Review

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Women's Health

The importance of pelvic nerve fibers in endometriosis

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Several lines of recent evidence suggest that pelvic innervation is altered in endometriosis-affected women, and there is a strong presumption that nerve fibers demonstrated in eutopic endometrium (of women with endometriosis) and in endometriotic lesions play roles in the generation of chronic pelvic pain. The recent observation of sensory C, sensory A-delta, sympathetic and parasympathetic nerve fibers in the functional layer of endometrium of most women affected by endometriosis, but not demonstrated in most women who do not have endometriosis, was a surprise. Nerve fiber densities were also greatly increased in myometrium of women with endometriosis and in endometriotic lesions compared with normal peritoneum. Chronic pelvic pain is complex, and endometriosis is only one condition which contributes to this pain. The relationship between the presence of certain nerve fibers and the potential for local pain generation requires much future research. This paper reviews current knowledge concerning nerve fibers in endometrium, myometrium and endometriotic lesions, and discusses avenues of research that may improve our knowledge and lead to enriched understanding and management of endometriotic pain symptoms.

Keywords: C-fibers • endometriosis • endometrium • nerve fibers • neurogenesis • pelvic pain • unmyelinated

Background

It has been known for more than a decade that nerve fibers are present in endometriotic lesions, especially deep invasive endometriosis [1–5], and also ovarian endometriomas [6] and peritoneal lesions [2,7–12]. Peritoneal lesions have been studied the most extensively and their innervation and densities are similar to eutopic endometrium [1,7]. It has been suggested that abnormal nerve fibers may also grow into a uterus and cervix that have been damaged by uterine trauma, for example, following childbirth or iatrogenic surgical damage to the cervix or uterine body [13].

Only recently, it was recognized that nerve fibers are rarely present in the functional layer of endometrium of unaffected women [7–8,14–15]. This discovery is so striking that it may be the only vascularized tissue in the body that is not normally innervated [16]. At the same time that this was recognized, it was demonstrated that the functional layer of endometrium in women with endometriosis contained substantial numbers of unmyelinated C-type nerve fibers [8,14]. It is thought that these may play a role in the mechanisms involved in the generation of pain signals within the pelvis of women with endometriosis [7,16–18].

Nerve fibers in endometrium

Detailed study of the typology and regional distribution of nerve fibers in endometrium, myometrium and lesions became possible due to the availability of robust pan-neuronal immunohistochemical markers, in particular antiprotein gene product 9.5 (PGP9.5), which labels both myelinated and unmyelinated nerve fibers. Using this pan-neuronal marker, the presence, abundance and distribution of nerve fibers within the reproductive tissues of

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women with and without endometriosis have now been explored by a number of authors [1,6–9,11–16,18–23]. These data have highlighted the unique differences in nerve fiber presence within the layers of endometrium and myometrium in women with endometriosis, compared with others, and within animal models.

The endometrium appears to be the only mucosal tissue in the body that is not normally innervated. Since this discovery, we have almost universally been able to demonstrate small C nerve fibers in both layers of endometrium and in increased density in myometrium in virtually all women with endometriosis [1]. However, we have been unable to demonstrate these nerve fibers in the functional layer of endometrium in any other gynecological condition, including adenomyosis, endometrial polyps, chronic pelvic inflammatory disease and endometrial hyperplasia [Al-Jefout M *et al.*, Unpublished Data]. However, Zhang *et al.* [23] found nerve fibers in the functional layer of the endometrium in women with endometriosis and/or adenomyosis or uterine fibroids. Interestingly, in some women with endometriosis, relatively thick trunks of nerve fibers have been identified running along the endometrial-myometrial interface (Figure 1), but have not been observed in women without endometriosis or pelvic pain [14]. This is perhaps not surprising since high densities of fine nerve trunks and axons are often seen in the endometrial basal layer [3]. Low-power inspection of tissue from the uterus (removed at hysterectomy) from women with endometriosis has shown that the endometrial nerve fibers enter the basal and functional layers in clusters, while many other areas of the endometrium seem to be devoid of nerve fibers (Figure 2) [Fraser IS, Tokushige N, Markham R, Unpublished Data]. This may contribute to variability in reported nerve fiber presence and density in the literature.

Microanatomical demonstration of the distribution of different nerve fibers in the endometrium

Through the identification and study of other neuronal markers, different types of nerve fibers have been identified in the endometrium and lesions of women with endometriosis [8]. These include substance P and calcitonin gene-related peptide to identify unmyelinated sensory C nerve fibers. Neurofilament was used to identify myelinated nerve fibers. Neuropeptide Y recognizes sympathetic (adrenergic) nerve fibers and vasoactive intestinal polypeptide identifies parasympathetic (cholinergic) nerve fibers. Growth-associated protein-43 is expressed on growing nerve fibers [16]. Together, these markers identify the presence of autonomic, sensory and myelinated nerve fibers in endometrium.

The great majority of endometrial nerve fibers are unmyelinated C fibers (sensory, sympathetic and parasympathetic), while a very small number in the basal layer are myelinated A-delta fibers. There may be an imbalance between the sensory and sympathetic nerve fibers in peritoneal endometriosis. Arnold *et al.* [19] have demonstrated an increased density of sensory and a decreased density of sympathetic nerve fibers in lesions. Only in endometriosis-affected women were sensory C and autonomic nerve fibers expressed in the functional layer of the endometrium. Densities were even higher in the basal layer [8]. Almost certainly, these fibers are neurites being stimulated by neuronal growth factors and growing by a branching process from preexisting nerves arising within myometrium. Nerve fiber densities in myometrium are much lower than endometrial densities in endometriosis sufferers, but are actually much higher than the densities in myometrium of women without endometriosis.

Neurotropins and their receptors are likely to play an important role in the complex process of neurogenesis and vascularization of nerve fibers in any tissue. Neurogenesis largely occurs through the regulation of axonal growth cones by neurotropic factors and other molecules that interact with specific substrates to regulate signaling and activation pathways [24]. For example, NGF is essential for the development and survival of sympathetic and sensory neurons. When NGF binds to the tyrosine kinase A (TrkA) receptor, it mediates growth and survival of sympathetic and sensory axons [25]. The immunohistochemical expression in endometrium of neurotropins and their receptors (especially NGF and its receptors, TrkA and p75) is greatly increased in women with endometriosis compared with unaffected women [26], although not all investigators have been able to demonstrate such neurotropic activity [27]. The increased expression of neurogenic promoters in endometrium may contribute to local activation of nerve fibers, sensitization of nociceptors and potentially contribute to pain symptoms in women with endometriosis.

Endometriosis is an estrogen-dependent disease and recent findings have shown an association between estrogen and progesterone receptor expression and the level of vascularization and proliferation of nerve fibers in endometriotic lesions, indicating that hormonal input may contribute to their genesis and maintenance [28]. The specific mechanism by which estrogen, in particular, regulates innervation in the endometrium is unclear, but estrogen does influence the activity of NGF, TrkA and p75 [29]. In contrast, the expression of NGF and its receptors in endometrium has been shown to be significantly suppressed in women with endometriosis who have been hormonally treated with progestogen or combined oral contraceptives [26]. Progestogen-only agents and oral contraceptives suppress ovarian estrogen production and serum estrogen levels. Combined oral contraceptives

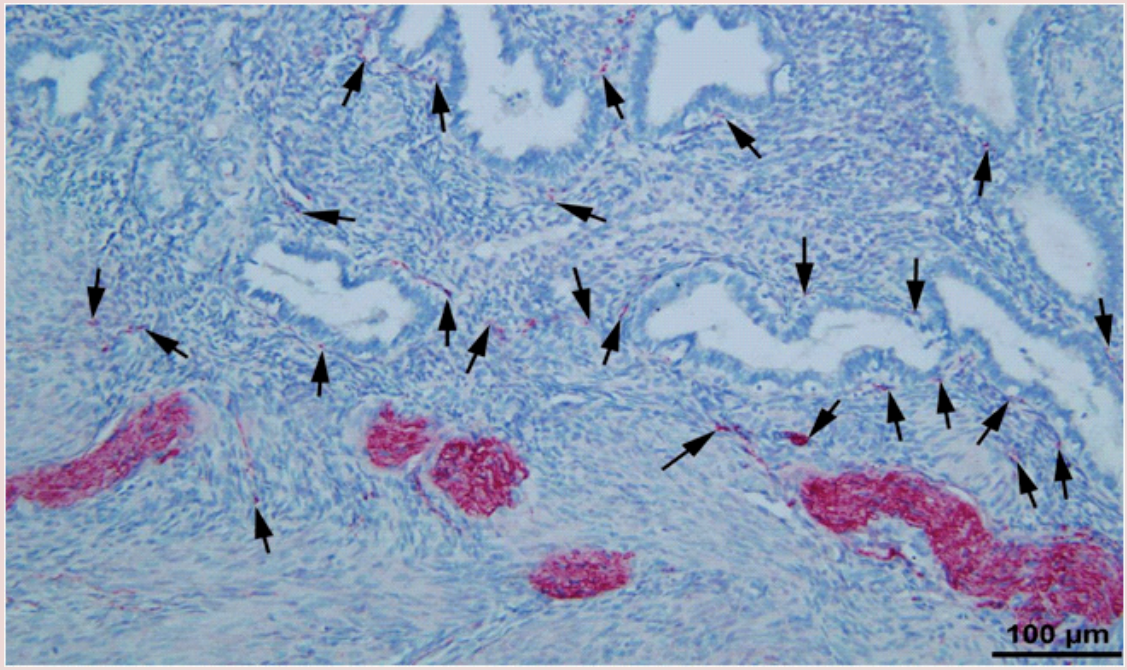

Figure 1. Large nerve trunk at the endometrial-myometrial interface and multiple small nerve fibers (arrows) in basal layer of endometrium in biopsy-confirmed endometriosis (PGP9.5; Chromagen: Fast-Red; 200×). Such large nerve trunks are never seen in this situation in women without endometriosis.
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suppress luteinizing hormone and follicle-stimulating hormone, inhibiting ovulation, which is associated with increased apoptosis and thinning in the eutopic endometrium [30]. These effects may explain why nerve fiber density in peritoneal lesions was significantly lower in hormonally treated, endometriosis-affected women, yet does not vary in lesions with normal menstrual cycle phases [20].

Asante and Taylor [31] have proposed the theory of neuroangiogenesis within endometriotic lesions to explain the common coincidence of nerves and blood vessels traveling together into new tissues. It appears generally that nerves and vessels do travel together as they traverse subperitoneal tissues, but diverge as they enter lesions. This divergence also occurs as nerves enter eutopic endometrium. This divergence may be associated with the highly significant decrease in expression of neuropilins (axonal and vascular guidance molecules; receptors for VEGF) in eutopic endometrium [32].

Potential utilization of nerve fiber detection in an endometrial biopsy as a diagnostic test for endometriosis

The preliminary findings of nerve fibers in the functional layer of endometrium in women with endometriosis suggested that an endometrial biopsy or endometrial curetting could potentially be used as a less invasive alternative to laparoscopy for diagnosis of endometriosis [7,14]. A pilot study compared the use of a narrow, disposable endometrial biopsy suction cannula 'Endosampler' to surgically obtained curettage specimens from women who were not on any hormonal treatment, to determine whether either approach would be feasible and reliable for diagnosis or exclusion of endometriosis [22]. The results were promising and suggested that endometrial biopsy could be a potential tool for initial diagnosis, although laparoscopy would still be required later, if precise staging, assessment and surgical excision were required [22]. These results were replicated with a double blind study that employed the same techniques, suggesting that endometrial office sampling might provide a reliable diagnosis of endometriosis [15]. Bokor *et al.* [21] have provided further validation for the use of a minimally invasive diagnostic test using an endometrial biopsy and immunohistochemical staining of nerve fibers.

These studies suggest that collection of a high-quality endometrial biopsy and robust research-standard nerve fiber immunohistochemical staining would be crucial for a reliable diagnosis of endometriosis using this approach [15,22]. This technique is unlikely to gain use as a diagnostic tool as it requires collection of a 'good-quality' endometrial biopsy, in addition to a well-optimized PGP9.5 immunohistochemical

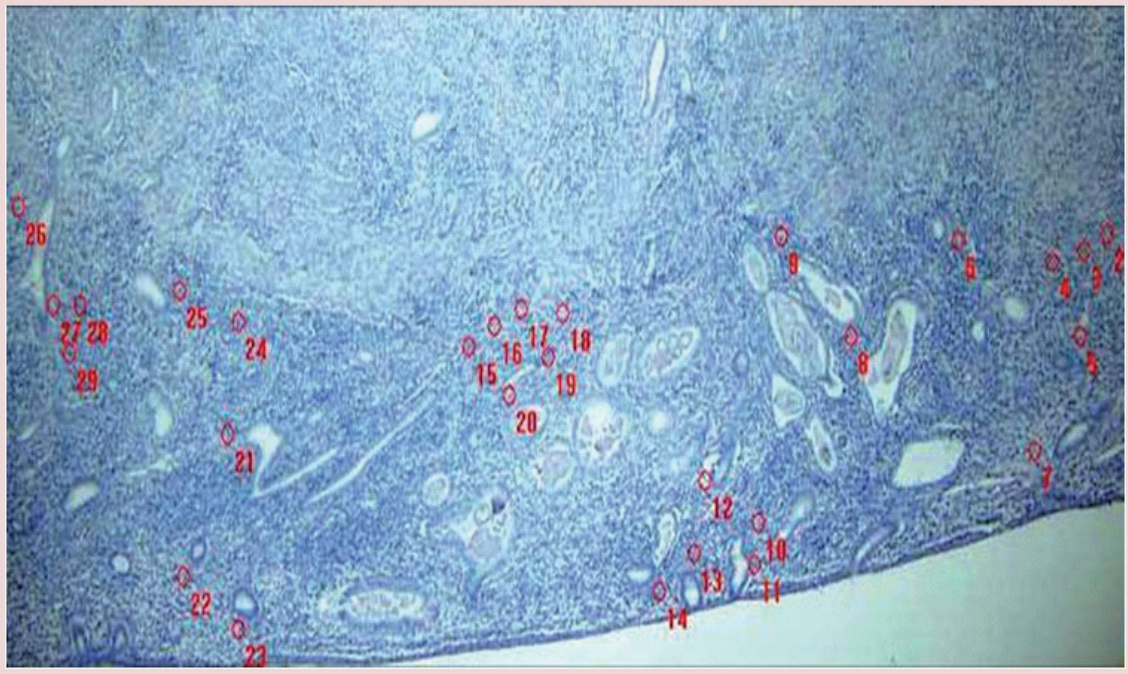

Figure 2. Full thickness section through the endometrium and inner myometrium at low power. Each individual nerve fiber has been circled in red and numbered. It is clear that most nerve fibers appear in small clusters, and at every level through the basal and functional layers up to the immediate subepithelial level. Myometrial nerve fibers have not been marked.

assay in a research standard pathology laboratory (see below).

Nerve fibers & endometriotic lesions

The nerve trunks in endometriotic lesions contain sensory, sympathetic and parasympathetic fibers. Peritoneal lesions contain substantially and significantly greater density of nerve fibers compared with normal peritoneum [7,14], and deep-infiltrating lesions involving bowel are the most densely innervated of all lesion types, with up to ten-times the density found in peritoneal lesions [1]. Lesions must be vascularized to attach and sustain themselves, and the blood vessels that aid this angiogenesis are presumably innervated by sensory and sympathetic fibers [33]. This innervation enables angiogenic and neurotropic factors to act on both blood vessels and nerve fibers, so that when blood vessels branch and vascularize to form lesions, the nerves innervating those blood vessels may also branch leading to nerve fiber infiltration of lesions [34]. This scenario is supported by data from baboon and rat models where nerve fiber density in peritoneal lesions was much greater 12 months after lesion initiation compared with 3 months after lesion initiation [Manconi F *et al.*, Unpublished Data].

If endometriosis lesions attract their own nerve supply when invading the peritoneum or deep tissue, branching and proliferation of these nerve fibers likely develop from the adjacent enteric (bowel) nerve plexus or more distant subperitoneal trunks [16]. This attraction is likely to be nurtured by neurotropins, particularly NGF and its receptors, which are involved in the growth and maintenance of sensory nerves [6,8,35], and this enriched innervation may play a key role in hyperalgesia or other pain generation [35]. Neurotropins and their receptors, such as NGF, TrkA and p75, are expressed in the stroma of peritoneal, ovarian and deep-infiltrating lesions [1,6–7,10] and the strongest expression is in deep-infiltrating lesions, which correlates with the high nerve fiber density and incidence of patient-reported pain [1,6].

Possible functions of the new nerve fibers in eutopic endometrium & endometriotic lesions

The specific functions of these new nerve fibers (sensory, sympathetic and parasympathetic) are unknown. It is hypothesized that they play a major role in the triggering of impulses that contribute to the generation of acute and chronic pelvic pain symptoms. Such stimuli will almost certainly be mediated by the nociceptors which comprise the sensory nerves in these newly innervated tissues. The presence of the sensory C fibers, which are nociceptors, can be inferred

from the demonstration of substance P and calcitonin gene-related peptide in these tissues.

Pelvic nociceptors appear to be sensitized by estrogen (which can be elevated locally in the eutopic endometrium and ectopic endometriotic lesions by local aromatase enzyme activity), and by NGF (which is also expressed locally within stromal cells of eutopic endometrium), but the actual triggering mechanisms are unclear. It is likely that specific leukocytes are again involved, since endometriosis is an intensely inflammatory condition. Nociceptor stimulation may occur around menstruation, when leukocyte infiltration is also increased and when tissue breakdown is occurring, with the products of both leukocytes and tissue damage activating nearby nerve fibers, either those adjacent to the surface epithelium in eutopic endometrium or that have grown into lesions. Mast cells, macrophages, dendritic cells, uterine natural killer cells and T-cells may play potential roles in these processes of neuronal activation [36–41].

Endometriosis is an inflammatory condition and several immune cells may play a role in the growth and development of lesions, and subsequently in the generation of pain through direct interaction with nerve fibers. Increased densities of immune cell populations such as macrophages, uterine natural killer cells and dendritic cells [36–39] and degranulation of mast cells [36] may contribute to the neurogenic factors at play [42].

Deep-infiltrating lesions are generally the most painful of the three lesion types and there is a close histological relationship between mast cells, which play an important role in the pathogenesis of many types of chronic pain, and the presence of nerve fibers [36]. These data suggest that mast cells are quite possibly contributing to the development of pain and hyperalgesia through a direct influence on nerve structures present both in lesions and myometrium/endometrium of women with endometriosis [35,36].

Innervation of the pelvis

The innervation of the pelvis is complex. Most pelvic organs are innervated by axons that have their cell bodies in the dorsal root ganglia near the spinal cord. Complex connections with neurons in the spinal cord permit local signaling and processing prior to relay to the spinothalamic tracts and higher centers. Many nerve fibers develop branches within the pelvis and form loose plexuses in specific parts of the pelvis, usually in association with major blood vessels. Some nerve cell bodies are present in these plexuses, and the presence of multiple branching nerve fibers and nerve cell bodies provides the opportunity for complex exchange of sensory and other neuronal information and effector actions. The anatomical connections of these plexuses are well described by [43], yet the functioning of these plexuses is poorly understood. In particular, there is little understanding of the way in which their interconnections may influence the potential of new sensory and sympathetic neurites in eutopic endometrium and endometriotic lesions to contribute neuronal signals to the persistent generation of pelvic pain symptoms. This is a fertile field for future research.

Our hypothesis is that these nerves develop as neurites, branching out from preexisting neurons, and stimulated by NGF acting on the high-affinity TrkA receptors. We believe that the greatly increased expression of NGF and other neurotropins in the endometrium and endometriotic lesions arises from secretion by several types of leukocyte. Current research suggests that innervation of endometriotic lesions follows similar principles with stimulation of extensive neurite ingrowth from local peritoneal nerves by increased local expression of NGF and its receptors [7].

Technical challenges of immunohistochemistry to study endometrial nerve fibers

Several research groups around the world have experienced difficulty in demonstrating nerve fibers in endometrium, and we have also sometimes experienced difficulty with tissues collected, fixed and blocked elsewhere for transfer to Sydney. There are several factors we consider important to success, these include antibody selection and optimization, retrieval and detection systems used and the sample quality.

The immunohistochemical conditions for the pan-neuronal marker, protein gene product 9.5 (PGP9.5), need to be optimized for each laboratory. In our laboratory, we have success with the use the specific DAKO polyclonal rabbit anti-PGP9.5 antibody at a 1 in 700 dilution (DAKO Cytomation, Sydney) compared with antibodies from other sources. Antigen retrieval is required using DAKO Target Retrieval Solution, pH 9.0, in a boiling water bath for 20 min. We also use a ENVISION Detection System that improves the sensitivity to detect nerve fibers and stain with Permanent Fast-Red Chromagen (DAKO Cytomation, Sydney), which aids in the detection of fine nerve fibers.

The best quality endometrial biopsy specimens are single, straight strips of endometrium, which in our institution are collected with a slow, smooth withdrawal action. Thin-fragmented, blood-filled samples do not allow examination of the endometrial stromal architecture. Samples are fixed in 10% neutral buffered formalin that is relatively fresh, for at least 18–24 h. Prolonged formalin exposure appears to make antigen retrieval less effective. We and other research groups

who have followed these detailed procedures have generally been able to identify PGP 9.5-positive neurons with reasonable reliability.

Conclusion

Extensive evidence now exists to confirm that pelvic innervation is altered and dysregulated in women with endometriosis. Greatly increased densities of sensory C, sensory A-delta, sympathetic and parasympathetic nerve fibers are present in endometrium, myometrium and endometriotic lesions. It is a presumption that these nerve fibers play a role in the generation of chronic pelvic pain, but the exact mechanisms are not delineated. Much research is ongoing by several groups and it is anticipated that improved knowledge will lead to improved management of endometriotic pain symptoms.

Future perspective & research questions

The endometrium is a remarkably complex cellular environment. It is clear that a range of disturbed cellular and molecular activities affect the eutopic endometrium in women with endometriosis. Nerve fibers have been identified in endometriosis lesions by several groups and in endometrium of women with endometriosis by some others, but their roles in pain, infertility and reproduction are not yet delineated. It is hypothesized that these nerves play important roles in the generation of neuronal signals contributing to the symptoms of chronic pelvic pain. However, better understanding of local neurogenesis and angiogenesis, and the role nerves play in pain generation is needed and is critical to developing better treatments. We do not understand what happens to these superficial endometrial nerve fibers during and after menstruation or how different hormonal treatments affect nerve fiber growth and function. The roles of various immune and other cellular components in endometrium and in lesions need urgent exploration.

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Executive summary

- Unique morphology of pelvic innervation is a key feature of endometriosis.
- Sensory C, sensory A delta, sympathetic and parasympathetic C nerve fibers are present in high density in eutopic (endometriotic) endometrium, myometrium and endometriotic lesions.
- Exact functions of these nerve fibers are unknown, but are presumed to have a central role in the generation of pelvic pain signals through nociceptor function.
- Several different types of leukocytes appear to play major roles in eutopic endometrial and endometriotic lesion function, and probably have a significant influence on nerve fiber and nociceptor function.
- Some women with persistent endometriosis appear to have a neuropathic component to their pain symptoms.
- Additionally, there are substantial alterations in central pain processing mechanisms in many women with endometriosis.

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