FACTORS ASSOCIATED WITH UTERINE FUNCTIONS IN THE MARE

Sung-Eun Bae

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Declaration

This thesis is submitted by the undersigned for examination for the degree of Doctor of Philosophy to the University of Edinburgh. This Thesis has not been submitted for the purposes of obtaining any other degree or qualification from any other academic institution.

I hereby declare that the composition and experiments of this thesis and the work presented in it are entirely my own exception of the plasma samples used in chapter 6 (experiment 2) which were collected by Dr. Elias Nikolakopoulos.

Sung-Eun Bae

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To all involved: Live long, prosper, and beloved.....

To

My grandmother, Soon-Oak Kim

For leading her grandchildren into beloved life

and intellectual pursuits

My parents, Chang-jung Bae and Gyui-sook Kwon

for their abundant support, for their patience and understanding

and for their love.

in the loving memory

of my beloved father

ABSTRACT

Delayed uterine clearance is the most common cause of subfertility in the mare. Previous studies have shown that mares that accumulate intrauterine fluid have impaired myometrial activity. Uterine contractility is a complex mechanism controlled by coordination of myogenic, neurogenic and hormonal factors. Despite the importance of the nervous system in controlling uterine contractility, there have been no studies on uterine innervation in the mare. Furthermore, there have been no detailed information on oxytocin release in mares during oestrus, the time when the uterus is challenged by breeding. The aims of this study were to investigate the factors which are associated with uterine contractility in mares. This study was designed to describe uterine innervation, to identify the presence and location of oxytocin in the equine endometrium, and to measure circulating concentrations of oxytocin around oestrus and after ovulation. The uterus of the mare was well supplied by a variety of nerve fibres. Using general neuronal marker, PAN-N and PGP 9.5, it showed a general view of equine uterine innervation. Within the regions of the uterus, nerve density score for both PGP-immunoreactive (IR) and PAN-N-IR was greatest in the myometrium. There were no marked differences between the supply to uterine horn or body, but nerve density was significantly greater ($P<0.05$) in the cervix. The nerve supply was predominantly adrenergic and was distributed throughout all regions of the uterus. With adrenergic nerves, the density score was the greatest in the myometrium within the structure and in the cervix within the regions. Peptidergic nerves were also seen and were slightly denser in the cervix than in the uterine horn or uterine body. Among the peptidergic nerves, neuropeptide Y

was the most abundant, whereas vasoactive intestinal polypeptide and calcitonin gene-related peptide were less frequent. Substance P was rarely observed. The presence of oxytocin and neurophysin in the uterus was demonstrated using immunohistochemistry. Ultrastructural studies showed that these hormones were stored in the secretory vesicles of the luminal secretory cells and the secretory cells in the superficial endometrial glands. Ciliated cells in the luminal epithelium and endometrial glands did not show any positive staining for either oxytocin or neurophysin. Mean plasma oxytocin concentrations in genitally-normal mares (n=5) were significantly higher ($P<0.02$) in oestrus (day –5 to day –2) than the day of ovulation (day 0). On the day 1 post ovulation, mean plasma oxytocin concentrations were the lowest level. Plasma oxytocin concentrations in day 2 oestrus were significantly higher ($P<0.01$) in genitally-normal mares (n=5) than in mares with delayed uterine clearance (n=5). This study has provided fundamental information on factors associated with uterine contractile function. This information will be used in further studies to investigate mares with dysfunction in uterine contractility. Uterine innervation is a main key function to regulate myometrial contractility and is coordinated by hormones such as sex steroid hormones, oxytocin and $PGF_{2\alpha}$. Studies are now needed to investigate further differences in uterine innervation, the distribution of α and β adrenergic receptors, uterine oxytocin and oxytocin receptors in mares between genitally-normal mares and mares with delayed uterine clearance.

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Chapter 1

Literature Review

Uterus - an overview

The uterus is a unique mammalian organ, essential to reproduction but not essential to the life of a female. The uterus is the place where embryos develop, establish a means of physiological exchange with the mother's blood stream, and where they are protected and nourished until they are ready to be delivered to the outside world. All subsequent embryonic and fetal development occurs within the uterus that undergoes dramatic physical and functional changes during pregnancy and at the time of parturition.

Endometritis is a common reproductive problem that affects in the endometrium of the mare. The main factor responsible for endometritis is thought to be impaired drainage of intrauterine fluids from the uterus *via* the cervix (Evans *et al.*, 1987). This failure of intrauterine clearance may result from dysfunction of the control mechanisms, including neurogenic and hormonal factors, which regulate uterine contractility.

1.1. Anatomy of the uterus

1.1.1. Structure of the uterus

The uterus of the mare is composed of two horns, a body, and a cervix. It is the part of the reproductive tract that displays the most striking differences between species. These differences are accounted for by the manner of formation of the reproductive tract from two paramesonephric ducts that grow caudally to meet and fuse with each other and with the median urogenital sinus. In some species, including many rodents and rabbit, fusion of the ducts is limited to the most caudal portions, which contribute to the vagina; the more cranial parts remain distinct, and the uterus thus

consists of paired tubes that open separately into the vagina (uterus duplex). In contrast, in women and most other primates, fusion is much more extensive and only the uterine tubes remain paired, with a median uterus with a simple undivided lumen (simple uterus). In the intermediate variety (bicornuate uterus) found in all major domestic species such as the mare, sow, bitch and cat, the uterus comprises a caudal median part from which paired horns diverge cranially to continue as the uterine tubes (Pineda, 1988).

The uterus of the mare has been described as T-shape or Y-shape when viewed dosally in its natural position (Figure 1.1). The uterus is suspended within the pelvic cavity and abdomen by the broad ligament (Figure 1.2). The uterine horns of the mare are entirely within the abdominal cavity and are floating on intestinal viscera. The thickness of the uterine walls, and the tone of the myometrium vary with the reproductive state and age of the mare (Dyce *et al.*, 1996; Blanchard, 1998). Uterine tone increases during dioestrus and remarkably during pregnancy, while it decreases during oestrus (Ginther, 1992a; Dales and Hughes, 1993).

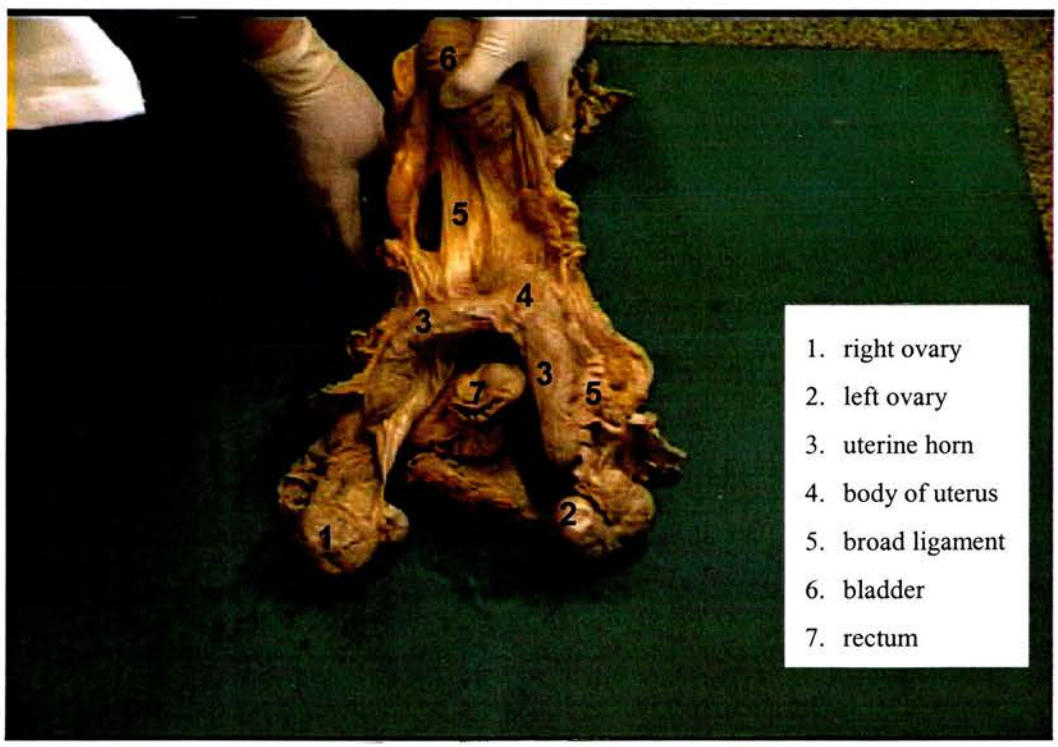


Figure 1.1 Ventral view of the equine reproductive tract.

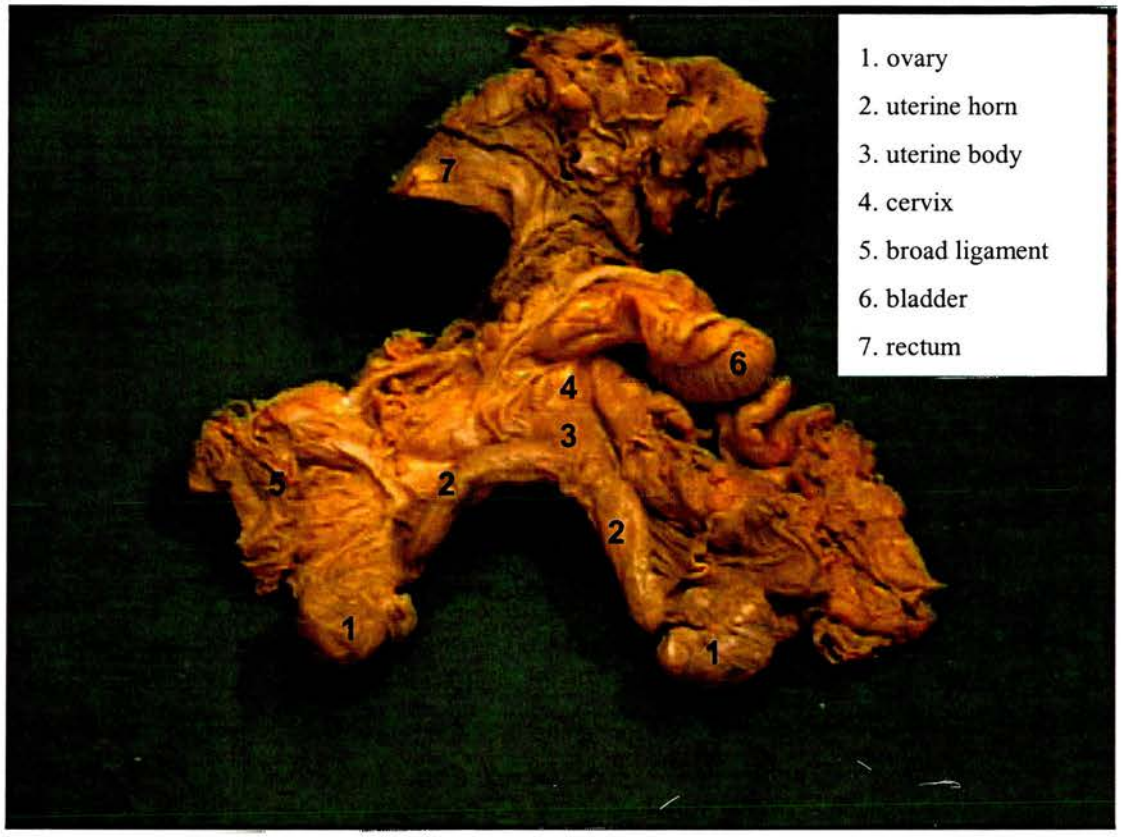


Figure 1.2. Ventral view of the reproductive organ (mare).

Histologically, the uterus is composed of three layers (Figure 1.3): (1) the *perimetrium*, which is an extension of the peritoneum, (2) the *myometrium* consisting of outer longitudinal muscle layer, vascular layer and inner circular muscle layer, and (3) the *endometrium* consisting of luminal epithelial layer and lamina propria (Kenny, 1978; Priedkalns and Leiser 1998).

The vascular, lymphatic, and nervous systems bring the reproductive system into functional relationship with other body systems. The vascular system of the reproductive tract performs the function of delivering products of the digestive, respiratory, and endocrine systems to the reproductive tract and of carrying reproductive tract products to the renal and endocrine systems. The vascular system complies with metabolic needs and transports regulatory substances such as hormones through the systemic circulation from one organ to another. The vessels of

the uterus undergo an expansion during pregnancy unequalled anywhere else in the vascular system. The uterus is supplied on each side by three arteries: the uterine branch of the vaginal artery, the uterine artery, and the uterine branch of the ovarian artery. The uterine artery is the main arterial supply. The uterus is drained on each side through three veins: uterine branch of vaginal vein, uterine vein and uterine branch of ovarian vein. The courses of the three veins and the areas drained are comparable with those of the corresponding arteries. The main venous drainage is through the uterine branch of the ovarian vein (Ginther, 1992a; Dyce *et al.*, 1996).

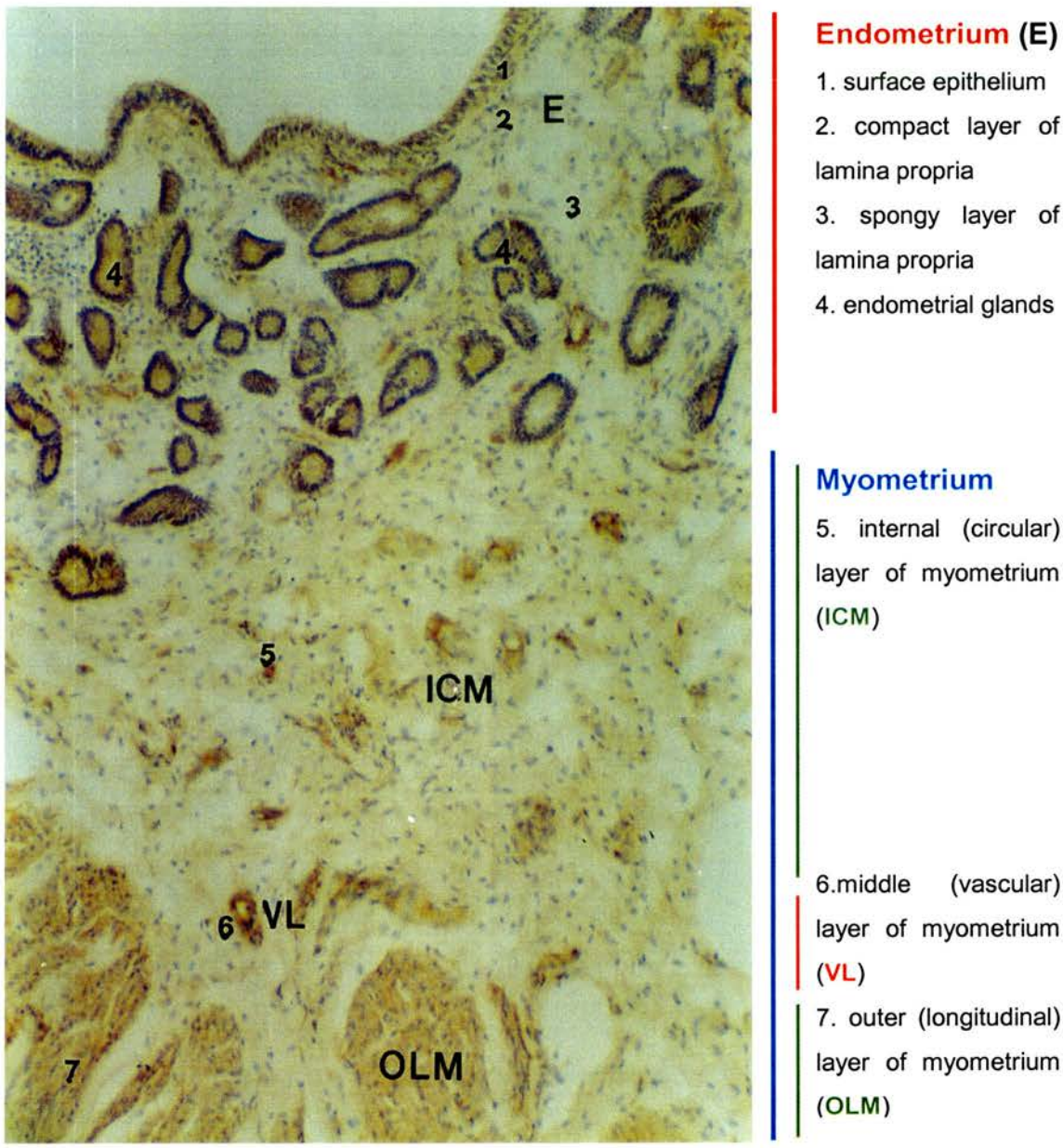


Figure 1.3. Histology of uterus (x10) showing endometrium and myometrium.

1.1.2. Structure of the myometrium

The myometrium is composed of smooth muscle cells, fibroblasts, nerves, immune cells, and blood vessels that lie in an extracellular matrix containing large numbers of collagen fibres and a lesser number of elastic fibres. The myometrium has two muscle layers with differing origin, structure, and function known as *the circular (inner)* and *the longitudinal (outer) muscle layers.* These are responsible for myometrial contractility during uterine clearance, sperm and egg transport and lymphatic drainage as well as during and after parturition. The outer longitudinal muscle layer consists of a network of bundles of smooth muscle cells that are generally oriented along the long axis of the uterus. Muscle cells of the circular muscle layer are arranged concentrically around the longitudinal axis of the uterus, and the bundle arrangement is different from that of the longitudinal muscle layers (Bengtsson, 1982; Garfield and Yallampalli, 1994; Priedkalns and Leiser, 1998).

Unlike other smooth muscle organs, the myometrium is highly functional for only a brief period following gestation and especially at the onset of parturition. During the later stages of gestation as a result of hyperplasia (an increase in the number of smooth muscle cells), and hypertrophy (an increase of cell size), smooth muscle cells of the myometrium increase in size and number. Their size and number are regulated by steroid hormones and distension. After parturition, there is destruction of some smooth muscle cells, a reduction in the size of the others and an enzymatic degradation of the collagen (Garfield and Yallampalli, 1994).

1.1.3 Control mechanisms of uterine smooth muscle cells

Uterine smooth muscle cells contain actin and myosin and they are much smaller than skeletal muscle cells. Contraction of uterine smooth muscle cells is dependent upon the interaction of thick (myosin) and thin (actin) myofilaments. Uterine smooth muscle cells do not contain transverse tubules, because their actin and myosin

molecules are close enough to the outer cell membrane to be influenced directly by the sarcolemma's action potential and transmembrane diffusion of Ca^{2+} ions. The interactions between calcium and contractile proteins are different from those in skeletal muscle (Kamm and Stull, 1985). In particular, Ca^{2+} ions react with calmodulin in the smooth muscle instead of troponin C. An increase in cytosolic free Ca^{2+} ion concentration has been considered to be the primary signal for smooth muscle contraction of the uterus (Thronton *et al.*, 1992). The Ca^{2+}-calmodulin complex activates a protein kinase that phosphorylates a myosin light chain. Once phosphorylated, the myosin can react with actin to form contractile linkages. This interaction occurs in a cyclic pattern involving attachment followed by detachment of cross-bridges and the splitting of one mole of ATP in each cycle.

In smooth muscle, extracellular calcium plays a dominant role in the activation of the uterine contractile process (Batra, 1994) as well as potassium ions (K^+) and sodium ions (Na^+). Additional mechanisms involving intercellular communications are mediated by gap junctions (Garfield *et al.*, 1980; Daniel, 1991).

1.1.4. Structure of the endometrium

The endometrium is a complex layer which lies nearest to the uterine lumen and undergoes a series of cyclical changes in both structure and function in response to endocrine events. The equine endometrium consists of *lamina propri*a and *luminal epithelial layer*. The epithelial layer is composed of high columnar cells that are secretory in nature, in contrast to other animals that have pseudostratified epithelium. The lamina propria consists of two layers; the *stratum compactum* containing densely packed stellate stromal cells and the *stratum spongiosum* containing a loose arrangement of interconnecting cells. The lamina propria of the uterus has apparent glands openings on the luminal epithelium and the glands extend down to the stratum spongiosum. These endometrial glands are branched and coiled. The epithelium of the endometrial glands comprises simple columnar cells (Kenny, 1978).

Characteristic changes in the appearance of the uterus occur at different stages of the oestrous cycle and during pregnancy. In the mare, the luminal epithelium reaches its maximal height during early oestrus; this decreases in late oestrus, dioestrus and early prooestrus. During oestrus, the luminal epithelium is tall columnar with vacuoles in the cytoplasm. These changes in the luminal epithelium result from increased cellular activity and oedema. During dioestrus, the endometrial glands become highly coiled, whereas during oestrus they appear straight and less dense. (Kenny, 1978; Britton, 1982; Ginther, 1992a). A remarkable feature in the early months of pregnancy in the mare is the presence of a ring or horseshoe of endometrial cups outlining the attachment of the yolk-sac placenta (Dyce *et al.*, 1996). Endometrial cups are formed by hypertrophy of endometrial glands and the subsequent invasion of the endometrium by chorionic epithelial cells (Allen *et al.*, 1973). These cells develop into cup cells, which produce equine chorionic gonadotrophin (eCG).

1.1.5. Ultrastructure of the endometrium

The luminal epithelium of the equine endometrium consists of three main cell types, which vary in proportion according to the stage of the oestrous cycle (Samuel *et al.*, 1979).

1) *Nonciliated microvillous cells* are polygonal in surface outline and are the most common cell types throughout the stages of the cycle. These polygonal secretory cells contain smooth apical blebs in which a number of cavities are visible.
2) *Ciliated cells* are rounded in surface outline. The cilia are 6-10μm long and are clearly separated from each other.
3) *Prociliated cells* have short surface projections that are identified as cilia in the early stages of development of the cell.

Using transmission electron microscopy, Tunon *et al* (1995) showed that the luminal epithelium of the healthy mare endometrium at oestrus was simple columnar containing both nonciliated (secretory) cells and ciliated cells. In oestrus, the glandular and surface epithelium were characterized by hypertrophy and differentiation of apparent mucus-secreting cells as shown by intense cellular vacuolation, an increased number of polyribosomes and predominant granular endoplasmic reticulum (Keenan *et al.*, 1991). The epithelial cells were anchored to each other apically by cell-to-cell junctions consisting mostly of tight junctions and numerous desmosomes. All cells had round to oval nuclei, located from the medial to the basal part of the cell. The basal plasmalemma was apposed to the basement membrane, but frequently the cells were separated by large intercellular spaces, that appeared in the light micrographs as basally located 'vacuoles'. The secretory cells had either microvilli or microvilli with a number of apical blebs (Motta and Andrews, 1976; Samuel *et al.*, 1979). The secretory cells presented a cytoplasm rich in organelles, particularly mitochondria, rough endoplasmic reticulum, Golgi apparatus, and numerous secretory vesicles containing electron-dense amorphous material at the apical, adluminal area. The ciliated cells had several organelles evenly distributed in the cytoplasm but lacked the secretory vesicles seen in the nonciliated cells in the mare (Samuel *et al.*, 1979; Ferreira-Dias *et al.*, 1999). Lysosome-like vesicles and phagosomes were often found in the ciliated cells. It has been suggested that the cilia are involved in the dispersion and direction of the flow of uterine fluids along the endometrial surface (Motta and Andrews, 1976).

The luminal epithelium, particularly in the uterine body, often contained granulocytes and intraepithelial macrophages intermingled with the epithelial cells (Tunon *et al.*, 1995). During oestrus, and in the early luteal phase, some of the nonciliated epithelial cells of the endometrium underwent transformation and lost their microvilli (Keenan *et al.*, 1991). Secretory cells declined rapidly in number after oestrus and few could be identified in dioestrus. By contrast, ciliated cells reached maximum density during mid-dioestrus and declined towards the end of the period (Ricketts, 1975; Samuel *et al.*, 1979; Keenan *et al.*, 1991). During dioestrus, the

surface of the endometrium was densely populated with ciliated cells.

The endometrial glands were composed of two distinct cell types, *dark cells* and *light cells*. Dark and elongated cells (89.3%) with a fusiform nucleus had many intact organells in the cytoplasm. The light cells (10.6%) were lighter in colour and had fewer organelles and a round nucleus with regular contour. This light cell type had numerous vacuoles (Ferreira-Dias *et al.*, 1999). The mitochondria in light cell types had a dilated semilunar shape. Intact ciliated cells were quite common on the surface epithelium and in the glandular lumen. The dark cells and light cells in the uterus have previously been reported for camels (Fetaih *et al.*, 1992) and primates (Beier *et al.*, 1971). The epithelial cells of the uterine glands varied according to their location in the gland. The gland duct contained cells that were similar to the luminal surface epithelium, except that the nonciliated cells contained fewer secretory vesicles. The mid- and deep portions of the endometrial glands were, however, nonciliated cells and were loaded with apical secretory vesicles. Ciliated cells were seen less frequently here (Tunon *et al.*, 1995).

Ultrastructural analysis demonstrated the presence of gap junctions in the subapical membranes of epithelial cells from uterine glands and luminal epithelium in equine endometrium (Brady *et al.*, 1995). Gap junctions are intercellular channels that connect the interiors of two cells by allowing the passage of inorganic ions and small molecules. The channels are composed of proteins, connexins, which span the plasma membrane to form a pore. Each channel is constructed from six connexin proteins in one cell aligned symmetrically with six connexins in the adjacent connected cell (Garfield and Yallampalli, 1994). Small gap junctional contacts were observed in the subapical membranes between the epithelial cells of endometrial glands in tissue from mares at all stages of the cycle. Gap junctions were less frequently observed within luminal epithelium. Unlike rodents which apparently do not express α_1 (Cx43) and possess β_2 (Cx26) connexins within endometrial epithelial cells, equine endometrium expressed α_1 (Cx43) as the predominant connexin (Brady *et al.*, 1995; Doualla-Bell *et al.*, 1995) in the epithelial cells. These differences

between species could be associated with differences in uterine physiology. These mechanisms involving cell-cell communication pathways mediated by gap junctions are thought to be an important factor in the integration of uterine function during pregnancy (Burghardt and Fletcher, 1990). In addition, the diversity of connexin phenotypes suggests that connexins may be functionally specialized and have specific functions in certain tissues (Beyer *et al.*, 1990). It has been demonstrated that certain connexins (including α_1 and β_1 but not β_2) are phosphoproteins and have phosphorylation sites that potentially provide additional regulatory control over gap junction permeability (Musil and Goodenough, 1993; Stagg and Fletcher, 1990).

1.1.6. Mode of release of the secretory product in endometrium

The mode by which the secretory products are released from the cell forms the basis for the classification of the glands. There are four different modes of secretion: merocrine, apocrine, holocrine, and cytocrine (Frappier, 1998).

1) During *the merocrine mode of secretion*, the contents of small secretory granules are released as the secretory product. The secretory granules are usually enclosed within a membrane. When the secretory granule reaches the cell surface, its membrane fuses with the plasmalemma, thereby discharging the secretory product via exocytosis.
2) In *the apocrine mode of secretion*, a large, single, intracellular secretory granule is also surrounded by a membrane. As the secretory granule migrates into the cell apex, the plasmalemma, and a portion of the neighbouring cytoplasm, surround the granule. Eventually, the plasmalemma constricts beneath the granule, causing the granule, and some surrounding cytoplasm, to bulge into the gland lumen. Examples of apocrine glands include the mammary gland and the sweat glands.
3) In *the holocrine mode of secretion*, entire cells are released as the secretory product. The sebaceous glands of the skin are typical holocrine glands. The cells become filled with lipid granules and move toward the duct; the cells then disintegrate, and their contents are extruded into the duct.

4) In *the cytocrine mode of secretion*, secretory material is transferred from one cell to the cytoplasm of another cell. An example of cytocrine secretion is the epidermis, where melanocytes transfer the brown pigment, melanin into the cytoplasm of the keratinocytes.

In the equine endometrium, previous studies during anoestrus reported that the luminal epithelium had a slightly undulating shape, and there was little secretory activity. However, during oestrus and in the early luteal phase, some of the nonciliated luminal epithelial cells went through transformation, and were thought to play a secretory role. Both apocrine and merocrine secretion were observed in the surface epithelial cells in the equine uterus (Samuel *et al.*, 1979; Keenan *et al.*, 1991), although Tunon *et al* (1995) only reported merocrine in the mare. Only merocrine secretion was found in the endometrial glandular epithelium (Samuel *et al.*, 1979; Keenan *et al.*, 1991).

1.1.7. Endometrial biopsy

In the mare, endometrial biopsy is an important procedure recommended to assess fertility. Several classification schemes have been developed. The most widely used technique is the grading system established by Kenny (1978). In this system, *category I* endometrium is essentially healthy tissue. *Category II* includes inflammation or fibrosis and is subdivided in to IIA and IIB. *Category III* is associated with widespread pathologic changes that drastically reduce the chances of carrying a foal to term. For the interpretation of any endometrial biopsy in the mare, various factors must be considered: these include the stage of the oestrous cycle, ovulatory and anovulatory season, age of the animal, sites of the biopsy, changes associated with the procurement of the tissue samples, and fixation (Ricketts, 1975).

1.2. Functions of the uterus

Functionally, the uterus is an essential female reproductive organ. The main function of the uterus is to serve as a conduit for transport and maturation of spermatozoa, to provide a unique embryotrophic environment required to support conceptus development and fetal growth throughout gestation. Therefore, the uterus is a relatively quiescent organ during pregnancy. During pregnancy, the uterus acts as a barrier to the maternal circulation and prevents immunological rejection of the embryo or fetus. In contrast, at the time of labour, it enters a very active state, characterised by forceful, rhythmic, and synchronous contractions. The uterus generates much of the expulsive force required to deliver the fetus and placenta at parturition (Rossdale, 1997).

The uterus is also required to support the normal ovarian cycle (Pineda, 1988). The uterus and associated structures perform endocrine functions in both the cycling and pregnant mare (Dyce *et al.*, 1996). The nongravid uterus is relatively resistant to infection while it is under the influence of oestrogen. Local antibody production and phagocytosis via opsonins are important defence mechanisms in limiting the duration of uterine infections. The cervix also provides an efficient protective barrier for the uterus. Recently, it is reported that the equine endometrium produces oxytocin locally (Watson *et al.*, 2000) as well as prostaglandin $F_{2\alpha}$.

1.3. Modulation of uterine contractility

Hormones are very important in modulation of uterine contractility by their direct effects on uterine smooth muscle as well as through indirect effects on the other neurohormonal factors (Putnam *et al.*, 1991; Batra, 1994). Progesterone suppresses the formation of gap junctions thereby decreasing the coupling between the myometrial cells. Oestrogen, in contrast, induces the formation of gap junctions, depolarizes the membrane, increases prostaglandin production and enhances the expression of oxytocin receptors. Prostaglandins enhance uterine contractility by causing membrane depolarization, influx of calcium, release of calcium from intracellular stores, and inhibition of calcium extrusion. Prostaglandins play a role in increasing oxytocin release and inhibiting progesterone synthesis. Indomethacin and meclofenamate which are known to be prostaglandin synthesis inhibitors alter the area of gap junctions in the myometrium indicating that prostaglandins are involved in the control of the junctions (Garfield and Yallampalli, 1994). They also lead a reduction in the number of gap junctions (Garfield *et al.*, 1980). Oxytocin stimulates uterine contraction by increasing the calcium influx, releasing arachidonic acid and producing prostaglandin $F_{2\alpha}$ (Torok and Csapo, 1976; Al-Eknah and Homeida, 1992). The sensitivity of the uterine smooth muscle to oxytocin is increased by upregulation of oxytocin receptors.

In a previous study, the two muscle layers of the myometrium showed differing patterns of contraction in the rat (Bengtsson, 1982). In the outer longitudinal layer, there was a gradual increase in frequency and coordination of contractions towards the end of the gestation. The inner circular muscle contracted with high frequency and low amplitude until 3-4 days before parturition when the contractions become more regular, less frequent and greater in amplitude. Recently Liu (1998) demonstrated using an *in vitro* mechanical testing procedure for the study of spontaneous contractile activity of the smooth muscle layers of the equine myometrium, that both muscle layers contracted independently of one another and appeared to be modulated by endocrine factors. The duration of circular muscle

contractions were significantly longer during the follicular phase than in the luteal phase, and significantly longer than those of longitudinal muscle during the follicular phase.

Uterine motility has been investigated in nonpregnant, early pregnant, and late pregnant mares. By ultrasonography, in pregnant mares, maximum uterine contractile activity occurred during the reported time of maximum mobility of the embryonic vesicle (Day 11 to 14). In cyclic nonpregnant mares, maximum uterine motility was detected on day 14 to 18 postovulation, during the reported time of luteolysis (Cross and Ginther, 1988). Myometrial contractility has been measured in the mare not only by ultrasonography (Cross and Ginther, 1988; Gastal *et al.*, 1998) but also by electromyographic activity (Taverne *et al.*, 1979; Jones *et al.*, 1991; Madill *et al.*, 2000), intraluminal pressure transducers (Ko *et al.*, 1989; De Lille *et al.*, 2000; Gutjahr *et al.*, 2000), and scintigraphy (Cadario *et al.*, 1995; LeBlanc *et al.*, 1998).

1.3.1. The effect of hormonal factors on modulation of uterine contractility

Myometrial activity is highly influenced by hormonal conditions throughout the oestrous cycle and during pregnancy to keep the fetus in the uterus, and by a variety of stimulants (i.e. oxytocin, prostaglandins, catecholamines etc.) (McNutt and Ducsay, 1991; Gilbert *et al.*, 1992; Gastal *et al.*, 1998) which promote expulsion of the fetus at the time of parturition.

(a) Ovarian steroid hormones

Oestrogens are secreted into the blood stream by the ovarian follicle in the mare (Ginther, 1992b). Concentrations of oestrogen are low during most of the cycle but rise in early oestrus to reach peak values 48 hours before ovulation. Progesterone is secreted by the corpus luteum. Concentrations of progesterone start to rise after ovulation and reach a peak 5 to 9 days later, plateau, and then fall rapidly at day 14-15 until the low oestrous values are reached (Townsend *et al.*, 1989). Concentrations of progesterone and oestrogen receptors are highest during oestrus, have declined by day 6 after ovulation and are lowest at day 12.

The myometrium and the smooth muscle of the uterine vessels are the major targets for ovarian steroids. Oestrogen treatments increase uterine contractility in the rat (Fuchs *et al.*, 1983) and in cows (Soloff and Fields, 1989). In contrast, progesterone decreases uterine contractility during pregnancy and in the luteal phase of the oestrous cycle (Putnam *et al.*, 1991). It is suggested that uterine membrane receptors for progesterone act *via* G protein which provides a regulatory link between action at a receptor site and intracellular activity (Nimmo *et al.*, 1991).

It is known that ovarian steroid hormones control oxytocin receptor numbers and sensitivity in the uterus (Gainer *et al.*, 1988; Sharp *et al.*, 1997) and also interact modulating gap junction formation in the myometrium (Garfield *et al.*, 1980). Oestrogen increases oxytocin receptors nine-fold in the myometrium of the nonpregnant rat, but progesterone blocks the increase. In addition, oestrogen stimulation leads to the accumulation of receptors for progesterone, oestrogen and oxytocin in the uterine target cells. Progesterone reduces nuclear oestrogen receptors with a subsequent decline in oxytocin receptors, and directly inactivates or degrades the oxytocin receptors (Fuchs *et al.*, 1983).

(b) Oxytocin

Structure, biosynthesis, and metabolism

Oxytocin is a nine amino acid residues (Cys-Tyr-Ile-Glu-Asp-Cys-Pro-Leu-Gly), which has a cyclic disulphide cystein-cystein bond containing one molar equivalent each of eight amino acid and three equivalents of ammonia, with a molecular weight of approximately 1007d (du Vigneaud *et al.,* 1953; Tuppy, 1953). There is a considerable overlap in biological characteristics between oxytocin and vasopressin. A more basic group in position 8 increases the vasopressor activity of the molecule, whereas a less basic group in that position enhances its oxytocic activity (Boissonnas, 1960).

The main synthesis of oxytocin occurs in the magnocellular neurons in the supraoptic and paraventricular nuclei of the hypothalamus in the horse (Melrose and Knigge, 1989). The exons of the oxytocinergic neurons from both these regions project through the median eminence, extend down the infundibulum, and terminate in the posterior pituitary. Oxytocin is bound to its carrier protein, neurophysin, and this complex passes down the axons of hypothalamic nuclei to be stored in nerve terminals in the posterior pituitary. The nerve terminals in the posterior pituitary release the biologically active oxytocin and neurophysin into blood vessels by *exocytosis* following appropriate stimulation (Brooks *et al.*, 1966; Brownstein *et al.*, 1980; Poulain and Wakerley, 1982). The kidney, liver and the splanchnic organs play an important role in removing oxytocin from blood. Oxytocin is mainly excreted in the urine in a biologically inactive form (Dawood and Khan-Dawood, 1985).

Oxytocin and neurophysin appear stimultaneously in the blood. It is known that the release of oxytocin into the peripheral circulation occurs in a pulsatile fashion. This phasic nature of the oxytocin release pattern is due to the unusual morphological and electrical nature of oxytocinergic cells in the paraventricular

and supraoptic nuclei of the hypothalamus. The formation of gap junctions in the cell membrane results in the pulsatile release of oxytocin into the peripheral circulation (Leng and Brown, 1997). Due to its fluctuations, plasma oxytocin concentrations in the circulation may vary tenfold or more over a relatively short period of time. This process has been described as 'spurt' release (Gibbens and Chard, 1976; Dawood, 1983).

Oxytocin circulates in the blood as a free peptide. Its biological half-life has been variously estimated between species; in goats it is about 22 minutes (Homeida and Cooke, 1984), in sheep it is 1 minute (Fitzpatrick, 1961), and in human 3 to 17 minutes (Chard, 1994). The half-life of exogenous oxytocin in mares was determined to be around 7 minutes (Paccamonti *et al.,* 1999). Concentrations of plasma oxytocin in cycling mares have been reported to be highest at oestrus (Burns *et al.*, 1981), high in early or late dioestrus (Tetzke *et al.*, 1987; Burns *et al.,* 1981) or to remain at very low concentrations throughout the cycle (Stevenson *et al.*, 1991). These contradictory studies have also shown differences in the amplitude and frequency of patterns of oxytocin release in the mare. These confusing results may be due to the short half-life of oxytocin and its pulsatile pattern of release, therefore frequent sampling is required to study the secretion of oxytocin. Oxytocin release is also reported following some types of stimulation such as stallion calls, teasing, natural service and artificial insemination (Burns *et al.*, 1981; Alexander *et al.*, 1995; Madill *et al.*, 2000; Nikolakopoulos and Watson, 2000b).

Extrahypothalamic sources of oxytocin

Oxytocin release from the hypothalamus is thought to be responsible for increases in circulating plasma oxytocin concentrations during the reproductive cycle. However, in other species oxytocin has also been identified in peripheral sites such as the thymus (Geenen *et al.*, 1986; Argiolas *et al.*, 1990), adrenal glands (Nicholson *et al.*, 1984), placenta (Fields *et al.*, 1983; Lefebvre *et al.*, 1992a),

fetal membrane (Chibbar *et al.*, 1993), ovary (Fields *et al.*, 1983; Watkins, 1983; Guldenaar *et al.*, 1984; Kruip *et al.*, 1985), uterus (Lefebvre *et al.*, 1992b) and male reproductive tract (Nicholson *et al.*, 1984; Einspanier and Ivell, 1997). In these tissues, oxytocin is expressed in such a low concentration that it is probably not released into the blood and, therefore, functions in a paracrine rather than an endocrine action (Zeeman *et al.,* 1997). In the mare, mRNA encoding oxytocin as well as the peptide has been identified in the uterus (Behrendt *et al.*, 1999; Watson *et al.,* 2000) but not in the ovary (Stevenson *et al.*, 1991; Watson *et al.,* 1999a).

1) Uterus

The oxytocin gene has been shown to be expressed in the rat uterus (Lefebvre *et al.*, 1992b). Small amounts of oxytocin mRNA have been found in the uteri of the nonpregnant rat, but a 150-fold increase in oxytocin mRNA was found during pregnancy, and the rise in oxytocin mRNA was very rapidly reversed after delivery. This suggests that oxytocin may act primarily as a local mediator. In the uterus of the sow, mRNA for oxytocin was also found (Trout *et al.*, 1995; Boulton *et al.,* 1996) and the concentrations of oxytocin mRNA were greater at oestrus than during the luteal phase or at any stage of pregnancy. This suggests that local uterine synthesis of oxytocin may be more important in control of the oestrous cycle than in pregnancy or at parturition in pigs.

In the equine endometrium both mRNA for oxytocin and oxytocin itself have been reported (Behrendt *et al.*, 1999; Watson *et al.*, 2000) as well as its prohormone, neurophysin. In the mare, most intense immunostaining for oxytocin was seen in early pregnancy followed by oestrus (Watson *et al.*, 2000). The local paracrine or autocrine effects of oxytocin on its endometrial receptors to produce and release prostaglandin may be sufficient to initiate uterine activity (Watson *et al.,* 2000). In addition, uterine oxytocin may act in a paracrine manner to drive luteolysis in the cycling mare (Stout *et al.*, 2000).

2) Ovary

The first evidence for an extra-hypothalamic source of oxytocin came from studies on the ovine corpus luteum (Wathes and Swann, 1982). Oxytocin has been identified immunocytochemically within the corpus luteum, especially the giant cells in sheep (Fields *et al.*, 1983; Watkins, 1983), cows (Fields *et al.*, 1983; Wathes *et al.*, 1983; Guldenaar *et al.*, 1984; Kruip *et al.*, 1985; Fields *et al.*, 1992), rats (Viggiano *et al.*, 1989) and women (Khan-Dawood, 1987). However, in contrast to the other species, the ovary is not a source of oxytocin in the mare (Murray *et al.*, 1991; Stevenson *et al,* 1991; Watson *et al.*, 1999a).

In ruminants, oxytocin secreted by the corpus luteum stimulates the pulsatile release of $PGF_{2\alpha}$ from the endometrium that brings about cyclical luteolysis (McCracken *et al.*, 1984) *via* the upregulation of oxytocin receptor. In the mare, the events that control luteolytic $PGF_{2\alpha}$ release are less clearly understood and previous studies indicated that oxytocin was not involved in the luteolytic pathway (Arthur, 1975; Neely *et al.*, 1979). However, Stout *et al* (1999) found that continuous, high-dose, systemic administration of oxytocin from day 8 after ovulation prevented luteolysis in mares, similar to other reports in sheep (Flint and Shedrick, 1985; Ayad *et al.*, 1993), and cattle (Gilbert *et al.*, 1989), and this result suggested that oxytocin is involved in the luteolytic pathway in mares (Stout *et al.*, 2000). Goff *et al* (1987) showed that administration of oxytocin in mid-dioestrus shortened the duration of the cycle.

3) The fetoplacental unit

Studies on fetal pituitary oxytocin (Khan-Dawood and Dawood, 1984) and fetal urine oxytocin output (Dawood *et al.*, 1978; Dawood, 1983) have indicated that the fetus can secrete oxytocin toward the maternal side during the first stage of labour that maternal plasma oxytocin concentrations do not increase. Chibbar *et*

al (1993) demonstrated synthesis of oxytocin mRNA in amnion, chorion and decidua and these tissues would be additional intrauterine sources of oxytocin during labour. They also suggested that there might be a paracrine system involving oxytocin and sex steroids within intrauterine tissues wherein significant changes in concentrations could occur without being reflected in the maternal circulation.

Regulation of oxytocin synthesis

Oxytocin can stimulate its own release. Positive feedback of oxytocin may contribute to the pulsatile release which is characteristic of this hormone. Activity of oxytocinergic neurons and the release of oxytocin depend upon the interaction of a variety of other hormones and neurochemicals (Wakerley *et al.*, 1988).

Ovarian steroid hormones are the main factors to control the hypothalamic and peripheral oxytocinergic system. Steroid hormones influence oxytocin gene expression in magnocellular oxytocin cells indirectly, by actions on afferent cells or by membrane actions during pregnancy (Leng and Brown, 1997). Oestrogens activate oxytocinergic neurons and increase oxytocin secretion into the circulation. Progesterone, in contrast, appears to be inhibitory (Pfaff, 1988; Putnam *et al.*, 1991). Behrendt-Adam *et al* (2000) also reported the effect of ovarian steroid hormones on the expression of the oxytocin-mRNA in the equine endometrium. A glucocorticoid responsive element and a thyroid responsive element are also involved in the regulation of oxytocin synthesis. The catecholamine and acetylcholamine also activate oxytocinergic system, although this effect may be receptor specific (Crowley *et al.*, 1978; Wikland *et al.*, 1985).

Biological functions of oxytocin

Oxytocin has a wide variety of biological actions. It acts not only as a hormone in control of uterine contractility in parturition and lactation, but also as a neuromodulator/neurotransmitter in the central nervous system where it plays an important role. Several studies have indicated that oxytocin regulates either the motivation for or the performance of sexual behaviour. In female rodents, oxytocin mRNA levels increases at oestrus relative to other stages in the oestrous cycle (van Tol *et al.*, 1988). This increase in oxytocin gene expression is associated with alterations in oxytocin content and release. In addition, in pigs and mares, oxytocin mRNA concentrations are higher in oestrus than in dioestrus (Boulton *et al.,* 1996; Behrendt-Adam *et al.*, 1999). Oxytocin also has a role in memory and learning processes, maternal behaviour, yawning, tolerance and dependence mechanisms, feeding, grooming, cardiovascular regulation and thermoregulation (Argiolas and Gessa 1990; Carter, 1992; Insel, 1992).

The most important role of oxytocin is in stimulating uterine contractility during parturition (Brownstein *et al.*, 1980; Fuchs and Dawood, 1980; Fuchs *et al.,* 1992) and regulating the oestrous cycle (Okano *et al.*, 1996). It is thought that oxytocin influences oviduct and myometrial contractile activity during oestrus including at the time of gamete transport (Gilbert *et al.*, 1992). Furthermore, there are various differences in the concentration of uterine oxytocin mRNA between species during parturition. There is a significant increase of uterine oxytocin mRNA concentration in the rat (Lefebvre *et al.*, 1992b), but there is no significant increase in uterine oxytocin mRNA concentration in pigs (Boulton *et al.*, 1996) and sheep (Wathes *et al.*, 1996).

Oxytocin also plays an important role in lactation. Somatosensory stimulation of the mammary glands results in an increase in the release of oxytocin from the posterior pituitary. *In vivo* microdialysis reveals that there is an increase in oxytocin release in the supraoptic nuclei during parturition and in the supraoptic

and paraventricular nuclei of the hypothalamus during suckling. The milk in the lactating mammary gland can be extracted only by sucking or milking. The contraction of special myoepitherial cells of the mammary gland causes 'milk let-down' and this contractile response is very sensitive to oxytocin (Berde and Cerletti, 1957). However, unlike other animals sucking is not significantly related to oxytocin release in the mare (Vivrette *et al.*, 2000).

Oxytocin plays a physiological role in luteolysis in several species (Flint and Sheldrick, 1985; King and Evans, 1987; Tetzke, *et al.*, 1987; Stout *et al.*, 1999) but precisely what this role is has not yet been determined. Oxytocin reacts with its endometrial receptor in the late luteal phase stimulating the synthesis and release of uterine $PGF_{2\alpha}$ in the mare (Sharp *et al.*, 1997)..

Oxytocin gene expression

The oxytocin gene is composed of three exons, with two intervening sequences. Exon A encodes the signal peptide, the hormone moiety, and the N-terminal part of neurophysin. Exon B encodes the central part of neurophysin, and exon C encodes the C-terminal part of neurophysin (Zeeman *et al.*, 1997). There are oxytocin-specific enhancers localized within or in the vicinity of the vasopressin gene because the oxytocin gene can be expressed only when it is linked to the vasopressin gene in a minilocus (Young *et al.*, 1990).

Oxytocin receptors

Oxytocin receptors have been identified in a variety of neural tissues, including the ventromedial hypothalamus (VMH), bed nucleus of the stria terminalis (BNST), central amygdala, anterior olfactory nucleus, lateral septum, ventral subicculum, and dorsal motor nucleus of the vagus in the rat (Insel, 1992).

Patterns of oxytocin receptors and relative concentrations of these receptors are species specific. Both the gene for oxytocin and oxytocin receptor induction are regulated by steroid hormones.

Uterine sensitivity to oxytocin is dependent on its receptor density. There have been differences in the location of the uterine oxytocin receptors reported between species (Soloff, 1990). In rats (Soloff *et al.*, 1977; Fuchs *et al.*, 1983; Pliska, 1991), cows (Fuchs *et al.*, 1992) and sows (Soloff and Swartz, 1974), oxytocin receptors were found only in the myometrium. However, in the ewe (Roberts *et al.*, 1976), mare (Stull and Evans, 1987) and human (Fuchs *et al.*, 1985; Kimura *et al.*, 1992), oxytocin receptors were found in both endometrium and myometrium. In the mare, the number of oxytocin receptors in the myometrium was greater than that in the endometrium (Stull and Evans, 1987). In the endometrium, oxytocin receptor concentration was lowest in mares during oestrus, increased in the luteal phase in cycling mares, and was highest in nonpregnant mares on day 14-16 (Sharp *et al.*, 1997). The affinity of oxytocin receptors was lower in pregnant than in nonpregnant mares. By contrast, in the ewe studies have shown that concentrations of oxytocin receptors increased from low mid-luteal concentrations to reach a peak at oestrus followed by a decline to previous values (Sheldrick and Flint, 1985; Ayad *et al.*, 1990).

Oxytocin-induced uterine contraction

With regard to uterine contractile activity, there is a complex coordination of endocrine, paracrine and autocrine cascades. However, the most powerful factor in stimulating uterine contractile activity is oxytocin. The relationship between oxytocin and its receptors provides the overall balance in uterine contractile activity by maintaining uterine quiescence in nonpregnancy or stimulating forceful contractility during parturition.

Oxytocin stimulates both the frequency and force of uterine contractile activity.

Oxytocin plays a dual role in the uterine contraction: acting directly on oxytocin receptor-mediated, voltage-mediated calcium channels to affect intracellular biochemical pathways for uterine contractions and indirectly through stimulation of $PGF_{2\alpha}$ and PGE_2 synthesis (Zeeman *et al.*, 1997). Small doses of oxytocin cause a slight lowering of the membrane potential, thus supporting the spread of the excitory waves over the uterus.

This oxytocin induces an increase of internal Ca^{2+} ions by (a) inhibiting Ca^{2+} extrusion by suppression of Ca^{2+} ATPase (the Ca^{2+} pump), (b) opening Ca^{2+} channels, and (c) stimulating IP_3, which releases internally stored Ca^{2+} ions (Riemer and Roberts, 1986; Kao, 1989). In the myometrium, influx of Ca^{2+} ions is important not for only contraction (Berridge, 1985) but also in activation of oxytocin receptors (Fuchs and Dawood, 1980). In the endometrium, there is also an increase in Ca^{2+} ions as a result of oxytocin receptor stimulation. Oxytocin receptor stimulation in the uterus results in a series of intracellular changes including G-protein activation (Wen *et al.*, 1992), increase of phospholipase C activity (Olins and Bremel, 1982; Flint *et al.,* 1986; Okawa *et al.*, 1993), and an influx of extracellular calcium. The results of these changes are increased myometrial contractility and elevated $PGF_{2\alpha}$ release from the endometrium.

Prostaglandins released from the uterus as a consequence of oxytocin-induced contractions act synergistically with oxytocin to change myometrial contractility (Brummer, 1971; Liggins, 1973). Oxytocin also influences the formation of gap junctions (Garfield *et al.*, 1980). In addition, oxytocin-induced contraction is highly dependent on an increase of oxytocin receptors (Soloff *et al.*, 1977; Soloff, 1990).

(c) Prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$)

Prostaglandin $F_{2\alpha}$ is produced by the endometrium at all stages of the oestrous cycle (King and Evans, 1987). In the process of the production of $PGF_{2\alpha}$, progesterone, oestrogens and oxytocin are involved. Vernon *et al* (1981) reported that in the cycling mare, $PGF_{2\alpha}$ production increased from Day 4 to Day 16 after ovulation (Day 0) and then declined on day 20 indicating that maximal $PGF_{2\alpha}$ production corresponded to the expected time of luteolysis (King and Evans, 1987). Maximal endogenous $PGF_{2\alpha}$ production is dependent upon a period of circulating increased concentration of systemic luteal progesterone (Sharp and Black, 1973; Douglas and Ginther, 1976). Vernon *et al* (1981) suggested that progesterone may be responsible for the production of the prostaglandin synthetase system and/or the sequestration of this enzyme system, or that the recruitment of precursors may be slow and require continuous, long-term progesterone exposure. Oestrogen, on the other hand, may trigger the prostaglandin synthetase system in a response that is rapid and requires only short-term exposure.

Oxytocin binding sites are greatest in the endometrium and myometrium on day 14 to 17 (Stull and Evans, 1987) and like other species, the increase in oxytocin binding sites during days 14-17 is thought to enhance the production of $PGF_{2\alpha}$. This may be due to the increasing concentration of oestradiol, which have a stimulatory effect on oestrogen and oxytocin binding sites. Oxytocin stimulates the uterus to release $PGF_{2\alpha}$ *via* oxytocin receptors (Sharp *et al.*, 1997). Oxytocin appears to work in conjunction with progesterone and oestrogen to accomplish luteolysis through stimulation of endometrial $PGF_{2\alpha}$ release. However, regulation of $PGF_{2\alpha}$ secretion is different in mares compared to ruminants because of the lack of local oxytocin synthesis from the ovary.

Prostaglandin $F_{2\alpha}$ is known to stimulate the myometrium of the mare (Capraro *et al.*, 1977). It is reported that after administration of $PGF_{2\alpha}$ there is an increase in myometrial electrical activity (Taverne *et al.*, 1979; Troedssen *et al.*, 1995a) and

intrauterine pressure (Goddard *et al.,* 1985) in mares. Inhibition of $PGF_{2\alpha}$ synthesis with phenylbutazone dramatically decreases the ability of the mare's uterus to evacuate intrauterine fluid (Cadario *et al.*, 1995). Manipulation and distension of the reproductive tract causes release of $PGF_{2\alpha}$ in the mare (Betteridge *et al.*, 1985; Watson *et al.*, 1988) and exogenous $PGF_{2\alpha}$ enhances clearance of intrauterine fluid in the mare (Cadario *et al.*, 1995). In addition, both oxytocin and $PGF_{2\alpha}$ have been used as ecbolic agents in mares (Troedsson *et al.*, 1995a).

1.4. Neuronal factors affecting uterine contractility

The female reproductive organs are supplied mainly by the autonomic nervous system comprising adrenergic (sympathetic) (Owman *et al.*, 1967; Mustafa *et al.*, 1987; Alm *et al.*, 1988; Alm and Lundberg, 1988) and cholinergic (parasympathetic) nerves (Mustafa *et al.*, 1987; Garfield, 1986). Previous studies on uterine innervation have reported that the uterus is mainly supplied by adrenergic nerves. By contrast, cholinergic nerves are sparse or absent.

1.4.1. Adrenergic innervation

The sympathetic (adrenergic) nerves originate in the 3rd to 5th lumbar segments of the spinal cord, via the caudal mesenteric ganglia, and then continue in the inferior hypogastric nerves (Rang *et al.*, 1995) (Figure 1.4). The caudal mesenteric ganglion is located at the origin of the caudal mesenteric artery. The internal spermatic nerve accompanies the ovarian artery and supplies the ovary, oviducts, and uterine horn (similar to distribution of the ovarian artery) (Ginther, 1992a). Although the autonomic nervous system has been extensively studied, the exact neuronal input to the physiological activity of the uterus is still not clear.

The adrenergic innervation in the female reproductive tract in guinea-pigs (Alm and Lundberg 1988; Mustafa, 1988; Mitchell and Ahmed, 1992; Zoubina *et al.*, 1998), cats (Alm *et al.*, 1986) and women (Owman *et al.*, 1967) is well established. Compared to other organs the uterine adrenergic innervation is unique with respect to the hormonal influences on transmitter levels which fluctuate during the oestrous cycle (Thorbert *et al.*, 1978). Also the degenerative and regenerative changes that occur in the uterine nerves during pregnancy and after parturition are unique to the uterus (Thorbert, 1978; Alm *et al.*, 1979; Mustafa, 1988). The adrenergic nerves are considered to be important for the neuronal control of uterine blood flow, for the contractility of the myometrium, and for endometrial secretory function.

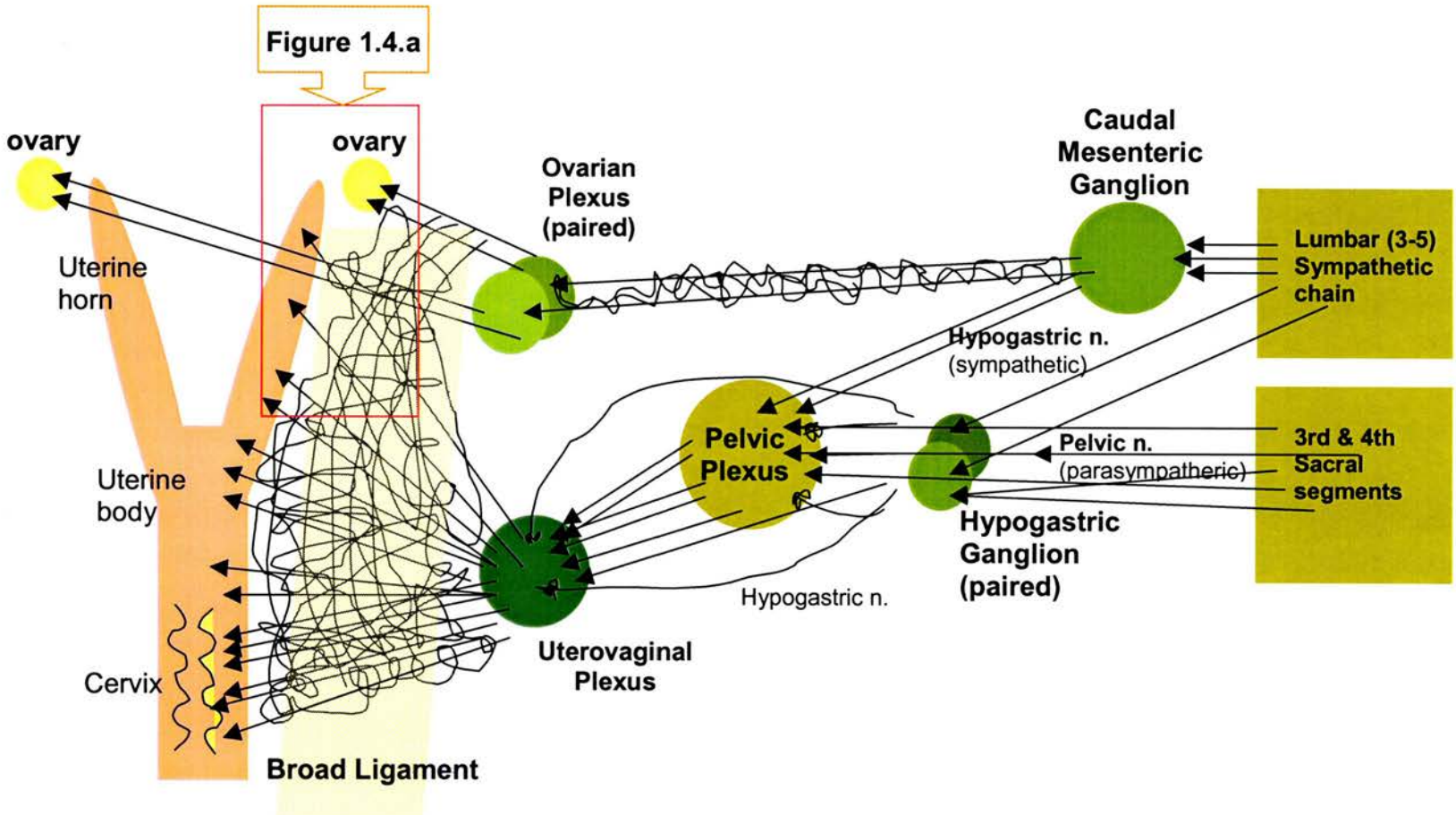


Figure 1.4. Distribution of sympathetic and parasympathetic nervous system in the reproductive tract.

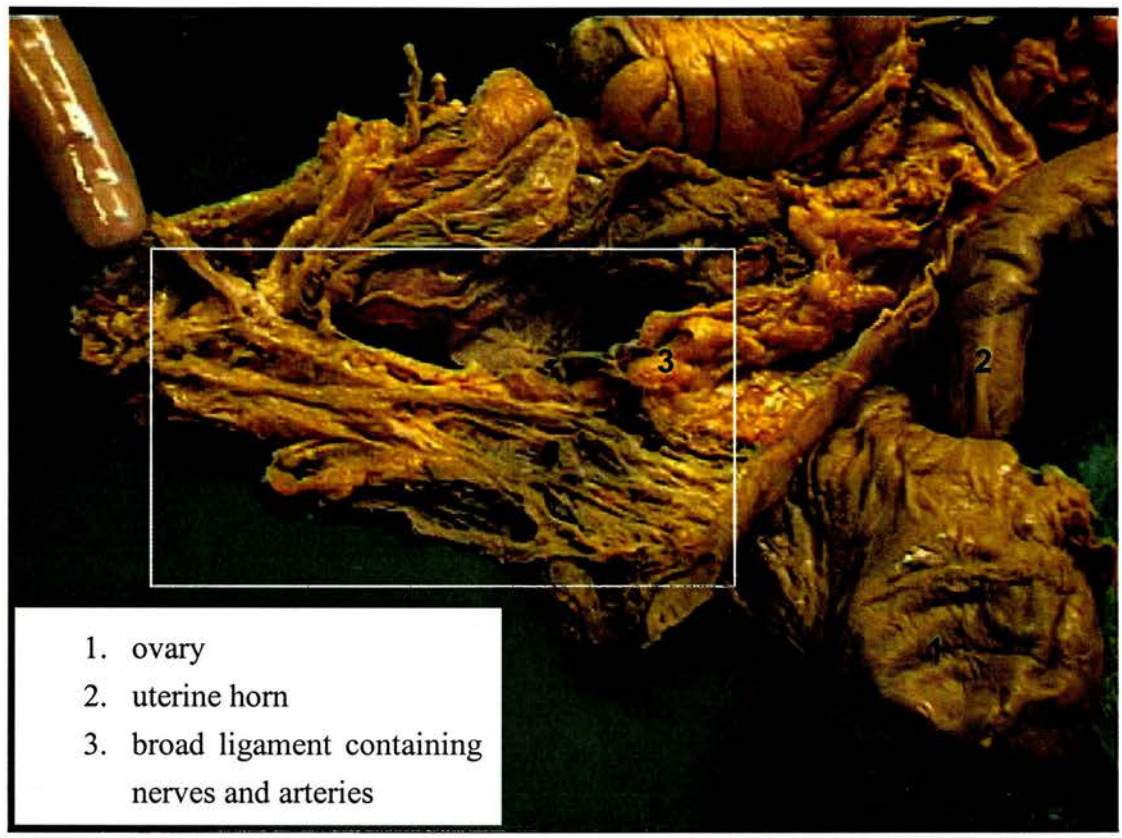


Figure 1.4.a. Photographs of reproductive tract (mare) showing broad ligament containing fine nerve branches (n) and arteries (a).

To visualize the adrenergic innervation, tyrosine hydroxylase (TH) and dopamine β hydroxylase (DβH) have been used as markers for adrenergic nerves by immunohistochemistry. TH and DβH are catecholamine-synthesizing enzymes which can be demonstrated in noradrenergic neurons (Figure 1.5).

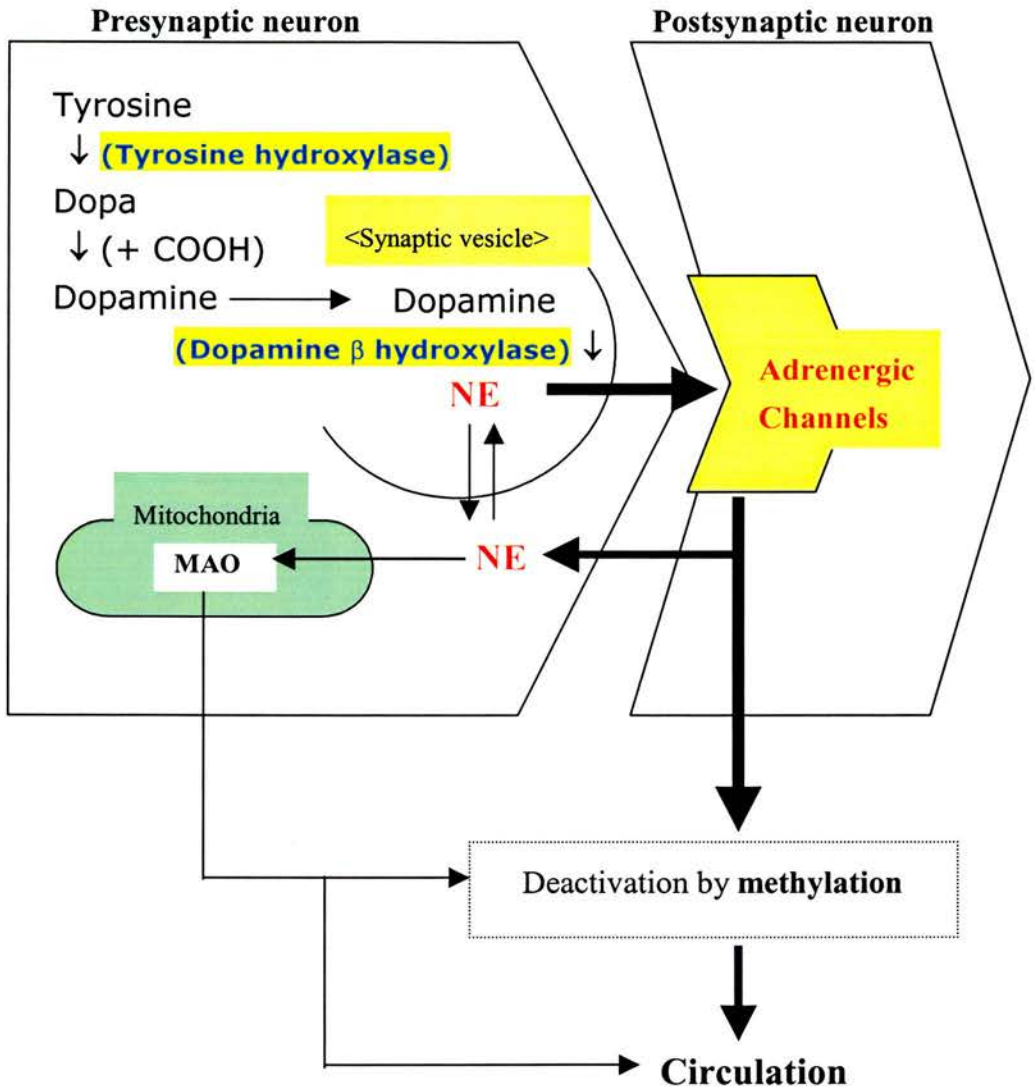


Figure 1.5. Transmitter chemistry at an adrenergic synapse. Norepinephrine (NE) is synthesized from the amino acid phenylalanine *via* tyrosine and stored in synaptic vesicles. After release, some NE is taken back up into the presynaptic terminal and some is deactivated by methylation and carried away in the blood. Cytoplasmic NE is either taken up into a synaptic vesicle or degraded by monoamine oxidase (MAO) (adapted from Mountcastle and Baldessarini, 1968).

1.4.2. Peptidergic innervation

When discussing the uterine innervation we have to consider not only the adrenergic and cholinergic, but also the peptidergic nerves containing neuropeptides (Heinrich *et al.*, 1986; Alm and Lundberg, 1988; Reinecke *et al.*, 1989).

Neuropeptides

Neuropeptides are peptides believed to have a neurotransmitter or neuromodulator function. Different neuropeptides have been reported to exist and/or coexist in nerve fibres of the female reproductive tract in various species. They may play an important role in the regulation of myometrial activity and possibly other reproductive functions. The neuronal regulation of uterine smooth musculature is highly complex. Neuropeptides have various functions not only neurotransmission or neuromodulating but also they may act as growth regulators (Woll and Rozengurt, 1989). The distribution of neuropeptides in the uterus may therefore be important in relation to the changes in growth patterns that the organ undergoes in the pre- and post-pubertal periods (Mitchell and Ahmed, 1992). In addition, changes in the distribution of the adrenergic nerve fibres and neuropeptides have been reported during the oestrous cycle (Thorbert *et al.*, 1978; Zoubina *et al.,* 1998) and pre- and post-pregnancy (Thorbert, 1978; Fried *et al.*, 1985; Alm *et al.*, 1988). The most commonly demonstrated neuropeptides are calcitonin gene-related peptide (CGRP), vasoactive intestinal peptide (VIP), substance P (SP), neuropeptide Y (NPY), galanin (GAL), gastrin-releasing peptide (GRP), enkephalins (ENK) and bombesin.

1) Vasoactive intestinal polypeptide (VIP)

Vasoactive intestinal polypeptide (VIP) is a 28-amino acid peptide (His-Ser-Asp-Ala-Val-Phe-Thr-Asp-Asn-Tyr-Thr-Arg-Leu-Arg-Lys-Gln-Met-Ala-Val-Lys-Lys-Tyr-Leu-Asn-Ser-Ile-Leu-Asn (NH_2)) which was originally isolated from porcine small intestine (Said and Mutt, 1970). By immunohistochemical and radio-immunochemical studies, VIP has been shown to be widely distributed in the central and peripheral nervous systems. It is found in brain (Roberts *et al.*, 1980), gastrointestinal (Bryant *et al.*, 1976; Polak and Bloom, 1978), respiratory (Said and Mutt, 1977; Uddman *et al.*, 1978) and reproductive tracts (Lynch *et al.*, 1980; Ottensen *et al.*, 1983a; Ottensen *et al.*, 1995; Bredkjcer *et al.*, 1997) associated with smooth muscle. It has a broad range of biological actions, including relaxation of vascular and non-vascular smooth muscle and stimulation of exocrine gland secretion (Said, 1982).

The female reproductive tract receives extensive innervation by VIP-containing nerves in women (Lynch *et al.*, 1980; Ottensen *et al.*, 1987; Bredkjcer *et al.*, 1997), cats (Alm *et al.*, 1986), and guinea-pigs (Huang *et al.*, 1984; Zoubina *et al.*, 1998). In addition to their perivascular position, the nerve fibres run in the submucosa and in the myometrium, where they are closely associated with non-vascular smooth muscle cells. There are considerable species differences in the extent of VIP-nerve supply to the myometrium (Ottensen and Larsen, 1981), but the pattern of distribution is common. They are particularly dense in the cat (Ottensen and Larsen, 1981; Alm *et al.*, 1986), but there are relatively few fibres in women (Helm *et al.*, 1981). Vasoactive intestinal polypeptide immunoreactive fibres were observed in ovary, uterus, and vagina associated with blood vessels and smooth muscle. It has been shown that the concentration of VIP was greater in the cervix than any other area of the reproductive organs in pigs and women (Lakomy *et al.*, 1994; Ottensen and Fahrenkrug, 1995).

The demonstration of VIP containing nerve fibres within the female and male genital tract indicated a putative role for this peptide in the local nervous control of reproductive functions (Ottensen and Fahrenkrug, 1995). Vasoactive intestinal polypeptide has been shown to participate in the nervous control of biological events in the reproductive tract, e.g. blood flow, smooth muscle contractility and ovarian function. In the ovary, VIP seems to play an important role as regulator and/or modulator of folliculogenesis and stimulation of steroidgenesis in human (Ottensen *et al.*, 1995) and rats (Ahmed *et al.*, 1986) as well as stimulation of ovulation in rats (Schimidt *et al.*, 1990).

Vasoactive intestinal polypeptide is a potent dilator of the uterine artery and may contribute to the noncholinergic autonomic dilation of the uterine vessel (Stjernquist *et al.*, 1985; Morris, 1990). Vasoactive intestinal polypeptide inhibits spontaneous activity as well as neuronally evoked contractions of the uterine cervix, but leaves the resting tension unaffected.

2) Neuropeptide Y (NPY)

Neuropeptide Y (NPY) is a pancreatic polypeptide composed of a 36-amino acid sequence (Tyr-Pro-Ser-Lys-Pro-Asp-Asn-Pro-Gly-Glu-Asp-Ala-Pro-Glu-Asp-Leu-Ala-Arg-Tyr-Tyr-Ser-Ala-Leu-Arg-His-Tyr-Ile-Asn-Leu-Ile-Leu-Arg-Gln-Arg-Tyr(NH_2)) originally isolated from porcine brain (Tatemoto and Calquist, 1982). Neuropeptide Y is widespread in the central and in the peripheral autonomic nervous system, generally colocalised with noradrenaline (NA) in neurons of the sympathetic ganglia (Lundberg *et al.*, 1990). It has been demonstrated in the respiratory system (Uddman *et al.*, 1984), bladder (Matiasson *et al.*, 1985), spleen (Lundberg *et al.*, 1985), eye (Zhang *et al.*, 1984) and female reproductive tract (Owman *et al.*, 1986; Häppölä *et al.*, 1991; Jørgensen, 1994; Serghini *et al.*, 1997).

The distribution of NPY immunoreactive nerve fibres in the various regions of female reproductive tract has been studied by immunoassays and immunohistochemistry. The presence of NPY in the female reproductive tract was reported in rats (Atke *et al.*, 1996; Serghini *et al.*, 1997; Houdeau *et al.*, 1997), guinea-pigs (Huang *et al.*, 1984; Heinrich *et al.,* 1986; Alm *et al.*, 1988; Mitchell and Ahmed, 1992), sows (Häppölä *et al.*, 1991; Lakomy *et al.,* 1994), and women (Owman *et al.*, 1986; Jørgensen, 1994). These studies showed that NPY is present in nerve fibres innervating the vascular and extravascular smooth muscle layers of the reproductive tract. NPY-IR nerve fibres were particularly abundant in the uterine cervix and vagina of guinea-pigs, rats, and humans (Heinrich *et al.*, 1986; Owman *et al.,* 1986; Serghini *et al.*, 1997).

The NPY-containing nerves are predominantly located around blood vessels, and seem to co-exist with noradrenaline (NA) in sympathetic vasoconstrictor neurons (Morris, 1989). Similar to VIP, NPY is located in the three cranial parasympathetic ganglia and stimulates glandular secretion (Moore and Black, 1991). Neuropeptide Y is a potent inhibitor of VIP- induced relaxation (Jørgensen, 1994). The localisation of NPY in both vasoconstrictor and vasodilator autonomic neurons may suggest that NPY has more than one action. Neuropeptide Y induces vasoconstriction both through a direct effect and *via* potentiation of the contractile effect of noradrenaline though NPY has also been shown to suppress the release both of noradrenaline and acetylcholine from pre-synaptic nerve terminals (Stjernquist and Owman, 1987). Neuropeptide Y inhibits the electrically induced smooth muscle contractions of the vas deferens (Lundberg *et al.*, 1984), urinary bladder (Lundberg *et al.*, 1984) and the cervix (Stjernquist *et al.,* 1983).

In the female reproductive tract, NPY is able to produce a long lasting contraction of the relaxed segment of the uterine artery in guinea-pigs (Morris and Murphy, 1988; Morris, 1990) and has no effect on spontaneous smooth

muscle activity (Stjernquist *et al.,* 1986). Vasoactive intestinal polypeptide-containing neurons have also been localised along with NPY. The coexistence of NPY and VIP was surprising in the light of the reported contrasting functions (VIP vasodilates, whereas NPY vasoconstricts). It was suggested that one of these neuropeptides acted as a neuromodulator in this situation, and drew analogy with other systems where NPY enhanced VIP release.

3) Calcitonin gene-related peptide (CGRP)

Calcitonin gene-related peptide (CGRP) is a 37-amino acid peptide (Ser-Cys-Asn-Thr-Ala-Thr-Cys-Val-Thr-His-Arg-Leu-Ala-Gly-Leu-Leu-Ser-Arg-Ser-Gly-Gly-Val-Val-Lys-Asp-Asn-Phe-Val-Pro-Thr-Asn-Val-Gly-Ser-Glu-Ala-Phe(NH_2)) encoded in the calcitonin gene. Immunoreactive CGRP has been demonstrated in the peripheral and central nervous systems (Skofitsch and Gacobowits, 1985; Häppölä and Lakomy, 1989). It is well established that CGRP is widespread in cardiovascular tissue (Mulderry *et al.*, 1985), urinary tract and bladder (Yokokawa *et al.*, 1986), gastrointestinal tract (Takaki *et al.*, 1989), airways and respiratory tract (Corcoran, 1996), and reproductive tract (Häppölä and Lakomy, 1989; Shew *et al.*, 1990). Calcitonin gene-related peptide partly coexists with substance P (SP) (Gibson *et al.*, 1984; Alm and Lundberg, 1988; Shew *et al.,* 1991) and sometimes also with galanin (GAL) (Shew *et al.*, 1992) and influences smooth muscle (Reinecke *et al.*, 1989). The distribution of CGRP in the central and peripheral nervous system and its colocalisation in some neurons with SP or acetylcholine suggests several possible roles in autonomic, sensory and motor function (Goodman and Iversen, 1986).

Calcitonin gene-related peptide is a potent vasodilator (Brain *et al.*, 1985; McCulloch *et al.*, 1986). With regard to the female reproductive system, CGRP inhibits spontaneous as well as electrically stimulated and chemically induced myometrial contraction in the rat (Shew *et al.*, 1990; Shew *et al.,* 1992; Shew *et*

al., 1993) and human (Samuelson *et al.*, 1985; Chan *et al.*, 1997). It has been suggested that it may play a regulatory role during parturition (Shew *et al.,* 1990; Chan *et al.*, 1997). Also CGRP's vasodilatory and uterine relaxant characteristics have supported its role in pregnancy. In a series of experiments, it has been shown that CGRP inhibits acetycholine- and substance P-, and galanin-stimulated rat myometrial contractions (Shew *et al.*, 1991; Shew *et al.*, 1992; Shew *et al.*, 1993). They also suggested that CGRP in the rat uterus may play an important role in other reproductive functions including coordination of muscular activity important in transport of sperm and ova, transport and spacing of the conceptus, maintenance of pregnancy, and parturition.

In addition to its localisation in the uterus, CGRP immunoreactivity was also found in the ovary, where it was distributed in medullary stroma, in close contact with blood vessels and between follicles of different stages of development (Ghatei *et al.*, 1985; Häppölä and Lakomy, 1989) suggesting its possible role in ovulation, folliculogenesis, and ovarian function.

4) Substance P (SP)

Substance P (SP) is a 11-amino acid peptide (Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met (NH_2)), firstly isolated by von Euler and Gaddum (1931) in extracts from gut and brain. Substance P is widely distributed in both central and peripheral system, and is also present in primary afferent sensory neurons (Moore and Black, 1991). Its localisation in C-fibres of both central and periphery sensory neurons suggests that it acts as a pain neurotransmitter. Substance P-immunoreactive fibres are present in the vascular system, including major cerebral blood vessels, and in many intrinsic neuronal pathways of the brain.

Substance P has been found in the female reproductive tract of the guinea-pigs (Heinrich *et al.*, 1986), cats (Alm *et al.*, 1986), cows (Majewski *et al.*, 1995),

sows (Lakomy *et al.*, 1994; Majewski *et al.*, 1995), and rats (Alm *et al.*, 1978; Traurig *et al.*, 1991). However, compared to other neuropeptides, SP-immunoreactive nerves were rarely found in the female reproductive tract. In pigs, SP-immunoreactive nerve fibres were predominantly found in the vagina, whereas very few of these fibres occurred in the cervix (Lakomy *et al.*, 1994). With regard to the female reproductive system, nerves immunoreactive for SP form a moderate plexus and varicose terminals throughout the uterus in rat (Traurig *et al.*, 1991). Immunoreactive SP fibres were shown in subepithelial plexuses in the endometrium, especially the mucosa of the cervix. Immunoreactive SP fibres were also present in the myometrial and mesometrial smooth muscle layers associated with blood vessels (Alm *et al.*, 1978; Heinrich *et al.*, 1986; Reinecke *et al.*, 1989; Lakomy *et al.*, 1994; Majewski *et al.*, 1995). These results suggest that it may play a role in the regulation of uterine blood flow, myometrial contractions and serve sensory functions (Papka *et al.*, 1985).

Substance P relaxes vascular smooth muscle, increases uterine blood flow, stimulates nonvascular smooth muscle and causes a dose-dependent increase in mechanical and myoelectrical activity (Ottensen *et al.*, 1983a). Substance P co-exisits with CGRP in capsaicin-sensitive nerves within the uterus but they have different actions. Shew *et al* (1991) suggested that SP and CGRP could be corealeased from afferent fibres in an efferent fashion, and influence uterine contractility, SP having a contractile effect and CGRP having a relaxing effect.

1.5. Equine grass sickness (EGS)

Equine grass sickness is a primary dysautonomia characterised pathologically by damage to neurons of the enteric and autonomic nerve system resulting in a wide range of clinical signs including tachycardia, muscle trembling, ptosis and rhinitis sicca (Doxey *et al.*, 1992; Pollin and Griffiths, 1992). Although the cause of EGS is unknown, it is characterized by dysfunction of the autonomic nervous system, primarily affecting the alimentary tract (Greig, 1928; Doxey *et al.*, 1995). The gross pathological findings are largely non-specific but there is usually evidence of severe gastro-intestinal disturbance, such as impaction of faeces in the large intestine and distension of the stomach in horses. It is reported that grass sickness affects horses aged between mostly 4-20 years of age and the peak incidence of diagnosis for grass sickness in the UK is from April to July (Wood *et al.* 1997). Equine grass sickness occurs in all months of the year, but between April and July the mortality rate approaches 90 per cent (Doxey *et al.*, 1991). The condition occurs in three clinical forms, acute, sub-acute and chronic forms, which were considered by Greig (1928) to be degrees of "sympathicotonia", or related to a depression of the parasympathetic system as opposed to a purely primary sympathetic stimulation (Pollin and Griffiths, 1992). The acute and subacute forms are invariably fatal and a few chronic cases recover.

The autonomic nervous system is a physiologic and anatomic system which includes the only motor system innervating those structures whose functions are primarily outside voluntary control. It is divided into sympathetic and parasympathetic divisions on the basis of physiologic, anatomic and pharmacologic differences of component neurons. Neuronal damage of equine grass sickness is observed not only in the alimentary tract but also in the enteric nervous system with the greatest damage to neurons in the ileum (Scholes *et al.*, 1993). Murray *et al* (1994) reported that there was an impaired motility in the equine small intestine in horses with equine grass sickness which resulted from the enteric neuronal degeneration in these horses. This study indicated that the loss of enteric neurons in

equine grass sickness leads to a reduction in the release of acetylcholine by cholinergic neurons that accordingly reduces intestinal motility, the greatest detrimental effect occurring in acute grass sickness. The ileum is affected to a greater extent than the duodenum (Scholes *et al.*, 1993).

1.6. Endometritis

Endometritis is a major cause of subfertility in broodmares (Asbury, 1987). Endometritis is the acute or chronic inflammatory changes that occur in the endometrium. Persistent endometritis is ranked by equine practitioners as the third most common medical problem in horses after by colic and viral respiratory disease (Traub-Dargatz *et al.*, 1991). Endometritis can occur in association with microbacterial infection or can be of a non-infectious nature.

Mares who develop persistent endometritis have undergone definite alterations to their normal uterine defence mechanisms. These may be related to changes to the physical barriers that enable entry into and/or removal of micro-organisms from the uterus, defects in the competence of uterine polymorphonuclear leucocytes, lack of endometrial immunoglobulins to effect bacterial killing, or an exaggerated decline in the resistance of the endometrium to infection while under the influence of progesterone (Hughes and Loy, 1969). It is also known that impaired myometrial activity in response to intrauterine bacterial challenge is an overriding causative factor in the whole syndrome of uterine susceptibility to infection in mares (Troedsson *et al.*, 1993a).

Endometritis has been subdivided into four categories, based on aetiology and pathophysiology (Troedsson *et al.*, 1995); a) endometrosis (chronic degenerative endometritis, b) sexually transmitted diseases, c) persistent mating-induced endometritis (PMIE), and d) chronic infectious endometritis. In contrast to infectious

endometritis (sexually transmitted endometritis and chronic infectious endometritis), mating induced endometritis has a known starting point and treatment can be initiated before the infection or inflammation has established itself in the endometrium (Troedsson, 1997).

1.6.1. Genitally-normal mares

Mares are classified as "genitally-normal" mares with a reproductive history of high fertility, ability to clear introduced intrauterine infection within 48 hours from the time of natural service and an endometrial biopsy score of 1 to 2A (Kenney, 1978). In addition, it is also important to confirm that mares have normal genital conformation. With correct conformation, there would be little or no faecal contamination of the vulva during defecation. It is normally vertical with firmly closed lips. Correct vulvar seal prevents pneumovagina from aspiration of air into the vagina.

1.6.2. Persistent mating-induced endometritis (PMIE)

After mating by natural service or by artificial insemination, semen provides an excellent opportunity for uterine contamination. Mating acts as a major source of bacteria, particulates, and irritants, as stallion semen is deposited directly into the uterine lumen and natural service also produces some degree of irritation to the vaginal tract and cervix (Watson, 2000). It is reported that spermatozoa without bacterial contamination also induce a uterine inflammatory response (Troedsson *et al.*, 1995b). Some degree mating-induced endometritis is therefore, likely to be a physiological reaction that serves to remove excess spermatozoa, seminal plasma, and possible bacterial contamination from the uterus before the embryo descends into the uterine lumen. Intrauterine fluid is composed of inflammatory mediators, neutrophils, and plasma proteins including immunoglobulins, complement and enzymes (Watson *et al.*, 1987; Pycock and Allen 1990; Troedsson *et al.*, 1993). The

uterus must be capable of clearing intrauterine fluid within a few days after mating/conception in order for a pregnancy to develop normally.

A recent study showed that 15% of Thoroughbred mares (Zent *et al*, 1998) and 43 % of a mixed population of mares in the UK (Newcombe, 1997) developed significant amounts of intrauterine fluid after mating. It is known that these mares that accumulate intrauterine fluid within 48 h after mating have a significant decrease in pregnancy rate (49%) compared with mares without post-breeding fluid (62%) (Pycock and Newcombe, 1996b). Furthermore, pregnancy loss by day 30 was higher in mares with persistent uterine fluid after mating (Newcombe, 1997).

1.6.3. Diagnosis of persistent mating-induced endometritis (PMIE)

A detailed reproductive history should be obtained. Mares should be checked for 1) the anatomy of the perineal and vulvar conformation (Troedsson and Liu, 1995), 2) cervical function, 3) the presence of intrauterine fluid using transrectal ultrasonography (Ginther and Pierson, 1984; Adams *et al.*, 1987; Pycock and Newcombe, 1996b), 4) endometrial smear and culture to detect neutrophils (Brook, 1985) or any significant growth of known uterine pathogens, and 5) endometrial biopsy to examine any histopathological changes (Kenney, 1978).

Mares who can clear uterine fluid ± infection after mating, resulting in a normal uterine environment are classified as 'resistant', whereas mares which fail to clear uterine fluid ± infection are classified as 'susceptible' (Hughes and Loy, 1969; Peterson *et al.*, 1969). After mating, a mare that retains intrauterine fluid for more than 12 h is considered to have persistent mating-induced endometritis (PMIE) (Troedsson, 1997). In mares, susceptibility to PMIE can be suspected from a history of: 1) repeated uterine bacterial infections, 2) repeated failure of breeding, and 3) accumulation of intrauterine fluid.

1.6.4. Clinical signs of persistent mating-induced endometritis (PMIE)

Between 11 and 39 % of mares accumulate fluid at oestrus (Pycock and Newcombe, 1996b; Reilas *et al.*, 1997), and this merocrine endometrial secretion and oedema is probably normal during oestrus (Tunon *et al.*, 1995). The secretions and intrauterine fluid should be effectively drained via the lymphatic system and the cervix (Evans *et al.*, 1987). These clearance mechanisms are dependent on myometrial contractions (Guyton and Hall, 1996).

Normal mares may retain intrauterine fluid up to 6 hours after mating. However, if uterine fluid is present at 12 hours or more after mating, the mare should be considered to have a PMIE. In contrast to the transient post-mating endometritis, PMIE has severe consequences for the fertility of affected mares. Newcombe (1997) reported that pregnancy rates were 49% in mares with delayed intrauterine fluid within 48 h after mating compared with 62% in mares without intrauterine fluid. In addition, a persistent inflammation results in premature luteolysis and embryonic loss in response to increased prostaglandin $F_{2\alpha}$ concentrations (Troedsson, 1997).

The presence of free intrauterine fluid prior to mating strongly suggests susceptibility to PMIE (Pycock and Newcombe, 1996a). A mare with a history of chronic infectious endometritis is also likely to develop PMIE. It has been concluded that in order for a mare to be considered normal (resistant), >50% of inoculated radiocolloids need to be cleared from the uterus within 2 hours of inoculation as detected by scintigraphy (LeBlanc *et al.*, 1994).

In mares with acceptable anatomical vulvar and perineal conformation, susceptibility to endometritis and uterine fluid accumulation is often associated with a defect in uterine contractility. Impaired myoelectrical activity in response to inflammation is reported in mares with delayed uterine clearance (Troedsson *et al.*, 1993a).

1.6.5. A defect of uterine contractility in the mares with PMIE

It is reported that mares with delayed uterine clearance have impaired electrical myometrial activity in response to intrauterine infusion of bacteria (Troedsson *et al.*, 1993a). Pharmacological depression of myometrial activity by administration of a beta-adrenergic agonist resulted in persistent fluid accumulation after intra-uterine bacterial challenge, effectively converting normal resistant mares into susceptible mares (Nikolakopoulos and Watson, 1999). LeBlanc *et al* (1994) reported that using scintigraphy, delayed mechanical clearance of the uterus contributed to susceptibility of mares to recurrent endometritis but not all mares susceptible to endometritis exhibit a delay in uterine clearance if cervical dilation is adequate. This result clearly demonstrates the importance of uterine contractility in the expulsion of intrauterine fluid.

Myometrial contractility is mediated by changes in membrane potentials and the intracellular calcium levels, which is under hormonal (oxytocin and prostaglandins: Riemer and Roberts, 1986; Kao, 1989), neuronal (adrenergic: Marshall, 1981; Guyton and Hall, 1996) and myogenic control (gap junctions: Garfield *et al.*, 1980). The female reproductive tract contains a well-developed plexus of adrenergic nerve terminals innervating both the non-vascular smooth muscle and the blood vessels. Dysfunction of myometrial nerves such as absence of neurotransmitters at nerve terminals may decrease myoelectrical activity in mares with PMIE. Such damage to the nervous system results in upregulation of receptors in the postsynaptic membranes of effector cells and is referred to as denervation hyper- or super-sensitivity (Guyton and Hall, 1996).

1.7. Objectives

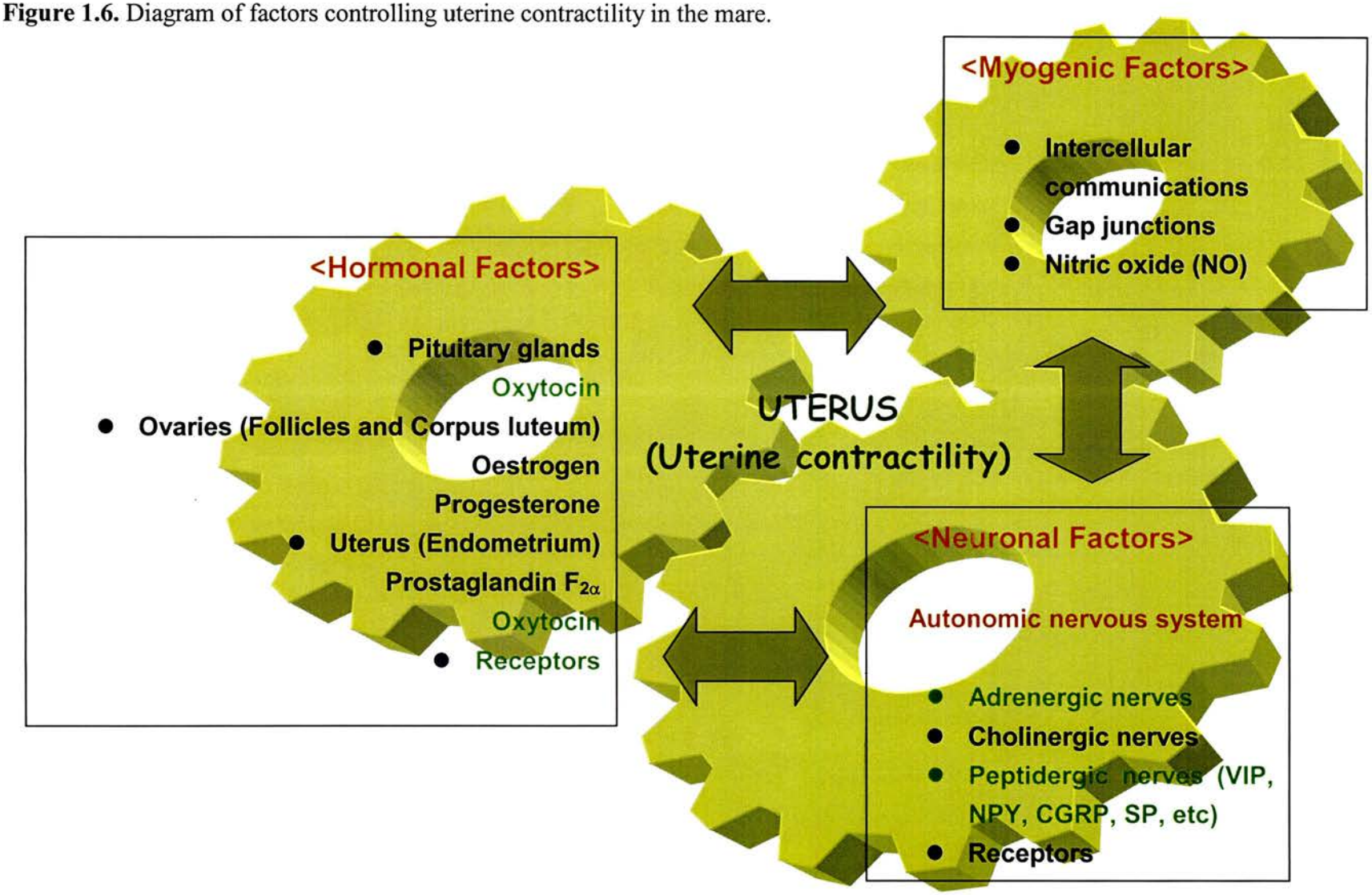
There has been no published information on the causes of defective uterine contractility in mares susceptible to PMIE. As already shown in this chapter, uterine contractility is a very complex mechanism (Figure 1.6). Although release of pituitary oxytocin is the main factor regulating myometrial contractility, there have been contradictory results on plasma concentrations in mares. Moreover, previous studies reported the presence of oxytocin in the equine endometrium (Watson *et al.*, 2000). However, the precise location of uterine oxytocin in the endometrium was not clearly determined. In addition, oxytocin acts not only *via* oxytocin receptors but also *via* nerve fibres in the uterus to play a role in myoelectrical activity. These nerves are composed of long postganglionic neurones originating in the lumbar and mesenteric ganglia and of short postganglionic neurones originating in the pelvic ganglia. Hormones such as oestrogen, progesterone, oxytocin and $PGF_{2\alpha}$ also modulate the myometrial contractile response to noradrenaline by their action on myometrial adrenergic receptors. Although uterine innervation is well documented in other species playing many roles in controlling myometrial contractility, uterine blood flow, endometrial secretions, ovarian functions, there has been no detailed information on adrenergic and peptidergic innervation in the equine reproductive tract.

Therefore, this study was designed 1) to investigate the distribution of uterine innervation and more precisely adrenergic and peptidergic nerve fibres in cycling mares, 2) to investigate whether neuronal damage is evident in the uterus of mares with dysautonomia, 3) to identify the presence and location of oxytocin in the equine endometrium, and 4) to measure circulating concentrations of oxytocin during oestrus using an intensive blood sampling regimen.

The objectives of the research that will be described in the following chapters are:

1) to investigate general uterine innervation by immunostaining PGP 9.5 and PAN-N in the mare,
2) to investigate adrenergic innervation by immunostaining TH and DβH and peptidergic (VIP, NPY, CGRP, and SP) innervation in the uterus of the mare
3) to examine neuronal damage of the uterine innervation of mares with equine grass sickness (dysautonomia)
4) to investigate the location of oxytocin and neurophysin in the equine endometrium at the ultrastructural level by immunohistochemistry and immunogold labelling using transmission electron microscopy, and
5) to investigate circulating plasma oxytocin concentrations in genitally-normal mares during oestrus and after ovulation

Figure 1.6. Diagram of factors controlling uterine contractility in the mare.



Chapter 2

Organization of Uterine Innervation in the Mare: Distribution of immunoreactivities for the general neuronal markers protein gene product 9.5 and PAN-N

Introduction

It has been well documented that the mammalian female reproductive tract receives an extensive nerve supply from different populations of nerves that can be visualized by specific immunohistochemical techniques. The nerve supply comprises not only autonomic nerves including adrenergic and cholinergic, but also peptidergic nerves (Papka *et al.* 1985; Wrobel and Kujat, 1993). However, the density and the populations of these nerves seem to vary between species (Huang *et al.* 1984; Renegar and Rexroad, 1990; Majewski *et al.* 1995). Previous studies have demonstrated that uterine innervation is influenced by a large number of mechanical, physiological and hormonal factors (Marshall, 1981; Lundberg *et al.*, 1989; Massmann *et al.*, 1992). The uterine nerve supply decreases significantly during pregnancy in cats (Alm *et al.* 1986), guinea pigs (Thorbert, 1978; Alm *et al.*, 1979; Lundberg *et al.* 1988), rats (Moustafa, 1988), and sheep (Renegar and Rexroad, 1990), and also changes throughout the oestrous cycle in the rat (Zoubina *et al.* 1998).

The female reproductive organs are supplied mainly by the autonomic nervous system comprising adrenergic (Owman *et al.*, 1967; Mustafa *et al.*, 1987; Alm *et al.*, 1988; Alm and Lundberg, 1988) and cholinergic nerves (Mustafa *et al.*, 1987; Garfield, 1986). The parasymphathetic nerves originate in the 3rd to 4th sacral roots, forming two pelvic nerves, and meet the sympathetic nerves in the paracervical ganglia which lie close to the uterine cervix (Rang *et al.*, 1995). The sympathetic innervation comes from the caudal mesenteric ganglion via the hypogastric nerves and pelvic plexus (Nickel *et al.*, 1973). The caudal mesenteric ganglion is located at the origin of the caudal mesenteric artery. Two pairs of nerves proceed caudally from it. The internal spermatic nerve accompanies the ovarian artery and supplies the ovary, oviducts, and cranial portion of the uterine horn (similar to distribution of the ovarian artery). The other pair of nerves from the ganglion follow the aorta and enter the pelvic cavity. They anastomose with each other and with branches of the sacral nerves (3^{rd} and 4^{th}) and ramify on the pelvic organs (Ginther, 1992a). Although the autonomic nervous system has been extensively studied, the exact neuronal input to

the physiological activity of the uterus is still not clear.

Protein gene product 9.5 (PGP), a cytoplasmic protein in neurones and neuroendocrine cells, has been used as a general cytoplasmic marker demonstrating all types of efferent and afferent nerve fibres, unlike neuron specific enolase (NSE) and neurofibrillary protein (NF) (Thompson *et al.*, 1983; Gulbenkian *et al.*, 1987). Protein gene product 9.5 is a neuronal cytoplasmic protein unrelated to NSE and has a molecular weight of about 27kDa (Doran *et al.*, 1983). Protein gene product 9.5 has been used as a pan-neuronal marker to identify all neuronal parts of the extrinsic and intrinsic uterine innervation, including different subpopulations of nerves in the rat (Zoubina *et al.*, 1998), and guinea pigs (Alm *et al.*, 1988; Lundberg *et al.*, 1988; Lundberg *et al.*, 1989).

Another general neuronal marker is PAN-N which is a convenient marker for neuronal cells and processes in general and in their differentiation from non-neuronal cells. The antibody for PAN-N is composed of three rabbit polyclonal sera which react with the low, medium and high molecular weight neuronal filament protein (NF-L [68-70kDa], NF-M [150kDa] and NF-H [200-210kDa]). Therefore, PAN-N is also a convenient marker for neuronal cells and processes in general and in their differentiation from non-neuronal cells. The use of both PGP 9.5 and PAN-N could provide a detailed visualization of the entire uterine innervation in the mare as already shown in other animals (Lundberg *et al.*, 1988; Zoubina *et al.*, 1998).

In the rat, Zoubina *et al* (1998) have shown that PGP-immunoreactive nerves are found in greatest abundance in the vascular zone of the myometrium, which is the area of blood vessels and connective tissue that is interposed between the outer longitudinal and inner circular smooth muscle layers of the uterus. The circular and longitudinal smooth muscle layers themselves contain moderate numbers of nerve fibres, whilst innervation of the endometrium was significantly lower than in other areas.

Although its importance is well recognised, there has been little information on uterine innervation in the mare. Therefore, the present study was undertaken with the aim of visualizing general uterine innervation using PGP and PAN-N before investigating uterine innervation in detail.

Materials and Methods

Sample collection:

Tissue samples were obtained at post-mortem from 13 cycling mares (6 oestrous and 7 dioestrus) aged 3 to 18 years. The stage of the cycle was established by peripheral blood progesterone levels (oestrus: <1ng/ml) as well as the presence or absence of a corpus luteum in the ovaries, the presence of a follicle size >30 mm in diameter and uterine oedema. The reproductive tract was removed within 20 min of euthanasia, was gently washed and then dissected in 0.1M phosphate-buffered saline (PBS). Four segments, size approximately 1.5 cm x 1.5 cm, were collected from each of the following regions: proximal part of uterine horn (left and right), body and the cervix. Samples were then fixed immediately in cold 4% paraformaldehyde (pH 7.2) for 14-16 h at 4°C. Samples were transferred into 0.1M PBS (pH 7.2) containing 30% sucrose and stored for 24 h at 4°C. Tissues were snap frozen in O.C.T (optimum cutting temperature) compound (Miles Inc, Elkhart, IN, USA) in an isopentane/dry-ice slurry, then stored at -70°C.

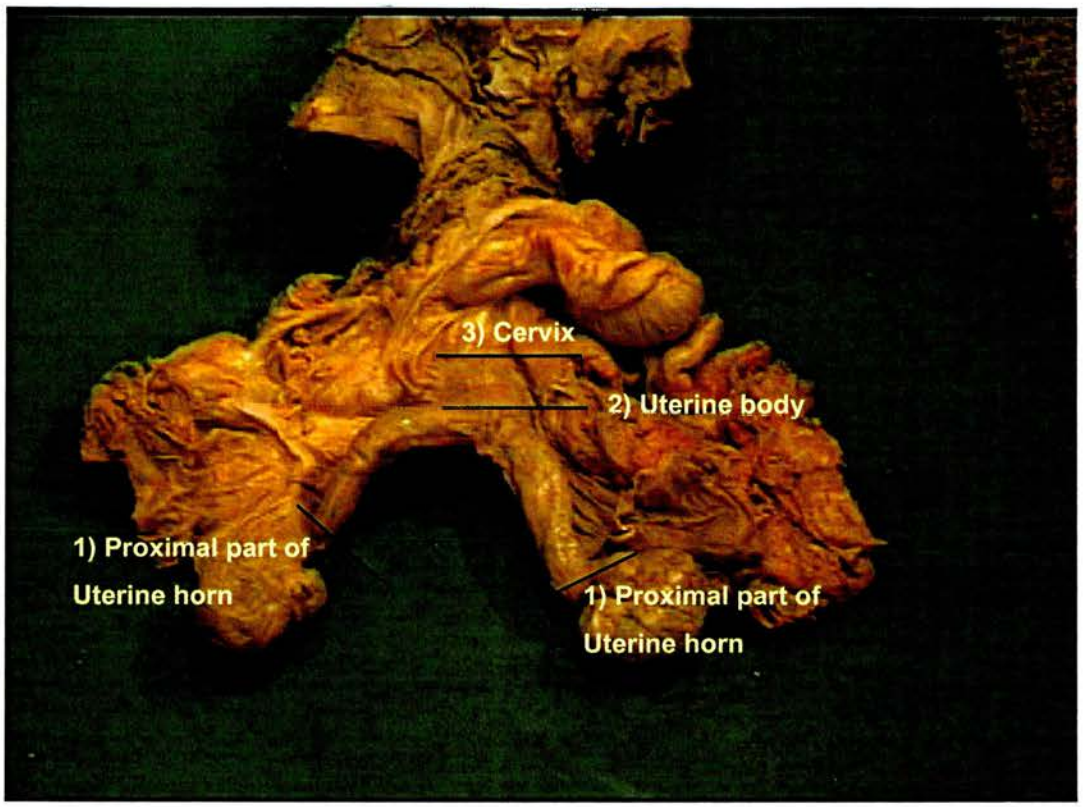


Figure 2.1 Regions of collected uterine tissues used in this study.

Immunohistochemical procedure

Cryostat sections (12-15μm) were cut and then processed for immunohistochemistry using the avidin-biotin complex method (Vector Laboratories, Peterborough, Cambridge, UK) described previously (Watson and Thompson, 1996). Briefly, sections were air-dried for 60-90 min at room temperature, rinsed in 0.01M PBS (pH 7.2) and blocked with 3% H_2O_2 in methanol for 30 min to reduce non-specific binding. Slides were then preincubated with 1% goat antiserum in 0.01M PBS for 1 h. Primary antibodies (Affinity Research Products, Exeter, UK) raised in rabbits were used at a dilution of 1:8000 for both PGP and PAN-N. The specificity of these antibodies had been characterized (Thompson *et al.* 1983; Wilson *et al.* 1988). The sections were incubated with primary antibodies overnight at 4°C. Sections were then washed three times in PBS. The sections were incubated with the second antibody (goat anti- rabbit; Vector Laboratories, Peterborough, Cambridge, UK) diluted in PBS containing 1% normal

for 2 h at room temperature. The sections were thoroughly washed in PBS, then incubated with the avidin-biotin complex for a further 30 min, and rinsed three further times with PBS. Chromagen (3-amino-9-ethylcarbazole: AEC; Vector Laboratories, Peterborough, Cambridge, UK) was added as the final substrate (red reaction product) and was incubated at room temperature in the dark for 10-15 min. Each slide was then rinsed carefully with distilled water. The sections were normally counterstained with Meyer's haematoxylin, however, sometimes sections with fine nerve fibres were excluded from this step. Slides were mounted and examined by light microscopy under magnification between x10 to x20.

To test the specificity of the primary antisera: a) sections were incubated with antibody which had been preabsorbed with its synthetic antigen (10 μg of antigen / ml of diluted antiserum), b) the primary antibody was omitted from the incubation, or c) normal rabbit serum was substituted for the primary antibody. Positive controls comprised equine jejunum and feline uterus incubated with each of the primary antibodies. As negative controls, sections were included in which the first antibody was replaced by normal rabbit serum.

Subjective evaluation of uterine innervation

Relative frequency of uterine nerve fibres and bundles was assessed using a subjective scoring system (Table 2.1) in immunostained sections obtained from uterine horn, uterine body, and the cervix. For each section, nerves were evaluated under magnification (x10 to x20) with respect to their frequency within three separate structures: myometrium, endometrium and vascular layer (between muscular layers). For each structure, numbers of nerves were assigned a score from 0 to 5 (See Appendix) (Alm *et al.*, 1988; Mitchell and Ahmed, 1992). Four sections from each of the three regions from each animal were included for analysis. Each section contained all three structures. It should be noted that because this study does not allow us to distinguish between fibres of passage and terminal innervation, the term

nerve fibre distribution includes all identified nerves either nerve bundles or fibres of whether they are terminations or pre-terminal axons.

Score	Description of innervation
0	no innervation
1	<5 nerve fibres
2	<20 nerve fibres
3	<50 nerve fibres
4	small number of nerve bundles with large number (>50) of nerve fibres
5	large number of nerve bundles with large number (>50) of nerve fibres

Table 2-1. A subjective scoring system used in this study.

Statistical analysis

Score for four sections from three regions and each structure from each animal were included for analysis. Scores for every section were pooled. Score values are presented as the mean ± SEM (as a general error term). Data were analysed by a general linear model analysis of variance (ANOVA) (SPSS 9.0) to determine the effect of 1) stages of the cycle, 2) regions, 3) structures and 4) regions*structures (factor). A probability of <0.05 was taken to indicate a statistically significant difference.

Results

Statistical analysis showed no difference between stage of cycle (oestrus vs dioestrus) in both PAN-N (P=0.6) and PGP (P=0.5). Within the regions of the uterus, average

score for both PGP-IR and PAN-N-IR was greatest in the myometrium (P <0.01), intermediate in the intramyometrial vascular layers and least in the endometrium (Table 2.2). There were no marked differences between the supply to uterine horn or body, but average nerve score was greater (P <0.01) in the cervix (Table 2.3). With adjacent sections stained for PGP-IR and PAN-N-IR neurofilaments, locations of the immunostaining were similar, however it appeared that more nerve bundles were positive for PAN-N-IR and immunostaining for PGP-IR identified more individual nerve fibres in the endometrium and near blood vessels. There was no significant interaction between factors (region*structure).

Regions	*PAN-N*	*PGP 9.5*
Uterine horn	3.59 ± 0.03^{a}	3.59 ± 0.03^{a}
Uterine body	3.59 ± 0.03^{a}	3.59 ± 0.03^{a}
Cervix	3.80 ± 0.03^{b}	3.79 ± 0.03^{b}
Structures	***PAN-N***	***PGP 9.5***
Myometrium	4.23 ± 0.03^{a}	4.21 ± 0.03^{a}
Endometrium	3.08 ± 0.03^{b}	3.09 ± 0.03^{b}
Vascular layer	3.67 ± 0.03^{c}	3.66 ± 0.03^{c}

Table 2.2 Relative frequency of subjectively graded distribution of PAN-N- and PGP 9.5- immunoreactive nerves within regions: uterine horn, uterine body and the cervix, and within structures: myometrium, endometrium, and vascular layer. Data show Mean ± SEM. ^{a,b,c} Groups with different superscripts within column are different (P <0.05).

Regions	Structures	PAN-N	PGP 9.5
Uterine horn	Myometrium	4.04 ± 0.05^{a}	4.02 ± 0.05^{a}
	Endometrium	3.06 ± 0.05^{b}	3.06 ± 0.05^{b}
	Vascular layer	3.67 ± 0.05^{c}	3.67 ± 0.05^{c}
Uterine body	Myometrium	4.02 ± 0.05^{a}	4.02 ± 0.05^{a}
	Endometrium	3.10 ± 0.05^{b}	3.12 ± 0.05^{b}
	Vascular layer	3.65 ± 0.05^{c}	3.64 ± 0.05^{c}
Cervix	Myometrium	4.62 ± 0.05^{a}	4.60 ± 0.05^{a}
	Endometrium	3.10 ± 0.05^{b}	3.10 ± 0.05^{b}
	Vascular layer	3.70 ± 0.05^{c}	3.67 ± 0.05^{c}

Table 2.3 Relative frequency of subjectively graded distribution of PAN-N- and PGP 9.5- immunoreactive nerves within structures from each region. Data show Mean ± SEM. ^{a,b,c} Groups with different superscripts within column and region are different (P<0.05).

Protein gene product 9.5 immunoreactivity

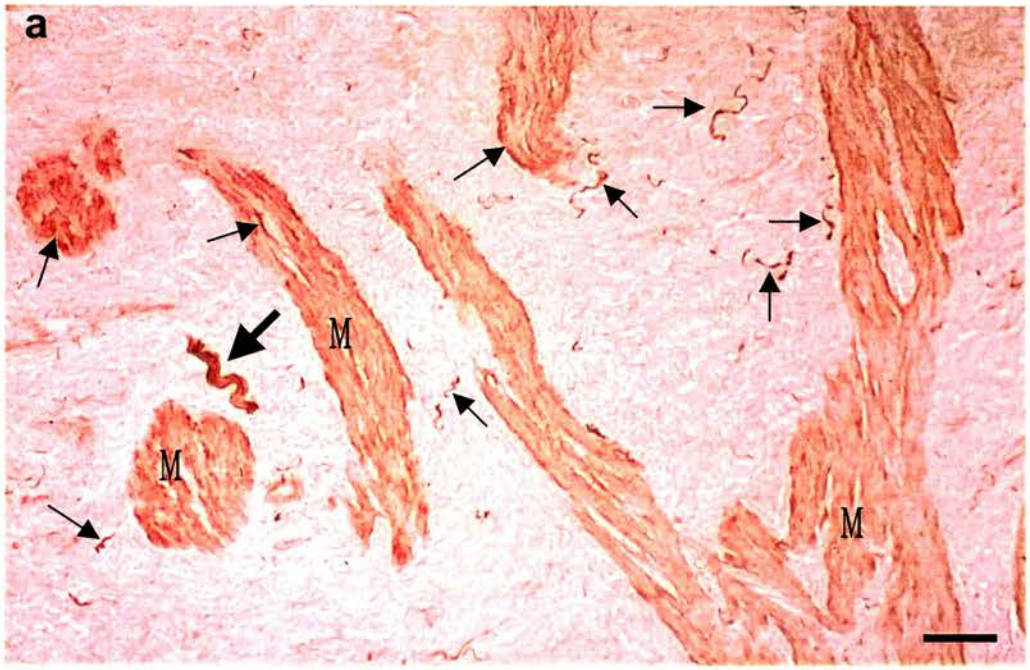
A large number of PGP-IR nerve bundles and nerve fibres were located in all regions of the uterus (Table 2.2, Table 2.3). The innervation was significantly greater in the cervix ($P<0.05$). In uterine horn and uterine body, the majority of PGP-IR nerve bundles were seen in the longitudinal and circular muscle layers (Figure 2-3-a, b) as well as around the blood vessels and the density was greater in the cervix (Figure 2-3-c). PGP-IR nerve bundles and fine nerve fibres were often oriented in parallel to uterine smooth muscle fibres. PGP-IR nerve fibres also reached the endometrium around the endometrial glands by travelling along arterial branches of the intermuscular vascular plexus (Figure 2-3-d) that supplied the endometrium. Comparing regions, innervation was significantly greater ($P<0.05$) in myometrium than the vascular layer or the endometrium (Table 2-2).

PAN-N immunoreactivity

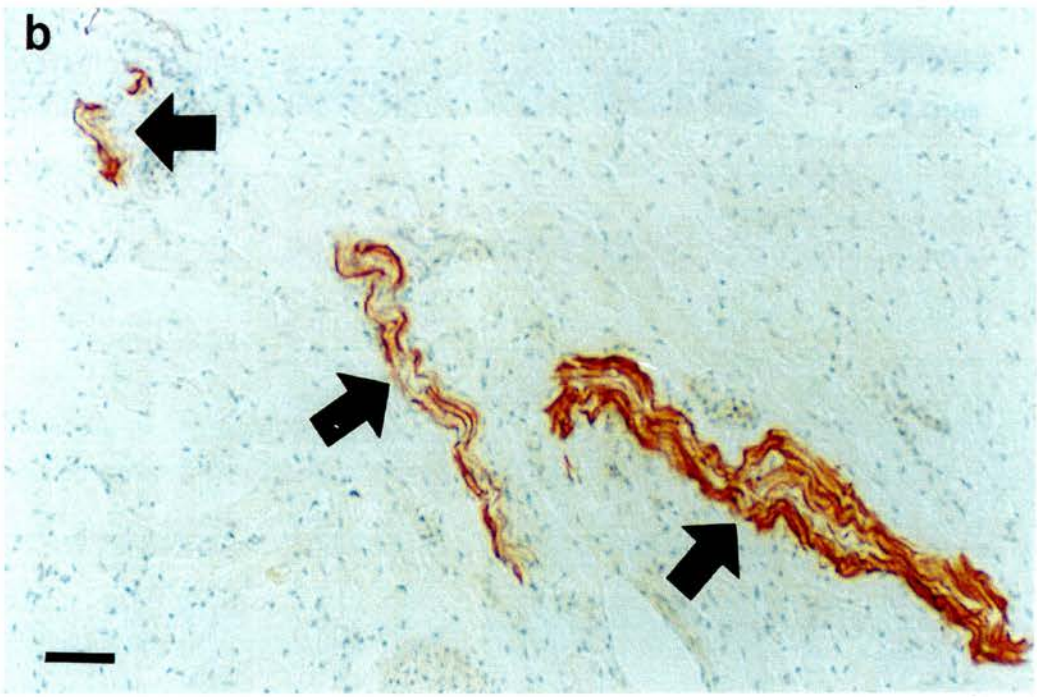
The greatest density of PAN-N-IR nerve bundles and fibres was observed in the cervix (Table 2.2, Table 2.3). In the myometrium, PAN-N-IR nerve bundles (Figure 2-4-a) and fibres travelled parallel to myometrial smooth muscle layers (Figure 2-4-b) often associated with blood vessels (Figure 2-4-c). PAN-N-IR nerves were present mostly as fine nerve fibres in the luminal epithelium and around endometrial glands (Figure 2-4-d). Comparing regions, like PGP-IR nerves, PAN-N-IR nerves were significantly greater ($P<0.05$) in myometrium than vascular layer or the endometrium (Table 2-2).

For both primary antibodies, the positive control sections showed clear positive staining of immunoreactive nerves (Figure 2-5-a, b) and negative control sections failed to demonstrate any positive staining (Figure 2-5-c).

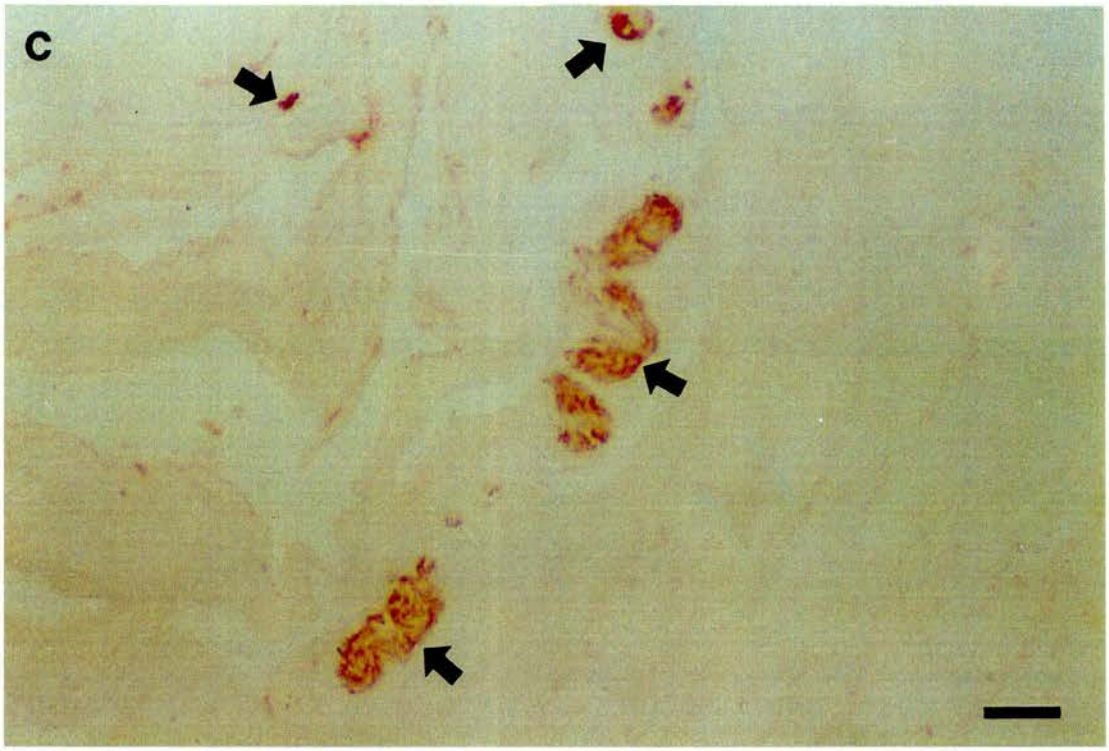
Figure 2-2. Protein gene product 9.5 immunoreactive nerve bundles and fibres in the equine uterus.



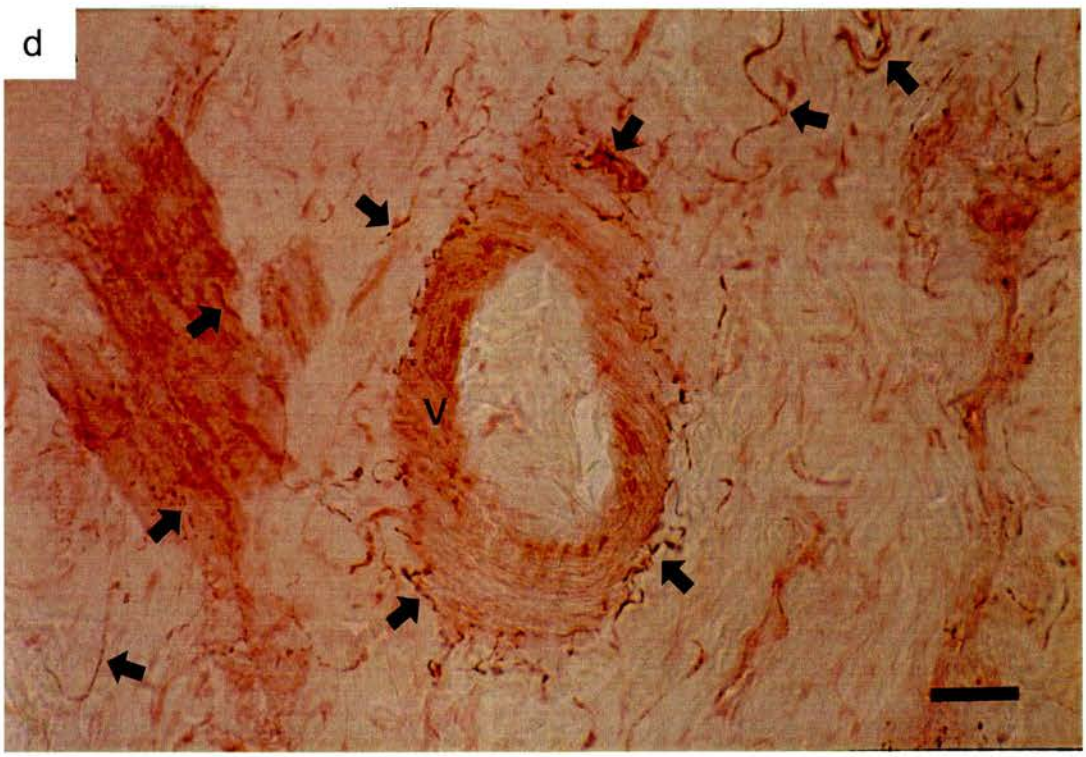
a. PGP-IR nerve bundles (➔) and nerve fibres (→) in the myometrium of the uterine horn. Smooth muscle bundles (M). Scale bar = 250 μm



b. PGP-IR nerve bundles and fibres (➡) in the myometrium of the uterine body. Counterstained with haematoxylin. Scale bar = 50 μm

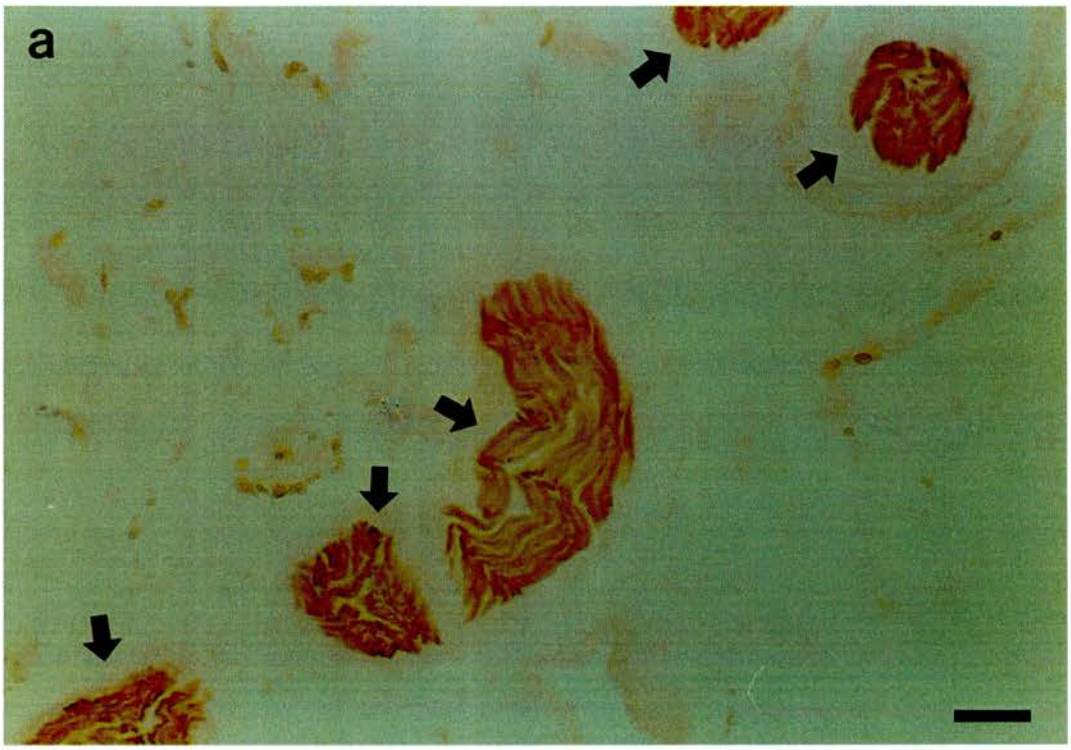


c. PGP-IR nerve bundles (➡) in the cervix. Scale bar = 100 μm

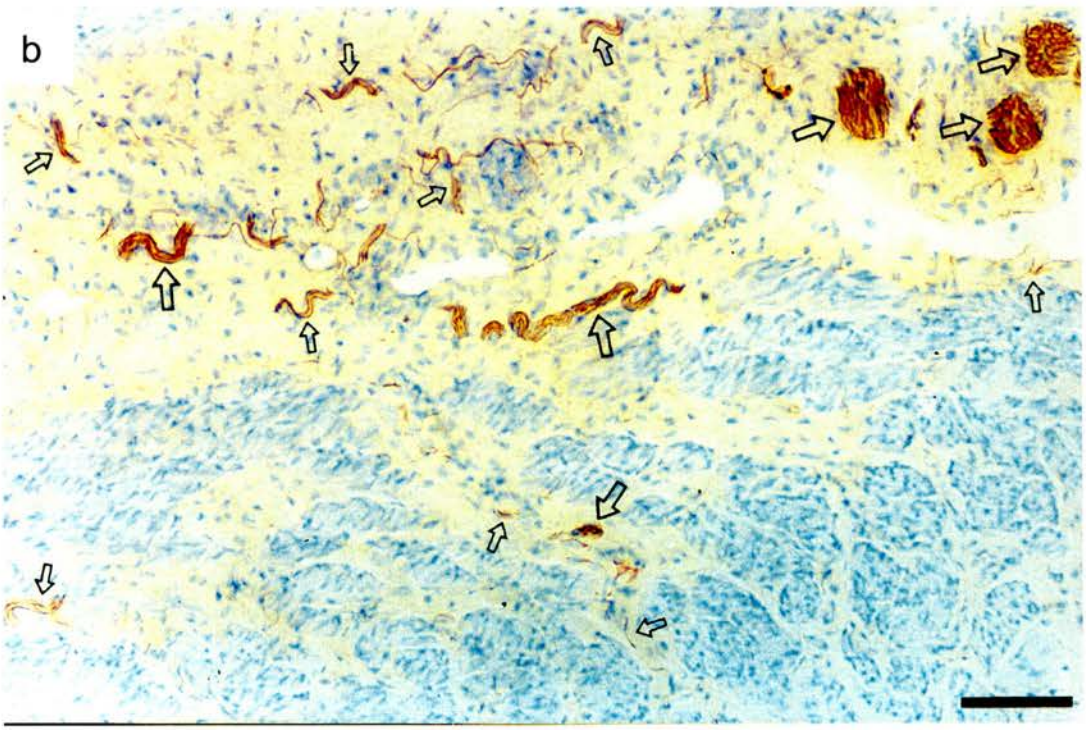


d. PGP-IR nerve fibres (➡) in the cervix associated with blood vessels (V). Scale bar = 50 μm

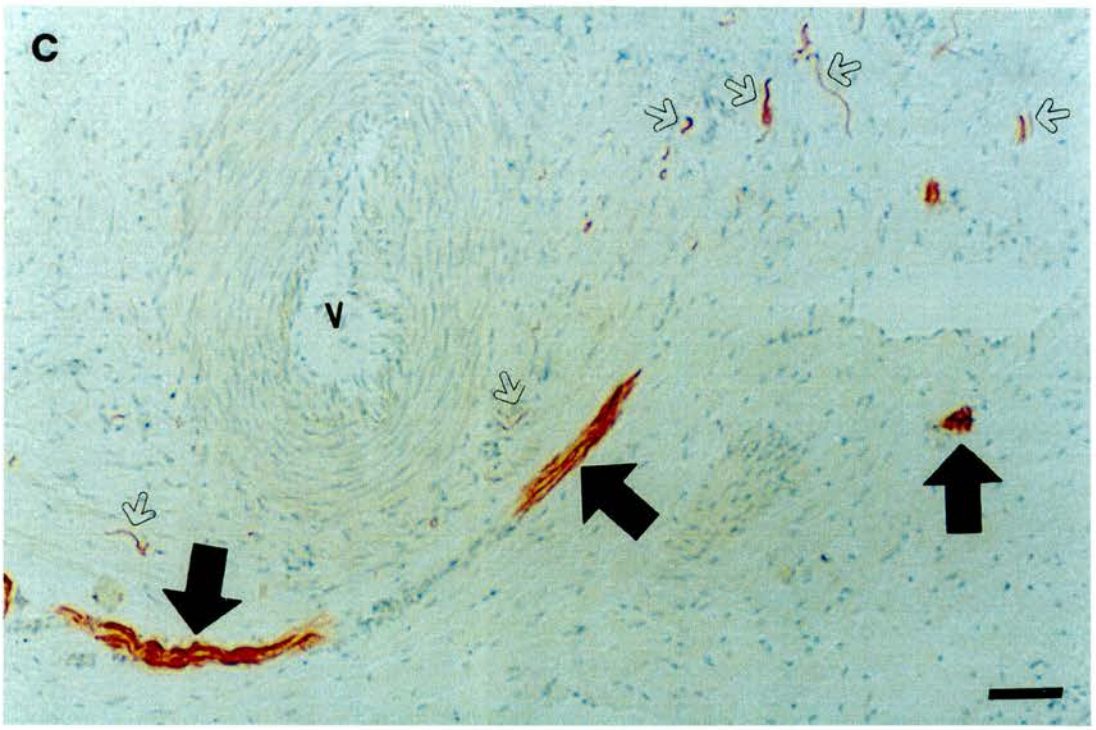
Figure 2-3. PAN-N-immunoreactive nerve bundles and fibres in the equine uterus



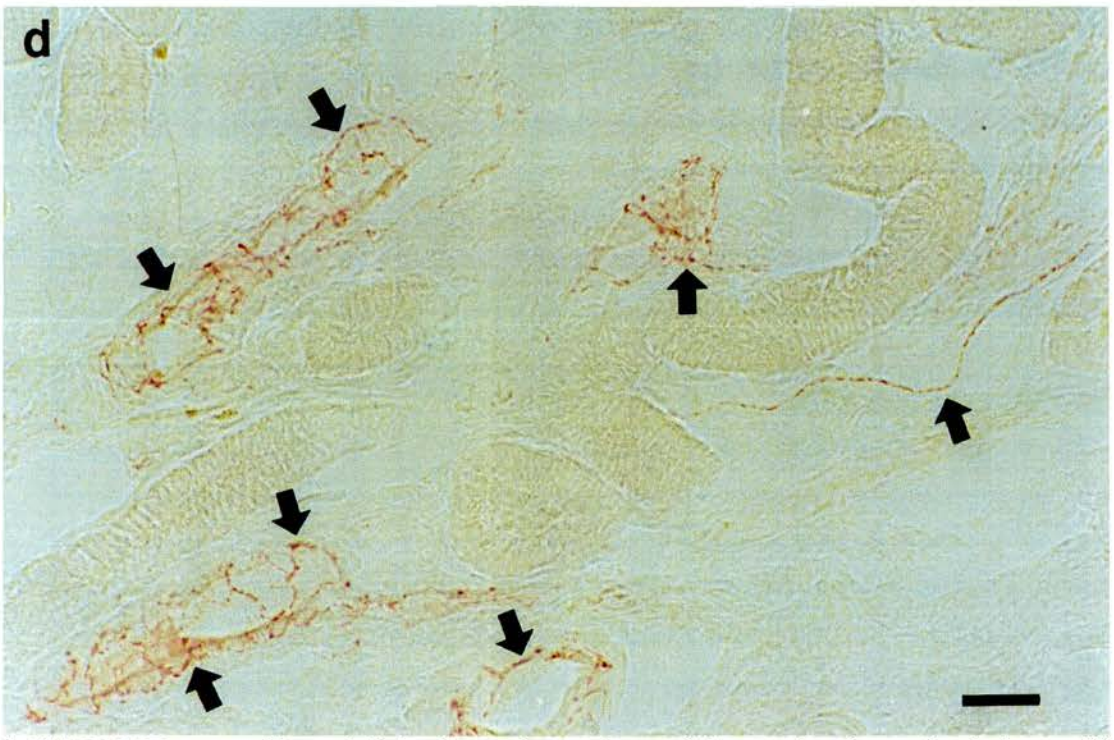
a. PAN-N-IR nerve bundles (➡) in the cervix. Scale bar = 50 μm.



b. PAN-N-IR nerve bundles and fibres (⇨) in the myometrium of the uterine body. Scale bar = 100 μm.

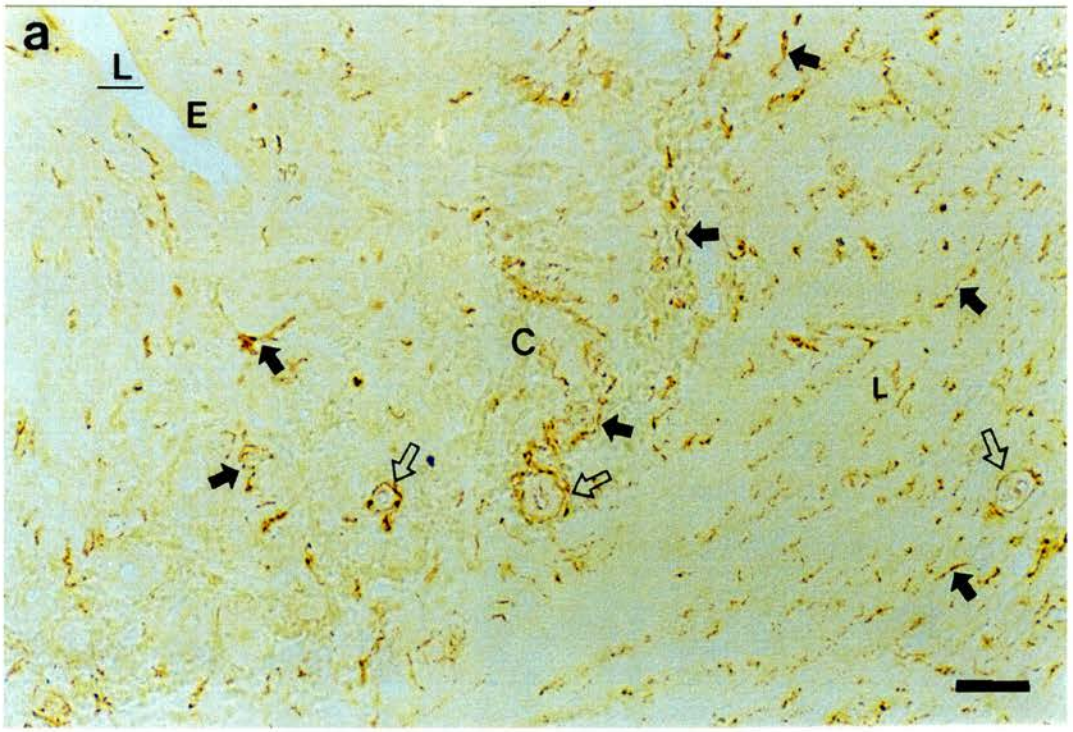


c. PAN-N-IR nerve bundles (➡) and fibres (⇨) in the cervix associated with blood vessels (V). Scale bar = 100 μm.

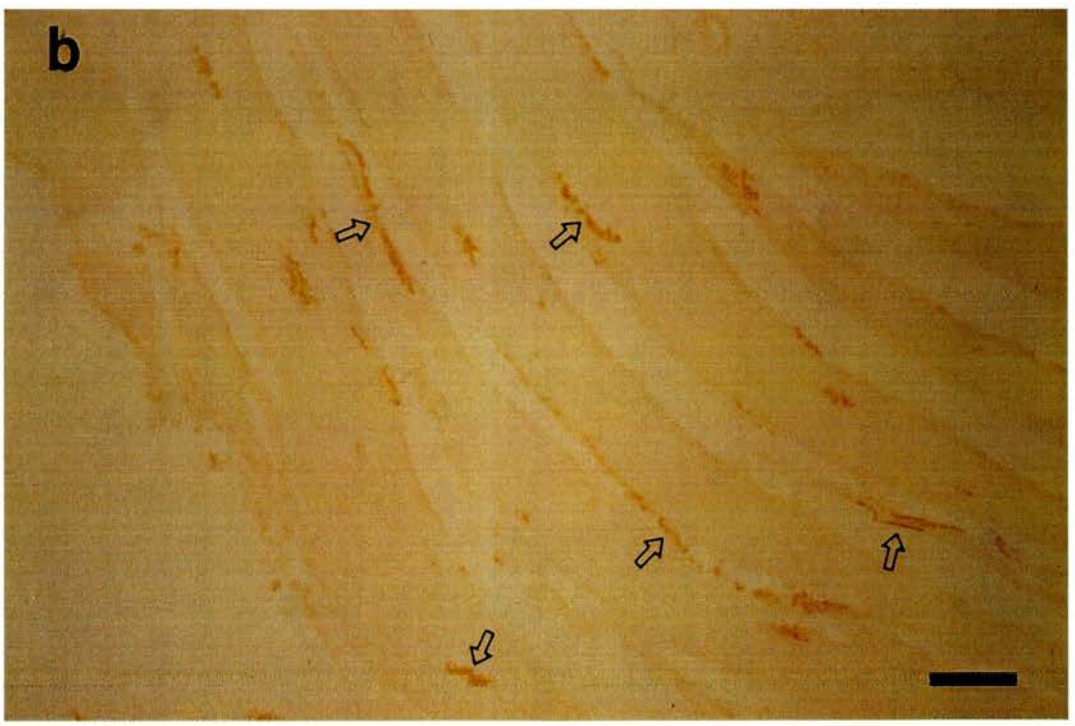


d. PAN-N-IR nerve fibres (➡) in the endometrium of the uterine horn. Scale bar = 50 μm

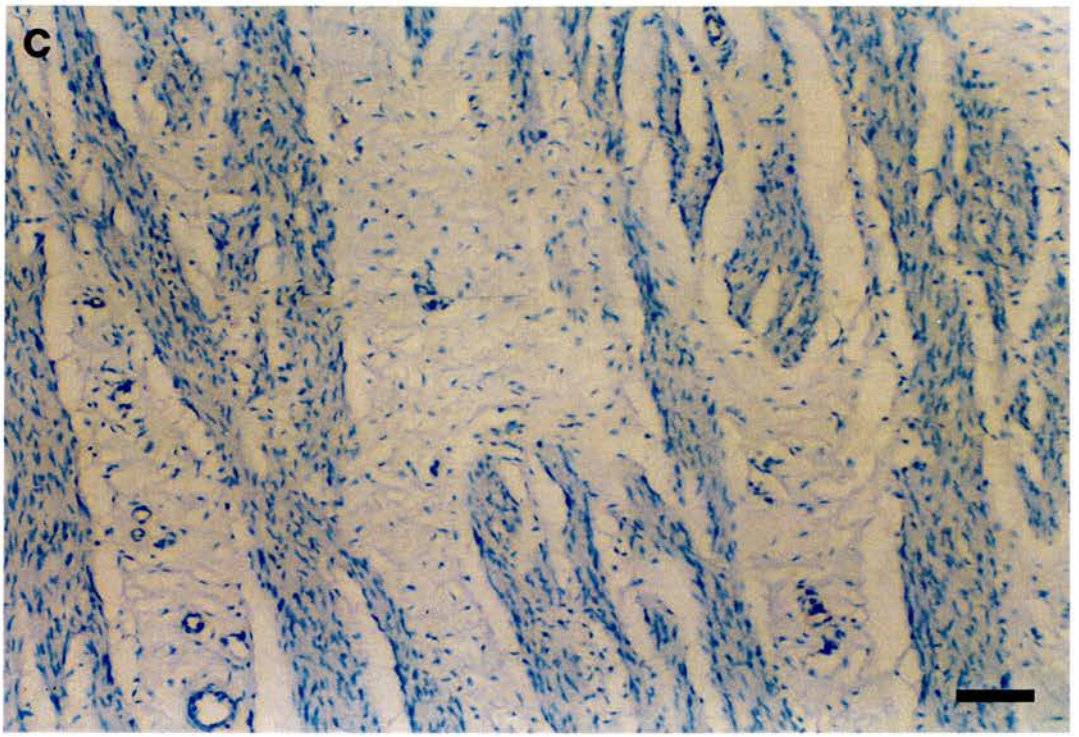
Figure 2-4. Positive and negative controls



a. PGP-IR nerves (➡) associated with blood vessels (⇨) in the uterus of the cat. L: lumen, E: endometrium. C: circular muscle layer, L: longitudinal muscle layers. Positive control. Scale bar = 100 μm



b. PAN-N-IR nerves (⇨) in the equine jejunum. Positive control. Scale bar = 100 μm



c. Myometrium of the uterine body. Negative control. Scale bar = 100 μm

Discussion

In this study, the general uterine innervation in the mare was investigated using PGP 9.5 and PAN-N and showed that, as in other species, the equine uterus receives an abundant nerve supply. The results indicated that the myometrium received the most abundant nerve supply following vascular layer and the endometrium. Of the regions studied, the cervix received the most abundant nerve supply. Two different general neuronal markers were used because PGP can pick up small nerve fibres better than PAN-N, while PAN-N is more likely to miss small diameter fibres than large fibres. Thus, in this study, more nerve bundles were immunoreactive for PAN-N, whereas more nerve fibres tended to be positive for PGP-IR. Sensitivity of positive immunostaining was similar in both PAN-N and PGP.

In this study, samples were collected from cycling mares (6 oestrous and 7 dioestrous) but there was no significant difference in innervation scores between oestrous and dioestrous regions or structures detected in this study ($P>0.01$). It may be that any changes in innervation are undetectable by immunohistochemistry. To compare putative differences in detail, quantitative analysis is needed as in other studies (Huang *et al.*, 1984; Blank *et al.*, 1986; Zoubina *et al.*, 1998). In addition, there might be differences between multiparous mares and young mares, however, mares used in this study did not have a record of their reproductive history, so age or parity were not taken into account.

A large number of immunoreactive nerve bundles and nerve fibres were present in the uterus and cervix. The difference in nerves around blood vessels or endometrial glands versus positions in muscle bundles was 1) lower density in the endometrium, most nerve fibres present travelled freely between glands, 2) moderate density in the vascular layers, nerve bundles and fibres were present near the blood vessels or in the blood vessel wall, and 3) abundant density in the myometrium, both nerve bundles and nerve fibres travelled following the muscle layers: the outer myometrial smooth muscle layers appeared to contain predominantly nerve bundles, whilst inner myometrial smooth muscle layers appear to contain more nerve fibres. This is in agreement with previous studies in the rat (Zoubina *et al.*, 1998) and guinea pig (Alm *et al.*, 1998; Lunberg *et al.*, 1988). In this study, the subjective density of uterine innervation was greatest in the myometrium and in the intramyometrial vascular layer, and was least dense in the endometrium. The results were in agreement with other reports which demonstrated that the density of innervation was greater in the cervix than in the uterus of guinea pigs (Mitchell and Ahmed, 1992), pigs (Lakomy *et al.*, 1994), and rats (Zoubina *et al.*, 1998). It has been shown that the cervix receives an extensive nerve supply from both the caudal mesenteric ganglion and the paracervical ganglion (Traurig *et al.*, 1991; Rang *et al.*, 1995).

Nerve fibres associated with blood vessels and myometrial smooth muscle probably function in the regulation of blood flow and uterine contractility,

respectively (Ottensen *et al.*, 1983; Papka *et al.*, 1985). Taneike *et al* (1991) reported that there are layer-specific variations in the functional innervation of the myometrium in the pig uterus: the circular muscle layer is primarily endowed with cholinergic innervation and the longitudinal muscle layer with adrenergic innervation. The regulation by neuronal factors is controlled by α- and β- adrenergic receptors whose expression is modulated by steroid hormones (Owman and Sjöberg, 1973; Thorbert, 1978; Marshall, 1981; Ford *et al.*, 1984). In addition, cholinergic stimulation of the uterus causes contraction of nonvascular smooth muscle and vasodilation.

The function of nerves associated with the endometrial glands and surface epithelium in the endometrium is still not clear, although nerves have been observed in these areas in most species (Lundberg *et al.*, 1988; Renegar and Rexroad, 1990; Mitchell and Ahmed, 1992). It is reported that endometrial nerves have a role in regulating secretomotor function (Hammarström, 1980). Massmann *et al* (1992) suggested that these nerves, especially adrenergic nerves, control the endocrine and paracrine functions of the endometrium, because the endometrium has oxytocin receptors and also is the main source of endometrial prostaglandin production. The large number of nerve fibres in the cervix may have a role in regulating cervical tone in dioestrus, during pregnancy, and at parturition in the mare.

Uterine motility is known to play an important role in fertility in the mare (Troedsson *et al.*, 1993a; LeBlanc *et al.*, 1994). Persistent mating-induced endometritis (PMIE) is the most common cause of subfertility in broodmares, it is important to understand the role of neuronal control of uterine motility in the equine reproductive system. Impaired uterine contractility results in delayed clearance of intrauterine fluids after mating and low pregnancy rates (LeBlanc *et al.*, 1998). Myoelectrical studies of the equine myometrium indicate that neuronal factors control myometrial activity; xylazine, a α_2 agonist, increases myometrial activity, whereas acepromazine, an α_1 antagonist, suppresses myometrial activity (Gibbs and Troedsson, 1995; De Lille *et al.*, 2000). Damage to myometrial nerves may

contribute to decreased myometrial activity in mares with persistent mating-induced endometritis. Dysfunction of uterine contractility in the mare arising from problems with neuronal control mechanisms could potentially lead to reproductive problems including delay in clearance of intrauterine fluid after mating, and problems during parturition.

In conclusion, the uterus of the mare receives an extensive nerve supply with the greatest density in the myometrium of the cervix. This study was performed using an immunohistochemical approach. The next step in this study would be to collect materials from uteri from different age groups with detailed reproductive records (e.g. pregnancy) and do quantitative assays including radioimmunoassay of neurotransmitters. Future studies will utilise detailed and quantitative morphometric studies to investigate changes in uterine innervation in mares displaying defects in myometrial contractility, such as mares with persistent mating-induced endometritis. In the next chapter, adrenergic nerves, DβH and TH, and peptidergic innervation such as NPY, VIP, CGRP, and SP, in the uterus of genitally-normal mares will be investigated.

Chapter 3

An immunohistochemical study of the adrenergic and peptidergic innervation in the uterus of the mare

: The distribution of dopamine β hydroxylase (DβH), tyrosine hydroxylase (TH), vasoactive intestinal polypeptide (VIP), neuropeptide Y (NPY), calcitonin gene-related peptide (CGRP) and substance P (SP)

Introduction

Uterine contractility is regulated by the coordination and interaction of myogenic, neurogenic, and hormonal control mechanisms. The main external factors that control uterine contractility are the sex steroid hormones, oestrogen and progesterone, and also oxytocin and prostaglandins. In the mare, uterine contractility is of key importance in uterine clearance after mating (Troedsson *et al.*, 1993a). It has been suggested that mares susceptible to persistent mating-induced endometritis have impaired uterine contractile activity (Troedsson *et al.*, 1993a; Nikolakopoulos *et al.*, 2000a). It is possible that defects in innervation or nerve function may contribute to this condition. However, to date there have been no detailed studies of innervation of the equine uterus.

Previous studies on innervation of the mammalian female reproductive tract have demonstrated that it is well supplied by the autonomic nervous system. Both adrenergic and cholinergic nerve fibres are present in the reproductive tract of a variety of species such as in the cats (Alm *et al.*, 1986), guinea pigs (Alm and Lundberg, 1988; Mitchell and Ahmed, 1992), rats (Moustafa, 1988), and women (Stjernquist and Sjöberg, 1994), with adrenergic innervation predominating (Thorbert *et al.*, 1977). In addition to adrenergic and cholinergic nerves, it has been shown that the mammalian reproductive tract is supplied by peptidergic nerves containing a variety of neuropeptides such as neuropeptide Y (NPY) (Lundberg *et al.*, 1990; Jørgensen, 1994; Serghini *et al.*, 1998), vasoactive intestinal polypeptide (VIP) (Majewski *et al.*, 1995; Bredkjcer *et al.*, 1997), calcitonin gene-related peptide (CGRP) (Ghatei *et al.*, 1985; Shew *et al.*, 1992), substance P (SP) (Traurig *et al.*, 1991), bombesin (Mukai *et al.*, 1991), galanin (Lakomy *et al.*, 1995), and gastrin-releasing peptide (GRP) (Häppölä and Lakomy, 1989).

Tyrosine hydroxylase (TH) and dopamine β hydroxylase (DβH) are catecholamine-synthesizing enzymes (Figure 1.10) and their detection by immunohistochemistry has been used to demonstrate the adrenergic contribution to uterine innervation (Alm and Lundberg, 1988; Wrobel and Kujat, 1993). In contrast to other organs, previous studies revealed that uterine adrenergic innervation is unique in that it is affected by ovarian steroid hormones during the oestrous cycle and pregnancy (Owman and Sjöberg, 1973; Marshall, 1981; Zoubina *et al.,* 1998). It has been suggested that autonomic innervation in the reproductive organs has an important role in ovulation, ovum transport, and pregnancy (Owman *et al.,* 1986). Adrenergic innervation is also important in the neuronal control of uterine blood flow, myometrial contractility, and endometrial secretory function (Heinrich *et al.,* 1986) and exerts biological effects consistent with a regulatory function in female reproduction (Taneike *et al.,* 1991).

It is well known that peptidergic nerves containing neuropeptides are present in varying abundance not only in different organs but also in different regions of the same organ (Mitchell and Ahmed, 1992; Bredkjcer *et al.,* 1997). Moreover, these peptidergic nerves are known to vary in type and density between species (Huang *et al.*, 1984; Heinrich *et al.*, 1986). Neuropeptides have a neurotransmitter or neuromodulatory function. Different neuropeptides have been reported to exist and/or coexist in nerve fibres of the female reproductive tract and these nerves are thought to be involved in the neural control of these reproductive organs. NPY, VIP, CGRP and SP are thought to play an important role in control of myometrial contractility (Mukai *et al.*, 1991; Bredkjcer *et al.*, 1997) and uterine blood flow (Ekesbo *et al.*, 1991; Ottensen and Fahrenkrug, 1995). The distribution of neuropeptides in the uterus may be important in the changes that the uterus undergoes during pre- and post-pubertal periods (Woll and Rozengurt, 1989) and during pregnancy (Alm *et al.*, 1988). Other workers have suggested that as these nerves are particularly abundant in the lower genital tract, they may also play a role in regulation of uterine activity during pregnancy and parturition (Majewski *et al.*, 1995; Houdeau *et al.*, 1997). At parturition, the release of NPY and VIP could

contribute to the relaxant mechanisms that are necessary for passage of the foetus through the endocervical canal (Houdeau *et al.*, 1997).

However, information on the uterine innervation of the equine reproductive tract is lacking. The present study investigated adrenergic innervation of the uteri of genitally normal mares using the catecholamine enzymatic markers tyrosine hydroxylase and dopamine β hydroxylase. The distribution of nerve fibres immunoreactive for the peptide neurotransmitters NPY, CGRP, VIP, and SP were also investigated.

Materials and Methods

Animals:

The uterine tissue samples were collected from 13 cyclic mares (6 oestrous and 7 dioestrus), aged between 3 and 18 years and weighed 390 to 520 kg, that were euthanased for reasons other than reproductive problems. The stage of the cycle was established by peripheral blood progesterone levels (oestrus: <1ng/ml) as well as the presence or absence of a corpus luteum in the ovaries, the presence of a follicle size >30 mm in diameter and uterine oedema. The reproductive tracts of these mares were removed within 20 min of euthanasia and were gently washed and dissected in 0.1M phosphate-buffered saline (PBS; pH 7.2). Four segments, size approximately 1.5 cm x 1.5 cm, were collected from the uterine horn, body and the cervix from each mare. Samples were then fixed immediately in 4% paraformaldehyde (pH 7.2) for 14-16 h at 4°C. Samples were transferred into 0.1M PBS (pH 7.2) containing 30% sucrose (v/v) and stored in this solution for at least 24 h at 4°C, with four changes of buffer. Tissues were snap frozen in optimum cutting temperature compound (Miles Inc, Elkhart, IN) in an isopentane/dry-ice slurry and then stored at -70°C before sectioning.

Immunohistochemical procedure:

Cryostat sections, 12-15μm thick, were cut and mounted on Bio-Bond (British BioCell International, Cardiff, UK) coated slides. Sections were air-dried for 60-90 min at room temperature, rinsed in 0.01M PBS (pH 7.2) for 10 min and blocked with 3% H_2O_2 in methanol for 30 min to reduce non-specific binding. Slides were then preincubated with 1% goat serum in 0.01M PBS for 1 h. The excess serum was blotted and the sections were then incubated with primary antibody at the appropriate dilution overnight at 4°C before washing three times in 0.01M PBS. Primary antisera were raised in rabbits, and were used at the following dilutions in PBS: TH (1:6000, Affiniti Research Products, Exeter, UK), DβH (1:4000, Affiniti Research Products, Exeter, UK), NPY (1:4000, Affiniti Research Products, Exeter, UK), VIP (1:4000, Peninsula Laboratories Europe, St Helens, UK), CGRP (1:4000, Peninsula Laboratories Europe, St Helens, UK), and SP (1:6000, Peninsula Laboratories Europe, St Helens, UK). The sections were incubated with the second antibody (goat anti- rabbit) in PBS containing 1% normal goat serum, for 2 h at room temperature. The sections were thoroughly washed three times in PBS, then incubated with the avidin-biotin complex (Vector Laboratories, Cambridges, UK) for a further 30 min, and rinsed in PBS. Chromagens (3-amino-9-ethylcarbazole: AEC - red reaction product or 3,3'-diaminobenzidine: DAB - brown reaction product, Vector Laboratories, Cambridge, UK) were added as the final substrate, and were incubated at room temperature in the dark for 10-15 min. Each slide was then rinsed carefully with distilled water. Slides were mounted and examined by light microscopy under magnification between x20 to x40.

To test the specificity of the primary antisera: a) sections were incubated with antibody which had been preabsorbed with its synthetic antigen (10 μg of antigen / ml of diluted antiserum) in our laboratory (Corcoran, 1996), b) the primary antibody was omitted from the incubation, or c) normal rabbit serum was substituted for the primary antibody. Positive controls comprised equine jejunum (Pearson, 1994) and feline (Alm *et al.*, 1986) uterus incubated with each of the primary antibodies.

Subjective evaluation of uterine innervation

Relative frequency of uterine nerve fibres and bundles was assessed using a subjective scoring system (See previous chapter) in immunostained sections obtained from uterine horn, uterine body, and the cervix. For each section, nerves were evaluated under magnification (x10 to x20) with respect to their distribution within three separate structures: myometrium, endometrium and vascular layer (between myometrial layers). For each structure, numbers of nerves were assigned a score from 0 to 5 (See Appendix) (Alm *et al.*, 1988; Mitchell and Ahmed, 1992). Four sections from each of the three regions from each animal were included for analysis. The term nerve fibre distribution includes all identified nerves either nerve bundles or fibres; whether they are terminations or pre-terminal axons.

Statistical analysis

Score for four sections from three regions and each structure from each animal were included for analysis. Scores for every section were pooled. Score values are presented as the mean ± SEM (as a general error term). Data were analysed by a general linear model analysis of variance (ANOVA) (SPSS 9.0) to determine the effect of 1) stages of the cycle, 2) regions, 3) structures and 4) regions*structures (factor). A probability of <0.01 was taken to indicate a statistically significant difference.

Results

Statistical analysis showed no significant differences in regions and/or in structures ($P>0.01$) between oestrous and dioestrous mares. Therefore, all score data were pooled together. Within regions (uterine horn, uterine body, and the cervix), the

cervix region showed significantly higher scoring density of innervation in all nerves. Substance P was only seen in the cervix (Table 3.1). Total mean value of density showed adrenergic nerves immunoreactive for TH and DβH, nerve frequency was much greater (P<0.01) than peptidergic nerves (Table 3.2). Within structure (myometrium, endometrium and vascular layer), higher frequency of adrenergic nerves immunoreactive for DβH, and NPY-immunoreactive nerves showed in the myometrium (P<0.01) than that in the endometrium, while VIP and CGRP showed no difference between myometrium and endometrium, but significantly less (P<0.01) in vascular layer (Table 3.3). There was no difference in SP within structures (P=1.0).

Regions	DβH	TH
Uterine horn	2.96 ± 0.03^{a}	3.00 ± 0.11^{a}
Uterine body	2.97 ± 0.03^{a}	2.99 ± 0.11^{a}
Cervix	3.29 ± 0.03^{b}	3.48 ± 0.11^{b}

Regions	NPY	VIP	CGRP	SP
Uterine horn	2.29 ± 0.04^{a}	0.59 ± 0.04^{a}	0.59 ± 0.04^{a}	0.00 ± 0.01^{a}
Uterine body	2.31 ± 0.04^{a}	0.57 ± 0.04^{a}	0.61 ± 0.04^{a}	0.00 ± 0.01^{a}
Cervix	2.60 ± 0.04^{b}	0.84 ± 0.04^{b}	0.92 ± 0.04^{b}	0.07 ± 0.01^{b}

Structures	DβH	TH
Myometrium	3.45 ± 0.03^{a}	3.48 ± 0.11^{a}
Endometrium	2.80 ± 0.03^{b}	2.81 ± 0.11^{b}
Vascular layer	2.97 ± 0.03^{c}	3.17 ± 0.11^{a}

Structures	NPY	VIP	CGRP	SP
Myometrium	2.65 ± 0.04^{a}	0.89 ± 0.04^{a}	0.90 ± 0.04^{a}	0.02 ± 0.01
Endometrium	2.16 ± 0.04^{b}	0.95 ± 0.04^{a}	1.01 ± 0.04^{a}	0.02 ± 0.01
Vascular layer	2.39 ± 0.04^{c}	0.15 ± 0.04^{b}	0.19 ± 0.04^{b}	0.02 ± 0.01

Table 3-1. Data show Mean ± SEM from subjectively graded scoring system (0 to 5) with adrenergic- and peptidergic-immunoreactive nerves in the equine uterus in each region and each structure. ^{a,b,c} Different superscripts indicate significant differences within column (P<0.01).

	Adrenergic innervation		*Peptidergic innervation*			
	DβH	*TH*	*NPY*	*VIP*	*CGRP*	*SP*
Grand mean	3.07 ± 0.02[a]	3.16 ± 0.07[a]	2.40 ± 0.03[b]	0.66 ± 0.03[c]	0.70 ± 0.03[c]	0.00 ± 0.01[d]

Table 3-2. Data show Mean ± SEM from subjectively graded scoring system (0 to 5) with adrenergic- and peptidergic-immunoreactive nerves in the equine uterus. [a,b,c,d] Different superscripts indicate significant differences within row ($P<0.01$).

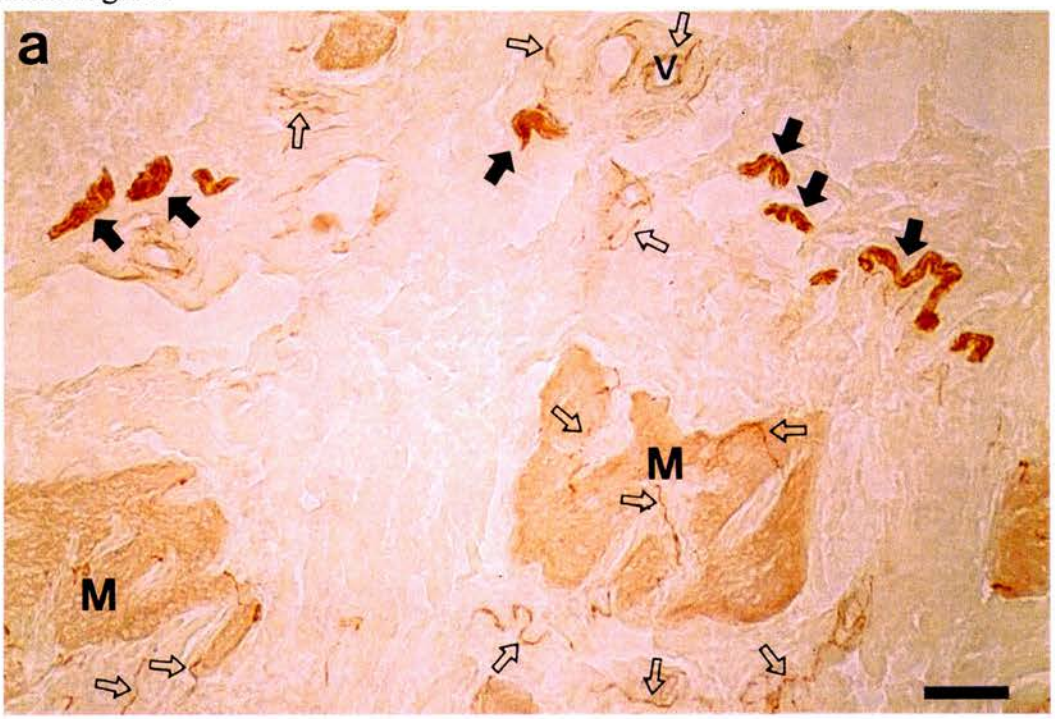
Distribution of adrenergic nerves (TH and DβH)

Nerves immunoreactive (IR) for TH and DβH were found throughout all regions of the uterus (Table 3.1). Statistical analysis showed no difference between stages of cycle (oestrus vs dioestrus) in both TH and DβH ($P=0.3$ and $P=0.9$, respectively). Numerous TH-IR nerve bundles and fibres (Fig 3-2-a) were found in the uterine horn, body and the cervix. In the muscle layers, TH-IR nerve fibres often travelled parallel to the muscle fibres. Nerve fibres immunoreactive for TH were distributed along the vessels (Fig 3-2-b) extending from the longitudinal to the circular smooth muscle layers of the myometrium. Some nerve fibres entered the endometrium close to endometrial glands (Figure 3-2-c). Nerves immunoreactive for DβH were also present in all regions of the uterus. An intense network of DβH-IR nerve bundles and fibres was found in the uterine horn (Figure 3-3-a), body (Figure 3-3-c) and the cervix (Figure 3-3-b). The density and distribution for DβH was similar to that for TH (Table 3.2). Within regions, the cervix received the most abundant adrenergic nerve supply ($P<0.01$) (Table 3.1). Within structures the myometrium received significant higher nerve supply ($P<0.01$) than that in the endometrium or in the vascular layers in DβH and TH (Table 3.1).

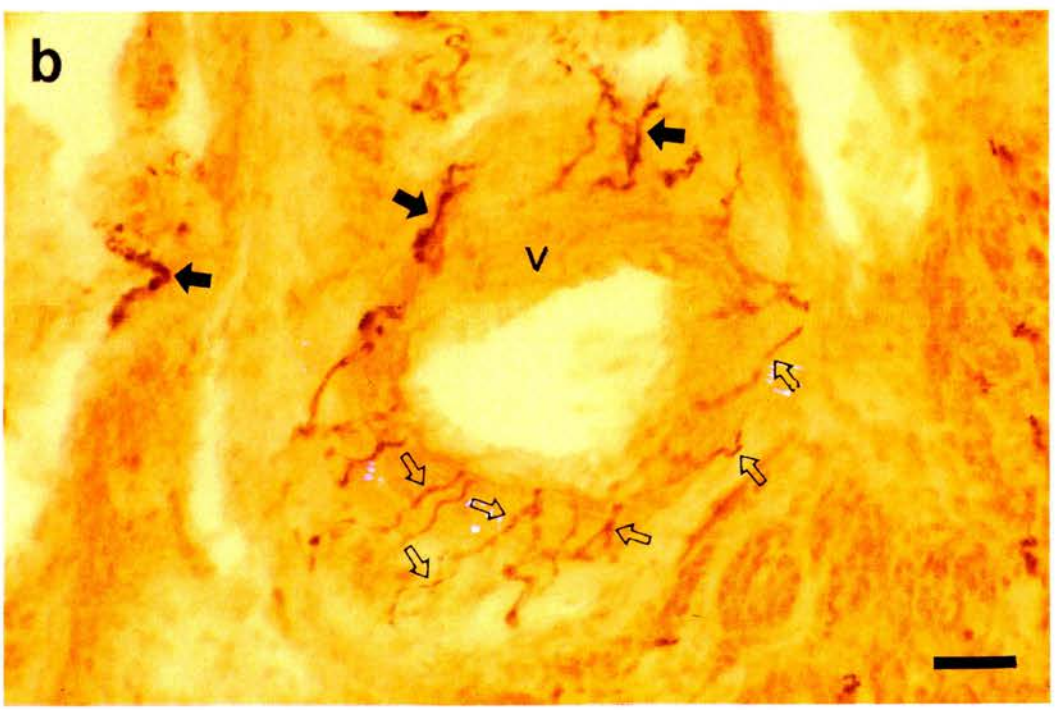
Table 3-3. Data show Mean ± SEM from subjectively graded scoring system (0 to 5) with adrenergic- and peptidergic-immunoreactive nerves in the equine uterus according to their structures.

Regions / Structure	Adrenergic innervation		Peptidergic innervation (Neuropeptides)			
	DβH	*TH*	*NPY*	*VIP*	*CGRP*	*SP*
Uterine horn						
Myometrium	3.25 ± 0.06	3.34 ± 0.2	2.49 ± 0.07	0.73 ± 0.07	0.72 ± 0.08	0.00 ± 0.02
Endometrium	2.75 ± 0.06	2.75 ± 0.2	2.16 ± 0.07	0.85 ± 0.07	0.87 ± 0.08	0.00 ± 0.02
Vascular layer	2.87 ± 0.06	2.93 ± 0.2	2.22 ± 0.07	0.18 ± 0.07	0.16 ± 0.08	0.00 ± 0.02
Uterine body						
Myometrium	3.31 ± 0.06	3.27 ± 0.2	2.44 ± 0.07	0.75 ± 0.07	0.77 ± 0.08	0.00 ± 0.02
Endometrium	2.73 ± 0.06	2.79 ± 0.2	2.16± 0.07	0.79 ± 0.07	0.90 ± 0.08	0.00 ± 0.02
Vascular layer	2.85 ± 0.06	2.90 ± 0.2	2.28 ± 0.07	0.17 ± 0.07	0.26 ± 0.08	0.00 ± 0.02
Cervix						
Myometrium	3.81 ± 0.06	3.84 ± 0.2	3.00 ± 0.07	1.13 ± 0.07	1.24 ± 0.08	0.09 ± 0.02
Endometrium	2.87 ± 0.06	2.87 ± 0.2	2.12 ± 0.07	1.21 ± 0.07	1.33 ± 0.08	0.09 ± 0.02
Vascular layer	3.16 ± 0.06	3.53 ± 0.2	2.63 ± 0.07	0.21 ± 0.07	0.27 ± 0.08	0.09 ± 0.02

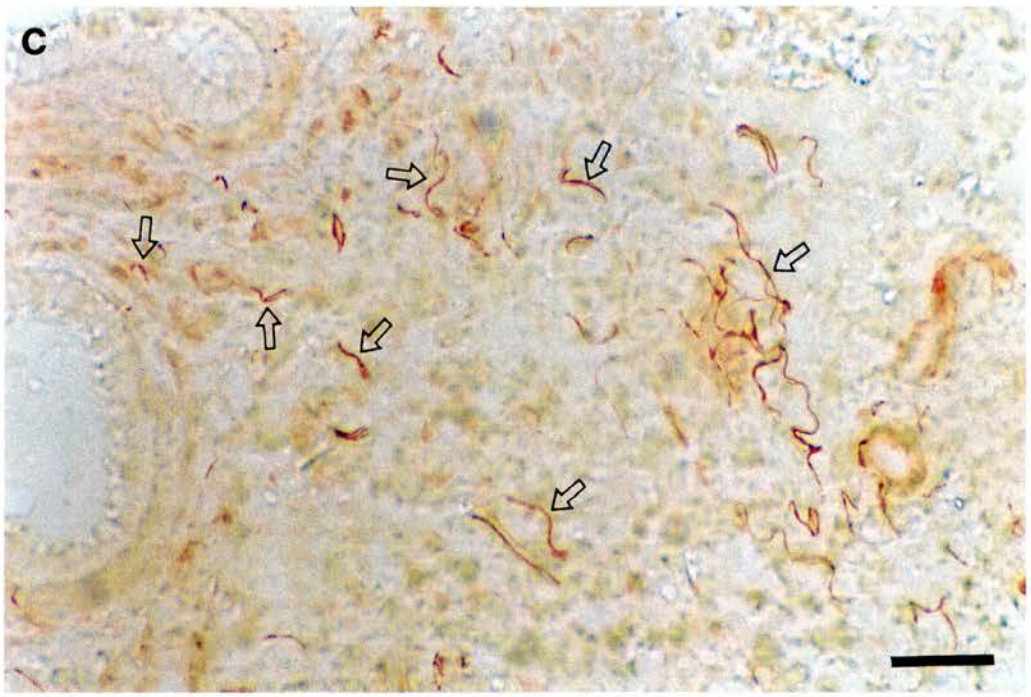
Figure 3-1. Tyrosine hydroxylase (TH)-immunoreactive (IR) nerve fibres in different uterine regions



a. TH-IR nerve bundles (➡) and nerve fibres (⇨) in the myometrium of the uterine body. M: Smooth muscle bundles. V : Blood vessel. Scale bar = 250 μm

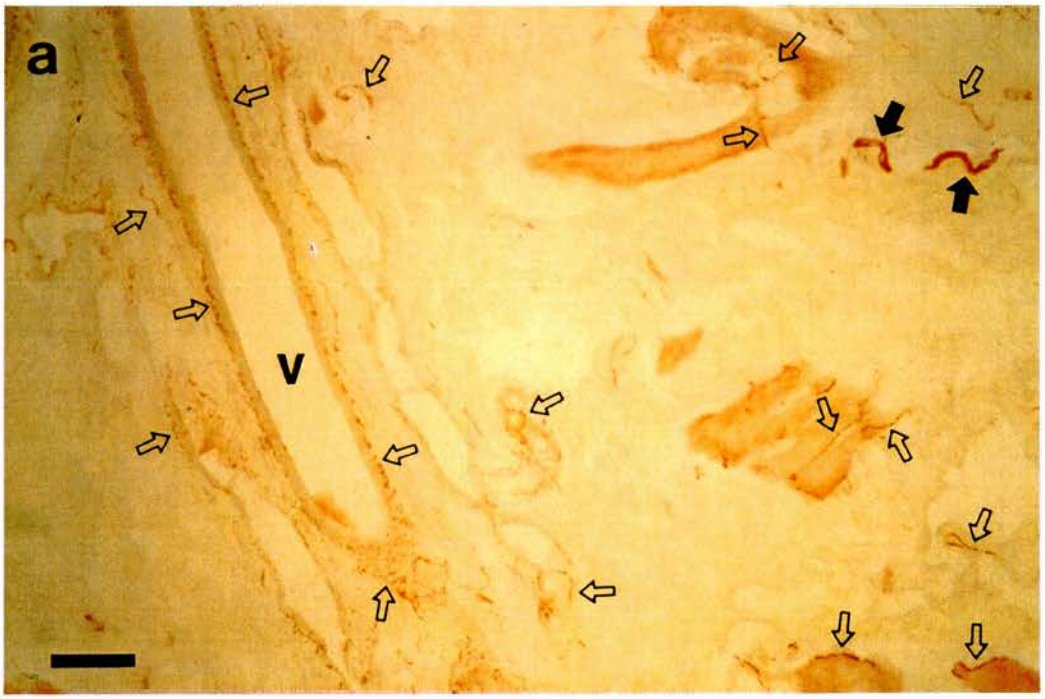


b. TH-IR nerve bundles (➡) and nerve fibres (⇨) in the myometrium of the uterine horn associated with blood vessels (V). Scale bar = 50 μm

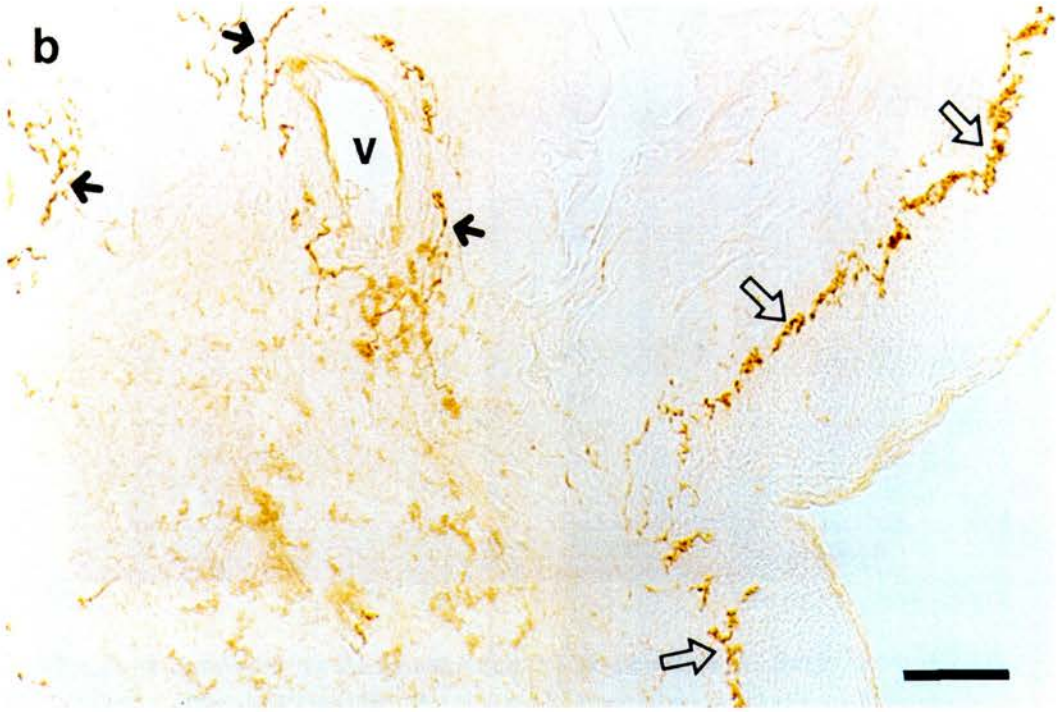


c. TH-IR nerve fibres (⇨) close to the endometrial glands in the endometrium of the uterine body. Scale bar = 50 μm

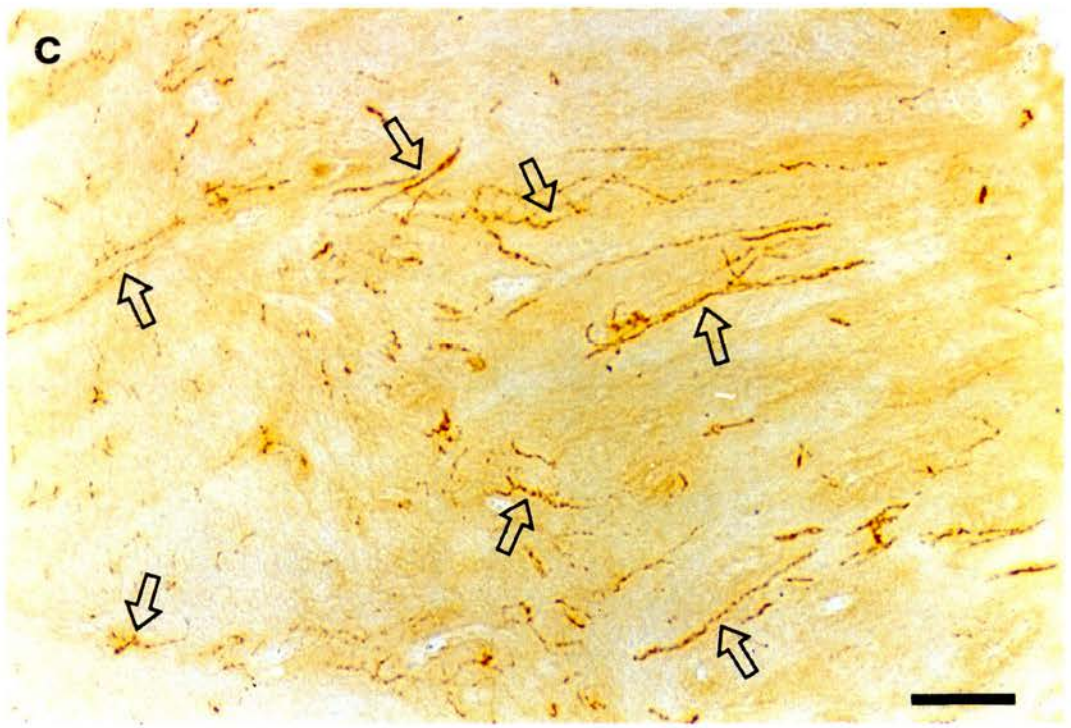
Figure 3-2. Dopamine β hydroxylase (DβH)-immunoreactive (IR) nerve fibres in different uterine regions



a. DβH –IR nerve bundles (➡) and nerve fibres (⇨) in the myometrium of the uterine horn. Scale bar = 250 μm



b. DβH – IR nerve fibres (⇨) in the cervix and nerve fibres (➔) associated with blood vessels (V). Scale bar = 50 μm



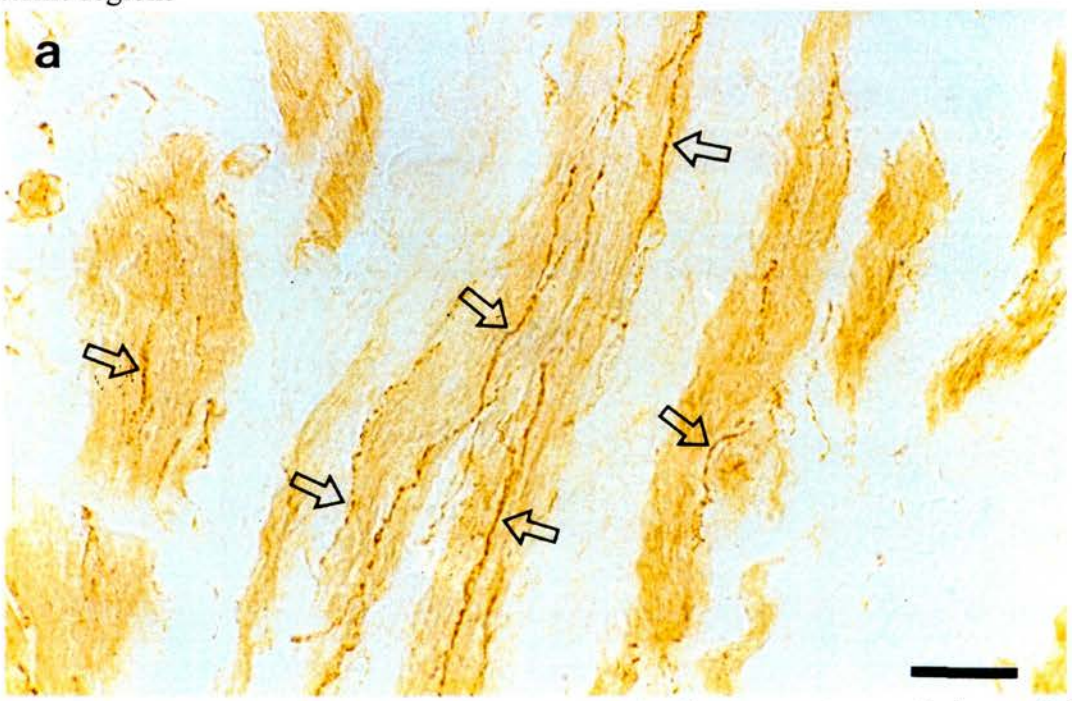
c. DβH – IR nerve fibres nerve bundles (⇨) in the smooth muscle layers of the uterine body. Scale bar = 100 μm

Distribution of peptidergic nerves

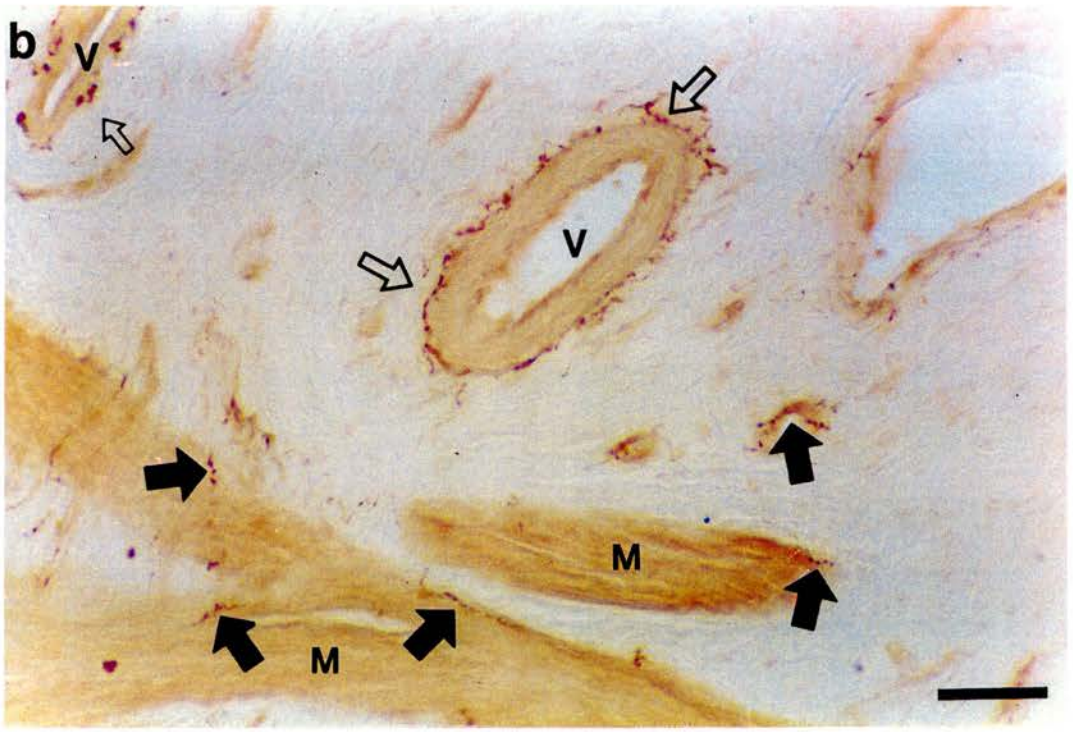
Nerves immunoreactive for NPY

Moderate numbers of NPY-IR nerve fibres were found in all regions of the uterus. Among the peptidergic nerves, NPY showed significantly higher density ($P<0.05$) than any other neuropeptides (Table 3.2). NPY-containing nerve fibres were present in both the circular and longitudinal muscle layers travelling in parallel to the long axis of the smooth muscle layers (Fig 3-4-a) and often associated with blood vessels forming vessel-surrounding plexiform networks (Fig 3-4-b). The density of NPY was significantly higher ($P<0.05$) in the myometrium than either in the vascular layer or in the endometrium (Table 3-3). NPY-IR nerve fibres were seen in the endometrium beneath the luminal epithelium, near endometrial glands (Fig 3-4-c) and submucosa. Statistical analysis showed no difference between stages of cycle ($P=0.7$).

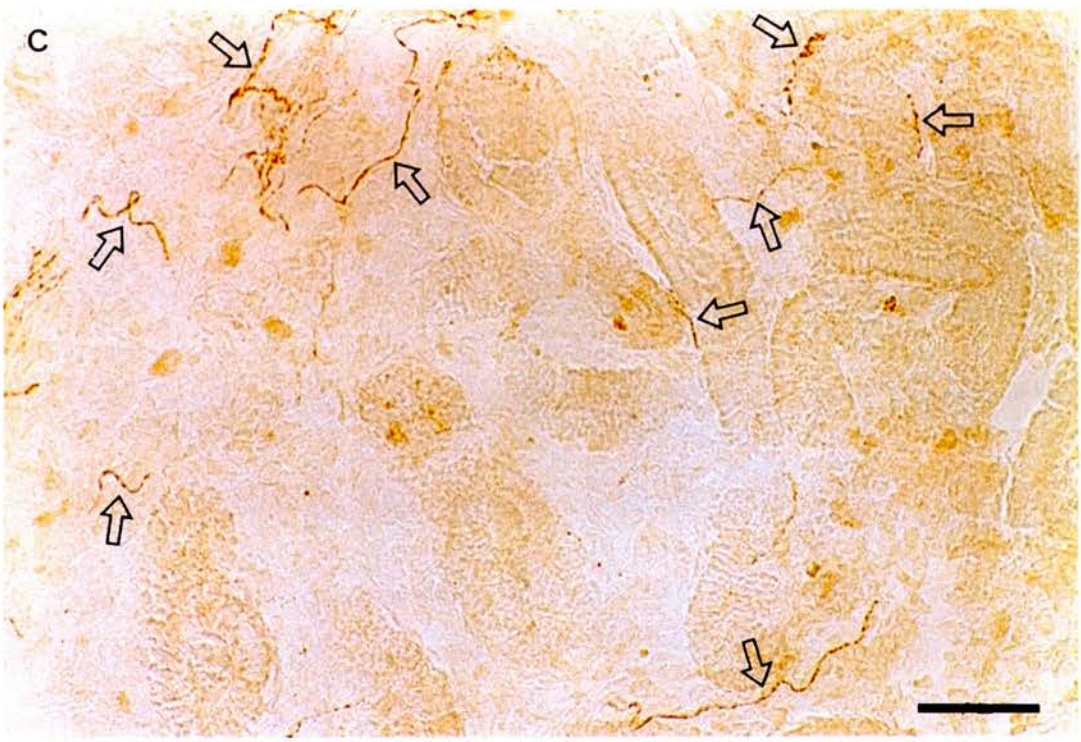
Figure 3-3. Neuropeptide Y (NPY)-immunoreactive (IR) nerve fibres in different uterine regions



a. NPY-IR nerve fibres (⇨) in the outer longitudinal smooth muscle layer of the uterine horn. Scale bar = 50 μm



b. NPY-IR nerve fibres (➡) in the smooth muscle bundles (M) and nerve fibres (⇨) associated with blood vessels (V) in the cervix. Scale bar = 50 μm

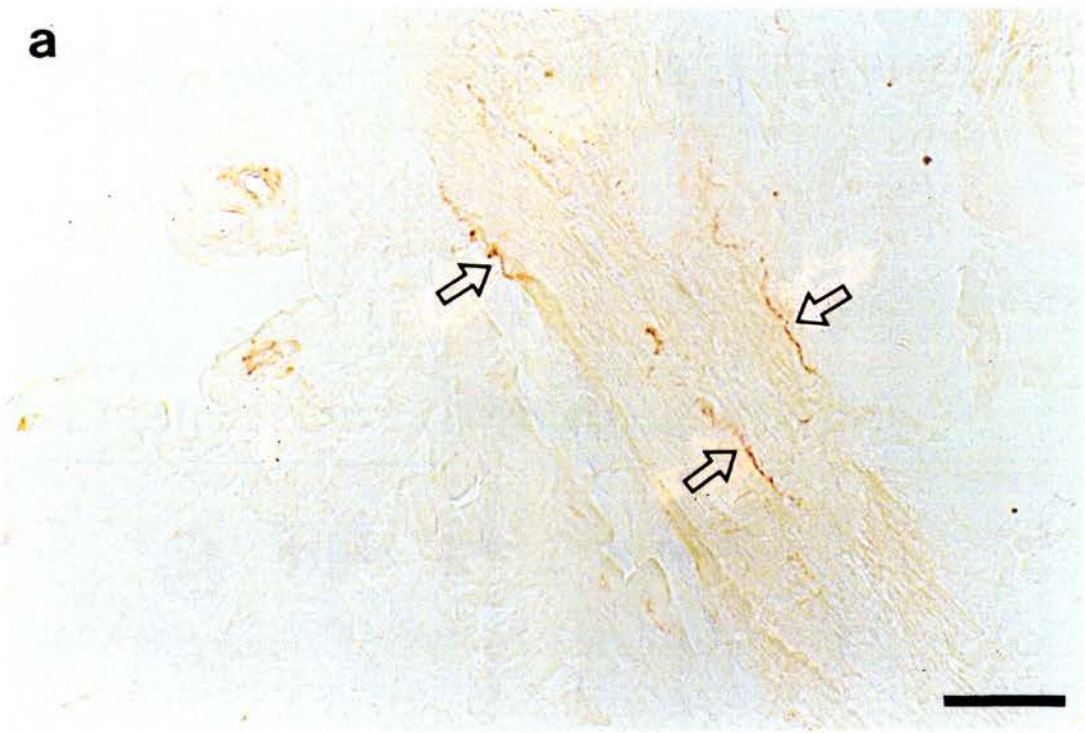


c. NPY-IR nerve fibres (⇨) close to the endometrial glands of the uterine body. Scale bar = 50 μm

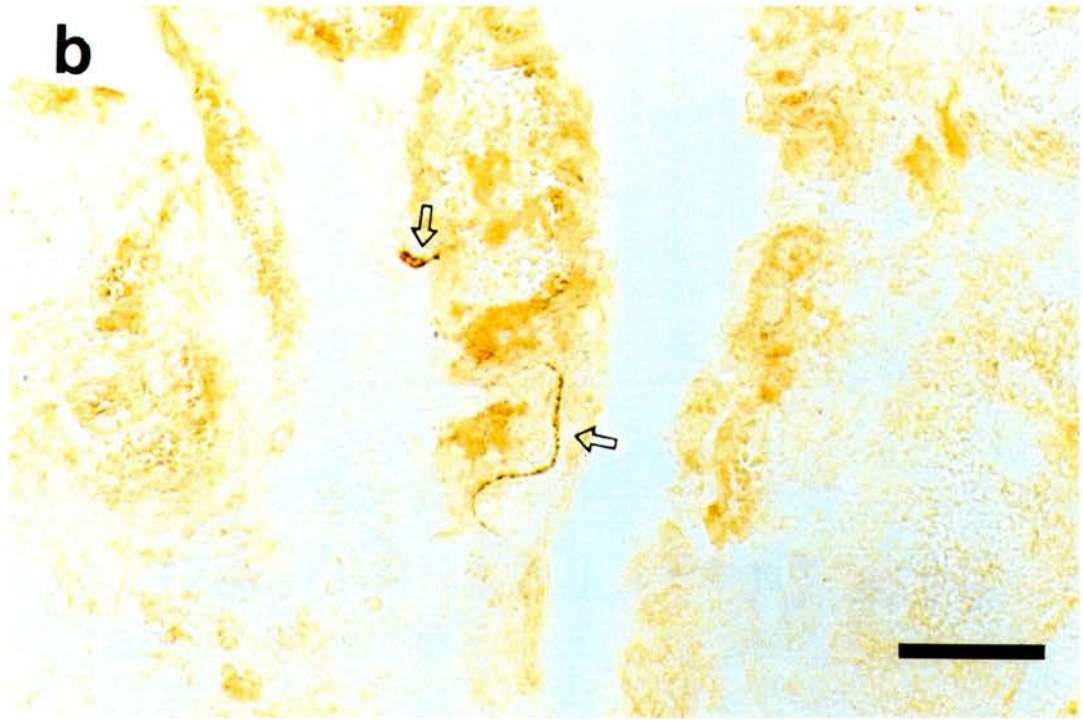
Nerves immunoreactive for VIP

The distribution of VIP-IR nerve fibres was found to be similar to NPY-IR nerve fibres, but smaller numbers of nerve fibres were seen. A small number of scattered VIP-IR nerve fibres were seen in the interstitial connective tissue and along the smooth muscle layers (Fig 3-5-a) sometimes associated with blood vessels. In the endometrium, VIP-IR nerve fibres were seen around the endometrial glands (Fig 3-5-b). The number of VIP-IR nerves was similar in the uterine horn and body, but was higher in the cervix (Table 3-1). Statistical analysis showed no difference between stages of cycle (P=0.2).

Figure 3-4. Vasoactive intestinal polypeptide (VIP)- immunoreactive (IR) nerve fibres in different uterine regions



a. VIP-IR nerve fibres (⇨) in the myometrium of the uterine body. Scale bar = 50 μm.

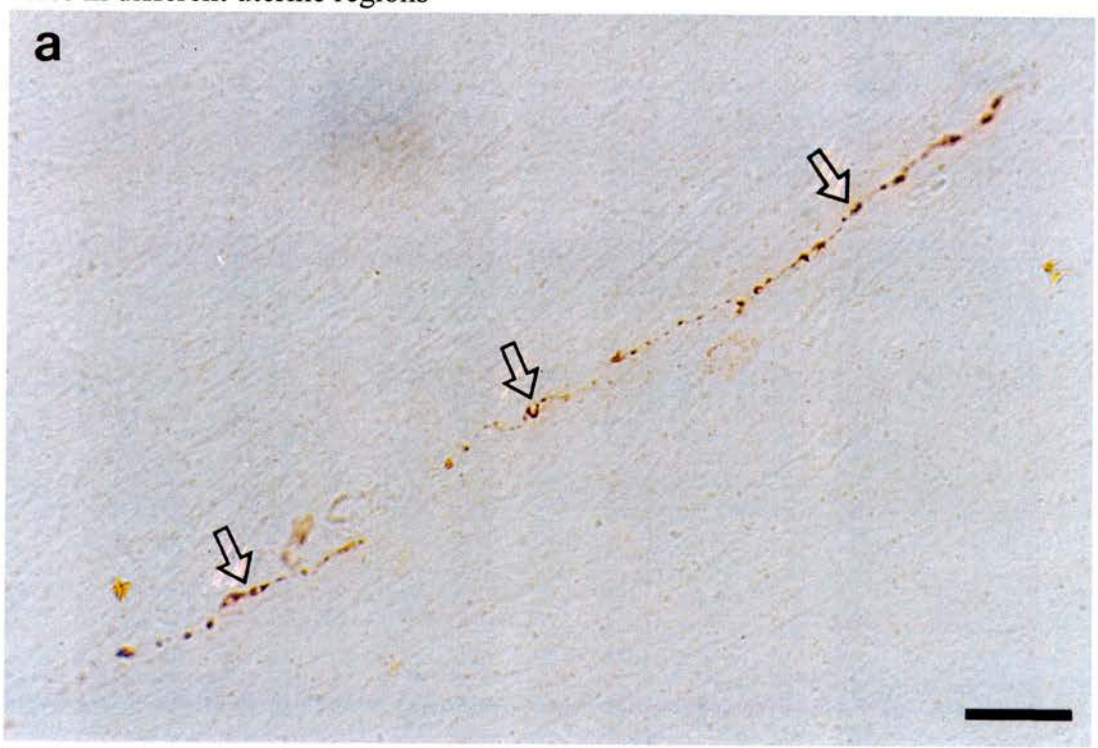


b. VIP-IR nerve fibres (⇨) in the endometrium of the uterine horn. Scale bar = 50 μm

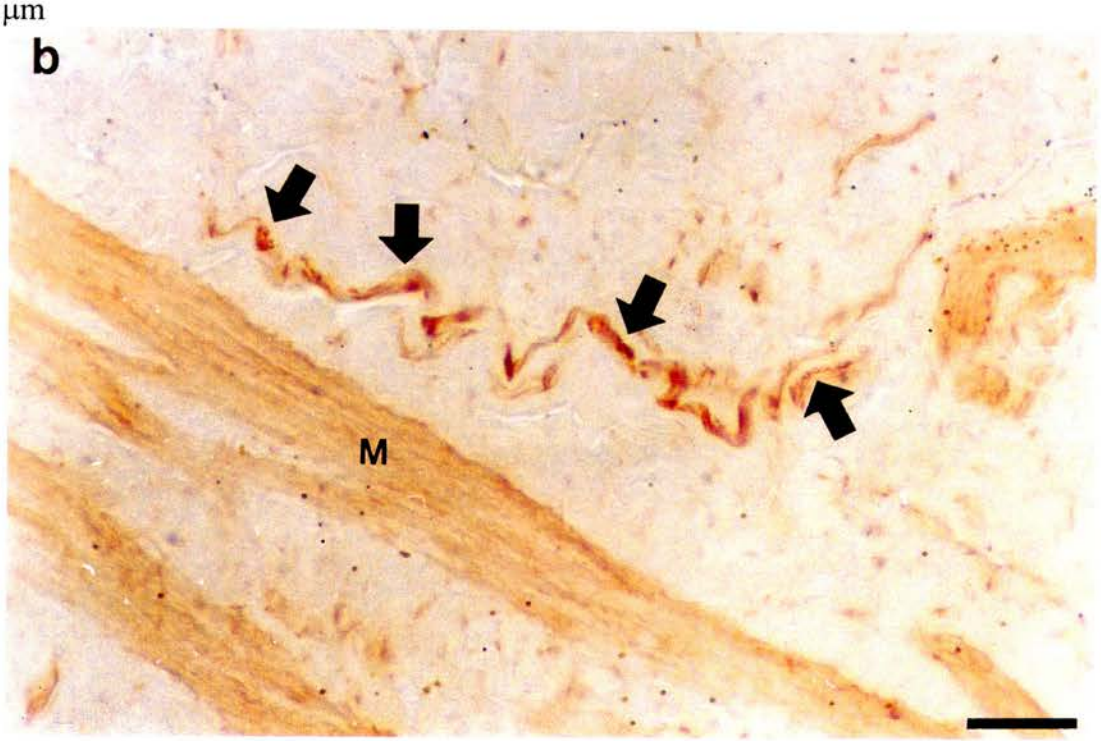
Nerves immunoreactive for CGRP

CGRP-IR nerve fibres were less numerous than nerves containing NPY, but were present in similar density to VIP. A small number of scattered CGRP nerve fibres were seen in the interstitial connective tissue and along smooth muscle layers (Fig 3-6-a) sometimes associated with blood vessels. Thick bundles of CGRP-IR nerve fibres were found in the cervix, travelling parallel to smooth muscle bundles (Fig 3-6-b). There were more CGRP-IR nerve fibres in the cervix than in other parts of uterus. Statistical analysis showed no difference between stages of cycle ($P>0.01$).

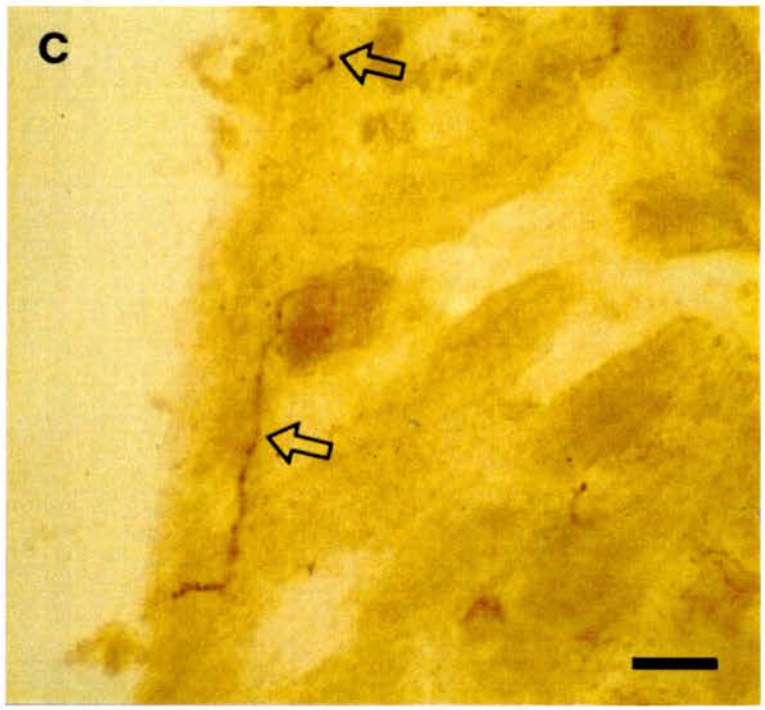
Figure 3-5. Calcitonin gene-related peptide (CGRP)– immunoreactive (IR) nerve fibres in different uterine regions



a. CGRP-IR nerve fibres (⇨) in the myometrium of the uterine body. Scale bar = 50 μm



b. Thin CGRP-IR nerve bundles and fibres (➡) in the cervix. M: Smooth muscle bundles. Scale bar = 50 μm



c. CGRP-IR nerve fibres (⇨) in the endometrium of the uterine horn. Scale bar = 50 μm

Nerves immunoreactive for SP

A few SP-IR nerve fibres were found only in the cervix. SP-IR nerve fibres travelled parallel to the long axis of the smooth muscle bundles, close to blood vessels (Fig 3-6). Statistical analysis showed no difference between stages of cycle (P=0.4).

Negative controls

Negative control sections (omission or preabsorption of primary antibody) failed to demonstrate any positive staining (Fig 3-7) and positive controls showed strong immunoreactivity

Figure 3-6. Substance P (SP)-immunoreactive nerve fibres (⇨) near blood vessel (V) in inner smooth muscle layer in the cervix. Scale bar = 50 μm

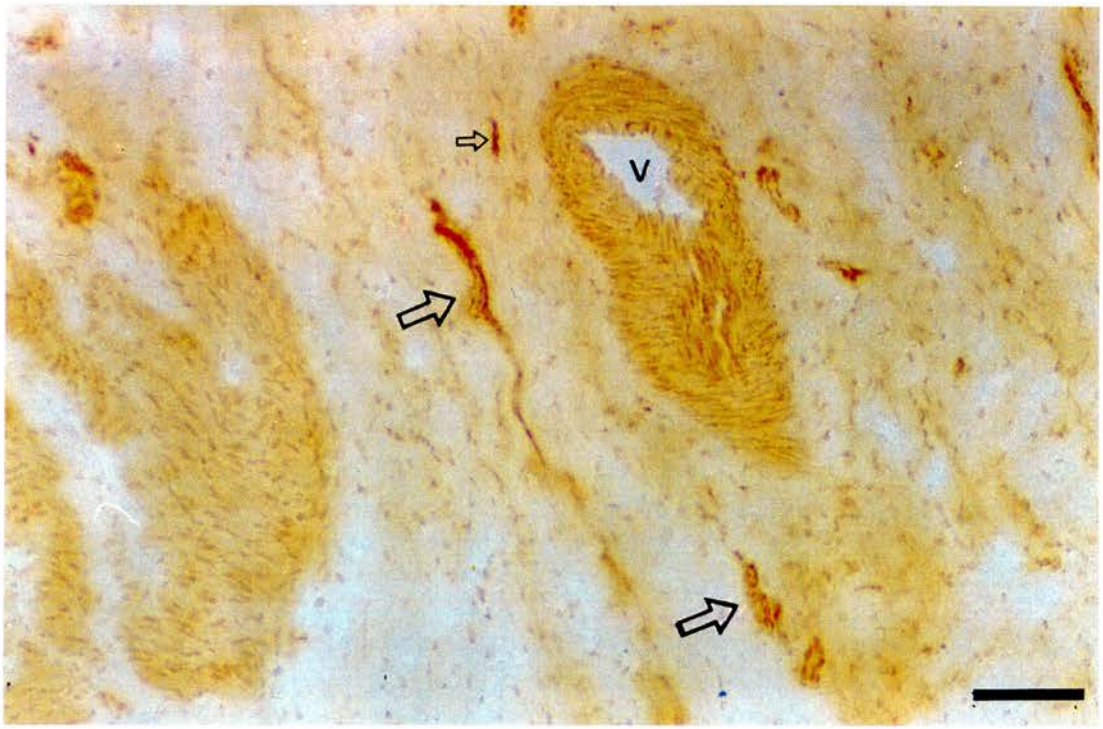


Figure 3-7. Negative control. Uterine horn. V: Blood vessel. Scale bar = 50 μm

Discussion

The present study is the first description of the distribution of adrenergic and peptidergic nerves in each structure from different regions of the equine uterus. The results provide immunohistochemical evidence for an abundant adrenergic innervation, as well as the presence of peptidergic innervation containing the neuropeptides, NPY, VIP, CGRP and SP in the uterus of the mare.

Previous studies have reported that the mammalian female reproductive tract receives several types of nerve fibres (Renegar and Rexroad, 1990; Melo and Machado, 1993). These include the classical autonomic nervous system (sympathetic and parasympathetic), and peptidergic nerves containing various neuropeptides. Although the relative densities of different classes of nerve fibres vary among different reproductive organs and between species, their distribution patterns tend to be similar.

These nerves are composed of long postganglionic nerve fibres whose cell bodies are in the lumbar and mesenteric ganglia and short postganglionic nerve fibres originating in the pelvic ganglia (Sjöberg, 1967). These "short" adrenergic nerves are believed to innervate predominantly the smooth muscle of the reproductive organs and they differ from the long nerve fibres anatomically. The most interesting feature of these short adrenergic nerves is the regulation of transmitter (noradrenaline) metabolism in these nerve fibres by ovarian steroid hormones (Owman and Sjöberg, 1973; Marshall, 1981). In cycling animals, the changes in uterine innervation vary between species. Zoubina *et al* (1998) reported that a reduction in the numbers of nerves occurred during oestrus with pan-neuronal marker protein gene product 9.5 and dopamine β hydroxylase, but not with CGRP and VIP in the rat, followed by structural remodeling of sympathetic nerves by way of retraction or degeneration of terminal nerve fibres. The structural loss of the terminal axon is preceded by depletion of catecholamine-synthesizing enzyme (Zoubina *et al.*, 1998). However, others reported that in the cycling rats, innervation

at uterotubal junction was nearly the same during oestrus and dioestrus regarding both the density of nerve fibres and intensity of the histochemical reactions (Melo and Machado, 1993). In this study, cycle stage appeared not to influence uterine or cervical innervation in the mare. This may result from subtle fluctuations in the uterine adrenergic and peptidergic innervation during the oestrous cycle which are undetectable by immunohistochemistry in the mare. It is also possible that although the uterine innervation remains similar throughout the oestrous cycle, the neuroeffector activity may fluctuate as a consequence of changes in the number of receptors. These receptors have been demonstrated to vary during the oestrous cycle in the uterus of rats (Krall *et al.*, 1978) and guinea pigs (Garris *et al.*, 1986) and are affected by hormones (Batra, 1994).

In the present study, TH- and DβH-IR nerves had a similar distribution and both enzymatic markers probably demonstrated the presence of noradrenaline. The enzyme tyrosine hydroxylase is rate limiting in the biosynthesis of the adrenergic transmitter noradrenaline (Levitt *et al.*, 1965). Since TH is localized intraneuronally in the adrenergic nerves (Hendry and Iversen, 1971), it has often been used as a specific marker for the demonstration of these nerves, as well as dopamine β hydroxylase. As in other species, intense adrenergic innervation was present in the myometrium, the vascular layers, and the endometrium. In the myometrium, abundant thick nerve bundles were located along with smooth muscle bundles and in the endometrium adrenergic nerve fibres were found in endometrial glands and near endometrial vessels, but nerve bundles were only rarely seen. These adrenergic nerve fibres associated with blood vessels, endometrial glands and myometrial smooth muscle probably function in the regulation of uterine blood flow, endometrial secretion and uterine contractility (Marshall, 1981; Renegar and Rexroad, 1990).

Previous studies have shown that during pregnancy, there is a reduction of uterine innervation in guinea pigs (Thorbert, 1978; Alm *et al.*, 1979), rats (Moustafa, 1988) and cats (Alm *et al.*, 1986). Marshall (1981) reported that during pregnancy additional factors including stretch-induced hypertrophy come into play and cause

degeneration of nerves in the uterine corpus. This degeneration may induce morphological changes in the muscle cell membrane. However, in the present study the history of pregnancy was not considered because samples were collected post-mortem. Therefore, in future studies the effect of previous pregnancies should be investigated.

The distribution of the peptidergic nerves in the equine uterus was similar to previous reports in guinea pigs (Alm and Lundberg, 1988; Mitchell and Ahmed, 1992), rats (Serghini *et al.*, 1997), sows (Taneike *et al.*, 1991; Majewski *et al.*, 1995), and women (Blank *et al.*, 1986; Jørgensen, 1994). Among the neuropeptides, NPY-IR nerve fibres were particularly numerous in the equine uterus. NPY-IR nerves appeared to have fewer nerve fibre bundles than the adrenergic nerves, however, their pattern of distribution was similar to that of adrenergic innervation. These findings suggest that most NPY-IR nerve fibres in the equine genital tract co-exist with TH-/DβH-IR nerves as documented in other species (Alm and Lundberg, 1988; Mitchell and Ahmed, 1992), but this needs to be confirmed by double-immunostaining. Previous quantitative studies have shown that NPY concentrations are greater in the cervix than in the uterine body of rats (Serghini *et al.*, 1998) and women (Blank *et al.*, 1986; Jørgensen, 1994), but in the guinea pig concentrations were greater in the uterine body (Huang *et al.*, 1984). By contrast, an immunohistochemical study showed that abundant NPY-IR nerves were present in all regions in the genital tract of the guinea pig (Alm and Lundberg, 1988). In the present study, I found that the distribution of NPY-IR nerve fibres were similar in the uterine horn, body and greater in the cervix in the equine uterus.

A small number of fine VIP- and CGRP-IR nerve fibres were found in the equine uterus. The density of these nerve fibres was greater in the cervical region. In the myometrium, nerve fibres containing VIP and CGRP were found in the smooth muscle layers often associated with blood vessels. In the endometrium, nerve fibres containing VIP and CGRP were found near endometrial glands. These findings are in agreement with reports on other species (Heinrich *et al.*, 1986; Ottensen and

Fahrenkrug, 1995). However, there are differences in the density of distribution of the neuropeptides reported between species. In the cats (Alm *et al.*, 1986), mice (Huang *et al.*, 1984) and women (Blank *et al.*, 1986), VIP-IR nerve fibres are known to be abundant in the uterus, while the guinea pig has only a small number of VIP-IR nerve fibres (Heinrich *et al.*, 1986; Alm and Lundberg, 1988). In contrast, the density of CGRP is similar in all species. The small number of CGRP nerve fibres found in the female reproductive tract have been reported to be slightly denser in the cervix than in the uterine horn and body in the sow (Lakomy *et al.*, 1994). In the present study the density of VIP and CGRP was similar in the endometrium and the myometrium and was less found in vascular layers.

SP is the least frequently reported neuropeptide transmitter in the female reproductive tract (Huang *et al.*, 1984; Majewski *et al.*, 1995). SP was rarely found in the uterus of women or guinea pigs (Heinrich *et al.*, 1986; Mitchell and Ahmed, 1992). In the present study, it showed that SP-immunoreactive fibres were present only in the cervix of the mare.

Many roles have been suggested for these neuropeptides in the female reproductive tract. The density of the peptidergic nerves is less than that of adrenergic nerves, but it is clear that neuropeptides have a role in controlling female reproductive function. NPY is known to coexist with noradrenaline in the majority of sympathetic vasoconstrictor nerve fibres (Lundberg *et al.*, 1990; Morris, 1990) and seems to have important biological functions in the regulation of vascular tone and myometrial contractility (Stjernquist and Owman, 1987; Atke *et al.*, 1996). The presence of VIP-containing nerves in the female reproductive tract, as well as the dose-dependent inhibitory effect of VIP on its smooth muscle activity, suggests that VIP may play a role in the local control of smooth muscle (Helm *et al.*, 1981; Ottensen *et al.*, 1983). VIP is also known to be a potent dilator of the uterine artery (Morris and Murphy, 1988). SP is known to produce an increase in myometrial blood flow and to stimulate both mechanical and myoelectrical activity (Ottensen *et al.*, 1983). CGRP and SP are present in, and coexist in some uterine afferent nerves and

influence uterine contractility; SP having a contractile effect, and CGRP having a relaxing effect (Shew *et al.*, 1991). The results showed peptidergic nerve fibres coursing beneath the luminal epithelium and endometrial glands in the endometrium in agreement with previous observations (Mitchell and Ahmed, 1992). This suggests a possible role for neurogenic mechanisms in the regulation of mucous secretion and endometrial secretion in the equine reproductive tract, as in other species (Blank *et al.*, 1986; Jørgensen, 1994; Atke *et al.*, 1996).

The presence and location of adrenergic and peptidergic innervation in the equine uterus and cervix suggest an important role for these neuronal factors in the regulation of uterine motility, endometrial secretion, and blood flow. The fact that the adrenergic and peptidergic nerves are predominantly located in the myometrium suggests that neurogenic factors have an important role in controlling uterine contractility. The high density of innervation in the cervix suggests a possible role in regulating cervical closure in dioestrus and during pregnancy, and in relaxation of the cervix at oestrus and during parturition in the mare. However, the exact functions of specific nerve fibres in the female reproductive tract are still unclear. Further studies are therefore required to clarify their precise physiological role in the regulation of the equine reproductive tract. In the next chapter, uterine innervation in mares with grass sickness was investigated compared to that in normal mares.

Chapter 4

Immunohistochemical study of neuronal changes in innervation in equine uterus of mares with grass sickness (dysautonomia)

Introduction

Equine grass sickness is a serious clinical disease in which horses suffer widespread dysautonomia, characterised by severe and extensive lesions in neurons of the autonomic and enteric nervous system and often restricted changes in sensory ganglia and specific central nervous system nuclei (Brownlee, 1959; Howell, 1974; Uzal *et al.*, 1992; Scholes *et al.*, 1993). Dysautonomia appears to be the result of initial damage to the protein synthetic pathway of a specific neuronal population, although there is no indication of the precise lesion or the nature of the causal agent. Numerous epidemiological studies have found no evidence of an infectious agent and a neurotoxin is suspected as the most probable aetiology (Gilmour, 1973). There are two probable routes by which the putative neurotoxins affect the neurons; by direct action on the perikaryon, or via the axon. An enteric toxin would have direct access to nerve terminals in the reproductive tract wall as in the intestinal wall. The toxin binds to specific receptor molecules at nerve terminals, enters the neuron and is possibly transported retrogradely up the exon (Griffiths *et al.*, 1994). Although the aetiology of this disease is still unclear, the neuropathology of grass sickness is well documented.

It is reported that grass sickness affects horses aged between 4-20 years and the peak incidence of diagnosis for grass sickness in the UK is from April to July. While 98% were on grass at the onset of clinical signs (Wood *et al.*, 1997). Equine grass sickness causes autonomic dysfunction in three clinical forms: acute (2 days), subacute (3-7 days), and chronic (weeks to months) (Milne and Mayhew, 1997). In the acute form there is massive distension of the stomach and small intestine with fluid and impaction of the large colon with hard dry material. In the chronic form the horses defecate infrequently and become emaciated. The reason for the abnormal motility in alimentary tract is still unclear. However, the importance of the enteric nervous system, that is the neurons whose cell bodies are intrinsic to the gut wall, in regulating gastrointestinal function has been recognised (Murray *et al.*, 1997).

It has been reported that there are marked reductions or disturbances in neurofilament proteins such as β-tubulin, neuron-specific enolase, and tyrosine hydroxylase in affected cells. Investigations into the cytopathology of the enteric neurons have focused on the regulatory peptide system. Using immunocytochemistry, a substantial loss of both peptide-containing ganglionic and mucosal endocrine cells and nerves was found in all affected horses, particularly in the ileum (Bishop *et al.*, 1984). Vasoactive intestinal polypeptides and substance P content, as well as bombesin and enkephalin, somatostatin and enteroglucagon-containing cells were reduced in the alimentary tract in cases of grass sickness with the most severe changes occurring in the ileum (Sabate *et al.*, 1983).

Previous research (Nikolakopoulos, unpublished data) has shown that mares with grass sickness have very low uterine motility; Grass sickness mares scored 0-2 out of 10 when motility was assessed by transrectal ultrasonography while normal mares scored 5-7. As already shown in previous chapters (chapter 2 and 3), the equine uterus also receives an extensive nerve supply, predominantly adrenergic but also peptidergic. Although there have been numerous studies on horses with grass sickness, there has been no information on neuronal damage in the reproductive tract.

We hypothesised that uterine dysfunction in mares with grass sickness is due to neuronal damage within the uterus, specifically the adrenergic uterine innervation is affected. Therefore, in the present study we checked uterine innervation in mares with grass sickness compared to that in mares with no history of reproductive failure.

Materials and Methods

Animals:

The tissue samples were obtained from mares (n=13) with no history of reproductive failure, aged between 3 and 18 years and weighed 390 to 520 kg, and mares with grass sickness (n=5) at post-mortem. The severity of illness was judged according to the clinical signs described by Doxey *et al* (1991) and diagnosis was confirmed at post-mortem (Table 4.1). The reproductive tract was removed within 20 min of euthanasia, gently washed and dissected in 0.1% PBS. Four segments, size approximately 1.5 cm x 1.5 cm, were collected from the uterine horn, body and the cervix from each mare.These samples were then fixed immediately in cold 4% paraformaldehyde (pH 7.2) for 14-16 hours at 4°C, transferred into 0.1% PBS, pH 7.2, containing 30% sucrose and stored for at least 24 hours at 4°C. The tissue samples were then removed from the sucrose and snap frozen with OCT in an isopentane/dry-ice slurry, and stored at -70°C until cryostat sectioning.

Horse (Case number)	Age (Year)	Clinical severity
99-336	1	Acute
98-263	3	Acute
99-469	4	Subacute
98-563	8	Subacute
98-244	7	Chronic

Table 4.1. Details of horses with grass sickness used in this study

Immunohistochemical procedure:

Cryostat sections (12-15μm) were cut and then processed for immunohistochemistry using the avidin-biotin complex method (Vector Laboratories, Peterborough, Cambridge, UK). Sections were air-dried for 60-90 min at room temperature, rinsed in 0.01M PBS (pH 7.2) and blocked with 3% H_2O_2 in methanol for 30 min to reduce non-specific binding. Slides were then preincubated with 1% normal goat serum in 0.01M PBS for 1 h. Primary antibodies raised in rabbits were used for general innervation with PAN-N (1:8000, Affinity Research Products, Exeter, UK) and presence of TH (1:6000, Affiniti Research Products, Exeter, UK), and DβH (1:4000, Affiniti Research Products, Exeter, UK) were used for adrenergic innervation. The sections were incubated with primary antibodies overnight at 4°C. Sections were then washed three times in PBS. The sections were incubated with the second antibody (goat anti- rabbit; Vector Laboratories, Peterborough, Cambridge, UK) diluted in PBS containing 1% normal goat serum for 2 h at room temperature. The sections were thoroughly washed in PBS, then incubated with the avidin-biotin complex for a further 30 min, and rinsed three further times with PBS. Chromagen (3-amino-9-ethylcarbazole: AEC; Vector Laboratories, Peterborough, Cambridge, UK) was added as the final substrate (red reaction product) and was incubated at room temperature in the dark for 10-15 min. Each slide was then rinsed carefully with distilled water. The sections were normally counterstained with Meyer's haematoxylin, however, sometimes sections with fine nerve fibres were excluded from this step. As negative controls, sections were included in which the first antibody was replaced by normal rabbit serum. Sections of equine jejunum and feline uterus were included on each slide to act as positive controls.

Subjective evaluation of uterine innervation

Relative frequency of uterine nerve fibres and bundles was assessed using a subjective scoring system (Table 2.1) in immunostained sections obtained from uterine horn, uterine body, and the cervix. For each section, nerves were evaluated under magnification (x10 to x20) with respect to their frequency within three separate structures: myometrium, endometrium and vascular layer (between muscular layers). For each structure, numbers of nerves were assigned a score from 0 to 5 (See Appendix) (Alm *et al.*, 1988; Mitchell and Ahmed, 1992). Four sections from each of the three regions from each animal were included for analysis. Each section contained all three structures.

Statistical analysis

Score for four sections from three regions and each structure from each animal were included for analysis. Scores for every section were pooled. Score values are presented as the mean ± SEM (as a general error term). Data were analysed by a general linear model analysis of variance (ANOVA) (SPSS 9.0) to determine the effect of 1) regions, 2) structures and 3) regions*structures (factor). Scores for every section were pooled. To compare mean value of each region and each structure, paired-samples t-test was used. A probability of <0.05 was taken to indicate a statistically significant difference.

Results

General uterine innervation

In genitally normal mares, general uterine innervation using PAN-N showed a great number of PAN-N-immunoreactive (IR) nerve bundles and nerve fibres in all regions of the uterus (Table 4.2). PAN-N-IR nerve bundles were filled with compactly arranged neurofilament proteins (Fig 4-1-a, c) (See chapter 2). In contrast to normal mares, degeneration and loss of nerve fibres was observed in the uterus of all five mares with equine grass sickness (Figure 4-1-b, d, Table 4.2). Although nerve bundles showed a mixture of morphologically normal and abnormal features, the number of nerve bundles and fibres were significantly reduced ($P<0.05$) in the myometrium and the vascular layers of mares with grass sickness (Table 4.3). Affected nerve bundles showed marked disturbances in neurofilament protein and vacuolation.

	PAN-N		DβH		TH	
Regions	**Normal**	**GS**	**Normal**	**GS**	**Normal**	**GS**
Uterine horn	3.6 ± 0.0^{a}	$2.6 \pm 0.1^{*a}$	3.0 ± 0.0^{a}	$1.8 \pm 0.1^{*}$	3.0 ± 0.1^{a}	$1.8 \pm 0.1^{*}$
Uterine body	3.6 ± 0.0^{a}	$2.7 \pm 0.1^{*a}$	3.0 ± 0.0^{a}	$1.8 \pm 0.1^{*}$	3.0 ± 0.1^{a}	$1.9 \pm 0.1^{*}$
Cervix	3.8 ± 0.0^{b}	$3.0 \pm 0.1^{*b}$	3.3 ± 0.0^{b}	$2.2 \pm 0.1^{*}$	3.5 ± 0.1^{b}	$2.2 \pm 0.1^{*}$
Structures	**Normal**	**GS**	**Normal**	**GS**	**Normal**	**GS**
Myometrium	4.2 ± 0.0^{a}	$3.0 \pm 0.1^{*a}$	3.0 ± 0.0^{a}	$2.0 \pm 0.7^{*}$	3.5 ± 0.1^{a}	$2.1 \pm 0.7^{*}$
Endometrium	3.1 ± 0.0^{b}	$2.5 \pm 0.1^{*b}$	3.0 ± 0.0^{b}	$1.9 \pm 0.7^{*}$	2.8 ± 0.1^{b}	$1.9 \pm 0.7^{*}$
Vascular layer	3.7 ± 0.0^{c}	$2.8 \pm 0.1^{*a}$	3.3 ± 0.0^{c}	$1.9 \pm 0.7^{*}$	3.2 ± 0.1^{a}	$1.9 \pm 0.7^{*}$

Table 4.2. Data show Mean ± SEM of subjectively graded distribution of general (PAN-N-IR) and adrenergic (DβH-IR and TH-IR) nerves in different regions. Nerve density was scored from 0 to 5. ^{a,b,c} Groups with different superscripts within column are different ($P<0.05$). Asterisks indicate significant differences (*: $P<0.05$) between normal mares and mares with grass sickness.

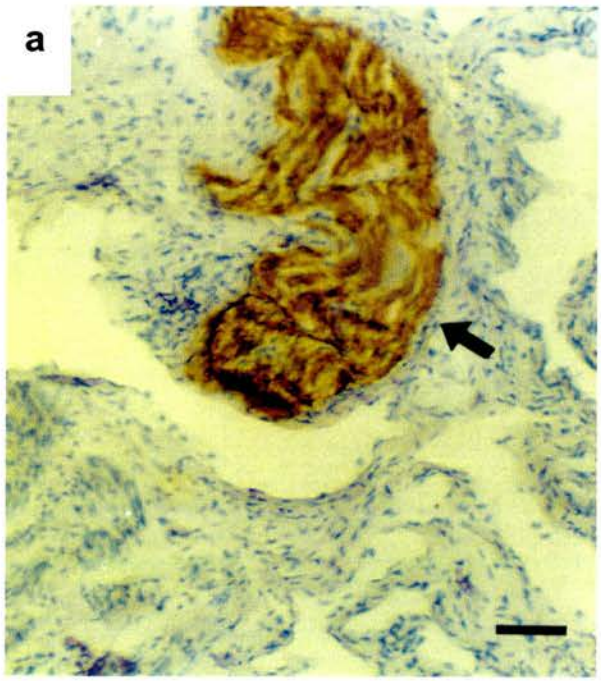
Regions Structures	Normal mares	Mares with grass sickness
Uterine horn		
Myometrium	4.04 ± 0.06	2.75 ± 0.10*
Endometrium	3.06 ± 0.06	2.35 ± 0.10*
Vascular layer	3.67 ± 0.06	2.65 ± 0.10*
Uterine body		
Myometrium	4.02 ± 0.06	2.80 ± 0.10*
Endometrium	3.10 ± 0.06	2.45 ± 0.10*
Vascular layer	3.65 ± 0.06	2.75 ± 0.10*
Cervix		
Myometrium	4.62 ± 0.06	3.35 ± 0.10*
Endometrium	3.10 ± 0.06	2.65 ± 0.10*
Vascular layer	3.70 ± 0.06	3.00± 0.10*

Table 4.3. Data show Mean ± SEM of arbitrarily graded distribution of general uterine innervation, PAN-N-immunoreactive nerves, within structures in normal mares (n=13) and in mares with equine grass sickness (n=5). Nerve density was scored from 0 to 5. Asterisks indicate significant differences (*: $P<0.05$) between normal mares and mares with grass sickness.

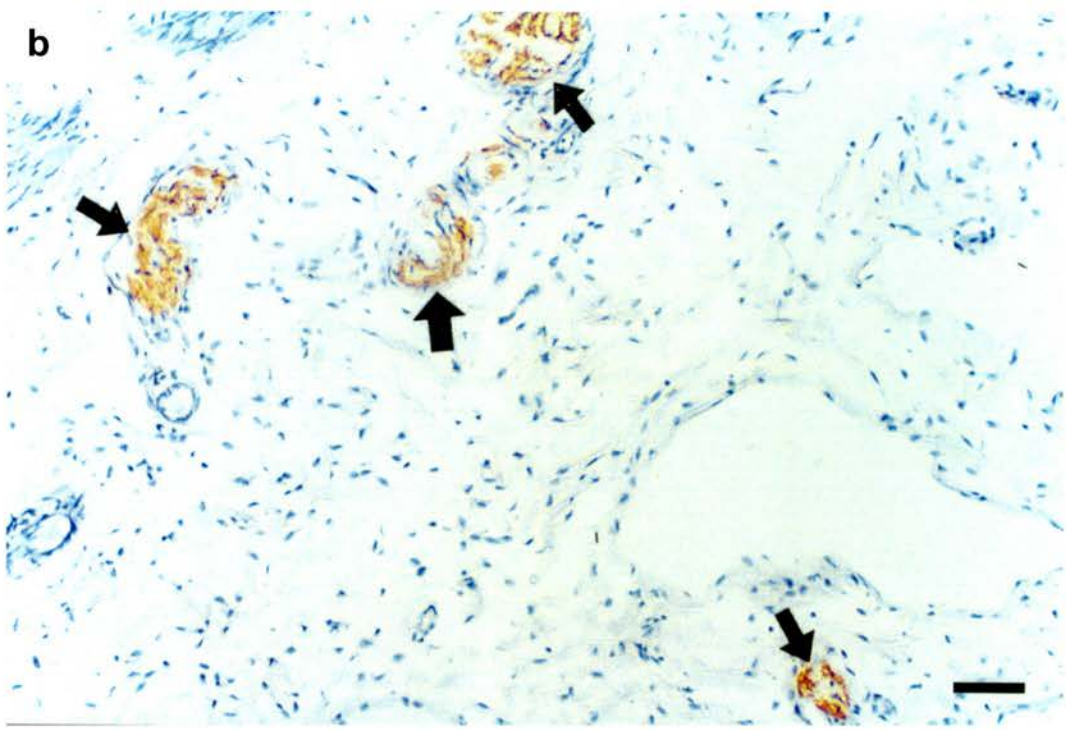
Adrenergic uterine innervation

With adrenergic nerves, normal mares showed large numbers of nerve bundles and fibres immunoreactive with both DβH (Figure 4-2-a) and TH (Figure 4-2-e, g) observed in all regions of the uterus (Table 4.2) (See chapter 3). Mares with grass sickness showed a significant reduction of adrenergic nerves in all structure in all regions (Table 4.4). With adrenergic nerves, there was no significance difference ($P>0.05$) within regions or within structures (Table 4.2). Changes varied between mares from occasional loss of nerve

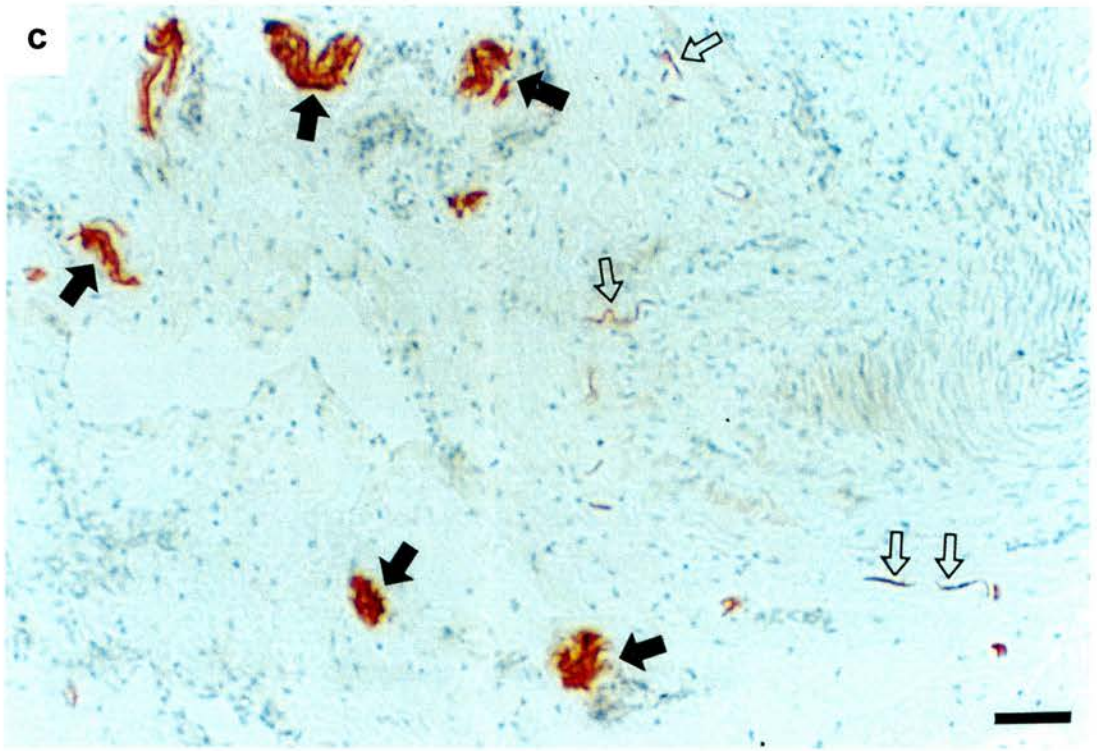
Figure 4-1. Photomicrographs illustrating general innervation immunoreactive to PAN- N in the uterus of genitally-normal mares and mares with equine grass sickness (dysautonomia).



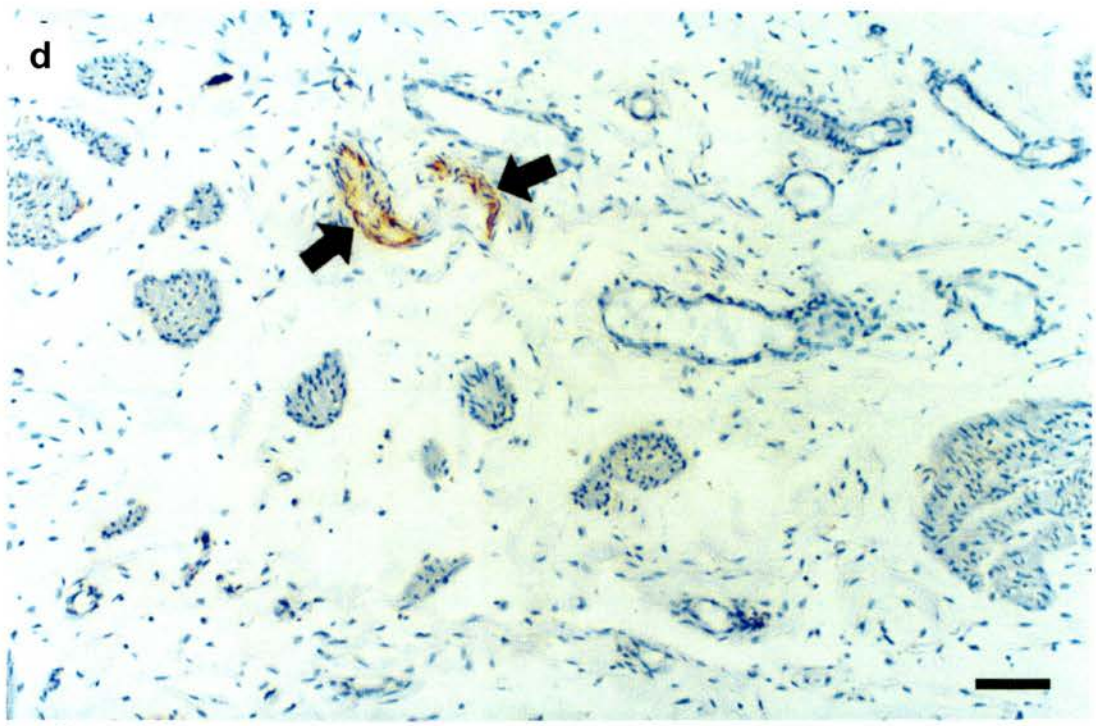
a. Nerve bundle filled with firmly dense PAN-N-immunoreactive (IR) neurofilaments protein (➡) in the uterine horn of a genitally-normal mare. Scale bar = 100 μm.



b. PAN-N-IR nerve bundles in the uterine horn of a mare with subacute grass sickness (99-469). Affected nerve bundles showed depleted nerve fibres (➡). Scale bar = 100 μm.



c. PAN-N-immunoreactive (IR) nerve bundle (➡) and fibres (⇨) in the uterine body of a genitally-normal mare. Scale bar = 100 μm.



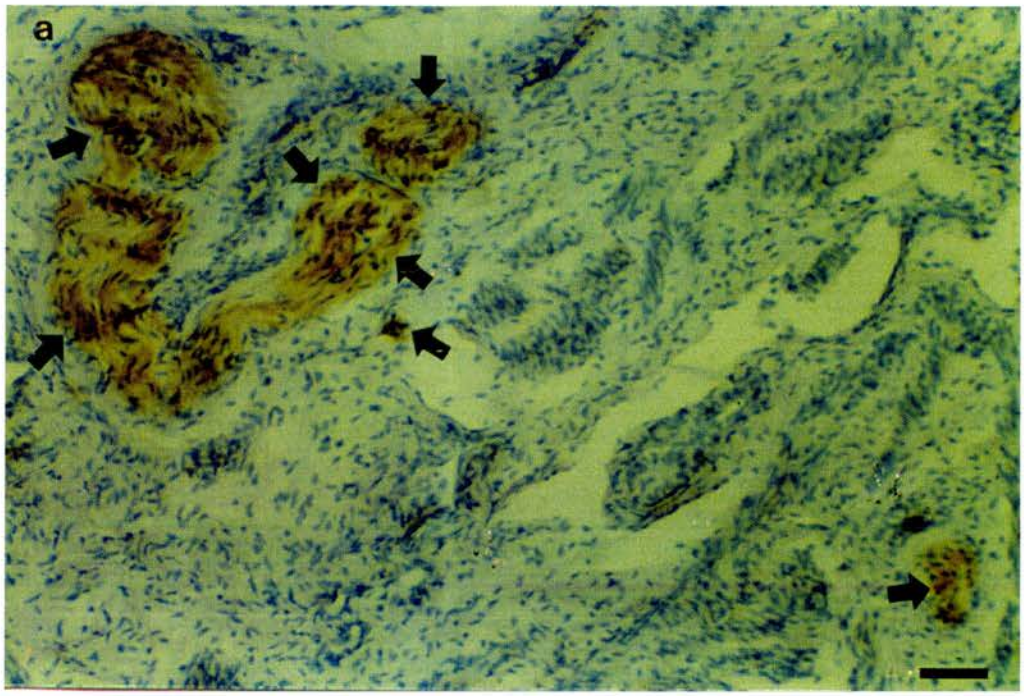
d. Affected nerve bundles in the uterine body of a mare with chronic grass sickness (98-244). There is marked depletion of PAN-N-IR neurofilaments (➡) within the nerve bundles. Scale bar = 100 μm

fibres to conspicuous degenerated nerve bundles. Degenerated nerve bundles immunoreactive with DβH, which contained reduced number of nerves and depletion within nerve bundles, were observed (Figure 4-2-b, c, d). Nerve fibres immunoreactive with TH appeared short and disrupted (Figure 4-2-f, h) compared to the longer and well-arranged nerve fibres in normal healthy mares (Figure 4-2-g). Negative control sections failed to demonstrate any positive staining (Figure 4-2-i).

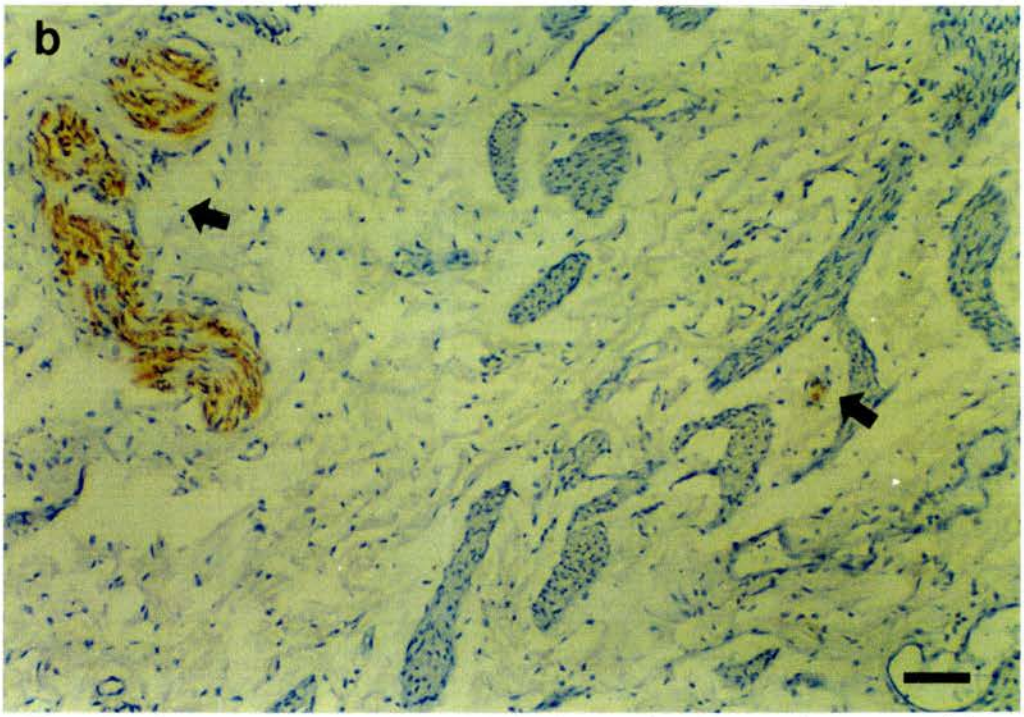
Region / Structure	DβH		TH	
	Normal	GS	Normal	GS
Uterine horn				
Myometrium	3.25 ± 0.06	1.95 ± 0.10*	3.35 ± 0.17	1.90 ± 0.27*
Endometrium	2.77 ± 0.06	1.75 ± 0.10*	2.77 ± 0.17	1.70 ± 0.27*
Vascular layer	2.87 ± 0.06	1.80 ± 0.10*	2.90 ± 0.17	1.75 ± 0.27*
Uterine body				
Myometrium	3.29 ± 0.06	1.95 ± 0.10*	3.25 ± 0.17	2.00 ± 0.27*
Endometrium	2.77 ± 0.06	1.75± 0.10*	2.83 ± 0.17	1.75 ± 0.27*
Vascular layer	2.65± 0.06	1.75 ± 0.10*	2.90 ± 0.17	1.80 ± 0.27*
Cervix				
Myometrium	3.81 ± 0.06	2.20 ± 0.10*	3.85 ± 0.17	2.3 ± 0.27*
Endometrium	2.87 ± 0.06	2.15± 0.10*	2.87 ± 0.17	2.1 ± 0.27*
Vascular layer	3.19 ± 0.06	2.15 ± 0.10*	3.73 ± 0.17	2.2 ± 0.27*

Table 4.4. Data show Mean ± SEM of subjectively graded distribution of adrenergic innervation, DβH and TH- immunoreactive nerves in normal mares (n=13) and in mares with equine grass sickness (n=5). Nerve density was scored from 0 to 5. Asterisk indicates significant differences (*: $P < 0.05$) between two groups, normal mares (Normal) and mares with grass sickness (GS).

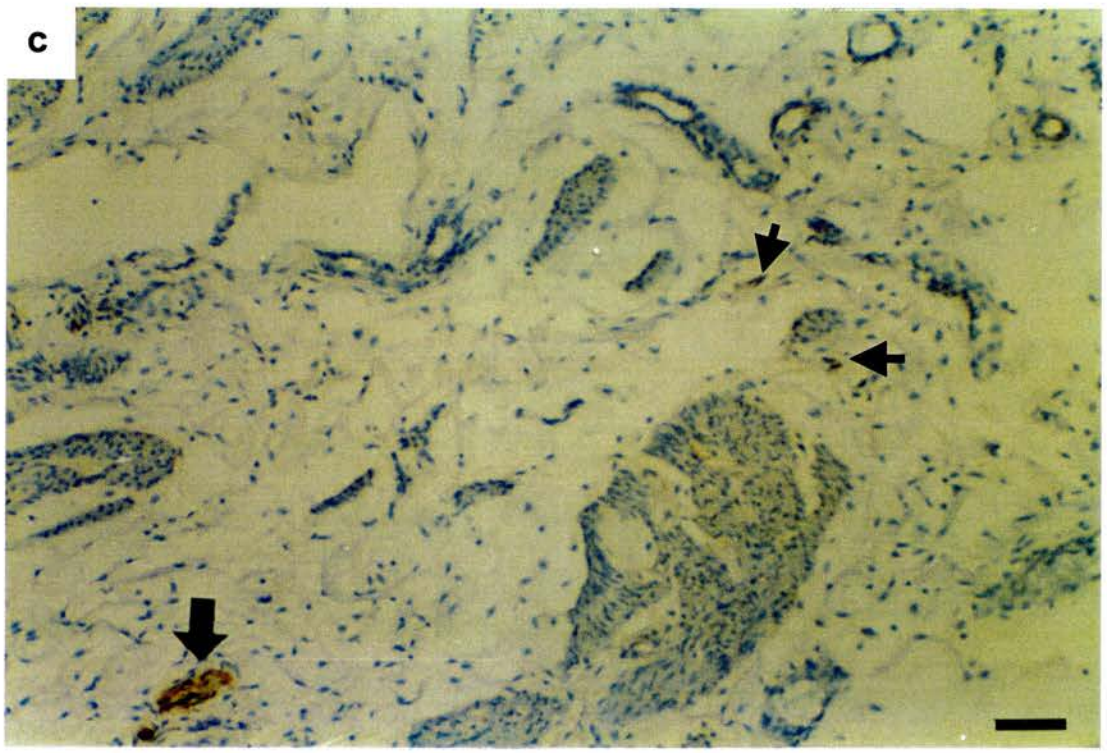
Figure 4-2. Photomicrographs illustrating adrenergic innervation immunoreactive to DβH and TH in the uterus of genitally-normal mares and mares with equine grass sickness (dysautonomia).



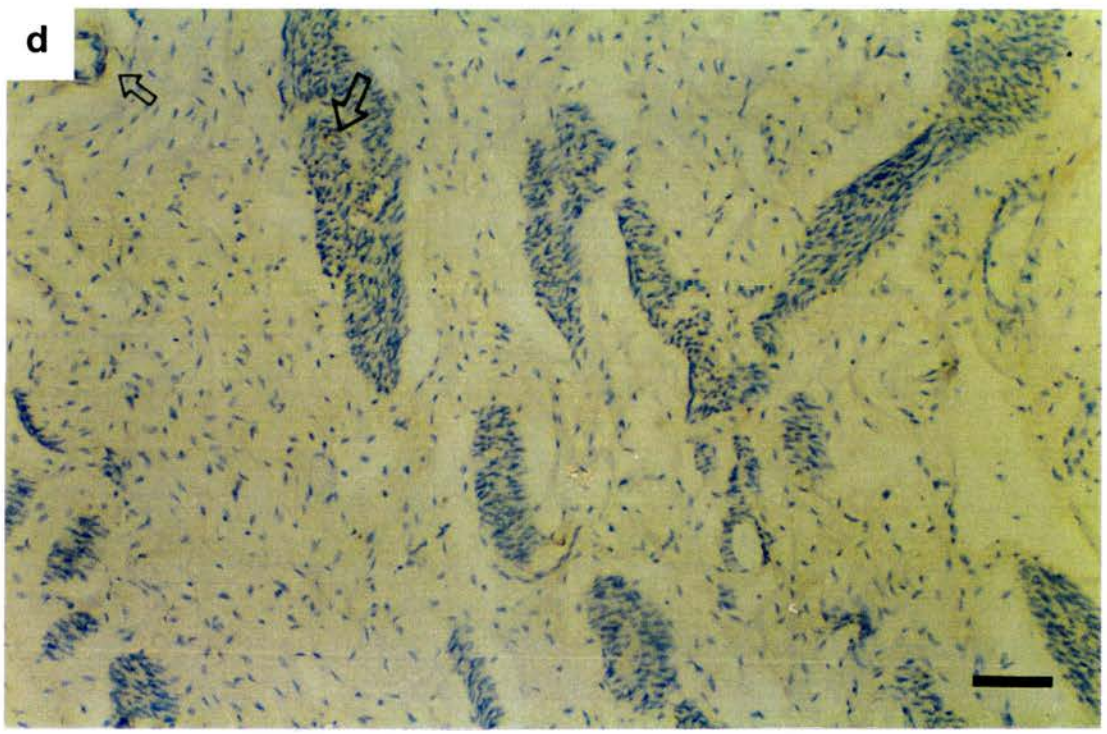
a. DβH-IR nerve bundles (➡) in the uterine horn of a normal mare. Nerve bundles show strong immunoreactivity with compactly arranged DβH-IR nerve fibres. Scale bar = 100μm.



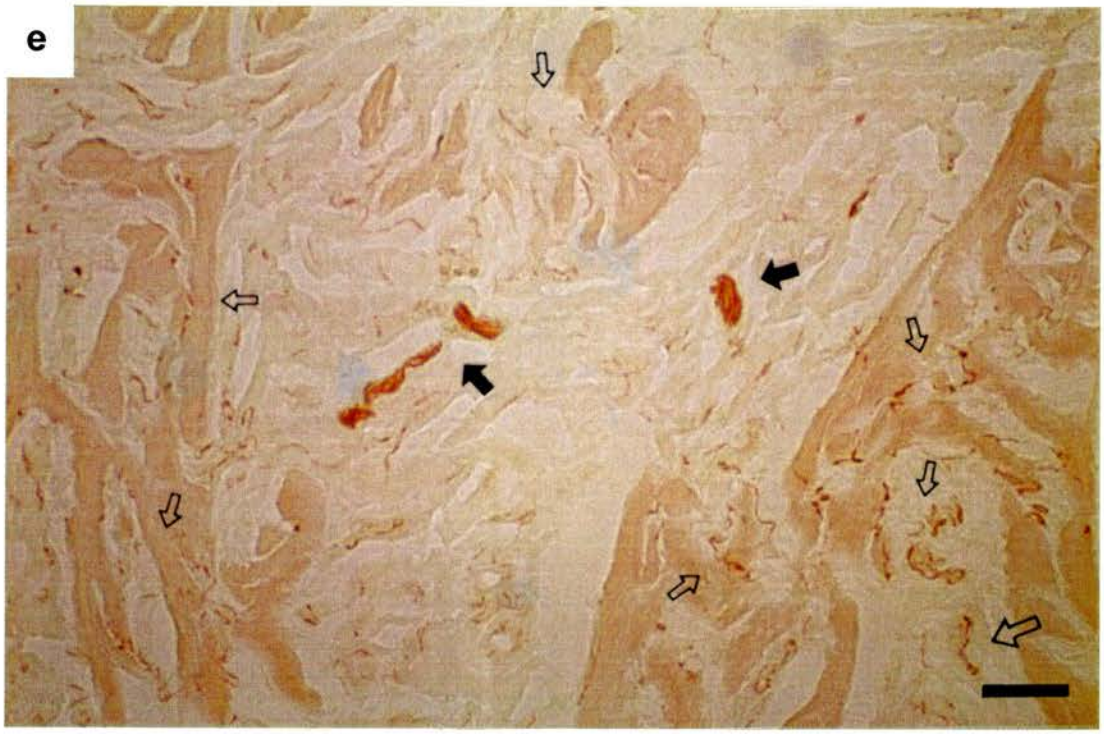
b. Affected DβH-IR nerve bundles (➡) in the uterine horn of a mare with acute grass sickness (99-336). Scale bar = 100 μm.



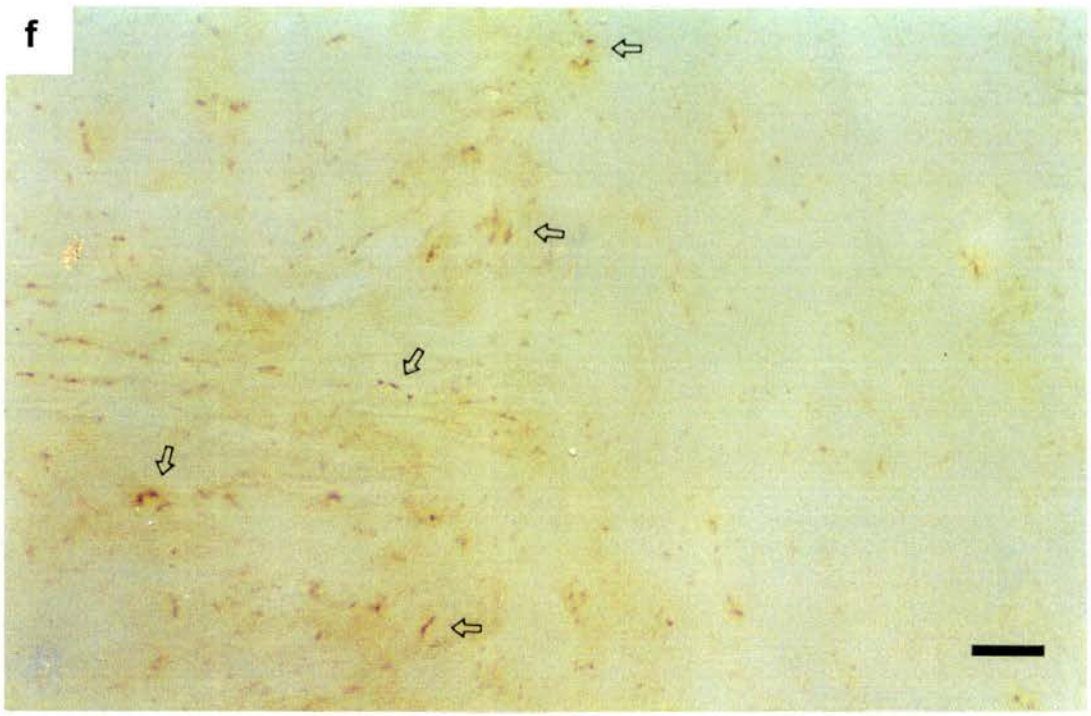
c. DβH-IR nerve bundle (➡) in the cervix of a mare with subacute grass sickness (98-469). There is a marked reduction in number of nerve bundles. Scale bar = 100 μm



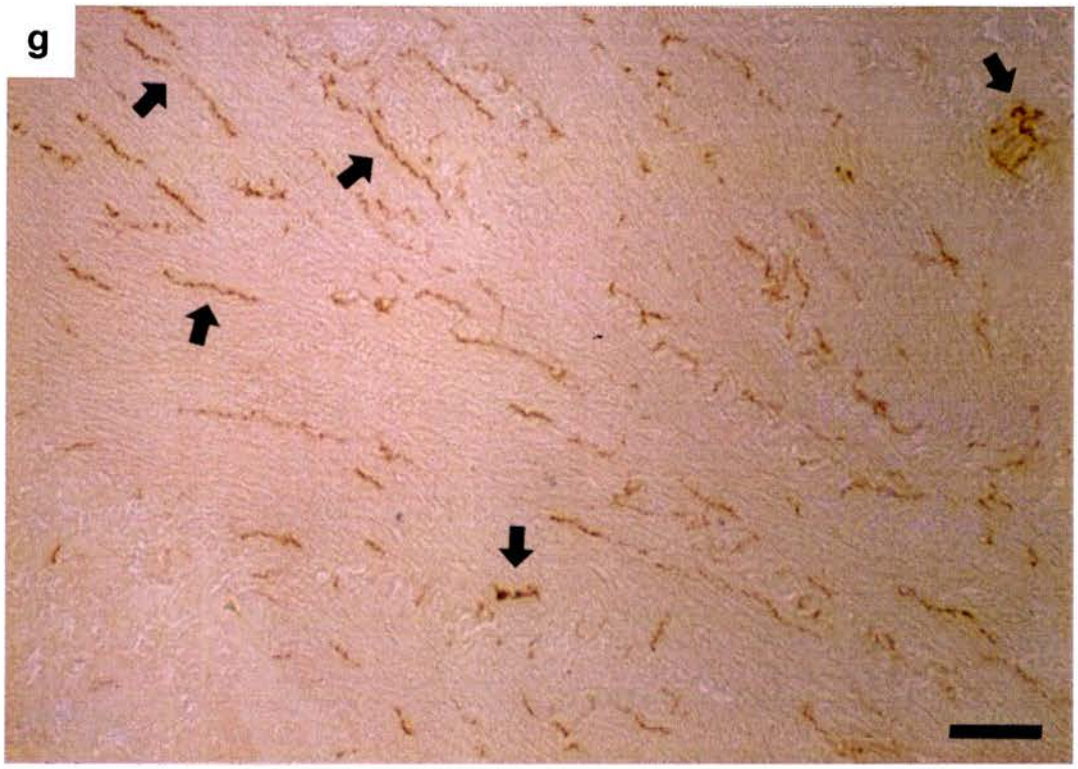
d. There is a marked reduction of DβH-IR (⇨) nerve fibres in the uterine horn of a mare with chronic grass sickness (98-244). Scale bar = 100 μm.



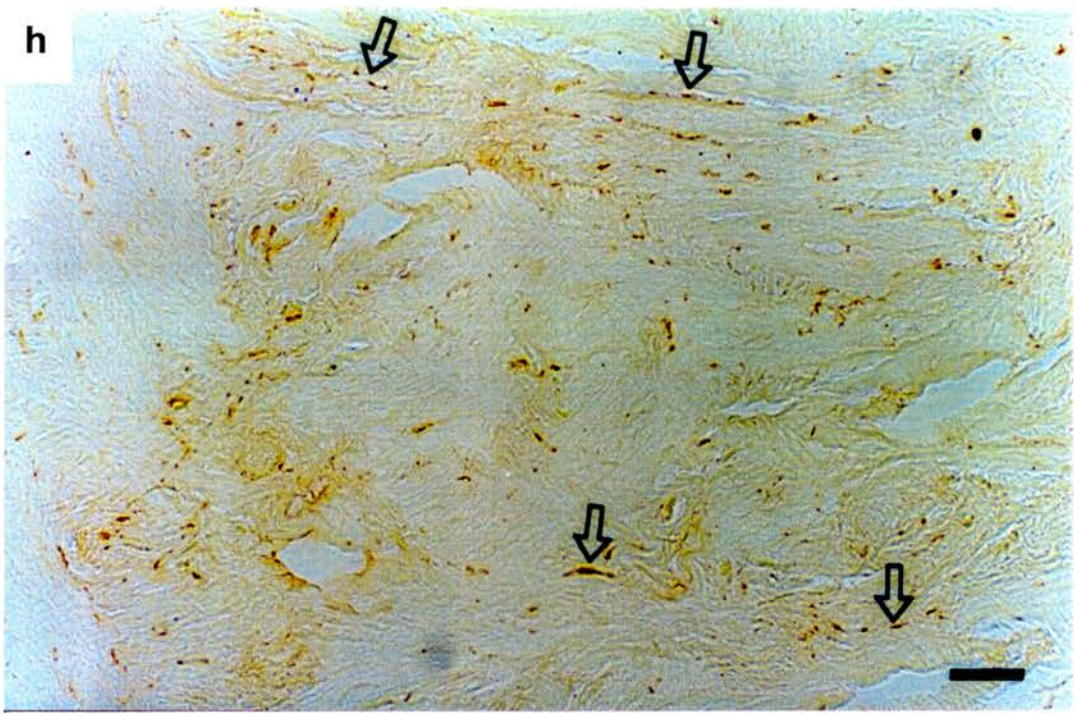
e. TH-IR nerve bundles (➡) and nerve fibres (⇨) in the uterine horn of a normal mare. Scale bar = 250 μm



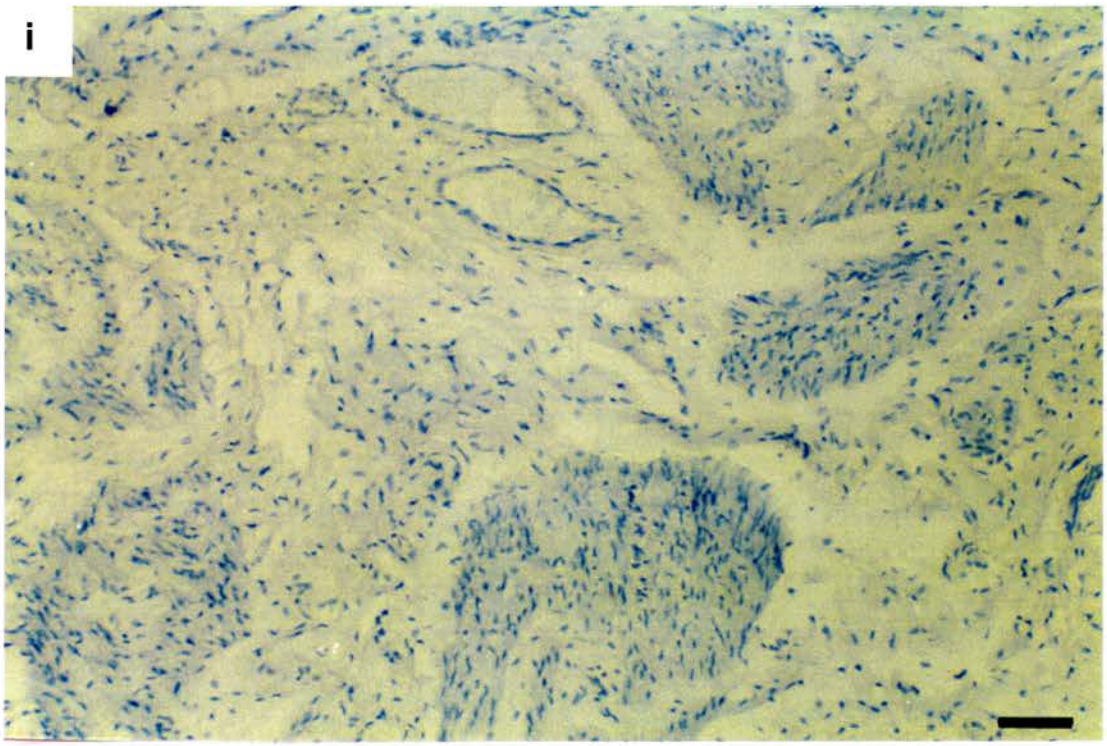
f. TH-IR-nerve fibres (⇨) in the uterine horn of a mare with subacute grass sickness (98-563). There is marked reduction of nerve fibres and these nerve fibres appeared short and disrupted. Scale bar = 100 μm.



g. TH-IR-nerve fibres (➡) in the uterine body of a normal mare. Scale bar = 100 μm.



h. TH-IR-nerve fibres (⇨) in the uterine body of a mare with acute grass sickness (99-336). There is marked reduction of nerve fibres and these nerve fibres appeared short and disrupted. Scale bar = 100 μm.



i. Negative control. Uterine horn. Scale bar = 100 μm.

Discussion

In this chapter, it is shown that mares with grass sickness appear to have neuronal damage in the uterus compared with the observations of uterine innervation in normal mares described in previous chapters (Chapter 2&3). This study demonstrated that mares with grass sickness appeared to have shortened nerve fibres and depleted nerve bundles in all regions of the uterus. This study also provides the evidence of widespread damage of autonomic nervous system including the female reproductive tract in equine grass sickness.

It is known that uterine innervation arises from the pelvic plexus that receives from caudal mesenteric, hypogastric and paracervical ganglion. The adrenergic and cholinergic as well as peptidergic nerves supplying the female reproductive tract converge at uterovaginal plexus which lie close to the uterine cervix. The present results are in agreement with other studies that report neuronal damage in the

autonomic ganglia (cervical, stellate and coeliaco-mesenteric) in horses with grass sickness (Gilmour, 1973; Doxey *et al.*, 1991; Doxey *et al.*, 1992; Pogson *et al.*, 1992; Scholes *et al.*, 1993). A previous study also found that there is a failure in transport of adrenergic neurotransmitters transportation, DβH and TH, down the axon (Gilmour, 1973).

Although equine grass sickness is a neuropathy of unknown aetiology, the overall pathology is felt to be compatible with neurotoxicosis. It is proposed that the ileum could be the main site of entry of a putative ingested neurotoxin (Gilmour, 1973). The circulating toxin present in the serum probably results from gastrointestinal absorption and it would have direct access to virtually all tissues except the central nervous system, which is protected by a blood-tissue barrier (Griffiths *et al.*, 1994a). The results showed that there appeared to be a marked reduction and degeneration of nerves in all regions of the uterus, and within layers in the myometrium and the vascular layers were more affected than that in the endometrium. As already discussed in previous chapters, neuronal mechanisms are important in regulating reproductive functions including ovarian function (Schimidt *et al.*, 1990), uterine contractility (Stejernquist *et al.*, 1983; Reinecke *et al.*, 1989; Shew *et al.*, 1993), uterine blood flow (Ford *et al.*, 1984; Morris, 1990; Majewski *et al.*, 1995), and endometrial secretory function (Hammarström, 1980; Massmann *et al.*, 1992). Therefore, degeneration of uterine innervation can cause many reproductive problems. It could cause delayed uterine clearance due to impaired uterine contractility as already shown for the small intestine (Murray *et al.*, 1994). Murray *et al* (1994) reported that *in vitro* preparation of equine small intestine demonstrated the presence of impaired neuronal mechanisms. These impaired neuronal mechanisms result from the loss of enteric neurons, which leads to a reduction in the release of acetycholine and reduces intestinal motility, as well as changes in the sensitivity of muscarinic receptors. These damaged neuronal factors could affect uterine contractility directly or indirectly by interfering with hormonal release or action, or formation of cAMP. Previous research (E. Nikolakopoulos, unpublished data) demonstrating low uterine motility in mares with grass sickness could be explained by damaged neuronal mechanisms. Neuronal loss in the endometrium

could cause dysfunction in regulating other aspects of reproductive physiology, not only the secretion of mucus or immunoglobulins, but also production of uterine oxytocin and prostaglandin $F_{2\alpha}$.

In conclusion, mares with grass sickness appear to have degeneration of uterine innervation, as already shown in neuronal damage of gastrointestinal tract, which may cause decreased uterine contractility as seen in previous study (Nikolakopoulos, unpublished data). This study did not show severity of disease linked with severity of neuronal changes. Neurogenic mechanisms are of primary importance in control of smooth muscles in the uterus in combination with hormonal and myogenic systems. Further studies are needed to determine 1) whether there is any damage to neurogenic control in other mares with delayed uterine clearance and 2) whether depletion lead to atrophy of myometrium. In the next chapter, the presence of oxytocin and neurophysin in the equine endometrium will be investigated at the ultrastructural level.

Chapter 5

An Ultrastructural Study on the Presence and Location of Oxytocin and Neurophysin in the Equine Endometrium

Introduction

In oestrus, the glandular and surface epithelium in the equine endometrium were characterized by hypertrophy and differentiation of apparent secreting cells as shown by intense cellular vacuolation, an increased number of polyribosomes and predominant granular endoplasmic reticulum (Keenan *et al.*, 1991).

Oxytocin is a neuropeptide hormone produced mainly by magnocellular neurons of nuclei in the hypothalamus, which are located in the supraoptic paraventricular nuclei and the arcuate nuclei of the horse (Melrose and Knigge, 1989). It is synthesized and stored in secretory vesicles of the posterior pituitary along with its "carrier protein" neurophysin (Brownstein *et al.*, 1980). It is secreted into the blood stream in a pulsatile manner and acts on the myometrium to cause uterine contractions (Gilbert *et al.*, 1992; Dickson, 1993). Uterine contractile activity is responsible for mechanical drainage of cellular debris and uterine fluid after mating, and failure to clear this intrauterine fluid is a major cause of subfertility in the mare (Pycock and Newcombe, 1996a; Newcombe, 1997). Oxytocin also controls uterine contractility at parturition in the mare (Vivrette *et al.*, 2000). Because of the important role of oxytocin in control of uterine contractility, both directly and indirectly, it is important to clarify sources and control of secretion of this hormone in the mare.

Several workers have reported the presence of immunoreactive oxytocin outside the central nervous system including the thymus (Geenen *et al.*, 1986; Argiolas *et al.*, 1990), adrenal medulla, male reproductive tract (Nicholson *et al.*, 1984; Einspanier and Ivell, 1997; Watson *et al.*, 1999b) and placenta (Lefebvre *et al.*, 1992b; Chibbar *et al.*, 1993). There has been much research on the local synthesis of oxytocin within the female reproductive tract, particularly the ovary. Oxytocin has been identified in the corpus luteum of cattle (Fields *et al.*, 1983; Wathes *et al.*, 1983; Guldenaar *et al.*, 1984; Kruip *et al.*, 1985), goats (Cooke and Knifton, 1981), sheep (Watkins, 1983), pigs (Pitzel *et al.*, 1984; Jarry *et al.*, 1990), rabbits (Miller *et al.*, 1983), and women (Takemura *et al.*, 1993) and is thought to have a role in regulating luteolysis (Flint

and Sheldrick, 1983), ovulation (Viggiano *et al.*, 1989) and possibly luteinization (Einspanier *et al.*, 1997). By contrast, the ovary of the mare does not appear to be a source of oxytocin (Murray *et al.*, 1991; Gilbert *et al.*, 1992; Stock *et al.*, 1995; Watson *et al.*, 1999a).

Previous studies have shown that oxytocin-mRNA and/or protein is present in the uterus of rats (Lefebvre *et al.*, 1992a) and pigs (Trout *et al.*, 1995; Boulton *et al.*, 1996). *In situ* hybridization has shown that luminal epithelium and myometrium of the porcine uterus and luminal epithelium of the rat uterus contain mRNA for oxytocin. The amount of mRNA encoding oxytocin in the luminal epithelium of the porcine uterus was higher at oestrus than any other stage of the cycle or during pregnancy (Boulton *et al.*, 1996) whereas levels were highest in pregnancy in the rat (Lefebvre *et al.*, 1992a). Oxytocin-mRNA has also been identified in the equine endometrium and levels tend to be higher during oestrus and late dioestrus than at other stages of the cycle or early pregnancy (Behrendt-Adam *et al.*, 1999). Our previous studies have shown that positive immunostaining for oxytocin was present in the luminal epithelium and the epithelium of the superficial glands of the endometrium of the mare (Watson *et al.*, 2000). Immunostaining of oxytocin was stronger in oestrus than in dioestrus, but was more intense on day 14 of pregnancy than at any other stage examined, in contrast to the relatively low abundance of oxytocin-mRNA (Behrendt-Adam *et al.*, 1999).

There is very limited information on localisation of oxytocin and neurophysin to specific cell types within the uterus or their location within cells. The aim of this study was to investigate the presence and location of oxytocin and neurophysin in the equine endometrium at the ultrastructural level.

Materials and Methods

Tissue preparation for TEM

Samples of equine endometrium were obtained from five mares by transcervical biopsy. These mares were aged 3 to 19 years and weighed 380 to 520 kg. Samples were collected during oestrus when uterine oxytocin immunostaining is intense (Watson *et al.*, 2000). The tissues were rinsed in 0.1M sodium cacodylate buffer solution (pH 7.2) to remove mucus and blood from the surface. The tissues were cut into pieces of approximately $1mm^3$. The tissues were then fixed in a mixture of 4% paraformaldehyde /0.1% glutaraldehyde in 0.1M sodium cacodylate buffer and were embedded in acrylic resin (Unicril; British Biocell International, Cardiff, UK). To select areas of tissue for study, semi-thin sections (1 μm) were stained with 1% methylene blue for light microscopy (Figure 5-3-a). After selecting sections, ultrathin, 60nm light gold-coloured sections were cut and mounted on coated gold single-slot grids.

Tissue preparation for light microscope

To confirm the presence of oxytocin and neurophysin at light microscope level, tissues were snap frozen in O.C.T. compound (Miles Inc, Elkhart, IN, USA) in an isopentane/dry ice slurry and then stored at –70 °C before sectioning.

Antibodies

The anti-oxytocin (UCB A481/R4V; Accurate Chemical and Scientific Co. Westbury, New York, USA) and the anti-neurophysin (NCL-NP_P, Vector Laboratories, Peterborough, UK) were used. These antibodies were specific for either oxytocin or neurophysin and did not cross-react with other pituitary hormones or related peptides.

The second antibody used for immunogold labelling was EM goat anti-rabbit gold IgG (particle size: 10nm) (British BioCell International, Cardiff, UK).

Immunohistochemistry

To confirm the presence of oxytocin and neurophysin at light microscope level, immunohistochemistry was performed using the avidin-biotin complex method (Vector Laboratories, Peterborough, UK) with 3-amino-9-ethylcarbazole (AEC). Cryostat sections, 7μm thick, were cut and mounted on Bio-Bond (British BioCell International, Cardiff, UK) coated slides. Sections were air-dried for 60-90 min at room temperature, rinsed in 0.01M PBS (pH 7.2) for 10 min and blocked with glucose oxidase for 60 min at 37°C to block any endogenous peroxidase staining. Slides were then preincubated with 1% normal goat serum in 0.01M PBS for 1 h. The excess serum was blotted and the sections were then incubated with primary antibody at the appropriate dilution overnight at 4°C before washing three times in 0.01M PBS. The sections were incubated with the second antibody (goat anti-rabbit) in PBS containing 1% normal goat serum, for 2 h at room temperature. The sections were thoroughly washed three times in PBS, then incubated with the avidin-biotin complex (Vector Laboratories, Cambridges, UK) for a further 30 min, and rinsed in PBS. Chromagens (3-amino-9-ethylcarbazole: AEC; Vector Laboratories, Cambridges, UK) were added as the final substrate, and were incubated at room temperature in the dark for 10-15 min. Each slide was then rinsed carefully with distilled water. The sections were counterstained with Meyer's haematoxylin. Slides were mounted and examined by light microscopy.

Immunostaining at ultrastructure level

Immunohistochemistry was performed using the avidin-biotin complex method with DAB (3'-3'diaminobenzidine dihydrochloride) as the substrate. For all incubations, gold single-slot grids were floated on droplets (40μl) of the solutions on Parafilm in a humidified Petri dish. Sections were incubated with glucose oxidase for 60 min at 37°C to block any endogenous peroxidase staining. Sections were then rinsed four times in phosphate buffered saline (PBS; pH 7.2) and were incubated for 30 min with 1% normal goat serum in PBS to block nonspecific staining before incubating with the primary antibodies. Sections were then transferred to the primary antiserum, both oxytocin and neurophysin, (diluted 1:1000 in PBS), and were floated overnight at 4°C. Sections were then washed in PBS. The sections were then incubated with the second antibody (goat anti- rabbit; 1:100) in PBS containing 1% normal goat serum, for 1 h at room temperature Avidin-biotin complex was added after washing and the sections were incubated for a further 30 min. Chromagen, DAB was added for 10 min. Sections were rinsed with distilled water followed by complete drying. Counterstaining was performed with 2% osmium tetroxide for 10 min.

Immunogold labelling

Immunogold labelling was performed following a protocol described previously by Rahmanian *et al* (1997). For all incubations gold single-slot grids were floated on the 40 μl droplets of the solutions on Parafilm in a humidified Petri dish. Sections were incubated for 10 min in Tris buffered saline (TBS; pH 8.2) containing 0.02M glycine to quench any aldehyde left in the tissue. Sections were then incubated for 30 min in blocking solution (TBS containing 1% BSA plus 10% normal goat serum, pH 8.2) to reduce non-specific binding. Sections were then transferred to the primary antiserum diluted in blocking solution (anti-oxytocin 1:200 and anti-neurophysin 1:100) and floated overnight at room temperature. Sections were rinsed four times in TBS (containing 1% normal goat serum, 0.1% Tween 20, 1% bovine serum albumin and

0.1% sodium azide, pH 8.2) and transferred to a drop of the blocking solution for 10 min. They were then incubated in the secondary antiserum diluted in blocking solution (1:100) for 90 min. Sections were then washed in TBS containing 2.5M NaCl for 10 min and rinsed four times in distilled water. Sections were finally incubated in 0.5% formaldehyde for 10 min and rinsed with distilled water followed by complete drying. Sections were counterstained with 1% uranyl acetate oxalate.

Control sections

Control sections were incubated in antisera (200 μl) preabsorbed with 1, 5, 10 μM of its respective synthetic antigen. Other negative control sections were included in which the antiserum was replaced by normal rabbit serum, or in which the antiserum was omitted. Equine posterior pituitary gland was included as a positive control.

Transmission electron microscopy

Sections were viewed and photographed using a Philips CM12 Transmission Electron Microscope.

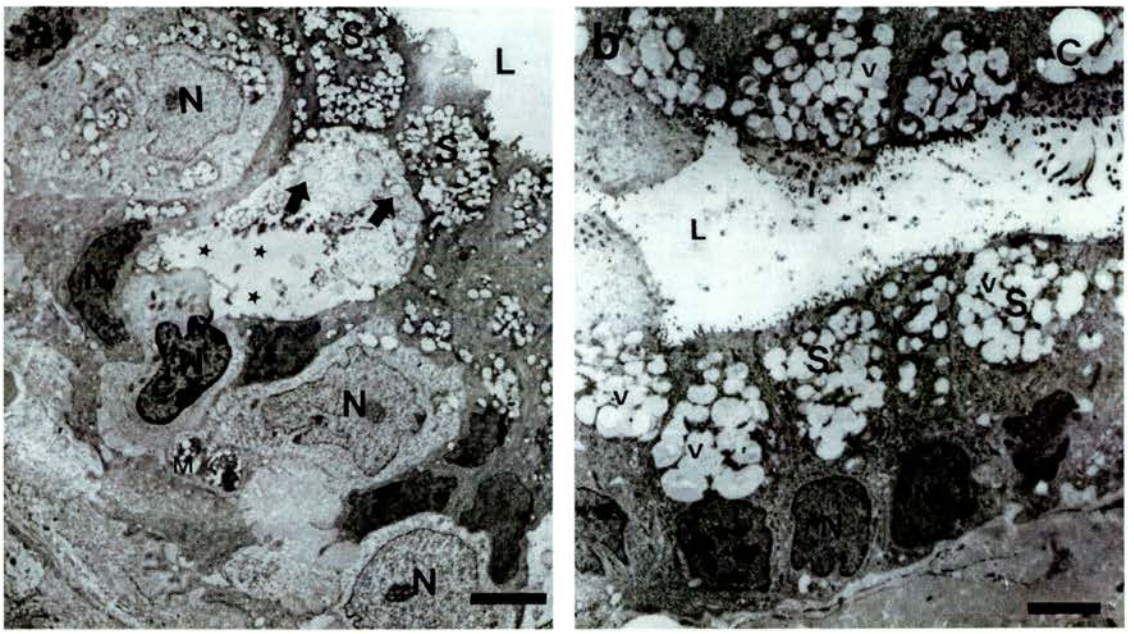
Results

Ultrastructural morphology of the endometrium

The ultrastructure of the endometrium was studied before immunohistochemistry was performed. The luminal epithelium and epithelial cells in the endometrial glands consisted predominantly of nonciliated cells containing numerous secretory vesicles in the apical part of the cells (Fig 5-1-a, b). Very few ciliated cells were present in the

luminal epithelium. The nuclei were located mainly in the basal to middle regions of the cells and were round to oval in shape (Figure 5-1-a). In the luminal epithelium, dark cells had a fusiform nucleus and light cells showed more round shape of nucleus (Figure 5-1-a). Intense cellular vacuolation, a high number of polyribosomes and a large amount of rough endoplasmic reticulum were present in the cells of the luminal surface and glandular epithelium. In the luminal epithelium, intercellular spaces were also seen (Figure 5-1-a). The subepithelial/superficial stroma contained blood vessels and lymphatic vessels, macrophages, mast cells, leukocytes and plasma cells (Fig 5-1-a). The superficial endometrial glands comprised predominantly nonciliated cells and tended to have larger lumens than the deeper endometrial glands (Fig 5-1-b). The deep basal endometrial glands contained a higher cell density and a small or nonexistent lumen.

Figure 5-1. Transmission electron micrographs of equine endometrium during oestrus showing the luminal epithelial cells and the epithelial cells of the endometrial glands

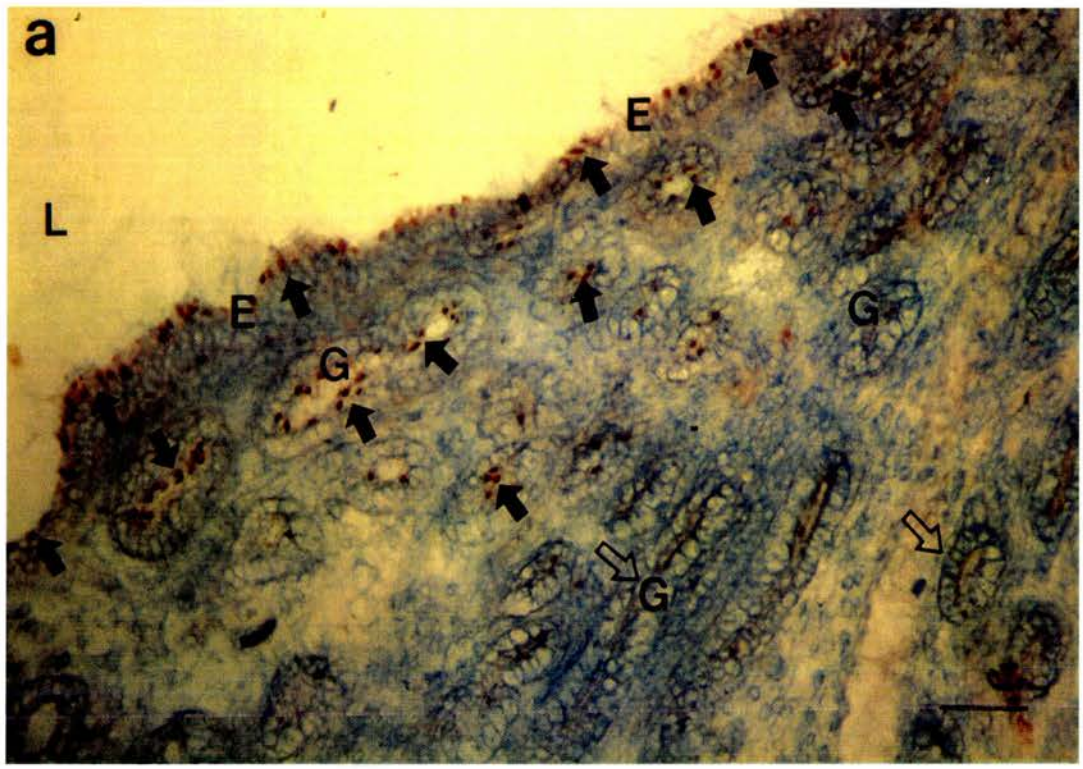


a. Row of secretory cells in the luminal epithelium with nuclei (N) located in basal to mid-region, secretory vesicles (S) and lumen (L). Note the large intercellular spaces (★), vacuolation (➡), and macrophages (M) intermingled with epithelial cells. Scale bar = 3.33 μm. b. Transverse section of an excurrent duct showing ciliated cells (C) and secretory cells (S). N: nucleus, L: lumen, v: secretory vesicles. Scale bar = 3.33 μm.

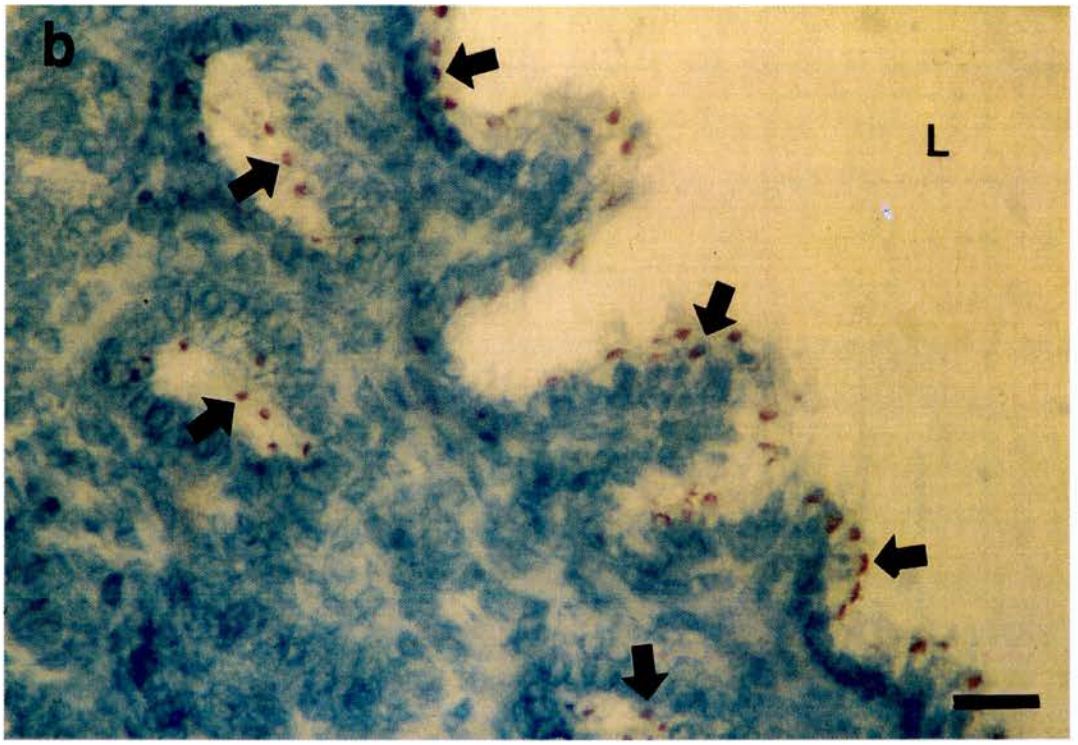
Immunohistochemistry

At light microscopic level, positive immunostaining for oxytocin and neurophysin was seen in the luminal epithelium and the epithelial cells of the superficial glands of the endometrium (Figure 5-2-a, b).

Figure 5-2. Equine endometrium showing the presence and location of oxytocin and neurophysin by immunohistochemistry.



a. Photomicrograph of frozen equine endometrium showing the location of oxytocin (➡) by immunohistochemistry. Notice the mid-endometrial glands do not show any positive staining (⇨). Scale bar = 0.1mm

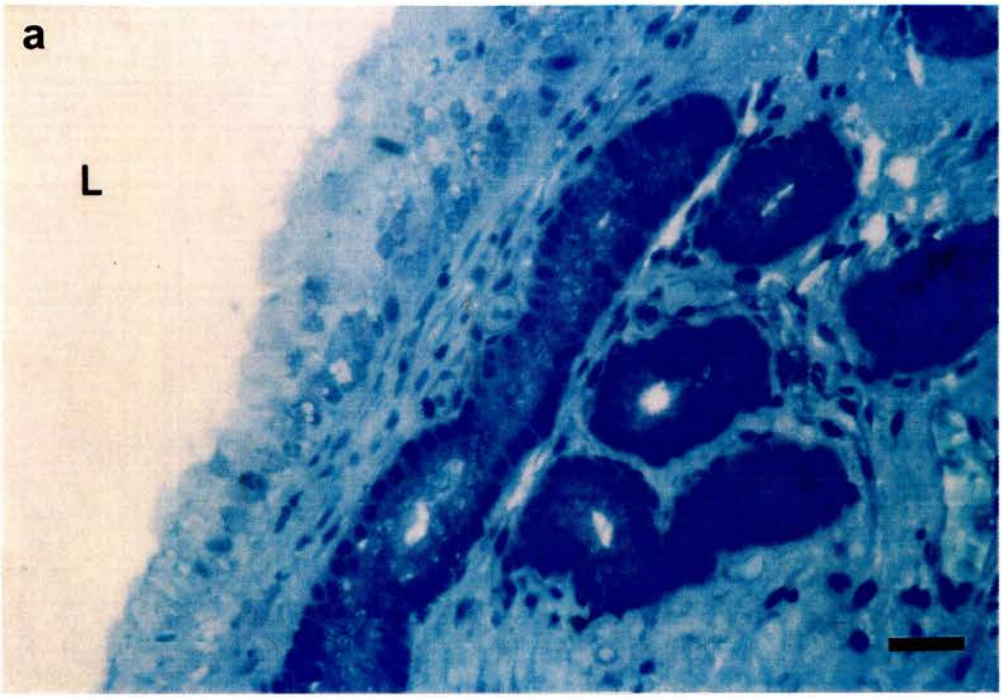


b. Photomicrograph of frozen equine endometrium showing the location of neurophysin (➡) by immunohistochemistry. Scale bar = 0.05 mm

Immunostaining at the ultrastructural level

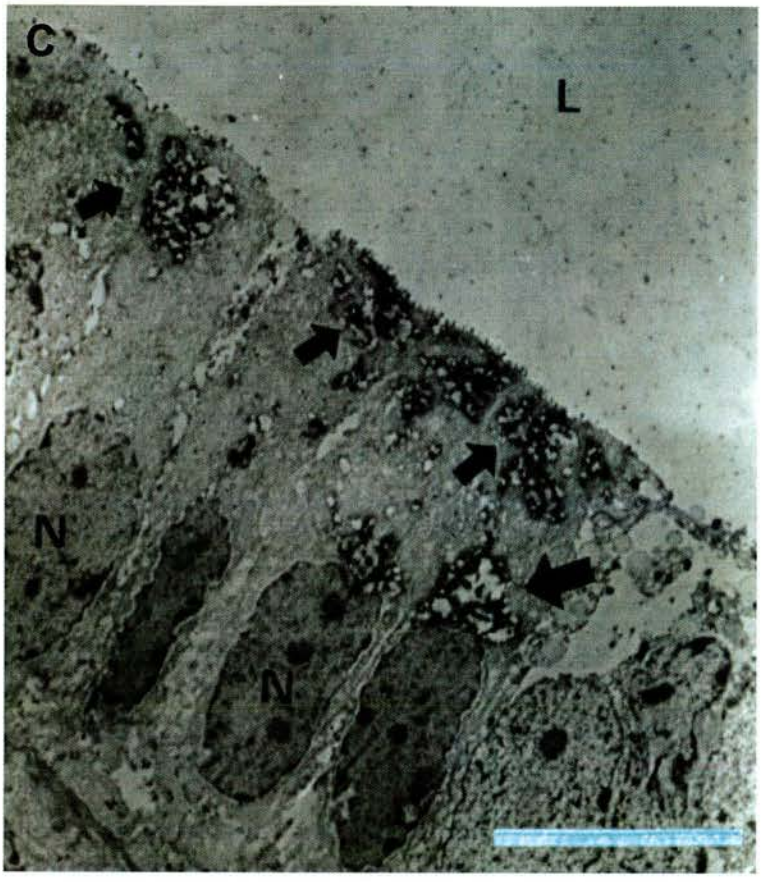
Strong positive immunostaining for oxytocin and neurophysin was present in the secretory (nonciliated) luminal epithelium (Fig 5-3-b,c) and in the secretory cells of the superficial endometrial glands (Fig 5-3-d, e) at ultrastructural level. The staining was located near the apical border of the secretory epithelial cells where the secretory vesicles were located. There was no staining in the ciliated cells in either the luminal epithelium or superficial endometrial glands (Fig 5-3-d, e). No staining was present in the deep glands or in the endometrial stroma and connective tissues for either of the hormones (Fig 5-3-f). No staining was present in negative control sections (Fig 5-3-g, h).

Figure 5-3. Sections of the equine endometrium.

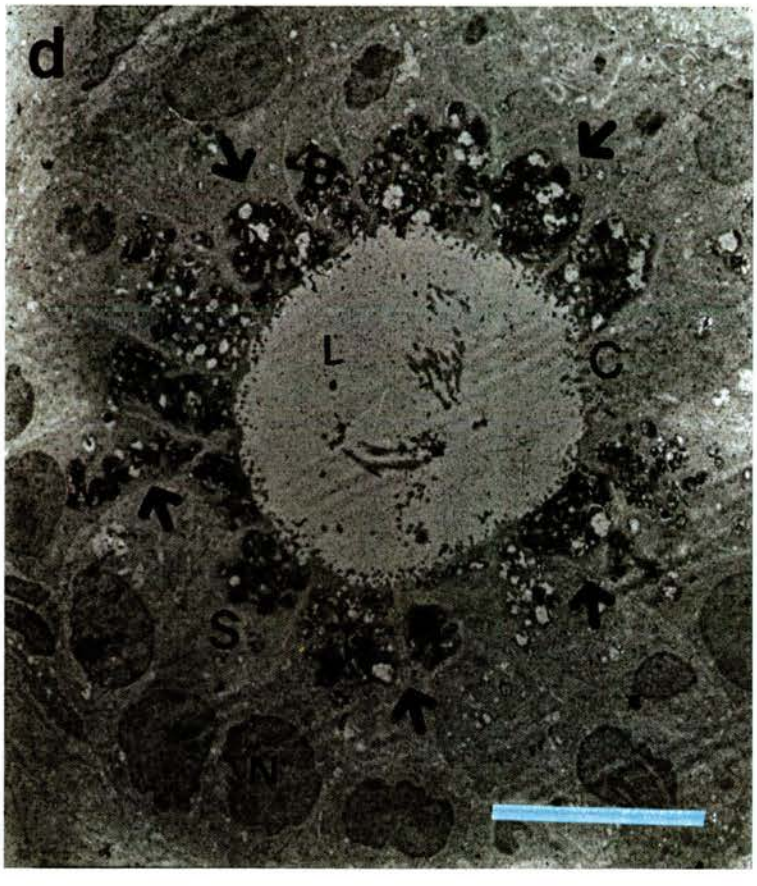


a. Semi-thin section (1 μm) of equine endometrium stained with 1% methylene blue. L: lumen. Scale bar = 100μm.

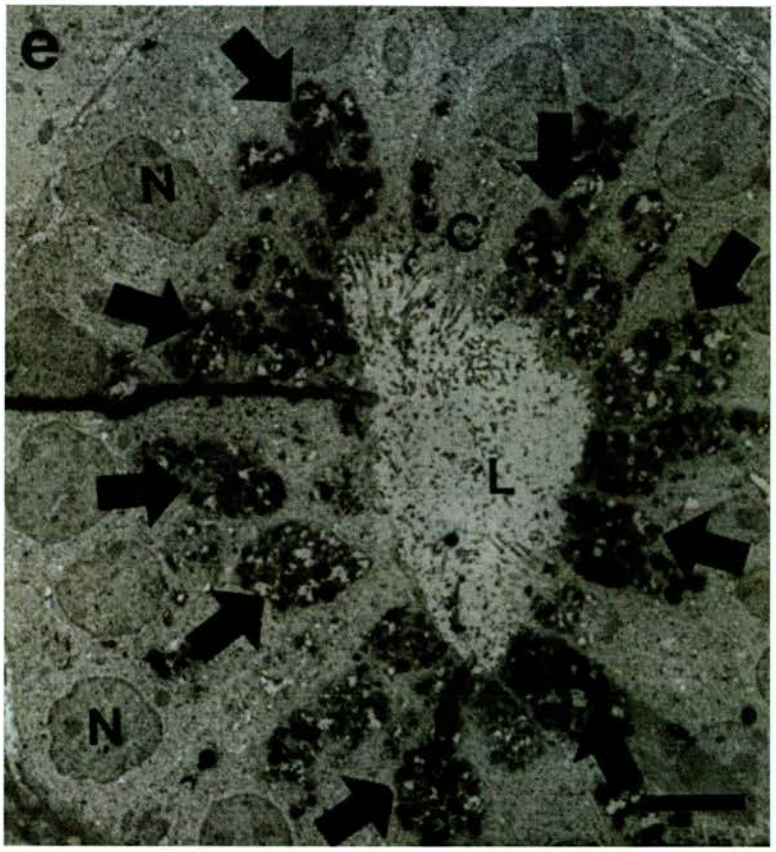
b. Electron micrograph of equine endometrium showing the location of oxytocin (➡) at ultrastructural level. Both luminal and glandular epithelium are present. Scale bar = 10 μm.
E: luminal epithelium,
G: endometrial glands



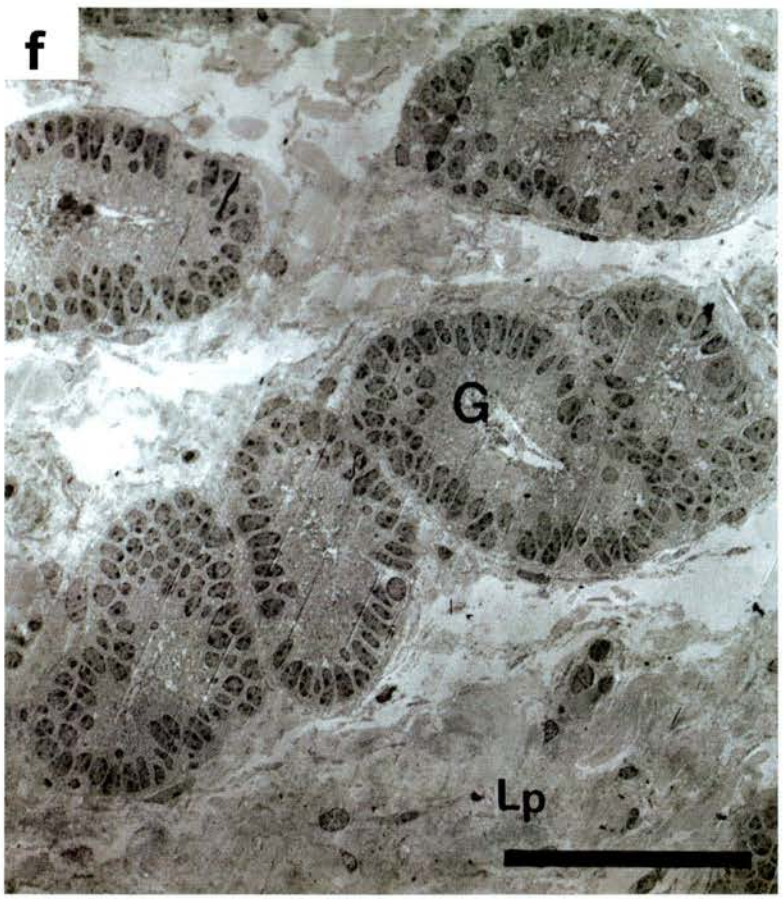
c. Secretory cells of the luminal epithelium immunostained for neurophysin in the region of the secretory vesicles near the uterine lumen and also around the smooth endoplasmic reticulum near the nucleus (➡). Scale bar = 0.5 μm. L: lumen, N: nucleus.



d. Superficial endometrial glands showing the location of neurophysin (➡) in the secretory cells. Scale bar = 1 μm. C: ciliated cells, L: lumen, N: nucleus, S: secretory cells.



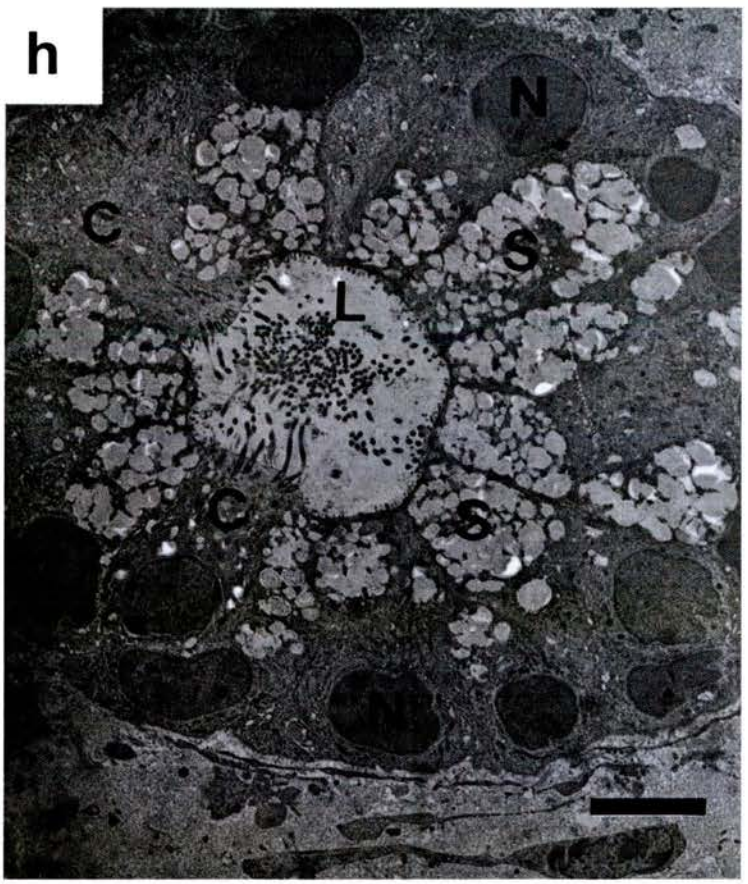
e. Superficial endometrial glands showing the location of oxytocin (➡) in the secretory cells. Scale bar = 1 μm. C: ciliated cells, L: lumen, N: nucleus.



f. Electron micrograph of deep endometrial glands. No positive immunostaining for oxytocin was present. Scale bar = 50 μm. G: endometrial glands, Lp: lamina propria.

g. Electron micrograph of negative control (antiserum was replaced by normal rabbit serum) showing luminal epithelial cells. Scale bar = 0.5 μm.

L: lumen, N: nucleus.

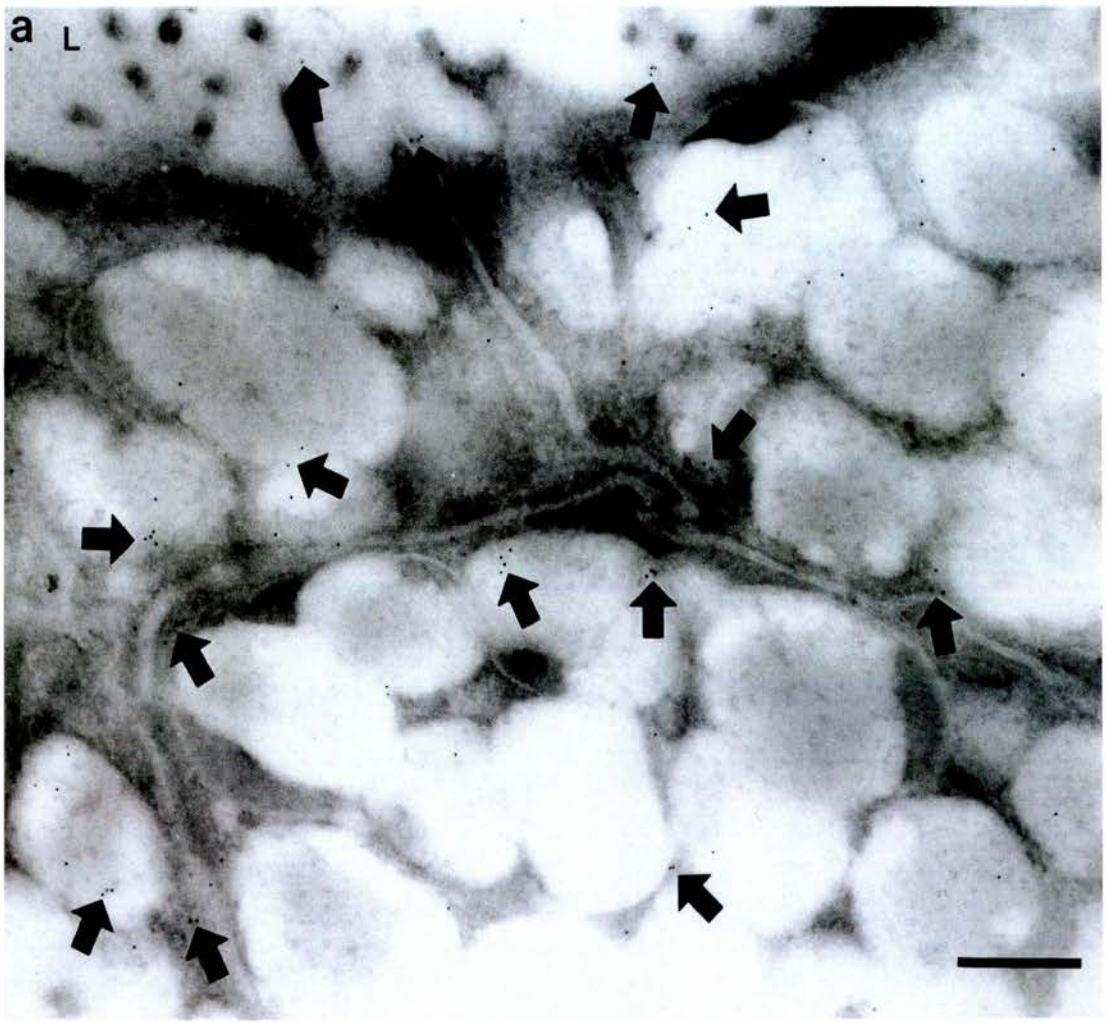


h. Electron micrograph of negative control (antiserum was replaced by normal rabbit serum) showing a superficial endometrial gland. Scale bar = 10 μm. C: ciliated cells, N: nucleus, S: secretory cells

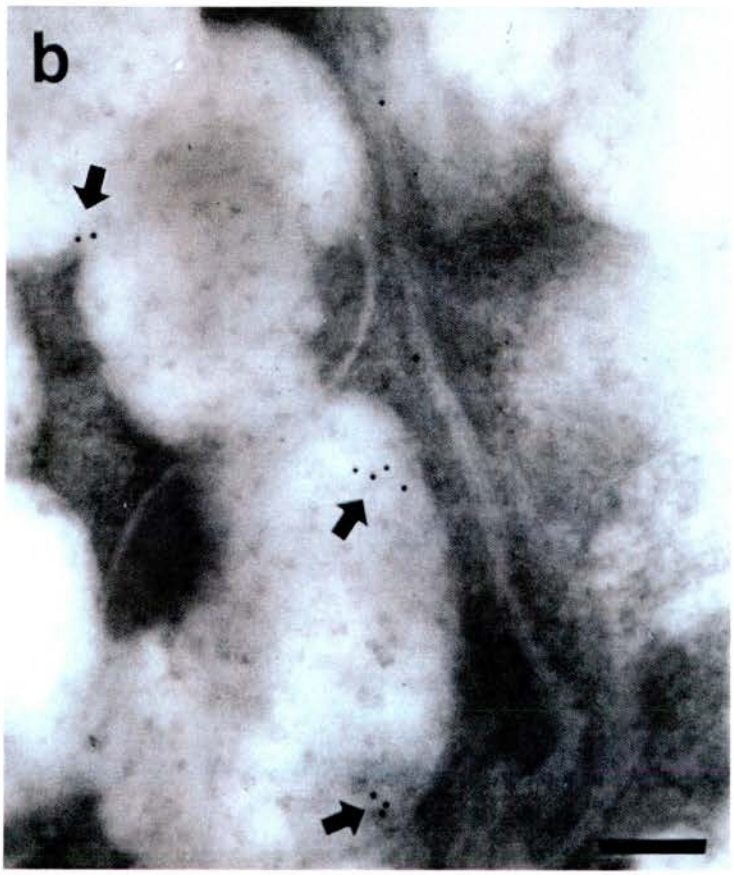
Immunogold labelling

Positive immunogold labelling for oxytocin (Figure 5-4-a) and neurophysin was present in the majority of the secretory vesicles in the secretory luminal epithelial cells and the secretory epithelial cells of the superficial endometrial glands. The immunogold labelled oxytocin and neurophysin was present in the secretory vesicles (Figure 5-4-b, c) but only oxytocin was present in the lumen around the microvilli of the secretory epithelial cells (Figure 5-4-a). The immunogold labelled oxytocin and neurophysin sometimes around the smooth endoplasmic reticulum (SER) (Fig 5-4-d). No positive immunogold labelling was present in secretory cells of the deep glands or in the stroma. Oxytocin was also found in collagen fibres and in the extracellular matrix beneath the luminal epithelium and near the endometrial glands. Positive controls showed very strong immunogold labelled with both oxytocin and neurophysin (Fig 5-4-e). No staining was present in negative control sections (Figure 5-4-f, g).

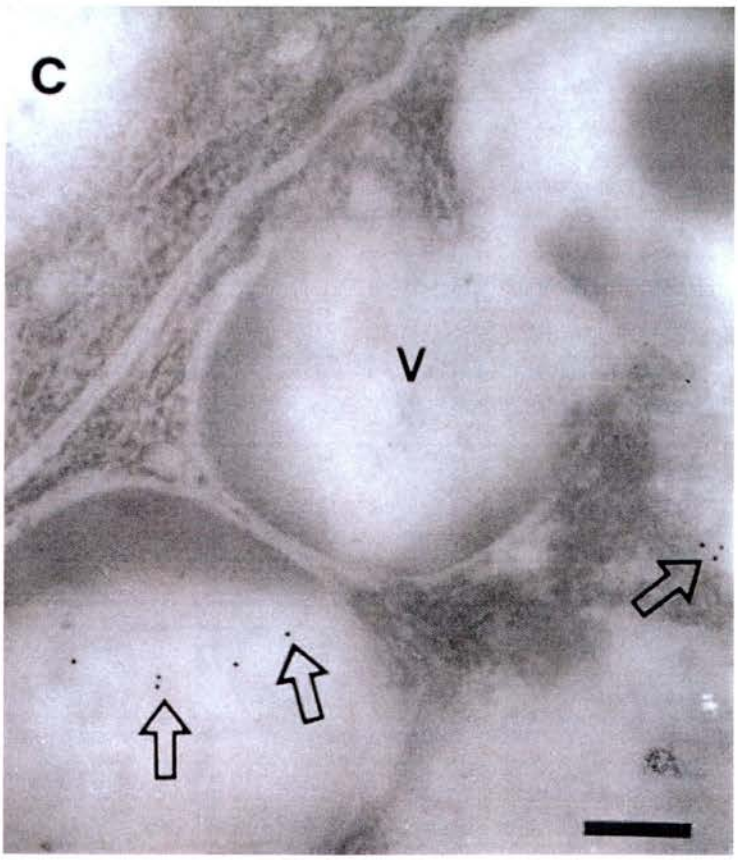
Figure 5-4. Transmission electron micrographs of the equine endometrium during oestrus, showing oxytocin and neurophysin visualized with immunogold (10nm) labelling



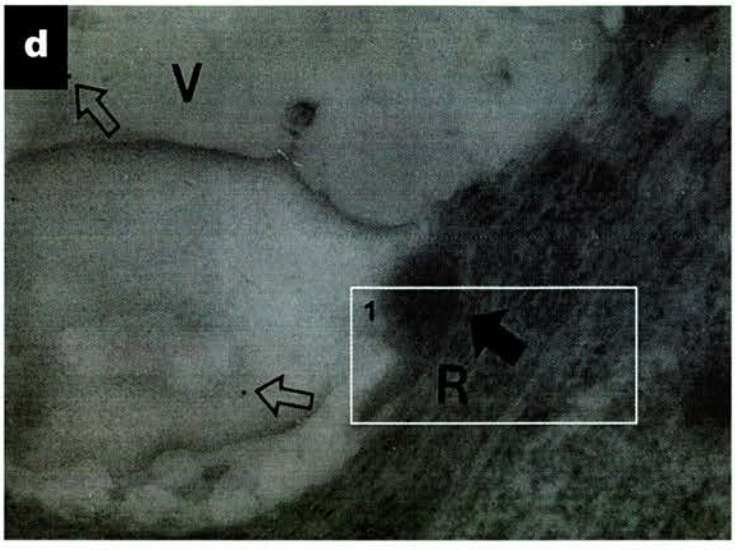
a. Secretory cells showing immunogold labelled oxytocin (➡) in the secretory vesicles and around the microvilli.
L: lumen. Scale bar = 1.8 μm



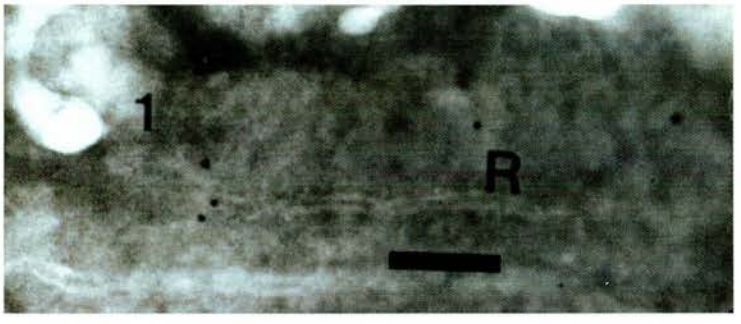
b. High power view of secretory vesicles showing immunogold labelled oxytocin (➡). Scale bar = 0.17 μm.



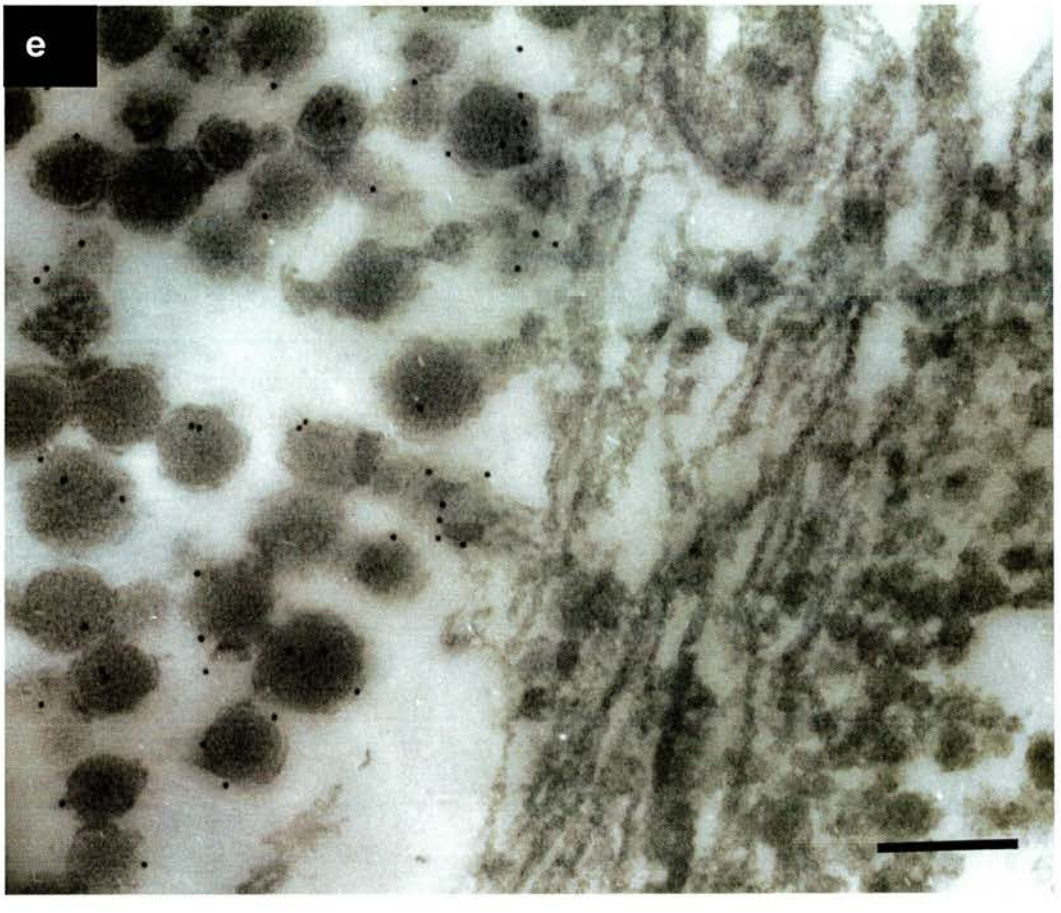
c. High power view of secretory vesicles showing immunogold labelled neurophysin (⇨). Scale bar = 0.17 μm. V: secretory vesicles

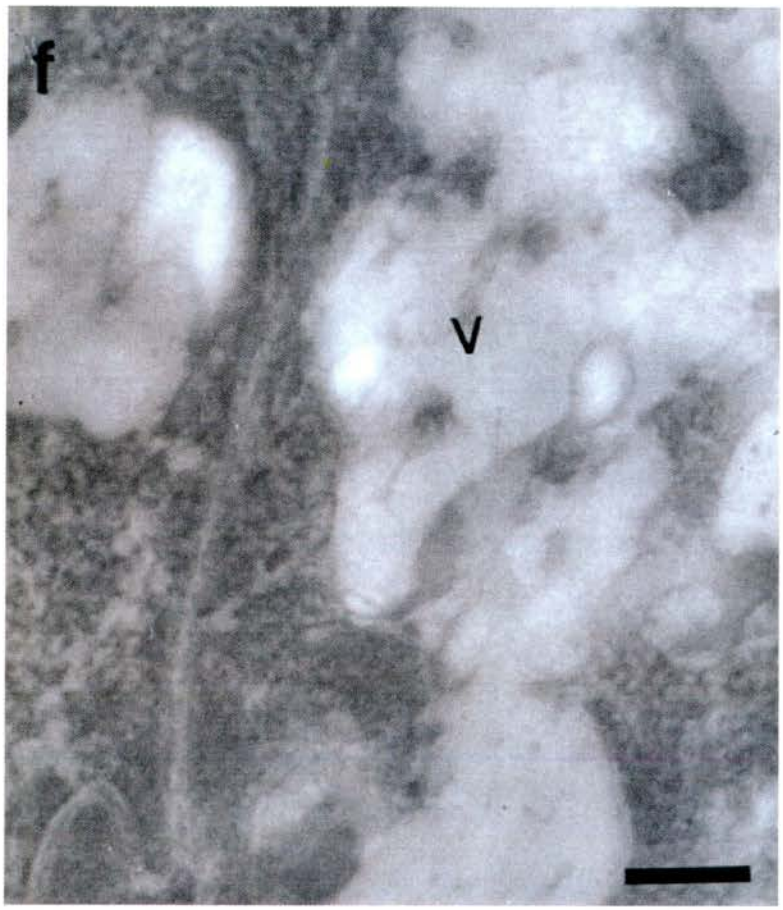


d. Immunogold labelled oxytocin in the secretory vesicles (V) (⇨) and around the smooth endoplasmic reticulum (R) (➡). Scale bar = 0.22 μm. 1. High magnification scale bar = 0.17 μm

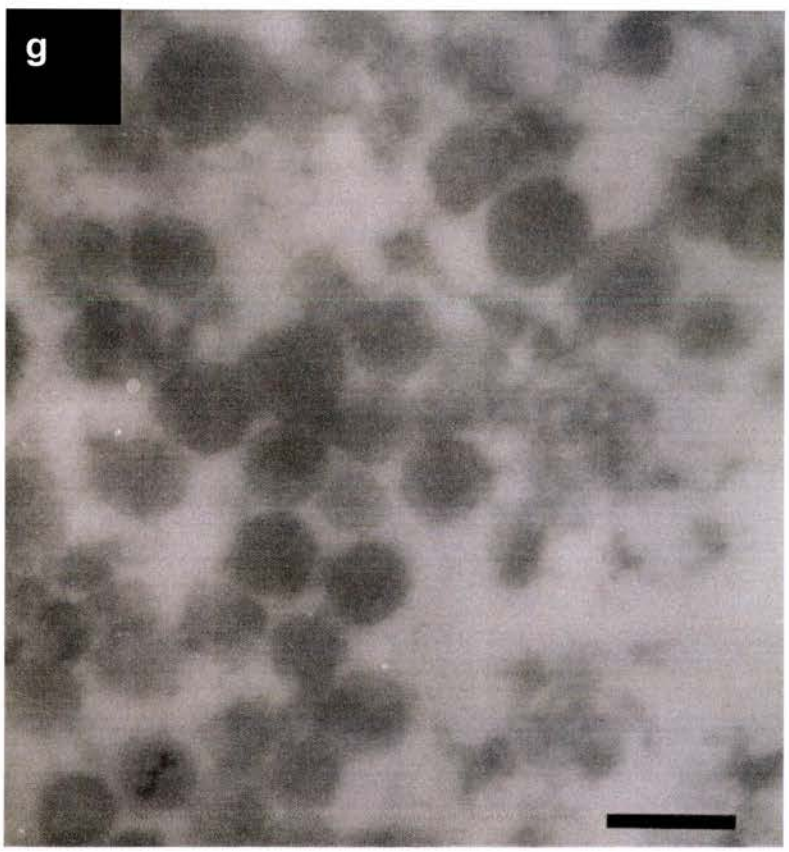


e. Positive control. Equine posterior pituitary glands showing immunogold labelled oxytocin. Scale bar = 0.17 μm





f. Negative control. Secretory vesicles (V) in the secretory epithelial cells.
Scale bar = 0.17 μm



g. Negative control. Equine pituitary gland showing no sign of immunogold.
Scale bar = 0.17 μm

Discussion

The present results using transmission electron microscopy demonstrate the presence and location of oxytocin and neurophysin in the equine endometrium. The detection of both immunoreactive oxytocin and neurophysin supports the hypothesis that they are locally synthesized in the equine endometrium which is in agreement with previous studies (Behrendt-Adam *et al.*, 1999; Watson *et al.*, 2000).

The production and secretion of oxytocin has been well documented in the magnocellular neurons in the supraoptic, paraventricular and arcuate nuclei of the equine hypothalamus (Melrose and Knigge, 1989). Previous researchers have suggested that the protein and peptide hormones are synthesized on mRNA templates of the polyribosomes of the rough endoplasmic reticulum (RER) and accumulate within the reticulum. From the RER, the polypeptides pass into the smooth, polysome-free portion of the endoplasmic reticulum, known as transitional elements, where the membrane of the reticulum buds off, encapsulating the secretory product. These vesicles then migrate to the Golgi complex. The mature secretory vesicles eventually reach the plasma membrane to await the appropriate signal to release their inclusions to the cell exterior (Brownstein *et al.*, 1980; Eckert *et al.*, 1988). Ultrastructural analysis in the rat showed that the transcripts of mRNA encoding for oxytocin are associated with secretory granules in magnocellular neuronal axons and nerve terminals and are not present in the surrounding pituitary cells (Jirikowski *et al.*, 1990).

During oestrus, the secretory cells of the equine endometrium have a cytoplasm rich in organelles, particularly mitochondria, rough endoplasmic reticulum, Golgi apparatus, and numerous secretory vesicles in the apical, adluminal area (Tunon *et al.*, 1995). In the present study in which endometrium was collected at oestrus, the majority of immunostaining for oxytocin and neurophysin was located in the secretory vesicles. This indicates that oxytocin synthesis in the equine endometrium is associated with secretory granules as already shown in the rat pituitary, and that it

is stored within the secretory vesicles, similar to the pathway of its synthesis in magnocellular neuronal axons and nerve terminals of the hypothalamus.

A previous study at light microscope level (Watson *et al.*, 2000) showed strong positive immunostaining for oxytocin and neurophysin in the luminal epithelium and in the epithelium of superficial glands, however, within the epithelial cells the staining appeared to be discrete. In the present study, the immunoreactive oxytocin and neurophysin appeared to be in secretory vesicles which are involved in intracellular transport of newly synthesised protein and exocytosis in the secretory cells (Cheville, 1994). In addition, the possible finding of oxytocin around and in the microvilli of the luminal epithelial cells suggests that oxytocin is secreted into or absorbed from the lumen of the uterus. It is thought that uterine oxytocin stored in endometrial cells and may not be released into the blood stream but released into the uterine lumen. Ciarochi *et al* (1985) suggested that oxytocin was secreted into the uterine lumen in rats because of high concentrations of these hormones in uterine fluid. Similarly, in the mare oxytocin is present in uterine lavage fluid (Watson *et al.*, 2000) and reaches very high concentrations during late dioestrus and early oestrus (Stout *et al.*, 2000). The possibility of positive immunostaining for glycogen was confirmed to have negative control sections which did not show any positive staining.

Immunogold labelling possibly indicated that immunoreactive oxytocin can be found in the secretory luminal epithelial cells of the uterus of the mare. This could suggest that the mechanism of secretion of uterine oxytocin in the equine endometrium is merocrine. In merocrine secretion, the contents of small secretory granules are released as the secretory product. It is suggest that in the synthesis of uterine oxytocin, oxytocin in the trans Golgi network is diverted into secretory vesicles where the proteins are concentrated and stored until an extracellular signal, possibly ovarian steroid hormones (Behrendt-Adam *et al.*, 2000), stimulates their secretion. When the secretory granule reaches the cell surface, its membrane fuses with the plasmalemma, and discharges the secretory product by exocytosis into the uterine lumen. The exact role of uterine oxytocin being secreted into the uterine

lumen is still not clear. In addition, there is a possibility that immunogold labelled technique may not have been optimized.

Previous studies showed that the ratio of secretory (nonciliated) and ciliated cells in the equine endometrium varies throughout in the cycle. Secretory cells declined rapidly in number after oestrus and few could be identified in dioestrus. By contrast ciliated cells reached maximum density during mid-dioestrus and declined towards the end of the period (Ricketts, 1975; Samuel *et al.*, 1979; Keenan *et al.*, 1991). The changes in predominant cell type with cycle stage probably explains the finding the endometrial oxytocin immunostaining is greater at oestrus than dioestrus (Watson *et al.*, 2000) when there are more secretory cells than ciliated cells. In addition, it has been shown that oxytocin-mRNA levels are positively correlated with serum oestradiol levels in mares (Behrendt-Adam *et al.*, 1999), probably reflecting a change in epithelial cell type.

Equine uterus has oxytocin binding sites in both endometrium and myometrium (Stull and Evans, 1987). In this study, oxytocin was found in collagen fibres and the extracellular matrix. Connective tissues, including extracellular matrix and collagen fibres, are connected via the basement membranes of epithelium, blood vessels, nerves and endometrial glands. It is known that the extracellular matrix can play a role in the fluid dynamics of a tissue, and provide mechanical support for tissues. Furthermore, nutritional materials, hormones, and other extracellular signals are often required to traverse the extracellular matrix to reach target cells (Hay, 1991). Stored uterine oxytocin in the endometrial cells might be transported from the endometrium to the myometrium via these connective tissues and may have a role in controlling myometrial contractility. However, it is also possible that oxytocin detected in endometrial cells may be secreted into or absorbed from the uterine lumen.

Technical difficulties were encountered in performing immunohistochemistry and immunogold labeling for TEM. With fixation, after trying various resins: unicryl, araldite, and durcupan, unicryl was chosen because it showed the best structure, wet

easily and had lower cross linking. This may have impacted on sensitivity of immunogold labelling. Use of a meshed grid created difficulty to obtain the specific area (e.g. luminal epithelial area), therefore a grid containing a hole in the middle was chosen in this study. However, after changing it to a grid containing a hole in the middle, tissues became very fragile and sensitive especially when the magnification was changed or when taking photos.

The exact functional role of uterine oxytocin remains unclear. However, the findings that uterine oxytocin is stored in the secretory vesicles of the luminal epithelium and superficial endometrial glands in the endometrium could suggest that uterine oxytocin may only act on the uterus. Oxytocin gene expression in the equine endometrium tends to be higher during oestrus and late dioestrus than during early to mid-dioestrus or early pregnancy (Behrendt-Adam *et al.*, 1999). Stout *et al* (2000) have suggested that uterine oxytocin may act in a paracrine manner to elicit endometrial prostaglandin $F_{2\alpha}$ synthesis at luteolysis. At oestrus, uterine oxytocin may increase uterine motility via myometrium during egg and sperm transport (Gilbert *et al.*, 1992) and facilitate uterine clearance mechanisms after mating. Maximum uterine contractility in the early pregnant mare occurs on day 11-14 during maximum mobility of the embryonic vesicle (Cross and Ginther, 1988), when previous studies have reported high levels of immunostaining for oxytocin in the endometrium (Watson *et al.*, 2000). The increased uterine contractions and extensive embryo mobility on day 11 through day 14 could be explained by the secretion of oestrogen by the equine conceptus (Flood *et al.*, 1979; Zavy *et al.*, 1979) increasing the synthesis of oxytocin via upregulation of oxytocin mRNA. However, mRNA encoding oxytocin is reported to be only present a low levels in early pregnancy (Behrendt *et al.*, 1999) and low concentrations of oxytocin were measured in uterine lavage fluid from pregnant mares (Watson *et al.*, 2000). This would indicate that oxytocin was being sequestered in the endometrium at this time rather than being actively synthesised and secreted.

In conclusion, this chapter has shown the presence of oxytocin and neurophysin in

the secretory vesicles of the secretory cells in the luminal epithelium and superficial endometrial glands in oestrous uteri. To my knowledge, this is the first detailed report on location of oxytocin and neurophysin in specific uterine cells in any species. Further studies are needed 1) to optimize the immunogold labeling (e.g. bigger size of immunogold particle to have better density and the location of neurophysin and oxytocin in the endometrium and 2) to define the exact reproductive physiological role of uterine oxytocin at oestrus and during early pregnancy in the mare and to look in dioestrus as well as in mares with delayed uterine clearance. In the next chapter, plasma oxytocin concentrations around oestrus and after ovulation in normal mares will be determined. These concentrations will be compared with mares with delayed uterine clearance.

Chapter 6

The concentrations of circulating plasma oxytocin and the pattern of oxytocin release in mares during oestrus and after ovulation

Introduction

Oxytocin is known to be produced mainly by the magnocellular neurons in the posterior pituitary (Bargmann and Scharer, 1951; Melrose and Knigge, 1989) and is transported via the blood to the uterus, stimulating the release of prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) from the endometrium (Roberts *et al.*, 1976). Oxytocin is central in controlling myometrial contractility and forms a positive feedback loop with $PGF_{2\alpha}$. In the previous chapter, the possibility of uterine oxytocin acting in a paracrine manner to elicit endometrial $PGF_{2\alpha}$ is also suggested.

As with all hypothalamic hormones, oxytocin is released discontinuously, and serial measurements reveal that plasma oxytocin levels fluctuate in the circulation, known as 'spurt' release (Gibbens and Chard, 1976; Dawood, 1983; Fuchs *et al.*, 1991). Previous studies on the plasma concentrations of oxytocin in mares gave contradictory results because of its pulsatile manner of secretion and differences in assay methodology. Peripheral concentrations of oxytocin have been measured during the oestrous cycle. Burns *et al.* (1981) collected samples from four different stages of the oestrous cycle and showed that concentrations of oxytocin were highest on day 2 of oestrus, and were greater on day 5 post-ovulation than on day 10 and day 15 post-ovulation. In contrast, Tetzke *et al* (1987) showed that plasma concentrations of oxytocin were greater on day 15 post-ovulation than on the day of ovulation or days 3 and 7 post-ovulation. By frequent sampling, these workers also showed a very irregular pattern of pulsatile secretion. Stevenson *et al.* (1991) measured plasma oxytocin concentrations throughout the oestrous cycle but mean oxytocin concentrations remained at very low concentrations with no evidence of cyclic variation. Recently, Vanderwall *et al.* (1998) collected samples from the intercavernous sinus of the mares during luteolysis and showed a clear pulsatile mode of oxytocin secretion.

Mares with delayed uterine clearance (e.g. persistent mating-induced endometritis; PMIE) are subfertile. Previous research showed that mares susceptible to PMIE accumulated six times more intrauterine fluid after bacterial challenge than mares

resistant to PMIE (Troedsson and Liu, 1992). The main factor causing delay in drainage of intrauterine fluid is thought to be impaired myometrial contractility in these susceptible mares (Troedsson *et al.,* 1993a). As oxytocin has a major influence on uterine contractility, these results suggest that there may be differences in susceptible and resistant mares in the pattern of oxytocin release, plasma oxytocin concentrations, the concentrations of the uterine oxytocin receptors, or in post-receptor mechanisms (Nikolakopoulos *et al.*, 2000a).

The objective of the present study was to determine changes in the plasma oxytocin concentrations during oestrus in genitally-normal mares by collecting frequent blood samples and to compare the baseline circulating oxytocin concentrations in genitally normal mares and in mares with delayed uterine clearance. The experiment was designed to determine: 1) plasma oxytocin concentrations during oestrus and after ovulation and 2) the differences in the concentrations of plasma oxytocin between two groups, genitally-normal mares and mares with delayed uterine clearance.

Materials and methods

Experiment 1. Concentrations of plasma oxytocin during oestrus until three days after ovulation in genitally-normal mares (n=5)

Animals:

Five genitally-normal mares according to their reproductive history of high fertility, ability to clear introduced uterine infection within 48 hours from the time of natural service and endometrial biopsy scores of 1 to 2A, aged x = 10 ± 2.7; range 6-13 years, weighing 410-520 kg were used. None of the mares had any detectable intrauterine fluid accumulation prior to experiments. Oestrus was defined by transrectal ultrasonographic examination of the genital tract as the presence of an ovarian

follicle of >30 mm in diameter and uterine oedema, with the mare responding positively to teasing with a stallion. Ovulation was detected ultrasonographically by the disappearance of the follicle and the presence of a corpus luteum on the ovary. Day of ovulation was designated as day 0.

Blood sampling:

On the first day that the mares were considered to be in oestrus, a catheter (either 13 gauge, Presidio Medico, Ecouen, France, or Central Venous Catheter Set with peel-away sheath introduction technique, V7854 ,Cook®, Australia) was placed in the jugular vein after asceptic preparation of the area and local anesthesia. The catheter was kept patent by flushing every 30 min with 20 ml heparin-saline (5,000 iu/l). Two blood samples of approximately 7 ml each were collected into evacuated heparinised tubes every 5 min for four hours between 0900 h and 1400 h on days during oestrus and for three consecutive days starting from day 0 (ovulation).

Experiment 2. Concentrations of plasma oxytocin in genitally-normal mares (n=5) and in mares clinically shown to have delayed uterine clearance (n=5) on day 3 of oestrus

Animals:

Five mares of mixed breeding, aged x = 10 ± 1.4 years; range 5-13 years, weighing 370-520 kg and classified as genitally-normal mares, and five mares, aged x=14 ± 1.9 years; range 7-18 years, weighing 370 to 620 kg and classified as mares with delayed uterine clearance from their previous clinical history of subfertility related to uterine fluid accumulation for at least 48 hours after mating were used. Oestrus was defined by transrectal ultrasonographic examination of the genital tract as the presence of a follicle of >30 mm in diameter and uterine oedema, with the mare responding positively to teasing with a stallion.

Blood sampling:

Blood samples were collected on day 3 of oestrus. Jugular blood samples (15ml) were collected by venipuncture as described in *Experiment* 1 from genitally-normal mares (n=5) and mares with delayed uterine clearance (n=5), every 5 min for 30 min.

Sample Handling

Blood samples were immediately placed on ice until separation. The samples were centrifuged at 2000 × *g* for 15 min at 4°C. An aliquot of plasma (4ml) was acidified with 10M acetic acid (10 µl per 1ml plasma) for oxytocin assay to improve oxytocin recovery rates as already shown in the sow (de Winter *et al.*, 1995) and goat (Homeida and Cooke, 1984b). All plasma samples were frozen at -70°C and subsequently stored at -20°C until assayed.

Oxytocin Assay (See Appendix)

Oxytocin was extracted from plasma using C_{18} SepPak cartridges (Waters Chromatography, Milford, MA, USA) and the radioimmunoassay (n=12) carried out in duplicate samples as described by Thornton *et al* (1986) using an antiserum (kindly donated from Prof. Flint) described previously (Sheldrick and Flint, 1981). The extraction recovery rate was 75 %. Three quality control samples (2 pg/ml, 5 pg/ml and 10 pg/ml) were included in each assay. The detection limit for the assay was 0.7 pg/ml. The intra- and inter-assay coefficients of variation were 3.7 % and 7.9 %, respectively.

Oxytocin Pulse Analysis

The oxytocin data from Experiment 1 was analyzed with the Munro algorithm (Taylor, 1987; Skinner *et al.*, 1995). The smoothing, nadir, and pulse interval windows were set at 200, 30, and 30 mins, respectively, whereas the rise threshold was fixed at 2 $_{SD}$. The amplitude (peak minus preceding nadir) was calculated for each pulse, and frequency, baseline, and mean level were estimated.

Statistical Analysis

All plasma oxytocin concentrations were calculated individually in both *Experiment* 1 and *Experiment* 2. Concentrations in samples below the detection limit of the assay were designated as equivalent to the detection limit of the respective assay. Data expressed as mean ± standard errors of the means (SEM).

In *Experiment* 1, the statistical significance of the differences between day of ovulation (day 0) and days in oestrus and day 1 & day 2 postovulation was examined by analysis of variance (ANOVA). The source of variation that was used in SEM in each day of mean plasma oxytocin concentrations was the interaction term between horses and days. In this case, the variation was 1.419 (degrees of freedom = 21). The calculation for ANOVA was performed by the statistical computer package Genstat 5. Overall mean concentrations of plasma oxytocin on each day during oestrus and after ovulation were compared with mean plasma oxytocin concentrations on day of ovulation (day 0) using a *t*-test. Frequency, amplitude and baseline of oxytocin pulse data were also examined by ANOVA using statistical computer package SPSS 9.0 to compare any difference between days.

In *Experiment* 2, difference in mean plasma oxytocin concentrations between genitally-normal mares and mares with delayed uterine clearance on day 3 of oestrus was compared using a *t*-test: Each data (7 data from each mare) was pooled. In all cases, a P value of <0.05 was considered significant.

Results

Experiment 1

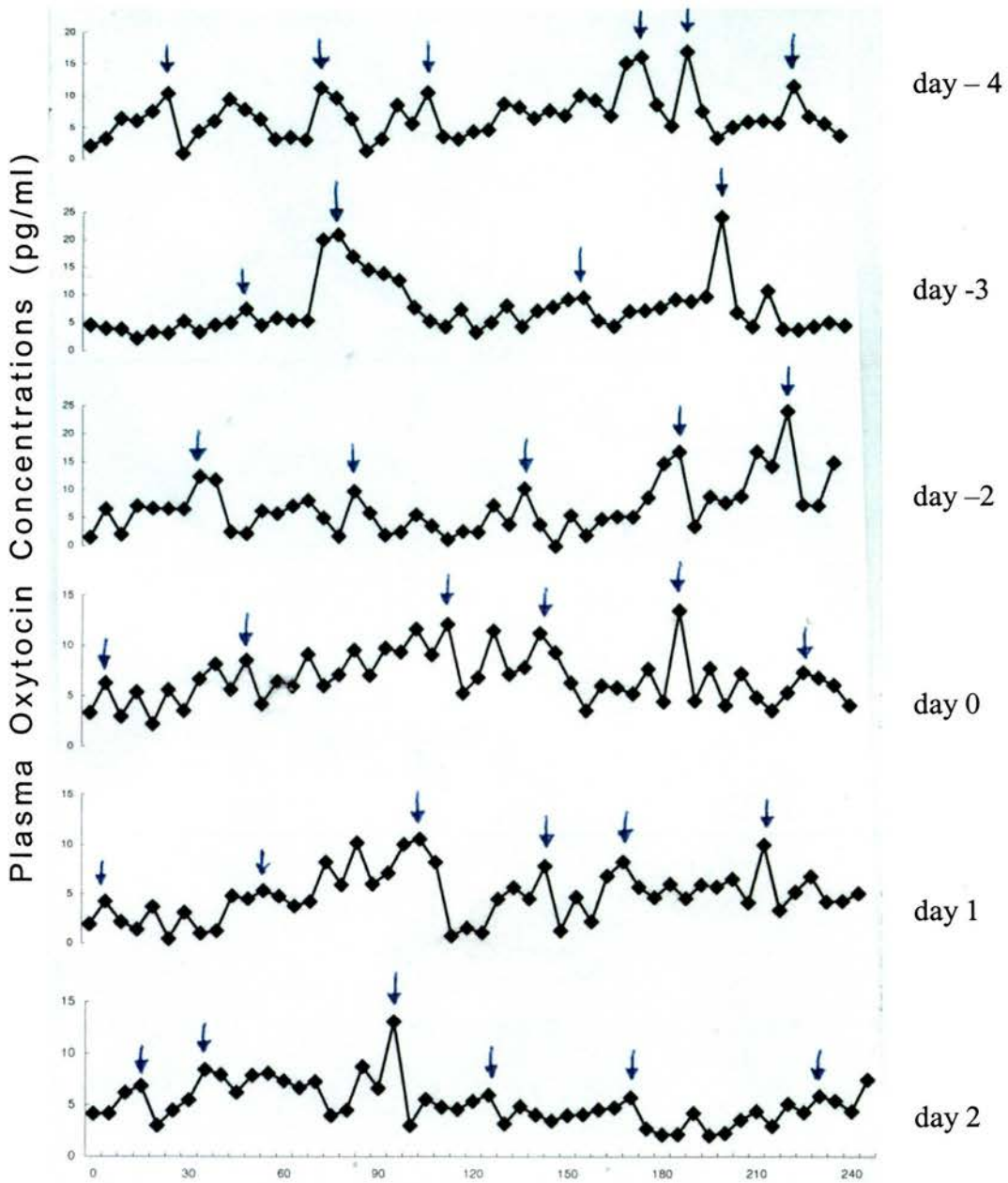
Pulsatile secretion of oxytocin was observed in all mares (Figure 6.1). However, there was no significant difference between days in frequency, amplitude and baseline of oxytocin pulses (P>0.05) (Table 6.1). Mean plasma oxytocin concentrations were significantly higher (P <0.02) in oestrus (day –5 to day –2) than the day of ovulation (day 0) (Figure 6.2). After ovulation, plasma oxytocin concentrations gradually increased (day 2 versus day 0: P=0.07).

	Day –5 postovulation	Day –4 postovulation	Day –3 postovulation	Day –2 postovulation
Frequency (pulses/per 4 hours)	7.00 ± 1.00	6.75 ± 0.25	6.00 ± 1.15	6.40 ± 0.40
Amplitude (pg/ml)	9.32 ± 4.95	5.77 ± 1.17	8.94 ± 3.32	8.46 ± 2.49
Mean level (pg/ml)	11.94 ± 1.90**	8.84 ± 0.74**	9.00 ± 0.72**	8.50 ± 0.68**
Baseline (pg/ml)	7.70 ± 0.99	5.93 ± 1.15	6.10 ± 1.44	5.13 ± 0.87
	Day –1 postovulation	**Day 0 ovulation**	**Day 1 postovulation**	**Day 2 postovulation**
Frequency (pulses/per 4 hours)	6.25 ± 0.25	6.60 ± 0.24	6.20 ± 0.20	6.00 ± 0.00
Amplitude (pg/ml)	6.76 ± 1.16	6.38 ± 0.69	7.14 ± 1.31	6.42 ± 1.12
Mean level (pg/ml)	7.78 ± 0.26*	6.58 ± 0.37	6.48 ± 0.49	7.64 ± 0.80*
Baseline (pg/ml)	4.48 ± 0.45	3.61 ± 0.82	4.00 ± 0.90	4.57 ± 0.70

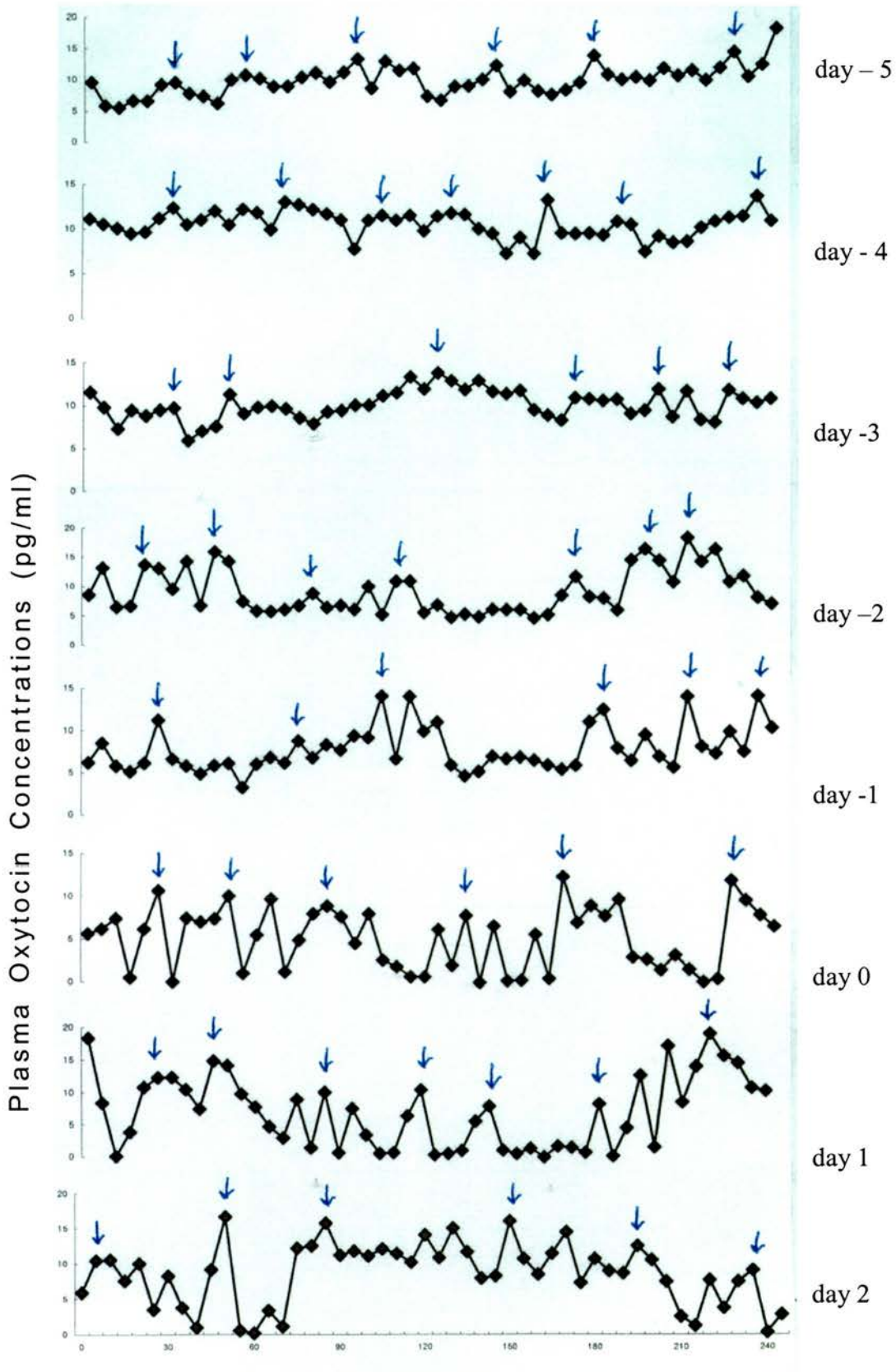
Table 6.1 Mean ± SEM. Frequency, amplitude, mean level and baseline for oxytocin throughout the oestrous cycle and days after ovulation. Asterisks indicate significant differences (**: P <0.02, *:P<0.1) between the day of ovulation (day 0) and each day during oestrus and after ovulation (day 2).

Figure 6.1 Plasma oxytocin concentration from individual mares (n=5) showing pulsatile secretion of oxytocin. Arrows represent pulses.

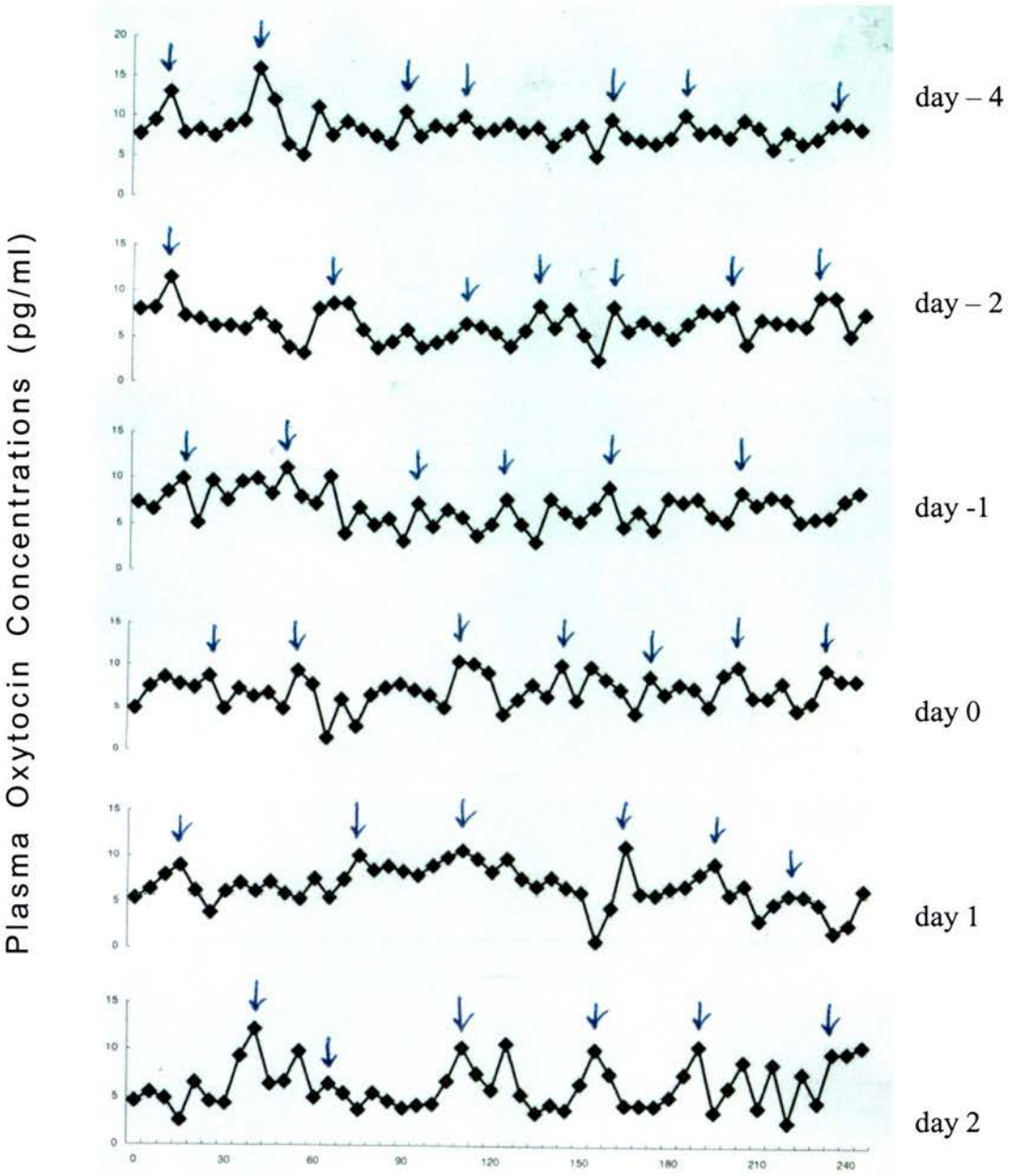
(A) Blondie



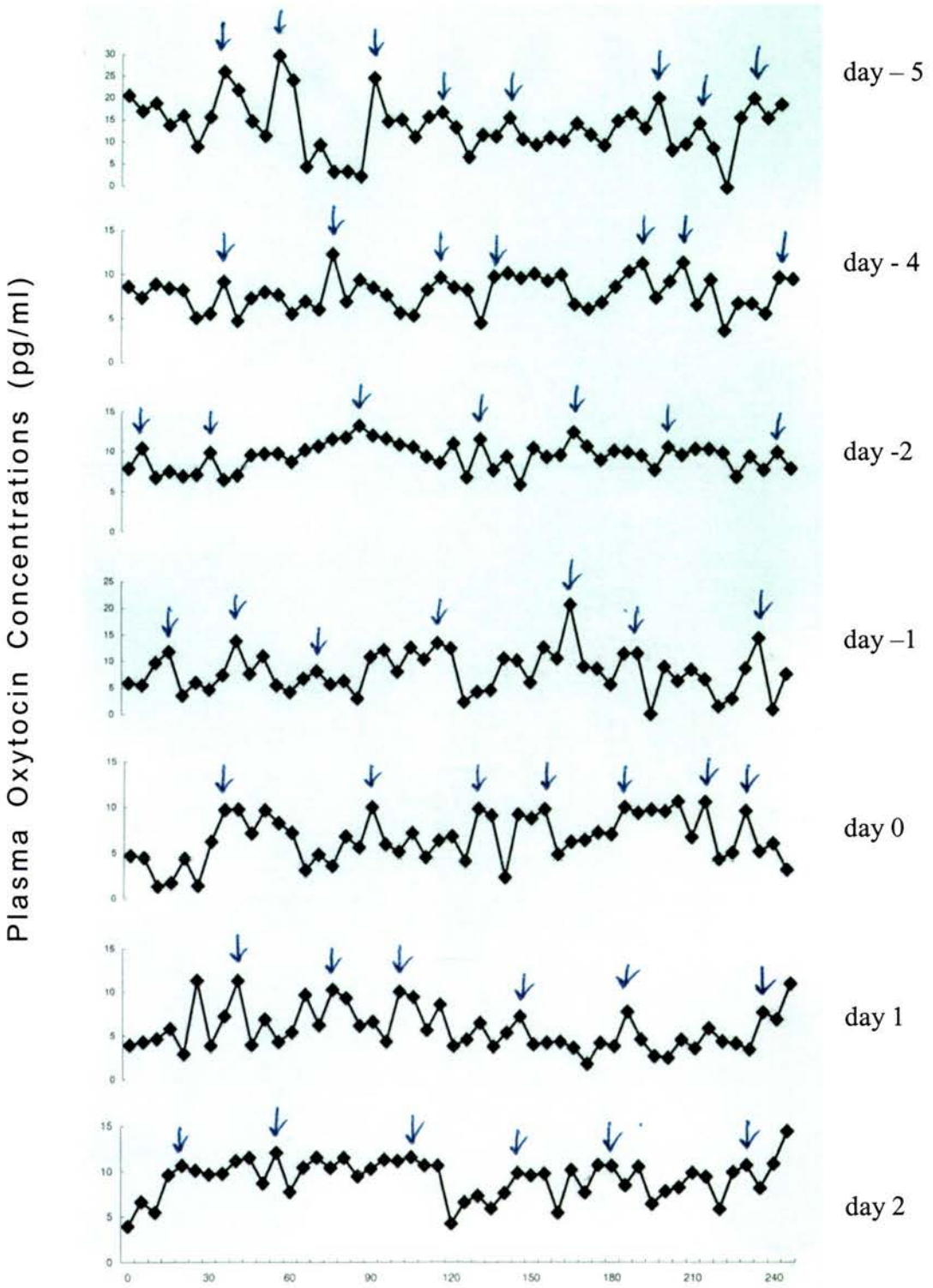
(B) Kim


Plasma Oxytocin Concentrations (pg/ml)
day – 5
day - 4
day -3
day –2
day -1
day 0
day 1
day 2
0
30
60
90
120
150
180
210
240

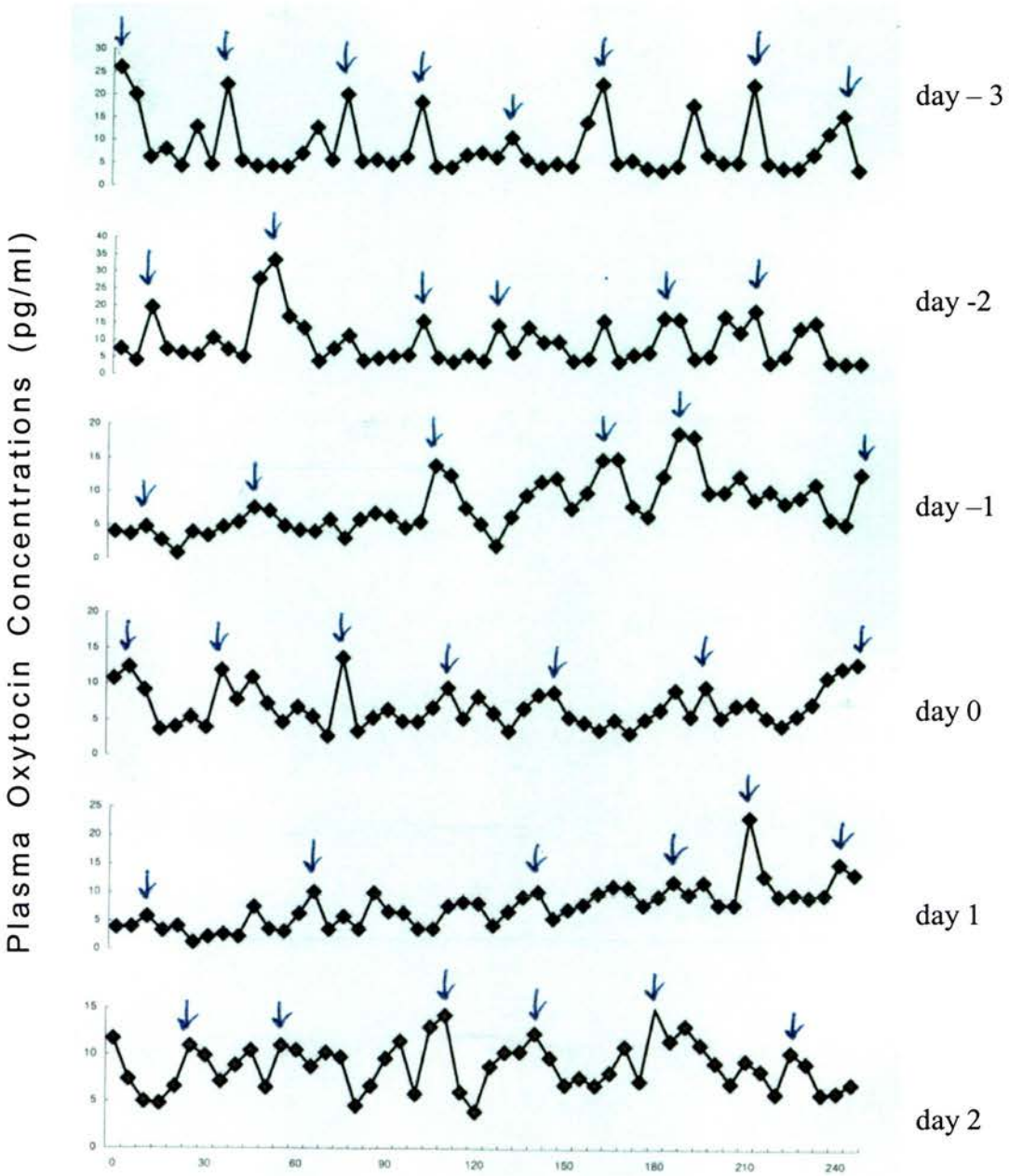
(C) Nora


Plasma Oxytocin Concentrations (pg/ml)
day – 4
day – 2
day -1
day 0
day 1
day 2
0
30
60
90
120
150
180
210
240

(D) Poppy


Plasma Oxytocin Concentrations (pg/ml)
day – 5
day - 4
day -2
day –1
day 0
day 1
day 2
0
30
60
90
120
150
180
210
240

(E) Vanessa


Plasma Oxytocin Concentrations (pg/ml)
day – 3
day -2
day –1
day 0
day 1
day 2
0
30
60
90
120
150
180
210
240

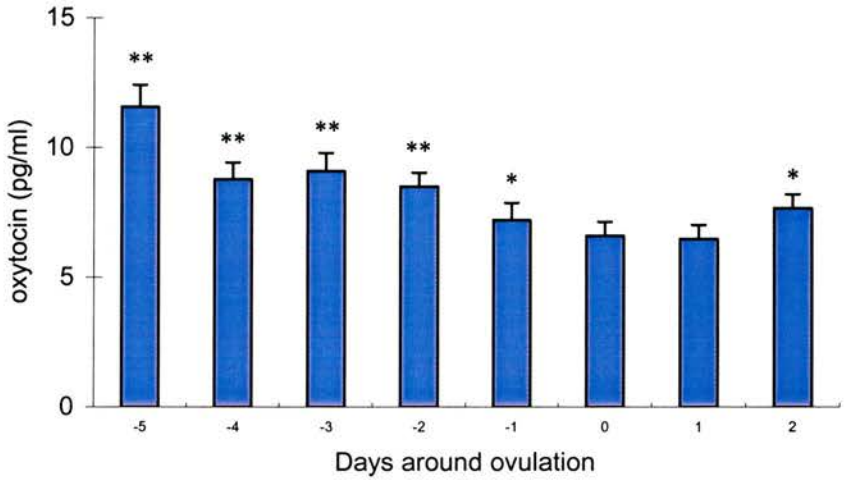


Figure 6.2 Mean (± SEM) plasma oxytocin concentrations from days during oestrus and after ovulation in genitally-normal mares. Asterisks indicate significant differences (**:P <0.02, *:P<0.1) between the day of ovulation (day 0) and each day during oestrus and after ovulation (day 2).

Experiment 2

Plasma oxytocin concentrations were significantly higher (P <0.01) in genitally-normal mares (n=5) than in mares with delayed uterine clearance (n=5) on day 3 of oestrus (Figure 6.3).

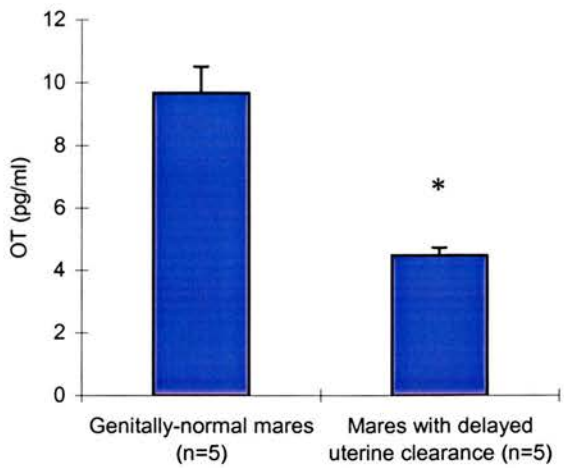


Figure 6.3 Mean (± SEM) concentrations of plasma oxytocin in genitally-normal mares (n=5) and in mares with delayed uterine clearance (n=5) on day 3 of oestrus. The asterisk (*) indicates significant difference (P <0.01).

Discussion

Uterine contractility is mediated by hormonal factors in association with neurogenic and myogenic factors. In this chapter, I have studied changes in circulating oxytocin, one of the hormonal factors controlling uterine contractility. Plasma oxytocin concentrations were significantly higher during oestrus, a period chracterised by low progesterone and high oestrogen levels (Noden *et al.*, 1975; Mienecke *et al.*, 1987) than on day 0 (ovulation). Plasma oxytocin concentrations tended to increase on day 2 after ovulation. A strong correlation between oxytocin release and uterine contractility has been reported in the mare (Madill *et al.*, 2000). This correlation between oxytocin and uterine contractility has been studied using electromyography (EMG) (Troedsson *et al.*, 1995a; Madill *et al.*, 2000) and intrauterine pressure (IUP) (Ko *et al.*, 1989; Gutjahr *et al.*, 2000). Ko *et al* (1989) reported that IUP was higher 3 days before ovulation, decreased towards ovulation (day 0) and increased after ovulation in normal mares. These IUP results are in agreement with our plasma oxytocin concentrations. Increased uterine contractility during oestrus is involved in sperm transport and facilitates uterine clearance mechanisms. Since oestrogen has an excitatory effect on paraventricular neurons, oestrogen may increase pituitary oxytocin secretion. Behrendt-Adam *et al* (2000) also showed effects of steroid hormones on endometrial oxytocin in the mare and demonstrated that oestrogen upregulated expression of the endometrial oxytocin gene.

Previous studies on plasma concentrations of oxytocin in mares have produced contradictory results. These differences probably result from methodology including different radioimmunoassay procedures, methods of oxytocin extraction from plasma, the limit of detection of the assay as well as the frequency of blood sampling. The problems with previous methodology relating to the assay procedure were; Burns *et al* (1981) used unextracted plasma, whereas Tetzke *et al* (1987) employed an acetone-HCl extraction procedure with Fuller's earth. The majority (68%) of Stevenson *et al* (1991)'s results were below detection limit of the assay. It is possible that these procedures produced high levels of nonspecific binding; or the differences could relate to the different antisera used. Because of its pulsatile manner of release

and short half-life, 6.78 min (Paccamonti *et al.*, 1999), the frequency of blood sampling could affect plasma oxytocin levels. Previous studies only collected one daily samples (Stevenson *et al.*, 1991) or small numbers of samples (Burns *et al.*, 1981). In this study, we used frequent blood sampling (5-min interval), longer periods of sampling (4 hours), larger numbers of mares (n=5), and a well-validated and sensitive radioimmunoassay. Therefore, the present data could be used as a reliable baseline of plasma oxytocin concentrations for further studies.

The results showed that the mean amplitude and frequency of oxytocin pulses did not differ during oestrus and until day 2 postovulation. However, mean concentrations were different: perhaps smaller differences in amplitude or frequency were not picked up in this study. Tetzke *et al* (1987) showed that mean amplitudes were greater on day 7 postovulation than on day 0 (ovulation), or day 3 and day 15 postovulation. Pulsatile oxytocin secretion has been observed in the ewe (Mitchell *et al.*, 1982), cow (Walters *et al.*, 1984) and mare (Tezke *et al.*, 1987) in agreement with 'spurt release' of oxytocin (Gibbens and Chard, 1976; Daawood, 1983).

The fact that plasma oxytocin concentrations are high during oestrus when uterine contractility at its most active (Nikolakopoulos, 1999) suggests a correlation between oxytocin/oxytocin receptors and sex steroid hormones. Oestrogen and progesterone control oxytocin receptor concentration/density and their sensitivity/affinity to oxytocin in the uterus in most species (Fuchs *et al.*, 1983; Soloff *et al.*, 1983; Soloff *et al.*, 1990; Maggie *et al.*, 1991). The present results suggest that the increased uterine contractility during oestrus may be related to the elevated concentrations of oxytocin. Oxytocin measured in plasma during oestrus comes mainly from posterior pituitary gland. It is also reported that the levels of uterine oxytocin are high in oestrus (Watson *et al.*, 2000) suggesting another possible source (See previous chapter). Jones *et al* (1991) measured uterine motility by electromyography and found that mares responded to oxytocin in all cycle stages, but they found a less pronounced response during dioestrus. Troedsson *et al* (1995a) also reported that an increased number of uterine myoelectrical activity bursts after oxytocin treatment was observed in oestrous mares compared to dioestrous mares.

They also suggested that the different characteristics of myometrial activity between oestrus and dioestrus are the result of hormone-dependent changes in myometrial cell communication such as the formation of gap junctions.

At the time of mating (oestrus) in mares, semen and bacteria are introduced into the uterus, resulting in a uterine inflammatory response (Troedsson *et al.*, 1995a). Most mares clear this inflammatory reaction from the uterus within 24-36 h of mating. However, inability to clear this inflammatory product from the uterus is a major cause of infertility. Between 11% and 39% of mares accumulate fluid at oestrus (Pycock and Newcombe, 1996b; Reilas *et al.*, 1997). Previous studies have shown that accumulation of intrauterine fluid in dioestrus is associated with a significant reduction in pregnancy rates and an increase in embryonic loss rate (Adams *et al.*, 1987). It is also reported that oestrous intrauterine fluid reduces spermatozoal motility in vitro, reduces embryo recovery rates (Squires *et al.*, 1989) and pregnancy rates (Pycock and Newcombe, 1996b). In this study, I have shown that there is a significant difference in the circulating plasma oxytocin concentrations between normal mares and mares with delayed uterine clearance. Previous studies showed that normal oestrous mares have significantly higher uterine contractile activity ($P<0.001$) than normal dioestrous mares and mares with delayed uterine clearance in both oestrus and dioestrus (Nikolakopoulos, 1999a). A recent study showed that there is no difference in endometrial oxytocin receptor concentrations between normal mares and mares with delayed uterine clearance (Cadario *et al.*, 1998), however myometrial receptors were not measured in that study. The results suggest therefore that low uterine contractility in the mares with delayed uterine clearance may be related to low plasma oxytocin concentrations, rather than oxytocin receptor concentrations. However, there may also be differences in these two groups of mares in post-receptor mechanisms as suggested in the study by Nikolakopoulos *et al* (2000a) where mares with delayed uterine clearance had significant lower release of $PGF_{2\alpha}$ after oxytocin administration. Vanderwall *et al* (1998) showed there is an eightfold difference between concentrations of oxytocin observed in blood samples from the intercavernous sinus versus the jugular vein. Therefore, the differences in pituitary oxytocin levels between normal mares and mares with

delayed uterine clearance are larger than plasma oxytocin levels. The low oxytocin levels in the mares with delayed uterine clearance may lead to defective myometrial contractility which causes the accumulation of uterine fluid after mating, in contrast to genitally normal mares that evacuate the intrauterine fluid in oestrus.

A previous study (Carnevale and Ginther, 1992) reported that old age was associated with increased endometrial inflammation, reduced pregnancy rate, high embryo-loss rate, and uterine contractile score as well as uterine tone was lower in the old than in the young mares. Although there was no significant difference in ages ($P>0.05$) between the two groups, mares with delayed uterine clearance used in this study were older than genitally-normal mares and had lower plasma oxytocin concentrations than the genitally-normal mare group. It is suggested that the pathogenesis of low pregnancy and high incidence of embryonic loss may involve the following progression: 1) reduced uterine contractility; 2) reduced clearance of foreign material from the uterus; 3) increased incidence of endometritis and intrauterine inflammatory exudates; 4) increased spermicidal (Squires et al., 1989) and embryocidal effects; and 5) reduced pregnancy rates and increased embryo-loss rates (Carnevale and Ginther, 1992). Rigby *et al* (2001) reported that the cellular defect for the contractile dysfunction of myometrium from older susceptible mares might be beyond the calcium regulatory system. This might include reductions in calmodulin protein expression, myosin light-chain kinase activity, or myofibrillar protein content (Marston and Pritchard, 1990). Previous studies also suggested that there may be a difference between genitally-normal mares and mares with PMIE at the level of the uterine oxytocin receptor, or post-receptor mechanisms (Nikolakopoulos *et al.*, 2000a).

Uterine contractility is an intricate interaction comprising myogenic (Guyton and Hall, 1996), neurogenic (Shew *et al.*, 1991; Shew *et al.*, 1992; Guyton and Hall, 1996) and hormonal factors (Garfield, 1984; Cadario *et al.*, 1995). In this chapter, I have reported reliable basal oxytocin levels in mares. Data showed there is a significant difference in plasma oxytocin concentrations between genitally-normal mares and mares with PMIE. These results suggest a hypothesis that there may be

differences in these two groups in mean plasma oxytocin concentrations level that might predispose to differences in uterine contraction after challenge, although no difference was found in oxytocin response to artificial insemination (Nikolakopoulos *et al.*, 2000b) in these two groups.

Further studies are required to determine the exact nature of the relationship between pituitary oxytocin secretion, uterine oxytocin secretion, and oxytocin receptors in myometrium and uterine contractility in the mares with delayed uterine clearance.

Chapter 7

General Discussion

The uterus functions primarily for gamete transport, provision of a suitable environment for fetal development, regulation of ovarian function, and pregnancy. The uterus has autonomous, spontaneous, and rhythmic contractions and relaxation of its smooth muscle, the myometrium. Clinical investigations on the organ have focused on its physiological role and the pathology that affects its function. Previous research has investigated uterine functions and dysfunctions as a basis for sub- or infertility in the mare. The pathogenesis of subfertility is based upon many factors. It is known that these clearance mechanisms are dependent on myometrial contractility.

Although the functions of each are important, the events related to uterine contractility have received most attention in equine reproduction. Uterine tone is controlled by the sympathetic and parasympathetic nervous system. As already shown in chapter 2 and 3, the equine uterus is innervated by a variety of nerves entering from uterovaginal plexus, not only adrenergic, but also various peptidergic nerves. The importance of adrenergic and peptidergic nerves in regulation of the female reproductive functions has been reported (Helm *et al.*, 1981; Blank *et al.*, 1986; Stejernquist and Owman, 1987; Renegar and Rexroad, 1990; Shew *et al.*, 1991; Atke *et al.*, 1996). Neurogenic mechanisms are important in regulation of ovarian function (Ottensen *et al.*, 1985; Schimidt *et al.*, 1990), myometrial contractility (Stejernquist *et al.*, 1983; Reinecke *et al.*, 1989; Shew *et al.*, 1993), endometrial secretion (Hammarström, 1980; Massmann *et al.*, 1992), and uterine blood flow (Morris, 1990; Majewski *et al.*, 1995). As in other species, our results showed that the myometrium, the endometrium and the vascular layers receive the same types of nerve supply, with the greatest density in the myometrium (See chapter 2 and 3). Taneike *et al* (1991) reported that there are layer-specific variations in the

functional innervation of the myometrium in the swine uterus: circular muscle layer is endowed with cholinergic innervation and the longitudinal muscle layer with adrenergic innervation. Liu *et al* (1998) using *in vitro* mechanical testing procedure for smooth muscle layers of the equine myometrium, reported that both circular and longitudinal muscle layers contract independently of one another and appear to be modulated by endocrine factors. Oestrogen and progesterone modulate the myometrial contractility response to noradrenaline (NA) by their action on myometrial adrenergic receptors, α and β (Figure 7.1). In the mare, the types and distribution of adrenergic receptors are not known. It is known that ovarian steroid hormones alter periarterial sympathetic nerve function (Ford *et al.*, 1984). Progesterone increases the uterine arterial contractility through an increase in the concentration of α_1 receptors, whereas oestrogen decreases contractility by decreasing receptor numbers. The importance of neurogenic factors in control of uterine contractility is shown by the use of clenbuterol, a β_2 sympathomimetic smooth muscle relaxant (Card and Wood, 1995; Gastal *et al.*, 1998), the α-adrenergic receptor antagonist, acepromazine, and the α-adrenergic receptor agonists, detomidine and xylazine (Gibbs and Troedsson, 1995). Studies showed that acepromazine, detomidine, xylazine and clenbuterol all significantly affect myometrial activity: clenbuterol (Gastal *et al.*, 1998; Nikolakopoulos and Watson, 1999) and acepromazine suppress myometrial activity while detomidine and xylazine stimulate the myometrial activity (Gibbs and Troedsson, 1995; DeLille *et al.*, 2000).

The results showed that among peptidergic nerves containing neuropeptides, NPY, VIP, CGRP, and SP, NPY was the most abundant neuropeptide in the equine uterus. These results are in agreement with other species such as guinea-pigs (Heinrich *et al.*,

1986; Alm and Lundberg, 1988; Mitchell and Ahmed, 1992), pigs (Lakomy *et al.*, 1994), and humans (Owman *et al.*, 1986). A previous study suggested that these neuropeptides have various functions not only neurotransmitter or neuromodulatory but also act as growth regulators (Woll and Rozengurt, 1989). Although there were no significant changes in uterine innervation between estrus and dioestrus mares in this study, changes in the distribution of innervation, adrenergic and peptidergic, during the oestrous cycle in the rat (Zoubina *et al.*, 1998) and pre- and post-pregnancy (Thorbert, 1978; Alm *et al.*, 1988) were reported. Therefore, in future work a quantitative study including radioimmunoassay would give more detailed information on changes in uterine innervation at different stages of the cycle.

Neuropeptides are found to colocalise and have reciprocal actions: Neuropeptide Y is a potent inhibitor of VIP-induced relaxation (Ottensen *et al.*, 1983; Jørgensen, 1994) with SP having a contractile effect and CGRP having a relaxing effect (Shew *et al.*, 1991). Therefore, future study needs to examine whether mares with delayed uterine clearance have different innervation compared with normal mares and whether there is any dysfunction in the actions of the neuropeptides.

It is reported that uterine contractility is important in clearing intrauterine fluid but the most important action is during parturition. Both events occur through the cervix. The high density of innervation in the cervix suggests a possible role in regulating cervical closure in dioestrus and during pregnancy, and in relaxation of the cervix at oestrus and during parturition in the mare.

In this study, using samples from mares with grass sickness it showed that

decreased number of nerves or disturbance in neurotransmitters in the nervous system could affect uterine contractility as already shown in the alimentary tract (Murray *et al.*, 1994). These damaged neuronal factors could affect uterine contractility directly or indirectly by interfering with hormonal factors or formation of cAMP (Figure 7.1).

The evidence of oxytocin production in the equine endometrium (See chapter 5) shows that uterine oxytocin appears to play an important role in regulation of either uterine contractility or endometrial secretion in the mare. The fact that oxytocin-mRNA levels were higher during oestrus (Behrendt-Adam *et al.*, 1999) and immunostaining for oxytocin was also stronger in oestrus than in dioestrus (Watson *et al.*, 2000) could explain the possible role of uterine oxytocin in increasing uterine contractility during egg and sperm transport (Gilbert *et al.*, 1992) and facilitate uterine clearance mechanisms after breeding. Stout *et al* (2000) also suggested that oxytocin may act in a paracrine manner to elicit endometrial $PGF_{2\alpha}$ synthesis at luteolysis. The possible role as an initiating stimulus for parturition was also suggested (Lefebvre *et al.*, 1992b; Chibbar *et al.*, 1993; Boulton *et al.*, 1996). The neuronal damage could also affect endometrial oxytocin, not only through endometrial secretion, but also production of uterine oxytocin and $PGF_{2\alpha}$, and sensitivity of hormonal receptors.

The data also showed significantly higher concentrations of plasma oxytocin during oestrus ($P<0.05$), a period characterised by low progesterone and high oestrogen levels (Mienecke *et al.*, 1987) than the day of ovulation. The ability of oxytocin to control myometrial contractility depends not only on its plasma

concentrations, but also on the number of its receptors in the myometrium (Soloff, 1990). This correlation is also involved in sex steroid hormones, oestrogen and progesterone, and nerves and receptors. A field fertility study including more than 400 mares revealed that persistent post-mating induced endometritis (PMIE) affects approximately 15% of mares (Zent *et al.*, 1998) and results in reduced fertility (Pycock and Newcombe, 1996a; Newcombe, 1997). Impaired uterine contractility is thought to contribute to the inability of mares to clear uterine fluid (Troedsson *et al.*, 1993a; LeBlanc *et al.*, 1994), in association with other factors such as functional disturbances of the endometrium (Schoon *et al.*, 1998) or uterine positioning (LeBlanc *et al.*, 1998). Mares that are susceptible to PMIE have delayed uterine clearance associated with reduced uterine contractility. The mechanism for reduced uterine contractile activity remains an enigma. Mares that are considered to be susceptible to PMIE are older mares (Carnevale and Ginther, 1992) that have delayed physical clearance of uterine contents (Evans *et al.*, 1987; Troedsson and Liu, 1992), which is associated with dysfunctional uterine contractile activity (LeBlanc *et al.*, 1994). The reasons for this dysfunction in contractile mechanisms could include 1) changes in the release, either systemically or locally, of oxytocin and prostaglandins; 2) altered production of neurotransmitters that affect myometrial activity; or 3) intrinsic change within the uterine muscle that renders it incapable of responding with normal contractile force.

It is suggested that structural changes within the uterus of mares with delayed uterine clearance may result from repeated, prolonged stretching of the myometrium during pregnancy. De Lille *et al* (2000) reported that compared to normal mares, mares with delayed uterine clearance responded to oxytocin treatment with enhanced

intrauterine pressure, measured in vivo, if they had been pretreated with sedatives that activated α-adrenergic receptors on the myometrium. They suggested that denervation supersentitivity of the myometrium may result from damage to uterine nerve fibres caused by repeated, prolonged stretching of the uterus during pregnancy. Other structural changes that have been reported to occur within the uterus with successive pregnancies include increased myometrial collagen and elastin content in the nongravid human uterus (Guna-Smith and Woessner, 1985). Delayed uterine clearance is associated with older brood mares (Carnevale and Ginther, 1992), and increased collagen deposition within the endometrium of older mares also is well documented (Kenny, 1978). However, increased age is not necessarily associated with the decreased intrinsic contractility seen in myometrium from older mares with delayed uterine clearance (Rigby *et al.*, 2001). In this study, it appeared that there was an intrinsic contractile dysfunction in the myometrium from mares with delayed uterine clearance that was not dependent on age or parity. Our study showed that there is a significant difference ($P<0.01$) in plasma oxytocin concentrations between genitally-normal mares and mares developing delayed uterine clearance. This demonstrates the importance of plasma oxytocin levels in regulating uterine contractility. Further studies will need to investigate the differences in oxytocin receptors in both the endometrium and the myometrium, between these two groups.

In order to understand myometrial contractility, the interaction of actin and myosin which is regulated by myosin light chain phosphorylation (in contrast to skeletal muscles in which regulation occurs through troponin/tropomyosin associated with the actin filament) needs to be explained. Myosin light chain kinase (MLCK) is the key enzyme for myosin phosphorylation and for regulation of myometrial

contractility (Olins and Bremel, 1982; Walsh, 1985; Huszar and Walsh, 1989). In the case of the myometrium, regulation is more complex, because the cellular events of myometrial contractility are modulated by the endocrine events of the cycle as well as nervous system (Figure 7.1). With respect to myogenic factors, in cellular regulation of myometrial contractility, the MLCK is regulated through the calcium (Ca^{2+}) and cyclic adenosine monophosphate (cAMP) pathways. Muscle cells in the myometrium are interconnected as a functional unit in simultaneous action. The essential parts in the functional/metabolic coordination of contraction are the gap junctions (Garfield *et al.*, 1980; Garfield, 1984). Modulation of coordinated contraction among the myometrial smooth muscle cells through gap junctions, along with other associated cellular events, are regulated by oestrogen and progesterone, as well as oxytocin, prostaglandins, cAMP and calcium (Garfield *et al.*, 1980; Olins and Bremel, 1982; Fuchs *et al.*, 1983; Flint *et al.*, 1986; Nimmo *et al.*, 1991; Okawa *et al.*, 1993; Sharp *et al.*, 1997). Therefore, due to the complex nature of regulation of uterine contractility, studies that aim to understand impaired myometrial contractility have to investigate all interacting factors, myogenic, neurogenic and hormonal, not each factor individually. Furthermore, the dysfunction of contractile activity was not receptor dependent and did not result from altered regulation of intracellular Ca^{2+} concentration (Rigby *et al.*, 2001).

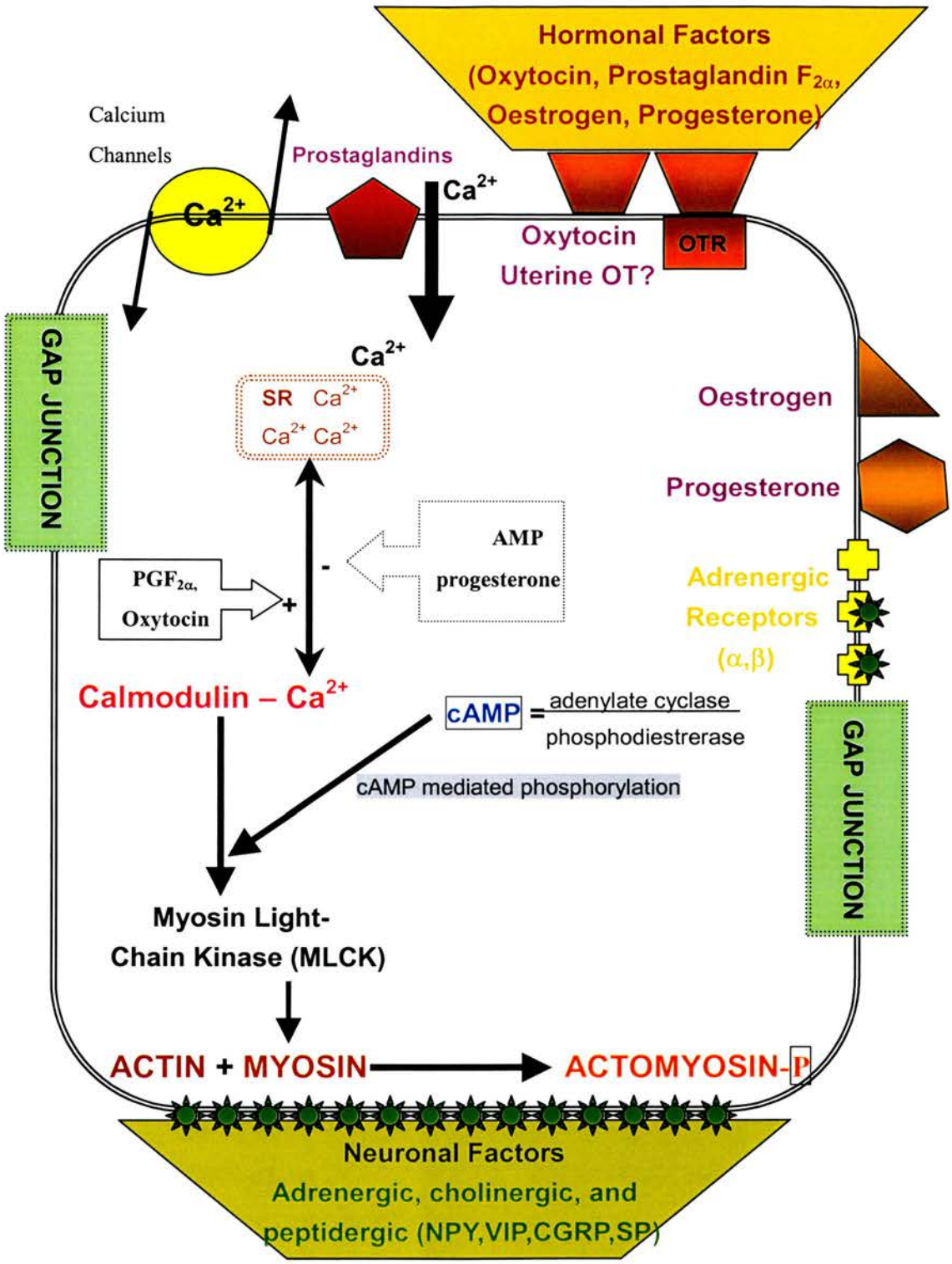


Figure 7.1. Modulation of myometrial contractility in a smooth muscle cell. The uterine contractility is regulated by interaction/coordination of myogenic, hormonal and neuronal factors. There could be dysfunction of uterine contractility if there is damage to any of these factors.

In conclusion, to regulate uterine contractility, all factors, neurogenic, myogenic, and hormonal factors need to coordinate/interact with each other. This thesis presented factors associated with uterine functions, the neuronal and hormonal factors, in the mare. The equine uterus receives a variety of nerve supply, mainly adrenergic as well as peptidergic mostly in the cervix within regions and mostly in the myometrium within structures. Among the neuropeptides, neuropeptide Y was the most abundant with similar distributive pattern to adrenergic nerves. Vasoactive intestinal polypeptide and calcitonin gene-related peptide were less abundant and substance P was sparse. With hormonal factors, equine endometrium produces uterine oxytocin and stores them in the secretory vesicles of the secretory cells in the luminal epithelium and in superficial endometrial glands. Circulating plasma oxytocin levels were higher ($P<0.05$) in oestrus than on the day of ovulation. In addition, plasma oxytocin levels were significantly higher ($P<0.01$) in genitally-normal mares (9.67 ± 0.84 pg/ml) compared to that in mares with delayed uterine clearance (4.48 ± 0.24 pg/ml).

Future work would be:

- to colocalise adrenergic and peptidergic nerves (e.g. NPY and adrenergic, NPY and VIP, and CGRP and SP) and to clarify the precise physiological role of peptidergic nerves in the regulation of the equine reproductive tract,
- to examine the uterine innervation of mares with delayed uterine clearance to determine whether these mares have a problem in distribution of nerves with quantitative analysis,
- to examine the types and distribution of adrenergic receptors (α and β) in genitally-normal mares and in mares with delayed uterine clearance,

- to investigate whether there is differences between quantity of uterine oxytocin between normal mares and mares with delayed uterine clearance,
- to obtain more samples from mares with delayed uterine clearance through the oestrous cycle and to examine oxytocin and $PGF_{2\alpha}$ concentrations, and
- to compare the distribution and the number of oxytocin receptors in the myometrium and the endometrium of genitally-normal mares and mares with delayed uterine clearance.

Appendix

1. Immunohistochemistry techniques

1.1 Reagent and buffers

Sources of specific chemicals and avidin-biotin complex kits are described in the relevant protocols. All other chemicals used were purchased from Sigma Chemical Co. Ltd., Poole, Dorset, UK., British BioCell International, Cardiff, UK or Vector Laboratories, Cambridge, UK.

1.1.1. PBS: Phosphate buffered saline (pH 7.2)

Na_2HPO_4 1.48 g

KH_2PO_4 0.43 g

NaCl 7.2 g

NaN_3 1.3 g

Made up to 1litre with distilled water then adjusted to pH 7.2.

1.1.2. TBS: Tris buffered saline (pH 8.2)

Add 100 ml distilled water

+ 0.242 g (20 mM) Tris (tris-hydroxymethyl-aminomethane)

+ 0.13 g (20 mM) NaN_3 (preservative)

+ 0.9 g (225 mM) NaCl Adjusted to pH 8.2.

1.1.3. Sodium cacodylate buffer solution (0.1 M, pH 7.2)

Dissolve 10.7 g of sodium cacodylate in 1 litre of distilled water.

Adjust to pH 7.2 with HCl.

1.1.4. 4% Paraformaldehyde

1. Heat 100 ml PBS to 60 °C.
2. Add 8 g of paraformaldehyde.
3. Add NaOH until paraformaldehyde dissolves.
4. Make up to 200 ml and cool under tap.
5. Adjust pH to 7.0.
6. Store at 4°C (up to a week)

1.1.5. Blocking agent (Glucose oxidase)

Stock solutions

B-D Glucose	1.802 g/100 ml PBS (100 mM)
Glucose oxidase	0.0054 g/1 ml PBS (100 units/ml)
Sodium Azide	0.065 g/100 ml PBS (10 mM)
PBS	0.1 M (pH 7.3)

Working solutions

B-D Glucose stock	1 ml
Sodium Azide stock	1 ml
Glucose oxidase	0.5 ml (made up fresh each time)
PBS stock	7.9 ml

1.1.6. Incubation buffer (Buffer A)

PBS

+ 1% Normal goat serum

+ 0.1% Tween 20

+ 1% BSA

+ 0.1% sodium azide adjusted to pH 8.2.

1.2. Tissue processing for EM

1) Fixation: a mixture of 4% paraformaldehyde/0.1% glutaraldehyde in 0.1M sodium cacodylate buffer for 2 h.
2) Wash: 0.1M sodium cacodylate buffer 3 x 20 min.
3) Post-fixation: 1% osmium tetroxide in 0.1M sodium cacodylate buffer for 1h.
4) Wash: distilled water 3 x 20 min.
5) Dehydration:

   50 % Acetone 1 x 10 min

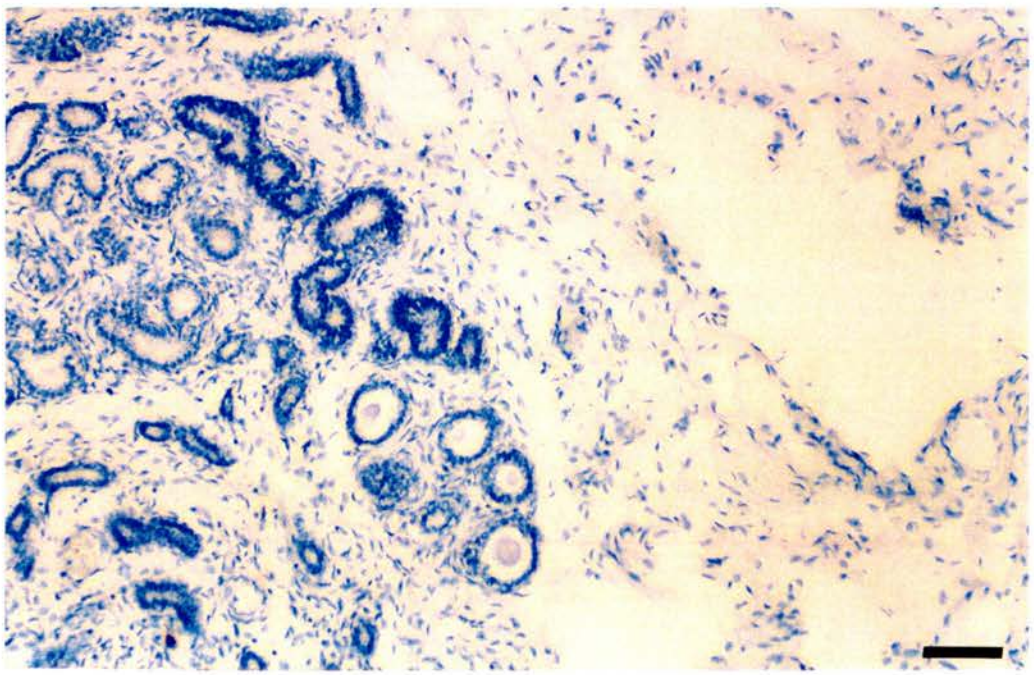
   70 % Acetone 1 x 10 min

   90 % Acetone 1 x 10 min

   100 % Acetone 3 x 10 min
6) Embedding: Unicril (overnight) then gelatine capsule
7) Ultrathin sectioning

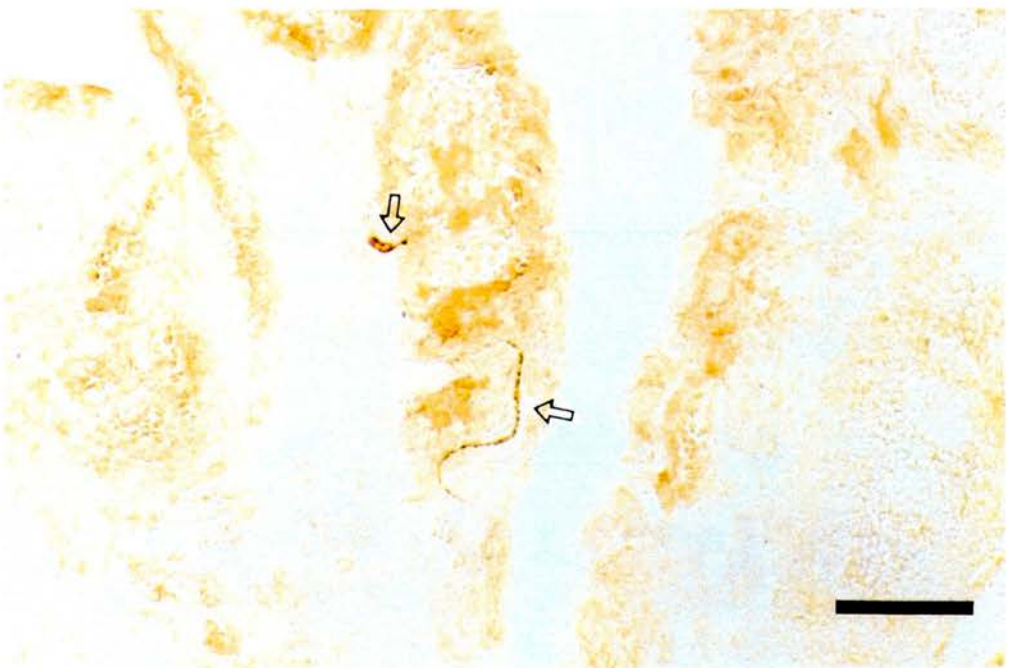
2. Illustration for a subjective graded scoring system (0-5)

1) Score 0: no nerve fibres



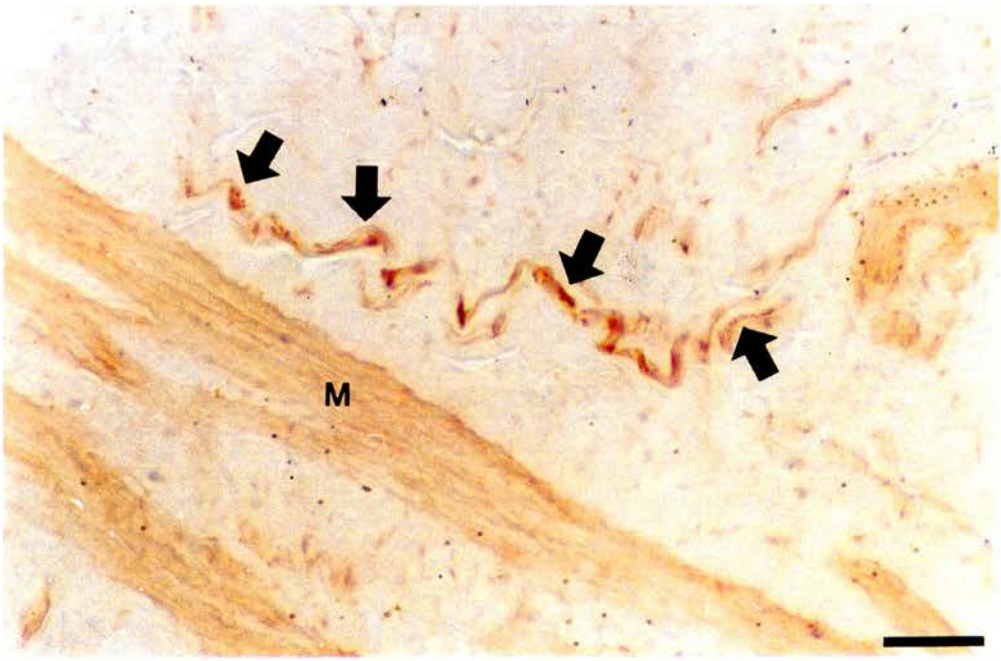
Substance P in the uterine body, counterstained with haematoxylin. Scale bar = 100μm.

2) Score 1: <5 nerve fibres



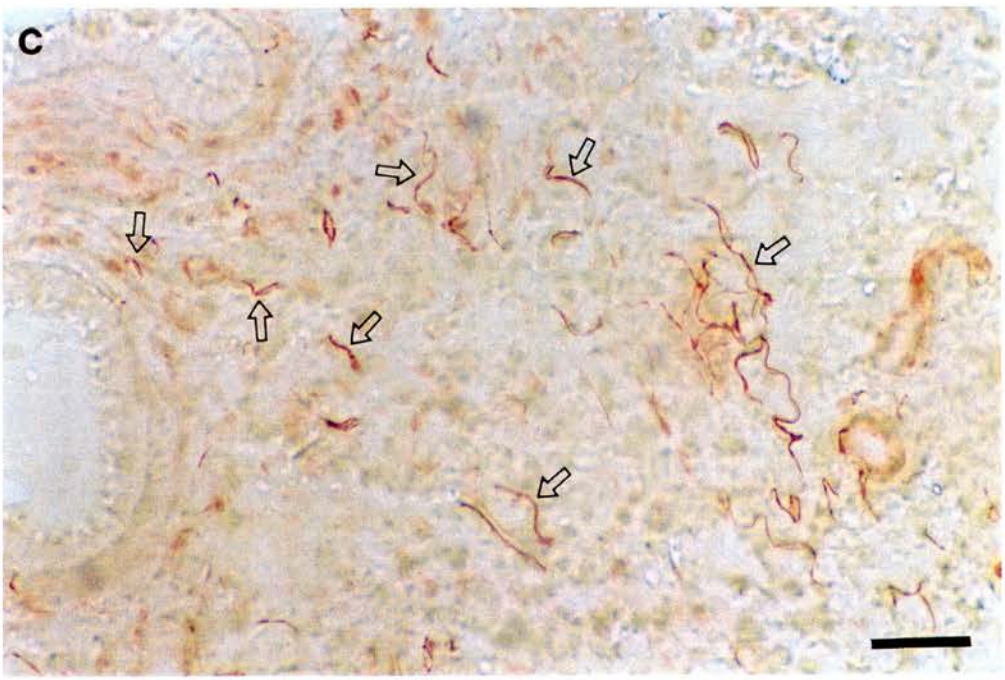
VIP-IR nerve fibres (⇨) in the endometrium of the uterine horn. Scale bar=50μm.

3) Score 2: <20 nerve fibres



CGRP-IR nerves (➡) in the cervix. M: smooth muscle bundles. Scale bar=50μm.

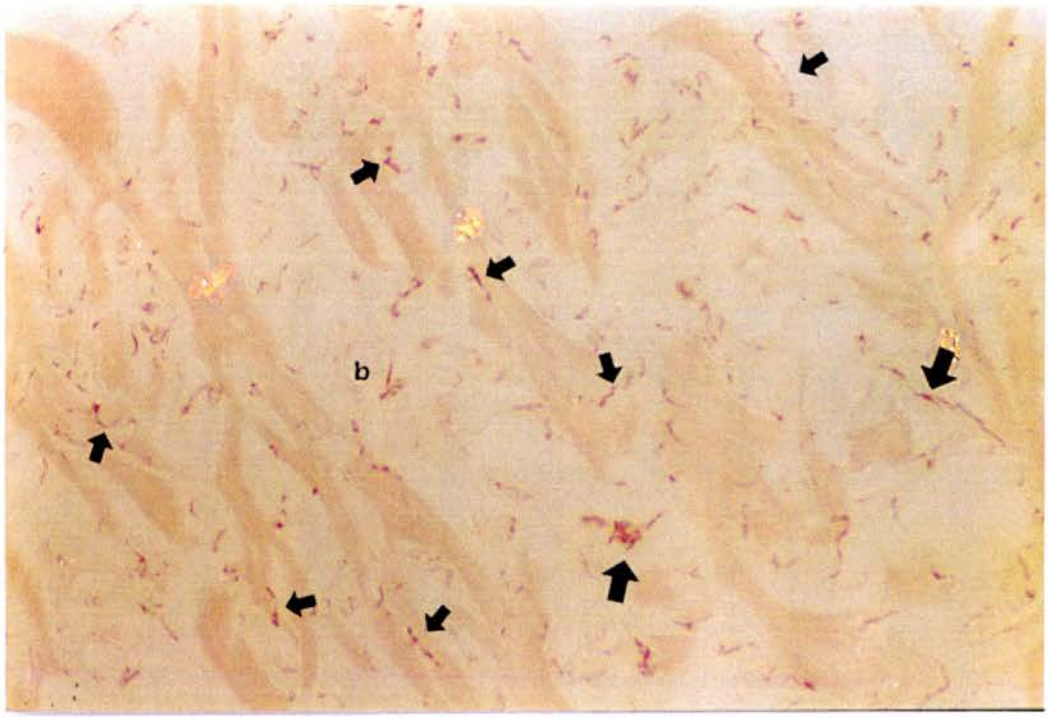
4) Score 3: <50 nerve fibres



(x20)

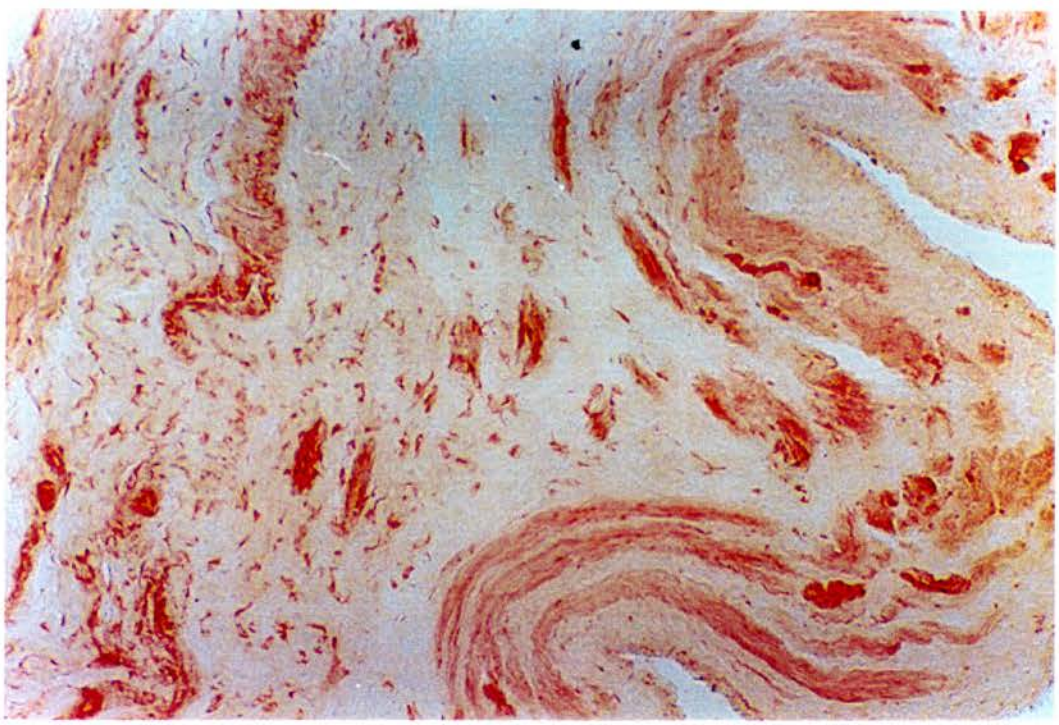
TH-IR nerves (⇨) in the endometrium of the uterine body. Scale bar=50μm.

5) Score 4: >50 nerve fibres and a small number of nerve bundles



TH-IR nerves (➡) in the myometrium of the uterine horn (x4).

6) Score 5: >50 nerve fibres and a large number of nerve bundles



PAN-N-IR nerves in the broad ligament (x4).

3. Oxytocin assay

3.1. Reagents required

Veronal Buffer (pH 9.0)

	per litre	per 500 ml
Barbituric Acid	2.56 g	1.28 g
Sodium Chloride	8.28 g	8.28 g
L-Cysteine	0.016 g	0.008 g
EDTA disodium salt	3.72 g	1.86 g
Bovine γ-globulin	2.0 g	1.0 g

Made up to 1 litre, adjust pH to 9.0 with 40% NaOH

Store at 4 °C until use

3.2. Antisera (A/S)

GJ137 used at 1:20,000. (A/S raised in sheep by Dr. Tony Flint.)

From lyophilised stock make up 1:50 dilution, store in 0.5 ml aliquots at -20°C. To use in assay take one of these aliquots and make up to 200 ml in Veronal buffer.

Store at +4°C prior to use.

3.3. Count

Oxytocin iodinated using the Chloramine T method.

Make up counts in buffer to give approximately 4000-4500 *g* per tube (i.e. per 100 µl)

3.4. OT Standards

Oxytocin (Peninsular lab) stock 500 µg/ml

20µl from the stock and make up to 1ml with assay buffer (Veronal buffer pH 9.0).

This will give a stock solution of 10 µg/ml which = **10,000 ng/ml.**

(1)	Diluting stock 1:500 = 10 µl made up to 5 ml = 20 ng/ml = 2 ng/tube (each tube has a volume of 100 µl)		
(2)	dil (1)	1:1 =	2 ml of (1) made up to 4 ml = 10 ng/ml = 1 ng/tube
(3)	dil (2)	1:1 =	2 ml of (2) made up to 4 ml = 5 ng/ml = 0.5 ng/tube
(4)	dil (3)	1:1 =	2 ml of (3) made up to 4 ml = 2.5 ng/ml = 0.25 ng/tube
(5)	dil (4)	1:1 =	2 ml of (4) made up to 4 ml = 1.25 ng/ml = 0.125 ng/tube

3.5. Radioimmunoassay (RIA) for oxytocin

3.5.1. Equipment and materials

Sample concentrator: Centrifugal Evaporator RC 10-22 (Jouan, Herts, UK)

Vaccum manifold (Vac-Elut) and reservoirs

Extraction cartridges: SepPak C18 (Waters, Milford, USA)

Poly-prep chromatography columns (Bio-Rad Laboratories, Hercules, USA)

Glass rimless tubes 60 x 12 mm

Methanol

Acetic Acid

Urea

3.5.2. Extraction Methods

- thaw samples
- centrifuge samples (4°C, 2500 *g*, 15 min)
- extraction

1) 5 ml 100% Methanol (Vacuum On)
2) 20 ml H_20 (Vacuum On)
3) 2 ml Samples (Vacuum On)
4) 20 ml 4% Acetic acid (Vacuum On)
5) Vaccum Off then glass-tubes in the Vac-Elut for sample collection
6) 20 ml 80% Methanol/Acetic acid (Vacuum On)
7) Glass-tubes out (Vacuum Off) then Sample concentrator: using Centrifugal Evaporator for overnight
8) 5 ml 8 M Urea (Vacuum On)
9) 20 ml H_2O, 2 times (Vacuum On)

3.5.3. Radioimmunoassay procedure for oxytocin

Assay performed in polyestyrene LP3 tubes.

50µl standard or sample (0,1.56, 3.13, 6.25, 12.5, 25, 50, 100, and 200 pg/ml)

100µl Antiserum (diluted with Veronal buffer in 1:20,000)

100µl Counts < I^{125} labelled oxytocin was made up in Veronal buffer to give 40,000 cpm in 100 µl>

PEG 6000 (Supplied by BDH and made up in distilled water to give a 30% solution)

Day 1

- Standards: 50 µl from standard vials + 100 µl antisera
- Non-specific binding (NSB): 50 µl from standard + 100 µl buffer
- Evaporated Samples: a) add 150 µl buffer (vortex individually)
- Samples: 50 µl from a) +100 µl antisera
- All tubes were vortexed and incubated overnight at 4°C

Day 2

- Add 100 µl I^{125} to standards, NSB, and samples
- Include 2 glass vials with 100 µl I^{125} (use plastic tops)
- All tubes were vortexed and incubated overnight at 4°C

Day 3

- Add 200 µl 30% PEG 6000 to standards, NSB, and samples
- Shake well, centrifuge for 20 min at 2500 *g*
- Supernatant was carefully decanted and the activity in the precipitate counted using a Clinigamma 1272, LKB-Wallac (E.G. & G. Wallac, Milton Keynes,

UK)

The extraction recovery rate was 75 %. The detection limit for the assay was 0.7 pg/ml. The intra- and inter-assay coefficients of variation were 3.7 % and 7.9 %, respectively.

Publications

List of Publications

1.**S-E Bae, BM Corcoran and ED Watson** (2001) Organisation of uterine innervation in the mare: distribution of immunoreactivies for the general neuronal markers; protein gene product 9.5 and PAN-N *Equine Veterinary Journal* **33**:323-325

2. **S-E Bae, BM Corcoran and ED Watson** (2001) An immunohistochemical study of the distribution of adrenergic and peptidergic innervation in the equine uterus and the cervix *Reproduction* **122:**275-282

Abstracts

1. **S-E Bae, BM Corcoran and ED Watson** Immunohistochemical localisation of adrenergic and peptidergic nerves in the uterus of the mare. *Journal of Reproduction and Fertility* (1999) Abstract series **23:**59

2. **S-E Bae and ED Watson** Immunohistochemical localisation of oxytocin and neurophysin in the equine endometrium using transmission electron microscopy. *Journal of Reproduction and Fertility* (1999) Abstract series **24:**65

3. **S-E Bae and ED Watson** Immunohistochemical localisation of oxytocin and neurophysin in the equine endometrium using transmission electron microscopy. *14th International Congress on Animal Reproduction* (2000) **1:**Abstract 55

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