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Effects of sex and ovarian hormones on reflex regulation of renal sympathetic nerve activity

With focus on sympathetic response to myocardial infarction

Maximilian Ichabod Pinkham

Abstract

The physiological mechanisms contributing to sex differences following myocardial infarction (MI) and subsequent heart failure are poorly understood. Ovarian hormones, particularly estradiol, have been shown to be cardioprotective but the mechanisms of action are not well known. Increases in sympathetic nerve activity (SNA) following MI and in heart failure are associated with pathophysiological changes that provide the basis for beta-blockers as a successful treatment strategy. There are significant sex differences throughout the sympathetic nervous system, suggesting that sex differences may exist in the regulation of SNA in disease states. Given the strong relationship between sympathetic nerve activity (SNA) and outcome, I hypothesized that female sex hormones will attenuate the dysregulation of SNA, and sympathoexcitation, post-MI. To investigate this, experiments were performed in anesthetized male, female and ovariectomized female (OVX) Wistar rats that were instrumented to record arterial pressure, heart rate and renal SNA. Specifically, the arterial baroreceptor and cardiac afferent reflexes were investigated as reflexes implicated in driving both short and long term changes in SNA post-MI.

In the first series of experiments the effects of sex and ovarian hormones on the initial sympathetic response to MI was investigated. It was found that males and OVX, but not ovary-intact females, displayed significant elevations in renal SNA in the acute period following MI. Furthermore, the findings suggest that in males, but not females, the arterial baroreflex has a predominant influence on the initial changes in renal SNA post-MI. To investigate a possible mechanism mediating the female responses to MI, the second series of experiments investigated the effects of sex and ovarian hormones on the cardiac afferent reflex. Ovarian hormone dependant and independent sex differences in the cardiac afferent reflex were observed. Estradiol in females augmented cardiac afferent reflex mediated sympathoinhibition and attenuated reflex mediated sympathoexcitation. Furthermore, compared to males, all females regardless of the state of circulating ovarian hormones displayed greater variations in their cardiac afferent reflex mediated changes in renal SNA. The results suggest that sex differences in the cardiac afferent reflex may mediate, at least in part, the sex differences observed in the initial sympathetic response to MI. In the final series of experiments the effects of sex and ovarian hormones on the changes in arterial baroreceptor and cardiac sympathetic afferent reflex control of renal SNA in MI-induced heart failure were investigated. The results indicate that ovarian hormones in females are capable of protecting reflex regulation of renal SNA in MI-induced heart failure.
The studies in this thesis provide important information regarding sex differences in the reflex regulation of renal SNA in MI and heart failure. The current findings suggest that sex differences in the regulation of SNA likely contribute to sex differences in patient presentation and progression following MI and subsequent heart failure.
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Thanks to family and friends, it is the time outside that made the work inside possible.

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### Abbreviations

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<thead>
<tr>
<th>Symbol</th>
<th>Term</th>
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<tbody>
<tr>
<td>ACE</td>
<td>Angiotensin converting enzyme</td>
</tr>
<tr>
<td>ANG I</td>
<td>Angiotensin I</td>
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<tr>
<td>ANG II</td>
<td>Angiotensin II</td>
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<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>ANP</td>
<td>Atrial natriuretic peptide</td>
</tr>
<tr>
<td>AT1R</td>
<td>Angiotensin-1 receptor</td>
</tr>
<tr>
<td>BD</td>
<td>Barodenervated</td>
</tr>
<tr>
<td>BP</td>
<td>Blood pressure</td>
</tr>
<tr>
<td>BP50</td>
<td>Mean arterial pressure curve midpoint</td>
</tr>
<tr>
<td>bpm</td>
<td>Beats per minute</td>
</tr>
<tr>
<td>CAD</td>
<td>Coronary artery disease</td>
</tr>
<tr>
<td>cm</td>
<td>Centimeter</td>
</tr>
<tr>
<td>CSAR</td>
<td>Cardiac sympathetic afferent reflex</td>
</tr>
<tr>
<td>CSN</td>
<td>Carotid sinus baroreceptors denervated</td>
</tr>
<tr>
<td>CVD</td>
<td>Cardiovascular disease</td>
</tr>
<tr>
<td>Cx</td>
<td>Left circumflex artery</td>
</tr>
<tr>
<td>ECG</td>
<td>Electrocardiogram</td>
</tr>
<tr>
<td>ERα</td>
<td>Estrogen receptor alpha</td>
</tr>
<tr>
<td>ERβ</td>
<td>Estrogen receptor beta</td>
</tr>
<tr>
<td>g</td>
<td>Grams</td>
</tr>
<tr>
<td>GABA</td>
<td>Gamma-aminobutyric acid</td>
</tr>
<tr>
<td>HR</td>
<td>Heart rate</td>
</tr>
<tr>
<td>HRT</td>
<td>Hormone replacement therapy</td>
</tr>
<tr>
<td>IMC</td>
<td>Intermediolateral cell column</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>--------------</td>
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<tr>
<td>i.p.</td>
<td>Intraperitoneal</td>
</tr>
<tr>
<td>kg</td>
<td>Kilogram</td>
</tr>
<tr>
<td>LAD</td>
<td>Left anterior descending coronary artery</td>
</tr>
<tr>
<td>LH</td>
<td>Luteinising hormone</td>
</tr>
<tr>
<td>L-NAME</td>
<td>L-Nitro-Arginine Methyl Ester</td>
</tr>
<tr>
<td>MAP</td>
<td>Mean arterial pressure</td>
</tr>
<tr>
<td>MI</td>
<td>Myocardial infarction</td>
</tr>
<tr>
<td>ml</td>
<td>Milliliter</td>
</tr>
<tr>
<td>mm</td>
<td>Millimeter</td>
</tr>
<tr>
<td>MSNA</td>
<td>Muscle sympathetic nerve activity</td>
</tr>
<tr>
<td>NA</td>
<td>Noradrenaline</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric oxide</td>
</tr>
<tr>
<td>NOS</td>
<td>Nitric oxide synthase</td>
</tr>
<tr>
<td>NTS</td>
<td>Nucleus tractus solitaries</td>
</tr>
<tr>
<td>OVX</td>
<td>Ovariectomized female</td>
</tr>
<tr>
<td>PE</td>
<td>Phenylephrine</td>
</tr>
<tr>
<td>PVN</td>
<td>Paraventricular nucleus</td>
</tr>
<tr>
<td>RAS</td>
<td>Renin-angiotensin system</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>RSNA</td>
<td>Renal sympathetic nerve activity</td>
</tr>
<tr>
<td>RVLM</td>
<td>Rostral ventrolateral medulla</td>
</tr>
<tr>
<td>SAD</td>
<td>Sinoaortic baroreceptors denervated</td>
</tr>
<tr>
<td>S.E.M.</td>
<td>Standard error of the mean</td>
</tr>
<tr>
<td>SNA</td>
<td>Sympathetic nerve activity</td>
</tr>
<tr>
<td>SNP</td>
<td>Sodium nitroprusside</td>
</tr>
<tr>
<td>SNS</td>
<td>Sympathetic nervous system</td>
</tr>
<tr>
<td>TPR</td>
<td>Total peripheral resistance</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<td>--------------</td>
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<tr>
<td>VLM</td>
<td>Ventrolateral medulla</td>
</tr>
<tr>
<td>VR1</td>
<td>Vanilloid receptor 1</td>
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<tr>
<td>Vs.</td>
<td>Versus</td>
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Chapter 1: General Introduction

This thesis examines the impact of sex on the reflex regulation of the sympathetic nervous system (SNS) before and following myocardial infarction (MI) and as heart failure develops. The fundamental hypothesis of this thesis states that female sex and ovarian hormones alter the regulation of the SNS thereby altering the sympathetic response to MI. Two sympathetic reflexes that drive changes in sympathetic nerve activity (SNA) following MI will be focused on: the arterial baroreceptor reflex and the cardiac afferent reflex. Ultimately, to obtain greater success in treatments for cardiovascular disease (CVD) it is important that we better understand how sex and sex hormones alter the regulation of the cardiovascular system.

In New Zealand men and women, cardiovascular disease is the leading cause of both overall death and premature mortality, accounting for 33% of life years lost between the ages of 45 and 64 years old (Hay 2004). In New Zealand, coronary artery disease (CAD), the primary cause of MI, is the leading CVD accounting for 91 deaths per 100,000 people (Hay 2004). Those who survive the initial CAD event are likely to develop heart failure which carries a dire prognosis with no outright successful treatment (Kannel 2000, Velagaleti et al. 2008). The increasing age of populations means the incidence of both MI and heart failure are fast becoming major health concerns (Stewart et al. 2003, Cornwall et al. 2004, Velagaleti et al. 2008). As will be discussed, significant sex differences exist in relation to MI and heart failure, from the rates and age of occurrence right through to rates and timelines of morbidity and mortality. Currently, sex differences in CVD do not appear to be formally recognized in New Zealand (Crooke 2007).

As it will be argued in this thesis, sex differences in the regulation of SNA suggest that sex-specific changes to SNA following MI and in the development of heart failure are a real possibility. The introduction of this thesis will give information that highlights pathways by which ovarian hormones may be acting to alter sympathetic regulation following MI and in subsequent development of heart failure. To the best of my knowledge, no study has directly compared the regulation of SNA in heart failure between males and females. To provide context and framework for the current work, the nitric oxide (NO) and renin-angiotensin systems (RAS) have been reviewed in the introduction as being systems that mediate the regulation of SNA in health and disease and also as systems that may mediate the effects of sex and ovarian hormones on sympathetic regulation.
1.1. Are ovarian hormones cardioprotective?

Women are at high risk for cardiovascular disorders yet medical history demonstrates an overwhelming bias towards the use of male subjects in cardiovascular research (Huxley 2007). Recently, research has begun to focus on the role of sex in cardiovascular regulation due to significant sex differences in the occurrence and prognosis in disease states.

Men develop most but not all CVD at an earlier age compared to women (Mikkola et al. 2013). In women, the occurrence of CVD increases significantly during and following menopause (when circulating estrogens significantly diminish) suggesting a cardioprotective role for estrogens (Kannel et al. 1976, van der Schouw et al. 1996, Mikkola et al. 2013). Over the last 25 years, there has been numerous observational and randomized control trials investigating the effects of hormone replacement therapy (HRT) on cardiovascular health in menopausal women with conflicting results (Bush 1990, Stampfer et al. 1991, Grady et al. 1992, Hulley et al. 1998, Rossouw et al. 2002, Anderson et al. 2004, Lawlor et al. 2004, Manson et al. 2013). A recent randomized control trial investigated the effects of HRT in peri-menopause or shortly after menopause and followed the subjects for 10 years (Schierbeck et al. 2012). Preliminary findings by Schierbeck et al. show that when HRT in women is begun during or close to menopause, HRT confers a significantly reduced risk of mortality, heart failure or MI with no increased risk of cancer or stroke (Schierbeck et al. 2012).

In experimental studies, estrogenic actions are associated with a plethora of favorable biological and physiological processes further contributing to evidence that estrogen is in some way cardioprotective (Mendelsohn 2002, Konhilas et al. 2007, Murphy 2011, Vaccarino et al. 2011, Zimmerman et al. 2013). In animals, the administration of estrogen both prior to and following MI in males and females reduces infarct size and protects cardiac function post-MI (Cavasin et al. 2003, Cavasin et al. 2004). Furthermore, the knock-out of estrogen receptor (ER)-β in mice not only increases mortality following MI but also aggravates the development of heart failure in those animals that survived the initial MI (Pelzer et al. 2005). These findings suggest that estrogen can be cardioprotective although the mechanisms of action are poorly understood.
1.2. Sex differences following myocardial infarction

There is a sex-age interaction in both the occurrence of MI and the progression of the disease (Vaccarino et al. 1999, Champney et al. 2009). Pre- or peri-menopausal women (<55 years old) are significantly less likely to experience an MI than age-matched males. However, when pre-menopausal women do experience an MI they are at greater risk of dying (Vaccarino et al. 1998, Vaccarino et al. 1999, Champney et al. 2009). As shown in Figure 2.1, from the ages of 75 years and above sex differences in occurrence and mortality post-MI become obsolete and perhaps even favor males (Vaccarino et al. 1998, Vaccarino et al. 1999, Champney et al. 2009). Therefore, on average, women are 9 years older than men when they first present with MI (Vaccarino et al. 2011).

In response to MI, there appears to be significant differences between males and females in the perception of cardiac pain (Culic et al. 2002). Women are more likely to experience atypical symptoms such as nausea or tiredness whereas men are more likely to experience chest pain (Meischke et al. 1998, Coventry et al. 2011). Women are also less likely than men to recognise their symptoms as being an MI and are on average much later in seeking treatment (Meischke et al. 1999, Berg et al. 2009, Collins 2011, Coventry et al. 2011). Women generally receive less effective treatment than men and are more likely to die when in hospital (Dellborg et al. 1993, Vaccarino et al. 1999, de Gevigney et al. 2001). However, men are more likely to die acutely in response to MI prior to reaching hospital (Lerner et al. 1986, Maclntyre et al. 2001, Andreotti et al. 2003). The current knowledge is limited in regard to sex-based differences in physiology that lead to sex differences in patient risk, presentation, treatment and prognosis with regards to MI.

Estrogen is capable of modifying many different processes involved in cardiovascular regulation (Mendelsohn 2002, Konhilas et al. 2007, Murphy 2011, Vaccarino et al. 2011, Zimmerman et al. 2013). Despite its well-known importance in driving changes post-MI, the SNS has received very little attention as a system mediating sex-specific prognoses. There is currently one published study in humans and none in animals that have specifically investigated sex-specific changes in SNA post-MI (Hogarth et al. 2009). Hogarth et al. recorded muscle SNA in male and post-menopausal female patients for 9 months following acute MI and compared levels of resting muscle SNA in post-MI patients with healthy, age-matched controls (Hogarth et al. 2009). The results suggest that following MI, muscle SNA increased more in post-menopausal women than in age-matched men (Hogarth et al. 2009). The remainder of this thesis focuses on the regulation of the SNS before and after MI, as heart failure develops and the role ovarian hormones may play.
1.3. The importance of the sympathetic nervous system in health and following myocardial infarction

The SNS is one side of the autonomic nervous system and is most often described as the driving force behind the *fight or flight* response. Importantly, the SNS plays a critical role in the development and progression of many cardiovascular diseases including MI and heart failure and is a primary treatment target for these diseases.

SNA is altered following MI, with increased SNA contributing to worsening morbidity and mortality in patients (Hasking et al. 1986, Leimbach et al. 1986, Grassi et al. 1995, Grassi et al. 2001). Increased adrenergic activity in response to MI can initiate a feed-forward cycle of increased oxygen demand and worsening ischemia within the heart. In addition, the pro-arrhythmic and vasoconstrictive effect of increased SNA can enhance the risk for worsening morbidity and mortality during and following the ischemic episode (Jardine et al. 2007). The deleterious actions of SNA in MI and heart failure have been extensively...

Preferential activation of SNA to the heart and kidneys appears to be of fundamental importance in the heart failure state (Hasking et al. 1986). Of particular importance to this thesis is the role of renal SNA in driving the development and progression of heart failure. It is known in both humans (Hasking et al. 1986, Rundqvist et al. 1997, Petersson et al. 2005) and animals (Sano et al. 1990) with heart failure that renal noradrenaline (NA) spillover is elevated. As shown in Figure 1.2, heart failure patients with a relatively high level of renal NA spillover predicts the enhanced progression of morbidity and mortality compared to heart failure patients with relatively low levels of renal NA spillover (Petersson et al. 2005). In heart failure, the failing left ventricle has been shown to be highly sensitive to afterload conditions (Sonnenblick et al. 1963, Ross et al. 1964, Cohn 1973). Increased SNA acting on the kidney can significantly impact on afterload conditions by increasing fluid volume and activation of the RAS (Nozawa et al. 2002, DiBona et al. 2003, DiBona et al. 2004, Petersson et al. 2005). Although the time course of changes in renal SNA following MI and as heart failure develops and progresses are not well understood, it is typically believed that cardiac SNA activation occurs early in the development of heart failure whereas renal SNA activation occurs later (Rundqvist et al. 1997, Ramchandra et al. 2009).

![Renal noradrenaline spillover vs. Total body noradrenaline spillover](image)

**Figure 1.2: Renal noradrenaline spillover is associated with outcome in heart failure**

Kaplan-Meir graphs showing how long term outcome in heart failure patients as measured by need for heart transplantation and mortality is independently associated with renal sympathetic nerve activity, as measured by renal noradrenaline (renal NA) spillover (A). Patients that had levels of noradrenaline spillover to the kidney above the median level upon diagnosis displayed significantly worse deterioration in heart function. No significant prognostic value was demonstrated by total body NA spillover (B) (Petersson et al. 2005).
1.3.1. Anatomy of the sympathetic nervous system

The anatomy of the SNS has been well described (Jansen et al. 1995, Malpas 2010) and is summarised briefly in this section. NA, acting on adrenergic receptors, is released by sympathetic postganglionic neurons and is the final step in the output of the SNS. Unmyelinated sympathetic postganglionic neurons receive input via the neurotransmitter acetylcholine from myelinated sympathetic preganglionic neurons that largely arise from the intermediolateral cell column (IMC) of the spinal cord. The IMC cells receive input via pre-motor neurons from brain regions responsible for generating sympathetic tone.

With the use of horseradish peroxidise retrograde labelling in cats, Amendt et al. first identified the nucleus of solitary tract (NTS), raphe nucleus and the ventrolateral reticular formation as being areas of origin within the brainstem for pre-motor sympathetic neurons projecting to the IMC (Amendt et al. 1978). Strack et al. further defined the specific brainstem regions of origin for sympathetic pre-motor neurons as being the A5 noradrenergic cell group, caudal raphe region, rostral ventrolateral medulla (RVLM), and ventromedial medulla (VMM) (Strack et al. 1989). In addition, pre-motor neurones originating from the hypothalamus (paraventricular nucleus (PVN)) have been observed (Guyenet 2006, Pyner 2009). Of the brainstem regions regulating SNA, the RVLM is identified as a primary contributor to tonic sympathetic tone (Guyenet 2006). Anatomically, neurons originating from the RVLM project to the IMC of the spinal cord (Blessing et al. 1981, Barman et al. 1985). Furthermore, discrete electrolytic lesioning of the RVLM reduces blood pressure levels similar to that seen in animals following cervical spinal cord transection (Reis et al. 1989).

The NTS within the medulla is the primary destination for both primary afferent and secondary spinal neurons, including input from the arterial baroreceptor and cardiac sensory receptor afferents (Palkovits et al. 1977). Sensory afferent neurons are not the only peripheral input that is capable of altering sympathetic outflow. The circumventricular organs, namely the subfornical organ, organum vasculosum lamina terminalis and area postrema, lack a blood brain barrier and therefore circulating hormones such as angiotensin II (ANG II) can act directly on central neurons to alter central regulation of the SNS (Osborn et al. 2007).
1.3.2. Sympathetic Nerve Activity

Postganglionic sympathetic nerves are comprised of thousands of unmyelinated fibres and thus the overall level of SNA represents the summated activity of individual sympathetic fibres (Malpas 2010). Postganglionic SNA is generally recorded as multi-fibre discharges, although it is possible to record from individual fibres. Individual postganglionic sympathetic fibres are synchronously activated to give the nerve signal a ‘bursting’ characteristic. Fibres that are sensitive to input from the arterial baroreceptors such as those to kidney or muscle display activation at the same point of the cardiac cycle, a phenomenon called ‘entrainment’. Entrainment of SNA provides a good way to validate multi-fibre recordings of SNA as it is possible to overlap time-averaged SNA and arterial pressure and observe whether the ‘bursts’ observed in the SNA signal are ‘entrained’ to the cardiac cycle. This is made possible by the tonic nature of SNA meaning that at rest, SNA conserves the ability to either increase or decrease when appropriate (Malpas 2010). In addition to a cardiac related rhythm, input via vagal afferents affect a respiratory rhythm in SNA and there are also higher and lower frequencies that are not entirely understood but are thought to be centrally generated (Ando et al. 1997, Malpas 2010).

The amplitude of the SNA signal refers to the level of activation at a given time and therefore the voltage level of the recorded signal. It is generally hypothesized that the amplitude is determined by the number of individual sympathetic fibres recruited during the specific ‘burst’ of SNA (Ninomiya et al. 1993). Amplitude and frequency appear to be differentially regulated as inputs from baroreceptors appear able to preferentially affect frequency whereas inputs from chemoreceptors preferentially affect amplitude (Malpas et al. 1992, Malpas et al. 1992). The ability to differentially alter the frequency and amplitude of SNA no doubt extends the range of functional effects, although this has not been well characterized (Malpas 2010).

1.3.2.1. What does sympathetic nerve activity do?

Among the many neurotransmitters released by the sympathetic nerves, the most recognized and predominant is NA. NA acts on adrenoceptors located throughout the cardiovascular system of which there are 3 major sub-types: β, α1 and α2. Adrenoceptors are G-protein linked receptors that act via the classic Gs–adenylyl cyclase-cAMP-PKA signalling pathway (Dzimiri 1999). The specific β-subtype β1 constitutes approximately 80% of β-adrenoceptors expressed within the heart (Brodde 1991). In mice lacking β1
adrenoceptor, catecholamine stimulation fails to elicit its typical increases in heart rate and cardiac contractility, suggesting the $\beta_1$-adrenoceptor is predominantly responsible for SNA-mediated modulation of inotropy and chronotropy (Rohrer et al. 1996). $\alpha_1$ and $\alpha_2$ adrenoceptors are located throughout smooth muscle within the vasculature. Activation of $\alpha$-adrenoceptor G-protein signalling by NA initiates smooth muscle contraction and therefore constriction of the blood vessels (Docherty 1998). In summary, SNA acts to raise arterial pressure by increasing cardiac contractility, heart rate and total peripheral resistance.

1.3.2.2. Renal Sympathetic Nerve Activity

SNA is implicated in maintaining long-term blood pressure homeostasis via regulating kidney function. The experiments in this thesis focus on postganglionic recordings of SNA to the kidney (i.e. renal SNA). The kidney achieves long-term blood pressure homeostasis through the regulation of fluid and solute excretion that ultimately determine blood volume. Blood volume is a key determinant of long-term blood pressure by affecting total peripheral resistance and cardiac output (Guyton 1991).

Retrograde labelling has identified that renal sympathetic preganglionic neurons originate from the IMC, synapsing with the renal sympathetic postganglionic neurons somewhere in the T13 – L1 sections of the spinal cord (Li et al. 1992, Taylor et al. 1992). Postganglionic renal nerves enter the kidney alongside the renal artery and vein and are subsequently distributed throughout the intrarenal vasculature within the renal cortex and outer medulla (Barajas et al. 1992). Renal denervation produces a significant (~95%) reduction in NA within the renal tissue whereas nerve stimulation produces significant increases in NA within the renal veins, providing the evidence that NA is the primary neurotransmitter of the renal nerves (Bello-Reuss et al. 1975, Bradley et al. 1984). Both $\alpha_1$- and $\alpha_2$-adrenoceptors are located throughout the renal vasculature and elicit smooth muscle contraction and therefore vasoconstriction. $\beta_1$ adrenoceptors are located in the juxtaglomerular cells of the kidney, with NA mediated activation initiating a cAMP/PKA transduction cascade resulting in increased renin secretion from these cells (Barajas et al. 1973, Kopp et al. 1980). Anatomical and functional evidence demonstrate that elevations in renal SNA result in a reduction in urinary sodium and water excretion by increasing renal tubular water and sodium reabsorption throughout the nephron, attenuating renal blood flow and glomerular filtration rate by constricting renal vasculature, and increasing the activity of the RAS via

Differential regulation of SNA indicates that it is necessary to record directly from the nerve of interest in order to be certain of the changes and regulation to that particular nerve (Morrison 2001). Given the importance of renal SNA in maintaining long-term blood pressure regulation, it is important to directly study renal SNA which is currently only possible in animals.

1.4. Reflex regulation of sympathetic nerve activity

Sensory receptors located within the cardiovascular system send information via afferent neurons to central autonomic nuclei. These peripheral receptors include pressure sensitive receptors (located within the heart, lungs and arteries) and chemically sensitive receptors (located in the heart and arteries) (Guyenet 2006). The specific reflex pathways investigated in this thesis are the arterial baroreceptor reflex and the cardiac afferent reflex. Previous research has implicated the arterial baroreceptor and cardiac afferent reflexes as being important in driving short- and long-term changes in SNA following MI (Thames et al. 1979, Wang et al. 2000). The peripheral and central processing of reflex regulation of SNA is further subject to the influences of neurohormonal factors. In particular, this thesis investigates the neuromodulatory actions of ovarian hormones in affecting alterations in sympathetic regulation. The sympathoregulatory actions of ovarian hormones and in particular estradiol via affecting changes in the NO and RAS will be focused on as these are important neuromodulators implicated in driving pathological increases in renal SNA following MI and subsequent development of heart failure (Zucker et al. 2004).

1.4.1. The Arterial Baroreceptor Reflex

The arterial baroreceptor reflex has a predominant influence on the beat-to-beat regulation of renal SNA. Baroreceptors respond to alterations in blood vessel distortion sending corresponding neuronal signals to the NTS in the brainstem (Palkovits et al. 1977). The arterial baroreceptor reflex helps maintain mean arterial pressure by responding to oscillations in arterial pressure via reciprocal changes in efferent sympathetic and parasympathetic activity. For example, an increase in arterial pressure will result in arterial baroreceptor reflex-mediated decreases in total peripheral resistance via SNA and
decreases in heart rate via activation of parasympathetic nerve activity, thereby acting to return arterial pressure to the predetermined set-point. The arterial baroreceptor reflex control of SNA is typically studied by acutely altering arterial pressure and plotting the resulting changes in SNA. As shown in Figure 1.3, the relationship between SNA and arterial pressure is typically represented by a sigmoidal curve that can be characterized by the resting point, gain (i.e. slope of the curve) and the lower and upper plateaus (Kirchheim 1976).

![Figure 1.3: The 'classic' arterial baroreceptor reflex](image)

Schematic showing the ‘classic’ arterial baroreceptor reflex curve of an inverse sigmoidal relationship between either sympathetic nerve activity or heart rate and arterial pressure

1.4.1.2. Sensory Component of the Arterial Baroreceptor Reflex

Baroreceptors consist of unencapsulated nerve endings that are located predominantly within the medial-adventitia border of the arterial wall of the aortic arch and carotid sinus (Kirchheim 1976). These receptors are capable of responding to deformation of the arterial wall by altering afferent neuronal firing (Neil 2011). Confirming that the arterial baroreceptors respond to deformation of the arterial wall, arterial baroreflex mediated decreases in arterial pressure in response to increases in carotid sinus pressure is abolished by fixing the carotid arteries in a plaster cast (Kirchheim 1976). Mechanical stretch of the baroreceptors is converted to a membrane depolarization and consequently afferent neuronal firing in a process that is not clearly understood (Schild et al. 2012). Baroreceptor deformation and therefore afferent neuronal firing are subsequently very reliant on vessel wall geometry and tissue composition (i.e. the thickness and elasticity of the arterial wall) (Kirchheim 1976). Baroreceptor afferent fibres are made up of low-threshold, myelinated A-fibres and high-threshold, unmyelinated C-fibers (Fidone et al. 1969). The different thresholds and conduction velocities of the baroreceptor afferent fibres provide an anatomical basis underlying the ability of the arterial baroreflex to respond to various arterial pressures with different rates and intensities of neuronal discharge.
Baroreceptor afferent nerve activity at rest is tonic and conserves the ability to both increase and decrease, thereby maintaining the ability to respond appropriately within its functioning range to fluctuations in arterial pressure (Bronk 1932, Seagard et al. 1990). Figure 1.4 gives a schematic overview of the afferent component of the arterial baroreceptor reflex pathway.

![Schematic Diagram of Arterial Baroreceptor Reflex Components and Pathways](image)

**Figure 1.4: Arterial Baroreceptor Reflex Components and Pathways**
Schematic showing the location and pathways of the arterial baroreceptors and arterial baroreceptor afferents. Arterial baroreceptor afferents arising from the aortic arch and carotid sinuses project to the nucleus tractus solitarius (NTS) in the brainstem.

### 1.4.1.3. Central Component of the Arterial Baroreceptor Reflex

The NTS, CVLM and RVLM form the essential central circuitry of the arterial baroreceptor reflex (Spyer 1981). A lesion in any one of these three nuclei abolishes arterial baroreceptor reflex SNA responses to changes in arterial pressure (Aicher et al. 2000). Complex anatomical and electrophysiological studies have successfully characterized the order and function of the central nuclei essential for the baroreceptor reflex. The NTS is the site of termination of baroreceptor afferent fibers where they form excitatory connections (i.e. release glutamate) with second order neurons (Ciriello 1983). Baroreceptor reflex sensitive second order NTS neurons project to the CVLM where they release glutamate from the synaptic efferent terminals onto gamma-aminobutyric acid
Excitation of GABAergic, RVLM projecting neurons results in GABA-mediated inhibition of baroreceptor sensitive, pre-motor neurons projecting from the RVLM to the IMC (Agarwal et al. 1990). Pre-motor neurons projecting from the RVLM are tonically active; the input from arterial baroreceptor afferents is therefore capable of either increasing or decreasing pre-motor neuronal discharge (Caverson et al. 1983, Brown et al. 1984). Figure 1.5 shows the essential central components of the arterial baroreceptor reflex; a lesion in any of the regions illustrated completely abolishes the arterial baroreceptor reflex SNA response (Spyer 1981).

Apart from the NTS, CVLM and RVLM, there are numerous brain nuclei that have modulatory actions on the arterial baroreceptor reflex. Studies examining neuronal activation by imaging c-FOS expression in conscious rats and rabbits in which arterial pressure is lowered or increased have identified both essential and non-essential brain regions involved in the modulation of the arterial baroreceptor reflex. In response to sustained hypotension, c-FOS expression is elevated in the NTS, CVLM, RVLM, area...
postrema, A5 area, locus coeruleus, midbrain periacquiductal gray, PVN and the supraoptic nucleus (Graham et al. 1995, Dampney et al. 2003). In response to sustained hypertension, c-FOS expression is elevated in the NTS, CVLM, RVLM and area postrema (Graham et al. 1995, Dampney et al. 2003). Autonomic nuclei that are not responsive to baroreceptor afferent input still maintain an ability to modulate the arterial baroreceptor reflex SNA response. For example, the parabrachial nucleus is thought to be a primary site for the relaying of visceral sensory information to the forebrain and contains numerous projections to and from the NTS and RVLM and in certain settings can alter baroreflex function (Paton et al. 1990, Jhamandas et al. 1992). Furthermore, strong connections between the circumventricular organs and autonomic nuclei that regulate the arterial baroreceptor reflex such as the NTS, RVLM and PVN form the anatomical substrate for circulating hormones such as ANG II to affect arterial baroreceptor reflex function (Potts et al. 1999, Guyenet 2006, Osborn et al. 2007, Guild et al. 2012).

### 1.4.2. The Cardiac Afferent Reflex

In the 1860’s, Von Bezold first identified chemically stimulated, vagal afferent mediated, decreases in heart rate and blood pressure, work that was continued in the 1930’s by Jarisch to characterize what is now known as the Bezold-Jarisch reflex (Dawes et al. 1954). Chemosensitive nerve endings within the heart were first identified in the ventricles in 1964 (Coleridge et al. 1964) and then subsequently within the atria (Coleridge et al. 1973). Since initial studies, it is now known that chemosensitive nerve endings, located within the heart, convey sensory information via vagal and sympathetic afferent pathways that converge within central autonomic regulatory regions (Staszewska-Woolley et al. 1986).

The role of the chemically sensitive cardiac afferent reflex in affecting SNA is typically studied by either placing a stimulatory substance (such as bradykinin or capsaicin) onto the surface of the left ventricle or injecting the stimulatory substance into the coronary vasculature (Coleridge et al. 1964, Coleridge et al. 1973, Reimann et al. 1980, Schultz et al. 1998). Typically, activation of cardiac vagal and sympathetic afferents causes an decrease or increase in efferent SNA respectively (Reimann et al. 1980, Weaver 1981, Schultz et al. 1996). Substances that excite chemosensitive cardiac fibres include those associated with pain and myocardial oxygen supply/demand imbalance. These include bradykinin, substance P, 5-HT, prostaglandins, capsaicin, potassium, hydrogen ions and lactic acid (Longhurst 1984). The capsaicin receptor vanilloid receptor 1 (VR1) has been located in the heart and dorsal root ganglia (Zahner et al. 2003). Activation of the VR1
causes an opening of a non-selective cationic channel resulting in an inward current thereby depolarizing the neuron (Caterina et al. 1997). Capsaicin applied directly to the heart surface activates both sympathetic and vagal chemosensitive fibres and a resulting SNA-mediated change in blood pressure and heart rate (Reimann et al. 1980, Schultz et al. 1996, Ustinova et al. 2000, Zahner et al. 2003). As shown in Figure 1.6, the vagal and sympathetic afferent projections converge in the NTS (Tjen et al. 1997).

![Figure 1.6: The Cardiac Afferent Reflex Pathway](image)

Figure 1.6: The Cardiac Afferent Reflex Pathway
Schematic showing the chemosensory cardiac vagal and sympathetic afferent pathways of the cardiac afferent reflex. The cardiac vagal afferents travel bilaterally in the vagus and the cardiac sympathetic afferents travel in the contralateral spinothalamic tract of the spinal column. Both cardiac vagal and sympathetic afferents project to the nucleus tractus solitarius (NTS) in the brainstem where the signals converge.

### 1.4.2.1. The Cardiac Vagal Afferent reflex

Activation of vagal afferents with sensory nerve endings in the cardiac wall results in bradycardic and depressor responses elicited by increased efferent vagal activity to sinoatrial pacemaker cells and a withdrawal of peripheral SNA respectively (Kunze 1972, Kaufman et al. 1980, Schultz et al. 1998). Cardiac vagal afferent fibres travel bilaterally in the vagus terminating in the NTS with cell bodies in the nodose ganglion (Xie et al. 1999). Typically it is understood that cardiac vagal afferent nerve endings are preferentially
located closer to the sub-endocardial layer of the infero-posterior left ventricular wall (Wei et al. 1983, Ludbrook 1990). The primary evidence for this comes from the observation that in response to myocardial ischemia or infarction, patients or animals with ischemia in the infero-posterior region of the left ventricle are more likely to show decreases in heart rate and arterial pressure. In contrast, ischemia in the anterior surface is most likely to produce increases in heart rate and blood pressure (Adgey et al. 1968, Webb et al. 1972, Wei et al. 1983). The hypotension that occurs in response to an MI in the infero-posterior region of the left ventricle is accompanied by bradycardia despite the presence of the arterial baroreceptor reflex suggesting that it is the cardiac vagal afferents driving these changes (Webb et al. 1972, Wei et al. 1983). Furthermore, the influence of the site of MI on the initial hemodynamic changes suggests that these responses are unlikely to be derived solely from cardiac mechanoreceptors (Adgey et al. 1968, Thoren 1972, Webb et al. 1972, Wei et al. 1983).

Evidence from single fibre recordings in anesthetized animals suggests that the majority of chemosensitive vagal afferents of cardiac origin selectively respond to chemical stimuli and only respond to mechanical stimuli if it is sufficiently profound (Kaufman et al. 1980, Ludbrook 1990). Cardiac vagal afferents are predominantly unmyelinated, C-type fibers with relatively slow conduction rates (Ludbrook 1990). Centrally, the cardiac sympathetic and vagal afferents converge within the NTS where it has been shown, using single unit recordings in anesthetized cats, that cardiac vagal afferent excitation can either inhibit or facilitate cardiac sympathetic afferent mediated neuronal excitation in the NTS (Tjen et al. 1997). The predominant effect is for the cardiac vagal afferents to inhibit NTS neurons which is associated with either inhibition of or attenuated excitation of SNA compared to when only sympathetic afferents are selectively stimulated (Tjen et al. 1997).

1.4.2.2. The Cardiac Sympathetic Afferent Reflex

Cardiac sensory nerve endings with afferent fibres travelling along the sympathetic pathway are responsible for tachycardic and pressor responses via elevating SNA, generally referred to as the cardiac sympathetic afferent reflex (CSAR) (Weaver 1981). The sensory nerve endings of the cardiac sympathetic afferent fibres are preferentially located in the left ventricle and are concentrated closer to the epicardial surface of the left ventricular wall (Wei et al. 1983, Ludbrook 1990). The cell bodies of the cardiac sympathetic afferents lie within the dorsal root ganglia C₈ to T₉, being most concentrated between T₂ to T₆, with the afferents terminating predominantly in dorsal horn spinal laminae
From here, the signals ascend via Lissau's Tract and the spinothalamic tract where it is thought that the secondary spinal afferents conveying information for the autonomic nervous system synapse in the NTS (Tjen et al. 1997). The CSAR also has a spinal reflex component that is not dependent on input from the brainstem, although its relative contribution to reflex mediated changes in SNA is unknown (Weaver 1981, Weaver et al. 1983). Unlike chemosensitive cardiac vagal afferents, chemosensitive cardiac sympathetic afferents can be either unmyelinated or myelinated (Nishi et al. 1977).

With the use of c-Fos labelling following stimulation of the CSAR in anesthetized cats using epicardial capsaicin application, areas of the brain responsible for processing the CSAR have been identified. These include the ventral lateral medulla, NTS, vestibular nucleus and lateral tegmental field within the medulla; the parabrachial nucleus, kolliker nucleus and locus coeruleus within the pons; and the locus coeruleus, dorsal nucleus of raphe, and the periacquiductal gray within the mid brain (Guo et al. 2002, Guo et al. 2002).

Single unit recordings of neurons in animal preparations have shown the NTS to be an important site for integrating peripheral inputs. For example, activation of the CSAR by epicardial application of capsaicin in rats augments the peripheral chemoreflex response and attenuates arterial baroreflex control of renal SNA (Gao et al. 2005, Wang et al. 2007, Wang et al. 2008). This effect can be directly observed in the NTS as CSAR activation increases the excitation of arterial chemoreceptor sensitive NTS neurons and decreases the sensitivity of arterial baroreceptor sensitive NTS neurons (Wang et al. 2007, Wang et al. 2008). Furthermore, neurons in the RVLM that are excited by the CSAR are also baroreceptor sensitive suggesting the RVLM is a site of central convergence of these peripheral reflexes (Li et al. 2000). There is considerable overlap in the central autonomic regions regulating the arterial baroreceptor reflex and the cardiac afferent reflex. Therefore, it is likely that alterations in circulating and local neuromodulators will impact simultaneously on both the CSAR and arterial baroreceptor reflex control of SNA.

1.5. Nitric oxide

NO is present throughout the SNS and appears to play an important role in the peripheral and central regulation of both the arterial baroreceptor- and cardiac afferent reflexes (Zanzinger 1999). NO is generated from 3 isoforms; neuronal nitric oxide synthase (NOS), inducible NOS and endothelial NOS (Schultz 2009). NO is essentially a gas that acts as a
neuromodulator in a large variety of tissues (Lancaster 1997). To produce NO, electrons are donated by NADPH to the NOS enzyme where they interact with haem iron and BH4 to catalyze the reaction of oxygen with L-arginine, thereby generating L-citrulline and NO as products (Moncada et al. 1993). The primary signalling pathway of NO is the activation of soluble guanylyl cyclase and generation of cyclic GMP (Moncada et al. 1991, Kennedy 2000). NO in the peripheral vasculature is a potent vasodilator and is important for the intrinsic regulation of vascular tone (Moncada et al. 1993, Kennedy 2000). A loss of endothelial NO is implicated in causing endothelial dysfunction in disease states such as heart failure (Bauersachs et al. 2004). Not only can NO modulate vascular tone but it also acts as a neuromodulator in the viscerosensory afferent neurons (Matsuda et al. 1995, Li et al. 1998, Tjen et al. 2001). For example, NO has been shown to directly alter baroreceptor afferent sensitivity, thereby altering arterial baroreflex control of SNA (Matsuda et al. 1995, Li et al. 1998, Meyrelles et al. 2003). However it is in the brain that NO appears to have a highly significant impact on the regulation of SNA.

1.5.1 Central actions of nitric oxide

Central-acting NO is produced locally in the brain (Moncada et al. 1991, Pyner 2009, Schultz 2009). Neuronal inhibition by NO appears to be largely mediated via GABAergic neurotransmission (Wang et al. 2007). NO is also implicated in mediating a decrease in the post-synaptic sensitivity to glutamate (Wang et al. 2007). Although NO is generally considered to be sympathoinhibitory, the central actions of NO and resulting outcomes are regionally specific and complex. For example, in anesthetized rabbits and rats, the microinjection of an NO inhibitor or donor into the RVLM causes an acute increase or decrease in baseline renal SNA respectively, although the opposite effect has also been observed (Harada et al. 1993, Hirooka et al. 1996, Tseng et al. 1996). In humans, peripheral administration of a NOS inhibitor causes an acute increase in resting skin SNA and arterial pressure providing evidence in humans that NO provides tonic sympathoinhibition (Young et al. 2009).

Central NO neuromodulation appears to be an important signalling pathway of both the arterial baroreceptor and cardiac afferent reflex arcs (Liu et al. 1996, Lo et al. 1996, Guo et al. 2003, Talman et al. 2004, Zhu et al. 2004, Dias et al. 2005, Mayorov 2005, Guo et al. 2009, Lin et al. 2012). In normal, healthy male rats, the microinjection of a specific neuronal NOS inhibitor into the RVLM attenuates the CSAR mediated increases in renal SNA (Guo et al. 2009). In comparison, a decrease in NO signaling in the RVLM in male rats with heart
failure is associated with enhanced CSAR mediated increases in renal SNA (Zhu et al. 2004). Therefore despite much research on the topic, the precise role of NO in mediating the sympathetic reflexes is still poorly understood.

In heart failure, a decrease in central NO has been associated with increased baseline renal SNA and altered reflex control of renal SNA (Zhang et al. 2001, Zhu et al. 2004, Zheng et al. 2005, Schultz 2009). Generally, NO is considered to be antagonistic towards the actions of ANG II which is thought to be mediated at least in part by attenuating the production of reactive oxygen species (ROS). ROS appears to form a crucial component of the ANG II signaling pathway (Gao et al. 2004, Campese et al. 2005, Gao et al. 2005). In heart failure, elevated ROS in central autonomic regions has been implicated in augmenting sympathoexcitation and impairing reflex control of SNA (Gao et al. 2004, Gao et al. 2005, Han et al. 2007). Based on the evidence in brief, NO is an important neuromodulator that has significant effects on the arterial baroreceptor and cardiac afferent reflex control of SNA in both health and disease.

1.6. Renin-Angiotensin System

The RAS is an important hormonal pathway regulating body fluid homeostasis and arterial pressure. Renin released from the juxtaglomerular cells of the kidney represents the rate limiting step in the activation of the circulating RAS. Renin is released in response to 1) decrease in pressure in the afferent arteriole of the kidney, 2) decrease in sodium delivery to the macula densa cells and 3) sympathetic nerve stimulation (DiBona 2000, DiBona 2005). Renin converts angiotensinogen to angiotensin I (ANG I) which in turn is converted by angiotensin converting enzyme (ACE) into angiotensin II (ANG II). ANG II is a potent vasoconstrictor and helps maintain fluid balance and arterial pressure by blood vessel constriction, increased sodium and water reabsorption, aldosterone release and vasopressin release (Peti-Peterdi et al. 2010). The interaction between SNS and RAS is bidirectional as not only is renin release and therefore RAS activation directly affected by renal SNA but also ANG II is intricately involved in both the peripheral and central regulation of the SNS (Osborn et al. 2007). The role of the RAS in cardiovascular and SNS regulation has been complicated in recent years by the characterization of numerous active downstream peptides of ANG I and ANG II that include angiotensin III, angiotensin (5-8) and angiotensin (1-7) (Ferrario et al. 1998 514). Angiotensin (1-7) in particular appears to have almost opposite effects compared to ANG II (Ferrario et al. 1998). In addition to the classic RAS, there is some evidence to suggest that ANG II is formed locally within brain
nuclei although how ANG II is formed centrally is not well understood (Culman et al. 2001, Kaschina et al. 2003, Ramchandra et al. 2013). The angiotensin type-1 (AT-1) receptor appears to be the predominant receptor mediating the classic effects of ANG II (Kaschina et al. 2003).

1.6.1. Angiotensin II and the central regulation of the SNS

The AT-1 receptor is highly expressed in central nuclei known to be important in the regulation of the sympathetic reflex pathways (Lenkei et al. 1997). AT-1 receptor expression in the circumventricular organs that include the area postrema, subfornical organ and lamina terminalis provide the anatomical substrate for circulating ANG II to affect central SNS regulation (Lenkei et al. 1997). Infusion of ANG II into the central ventricles of experimental animals provides a complex picture of circulating ANG II action on the SNS. Acute intracerebroventricular infusion of ANG II produces a sympathetically mediated pressor response with an accompanying baroreflex-independent inhibition in renal SNA in anesthetized and conscious healthy rats and sheep (Jin et al. 1989, Kannan et al. 1991, May et al. 1997). However, when ANG II is acutely injected into the fourth ventricle of conscious healthy rabbits, an increase in renal SNA is observed which appears to be dependent on an intact area postrema (Joy et al. 1970, Dorward et al. 1991). Chronic intravenous infusion of a sub-pressor dose of ANG II in rabbits causes a significant increase in renal SNA suggesting that chronically elevated levels of circulating ANG II can be sympathoexcitatory (Guild et al. 2012, Moretti et al. 2012). In disease states such as hypertension and heart failure which are associated with increases in renal SNA and circulating ANG II, antagonism of the AT-1 receptor by chronic peripheral or central infusion of losartan has been shown to decrease baseline renal SNA and improve reflex control of SNA (DiBona et al. 1995, Zhang et al. 1999, Bechir et al. 2005). For example, in rats with MI induced heart failure, chronic intracerebroventricular infusion of losartan normalizes arterial baroreceptor reflex control of renal SNA to be comparable with sham animals and does not affect the sham animal baroreflex control of renal SNA (Zhang et al. 1999). The findings discussed in brief suggest that circulating ANG II acting on the circumventricular organs is capable of altering baseline and reflex control of renal SNA both acutely and over a long period of time in both health and disease.
1.7. Ovarian hormones

Ovarian hormones consist of estrogens (estrone, estriol and estradiol) and progesterone. Estradiol is the most active estrogen and has been the main focus of research in regards to cardiovascular control. The ovaries are the primary source of estradiol, where it is synthesized by aromatization of androgen precursors. Local estradiol biosynthesis can also occur throughout the body including the vasculature, fat cells and the brain (Simpson et al. 1999). Estradiol levels vary throughout the menstrual cycle of women (known as the estrous cycle in rat) with levels being highest during the pre-ovulatory peak and mid-luteal phase in women and lowest in the early-follicular phase (Abraham et al. 1972). Figure 1.7 demonstrates in rats that estradiol levels are significantly higher during proestrus compared to metestrus, diestrus and estrus (Butcher et al. 1974). Circulating levels of progesterone tend to reach its peak following the spike in estradiol levels (Butcher et al. 1974, Goldman et al. 2007).

![Figure 1.7: Estrous cycle in rat](image)

Schematic pattern of the 4-day estrus cycle in the rat depicting serum estradiol and progesterone concentrations as they relate in time to the surge of luteinizing hormone (LH). Ovulation will typically occur during the early morning hours of estrus, approximately 10-12 hours after the rise in LH. Shaded blocks at the base of the figure indicate the dark portion of a 14:10hr light/dark photoperiod (Goldman et al. 2007).

Following menopause, the level of circulating estradiol in women is significantly decreased and is similar to circulating levels in men (Bjornerem et al. 2004). Estradiol can affect gene expression by binding to estrogen receptor alpha (ER)-α and ER-β receptors located within the nucleus (effects can occur within 2-8 hours) or produce more rapid actions via
membrane-bound ERα, ERβ and G protein-coupled estrogen receptor 30 (effects can occur within minutes) (Zhang et al. 2006). ERα and ERβ are found throughout the body and importantly in peripheral and central areas of the SNS (Papka et al. 1997, Shughrue et al. 1997, Papka et al. 2001, Brailoiu et al. 2007, Dun et al. 2009).

Sex differences have been described throughout the SNS (Bengtsson et al. 1983, Gisclard et al. 1987, Riemer et al. 1988, Karkanias et al. 1993, Sudhir et al. 1997, Moura et al. 2001) however this thesis is focused on the regulation of SNA. Previous research, as described below, has shown that ovarian hormones are capable of modulating peripheral and central actions of NO and ANG II in relation to the regulation of the SNS. Of the ovarian hormones, estradiol has been studied the most extensively in relation to the SNS and will be specifically focussed on in this introduction. In relation to the SNS, progesterone has been studied relatively little. It is acknowledged that progesterone has the ability to affect sympathetic regulation although the actions of progesterone will not be discussed in depth in this thesis (Heesch et al. 1995, Masilamani et al. 1997, Heesch et al. 2001).

1.8. Evidence for sex differences in the regulation of SNA

Ovarian hormones do affect the regulation of SNA but the underlying mechanisms are poorly understood. Recording of muscle SNA measured directly with microneurography indicates that resting sympathetic tone in pre-menopausal women is lower than age-matched men but following menopause resting muscle SNA becomes equal or higher in women compared to age-matched men (see Figure 1.8) (Matsukawa et al. 1998, Narkiewicz et al. 2005). Furthermore, most studies have demonstrated that resting muscle SNA changes across the menstrual cycle of women (Minson et al. 2000, Middlekauff et al. 2012). For example, Carter et al. observed that changes in resting muscle SNA throughout the menstrual cycle were negatively and significantly correlated with circulating estradiol levels and positively but insignificantly correlated with progesterone levels (Carter et al. 2012). The ratio of estradiol-to-progesterone within the circulation displayed the greatest relationship to resting SNA, suggesting that circulating estradiol has an inhibitory effect on resting muscle SNA whereas progesterone may have a sympathoexcitatory effect (Carter et al. 2012). Estrogen treatment in post-menopausal women can suppress muscle SNA, further confirming an inhibitory action of estrogen on resting SNA (see Figure 1.9) (Vongpatanasin et al. 2001, Weitz et al. 2001). The general consensus is that estrogens shift the sympathovagal balance in greater favour of the vagal side (Saleh et al. 2000) and therefore the significant decrease in circulating estrogens following menopause is cited as
causing the relatively greater increases in resting SNA and related changes in sympathetic regulation in older women (Hinojosa-Laborde et al. 1999).

Reflex regulation of renal SNA in female rats has been observed to be significantly affected by both sex and ovarian hormones. As shown in Figure 1.10, in conscious ovary-intact female rats, the arterial baroreflex mediated activation of renal SNA is significantly reduced compared to male rats (Foley et al. 2005). Furthermore, conscious female rats in proestrus display greater arterial baroreflex gain and excitation of renal SNA compared to rats in diestrus/estrus (Goldman et al. 2009). The majority of evidence suggests that estrogen is the hormone responsible for alterations in the arterial baroreceptor reflex and that it does so by acting centrally (Heesch et al. 2001, Tanaka et al. 2003, Brooks et al. 2012). However, the actions of ovarian hormones on SNS regulation appear to be complicated as concurrent evidence in female rats has shown that ovariectomy does not affect changes in the arterial baroreflex control of renal SNA in conscious animals (He et al. 1999). Because this thesis is focused on the effects of ovarian hormones on reflex control over efferent SNA, specifically the arterial baroreflex and cardiac afferent reflex, this introduction will focus on the known effects of estradiol on components of the reflex arcs that are directly applicable to the studies within this thesis.
1.8.1. Actions of estradiol on peripheral component of sympathetic reflexes

There is a lack of evidence regarding the direct actions of ovarian hormones on peripheral components of sympathetic reflexes. In regard to the arterial baroreceptor reflex, acute administration of estradiol in male rats appears to inhibit carotid sinus nerve sensitivity in response to alterations in arterial pressure (Wang et al. 2001). These actions appear to be dependent on NO signalling, as intravenous administration of an NO inhibitor completely removed any effects of estradiol on arterial baroreceptor sensitivity (Wang et al. 2001). It is well recognized that estradiol augments NO expression within the vasculature, suggesting a role for estradiol in altering endothelial function (Kullo et al. 1997, Cannon 1998, Mendelsohn 2002, Murphy et al. 2007, Novella et al. 2012). Furthermore, neuronal NOS is present in afferent sensory nerves with direct NO actions able to inhibit carotid baroreceptor afferent sensitivity to changes in arterial pressure and also causing a shift to higher arterial pressures in the responsiveness of the carotid baroreceptor afferents in rabbits (Wang et al. 1993, Matsuda et al. 1995, Meyrelles et al. 2003). It is possible that
estradiol may alter the arterial baroreceptor reflex control of SNA by altering arterial baroreceptor afferent sensitivity.

**Figure 1.10: Arterial baroreflex curves in a female or male rat**
Baroreflex curves obtained in the female (on left) and male (on right) conscious rat by increasing or decreasing mean arterial pressure and monitoring RSNA directly. It is noticeable that the female rat baroreflex curve has a lower upper plateau and a slightly smaller gain indicating that the baroreflex mediated increase in RSNA in response to a fall in arterial pressure is attenuated (Foley et al. 2005).

The effect of estradiol on the cardiac afferent reflex has not been investigated. However given the close association of this reflex with cardiac pain, some inferences from the current literature can be made. Sex differences exist between men and women in the sensation of pain with a low level of circulating estradiol being pronociceptive within women (Smith et al. 2006).

Estradiol receptors have been located within the dorsal root ganglion (Papka et al. 2001) and nodose ganglion (Dun et al. 2009). Within the dorsal root ganglion, estradiol appears to inhibit the ATP-induced increase in intracellular calcium suggesting a role for estradiol in modulating sensory afferent sensitivity at the primary afferent level (Chaban et al. 2011). With the use of whole cell patch clamping in neurons taken from the dorsal horn along with the combination of stimulation of Lissauers tract and field potential recordings, researchers have demonstrated estradiol is able to facilitate afferent synaptic transmission in a manner related to NMDA receptor signalling, increased pre-synaptic glutamate release from primary afferent terminals and augmenting spinal cord long-term potentiation (Zhang et al. 2012). As mentioned previously, VR1 channels mediate the response to capsaicin (an exogenous substance that induces pain). Estradiol has been demonstrated to potentiate the VR1 mediated cationic-current in rat dorsal root ganglia (Lu et al. 2009).
Furthermore, it has been shown that ovariectomy significantly reduces the excitability of a sex-specific subset of vagal afferents, with excitability being restored by acute estradiol replacement into the bath mixture that the cells are held while performing the patch clamp (Qiao et al. 2009). It has been demonstrated that the peripheral sensory neurons express both ER-\(\alpha\) and ER-\(\beta\), with ER-\(\alpha\) being selectively localized in small-diameter neurons (Taleghany et al. 1999). A high level of expression of sodium voltage gated 1.7, 1.8 and 1.9\(\alpha\) sub-unit mRNA in nodose ganglia and dorsal root ganglia has been shown (Kwong et al. 2008). In particular, the selective silencing of sodium voltage gated 1.7 ion channel using adeno virus decreases neuronal excitability and conduction block in sensory vagal afferent fibres suggesting that it is this sub-type of the sodium channel that mediates peripheral neuronal excitability (Muroi et al. 2011). Estradiol is capable of regulating the gene expression of voltage gated sodium channels (Hu et al. 2012). Therefore, estrogen can potentially alter the nociceptive process at the primary afferent level.

Furthermore, it has been demonstrated that pregnancy in female rats can attenuate cardiac afferent responsivity to atrial distension compared to virgin rats, with the mechanisms of action not understood (Deng et al. 1995, Storey et al. 2004). Together, these findings suggest that changes in endogenous hormones in females have the potential to alter excitability of both chemosensory and mechanosensory afferents.

### 1.8.2. Central sites of estradiol action

Estradiol and progesterone are lipophilic in nature enabling these steroid hormones to readily cross the blood-brain barrier and act within central brain regions (Curtis 2009). A single peripheral administration of estradiol can cause an acute decrease in muscle SNA in post-menopausal women (Weitz et al. 2001) and renal SNA in anesthetized and conscious, ovariectomized female rats (He et al. 1998, Saleh et al. 2000). In the female rat, the decrease in renal SNA observed following peripheral estradiol administration is centrally mediated as a prior intrathecal injection of an estradiol receptor antagonist abolishes estradiol mediated sympathoinhibition (Saleh et al. 2000). Knowledge regarding mechanisms of action of central estradiol signalling in affecting sympathetic outflow is still highly nonspecific and therefore poorly understood.

Estradiol receptors are expressed in most, if not all, regions of the brain involved in the central regulation of SNA (Shughrue et al. 1997, Brailoiu et al. 2007). Central estrogen receptor expression appears to fluctuate across the estrous cycle of the rat in a regional
specific manner (Milner et al. 2008). The topic of estradiol signalling in the brain is too complex to cover completely in this thesis. To convey some of the possible central actions of estradiol, I will focus on the NTS, RVLM and PVN as being primary sites responsible for regulating both the arterial baroreceptor reflex and cardiac afferent reflex.

1.8.2.1. Estradiol in the nucleus tractus solitaries

As mentioned previously, the NTS is the primary termination site for sensory afferents conveying information from baroreceptors and chemoreceptors (Palkovits et al. 1977). As stated, estradiol receptors are located in the NTS (Shughrue et al. 1997). Direct microinjection of estradiol into the NTS causes an acute decrease in renal SNA and arterial pressure in anesthetized rats, which is accompanied by an augmented baroreflex mediated inhibition of renal SNA in response to a fixed, bolus dose of phenylephrine (Saleh et al. 2000). However, the role of estradiol in mediating sympathetic neurons in the NTS has not been extensively studied. Estradiol in the NTS appears capable of mediating hypotension via NO (Li et al. 2009). Furthermore, estradiol replacement in ovariectomized rats enhances the bradycardic responses to orexin directly injected into the NTS (de Oliveira et al. 2003). Estradiol directly injected into the NTS inhibits neuronal activity and inhibits the responsiveness of NTS neurons to glutamate but not GABA, suggesting that estradiol may alter NTS neuronal responsivity to excitatory afferent input (Xue et al. 2003). Therefore, the small number of studies suggests that estradiol acting in the NTS likely has the ability to affect changes in sympathetic regulation.

1.8.2.2. Estradiol in the rostral ventrolateral medulla

The RVLM consists of a significant portion of preganglionic neurons contributing to tonic sympathetic tone and is a primary site for the processing and integration of sensory afferent input (Blessing et al. 1981, Barman et al. 1985, Guyenet 2006). ERα and ERβ are both expressed in the RVLM but show regional homogeneity (Wang et al. 2006). With the use of retrograde labelling and electron microscopic immunocytochemistry in rats, ERβ expression has been largely located within pre-sympathetic, tyrosine hydroxylase-containing, C1 adrenergic bulbo spinal neurons (Wang et al. 2006, Wang et al. 2008). ERα expression was found to be located on non-tyrosine hydroxylase expressing neurons with dendritic connections to tyrosine hydroxylase-containing neurons suggesting a possible
indirect action of ERα activation on pre-sympathetic neurons (Wang et al. 2006, Wang et al. 2008).

In male and female rats, the microinjection of estradiol into the RVLM causes an acute decrease in arterial pressure and renal SNA that appears to be ERβ-dependent (Saleh et al. 2000, Shih 2009). In male rats, an inducible NOS inhibitor abolishes the decrease in blood pressure following microinjection of estradiol into the RVLM suggesting that the centrally mediated depressor effect of estradiol is NO-dependent (Shih 2009). This finding corresponds well with the observation that inducible NOS is responsible for NO mediated sympathoinhibition in the RVLM (Kagiyama et al. 1997, Chan et al. 2001).

The actions of estradiol in the RVLM discussed so far have been investigated in an acute experimental setting. In contrast, Subramanian et al. investigated the effects of chronic peripheral estradiol treatment of a relatively low-dose for 90 days in ovary-intact female rats therefore causing higher than normal levels of circulating estradiol (Subramanian et al. 2011). This study by Subramanian et al. observed that chronically elevated levels of estradiol caused hypertension in conscious ovary-intact female rats, measured by telemetry, which was accompanied by a significantly increased level of superoxide in the RVLM, suggesting the possibility that the resulting hypertension was centrally mediated (Subramanian et al. 2011). Both NO and ROS have been shown to affect the arterial baroreceptor and cardiac afferent reflex control of SNA in health and disease (Wang et al. 2003, Gao et al. 2004, Zucker et al. 2004, Mayorov 2005, Guo et al. 2009). The study by Subramanian et al. suggests that the levels and timeline changes of circulating estradiol likely has a significant impact on the subsequent effects of estradiol on cardiovascular control.

1.8.2.3. Estradiol in the paraventricular nucleus

The PVN contains RVLM and IMC projecting neurons and plays an important role in sympathetic regulation, particularly in relation to volume regulation (Coote et al. 1998, Pyner 2009). The PVN contains preautonomic parvocellular neurons projecting to the RVLM that display ERβ expression thereby suggesting that estradiol may directly act on the PVN-to-RVLM projecting neurons (Stern et al. 2003). In general, the PVN appears to have an excitatory output on sympathetic excitatory neurons (Coote et al. 1998). For example, the direct stimulation of the PVN in vivo using glutamate (Martin et al. 1992), electrical current (Martin et al. 1993) and bicuculline (presumably by inhibition of basal

In anesthetized rats, the direct injection of estradiol into the PVN causes an acute decrease in arterial pressure suggesting a direct sympathoinhibitory action (Gingerich et al. 2006). Estradiol in the PVN appears capable of increasing NO production (Gingerich et al. 2005). In anesthetized rats, estradiol significantly attenuates the pressor response caused by direct microinjection of glutamate into the PVN, an effect that is mediated via the ERβ and NO (Gingerich et al. 2006). NO in the PVN has been shown to be sympathoinhibitory and is intricately involved in modulating neuronal release and sensitivity to GABA and glutamate (Li et al. 2001, Wang et al. 2006, Biancardi et al. 2011). The studies described in this section suggest the probability that estradiol within the PVN can alter sympathetic regulation and is most likely sympathoinhibitory.

1.8.3. Estradiol, nitric oxide and sympathetic regulation

Normal levels of central NO appear to have a small, insignificant effect on resting vasomotor tone in both conscious male and female rats (Xue et al. 2009). However, resting NO levels in conscious female rats appear to have a greater inhibitory action on the centrally mediated pressor response to peripherally administered ANG II when compared to males, a difference that is abolished by ovariectomy (Xue et al. 2009). To elaborate, chronic, peripheral administration of ANG II in conscious male rats results in significantly greater increases in blood pressure when compared to ovari-intact female rats, a difference that is abolished by ovariectomy (Xue et al. 2009). In the same study, central inhibition of NO with L-NAME did not alter the resting blood pressure in response to chronic administration of angiotensin II in male and ovariectomized female rats but did augment the blood pressure response to ANG II in ovari-intact females (Xue et al. 2009). These functional results were related to neuronal NOS protein expression in the subfornical organ and PVN where ovari-intact females displayed significantly higher levels of neuronal NOS expression compared to males and ovariectomized females, with only ovari-intact females displaying an increase in neuronal NOS expression in response to chronic ANG II infusion.
The findings discussed suggest that estradiol not only augments central NO signalling but also attenuates the central sympathoexcitatory actions of ANG II.

1.8.4. Estradiol, renin-angiotensin system and sympathetic regulation

A positive correlation between estradiol and AT1R expression in central autonomic regions has been observed (Wang et al. 2008, Pierce et al. 2009). Estradiol replacement in ovariectomized rats results in greater AT1R expression in bulbospinal neurons of the RVLM compared to both ovariectomized female and male rats (Wang et al. 2008). Binding of ANG II to AT1R leads to the phosphorylation of the cytoplasmic NADPH oxidase subunit p47, inducing the production of ROS (Infanger et al. 2006). However, the increase in AT1R expression in the RVLM due to estradiol is not associated with a concomitant increase in NADPH oxidase as would be expected suggesting one action of estradiol is to limit the production of ROS in response to ANG II signalling (Hanna et al. 2002, Infanger et al. 2006, Wang et al. 2008). Therefore the results suggest that estradiol can attenuate central ANG II actions by limiting the subsequent expression of ROS, a key element involved in ANG II signalling and central ANG II mediated sympathoexcitation (Gao et al. 2005, Zhang et al. 2006).

Estradiol appears capable of directly affecting ANG II mediated neuronal activation in the circumventricular organs. Extracellular single unit recordings of PVN-projecting neurons in the subfornical organ suggest that estradiol is capable of inhibiting the excitation of these neurons in response to circulating ANG II (Tanaka et al. 2001, Ciriello et al. 2013). Estradiol also appears capable of attenuating the excitatory actions of ANG II on neurons in the area postrema (Pamidimukkala et al. 2003). ANG II sensitive neurons in the area postrema have been shown to attenuate the excitation of baroreceptor sensitive neurons in the NTS, suggesting a possible pathway by which circulating ANG II and estradiol may alter arterial baroreflex function (Matsukawa et al. 1990, Cai et al. 1994). Furthermore, female sex in animal studies is associated with a shift in the axis towards the depressor arm of the RAS that is estrogen dependent (Sampson et al. 2012). For example, ovary-intact females display greater production of the angiotensin 1-7 peptide compared to males (Bhatia et al. 2013, Hilliard et al. 2013). Angiotensin 1-7 has been demonstrated to have opposing effects to those of ANG II by augmenting central NO expression (Zheng et al. 2011) and protecting reflex regulation of SNA in disease states such as heart failure (Kar et al. 2011).

To summarize, both the expression of estrogen receptors and the functional data suggests estradiol is capable of affecting central sympathetic regulation, both directly and indirectly,
via modulating the expression of and responsiveness to neurohormones such as NO and the RAS in central autonomic nuclei.

1.9. Changes in sympathetic nerve activity following myocardial infarction

As stated previously SNA is in general elevated following MI, which contributes to worsening cardiac function and the development of heart failure (Hasking et al. 1986, Leimbach et al. 1986, Grassi et al. 1995, Grassi et al. 2001, Nozawa et al. 2002, Jardine et al. 2005, Jardine et al. 2007). In the first 24 hours following a MI, patients are prescribed beta-blockers and advised to maintain on beta-blocker treatment indefinitely (Viscoli et al. 1993, Godlee 2000). Beta-blocker treatment directly following MI is associated with a significant decrease in the chance of sudden death, highlighting the fact that SNA, at least to the heart, is elevated acutely following MI which can have severe consequences (Godlee 2000, Jardine et al. 2005, Jardine et al. 2007). It is not possible to record SNA to any organ during an MI in patients, as the occurrence of MI is not possible to predict. Therefore studies in animals are necessary to investigate the changes in SNA in direct response to experimental MI.

There are no studies that have been able to investigate long term changes in SNA to the kidney following MI in the same person or animal. Changes in renal SNA following MI are particularly important to consider because increased renal SNA can increase fluid volume and circulating ANG II which are both changes that are adversely associated with heart failure development and progression (Nozawa et al. 2002, DiBona et al. 2003, DiBona et al. 2004, Petersson et al. 2005). Experimental studies suggest that renal SNA is elevated acutely in male animals in response to MI (Thames et al. 1979). Renal SNA is strongly under arterial baroreceptor reflex control and therefore it would be expected that changes in renal SNA following MI would be, at least partly, governed by this reflex. In anesthetized dogs, denervation of the arterial baroreceptors abolishes any sympathoexcitatory response in the first minute of occlusion of the left anterior descending coronary artery, providing strong evidence that the arterial baroreceptor reflex has a dominant influence on the initial changes in renal SNA post-MI (Thames et al. 1979). As previously discussed, the chemosensitive cardiac afferent reflex also plays a role in driving sympathetic and hemodynamic changes in the acute response to MI (Webb et al. 1972, Weaver et al. 1981, Wei et al. 1983). Chemically sensitive cardiac sympathetic and vagal afferents are excited in direct response to myocardial ischemia in anesthetized animals (Minisi et al. 1991,
Schultz et al. 1996, Pan et al. 2002). In animals with either cardiac sympathetic afferent or cardiac vagal denervation, the renal SNA response to short-term myocardial ischemia shows either an increase or decrease respectively (Weaver et al. 1981). As shown in Figure 1.11, alongside input from the arterial baroreceptor reflex, the activation of the two parallel yet opposing cardiac afferent pathways will have some determining impact on the changes in renal SNA in response to acute MI (Webb et al. 1972, Felder et al. 1979, Thames et al. 1979, Weaver et al. 1981, Wei et al. 1983). Whether sex and ovarian hormones can affect the sympathetic response is unknown.

![Figure 1.11: Initial sympathetic response to myocardial infarction](image)

A modified graph from Thames et al (1979) showing change in mean arterial pressure (MAP) and percentage change in renal sympathetic nerve activity (RSNA) resulting from 60 second occlusion of the left anterior descending coronary artery (LAD) or left circumflex coronary artery (Cx) in the intact dog, in dogs with carotid sinus baroreceptors denervated (CSN section), in dogs with sino-aortic baroreceptors denervated (SAD), and dogs with carotid sinus and sino-aortic baroreceptors denervated and with removal of vagus nerve (CSN+vagi sectioned) (Thames et al. 1979).
1.9.1. Can female sex and ovarian hormones affect the initial renal sympathetic nerve activity response to myocardial infarction?

In humans, it has been demonstrated that in response to a two-minute balloon occlusion of a coronary artery, women compared to men display a greater likelihood of reductions in both heart rate and arterial pressure suggesting a greater efferent vagal activation and/or attenuated sympathetic activation in women in response to myocardial ischemia (Airaksinen et al. 1998). Du et al. have demonstrated in anesthetized female rats that coronary ligation of the left anterior descending coronary artery results in significant decreases from baseline in heart rate over the first 30 minutes post-MI in females compared with a slight increase in heart rate in males (Du et al. 1995). Vagotomy abolished the decrease in heart rate in female rats post-MI suggesting that the vagal afferent and/or efferent pathway is responsible (Du et al. 1995). Furthermore, in conscious rats, beta-blockade did not have a significant effect on time to ventricular tachycardia in ovari-intact females in response to MI but did significantly increase time to ventricular tachycardia in male and ovariectomized female rats (Lujan et al. 2008). The results discussed suggest the autonomic nervous system may mediate the sex differences in the hemodynamic responses to MI (Du et al. 1995, Airaksinen et al. 1998, Lujan et al. 2008).

One study that has directly investigated sex specific changes in SNA following MI was performed in humans recording muscle SNA. Hogarth et al demonstrated that when compared to men post-menopausal women displayed relatively greater increases in muscle SNA in the first 9 months following MI when compared to a single recording of resting muscle SNA in healthy sex and age matched subjects (Hogarth et al. 2009). As discussed previously in this introduction, the resting levels of muscle SNA can differ between men and women and can be differentially affected by circulating ovarian hormones and age. Different pre-MI levels of muscle SNA in males and females may potentially explain the sex differences in resting muscle SNA when comparing between patients with MI and healthy controls which further highlights the limitations of acute recordings of SNA (Hogarth et al. 2009). Based on the findings discussed in this introduction, it would seem plausible that there exist sex differences in the sympathetic response to MI. In particular, given the sympathoinhibitory actions of estradiol, it would seem plausible to hypothesize that estradiol is capable of attenuating increases in renal SNA post-MI. Animal experiments in which SNA can be recorded both before and after MI in the same animal provide an opportunity to determine what effects, if any, female sex and ovarian hormones have on the initial sympathetic response to MI and the mechanisms responsible.
1.10. Changes in renal sympathetic nerve activity in the development and progression of heart failure

In both humans and animals, an increase in renal SNA in heart failure development and progression is associated with worsening cardiac function and mortality (Nozawa et al. 2002, Petersson et al. 2005, Hu et al. 2012). It is generally considered that renal SNA increases in the later stages of heart failure, once heart failure is established (Rundqvist et al. 1997). However, the evidence in rats suggest that acute increases in renal SNA in the first few weeks following MI contribute to adverse changes in volume regulation and heart function, as shown by the beneficial effects of renal denervation in the first 4 weeks post-MI (Nozawa et al. 2002, Hu et al. 2012). Therefore, it is likely that increases in renal SNA not only contribute to heart failure progression but also in heart failure development, at least in ischemic heart failure. In addition to elevated baseline SNA, there appears to be fundamental alterations in the firing patterns in single postganglionic sympathetic neurons in heart failure patients that may contribute to the adverse actions related to SNA, independant of changes in total nerve activity (Elam et al. 2002). The mechanisms responsible for the adverse changes in renal SNA in heart failure have not been definitively investigated.

1.10.1. What drives increases in renal sympathetic nerve activity in the development of heart failure?

It is generally understood that a complex interaction between sensory autonomic reflexes and circulatory control systems drive pathological changes in SNA (May et al. 2013). In both humans (Grassi et al. 2001) and animals (DiBona et al. 1994, Zhang et al. 1999, Zhu et al. 2002) the arterial baroreceptor reflex control of SNA has been demonstrated to be impaired in severe heart failure compared to healthy controls. However, there is some debate as to whether the arterial baroreflex is indeed impaired in heart failure as the arterial baroreflex control of muscle SNA in patients and cardiac/renal SNA in sheep has been demonstrated to be functioning normally (Ferguson et al. 1992, Floras 2001, Ramchandra et al. 2009). Impairment of the arterial baroreflex in heart failure has typically been presented as a decrease in the sensitivity (rate of change in SNA for a given change in arterial pressure) and/or a decreased range of control (DiBona et al. 1994, Zhang et al. 1999, Grassi et al. 2001, Zhu et al. 2002). It is possible that the severity of heart failure
impacts on changes in the arterial baroreflex control of SNA thereby contributing to discrepancies in the clinical and experimental literature (May et al. 2013).

Desensitization of the arterial baroreceptor reflex, which is sympathoinhibitory in nature, may result in SNA being more responsive to excitatory inputs (DiBona et al. 1995). In addition, sensitization of the chemosensitive cardiac sympathetic afferent reflex in the setting of heart failure may contribute to elevated sympathetic outflow. Figure 1.12 demonstrates that in animals with heart failure, the CSAR drives greater increases in renal SNA when compared to controls in response to epicardial application of a given dose of an activating substance, such as capsaicin or bradykinin (Wang et al. 1996, Ma et al. 1999, Wang et al. 2000, Zhu et al. 2002, Zhu et al. 2004, Zhu et al. 2004). Based on findings that have both recorded from and stimulated the sensory afferent neurons, the changes in the CSAR in heart failure are considered to be mediated by changes both in the afferent component of the reflex and centrally (Ma et al. 1997, Ma et al. 1999, Wang et al. 1999). There is also conflicting data regarding the location responsible for alterations in the arterial baroreceptor reflex pathway in heart failure (Dibner-Dunlap et al. 1989, DiBona et al. 1994, Zhang et al. 1999). Given the overlap of central autonomic nuclei governing the arterial baroreflex and CSAR, it is likely that central changes responsible for altering these two sympathetic reflexes are related (Gao et al. 2005, Wang et al. 2007).

**Figure 1.12: Example traces showing sensitized cardiac sympathetic afferent reflex responses in heart failure**

Recordings of arterial pressure (top row) and renal sympathetic nerve activity (RSNA; bottom row) in response to epicardial administration of bradykinin (50 mg) in a sham dog (A) and a dog with pacing induced heart failure. Horizontal bars on bottom indicate when bradykinin was applied. Sex and age of the subjects is unknown (Wang et al. 1996).
1.10.2. Can female sex and ovarian hormones protect reflex regulation of renal SNA in heart failure?

To date, studies investigating the regulation of the SNS in heart failure have used either male or mixed sex groups. Studies performed using mixed sex groups have typically used rabbits, cats, or dogs whereas studies performed solely in males are typically performed in rats. Mixed sex animal studies commonly present the total group numbers and weights of the experimental animals: therefore the ratio of males to females or state of reproductive development of the animals cannot be addressed. Rabbits, cats and dogs display estrous cycles that vary quite significantly from the menstrual cycle in women whereas the rat estrous cycle appears to correspond more closely albeit at a much more rapid rate (i.e. 4 days in rat compared to 30 days in women) (Goldman et al. 2007). The failure to address the possibility of sex specific effects on sympathetic regulation in states such as heart failure appears to be a limitation of the current literature.

Figure 1.13 demonstrates that in male rats with ischemic heart failure, the ability of the arterial baroreceptor reflex to appropriately inhibit renal SNA is significantly impaired along with a reduced gain of the arterial baroreflex curve (DiBona et al. 1994, DiBona et al. 1995). Sensitization of the CSAR has also been demonstrated in anesthetized male rats with MI-induced heart failure (Wang et al. 2000). Therefore the current understanding that has been developed predominantly in male rats is that reflex regulation of renal SNA is altered to favor sympatheexcitation and attenuate reflex mediated sympathetic inhibition. Whether the same is true for females is unknown.

The only studies investigating reflex regulation of renal SNA in heart failure specifically in females have been performed in sheep. Ewes, as opposed to rams, are used because they are more compliant and easier to handle compared with their male counterparts. The sheep model offers a large animal model in which SNA to various organs can be recorded in the conscious state. Using a pacing induced model of heart failure, Ramchandra et al. have demonstrated that the arterial baroreceptor reflex control of renal SNA is unchanged when compared to shams in conscious female sheep with mild to moderate heart failure (Ramchandra et al. 2009). The results by Ramchandra et al. have been used to explain why renal NA spillover in patients with mild to moderate heart failure are not significantly elevated when compared with healthy individuals (Rundqvist et al. 1997, Ramchandra et al. 2009). However, another possibility is that the female sex may be acting to attenuate alterations in the arterial baroreceptor reflex control of renal SNA (Ramchandra et al. 2009). It is difficult to compare results obtained in different species using different models of heart
failure. Typically, heart failure in rats induced by MI tends to be more severe than the pacing induced model of heart failure performed in sheep, so it may be the severity of heart failure that determines the changes in baseline SNA and reflex control (May et al. 2013).

Figure 1.13: Arterial baroreflex curves in male rats with or without heart failure
Sigmoidal curves describing the relationship between changes in efferent renal sympathetic nerve activity (ERSNA, as a percentage (% change from baseline) in relation to changes in mean arterial pressure (MAP, mmHg)) in anesthetized, male rats with myocardial infarction induced heart failure (CHF, dashed line) or control rats (straight line) (DiBona et al. 1994).

As previously discussed, ovarian hormones, particularly estradiol, have the ability to alter sympathetic regulation and do so, at least in part, by mediating central neuromodulation in important autonomic nuclei. A decrease in central NO signalling and an increase in central ANG II signalling, in general, appear to contribute to dysregulation of the reflex control of SNA in heart failure (Zhang et al. 1999, Wang et al. 2003, Zhu et al. 2004, Zhu et al. 2004, Zucker et al. 2004, Pyner 2009, Zheng et al. 2009, Zheng et al. 2011, Zucker et al. 2004, Pyner 2009, Zheng et al. 2009, Zheng et al. 2011). In contrast, as previously discussed, estradiol in females appears to act centrally in almost the opposite fashion in that it can increase central NO and decreases central ANG II actions (Tanaka et al. 2001, Pamidimukkala et al. 2003, Wang et al. 2008, Shih 2009, Xue et al. 2009, Sampson et al. 2012, Ciriello et al. 2013). For example, Guild et al. have shown in conscious rabbits that a chronic, intravenous sub-pressor dose of ANG II can drive long-term increases in baseline renal SNA which is associated with an impaired ability of the arterial baroreflex to appropriately inhibit renal SNA (Guild et al. 2012). In conjunction, Xue et al. have demonstrated an attenuation of the centrally mediated ANG II pressor effect by ovarian hormones in females in a manner related to augmented central NO signalling (Xue
et al. 2009). Therefore, my hypothesis is that ovarian hormones in females will protect the arterial baroreceptor reflex and cardiac sympathetic afferent reflex control of renal SNA in heart failure.

1.11. Specific aims and hypotheses of this thesis

The overall aim of this thesis was to investigate the effects of sex and ovarian hormones on changes in renal SNA and the regulation thereof following MI and in subsequent heart failure. The first aim was to investigate the effects of sex and ovarian hormones on the initial sympathetic response to MI. Based on the findings from the first aim, the second aim was to determine the effects of sex and ovarian hormones on the cardiac afferent reflex as a possible contributor to sex-specific changes in autonomic activity in response to MI. The third and final aim of this thesis was to investigate the effects of sex and ovarian hormones on changes in the arterial baroreceptor reflex and CSAR control of renal SNA in heart failure.

Hypotheses;

1) Ovarian hormones in female rats will attenuate the increase in renal SNA observed in response to MI, in a manner related to attenuated arterial baroreflex mediated increases in renal SNA

2) In female rats, estradiol will augment the cardiac vagal afferent reflex mediated inhibition of renal SNA and attenuate the cardiac sympathetic afferent reflex mediated excitation of renal SNA.

3) Ovarian hormones in female rats will protect the arterial baroreceptor reflex and CSAR control of renal SNA in MI-induced heart failure

Using anesthetized rats instrumented to record arterial pressure, heart rate and renal SNA, the specific aims of this thesis were investigated.
Chapter 2: General Methods

2.1. Introduction

This chapter describes the general methodology used for the experiments outlined in this thesis, although specific methodologies are described in the individual chapters. All procedures were approved by the Animal Ethics Committee of the University of Auckland, New Zealand (AEC number 815).

2.2. Subjects

The experiments within this thesis are performed on male and female Wistar rats. The Wistar rat provides a standardized research model that enables the current results to be confidently related to past and future research (Janig et al. 2003). The use of the rat as an experimental model to investigate the SNS is justified when, as is the case in this thesis, the experiments cannot be performed in humans and will improve the understanding of disease and treatment.

In the current thesis, the age of the subjects and the state of circulating sex hormones are important considerations. The developmental stages of different rat strains appear to vary. It has been reported that puberty, defined as the time in which reproduction is first possible, is attained in female Wistar rats between 36 and 66 days with an average of 47 days following birth, with a similar age of puberty observed in males (Freudenberger 1932). Puberty is associated with significant increases in circulating sex hormones in both humans and animals. Males do not experience cyclic fluctuations in sex hormones.

Women experience menopause, the period when menstrual cycling ceases, and is associated with a precipitous fall in the amount of circulating estrogens and progesterone (Bjornerem et al. 2004). Female rats do not necessarily display a similar menopausal event as humans, but there are age-related changes in cycling and hormone levels (Chakraborty et al. 2004). Due to time and cost limitations, menopause is typically modelled in female rats by removing the ovaries (referred to as ovariectomy) thereby causing a significant reduction in the levels of circulating ovarian hormones (Strom et al. 2008, Goldman et al. 2009, Strom et al. 2010). Ovariectomy in the rat is a well-characterized and widely used model to reduce circulating levels of female ovarian hormones (Kalu 1991, Chakraborty et
In this thesis, all ovariectomies were performed in female rats aged between 4-6 weeks old. These rats were not checked to see whether they had begun cycling prior to ovariectomy surgery. It is acknowledged that inducing artificial menopause in rats that are so young may not accurately reflect the real life situation and may have interfered with developmental effects of estrogens and progesterone (Chakraborty et al. 2004). All experiments performed in this thesis were done in both male and female rats aged between 8 to 18 weeks old, the age range in which both male and female rats display the highest levels of circulating sex hormones (Lu et al. 1979).

2.3. Anesthetized rat model for recording SNA

The experiments in this thesis focus on extracellular recordings of postganglionic efferent renal sympathetic nerves in anesthetized rats. It is understood that recording SNA in conscious, freely moving and well recovered animals is the gold standard experimental technique. However, limitations in recording SNA in conscious, freely moving animals can justify the use of the anesthetized model. For example, the studies in this thesis required direct access to the heart whilst recording renal SNA which is not possible in the conscious rat. Furthermore, activation of the CSAR is associated with the perception of pain, making the anesthetized model appropriate on an ethical basis.

Anesthesia can affect the regulation of SNA in ways not wholly understood; however the small amount of evidence available does suggest that the choice of anesthesia is critical in ensuring that reflex regulation of renal SNA remains viable. In male rats, α-chloralose and urethane, but not sodium pentobarbital, appear to cause significant increases of around 100% of the pre-anesthetic level in renal SNA by 5 hours post-infusion (Shimokawa et al. 1998). The arterial baroreceptor reflex control of renal SNA appears to be unaltered significantly by either sodium pentobarbital, α-chloralose or urethane at 5 hours post-infusion of anesthetic (Shimokawa et al. 1998). Therefore at 5 hours post anesthetization, the arterial baroreceptor reflex curves plotted as renal SNA over arterial pressure are unaltered when compared to conscious animals but the resting values sit closer to the upper plateau (Shimokawa et al. 1998). With the evidence in the literature in mind, the investigation of the reflex regulation of renal SNA in the studies performed in this thesis took place at least 4 hours following initial anesthetization of the rat.
2.3. Myocardial infarction model in rat

In the first and third studies described in this thesis, the coronary ligation model of MI in rats has been used. The coronary ligation model of MI in the rat has been well defined (Goldman et al. 1995, Francis et al. 2001, Krzeminski et al. 2008). It has been well characterized that when the MI is above a certain size, generally around 30% of the left ventricular wall, the rat develops signs and symptoms of congestive heart failure over the following weeks (Goldman et al. 1995, Hasenfuss 1998, Francis et al. 2001). Following experimental MI, neurohormonal activation is comparable between rats and humans (Goldman et al. 1995, Hasenfuss 1998, Francis et al. 2001). In particular, the level of circulating atrial natriuretic peptide (ANP) is inversely correlated with cardiac function in humans and is therefore a good indicator of the severity of heart failure (Riegger et al. 1986, Hara et al. 1987). The significant elevations in ANP in the rat following chronic, large MI suggest this experimental technique results in attenuated cardiac function similar to the clinical setting (Riegger et al. 1986, Hara et al. 1987, Francis et al. 2001). Specifically the MI model produces heart failure characterized by systolic failure meaning that the ability of the left ventricle to pump blood is significantly attenuated (Philbin et al. 2000, Kitzman et al. 2002, Bursi et al. 2006). Therefore the coronary ligation model of MI in the rat is appropriate for the investigation of SNA in MI and systolic heart failure settings.

2.4. Preparatory surgery

2.4.1. Ovariectomy

Bilateral ovariectomy was performed in 4-6 week old female rats lying in the ventral recumbent position under isoflurane anesthesia (2% in oxygen). At the beginning of surgery, all animals were given antibiotics (12.5 mg/kg enrofloxacin, Baytril, Bayer, NZ) and analgesia (20 µg/kg buprenorphine, Temgesic, Reckitt Benckiser, NZ) and again 24 hours later. A dorsal midline skin incision was made, beginning approximately 5 centimeter (cm) from the base of the head and ending 2cm from the base of the tail. Bilateral openings of approximately 1cm was created through the muscle layers located on the border of the regio lumbalis and regio abdominis lateralis, directly caudal of the regio tuburis coxae. The ovaries and oviduct were externalized; the oviduct was tied and then sectioned on the peripheral side of the tie at a distance of approximately 1cm from the ovaries, which were subsequently removed. The remaining tissue was replaced in the peritoneal cavity and the
openings were sutured closed. As soon as the rats regained consciousness they were returned to their home cages. A heating pad was placed under the cage for 24 hours after the surgery.

2.4.2. Hormone replacement

Chronic hormone replacement was achieved at the time of ovariectomy via sub-cutaneous implantation at the dorsal midline incision via silastic capsules (see Figure 2.1) that contained either 17β-estradiol (Sigma-Aldrich Inc. USA) or progesterone (Sigma-Aldrich Inc. USA) using a widely used and well characterized technique (Bridges 1984, Strom et al. 2008). The capsules were silicone tubing with an internal diameter of 0.93 mm and an outer diameter of 3.23 mm (Dow Corning, VWR International, Buffalo Grove, IL, USA). For 17β-estradiol administration, the tubing was cut at 20 millimeter (mm) lengths and for progesterone administration the tubing was cut at 30mm lengths.

![Silicone Tube](image)

**Figure 2.1 Hormone replacement capsule**
Schematic diagram of the silastic capsules that contained either estradiol or progesterone and were implanted subcutaneously in the rat.

17β-estradiol was initially dissolved in 100% ethanol at a concentration of 50 milligrams (mg) per milliliter (ml). Once dissolved, the ethanol containing the dissolved 17β-estradiol was diluted in peanut oil at a concentration of 200 microgram (µg) of 17β-estradiol per ml of peanut oil/ethanol mixture and mixed well. The 17β-estradiol solution was syringed into the silastic tubes, which were sealed at either end with silicone sealant as seen in Figure 2.1 (Selleys glass silicone, NZ). Progesterone was packed tightly into the silicone tubing in crystalline form and each end of the 30mm silastic capsule was sealed with silicone sealant (Selleys glass sealant, NZ). Prior to implantation, the ready-made silastic capsules containing either 17β-estradiol or progesterone were sterilized by placing in 70% ethanol for 10 minutes, they were then rinsed with sterile saline and left to soak in sterile saline for at least 4 hours, if not overnight, prior to implantation. Soaking the silastic capsules prior to implantation is an important step as there is an initial surge in hormone release when these capsules are first exposed to a fluid environment. Once the initial surge in release
subsides, the release of hormone appears to be stable over time (Bridges 1984, Strom et al. 2010). The ready-made silastic capsules were implanted subcutaneously at the site of the dorsal midline skin incision that was made during ovariectomy surgery. Animals that did not receive hormone replacement did not have silastic capsules implanted at the time of ovariectomy.

2.5. Experimental preparation

2.5.1. Anesthesia

On the day of experiment, a new batch of anesthetic was made up of 20mg/ml α-chloralose (Sigma-Aldrich Inc, USA) and 250mg/ml urethane (Sigma-Aldrich Inc, USA) in 10ml of saline. The anesthetic was administered intraperitoneally (I.P.) to effect over the course of an hour. The initial volume of anesthetic was 3.3ml per kilogram (kg) of body weight making the average initial dose of anesthetic 66mg α-chloralose and 825mg urethane per kg of body weight. Subsequent doses averaged 20mg α-chloralose and 250mg urethane per kg of body weight at 10 minute intervals. Adequate anesthesia was generally obtained over the course of the hour following the gradual administration of a total volume of 6ml of anesthetic per kg body weight making the total average dose of anesthetic 120mg α-chloralose and 1500 urethane per kg of body weight. Sufficient anesthesia was confirmed by testing for the complete removal of the hind-limb reflex, tail pinch and blink reflex. Anesthesia was maintained throughout the experiment by intravenous infusion or I.P. injection of anesthetic (10% of initial dose every hour).

2.5.2. Determining estrous phase

The estrous phase in the ovary-intact females was observed on the day of experiment by examining the cytology of vaginal smears. To obtain the vaginal smear, a pipette tip containing 50µl of saline was inserted into the rat’s vagina and the saline was flushed into the opening. The fluid was then brought back into the pipette tip, placed onto a microscope slide and was left to evaporate. Once dry, the cells left on the slide were bathed in hematoxylin for one minute before the stain was gently washed off with water. Once the slide had dried, a coverslip was placed over the stained cells and were then observed using a standard light microscope. Fluctuating hormone levels during the cycle results in changes
in the number and types of cells present in the vagina that can then be used to determine estrous stage of the rat (Hubscher et al. 2005, Goldman et al. 2007).

2.6. Surgical preparation

2.6.1. Tracheotomy

Artificial ventilation was required in these experiments because anesthetics can depress breathing and an open chest was required. Once sufficiently anesthetized, the rat was placed in the dorsal recumbent position. A ~2cm skin incision was made in the region trachealis. The muscle surrounding the trachea was separated with forceps. The trachea was cannulated with plastic tubing with an inner diameter of 1.77mm, an outer diameter of 2.8 mm and a length of ~3cm. Artificial ventilation was maintained throughout the course of the experiment with a Harvard rodent ventilator (model 680; Harvard apparatus, Holliston, MA). The inspirate gas was enriched with O₂ (~50% O₂) and the ventilator settings were adjusted (tidal volume ~3-4ml; breathing rate ~70-80/min) to maintain arterial Pco₂ normocapnic.

2.6.2. Arterial and venous lines

A ~5cm skin incision was made in the inguinal region. The right femoral artery was cannulated (polyethylene-50) to record arterial pressure. The tip of the cannula was advanced into the lower aorta and tied into position. The arterial catheter contained heparinized saline (50 U/ml). The catheter was attached to a pressure transducer (PX6001, Edwards lifesciences LLC, USA) external to the body. The right femoral vein was cannulated (polyethylene-50) in order to provide the animal with a constant infusion of saline (0.05 to 0.1 ml/hr) and for use in baroreceptor reflex curve protocol. The venous catheter contained saline and was attached to an infusion pump.
2.6.3. Renal nerve electrode implant

Following tracheotomy and blood vessel cannulation, the rat was placed in the right lateral recumbent position. A ~5cm skin incision along the border of the left regio lumbalis and regio abdominis lateralis was made. A retroperitoneal incision through the muscle was made along the left border of the erector spinae muscles exposed. The kidney was retracted to better expose the renal artery and renal nerves. The electrodes used to record renal SNA were made of individually insulated stainless steel wires that were further encased in silicone tubing. At one end, each wire was soldered onto a gold pin to form the connection to the recording equipment. At the recording end of each stainless steel wire, the wire was stripped of its insulation and the bare wire was coiled twice each around the barrel of a 20 gauge needle. Once the exposed recording wires were coiled, the openings in the silicone tubing (that the recording wires were encased within) were sealed using silicone sealant (Selleys glass sealant, NZ) to prevent fluid from potentially contacting exposed wire. The two coils were roughly 2-3mm apart from each other and formed the site of the nerve-electrode interface. As shown in Figure 2.2, the renal nerve electrodes were securely tied onto the walls of the aorta and renal artery and a branch of the renal nerve was placed over the wire coils in a manner that has previously been described (Dorward et al. 1985).

![Diagram of renal nerve electrode implant]

Figure 2.2: Schematic showing the equipment and set-up used for recording renal sympathetic nerve activity.
Once the renal nerve was making good contact with the wire coils, the surrounding tissue was dried. Once sufficiently dry, the nerve-electrode bundle was insulated with silicone gel (Kwik-sil, World Precision Instruments, Sarasota, FL) in order to completely insulate the nerve-electrode bundle from surrounding tissue. Once the silicone insulation had set properly, the retroperitoneal cavity was closed and the skin incision stapled shut. The non-recording end of the nerve electrode wires were externalized through the same incisions made for the renal nerve surgery. Following renal nerve electrode implantation, the rat was returned to the dorsal recumbent position for the remainder of the experiment.

Renal SNA was wirelessly recorded via a high gain amplifier (model TR46SP, Telemetry Research Ltd., Auckland, NZ) with the signal transmitted to a receiver (TR162, Telemetry Research Ltd., Auckland, NZ). The wireless recording transmitter sat external to the rat and was attached to the recording electrodes via gold attachments. An earth electrode that was permanently connected to the transmitter was placed subcutaneously close to the recording site. Aluminium foil was used to reduce external noise on the signal by shielding the transmitter.

2.6.4. Opening Chest

In most of the experimental protocols described in this thesis, a direct access to the heart was required. To open the chest, a ~4cm skin incision was made in the regio sternalis. The left pectoris and external oblique muscles were separated from surrounding tissue to expose the left rib cage. An opening was made either in the left 4th or 5th intercostal space to expose the heart. At this point, positive end pressure of approximately 3-4mmHg was applied to the artificial respirator in order to maintain adequate pressure within the chest area. The opening in the intercostal space was maintained by surgical retractors. Once the heart was exposed, the pericardium was removed using forceps.

2.6.5. Arterial baroreceptor denervation

In a subset of animals, the arterial baroreceptor afferents were denervated to remove arterial baroreflex mediated responses to changes in arterial pressure caused by either MI or cardiac afferent reflex activation. Access to the arterial baroreceptor afferents was achieved via the same incision used during the tracheotomy. Arterial baroreceptors were denervated by cutting the carotid sinus and aortic depressor nerves. The carotid sinus was
exposed and denervated bilaterally by cutting all visible nerves between the internal and external carotid arteries and stripping these vessels. The aortic depressor nerve was located at the point where the superior laryngeal nerve meets the vagal nerve and transected bilaterally. Baroreceptor denervation was confirmed by the absence of changes in renal SNA and heart rate (HR) when arterial pressure was increased by intravenous infusion of phenylephrine (PE, 20µg/kg). Figure 2.3 demonstrates an example of the renal SNA response to an increase in arterial pressure in a single animal before and after baroreceptor denervation.

### 2.6.6. Vagal denervation

In a subset of animals, bilateral vagal denervation was performed in addition to baroreceptor denervation by cutting the cervical vagal nerves running alongside the left and right carotid arteries.

![Figure 2.3: Example traces demonstrating lack of renal SNA response to changes in arterial pressure in baroreceptor denervated rat](image)

Example traces showing arterial pressure (mmHg), original renal sympathetic nerve activity (SNA, µV) and integrated renal SNA (µV) in a single animal at baseline (left graph) and during arterial baroreceptor reflex stimulation by intravenous infusion of phenylephrine before (middle graph) or after (right graph) arterial baroreceptor denervation.
2.7. Data acquisition

All signals were passed through an analog-to-digital conversion and displayed, and recorded, on computer via the program Universal Acquisition (Universal Acquisition and Analysis, ver. 11. University of Auckland, Auckland, NZ). All data was sampled at 500 Hz using an analog-to-digital data acquisition card (AT-MIO64E-3 National Instruments, Austin, TX). All subsequent data analysis was performed using a data acquisition program (Universal acquisition and analysis, ver. 11; Telemetry Research, Limited, Auckland, New Zealand). HR was derived from the arterial pressure trace. The 2-s averages of mean arterial pressure, HR and renal SNA were saved continuously throughout the experiment at a sampling frequency of 500Hz and these were used for analysis of timeline changes and arterial baroreflex data. The SNA signal was passed through a band-pass filter located in the receiver with a high pass cut-off frequency of 50Hz and low pass cut-off frequency of 2000Hz. The original renal SNA signal was amplified, full-wave rectified and integrated using a low pass filter with a 20ms time constant.

2.8. General experimental protocols

The experimental protocol was begun at least 30 minutes following surgical preparation and once renal SNA recordings had stabilized.

2.8.1. Arterial baroreceptor reflex curves

Arterial baroreceptor reflex responses were obtained by acutely decreasing and increasing arterial pressure while simultaneously recording arterial pressure, HR and renal SNA as shown in Figure 2.4. Arterial pressure was lowered by infusing sodium nitroprusside (SNP, 5-20µg, rate of change is 1 to 2mmHg per second) either by hand or infusion pump into the femoral vein. Arterial pressure was increased by infusing phenylephrine (PE, 20 to 80 µg, rate of change is 1 to 2mmHg per second) either by hand or infusion pump into the femoral vein. Typically it was easier to control the decrease in arterial pressure compared to increasing arterial pressure. SNP and PE were applied sequentially and a period of time was allowed between each grouped infusion of SNP and PE to allow renal SNA and arterial pressure to return to baseline levels before another baroreceptor reflex response was initiated. Arterial baroreceptor reflex curves were created from the arterial pressure and
renal SNA responses to SNP and PE according to the work performed by Ricketts and Head (Ricketts et al. 1999). In brief, a 5-parameter logistic equation was used to fit the individual data points obtained from the concurrent levels of arterial pressure and renal SNA obtained at specific time points with a nonlinear regression curve as demonstrated in Figure 2.4. The 5 parameters used were the level of maximum inhibition of renal SNA during elevated arterial pressure (lower plateau), range of baroreflex mediated change in renal SNA, level of arterial pressure at the midway between minimum and maximum levels of renal SNA (BP_{50}), and the upper and lower curvatures.

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**Figure 2.4: Example traces demonstrating arterial baroreflex responses**

A: Example traces showing arterial pressure (mmHg), original renal sympathetic nerve activity (SNA, µV) and integrated renal SNA (µV) before and during the intravenous infusion of sodium nitroprusside (SNP, left graph) and phenylephrine (PE, right graph) in a single rat. B: Arterial baroreceptor reflex curve derived from the raw data in A. Each individual square represents the level of renal SNA in relation to the concurrent level of arterial pressure at that specific time point, a 5-parameter non-linear regression curve is fitted to the data points (Ricketts et al. 1999). The large unfilled square represents the level of arterial pressure and renal SNA at rest.
2.8.2. Cardiac afferent reflex responses

As demonstrated in Figure 2.5, to obtain cardiac afferent reflex responses, capsaicin (Sigma-Aldrich Inc. USA) was placed onto the anterior surface of the left ventricle via a 3x3mm sized piece of filter paper. On the day of experiment, capsaicin was first dissolved in 100% ethanol at a concentration of 1 mg capsaicin per 100 µl ethanol. Tween-80 (Sigma-Aldrich Inc. USA) was then applied to help keep the capsaicin dissolved in solution. The capsaicin solution was then diluted with saline to make the final concentration of 1mg capsaicin per 0.89ml saline/0.1ml ethanol/0.01ml Tween-80. In each experiment, different dose concentrations were applied in random order to determine whether the responses to epicardial capsaicin application were dose-dependent. The precise doses for the specific study are described in the methods of the relevant results section.

Although capsaicin successfully activates the cardiac afferent reflex, it is an exogenous substance. Bradykinin, an endogenous substance, was also applied to the surface of the left ventricle in many experiments. However, as shown in Figure 2.6, bradykinin did not produce reliable renal SNA responses whereas the response produced by capsaicin was very consistent. The failure for bradykinin to activate the cardiac afferent reflex is perhaps due to a loss of potency during the shipping and storing of the bradykinin. Many attempts at different ways of storing and dissolving bradykinin were made but none were successful. Therefore only the results obtained from capsaicin have been presented.

**Figure 2.5: Experimental set-up when performing cardiac afferent reflex protocol**
Schematic showing the experimental set-up when performing the cardiac afferent reflex protocol. A pipette was used to apply capsaicin, via a piece of filter paper, onto the anterior surface of the left ventricle in an anesthetized rat.
Figure 2.6: Renal SNA and arterial pressure responses to epicardial application of bradykinin

Example traces showing arterial pressure (mmHg) and integrated renal sympathetic nerve activity (SNA, µV) for 10 seconds before and 40 seconds following the application of bradykinin (10µg per 10µl saline) onto the epicardial surface of the left ventricle in two separate males (top row) and two separate females (bottom row) that had total intact cardiac innervation.

Previous investigations on the cardiac afferent reflex have indicated that desensitization of the afferent fibers can occur in response to capsaicin. Therefore capsaicin was only applied to the heart surface for 40 seconds before being washed off with warm saline (~50mls). The saline used to rinse the heart during the cardiac afferent reflex protocol was maintained at 37 degrees Celsius by storing in a water bath when it was not being used. The application of capsaicin for just 40 seconds has been shown to be an appropriate length of time to obtain maximum cardiac afferent reflex mediated SNA changes (Reimann et al. 1980). At least 15 minutes was allowed between successive applications of capsaicin to avoid tachyphylaxis.
2.9. Data analysis

Data analysis specific to the studies are outlined in the appropriate methods sections in individual chapters. General calculations and confirmations of signals are outlined in this section.

2.9.1. Analysis of RSNA

To confirm the existence and quality of the renal SNA signal visual confirmation, listening to the signal and inducing arterial baroreflex responses to acutely altering arterial pressure were performed. Furthermore, one second ‘sweeps’ of integrated renal SNA and arterial pressure data were averaged using systolic peak as a trigger, for one or two hundred seconds. The traces were visually assessed for an arterial pressure waveform related cyclic pattern in renal SNA discharge as demonstrated in Figure 2.7.

![Figure 2.7: Cardiac cycle related sympathetic bursts](image)

Systolic pressure triggered averaged records of arterial pressure (straight line) and integrated renal sympathetic nerve activity (SNA, dashed line). Figure shows the pressure related changes in mean averaged activity of renal SNA.
2.9.2. Normalization of renal sympathetic nerve activity

In order to appropriately discuss the functional consequences of a change in SNA, the quantification of the signal must 1) reflect the physiological actions of SNA and 2) be a consistent and reliable means of comparison between animals.

The baseline level of sympathetic nerve activity being recorded is dependent on a number of physiological and technical factors. Biologically, resting SNA has been shown to vary significantly between individuals and is altered in disease states. The SNA signal is also affected by numerous technical issues such as the number of many fibres being recorded from, the distance of each recorded fibre from the recording wire, whether fluid is contacting the recording wires and how much the nerve is insulated within connective tissue. Therefore, the skill and knowledge of the person performing the nerve recording surgery can significantly impact the type of signal achieved. The relatively low voltage of a recorded SNA signal means that the determined noise level can significantly impact the determined level of baseline SNA and any normalized changes due to an intervention. ‘Noise’ in the SNA signal can refer to electrical noise, movement or electrocardiogram artefact (Stocker et al. 2013). The noise in the signal must be accurately estimated and deducted from the total recorded signal. The method for estimating noise level used within the current work has been to average a number of ‘quiet’ periods in the integrated SNA signal (Guild et al. 2010, Stocker et al. 2013). Provided that the signal is stable, the ‘noise’ should be represented as the area beneath the signal during the ‘quiet period’ between distinct bursts of SNA (Guild et al. 2010, Stocker et al. 2013).

Although it has been argued that using the raw microvolt (µV) levels is appropriate when analysing SNA data obtained from whole nerve recordings (Guild et al. 2010), the technical issues surrounding the recording from whole nerve bundles means that comparisons between animals using raw microvolt levels results in a large variation in preparation. It is likely that with enough experimental numbers and a consistent approach to the nerve surgery, the microvolt levels can be used (Guild et al. 2010). However, using raw microvolt levels represents an unprocessed reflection of the recorded nerve activity because the contact between nerve and recording wires is such a fundamental determinant of the absolute signal and thus normalization is necessary. As shown in Figure 2.8, the absolute arterial baroreflex range of control over renal SNA as measured in microvolts is significantly correlated to the microvolt level of baseline SNA. Baseline SNA is significantly affected by the amount of fibres being recorded from and therefore it would be assumed that the absolute microvolt changes in response to a stimulus will reflect the number of fibres being
recorded from. Therefore I believe that normalizing the change in renal SNA as a percentage change from baseline is appropriate.

![Graph showing the relationship between change in renal SNA (µV) and baseline renal SNA (µV)](image)

$r^2 = 0.8802 (P<0.01)$

![Graph showing the relationship between change in renal SNA (%) and baseline renal SNA (µV)](image)

$r^2 = 0.018 (P>0.05)$

**Figure 2.8: Effect of baseline renal SNA on arterial baroreflex response**

Maximum ability of the arterial baroreflex to change total renal sympathetic nerve activity (SNA) in response to both increasing and decreasing arterial pressure (i.e. range of control of arterial baroreflex over renal SNA) represented as an absolute change in microvolts (µV; top graph) or as a percentage change from baseline (%; bottom graph) plotted in relation to resting renal SNA (µV) prior to beginning arterial baroreflex protocol. Data is taken from normal, healthy male and female rats. P-value of less than 0.05 is considered to be a significant effect.

The equation used in this thesis to normalize the change in SNA is:

$\frac{\text{Change in SNA (µV)}}{\text{baseline SNA (µV)}} \times 100 = \% \text{ change in SNA}$

### 2.10. Post-mortem protocol

At the end of the experiment the animal was killed by an overdose of chloralose-urethane anesthetic that was infused intravenously. This method is in accordance with the New Zealand Animal Welfare Act 1999.
Chapter 3: Initial Sympathetic Response to MI

3.1. Introduction

The incidence of myocardial infarction (MI) in women increases significantly post-menopause and is a leading cause of death in women in industrialized countries (Lerner et al. 1986). Age-related changes in rates of morbidity and mortality following MI in men and women are disparate, suggesting an effect of changes in sex hormone levels due to menopause (Vaccarino et al. 1998). Furthermore, significant differences exist between men and women in the presentation of symptoms, treatment and early survival following myocardial infarction (Chandra et al. 1998). Currently, the physiological mechanisms contributing to sex differences following MI are poorly understood (Chandra et al. 1998).

Elevations in SNA, particularly to the heart and kidneys, are hallmarks of cardiovascular disease (Esler et al. 1998), with increases in SNA after MI adversely related to patient outcome (Sigurdsson et al. 1993). The successful use of beta-adrenergic antagonists in treating patients highlights the deleterious nature of sustained sympathetic activation (Gottlieb et al. 2001, Heras et al. 2006, Setoguchi et al. 2008). In the immediate period following MI, elevations in SNA are thought to play a critical role in the development of ventricular arrhythmias, fibrillation and sudden death with sustained increases in renal SNA contributing to sodium and fluid retention and increases in angiotensin II (Ammons et al. 1982, Jardine et al. 2007). Higher occurrences of arrhythmias and early sudden death within males, compared to females, post-MI suggests possible differences between the sexes in initial changes in SNA (Lerner et al. 1986, Chandra et al. 1998). In animal studies, where the confounding effects of age and co-morbidities are controlled, ovary-intact females display better preservation of cardiac function following MI compared to males, whereas ovariectomy removes this sex difference (Cavasin et al. 2003). In males, MI causes a significant increase in SNA both acutely and in the long-term, with changes in renal SNA being positively associated with morbidity and mortality in the progression of heart failure (Thames et al. 1979, Graham et al. 2002). Whether changes in renal SNA post-MI are similar in females is unclear.

The initial change in SNA post-MI is dependent on sympathetic reflexes (Thames et al. 1979, Minisi et al. 1991, Xu et al. 2011). In anesthetized dogs, denervation of the carotid sinus and aortic depressor nerves abolished the excitatory changes to renal SNA in the first minute of myocardial ischemia that was initially observed with an intact arterial
baroreflex (Thames et al. 1979). Arterial baroreflex responses have been found to differ between males and females (Frey et al. 1988, Convertino 1998, Shoemaker et al. 2001, Foley et al. 2005, Kimmerly et al. 2007). The current understanding is that females, in relation to males, exhibit attenuated sympathetic activation during baroreceptor unloading (Hinojosa-Laborde et al. 1999), although this is not a universal finding (Tank et al. 2005).

In the current study we have set out to define the initial changes to renal SNA in the first few hours following MI in males and females with ovaries-intact and ovaries-removed. The role of the arterial baroreflex in the initial changes to renal SNA post-MI was specifically investigated by creating arterial baroreflex curves before and following MI and by observing the initial renal SNA response to MI in arterial baroreceptor denervated animals. Based on the background literature, I hypothesized that ovarian hormones in females would attenuate the initial sympathoexcitation post-MI in a manner related to attenuated arterial baroreceptor reflex response to a decrease in arterial pressure.
3.2. Methods

3.2.1. Experimental preparation

Experiments were conducted in 64 Wistar rats of male and female sex. Briefly, in a subset of the female rats weighing 120-150g and aged between 5 and 6 weeks old, ovariectomy (OVX) surgery was performed (see Chapter 2). Prior to the experimental day all rats were housed 2-4 per cage with water and food *ad libitum* in a room of constant temperature (22°C) with a 12h:12h light:dark cycle. Experiments were performed 4-6 weeks following ovariectomy. On the day of experiment animals between 8 and 12 weeks old were anesthetized following the protocol described in Chapter 2.

3.2.2. Surgical Preparation

The surgical preparation for the current study is described in Chapter 2. In addition, electrocardiograph (ECG) was recorded using a telemetry unit (TR70B, Telemetry Research Ltd., Auckland, NZ) with a positive and a negative lead placed on opposite sides of the chest beneath the skin. Once arterial pressure, heart rate, renal SNA and ECG recordings were obtained in all rats the chest was then opened via an incision through the 4th intercostal space and the pericardium removed. A positive end pressure of 3-4mmHg was used to ensure the lungs remained inflated. Mean arterial pressure (MAP), HR, renal SNA and ECG were recorded throughout the course of the experiment in all groups.

3.2.3. Experimental protocol

3.2.3.1. Series 1: Timeline data

Animals were divided into 6 groups; male MI group (n=10), female MI group (n=9), ovariectomized female (OVX MI) group (n=7), male sham group (n=6), female sham group (n=7), ovariectomized female sham (OVX sham) group (n=5). A minimum of 20 minutes after completion of preparatory surgery and once MAP, HR and renal SNA recordings were stable the first set of arterial baroreflex curves were obtained. MAP, HR, and renal SNA were then allowed to recover for a minimum of 20 minutes before baseline data were collected for 10 minutes. MI or sham surgery was then performed: In the MI groups, MI
was induced by tying off the left anterior descending coronary artery (LAD) 2-3 mm from origin using a 6-0 silk suture. In the sham groups a suture was passed through the heart wall but the LAD was not tied off. MAP, HR and renal SNA were then recorded continuously for a further 2 hours.

### 3.2.3.2. Arterial baroreflex responses

Arterial baroreflex responses were assessed before and 60 minutes after MI or sham surgery. Arterial baroreflex control of HR and renal SNA were obtained as described in Chapter 2.

### 3.2.3.3. Series 2: Arterial baroreceptor denervation

Upon completion of the preparatory surgery as above, arterial baroreceptor denervation was performed in a separate group of males (n=6, male BD MI), ovary-intact females (n=7, female BD MI) and ovariectomized (OVX) females (n=7, OVX BD MI) to assess the sympathetic response to MI without the influence of the arterial baroreceptor reflex. Arterial baroreceptors were denervated as described in Chapter 2.

MAP, HR, and renal SNA were then allowed to recover for a minimum of 20 minutes before baseline data were collected for 10 minutes. Once baseline data were obtained, MI was induced in both groups as described in Series 1. MAP, HR and renal SNA were then recorded continuously for 30 minutes post-MI.

### 3.2.3.4. Determination of myocardial infarction

In all animals, MI was confirmed by observing changes to the ECG and by intravenous (IV) infusion of Evans Blue dye at the conclusion of experiment. At the end of the experiment the rat was euthanized with an intravenous overdose of chloralose-urethane and the heart excised and frozen. The frozen heart was cut into 4 to 5 transmural slices parallel to the base, and then photographed. The area where the dye failed to perfuse was considered to represent the area at risk of MI.
3.2.3.5. Data Collection

Data was collected as described in Chapter 2. Analysis of the arterial baroreceptor reflex responses was performed using a 5-parameter nonlinear regression equation to fit the collected MAP-renal SNA and MAP-HR data to produce arterial baroreflex curves (Ricketts et al. 1999). Two-second averages of MAP, HR, and renal SNA were saved continuously throughout the experiment. Sympathetic nerve values were normalized as a percentage change from resting level, which was the average renal SNA value over ten minutes prior to MI or sham surgery. The noise level was taken to be the integrated SNA value when SNA was at the lowest level between two distinct bursts of nerve activity.

3.2.3.6. Statistical analysis

Within the six groups of rats with intact arterial baroreceptors; timeline changes to MAP, HR, and renal SNA were assessed as 1 minute averages for the 10 minutes of baseline and 60 minutes following MI or sham surgery. From then a 5-minute average was taken at the selected points 75, 90 and 120 minutes post MI or sham. In the three baroreceptor denervated groups of rats, timeline changes to MAP, HR and renal SNA were assessed as 1 minute averages for the 10 minutes of baseline and 30 minutes post-MI. Repeated measures ANOVA were used to analyze timeline data. Two-way ANOVA with Bonferroni post-hoc test was used to compare individual time-points. Data are shown as the means +/- SEM. P values less than (<) 0.05 were considered significant.
3.3. Results

3.3.1. Baseline characteristics

Baseline variables for the eight groups are shown in Table 3.1. Three of the male MI rats and 1 OVX MI rat died within the first 60 min post MI after experiencing ventricular arrhythmias (at 5, 10, 25, and 60 minutes post-MI). Data from these rats were excluded from the time-line analysis. However as arterial baroreflex data was obtained prior to MI or sham-MI the data from the animals that died were included within the comparison of the arterial baroreflex between males, ovary-intact females and OVX females. No deaths were observed in the ovary-intact female MI or within any of the sham groups.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Male Sham</th>
<th>Male MI</th>
<th>Female Sham</th>
<th>Female MI</th>
<th>OVX Sham</th>
<th>OVX MI</th>
<th>Male BD MI</th>
<th>Female BD MI</th>
<th>OVX BD MI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Weight (grams)</td>
<td>318 ± 20</td>
<td>308 ± 18</td>
<td>269 ± 8</td>
<td>278 ± 11</td>
<td>295 ± 12</td>
<td>307 ± 9</td>
<td>275 ± 4</td>
<td>278 ± 11</td>
<td>305 ± 10</td>
</tr>
<tr>
<td>MAP, mmHg</td>
<td>73 ± 5</td>
<td>73 ± 7</td>
<td>85 ± 5</td>
<td>76 ± 5</td>
<td>77 ± 4</td>
<td>78 ± 8</td>
<td>83 ± 3</td>
<td>78 ± 5</td>
<td>79 ± 6</td>
</tr>
<tr>
<td>HR, bpm</td>
<td>350 ± 26</td>
<td>390 ± 22</td>
<td>367 ± 35</td>
<td>355 ± 18</td>
<td>331 ± 14</td>
<td>377 ± 14</td>
<td>444 ± 10*</td>
<td>401 ± 18</td>
<td>390 ± 15</td>
</tr>
<tr>
<td>RSNA, uV</td>
<td>14 ± 1</td>
<td>12 ± 2</td>
<td>12 ± 2</td>
<td>13 ± 3</td>
<td>12 ± 2</td>
<td>12 ± 2</td>
<td>14 ± 2</td>
<td>11 ± 2</td>
<td>11 ± 1</td>
</tr>
<tr>
<td>Area at risk of myocardial infarct, %</td>
<td>-</td>
<td>40 ± 4</td>
<td>-</td>
<td>39 ± 5</td>
<td>-</td>
<td>38 ± 4</td>
<td>35 ± 3</td>
<td>39 ± 5</td>
<td>38 ± 5</td>
</tr>
</tbody>
</table>

Table 3.1: Baseline variables prior to myocardial infarction
Baseline (before MI or sham surgery) weight (grams), mean arterial pressure (MAP), heart rate ((HR) beats per minute (bpm)), and area at risk of myocardial infarct (% of left ventricular wall) of the eight groups. Abbreviations: MI – myocardial infarction, OVX – ovariectomised, BD – baroreceptor denervated. Data are mean±SEM. * indicates significant difference between Male BD MI group and OVX sham group, p<0.05.

3.3.2. Series 1: Timeline response of males, ovary-intact females and ovary-removed (OVX) females in response to MI

Raw traces of recordings within an individual animal representative of male MI, ovary-intact and OVX female MI groups taken pre-MI, 1 minute post-MI and 120 minutes post-MI are provided in Figure 3.1.
Figure 3.1: Example traces showing response to myocardial infarction

Raw traces of arterial pressure (mm Hg), original renal SNA (uV) and integrated renal SNA (uV) taken pre-myocardial infarction (MI), 1 minute post-MI and 120 minutes post-MI in an individual animal from the male MI group (top row), female MI group (middle row) and OVX MI group (bottom row).
Timeline changes to renal sympathetic nerve activity (SNA), heart rate (HR), and mean arterial pressure (MAP) for 10 minutes before and 120 minutes following myocardial infarction (MI) at time zero in three MI groups; male MI (n=7) and female MI (n=9) and ovariectomized (OVX) female MI (n=6). Data are shown as mean±S.E.M. * indicates significant difference from before MI in male MI group response, + indicates significant difference from before MI in female MI group response and # indicates significant difference from before MI in OVX MI group response, P<0.05.

Figure 3.2: Timeline responses to myocardial infarction
The mean hemodynamic and renal SNA data for males, ovary-intact and OVX females before and for 120 minutes after MI surgery are shown in Figure 3.2. In males, coronary occlusion elicited a significant increase in renal SNA of 30±6% within the first minute when compared to male sham response and as a change from baseline (Figure 3.2). Following the initial large increase within the male MI group, renal SNA made a partial recovery back towards baseline level; however renal SNA remained significantly elevated at 2 hours post-MI (at 120 min post-MI male renal SNA was 18±7% above baseline vs. -1±3% above baseline in the male shams, P<0.05). In contrast to males, renal SNA was unchanged in ovary-intact females for the entire two hours following MI (at 120 min ovary-intact female MI renal SNA was 1±4% change from baseline vs. 2±3% change from baseline in female shams, P>0.05). In the OVX MI group, coronary occlusion elicited a significant increase in renal SNA of 43±13% within the first minute (Figure 3.2). Following the initial large increase within the OVX MI group, renal SNA made a partial recovery back towards baseline level, however renal SNA remained significantly elevated for the remaining two hours (at 120 min post-MI OVX renal SNA was 21±7% change from baseline vs. -5±5% in the sham MI OVX rats, P<0.05 (Figure 3.2)). The sham-MI groups showed no changes in renal SNA over time. When comparing between groups, the increase in renal SNA post-MI within the male MI group was significantly greater than that in both the ovary-intact female MI group and male sham group but was similar to that in OVX females (Figure 3.3). The renal SNA response to coronary occlusion in the OVX MI group was significantly different to both ovary-intact females and OVX sham group and was similar to that within the male MI group (Figure 3.3).

MAP significantly decreased from baseline in the first minute after coronary occlusion by -14±5 mm Hg in males, -16±3 mm Hg in ovary-intact females and -16±5 mm Hg in OVX females (P<0.05 vs baseline, Figure 3.2). Despite similar changes to MAP post-MI, only the MAP response at 1 minute post-MI in the ovary-intact female MI group was statistically significantly different compared to the shams (at 1 min MAP in ovary-intact female MI was -16±3 mm Hg compared to baseline whereas MAP in female sham was -4±3 mm Hg compared to baseline, P<0.05 (Figure 3.3)). Males and OVX females appeared to recover MAP to a greater extent than the ovary-intact females over the two hours following MI. MAP in ovary-intact females remained significantly below baseline value following the initial 10 minutes post-MI and was -10±3 mm Hg below baseline at 120 minutes post-MI (P<0.05 (Figure 3.2). In contrast no significant change from baseline value was observed following the initial 10 minutes post-MI in males and OVX females (Figure 3.3).
HR tended to display an initial increase, followed by a gradual decrease in the male, ovary-intact and OVX female MI groups; however the large variations meant none of the heart rate responses reached statistical significance (Figure 3.3).

**Figure 3.3: Comparing male, female and OVX responses to acute myocardial infarction**

Changes to renal sympathetic nerve activity (SNA), heart rate (HR, beats per minute (bpm)) and mean arterial pressure (MAP) in six groups: male sham (n=6), male MI (n=7), ovary-intact female sham (n=6), ovary-intact female MI (n=9), Ovary-removed (OVX) sham (n=5) and OVX MI (n=6) at 1, 10, 30 and 120 minutes following myocardial infarction (MI) or sham-MI. All data are presented as a change from the 10 minute baseline period immediately prior to MI or sham-MI. Data are shown as mean±S.E.M. * indicates statistically significant difference from sham, P<0.05. # indicates statistically significant difference from Female MI renal SNA response, P<0.05.
3.3.3. Arterial baroreflex control of HR and renal SNA after MI

Arterial baroreflex control showing the relationship between arterial pressure and renal SNA or HR in males, females and OVX females prior to MI or sham surgery is shown in Figure 3.4. There were noticeable differences between male and female arterial baroreflex control over renal SNA (Table 3.2). Both ovary-intact and OVX female groups displayed a reduced ability to maximally increase renal SNA in response to a decrease in arterial pressure when compared to males resulting in a significantly lower upper plateau in both female groups compared to males (Table 3.2). No differences were observed between ovary-intact female and OVX female arterial baroreflex control over renal SNA. Ovary-intact and OVX females displayed a significantly higher MAP curve mid-point than males. Ovariectomy tended to reduce the arterial baroreflex range of control over heart rate within females but this did not quite reach significance (female baroreflex range of control over heart rate, 103±12 beats per minute vs. OVX baroreflex range of control over heart rate, 72±6 beats per minute; P>0.05).

Arterial baroreflex control showing the relationship between arterial pressure and renal SNA before and after MI in males, ovary-intact females and OVX females are shown in Figure 3.5. No significant changes to arterial baroreflex control of renal SNA were observed at 1 hour after MI or sham surgery in either the males or ovary-intact females (Table 3.3). Following MI, the upper plateau within the OVX females was elevated but this did not reach significance.

Neither sham surgery nor MI had a significant effect on baroreflex control over HR one hour after intervention as shown in Table 3.3. The MAP curve mid-point (BP50) tended to be lower following MI in males, but the large variation meant this did not reach significance (Pre-MI male BP50, 125±22 mm Hg vs. Post-MI male BP50, 102±11 mm Hg). The magnitude of arterial baroreflex modulation (range) of heart rate tended to display a small decrease following MI in males and females but again these changes were not significant.
Figure 3.4: Arterial baroreflex curves in males, females and OVX females
Mean curves describing arterial baroreflex control over renal sympathetic nerve activity (SNA) (top graph) and heart rate (HR, beats per minute (bpm)) (bottom graph) in males (solid line, n=13), females (dashed line, n=15) and ovariectomized (OVX) (dotted line, n=12). Resting mean arterial pressure and renal SNA or heart rate at time arterial baroreflex curves were obtained are indicated by ○ for males, Δ for females and □ for OVX. Data are shown as mean±S.E.M. * indicates significant difference between males and females in the arterial baroreflex range of control and the BP50 (indicated by arrow), P<0.05. # indicates significant difference between males and OVX in the arterial baroreflex range of control and BP 50 (indicated by arrow), P<0.05.
### Chapter 3 – Initial Sympathetic Response to MI

<table>
<thead>
<tr>
<th>Variable</th>
<th>Male</th>
<th>Female</th>
<th>OVX</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean arterial pressure, mm Hg</td>
<td>79±4</td>
<td>80±6</td>
<td>84±5</td>
</tr>
<tr>
<td>Renal sympathetic nerve activity, % from baseline</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Heart rate, bpm</td>
<td>378±20</td>
<td>369±15</td>
<td>355±11</td>
</tr>
<tr>
<td>Renal sympathetic nerve activity parameters</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Upper plateau, % from baseline.</td>
<td>130±5</td>
<td>110±4*</td>
<td>108±3*</td>
</tr>
<tr>
<td>Lower plateau, % from baseline.</td>
<td>40±5</td>
<td>39±4</td>
<td>40±4</td>
</tr>
<tr>
<td>Range, % from baseline.</td>
<td>89±6</td>
<td>70±4*</td>
<td>68±3*</td>
</tr>
<tr>
<td>Lower plateau curvature, % / mm Hg</td>
<td>-0.11±0.01</td>
<td>-0.11±0.02</td>
<td>-0.15±0.03</td>
</tr>
<tr>
<td>BP&lt;sub&gt;50&lt;/sub&gt;, mm Hg</td>
<td>87±3</td>
<td>110±3*</td>
<td>113±3*</td>
</tr>
<tr>
<td>Upper plateau curvature, % / mm Hg</td>
<td>-0.10±0.03</td>
<td>-0.10±0.03</td>
<td>-0.14±0.02</td>
</tr>
<tr>
<td>Maximum gain</td>
<td>-0.36±0.08</td>
<td>-0.57±0.11</td>
<td>-0.52±0.10</td>
</tr>
<tr>
<td>Heart rate parameters</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Upper plateau, bpm</td>
<td>394±13</td>
<td>379±14</td>
<td>366±10</td>
</tr>
<tr>
<td>Lower plateau, bpm</td>
<td>316±15</td>
<td>276±15</td>
<td>294±13</td>
</tr>
<tr>
<td>Range, bpm</td>
<td>78±10</td>
<td>103±12</td>
<td>72±6</td>
</tr>
<tr>
<td>Lower plateau curvature, bpm/mm Hg</td>
<td>-0.28±0.15</td>
<td>-0.19±0.05</td>
<td>-0.18±0.03</td>
</tr>
<tr>
<td>BP&lt;sub&gt;50&lt;/sub&gt;, mm Hg</td>
<td>122±12</td>
<td>129±4</td>
<td>120±4</td>
</tr>
<tr>
<td>Upper plateau curvature, bpm/mm Hg</td>
<td>-0.13±0.03</td>
<td>-0.19±0.08</td>
<td>-0.16±0.05</td>
</tr>
<tr>
<td>Maximum gain, bpm/mm Hg</td>
<td>-2.32±0.52</td>
<td>-3.32±0.66</td>
<td>-2.74±0.48</td>
</tr>
</tbody>
</table>

**Table 3.2: Baroreflex parameters comparing males, females and OVX females**

Comparison of male, female, and OVX female arterial baroreflex. Baroreflex parameters obtained in males (n=13), females (n=15), and OVX females (n=12). Abbreviations: bpm = beats per minute, BP<sub>50</sub> = mean arterial pressure curve mid-point. Data are mean±SEM. * indicates statistical significance compared to males, P<0.05.
Figure 3.5: Arterial baroreflex curves before and 1 hour after myocardial infarction

Mean curves describing arterial baroreflex control over renal sympathetic nerve activity (SNA) before and 60 minutes following myocardial infarction (MI) in the male MI group (top graph, n=7), female MI group (middle graph, n=9) and OVX MI group (bottom graph, n=6). Resting mean arterial pressure and renal SNA at time arterial baroreflex curves were obtained are indicated by • for pre-MI and ▼ for 60 minutes post-MI. Data are shown as mean±S.E.M.
## Table 3.3: Baroreflex parameters before and 1 hour after myocardial infarction

Baroreflex parameters obtained in Male sham (n=6), Male MI (n=7), female sham (n=6), female MI (n=9), OVX sham (n=5) and OVX MI (n=6) before and 1 hour post-myocardial infarction (MI). Abbreviations: bpm beats per minute, SNA sympathetic nerve activity, BP<sub>50</sub> mean arterial pressure curve mid-point. Data are mean±SEM, * indicates significant difference from pre-MI P<0.05.

<table>
<thead>
<tr>
<th></th>
<th>Pre-MI</th>
<th></th>
<th></th>
<th>Female MI</th>
<th>OVX sham</th>
<th>OVX MI</th>
</tr>
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<tbody>
<tr>
<td><strong>Mean arterial pressure, mm Hg</strong></td>
<td>82 ± 6</td>
<td>76 ± 5</td>
<td>87 ± 5</td>
<td>82 ± 6</td>
<td>80 ± 7</td>
<td>89 ± 7</td>
</tr>
<tr>
<td><strong>Renal SNA, % from baseline</strong></td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td><strong>Heart rate, bpm</strong></td>
<td>349 ± 33</td>
<td>404 ± 15</td>
<td>368 ± 30</td>
<td>369 ± 16</td>
<td>338 ± 20</td>
<td>371 ± 13</td>
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</tbody>
</table>

### Renal SNA parameters

<table>
<thead>
<tr>
<th></th>
<th>Upper plateau, % from baseline</th>
<th>Lower plateau, % from baseline.</th>
<th>Range, % from baseline.</th>
<th>Lower plateau curvature, % / mm Hg</th>
<th>BP&lt;sub&gt;50&lt;/sub&gt; mm Hg</th>
<th>Upper plateau curvature, % / mm Hg</th>
<th>Maximum gain, % / mm Hg</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>135 ± 7</td>
<td>43 ± 8</td>
<td>93 ± 7</td>
<td>-0.09 ± 0.01</td>
<td>87 ± 4</td>
<td>-0.08 ± 0.02</td>
<td>-0.31 ± 0.09</td>
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### Heart rate parameters

<table>
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<tr>
<th></th>
<th>Lower plateau, bpm</th>
<th>Range, bpm</th>
<th>Lower plateau curvature, bpm/mm</th>
<th>BP&lt;sub&gt;50&lt;/sub&gt; mm Hg</th>
<th>Upper plateau curvature, bpm/mm</th>
<th>Maximum gain, bpm/mm</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>290 ± 30</td>
<td>86 ± 17</td>
<td>0.17 ± 0.09</td>
<td>118 ± 7</td>
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<td>-2.6 ± 1.03</td>
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### Post-MI

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<tr>
<th></th>
<th>Male sham</th>
<th>Male MI</th>
<th>Female sham</th>
<th>Female MI</th>
<th>OVX sham</th>
<th>OVX MI</th>
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</thead>
<tbody>
<tr>
<td><strong>Mean arterial pressure, mm Hg</strong></td>
<td>79 ± 3</td>
<td>65 ± 6</td>
<td>85 ± 7</td>
<td>69 ± 5</td>
<td>68 ± 5</td>
<td>77 ± 8</td>
</tr>
<tr>
<td><strong>Renal SNA, % from baseline</strong></td>
<td>94 ± 7</td>
<td>112 ± 8</td>
<td>103 ± 2</td>
<td>106 ± 7</td>
<td>104 ± 8</td>
<td>120 ± 8*</td>
</tr>
<tr>
<td><strong>Heart rate, bpm</strong></td>
<td>337 ± 27</td>
<td>411 ± 16</td>
<td>378 ± 33</td>
<td>342 ± 13</td>
<td>316 ± 17</td>
<td>384 ± 14</td>
</tr>
</tbody>
</table>

### Renal SNA parameters

<table>
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<tr>
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<th>Upper plateau, % from baseline</th>
<th>Lower plateau, % from baseline.</th>
<th>Range, % from baseline.</th>
<th>Lower plateau curvature, % / mm Hg</th>
<th>BP&lt;sub&gt;50&lt;/sub&gt; mm Hg</th>
<th>Upper plateau curvature, % / mm Hg</th>
<th>Maximum gain, % / mm Hg</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>133 ± 9</td>
<td>44 ± 5</td>
<td>89 ± 8</td>
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<td>83 ± 7</td>
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<td>-0.42 ± 0.04</td>
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### Heart rate parameters

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<th>Range, bpm</th>
<th>Lower plateau curvature, bpm/mm</th>
<th>BP&lt;sub&gt;50&lt;/sub&gt; mm Hg</th>
<th>Upper plateau curvature, bpm/mm</th>
<th>Maximum gain, bpm/mm</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>277 ± 27</td>
<td>107 ± 24</td>
<td>-0.07 ± 0.04</td>
<td>130 ± 4</td>
<td>-0.09 ± 0.01</td>
<td>-1.9 ± 0.17</td>
</tr>
</tbody>
</table>
3.3.4. Series 2: Timeline response to MI in baroreceptor denervated rats

Raw traces of recordings within an individual animal representative of male BD MI, female BD MI, and OVX BD MI groups taken pre-MI, 1 minute post-MI and 30 minutes post-MI are provided in Figure 3.6. The mean hemodynamic and renal SNA data of the male, ovary-intact female and OVX baroreceptor denervated groups at 1, 10 and 30 minutes post-MI are shown in figure 3.7. MI failed to elicit any significant changes to renal SNA in both male and ovary-intact female arterial baroreceptor denervated groups. MI elicited a significant increase in renal SNA from baseline within the OVX BD MI group at 1 minute post-MI (27±7% change from baseline, p<0.05) and 30 minutes post-MI (25±6% change from baseline, P<0.05). The changes to HR and MAP post-MI in male (male BD MI) female (female BD MI), and OVX baroreceptor denervated groups were not significantly different from the HR and MAP changes seen in the baroreceptor intact groups. Renal SNA changed by -5±2% from baseline in the Male BD MI group in the first minute of MI compared to the 30±6% increase from baseline in renal SNA displayed by the Male MI group with intact arterial baroreceptors (p<0.05, Figures 3.3 and 3.7). In the male baroreceptor denervated group no change from baseline to renal SNA was observed at 30 minutes post-MI (at 30 min renal SNA in male BD was -2±4%). The renal SNA response to MI in ovary-intact females was not different with or without arterial baroreceptors intact. Within the OVX females, the renal SNA excitatory response to MI was blunted slightly in baroreceptor denervated OVX animals but this was not a significant effect when compared to OVX females with intact arterial baroreceptors (at 1min post-MI renal SNA in OVX MI group was 43±13% change from baseline vs. 27±7% change in the OVX BD MI group, p>0.05, figures 3.3 and 3.7).
Figure 3.6: Example traces of response to myocardial infarction in baroreceptor denervated animals

Raw traces of arterial pressure (mm Hg), original renal SNA (uV) and integrated renal SNA (uV) in barodenervated (BD) animals taken pre-myocardial infarction (MI), 1 minute post-MI and 30 minutes post-MI in an individual animal from the male BD MI group (top row), female BD MI group (middle row) and OVX BD MI group (bottom row).
Figure 3.7: Renal SNA, HR and arterial pressure responses to acute myocardial infarction in baroreceptor denervated animals

Changes to renal sympathetic nerve activity (SNA), heart rate (HR) and mean arterial pressure (MAP) in the three baroreceptor denervated groups; male BD MI (n=7), Female BD MI (n=7) and OVX BD MI (n=7) at 1, 10 and 30 minutes following myocardial infarction (MI). All data are presented as a change from the 10-minute baseline period immediately prior to MI. Data are shown as mean±S.E.M. + indicates statistically significant difference from Male BD MI renal SNA response, P<0.05. # indicates statistically significant difference from Female BD MI renal SNA response, P<0.05. * indicates statistically significant difference from baseline value pre-MI, P<0.05.
3.3.5. Validation of MI

ECG was used primarily as a qualitative method for evaluating at the time of surgery whether MI surgery was successful. Occlusion of the LAD resulted in pathologic changes to the ST segment. Changes to ECG within the six MI groups corresponded well with known changes to the ECG signal upon MI (Ross 1976). At the conclusion of the experiment staining of the heart allowed estimation of area at risk of infarct. Figure 3.8 shows an example of a sham MI or MI as measured by testing perfusion of dye within the heart muscle. Using this method no significant difference between average estimated area at risk of infarct within the left ventricular walls between groups was observed (Table 3.1) (calculated area at risk of MI within left ventricular wall; Male MI – 40±4%, Female MI - 39±5%, OVX MI - 41±4, Male BD MI - 35±3, Female BD MI - 39±5%, OVX BD MI - 38±6%).

![Example images of myocardial infarction](image)

**Figure 3.8: Example images of myocardial infarction**

Example of a sham-myocardial infarction (MI, on left) or MI (on right) as measured by testing perfusion of dye within the heart muscle. The dark coloured area indicates the heart muscle where dye was able to perfuse thus indicating the area of tissue still receiving a blood supply whereas the red area represents the unstained area of heart muscle where the dye failed to perfuse indicating site of infarct. The image on right is an example taken from an MI group with an estimated area at risk of infarct size of 54%.
3.4. Discussion

This study demonstrates a significant impact of sex and ovarian hormones on the initial sympathetic response to myocardial infarction. The current study found that ovary-intact females failed to elicit any changes in renal SNA in response to MI, whereas females with ovaries removed and males elicited significant increases in renal SNA post-MI. In comparison to females, the male arterial baroreceptor reflex displayed an augmented ability to increase renal SNA in response to a decrease in arterial pressure and removal of the ovaries failed to alter this difference. Denervation of the arterial baroreceptor reflex eliminated the initial excitatory increase in renal SNA post-MI within males. However, within ovary-removed females arterial baroreceptor denervation failed to abolish the initial sympathetic excitation in response to MI. The findings suggest that sex hormones are important in determining the sympathetic response to MI and that the importance of the arterial baroreflex in mediating the initial sympathetic activation post-MI is different between the sexes.

This is the first study to demonstrate sex specific renal SNA responses to MI. Renal SNA plays an important role in regulating fluid volume via its direct and indirect actions on the kidney. In male rats at least, renal denervation improves maintenance of cardiac function following myocardial infarction, in a manner related to improved sodium and urine excretion (Nozawa et al. 2002). Renal SNA also mediates renin release from the juxtaglomerular cells. Renin secretion is the rate limiting step for production of Angiotensin II with inhibitors of Angiotensin II action successful in treating MI patients (Werner et al. 2008). Changes in renal SNA are particularly important in driving pathological changes leading to the development and progression of heart failure (Petersson et al. 2005). The present experiments suggest that sex specific renal sympathetic responses to MI may have a role to play, at least initially, in affecting sex specific rates of morbidity and mortality following myocardial infarction (Vaccarino et al. 1998).

A number of studies have investigated the initial changes in renal SNA following myocardial infarction/ischemia and the mechanisms driving such changes. Changes to SNA in the initial first few minutes post-infarction appear reliant on the arterial baroreceptor reflex as denervation of the arterial baroreflex largely abolishes the initial excitatory changes in SNA (Felder et al. 1979, Thames et al. 1979). In the current study, denervation of the arterial baroreceptor reflex abolished the sympathoexcitatory response to MI in males and left the ovary-intact female renal SNA response unchanged, thereby removing the difference in SNA response to MI between these two groups. Compared to females, males displayed
an augmented ability to increase renal SNA in response to decreases in arterial pressure. Similar sex differences in the baroreflex responses have been previously reported in the conscious rat, where although resting SNA was nearer the middle of the response curve, the maximum increase in renal SNA was much smaller in females versus males (Foley et al. 2005). A study by Ustinova et al. within a similar rat preparation as the current study but in males only confirm the current findings that denervation of the arterial baroreceptors abolishes any excitatory sympathetic response within the first few minutes of myocardial infarction (Ustinova et al. 1996).

However, while on first appearances it would seem possible to conclude that the increase in renal SNA post MI is entirely dependent on the arterial baroreflex, the findings in ovariectomized females suggest the response is not as simple as this. Ovariectomy resulted in a significant increase in renal SNA post MI not observed in the intact females, but had no effect on the arterial baroreceptor reflex control of renal SNA. Furthermore, OVX females with denervated arterial baroreceptors still maintained a significant sympathoexcitatory response to MI. This suggests that in ovariectomized females a mechanism other than the arterial baroreceptor reflex is responsible for the initial elevations in SNA post-MI. One such mechanism is the cardiac afferent reflex that has the ability to alter renal SNA in response to myocardial infarction (Weaver et al. 1981, Xu et al. 2011). The possibility is that, within females, ovarian hormones may affect the relative activation of vagal and sympathetic pathways in response to myocardial infarction. Given the similar changes in arterial pressure post-MI between baroreceptor-intact and baroreceptor-denervated animals it could be argued that the arterial baroreflex was not responsible for the recovery in arterial pressure post-MI. The heart muscle retains the ability to compensate for a loss of functioning cardiac myocytes and it may be this that is the main cause for the recovery in arterial pressure.

Female sex and sex hormones have been shown to significantly impact on resting SNA levels and on the arterial baroreceptor reflex control of SNA (Minson et al. 2000, Saleh et al. 2000, Vongpatanasin et al. 2001). In anesthetized, ovariectomized female rats the acute administration of 17β-estradiol lowers resting renal SNA and depresses the arterial baroreceptor reflex driven tachycardia in response to sodium-nitroprusside induced decreases in arterial pressure (Saleh et al. 2000). In women who are using the oral contraceptive pill, baroreceptor driven increases in muscle SNA in response to decreases in blood pressure are reduced during the period in which high concentration of estrogens are taken compared to the period during which the placebo pill is taken (Minson et al. 2000). In contrast to findings with exogenously applied estrogens, it has been shown in
both humans and animals that baroreflex driven sympathoexcitation is greater during the phases of the menstrual/estrous cycles in which circulating estrogens are highest (Fu et al. 2009, Goldman et al. 2009). The precise effects of estrogens on the arterial baroreflex are poorly understood. A limitation of the current study is that the estrous phase of the ovary-intact females was not determined; however given MI did not result in an increase in renal SNA in any of the intact females, there is no evidence to suggest the estrous phase is important in this regard. The current study also did not find any effect of removing the ovaries on the female arterial baroreflex control of renal SNA. This finding was surprising considering the evidence showing significant impacts of circulating estrogens on the regulation of SNA (Minson et al. 2000, Saleh et al. 2000, Vongpatanasin et al. 2001, Fu et al. 2009, Goldman et al. 2009). We did not measure changes in ovarian hormones caused by ovariectomy which is a limitation. Previous studies have performed the same approach in removing the ovaries within rats as the current study and have shown that estrogens are significantly diminished compared to ovary-intact females (Goldman et al. 2009) and it can be assumed that the same occurred within the ovariectomized rats within the current study. Concurrently, the age of the females with ovaries intact within the current study suggest that estrogens would be at normal, high levels within these animals (Lu et al. 1979).

It should be acknowledged that in the present study, due to the animals being anesthetized and with an open chest, the resting arterial pressure was lower than would be the case in a conscious state. The variation within the hemodynamic variables within the current study did not allow for any clear differences between males, females and ovariectomized females to be observed in their response to MI. In addition we accept that the anesthesia may well have affected the baroreflex responses. Sex differences in the initial hemodynamic response to coronary occlusion have been demonstrated previously where females displayed greater decreases in arterial pressure and heart rate compared to males (Du et al. 1995). Du et al. demonstrated that vagotomy abolished the sex differences in the hemodynamic response to coronary occlusion further suggesting a role for the autonomic nervous system in driving these changes (Du et al. 1995).

An initial, significant, decrease from baseline in arterial pressure in all MI groups would suggest that cardiac function was significantly impaired in all MI groups. However, it should be noted we did not directly determine cardiac function that measuring the ischemic area with the use of Evans blue dye is only an estimation of infarction size. Sex and sex hormones may impact on the size of infarction thus the possibility exists that the infarction area may differ between the sexes (Smith et al. 2000, Cavasin et al. 2003). A positive relationship has been previously observed between plasma norepinephrine levels and
myocardial infarct size (Karlsberg et al. 1981). However the causal direction of this relationship is obscured by the findings that increases in cardiac sympathetic nerve activity post-MI may affect infarct size (Flatley et al. 1985). The current findings suggest the possibility that blunted sympathetic activation within ovary-intact females, compared to ovary-removed females and males, may contribute to the previously observed findings that female sex hormones are able to reduce myocardial infarct size (Cavasin et al. 2003).

3.4.1. Potential significance

Elevations in SNA are well recognized as being detrimental in cardiovascular disease. Clearly more work is required to understand the origin of the sympathetic activation in both males and females. Finally, whether a sex difference also exists over longer time periods remains unclear. Elevations in sympathetic tone are closely related to the development of heart failure post MI, with patients with high noradrenaline levels for a prolonged period more likely to develop clinical heart failure (Sigurdsson et al. 1993). Females that experience an MI are at a high risk of developing heart failure, suggesting that while renal SNA may not be elevated post-MI acutely, elevations in SNA may occur over the ensuing days to months. Elucidating the long-term changes to SNA in females as well as males may help in the understanding of differences post-MI observed between the sexes and aid in the overall understanding about the role that SNA has post-MI.
Chapter 4: Ovarian hormones and the Cardiac Afferent Reflex

4.1. Introduction

In response to myocardial infarction (MI), men and women display significant differences in the rates and timelines of morbidity and mortality. The pro-arrhythmic and vasoconstrictive effects of increased sympathetic nerve activity (SNA) following MI can enhance the risk for worsening morbidity and mortality (Sigurdsson et al. 1993, Jardine et al. 2007).

The cardiac chemosensitive afferent reflex is implicated in driving changes in SNA and hemodynamics in response to MI (Weaver et al. 1981). MI results in the release of metabolites within the heart muscle, which in turn stimulates both cardiac sympathetic and vagal chemosensory afferents. The cardiac sympathetic afferent fibres travel via the middle cardiac nerve, with cell bodies in the dorsal root ganglion and project via secondary spinal afferents to the nucleus tractus solitarii (NTS). The cardiac vagal afferent fibres travel bilaterally via the vagus, with cell bodies in the nodose ganglion to the NTS. Activation of cardiac sympathetic or vagal afferents results in increases and decreases in SNA respectively, therefore the autonomic changes that occur in response to myocardial infarction depend on the balance of activation between the two parallel, yet opposing cardiac afferent pathways (Weaver et al. 1981). Clinical and experimental evidence suggest that sex and ovarian hormones can influence the autonomic response to myocardial infarction (Hogarth et al. 2009, Pinkham et al.). In Chapter 3, it was shown that ovarian hormones in females can inhibit the sympathetic excitation in response to MI, independent of the arterial baroreceptor reflex. The effects of sex and ovarian hormones on the chemosensitive cardiac afferent reflex have not been investigated.

Ovarian hormones appear to affect the regulation of sympathetic activity. For example, resting muscle SNA varies throughout the menstrual cycle and increases significantly following menopause (Narkiewicz et al. 2005, Carter et al. 2012). In anesthetized rats, the microinjection of estradiol directly within areas governing sympathetic regulation such as the nucleus tractus solitarius (NTS) and rostral ventrolateral medulla (RVLM) produces an acute decrease in renal SNA suggesting that estradiol has central sympathoinhibitory actions (Saleh et al. 2000). Importantly, receptors for estrogens have been found in areas associated with the cardiac afferent reflex pathway including sympathetic and vagal
afferent fibres and central autonomic regions (Papka et al. 1997, Shughrue et al. 1997, Brailoiu et al. 2007, Dun et al. 2009). Whether progesterone affects the regulation of SNA has not been fully studied as estradiol. In humans, it is suggested that progesterone may have an excitatory influence over sympathetic regulation, although this has not been widely characterized (Carter et al. 2012).

The current study set out to determine the impact of sex and ovarian hormones on the cardiac afferent reflex in baroreceptor denervated rats. Specifically, the impact of both the estrous cycle in ovary-intact females and hormone replacement in ovariectomized females on the cardiac afferent reflex were investigated and compared to responses in males. Based on the background literature and the results obtained in Chapter 3, I hypothesized that estrogen would augment the cardiac vagal afferent mediated sympathoinhibition and attenuate cardiac sympathetic afferent mediated sympathoexcitation.
4.2. Methods

4.2.1. Experimental preparation

Experiments were conducted in 70 Wistar rats. Ovariectomy surgery (see Chapter 2) was performed in a subset of female rats weighing 120-150 grams and aged between 5-6 weeks old. Chronic hormone replacement began at the time of ovariectomy and was achieved as described in Chapter 2. Rats with no hormone replacement did not receive silastic capsule implantation. Prior to the experimental day all rats were housed 2-4 per cage with water and food ad libitum in a room of constant temperature (22±1°C) with a 12h:12h light:dark cycle. Experiments were performed 4-6 weeks following ovariectomy.

On the day of experiment, female animals with intact ovaries had their stage of estrous cycle determined by examining the cytology of vaginal smears (see Chapter 2). Animals were divided according to sex, state of ovarian hormones and state of cardiac innervation. In regard to state of cardiac innervation, either the animal had completely intact cardiac innervation or the animal underwent bilateral vagotomy thereby leaving the heart with only sympathetic innervation. In some cases animals already used in the total cardiac innervation group subsequently underwent bilateral vagotomy and were subsequently used in the vagal denervation group. The animals, aged between 8-14 weeks old, were anesthetized as per Chapter 2.

4.2.2. Surgical Preparation

The surgical preparation for the current study is described in Chapter 2. Arterial pressure, heart rate and renal sympathetic nerve activity recordings were obtained in all rats and once recordings had stabilized the experimental protocol began. Arterial baroreceptors were denervated in all animals as per Chapter 2. In a subset of rats, vagal denervation was also performed in addition to baroreceptor denervation by cutting the cervical vagal nerves running alongside the carotid artery (see Chapter 2). The chest was then opened via an incision through the 4th intercostal space and the pericardium removed. A positive end pressure of 3-4 mmHg was used to ensure the lungs remained inflated. Mean arterial pressure (MAP), heart rate (HR) and renal SNA were recorded throughout the course of the experiment in all groups.
4.2.3. Experimental protocol

4.2.3.1. Cardiac afferent reflex responses to capsaicin

Following preparatory surgery and prior to beginning the experimental protocol a minimum of 30 minutes was allowed to ensure all recorded variables were stable. To test the cardiac afferent reflex in anesthetized rats, dose-dependent responses to capsaicin (1, 5 and 10µg per 10µl of saline (10% ethanol and 1% Tween 80)) were applied via a ~3x3mm piece of filter paper onto the epicardial surface of the left ventricle. MAP, HR and renal SNA responses were recorded for 40 seconds post-capsaicin application. Previous studies (Reimann et al. 1980) and our own observations (not shown) have identified that maximum sympathetic excitation following the epicardial application of capsaicin is achieved by 40 seconds. After 40 seconds the filter paper was removed and the heart rinsed in warm saline (~50ml). A recovery period of at least 15 minutes was allowed between applications of the different doses of capsaicin in each rat. Application of vehicle or saline alone onto the epicardial surface of the left ventricle had no effect on hemodynamics and renal SNA.

4.2.3.2. Vagal Stimulation

In a subset of baroreceptor denervated male, female and OVX rats the vagal control of renal SNA, arterial pressure and heart rate was investigated. For vagal afferent stimulation the left vagus was cut distally, to eliminate effects of vagal efferent stimulation on heart rate, and mounted on a bipolar stainless steel electrode connected to a stimulator (Universal Stimulator, ver. 11. University of Auckland, Auckland, NZ). Renal SNA, HR and arterial pressure were continuously measured during alternating 4 minute periods of control and stimulations of the vagal afferent nerve (20s train, 4.0 V and 2.0 ms pulses at 0.5, 1, 2, 4 and 8 Hz).

Once the vagal afferent stimulation protocol was completed the vagal efferent protocol was commenced. For vagal efferent stimulation, the right vagus was cut centrally, to eliminate central effects, and mounted on a bipolar stainless steel electrode connected to a stimulator (Universal Stimulator, ver. 11. University of Auckland, Auckland, NZ). Heart rate and arterial pressure were continuously measured during alternating 6 minute periods of control and stimulations of the vagal efferent nerve (20s train, 4.0 V and 2.0 ms pulses at 1, 2, 4 and 8 Hz).
4.2.3.3. Data Collection

Data was collected as per Chapter 2. Sympathetic nerve activity was normalized as a percentage change from resting level. Changes in arterial pressure and HR were taken as absolute values of the change from resting level. The resting level for each variable was taken as a period of 60 seconds directly prior to capsaicin application.

4.2.3.4. Statistical analysis

For comparison between the groups of the cardiac afferent reflex responses, the changes from baseline in arterial pressure (mmHg), HR (beats per minute, BPM), and renal SNA (\%) were assessed at two distinct time points following the application of capsaicin onto the anterior surface of the left ventricle. The sympathoinhibitory phase was taken as the average over a period of 5 seconds of recording between 2-7 seconds following capsaicin application. The sympathoexcitatory phase was taken as the average over a period of 10 seconds of recording between 30 to 40 seconds following capsaicin application (see results for further explanation). For the analysis of timeline changes in individual animals the changes from baseline in renal SNA were assessed as periods of 5 seconds of recording during maximum inhibitory phase (which was between 2-7 seconds following capsaicin application) and then at 10, 20, 30 and 40 seconds following capsaicin application. For effects of vagal stimulation, a 5 second period of recording of renal SNA, HR and arterial pressure was averaged during the period of greatest change during vagal stimulation. Two-way ANOVA with Bonferroni post-hoc analysis was used to analyze data. Data are shown as the means +/- SEM. P values < 0.05 were considered significant.
### Table 4.1: Baseline variables prior to cardiac afferent reflex stimulation

Baseline body weight, mean arterial pressure (MAP, mm Hg), heart rate (HR, beats per minute (BPM)), and renal sympathetic nerve activity (RSNA (µV)) prior to the application of capsaicin onto the epicardial surface of the left ventricle in males, females in metestrus/estrus, females in proestrus, ovariectomized females: without hormone replacement (OVX), with estradiol replacement (OVX+E), or with progesterone replacement (OVX+P) with total cardiac innervation (Total inner.) or vagal denervated and only cardiac sympathetic innervation intact (Vagal den.). * indicates significant difference compared to OVX and OVX+P groups, P<0.05.
**Total Cardiac Innervation**

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**Vagal Denervated**

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**Figure 4.1: Example traces of cardiac afferent reflex responses**

Example traces of arterial pressure (mm Hg), original renal sympathetic nerve activity (RSNA, µV) and integrated RSNA (µV) showing 10 seconds before and 40 seconds following epicardial application of capsaicin (time of application indicated by arrow) onto the left ventricle in a male, ovary-intact female, ovariectomized female (OVX), OVX with estradiol replacement (OVX+E) and OVX with progesterone replacement (OVX+P) with either total intact cardiac innervation (top panels) or vagal denervated and only cardiac sympathetic innervation (bottom panels).
4.3 Results

4.3.1. Baseline hemodynamics

Baseline variables in the 12 groups are shown in Table 4.1. Baseline mean arterial pressure (MAP), HR and renal SNA were not significantly different between the 12 groups. Despite being the same age, ovariectomized (OVX) female rats with estradiol replacement (OVX+E) had significantly lighter body weights than OVX females with no hormone replacement (OVX+E, 223±7 grams Vs. OVX, 280±7 grams, P<0.05) and OVX females with progesterone replacement (OVX+P) (OVX+E, 223±7 grams Vs. OVX+P, 310±7 grams, P<0.05, Table 4.1).

4.3.2. Cardiac afferent mediated responses in males with intact cardiac innervation

Figure 4.1 illustrates raw recordings of cardiac afferent reflex mediated changes in arterial pressure and renal SNA in response to capsaicin placed onto the anterior surface of the left ventricle. In male rats with intact cardiac sympathetic and vagal innervation, the epicardial application of capsaicin caused a biphasic response with an initial sympathoinhibitory phase and hypotension, followed by a secondary sympathoexcitatory phase and hypertension consistent with previous observations (Ustinova et al. 2000). Bilateral vagotomy abolished initial inhibition of renal SNA and hypotension confirming these responses were driven by cardiac vagal afferents.

4.3.3. Effect of estrous cycle on cardiac afferent reflex in females

Figure 4.1 illustrates that, like males, ovary-intact females with intact cardiac sympathetic and vagal innervation displayed a bi-phasic renal SNA and arterial pressure. In ovary-intact females bilateral vagotomy abolished the initial cardiac afferent reflex mediated sympathoinhibition confirming that the cardiac vagal afferents were responsible for the sympathoinhibitory phase (Figure 4.1). As shown in Figure 4.2, there was no effect of estrous cycle on the cardiac afferent reflex mediated inhibition and excitation of renal SNA (Figure 4.2). There was also no effect of estrous cycle on the cardiac afferent reflex mediated changes in arterial pressure and heart rate (data not shown). Because the
The estrous cycle did not significantly affect the cardiac afferent reflex responses, ovary-intact females were grouped together regardless of estrous phase for subsequent analysis.

**Figure 4.2: Effect of estrous cycle on cardiac afferent reflex**

Changes in renal sympathetic nerve activity (SNA) as a percentage (%) change from baseline during sympathoinhibitory phase (on left) or during secondary sympathoexcitatory phase (on right) of biphasic renal SNA response (as illustrated in figure 1) following application of capsaicin (10µg per 10µl saline) onto the epicardial surface of the left ventricle in ovary-intact females in the metestrus/estrus phases (met/est) or proestrus phase (pro) of the estrus cycle with total cardiac innervation (white bars) or with vagal denervation and only cardiac sympathetic innervation (black bars). Data are presented as mean ± SEM. * indicates significant effect of vagal denervation when compared to respective group with total cardiac innervation, P<0.05.

### 4.3.4. The cardiac afferent reflex in females

Males and females displayed dose-dependent renal SNA responses to epicardial capsaicin application as shown in Figure 4.3. Males and ovary-intact females displayed similar changes in renal SNA during the sympathoinhibitory phase of the cardiac afferent reflex (Figure 4.3). However, in the groups with total cardiac innervation and in response to epicardial application of 10 µg capsaicin, ovary-intact females (renal SNA increased 51±10%), compared to males (renal SNA increased 93±7%) displayed a significantly attenuated sympathoexcitatory phase (P<0.05). Vagal denervation did not alter the difference between males and females in the sympathoexcitatory phase of the cardiac afferent reflex (during sympathoexcitatory phase post-capsaicin (10 µg), renal SNA in females with vagal denervation was 62±9% above baseline vs. 100±13% above baseline in males with vagal denervation, P<0.05).
In males and females the changes in arterial pressure and heart rate mirrored the changes observed in renal SNA (Figure 4.4). Females with intact cardiac innervation, when compared to males, displayed attenuated cardiac afferent reflex driven hypertension but the effect was not significant (in response to capsaicin (10 µg), arterial pressure increased 22±6 mmHg in females vs. 40±6 mmHg in males, P>0.05, Figure 4.4). There were no differences in the cardiac afferent reflex mediated changes in heart rate when comparing between ovary-intact females and males (Figure 4.4).

**Figure 4.3: Dose response curves in males and ovary-intact females**
Dose response curves showing the renal sympathetic nerve activity (RSNA) response during the initial sympathoinhibitory phase (A and C) or during the secondary sympatoexcitatory phase (B and D) of the biphasic response (as illustrated in figure 1) following epicardial application of capsaicin at three different dose concentrations (1, 5 and 10µg per 10µl saline) in male and female groups. Graphs A and B on top row are from animals with total cardiac innervation and graphs C and D on bottom row are from animals with vagal denervation and only cardiac sympathetic innervation. Data are presented as mean ± SEM. Δ indicates significant difference between ovary-intact female and male groups.
4.3.5. Effect of ovariectomy on the cardiac afferent reflex

In response to the application of 10 µg capsaicin onto the anterior surface of the left ventricle, ovariectomy (renal SNA decreased -18±7%) significantly attenuated the cardiac afferent reflex mediated initial inhibition of renal SNA when compared to ovary-intact females (renal SNA decreased 53±4%, P<0.05) and significantly augmented the subsequent excitation of renal SNA (renal SNA increased 104±16% in OVX females Vs. 50±10% in ovary-intact females, P<0.05) as shown in Figure 4.4. The increases in renal SNA during the sympathoexcitatory phase of the cardiac afferent reflex were comparable between OVX females and males (Figure 4.4). In contrast to males and ovary-intact females, vagal denervation failed to significantly alter the initial sympathoinhibitory phase in OVX females when compared to the response in OVX females with intact cardiac innervation (during sympathoinhibitory phase post-capsaicin (10µg), renal SNA was -9±2% below baseline in OVX with vagal denervation vs. -18±7% below baseline OVX with total cardiac innervation, P>0.05, Figure 4.4).

Ovariectomy significantly attenuated the cardiac afferent reflex mediated decreases in arterial pressure and heart rate when compared to ovary-intact females (Figure 4.4). Compared to ovary-intact females, ovariectomy significantly augmented the cardiac afferent reflex mediated hypertension following epicardial application of capsaicin (Figure 4.4).

4.3.6. Effect of hormone replacement on the cardiac afferent reflex

As shown in Figure 4.5, OVX females with or without hormone replacement displayed dose-dependent renal SNA responses to the application of capsaicin onto the anterior wall of the left ventricle. In response to the application of 10 µg capsaicin onto the anterior wall of the left ventricular surface, chronic estradiol replacement (OVX+E) significantly augmented cardiac afferent reflex mediated inhibition of renal SNA compared to OVX with no hormone replacement (renal SNA decreased -43±7% OVX+E vs -18±7% in OVX, P<0.05) and significantly attenuated cardiac afferent reflex mediated sympathetic excitation (renal SNA increased 40±15% in OVX+E vs. 104±16% above baseline in OVX, p<0.05). Progesterone replacement in OVX rats had no effect on the renal SNA responses to epicardial application of capsaicin when compared to OVX rats with no hormone replacement (Figure 4.5). The cardiac afferent reflex mediated inhibition and excitation of
renal SNA were similar when comparing between OVX+E and ovary-intact females (Figure 4.4).

In OVX rats, chronic estradiol replacement, but not progesterone, significantly augmented the cardiac afferent reflex mediated decreases in both heart rate and arterial pressure. Therefore, estradiol replacement resulted in similar cardiac afferent reflex mediated decreases in heart rate and arterial pressure when comparing with ovary-intact females (Figure 4.4).

4.3.7. Sex differences in the cardiac afferent reflex

Figure 4.6 illustrates individual timeline responses in cardiac afferent reflex mediated changes in renal SNA within each group. As illustrated in Figure 4.1 males reached maximum sympathoexcitation around 40 seconds post-capsaicin application. In comparison to males, all female groups displayed a relatively faster time in reaching maximum sympathoexcitation following the epicardial application of capsaicin (Figure 4.1 and 4.6). In animals with intact cardiac innervation, all female groups when compared to males displayed greater variation in the cardiac afferent reflex mediated sympathoexcitatory responses (during sympathoexcitatory phase in intact cardiac innervation groups the standard deviation in renal SNA response was 19% in males vs. 39% in ovary intact females, 45% in OVX, 39% in OVX+E and 36% in OVX+P, Figure 4.6). Following bilateral vagotomy, the male sympathoexcitatory responses to epicardial capsaicin application became more variable whereas the variability in the female responses was unaltered, resulting in a loss of sex differences in both the timeline changes and variation of responses during cardiac afferent reflex mediated excitation of renal SNA (Figure 4.6).
Figure 4.4: Cardiac afferent reflex responses in males and females
Changes in renal sympathetic nerve activity (SNA, as percentage (%) change), arterial pressure (mm Hg) and heart rate (beats per minute (bpm)) from baseline during initial sympathoinhibitory phase (on left) or secondary sympathoexcitatory phase (on right) of biphasic response (illustrated in figure 1) following application of 10µg capsaicin onto epicardial surface of left ventricle in males, females, ovariectomized females (OVX), OVX with estradiol replacement (OVX+E), and OVX with progesterone replacement (OVX+P). White bars indicate animals with total cardiac innervation and black bars indicate animals with vagal denervation and only cardiac sympathetic innervation. Data are presented as mean ± SEM. # indicates significant difference with male group with same cardiac innervation, P<0.05. Δ indicates significant difference with female group with same cardiac innervation, P<0.05. + indicates significant difference with OVX+E group with same cardiac innervation, P<0.05. * indicates significant effect of vagal denervation when compared to respective group with total cardiac innervation, P<0.05.
**Figure 4.5: Dose response curves in OVX females**

Dose response curves showing the renal sympathetic nerve activity (RSNA) response during the initial sympathoinhibitory phase (A and C) or during the secondary sympathoexcitatory phase (B and D) of the biphasic response (as illustrated in figure 1) following epicardial application of capsaicin at three different dose concentrations (1, 5 and 10µg per 10µl saline) in ovariectomized females (OVX), OVX with estradiol replacement (OVX+E) and OVX with progesterone replacement (OVX+P) groups. Graphs A and B on top row are from animals with total cardiac innervation and graphs C and D on bottom row are from animals with vagal denervation and only cardiac sympathetic innervation. Data are presented as mean ± SEM. * indicates significant difference between OVX+E and OVX groups, p<0.05. + indicates significant difference between OVX+E and OVX+P groups, p<0.05.
Figure 4.6: Cardiac sympathetic afferent reflex mediated change in renal SNA in individual animals

Individual timeline changes in renal sympathetic nerve activity (SNA), as a percentage (%) change from baseline, for 40 seconds following the application of capsaicin (10µg per 10µl) onto the epicardial surface of the left ventricle at time zero in male, female, ovariectomized female (OVX), OVX+estradiol replacement (E) and OVX+progesterone replacement (P) groups with either total cardiac innervation (on left) or vagal denervated (on right).
4.3.8. Vagal afferent stimulation in males and females

To investigate whether there was a sex difference in the central processing of vagal afferent input, vagal afferent stimulation was performed in male, female and OVX. In all groups, the stimulation of the left afferent vagus caused frequency dependant changes in renal SNA, arterial pressure and heart rate (Figure 4.7). No significant differences were observed between males, females and OVX in the renal SNA, arterial pressure and heart rate responses to vagal afferent stimulation (Figure 4.7, data for arterial pressure and heart rate not shown).

![Graph](image-url)

**Figure 4.7: Renal SNA response to vagal afferent stimulation**
Changes from baseline in renal sympathetic nerve activity (SNA, as a percentage (%) change; top graph) in response to the electrical stimulation of the left afferent vagus at different frequencies (Hz) in arterial baroreceptor denervated male, female and ovariectomized females (OVX).
4.3.9. Vagal efferent stimulation in males and females

To investigate whether there was a sex difference in the efferent vagal control of heart rate, vagal efferent stimulation was performed in male, female and OVX. In all groups, stimulation of the right efferent vagus caused frequency dependent changes in heart rate (Figure 4.8). No significant differences were observed between males, female and OVX in the heart rate responses to vagal efferent stimulation (Figure 4.8).

![Figure 4.8: Heart rate response to vagal efferent stimulation](image)

Changes from baseline in renal heart rate (beats per minute, bpm) in response to the electrical stimulation of the right efferent vagus at different frequencies (Hz) in arterial baroreceptor denervated male, female and ovariectomized females (OVX).
4.4. Discussion

The current study is the first to describe an effect of sex and ovarian hormones on the cardiac afferent reflex. Compared to males, ovary-intact females displayed attenuated cardiac afferent reflex mediated sympathetic excitation, throughout the estrous cycle. Ovariectomy in females augmented cardiac afferent reflex driven sympathoexcitation and attenuated reflex mediated sympathoinhibition, effects that were abolished by chronic estradiol replacement. The current results demonstrate that estradiol is capable of altering the cardiac afferent reflex in females by augmenting reflex mediated sympathoinhibition and attenuating reflex mediated sympathoexcitation.

Previous studies have only investigated the cardiac afferent reflex in either males or mixed sex groups. The cardiac afferent reflex is implicated in driving changes in SNA in cardiovascular disease states such as MI and heart failure (Weaver et al. 1981, Wang et al. 1996). Following MI, pathological increases in SNA have been shown to drive arrhythmia, fibrillation and sudden death (Jardine et al. 2007), whilst elevated peripheral SNA increase total peripheral resistance, fluid retention and renin release from the juxtaglomerular cells of the kidney thereby adversely altering both preload and afterload (DiBona 2000, Nozawa et al. 2002). In animal studies, ovarian hormones have been implicated in providing cardioprotection following MI and in heart failure (Cavasin et al. 2003, Cavasin et al. 2004). Furthermore, the findings in Chapter 3, alongside previous studies suggest that ovary-intact females may respond to myocardial infarction with less sympathetic activation and greater vagal activation compared to males (Du et al. 1995, Airaksinen et al. 1998). The findings in this study provide a possible mechanism by which sex and ovarian hormones may influence SNA changes in cardiovascular disease.

In the current study the cardiac afferent reflex responses observed in males were consistent with previous findings (Ustinova et al. 2000). Compared to males, ovary-intact females displayed significantly attenuated cardiac afferent reflex mediated increases in renal SNA whilst reflex-mediated inhibition of renal SNA was not different between males and ovary-intact females. Furthermore, ovariectomy significantly augmented the cardiac afferent reflex mediated sympathoexcitation in females and attenuated the cardiac vagal afferent mediated inhibition of renal SNA. Estradiol appears to be the ovarian hormone responsible for mediating changes in cardiac afferent reflex mediated sympathetic inhibition and excitation in females as chronic estradiol replacement, but not progesterone replacement, restored the cardiac afferent reflex responses to be similar to ovary-intact females. It is currently unclear what possible locations and mechanisms may underlie
actions of estradiol on the cardiac afferent reflex. In anesthetized rats and cats it has been shown that cardiac vagal afferent input antagonizes the sympathoexcitatory actions of cardiac sympathetic afferents (Reimann et al. 1980, Tjen et al. 1997, Ustinova et al. 2000). Therefore the relative activation of the chemosensitive cardiac vagal and sympathetic afferents will affect the overall change in sympathetic outflow. Ovariectomy significantly attenuated the vagal component of the cardiac afferent reflex, therefore it may be that the attenuation of the cardiac vagal component augmented the subsequent sympathoexcitation. Further studies in animals with only cardiac vagal afferents intact would further clarify the effects of estradiol on this pathway.

Estradiol receptors are expressed in the heart, dorsal root ganglia, nodose ganglia, spinal afferent pathways and in central brain regions involved in the cardiac afferent reflex pathway (Rees et al. 1980, Shughrze et al. 1997, Papka et al. 2001, Dun et al. 2009, Brailoiu et al. 2013). Furthermore capsaicin sensitive afferent neurons are immunoreactive for estradiol receptors-α and –β (Papka et al. 2001). Estradiol appears capable of directly modulating the function of peripheral sensory fibers as shown by studies investigating sex differences in pain perception. For example, estradiol potentiates the VR1-mediated cationic current in rat dorsal root ganglia thereby potentiating capsaicin induced sensory sympathetic afferent activation (Lu et al. 2009). Conversely, estradiol has been shown to be capable of inhibiting sympathetic afferent sensitivity by inhibiting ATP-induced increases in intracellular calcium within the dorsal root ganglia (Chaban et al. 2011). Centrally, microinjection of estradiol into the nucleus tractus solitarius and rostral ventrolateral medulla causes an acute inhibition in efferent renal SNA and an increase in efferent vagal activity suggesting that central estradiol receptors can alter sympathetic regulation (Saleh et al. 2000). Given the widespread location of estradiol receptors within the reflex pathway and the complicated actions of estradiol it is not possible to comment on the precise mechanisms by which estradiol is capable of altering the cardiac afferent reflex.

The current study failed to observe a significant impact of the estrous cycle on the cardiac afferent reflex. However, this is not to say that changing levels of circulating estrogens within ovary-intact females do not alter the cardiac afferent reflex, as the level of estradiol and progesterone were not measured. Ovary-intact females were divided into two groups in order to separate females according to estrous phases with high levels of estrogen (proestrous) and low levels of estrogen (metestrous and estrous). The estrous cycle in the rat occurs over four days and in proestrous the spikes in estrogens happen over a relatively short time, it may be that in the females in proestrous the level of estrogens were not
significantly increased at the time of experiment. Previously, it has been demonstrated that the estrous cycle in rat and menstrual cycle in women affects baroreflex control of SNA and we had expected to observe a similar effect on the cardiac afferent reflex (Minson et al. 2000, Goldman et al. 2009).

Alongside significant effects of ovarian hormones on the magnitude of renal SNA responses to cardiac afferent reflex stimulation, there were some differences that appeared to be independent of circulating ovarian hormones. Similar with previous observations, males with intact cardiac sympathetic and vagal afferents reached maximum sympathetic excitation at 40 seconds following the application of capsaicin (Reimann et al. 1980, Ustinova et al. 2000). In contrast, in females with intact cardiac innervation the maximum sympathetic excitation was reached by 10 seconds. Furthermore, compared to males all female groups with intact cardiac innervation tended to display larger variations in the cardiac afferent reflex mediated sympathetic excitation. The sex differences in timeline changes and variation in the cardiac afferent reflex mediated sympathoexcitation were abolished by vagotomy, suggesting that sex differences in the cardiac vagal afferent input on central sympathetic regulation may be responsible. In the current study there were no effects of sex or ovarian hormones on the central processing of vagal afferent input on changes in renal SNA. Consistent with the current findings, a previous study also found no sex differences in the effect of vagal stimulation on changes in heart rate and arterial pressure in anesthetized male and female rats (Du et al. 1994). Female rats appear to have 50% more myelinated vagal afferents when compared to male rats, a difference contributed to by a sex-specific sub-population of low-threshold myelinated fibers in females (Li et al. 2008). This sex-specific sub-population of low-threshold myelinated fibers becomes inactive or excitable in the absence or presence of 17ß-estradiol respectively (Qiao et al. 2009). Furthermore, the sensitivity of mechanosensory vagal afferent fibres of cardiac origin can be altered by circulating ovarian hormones, as shown by a loss of sensitivity in mechanosensory cardiac vagal afferents during pregnancy in rats (Deng et al. 1995, Storey et al. 2004). Based on the current findings it would appear likely that the sex differences observed in the cardiac afferent reflex are at least partly mediated by sex differences in the vagal afferent pathway, however future research is required to confirm this.

The current study highlights sex specific changes in SNA in response to activation of chemosensory cardiac afferents. It is known that the chemosensitive cardiac afferent reflex is an important proponent of initial changes in autonomic balance in response to MI (Weaver et al. 1981). Following MI, a shift in the sympathovagal balance towards
sympathetic predominance exposes the patient to a higher likelihood of ventricular fibrillation and sudden death whereas vagal predominance is cardioprotective. In other disease states, such as hypertension, heart failure, and renal disease the cardiac afferent reflex may also be important in eliciting changes in SNA. The current experiments were performed under chloralose-urethane anesthesia, as it is not possible to perform these experiments in conscious rats and therefore how the current results relate to the conscious state is not known. Whether sex differences in cardiac afferent reflex activation of renal SNA remain in the conscious state is unknown. Furthermore, it remains to be seen whether the effect of sex and ovarian hormones on the cardiac afferent reflex can impact on changes in SNA in cardiovascular disease states.
Chapter 5: Ovarian hormones, reflex regulation and heart failure

5.1. Introduction

Significant sex differences exist in myocardial adaptations to hemodynamic overload in the setting of heart failure (Adams et al. 1999, Luchner et al. 2002, Ghali et al. 2003). Compared to men, women with heart failure have better preserved cardiac function and reduced hypertrophy while concurrently receiving less medication and displaying better survival rates (Adams et al. 1999, Luchner et al. 2002, Ghali et al. 2003). Similarly, in experimental animals where surgically induced myocardial infarction (MI) has been used to induce heart failure, compared to males, females display preserved cardiac function and lower mortality rates (Cavasin et al. 2003, Cavasin et al. 2004). Evidence suggests that ovarian hormones in females are in some way cardioprotective in the development and progression of heart failure following MI (Cavasin et al. 2003, Cavasin et al. 2004, Pelzer et al. 2005). Despite well recognized sex differences in the development and progression of heart failure the mechanisms responsible are not well understood.

Sympathetic nerve activity (SNA) is pathophysiologically altered in heart failure which forms the basis for successful use of beta-blockers in treating heart failure patients (Metra et al. 2001, Maack et al. 2003). Particularly, in both humans and animals, an increase in renal SNA in heart failure is associated with worsening cardiac function, morbidity and mortality (Nozawa et al. 2002, Petersson et al. 2005). In heart failure, attenuation of the arterial baroreflex control of renal SNA and sensitization of the cardiac sympathetic afferent reflex (CSAR) is hypothesized to contribute to the pathophysiological increases in renal SNA. For example, male rats with MI induced heart failure display a reduced sensitivity and range of arterial baroreflex control of renal SNA (DiBona et al. 1994, Zhang et al. 1999) and also display augmented CSAR mediated increases in renal SNA in response to a given dose of a stimulating substance (Zhu et al. 2002, Zhu et al. 2004, Zhu et al. 2004, Gao et al. 2005). Alterations in reflex control of SNA in heart failure are not always observed in humans and other experimental models (Floras 2001, Ramchandra et al. 2009). For example, it has been demonstrated that the arterial baroreflex control of muscle SNA, represented by the gain of the arterial baroreflex, is unaltered in heart failure patients when compared to healthy controls (Ferguson et al. 1992). Furthermore, Ramchandra et al. have demonstrated in female sheep with mild to moderate heart failure that the arterial baroreflex control of renal SNA is unaltered in this particular experimental setting (Ramchandra et al. 2009). It has not been investigated whether female sex and ovarian hormones affect the
changes that occur in the arterial baroreceptor reflex and CSAR control of renal SNA in heart failure.

Ovarian hormones, in particular estrogen, have been shown to alter reflex regulation of SNA in humans and animals (Minson et al. 2000, Shoemaker et al. 2001, Fu et al. 2009, Goldman et al. 2009). Receptors for estrogen are located throughout peripheral and central pathways governing both the arterial baroreceptor reflex and CSAR (Papka et al. 1997, Shughrue et al. 1997, Papka et al. 2001, Brailoiu et al. 2007, Dun et al. 2009). Estrogen is generally observed to attenuate sympathetic excitation, an action that is in opposition to the effect that heart failure is considered to have on sympathetic regulation (Hinojosa-Laborde et al. 1999, Zucker et al. 2004). In heart failure, attenuated nitric oxide and augmented angiotensin II signaling has been credited for causing adverse alterations in both the arterial baroreflex and CSAR in experimental heart failure (Zhang et al. 1999, Zhu et al. 2004, Zhu et al. 2004, Zucker et al. 2004). In contrast, estrogen has been shown to augment central nitric oxide signaling and attenuate central angiotensin II signaling (Tanaka et al. 2001, Wang et al. 2008, Shih 2009, Xue et al. 2009, Ciriello et al. 2013). Therefore it was hypothesized that ovarian hormones in females may attenuate heart failure-mediated alterations in the reflex regulation of renal SNA (DiBona et al. 1994, Zhu et al. 2002, Zhu et al. 2004). In particular, the current study investigated the arterial baroreceptor reflex and CSAR control of renal SNA in male, female and ovariectomized female rats with MI induced heart failure. Based on the background literature, I hypothesized that female sex hormones would protect the reflex regulation of renal SNA in MI-induced heart failure.
5.2. Methods

5.2.1. Experimental preparation

Experiments were conducted in 49 Wistar rats. Ovariectomy surgery (see Chapter 2) was performed in a subset of female rats weighing 120-150 grams and aged between 5-6 weeks old; 2 weeks prior to myocardial infarct or sham surgery. MI was used as the model to induce heart failure. Briefly, MI was performed under isoflurane anesthesia (2% in oxygen), whilst the rat was artificially ventilated (model 680; Harvard apparatus, Holliston, MA). At the beginning of surgery, all animals were given antibiotics (12.5 mg/kg enrofloxacin, Baytril, Bayer, New Zealand,) and analgesia (20 µg/kg buprenorphine, Temgesic, Reckitt Benckiser, New Zealand and again 24 hours later). The chest was opened via an incision through the 4th intercostal space and the pericardium removed. In the MI groups, MI was induced by tying off the left anterior descending coronary artery (LAD) 2-3 mm from origin using a 6-0 silk suture. In the sham groups a suture was passed through the heart wall but the LAD was not tied off. At the conclusion of the surgery the lungs were reinflated and then the chest was sutured closed. As soon as the rats regained consciousness they were returned to their home cages. A heating pad was placed in the cage for 24 hr after the surgery. Prior to the experimental day all rats were housed 2-4 per cage with water and food ad libitum in a room of constant temperature (22°C) with a 12h:12h light:dark cycle. Experiments were performed 6-7 weeks following MI. On the day of experiment the animals were anesthetized as per the protocol described in Chapter 2. Body temperature was maintained at 37°C by a heating pad and heating lamp. Animals were divided into 9 groups representing males, females or ovariectomized females (OVX) who had undergone a sham MI surgery or who had an MI less than, or greater than 25% of the left ventricular wall (as determined post-mortem). Animals with an MI size of less than- or greater than 25% of the left ventricular wall were grouped into ‘small MI’ and ‘large MI’ groups respectively.

5.2.2. Surgical Preparation

The surgical preparation for the current study is described in Chapter 2. Arterial pressure, heart rate and renal SNA recordings were obtained in all rats and once recordings had stabilized the experimental protocol began. Once arterial baroreceptor reflex responses had been obtained the CSAR was investigated. To investigate the CSAR; baroreceptor
and vagal denervation was performed (see Chapter 2) in all animals and the chest opened. When the chest was opened a positive end pressure of 3-4mmHg was applied to the outflow of the ventilator to ensure the lungs remained inflated. Once all recordings were stable, the CSAR protocol began. Renal SNA, heart rate (HR) and arterial pressure were recorded throughout the entire experiment.

### 5.2.3. Experimental protocol

#### 5.2.3.1. Arterial baroreceptor reflex responses

Once renal SNA, HR and arterial pressure recordings had stabilized the arterial baroreceptor reflex protocol was begun. The protocol for arterial baroreceptor reflex responses is described in Chapter 2.

#### 5.2.3.2. Cardiac sympathetic afferent reflex responses to capsaicin

In arterial baroreceptor and vagal denervated open chest animals and once recordings had stabilized the CSAR responses were obtained by placing capsaicin (2.5µg and 5µg capsaicin per 10µl solution) onto the surface of the left ventricle via a piece of 3x3mm filter paper. Once applied, the capsaicin was left on the heart surface for 40 seconds and then washed off with ~50ml warm saline. At least 15 minutes was allowed between each application of capsaicin (refer to Chapter 2 for more information regarding the epicardial application of capsaicin). Each dose of capsaicin was applied 2-3 times in random order in each animal.

#### 5.2.3.3. Heart and lung weights

Once the experimental protocols were completed the animal was euthanized with an overdose of chloralose-urethane. The heart and lungs were excised and total heart weight and lung weight were recorded for each animal.
5.2.3.4. Determination of myocardial infarct size

Once the heart had been weighed it was frozen and then cut into 4-5 transmural slices. The heart slices were stained using triphenyltetrazolium chloride (Sigma-Aldrich, USA). Following 20 minutes of incubation in the triphenyltetrazolium chloride solution the heart slices underwent a bleach cycle in formaldehyde for 30 minutes. The heart slices were subsequently photographed and the infarct size was determined as a percentage of the total left ventricular wall.

5.2.3.5. Data Collection

Data was collected as described in Chapter 2. Analysis of the arterial baroreceptor reflex responses was performed using a 5-parameter nonlinear regression equation to fit the collected mean arterial pressure (MAP)-renal SNA and MAP-HR data to produce arterial baroreflex curves (Ricketts et al. 1999). CSAR mediated renal SNA responses were analyzed as a percentage change from baseline (baseline was determined as 30 seconds of renal SNA directly prior to the application of epicardial capsaicin). CSAR mediated arterial pressure (mmHg) and HR (beats per minute (BPM)) responses were analyzed as an absolute change from baseline. Changes from baseline in arterial pressure (mmHg), HR (BPM), and renal SNA (%) were assessed as the average change over the period of time between 20 to 40 seconds of recording following the application of capsaicin. The averaged arterial pressure (mmHg), HR (BPM) and renal SNA (%) response to 2-3 applications of each dose of capsaicin are presented. The noise level was taken to be the integrated SNA value when SNA was at the lowest level between two distinct bursts of nerve activity.

5.2.3.6. Statistical analysis

Two-way ANOVA with Bonferroni post-hoc analysis was used for comparisons among groups. Data are shown as the means +/- SE. P values < 0.05 were considered significant.
5.3. Results

5.3.1. Survival following myocardial infarction in males and females

Ovary-intact female rats displayed reduced mortality rates following MI when compared to males (mortality rate post-MI in ovary-intact females was 24% vs. 38% in males) and OVX females (mortality rate post-MI in OVX females was 50% vs. 24% in ovary-intact females, Figure 5.1).

![Mortality rates in response to myocardial infarction](image)

**Figure 5.1: Mortality rates in response to myocardial infarction**
Mortality rate as a percentage (%) of total number of animals in which myocardial infarction surgery was performed in males, females, and ovariectomized females (OVX).

5.3.2. Baseline hemodynamics

Body weights in the ovary-intact female groups were typically lower when compared to male and OVX female groups as shown in Table 5.1. Examples of cross-sectional images of a sham, small or large MI are shown in Figure 5.2. MI size in the male, female and OVX large MI groups were significantly greater compared to MI sizes in the small MI groups (Table 5.1). MI size was not different between the male, female and OVX large MI groups (Table 5.1). Heart-weight to body-weight ratio was significantly elevated in all large MI groups but not in small MI groups, when compared to sham groups (Table 5.1). Lung-
Chapter 5 – Ovarian hormones, reflex regulation and heart failure

Weight to body-weight ratios were significantly elevated in female large MI group and OVX female large MI group but not in male large MI group and all small MI groups, when compared to sham groups (Table 5.1).

Baseline MAP, HR and renal SNA were not significantly different when comparing between sham groups and when comparing between sham and small MI groups (Table 5.1). Baseline renal SNA was significantly higher in ovary-intact female large MI group but not in male large MI group and OVX large MI group, when compared to sham groups (Table 5.1).

Figure 5.2: Example images of myocardial infarction
Heart sections from sham animal (left), animal with a small myocardial infarction (middle) or animal with large myocardial infarction (right) of the left ventricular wall.
**Table 5.1: Baseline variables in male, female and OVX with or without chronic myocardial infarction**

Baseline body weight, heart weight, lung weight, mean arterial pressure, heart rate, renal sympathetic nerve activity (SNA, µV) and infarct size measured as a percentage (%) of the left ventricular wall. Abbreviations: MI – Myocardial infarction, OVX – ovarectomized female. Data are mean±SEM. * indicates significant difference from respective sham, P<0.05, Δ indicates significant difference from respective Small MI group, P<0.05, ^ indicates significant difference from respective Male group, P<0.05, and # indicates significant difference from respective OVX group, P<0.05.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Male Sham</th>
<th>Male Small MI</th>
<th>Male Large MI</th>
<th>Female Sham</th>
<th>Female Small MI</th>
<th>Female Large MI</th>
<th>OVX sham</th>
<th>OVX Small MI</th>
<th>OVX Large MI</th>
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<tr>
<td>Body weight (g)</td>
<td>473 ± 13</td>
<td>479 ± 29</td>
<td>481 ± 11</td>
<td>337 ± 10</td>
<td>267 ± 14</td>
<td>297 ± 19</td>
<td>379 ± 9</td>
<td>388 ± 24</td>
<td>339 ± 9</td>
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<tr>
<td>Wet whole heart weight</td>
<td>1.4 ± 0.06</td>
<td>1.8 ± 0.15</td>
<td>2.0 ± 0.13</td>
<td>1.1 ± 0.04</td>
<td>1.0 ± 0.04</td>
<td>1.3 ± 0.10</td>
<td>1.1 ± 0.04</td>
<td>1.2 ± 0.08</td>
<td>1.5 ± 0.23</td>
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<tr>
<td>Wet whole heart weight (mg/g)</td>
<td>2.9 ± 0.14</td>
<td>3.9 ± 0.35</td>
<td>4.2 ± 0.17</td>
<td>3.2 ± 0.10</td>
<td>3.8 ± 0.22</td>
<td>4.4 ± 0.17</td>
<td>2.9 ± 0.10</td>
<td>3.0 ± 0.19</td>
<td>4.3 ± 0.60*</td>
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<tr>
<td>Wet whole lung weight (g)</td>
<td>1.7 ± 0.06</td>
<td>1.8 ± 0.18</td>
<td>2.3 ± 0.41</td>
<td>1.7 ± 0.09</td>
<td>1.3 ± 0.07</td>
<td>2.3 ± 0.20</td>
<td>1.5 ± 0.11</td>
<td>1.6 ± 0.10</td>
<td>2.5 ± 0.54*</td>
</tr>
<tr>
<td>Wet whole lung weight (mg/g)</td>
<td>3.7 ± 0.16</td>
<td>3.8 ± 0.49</td>
<td>4.7 ± 0.9</td>
<td>4.8 ± 0.22</td>
<td>4.8 ± 0.21</td>
<td>7.7 ± 0.64</td>
<td>4.1 ± 0.30</td>
<td>4.2 ± 0.20+</td>
<td>7.4 ± 1.45*</td>
</tr>
<tr>
<td>Mean arterial pressure, mmHg</td>
<td>92 ± 5</td>
<td>84 ± 5</td>
<td>87 ± 7</td>
<td>94 ± 7</td>
<td>97 ± 3</td>
<td>85 ± 7</td>
<td>92 ± 5</td>
<td>94 ± 12</td>
<td>90 ± 2</td>
</tr>
<tr>
<td>Heart rate, bpm</td>
<td>393 ± 12</td>
<td>388 ± 32</td>
<td>436 ± 31</td>
<td>393 ± 14</td>
<td>361 ± 14</td>
<td>363 ± 31</td>
<td>351 ± 14</td>
<td>341 ± 32</td>
<td>396 ± 21</td>
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<tr>
<td>Renal SNA, µV</td>
<td>5.7 ± 1.67</td>
<td>5.2 ± 1.59</td>
<td>8.0 ± 1.90</td>
<td>4.2 ± 0.89</td>
<td>9.9 ± 2.42</td>
<td>11.7 ± 2.63</td>
<td>9.6 ± 2.11</td>
<td>5.9 ± 1.69</td>
<td>6.2 ± 1.79</td>
</tr>
<tr>
<td>Infarct size as a % of left ventricle</td>
<td>-</td>
<td>12 ± 3</td>
<td>34 ± 3*</td>
<td>-</td>
<td>13 ± 5</td>
<td>31 ± 2*</td>
<td>-</td>
<td>11 ± 3</td>
<td>30 ± 1*</td>
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</table>
Arterial Baroreflex and CSAR responses in a single sham animal

Figure 5.3: Example traces of arterial baroreflex and cardiac sympathetic reflex responses in the single animal

Example traces showing arterial pressure (mmHg), original renal sympathetic nerve activity (SNA, µV) and integrated renal SNA (µV) in a single sham animal during arterial baroreceptor reflex stimulation (top graph) and cardiac sympathetic afferent reflex (CSAR) stimulation (bottom figure). Arterial baroreceptor reflex stimulation was achieved by lowering or increasing arterial pressure (mmHg) using ramp infusions of sodium nitroprusside and phenylephrine respectively. CSAR stimulation was achieved by placing capsaicin (5µg per 10µl saline) onto the epicardial surface of the left ventricle following total baroreceptor- and bilateral vagal-denervation.
5.3.3. The arterial baroreceptor reflex in males and females with heart failure

Example raw recordings representing arterial baroreceptor reflex and CSAR renal SNA responses in the same animal are shown in Figure 5.3. Arterial baroreceptor reflex curves showing the relationship between arterial pressure and renal SNA for male, female and OVX groups are shown in Figures 5.5, 5.6 and 5.7 respectively.

There were no significant differences in arterial baroreceptor reflex control of renal SNA between the three sham groups, or between the sham and small MI groups (Table 5.2). Male and OVX large MI groups compared to male and OVX sham groups respectively, displayed an impaired ability of the arterial baroreflex to appropriately inhibit renal SNA, characterized by a significantly elevated lower plateau of the arterial baroreflex curve (lower plateau in male large MI group was 60±4% vs. 36±5% in male sham group, p<0.05, and lower plateau in OVX large MI group was 62±4% vs. 37±4% in OVX sham group, p<0.05, Figures 5.4 and 5.6). The arterial baroreflex range of control over renal SNA was significantly reduced in both male and OVX large MI groups when compared to male and OVX sham groups respectively (baroreflex range of control over renal SNA in male large MI group was 47±6% vs. 79±7% in male sham group, p<0.05, and baroreflex range of control over renal SNA in OVX large MI group was 41±5% vs. 73±6% in OVX sham group, p<0.05, Table 5.2). The ability of the arterial baroreflex to inhibit renal SNA was unaltered by heart failure in the ovary-intact females (lower plateau in female large MI group was 47±7% vs. 42±5% in female sham group, p>0.05, Figure 5.5) as was the arterial baroreflex range of control over renal SNA (baroreflex range of control over renal SNA in female large MI group was 70±9% vs. 62±5% in female sham group, p>0.05, Table 5.2). Both ovary-intact female large MI group and female small MI group did display a significantly lower MAP baroreflex curve mid-point when compared to female sham group (Figure 5.5). The arterial baroreflex control over renal SNA was not different between male, female and OVX small MI groups compared to male, female and OVX sham groups respectively (Table 5.2).

The arterial baroreflex control of heart rate displayed large variations within groups. The arterial baroreflex control of heart rate was not significantly affected by large MI in males and OVX females. Ovary-intact females with large MI did display a significantly reduced baroreflex range of control over HR when compared to female sham group (baroreflex range of control over HR in female large MI group was 41±4 bpm vs. 81±17 bpm in female sham group, p<0.05, Table 5.2).
Figure 5.4: Example traces showing arterial baroreflex responses in males and females with or without chronic myocardial infarction
Example traces showing arterial pressure (mmHg), original renal sympathetic nerve activity (SNA, µV) and integrated renal SNA (µV) in male, female and ovariectomized female (OVX) rats with either a sham myocardial infarction (MI, on left) or large MI (on right) during arterial baroreceptor reflex stimulation. Arterial baroreceptor reflex stimulation was achieved by lowering or increasing arterial pressure (mmHg) using ramp infusions of sodium nitroprusside (SNP) and phenylephrine (PE) respectively.
Male Arterial Baroreflex Curves

Figure 5.5: Arterial baroreflex curves in males with or without chronic myocardial infarction
Mean curves describing arterial baroreflex control over renal sympathetic nerve activity (SNA) as a percentage (%) change from baseline in sham group (solid line), small MI group (dashed line) or large MI group (dash-dot line) in males. Resting mean arterial pressure and renal SNA at time baroreflex curves were obtained are indicated by ○ for sham group Δ for small MI group □ for large MI group. * indicates significant difference between large MI group and sham group, P<0.05. Data are shown as mean±SEM.
Figure 5.6: Arterial baroreflex curves in females with or without chronic myocardial infarction

Mean curves describing arterial baroreflex control over renal sympathetic nerve activity (SNA) as a percentage (%) change from baseline in sham group (solid line), small MI group (dashed line) or large MI group (dash-dot line) in ovary-intact females. Resting mean arterial pressure and renal SNA at time baroreflex curves were obtained are indicated by ○ for sham group △ for small MI group ■ for large MI group. * indicates significant difference between large MI group and sham group and # indicates significant difference between small MI group and sham group, P<0.05. Data are shown as mean±SEM.
Figure 5.7: Arterial baroreflex curves in OVX females with or without chronic myocardial infarction
Mean curves describing arterial baroreflex control over renal sympathetic nerve activity (SNA) as a percentage (%) change from baseline in sham group (solid line), small MI group (dashed line) or large MI group (dash-dot line) in ovariectomized (OVX) females. Resting mean arterial pressure and renal SNA at time baroreflex curves were obtained are indicated by ○ for sham group △ for small MI group □ for large MI group. * indicates significant difference between large MI group and sham group, P<0.05. Data are shown as mean±SEM.
Table 5.2: Baroreflex parameters in males and females following chronic myocardial infarction.

Baroreflex parameters obtained in Male sham (n=6), Male Small MI (n=5), Male Large MI (n=5) Female Sham (n=6), Female Small MI (n=4), Female Large MI (n=5), OVX Sham (n=8), OVX Small MI (n=5) and OVX Large MI (n=5). Abbreviations: bpm = beats per minute, SNA = sympathetic nerve activity, BP_50 = mean arterial pressure curve mid-point. Data are mean±SEM, * indicates significant difference from respective sham, P<0.05, Δ indicates significant difference from respective MI<25%, P<0.05.
5.3.4. The cardiac sympathetic afferent reflex in males and females with heart failure

An example raw recording representing renal SNA and arterial pressure response to epicardial administration of capsaicin in a baroreceptor and vagal denervated sham animal is shown in Figure 5.3. Cardiac sympathetic afferent reflex mediated changes in renal SNA, HR and arterial pressure in male, female and OVX female groups are shown in Figure 5.7.

There were no significant differences in the CSAR mediated changes in renal SNA in response to epicardial capsaicin when comparing between male, female and OVX sham groups. Males and OVX females with large MI displayed a tendency for greater increases in renal SNA following epicardial application of capsaicin when compared to male and OVX sham groups, respectively, but these differences did not reach statistical significance (Figure 5.8). Male large MI and OVX large MI groups did display significantly augmented CSAR mediated increases in renal SNA when compared with male small MI and OVX small MI groups respectively (male large MI group renal SNA response to epicardial application of 5µg capsaicin onto left ventricle was 41±3% vs. 23±4% in male small MI group, P<0.05, and OVX large MI group renal SNA response to epicardial application of 5µg capsaicin onto left ventricle was 44±7% vs. 14±1% in OVX small MI group, P<0.05). Male large MI and OVX large MI groups displayed significantly augmented CSAR mediated increases in renal SNA when compared with female large MI group (male large MI group renal SNA response to epicardial application of 5µg capsaicin onto left ventricle was 41±3% vs. 19±4% in female large MI group, P<0.05, and OVX large MI group renal SNA response to epicardial application of 5µg capsaicin onto left ventricle was 44±7% vs. 19±4% in female large MI group, P<0.05). CSAR mediated changes in renal SNA were the same when comparing female large MI group and female sham group (female large MI group renal SNA response to epicardial application of 5µg capsaicin onto left ventricle was 19±4% vs. 25±5% in female sham group, P>0.05).

CSAR mediated changes in arterial pressure mirrored changes in renal SNA in all groups (Figure 5.8). The OVX sham group displayed significantly greater increases in arterial pressure following epicardial administration of capsaicin when compared to the male sham group (OVX sham group arterial pressure response to epicardial application of 5µg capsaicin onto left ventricle was 37±6 mmHg vs. 19±5 mmHg in male sham group, p<0.05). Compared to shams, males with a large MI displayed significantly augmented CSAR mediated increases in arterial pressure (Male large MI group arterial pressure response to epicardial application of 5µg capsaicin onto left ventricle was 47±10 mmHg vs. 19±5 mmHg.
in male sham group, p<0.05). The female CSAR arterial pressure response to epicardial application of capsaicin was not altered by large MI (Female large MI group arterial pressure response to epicardial application of 5µg capsaicin onto left ventricle was 17±7 mmHg vs. 20±4 mmHg in female sham group, p<0.05). Male large MI and OVX large MI groups displayed greater CSAR mediated increases in arterial pressure when compared to female large MI group (male large MI group arterial pressure response to epicardial application of 5µg capsaicin onto left ventricle was 47±10 mmHg vs. 17±7 mmHg in female large MI group, p<0.05, and OVX large MI group arterial pressure response to epicardial application of 5µg capsaicin onto left ventricle was 51±5 mmHg vs. 17±7 mmHg in female large MI group, p<0.05). There were no differences between groups when comparing the heart rate responses to epicardial capsaicin application.
Cardiac Sympathetic Afferent Reflex Responses

2.5µg capsaicin

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Figure 5.8: Cardiac sympathetic afferent reflex responses following chronic myocardial infarction

Changes in renal sympathetic nerve activity (SNA), as percentage (%) change from baseline, and change in heart rate (beats per minute (bpm)) and arterial pressure (mmHg) as an absolute change from baseline following the application of 2.5µg (left column) or 5µg (right column) capsaicin per 10µl saline placed onto the epicardial surface of a non-infarcted part of the left ventricle in Males, Females and Ovariectomized (OVX) females with either sham myocardial infarction (MI), small MI or large MI. Data is mean ± SEM. * indicates significant difference from respective Small MI group. # indicates significant difference from female large MI group. + indicates significant difference from male sham group.
5.4. Discussion

The current results are the first to show that ovarian hormones in female rats protects the arterial baroreflex and CSAR control of renal SNA in the heart failure state, caused by MI. It was found that males and ovariectomized females, but not females with ovaries-intact, had attenuated arterial baroreflex control of renal SNA following a chronic, large, MI. Furthermore, compared to ovary-intact females with large MI, males and OVX females with large MI displayed augmented CSAR mediated increases in renal SNA and arterial pressure.

Large MI in the rat has been well characterized to cause significant impairment in cardiac function leading to the development of heart failure and subsequent neurohormonal changes that are similar to what occur clinically (Goldman et al. 1995, Hasenfuss 1998, Francis et al. 2001). It is known that renal SNA is elevated in the heart failure state and that this elevation contributes to morbidity and mortality (Nozawa et al. 2002, Petersson et al. 2005). Chronic elevations in renal SNA contribute to increased sodium and fluid retention and activation of the renin-angiotensin system that are hallmarks of heart failure (DiBona 2000, Nozawa et al. 2002, DiBona 2003). However, the mechanisms driving detrimental increases in renal SNA following MI and as heart failure develops and progresses are not well known. In heart failure, impairment of the arterial baroreflex may reduce the inhibitory input of this reflex on renal SNA outflow and leave SNA regulation more responsive to excitatory stimuli (DiBona et al. 1994, DiBona et al. 1995). The current study observed that in ovary-intact females with MI-induced heart failure the arterial baroreflex control of renal SNA was functioning normally. In contrast males and ovariectomized females with a large, chronic MI displayed an impaired ability of the arterial baroreflex to appropriately inhibit renal SNA and a reduced range of control over renal SNA, a finding consistent with previous evidence by other groups using similar experimental models (DiBona et al. 1994, Zhang et al. 1999).

The CSAR is an excitatory reflex that responds to changes in the chemical milieu within the heart tissue, driving increases in renal SNA. Current evidence in animals suggests the CSAR becomes sensitized in experimental heart failure which may contribute to increases in baseline SNA (Wang et al. 1996, Ma et al. 1997, Ma et al. 1999, Zhu et al. 2004, Gao et al. 2005). In the present study males and OVX females with large MI displayed greater CSAR mediated increases in renal SNA when compared to male and OVX females with small MI. In contrast, the size of MI did not alter the CSAR mediated changes in renal SNA and arterial pressure in ovary-intact females. The current results do show CSAR driven
increases in renal SNA and arterial pressure are significantly greater in male and OVX females with heart failure when compared to ovary-intact females with heart failure. Therefore the current findings suggest that ovarian hormones are capable of attenuating the CSAR activation of renal SNA in heart failure.

In the present study, alterations in the arterial baroreflex in males and ovariectomized females with heart failure were characterized by an inability to appropriately inhibit renal SNA and a reduced range of control over renal SNA when compared to sham animals. There was no effect of heart failure on the gain of the arterial baroreflex curve observed in the present study, a finding not consistent with previous experimental studies (DiBona et al. 1994, Zhang et al. 1999). In heart failure patients, it has been demonstrated in some studies, but not all, that the arterial baroreflex control of muscle SNA is unaltered as shown by no alteration in sensitivity (i.e. gain) of the arterial baroreflex (Floras 2001). Human studies are limited to investigating the operational area in the arterial baroreflex range and not the lower and upper limits of the arterial baroreflex control of SNA as was done in the present study.

Evidence in humans and sheep directly recording the arterial baroreflex control of muscle SNA or cardiac and renal SNA, respectively, have challenged the concept that increases in baseline SNA are associated with impairment of the arterial baroreflex (Floras 2001, Watson et al. 2007, Ramchandra et al. 2009). For example, patients with heart failure display significantly elevated baseline muscle SNA but still maintain appropriate arterial baroreflex responses to both natural and experimentally induced changes in arterial pressure (Floras 2001). In the current study, ovary-intact females with heart failure displayed a conserved ability of the arterial baroreflex to respond to acute changes in arterial pressure whilst displaying significantly elevated baseline renal SNA when compared to shams. In contrast, ovariectomized females with heart failure displayed an impaired arterial baroreflex control of renal SNA but did not display elevated baseline renal SNA compared to shams. Whilst in males there was a tendency for baseline renal SNA to be elevated whilst arterial baroreflex control of renal SNA was significantly impaired. Acute, whole nerve recordings of SNA, as performed in the current study, are influenced by inherent technical limitations. The low experimental numbers along with high variability of renal SNA signals within each group makes comparing the microvolt levels difficult. Previously, it has been demonstrated in conscious female sheep with pacing induced heart failure that the arterial baroreflex control of renal SNA is unaltered when compared to shams (Ramchandra et al. 2009), a finding not supported by evidence in male rats with MI-induced heart failure (DiBona et al. 1994, Zhang et al. 1999). Based on the current findings,
it is possible that discrepancies in the literature investigating the effects of heart failure on the arterial baroreflex control of SNA may be influenced by sex and sex steroids.

When compared to sham animals it appeared that in males and ovariectomized females, but not ovary-intact females, with large MI there was a tendency for the CSAR to be sensitized but this effect was not significant. The failure to observe a significant effect of MI-induced heart failure on the CSAR control of renal SNA in both males and females when comparing to sham animals was surprising (Wang et al. 1996, Ma et al. 1997, Zhu et al. 2002, Gao et al. 2004, Zhu et al. 2004). In the current study capsaicin was placed onto the anterior surface of the left ventricle to stimulate the CSAR; however in animals with MI, the access to the anterior surface of the left ventricle was significantly restricted when compared to animals with no MI. The greatest proportion of chemosensitive cardiac sympathetic afferent fibers originate from the anterior wall of the left ventricle (Ludbrook 1990), therefore the CSAR mediated changes in renal SNA are limited by the area of the heart surface that is accessible. This may help explain why in the current study there was a significant difference in the CSAR mediated renal SNA responses when comparing male and OVX females with large MI with male and OVX females with small MI, respectively. To elaborate, the CSAR would not be expected to be sensitized in animals with a small MI as these animals were not in heart failure while concurrently the access to the anterior wall of the left ventricle was restricted. Activation of the CSAR in heart failure has been implicated in attenuating arterial baroreflex control of renal SNA as silencing of the cardiac sympathetic afferents normalizes arterial baroreflex control of renal SNA in male rats with heart failure (Gao et al. 2005). Therefore it was expected that changes in the arterial baroreflex would reflect changes in the CSAR (Gao et al. 2005). Considering that the arterial baroreflex and CSAR share common central and efferent pathways it is likely that ovarian hormones are acting at least in part to affect both reflexes similarly (Gao et al. 2004, Gao et al. 2005).

Mortality rates post-MI were lower in ovary-intact females compared to males, a finding consistent with previous studies and unlikely to be a result of bias created by differing sizes of MI (Cavasin et al. 2004). Evidence in female mice suggests that the estrogen receptor-β (ERβ) may mediate at least some of the protective effect of ovarian hormones post-MI (Pelzer et al. 2005). Knock-out of ERβ causes a significant increase in mortality rates post-MI in female mice and an aggravation of heart failure in those animals that survived the initial MI, results that are similar when comparing the mortality rates between ovariectomized females with ovary-intact females in the current study (Pelzer et al. 2005).
Receptors for estrogen are located throughout peripheral and central pathways governing both the arterial baroreceptor reflex and CSAR (Papka et al. 1997, Shughrue et al. 1997, Papka et al. 2001, Brailoiu et al. 2007, Dun et al. 2009). It is understood that estrogen has an inhibitory action on sympathetic outflow that is mediated centrally, both in the resting state and in response to stressors (Saleh et al. 2000). Furthermore, estrogen appears to exert at least some of its actions on sympathetic regulation via nitric oxide and angiotensin II (Tanaka et al. 2001, Wang et al. 2008, Shih 2009, Xue et al. 2009, Ciriello et al. 2013). Both nitric oxide and angiotensin II are intricately linked in the reflex regulation of renal SNA and both have been shown to be altered in heart failure (DiBona et al. 1995, Zhang et al. 1999, Zhu et al. 2004, Zucker et al. 2004). For example, central antagonism of the angiotensin receptor 1 (AT1R) with losartan normalizes the arterial baroreflex control of renal SNA in male rats with MI-induced heart failure (DiBona et al. 1995, Zhang et al. 1999). Estrogen appears capable of attenuating the central actions of angiotensin II by limiting the production of reactive oxygen species and augmenting the sympathoinhibitory actions of nitric oxide (Wang et al. 2008, Xue et al. 2009). The available evidence suggests that estrogen may be capable of protecting the reflex regulation of renal SNA in heart failure by direct actions on autonomic pathways.

However, it may be that protection of cardiac function post-MI by estrogen limited the changes in the arterial baroreflex control of renal SNA, independent of direct actions of estrogen on sympathetic regulation. Furthermore, it may be that estrogen is acting to conserve endothelial function in females with heart failure thereby limiting the loss of arterial distensibility in areas where the arterial baroreceptors are located (Mendelsohn 2002, Murphy et al. 2007). There is conflicting experimental evidence regarding where in the arterial baroreflex pathway impairment occurs in heart failure as there is evidence suggesting that it is in the afferent arm of the arterial baroreflex (Dibner-Dunlap et al. 1989, DiBona et al. 1994), or that the impairment occurs centrally (DiBona et al. 1995, Zhang et al. 1999). In the current study, it was not investigated where in the arterial baroreflex pathway the alterations caused by heart failure occurred and where in the reflex pathway estrogen was acting to protect arterial baroreflex function in heart failure.

Based on previous evidence it is expected that the arterial baroreflex control of renal SNA is not significantly different between the anesthetized and conscious rat (Shimokawa et al. 1998), however it is not known how the current results will translate to the conscious animal. In the current study, the heart rate responses in all groups during both the arterial baroreflex and CSAR protocols were highly variable. Previous evidence in both humans and animals with heart failure have consistently shown an impairment of arterial baroreflex
control over HR and so this was expected to be observed in the present study (Brandle et al. 1996, Zhang et al. 1999, Floras 2001). However, we did not observe any significant effect of heart failure on the arterial baroreflex control of HR in males and ovariectomized females. Ovary-intact females with heart failure did display a significant decrease in the range of arterial baroreflex control over HR when compared to female sham group, a finding consistent with evidence in female sheep with heart failure (Watson et al. 2007). Because of the large variations in the heart rate data, conclusions regarding both arterial baroreflex and CSAR control of heart rate are not possible and further investigations are required.

The current study is the first to describe a protective effect of ovarian hormones in females on the reflex control of renal SNA in heart failure, caused by chronic MI. It is possible that a direct, protective action of estrogen on sympathetic regulation post-MI and as heart failure develops may help to at least partly explain the cardioprotective actions of estrogen. Furthermore, the findings suggest that studies investigating the regulation of the sympathetic nervous system in cardiovascular disease states, such as MI and heart failure, need to acknowledge the possible impact of sex and sex hormones. It remains to be investigated whether the protective actions of ovarian hormones on the arterial baroreflex control of renal SNA observed in the current study are a cause or consequence of the cardioprotective actions of ovarian hormones post-MI and subsequent heart failure.
Chapter 6: General Discussion

6.1. Introduction

Despite the importance of renal SNA in mediating the hemodynamic responses to MI, sex differences in the changes and regulation of renal SNA post-MI have not been previously studied. The work in this thesis has addressed this by investigating reflex control of renal SNA before and following MI in male and female rats. My results show that in females, ovarian hormones attenuate the initial excitation of renal SNA following acute MI. Sex differences in the arterial baroreceptor and cardiac afferent reflex control of renal SNA may explain the sex differences in the initial sympathetic response to MI. Specifically female sex was associated with attenuated arterial baroreceptor reflex mediated sympathoexcitation. Furthermore, it was shown that estradiol in females attenuated cardiac afferent reflex mediated sympathetic excitation, while augmenting the reflex mediated sympathoinhibition. Furthermore, I found that ovarian hormones in females protect the arterial baroreceptor and cardiac afferent reflex control of renal SNA in heart failure; a condition previously characterized, in males, by loss of normal reflex function. The findings within this thesis support my overall hypothesis that ovarian hormones acting via the SNS may contribute to estrogen related cardioprotection in both the acute and chronic phases’ post-MI.

6.2. Sex differences in sympathetic excitation post-myocardial infarction

Previous evidence in both humans and animals has shown that SNA is elevated following MI (Thames et al. 1979, Graham et al. 2002, Graham et al. 2004, Jardine et al. 2005, Schwenke et al. 2008, Hogarth et al. 2009). Of particular importance, an increase in SNA to the kidney following MI contributes to pathological changes in cardiac function by adversely altering both preload and afterload conditions (Nozawa et al. 2002, DiBona 2003, DiBona et al. 2004, Petersson et al. 2005). The findings in Chapter 3 of this thesis showed that sex and ovarian hormones in females can alter the initial changes in renal SNA post-MI. Specifically, males and ovariectomized females, but not ovary-intact females, displayed significantly elevated renal SNA in direct response to acute MI. Previously, it has been shown that the excitation of renal SNA in response to a 1-minute occlusion of the left
anterior descending coronary artery is largely driven by the arterial baroreceptor reflex, therefore the role of the arterial baroreceptor reflex was investigated (Thames et al. 1979, Weaver et al. 1981). Compared to males, female sex attenuated arterial baroreceptor reflex mediated excitation of renal SNA, consistent with previous evidence in conscious rats (Foley et al. 2005). In females, ovariectomy did not alter the arterial baroreceptor reflex control of renal SNA but did significantly augment the initial excitatory renal SNA response to MI. Arterial baroreceptor denervation abolished the initial excitation in renal SNA in response to acute MI in males but not in ovariectomized females. The findings in Chapter 3 partially support the 1st hypothesis made in Chapter 1 by showing that the arterial baroreceptor reflex was at least partly responsible for sex differences in the sympathetic response to MI. However, the observation that ovariectomy, in females, does not affect arterial baroreceptor reflex control of renal SNA suggests that ovarian hormones can alter sympathetic excitation post-MI via another pathway.

Following on from the work in Chapter 3, I investigated the effects of sex and ovarian hormones on the cardiac afferent reflex. Following MI, the cardiac afferent reflex responds to changes in the chemical milieu in the heart wall (Poole-Wilson 1978, Pinto et al. 1999, Tschope et al. 1999, Longhurst et al. 2001, Fu et al. 2002) driving either decreases, increases or no change in SNA (Webb et al. 1972, Weaver et al. 1981, Wei et al. 1983, Ustinova et al. 1996). The results in Chapter 4 of this thesis show that in females, estradiol augments the cardiac vagal afferent reflex mediated inhibition of renal SNA and attenuates the cardiac sympathetic afferent reflex (CSAR) mediated excitation of renal SNA, confirming the 2nd hypothesis made in Chapter 1. However, based on previous evidences I had expected to observe a greater cardiac vagal afferent mediated inhibition of SNA in ovary-intact females when compared to males, which was not observed, and, furthermore, the cardiac vagal afferent mediated inhibition in ovariectomized females would then become similar to males. In Chapter 4, the observation that the cardiac vagal afferent mediated sympathoinhibition was nearly abolished in females following ovariectomy was unexpected and deserves future investigation. The findings in this thesis present the first study to investigate the effects of sex and ovarian hormones on the cardiac afferent reflex. Therefore, exactly where and how estradiol is acting to affect the cardiac afferent reflex is not known. The brief literature review presented in Chapter 1 of this thesis suggests that estradiol has the potential to be acting throughout the cardiac afferent reflex pathway, with estradiol receptors located both in the peripheral components (Papka et al. 1997, Papka et al. 2001, Dun et al. 2009) and central components (Shughrue et al. 1997, Brailoiu et al. 2007) of the reflex.
In the results from Chapter 4, it was shown that vagotomy abolished sex differences in the CSAR mediated changes in renal SNA. Additionally, there were no effects of sex and ovarian hormones on changes in renal SNA caused by vagal afferent stimulation, suggesting that the central processing of the vagal afferents, in relation to sympathetic outflow, was not affected by sex. It has been shown that sex and ovarian hormones can affect the afferent processing of both chemosensory (Lu et al. 2009) and mechanosensory input (Deng et al. 1995, Storey et al. 2004). Furthermore, the expression of a sex-specific sub-population of estradiol sensitive, myelinated vagal fibres, alongside the expression of ER-like immunoreactivity in the nodose ganglion provide an anatomical substrate for actions of ovarian hormones on vagal afferents (Papka et al. 1997, Papka et al. 2001, Li et al. 2008, Qiao et al. 2009). Furthermore, a previous study recording heart rate and arterial pressure in male and female rats found that the sex difference in the hemodynamic response to 30 minutes of MI was abolished by vagotomy (Du et al. 1995). Vagal afferents are capable of affecting the sympathetic processing of chemosensory and mechanosensory stimuli (Randich et al. 1992, Tjen et al. 1997, Zagon et al. 1999, Verberne et al. 2003). The results from Chapter 4 alongside previous findings do suggest that female sex alone or female sex and ovarian hormones are capable of affecting the vagal afferent component of the cardiac afferent reflex mediated changes in renal SNA (Deng et al. 1995, Papka et al. 1997, Storey et al. 2004, Li et al. 2008).

In males, the importance of the arterial baroreceptor reflex in mediating the initial changes in renal SNA post-MI suggests this to be a compensatory response helping to maintain arterial pressure due to loss of ventricular function (Gorfinkel et al. 1972, Thames et al. 1979). In contrast to the well known homeostatic actions of the arterial baroreflex, the physiological role of the cardiac afferent reflex is not so well understood. In the ovariectomized females the cardiac afferent reflex would appear to play a dominant role in mediating the initial increases in renal SNA post-MI, this therefore raises the question as to the physiological nature of this response. In anesthetized dog the CSAR mediated excitation of renal SNA does cause an acute decrease in fluid and sodium excretion (Meckler et al. 1981). Arterial baroreceptor and chemoreceptor afferent inputs can differentially alter sympathetic neuronal outflow, both in terms of recruitment of individual fibres and rate of neuronal firing (Malpas et al. 1992, Malpas et al. 1992). Sex differences in the contributing mechanisms to changes in sympathetic outflow to the kidney following MI raises the possibility that there may also be sex differences in the SNA mediated changes in kidney function post-MI (DiBona 2000, Nozawa et al. 2002, Malpas 2010).
The evidence in this thesis has focussed on effects of sex and ovarian hormones on the regulation of renal SNA but it is possible that sex and ovarian hormones may differentially alter the regulation of SNA to various organs. Selective blockade of β₁-adrenoceptors with metoprolol increases the time to arrhythmia and fibrillation following MI in conscious male and ovariectomized, but not ovary-intact, female rats suggesting the possibility that changes in cardiac SNA post-MI may be influenced by sex and ovarian hormones (Lujan et al. 2008). The CSAR has been shown to have a more profound effect on cardiac SNA than renal SNA, thus suggesting the sex differences in the cardiac afferent reflex observed in this thesis could drive sex differences in the changes in cardiac SNA in response to MI (Malik et al. 1997). Ovarian hormones appear capable of altering noradrenaline release (Bengtsson et al. 1983, Karkanias et al. 1993) and adrenergic receptor sensitivity (Gisclard et al. 1987, Riemer et al. 1988, Sudhir et al. 1997, Moura et al. 2001) therefore there is vast complexity in the actions of ovarian hormones. Therefore, whether the same changes in SNA cause the same functional changes in males and females remains unknown.

6.3. Changes in the arterial baroreceptor reflex control of renal SNA following myocardial infarction and in subsequent development of heart failure

In Chapter 5 I presented the findings that male and ovariectomized female rats following a large MI had significantly impaired arterial baroreceptor reflex control of renal SNA, largely characterized by an attenuated ability of the arterial baroreflex to inhibit renal SNA. The findings in heart failure male rats were similar, but not the same, as previous evidences in very similar experimental studies (DiBona et al. 1994, Zhang et al. 1999). A distinct difference between previous evidence in similar animal models and the findings in Chapter 5 of this thesis was that the gain of the arterial baroreflex control over renal SNA was unchanged in animals with heart failure in the results in this thesis (DiBona et al. 1994, Zhang et al. 1999). It has been strongly argued that in humans with heart failure the gain of the arterial baroreflex control of muscle SNA is unaltered (Floras 2001). In humans, the full range of arterial baroreflex control of SNA cannot be investigated and therefore the way of quantifying arterial baroreflex gain is different compared to animal studies (Floras 2001).

The current method used in animal studies to quantify arterial baroreflex control of renal SNA is based on the observation that the arterial pressure-SNA relationship is sigmoidal (Dorward et al. 1985). In the majority of experimental studies the quantifiable variables of arterial baroreflex function such as gain and range are reliant on a non-linear regression curve being fitted to the data which is then subsequently normalized, generally as a percentage change from either baseline or the upper/lower plateau. Effectively, the height (range of control over SNA/HR), width (operating range of arterial pressures) and upper and lower curvatures of the arterial baroreflex curve will determine the gain of the curve, which represents the sensitivity of the baroreceptor reflex. It was observed in Chapter 5 of this thesis that both the height and width of the normalized arterial baroreflex curves were reduced in males and ovariectomized females with heart failure compared to male shams, whereas the normalized slope (representing the gain) of the arterial baroreflex curve was unaltered. Figure 6.1 gives an example of arterial baroreflex curves derived from an individual sham or heart failure male rat and the range of arterial pressures that the arterial baroreflex was operational. A strength of the current studies was the ability to alter arterial pressure over a large range, enabling an accurate determination of the lower and upper plateaus of the arterial baroreflex curve. As shown in Figure 6.1, the operational range of arterial pressure of the arterial baroreflex was quite large in sham animals. Therefore, it is possible that in previous investigations where the ranges of arterial pressure investigated were smaller, the estimation of the arterial pressure/SNA relationship may be limited.
6.4. Ovarian hormones protect arterial baroreceptor reflex control of renal SNA in heart failure

A major, novel finding of the work in this thesis is that ovarian hormones in female rats appear capable of protecting normal arterial baroreflex control of renal SNA in MI-induced heart failure, supporting the 3rd hypothesis made in Chapter 1. Previously, it has been shown in male rats with MI-induced heart failure that the attenuated ability of the arterial baroreflex to inhibit renal SNA and the decreased gain of arterial baroreflex control of renal SNA is related to central actions of angiotensin II (ANG II), with intracerebroventricular infusion of the AT1R normalizing arterial baroreflex function (DiBona et al. 1995, Zhang et al. 1999). Therefore it is possible that ovarian hormones may somehow attenuate the adverse actions of central ANG II signalling on arterial baroreflex function. Estradiol is capable of attenuating ANG II mediated neuronal activation in circumventricular organs (Ciriello 1983, Tanaka et al. 2001, Pamidimukkala et al. 2003, Xue et al. 2009). Female rats with intact ovaries display reduced central-mediated pressor responses to low-dose ANG II infusion compared to both males and ovariectomized females; a response mediated at least in part by relatively higher levels of central NO, decreased production of reactive oxygen species (ROS) and a shift in the axis towards the depressor arm of the renin-angiotensin system (RAS) (e.g. increased production of angiotensin 1-7) (Xue et al. 2009, Sampson et al. 2012, Bhatia et al. 2013). It would appear that whatever modulatory factor
is responsible for changes in the arterial baroreflex function in chronic MI-induced heart failure is of a chronic nature. This idea is supported by the findings in Chapter 3 of this thesis which did not find any alteration in arterial baroreflex control of renal SNA in males and females at 1 hour post-MI.

One hypothesis explaining the pathological elevation in SNA in heart failure is that an increased release of renin from the juxtaglomerular cells of the kidney increases ANG II mediated sympathoexcitation (Zhang et al. 1999, Zucker et al. 2007, Guild et al. 2012). The potential is for a feed-forward cycle of increased SNA to the kidney increasing renin release which in turn would further enhance ANG II mediated sympathoexcitation and impairment of the arterial baroreflex (Barajas et al. 1973, Kopp et al. 1980, DiBona 2000, Zucker et al. 2007). The findings in Chapter 3 of this thesis raise the possibility that in ovary-intact females, compared to males, attenuated excitation of renal SNA following MI may limit renal sympathetic nerve mediated release of renin thereby limiting levels of circulating ANG II and subsequent changes in arterial baroreceptor reflex function (Barajas et al. 1973, Kopp et al. 1980, DiBona et al. 1995, DiBona 2000, Zucker et al. 2007). Evidence to support this comes from a study performed in ovary-intact female rats found no significant increase in plasma renin and plasma arginine vasopressin at 4 weeks after MI and also no observable change to sodium handling during this time (Hodsman et al. 1988). In comparison a similar study in male rats found significant increases in both plasma renin and plasma arginine vasopressin 4 weeks after MI with male rats displaying reduced sodium and urine excretion (Francis et al. 2001). Neither study recognized the influence of sex on the results.

Ovarian hormones, particularly estradiol, have been shown to be cardioprotective in a number of ways, therefore preserved cardiac function following MI may have limited changes in the arterial baroreflex regulation of SNA, independent of direct actions of ovarian hormones on the regulation of the SNS (Mendelsohn 2002, Cavasin et al. 2003, Konhilas et al. 2007, Vaccarino et al. 2011, Zimmerman et al. 2013). Furthermore, the actions of ovarian hormones on other circulating neuromodulatory hormones such as vasopressin (Hasser et al. 1997, Morrissey et al. 1997, Nylen et al. 2001, Mecawi et al. 2011), aldosterone (Oelkers 1996, Yu et al. 2008), or the natriuretic peptides (Kuroski de Bold 1999, Lam et al. 2011, Wong et al. 2013) may have a role to play in the effects of ovarian hormones on reflex regulation of renal SNA in heart failure.
6.5. The origin of action for ovarian hormones on the sympathetic nervous system

The question remains as to how do ovarian hormones affect changes in the regulation of SNA in MI and heart failure? The most obvious point of difference between men and women in terms of ‘programmed’ cardiovascular events is pregnancy. The female body undergoes significant cardiovascular changes during pregnancy that are initiated by changes in ovarian hormones (Brunton et al. 2010, Brunton et al. 2011). The receptors and subsequent pathways that mediate these events therefore must be in place in order for the actions of ovarian hormones to be effective. The incidence of cardiovascular disease in women has increased in recent times due to the increasing age of populations and changes in lifestyles (Mair et al. 1996, Roger et al. 2004, Jayawardena et al. 2013, Kelly et al. 2013, Vadiveloo et al. 2013), therefore contributing to the relatively recent emergence and importance of the ‘cardiovascular protective’ actions of ovarian hormones (Stevens et al. 2013). Most probably, there exists a very complex interaction between the various sex hormones (including testosterone), ‘classical’ neuromodulators and the SNS that are beyond the current level of understanding. However, based on the evidences in this thesis estrogen will be put forward as the ovarian hormone responsible for at least some of the effects of ovarian hormones on the SNS. Therefore, I propose two theories that may help to explain the results in this thesis.

One possibility is that estrogen is acting directly on important sympathetic regulatory neurons in response to an acute loss of left ventricular function. In pregnancy maternal blood volume is significantly increased whilst blood pressure is lowered, driven by relative changes in ovarian hormones and thus ovarian hormones clearly do play a homeostatic role in cardiovascular regulation (Fisch et al. 1972, Wilson et al. 1980, Longo 1983). In support of this concept is the observation that estrogen receptors are located throughout the important peripheral and central regions regulating the SNS (Papka et al. 1997, Shughrue et al. 1997, Papka et al. 2001, Brailoiu et al. 2007, Dun et al. 2009). It is possible that sex differences in anatomical and physiological substrates exist that result in females responding differently than males to particular cardiovascular stressors caused by MI and heart failure, such as a decrease in cardiac output, increase in cardiac stretch or increase in circulating blood volume. It may be that these processes act via direct actions of estrogen on neurons regulating SNA or that estrogen regulates neuromodulatory hormones, for example ANG II or vasopresin, that in turn create sex differences in changes in sympathetic regulation.
Another possibility is that estrogen acting in certain regions such as within the hypothalamic-pituitary-gonadal system or the endothelium can then have a spillover effect onto important sympathoregulatory regions. For example, NO is capable of diffusing across tissue and is a neuromodulator in both the ovarian cycle and sympathetic regulation (Tsukamura et al. 1994, Watson et al. 1995, Nagatani et al. 1996, Kallo et al. 2001, Pyner 2009, Schultz 2009). It could be that estrogen mediated increases in NO acting on neurons involved in the hypothalamic-gonadal pathway, such as the PVN, can then diffuse across to adjacent sympathoregulatory neurons (see Figure 6.2) (Tsukamura et al. 1994, Watson et al. 1995, Nagatani et al. 1996, Geary et al. 1998, Kallo et al. 2001, Scordalakes et al. 2002, Xia et al. 2004, Pyner 2009). Furthermore, It is possible that estrogen mediated production of NO in the endothelium of the cerebral blood vessels can act on adjacent brain regions to alter sympathetic regulation (Figure 6.2) (Geary et al. 1998, Paton et al. 2001, McNeil et al. 2002, Momoi et al. 2003, Biancardi et al. 2011). Based on the available evidence, it is likely that greater NO actions in sympathoregulatory regions in disease states such as heart failure would lead to attenuated sympathoexcitation and protection of arterial baroreceptor and CSAR function in heart failure (Lancaster 1997, Zhang et al. 1997, Zhang et al. 2001, Wang et al. 2003, Pyner 2009, Schultz 2009, Tsuda 2013). Furthermore, greater production of NO in the endothelium would likely protect from endothelial dysfunction, a possible contributor to arterial baroreceptor reflex dysfunction in heart failure (Kirchheim 1976, Bauersachs et al. 2004, Yung et al. 2011).

Another inadvertent action of female sex on sympathetic regulation may be via acting on the pain pathways. During pregnancy the pain threshold rises which is no doubt useful when considering the corresponding changes that occur during pregnancy and labour (Gintzler 1980, Cogan et al. 1986). It may be that a residual effect of the female bodies preparedness for pregnancy comes in the form of ovarian hormones altering sympathetic afferent neuronal pathways that show similar characteristics and anatomical locations to pain pathways (Nishi et al. 1977, Malliani et al. 1981, Malliani et al. 1983, Zhang et al. 2012). This may help to explain the findings in Chapter 4 where sex and ovarian hormones significantly affected the cardiac afferent reflex, which is an autonomic reflex known to be associated with the sensation of cardiac pain (Nishi et al. 1977, Malliani et al. 1981, Malliani et al. 1983).
Figure 6.2: Examples of possible inadvertent actions of estrogen on the SNS
Top graph: Estrogen mediated nitric oxide (NO) release from a neuron that is not involved in sympathetic nerve activity regulation may then diffuse across to an adjacent neuron that is involved in sympathetic regulation. Bottom graph: Estrogen mediated release of NO from endothelial cells in the cerebral vasculature may then diffuse into adjacent sympathoregulatory regions. Abbreviations: BBB – blood brain barrier.

The ideas discussed in this section are examples of how ovarian hormones may be acting to mediate sympathetic regulation following MI and in heart failure. The precise type, locations and mechanisms of action of ovarian hormones that are acting to affect changes in the regulation of SNA in heart failure remain to be elucidated. Therefore there is much scope for future work investigating the actions of sex hormones on sympathetic regulation in both normal and cardiovascular disease states.

6.6. Limitations

Major limitations in the work performed in this thesis include, 1) experiments were in anesthetized animals, 2) use of exogenous stimulant (i.e. capsaicin) for the cardiac afferent
reflex in Chapters 4 and 5, 3) failure to measure hormone levels in female animals, and 4) the difficulty in characterizing heart failure in Chapter 5.

In Chapter 2 I described some evidence to suggest that reflex control of renal SNA in the rat is not significantly altered by alpha-chloralose and urethane anesthetic (Shimokawa et al. 1998). However, baseline renal SNA is significantly increased thereby causing the resting level of renal SNA to sit closer to the upper plateau, which is consistent with the findings in this thesis (Shimokawa et al. 1998). In conscious animals the baseline renal SNA is located in the middle or closer to the lower plateau of the arterial baroreflex curve (Guild et al. 2012). How anesthesia affects the cardiac afferent reflex is not known, but at the very least an increased baseline SNA is likely to affect the normalized changes in SNA following cardiac afferent reflex activation. It is unknown if the anesthetics used in the current work affect the regulation of SNA in males and females differently, but this is a possibility.

Heart rate responses in all studies in this thesis were highly variable and as such have not been discussed in depth. However, there are some major discrepancies between my work and previous work in regards to arterial baroreflex control of heart rate in normal and heart failure settings. The failure to observe significant effects of sex and ovarian hormones on the arterial baroreflex control of heart rate was surprising given previous evidence (Saleh et al. 2000, Saleh et al. 2000, Goldman et al. 2009). Furthermore, the failure to observe significant impairment in arterial baroreflex control of heart rate in heart failure is not consistent with previous findings in both humans and animals of different species and heart failure models (DiBona et al. 1994, Brandle et al. 1996, Zhang et al. 1999, Floras 2001, Ramchandra et al. 2009, Kar et al. 2011). Previous studies have consistently shown that both the range and gain of arterial baroreflex control of heart rate is significantly attenuated in heart failure (DiBona et al. 1995, Brandle et al. 1996, Zhang et al. 1999, Floras 2001, Ramchandra et al. 2009, Kar et al. 2011). Altered arterial baroreflex control of heart rate is a prognostic indicator of morbidity and mortality in heart failure patients (La Rovere et al. 1998). In ovary-intact females, heart failure did cause a significant reduction in the range of arterial baroreflex control of heart rate, and along with an intact arterial baroreflex control of renal SNA the findings are consistent with observations in female sheep with heart failure (Ramchandra et al. 2009). However, large variations in arterial baroreflex control of heart rate in the current studies highlight this as a major limitation in my work, and suggests that there is benefit in performing future investigations in conscious animals (Shimokawa et al. 1998).
It is possible to obtain arterial baroreflex data in conscious rats (DiBona et al. 1995, Zhang et al. 1999, Goldman et al. 2009). The success rate in obtaining viable recordings of renal SNA in conscious rats is much lower compared with anesthetized rats. In regards to the work in Chapter 5, it made sense to obtain evidence that ovarian hormones can affect arterial baroreflex function in heart failure before progressing to more expensive and time consuming protocols, especially given the high death rates post-MI. It would be ideal to investigate the effects of sex hormones on the regulation of SNA following MI and heart failure in freely moving conscious animals that are well recovered from the required surgeries (Barrett et al. 2001, Stocker et al. 2013).

The use of the anesthetized preparation was required in order to have an open chest preparation, enabling the investigation of changes in SNA in direct response to MI and the cardiac afferent reflex. In combination with the anesthetic, an open chest meant that maintaining a stable and appropriate blood pressure was difficult, especially in heart failure animals, and therefore lower blood pressures when compared to conscious animals may have influenced the results. Capsaicin was used to stimulate the chemoreceptors in the heart. Although capsaicin is widely used in the literature it is not released endogenously and therefore the use of an endogenous stimulant would have been preferable. As briefly described in Chapter 2, bradykinin which is an endogenous substance was applied to the heart in some experiments but failed to elicit any cardiac afferent reflex mediated changes in renal SNA, despite a large body of evidence suggesting bradykinin consistently activates this reflex (Wang et al. 2000, Longhurst et al. 2001). In the studies in Chapters 4 and 5, capsaicin did produce very consistent and reliable cardiac afferent reflex responses in males, strengthening the comparisons with females.

Another possible limitation is the doses of capsaicin that were used in Chapters 4 and 5. The relatively high dose of capsaicin given and therefore subsequent large hemodynamic changes may have caused secondary activation of mechanoreceptors, however this is unlikely (Nishi et al. 1977, Kaufman et al. 1980). Furthermore, in Chapter 5 the highest dose of capsaicin was chosen as 5µg, a dose that when used in the study in Chapter 4 hadn’t resulted in sex differences in the cardiac afferent reflex responses. While performing the experiments presented in Chapter 4 it had been observed that 10µg capsaicin in some animals, mostly ovariectomized females, resulted in a destabilization of the hemodynamic variables most likely due to activation of SNA that sometimes resulted in death. It was thought wise not to apply too high a dose in heart failure animals because it was hypothesized the CSAR would be sensitized therefore leading to greater sympathetic activation and perhaps increased likelihood of death (Du et al. 1999, Longhurst et al. 2001).
In the experiments in this thesis the levels of ovarian hormones in the females were not measured. The techniques used to manipulate the levels of ovarian hormones have been well characterized and the hormone replacement protocols were followed according to correspondence regarding a previously validated protocol (Kalu 1991, Chakraborty et al. 2004, Brown et al. 2008, Strom et al. 2008, Strom et al. 2010). It could be argued that the significant differences obtained between ovary-intact and ovariectomized rats in this thesis indicate that the ovariectomy and hormone replacement procedures were successful. Furthermore, the differences in body weight gain between ovariectomized females with or without estradiol replacement suggests that the estradiol replacement was successful, as estradiol attenuates weight gain in female rodents (Blaustein et al. 1976, Landau et al. 1976, Roesch 2006).

In Chapter 5 coronary ligation-induced MI was used to induce heart failure, which is a well characterized experimental model with functional and neurohormonal changes being similar to heart failure patients (Goldman et al. 1995, Hasenfuss 1998, Francis et al. 2001). However, the clinical classifications of heart failure tend to be symptom based; therefore the precise quantification of heart failure is difficult in an animal model. The significant differences between males and OVX females with large MI and small or sham MI do suggest that the MI were of large enough size to produce significant effects on cardiovascular regulation. Further characterizing aspects of heart failure such as measuring ventricular pressures would have provided more evidence confirming the animals were indeed in heart failure.

6.7 Significance

Historically, women compared to men have received less aggressive treatments which may explain their higher death rate in-hospital (Dellborg et al. 1993, Vaccarino et al. 1999, de Gevigney et al. 2001). Following MI, hemodynamic variables are used as a means to determine the course of treatment. For example, beta-blockers may be contraindicated in a patient with hypotension and bradycardia. Based on the findings in Chapter 3 it is possible that females display different autonomic responses to MI. Furthermore, in Chapter 4 the variable cardiac afferent reflex mediated SNA changes in females may suggest a mechanism explaining why women, compared to men, display more variable symptomatic and hemodynamic responses to MI (Airaksinen et al. 1998, Meischke et al. 1998, Meischke et al. 1999, Berg et al. 2009, Collins 2011, Coventry et al. 2011). Therefore, it is possible that sex specific autonomic responses to MI may affect the diagnosis and subsequent treatment of the patient. The reality is that the vast majority of work investigating the sympathetic response to MI has been performed in males, providing an unfavourable bias for females in regards to treatment (Huxley 2007).

In heart failure the loss of appropriate regulation of SNA has been attributed to driving adverse changes in morbidity and mortality (Hasking et al. 1986, Rundqvist et al. 1997, Petersson et al. 2005). The work in Chapter 5 is the first to demonstrate that ovarian hormones in females act to maintain normal reflex regulation of renal SNA in heart failure. It is unknown how these sex differences in regulation of renal SNA may relate to kidney function, and therefore cardiovascular homeostasis, in both health and disease. It is currently unknown where in the SNS the ovarian hormones are acting to produce these favourable effects on sympathetic regulation. Future work investigating how ovarian hormones are capable of protecting sympathetic regulation may lead to new treatment strategies for heart failure. Based on the current findings it is possible that current treatment strategies that target the SNS such as beta-blockers, or possible future treatment strategies such as renal denervation may require sex specific tailoring.
Chapter 7: List of References


Ciriello, J. and S. Roder (2013). 17beta-Estradiol alters the response of subfornical organ neurons that project to supraoptic nucleus to plasma angiotensin II and hypernatremia. Brain Res 1526: 54-64.


