CHAPTER 5

GENERAL DISCUSSION

This study details for the first time the entire sympathetic, parasympathetic and sensory innervation of the non-pregnant rat uterus. The uterine innervation was revealed by an immunoperoxidase method for staining whole mount preparations that was developed during the course of this project. The sympathetic, parasympathetic and sensory innervations of 20 day pregnant rat uterus were studied using the same method.

The non-pregnant rat uterus was found to be densely innervated by sympathetic, parasympathetic and sensory nerves. Intensely stained plexuses of sympathetic and parasympathetic innervation surrounded blood vessels. Sensory nerves were found in similar densities in the muscle and endometrium of the non-pregnant uterus. Linea uteri, thick bundles of longitudinal muscle opposite the mesometrium were seen in the non-pregnant rat uterus from different strains of rats. This is the first study to examine the innervation of linea uteri and has shown that linea uteri have more TH-, NPY-, CGRP- and VAChT-positive axons than the rest of the uterine smooth muscle. Near the circular muscle-endometrium boundary, a network of fine varicose axons immunoreactive for TH, NPY, CGRP, SP, NOS and VAChT oriented parallel to the circular smooth muscle cells were present on the mesometrial side of the uterus. However, this network did not occur under the linea uteri on the anti-mesometrial side.

The uterus at day 20 of a 21 to 22 day pregnancy showed almost complete sympathetic, parasympathetic and sensory denervation. Rare nerves immunoreactive for TH and NPY were seen at the mesometrial side in the ovarian region of the pregnant uterus. TH axons were slightly more numerous in the cervical region compared to the

ovarian region of the pregnant uterus. Very few SP, CGRP, NOS and VAChT axons occurred in the ovarian region of the pregnant uterus.

The pregnant rat uterus stretched much more than the non-pregnant rat uterus. In whole mount preparations of pregnant uterus, the uterine wall was much thinner than in non-pregnant rats, probably because of this stretchiness. The pregnant rat uterus was 4 times larger in length, 3.5 times larger in width, 15 times larger in area, and 11 times heavier than the non-pregnant rat uterus. The increase in size of the pregnant uterus is necessary in order to accommodate the growing foetuses.

Whole mount preparations

Most of the published observations on uterine innervation are from sections. In sections, only very small portions of uterine tissue can be examined. The orientation and paths that axons take cannot be seen in sections unless many sections are cut serially and all are examined, a process that requires extensive labour and time. Earlier studies that used whole mount preparations have reported difficulties in assessing innervation. For example, Papka et al. (1985) were unable to extensively analyse whole mounts because of their thickness and the many different focal planes that needed to be photographed. Haase et al. (1997) examined acetylcholinesterase (AChE)-stained uterine whole mounts in Petri dishes containing glycerine using a dissecting microscope and needed careful manipulation of light sources for assessment. As a result, these authors were able to publish only low magnification diagrams of AChE staining, which contained little detail about the actual trajectories of cholinesterase-positive nerve bundles and axons. In the current project, a method for peroxidase immunostaining of uterine whole mounts and embedding them in resin was developed. This method enabled me to successfully assess and photograph the innervation of entire uterine horns without any of the difficulties experienced previously.

Whole mount preparations enabled me to assess the innervation of linea uteri for the first time. Densities of axons in each layer of the uterus and their associations with blood vessels could be identified without difficulty. Whole mounts also made it possible for axons to be mapped in their entry until they terminated. Axonal paths could be traced from nerve bundles associated with blood vessels in the myometrium to the endometrium and in some instances close to uterine glands. This sort of data in particular would have been impossible to collect in sections. Finally, whole mounts made possible low magnification micrographs showing the actual courses of clearly visible large and small nerve bundles and more detailed high magnification micrographs of the smallest nerve bundles and individual axons.

Linea uteri

Linea uteri were found in the non-pregnant uteri in all three rat strains examined in this project. Only the conductive, functional, pharmacological and morphological properties of linea uteri have previously been reported (Melton & Saldivar, 1967; Borda et al., 1978). Surprisingly, the presence of linea uteri has not been noted in any of the previous studies on uterine innervation. Consequently, this study is the first to investigate the innervation of linea uteri and has shown that, in the non-pregnant rat uterus, linea uteri are more heavily innervated by TH-, NPY-, CGRP- and VAChT-immunoreactive axons compared to the rest of the uterine smooth muscle. This increased density of innervation may be due to the presence of more longitudinal muscle cells in the linea uteri and may have an important relationship to conductivity (Melton & Saldivar, 1967) in the non-pregnant rat uterus. In linea uteri, the conduction velocity, frequency and amplitude of contractions are greater than in the rest of the uterine smooth muscle (Melton & Saldivar, 1967). Linea uteri were not seen in the uteri of the four rats examined at day 20 of a 21 to 22 day pregnancy.

Innervation of the non-pregnant uterus

The results of this study agree with previously published data on the sympathetic innervation of the non-pregnant rat uterus. Sympathetic innervation has been extensively studied in the non-pregnant rat uterus using TH-, NPY- or DBH-immunoreactivity and using formaldehyde- and glyoxylic acid-induced fluorescence. Sympathetic nerves have been documented in all layers of the uterus and occur in particularly high density around blood vessels (Zoubina et al., 1998; Bae et al., 2001). In the myometrium, sympathetic nerves follow the orientation of smooth muscle cells (Bae et al., 2001). With the use of whole mounts, I have been able to show for the first time that there is a difference in the density of innervation by TH and NPY axons in the linea uteri, which are more heavily innervated that the rest of the uterine smooth muscle. Also for the first time, rare TH and NPY axons close to uterine glands have been demonstrated. The gradual decrease in the density of sympathetic axons from their entry into the uterus until their termination was clearly visible in the whole mount preparations. Tracing the trajectory of axons from nerve bundles associated with blood vessels in the myometrium to the endometrium has also revealed that some sympathetic axons take crooked paths through many different focal planes before terminating deep in the endometrium. In this project, only rats at the estrous or late estrous/early diestrous stage of the estrous cycle were used. It was therefore not possible to confirm that sympathetic innervation varies with the estrous cycle (Zoubina et al., 1998).

Parasympathetic nerves revealed by VIP- and NOS-immunoreactivity have been reported to occur in all layers of the uterus. Parasympathetic nerves do not change in density during the estrous cycle and densely innervate blood vessels (Zoubina et al., 1998; Bae et al., 2001; Majewski et al., 1995). In this project, I have used NOS- and VAChT-immunoreactivity to assess parasympathetic innervation in uterine whole mounts. NOS-

and VAChT-containing axons were found in all layers of the non-pregnant rat uterus. For the first time, I have shown that the linea uteri are more heavily innervated by VAChT-immunoreactive axons compared to the rest of the uterine smooth muscle. NOS-immunoreactive axons did not show any difference in innervation between the linea uteri and the rest of the smooth muscle. NOS- and VAChT-positive axons heavily innervated blood vessels. Fine varicose axons arising from nerve bundles associated with blood vessels in the myometrium were seen in the endometrium. For the first time, the paths of NOS- and VAChT-containing axons could be followed from their entry into the uterus at the mesometrium until they terminated in the endometrium.

Sensory nerves have been shown to innervate the non-pregnant rat uterus. Sensory nerves revealed by CGRP-, SP- or NKA-immunoreactivity occur in all layers of the uterus (Papka et al., 1985; Zoubina et al., 1998; Bae et al., 2001; Alm & Lundberg, 1988). Sensory neurons in the dorsal root ganglia (DRG) express SP, CGRP, NKA, VIP, SOM, galanin, opioid peptides and atrial natriuretic peptide (Lawson, 1995). Sensory neurons with unmyelinated (C fiber) axons express SP and CGRP. In general, SP and CGRP are released by neurons in DRG in response to pain (Kingsley, 2000). In the uterus, SP regulates contraction of the myometrium and is vasodilatory; CGRP is also vasodilatory and relaxes the smooth muscle (Traurig & Papka, 1993; Yallampalli et al., 2002; Gangula et al., 2003). In this project, I used CGRP and SP to identify sensory nerves in uterine whole mounts. CGRP-immunoreactive axons are more numerous in the linea uteri; SP-immunoreactive axons did not show any difference in density between the linea uteri and the rest of the uterine smooth muscle. The density and the number of nerve bundles containing SP- and CGRP-immunoreactive axons associated with blood vessels was less compared to perivascular sympathetic innervation in the non-pregnant uterus. More CGRP-positive axons were found close to the uterine glands compared to TH and NPY.

Both SP and CGRP-containing axons occurred deep in the endometrium. These axons arose from nerve bundles associated with blood vessels in the myometrium. Like TH, NPY, NOS and VAChT, SP and CGRP axons could also be mapped from their entry into the uterus until the axons terminated. This type of assessment has not been possible using sections.

Innervation of the pregnant uterus

Sympathetic denervation of the uterus during pregnancy has been studied in rats (Klukovits et al., 2002; Chavez-Genaro et al., 2006), guinea pigs (Fried et al., 1985) and humans (Nakanishi et al., 1969; Fried et al., 1986). All of these studies, which used either glyoxylic acid fluorescence or immunohistochemistry for TH and NPY, have shown that the innervation of the uterus is dramatically reduced during pregnancy. In this project TH- and NPY-immunoreactivity were used in whole mount preparations to extensively examine sympathetic denervation during pregnancy. Rare TH axons were found around blood vessels at the mesometrium but gradually disappeared completely. In the ovarian end of the pregnant uterus, TH and NPY axons were only seen in the mesometrial region in very small numbers. The anti-mesometrial side of the ovarian region did not show any TH or NPY immunoreactivity. TH and NPY axons occurred between the circular muscle layer and the anti-luminal side of the endometrium. TH and NPY axons were not found deep in the endometrium or in the muscle layers in the pregnant rat uterus. There was a difference in TH and NPY innervation in the pregnant uterus. Although innervation was dramatically reduced, TH axons were more numerous than NPY axons in the cervical region of the pregnant rat uterus, indicating that the co-localization of TH and NPY may not be complete in the pregnant rat uterus.

The present project has demonstrated that there is a massive reduction in SP- and CGRP-immunoreactive axons in the pregnant rat uterus. This is the first study to show

pregnancy-induced denervation in CGRP-containing axons in the rat. SP- and CGRP-positive axons were not seen in all layers of the pregnant uterus; they were absent in the muscle layers and deep in the endometrium. Very few SP and CGRP axons were found in the endometrium at the mesometrial side, where they entered the pregnant rat uterus. SP and CGRP axons were absent on the anti-mesometrial side. SP- and CGRP-immunoreactive axons were not found in association with blood vessels in the pregnant rat uterus. Alm and Lundberg. (1988) studied the innervation of the guinea pig uterus at full-term pregnancy and reported that there were no SP- or CGRP-containing axons at that stage. However, they examined only 8 to 20 15µm-thick sections per guinea pig from 6 to 10 guinea pigs. This is a very small sample considering the increase in size of the uterus during pregnancy and they could have easily missed rare SP- and CGRP-immunoreactive axons. Traurig et al. (1984) reported a reduction in SP-immunoreactivity in the rat uterus at day 19 of pregnancy compared to the non-pregnant rat uterus using radioimmunoassay (Traurig et al., 1984).

In this project, whole mounts have enabled assessment of parasympathetic nerves in pregnant rat uteri, using VAChT- and NOS-immunoreactivity as neurochemical markers for these nerves. Pregnancy significantly reduced the parasympathetic innervation of the rat uterus. Rare nerves immunoreactive for NOS and VAChT were seen at the mesometrial side as they entered the uterus. These axons gradually disappeared towards the anti-mesometrial side. NOS and VAChT axons were not seen around blood vessels or in all layers of the uterus. This is the first study to assess innervation of VAChT axons in the pregnant rat uterus. Immunohistochemistry for nNOS by Riemer et al. (1997) did not reveal any immunoreactive axons in the pregnant rat uterus. However, again, only small samples of 1 × 1 cm full thickness uterine tissue was examined.

Why does uterine innervation change during pregnancy?

Hormonal influence has been suggested as the possible cause for sympathetic neuronal changes observed during the estrous cycle and during pregnancy. When estrogen was administered to ovariectomized rats, a reduction in sympathetic innervation revealed by DBH-immunofluorescence was seen in the longitudinal and circular smooth muscle and endometrium (Zoubina & Smith, 2001). This influence of estrogen is mediated by estrogen receptor alpha (Zoubina & Smith, 2001). Other factors, such as neurotrimin (Ntm), a neural cell adhesion protein, are also involved in plastic changes in sympathetic innervation (Krizsan-Agbas et al., 2008).

Neurotrophic factors may also contribute to the denervation of the uterus during pregnancy (Brauer, 2008; Latini et al., 2008). The role of NGF in the sympathetic denervation of the uterus during pregnancy is not clearly understood. NGF levels decrease during pregnancy in the guinea pig. However, this decrease is not statistically significant, suggesting that NGF was not responsible for pregnancy-induced denervation of the uterus in guinea pigs (Brauer et al., 2000). Varol et al. (2000) reported an increase in NGF and NGF mRNA in the rat uterus during pregnancy whereas Lobos et al. (2005) found an increase in proNGF but a decrease in mature NGF during pregnancy in the rat (Lobos et al., 2005). However, the increase in proNGF is due to accumulation of proNGF (Lobos et al., 2005). Sympathetic neurons that project to the uterus decrease in size during pregnancy, probably due to axonal degeneration. (Richeri et al., 2005). Sympathetic denervation during pregnancy may also be caused by the presence of foetus as suggested by Alm et al. (1988). All of these factors have been discussed by others in relation to sympathetic denervation in the pregnant uterus.

Since sympathetic nerves have vasoconstrictive properties in the female reproductive system (Papka et al., 1985; Markiewicz et al., 2003), the degeneration of sympathetic

axons may be necessary to accommodate the increase in blood flow during pregnancy. However, it is not understood why parasympathetic and sensory nerves degenerate during pregnancy. Since the parasympathetic and sensory denervation during pregnancy has not been previously examined in detail, there is no information available about what might cause this denervation. Brain derived neurotrophic factor (BDNF) is a neurotrophin that is associated with the growth of peripheral sensory neurons (Landreth, 2006). The effect of BDNF in pregnancy-induced denervation has not been studied. Neurotrophin 3 and neurotrophin 4/5 belong to the family of neurotrophins that target both sympathetic and sensory neurons (Landreth, 2006). The effect of both neurotrophin 3 and neurotrophin 4/5 on the denervation of the pregnant uterus has not been examined. While the neurotrophin family (NGF, BDNF, NT3 and NT4/5) acts on both sympathetic and sensory neurons (Landreth, 2006); their involvement with parasympathetic neurons has not been studied. Responsiveness of neurotrophin receptors in sympathetic, parasympathetic and sensory neurons may have an influence on pregnancy-induced denervation of the uterus. A combination of neurotrophic factors, their receptors and hormones may be responsible for the denervation of the pregnant uterus.

Innervation changes in pre-eclamptic versus normal human pregnancy

Reduction in blood flow to maternal organs and to the placenta is a characteristic feature of pre-eclampsia, a hypertensive disorder of pregnancy. Determination of the causes of the sympathetic, parasympathetic and sensory denervation of the pregnant uterus will provide important insights into the possible cause for this characteristic of pre-eclampsia. In the pre-eclamptic human uterus, the sympathetic nerve supply to the uterus does not decrease as in normal pregnancy (Fried et al., 1986; Quinn, 2005). Parasympathetic and sensory innervations have not been examined in human pre-eclampsia.

Future Directions

The future directions of this project will include assessing the parasympathetic and sensory innervation of the middle and cervical regions of the 20-day pregnant rat uterus. In this project only the ovarian regions have been examined for parasympathetic and sensory denervation. Whether pregnancy also causes denervation of other maternal organs and uteri devoid of foetus is not known. The time courses for denervation and regeneration the innervation of pregnant uteri also need to be assessed. Factors influencing the regeneration of axons post-partum are also unknown. Whether BDNF or other members of the neurotrophin family have an influence on the denervation of the uterus during pregnancy should be investigated.

An insight into the causes for pregnancy induced-denervation may provide enough information to develop an animal model of pre-eclampsia. In pre-eclampsia, the sympathetic innervation is higher than in normal pregnancy. The changes in the innervation from parasympathetic and sensory nerves in the pre-eclamptic uterus are not known. Whether the innervation of other maternal organs is also higher in pre-eclampsia compared to a normal pregnancy also requires examination.

Studies of uterine innervation have not been carried out in gestational and chronic hypertensive conditions during pregnancy. Assessing the uterine innervation using the immunoperoxidase method on whole mount preparations from hypertensive pregnant rats should help to answer this question. It is also not known if changes in the innervation of the uterus have any relationship to adverse perinatal outcomes, such as premature delivery, small for gestational age infants and perinatal death, seen in hypertensive disorders of pregnancy. Innervation changes, if any, in gestational and chronic hypertensive uteri may provide clues to the causes of such adverse outcomes. These clues will come from

comparing the innervation of pregnant uteri from animals and humans with hypertension with the innervation of normal pregnant uteri.