6$ animals). The majority of lesions were found attached to connective tissues near the stomach/pancreas and uterus/colon, within the gonadal white adipose tissue, and on the anterior abdominal wall. **(*B*)** 1j-ENDO mice ($n = 6$ animals) developed most lesions in the same locations, as well as a large proportion on the posterior peritoneum and one lesion on the uterine surface. *CT* = connective tissue.

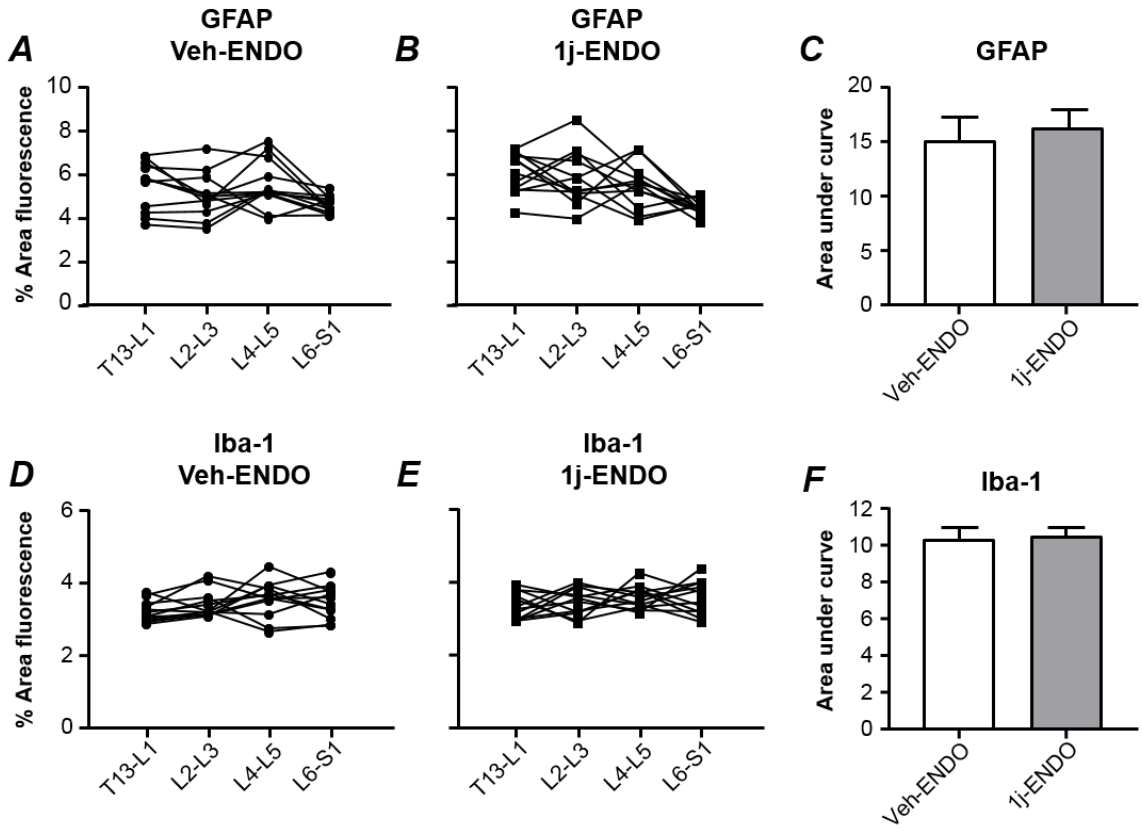

Figure 6.5 Glial immunoreactivity expression in the thoracolumbar spinal dorsal horn of Veh-ENDO and 1j-ENDO mice. (*A-B*) The area of astrocytic GFAP immunostaining in spinal dorsal horns (linked by horizontal bars) from Veh-ENDO and 1j-ENDO mice, respectively, were highly variable across spinal levels T13-L5 and relatively consistent in L6-S1. **(*C*)** Summarised data comparing AUC values for each dorsal horn indicate no statistical differences were detected between groups. **(*D-E*)** Percentage area of each dorsal horn occupied by Iba-1 immunostaining in was reasonably consistent across spinal levels T13-S1 for both Veh-ENDO and 1j-ENDO mice, respectively. **(*F*)** Summarised data comparing AUC values for each dorsal horn also indicate that Iba-1 values were unchanged between Veh-ENDO and 1j-ENDO mice. Y-axis labels in **(*B*)** apply to **(*A*)**; Y-axis labels in **(*E*)** apply to **(*D*)**. All groups n = 12 from 6 animals. Data in ***(C, F)*** expressed as mean ± SD.

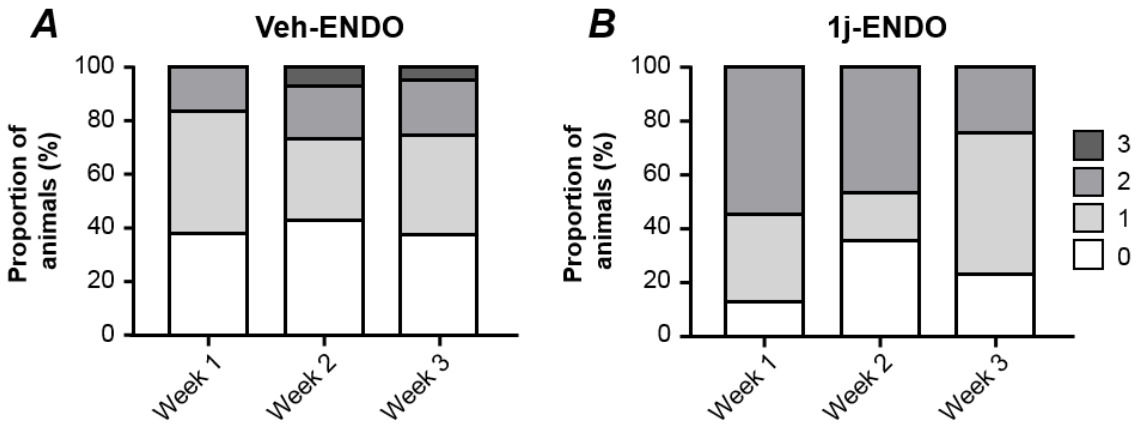

Figure 6.6 Facial pain scores following development of endometriosis-like lesions in Veh-ENDO and 1j-ENDO mice. (*A*) Veh-ENDO mice (n = 6 animals) had a median grimace score of 2 in the first week following endometriosis-like lesion induction, and a median score of 1 thereafter. **(*B*)** No statistical differences in spontaneous pain were detected in 1j-ENDO mice (n = 6 animals), which displayed median scores of 1 across the three-week developmental period.

6.5 Discussion

The principal finding from this study was that the occurrence of endometriosis-like lesions was enhanced by transient peritoneal blockade of the innate immune receptor, TLR4, during recapitulated retrograde menstruation. Firstly, these data support the large body of literature suggesting that an intact, competent immune system is necessary to minimise lesion development. Secondly, we have verified that the heightened incidence of endometriosis-like lesions observed in *TLR4*-knockout ENDO mice (Dodds *et al.*, 2018 - Chapter 5) can be reasonably well reproduced by pharmacological inhibition of TLR4 in wildtype animals. The method of TLR4 blockade in the present study most closely represents the wildtype donor to *TLR4*-knockout recipient mouse model examined previously. This similarity in results between studies suggests that the TLR4-associated effects observed in genetically-modified ENDO mice, at least regarding total lesion number, is a genuine consequence of reduced receptor activation with negligible genetic compensation.

Moreover, the increase in lesions observed in 1j-ENDO mice further demonstrates that peritoneal TLR4 activation can regulate the early induction stages of endometriosis. It has been reported that adherence of ectopic endometrial implants to mesothelial structures in mice can occur within 24 hours (Eggermont *et al.*, 2005), and it is therefore critical that the immune system is sufficiently responsive toward endometrial debris at the onset of retrograde menstruation. As mentioned earlier, TLR4 expression on peripheral immune cells is important for generating inflammatory responses following recognition of exogenous (pathogenic/infectious) and host-derived (damage/stress-related) factors. It is plausible that this mechanism is abnormal and contributes to the early development of endometriosis in women, given that altered expression of TLR4 and its various ligands (such as LPS, Hsp-70 and HMGB1) has been described in the peritoneum, eutopic

endometrium and ectopic lesions from endometriosis patients. In addition, a greater total yield of unviable necrotic (unattached, uncleared) lesions were found in 1j-treated ENDO animals compared to controls. Thus, TLR4-mediated detection of 'foreign' endometrium and subsequent initiation of inflammatory responses directed toward tissue elimination, may assist with restricting the initial formation of endometriosis lesions.

The proportional classifications of viable lesions (cystic and dense) and their respective average sizes were unaffected by administration of 1j in ENDO animals. A probable explanation for these observations is endometrial tissues that achieved peritoneal attachments were able to progress as standard once effectiveness of the TLR4 antagonist had expired. However, lesions retrieved from 1j-treated ENDO animals developed in several more locations compared to Veh-ENDO controls. As postulated in our study of *TLR4*-knockout ENDO mice, tissue clearance and/or endometrial cell proliferation may be altered due to the loss of TLR4 signalling pathways.

Regarding central consequences, spinal glial-related adaptations and spontaneous pain behaviour in ENDO mice were unaltered by acute blockade of peripheral TLR4. It is likely that development of inflammatory pain subsequent to lesion establishment largely occurred at a time when drug antagonism of TLR4 had subsided. Although the pharmacodynamics of 1j are yet to be fully characterised, it is nevertheless understood that (+)-Naltrexone (of which 1j is an analogue) is readily able to cross the blood-brain barrier, with a biological half-life of approximately 4-10 hours (Crabtree, 1984). Therefore, any central effects of 1j observed beyond 24 hours, comparable to (+)-Naltrexone, probably occurred in the presence of unabated spinal TLR4 activity. In other words, it is doubtful that a long-term reduction in central TLR4 activity, driven by acute systemic drug administration, was responsible for the lesion-associated pain behaviour observed in this study. Additionally, this finding supports the notion that central TLR4 activity contributes to pain behaviour

associated with endometriosis, as we previously found that genetic knockout of TLR4 resulted in reduced facial grimace scores despite an increase in lesion incidence.

1j-ENDO animals developed a greater total incidence of endometriosis-like lesions, yet with gross macroscopic characteristics that did not significantly differ from Veh-ENDO controls. Despite an ambiguous clinical relationship between the extent of endometriosis lesions and pain, in many other experimental inflammatory conditions, a greater disease burden generally correlates with an increase in pain behaviour (i.e. graded responses). It may therefore be speculated that the lack of differences in spinal neuroimmune adaptations and/or pain between groups was related to aspects of lesion pathology that were not examined. For instance, it is possible that acute blockade of TLR4 could alter the degree and/or early composition of inflammatory mediators within 1j-ENDO lesions, rendering them more innocuous to peritoneal afferent neurons and immune cells. In this state, reduced transmission of noxious inflammatory signals (albeit from a greater number of lesions) reaching the CNS might limit the degree of change in spinal glial reactivity, and associated pain. Alternatively, these results could imply a threshold effect, whereby additional development of endometriosis-like lesions beyond a specific quantity or inflammatory capacity does not cause further spinal or behavioural consequences. These issues require further investigation.

In any case, it remains relevant to explicitly determine the analgesic potential of directly blocking central TLR4 activity (e.g. intrathecal application) in mice with endometriosis-like lesion-induced pain. Future experiments may examine these effects over chronic time-courses and/or after long-term establishment of endometriosis-like lesions (e.g. from three weeks post-induction), to more accurately mimic the clinical situation. Such research will contribute not only to the knowledge of mechanisms involved in endometriosis pain, but potentially also other inflammatory conditions where TLR4-mediated neuroimmune

adaptations have been implicated. We can propose from our preliminary results that intraperitoneal application of TLR4 antagonists (for any reason) during menstruation in women would be unadvisable, as this might increase their risk of developing endometriosis lesions. However, the short- and long-term effects of systemic TLR4 blockade on pre-established lesions are unknown, as well as potential off-target effects on adjacent visceral systems, such as gut microbial populations and aspects of fertility (Schjenken *et al.*, 2015). Therefore, additional studies on the peripheral administration of 1j-like drugs for the treatment of endometriosis are necessary.

Statement of Authorship

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Publication Details	N/A

Principal Author

Name of Principal Author (Candidate)	Kelsi N. Dodds		
Contribution to the Paper	Designed experiments, performed all experiments, analysed and interpreted data, generated figures, and wrote manuscript.		
Overall percentage (%)	85%		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
Signature		Date	30/04/2018

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);
ii. permission is granted for the candidate in include the publication in the thesis; and
iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

Name of Co-Author	Elizabeth A. H. Beckett		
Contribution to the Paper	Supervised development of work, assisted with experimental design, data interpretation, and reviewed the manuscript.		
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Contribution to the Paper	Helped to interpret data, and to evaluate and edit the manuscript.		
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Contribution to the Paper	Supervised development of work, assisted with experimental design, data interpretation, and reviewed the manuscript.		
Signature		Date	30/04/2018

Chapter 7. Prior activation of Toll-like receptor 4 (TLR4) alters lesion development, neuroimmune adaptations and pain behaviour in a mouse model of endometriosis

This chapter is an unpublished and unsubmitted primary research study written in manuscript style.

7.1 Abstract

Chronic inflammation and persistent pain are cardinal features of endometriosis, and it is thought that aberrant inflammatory processes facilitate the development of endometriosis lesions. Enhanced production of proinflammatory mediators may result from immune cell priming, where an initial inflammatory episode sensitises peripheral macrophages and central glial cells to over-respond to new challenges. This can lead to perpetuation of peripheral inflammatory conditions, and an associated development of central sensitisation and pain. Priming of central glia may additionally contribute to peripheral inflammation by stimulating the antidromic propagation of neurogenic inflammatory signals. We therefore investigated whether peripheral or central priming (i.e. in two distinct, anatomically separate compartments) of the innate immune receptor, Toll-like receptor 4 (TLR4), could alter the development of endometriosis and associated pain. Endometriosis-like lesions (ENDO) were induced in female BALB/c mice via intraperitoneal injection of syngeneic donor endometrium, four hours following intraperitoneal (IP) or intrathecal (IT) administration of the TLR4 agonist, lipopolysaccharide (LPS) or vehicle (Veh). Compared to Veh-IP, lesions from peripherally primed LPS-IP ENDO mice were significantly increased in number and occupied a wider variety of peritoneal locations. LPS-IP ENDO mice also produced higher facial grimace scores; increased spinal immunoreactivity to microglial Iba-1; and altered spinal concentrations of IFN-γ, TNF-α, IL-10, IL-17A and GM-CSF. Centrally primed LPS-IT ENDO mice displayed alterations in spinal protein

expression of IFN-γ and IL-6, and developed endometriosis-like lesions that were significantly heavier in weight compared to Veh-IT. These data support that a prior TLR4-mediated peripheral inflammatory event can potentiate development of endometriosis-like lesions and enhance lesion-associated pain. In addition, proinflammatory signalling induced by central immune priming may promote lesion growth in the periphery.

7.2 Introduction

Endometriosis is a common gynaecological condition in women characterised by the development of endometrial-like lesions in extra-uterine locations. Typically, lesions form in the abdominopelvic cavity, and are associated with both focal and systemic chronic inflammatory processes. The aetiology of endometriosis is incompletely understood, though a primary hypothesis involves the retrograde transport and peritoneal deposition of endometrial debris during menstruation (Sampson, 1927). While this reflux mechanism may be necessary to provide the endometrial tissues that progress into mature lesions, it is not wholly sufficient to explain lesion pathogenesis. Hence, dysregulation of peritoneal immunity and the concomitant inflammation observed in endometriosis is thought to facilitate, as well as result from, the development of lesions (Herington *et al.*, 2011; Ahn *et al.*, 2015). Evidence additionally suggests that lesion-associated inflammatory processes may contribute to the frequent clinical manifestation of persistent pain, via peripheral and central sensitisation of nociceptive neural pathways (Greaves *et al.*, 2015; Chen *et al.*, 2016).

Inflammatory signalling mediated by the innate immune receptor, Toll-like receptor 4 (TLR4), has been implicated in endometriosis pathology (Khan *et al.*, 2018). TLR4 expressed by peripheral macrophages and central glial cells plays a major role in recognising exogenous pathogen and endogenous damage-associated molecular patterns. Ligand stimulation of TLR4, such as via bacterial lipopolysaccharide (LPS; or endotoxin),

elicits the secretion of proinflammatory mediators (including interleukin (IL)-1β and tumour necrosis factor (TNF)-α), which assist in resolving the immune insult. However, aberrant cytokine release attributed to inappropriate TLR4 activity can potentiate inflammatory disorders, as has been described in chronic inflammation of the gastrointestinal tract (Cario, 2010; Frosali *et al.*, 2015) and neuroinflammatory processes of the brain (Glass *et al.*, 2010; Lehnardt, 2010). Studies have thus far agreed that exaggerated TLR4-mediated inflammation may also contribute to the development of both endometriosis and adenomyosis (Guo *et al.*, 2016a), in part by augmenting cellular proliferation (Iba *et al.*, 2004; Khan *et al.*, 2008b; Khan *et al.*, 2010; Khan *et al.*, 2013b; Azuma *et al.*, 2017). It has therefore been posited that the inflammation produced by TLR4 activation in response to refluxed 'foreign' or damaged endometrial cells, could provide suitable conditions within the peritoneal microenvironment for lesions to establish.

These preliminary data suggest a simultaneous relationship between ectopic endometrial tissue and the initial activation of TLR4. However, it is unknown whether a prior or pre-existing TLR4-mediated inflammatory event may also enhance the adherence of endometrial cells to the peritoneum, and subsequent lesion growth. The paradigm of immune cell sensitisation or 'priming' is well established, where previous exposure or conditioning to a 'first-hit' immune insult can dramatically alter the strength and duration of responses to new 'second-hit' immune challenges (Morris *et al.*, 2015). Importantly, this can result in maladaptive inflammation, which culminates in the development of pathological conditions. Indeed, endometriosis patients often present with multiple comorbid inflammatory disorders, including painful bladder syndrome, irritable bowel syndrome and migraine (Tietjen *et al.*, 2007; Seaman *et al.*, 2008; Smorgick *et al.*, 2013).

Acute peripheral inflammatory episodes, such as those mediated by TLR4, can also lead to priming of glial cells (astrocytes and microglia) within the central nervous system (CNS)

(Guo & Schluesener, 2006; Hains *et al.*, 2010). Highly reactive glia are well known to facilitate central sensitisation and heightened pain responses, as their release of proinflammatory mediators can alter the molecular composition of adjacent spinal neurons; thereby increasing their capacity to transmit nociceptive signals to the brain (Dodds *et al.*, 2016 - Chapter 2). We have previously demonstrated this peripheral-to-central neuroimmune effect (or 'spinal consequence') in endometriosis, where adaptations in spinal glial reactivity and pain behaviour occur in association with peritoneal endometriosis-like lesions (Dodds *et al.*, 2018 - Chapter 4, 2018 - Chapter 5). We therefore hypothesise that a TLR4-primed peritoneal immune system ('first-hit') may over-respond to the 'second-hit' of endometrial debris, increasing both susceptibility to lesion development and further enhancing the accompanying pain.

Central sensitisation evoked by neuroimmune adaptations has also been proposed to antidromically stimulate afferent neurons, with the propagation and release of neuropeptide transmitters in peripheral tissues. In this scenario, glial priming not only affects projection neurons contributing to the heightened perception of pain, but may also activate sensory neurons and neurogenic inflammation that perpetuate chronic inflammatory conditions in the periphery. This mechanism appears to contribute to the progression of arthritis (Boyle *et al.*, 2006; Boettger *et al.*, 2010; Bressan *et al.*, 2010; Luo *et al.*, 2014), and sensitisation of both the colon and bladder (Majima *et al.*, 2018). The ability for central TLR4 priming to influence the growth of endometriosis lesions remains to be determined (McKinnon *et al.*, 2015; Dodds *et al.*, 2016 - Chapter 2; Dodds *et al.*, 2018 - Chapter 4).

This study sought to determine whether peripheral priming of TLR4 with LPS can alter the development of peritoneal endometriosis-like lesions in a minimally-invasive mouse model (Dodds *et al.*, 2017 - Chapter 3). Further, we assessed for spinal neuroimmune adaptations that may contribute to central sensitisation and lesion-associated pain (i.e. peripheral-to-

central consequence). In a second series of experiments endometriosis-like lesions were characterised following central priming of TLR4, to explore whether spinal neuroimmune manipulations can influence the development of lesions within the peritoneum (i.e. central-to-peripheral consequence). Our experimental paradigms thus used LPS to engage either the immune (peripheral TLR4) or immune-like (central TLR4) systems, respectively. Given this fundamental difference, direct statistical comparisons between peripherally and centrally TLR4-primed groups were not performed.

7.3 Methods

7.3.1 Animals

Female BALB/c mice (*n* =72) 8-13 weeks in age, weighing 19.3 ± 1.1 g, were obtained from the University of Adelaide Laboratory Animal Services. All animals underwent daily cervical smear testing to determine their oestrous cycle phase, as described previously (Dodds *et al.*, 2015). Each was selected for use only when in the pro-oestrus phase (high oestrogen; pre-ovulation), as this timing has been shown to support the development of endometriosis-like lesions in gonad-intact BALB/c mice (Dodds *et al.*, 2017 - Chapter 3). All animal use was conducted in accordance with the National Health and Medical Research Council Australian code for the care and use of animals for scientific purposes (8^{th} edition, 2013) and the University of Adelaide Animal Ethics Guidelines, and was approved by the University of Adelaide Animal Ethics Committee.

7.3.2 Minimally-invasive mouse model of endometriosis

Recipient mice (ENDO; *n* = 36) were primed with a single peripheral (intraperitoneal; IP) or central (intrathecal; IT) administration of the TLR4 agonist, LPS, or vehicle solution prior to the induction of endometriosis-like lesions (ENDO).

7.3.2.1 Experiment 1 – Peripheral TLR4 priming with LPS

Using a 30-gauge needle, the first cohort of recipient ENDO animals received a 100 µg/kg dose of LPS via IP injection, at the ventral midline between the left inguinal nipples (LPS-IP; *n* = 12). Control (vehicle-treated) ENDO animals (Veh-IP; *n* = 12) were injected with an equivalent volume of vehicle solution (sterile saline (0.9% NaCl)).

7.3.2.2 Experiment 2 – Central TLR4 priming with LPS

The second cohort of recipient ENDO animals were anaesthetised with inhaled isoflurane (1.5-2% in O^2), and a 2 cm^2 patch of hair was shaved from the lumbar region of the spine. The site was sterilised with 70% ethanol, and a 30-gauge needle attached to a 25 µl Hamilton syringe via 25 cm of PE-10 tubing was inserted approximately 5 mm between the L4-L6 vertebrae. Over 1 min, 500 ng LPS in 1 µl sterile saline plus 5 µl of sterile saline flush were injected into the IT space (LPS-IT; *n* = 6). Control (vehicle-treated) ENDO animals (Veh-IT; *n* = 6) were injected in the same manner with an equivalent volume of vehicle and flush solutions.

7.3.2.3 Induction of endometriosis-like lesions

Approximately three hours following LPS or vehicle priming of recipient ENDO animals, donor mice (*n* = 36) were sacrificed by cervical dislocation whilst under deep inhaled isoflurane anaesthesia. The uterus was removed placed into a sterilised glass Petri dish containing cold (4°C) 0.01 M phosphate-buffered saline (PBS). Each horn was opened along the mesometrial border and pinned flat to the Sylgard®-lined (Dow Corning; Michigan, USA) base of the Petri dish using entomology pins. The endometrium (40 mg) was then carefully removed by sharp dissection and further cut into segments of 2-3 mm^2. The resultant endometrial fragments were suspended in a 1-ml syringe containing 0.5 ml sterile saline and passed once through a 21-gauge needle, to ensure smooth delivery of

content to recipient ENDO animals.

At a total interval of four hours post-LPS or vehicle administration, recipient ENDO mice received the donor endometrial suspension, via a second IP injection at the same anatomical site (1 donor: 1 recipient). The timing of drug and ENDO administration was selected, as it is within the common peak duration for a sickness response elicited by systemic LPS exposure in mice (Norden *et al.*, 2016). Following 21 days of development, ENDO animals were deeply anaesthetised with isoflurane gas and decapitated (Fig. 7.1).

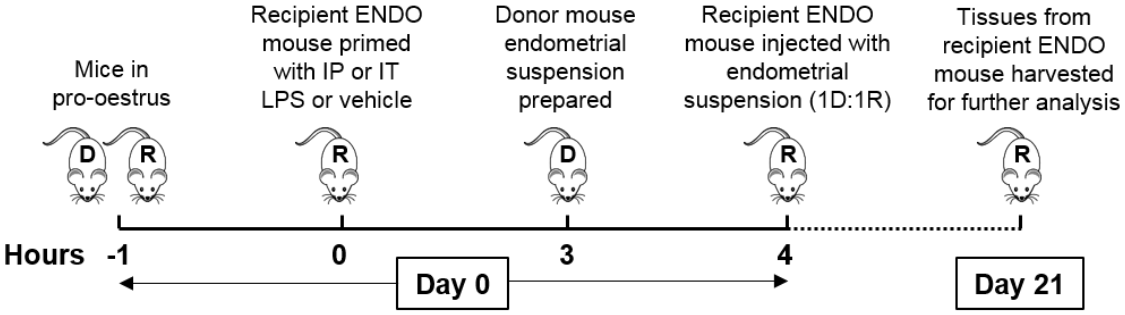

Figure 7.1 Experimental timeline for peripheral and central drug priming of TLR4 and the subsequent induction of endometriosis-like lesions in ENDO mice. Pro-oestrus recipient mice (ENDO) were primed by intraperitoneal (IP) or intrathecal (IT) injection of the TLR4 agonist, lipopolysaccharide (LPS) or vehicle (Veh), four hours prior to IP injection of pro-oestrus donor endometrial tissue. Following 21 days of endometriosis-like lesion development, recipient ENDO animals were sacrificed, and tissues of interest were collected for further analysis.

7.3.2.4 Assessment of endometriosis-like lesion characteristics

After thorough examination of the peritoneal cavity in all ENDO mice, the number and location of potential endometriosis-like lesions was recorded. Lesions from the first subset of ENDO animals in *Experiment 1* and all animals in *Experiment 2* (for multiplex ELISA analysis; see below) were pinned to the Sylgard®-coated base of a Petri dish containing cold (4°C) PBS, and photographed with a digital camera (iSight; Apple Inc.; California, USA) mounted on the eyepiece of a stereomicroscope (#SMZ445; Nikon; Tokyo, Japan). Lesions were then measured from the images using ImageJ Fiji software (Schindelin *et al.*,

2012), and classified based on their macroscopic appearance (Dodds *et al.*, 2017 - Chapter 3).

Endometriosis-like lesions and control tissues (lymph nodes, fat etc.) retrieved from the second subset of animals in *Experiment 1* (for immunohistochemical analysis; see below) were immersed in cold (4°C) 10% neutral-buffered formalin (Chem-Supply; South Australia, Australia) overnight (4°C). All tissues then underwent standard histological processing, sectioning and staining for hematoxylin and eosin to confirm endometriosis-like lesions, in which endometrial glands and stroma were present (Clement, 2007). The size and classification of confirmed lesions (cystic, dense or necrotic) was subsequently determined, as described previously (Dodds *et al.*, 2017 - Chapter 3).

Due to their variable shape, the size of endometriosis-like lesions from both cohorts of animals was defined as the maximum diameter of their greatest longitudinal surface. Only lesions classified as dense- or cystic-type were counted toward the total number of viable lesions obtained from an ENDO animal. Necrotic-type lesions, except where specified, were excluded from analysis due to inconsistencies in meeting the diagnostic criteria (Dodds *et al.*, 2017 - Chapter 3).

7.3.3 Multiplex enzyme-linked immunosorbent assay (ELISA) for cytokine protein quantification

7.3.3.1 Tissue processing

Proinflammatory cytokine expression was assessed in spinal cord segments T13-L1, L2-L3, L4-L5 and L6-S1 from the first subset of ENDO mice in *Experiment 1* and all animals in *Experiment 2* (n = 6 per group). For statistical analysis, cytokine concentrations from each spinal level were combined per animal. Endometriosis-like lesions (pooled per animal) were also examined. Each sample was weighed and snap frozen with liquid

nitrogen, then submerged in a 10 µl/mg.tissue^{-1} volume of radio-immunoprecipitation assay buffer (RIPA) supplemented with 1% protease inhibitor cocktail (#P8340 Sigma Aldrich; New South Wales, Australia). Samples were homogenised in the buffer for 30 s at room temperature (RT) using a motorised pellet pestle (components #Z359963 and #Z359971; Sigma Aldrich; New South Wales, Australia), and then centrifuged at 15,000 RPM for 30 min at 4°C. The resulting supernatant was isolated, aliquoted and stored at -80°C. Total protein concentration was quantified using the Pierce™ BCA Protein Assay Kit (#23225; ThermoFisher Scientific; Victoria, Australia) as per the manufacturer's instructions. A working concentration of 2 mg/ml for all samples was used for cytokine analysis.

7.3.3.2 Cytokine quantification

Cytokine concentrations (pg/mL) were measured using MILLIPLEX® MAP Mouse High Sensitivity T-cell Magnetic Bead Panel kit (#MHSTCMAG-70K; Merck Millipore; Darmstadt, Germany), prepared as per manufacturer's instructions. Cytokines analysed were: IL-1β, IL-2, IL-6, IL-10, IL-17A, interferon (IFN)-γ, TNF-α and granulocyte-macrophage colony stimulating factor (GM-CSF). Each 96-well plate included an 8-point standard curve and two quality controls provided by Merck Millipore. All standards, quality controls and samples were loaded onto plates in duplicates. Plates were read using a MAGPIX® Luminex xMAP® platform, and experimental data calibrated against the standard curves of each corresponding cytokine using a cubic spline algorithm calculated by MILLIPLEX® Analyst 5.1 software (Merck Millipore; Darmstadt, Germany).

7.3.4 Facial grimace scoring for assessment of pain behaviour

Pain behaviour exhibited by ENDO mice was measured in a blinded manner throughout the 21-day development period for all groups of ENDO mice. Once daily (between 09:00-

10:00 am) from 24 h post-tissue transfer, animals were individually weighed and 2-3 images of the face photographed using a digital camera (iSight; Apple Inc.; California, USA). Images were then de-identified, and scores were determined using a validated mouse grimace scale designed to measure spontaneously emitted pain (Langford *et al.*, 2010). The facial grimace scoring method measures five distinct criteria: orbital tightening, nose bulge, cheek bulge, ear position and whisker position; with each criterion counted as 0 = absent, 1 = moderate, and 2 = severe. Total daily scores for each animal were then grouped per week (1-3) of the experimental time-course.

7.3.5 Fluorescent immunohistochemistry for visualisation of glial markers

7.3.5.1 Tissue processing

The immunohistochemical protocol for visualising spinal glia in this study has been described in detail previously (Dodds *et al.*, 2018 - Chapter 4). Briefly, spinal cords (spanning thoracic to sacral, inclusive) from the second subset of ENDO animals in *Experiment 1* (n = 6 per group) were carefully removed and immersed in 4% paraformaldehyde fixative (PFA; pH 7.2) (4°C; overnight). Tissues were then washed and cryoprotected in 30% sucrose (4°C; two nights). Following dissection into regions T13-L1, L2-3, L4-5 and L6-S1, spinal segments were submerged into individual plastic moulds containing Tissue-Tek® OCT compound (#IA018; ProSciTech; Queensland, Australia) and snap frozen. Spinal segments were then cryostat sectioned (10 μm) in duplicate per antibody label, and collected onto SuperFrost® glass microscope slides (Menzel-Gläser; Braunschweig, Germany). After air-drying, slides were briefly rinsed before undergoing heat-mediated antigen retrieval (97°C for 10 min) with sodium citrate buffer (0.01 M with 0.05% Tween 20; pH 6.0), and cooled with 0.01% Tween 20.

To visualise astrocytes, sections were blocked with 10% normal donkey serum/0.01%

Triton X-100 (RT; 1 h). Sections were then incubated in Alexa Fluor® 488-conjugated mouse monoclonal anti-glial fibrillary acidic protein (GFAP) antibody (#53-9892-82, clone GA5; RRID: AB_10598515; 1 µg/ml; eBioscience; California, USA) (4°C; two nights). For microglial assessment, sections were blocked with 10% normal donkey serum/0.01% Triton X-100 (RT; 2 h). Slides were then incubated in rabbit polyclonal anti-ionised calcium-binding adaptor molecule 1 (Iba-1) (#019-19741; RRID: AB_839504; 0.5 µg/ml; WAKO; Osaka, Japan) (4°C; two nights). After washing, sections were then incubated with donkey anti-rabbit Alexa Fluor® 488 secondary antibody (#ab150073; RRID: AB_2636877; 2 µg/ml; Abcam; Cambridge, UK) (RT; 2 h). All sections were given a final rinse and mounted with Tris-based Fluoro-Gel medium (#IM030; ProSciTech; Queensland, Australia).

7.3.5.2 Image acquisition

Slides were viewed with a Leica TCS SP5 scanning confocal microscope (Leica Microsystems; Wetzlar, Germany) using appropriate excitation wavelengths at 20x magnification with oil immersion. Images were acquired using Leica Application Suite Advanced Fluorescence version 2.6.3 (Leica Microsystems; Mannheim, Germany). Final images are digital composites of 1-1.5 µm Z-series scans (approximately 8-14 optical sections through a depth of 10-16 µm). All images per antibody label were taken at the same gain and offset parameters between animals. Each spinal dorsal horn per section was imaged separately.

7.3.5.3 Image analysis

Semiquantitative analyses were performed on collected images using ImageJ Fiji software (Schindelin *et al.*, 2012). All images were measured blinded as to animal treatment. Maximized Z-stack of images were converted from Leica image files (.lif) to 8-bit

greyscale .tiff images, and signal pixels of positive staining areas in the region of interest (ROI) were selected. The ROI was an ellipsoid shape that remained a consistent size for each spinal level between animals, and was positioned over Rexed's laminae I-IV (Dodds *et al.*, 2018 - Chapter 4). The percentage of immunofluorescence in the ROI was calculated, and the duplicate area measurements for each dorsal horn were averaged to obtain a single percentage area value per dorsal horn, per spinal level, per animal.

7.3.6 Solutions and Drugs

LPS (*Escherichia coli* serotype O111:B4; #L2630; lot no. 091M4031V; Sigma Aldrich; Massachusetts, USA) was dissolved from powder in sterile deionised water to produce stock concentrations of 20 mg/ml, and frozen at -20°C. When required, aliquots were diluted using sterile saline to 20 µg/ml (for IP injections in *Experiment 1*) or 500 ng/µl (for IT injections in *Experiment 2*). PBS (pH 7.4) was composed of (in mM): NaCl 13.7; KCl 0.27; KH_2PO_4 0.15 and Na_2HPO_4 0.8. PFA, sodium citrate buffer and all antibody-related solutions were made with PBS as the solvent. RIPA was composed of (in mM): HEPES 50; NaCl 150; sodium deoxycholate 12; NaF 10; $Na_4P_2O_7$ 10; EDTA 5; in 1% Triton X-100; 0.1% SDS; and 5% ethylene glycol.

7.3.7 Statistical analysis

All data were compared using GraphPad Prism® 7 software (GraphPad Software Inc.; California, USA). A D'Agostino-Pearson omnibus K2 test was initially performed to assess normality. Total number of retrieved viable (cystic- and dense-type) and necrotic-type lesions were assessed using a Student unpaired two-tailed *t*-test. The proportions of cystic- and dense-type lesions between ENDO mice were determined by a Kruskal-Wallis test with Dunn multiple comparisons, and their sizes by a two-tailed Mann-Whitney test. The combined average weights, as well as cytokine concentrations within endometriosis-like

lesions, were assessed by a Student unpaired two-tailed *t*-test with or without Welch correction. A two-way ANOVA with Bonferroni post-hoc tests were used to determine differences in facial pain scores and spinal glial GFAP and Iba-1 immunolabelling values between appropriate ENDO groups. Area under the curve (AUC) scores for GFAP and Iba-1 were generated for each dorsal horn per animal, and compared between ENDO mice using a Student unpaired two-tailed *t*-test with or without Welch correction. For spinal cytokine concentrations, a two-tailed Mann-Whitney test was performed. Microsoft® Excel® 2013 (Microsoft Corporation; Washington, USA) was additionally used to generate F-test scores of variability around the standard deviation (SD) for glial immunolabelling values and cytokine concentrations. Unless otherwise specified, data in the text are expressed as mean ± SD, and *P* values of <0.05 were considered statistically significant.

7.4 Results

Both peripheral and central administration of LPS produced obvious sickness responses in all recipient ENDO animals at the time of endometriosis-like lesion induction. Observed clinical signs included hunched posture, ruffled fur, severe orbital tightening, tachypnoea, listlessness and reluctance to move. At necropsy following 21 days of development, it was confirmed that endometriosis-like lesions were successfully established in all ENDO mice.

7.4.1 Total number of endometriosis-like lesions developed by ENDO mice is significantly increased with peripheral but not central priming of TLR4

To reiterate, only lesions that were classified as cystic- and dense-type were counted toward the total number of viable lesions developed by each ENDO animal. Veh-IP ENDO mice developed 1-5 endometriosis-like lesions (n = 37 lesions from 12 animals; average 3.1 ± 1.4), with a mass of injected endometrial tissue 41.1 ± 1.9 mg in 27.8 ± 1.7 pieces. A

significantly greater number of lesions were retrieved from peripherally primed LPS-IP ENDO mice (P = 0.03) compared to Veh-IP, where 3-9 lesions (n = 56 lesions from 12 animals; average 4.7 ± 1.9) developed from 41.7 ± 0.9 mg in 27.2 ± 2.3 pieces (Fig. 7.2A). Veh-IT ENDO mice developed 3-5 endometriosis-like lesions (n = 23 lesions from 6 animals; average 3.8 ± 0.8) from 40.5 ± 0.8 mg donor endometrial tissue in 29.5 ± 1.6 pieces. No statistical difference was observed for the centrally primed LPS-IT ENDO group (P = 0.06) compared to Veh-IT, with 2-4 endometriosis-like lesions (n = 18 lesions from 6 animals; average 3.0 ± 0.6) developed from 42.0 ± 1.3 mg donor endometrium in 27.0 ± 0.7 pieces (Fig. 7.2B).

Necrotic-type endometriosis-like lesions have generally been considered unviable due to inconsistencies in demonstrating clear epithelial glands and stroma (Dodds *et al.*, 2017 - Chapter 3). However, the number of necrotic lesions was previously found to be significantly increased in *TLR4*-knockout ENDO mice (Dodds *et al.*, 2018 - Chapter 5); hence their incidence was also recorded in this study. An average of 2.6 ± 2.1 necrotic-type lesions were retrieved from Veh-IP ENDO animals, which was comparable to peripherally primed LPS-IP ENDO animals developing 2.0 ± 2.1 lesions (P = 0.5) (Fig. 7.2C). Veh-IT ENDO and centrally primed LPS-IT ENDO mice also developed a similar number of necrotic-type lesions, with averages of 1.8 ± 4.0 and 0.6 ± 0.9, respectively (P >0.9) (Fig. 7.2D).

7.4.2 Greater diversity of peritoneal endometriosis-like lesion locality in ENDO animals with peripheral but not central priming of TLR4

The peritoneal locations where endometriosis-like lesions established have been reported in the minimally-invasive mouse model previously (Dodds *et al.*, 2017 - Chapter 3, 2018 - Chapter 4, 2018 - Chapter 5, 2018 - Chapter 6).

For Veh-IP ENDO animals, the vast majority of lesions (30/37; 81.1%) attached to connective tissues associated with the stomach and pancreas, followed by connective tissues surrounding the colon and uterus (4/37 lesions; 10.8%). A single lesion was found on each of the gonadal white adipose tissue, the surface of the uterus, and the posterior peritoneal wall (2.7% per location) (Fig. 7.3A). Peripherally primed LPS-IP ENDO mice developed 26/56 endometriosis-like lesions (46.4%) associated with gastric/pancreatic connective tissue; 13/56 lesions (23.2%) in gonadal white adipose tissue; 7/56 lesions (12.5%) on the posterior peritoneal wall; 2/56 lesions (3.6%) in the surrounding colonic/uterine connective tissue; and one lesion on the uterus (1.8%). Additional locations included the anterior abdominal wall (6/56 lesions; 10.7%), and on the surface of the bladder (1/56 lesions; 1.8%) (Fig. 7.3B).

For Veh-IT and centrally primed LPS-IT ENDO mice, endometriosis-like lesions were comparably retrieved from the gastric/pancreatic connective tissues (14/23 lesions, 60.9%; and 10/18 lesions, 55.5%, respectively); the gonadal white adipose tissue (6/23, 26.1%; and 4/18, 22.2%); the anterior abdominal wall (1/23, 4.3%; and 2/18, 11.1%), and the uterine/colonic connective tissues (2/23, 8.7%; and 1/18, 5.6%). One lesion from the LPS-IT ENDO group was also located on the surface of the uterus (5.6%) (Fig. 7.3C-D).

7.4.3 Average mass of endometriosis-like lesions is enhanced by central TLR4 priming in ENDO mice while other phenotype characteristics are unchanged

Endometriosis-like lesions pooled and weighed for Multiplex ELISA analysis permitted the calculation of average lesion mass (n = 6 per group). For Veh-IP ENDO animals, lesions weighed an average of 2.7 ± 2.2 mg, which was similar to those collected from peripherally primed LPS-IP ENDO mice (average 3.0 ± 1.0 mg; P = 0.7) (Fig. 7.4A). However, lesions from centrally primed LPS-IT ENDO animals were significantly heavier (P = 0.03) and more variable in mass (P = 0.03) than Veh-IT ENDO animals, with an

average weight of 3.8 ± 1.3 mg compared to 1.9 ± 0.5 mg, respectively (Fig. 7.4B).

In all ENDO groups, cystic-type endometriosis-like lesions were more prevalent than dense-type lesions ($P \leq 0.03$), however the proportions of cystic- and dense-type lesions developed by peripherally and centrally TLR4-primed ENDO animals were similar to their respective Veh-ENDO controls. Cystic-type lesions comprised 87.5 ± 19.9% (33/37 lesions) in Veh-IP ENDO mice and 75.1 ± 27.7% (42/56 lesions) in peripherally primed LPS-IP ENDO ($P > 0.9$). Dense-type lesions accounted for 12.5 ± 19.9% (4/37 lesions) in Veh-IP ENDO animals and 24.9 ± 27.7% (14/56 lesions) in LPS-IP ENDO animals ($P > 0.9$) (Fig.7.4C). The proportion of cystic-type lesions in Veh-IT ENDO mice was 80.6 ± 15.5% (19/23 lesions) and in centrally primed LPS-IT ENDO 80.6 ± 22.2% (15/18 lesions) ($P > 0.9$). Dense-type lesions comprised 19.4 ± 15.5 % (4/23 lesions) from Veh-IT ENDO animals and for LPS-IT ENDO animals 19.4 ± 22.2 % (3/18 lesions) ($P > 0.9$) (Fig. 7.4D).

The average diameter of cystic-type lesions from Veh-IP ENDO mice (2.2 ± 0.9 mm) was similar to those from peripherally primed LPS-IP ENDO mice (2.1 ± 1.1 mm; $P = 0.4$) (Fig. 7.4E). Likewise, the diameter of dense-type lesions from Veh-IP ENDO mice (1.3 ± 0.5 mm) did not significantly differ to those from LPS-IP ENDO counterparts (1.6 ± 0.8 mm; $P = 0.7$) (Fig. 7.4F). Cystic-type lesions from Veh-IT ENDO mice averaged 1.9 ± 0.9 mm in diameter, and were comparable for centrally primed LPS-IT ENDO 1.7 ± 0.9 mm ($P = 0.6$) (Fig. 7.4G). Diameters of dense-type lesions were also unchanged between IT ENDO groups, with a mean of 0.8 ± 0.1 in Veh-IT and 1.3 ± 0.3 in LPS-IT ENDO mice; although this approached significance ($P = 0.05$) (Fig. 7.4H). The diameter of necrotic-type lesions was not recorded.

7.4.4 Cytokine profiles of endometriosis-like lesions from TLR4-primed ENDO animals altered compared to vehicle controls

Protein concentrations were detected for all cytokines of interest in pooled endometriosis-like lesions from each group of ENDO mice. To assist with readability, all data values are specified in Table 7.1. The variability in expression of GM-CSF from peripherally primed LPS-IP ENDO lesions was significantly reduced (P <0.0001) compared to those from Veh-IP ENDO animals, although unchanged in mean values (P = 0.1). Similarly, while means were unaffected (P = 0.9), variability in protein levels of IL-6 was reduced (P = 0.007) from Veh-IP to LPS-IP ENDO animals. No differences in mean values (all comparisons P ≥0.1) or variability (all comparisons P ≥0.08) were observed between Veh-IP and LPS-IP endometriosis-like lesions for IFN-γ, IL-1β, IL-2, IL-10, IL-17A or TNF-α (Fig. 7.5; Table 7.1A).

In endometriosis-like lesions from centrally primed LPS-IT ENDO mice, protein concentrations of GM-CSF were conversely increased in variability compared to Veh-IT ENDO (P = 0.01) and IFN-γ (P <0.0001); although mean values of these cytokines were not statistically different between groups (all comparisons P ≥0.06) (Fig. 7.5). In addition, the variability of IL-17A protein expression from LPS-IT lesions was decreased compared to Veh-IT ENDO (P <0.0001; mean comparison P = 0.5). Statistical differences in mean values (all comparisons P ≥0.3) and variability (all comparisons P ≥0.1) were not detected for IL-1β, IL-2, IL-6, IL-10 and TNF-α (Fig. 7.5; Table 7.1B).

7.4.5 Distinct spinal cytokine characteristics are observed in TLR4-primed ENDO animals

Protein concentrations were also detected for all cytokines of interest in the thoracolumbar spinal cords from each group of ENDO mice. To assist with readability, all data values are

specified in Table 7.2. Levels of spinal IFN-γ from peripherally primed LPS-IP ENDO animals were significantly increased in mean (P = 0.002) and variability (P <0.0001) compared to Veh-IP ENDO controls. Similarly, overall values for TNF-α were increased (P = 0.009) and of greater variability (P = 0.001) from spinal cords of LPS-IP ENDO than Veh-IP ENDO animals. An increased mean protein expression of spinal IL-10 was observed in LPS-IP ENDO compared with Veh-IP ENDO mice (P = 0.0005), as well as IL-17A (P = 0.004); although variability comparisons between the peripherally primed groups were unchanged (P ≥0.09). A heightened variability alone was additionally observed in LPS-IP ENDO animals for the spinal expression of IL-6 (P = 0.005) (mean comparison P = 0.1). No significant differences were detected in mean values (all comparisons P ≥0.2) or variability (all comparisons P ≥0.2) for GM-CSF, IL-1β or IL-2 (Fig. 7.6; Table 7.2A).

For centrally primed animals, a significant increase in mean spinal cytokine expression was only observed for IFN-γ (P = 0.01) in LPS-IT ENDO animals versus Veh-IT ENDO controls (variability comparison P = 0.1) (Fig. 7.6). A greater variability (P = 0.03) but no change in mean spinal IL-6 expression (P = 0.5) was detected between Veh-IT to LPS-IT ENDO animals. Statistical analysis did not determine any significant differences in mean (all comparisons P ≥0.1) or variability values (all comparisons P ≥0.1) for spinal GM-CSF, IL-1β, IL-2, IL-10, IL-17A or TNF-α (Fig. 7.6; Table 7.2B).

7.4.6 Spontaneous pain behaviour associated with early endometriosis-like lesion development is enhanced in ENDO animals with peripheral but not central priming of TLR4

Facial grimace criteria were assessed for all ENDO groups throughout the three-week experimental time-course, with the most common features of orbital tightening and changes in ear position. For peripherally primed LPS-IP ENDO mice, the median grimace scores

were 2, 1 and 1 (range 0-3) across weeks 1-3, respectively. Overall, such scores from LPS-IP ENDO mice were significantly increased compared to Veh-IP ENDO controls (median values 1, 2 and 1, respectively; range 0-3; $P = 0.04$), with the most significant difference in week 1 ($P = 0.01$; week 2 $P > 0.9$; week 3 $P = 0.2$) (Fig. 7.7A-B). Facial grimace scores developed by centrally primed animals were similar between groups over weeks 1-3, with median scores of 1, 1.5 and 1, respectively for LPS-IT ENDO animals (range 0-3) compared to a score of 1 across all weeks for Veh-IT ENDO (range 0-3; $P = 0.9$) (Fig. 7.7C-D).

7.4.7 Adaptations in spinal astrocytic GFAP-immunoreactivity are unchanged in ENDO animals with peripherally primed TLR4

Given the extent of spinal cytokine alterations observed in peripherally primed LPS-IP ENDO mice, we further examined the thoracolumbar spinal cords from a subset of these animals for alterations in glial reactivity. We have previously shown that astrocytic GFAP- and microglial Iba-1- (or CD11b)-immunoreactivities in the spinal dorsal horn of control mice (no endometriosis-like lesions) occupy a consistent total area across spinal levels, and between individual animals (Dodds *et al.*, 2018 - Chapter 4, 2018 - Chapter 5). Hence comparisons in the current study were between Veh-IP and peripherally primed LPS-IP ENDO mice only ($n = 6$ per group).

For Veh-IP ENDO animals, the expression of GFAP-immunoreactivity within the spinal dorsal horn showed a high degree of variability. In T13-L1, the average area occupied by GFAP-positive structures was 5.6 ± 0.7% (CV 13%); L2-L3 5.8 ± 1.5% (CV 27%); L4-L5 6.3 ± 1.2% (CV 20%); and L6-S1 5.0 ± 0.7% (CV 15%) (minimum value from any segment 3.6%; maximum value 8.1%) (Fig. 7.8A). GFAP-immunoreactivity in the spinal dorsal horn of LPS-IP ENDO animals was also variable, with expression in T13-L1 of 5.9 ± 0.9% (CV 15%); L2-L3 6.2 ± 1.1% (CV 18%); L4-L5 6.3 ± 1.2% (CV 25%); and L6-S1 5.0 ±

0.5% (CV 9%) (minimum value from any segment 3.8%; maximum value 8.2%) (Fig. 7.8B). No significant differences were detected between groups at any spinal level in mean values (all comparisons $P > 0.9$) or variability (all comparisons $P > 0.1$). Summarised AUC values further reiterated that overall, spinal astrocytic GFAP expression in LPS-IP ENDO animals (average AUC 17.8 ± 2.7) compared to Veh-IP ENDO controls (17.4 ± 3.1) was unchanged (mean $P = 0.7$; variability $P = 0.6$) (Fig. 7.8C).

7.4.8 Spinal microglial Iba-1-immunoreactivity is altered by peripheral priming of TLR4

The extent of microglial Iba-1 expression in the spinal dorsal horn of Veh-IP ENDO animals was found to be similar across spinal segments. The average area occupied by Iba-1-positive structures in T13-L1 was 3.5 ± 0.4% (CV 11%); L2-L3 3.6 ± 0.5% (CV 12%); L4-L5 3.9 ± 0.3% (CV 7%); and L6-S1 3.5 ± 0.3% (CV 8%) (minimum value from any segment 2.8%; maximum value 4.6%) (Fig. 7.8D). In LPS-IP ENDO mice, the area of Iba-1-immunoreactivity was 4.0 ± 0.5% (CV 14%) in T13-L1; 4.1 ± 0.5% (CV 12%) for L2-L3; 4.0 ± 0.4% (CV 11%) in L4-L5; and 3.9 ± 0.5% (CV 12% for L6-S1) (minimum value from any segment 3.0%; maximum value 4.9%) (Fig. 7.8E). An overall treatment effect was observed for LPS-IP ENDO animals compared to Veh-IP ENDO controls ($P = 0.01$), with the most significant increase in Iba-1-immunoreactivity at the level of L6-S1 ($P = 0.03$; T13-L1 $P = 0.05$; L2-L3 $P = 0.06$; L4-L5 $P > 0.9$). Statistical evaluation of Iba-1 variability between groups was unchanged for T13-L5 (all comparisons $P \geq 0.1$), although approached significance in L6-S1 ($P = 0.05$). A comparison of summarised AUC values further demonstrated that spinal microglial Iba-1 expression was increased in LPS-IP ENDO animals (average AUC 12.0 ± 1.2) compared to Veh-IP ENDO controls (11.0 ± 0.6; $P = 0.02$) and revealed an overall increase in variability ($P = 0.04$) (Fig. 7.8F).

7.4.9 Figures and tables

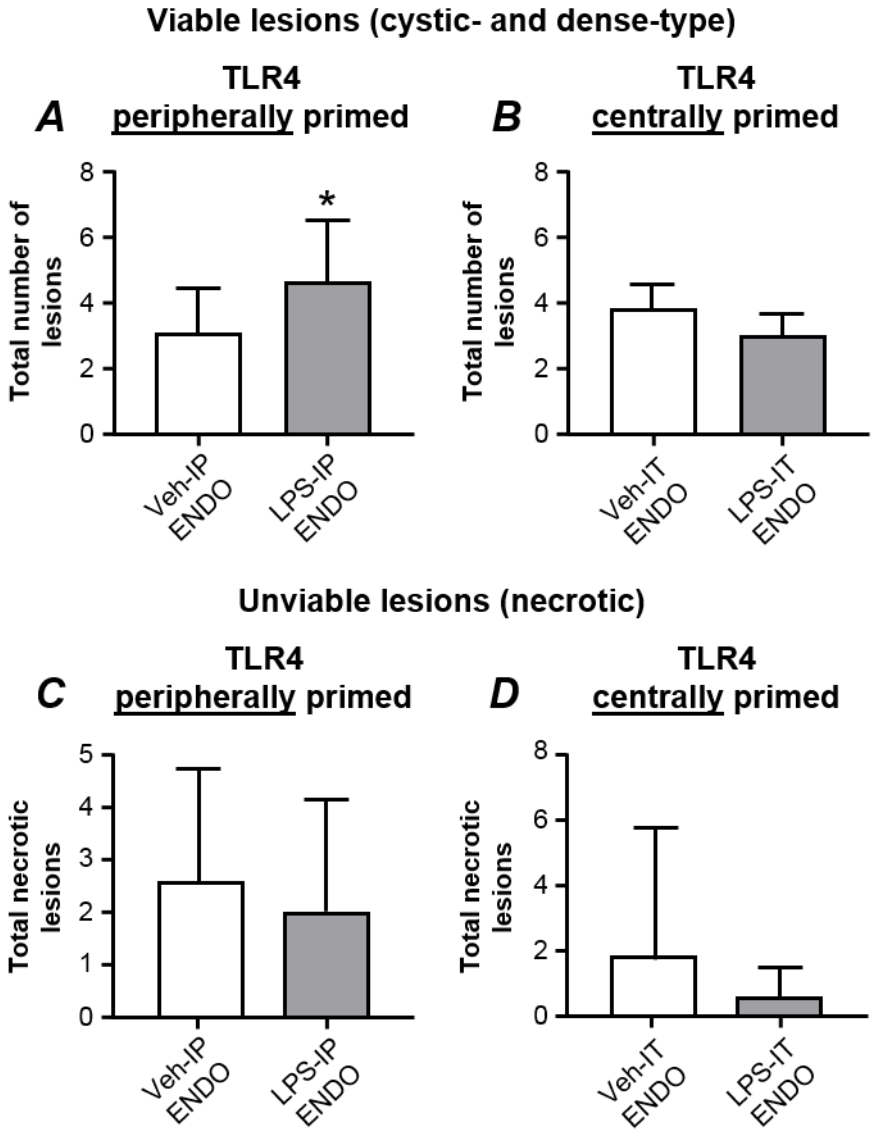

Figure 7.2 Total number of viable (cystic- and dense-type) and unviable (necrotic) endometriosis-like lesions developed by TLR4-primed ENDO mice. (*A*) Summarised data shows a significantly greater number of endometriosis-like lesions were retrieved from peripherally primed LPS-IP ENDO mice compared to Veh-IP ENDO controls (n = 12 animals per group). **(*B*)** The total number of lesions developed by centrally primed Veh-IT and LPS-IT ENDO animals was similar between groups (n = 6 animals per group). **(*C-D*)** Comparable numbers of residual necrotic endometrial tissues were retrieved from peripherally (Veh-IP and LPS-IP) and centrally (Veh-IT and LPS-IT) primed ENDO mice.* denotes mean comparison P <0.05. Data expressed as mean ± SD.

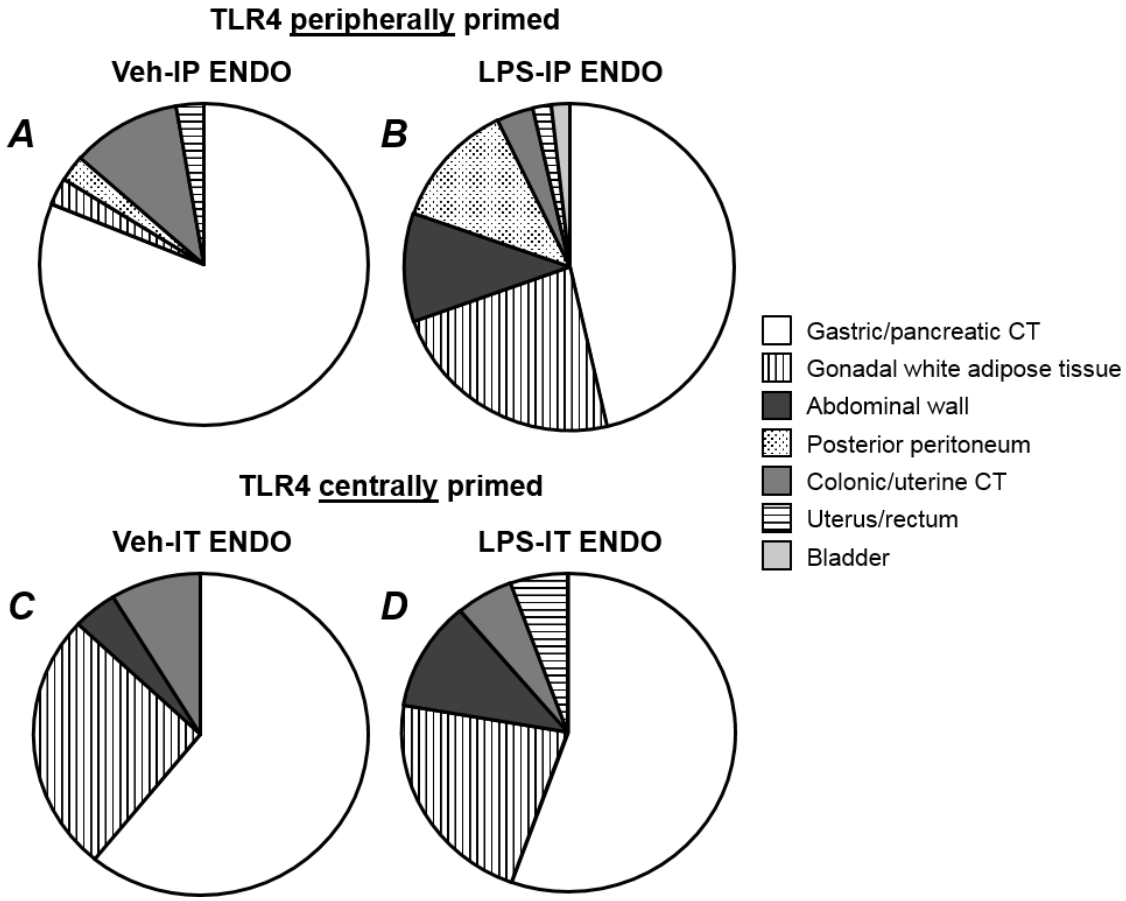

Figure 7.3 Diversity of anatomical locations of endometriosis-like lesions established in TLR4-primed ENDO mice. (*A-B*) Proportions of endometriosis-like lesions found in each peritoneal site show that lesions from peripherally-primed LPS-IP ENDO mice established in a greater range of locations compared to Veh-IP ENDO controls (n = 12 animals per group). **(*C-D*)** However, endometriosis-like lesions developed by centrally-primed LPS-IT ENDO mice were found in largely similar locations to those from control Veh-IT ENDO mice (n = 6 animals per group). *CT* = connective tissue.

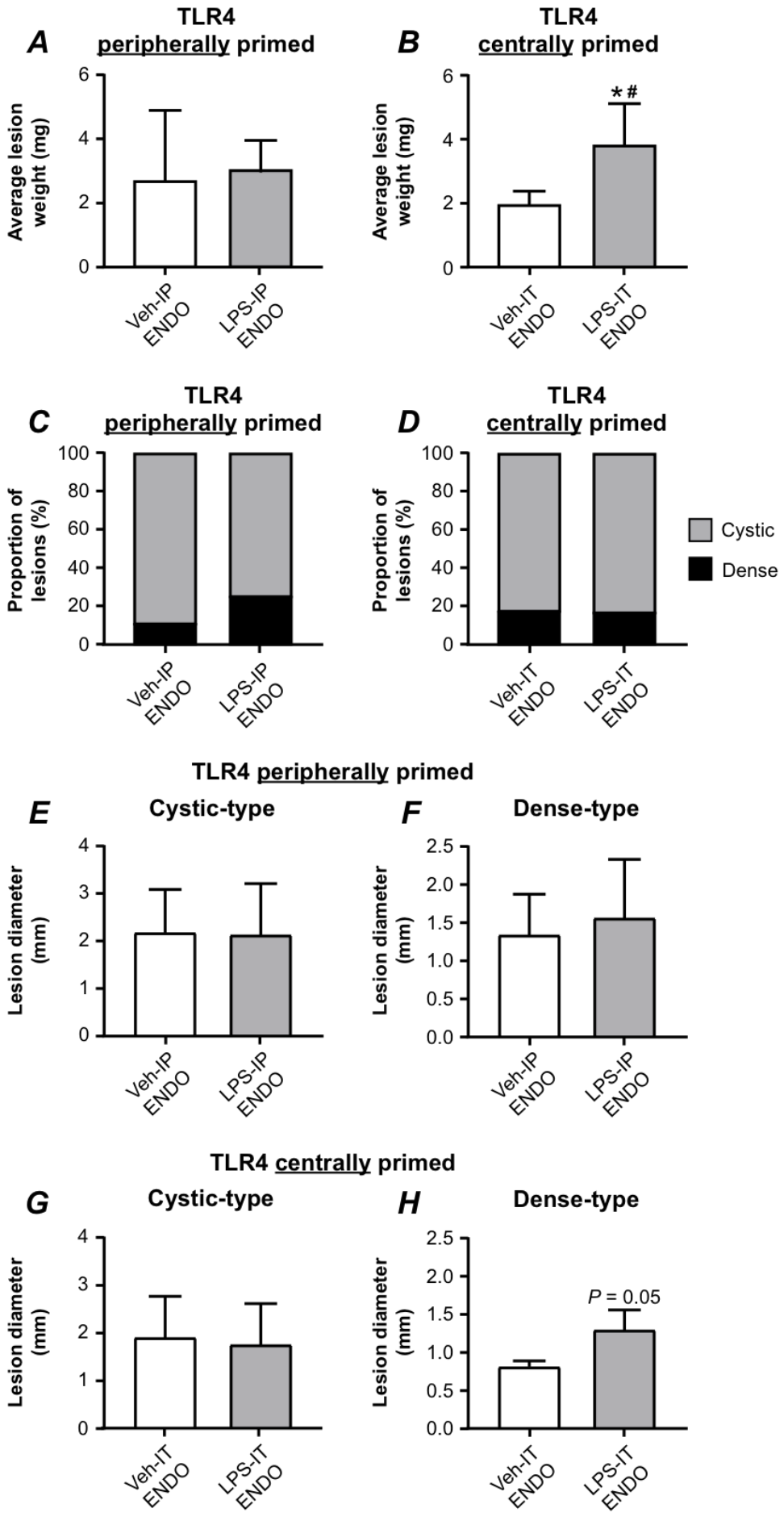
A
TLR4
peripherally primed
Average lesion weight (mg)
Veh-IP ENDO
LPS-IP ENDO
B
TLR4
centrally primed
*#
Veh-IT ENDO
LPS-IT ENDO
C
TLR4
peripherally primed
Proportion of lesions (%)
D
TLR4
centrally primed
Cystic
Dense
TLR4 peripherally primed
E
Cystic-type
Lesion diameter (mm)
F
Dense-type
TLR4 centrally primed
G
Cystic-type
H
Dense-type
P = 0.05

Figure 7.4 Phenotype profiles, diameter and mass measurements of endometriosis-like lesions developed by TLR4-primed ENDO mice. (*A*) Endometriosis-like lesions developed by peripherally primed LPS-IP ENDO mice had a similar average weight compared with Veh-IP ENDO controls. **(*B*)** In contrast, the average mass of lesions was significantly heavier and of greater variability in centrally primed LPS-IT than Veh-IT ENDO animals. **(*C*)** Proportions of cystic- and dense-type endometriosis-like lesions were similar between peripherally primed Veh-IP and LPS-IP ENDO animals. **(*D*)** Likewise, the quantities of lesion phenotypes retrieved from centrally primed Veh-IT and LPS-IT ENDO animals were unchanged. **(*E*)** The average sizes of cystic- and **(*F*)** dense-type lesions from peripherally primed LPS-IP ENDO mice were not statistically different from Veh-IP ENDO controls. **(*G*)** Cystic- and **(*H*)** dense-type lesion diameter measurements were also similar between centrally-primed Veh-IT and LPS-IT ENDO mice; although an increase in dense-type lesion size in LPS-IT ENDO animals approached significance ($P = 0.05$). * denotes mean comparison $P < 0.05$; # denotes variability comparison $P < 0.05$. For peripherally primed mice, $n = 12$ animals per group; centrally primed mice, $n = 6$ animals per group. Data in ***(A-B)*** and ***(E-H)*** expressed as mean ± SD.

A

Peripherally primed TLR4				
Cytokine (pg/mL)	Veh-IP (mean ± SD)	LPS-IP (mean ± SD)	Mean *P* value	Variability *P* value
GM-CSF	81.4 ± 70.3	20.0 ± 4.7	0.1	*<0.0001*
IL-1β	23.8 ± 18.2	33.3 ± 11.8	0.1	0.3
IL-2	5.6 ± 2.0	6.4 ± 3.1	0.7	0.3
IL-6	513.1 ± 659.9	273.9 ± 157.1	0.9	*0.007*
IL-10	2.7 ± 1.0	2.5 ± 1.2	0.7	0.6
IL-17A	0.5 ± 0.3	0.7 ± 0.6	0.5	0.2
IFN-γ	0.1 ± 0.1	0.1 ± 0.3	0.9	0.5
TNF-α	27.7 ± 16.7	11.3 ± 7.0	0.1	0.08

B

Centrally primed TLR4				
Cytokine (pg/mL)	Veh-IT (mean ± SD)	LPS-IT (mean ± SD)	Mean *P* value	Variability *P* value
GM-CSF	12.0 ± 5.7	30.7 ± 21.1	0.06	*0.01*
IL-1β	25.1 ± 24.2	23.9 ± 14.2	0.8	0.2
IL-2	6.0 ± 3.1	7.5 ± 3.7	0.3	0.6
IL-6	140.6 ± 78.8	120.9 ± 90.6	0.6	0.7
IL-10	1.9 ± 1.2	2.0 ± 1.4	0.8	0.9
IL-17A	1.8 ± 3.8	0.5 ± 0.3	0.5	*<0.0001*
IFN-γ	0.1 ± 0.1	1.0 ± 1.7	0.6	*<0.0001*
TNF-α	17.6 ± 21.4	12.1 ± 9.6	0.8	0.1

Table 7.1 Cytokine protein concentrations within endometriosis-like lesions obtained from TLR4-primed ENDO mice. (*A*) Cytokine and significance values obtained from Veh-IP and peripherally primed LPS-IP ENDO mice. **(*B*)** Cytokine and significance values obtained from Veh-IT and centrally primed LPS-IT ENDO mice. Statistically significant comparisons of mean and variability values between respective vehicle- and LPS-treated ENDO mice are italicised and underlined. All groups $n = 6$ animals.

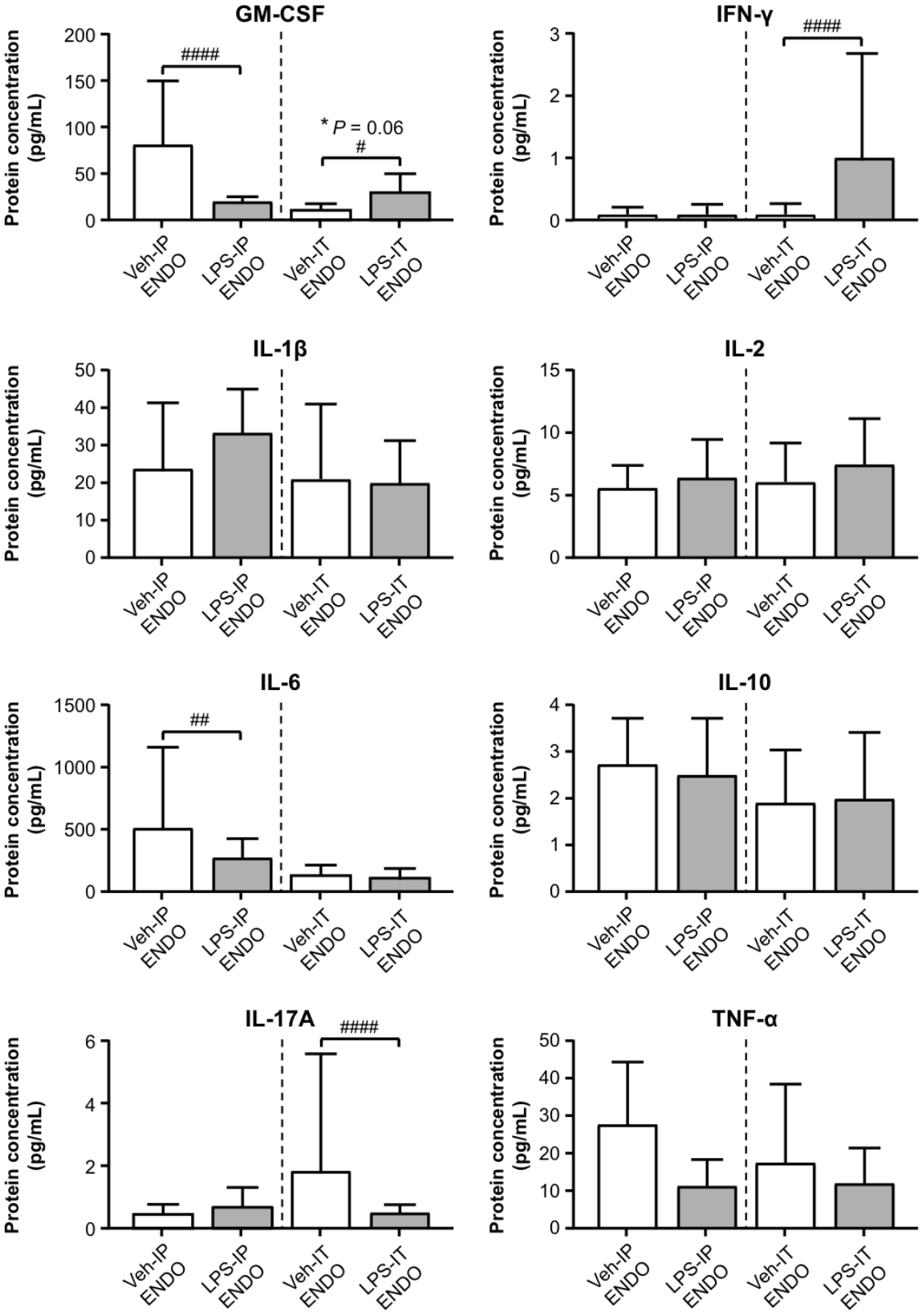
GM-CSF
Protein concentration (pg/mL)
200
150
100
50
0
####
* P = 0.06
#
Veh-IP ENDO
LPS-IP ENDO
Veh-IT ENDO
LPS-IT ENDO
IFN-γ
Protein concentration (pg/mL)
3
2
1
0
####
Veh-IP ENDO
LPS-IP ENDO
Veh-IT ENDO
LPS-IT ENDO
IL-1β
Protein concentration (pg/mL)
50
40
30
20
10
0
Veh-IP ENDO
LPS-IP ENDO
Veh-IT ENDO
LPS-IT ENDO
IL-2
Protein concentration (pg/mL)
15
10
5
0
Veh-IP ENDO
LPS-IP ENDO
Veh-IT ENDO
LPS-IT ENDO
IL-6
Protein concentration (pg/mL)
1500
1000
500
0
##
Veh-IP ENDO
LPS-IP ENDO
Veh-IT ENDO
LPS-IT ENDO
IL-10
Protein concentration (pg/mL)
4
3
2
1
0
Veh-IP ENDO
LPS-IP ENDO
Veh-IT ENDO
LPS-IT ENDO
IL-17A
Protein concentration (pg/mL)
6
4
2
0
####
Veh-IP ENDO
LPS-IP ENDO
Veh-IT ENDO
LPS-IT ENDO
TNF-α
Protein concentration (pg/mL)
50
40
30
20
10
0
Veh-IP ENDO
LPS-IP ENDO
Veh-IT ENDO
LPS-IT ENDO

Figure 7.5 Endometriosis-like lesion cytokine profiles from TLR4-primed ENDO mice. Endometriosis-like lesions from peripherally primed LPS-IP ENDO animals displayed a decrease in the variability of GM-CSF and IL-6 protein expression compared with Veh-IP ENDO animals. An increase in the variability of GM-CSF and IFN-γ, and a decrease in the variability of IL-17A, occurred in endometriosis-like lesions from centrally primed LPS-IT ENDO animals compared to Veh-IT ENDO animals. No differences were detected in endometriosis-like lesions between any vehicle- and LPS-treated groups for IL-1β, IL-2, IL-10 or TNF-α. * denotes mean comparison; # denotes variability comparison $P < 0.05$; ## $P < 0.01$; #### $P < 0.0001$. Statistical comparisons were only made within (not across) peripherally and centrally TLR4-primed groups. All groups $n = 6$ animals. Data expressed as mean ± SD.

A

Peripherally primed TLR4				
Cytokine (pg/mL)	Veh-IP (mean ± SD)	LPS-IP (mean ± SD)	Mean *P* value	Variability *P* value
GM-CSF	8.5 ± 4.5	9.3 ± 5.4	0.5	0.3
IL-1β	30.2 ± 15.7	37.9 ± 20.3	0.2	0.2
IL-2	175.4 ± 58.0	174.3 ± 62.9	0.9	0.6
IL-6	20.1 ± 22.2	38.3 ± 40.6	0.1	*0.005*
IL-10	10.6 ± 5.8	18.2 ± 8.3	*0.0005*	*0.09*
IL-17A	1.5 ± 0.5	2.0 ± 0.7	*0.004*	0.2
IFN-γ	22.3 ± 28.8	76.3 ± 80.2	*0.002*	*<0.0001*
TNF-α	0.9 ± 0.4	1.4 ± 0.8	*0.009*	*0.001*

B

Centrally primed TLR4				
Cytokine (pg/mL)	Veh-IT (mean ± SD)	LPS-IT (mean ± SD)	Mean *P* value	Variability *P* value
GM-CSF	9.2 ± 4.0	8.8 ± 3.0	0.6	0.1
IL-1β	44.6 ± 12.8	43.2 ± 12.0	0.8	0.7
IL-2	202.5 ± 51.2	186.0 ± 47.1	0.4	0.6
IL-6	31.8 ± 25.7	33.5 ± 16.5	0.5	*0.03*
IL-10	11.3 ± 7.0	15.0 ± 8.7	0.1	0.3
IL-17A	2.3 ± 0.6	2.2 ± 0.7	0.1	0.7
IFN-γ	34.2 ± 33.7	66.5 ± 47.8	*0.01*	0.1
TNF-α	2.2 ± 1.2	2.0 ± 0.9	0.7	0.3

Table 7.2 Cytokine protein concentrations within the thoracolumbar spinal cord of TLR4-primed ENDO mice. (*A*) Cytokine and significance values obtained from Veh-IP and peripherally primed LPS-IP ENDO mice. **(*B*)** Cytokine and significance values obtained from Veh-IT and centrally primed LPS-IT ENDO mice. Statistically significant comparisons of mean and variability values between respective vehicle- and LPS-treated ENDO mice are italicised and underlined. All groups $n = 6$ animals.

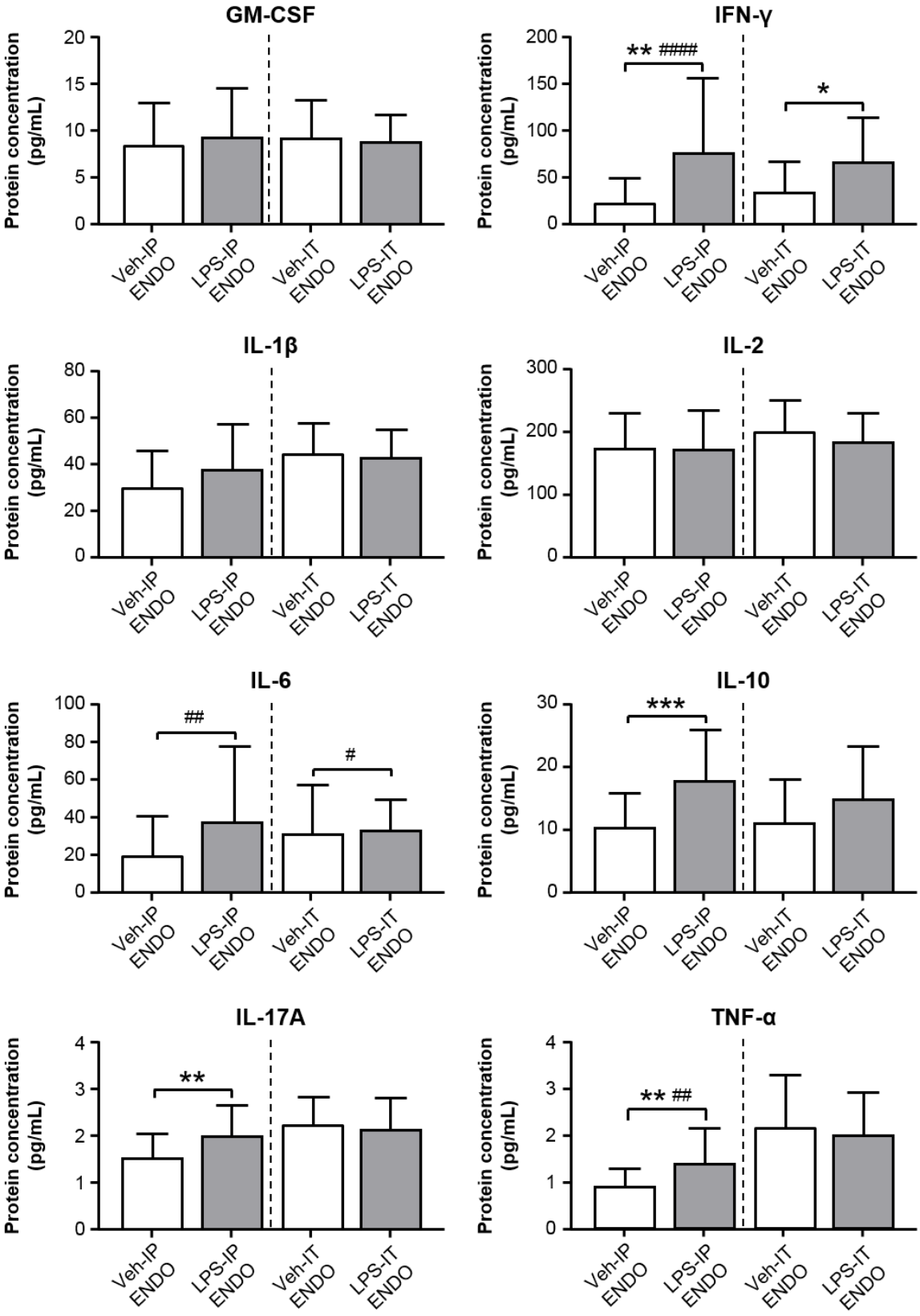
GM-CSF
IFN-γ
IL-1β
IL-2
IL-6
IL-10
IL-17A
TNF-α
Protein concentration (pg/mL)
Veh-IP ENDO
LPS-IP ENDO
Veh-IT ENDO
LPS-IT ENDO
** ####
*
##
#
***
**
** ##

Figure 7.6 Cytokine expression in the thoracolumbar spinal cords of TLR4-primed ENDO mice. Spinal cords from peripherally primed LPS-IP ENDO animals displayed a significant increase in mean protein levels and variability of IFN-γ and TNF-α compared to Veh-IP ENDO animals. An increase in the mean expression of IL-10 and IL-17A, and the variability of IL-6, was also observed. Centrally primed LPS-IT ENDO animals displayed an increase in mean spinal protein levels of IFN-γ and the variability of IL-6 compared with Veh-IT ENDO animals. No differences between any vehicle- and LPS-treated groups were observed for spinal concentrations of GM-CSF, IL-1β or IL-2. * denotes mean comparison P <0.05; ** P <0.01; *** P <0.001. # denotes variability comparison P <0.05; ## P <0.01; #### P <0.0001. Statistical comparisons were only made within (not across) peripherally and centrally TLR4-primed groups. All groups n = 6 animals. Data expressed as mean ± SD.

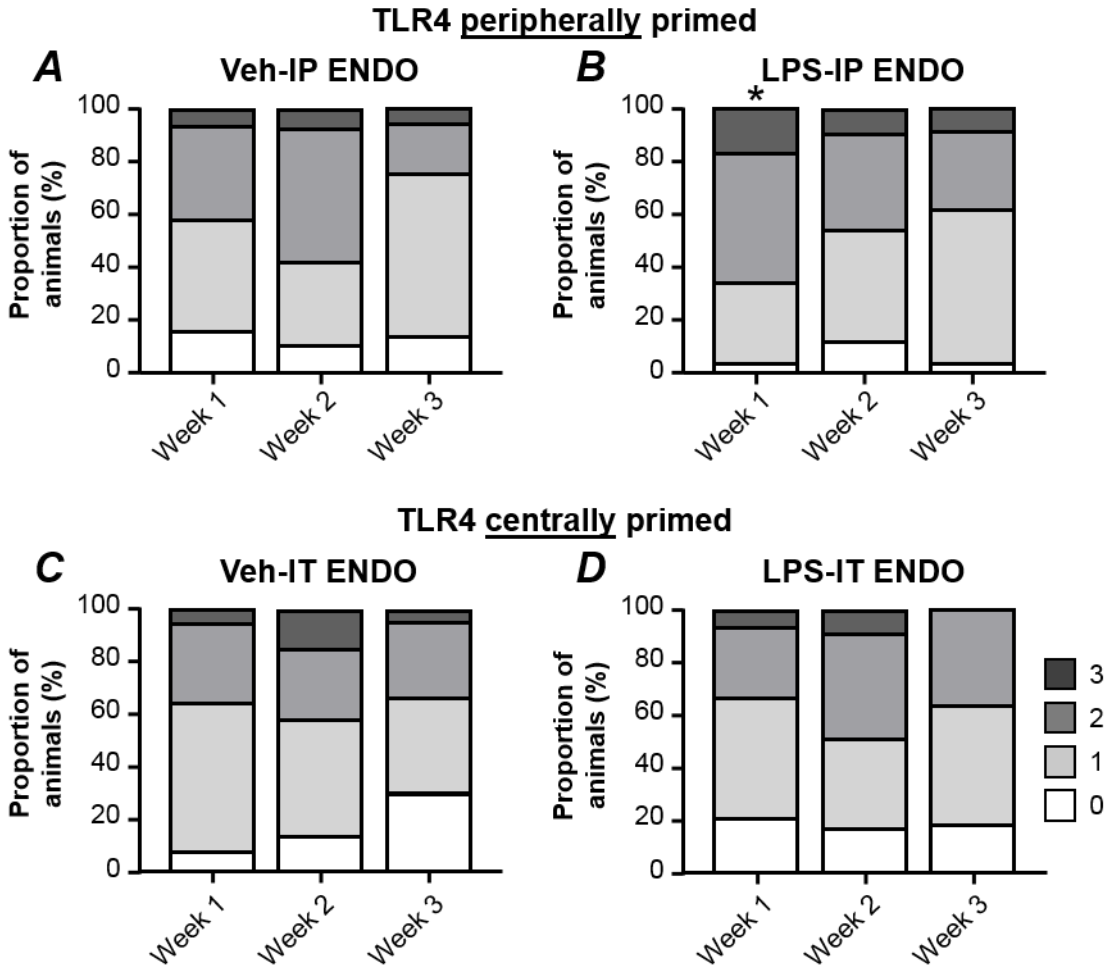

Figure 7.7 Facial pain scores following development of endometriosis-like lesions in TLR4-primed ENDO mice. (*A*) The majority of peripherally-primed Veh-IP ENDO control animals had median grimace scores of 1-2 over the three-week developmental period. **(*B*)** While LPS-IP ENDO mice also had median scores of 1-2, overall this group showed significantly more signs of pain; particularly in week 1 of endometriosis-like lesion development. **(*C*)** Centrally-primed Veh-IT ENDO controls also developed median scores of 1 across weeks 1-3, which were unchanged compared with **(*D*)** facial grimace scores from LPS-IT ENDO mice (median scores 1-1.5). * denotes $P < 0.05$. Asterisks in panel **(*B*)** refer to the corresponding weeks in panel **(*A*)**. All groups $n = 6$ animals.

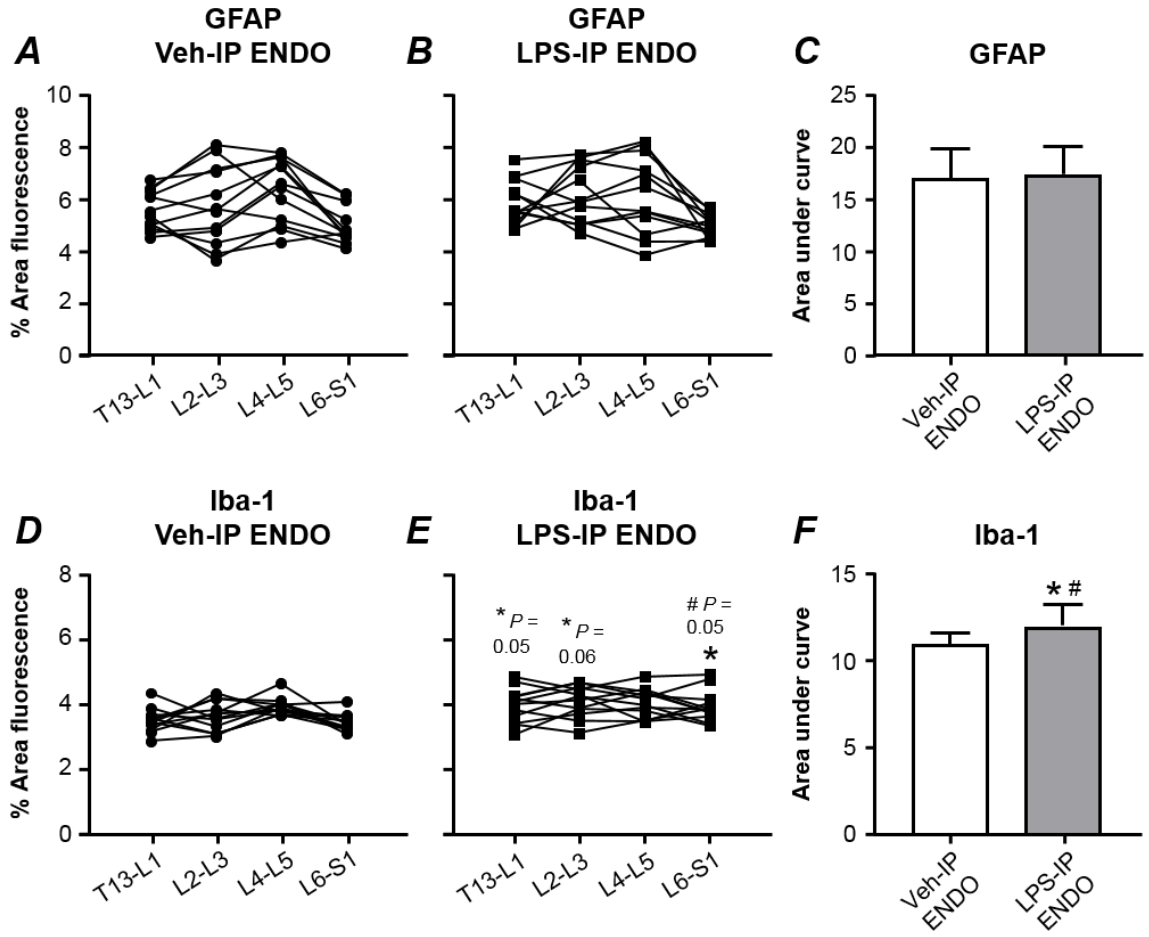

Figure 7.8 Glial immunoreactivity expression in the thoracolumbar spinal dorsal horn of peripherally TLR4-primed ENDO mice. (*A-B*) The area of astrocytic GFAP immunostaining in dorsal horns (linked by horizontal bars) from Veh-IP and peripherally primed LPS-IP ENDO mice, respectively, were variable across spinal levels T13-S1. **(*C*)** Summarised AUC data per dorsal horn indicate no statistical differences in mean values or variability for spinal GFAP immunostaining between groups. **(*D*)** Percentage area of each dorsal horn occupied by microglial Iba-1 immunostaining in Veh-IP ENDO controls was reasonably consistent across spinal levels T13-S1. **(*E*)** In contrast, a significant overall increase in mean Iba-1 expression was observed for LPS-IT ENDO mice, particularly in L6-S1. **(*F*)** Summarised data comparing AUC values for each dorsal horn further demonstrate an increase in mean values and heightened variability of Iba-1 immunostaining in LPS-IP ENDO mice compared with Veh-IP ENDO controls. ***** denotes mean comparison P <0.05; **#** denotes variability comparison P <0.05. Significance notations in panel **(*E*)** refers to corresponding spinal levels in panel **(*D*)**. Y-axis label in **(*B*)** applies to **(*A*)**; Y-axis label in **(*E*)** applies to **(*D*)**. All groups n = 12 from 6 animals. Data in ***(C, F)*** expressed as mean ± SD.

7.5 Discussion

The first series of experiments presented here support the hypothesis that an increase in peritoneal inflammation, mediated by TLR4 activation, can potentiate the formation of endometriosis-like lesions. An acute systemic administration of LPS shortly prior to endometrial tissue transfer was sufficient to increase total lesion incidence. In addition, long lasting adaptations in spinal glial reactivity, associated cytokine concentrations and enhanced pain were observed in these animals; indicative of heightened central sensitisation. In our second series of experiments, we are the first to provide evidence of a central-to-peripheral mechanism in endometriosis, with acute activation of spinal TLR4 associated with the increased mass of peritoneal endometriosis-like lesions.

7.5.1 Prior stimulation of peripheral TLR4 increases endometriosis-like lesion burden and associated neuroimmune-mediated pain

LPS-IP ENDO mice that were peripherally primed with LPS prior to the induction of endometriosis formed a greater total number of lesions, which adhered to a larger range of peritoneal locations. This finding is in agreement with other studies proposing that heightened TLR4-mediated proinflammatory signalling facilitates the pathogenesis of endometriosis (Khan *et al.*, 2008b; Khan *et al.*, 2010; Khan *et al.*, 2013b; Yun *et al.*, 2016; Azuma *et al.*, 2017), and that disease progression may be enhanced by a 'first hit' immune challenge (Hains *et al.*, 2010). In line with this, various anti-inflammatory treatments can attenuate the progression of endometriosis-like lesions (Barrier *et al.*, 2004; Efstathiou *et al.*; Zhou *et al.*, 2010; Mariani *et al.*, 2012; Wu *et al.*, 2014). Interestingly however, it has been reported that existing peritonitis may in fact reduce the subsequent formation of lesions (Nowak *et al.*, 2008). Since a chemical irritant (dissimilar to LPS) generated this opposing result, it implies that peritoneal immune responses orchestrated in response to diverse stimuli are not equal and may exert differential effects on the development of

lesions. Prospective studies will be required to determine the mechanisms by which acute LPS priming functionally sensitises peritoneal TLR4-expressing cells, and specifically how this contributes towards endometriotic lesion establishment. Careful characterisation of the immune cells and cytokines recruited by specific immune challenges (such as LPS priming) and comparisons with those caused by endometrial debris will be required.

Apart from total lesion incidence, no differences were observed in the phenotypic classifications or sizes of lesions from LPS-IP ENDO mice. In addition, negligible changes occurred in cytokine concentrations within lesions, with only a reduction in variability found for GM-CSF and IL-6. These findings intriguingly suggest that peripheral LPS-mediated TLR4 priming influences the establishment of endometriosis-like lesions but not necessarily any further growth (Nothnick *et al.*, 1994). There are suggestions in the literature that endometriosis-like lesions can embed within 24 hours of inoculation (Nisolle *et al.*; Eggermont *et al.*, 2005), thus it is possible many of the measures alluding to a role for TLR4 in this process may have regressed by the time of tissue collection (21 days post-lesion induction). Indeed, it has recently been shown that the early establishment of endometriosis-like lesions in mice occurs in two phases – an initial immune-dependent phase, followed by the later influence of steroid hormones (Burns *et al.*, 2018). Therefore, future research examining earlier time-points in this model are also necessary to characterise the role of TLR4 in the initial stages of lesion development.

We have previously shown that endometriosis-like lesions can induce spinal adaptations in the expression of glial cells and cytokines, and enhance the presentation of pain behaviours (i.e. a peripheral-to-central effect) (Dodds *et al.*, 2018 - Chapter 4, 2018 - Chapter 5, 2018 - Chapter 6). In peripherally primed LPS-IP ENDO mice the increase in lesions coincided with a greater degree of change in all of these measures associated with central sensitisation. Such observations most likely reflect nerve- and/or immune cell-mediated

signalling from lesions and their surrounds, as it is known that, when administered systemically, LPS has a limited ability to cross the blood brain barrier (Banks & Robinson, 2010). While it is established that a transient systemic injection of LPS alone can alter microglial reactivity and pain behaviours (Kondo *et al.*, 2011; Yoon *et al.*, 2012), in the current context of priming it appears to produce an additive effect on the central adaptations associated with endometriosis-like lesions.

The first major possibility that may account for the central findings in LPS-IP ENDO mice is an increase in nociceptive neurotransmission arising from the lesions. Noxious activation of afferent neurons with the release of neurotransmitter in the spinal dorsal horn can directly stimulate second-order neurons projecting to high brain centres, as well as neighbouring glia; thereby initiating an increase in glial reactivity, heightened proinflammatory signalling, and enhanced pain (Dodds *et al.*, 2016 - Chapter 2). A greater number of endometriosis-like lesions in LPS-IP ENDO mice therefore improves the opportunity for neurite outgrowth (i.e. more sensors) and inflammation associated with lesions (i.e. more stimuli). The potential for residual proinflammatory signalling by peritoneal immune cells, as mentioned above, could further exacerbate this process by modulating neuronal plasticity and excitability. In addition, nociceptive neurons have been shown to express TLR4, and therefore may be directly stimulated by priming with LPS (Diogenes *et al.*, 2011; Due *et al.*, 2012; Helley *et al.*, 2015). Ultimately, these factors may result in an increase in nociceptive information reaching the CNS, and a strengthening of activity-dependent central sensitisation processes.

Migration of activated peritoneal immune cells to the CNS is another potential mechanism underlying the central effects observed in LPS-IP ENDO mice. It is now recognised that peripheral macrophages and T-cells (as well as humoral factors (Tegeder *et al.*, 2006)) can infiltrate CNS tissues, produce inflammatory responses, and facilitate persistent pain

within such locations (Costigan *et al.*, 2009; Kim *et al.*, 2011; Du *et al.*, 2018). The finding that cytokine alterations within LPS-IP ENDO spinal cords were not identical to those from within peritoneal lesions suggests these mediators were derived from immune cells within the spinal cord, as opposed to a direct dissemination from peripheral to central tissues.

7.5.2 Prior stimulation of central TLR4 alters spinal cytokine composition and enhances growth of peritoneal endometriosis-like lesions

The most prominent observation regarding endometriosis-like lesion pathology in centrally primed LPS-IT ENDO mice was that lesions displayed a heavier average mass, independent of total lesion incidence. To our knowledge, this is the first study to demonstrate that spinal manipulation can influence the development of endometriosis (i.e. a central-to-peripheral effect). Recently, it was discovered that the CNS meninges have direct connections with lymphatic vasculature, which may promote the generation of peripheral immune responses (Aspelund *et al.*, 2015; Louveau *et al.*, 2015). It is therefore possible that reactive inflammatory cells and their products could disseminate from the CNS via this system to perpetuate peripheral inflammation and endometriosis-like lesion growth. However, given the mounting evidence supporting a relationship between glial-mediated central sensitisation, neurogenic inflammation, and peripheral inflammatory conditions, neuroimmune signalling pathways are likely to be involved.

Spinal IFN-γ was elevated and the variability of IL-6 expression was reduced in LPS-IT ENDO mice. Both cytokines have been strongly implicated in glial-mediated central sensitisation, and activation of neuronal signalling. It is known that TLR4 stimulation can induce the release of IFN-γ and IL-6 from immune cells (Weighardt *et al.*, 2004; Spiller *et al.*, 2008; Greenhill *et al.*, 2011; Kraaij *et al.*, 2014), and levels are upregulated in the spinal cords of animals with neuropathic pain (Tanga *et al.*, 2005; Schoeniger-Skinner *et al.*, 2007; Latremoliere *et al.*, 2008; Costigan *et al.*, 2009). IFN-γ receptors are located on pre-

and post-synaptic neurons in the superficial dorsal horn (Vikman *et al.*, 1998); expressed in close association with the neuropeptide, substance P, in dorsal root ganglia (Neumann *et al.*, 1997; Vikman *et al.*, 1998); and stimulation of peripheral neurons by IFN-γ can alter the gene expression of certain ion channels and neurotransmitters (Toledo-Aral *et al.*, 1995; Jonakait *et al.*, 1996). Interestingly, IFN-γ levels were also found to be higher within LPS-IT ENDO lesions (compared to Veh-IT ENDO lesions), which may further facilitate (and indicate) the occurrence of neurogenic inflammatory processes. Intrathecal delivery of IFN-γ or IL-6 is known to increase neuronal activity and pain behaviour (DeLeo *et al.*, 1996; Robertson *et al.*, 1997; Vikman *et al.*, 2005), and stimulation of microglia by IFN-γ can potentiate neuronal signalling via CCL2 and iNOS (Racz *et al.*, 2008; Sonekatsu *et al.*, 2016). IL-6 activity can also directly sensitise nociceptive neurons (Obreja *et al.*, 2002; Brenn *et al.*, 2007), and has been demonstrated to contribute to neurally-evoked joint inflammation associated with rheumatoid arthritis (Ebbinghaus *et al.*, 2015). Hence, there is potential for altered spinal expression of IFN-γ and IL-6 to contribute to central sensitisation and lesion-associated pain, as well as neurogenic inflammation and lesion-associated growth. Of course, given the single time-point at which tissues were collected in this study, it is unknown whether the postmortem cytokine levels persisted for the entire development period, or if transient fluctuations in other inflammatory mediators contributed to the observed changes in lesion pathology.

It is also noteworthy that an increase in the size of dense-type lesions from LPS-IT ENDO mice approached statistical significance ($P = 0.05$), which may underlie the enhanced average lesion mass observed in these animals. Interestingly, in an earlier study we found that genetic knockout of *TLR4* conversely resulted in the development of smaller dense-type lesions (Dodds *et al.*, 2018 - Chapter 5). Thus TLR4-mediated signalling appears to specifically influence growth of this lesion type in our ENDO model. Future determination

of the specific contributions of cytokines and growth factors intrinsic to these lesions (as mentioned above) may provide insights as to why dense-type lesions, as opposed to cystic-type lesions, are afflicted by manipulations of TLR4. Despite changes in lesion mass, facial grimace assessment did not detect an increase in pain behaviour of centrally primed LPS-IT ENDO animals (compared to Veh-IT ENDO). At this stage, we can only speculate that the increase in endometriosis-like lesion weight was not sufficient to modify associated nociceptive activity; that pain in this model is determined more significantly by cystic- rather than dense-type lesions; or that the method employed to score pain was unable to discriminate fine changes between IT ENDO animals.

7.5.3 Conclusions

Amongst researchers and clinicians, there is a general consensus that immune activity is pivotal to the development of endometriosis lesions. The causal relationship between immune-mediated inflammation and endometriosis does, however, remain contentious. Here, we have demonstrated that a prior inflammatory response evoked by the TLR4 agonist, LPS, can alter the dynamics of endometriosis-like lesion development. When administered peripherally, LPS enhanced lesion incidence and central adaptations known to be related to hyperalgesic behaviour. When administered centrally, LPS induced an increase in lesion weight; possibly via the conduit of neurogenic inflammation. The formation and progression of endometriosis thus appears to involve bidirectional communication between peritoneal lesions and the CNS. As TLR4-mediated signalling processes may result following a range of stimuli, aetiological factors may extend beyond that caused by bacterial infection, owing to the possibility of 'sterile inflammation' evoked by host danger-associated ligands (Kajihara *et al.*, 2011). Crucially, episodes of stress and adverse early-life events that release these endogenous TLR4 mediators have been implicated in immune sensitisation, with an increased susceptibility to other persistent pain

conditions. Our study demonstrates that research efforts directed toward TLR4 activity and endometriosis will improve our knowledge of the immunobiological contributions to lesion pathology.

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Chapter 8. General discussion

This thesis aimed to bridge a number of gaps in the knowledge of endometriosis regarding its pathogenesis, biological consequences, and the limitations of current animal models used to study such processes. Of significance, the current results offer novel hypotheses relating to activity of the immune-like glial cells within the CNS, and the innate immune receptor, TLR4. Thus, the collection of work presented here contributes several world-first findings to the endometriosis literature, which will shape future investigations. Specifically, the key results from these studies have determined:

- Histologically relevant endometriosis-like lesions successfully establish in a minimally-invasive mouse model of endometriosis, and develop in an oestrogen-, strain- and tissue volume-dependent manner (Aim 1);
- Adaptations in the expression of spinal glial cells occur in the presence of endometriosis-like lesions, and the spinal levels most affected by these changes correspond to the peritoneal locations where lesions have developed (Aim 2);
- Genetic knockout of *TLR4* promotes the development of peritoneal endometriosis-like lesions, which are contrarily associated with reduced pain behaviour despite showing further changes in spinal glial reactivity and altered cytokine expression (Aim 3);
- Acute *peripheral blockade* of TLR4 during the early phase of induction can replicate the increased lesion incidence observed in knockout mice, and this is accompanied by standard adaptations in spinal glial reactivity and pain behaviour (Aim 4);
- Acute *peripheral stimulation* of TLR4 prior to the induction of endometriosis enhances lesion development, causes further changes to spinal glial reactivity, alters cytokine expression, and heightens pain behaviour (Aim 5); and

- Acute *central stimulation* of TLR4 prior to the induction of endometriosis increases the mass of established lesions and alters the expression of spinal cytokines, while pain behaviour remains unchanged (Aim 6).

8.1 Implications of altered spinal glial reactivity, cytokines and pain behaviour that occur in association with endometriosis

Current drug treatments available to women with endometriosis often provide suboptimal pain relief. These include hormonal medications that suppress oestrogen activity, and NSAIDs that target the production of PGE_2 – both of which reduce lesion-associated inflammation that can stimulate nociceptive neurotransmission. However, where pain pathways have undergone central sensitisation, the largely peripheral actions of these drugs are likely to be ineffective. Therefore, the identification of cellular or molecular targets within CNS pain pathways may provide opportunities to enhance analgesia relating to endometriosis. Using the minimally-invasive mouse model described in Chapter 3, experiments in Chapters 4-7 have demonstrated for the first time that the expression of spinal glial cell populations (astrocytes ± microglia) become altered in the presence of endometriosis. These adaptations are associated with changes in the levels of various spinal cytokines and facial grimace behaviours indicative of pain. Previously, the sensitisation of CNS pain pathways in endometriosis was thought to simply involve neuronal adaptations. We can now appreciate that changes in spinal glial reactivity might also play a role in generating endometriosis-related pain, which will shift the current paradigm from being considered an exclusively neural to a neuroimmune-mediated phenomenon. Thus, glial cells and neuroimmune signalling pathways represent new central targets to be explored for intervention in patients whose pain remains recalcitrant to existing therapies.

Now that central neuroimmune-related changes have been demonstrated in our endometriosis model, prospective studies should focus on providing direct functional links

between endometriosis-like lesions, spinal glial reactivity and pain. Such experiments could include examination of endometriosis-related pain behaviour in the presence of known glial inhibitors, such as ibudilast (global), L-α-animoadipate (astrocytes) and minocycline (microglia), allowing researchers to specifically determine whether blocking the activity of glial cells has therapeutic potential for this condition. Indeed, the spinal administration of L-α-animoadipate or minocycline has already been shown to alleviate pain in several other models of visceral inflammation (Feng *et al.*, 2010; Liu *et al.*, 2012b; Kannampalli *et al.*, 2014; Liu *et al.*, 2016a). Experiments evaluating the antagonism of glial-related cytokines upregulated in our wildtype BALB/c mice with endometriosis-like lesions (Chapter 5), such as GM-CSF (Nicol *et al.*, 2018) and TNF-α (Svensson *et al.*, 2005; Bas *et al.*, 2015), have also exemplified the importance of these mediators as possible central pain targets (Lees *et al.*, 2013). While the clinical translation of neuroimmune-mediated therapies remains to be established, ibudilast is currently used in humans to treat various conditions (e.g. asthma, post-stroke dizziness) and is thus reasonably well tolerated (Ledeboer *et al.*, 2007). With further investigations, these agents may provide opportunities as adjuvant analgesics that are crucially needed for many women with endometriosis.

A further caveat of the experiments in Chapters 5-7 was that facial grimace scoring was the only method used to determine endometriosis-related pain behaviour. Although this analysis was originally devised and validated by an esteemed pain research group (Langford *et al.*, 2010) and performed appropriately throughout our studies, there are some concerns surrounding its subjective nature and one-dimensionality to evaluate spontaneous pain. To support our findings it would be ideal to include supplementary analyses of pain, which were unfeasible during the experimental period owing to constraints with time and resources. Examples of additional methods currently used to examine spontaneous pain include animal immobility (i.e. less movement when experiencing pain), abdominal licking

(i.e. grooming painful area), and gait disturbances (i.e. more pressure on forepaws with abdominal pain). Place preference and evoked pain behaviours can also be examined, such as mechanical allodynia via von Frey probing of the abdomen or hindpaw, or heat hyperalgesia using the hotplate or tail flick tests. In future studies using our minimally-invasive mouse model, the addition of other behavioural tests will provide a more comprehensive characterisation of endometriosis-associated pain in these animals.

In Chapter 4, our experiments revealed that spinal cord levels showing prominent glial adaptations correlated with lesion locations in the peritoneum. Adaptations in glial reactivity are primarily thought to initiate from the release of nociceptive neurotransmitters by afferent terminals in the spinal dorsal horn. Lesions in our model developed in various, seemingly random locations, and therefore it is presumed that different afferents and thus spinal levels were affected in each animal. To determine whether changes in glial expression occurred in endometriosis, we examined spinal segments T13-S1 (Chapters 4-7) as they are known to innervate a large proportion structures in the abdominopelvic cavity. While our analysis provided basic associations between the site of lesions and affected spinal levels, much larger studies will be required to quantify specifically localised and graded glial adaptations according to lesion characteristics. For example, do cystic-type lesions cause greater changes in glial reactivity than dense-type lesions? Do lesions attached to connective tissues of the stomach alter glial reactivity more than those embedded within gonadal adipose tissues? Obtaining answers to such questions may be facilitated by retrograde tracing, which is a procedure largely pioneered by our colleagues at Flinders University studying neural connections between visceral organs and the CNS (Kyloh *et al.*, 2011; Herweijer *et al.*, 2014). In this technique, neurons supplying particular tissues take up a microinjected fluorescent lipophilic dye (e.g. DiI), which is transported back to the DRG and spinal cord. Therefore, applying this principle and tracing from

discrete lesions in our model would allow us to more accurately determine (and then predict) typical spinal levels affected by a certain type of lesion in a certain location. From here, the features of glial adaptations and sensitised neurons will be able to undergo more detailed interrogations. In addition, and of utmost importance, this technique may also allow researchers to explicitly state that endometriosis lesions establish direct connections with the CNS. It is known that lesions develop their own nerves, are linked to neural changes in the DRG, spinal cord and brain, and are associated with pain – a neurally-mediated signal. While these findings (and ours) strongly infer that nerves of lesions must project to the CNS, to our knowledge no studies have unequivocally demonstrated that this relationship exists.

8.2 The potential role of TLR4 activation in the development of endometriosis-associated pain

Inflammatory activity generated by spinal glial cells that promotes central sensitisation and pain may be stimulated via the expression of TLR4. A role for TLR4 signalling in glial-mediated pain has been demonstrated under numerous pathophysiological conditions, and our results from Chapter 5 suggest that central TLR4 activity may also be involved in pain associated with endometriosis. In these experiments, animals with genetic knockout of *TLR4* displayed less pain behaviour than wildtype animals, despite developing more endometriosis-like lesions and a further increase in spinal astrocyte expression. While there were no differences in basal spinal cytokine levels between wildtype and TLR4-deficient mice, their cytokine profiles became markedly different in the presence of endometriosis. Thus, the loss of TLR4 expression was associated with altered glial-mediated inflammatory activity, which corresponded with a reduction in endometriosis-related pain behaviour. This is supported by our findings in Chapters 6 and 7 where central TLR4 expression remained intact, and the various perturbations to endometriosis-like lesions resulted in

expected or heightened pain behaviour. Further, spinal microglia are considered to express higher levels of TLR4 than astrocytes (Bsibsi *et al.*, 2002), and microglial populations were only affected by endometriosis in wildtype animals with genetically 'normal' TLR4 (Chapters 4 & 7). Collectively these studies indicate that, in addition to glial modulation, specifically inhibiting TLR4 activity in the spinal cord might offer analgesic potential in endometriosis.

We successfully inhibited peripheral TLR4 activity in Chapter 6 using the compound, 1j, which is a newly synthesised analogue of (+)-Naltrexone (Selfridge *et al.*, 2015). (+)-Naltrexone is an enantiomer of (-)-Naltrexone, a μ-opioid receptor and TLR4 antagonist. Changing the stereochemistry from (-) to (+)-Naltrexone has rendered the compound devoid of μ-opioid receptor binding at physiological concentrations, and consequently (+)-Naltrexone is believed to selectively attenuate TLR4 signalling (Hutchinson *et al.*, 2010b; Wang *et al.*, 2016). In studies of persistent pain, both intrathecal and systemic administration of (+)-Naltrexone can reverse mechanical allodynia (Hutchinson *et al.*, 2008; Ellis *et al.*, 2014) and enhance opioid-induced analgesia (Powell *et al.*, 2002; Hayl *et al.*, 2011), which are associated with reduced glial activation (Hutchinson *et al.*, 2010b). While experimental data are currently limited for 1j, this drug has also demonstrated *in vivo* efficacy in potentiating morphine antinociception, with a significantly increased TLR4 antagonistic potency and duration of action compared to (+)-Naltrexone (Selfridge *et al.*, 2015). It therefore presents as a candidate drug to explore mechanisms of TLR4-mediated pain behaviour observed in our endometriosis model; at least examined via intrathecal delivery (i.e. to selectively inhibit central TLR4 signalling). Given the high oral bioavailability and long duration of action, it may also be possible for 1j to demonstrate therapeutic analgesia when administered via a less invasive, and therefore clinically-relevant, means. Unquestionably, however, for this to become a viable treatment option in

the endometriosis population, the benefit of pain relief will need to outweigh potential off-target costs of TLR4-mediated lesion suppression (Chapters 5-6). In this context, global glial-specific inhibitors (such as ibudilast) may demonstrate better patient application.

8.3 Future directions to study the dichotomous roles of TLR4 in the development of endometriosis lesions

Studies exploring the pathophysiological roles of TLR4 in the peripheral tissues have largely implicated its heightened activation and ensuing proinflammatory signalling as contributing factors in the development of endometriosis. Accordingly, we hypothesised that animals in Chapters 5 and 6 with reduced peripheral TLR4 expression and activity, respectively, would produce relatively fewer endometriosis-like lesions, whereas those in Chapter 7 with pre-stimulated TLR4 activity would generate many. The latter theory was supported in our experiments, in agreement with the wider literature. However, an unexpected result was obtained from animals with reduced TLR4, where lesion incidence was also found to increase. This led us to speculate that typical activation of TLR4 is necessary to limit the extent of endometriosis, but any significant deviations in its function – by either attenuating or enhancing activity – can promote lesion development. Studies on IBS have shown comparable findings of both hypo- and hyperresponsiveness of TLR4 that contributes to disease progression (Cario, 2010; Frosali *et al.*, 2015). Therefore, insights into the mutual requirement for balanced TLR4 activity in endometriosis and IBS may be useful for understanding the pathogenic mechanisms underlying each condition.

Although not directly compared, it appeared that the overall condition of endometriosis (regarding spinal glial adaptations, cytokine responses and pain behaviour) differed between groups with lowered or heightened activation of TLR4. Hence, while the physical development of lesions was not discernibly different, activity of the innate immune system during the early induction phase might influence the ability of lesions to produce

subsequent biological effects. This may be significant, because the macroscopic appearances of lesions in women with endometriosis do not consistently reflect the severity of symptoms (Fauconnier & Chapron, 2005; Vercellini *et al.*, 2007). In addition, the prospective ability to measure peripheral TLR4 activity may be an important tool to determine the individual risk of developing endometriosis and its associated pain, and to treat the condition by appropriately restoring TLR4 homeostasis.

Results obtained in Chapter 7 also demonstrated that heightened TLR4-mediated inflammatory activity in the CNS may alter the development of endometriosis-like lesions in the peritoneal cavity – two anatomically distinct compartments. Administration of LPS into the spinal cord produced lesions with a heavier mass and which trended toward an increase in size, suggesting that spinal neuroimmune signalling facilitates lesion growth. Thus, CNS adaptations and inflammatory signalling may not be just a consequence of endometriosis, but also a contributing factor in the development of lesions. Although this finding is perhaps too preliminary to suggest plausible clinical implications, concomitant TLR4-related conditions that largely arise within the CNS, such as stress (Liu *et al.*, 2014; Hernandez *et al.*, 2017; Cuevas *et al.*, 2018), may prove to influence endometriosis in this manner. This central-to-peripheral effect is a relatively new concept in neuroimmune research, and has thus far only been investigated in a few models of disease, such as arthritis (Boyle *et al.*, 2006; Boettger *et al.*, 2010; Bressan *et al.*, 2010; Luo *et al.*, 2014) and colon-bladder cross-sensitisation (Majima *et al.*, 2018). These studies postulate that the mechanistic link between spinal neuroimmune signalling and enhanced peripheral inflammation involves the antidromic propagation of neurogenic inflammation via afferent neurons. It will be interesting to elucidate in future studies whether inhibiting spinal TLR4 activity reduces lesion growth, in addition to improving the development of endometriosis-related central sensitisation and pain.

Collectively, these findings imply that the relationships between TLR4 activity and endometriosis are extremely complex, involving both peripheral immune and central immune-like signalling pathways, and require further in-depth investigations beyond the scope of this thesis. The hypothesised roles of spinal glia and TLR4 in the development of endometriosis and neuroimmune-associated pain, deduced from the work presented in Chapters 4-7, are summarised in Figure 8.1.

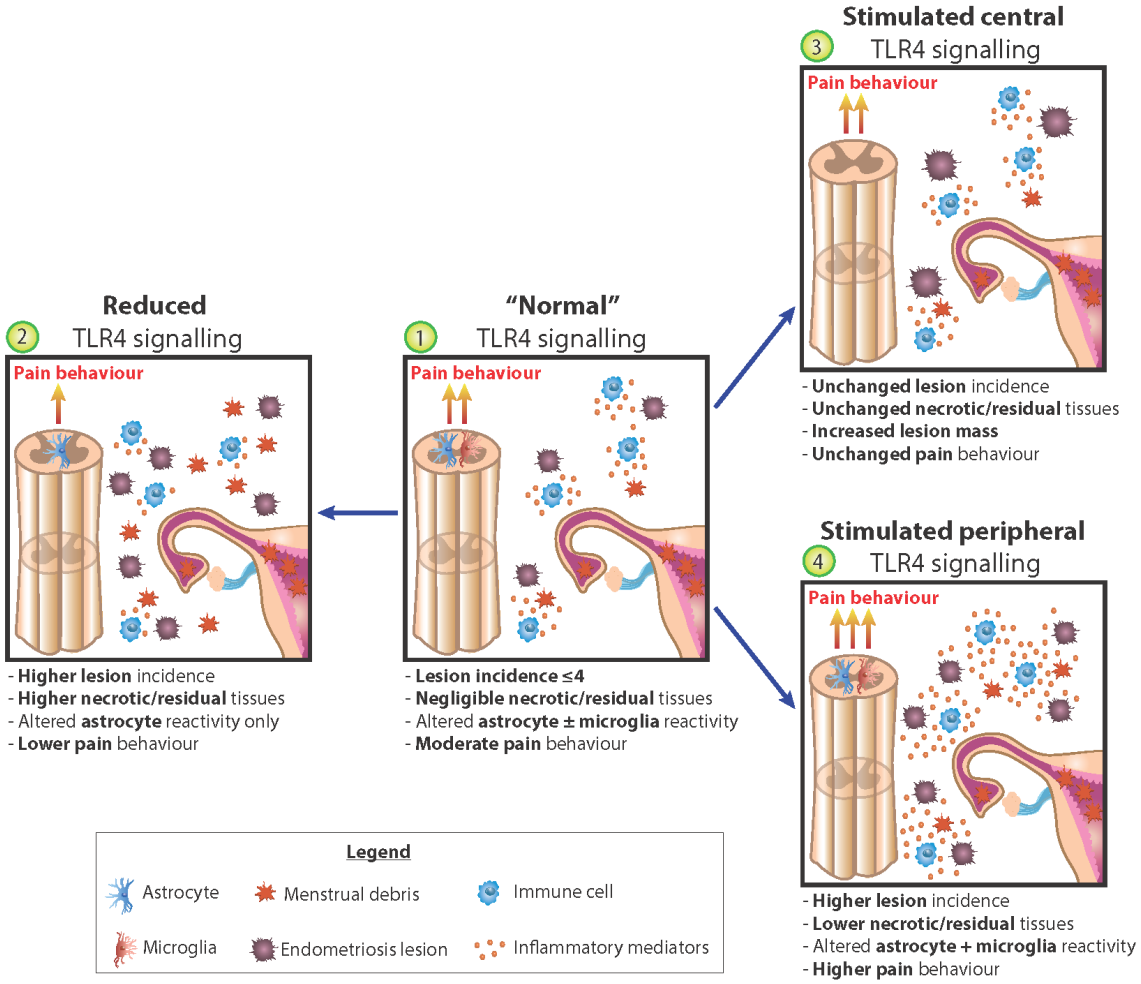
Stimulated central
3 TLR4 signalling
Pain behaviour
- Unchanged lesion incidence
- Unchanged necrotic/residual tissues
- Increased lesion mass
- Unchanged pain behaviour
Reduced
2 TLR4 signalling
Pain behaviour
- Higher lesion incidence
- Higher necrotic/residual tissues
- Altered astrocyte reactivity only
- Lower pain behaviour
"Normal"
1 TLR4 signalling
Pain behaviour
- Lesion incidence ≤4
- Negligible necrotic/residual tissues
- Altered astrocyte ± microglia reactivity
- Moderate pain behaviour
Stimulated peripheral
4 TLR4 signalling
Pain behaviour
- Higher lesion incidence
- Lower necrotic/residual tissues
- Altered astrocyte + microglia reactivity
- Higher pain behaviour
Legend
Astrocyte
Menstrual debris
Immune cell
Microglia
Endometriosis lesion
Inflammatory mediators

Figure 8.1 Diagrammatic summary of the hypothesised role for spinal glia and TLR4 in the development of endometriosis and neuroimmune-associated pain. (*1*) In wildtype ENDO animals with 'normal' TLR4 activity, few endometriosis-like lesions develop in the peritoneal cavity, and most ectopic endometrial debris is cleared by the immune system. These lesions are associated with an altered spinal expression of astrocytes (GFAP-immunoreactivity) and cytokines, with (C57BL/6) or without (BALB/c) microglial changes (CD11b or Iba-1-immunoreactivity), and a moderate degree of spontaneous pain behaviour (Chapters 4-5). **(*2*)** Compared to wildtype, ENDO animals with reduced TLR4 expression or activity display more lesions and residual endometrial tissues, due to a lack of peritoneal immune clearance. Only the expression of spinal astrocytes and cytokines are altered, and the exhibited pain behaviour is unchanged or attenuated compared to wildtype, despite the greater lesion incidence (Chapters 5-6). **(*3*)** Lesion incidence in ENDO animals with stimulated *central* TLR4 activity is similar to wildtypes, although the lesions themselves become heavier in mass (and potentially larger in diameter), suggesting that central immune aberrations may influence lesion growth in the periphery. Changes in glial reactivity are undetermined, but the few alterations observed in spinal cytokine levels do not culminate in a change of pain behaviour (Chapter 7). **(*4*)** ENDO animals with stimulated *peripheral* TLR4 activity develop the most severe lesion pathology and pain symptoms of all groups. Lesion incidence is higher compared to wildtypes, but most residual endometrial tissues are cleared by the immune system. These animals display alterations in the expression of both spinal astrocytes and microglia, as well as cytokines, and exhibit heightened pain behaviour (Chapter 7).

8.3.1 Reduced TLR4 signalling activity

The increased incidence of endometriosis-like lesions caused by genetic knockout of *TLR4* (Chapter 5) was replicated by acute receptor inhibition in the peritoneal cavity using the TLR4 antagonist, 1j (Chapter 6). Downstream signalling of TLR4 requires ligand recognition together with co-receptor, MD2. Simplistically, this triggers the subsequent activation of MyD88- or TRIF-dependent pathways; where stimulation of MyD88 leads to the transcription of traditional proinflammatory cytokines, and TRIF produces type-1 IFNs and anti-inflammatory cytokines. Although (+)-Naltrexone (and likely 1j) docks to the binding pocket of MD2 (Hutchinson *et al.*, 2010a; Northcutt *et al.*, 2015), its specific mechanism of action is thought to occur via biased antagonism of the TRIF-IRF3-dependent signalling pathway (Takeuchi & Akira, 2010; Wang *et al.*, 2016). This raises the possibility that 1j might also predominantly exhibit TLR4-TRIF antagonistic properties. As mentioned, aberrant peritoneal inflammation is believed to contribute to the development of endometriosis lesions, and alterations in various MyD88-associated transcription factors and proinflammatory cytokines, such as AP1 and IL-1β, have been observed in peritoneal fluid and ectopic endometrial tissues from endometriosis patients (Mori *et al.*, 1992; Bergqvist *et al.*; Sikora *et al.*, 2012; Beste *et al.*, 2014). Specific inhibition of TLR4-MyD88-dependent signalling may therefore offer more protection against endometriosis-like lesion formation than was observed in the present study.

Interestingly, a loss-of-function mutation in the TLR4 gene in women has been associated with an increased risk of developing endometriosis (Latha *et al.*, 2011). We hypothesise that the altered recognition of ectopic endometrial debris by peritoneal immune cells that normally express TLR4 may allow ectopic endometrial tissues to evade immune clearance, survive, and establish as mature lesions. Evaluation of TLR4-related mechanisms leading to other pathological conditions, such as tumorigenesis, may reveal clues to the

mechanisms involved in this relationship. For instance, while endometriosis lesions are considered to be benign, they do display tumour-like features such as local invasion and resistance to apoptosis, and have been associated with somatic endometrial mutations known to be involved in the development of cancer (Dinulescu *et al.*, 2005; Anglesio *et al.*, 2017). In parallel, recent evidence suggests that TLR4 signalling can play a critical role in tumour growth, including those derived from endometrial tissues (Husler *et al.*, 1998). This link is thought to involve secretion of DAMPs, such as HMGB1, by damaged tumour cells that interact with TLR4-expressing peripheral immune cells. The tumour antigen is then processed and presented to cytotoxic T-cells of the adaptive immune system for clearance. Thus in TLR4-deficient animals, increased tumour (and endometriosis lesion) susceptibility might be caused by the loss of antigen cross-presentation and subsequent anti-tumour T-cell mediated immunity (Apetoh *et al.*, 2007). Reports have also suggested that cancerous stem cells with low TLR4 expression may survive due to their ability to disregard innate inflammatory signals (Alvarado *et al.*, 2017).

TLR4 is expressed mostly, but not exclusively, by immune cells and thus can engage in non-immunological functions that may additionally alter the development of endometriosis-like lesions. Previous studies have suggested that TLRs may be involved in epithelial repair as described for the intestines (Frosali *et al.*, 2015) and lungs (Noble & Jiang, 2006). In damaged tissue, the extracellular matrix protein, hyaluron, is released, and its breakdown products are DAMPs that can stimulate TLR4-mediated cellular repair responses (Taylor *et al.*, 2004). However, hyaluron is also known to interact with the CD44 receptor on endometrial cells, which promotes their attachment to mesothelial surfaces such as the peritoneum (Dechaud *et al.*, 2001). It has therefore been proposed that unabated hyaluron-CD44 cellular binding, in the absence of hyaluron-TLR4 epithelial repair, may be involved in the early pathogenesis of endometriosis lesions (Dechaud *et al.*, 2001).

The loss of TLR4 activity may also indirectly affect other processes involved in endometriosis, such immune-endocrine interactions. For instance, bacterial sequencing of caecal contents from female *TLR4*$^{-/-}$ mice by researchers from our institution serendipitously revealed an increased population of β-glucuronidase-producing proteobacteria compared to BALB/c wildtypes (Wardill *et al.*, 2016). Intriguingly, β-glucuronidases are enzymes secreted by enteric proteobacteria that deconjugate oestrogens back into their active form. Hence, an increase in their abundance and/or activity may lead to higher levels of circulating oestrogens, and the risk of developing hyperoestrogenic pathologies. It has therefore been proposed that increased β-glucuronidase-producing bacteria in the gut microbiome of endometriosis patients may contribute to the elevated levels of oestrogen that drives lesion establishment (Baker *et al.*, 2017). Shifts in gut microbial profiles have been demonstrated in primates (Bailey & Coe, 2002) and mice (Yuan *et al.*, 2018) with endometriosis-like lesions, although the mechanisms connecting these findings remain unclear.

8.3.2 Heightened TLR4 signalling activity

Inflammatory mediators within the peritoneal environment and endometriosis lesions are believed to facilitate the condition via numerous processes, including ectopic endometrial cell adhesion, implantation and proliferation (Khan *et al.*, 2018). Although the causal relationship between inflammation and endometriosis are still uncertain, experiments in Chapter 7 demonstrated that a pre-existing peritoneal TLR4-mediated inflammatory response is sufficient to promote lesion development and enhance pain behaviour. As alluded to earlier, the prospective ability to determine TLR4 inflammatory profiles of women may assist with predictions of susceptibility to, or recurrence of, endometriosis lesions and pain. Previous studies have demonstrated upregulated TLR4-mediated cytokine output from peritoneal immune cells of endometriosis patients (Khan *et al.*, 2008b; Khan

et al., 2013b), although these cells can only be obtained from women via laparoscopy. Given the functional similarities in TLR4 signalling between immune cells of peripheral tissues, it should be determined whether an equivalent result can be obtained from more accessible sources, such as PBMCs. Several studies from our laboratories have already utilised this idea to successfully predict pain in various patient populations, where LPS-stimulated PBMCs produce greater cytokine output in people with persistent pain than those without (Kwok *et al.*, 2012; Kwok *et al.*, 2013). A similar finding has also been described in patients with bladder pain syndrome (Schrepf *et al.*, 2014; Schrepf *et al.*, 2015). The TLR4 responsiveness of PBMCs has therefore been proposed as a systemic biomarker for pain (Kwok *et al.*, 2013), which may be highly anticipated for endometriosis patients as there are no reliable non-invasive prognostic or diagnostic tests (for lesions or pain) currently in existence.

Of course, this suggestion stems from the assumption that our present results have emerged by directly stimulating peritoneal immune cells via IP LPS injection. However, as mentioned above, TLR4 is not exclusively expressed by immune cells; therefore, other non-immunological effects may have been involved. To clarify this point, we recommend experiments in which LPS-primed peritoneal immune cells are adoptively transferred into naïve animals prior to the induction of endometriosis. Our experiments in Chapter 7 also did not conclusively show that peritoneal immune or spinal glial cells were functionally sensitised by peripheral or central LPS, respectively, although acute LPS-mediated priming of these cells has been demonstrated extensively in previous studies (Guo & Schluesener, 2006; Pestka & Zhou, 2006; Hains *et al.*, 2010). Nevertheless, analysis of cytokines that were upregulated at the time of tissue collection may support that this occurred in our models, especially given the suggestion that circulating inflammatory mediators may be used as a proxy for immune memory (Nott & Glass, 2018). For example, animals centrally

administered with LPS and endometriosis in Chapter 7 showed a persistent increase in spinal levels of IFN-γ, and LPS-primed primary microglial cells have been shown to release IFN-γ *in vitro* (Makela *et al.*, 2010). Profiling of altered inflammatory mediators associated with TLR4 activation and endometriosis might additionally prove to be beneficial by someday contributing to the goal of personalised medicine. Neutralisation of TLR4-related cytokines, for example, has already shown therapeutic promise in animal models of endometriosis (Somigliana *et al.*, 1999; Barrier *et al.*, 2004; Khoufache *et al.*, 2012; Quattrone *et al.*, 2015; Liu *et al.*, 2016b; Miller *et al.*, 2017), with the potential for further efficacy when used in a tailored combination.

Finally, experiments in Chapter 7 used the most well-characterised TLR4 agonist, LPS. However, TLR4 can be stimulated by many other ligands and thus additional two-hit paradigms may be investigated. This could include any of the aforementioned DAMPs implicated in the development of endometriosis, such as Hsp-70, HMGB1 or metabolites of hyaluronic acid. The significance of these molecules is that they can stimulate TLR4 in the absence of obvious infection or injury, leading to an inconspicuous 'sterile' inflammatory response (Khan *et al.*, 2013a; Kobayashi *et al.*, 2014). In addition, successive immune challenges that are not necessarily specific to TLR4, but relevant to the clinical condition of endometriosis, may be of interest. For example, women with endometriosis display altered menstrual characteristics that increase the likelihood of peritoneal exposure to endometrial debris, including heavier and more frequent menstrual periods compared to healthy controls (Vercellini *et al.*, 1997). The development of endometriosis-like lesions could therefore be examined following multiple 'hits' of simulated retrograde menstruation, as might occur in humans. Moreover, the only available method for removing endometriosis is through surgical excision at laparoscopy or laparotomy, yet these procedures do not guarantee that the condition will not recur and often women undergo multiple surgeries (Cheong *et al.*, 2008). Laparotomy itself is significantly

associated with priming of immune cells and spinal glia (Hains *et al.*, 2010; Yoshida *et al.*, 2015), and therefore it remains to be determined whether this surgery – required for diagnosing and treating endometriosis – could paradoxically worsen a subsequent bout of lesions and pain (Long *et al.*, 2016). These examples, and our results, highlight that the increased susceptibility to endometriosis in a subset of women can involve an existing or sensitised inflammatory response, which has the potential to be instigated by diverse immune challenging stimuli.

8.4 Concluding remarks

Endometriosis remains an enigmatic condition associated with infertility and debilitating pelvic pain syndromes, which pose a substantial burden upon the health and wellbeing of millions of women, and their families and communities, worldwide. Like many developed countries, the significance of endometriosis-related pain has been largely overlooked and dismissed in Australia by primary health care clinicians, major medical research funding agencies, and government bodies at the local, state and federal levels. During the final year of this PhD candidature, several non-profit foundations, women's health advocates, researchers and members of parliament formed the Australian Coalition for Endometriosis (ACE). Their tenacious efforts to bring attention to this issue led to a formal apology by the current Australian Minister for Health, Greg Hunt, and the development of a National Action Plan for Endometriosis. In addition to improving the awareness and education of endometriosis in the community, this policy has pledged an initial $2.5 million in funds for research – a tremendous step toward furthering our understanding of the condition, optimising methods for timely diagnosis, and developing new treatment opportunities to enhance each patient's quality of life.

The studies presented in this thesis have provided evidence indicating that spinal neuroimmune adaptations occur in the presence of endometriosis. Specifically, we have

determined that the altered reactivity of glial cells and inflammatory signalling mediated via the innate immune receptor, TLR4, may contribute to endometriosis-related pain. Further appreciating the impact of these changes could facilitate the development of novel centrally-targeted analgesic therapies, potentially leading to useful clinical outcomes for patients with intractable pain symptoms. In addition, our studies support the overarching view that immune dysfunction is a crucial factor in the pathogenesis of endometriosis, by demonstrating the importance of TLR4-mediated inflammatory signalling in the development of lesions. Both reduced and heightened activation of TLR4, in the CNS or peripheral tissues, could facilitate lesion establishment or growth. Thus, in order to limit progression of lesions, a fine balance of TLR4 activity appears to be required. Although the wider implications of these findings remain unclear, the risk of lesion incidence or recurrence may someday be evaluated by the responsiveness of TLR4-expressing cells. Immunomodulatory therapies that target the lesion-promoting effects of TLR4 might also represent a new treatment approach. In any case, it is anticipated that present work will prompt future research efforts with a significant focus on improving the knowledge and treatment of endometriosis, and thus the livelihood of our resilient female friends, family members and colleagues living with this ‘invisible’ disease – I know they certainly look forward to it.

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Appendix 1. Publications arising from this thesis

This thesis was written as a combination of traditional (Chapters 1 & 8) and manuscript style (Chapters 2-7) sections. The peer-reviewed, formally published manuscripts (Chapters 2-4) are presented in their **original** format, with the exception of language conversions to Australian English. The references from each chapter have also been collated. Here, the final PDF (.pdf) files of these published manuscripts are presented.

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REVIEW

Glial contributions to visceral pain: implications for disease etiology and the female predominance of persistent pain

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In the central nervous system, bidirectional signaling between glial cells and neurons ('neuroimmune communication') facilitates the development of persistent pain. Spinal glia can contribute to heightened pain states by a prolonged release of neurokine signals that sensitize adjacent centrally projecting neurons. Although many persistent pain conditions are disproportionately common in females, whether specific neuroimmune mechanisms lead to this increased susceptibility remains unclear. This review summarizes the major known contributions of glia and neuroimmune interactions in pain, which has been determined principally in male rodents and in the context of somatic pain conditions. It is then postulated that studying neuroimmune interactions involved in pain attributed to visceral diseases common to females may offer a more suitable avenue for investigating unique mechanisms involved in female pain. Further, we discuss the potential for primed spinal glia and subsequent neurogenic inflammation as a contributing factor in the development of peripheral inflammation, therefore, representing a predisposing factor for females in developing a high percentage of such persistent pain conditions.

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FROM 'HYSTERIA' TO A MOLECULAR UNDERSTANDING OF FEMALE PAIN

Historical descriptions of chronic debilitating pain without obvious visible cause were originally restricted to females, and dated back over 2000 years to the era of renowned Greek physician Hippocrates (460–370 BC). Episodes of severe emotional and physical distress in women were diagnosed as 'hysteria', a condition attributed to the movement of the uterus outside of the pelvis (the 'wandering womb').[1] Towards the end of the nineteenth century, the stigma surrounding female hysteria diminished owing to accumulating evidence that men could also suffer from persistent pain, work which was largely pioneered by Sigmund Freud (1856–1939).[2] Considering pain as sex-independent in this context, along with general medical advances from the mid-twentieth century, has contributed to an immense expansion in our understanding of the mechanisms underlying the development of persistent pain. Notably, this is now known to involve bidirectional signaling between neurons and glia within the central nervous system (CNS).

However, a key discrepancy that remains in the literature is the clear over-representation of females among patients with persistent pain. There is an almost unanimous consensus that women are not only more sensitive in detecting painful stimuli, but are also the predominant sex with the most common painful disorders.[3–6] This includes, but is not limited to, conditions associated with neuropathic pain, musculoskeletal pain (such as back pain, fibromyalgia, osteoarthritis and complex regional pain syndrome), orofacial pain (including temporomandibular joint pain), abdominal and pelvic pain (such as irritable bowel syndrome, painful bladder syndrome and dyspareunia) and headache/migraine.[5]

Extensive epidemiological, clinical and experimental evidence implicates several biopsychosocial factors as contributing to the disparity in pain susceptibility across the sexes.[4] Despite this, a dichotomy exists in the pain research field at large, where the vast majority of preclinical studies have characterized pain models using male subjects only.[7] Moreover, evidence implicating neuroimmune signaling in the development of persistent pain has primarily been acquired using animal models of neuropathic and somatic inflammatory pain. This has included, but is not restricted to, muscle inflammation, spinal cord injury, peripheral nerve injury, arthritis, bone cancer and chemotherapy. Although many of these pathologies are important for understanding female pain, there is a lack of research into the large number of female-dominant conditions that stem from the viscera. Consequently, the specific biological mechanisms underlying the predisposition of females to persistent pain remain elusive.

It is possible that past research generalizing nociceptive mechanisms across the sexes has limited our approach in effectively treating female pain. Is it appropriate to assume that females process pain via identical mechanisms to males? Can we learn from, adapt and update aspects of the ancient Greek philosophy, by regarding female pain as a fundamentally distinct entity? And, to what extent do the sex-specific anatomical and neuroendocrine systems influence the heightened sensitivity of females to persistent pain?

To consider these questions, this review provides a summary of neuroimmune contributions, specifically those provided by astrocytes and microglia, to persistent pain signaling within the spinal

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cord. The concept that female sex hormones may modulate central neuroimmune signaling is then discussed, and that variations in these processes may have relevance for female-dominant pain conditions, as exemplified by several visceral inflammatory diseases. In addition, the dorsal root reflex is re-explored as a central driver of peripheral neurogenic inflammation, leading to the hypothesis that sensitized spinal glia might contribute to, and predispose, a subpopulation of females to persistent inflammatory pain.

PERSISTENT PAIN ARISES FROM CENTRAL SENSITIZATION

Pain is a complex, unpleasant sensory and emotional experience that arises in response to, or is described in terms of, tissue damage.[8] Distinct from the well-established protective and adaptive functions of acute pain, pain persisting beyond tissue healing is maladaptive and serves no known physiological function. In contrast to acute pain, the mechanisms involved in the development and maintenance of persistent pain are not fully understood. One potential mechanism that has received detailed investigation is the process of 'central sensitization', whereby long-lasting molecular changes cause amplification of pain signaling by nociceptive neurons within the CNS. Central sensitization can include conditions of both hyperalgesia (heightened pain to a previously noxious stimulus) and allodynia (pain caused by a normally innocuous stimulus).[9,10] It is now acknowledged that the development of central sensitization engages not only neuronal, but also glial processes. Hence, the following sections outline the rationale for considering persistent pain to be a 'gliopathy',[11] in addition to the previously described 'neuropathy'.

GLIA AND THE TETRAPARTITE SYNAPSE SUPPORT THE MAINTENANCE OF CNS HOMEOSTASIS

Glia are a non-neuronal, immune-like cell population that constitute the vast majority of cells within the CNS. They comprise satellite glial cells in the ganglia, and microglia, astrocytes and oligodendrocytes within the spinal cord and brain. The anatomical co-localization of astrocytes and microglia in the spinal cord, combined with pre- and postsynaptic neurons, forms a key site of interaction termed the 'tetrapartite synapse'.[12,13] Each cell within this functional unit reciprocally signals to another, contributing to a 'neuroimmune communication' that allows glia to respond rapidly to disruptions in neuronal signaling.[14,15] The reactivity state and control of astrocytes and microglia is therefore critical in maintaining healthy CNS activity.

DYSREGULATION OF HEALTHY GLIAL ACTIVITY CONTRIBUTES TO THE DEVELOPMENT OF PERSISTENT PAIN

Following injury and aberrant nociceptive events, microglia and astrocytes increase their expression and secretion of various proinflammatory cytokines and chemokines.[15] The stimulation of glial cells can occur by neurokine products released as a result of tissue injury, or by neurotransmitters released from activated neurons. Many of the proinflammatory responses of glia are important in protecting against challenges that disrupt the homeostatic balance of the CNS, such as during the sickness response—a constellation of adaptive behaviors and physiological responses that promote recovery from illness.[16] However, under certain conditions, glial reactivity is not advantageous and can instead be detrimental to neuronal function, such as during the manifestation of persistent pain.

In response to strong or persistent receptor stimulation, microglia switch from a surveillance state to an active response state, and astrocytes transition from a regulatory to reactive state.[11] Under these circumstances, the release of proinflammatory mediators by glia can contribute to ongoing nociception, by inducing long-lasting plastic changes of synaptic connectivity that enhances the transmission of ascending nociceptive information. As such, glia and their products are sufficient to create exaggerated pain. This has been shown where intrathecal transfer of highly reactive microglia alone, or injection or induction of their proinflammatory products (such as interleukin (IL)-1β and tumor necrosis factor-α (TNFα)) into naive animals, can induce symptoms of neuropathic pain.[17–19]

The downstream effects of enhanced glial reactivity are strengthened by the fact that immune mediators, including those released by glia, are substantially more potent in modulating neuronal signaling compared with classical neurotransmitters on a per molecule basis.[11] Glial proliferation, morphological changes and increases in protein expression can persist for months after initial injury, even beyond tissue healing.[20,21] Moreover, proinflammatory mediators and glial-derived neurotransmitters can reciprocally stimulate glia in an autocrine and paracrine manner, thereby amplifying a positive feedback loop of unfavorable activity.[22–24]

How do glia become activated?

Glia function as a product of their microenvironment, and as such the types of receptors they express vary from site to site, and many receptors can be upregulated to make glia more 'tuned' to ongoing stimulation. Within the spinal cord, microglia are sensitive to ATP that binds to ionotropic (for example, P2X4 and P2X7) and metabotropic (for example, P2Y6 and P2Y12) purinergic receptors.[25–28] Chemokine receptors, such as CX3CR1 (with CX3CL1/fractalkine as ligand) and CCR2 (activated by CCL2/MCP-1), also contribute to the microglial proinflammatory response,[29–32] as well as receptors for the sensory neuropeptide, calcitonin gene-related peptide (CGRP)[33] and interferons (IFN), such as IFNγ.[34] Akin to microglia, astrocytes can respond to ATP via the surface expression of P2X7 (refs 35,36) and P2Y1 (refs 25,37) and can be stimulated by IFNγ,[38] CGRP[39–41] and several mediators released by microglia themselves, including TNFα and IL-18 (for reviews, see refs 11,42). There is also evidence that astrocytes express tachykinergic NK1 receptors,[43] with substance P potentiating the IL-1β-mediated induction of IL-1β and prostaglandin E2 (PGE2) secretion from spinal cord astrocytes.[44]

Furthermore, a receptor family expressed by both glial cell types that has gained much recent attention, with regard to pain and immunity, are the Toll-like receptors (TLRs).[45] TLRs allow glia to sense the presence of pathogen- or microbial-associated molecular products. Importantly, some receptor subtypes, such as TLR4, can additionally recognize endogenous 'self' warning molecules. Numerous putative ligands have been identified for these so-called damage-associated molecular patterns in the processing of pain, including high mobility group box 1 protein,[46–48] heat-shock protein 90 (ref 49) and fibronectin.[50]

What proinflammatory products do glia release upon activation?

Glial-induced upregulation of proinflammatory signaling is achieved through the induction of gene expression by numerous second messenger-mediated pathways. This includes activation of transcription by phosphorylation of mitogen-activated protein kinases and nuclear factor-κB. Specifically, the mitogen-activated protein kinases implicated here are p38 in microglia,[51] c-Jun N-terminal kinase in astrocytes[52] and extracellular signal-regulated kinases (ERKs) in both glial cell types.[53,54] The proinflammatory products subsequently released from microglia include IL-1β, IL-6, IL-18, TNFα, PGE2, nitric oxide and brain-derived neurotrophic factor, and IL-1β, IL-6, TNFα, IFNγ, CCL2, CXCL1, CXCL21 and MMP9 from astrocytes (for reviews, see refs 55–58). In addition, astrocytes can increase their release of gliotransmitters, such as ATP,[59] glutamate and D-serine.[60]

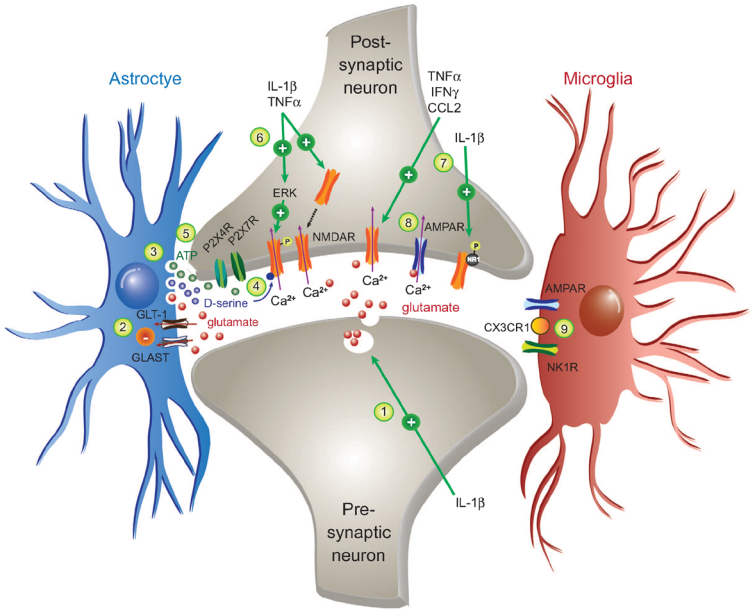

Figure 1. Schematic representation of the major proinflammatory glial-mediated alterations to excitatory synapses within the spinal dorsal horn that contribute to central sensitization. Strong or long-term noxious activation of astrocytes and microglia within the spinal dorsal horn can lead to the aberrant synthesis and release of proinflammatory mediators, such as TNFα and IL-1β. The overarching effect of these neurokine signals in excitatory synapses contributes to central sensitization and facilitates the transmission of nociceptive signals to the brain. Some of the major known adaptations include the following. (1) Increased release of the excitatory neurotransmitter, glutamate, from presynaptic nerve terminals. (2) Suppression of astrocytic glutamate reuptake via downregulation of GLT-1 and GLAST activity. (3) Release of the glutamate from astrocytes, which is capable of increasing the excitability of nearby neurons. (4) D-serine, also released from astrocytes, enhances Ca^{2+} influx via binding to glycine sites on NMDA receptors on postsynaptic neurons. (5) Astrocytic release of ATP also increases postsynaptic excitability via activation of ligand-gated purinergic receptors, P2X4R and P2X7R. (6) TNFα and IL-1β increase translocation of NMDA receptors to the postsynaptic membrane and increases their conductance via an ERK-dependent pathway. (7) IL-1β, TNFα, IFNγ and CCL2 increase NMDA receptor-mediated excitatory signaling; in the case of IL-1β, this is thought to involve the phosphorylation of receptor subunits including NR1, 2a and 2b. (8) Proinflammatory cytokines have been linked to increased expression and activation of AMPA receptors at excitatory synapses. (9) Reactive microglia have increased expression of receptors for various neurotransmitters and chemokines (for example, AMPARs, NK1Rs and CX3CR1), which can induce the further release of proinflammatory cytokines upon stimulation, thereby perpetuating neuronal excitation. ERK, extracellular signal-regulated kinase; IFN, interferon; IL, interleukin; TNFα, tumor necrosis factor-α.

As the discovery of neuroimmune contributions to pain more than two decades ago,[61–63] knowledge of glial-mediated molecular alterations in central sensitization has grown exponentially. Overall, their proinflammatory effects enhance excitatory tone and synaptic efficiency, thereby facilitating an exaggerated pain state. The sequelae of mediators released and resultant outcome are now realized to be highly dependent on the type of glial cell that is activated, the degree of its reactivity and the nature of the stimulus.[64,65] For this reason, we will provide a brief summary of the major known excitatory and inhibitory adaptations, and strongly encourage readers to explore other excellent in-depth reviews.[11,14,15,42,66,67]

GLIA ENHANCE EXCITATORY NOCICEPTIVE SIGNALING

Glial-derived proinflammatory mediators enhance nociceptive signaling in the spinal cord first by facilitating glutamatergic neurotransmission (Figure 1). IL-1β has been shown to increase presynaptic release of glutamate,[68] and IL-1β, TNFα, CCL2 and IFNγ increase postsynaptic N-methyl-D-aspartic (NMDA) and AMPA receptor currents.[69–74] Postsynaptic neurons may further be excited by the release of glutamate from reactive astrocytes.[75,76] TNFα can increase postsynaptic NMDA and AMPA-mediated activity by trafficking more receptor to the cell surface,[77] and by increasing subsequent Ca^{2+} conductance through phosphorylation of neuronal ERK.[78] In addition, IL-1β can induce SRC-1-mediated phosphorylation of the NR1 subunit on NMDA.[79,80] D-serine, a powerful neuromodulator released by reactive astrocytes, enhances depolarizing NMDA cation currents by binding to the NMDAR glycine site.[81] There is also a persistent decrease in astrocytic expression of GLAST and GLT-1;[82,83] loss of function of these glutamate transporters causes an elevation in extracellular glutamate concentrations within the synapse.[84,85] Thus, the resultant aberrant uptake and/or release of glutamate, as well as the enhanced activity of its postsynaptic receptors, can contribute to excessive nociceptive signaling reaching the brain.

In addition, increased exocytosis of ATP from reactive astrocytes[42] can directly stimulate neuronal excitation[86] or induce glutamate release from presynaptic neurons,[87] an effect that is facilitated by the upregulation of purinoceptors, such as

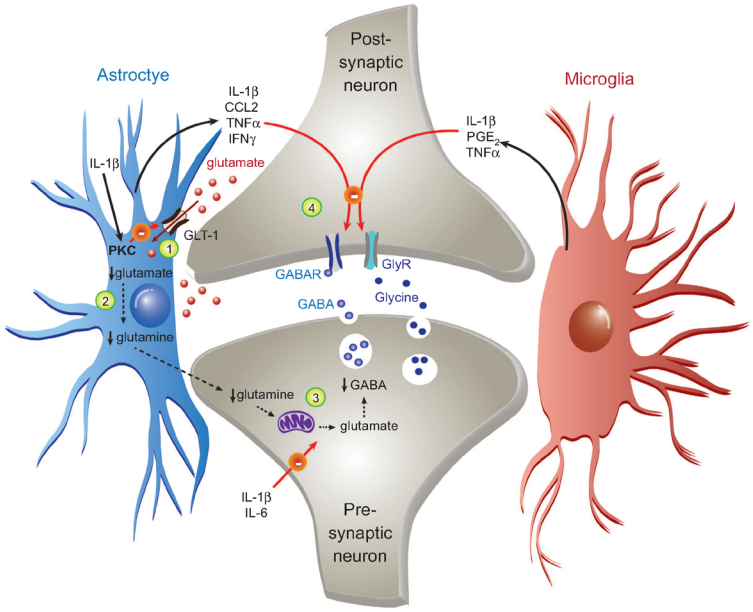

Figure 2. Schematic depicting the major proinflammatory glial-mediated changes to inhibitory synapses within the spinal dorsal horn that facilitate central sensitization. As mentioned in Figure 1, prolonged stimulation of astrocytes and microglia can lead to the increased synthesis and release of various proinflammatory cytokines and chemokines. Within inhibitory synapses of the spinal cord dorsal horn, the effects of these mediators ultimately lead to a reduction in inhibitory neurotransmission ('disinhibition'), which further facilitates central sensitization. For example: (1) IL-1β can mediate a decrease in the astrocytic uptake of glutamate, via a PKC-mediated suppression of glutamate transporter GLT-1. (2) The reduced uptake of glutamate via GLT-1 leads to decreased availability of glutamine for GABA synthesis. (3) IL-1β and IL-6 inhibit presynaptic GABA and glycine currents. (4) Last, IL-1β, PGE2, CCL2, TNFα and IFNγ decrease GABA and glycine receptor activity; in the case of IL-1β, this is thought to be mediated via a PKC-dependent pathway. IFN, interferon; IL, interleukin; PKC, protein kinase C; TNFα, tumor necrosis factor-α.

P2X4R,[50,88] P2X7R[89,90] and P2Y12R.[91,92] Levels of other cytokine and chemokine receptors are also upregulated, including IL-6-induced microglial CX3CR1 (refs 29,93) that enhances pain via IL-1β.[94] Under certain conditions, such as IL-1β stimulation, both glial cell types may increase NK1-receptor expression.[95] This potentiates the response to substance P,[43] in turn facilitating the release of astrocytic ATP[59] and proinflammatory cytokines, including TNFα, IL-6 and PGE2.[44,96,97] Last, TNFα, IL-1β and IL-6 can elicit long-term synaptic plasticity by inducing the phosphorylation of the transcription factor cAMP response element-binding protein (CREB),[70] which may lead to the CREB-mediated transcription of COX-2 and NK1.[98–100]

GLIA ATTENUATE THE INHIBITION OF NOCICEPTIVE SIGNALING

Heightened glial activation can also induce disinhibition; that is, a loss of inhibitory signals within the CNS that usually suppress nociceptive transmission, such as GABA and glycine signaling (Figure 2). The activation of microglial TLR4 by lipopolysaccharide (LPS) in rodent spinal slices induces IL-1β release, which suppresses postsynaptic GABA receptor function through the activation of protein kinase C.[101] IL-1β-induced protein kinase C activation also attenuates astrocytic GLT-1 activity, leading to increased glutamate within the synaptic cleft.[101] This not only drives a sustained excitation of postsynaptic neurons, but also a deficiency in the supply of glutamine, which is metabolized from glutamate following its reuptake. Consequently, glutamate–glutamine cycle-dependent GABA synthesis by the presynaptic neuron is attenuated.[102] Moreover, TNFα can prevent action potentials in inhibitory presynaptic neurons;[103] IL-1β and IL-6 suppress presynaptic GABA and glycine currents;[70] and PGE2, CCL2 and IFNγ can attenuate postsynaptic electrical activity mediated by GABA or glycine.[104–106] Thus, suppression of inhibitory influences within the spinal cord by glial-derived factors may exacerbate pain, by potentiating the transduction of nociceptive information.

FEMALE SEX HORMONES AND NEURONAL HYPOTHESES UNDERLYING THE SEXUAL DIMORPHISM OF PAIN

In addition to many pain syndromes having greater prevalence in females than males, other anecdotal evidence suggests that sex steroid hormones can have a direct influence on somatic and visceral persistent pain. In women, for instance, certain painful conditions typically occur during the menstrual years, and symptoms tend to fluctuate with the menstrual cycle.[107,108] Symptom severity of several visceral pain conditions, such as irritable bowel syndrome, has been reported to decrease following menopause,[109] and increase with hormone replacement therapy

in postmenopausal women.[110] Similarly, nociceptive stimuli in rodent visceral pain models are sensitive to both the changing steroid hormone levels throughout the estrous cycle,[111–113] and during hormone supplementation following ovariectomy.[114–116] Thus, it has been suggested that either elevated or fluctuating levels of sex hormones have a key role in exacerbating persistent pain.[117]

However, the mechanisms underlying this modulation remain unclear and, to date, much of the research has focused on sex steroid-mediated alterations in neural activity and/or molecular targets expressed by neurons. For example, antagonism of neuronal NMDA receptors, often co-expressed with estrogen receptor α (ERα), can attenuate the visceromotor reflex to colorectal distension with greater potency in untreated ovariectomized rats, compared with those with estradiol replacement.[118] Colorectal distension is correlated with an increase in PKA-mediated NMDAR NR1 subunit expression and phosphorylation in ovariectomized, estrogen-supplemented animals, compared with those not receiving estrogen.[118] Furthermore, intrathecal administration of estrogen or an ERα-selective agonist can cause an increase in distension-evoked dorsal horn neuron pERK expression, and reverse the decrease in distension-evoked visceromotor reflex produced by ovariectomized rats.[119]

DOES FEMALE SEX HORMONE MODULATION OF GLIAL REACTIVITY CONTRIBUTE TO THE FEMALE PREDOMINANCE OF PERSISTENT PAIN?

Despite our understanding of the tetrapartite synapse in facilitating nociceptive signaling, it is likely that the contribution of glia has not yet received sufficient attention with regard to the female susceptibility to persistent pain. Intriguingly, TLRs - which, as discussed previously, are one receptor family expressed by glia and have an important role in the immunological response to pathogenic stimuli—are well situated to serve as an important molecular target for persistent pain conditions. This is particularly true for hormonally regulated female pain, as estrogen appears to influence TLR4-mediated proinflammation and pain in various conditions. For instance, glucuronide metabolites (which typically have a longer half-life than the parent molecule) of estrogen cause potent activation of TLR4 *in vitro*, correlating with enhanced mechanical allodynia in rats *in vivo*.[120] The proinflammatory response to LPS is potentiated by estrogen in female but not male neonatal microglia.[121] Moreover, although adult hippocampal microglia from ovariectomized rats in *ex vivo* preparations show a downregulation in LPS-induced inflammation upon estrogen supplementation, IL-1β mRNA is potentiated when estrogen is administered *in vivo*.[121] Long-term estrogen exposure in ovariectomized mice promotes the expression of inflammatory mediators by CNS and peritoneal macrophages, in response to LPS activation *in vivo*[122] and *ex vivo*,[123] respectively. Intravenous administration of LPS in humans induces a similar decrease in visceral and musculoskeletal pain thresholds, although intriguingly a much more pronounced increase in circulating levels of plasma TNFα and IL-6 was evidenced in females compared with males.[124] A recent randomized control trial additionally showed that low-dose LPS was perceived to increase pain from suprathreshold noxious thermal stimuli in women only, and impaired conditioned pain modulation, a measure of endogenous pain inhibition.[125]

Other studies have reported that TLR-mediated responses are important in male but not female pain. Using LPS-induced (in TLR4 mutant mice)[126] and spinal nerve ligation (in TLR4 knockout mice)[127] models of pain enhancement, it was reported that mechanical allodynia is TLR4-dependent in males but TLR4-independent in females. Inhibition of spinal p38 MAP kinase has been effective in attenuating inflammatory and neuropathic pain in male, but not female mice.[128] It has further been proposed that female pain is independent of microglia in a rodent model of mechanical allodynia, alternatively involving the recruitment of T cells.[129] However, this argument bears further consideration given that males are comparable to females in the generation of autoimmune T cells, but the phenotype of regulatory T cells (Treg), which serve to suppress inflammatory processes, may be more aggressive in males.[130]

Perhaps these opposing results mirror the highly complex, and well recognized, nature of estrogen being both a pronociceptive and antinociceptive hormone (see reviews in refs 131–135). Regardless, it is evident that the effects of female sex hormones on TLR4-mediated signaling are multifaceted and, given the range of receptors and pathways utilized by glia, highlight the need for research into neuroimmune mechanisms that may be specific to pain in females.

SOMATIC VERSUS VISCERAL PAIN

Persistent pain is a cardinal feature of chronic inflammation of peripheral tissues; thus, our increase in knowledge of neuroimmune signaling has led to investigations of the link between glia and persistent pain associated with inflammation. These data have been primarily acquired using animal models of neuropathic and somatic inflammatory pain, with considerably less attention given to pain arising from the viscera. Although there are many commonalities in the processing of somatic and visceral pain, there are also several important clinical distinctions (for reviews, see refs 136–138). For instance, pain cannot be evoked from all viscera; visceral pain is diffuse and poorly localized, owing to relatively few visceral afferents with extensive receptive fields; visceral pain can often be referred to remote locations, attributable to visceral and somatic afferent pathways converging into shared spinal levels; injury to the viscera does not necessarily cause pain; and intense motor and autonomic reflexes, such as nausea and muscle tension, usually accompany visceral pain. This aside, the fundamental mechanisms leading to the perception of somatic and visceral pain are similar, where enhanced activity from peripheral nociceptors activates ascending central pathways to the brain. Consequently, the involvement of neuroimmune signaling in persistent pain attributed to visceral inflammation has gained interest in the past few years.[139]

NEUROIMMUNE CONTRIBUTIONS TO THE FEMALE PREDOMINANCE OF PAIN ASSOCIATED WITH INFLAMMATION OF THE PELVIC VISCERA

The viscera are also where sex divergences in pain processing become particularly intriguing, owing to the unique organization of the reproductive and pelvic anatomy in males and females. It has been estimated that women are at greater risk of developing persistent pain within the pelvis, currently affecting between 15 and 24% of women[140,141] (versus 1.8–12% in men[142,143]), including pain due to menstruation, intercourse, pregnancy and childbirth, and infection and inflammation via the vagina, cervix and uterus.[3,144,145] Spinal microglia been found to contribute to pain in male animals with chronic prostatitis.[146,147] To our knowledge, however, there are currently no comprehensive studies investigating glial contributions to pain associated with visceral diseases that have been restricted to, or with a substantial focus on, females. This alternative scope in research could reveal distinct female pain mechanisms that may be exploited to improve pain management.

Potential neuroimmune contributions to three visceral conditions that have a greater prevalence in, or are exclusive to, females are discussed below: inflammatory bowel disease (IBD), painful bladder syndrome and endometriosis. These pathologies share several features of neuropathic pain and somatic inflammation, such as heightened neural activity, decreased pain thresholds and increased pain behavior, indicating that central neuroimmune

adaptations are probably taking place. This is supported by evidence demonstrating that experimentally induced IBD, cystitis or endometriosis can result in the sensitization of adjacent pelvic organs (for example, intestines, bladder and uterus).[148–151] A similar phenomenon is observed clinically with the clustering of comorbidities in women with pelvic pain, such as patients with irritable bowel often presenting with viscero-visceral (for example, bladder or menstrual pain) or viscero-somatic (for example, pelvic muscle spasm, temporomandibular pain) complaints.

Inflammatory bowel disease

IBD comprises ulcerative colitis and Crohn's disease, both of which involve colonic inflammation; however, each has distinctive pathologic features.[152] Although the prevalence of ulcerative colitis in males and females is generally similar, the female–male ratio of Crohn's disease in adults is increased up to approximately 1.2–1.3 times.[153,154] The studies on glia and IBD have utilized rodent models of di- or trinitrobenzene sulfonic acid-induced colitis, and potential differences between the sexes have not been analyzed.[155–158] Nonetheless, marked increases in reactivity were described for microglia in the spinal cord and hippocampus,[155,156] and activated satellite glia in the dorsal root ganglia.[156] This is associated with an upregulation of TNFα levels,[155,156] and closer apposition between satellite glial cells and primary afferent neurons in the dorsal root ganglia[156] via enhanced neuron–glia gap junction coupling.[158] Associated centrally derived hyperalgesia was assessed by various methods, including increased visceromotor reflex activity[156] and abdominal withdrawal reflex,[157] to graded colonic distension. Intracerebroventricular,[155] intrathecal or systemic[156] minocycline or intrathecal administration of an anti-TNFα antibody[157] attenuated the respective pain behaviors examined.

Painful bladder syndrome

Contributions of neuroimmune overactivity to persistent pain have also been suggested in animal models of, and human patients with, painful bladder syndrome. Formally known as interstitial cystitis, painful bladder syndrome affects approximately 3–7% of adult females and 2–4% of males, encompassing a range of bladder disorders that involve persistent pelvic pain or discomfort, nonspecific urinary symptoms and often cystitis.[142,159,160] In a preliminary study using pooled data from male and female cats with spontaneous feline interstitial cystitis, the fluorescent intensity and number of GFAP-immunopositive astrocytes in the S1 spinal cord dorsal horn was increased compared with healthy unaffected cats.[161] In addition, it has recently been demonstrated that peripheral blood mononuclear cells from women with painful bladder have an increased proinflammatory response to TLR2 and TLR4 stimulation *in vitro*.[162] The magnitude of the proinflammatory response also positively correlated with the extent of pelvic and extra-pelvic pain, and the manifestation of comorbid conditions.[163] This observation has great importance, as the TLR responsivity of peripheral blood mononuclear cells could serve as a neuroimmune biomarker for persistent pain,[164] given the functional similarities between TLR signaling of immune cells in the periphery and in the CNS. Thus, the heightened TLR responsivity of peripheral immune cells in females with painful bladder syndrome may indicate that CNS sensitization involving neuroimmune modulation may be occurring in parallel, and remains to be explored further.

Endometriosis

Endometriosis is an estrogen-dependent, chronic, inflammatory medical condition in women, defined as the presence of endometrial tissue in extra-uterine locations, and commonly associated with painful pelvic symptoms. It affects an estimated 5–10% women of reproductive age,[165] and up to 60% women with persistent pelvic pain.[166] Endometriosis-associated pain is thought to solely arise from the presence of lesions, yet pain symptoms attributed to the disease can occur in women with lesions removed,[167] and the severity of experienced pain correlates poorly with the degree of lesions.[168,169] Thus, it exemplifies all that is female, from the unique visceral anatomy to the complex hormonal interplay, and the long-standing association with unexplained persistent pain.

Given that the conditions mentioned above affect the visceral organs present in both sexes, studying endometriosis (and indeed other female-specific conditions, such as vulvodynia) may provide further insight into subpopulation adaptations of neuroimmune-mediated pain. Neural changes have been studied in detail,[170,171] and it has been suggested that pain attributed to endometriosis is likely to involve neuronal processes leading to central sensitization.[115,170,172,173] However, a potential role for glia has yet to be investigated. Accumulating evidence nevertheless demonstrates that there are alterations in peripheral immune function in endometriosis patients.[174,175] LPS-stimulated peritoneal macrophages from women with endometriosis secrete significantly higher levels of proinflammatory cytokines (for example, IL-6 and TNFα) than non-diseased counterparts, an effect that can be attenuated by pre-treatment with a TLR4-neutralizing antibody.[176] TLR4 mRNA transcript expression is increased up to sixfold in endometriosis lesions compared with eutopic endometrium,[177] and TLR2 and TLR9 mRNA from peritoneal effusions are upregulated in endometriosis patients compared with healthy controls.[178] It remains to be determined whether the increased TLR levels are owing to an upregulation of the receptors per immune cell, or recruitment of TLR-bearing cells to the diseased area. There is now also solid evidence from multiple lines of investigation that the development and maintenance of endometriosis involves atypical peritoneal macrophage activity.[179,180]

Collectively, these data suggest that several alterations in neural, immune and neuroimmune functions exist in the female-predominant conditions of IBS, painful bladder and endometriosis. Studies that further investigate visceral disease-associated modifications in neuroimmune signaling are desirable. Such information would further our knowledge of persistent pain mechanisms, and may also identify a molecular basis of pain susceptibility in the subpopulation of females.

DOES THE DORSAL ROOT REFLEX AND NEUROGENIC INFLAMMATION CONTRIBUTE TO THE DEVELOPMENT OF VISCERAL INFLAMMATORY CONDITIONS?

Besides painful symptoms, many chronic inflammatory diseases present with visible tissue abnormalities and consequently a vast number of studies focus on characterizing and treating these lesions. However, attention has recently shifted to unraveling the complex molecular pathways that instead underlie disease etiology. This is particularly interesting in the example of endometriosis, which is generally attributed to the movement of menstrual debris through the fallopian tubes into the abdomino-pelvic cavity during menses (retrograde menstruation).[181] Although it is estimated that approximately 90% women aged 15–49 years will exhibit retrograde menstruation,[182] only around one in ten will develop endometriosis lesions. Similarly, in many patients, the onset of IBD follows a bout of gastroenteritis,[183] yet not all individuals with gastroenteritis will develop IBD. Thus it seems other factors affect the likelihood of disease formation in subsets of patients, leaving them susceptible to developing disease compared with their peers.

It is well established that sensitized sensory nerves can initiate or exacerbate inflammatory conditions by the release of

neuropeptides from peripheral nerve terminals, such as CGRP and substance P.[184–186] This results in edema, immune cell infiltrate and other sequelae reminiscent of inflammation; hence has been termed neurogenic inflammation.[187] The release of such peptides in the periphery is known to occur via two antidromic signaling mechanisms. Initially, there is strong local stimulation of peripheral nerve terminals at the site of disease, known as the 'axonal reflex'. With increased afferent input, the central terminals of sensory neurons within the spinal dorsal horn may also be excited, leading to anterograde propagation of action potentials back to the periphery (the 'dorsal root reflex').[188–190]

Centrally derived neurogenic inflammation via the dorsal root reflex contributes to pathology in several animal models of peripheral inflammation, mostly involving the skin[191–196] and joints,[197–199] but also colitis.[200] Compared with control animals receiving infused saline, colonic tissues from rats stimulated with intrathecal SP to the lumbar spine showed increased protein expression of the proinflammatory cytokine, migration inhibitory factor, mucosal edema and lymphocyte infiltration, effects that were attenuated by intrathecal pre-treatment with an NK1-receptor antagonist. The efferent propagation of inflammation via central dorsal horn activation has also been supported in humans, by observations that relapses in ulcerative colitis have been associated with electrical stimulation of the spinal cord.[201–203]

DOES CENTRAL GLIAL STIMULATION AND OVERACTIVITY TRIGGER PERIPHERAL NEUROGENIC INFLAMMATION OF THE VISCERA?

In addition to neuropeptides, it has been suggested that pro-inflammatory cytokines are able to stimulate dorsal horn afferents to influence the development of peripheral inflammation.[204,205] It has been reported that spinal IL-1β, associated with reactive astrocytes, can contribute to the induction and maintenance of temporomandibular arthritis and associated pain.[205] In these experiments, central disruption or inhibition of spinal IL-1 receptor type 1 (a receptor for IL-1β) signaling in mice with established arthritis, resulted in significant attenuation of joint pathology. Mice without previously established arthritis showed an upregulation of astrocyte reactivity within the dorsal horn following local spinal overexpression of IL-1β, as well as joint changes indicative of the initial stages of arthritic disease. Enhanced CGRP expression was observed in primary sensory fibers of mice with

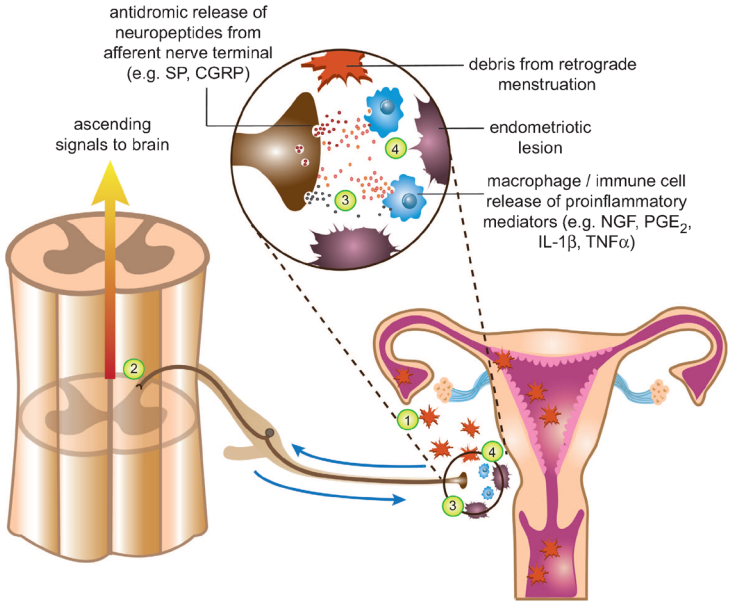

Figure 3. Possible involvement of centrally mediated neurogenic inflammation in the development of visceral inflammatory disease in the periphery: example for endometriosis. (1) During menstruation, endometrial debris passes both per vaginum and in a retrograde fashion through the fallopian tubes to the peritoneal cavity. (2) In certain women, the inflammatory events initiated by ectopic endometrial tissue activate sensory afferents innervating adjacent visceral structures, which transmit the noxious information to the spinal dorsal horn. In addition to exciting ascending neural signals projecting to the brain, afferent neurotransmitter release could potentially also activate spinal astrocytes and microglia, whose proinflammatory products contribute to the development of central sensitization and exaggerated pain (see Figures 1 and 2 for details). (3) Strong ongoing afferent stimulation associated with regular monthly menstruation and dysmenorrhea, as well as the excitatory environment created by reactive glia, may reciprocally activate the central terminals of sensory nerves. This can then induce the antidromic release of neuropeptides (such as SP and CGRP) at the peripheral site of disease (the 'dorsal root reflex'). (4) The subsequent induction of neurogenic inflammation, including the release of cytokines (IL-1β and TNFα), PGE2 and nerve growth factor (NGF) from local immune cells, may then contribute to an environment that encourages the implantation of endometrial debris onto the peritoneum, and the development of endometriotic lesions (including the associated neovascularization and sprouted innervation). CGRP, calcitonin gene-related peptide; IL, interleukin; PGE2, prostaglandin E2; TNFα, tumor necrosis factor-α.

IL-1β-overexpression (peripheral projections, dorsal root ganglia and central projections), which also displayed spontaneous behavior indicative of pain. It was suggested that bidirectional crosstalk between the CNS and peripheral joints, via spinal IL-1β stimulation of sensory afferents to release CGRP in the periphery, may have a role in the exacerbation of inflammation and pain.[205] Therefore, heightened spinal glial reactivity and proinflammatory signaling may contribute to ongoing peripheral inflammation, as well as enhancing pain by central sensitization.

This raises the interesting question as to whether centrally derived neurogenic inflammation, generated in part by neuroimmune signaling, contributes to the perpetuation of other inflammatory diseases. Indeed, neurogenic inflammatory processes have been implicated in the exacerbation of IBD, cystitis and endometriosis.[206–209] In endometriosis, neurogenic inflammation is thought to create an optimal peritoneal environment for ectopic lesion formation in the visceral tissues.[210,211] In this setting, enhanced afferent signaling in response to accumulating endometrial debris may facilitate lesion development by a positive feedback loop (Figure 3). Further research into the role of glia and the dorsal root reflex in the development of inflammation are recommended.

EARLY-LIFE STRESSORS AS CENTRAL GLIAL PRIMERS FOR VISCERAL INFLAMMATION

It is now realized that glia have the ability to be 'primed' by prior experience to over-respond to new immune challenges (a 'two-hit hypothesis'[14]). This is shown where laparotomy and intraperitoneal injection of LPS each individually cause modest increases in mechanical allodynia. However, allodynia is potentiated up to threefold when laparotomy and LPS are administered sequentially, with enhanced pain being associated with heightened microglial reactivity.[212]

Many studies are currently investigating the impact of early-life stressors, such as maternal separation or injury, on long-lasting glial alterations in the adult. Such events can be the 'first hit' that primes glia to over-respond and be detrimental in restoring 'second hit' immune challenges later in life. Visceral hyperalgesia can be enhanced by early adverse events,[213–216] although associations with glia have thus far been described only for somatic pain. For instance, incisional surgery of the neonatal rat hind paw caused an increase in the intensity of microglial activation and expression within the dorsal horn that persisted into adulthood.[20] This was associated with hyperalgesia following incisional surgery as an adult, and was prevented by intrathecal administration of minocycline at the time of adult injury. Thus, this suggests that early adverse life events provoking long-term heightened glial reactivity may lead to greater sensitivity to future harmful stimuli.

Priming of spinal glia may provide an explanation for why some subpopulations, such as females, are predisposed to developing certain painful conditions. If the neuroimmune communication has been primed before a persistent pain-triggering insult, then this mechanism may inherently increase disease burden in females (or males) due to the increased release of proinflammatory products, and may also be exacerbated by the activity of sex hormones, such as estradiol. Early aggravation of spinal glia might therefore contribute to the development of peripheral inflammation, via the dorsal root reflex or otherwise. Regarding endometriosis, clinical records from female monkeys have indicated that animals exposed to prior adverse life events, such as laparoscopic examination and cesarean section, were associated with an increase in the incidence of developing endometriosis.[217,218] The initial scenario of gastroenteritis preceding IBD could further represent the 'first hit' of irritation that sensitizes the neuroimmune system, later contributing to disease progression. Direct evidence linking early-life glial priming and the incidence of visceral inflammation in adulthood await to be studied.

BEYOND 'HYSTERIA' TOWARDS TARGETED TREATMENT OF FEMALE PAIN

Our current understanding of central sensitization leading to the development of persistent pain involves interactions between neurons and highly reactive glia. Studying alterations in these neuroimmune connections under various conditions provides enormous potential for meaningful new research discoveries and, given the significant female predominance of pain, may contribute to understanding the biological mechanisms that underlie sex differences in pain processes. Using both male and female subjects will be crucial for this future pain research. Exploring painful conditions of the viscera that are most prevalent or specific to each of the sexes, such as IBD, painful bladder syndrome and endometriosis in females and prostatitis in males, may additionally provide clues into the unique anatomical and neuroendocrine influences on pain sensitivity. Indeed, the potential contribution of neuroimmune and neurogenic signaling to inflammation and pain is a novel avenue for gynecological and urogenital research. Although much of this review has focused on female sex hormones and pain, male sex hormones may also have a critical role, where low testosterone levels are an emerging link to persistent pain states in both the sexes.[219,220] Thus, prospective studies comparing the root causes of sex-specific pain conditions may have important implications for both future pain prevention and treatment strategies.

As we unravel the molecular pathways involved in enhancing nociceptive transmission, this will provide opportunities for resultant drug discovery. New pharmacotherapies that aim to target glia to modulate their deleterious, proinflammatory contributions to pain are now steadily emerging.[14,221] This is emphasized by recent exciting studies that have for the first time demonstrated an upregulation of central glial cell reactivity in pain patients *in vivo*.[222–224] Although the translation of results from animals to humans has been variable in effectiveness, an issue plaguing the field of pain at large,[225,226] it is likely that the future analgesic success of these agents will be highly dependent on the type of injury or disease, the selection of drug and dosing regimen, the route of delivery and the timing of treatment. With continued investigations, the neuroimmune system represents a key target to decrease the burden of persistent pain.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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