CHARACTERISTICS, CAUSES AND FUNCTIONAL CONSEQUENCES OF BRAIN INFLAMMATION IN DIABETES AND HYPERTENSION

A thesis submitted in fulfilment of the requirements for the degree of doctor of philosophy

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Thesis summary

Diabetes and its complications, such as hypertension and diabetic cardiomyopathy, increase the risk of morbidity and mortality in human populations. Despite decades of research, the full aetiology of these complications still remains unclear. Strong evidence suggests there is dysfunction in the autonomic neurons system, including abnormal activity in the sympathetic nervous system and baroreflex impairment, which may contribute to the development of cardiovascular complications in diabetes; however, the mechanisms behind these abnormalities in diabetes are not well understood. Autonomic nuclei within the central nervous system (CNS) that are involved in the control of sympathetic nerve activity, the sensitivity of the baroreflex and the cardiovascular system are the paraventricular nucleus of the hypothalamus (PVN), the rostral ventrolateral medulla (RVLM) and the nucleus tractus solitarius (NTS). Inflammation and oxidative stress in these centres have been identified to contribute to the hyperactivity of the sympathetic nerves and baroreflex dysfunction in other diseases, but whether this true in diabetes is not clear. Microglia and astrocytes are the resident immune inflammatory cells within the CNS. Once they become activated in response to various stimuli, they can release pro-inflammatory molecules and reactive oxygen species; however, the role of these cells in the development of diabetic cardiovascular complications remains unclear. Drinking 1% NaCl has been shown to have biphasic effects on the development of diabetic complications in animals, but whether these effects are mediated via influencing brain inflammation has not been investigated. In addition, little is known about the influence of antioxidants and high fat feeding on microglial activation in central autonomic centres in diabetic animals. Thus, the aim of this thesis was to identify whether inflammation occurs in central autonomic centres in different species and models of diabetes and to explore the consequences of neuroinflammation in these animals.

In **chapter 3**, the effect of the inhibition of microglia in the PVN on blood pressure and heart rate in long-term (8 weeks) STZ-diabetic rats was investigated. Microglia and astrocytes were activated in the PVN in STZ diabetic rats. Pro-inflammatory cytokines released from both activated microglia and astrocytes have been implicated in spinal neuronal hyperexcitability. This suggested that the activation of microglia and astrocytes may be important for mediating inflammation in the PVN in diabetic rats, which may cause neuronal hyperactivity and then lead to increased sympathetic activity. Minocycline treatment inhibited PVN microglial activation but not the activation of astrocytes, suggesting that microglial activation was not responsible for astrocyte activation; however, in this study, the consequence of the inhibition of microglial activation could not be tested for several reasons, including morbidity and the lack of hypertension in STZ diabetic animals. Therefore, an alternative approach to this study was justified.

In most studies on long-term (6-8 weeks) STZ diabetic rats, unchanged or lowered blood pressure has been observed, which is consistent with the data presented in **chapter 3**. Drinking 1% NaCl, which may prevent dehydration, causes hypertension in diabetic rats within 2 weeks after STZ treatment, but how this occurs and whether brain inflammation contributes to this hypertension has not been investigated. Therefore, in **chapter 4,** we investigated the effects of 1% NaCl intake on blood pressure, baroreflex sensitivity and inflammation in the PVN, NTS and RVLM in 2-week STZ diabetic rats. In addition, we investigated whether the inhibition of microglia in the PVN can prevent hypertension in STZ diabetic rats given 1% NaCl. Diabetic rats given saline exhibited hypertension, dysfunction of the bradycardic baroreflex and signs of normalised blood volume in comparison with the control rats and the diabetic rats that drank water. Diabetic rats that drank 1% NaCl also showed increased microglial activation in the PVN, NTS and RVLM. The inhibition of activated microglia in the PVN via administering ICV minocycline prevented the

hypertension seen in diabetic rats given 1% NaCl, strongly suggesting that microglial activation plays an important role in the generation of hypertension in these animals. Despite this, the possibility that increased blood volume and/or baroreflex dysfunction are mechanisms by which 1% NaCl intake induces hypertension in STZ diabetic rats cannot be ruled out.

While it is clear from these studies that 1% NaCl intake can reduce baroreflex sensitivity in 2-week STZ diabetic rats, another study reported that the prolonged drinking of 1% NaCl causes improvement in *ex vivo* cardiac function in 6-week STZ diabetic rats. Whether treatment with 1% NaCl for longer periods produces similar beneficial effects on the baroreflex sensitivity is not known. Therefore, **in chapter 5,** we aimed to investigate the effects of 1% NaCl on the baroreflex sensitivity and to determine whether 1% NaCl intake influences inflammation cardiovascular centres in longer term (6 weeks) STZ diabetic rats. The diabetic rats showed dysfunction in the barorflex sensitivity. This dysfunction was associated with increased microglial activation in the NTS and PVN. Drinking 1% NaCl for 6 weeks restored the function of bradycardic baroreflex and also reduced the activation of microglia and neurons in the NTS in these animals. The data suggest that microglial activation in the NTS may be responsible for the baroreflex dysfunction seen in 6-week STZ diabetic animals. The data also suggest that drinking 1% NaCl can prevent cardiovascular complications through a reduction in microglial activation in longer term STZ diabetic rats.

In addition to hypertension and the baroreflex dysfunction, cardiomyopathy is a common form of diabetic complication and can occur independently of hypertension in diabetes. There is evidence that inflammation in the PVN is a potential contributing factor to the development of cardiomyopathy in other diseases. In **chapters 3 and 5,** evidence is provided that microglial cells are activated in the PVN in 6-8 STZ diabetic rats, but the role of these cells in diabetic cardiomyopathy has not been investigated. Therefore, in **chapter 6,** we aimed to

investigate the structural and functional parameters of the left ventricle in diabetic rats at 6 weeks following STZ injection and to determine whether the inhibition of microglial activation in the PVN could reverse any of the changes observed. Six-week STZ diabetic rats showed clear left ventricular dysfunction, including elevated end diastolic pressure, an increased internal diameter in the systole and diastole and a decreased E/A ratio when compared with the control rats. These animals also displayed marked activation of microglia and neurons in the PVN. Inhibition of microglial activation via administering ICV minocycline reduced the PVN neuronal activity and significantly normalised the left ventricular function. This study suggests that microglial activation in the PVN leads to PVN neuronal excitation in STZ diabetic rats. The data also confirm our proposal that microglial activation in the PVN plays a critical role in the pathogenesis of diabetic complications.

Drinking 1% NaCl for 6 weeks in STZ diabetic rats reduces cardiovascular complications, but the exact mechanism is still unclear. The data from **chapter 6** indicated that microglial activation in the PVN contributes to cardiac dysfunction in STZ diabetic rats; however, whether the beneficial effects of the prolonged drinking of 1% NaCl on the cardiac function are mediated by reducing the inflammatory response is not known. Therefore, in **chapter 7,** we investigated the effects of 1% NaCl on the cardiac function *in vivo* and whether 1% NaCl intake influences microglial activation in 6-week STZ diabetic rats. Drinking 1% NaCl restored elevated end diastolic pressure but not the other parameters of the left ventricle in these animals. The 1% NaCl intake also reduced the activation of microglia and neurons in the NTS and PVN when compared to the STZ diabetic rats given tap water. When the drug minocycline was accompanied by prolonged 1% NaCl intake, the STZ diabetic rats showed a further improvement in cardiac performance and a reduction in microglial and neuronal activation in PVN compared with STZ diabetic rats given saline alone. These results

indicated that changes in cardiac function are paralleled by the level of microglial activation in the PVN observed in diabetic animals.

Because the mouse is the most suitable animal model for genetic manipulations, it was important to examine whether microglia and neurons are also activated in the PVN in STZ diabetic mice as well as the time period of any activation. Hydrogen sulphide (H_2S) has been shown to inhibit microglial activation *in vitro* experiments, but the effect of the systemic infusion of H_2S on PVN function in STZ mice has not been investigated. Therefore, **in chapter 8**, we examined the dose and time dependence of microglial and neuronal activation and the effects of H_2S in STZ treated mice at 7 weeks. The microglia were activated in the PVN in STZ diabetic mice at 16 weeks after STZ injection but not at the 7-week time period compared to the control. In diabetic mice treated with a low dose of STZ, the microglia in the PVN were not activated at the 10-week time period; however, the PVN neuron activation was observed in STZ diabetic mice at all time periods as well as in diabetic mice treated with a low dose of STZ, suggesting neuronal activation precedes microglial activity. H_2S treatment attenuated the increase in neuronal activation in the PVN and prevented dehydration at 7 weeks following STZ injections. These findings suggest that H_2S treatment may play an important role in regulating kidney and PVN neuron function in diabetes.

A high fat diet is a major contributing factor in the pathogenesis of type 2 diabetes, which is the most common form of diabetes in humans. Whether PVN inflammation occurs in models of type II diabetes requires investigation. Thus, in **chapter 8,** we also examined the PVN inflammation in mice treated with a low dose of STZ and high fat feeding. No significant difference was observed in the percentage of microglia activated in the PVN in mice treated with STZ and high fat feeding when compared to the control; however, the neuronal activation was significantly increased in the PVN in these mice. The addition of a high fat diet to the STZ treatment attenuated the increase in neuronal activation in the PVN. These

findings suggest that the activation of microglia in the PVN does not occur in all animal models of diabetes.

In conclusion, microglial activation occurs in the PVN in long-term STZ-induced diabetic rats and mice but not in a mouse model of type II diabetes. Although the cause is not fully known, microglial activation in the PVN plays an important role in the pathogenesis of diabetic complications. While 1% NaCl prevents dehydration in short and long-term STZ diabetic rats, it has a biphasic effect on diabetic complications. This effect was mediated, at least in part, by changes in neuroinflammation. The beneficial effects of hydrogen sulphide on PVN function may be mediated via reducing neuroinflammation and/or oxidative stress in diabetes. Thus, new therapeutic approaches aimed to target neuroinflammation may be of clinical importance in preventing diabetic cardiovascular complications in humans.

Declaration

I certify that, except where due acknowledgement has been made, the work is that of the author alone; the work has not been submitted previously, in whole or in part, to qualify for any other academic award; the content of the thesis is the result of work that has been carried out since the official commencement date of the approved research program; any editorial work, paid or unpaid, carried out by a third party is acknowledged; and ethics procedures and guidelines have been followed.

Emad Alahmadi

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TO My father, Khalid Alahmadi

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Abbreviations

Ang II	Angiotensin II
Arc	Arcuate nucleus
ATP	Adenosine-5'-triphosphate
BDNF	Brain derived neurotrophic factor
BBB	Blood–brain barrier
CNS	Central nervous system
GABA	γ-aminobutyric acid
GFAP	Filament / Fibrillary Acidic Protein
H_2O_2	Hydrogen peroxide
H_2S	Hydrogen sulfide
I.C.V.	Intracerebroventricular
IL-6	Interleukin-6
IL-10	Interleukin-10
IL-1β	Interleukin-1 beta
IR	Immunoreactive

LPS	Lipopolysaccharides
NF-kB	Nuclear factor-kappaB
nNOS	Neuronal nitric oxide synthase
NO	Nitric oxide
NTS	Nucleus tractus solitarius
PBS	Phosphate buffered saline
PVN	Paraventricular hypothalamic nucleus
ROS	Reactive oxygen species
RVLM	Rostoventral medulla
SD	Sprague Dawley
SON	Supraoptic nucleus
STZ	Streptozotocin
TNF- α	Tumor necrotic factor-alpha

Publications and Communications

Journal publications

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Abstracts presented at meetings

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E. Alahmadi, G. Yildiz, J. Hart, and M. Stebbing (2014) Relationship between microglial activation, neuronal activation and dehydration in diabetes – role of antioxidant. Proceedings of Higher Degree Research Student Conference at RMIT.

E.Alahmadi, E. Badoer, OL. Woodman, MJ. Stebbing (2015) Drinking 1% saline causes hypertension in STZ-treated rats via increased microglial activation in CNS cardiovascular control centres. Proceedings of High Blood Pressure Research Council of Australia.

Chapter One: Literature Review

1.1 Overview

Diabetes mellitus is a chronic disease that affects around 382 million people worldwide both in developing and developed countries, according to the International Diabetes Federation (2013). Diabetes has been described as a lethal disease because it is estimated that 2.9 million deaths around the world in 2000 were attributable to diabetes, which is equivalent to 5.2% of all deaths (Roglic *et al.*, 2005). In fact, the death rates among diabetic patients are two times higher compared to people without diabetes (Deshpande *et al.*, 2008). Indeed, the life expectancy in people with diabetes is reduced by 5 to 10 years (Marshall & Flyvbjerg, 2006). Diabetes mellitus related cardiovascular complications are a major cause of morbidity and mortality. For example, in the United States, approximately 65% of deaths from diabetes are attributed to cardiovascular disease (Geiss LS, 1995; Engelgau *et al.*, 2004). Because of the high rate of death caused by diabetic cardiovascular complications, preventing these complications is an important objective.

During the last decade, improvements in insulin replacement treatment have caused the greatest change in diabetes therapy for type 1 and 2 diabetic patients. Insulin treatment in diabetic patients reduces injury to numerous internal organ systems via controlling elevated blood glucose concentrations. Well-designed clinical prospective studies have shown a reduction in the onset and progression of diabetic neuropathy, nephropathy and retinopathy in people with type 1 diabetes mellitus following intensive insulin treatment (Control & Group, 1993; Albers *et al.*, 1995; DiabetesControl & Group, 1998). Insulin therapy has also been shown to reduce some but not all cardiovascular diseases in people with type 1 diabetes (Group, 1995). Thus, treating diabetic patients with insulin does reduce and/or delay some diabetic complications but does not prevent the development and progression of these complications. Indeed, it appears that current insulin therapy is not enough to treat diabetes

and its complications in the long term, and a better understanding of the mechanisms of deleterious cardiovascular complications in diabetes is needed.

There is increasing evidence suggesting that an abnormal activation of the sympathetic nervous system contributes to the development of diabetic complications, including hypertension as well as cardiovascular and renal dysfunction (Perin *et al.*, 2001). For instance, the blocking activity of the sympathetic nervous system has been shown to reduce morbidity and mortality in patients with heart failure and diabetes (Shekelle *et al.*, 2003). The activity of sympathetic nerves is regulated by autonomic neurons within the central nervous system (CNS), including the paraventricular nucleus of the hypothalamus (PVN), the rostral ventrolateral medulla (RVLM) and the nucleus tractus solitarius (NTS). As such, pathological changes in these nuclei may result in hyperactivity of the sympathetic nerves, but the detailed mechanisms that underlie the dysfunction of these nuclei in diabetes are poorly understood.

Neuroinflammation in CNS cardiovascular control regions has been suggested to contribute to the hyperactivity of sympathetic nerves in heart failure (Kang *et al.*, 2009a; Felder, 2010) and hypertension (Kang *et al.*, 2009b; Colombari *et al.*, 2010; Shi *et al.*, 2010a; Waki *et al.*, 2010), both of which are consequences of diabetes. The resident immune cells, which mediate inflammation within the CNS when activated, are called microglia. Microglial activation in the PVN is associated with angiotensin II-induced hypertension, and the pharmacological inhibition of microglial activation has been shown to prevent the development of hypertension, decrease PVN cytokines and attenuate cardiac hypertrophy in response to angiotensin II infusion (Shi *et al.*, 2010a). Diabetes is known to cause pathological changes in peripheral nerves, which results in subsequent inflammation within the CNS (Pabreja *et al.*, 2011). Previously, we have demonstrated an increase in activated microglia in specific cardiovascular control regions of the brain in STZ diabetic rats (Rana *et al.*, 2014). Despite this evidence, the functional consequence of microglial activation and its

contribution to the development of diabetic cardiovascular complications has never been investigated.

In this review, the objective is to provide a general overview of diabetes and its complications and the role that autonomic dysfunction plays in their generation, highlighting current knowledge relevant to the potential contribution of central inflammation in the brain regions involved in autonomic control of the cardiovascular system. The rationale for the experiments in this thesis is also explained.

1.2 Diabetes mellitus

The prevalence of diabetes is growing worldwide in both developed and developing countries. In 1980, around 153 million people suffered from diabetes mellitus, and this number had increased to 347 million by 2008 (Danaei *et al.*, 2011). By the year 2030, it is estimated that over 439 million people worldwide will be diagnosed with diabetes mellitus (Shaw *et al.*, 2010). In Australia, 3.6 million people have diabetes or a pre-diabetic condition (Atlas, 2011). In 2000, around 1 million Australians aged ≥25 years suffered from diabetes, and its prevalence is expected to reach 2–2.9 million by 2025 (Magliano *et al.*, 2008). Diabetes is one of the top ten causes of death in Australia. In 2005, an estimated 3,529 Australians died as a consequence of diabetes and it complications. Diabetic complications not only increase mortality but also increase the cost of managing diabetes. For instance, type 1 diabetes costs an individual about $4,669 each year if they are healthy; however, once diabetic complications develop, the cost increases up to $16,698 (Colagiuri *et al.*, 2009). Thus, preventing or reducing the development of diabetic complications is an important objective to minimize the high prevalence of morbidity and mortality as well as the cost of treatments related to diabetes.

Diabetes mellitus is a metabolic disorder characterized by elevated blood glucose levels and associated disturbances of the metabolism of glucose, carbohydrates, proteins and lipids (Mwanri *et al.*, 2015). The elevation levels of glucose in the blood occur as a result of the inability of the pancreas to secrete insulin, defects in insulin action or both. Diabetes is classified into two main categories: type 1 diabetes and type 2 diabetes. Type 1 diabetes is characterised by hyperglycaemia due to the autoimmune destruction of pancreatic β cells, which secrete insulin to stimulate the metabolism of glucose in the body (Bluestone *et al.*, 2010). This type of diabetes accounts for 5% to 10% of all cases of diabetes. The risk factors for this type of diabetes are autoimmune, genetic and environmental factors (Deshpande *et*

al., 2008). In contrast, type 2 diabetes is characterised by the inability of the body to effectively respond to the insulin. Type 2 diabetes is responsible for around 90% to 95% of all cases of diabetes (Deshpande *et al.*, 2008). There are many factors that can contribute to the incidence of this form of diabetes. These include obesity, poor diet, western diet, physical inactivity, increasing age, family history of diabetes and ethnicity (Alberti *et al.*, 2007; Shaw *et al.*, 2010). The normal blood level of glucose in humans during fasting is between 4 to 6 mmol/L, and it is considered high when it reaches 12 mmol/L or above. High blood glucose is a defining feature of both types of diabetes. In the long term in both of these types of diabetes, the direct or indirect effects of hyperglycaemia increase the risk of the development of diabetes complications; however, correlation studies in humans can only go so far in understanding the mechanisms of generating diabetic complications via hyperglycaemia in animals.

1.2.1 Use of streptozotocin-induced diabetic models to study complications

The administration of streptozotocin (STZ) is a well-established technique to induce diabetes in animals through the selective destruction of the pancreatic β cells. As a consequence, insulin deficiency leads to hyperglycaemia and other characteristics observed in humans with diabetes mellitus, such as glycosuria, polyuria, polyphagia and polydipsia (Hebden *et al.*, 1986b); however, STZ induced diabetic animals do not show strong autoimmune features (Baxter & Duckworth, 2004). Because type 1 diabetes in humans results from the autoimmune destruction of pancreatic β cells, this model is not useful to study the mechanism that causes type 1 diabetes, but since an injection of STZ produces hyperglycaemia, it is a good model to study the mechanism that causes complications due to increased blood glucose levels. An injection of STZ also provides a robust and consistent hyperglycaemic in animals. In addition, since the hallmark symptom for humans with type 1 and 2 diabetes is hyperglycaemia, animal models injected with STZ may resemble both types of diabetes. STZ

induced hyperglycaemia rats display abnormalities in the cardiovascular system, which is a characteristic of humans with type 1 and 2 diabetes. Thus, STZ-induced diabetes in animals is an adequate model for investigating the mechanisms of the cardiovascular complications of diabetes. Indeed, an STZ rat model was the primary model used for this thesis.

1.3 Cardiovascular complications in diabetes

Diabetes mellitus is described as a primary risk factor for the development of cardiovascular disease (Grundy *et al.*, 1999; Fowler, 2008). Cardiovascular complications are the likely cause of morbidity and mortality in approximately 80% of diabetes mellitus patients (Carlson, Shelton *et al.*, 2000). Based on the differences in the size of the vessels injured, pathological mechanisms and risk factors, diabetic complications have been classified as either microvascular or macrovascular complications.

1.3.1 Microvascular complications

In diabetic patients, microvascular changes involve damage to small arteries, arterioles and capillaries, and these vascular abnormalities are suggested to lead to the following complications:

A- Diabetic retinopathy: destruction of the retina, leading to vision impairment and eventually blindness (Porta *et al.*, 2011).

B- Diabetic nephropathy: damage to the kidney, which progresses to renal failure. Diabetic nephropathy is associated with a decrease in the glomerular filtration rate and albuminuria (Van Buren & Toto, 2011).

C- Diabetic neuropathy: damage to the peripheral nerves, which results in weakness, tingling, numbness and chronic pain (Callaghan *et al.*, 2012). For instance, diabetic neuropathy can cause foot ulcers in diabetic patients. Neuropathy not only affects sensory neurons but also autonomic neurons, which may lead to a secondary dysfunction of the cardiovascular and renal systems (Tesfaye *et al.*, 2010).

These microvascular complications are closely related to the severity and duration of diabetes. During the last decade, the most studied mechanisms of these complications included but were not limited to 1) increased metabolism of glucose through alternative polyol and hexosamine pathways to produce damage in diabetes; 2) increased activation of protein kinase isoforms; and 3) increased formation of advanced glycation end-products (Brownlee, 2001; Giacco & Brownlee, 2010; Rambhade *et al.*, 2011). An alteration in any of these molecular pathways induced by hyperglycemia will cause increased oxidative stress, increased activity of the renin-angiotensin system and increased free radicals production in mitochondria, which in turn will result in an up-regulation of inflammatory cytokines and growth factors. Combined, these changes are suggested to lead to multiple vascular and neurological dysfunctions, including ischemia in the retina, glomerulosclerosis and proteinuria in the kidney and degeneration in peripheral nerves (Brownlee, 2001, 2005; Gologorsky *et al.*, 2012; Nguyen *et al.*, 2012). All of these abnormalities could play a role in the development of cardiovascular disease in people with diabetes (Kim *et al.*, 2011).

1.3.2 Macrovascular complications

Macrovascular complications in people with diabetes are caused by damage to medium or large arteries. These complications include coronary artery disease, myocardial infarction and stroke, which can impair the central nervous system and the heart (Krentz *et al.*, 2007; Fowler, 2008). Such complications are known to be the most common risk factors that increase the mortality rate in people with diabetes. The common pathological mechanism of these complications is thought to be the development of atherosclerosis. While the pathogenesis of atherosclerosis remains insufficiently understood, endothelia dysfunction and chronic inflammation are thought to be important contributors (Williams & Nadler, 2007; Forbes & Cooper, 2013).

There are others factors that may be involved in the pathogenesis of cardiovascular complications in diabetes, such as macrophage dysfunction, dyslipidaemia and impaired platelet function (Cade, 2008). All of the diabetic complications mentioned above are dependent on the damaging effects of hyperglycemia on the blood vessels. In contrast, cardiomyopathy is a common complication of diabetes that is suggested to occur independent of vascular damage.

1.3.3 Diabetic cardiomyopathy

Diabetic cardiomyopathy is characterized by structural and functional changes in the myocardium of diabetic patients in the absence of other confounding factors, including coronary artery disease, valvular heart disease or hypertension (Aneja *et al.*, 2008; Pappachan *et al.*, 2013). Diabetic cardiomyopathy is associated with a left ventricular hypertrophy and myocardial remodelling, leading to abnormal left ventricle filling and diastolic dysfunction at early stages (Schilling & Mann, 2012). The progression of diastolic dysfunction accelerates systolic dysfunction, which in turn contributes to reducing the ejection fraction and the later development of heart failure (Fonseca, 2003). In both type 1 and type 2 diabetic patients, cardiomyopathy is common (Danielsen *et al.*, 1987; Schannwell *et al.*, 2002; Diamant *et al.*, 2003; Korosoglou *et al.*, 2012).

There is also evidence that cardiac functions are compromised in several types of diabetic animals. For example, *ex-vivo* studies on isolated hearts from diabetic mice and rats demonstrated a reduction in the maximal rate of pressure increase (+dP/dt) and decrease (–dP/dt) in the left ventricle and a reduction in the left ventricular developed pressure and left ventricular systolic pressure (Choi *et al.*, 2002; El-Omar *et al.*, 2003; Belke *et al.*, 2004; Ligeti *et al.*, 2006; Cao *et al.*, 2012). Similarly, *in vivo* studies have reported cardiac abnormalities, such as a decreased +dP/dt and decreased –dP/dt in the left ventricle; a decreased left ventricular developed pressure and left ventricular systolic pressure; an

increased left ventricular end-diastolic pressure; and a decreased fractional shortening and ejection fraction in STZ diabetic rats (Cheng *et al.*, 2004; Borges *et al.*, 2006; Bidasee *et al.*, 2008; Arozal *et al.*, 2009; Wang *et al.*, 2011; Bhatt & Veeranjaneyulu, 2012; Tappia *et al.*, 2013; Guo *et al.*, 2014), in rats treated with STZ combined with high fat feeding and in obese mice, both of which are animal models of type 2 diabetes (Zhou *et al.*, 2011; Sen *et al.*, 2012). Thus, in terms of cardiac changes, it appears that diabetic animals mimic cardiac features observed in human diabetes. The pathology of diabetic cardiomyopathy has been linked to several mechanisms, which include lipid accumulation in the cardiomyocytes (Yokoyama *et al.*, 2004), defects in myocardial subcellular organelles (Dhalla *et al.*, 2012), impairment of Ca^{2+} handling (Lagadic-Gossmann *et al.*, 1996; Dhalla *et al.*, 2014), endothelial dysfunction and myocardial fibrosis (Tschöpe *et al.*, 2005; Asbun & Villarreal, 2006); however, the exact mechanism behind diabetic cardiomyopathy is still unclear. There is evidence that suggests that increased sympathetic activity can cause cardiac dysfunction in other diseases (Aronson & Burger, 2002; Guggilam *et al.*, 2008), but its role in diabetic cardiomyopathy has not been investigated.

1.3.4 Blood pressure

Hypertension is considered to be an important risk factor in the development of cardiovascular disease, which is the likely cause of morbidity and mortality in approximately 80% of diabetes mellitus patients (O'Keefe *et al.*, 1999). The risk of developing cardiovascular disease in persons with diabetes is increased when it occurs with hypertension (Epstein & Sowers, 1992). In addition, it has been suggested that up to 75% of cardiovascular diabetic complications can be attributed to hypertension (Bild & Teutsch, 1987). Well-designed clinical trials have shown a reduction in complications in both types of diabetes following aggressive treatment for hypertension through the use of renin-angiotensin system inhibitors, such as diuretics, angiotensin receptor blockers and angiotensin-converting

enzyme inhibitors (Group, 1998; Tuomilehto *et al.*, 1999; Association, 2003). Therefore, the presence of hypertension in diabetic patients is thought to contribute to the overall diabetes mortality, although the possibility exists that increased angiotensin II levels cause complications via another mechanism.

While several clinical studies have shown that patients with type 1 and type 2 diabetes are likely to show hypertension (Parving *et al.*, 1988; Turner *et al.*, 1993), blood pressure changes in diabetic animals are controversial. Studies showing hypertension in STZ-diabetic rats have mainly used the non-invasive tail cuff measurement method (Bunag *et al.*, 1982; Katayama & Lee, 1985; Hartmann *et al.*, 1988; Ramos, 1988). It has been suggested that the increased blood pressure observed in STZ rats with tail cuff measurements is spurious and is due to structural changes in the tail. Others studies have shown a decrease or no change in the blood pressure in conscious STZ diabetic animal studies using direct invasive measurements (Hebden *et al.*, 1987; Maeda *et al.*, 1995; Fazan *et al.*, 1997; Schaan *et al.*, 1997; Van Buren *et al.*, 1998; Fazan *et al.*, 1999; Dall'Ago *et al.*, 2002; Schaan *et al.*, 2004; Borges *et al.*, 2006; Farah *et al.*, 2007). The reasons for the difference between humans and rats in this aspect are not completely understood; however, several mechanisms have been postulated to be responsible for hypotension in STZ-diabetic rats, such as 1) a decreased cardiac output due to a dysfunction in the myocardium (Ren & Bode, 2000); 2) a differential sympathetic outflow to various organs; 3) hypovolemia due to osmotic diuresis (Teshima *et al.*, 2000); and 4) the impairment of sympathetic innervation of the heart and blood vessels (Borges *et al.*, 2006). For this thesis, the aim was to gain a better understanding of this difference, which will aid in the interpretation of the relevance of the STZ rat model to humans.

1.3.5 Salt-sensitivity of arterial blood pressure in diabetes

As discussed, the changes in blood pressure in STZ diabetic animals are different from the human condition, and the reason for the lack of an increase in blood pressure in rats treated

with STZ is a matter of speculation; however, it is possible that dietary factors relevant to hypertension in human diabetes are also required for the development of hypertension in STZ diabetic rats.

There is consensus that dietary salt is a contributing factor in the development of hypertension in both humans and animals, particularly in salt-sensitive individuals (Campese, 1994; Sacks *et al.*, 2001; Jones, 2004; Brooks *et al.*, 2005). A large number of clinical and animal experimental studies have shown that a reduction in dietary salt lowers blood pressure, while a high salt intake has been found to increase blood pressure in animal models (Campese *et al.*, 1982; Koolen & van Brummelen, 1984; Howe *et al.*, 1990; Law *et al.*, 1991; Wyss *et al.*, 1995). In addition, excessive dietary salt reduces the effectiveness of most antihypertensive drugs (Chobanian & Hill, 2000).

Although the role of dietary salt in the development of hypertension in humans with type 1 and 2 diabetes is well-documented, the relationship between salt intake and hypertension in STZ diabetic rats has not been fully studied. Nevertheless, two studies on STZ-induced diabetes using the direct recording of blood pressure have reported that 1% NaCl intake causes an increase in blood pressure (Santos *et al.*, 1995; Maeda *et al.*, 2007). Dietary salt has also been shown to further elevate the blood pressure levels in obese Zucker rats compared with non-diabetic, lean Zucker rats (Carlson *et al.*, 2000). Collectively, this data suggests that dietary salt may have an important role in the development of hypertension in both diabetic humans and animals. Because the administration of STZ to rats induces a diabetic state that is not accompanied by hypertension, using STZ diabetic rats with increased salt intake may produce an animal model more closely related to human diabetes and thereby lead to a better understanding of the effects of hyperglycaemia on cardiovascular functions. This model will also provide a better understanding of the link between diabetes and hypertension.

1.3.6 Diabetic cardiovascular complications: the role of sympathetic nerve activity

The mechanisms responsible for the development diabetic cardiovascular complications are complex and multifactorial. For example, many studies on the mechanisms of the development of type 1 diabetes complications have suggested an important role for endothelial dysfunction (Woodman *et al.*, 2008; Woodman & Malakul, 2009); however, a large body of evidence shows that the autonomic nervous system is impaired in diabetic patients. The peripheral autonomic nervous system functions to regulate the organ systems, such as the cardiovascular, digestive and renal systems. It is, in turn, regulated by the brainstem and the hypothalamus, which contain central autonomic control areas. Both the sympathetic and parasympathetic nervous systems are regulated by reflex arcs between sensory and motor neurons in the viscera and autonomic brainstem nuclei. The sympathetic and parasympathetic nervous systems work together in a complex interplay to regulate the cardiovascular system. For example, in response to environmental stress, the activity of the sympathetic nervous system elevates blood pressure and accelerates the heart rate (Floras, 2009; Triposkiadis *et al.*, 2009); however, the parasympathetic nervous system has restorative functions through exerting inhibitory input to the heart to decrease blood pressure and heart rate (Andresen & Kunze, 1994).

Diabetes mellitus imposes negative effects on both the function and structure of the autonomic nervous system, which may compromise its role in the maintenance of the body's internal environment (Baydas *et al.*, 2003; Lebed *et al.*, 2008). Autonomic neuropathy has been suggested to be the most serious complication of diabetes in terms of morbidity and mortality (Ewing *et al.*, 1980; Kempler *et al.*, 2002), and it may affect sensory and motor autonomic nerves. Still, the roles of the autonomic neuropathy and the dysregulation of the

autonomic nervous system in causing long-term diabetic complications, such as cardiovascular diseases, have not been fully explored.

As mentioned, diabetes affects not only the somatic sensory nerves but also autonomic sensory nerves, which modulate the control of the internal organs by sending signals regarding the state of the internal environment to the CNS, particularly into autonomic centres in the brainstem and hypothalamus (Perin *et al.*, 2001; Verrotti *et al.*, 2009). Thus, pathological changes in the peripheral nerves may result in the dysfunction of these autonomic centres, thereby causing adverse effects in the functionality of the cardiovascular, renal, endocrine and digestive systems. Any disturbance to the function of the autonomic nervous system may have substantial consequences for the health of the individual and mortality (Ewing *et al.*, 1980; Kempler *et al.*, 2002).

There is strong evidence that abnormal elevation of the sympathetic tone is a key mechanism underlying the development of cardiovascular diseases, including hypertension (Dampney *et al.*, 2005; Esler, 2011; Takahashi, 2012) and heart failure (Watson *et al.*, 2006; Kishi, 2012), which are major consequences of diabetes in the long term. Increased activity of the sympathetic nervous system has been reported in both humans and animals with heart failure (Packer, 1988; Zheng *et al.*, 2009; Patel *et al.*, 2011). Blocking the effect of increased activity with beta-blockers has been shown to decrease the mortality rate and improve outcomes for patients following a heart attack (Butler *et al.*, 2006); however, whether this approach is useful in diabetes has not been investigated.

Sympathetic nerve overactivity is known to occur in diabetic patients (Langer *et al.*, 1995; Stevens *et al.*, 1997; Perin *et al.*, 2001). Such overactivity has been shown to be involved in the development of diabetic complications, including hypertension (Frontoni *et al.*, 2005) and an increased risk of abnormal cardiac function and adverse cardiovascular events (Perin *et al.*, 2001; Manzella & Paolisso, 2005; Mori *et al.*, 2008) as well as chronic renal failure

(Schlaich *et al.*, 2009). Thus, based on the data, an impaired autonomic nervous system function in diabetes promotes adverse effects on various body organs, leading to substantial and serious consequences for the health and survival of the individual.

Interestingly, a study on STZ-induced diabetic rats demonstrated significantly elevated basal renal sympathetic nerve activity (Patel *et al.*, 2011). Despite this evidence, the brain regions and cellular mechanisms responsible for the increase in sympathetic nerve activity in diabetes remain unidentified; however, it is likely that the increase in sympathetic nerve activity originates in the CNS cardiovascular centres (Dampney, 1994; Sato *et al.*, 2000; Chen & Toney, 2009).

1.4 Cardiovascular centers in the CNS

As discussed, cardiovascular functions are regulated via several nuclei in the brainstem and hypothalamus (Guyenet, 2006). These nuclei control and influence the neurons in the intermediolateral cell column of the spinal cord, which are sympathetic preganglionic motor neurons. These motor neurons activate the sympathetic postganglionic neurons in the peripheral ganglion to regulate cardiovascular function (Guyenet, 2006). The paraventricular nucleus of the hypothalamus (PVN), the rostral ventrolateral medulla (RVLM) and the nucleus tractus solitarius (NTS) are the main areas of the brain that are involved in cardiovascular regulation.

1.4.1 The paraventricular nucleus (PVN)

The PVN is located on both sides of the third ventricle in the hypothalamus, and it consists of parvocellular and magnocellular neurons. The PVN regulates the autonomic nervous system, including its cardiovascular functions, as well as the endocrine system (Kannan *et al.*, 1989; Badoer, 2001). Magnocellular neurons project to the posterior pituitary and are involved in secreting oxytocin and vasopressin in response to osmotic and other stimuli (Kannan *et al.*, 1989). Thus, the PVN plays a critical role in the control of blood volume via the secretion of oxytocin and vasopressin. In contrast, parvocellular neurons project to the anterior pituitary as well as other regions in the CNS in which they play a role in the regulation of sympathetic nerve activity (Cechetto & Saper, 1988; Stern *et al.*, 2003).

These parvocellular neurons can influence sympathetic nerve activity directly and indirectly (Figure1-1). They project directly into the intermediolateral cell column of the spinal cord, and the stimulation of this connection increases blood pressure and renal sympathetic nerve activity through the secretion of neurotransmitters, including vasopressin (Kawabe *et al.*, 2009). Parvocellular neurons also project to other brain regions, such as the RVLM, which in

turn projects to the sympathetic preganglionic neurons, thereby indirectly influencing sympathetic nerve activity (Kannan *et al.*, 1989; Kantzides & Badoer, 2003). In addition, functional evidence indicates that the inhibition of the PVN by the microinjection of a GABA receptor agonist produces a reduction in renal sympathetic nerve activity and mean arterial pressure in the normotensive rat (Haselton & Vari, 1998; Zhang & Patel, 1998; Deering & Coote, 2000). These findings indicate that activation of the PVN can cause changes in cardiovascular functions through the modulation of sympathetic nerve activity. Therefore, it is important to determine whether the cellular and molecular changes in the PVN may contribute to the activation of the sympathetic nerve system in diabetes.

The activation state of PVN is stimulated by a variety of factors, including haemorrhage (Badoer & Merolli, 1998), increased plasma leptin (Huang *et al.*, 1998), increased plasma volume (Badoer *et al.*, 1997), increased plasma osmolarity (Charlton *et al.*, 1988; Brooks *et al.*, 1989), dehydration (Zheng *et al.*, 2002), stress (Imaki *et al.*, 1995), prostaglandins (Konsman *et al.*, 2002), nuclear factor-kappa B (Kang *et al.*, 2009b) and proinflammatory cytokines (Shi *et al.*, 2010a).

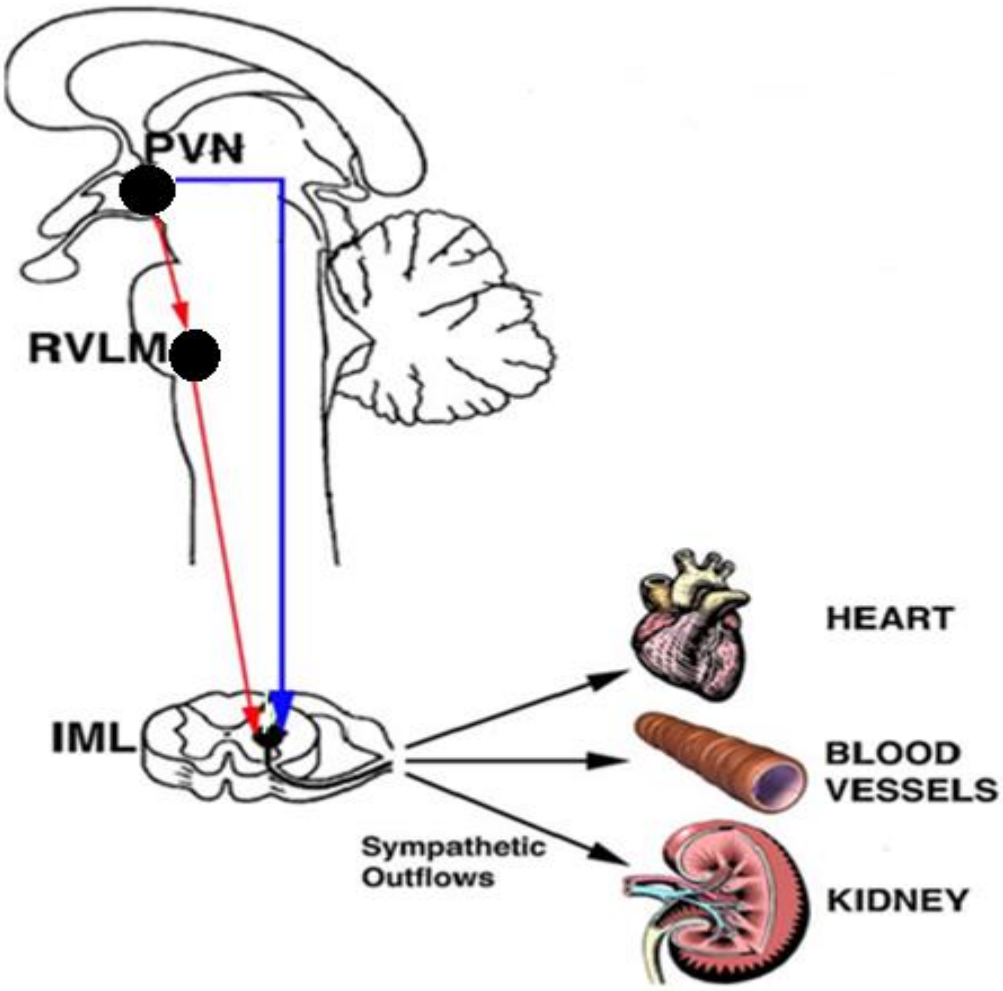


Figure 1-1. Diagrams illustrating the PVN neuron pathways involved in the control of sympathetic activity. The PVN sends projections to the IML of the spinal cord to influence sympathetic activity directly (blue) or sends projections to the RVLM neurons, which send projections to the IML to ultimately influence sympathetic activity (red). PVN, paraventricular nucleus; RVLM, rostral ventrolateral medulla; IML, intermediolateral column. Modified from (Pyner, 2009).

1.4.2 Nucleus tractus solitarius (NTS)

The nucleus tractus solitarius (NTS) is located in the dorsomedial medulla oblongata and consists of the caudal and rostral regions. The caudal region of NTS is the region most involved in cardiovascular regulation. This nucleus receives projections from other brain regions that are known to be involved in cardiovascular regulation, such as the PVN and RVLM (Andresen *et al.*, 2004). The NTS neurons play a pivotal role in regulating the cardiovascular system via modulating autonomic efferent activity (Paton, 1998; Silva-Carvalho *et al.*, 1998). The NTS is the main area of the brain that relays sensory input for baroreflex control. It receives sensory terminals from the carotid chemoreceptors, arterial baroreceptors and cardiopulmonary receptors in the heart and vessels, which is used for the appropriate adjustment of blood pressure (Miura & Reis, 1969; Van Giersbergen *et al.*, 1992; Machado *et al.*, 1997) (Figure1-2). Upon baroreflex activation, the sympathetic nerve activity to the heart and vessels increases, and the parasympathetic nerve activity to the heart decreases to maintain a normal blood pressure. Lesions of NTS neurons have been shown to abolish the baroreflex responses in anesthetized animals (Miura & Reis, 1972), and the electrical stimulation of these neurons also produced a significant increase in blood pressure in another study in rats (Doba & Reis, 1973). The microinjection of a GABA receptor agonist into the NTS caused a decrease in sympathetic nerve activity and mean arterial pressure in spontaneously hypertensive rats (Sato *et al.*, 2000). These data indicate that the NTS is crucial in the regulation of the cardiovascular system and that increased NTS neuronal activity may contribute to the elevation of arterial pressure.

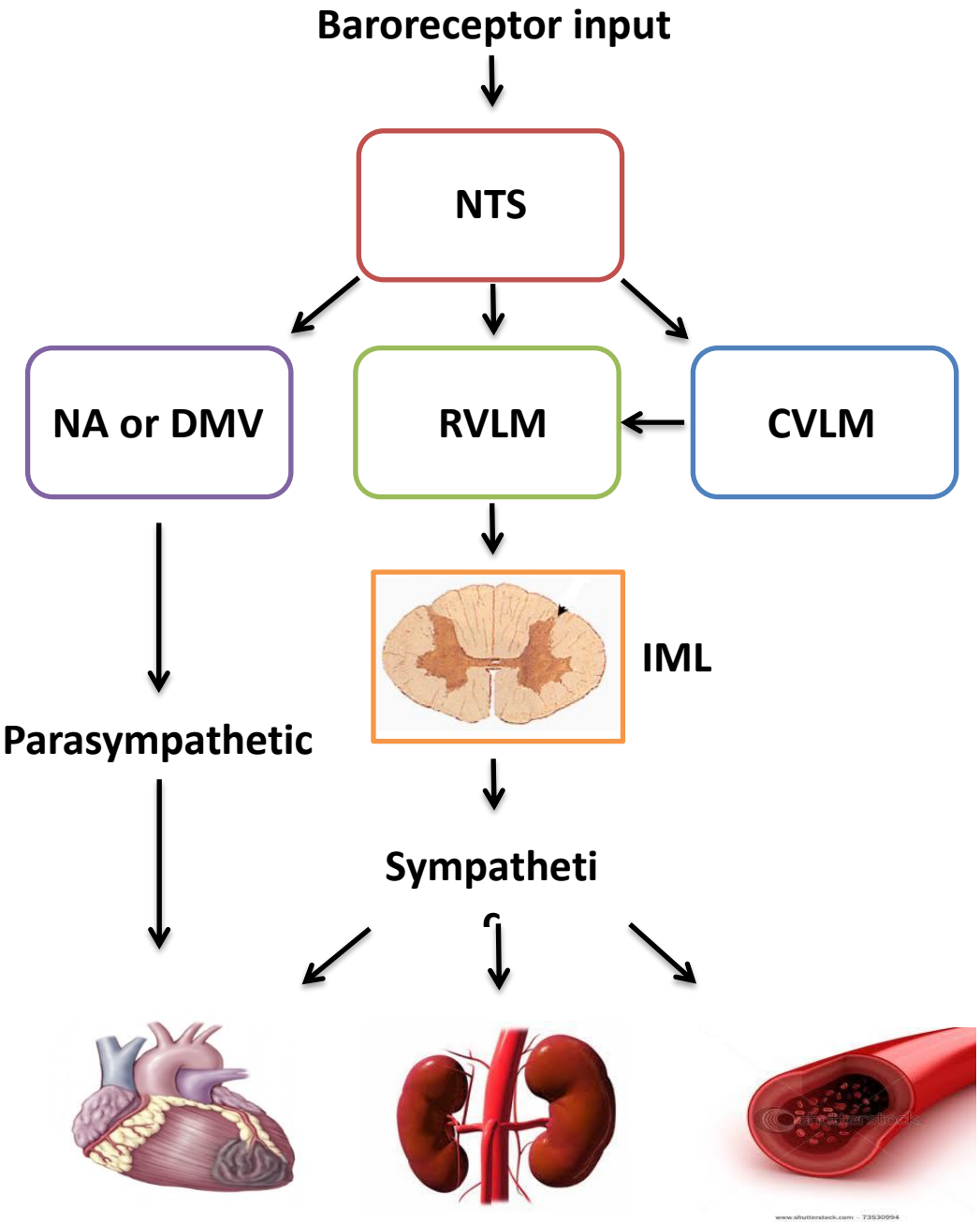


Figure 1-2. Diagram illustrating the neural pathways involved in the baroreceptor reflex. The NTS receives input from high and low-pressure baroreceptors and projects to the RVLM either directly or via the CVLM, which in turn projects to the RVLM before terminating at the IML of the spinal cord, which contains sympathetic post-ganglionic neurons, to ultimately influence sympathetic control of effector organs, including the heart, kidney and blood vessels. The NTS also projects to the NA and DMV, which mediate parasympathetic effects on the heart. NTS, nucleus tractus solitarius; RVLM, rostral ventrolateral medulla; IML, intermediolateral column; NA, nucleus ambiguous; DMV, dorsal motor nucleus of vagus.

1.4.3 Rostral Ventrolateral Medulla (RVLM)

The rostral ventrolateral medulla (RVLM) is the area of the brain stem situated ventral to the nucleus ambiguous and caudal to the facial nucleus. This nucleus also projects into the intermediolateral cell column of the spinal cord (Guertzenstein & Silver, 1974; Dampney, 1994; Card *et al.*, 2006) and receives a variety of inputs from the NTS, PVN (Nosjean *et al.*, 1998) and caudal ventrolateral medulla (CVLM), which in turn are involved in the regulation of sympathetic activity via the inhibitory effects on the RVLM (Badoer & Merolli, 1998; Pyner, 2009).

The RVLM neurons are thought to play a pivotal role in controlling the tonic excitation of sympathetic preganglionic neurons, and the functional integrity of these neurons is important for maintaining blood pressure and sympathetic vasomotor activity. Chemical or electrical stimulation of the RVLM neurons has been shown to increase arterial pressure and heart rates in animals (Guertzenstein & Silver, 1974; Ross *et al.*, 1984). On the other hand, the bilateral inhibition of the RVLM neurons causes a decrease in sympathetic nerve activity and blood pressure (Dampney, 1994; Guyenet, 2006). The RVLM neurons are also influenced indirectly by the NTS through peripheral inputs that adjust sympathetic nerve activity (Figure1-2). For example, the arterial baroreflex is mediated via the RVLM neurons. An increase in arterial pressure causes the activation of arterial baroreceptors, which in turn inhibits output from the NTS to inhibit activation of RVLM neurons, thereby reducing sympathetic nerve activity to normalize blood pressure (Pilowsky & Goodchild, 2002). RVLM neurons also modulate the somatic pressor reflex and chemoreflex, which are involved in the regulation of blood pressure (Kiely & Gordon, 1994; Koshiya & Guyenet, 1996). The activity of RVLM neurons can also be influenced via inputs from the PVN (Badoer, 2000). Thus, multiple brain regions contribute to blood pressure control, and almost all are reported to show inflammation in disorders with increased sympathetic nerve activity.

1.5 The role of microglia in inflammation of the CNS

There is increasing evidence suggesting that inflammation within the central nervous system (CNS) may be crucial in the pathogenesis of peripheral diseases such as diabetes (Tsuda *et al.*, 2008; Patel *et al.*, 2011). Various studies on diabetic rats have demonstrated inflammation in specific cardiovascular centres of the brain (Luo *et al.*, 2002; Zheng *et al.*, 2006; Rana *et al.*, 2014). Despite this, the functional consequence of inflammation has never been investigated. The major cell types involved in CNS inflammation are microglia and astrocytes. As microglia play a significant role in the pathology of several diseases, it is important to understand their origins.

1.5.1 The origin of microglia

The functionality of the CNS is dependent on glia cells, which mainly consist of oligodendrocytes, astrocytes and microglia. Microglia are the primary immune cells in the CNS and were first described in 1899 by Nissl (1899). In 1919, Pio del Rio-Hortega began to characterize their function and origin. He documented that in response to brain injury, microglia migrate to the damaged tissue, undergo morphological changes and engulf tissue debris, suggesting that microglia act as an immunocompetent cell. He also suggested that microglia originate from mesoderm, which is similar to macrophages (del Rio-Hortega & Penfield, 1932).

For some time, the precise origin of the microglia in the CNS was controversial, but the neuroectoderm was proposed as the microglial origin, as well as for other cells in the CNS (Hutchins *et al.*, 1990; Hao *et al.*, 1991). This view was also supported by experimental data from cell cultures that suggested astrocytes and microglia cells derived from a similar progenitor cell (Eglitis & Mezey, 1997; Fedoroff *et al.*, 1997); however, it is now widely accepted that microglial cells in the CNS are derived from the mesoderm. Several studies

suggested that postnatally, progenitor microglia originate from circulating monocytes, traverse through blood vessels and eventually invade the CNS and differentiate to be ramified microglia (Perry *et al.*, 1985; Rezaie & Male, 2002); however, microglial cells have been shown to develop in the brain before vascularization (Ashwell, 1991; Cuadros *et al.*, 1993) and the formation of monocytes in hemopoietic tissues (Sorokin *et al.*, 1992; Naito *et al.*, 1996), indicating that during development at least, microglia cells are not generated from monocytes.

1.5.2 Morphology and function of microglia

The CNS previously was considered an 'immune privileged organ', meaning it was not susceptible to inflammation or activation of the immune system, as the CNS was not thought to have lymphatic drainage and because of the presence of the blood–brain barrier (BBB); however, this notion has changed, as activated T-cells cross the BBB (Cross *et al.*, 1990). In fact, the CNS has its own resident immune cells (microglia and astrocytes), which are involved in inflammation mechanisms and protect the CNS from pathogens (Gehrmann *et al.*, 1995; Dong & Benveniste, 2001).

Microglia represent the predominant resident immune cells in the CNS, and similar to macrophages, microglia perform immune functions and communicate with other cells of the immune system. In the CNS, three general morphological states of microglia have been described, which are ramified, activated and amoeboid, but others have suggested that there are more than three. It is believed that these morphological states indicate different functional states of microglia. In a healthy brain, microglia exist in the ramified state, consisting of a small cell body with long and highly branched processes that extend outwards (Davalos *et al.*, 2005). These long microglial processes monitor the surrounding local environment, allowing microglia to function as sentinels that sense insults within the CNS and engulf tissue debris

via phagocytosis (Sierra *et al.*, 2010). Ramified microglia also play a homeostatic role in supporting neurons (van Rossum & Hanisch, 2004).

Once activated in response to infection, injury, or inflammation of the CNS, ramified microglia undergo morphological changes consisting of shortened processes and enlarged cell bodies. In this state, microglia have the potential to display a diverse set of functional changes. They express other molecules, such as cell adhesion molecules, which enable them to further detect and eliminate foreign organisms (Kreutzberg, 1996; Lee & Benveniste, 1999). They also act as antigen-presenting cells by expressing major histocompatibility complexes, such as class I and II antigens, which are essential for T-cell activation and for allowing the entry of T-cells across the BBB (Babcock *et al.*, 2003; Bailey *et al.*, 2006). Upon activation, microglia secrete a wide variety of cytokines and express cytokine receptors, which facilitate communication with other cells of the immune system (Ferrari *et al.*, 1996; Hide *et al.*, 2000; Hanisch, 2002; Inoue, 2006). These immune mediators also may be involved in the regulation of inflammation by recruiting further microglia and other immune cells and causing the activation of these cells. Cytokines are subdivided into either pro-inflammatory or anti-inflammatory groups. When microglia become activated, they release both pro-inflammatory and anti-inflammatory cytokines and change their expression of cytokine receptors, depending on the severity and type of stimulus. Furthermore, as modulators of immune function, microglia express various toll-like receptors, which are involved in the generation of innate immune responses and stimulate the secretion of pro-inflammatory cytokines (Carty & Bowie, 2011). Upon activation, microglia also secrete reactive oxygen species, which can induce the proliferation and migration of microglia to the site of neuronal injury to further induce inflammatory processes (Parvathenani *et al.*, 2003; Block *et al.*, 2007; Lenertz *et al.*, 2009).

Prolonged activation of microglia can result in their assuming amoeboid shapes with sparse processes, a cell type which is often called phagocytic microglia. They are also, however, capable of phagocytosis in their ramified state and this process plays an important role in maintaining the homeostasis of the CNS environment (Koizumi *et al.*, 2007; Fang *et al.*, 2009; Neumann *et al.*, 2009). Therefore, microglia cells are the principal immune cells in the CNS, play a significant role in normal brain function, host defence and serve as the link between the CNS and the peripheral immune system.

Several studies have reported activated microglia in pathologies of the CNS that are associated with brain inflammation, such as Alzheimer's disease, Parkinson's disease, multiple sclerosis, neuropathic pain and diabetes. While microglia play a complex and multifaceted role in response to CNS pathological challenges, the situations in which activated microglia can be either neuroprotective or neurotoxic are still controversial.

1.5.3 Microglial activation: the dual ability of neuroprotection and neurotoxicity

As discussed, in response to CNS pathological challenges, such as stressful stimuli, brain injury, infection, inflammation or neurodegenerative diseases, microglial cells initiate and regulate the immune response in the CNS. This includes releasing pro-inflammatory cytokines and the generation of reactive oxygen species. These secretions of microglia are beneficial to the host through the protection from infection in the CNS, but they also can be detrimental when inflammatory processes are induced chronically. It has been suggested that similar to systemic macrophages, activated microglia can be divided into two phenotypes. M1 microglia are the classic activated phenotype, which may be involved in the production of reactive oxygen species and pro-inflammatory cytokines; however, M2 or "alternatively activated" microglia are an anti-inflammatory phenotype, which release anti-inflammatory cytokines and neurotrophic factors (Fernandes *et al.*, 2014). In terms of morphology, no difference between these phenotypes has been observed and it is not yet clear whether so

called M2 microglia can participate in pathological processes. Therefore, additional research is needed to better define microglial activation phenotypes.

Activated microglia are commonly seen in the pathologies of the CNS, and their involvement in neuroprotection in various models of CNS injury has been reported. For example, studies on models of transient cerebral ischemia have shown that inhibition of microglial activation via minocycline reduces neurogenesis following brain ischemia (Yanagisawa *et al.*, 2008; Kim *et al.*, 2009). The secretion of the cytokine tumour necrosis factor (TNF) from activated microglia has been shown to protect neurons from ischemia-induced death (Lambertsen *et al.*, 2009). It has also been demonstrated that activated microglia are neuroprotective and stimulate remyelination in mice through the synthesis of the IL-1β (Mason *et al.*, 2001). Also, many studies on other diseases have reported that activated microglia can, in some circumstances, protect CNS cells through the removal of potentially excitotoxic glutamate without producing excessing amount of inflammatory mediators (Shaked *et al.*, 2005), the engulfment of harmful invading neutrophils (Neumann *et al.*, 2008) or the degradation of amyloid-beta peptides deposits (Lee & Landreth, 2010).

On the other hand, a large body of evidence indicates that in a manner parallel to inflammation, exaggerated immune responses of activated microglia under specific circumstances have the potential to exert detrimental effects. Studies have reported the contribution of activated microglia to the pathologies of CNS diseases through the production of a range of neurotoxins, which include reactive oxygen species (Colton & Gilbert, 1987; Mao & Liu, 2008), nitric oxide (Moss & Bates, 2001; Liu *et al.*, 2002) and pro-inflammatory cytokines (Bi *et al.*, 2005; Balasubramaniam *et al.*, 2009). Activated microglia are found in the CNS in various neurodegenerative disorders, and they are thought to play a critical role in the pathology of these diseases. Several experimental studies have indicated that the inhibition of microglial activation via the drug minocycline leads to reduced levels of

neurotoxins and improved neuronal survival in neurodegenerative diseases (Jackson-Lewis *et al.*, 2002; Biscaro *et al.*, 2012). In Alzheimer's and Parkinson's diseases, microglial activation is observed in various regions in the CNS, and their secretions, such as neurotoxic cytokines and reactive oxygen species, have been shown to promote neuronal damage (Teismann & Schulz, 2004; Mandrekar & Landreth, 2010). Further, chronically activated microglia have been observed in multiple sclerosis, and they have been implicated in contributing to the progression of the disease through the generation of nitric oxide (Hill *et al.*, 2004). These data suggest that activated microglia can exacerbate inflammation by producing toxic molecules and thereby contributing to CNS pathologies by exacerbating damage to neuronal tissue.

Even if activated microglia are not neurotoxic, they can still induce CNS dysfunction through increased neuronal excitability. For instance, activated microglia release brain-derived neurotrophic factor (BDNF) to induce neuronal survival, but increased levels of BDNF can induce neuronal excitability (Biggs *et al.*, 2010). Collectively, these studies have indicated the complexity of the role of activated microglia in regulating the CNS immune response and the difficulty in determining whether activated microglia exert pathological or protective effects. Therefore the role of activated microglia in diabetes, whether pathological or protective needs to be determined.

1.5.4 Consequences of microglial activation in the CNS in diabetes

Previous studies on STZ diabetic rats have reported increased neuronal activity in cardiovascular centres of the brain (Luo *et al.*, 2002; Zheng *et al.*, 2006; Rana *et al.*, 2014). Recently, our laboratory has shown that microglia become activated in these centres, including the PVN and NTS, but the functional consequence of this activation is not known (Rana *et al.*, 2014). In contrast, the consequences of microglial activation are better studied in other parts of the brain in diabetes.

Diabetes is known to cause pathological changes in the peripheral nerves and to result in subsequent inflammation within the CNS (Tsuda *et al.*, 2005). Chronic inflammation is also a major characteristic of neuropathic pain, which is a common complication of diabetes. Various animal models of neuropathic pain are characterised by activated microglia in the dorsal horn of the spinal cord and an increased expression of pro-inflammatory cytokines, such as TNF-α and IL-1β, which have been implicated in increased neuronal excitability (Coyle, 1998; Schwei *et al.*, 1999; Tanga *et al.*, 2004; Obata *et al.*, 2006; Vega-Avelaira *et al.*, 2007). It has been documented that microglia-released pro-inflammatory cytokines can exacerbate chronic and neuropathological pain via the alteration of neuronal excitability in the spinal cord in STZ diabetic rats (Tsuda *et al.*, 2005). Enhanced expression of TNF-α and IL-1β has been observed in the spinal cords of STZ diabetic rats. The inhibition of microglia has been shown to reduce TNF-α and IL-1β levels and neuropathic pain-like behaviours in these animals (Talbot *et al.*, 2010; Pabreja *et al.*, 2011).

Another possible mechanism by which activated microglia can cause detrimental consequences in diabetes is the production of neurotoxic agents. Hyperglycaemia is believed to induce the generation of reactive oxygen species (Nishikawa *et al.*, 2000; Brownlee, 2005). These chemicals have been documented to be involved in the pathogenesis of diabetic neuropathic pain because antioxidant treatment prevents mechanical hypersensitivity in STZ diabetic rats (Cameron *et al.*, 2001; Li *et al.*, 2005a). An increase in oxidative stress in the spinal cord of STZ diabetic rats was reported and was attributed to microglial activation because the drug minocycline, which inhibits microglial activation, prevented the development of neuropathic pain associated with a decrease in oxidative stress (Pabreja *et al.*, 2011). These findings suggest that activated microglia could be a potential source of molecules that induce neurotoxicity and/or hyperexcitability in the context of diabetes; however, it appears that the functional consequence of microglial activation in the cardiovascular centres of the brain has not received adequate attention, and the possibility

that activated microglia may actually contribute to pathological cardiovascular changes in diabetes has not been investigated.

1.6 Astrocytes another cell types involved in neuroinflammation

Another important type of glial cells in the CNS is the astrocyte. As the name implies, astrocytes are star- or satellite-shaped cells with a central cell body and long, branched processes that extend outwards. The processes of adjacent astrocytes are connected to each other by gap junction channels that allow them to create a cellular network (Liu *et al.*, 2011).. Although high in number, the functions of astrocytes in the CNS have been somewhat mysterious in the field of neuroscience. For decades, they have been viewed as supporting structural cells that hold neuronal tissue together, but in recent years, astrocytes have been shown to be the most multifunctional cells in the CNS. In their resting state, they play a vital role in the maintenance of the neuronal microenvironment, which is required for the proper functioning of the CNS. In fact, astrocytes have the ability to affect neuronal function by regulating extracellular potassium and pH levels, regulating blood flow, providing energy metabolites for neurons, participating in synaptic functions, contributing to the metabolism of neurotransmitters and facilitating cellular communication (Koehler *et al.*, 2006; Hamby & Sofroniew, 2010). Thus, neuronal functioning is strongly linked to astrocytes via all of these processes, and astrocyte dysfunction may have a negative impact on neuronal activity.

Astrocytes also express a diversity of receptors that enable them to respond to virtually all known neuroactive compounds, neuropeptides, growth factors, cytokines, small molecules and toxins. These receptors enable astrocytes not only to participate in signal processing but also to function as sentinels that sense insults within the CNS (Dong & Benveniste, 2001; Liberto *et al.*, 2004), a role that has previously been attributed solely to microglia. In pathological conditions, astrocytes are activated and have the potential to display a diverse set of functional changes, including morphological changes, chemotaxis and proliferation (Ridet

et al., 1997). Astrocytes also perform immune functions and much like macrophage and microglia, are capable of phagocytosis (Magnus *et al.*, 2002). It now seems certain that astrocytes have an important function in the initiation and regulation of the immune response of the CNS (Hauwel *et al.*, 2005; Farina *et al.*, 2007). Therefore, while microglia cells are the principal immune cells in the CNS and play a significant role in host defence, astrocytes are also considered to be one of the immune effecter cells in the CNS.

1.6.1 Astrocytes are key players in CNS immunity

In their basal state, astrocytes have a small cell body and long, highly branched processes; however, in response to CNS pathological challenges, such as stressful stimuli, brain injury, infection, inflammation or neurodegenerative diseases, they undergo a process in which they are recognised as activated astrocytes. This process is associated with morphological changes that vary according to the stimulus but include the enlargement of the soma (Pekny & Nilsson, 2005). Furthermore, cell-surface molecules and cellular components, such as glial fibrillary acidic protein (GFAP), are upregulated when astrocytes are activated (Lee & Brosnan, 1997). In the CNS, GFAP is specifically produced by astroglial cells, and it is widely used as a marker of astrocytes. Astrocyte activation causes a marked upregulation of GFAP that can be visualised with antibodies (Pekny & Pekna, 2004). Upon stimulation, astrocytes act as immune competent cells within the CNS, presenting antigens by expressing major histocompatibility complexes to activate T-cell activation (Benveniste, 1992; Kraus *et al.*, 1992; Montgomery, 1994). Furthermore, as modulators of immune function, astrocytes display expressions of toll-like receptors, such as TLR3, which are involved in the generation of innate immune responses (Farina *et al.*, 2007; Jacobs *et al.*, 2011), and they secrete a wide variety of chemokines and cytokines that act as immune mediators, such as IL -1, -2, and -6, alpha and beta interferons, TNF and prostaglandins (Montgomery, 1994; John *et al.*, 2003). Astrocytes also undergo other complex processes following activation, including an increase

in number; an upregulation of intermediate filament components (nestin and vimentin) (Lee & Brosnan, 1997); a release of neurotrophic factors; a release of various growth factors, including a basic fibroblast growth factor, glial-derived growth factor, platelet-derived growth factor and an insulin-like growth factor-I (Travis, 1994; Merrill & Jonakait, 1995); a release of antioxidants (Wilson, 1997; Dringen *et al.*, 2000); the generation of reactive oxygen species (Simmons & Murphy, 1992; Kugler & Drenckhahn, 1996; Marchetti & Abbracchio, 2005); and glial scar formation (Silver & Miller, 2004). Thus, activated astrocytes help orchestrate the immune responses of the CNS.

1.6.2 The role of activated astrocytes in pathological conditions

There is compelling evidence that activated astrocytes have the potential to protect neural functions and promote tissue repair. Their protective effects can be mediated through the removal of potentially excitotoxic glutamate (Bush *et al.*, 1999; Swanson *et al.*, 2004); the production of glutathione to protect the CNS from oxidative stress (Shih *et al.*, 2003; Vargas & Johnson, 2009); neuroprotection via adenosine release (Lin *et al.*, 2008); the protection of neurons from ammonia toxicity, which is implicated in CNS dysfunction (Rao *et al.*, 2005); the protection of CNS cells by the degradation of amyloid-beta peptides (Koistinaho *et al.*, 2004); the repair of the BBB following injury (Bush *et al.*, 1999); and the reduction of oedema following injury (Bush *et al.*, 1999).

Moreover, astrocytes' involvement in the regulation of CNS inflammation in response to pathological insults is of particular interest regarding the neuroprotective effects of activated astrocytes. A large body of experimental studies have indicated that the inhibition of activated astrocytes is associated with a rapid infiltration of inflammatory cells into neural tissue following spinal cord injury (Okada *et al.*, 2006; Herrmann *et al.*, 2008). Similarly, Bush et al. showed that an ablation of activated astrocytes leads to a prolonged infiltration of

leucocytes associated with the degeneration of neurons in the injured CNS due to the absence of the glia scar (1999).

Under pathological conditions, activated astrocytes may also result in neuronal death and/or hyperactivity due to their involvement in CNS inflammation. Activated astrocytes can interact with microglia cells, the key players in the immunological response of the CNS, and modulate microglial inflammatory responses by releasing pro-inflammatory or anti-inflammatory cytokines (Min *et al.*, 2006; Farina *et al.*, 2007). Both *in vivo* and *in vitro* studies have reported TNF-α and IL-1β secretion from astrocytes (as reviewed in Dong and Benveniste 2001). An enhanced expression of TNF-α and IL-1β has been observed in activated spinal astrocytes following peripheral nerve injury (Wei *et al.*, 2008). The inhibition of these pro-inflammatory cytokines has been shown to reduce neuropathic pain-like behaviours, suggesting that TNF-α and IL-1β contribute to increased neuronal excitability (Milligan *et al.*, 2001). Moreover, studies on transgenic models that selectively delete or inhibit inflammatory molecules in astrocytes, such as transcription factor NF-kB, showed a reduction in the expression of pro-inflammatory cytokines and an improvement in functional recovery following spinal cord injury (Brambilla *et al.*, 2005) or experimental autoimmune encephalomyelitis (Brambilla *et al.*, 2009). These findings suggest that activated astrocytes could induce or facilitate neurotoxicity in various diseases.

Interestingly, in animal models of neuropathic pain, the rapid activation of microglia has been observed in the spinal cord followed by a delayed activation of astrocytes (Sweitzer *et al.*, 1999; Winkelstein *et al.*, 2001). Astrocyte activation persisted for a longer period compared with microglial activation (Coyle, 1998; Milligan & Watkins, 2009; Benarroch, 2010). At early time periods, microglial inhibition following peripheral nerve injury is effective on pain behaviours. In contrast, this inhibition at later stages has been shown to be less effective on pain behaviours (Raghavendra *et al.*, 2003; Nakagawa *et al.*, 2007; Padi & Kulkarni, 2008)

when compared with the inhibition of astrocytes in these animals (Meller *et al.*, 1994; Okada-Ogawa *et al.*, 2009). It has been suggested that microglial activation may induce astrocyte activation. As such, we propose that astrocyte activation may also have a greater effect in causing long-term complications of diabetes than microglia; however, whether activated astrocytes in the PVN contribute to neuronal activation and thereby promote diabetic cardiovascular complications requires investigation. Activated astrocytes have been observed in the PVN of diabetic rats (Luo *et al.*, 2002), but the time course, mechanism and consequences of astrocyte activation in the PVN have not been investigated.

1.7 Inflammation in cardiovascular centres and its role in regulating sympathetic activity and the cardiovascular system

Although there is a lack of studies on the consequence of inflammation in the cardiovascular centres of diabetic rats, the consequences of inflammation in these areas is better studied in other cardiovascular diseases, such as hypertension and heart failure. A common cause of heart failure is myocardial infarction, which is characterized by an elevation in sympathetic nerve activity. Brain pro-inflammatory cytokines are implicated in a pathological increase in the sympathetic drive. An elevation of pro-inflammatory cytokines, such as TNF-α, IL-1β and IL-1, in the periphery has also been reported following myocardial infarction, and increased levels of these cytokines have been shown to correlate with the severity of heart failure (Tsutamoto MD *et al.*, 1998; Maeda *et al.*, 2000; Rauchhaus *et al.*, 2000). Moreover, there is a large body of evidence suggesting that pro-inflammatory cytokine levels in the hypothalamic PVN are also elevated in heart failure, thereby contributing to the abnormal sympathetic overactivity (Felder *et al.*, 2003; Francis *et al.*, 2004a; Francis *et al.*, 2004b). While the mechanisms responsible for increasing pro-inflammatory cytokines in the PVN are not known, it has been suggested that peripherally produced pro-inflammatory cytokines may cross the blood brain barrier (Banks *et al.*, 1995). Despite this, local production of cytokines also has been demonstrated because mRNA levels for pro-inflammatory cytokines are elevated in the PVN (Francis *et al.*, 2004b). In addition, in rats with acute myocardial infarction, an elevated expression of TNF-α and IL-1β has also been reported in the PVN. These cytokines induce the production of reactive oxygen species in the PVN and contribute to exaggerated neuronal activity and increased sympathetic activity (Guggilam *et al.*, 2007; Guggilam *et al.*, 2008). While microinjection of TNF-α into the PVN has been shown to increase renal sympathetic activity, most likely through the activation of PVN neurons (Felder *et al.*, 2003; Kang *et al.*, 2008), an infusion of a TNF-α blocker into the PVN of rats with heart failure decreased sympathetic hyperactivity (Kang *et al.*, 2010). We have

demonstrated that microglia are activated in the PVN following myocardial infarction in rats, suggesting that these cells may be a source of the local production of pro-inflammatory cytokines in the PVN (Rana *et al.*, 2010; Dworak *et al.*, 2012). Microglial activation was also observed in the NTS and RVLM following myocardial infarction in rats (Dworak *et al.*, 2014).

Hypertension is another condition for which neuroinflammation and increased activity in the sympathetic nervous system may be involved in the pathogenesis. TNFα and IL-1β mRNA were found to be increased in the PVN of spontaneously hypertensive rats (Agarwal *et al.*, 2011) and angiotensin II–induced hypertension rats (Kang *et al.*, 2009b). Other researchers revealed that an ICV infusion of recombinant IL-1β and TNF-a results in increased sympathetic nerve activity and elevated arterial blood pressure in conscious control animals (Kimura *et al.*, 1993; Kannan *et al.*, 1996; Ufnal *et al.*, 2005). Specifically, PVN stimulation with these mediators of inflammation has been shown to have the same effect in anesthetized rats (Shi *et al.*, 2011). These studies suggest that pro-inflammatory cytokines in the PVN play a key role in the regulation of the cardiovascular system through the modulation of PVN neuronal activity.

Previous data from our laboratory indicates that the injection of activated microglia into the PVN in naïve rats produces an increase in blood pressure. Interestingly, it has been postulated that microglia and their pro-inflammatory cytokines in the PVN participate in the development of Ang-II-induced hypertension. Chronic Ang II infusion has been shown to increase the mean arterial pressure and to activate microglia in the PVN (Shi *et al.*, 2010a). The inhibition of microglial activation via ICV minocycline treatment reduced arterial blood pressure, reversed cardiac hypertrophy and reduced the pro-inflammatory cytokine expression in the PVN in response to Ang II infusion (Shi *et al.*, 2010a). Moreover, the

microinjection of anti-inflammatory cytokine IL-10 into the PVN also resulted in attenuated microglial activation and reduced hypertension in this model (Shi *et al.*, 2010a).

Similarly, the NTS and RVLM regions, which are involved in the regulation of cardiovascular functions, have been found to exhibit abnormal inflammatory responses in spontaneously hypertensive rats. In the RVLM, the expression of TNFα and IL-1β are increased in hypertensive rats compared with normotensive rats (Agarwal *et al.*, 2011). There is also evidence of an increased reactive oxygen species production in the RVLM in hypertensive rats. These pro-inflammatory cytokines and reactive oxygen species are well-documented to induce neuronal activity via the transcription factor NFκB expression (Shi *et al.*, 2010b; Hirooka *et al.*, 2011). Thus, abnormal inflammatory responses seen in the RVLM may contribute to sympathetic overactivity in this model. The NTS of spontaneously hypertensive rats also exhibited signs of inflammation, including the expression of junctional adhesion molecule-1 (Waki *et al.*, 2007). In addition, Takagishi et al. reported that the microinjection of IL-6 into the NTS decreased baroreceptor sensitivity, a phenomenon associated with hypertension (2010).

Collectively, these findings support the theory that inflammation in cardiovascular centres induces the over-activation of sympathetic nerves, which disrupts the normal functioning of the heart and blood vessels and may contribute to cardiovascular complications in other diseases. Whether the same mechanisms are involved in the increase in sympathetic nerve activity in diabetes is not known.

1.8 Rationale and research questions

In summary, cardiovascular complications, such as hypertension and cardiomyopathy, are common in diabetes mellitus. It is well-known that an elevated sympathetic drive occurs in diabetes and may contribute to the development of diabetic cardiovascular complications. Although the cause of the abnormal activation of sympathetic nerves in diabetes is not well understood, there is increasing evidence that increased levels of pro-inflammatory cytokines at the level of cardiovascular centres play an important role in other cardiovascular disorders. Diabetes is known to cause pathological changes in the peripheral nerves and to result in subsequent inflammation within the CNS, at least in the spinal cord. Our previous data indicated that microglia become activated in the areas of the brain that control the cardiovascular system in STZ diabetic rats (Rana *et al.*, 2014). Because activated microglia are involved in mediating inflammation, we hypothesize that they may influence the cardiovascular control centres to cause increased sympathetic nerve activity, which in turn contributes to cardiovascular complications. Thus, we will also investigate whether the inhibition of activated microglia in the PVN can reduce or prevent cardiovascular changes in STZ diabetic rats.

Astrocytes also contribute to inflammation in the CNS, and published studies have demonstrated that activated astrocytes have the potential to increase neuronal hyperactivity and thereby exaggerate pathological processes and are potentially more important than microglia in the long-term complications of diabetes. Thus, we will investigate whether astrocytes are activated in the PVN of diabetic rats at different time points following STZ treatment and whether microglia induce astrocyte activation in the PVN in these animals.

As mentioned previously, giving STZ-treated diabetic rats 1% NaCl to drink causes a marked elevation in their blood pressure (Maeda *et al.*, 2007). The mechanisms underlying this hypertension are poorly understood but may be explained in part by an increased blood

volume. Interestingly, hypertension develops quickly at 2 weeks after STZ treatment in these animals; however, at this time period (2 weeks), STZ diabetic rats show no microglial activation in the cardiovascular centres (Rana *et al.*, 2014). Despite the possible effects of drinking 1% NaCl on blood volume, whether 1% NaCl also influences inflammation in cardiovascular centres to cause an increase in blood pressure in STZ diabetic rats is not known. Thus, we will investigate the effects of 1% NaCl on blood pressure, baroreceptor reflex, cardiac function and cardiovascular centre inflammation in STZ diabetic rats. In addition, we will investigate whether inhibition microglia in the PVN can reduce or prevent diabetic complications in STZ diabetic rats given 1% NaCl, as observed in the animal model of angiotensin II-induced hypertension (Shi *et al.*, 2010a).

Previously, we have reported the time range of microglial activation in the PVN in STZ diabetic rats (Rana *et al.*, 2014), but whether this activation also occurs in other species and other models of diabetes is not known. The mouse is the most suitable for genetic manipulations and can also be used in cellular and molecular studies. Thus, it is useful to examine whether microglia and neurons are activated in the PVN in STZ diabetic mice and the time course of any activation. This would also provide information about whether the results obtained in STZ diabetic rats are due to the effects of diabetes or are specific to the model tested.

Antioxidant treatment has been shown to prevent cardiovascular diseases in humans (Sugamura & Keaney, 2011). Systemic infusion of hydrogen sulphide (H_2S), which is known to have antioxidant properties, has been reported to reduce oxidative stress and blood pressure in an animal model of angiotensin II (Al-Magableh *et al.*, 2015), but whether the beneficial effect of H_2S is mediated via a central action is not clear. A deficiency in the levels of H_2S has been shown to occur in diabetic humans as well as animals (Jain *et al.*, 2010), and this deficiency contributes to the development of diabetic complications (Lefer,

2008; Szabo, 2012). In fact, H_2S also has anti-inflammatory actions, and several studies showed that H_2S reduces microglial and neuronal activation *in vitro* (Hu *et al.*, 2007; Umemura & Kimura, 2007; Hu *et al.*, 2009). Thus, we will investigate whether hydrogen sulphide influences PVN inflammation in STZ diabetic mice.

Type 2 diabetes in humans is also associated with cardiovascular disease and an overactivity of the sympathetic nerves that control the cardiovascular system. A high fat diet is a major contributing factor in the pathogenesis of type 2 diabetes, the most common form of diabetes in humans. If hyperglycaemia results in subsequent brain inflammation in STZ diabetic rats, it may be expected to also observe brain inflammation in type 2 diabetic animals. Thus, we will investigate whether microglia and neurons are activated in the PVN of mice treated with a low dose of STZ and fed a high-fat diet.

1.8.1 Project specific aims

The overall aim of this project is to determine whether inflammation within in the region of the hypothalamus that controls cardiovascular function can contribute to the development of cardiovascular and autonomic complications in diabetes. To achieve this, we address the following specific aims:

- To determine whether the inhibition of microglial activation or action in the PVN can ameliorate some of the cardiovascular complications of STZ diabetes.
- To investigate the time course of astrocyte activation in the PVN in STZ-treated diabetic rats and whether inhibiting the activation of microglia can prevent the activation of astrocytes in these animals.
- To investigate the effects of 1% NaCl on cardiovascular parameters and whether 1% NaCl influences inflammation in the CNS cardiovascular centres in STZ-diabetic rats and

whether the inhibition of microglial activation in the PVN can reverse any of the changes observed.

- To investigate whether microglia are activated in the cardiovascular centres of the brain in STZ diabetic mice and the time course of this activation.

- To investigate the effect a high-fat diet and hydrogen sulphide on PVN inflammation in STZ diabetic mice.

Chapter Two: Materials and Methods

2.1 Animals

All animal experiments were approved by the RMIT University animal ethics committee and were conducted in accordance with the National Health and Medical Research Council guidelines. Male Sprague Dawley rats and C57BL6/J mice were obtained from the Animal Resource Centre (Perth, Australia).

All animals were kept in standard high-top rat cages with environmental enrichment in a temperature-controlled room on a 12:12 hour light/dark cycle (lights on at 7:00 A.M.) in the Animal Facility (RMIT University, Victoria, Australia). Two male Sprague Dawley rats were housed per rat cage, and 4-5 C57BL6/J mice were housed per mouse cage. The plastic cages were cleaned and replaced once each week. Food and water were provided for all animals at all times. All animals were acclimatized for 4-7 days prior to undergoing any experimental procedure.

2.2 Animals models of diabetes

2.2.1 STZ / NaCl treatment

Male Sprague Dawley rats weighing 170-200 grams were exposed to a heat lamp for 2-3 minutes in a small animal cage to dilate the tail vasculature and then were given an intravenous injection of streptozotocin (STZ, 55mg/kg, Sigma) through the tail vein using a 27-gauge needle. The control rats were injected with a citrate buffer (0.42% in sterile saline, pH 4.5). Each week thereafter, blood was taken by heat lamp exposure followed by puncturing the tail vein with a 30-gauge needle to evaluate the blood glucose levels using a One Touch glucometer (Accu-Chek Perfoma). When the blood glucose exceeded 25 mM, the subcutaneous injection of insulin (3-4 units twice per week) was given. Two days after the induction of diabetes, the rats were placed in individual cages with free access to a 1% NaCl

solution **OR** tap water as a control. The amount of saline or water ingested was monitored daily by weighting the water bottles.

2.2.2 STZ / high fat diet treatment

Male C57BL/6J mice (10 weeks old) were provided standard standard lab chow diet (8% calories from fat, 21% calories from protein, and 71% calories from carbohydrate) or a high-fat diet (45% calories from fat, 20% calories from protein, and 35% calories from carbohydrate) for up to 10 weeks. Mice were then injected with either vehicle (saline) or a low dose of streptosotocin (STZ, 40 mg/kg/day) for five consecutive days in order to induce diabetes. Mice were then received an intraperitoneal injection with either or a citrate buffer or a low dose of streptosotocin (STZ, 40 mg/kg/day) for five consecutive days in order to induce type 2 diabetes.

2.2.3 STZ/ NaHS treatment

Male C57BL/6J mice aged 8 weeks (Animal Resource Centre, Western Australia) were randomly divided into three groups: Vehicle (Citrate buffer) control, diabetic and diabetic treated with NaHS. Type 1 diabetes was induced with streptozotocin (STZ, 60mg/kg/day, ip) for two weeks. The control group received an equivalent volume of the vehicle (0.1M citrate buffer, pH 4.5) daily for two weeks. NaHS (100µmol/kg/day, ip) was given daily for the final four weeks. Blood glucose measurement was performed at the beginning and the end of the experiment and twice a week during the experiment for diabetic mice. Insulin (0.1U, i.p, 50% isophane insulin (Protaphane) + 50% neutral insulin (Actrapid)) was given when the blood glucose level exceeded 30mM. Control group blood glucose was also measured during week 5. Blood samples were obtained from the tail vein and blood glucose concentration was measured using a one touch glucometer ACCU-CHEK Advantage (Roche, NSW, Australia) and glycated haemoglobin (HbA_{1c}) using an in2it (II) analyser (281-0000EX) (Bio-Rad,

NSW, Australia). Induction of diabetes was considered successful when the blood glucose level was greater than 20mM.

2.3 Surgical procedure

2.3.1 Blood Pressure and heart rate recording under anesthesia

At 2 or 6 weeks following STZ/citrate buffer treatment, the animals were anaesthetized using isofluorane (5% in air or oxygen using a small animal anaesthetic Vetquip machine) by placing the animal in a closed box. Immediately following induction, the animals were placed on a heat pad, and isofluorane (2-3% in air or oxygen) was continually provided via a mask. For cannulation, the right groin region was shaved and swabbed with 70% alcohol. A midline incision (4-5 mm) through the skin in the right groin region was then made to expose the femoral vein and artery. A blunt dissection was made to clear the subcutaneous fascia and to separate the femoral vein from the artery. Two sutures (silk 3/0, Dynek Pty, Australia) were placed under the femoral vein and artery and pulled tight to obstruct the blood flow during the insertion of a separate cannula. Both the femoral vein and artery were then pricked with very fine McPherson-Vannas 8cm scissors (World Precision Instruments Inc., FL, USA). The cut in the femoral vein and artery was expanded by inserting closed fine tweezers # 5 (World Precision Instruments Inc., FL, USA) into the cut to allow a cannula tip to be introduced into the vessel. The cannula, filled with heparinised saline (50U/mL), was inserted into the femoral vein and artery while the tweezer was withdrawn. The cannula was then threaded along the femoral vein and artery and secured with a sutuer. The skin incision was then closed using stiches (silk 0-USP, Dynek Pty, Australia). At this stage, the anaesthetic agent was changed to urethane (1-1.4 g/kg intravenously), which has the least effect on dampening the cardiovascular reflexes compared to the other anaesthetics (Barringer *et al.*, 1990). Supplemental doses of urethane were given as required. Every 5 minutes, the depth of anaesthesia was tested using the corneal and pedal reflexes. The arterial cannula was attached

to a transducer for monitoring blood pressure and heart rate. Prior to elicit the baroreceptor reflex, mean arterial pressure and heart rate were calculated from the arterial pressure pulse over a period of 5 minutes using labchart (ADInstruments, Bella Vista, NSW, Australia).

2.3.2 Conscious blood pressure recording via tail cuff

The systolic blood pressure in the restrained conscious animals was recorded via a tail cuff blood pressure measurement system (ML125 NIBP Controller and MLT125/R Pulse Transducer/Pressure Cuff for NIBP - Rat). At 2 weeks following STZ/buffer treatment and 10 days after minocycline or saline ICV infusion, the rats initially trained 2-3 times for recording blood pressure to acclimatize them to preheating, the restrainer and the inflation and deflation of the tail cuff. Prior to actual recording, the rats were exposed to a heat lamp for 5 minutes at 30°C and then placed in restrainers, and the tail cuff was placed around the tail of the rat for 10-15 minutes. Then, the systolic blood pressure was recorded.

2.3.3 Baroreceptor reflex testing

Before the beginning of the experiment recording, blood pressure and heart rat were allowed to stabilize for at least five minutes. The venous cannula was then flushed with saline (0.2ml), and five minutes later, four different doses of phenylephrine (1, 2, 5 and 10 μg/kg) were given I.V. These doses were given in sequence to elicit the baroreceptor reflex. For each dose, 0.2 ml of phenylephrine solution was injected followed by flushing with 0.2 ml of saline. From 5-8 minutes were allowed between each dose of phenylephrine to allow blood pressure and heart rate to return to their original baseline levels before the injection of the next dose. The peak values of mean arterial blood pressure and heart rate in response to increasing doses of phenylephrine were measured and recorded using a PowerLab data acquisition system (ADInstruments, NSW, Australia).

2.3.4 Implantation of brain infusion catheters and osmotic pumps

One week following STZ/citrate buffer treatment, the animals were anaesthetized using isofluorane as previously described. The head of the rat was shaved and swabbed with 70% alcohol before being placed in a David Koph stereotaxic frame, such that the bregma and lambda were positioned on the same horizontal plane to allow for the accurate placement of the cannula in the lateral ventricle using a coordinate system. A midline incision was then made through the skin to expose the skull. The bregma and lambda were identified, and a small hole (1-2 mm diameter approximately) was drilled, centred at 0.7 mm caudal and 1.8 mm lateral from the bregma. Another hole was then drilled at a distance of 5mm from the initial hole to allow for the insertion of jeweller screws to act as anchors. A guide cannula was then inserted into the lateral ventricle (stereotaxic coordinates: 0.7 mm caudal to bregma, 1.8 mm lateral to midline, and 3.7 mm ventral to the surface of the dura). It was then advanced approximately 4 mm down to the lateral brain ventricle using a micromanipulator attached to the stereotactic frame. After the insertion procedure, dental cement was applied to the skull, the cannula and the screw. The dental cement was then set using a UV light device. Next, the skin of the back was separated from the underlying tissue by blunt dissection to insert a small osmotic pump (Alzet, 2004, Weight 1.1g, volume 1ml) under the skin. The tube attached to the osmotic pump was then connected to the intraventricular cannula to allow direct infusion of either saline (0.9% NaCl) or minocycline (5 μg/h) over a period of 4 weeks. Upon completion of the implantation procedures, the incision was sutured (silk 0-USP, Dynek Pty, Australia), and the animal was allowed to recover.

2.3.5 Implantation of telemetric blood pressure monitoring devices

Three weeks after the STZ/citrate buffer treatment, the animals were anaesthetized using isofluorane as previously described. The abdominal wall was shaved and swabbed with 70%

alcohol before a midline incision was made through the skin. With the aid of retractors, the intestine and fat tissue were pulled aside to expose the abdominal aorta. Then, the abdominal aorta was dissected free from the surrounding tissue under an operating microscope. Two sutures (silk 3/0, Dynek Pty, Australia) were placed under the abdominal aorta to obstruct the blood flow during the introduction of a catheter. The aorta was then cut using McPherson-Vannas 8cm scissors or a 27-gauge needle followed by introducing the catheter and gluing using tissue glue. A piece of surgical mesh was applied and glued to the outside of the aorta to further stabilize the catheter. The body of the telemetry was covered with sterilised surgical mesh and placed in the abdominal cavity. Upon completion of the implantation procedures, the muscle layers and skin were sutured with absorbable sutures, and the animal was allowed to recover.

2.3.6 Echocardiographic measurements

Six weeks after the STZ/citrate buffer treatment, the animals were anaesthetized with pentobarbitone 40 mg/kg i.p. To assess the cardiovascular function in these animals, a Vivid e echocardiography machine with a 10-MHz phased array probe (GE Vingmed, Horten, Norway) was used. The left chest, abdomen and groin were shaved and swabbed with 70% alcohol followed by applying a layer of acoustic coupling gel. A two-dimensional and Doppler echocardiography were performed with the animal in the left recumbent position. M-mode echocardiograms were recorded from the parasternal short-axis view of the left ventricle at the mitral valve level to measure the left ventricular internal diameter in the systole (LVIDs) and the left ventricular internal diameter in the diastole (LVIDd). These parameters were assessed using at least five cardiac cycles from the M-mode image. Next, a flow velocity was assessed using pulsed-wave Doppler echocardiography from the apical four-chamber view. Mitral inflow velocities at early diastole (E) and late diastole (A) were evaluated, and their ratio (E/A ratio) was calculated. The Doppler images were obtained with

a heart rate at approximately 300-350 bpm and all measurements were obtained from an average of at least three consecutive heart beats.

2.3.7 Ventricular hemodynamics

Immediately after the echocardiography procedures, a midline incision was made through the neck skin to expose the right carotid artery. The artery was dissected free from the surrounding tissue under an operating microscope, and 2 fine sutures (silk 3/0, Dynek Pty, Australia) were placed under it and pulled tight to obstruct the blood flow. The carotid artery was then pricked with very fine McPherson-Vannas 8cm scissors (World Precision Instruments Inc., FL, USA). A telemetry transducer catheter was used to insert it into the carotid artery, while monotring the blood pressure trace. It was then advanced 4-5 mm approximately into the left ventricle to assess the left ventricular end diastolic pressure, dP/dt max and heart rate using a PowerLab data acquisition system (ADInstruments, NSW, Australia).

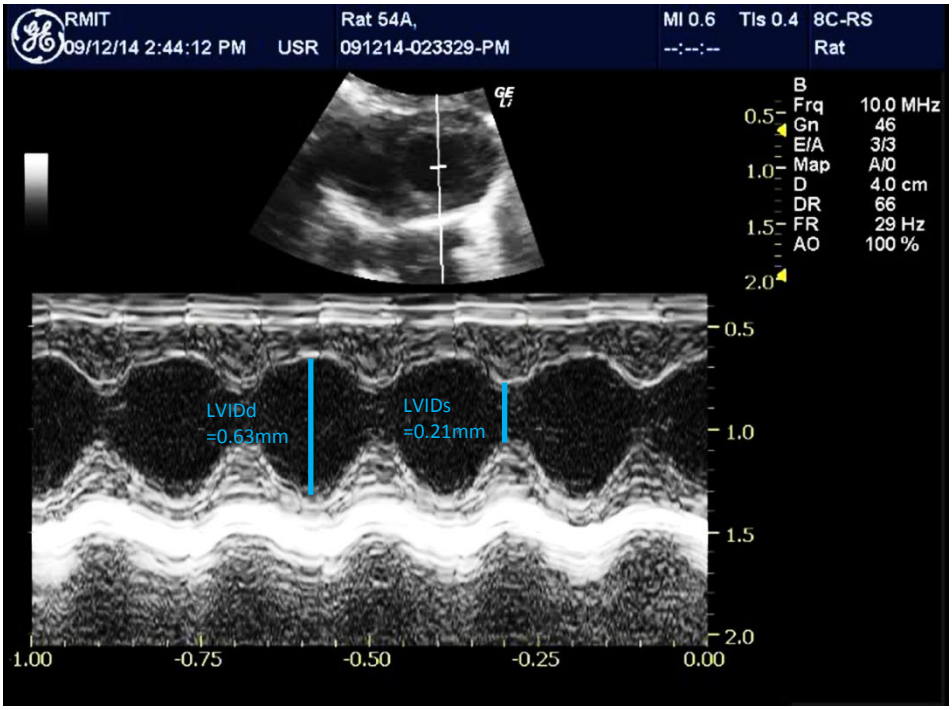


Figure 2-1. Echocardiogram- representative M-mode image and measurements of left ventricular internal diameter in systole (LVIDs) and left ventricular internal diameter in diastole (LVIDd) in control animals.

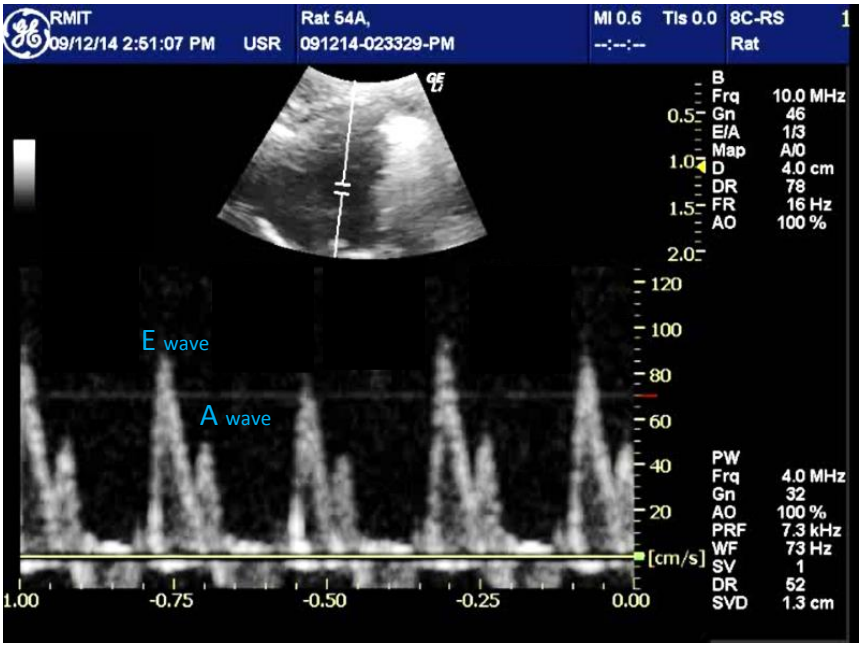


Figure 2-2. Functional echocardiography: apical four-chamber view pulsed-wave Doppler displaing E and A wave in control animals.

2.4 Blood collection

For blood collection, a 1ml syringe was flushed with 6% Ethylenediaminetetraacetic acid and 10 ul of the same solution was added to the syringe before connecting it to the venous cannula to collect blood while the animal was under deep anaesthesia. A minimum of 500ul of blood was collected in each syringe. The haematocrit and haemoglobin concentration were determined using ACTdiff 5 blood analyser (Beckman, USA). Blood samples were immediately centrifuged at 1500 rpm for 10 minutes. Plasma osmolarity was measured by using an osmometer (Gonotec Osmomat 030).

2.5 Tissue collection

2.5.1 Perfusion

Two, 6 or 8 weeks after the STZ/buffer treatment, the animals were anaesthetied with pentobarbital sodium (180mg/kg of body weight). The abdominal skin was cut to expose the end of the sternum, which was grasped and the diaphragm was cut laterally on both sides to access the rib cage. The pump needle (25-gauge) was inserted into the aorta through the left ventricle and clamped before the atrium was cut. Phosphate buffered saline (PBS) (200ml) was perfused at a pressure of up to 100 mmHg and when the blood had cleared from the body, the perfusing solution was changed to 4% paraformaldehyde solution.

2.5.2 Brain collection

The occipital bone and vertebrae were exposed via a midline incision in the skin of the head. With the aid of rongeurs, the occipital bone was carefully removed to expose the cerebellum and brainstem. The bone over the posterior part of the nasal cavity was then cut with the rongeurs. The dura mater over the cortex of the cerebellum, and the cerebral hemispheres were elevated slightly and cut by sharp scissors. The brain was elevated using a long thin spatula and the optic nerves cut to allow it to be removed and post-fixed in a 4%

paraformaldehyde solution for 4 hours at 4°C. It was then placed in a cryoprotection solution (30% sucrose in PBS) for at least 2 days at 4°C before sectioning processing.

2.5.3 Cryostat sectioning

A Leica CM1900 Cryostat was used for freezing and sectioning brain tissue. A few drops of embedding medium (OCT, **O**ptimal **C**utting **T**emperature) were placed on the surface of the specimen disc and the brain was positioned onto it. The tissue was frozen within the cryostat chamber at –20 °C for approximately 20 minutes. The transverse sections of the brain included were the paraventricular nucleus, rostral ventrolateral medulla and nucleus of the solitary tract, which were sectioned at 40 µm thickness and immediately mounted onto gelatine coated slides and left to dry for 2 hours at room temperature.

2.6 Immunohistochemistry

Immunohistochemistry was used to identify various markers of glial cells (microglia and astrocytes) and neurons in the cardiovascular centres of diabetic animals. CD11b and Glial Filament/Fibrillary Acidic Protein (GFAP) markers were used to show morphological changes in microglia and astrocytes, respectively. In addition, nuclear c-fos protein immunoreactivity was used to identify activated neurons in the brain tissue (Dworak *et al.*, 2014).

All sections of the brain were washed 3 times in PBS (each wash for 5 min), then the endogenous peroxidase activity was destroyed by incubating with 0.5% H_2O_2 (Sigma-Aldrich, Australia) for 30 minutes at room temperature. The sections were blocked with 10% normal horse serum for 1 hour at room temperature to prevent nonspecific binding and then washed 3 times in PBS. The PBS was then replaced and the sections were washed 3 times in PBS before incubation with 0.5% Triton X (Sigma-Aldrich, Australia) in PBS for 30 minutes

at room temperature to enhance antibody penetration, and then washed 3 times in PBS. The sections were then incubated with the appropriate primary antibodies.

A mouse monoclonal primary antibody (OX-42 produced in house diluted 1:150 in 2% normal horse serum and 0.3% Triton X-100 in PBS) was applied to the sections, and the sections were placed into a humidified environment and left for three nights at –4°C. The sections were then washed three times with PBS and incubated with a biotinylated antimouse secondary antibody (diluted 1:400 in 2% normal horse serum in PBS: catalogue number BA-2001; Vector Laboratories,Burlingame, CA, USA) for 2 hours at room temperature. Next, the sections were washed three times with PBS and incubated with extravidin (diluted 1:40 in 2% normal horse serum in PBS) for 2 hours at room temperature. The sections were then washed three times with PBS and the slides were placed in 0.05% 3,3-diaminobenzidine hydrochloride (DAB; Sigma-Aldrich, Australia) in PBS for 10 minutes. Then 5 μl of 30% hydrogen peroxide was added to start the reaction for 10 to 15 minutes at room temperature. The reaction was then stopped by washing them with PBS. The slides were left for 1 hour at room temperature to dry and then cover-slipped with Depex mounting medium (BDH Lab Supplies, Poole, UK).

To detect changes in astrocytes and c-fos activity, similar techniques were applied using a rabbit monoclonal primary antibody (GFAP Sigma-Aldrich, Australia: diluted 1:150 in 2% normal horse serum and 0.3% Triton X-100 in PBS) and a rabbit polyclonal primary antibody (Fos, diluted 1:400 in 2% normal horse serum and 0.3% Triton X-100 in PBS: c-Fos (K-25): sc-253, Santa Cruz Biotechnology, CA, USA), respectively and then incubated with the anti-rabbit secondary antibody (diluted 1:400 in 2% normal horse serum in PBS: catalogue number B8895, Sigma-Aldrich, Australia).

2.7 Quantification and morphological analysis

OX-42 antibody staining was used to identify morphological changes in microglia cells. The quantification and morphological analyses of microglia were performed as previously discussed (Rana *et al.*, 2014) with a light microscope using ×400 magnifications. On the basis of subjective classification, non-activated microglia were identified by their small soma from which there emanated extensive, highly branched, long, thin processes. However, activated microglia were defined by three main criteria; stronger immunohistochemical staining for the marker CD11b (clone OX-42), the presence of a clearly enlarged soma, and marked changes in the appearance of the processes which were now reduced in number, but considerably thicker and shorter giving a stubby appearance. The number of microglia was counted in squares measuring 0.2 × 0.2 mm in size in all examined regions. The numbers of normal and activated microglia in the PVN were counted in two squares to cover the region from 0-1 mm lateral to the edge of third ventricle. In the NTS and RVLM, a similar analysis was performed. The percentage of activated microglia was determined by counting the number of activated microglia and total microglia in all examined regions. To quantify the length of microglial processes, the longest process was measured in 10 randomly selected microglia in one square in each brain region examined. A similar analysis for accounting for the number of neuronal activation was performed. The quantification of microglial and neuronal histology was done with the experimenter blinded to the experimental conditions in two sections from each region in all animals.

2.8 Statistical analysis

The statistical analysis was performed using GRAPH PAD PRISM, version 6 (GraphPad Software Inc., San Diego, CA, USA). Data are expressed as the mean ± SEM. The significance between groups was evaluated using a one way ANOVA. If a statistical

difference was obtained, subsequent comparisons between all groups were made using Tukey's post hoc test.

Chapter Three: Neuroinflammation in the Paraventricular Nucleus in STZ Diabetic Rats

3.1 Introduction

Cardiovascular complications are common in diabetes mellitus (O'Keefe *et al.*, 1999). Evidence demonstrates that overactivation of the sympathetic nerve is crucial in the development of these complications (Perin *et al.*, 2001; Augustyniak *et al.*, 2002; Patel *et al.*, 2011). Increased activity of the sympathetic nervous system has also been reported in both humans and animals with type 1 diabetes (Perin *et al.*, 2001; Patel *et al.*, 2011), but how this occurs is not clear.

The paraventricular nucleus (PVN) is a region of the brain that controls sympathetic nerve activity and the cardiovascular system (Kannan *et al.*, 1989; Badoer, 2001). Our study and other previous studies on rats injected with STZ to induce chronic hyperglycaemia have indicated that pathological changes occur in the PVN, including abnormally increased neuronal activity (Krukoff & Patel, 1990; Zheng *et al.*, 2002; Rana *et al.*, 2014). The central mechanisms behind PVN neuronal activation are not well understood, but studies on other cardiovascular diseases have suggested that proinflammatory cytokines increase neuronal activity in the PVN (Rivest *et al.*, 1992; Shi *et al.*, 2010a) and contribute to increased sympathetic activity (Guggilam *et al.*, 2007; Guggilam *et al.*, 2008); however, the source of proinflammatory cytokines in diabetes is not known.

The resident immune cells within the CNS that mediate inflammation are microglia. These cells have been implicated in the development of various neurological diseases. It has been suggested that the inhibition of microglia is therapeutically useful in preventing the consequences of inflammation (Sweitzer *et al.*, 1999; Moalem & Tracey, 2006; Milligan & Watkins, 2009). Previously, we found that microglia were activated in the PVN in STZ diabetic rats (Rana *et al.*, 2014). Activated microglia can release proinflammatory molecules and cytotoxic factors, such as nitric oxide and TNF-α, which induce the activation of neurons.

Despite this evidence, whether the inhibition of microglial activation in the PVN can prevent the cardiovascular complications in STZ-induced diabetic rats has not been investigated.

Astrocytes also contribute to immune responses and inflammation in the CNS. Published studies have demonstrated that activated astrocytes in the CNS have the potential to exaggerate pathological processes (Meller *et al.*, 1994; Okada-Ogawa *et al.*, 2009). Furthermore, *in vivo* and *in vitro* studies have reported that activated astrocytes produce a variety of proinflammatory cytokines, including IL-1, IL-6 and TNFα, which may cause abnormal neuronal activity (Aloisi *et al.*, 1992; Lee *et al.*, 1993; Johnstone *et al.*, 1999). Following spinal nerve injury in rats, the rapid activation of microglia in the spinal cord is observed followed by a delayed activation of astrocytes, and this activation persists for a long period compared with microglial activation (Coyle, 1998; Milligan & Watkins, 2009; Benarroch, 2010). Hence, we hypothesised that activated astrocytes may have a greater effect in causing long-term complications of diabetes than microglia.

Regarding diabetic complications, in long-term STZ diabetic rats, hypertension has been demonstrated with the tail cuff measurement method (Bunag *et al.*, 1982; Katayama & Lee, 1985; Hartmann *et al.*, 1988; Ramos, 1988). In contrast, data from other studies that used the indirect blood pressure recordings are inconsistent (Fazan *et al.*, 1997; Van Buren *et al.*, 1998; Fazan *et al.*, 1999; Schaan *et al.*, 2004). The tail cuff measurement has been suggested to be inaccurate in long-term STZ diabetic rats due to morphological changes in the tail that lead to a change in the relationship between the pressure at the cuff and the pressure actually transmitted to the caudal artery. Therefore, in this study, a telemetry system was used to measure cardiovascular changes more precisely in conscious animals on a continuous basis, thereby also reducing the effect of handling induced stress.

Therefore, we aimed to investigate: (i) the effect of the inhibition of microglia in the PVN on blood pressure and heart rate in STZ diabetic rats, (ii) whether astrocytes are activated in the PVN in STZ diabetic rats (iii) and the mechanisms of astrocyte activation in these animals.

3.2 Results

3.2.1 Body weight and blood glucose measurements

Eight weeks after injection of Male Sprague Dawley rats with either STZ or citrate buffer, all groups exhibited a continuous growth in body weights, but as expected, the body weights of the control group were significantly higher ($P<0.001$) compared with diabetic animals with an ICV infusion of saline (STZ+saline) and the diabetic animals with an ICV infusion of minocycline (STZ+minocycline) (Figure 3-1A). Minocycline treatment did not significantly affect body weight in diabetic animals. The blood glucose levels were lower than 10 mmol/L in the control group and higher than 25 mmol/L in diabetic animals, indicating extreme hyperglycaemia (Figure 3-1B). The STZ+saline group exhibited significantly higher ($P < 0.0001$) blood glucose levels compared with the control group, as expected. This was also the case for blood glucose levels in the STZ+minocycline group when compared to the control group ($P < 0.0001$). Minocycline treatment had no effect on blood glucose in diabetic animals.

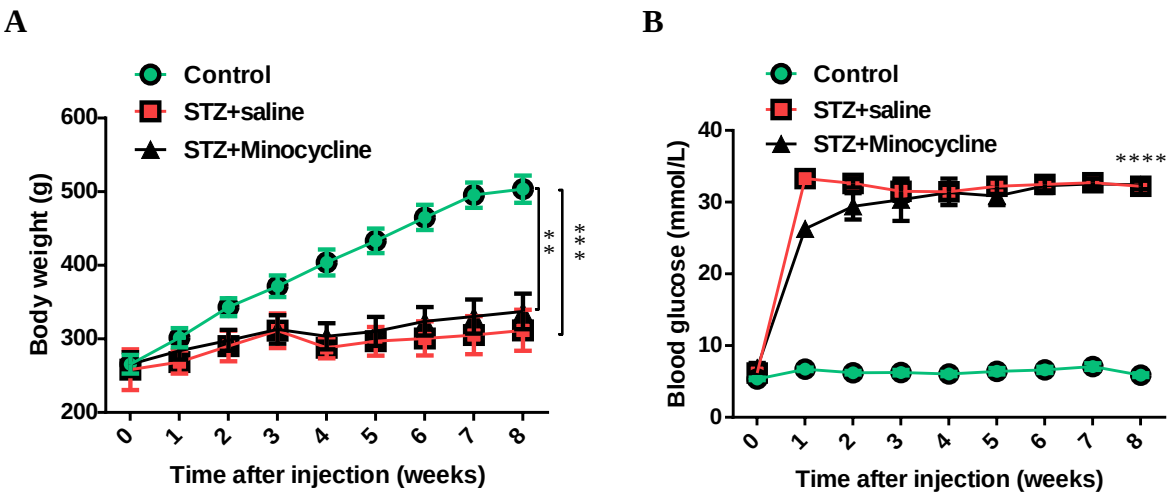


Figure 3-1. Effect of minocycline treatment on body weight and blood glucose of diabetic rats. A: Body weights; and B: blood glucose levels measured at weekly intervals after the injection of rats with either citrate buffer in control animals with an ICV infusion of saline (control) or STZ in diabetic animals with an ICV infusion of saline (STZ+saline) or minocycline (STZ+minocycline). Number of rats = 8, 4, 8 respectively. Data are expressed as the mean ± SEM. Significance was evaluated using one way ANOVA followed by Tukey's post hoc test for all comparisons (Control vs STZ+saline, control vs STZ+minocycline and STZ+saline vs STZ+minocycline) in this and all subsequent figures; * indicates P<0.05; ** indicates P<0.01; *** indicates P<0.001; **** indicates P < 0.0001 for comparisons between diabetic groups and controls; # indicates P<0.05; ## indicates P<0.0; ### indicates P<0.001; #### indicates P < 0.0001 for comparisons between diabetic groups.

3.2.2 Effect of minocycline on the mean arterial pressure and heart rate of diabetic rats

In this study, there were technical issues with the telemetric recording apparatus that resulted in a limitation in our ability to record the mean arterial pressure and heart rate in an adequate number of diabetic animals as well as to perform a statistical analysis. In addition, animals did not recover well following surgical implantation of the telemetry; however, our preliminary data indicated that the STZ+saline group (N=1) showed reduced blood pressure compared to the control group (N=6) (Figure 3-2A). Moreover, our results from the STZ+minocycline group (N=2) showed decreases in blood pressure similar to those seen in the STZ+saline group. The STZ+saline and STZ+minocycline groups showed a decrease in heart rate compared to the control group (N=6) (Figure 3-2B). Because the number of rats examined was small, we were unable to determine the effect of minocycline treatment on the blood pressure and heart rate of STZ diabetic animals.

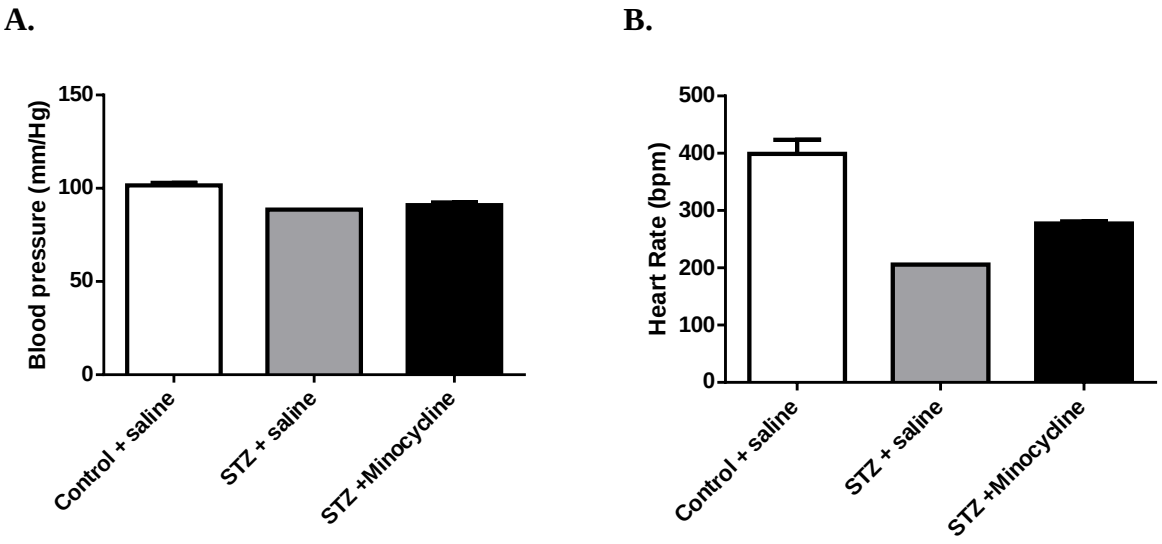


Figure 3-2. Effect of minocycline treatment on cardiovascular parameters in diabetic rats. A: The mean arterial blood pressure (MAP); and B: The heart rate of conscious control animals with an ICV infusion of saline (control+saline) and diabetic animals with an ICV infusion of saline (STZ+saline) or minocycline (STZ+minocycline). Number of rats = 6, 1, 2 respectively. Data are expressed as the mean ± SEM.

3.2.3 Effect of minocycline on microglial activation in the paraventricular nucleus of diabetic rats

The OX-42 antibody was used to identify morphological changes in microglia cells in the PVN in STZ diabetic rats. At eight weeks following vehicle injection, the microglia displayed normal morphology with small cell bodies and long, fine-branched processes throughout the PVN region in the control (Figure 3-3). In contrast, darkly stained microglia with shorter, thicker processes were clearly observed in the PVN in the STZ+saline group, which is consistent with our previous study (Rana *et al.*, 2014). As expected, minocycline treatment reversed the morphological changes of microglia that was seen in STZ+saline groups. The quantification of the percentage of activated microglia in the PVN in the STZ+saline group showed a significantly greater activation compared to the control group ($P<0.0001$) (Figure 3-4). Minocycline treatment in diabetic animals significantly ($P<0.0001$) reduced the percentage of activated microglia to a normal level seen in the control animals.

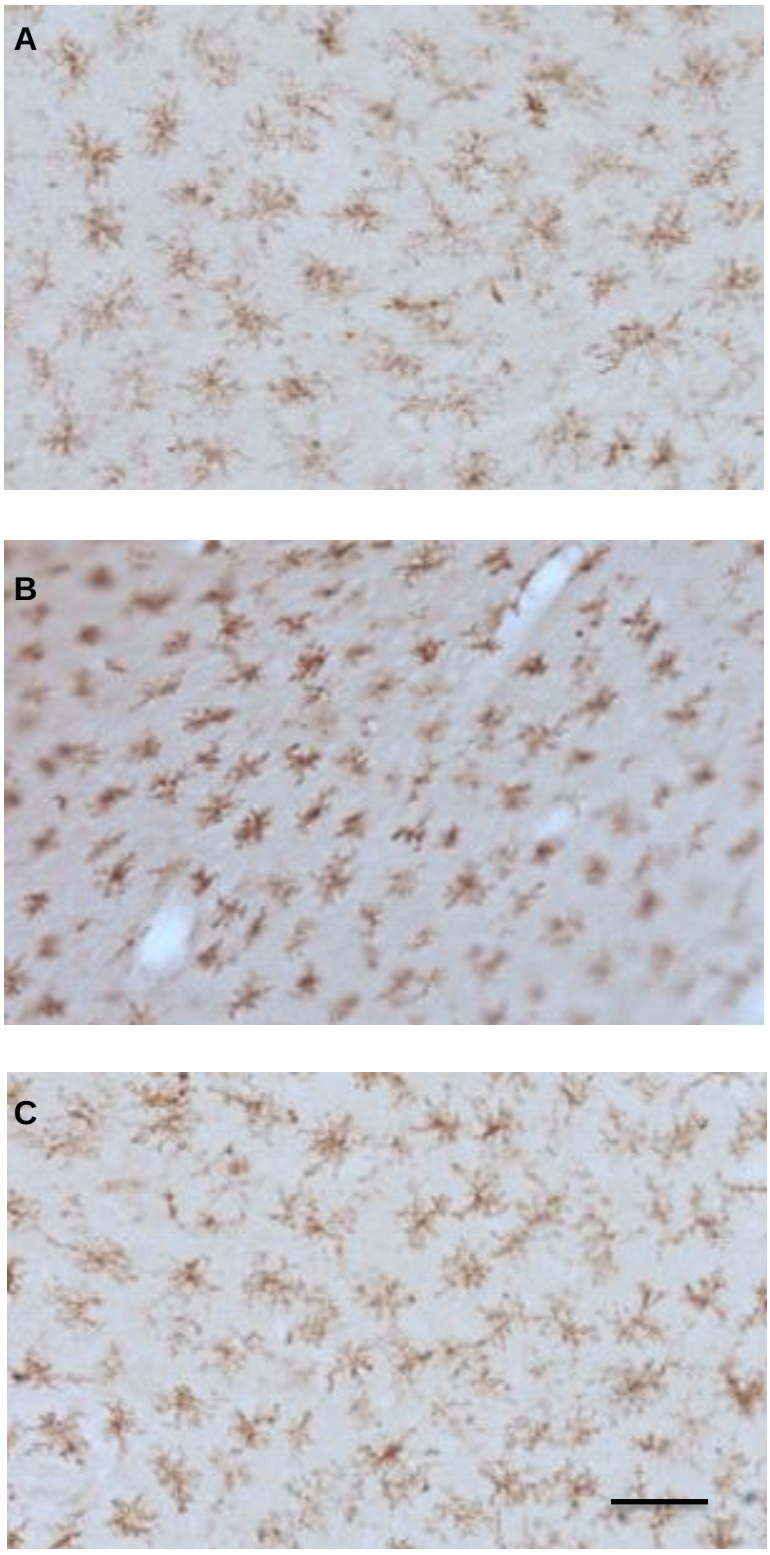

A
B
C

Figure 3-3. Morphology of microglia in STZ diabetic rats. Photomicrographs showing the morphology of CD11b (OX-42 clone) immunoreactive microglia in the paraventricular nucleus (PVN) in control animals with an ICV infusion of saline (A) and diabetic animals with an ICV infusion of saline (B) or minocycline (C). OX-42 antibody staining shows microglia with normal morphology with small cell bodies and long, fine-branched processes throughout the PVN region in the control and STZ+minocycline groups, while activated microglia showing larger cell bodies and shorter, thicker processes are seen in the STZ+saline group. Bar = 40 μm.

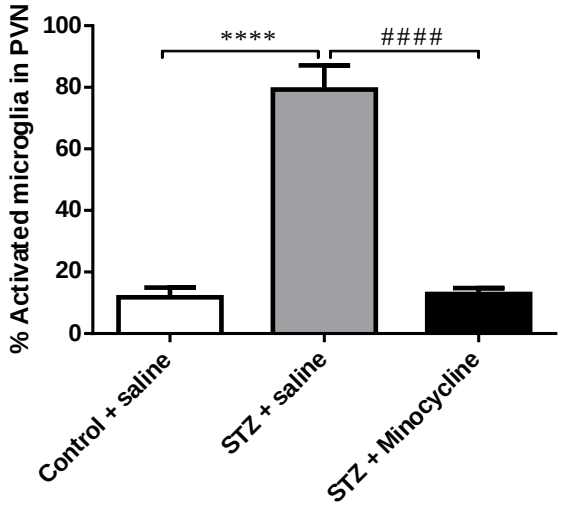


Figure 3-4. Effect of minocycline on microglial activation in the paraventricular nucleus of diabetic rats. The percentage of microglia showing activated morphology in the paraventricular nucleus (PVN) of control animals with an ICV infusion of saline (control+saline) and diabetic animals with an ICV infusion of saline (STZ+saline) or minocycline (STZ+minocycline). Number of rats = 4 in each group. Data are expressed as the mean ± SEM.

3.2.4 Astrocyte activation in the paraventricular nucleus of diabetic rats

Within the PVN, astrocytes were stained with a GFAP antibody in all groups (Figure 3-5). In control rats, astrocytes showed a normal morphology with a small cell body and very fine processes; however, in the STZ+saline group, astrocytes had swollen cell bodies with thicker processes as well as a marked upregulation of GFAP immunolabeling compared to the control. The same pattern was also observed in the STZ+minocycline group. the percentage of activated astrocytes in the PVN in the STZ+saline and STZ+minocycline groups was significantly greater ($P<0.0001$) compared to the control group (Figure 3-6). Minocycline treatment in STZ diabetic rats had no significant effect on the percentage of activated astrocytes in diabetic animals.

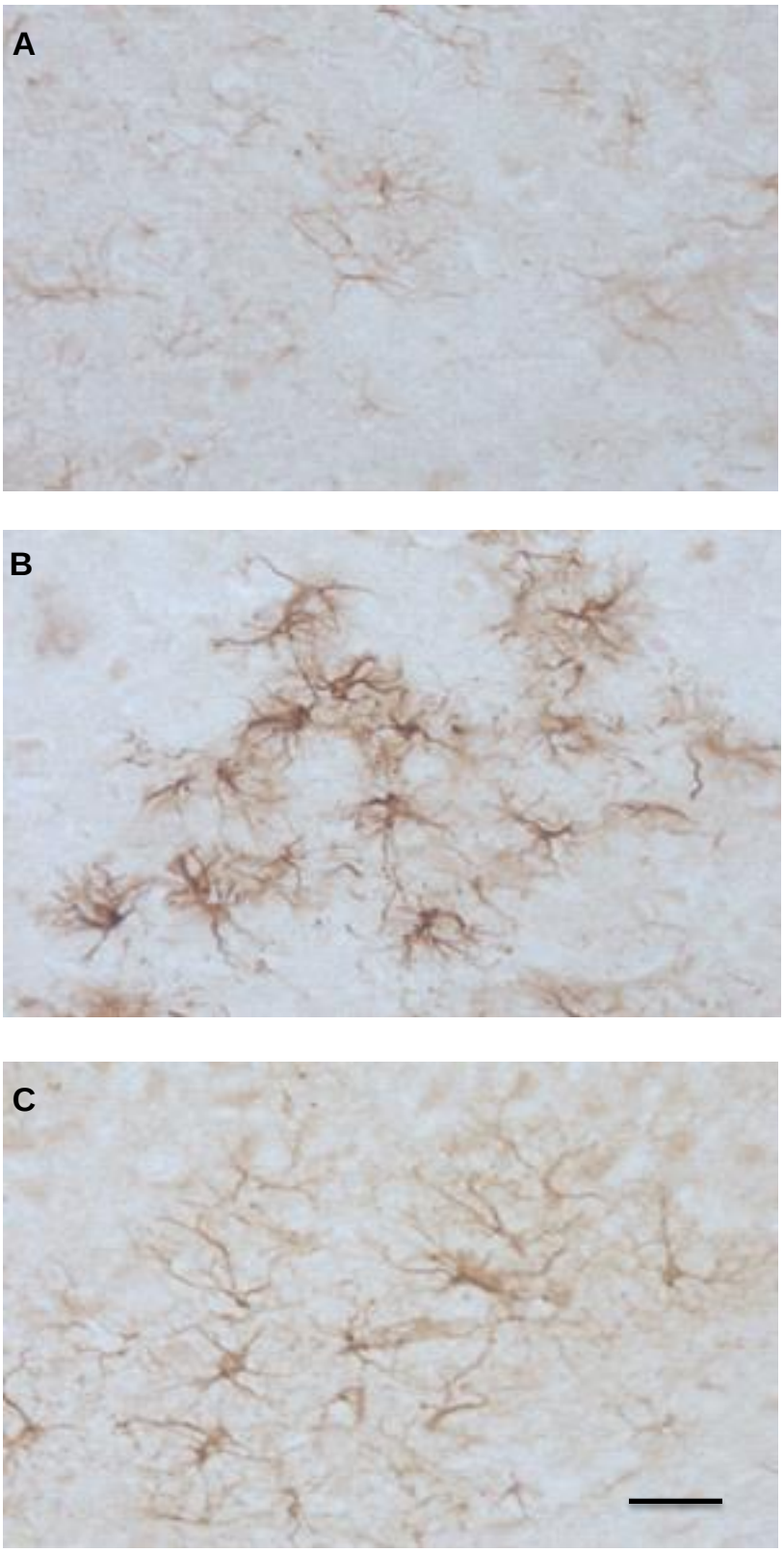

A
B
C

Figure 3-5. Morphology of astrocytes in STZ diabetic rats. Photomicrographs showing GFAP immunoreactive astrocytes in the paraventricular nucleus (PVN) in control animals with an ICV infusion of saline (A) and diabetic animals with an ICV infusion of saline (B) or minocycline (C). GFAP antibody staining shows astrocytes with normal morphology with small cell bodies and fine processes throughout the PVN region in the control animals, while activated astrocytes showing intense expression of GFAP, larger cell bodies and thicker processes are seen in the STZ+saline and STZ+minocycline groups. Bar = 40 μm.

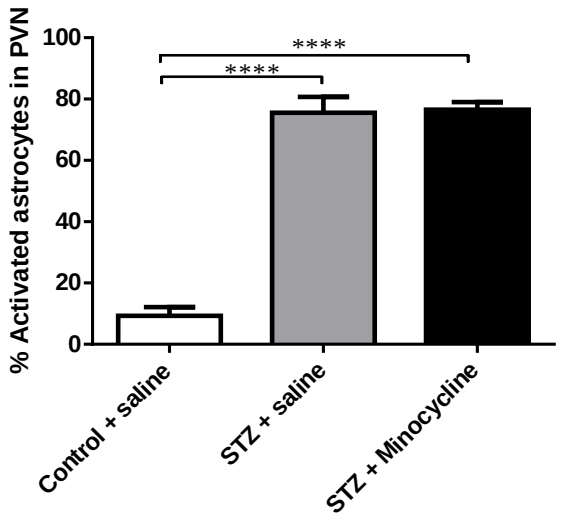


Figure 3-6. Astrocytes activation in the paraventricular nucleus in diabetic rats. A: The percentage of astrocytes showing activated morphology in the paraventricular nucleus (PVN) of control animals with an ICV infusion of saline (control+saline) and diabetic animals with an ICV infusion of saline (STZ+saline) or minocycline (STZ+minocycline). Number of rats = 4 in each group. Data are expressed as the mean ± SEM.

3.3 Discussion

We have previously found that microglia become activated in the PVN in STZ-diabetic rats at eight weeks following STZ injection (Rana *et al.*, 2014), but the consequence of this activation on diabetic complications has not been investigated. Thus, in this study, we aimed to investigate the effect of the inhibition of microglial activation in the PVN on the blood pressure and heart rate of long-term STZ diabetic rats and whether astrocytes are activated in these animals. The main findings of the present study were that (i) microglia and astrocytes are activated in the PVN in STZ diabetic rats at 8 weeks after STZ injection and (ii) minocycline treatment inhibited PVN microglial activation but not astrocyte activation.

In this study, we observed marked activation of microglia in the PVN in STZ diabetic rats when compared to the control animals, which is consistent with our previous study (Rana *et al.*, 2014). Microglial activation was specifically in the PVN and not seen in surrounding areas. The PVN region is well-known to play an important role in the regulation of the autonomic nervous system, including cardiovascular functions. Several lines of evidence suggest that an abnormal increase in sympathetic nerve activity is linked to the pathogenesis of the development of cardiovascular-related diseases, including hypertension (Allen, 2002), myocardial infarction and heart failure (Aronson & Burger, 2002; Guggilam *et al.*, 2008), which are major complications of diabetes. In the case of diabetes, over-activity of the sympathetic nervous system has been reported in humans and animals with type 1 diabetes hyperglycaemia (Perin *et al.*, 2001; Patel *et al.*, 2011), but the cause of this activation is not clear. Increased proinflammatory cytokines within the PVN are implicated in generating sympathetic hyperactivity in rats with heart failure (Guggilam *et al.*, 2008; Kang *et al.*, 2010). Once microglia undergo morphological changes, they can release proinflammatory cytokines. Hence, activated microglia seen in the PVN of STZ diabetic rats may be the source of proinflammatory cytokines. Combined with these studies, our data suggest that activated

microglia in the PVN may release proinflammatory cytokines, which may contribute to the observed changes in sympathetic nerve activity in diabetic animals.

Although the role of activated microglia in the pathological consequences of brain inflammation has been investigated, less is known about the role of astrocytes. Nevertheless, it is reported that an association exists between activated spinal astrocytes and neuropathic pain following nerve damage (Sweitzer *et al.*, 1999). Thus, we investigated whether astrocytes are activated in the PVN of diabetic rats. Our results showed that activated astrocytes exist in the PVN of diabetic rats. This finding is in agreement with a previous study that reported activated astrocytes in the PVN of diabetic rats (Luo *et al.*, 2002). While the consequences of this activation is unknown, an increased expression of astroglial protein (S100β) in the spinal cord after nerve injury has been reported, and the inhibition of astroglial protein has been shown to result in a reduction of neuropathic pain behaviours in rats (Tanga *et al.*, 2006). In this animal model, an enhanced expression of proinflammatory cytokines, such as TNF-α and IL-1β, are observed in activated spinal astrocytes following peripheral nerve injury (Wei *et al.*, 2008), and the inhibition of these proinflammatory cytokines has been shown to reduce neuropathic pain-like behaviours (Milligan *et al.*, 2001). Thus, the activation of the astrocytes may be important for mediating inflammation in the PVN of diabetic rats, which may cause neuronal hyperactivity and sympathetic over-activity.

Minocycline is a second generation of a tetracycline antibiotic and has been shown to have anti-inflammatory actions (Yrjänheikki *et al.*, 1999; Sapadin & Fleischmajer, 2006). Also, several studies have reported an inhibitory effect of minocycline on microglial activation (Krady *et al.*, 2005; Shi *et al.*, 2010a). In the present study, ICV minocycline treatment significantly reduced microglial activation in the PVN in STZ diabetic rats. In contrast, minocycline treatment was not able to inhibit astrocyte activation, which is in agreement with other previous studies (Yoon *et al.*, 2012; Sauvant *et al.*, 2014). In animal models of

neuropathic pain, astrocyte activation in the CNS was seen at later stages compared with microglial activation, suggesting that microglia may be responsible for astrocyte activation (Coyle, 1998; Milligan & Watkins, 2009; Benarroch, 2010); however, data obtained in this study suggested that astrocyte activation is not related to microglial activation. The mechanism behind astrocyte activation in the PVN in STZ diabetic rats is not clear, and further research is needed.

Regarding cardiovascular parameters, although the number of animals tested in this study was insufficient, we found that blood pressure tended to decrease in conscious STZ diabetic rats at eight weeks following STZ injection. This finding is in agreement with previous studies on long-term STZ-diabetic rats that used the direct technique for the measurement of blood pressure (Fazan *et al.*, 1997; Van Buren *et al.*, 1998; Fazan *et al.*, 1999; Schaan *et al.*, 2004). The mechanisms involved in hypotension seen in STZ diabetic rats have not been completely understood; however, there are several possible reasons for hypotension in these animals. It well-known that STZ diabetic rats excrete large amounts of urine and thereby they lose both water and salt, causing hypervolemia. Interestingly, in two other studies, giving STZ-treated diabetic rats 1% saline caused a marked elevation in their blood pressure, which is not seen when control rats are given saline (Santos *et al.*, 1995; Maeda *et al.*, 2007). Thus, hypotension seen in STZ diabetic rats could be related to hypovolima. The observation of decreased cardiac output due to a dysfunction in the myocardium (Ren & Bode, 2000) or the impairment of sympathetic innervation of the heart and blood vessels (Borges *et al.*, 2006) in STZ diabetic rats may also be possible explanations for this hypotension.

3.4 Conclusion

The present study demonstrated that microglia and astrocyte activation occur in the PVN in long-term STZ-induced diabetic rats. Minocycline treatment reduces microglial activation in the PVN but does not significantly affect the astrocyte activation seen in these animals. These

findings suggest that microglial activation does not precede astrocyte activation in the PVN in STZ diabetic animals. In this study, due to unintended morbidity and a lack of hypertension in STZ diabetic animals, we were unable to investigate the consequence of microglial activation. Thus, alternative routes of blood pressure recording with volume replacement therapy in STZ diabetic rats may be suitable for investigating the effect of PVN inflammation on diabetic complications.

Chapter Four: Drinking 1% NaCl for 2 Weeks Enhances Inflammation in the Cardiovascular Control Centers of the Brain in STZ Diabetic Rats

4.1 Introduction

Cardiovascular complications remain amongst the most serious long-term consequences of diabetes. Hypertension is the single most important risk factor for the development of cardiovascular disease, and it is associated with various pathological conditions, including diabetes (Epstein & Sowers, 1992). While many studies on the mechanisms of diabetic cardiovascular complications have suggested an important role for endothelial dysfunction (Woodman *et al.*, 2008; Woodman & Malakul, 2009), a large body of evidence shows that the autonomic nervous system, which functions to regulate the cardiovascular and renal systems, is impaired in diabetes (Baydas *et al.*, 2003; Lebed *et al.*, 2008). It is well established that an abnormal activation of the sympathetic nervous system is associated with various pathological conditions and is an important mechanism underlying the development of hypertension (Esler, 2000; Esler & Kaye, 2000). Interestingly, elevated renal sympathetic nerve activity is reported in humans with type 1 diabetes and animals with chronic hyperglycaemia (Perin *et al.*, 2001; Patel *et al.*, 2011). These changes in sympathetic nerve activity have been suggested to contribute to the development of diabetic complications, such as hypertension, heart failure and sudden cardiac death (Perin *et al.*, 2001), but so far, evidence is lacking.

In addition to the role of sympathetic nerve overactivity in the pathogenesis of hypertension, emerging evidence shows that the sensitivity of the baroreflex, which controls heart rate, is reduced in conditions associated with hypertension. It is believed that this change may precede and contribute to the development of hypertension and its consequences (Grassi *et al.*, 1998; Gao *et al.*, 2002; Salgado *et al.*, 2007; Maliszewska-Scislo *et al.*, 2008). A study on spontaneously hypertensive rats, which showed decreased baroreflex sensitivity, demonstrated a direct link between an improvement of baroreflex function and a reduced incidence of stroke (Liu *et al.*, 2007). Thus, the restoration of baroreflex sensitivity may be a

novel strategy for reducing blood pressure and lowering the cardiovascular disease risk in diabetic patients.

Baroreflex impairment has been shown to occur in diabetic humans (Eckberg *et al.*, 1986; Frattola *et al.*, 1997) as well as in STZ-induced diabetic rats (Fazan *et al.*, 1999; Gouty *et al.*, 2001; Schaan *et al.*, 2004). In addition, an induction of diabetes by STZ treatment in spontaneously hypertensive rats further depressed baroreflex function (Farah *et al.*, 2007). Dysfunction in the autonomic neurons system could explain both the decrease in baroreceptor reflex activity and the increase in sympathetic nerve activity seen in diabetic humans and STZ-induced diabetic rats. Thus, it is important to determine which pathological changes may be occurring in the central autonomic centers of diabetic animals.

Cardiovascular centers of the brain, such as the hypothalamic paraventricular nucleus (PVN), rostral ventrolateral medulla (RVLM) and nucleus tractus solitarius (NTS), play a pivotal role in autonomic cardiovascular regulation. Several studies on STZ-induced diabetic animals have reported abnormally increased neuronal activity in the cardiovascular centers of the brain involved in sympathetic and baroreflex regulation, but the central mechanisms behind this neuronal activation are not well understood (Krukoff & Patel, 1990; Zheng *et al.*, 2002; Rana *et al.*, 2014). Interestingly, other studies have suggested that inflammation in these areas may result in neuronal activation (Shi *et al.*, 2010a; Shi *et al.*, 2011).

We have recently reported activation of microglia in the PVN and NTS of STZ-induced diabetic animals at eight weeks following STZ injection (Rana *et al.*, 2014). Microglia are the primary immune cells in the brain and act to protect and support neurons but also have the potential to exaggerate pathological processes by causing inflammation. Activated microglia release proinflammatory molecules and cytotoxic factors, such as nitric oxide and TNF-α, which can damage neurons. In addition, in other CNS regions, secretion of purines and BDNF from activated microglia have been shown to cause neuronal hyperexcitability by

increasing excitation and decreasing inhibition (Lu *et al.*, 2009). While the effect of microglial activation in the PVN in diabetes is not known, in rats treated with angiotensin II, inhibition of microglial activation in the PVN has been shown to prevent the development of hypertension (Shi *et al.*, 2010a). Collectively, this data suggests that microglia may have an important role in generating enhanced sympathetic nerve activity and baroreflex dysfunction through releasing proinflammatory cytokines, neurotrophic factors and/or purines. Whether inhibition of microglial activation or action in the PVN can ameliorate some of the cardiovascular complications of STZ-induced diabetes has not been investigated.

While in human subjects, Type 1 diabetes is strongly associated with hypertension, rats treated with STZ to induce hyperglycaemia show unchanged or lowered blood pressure (Fazan *et al.*, 1999; Dall'Ago *et al.*, 2002), although there is evidence that their sympathetic nerves are more activated (Patel *et al.*, 2011). Interestingly, two studies on animal models of STZ-induced diabetes that used direct recording of blood pressure have reported that saline intake (1% NaCl) increases blood pressure at two and four weeks following STZ injections (Santos *et al.*, 1995; Maeda *et al.*, 2007). The mechanisms underlying this hypertension are not clear, but at early stages following STZ injections, diabetic rats showed unchanged baroreflex-mediated bradycardia (Maeda *et al.*, 1995; Van Buren *et al.*, 1998), and there was no microglial activation in the cardiovascular centers (Rana *et al.*, 2014). It was therefore hypothesised that saline intake causes hypertension by increasing inflammation in the cardiovascular control centres and causing baroreflex dysfunction, thereby increasing blood pressure in STZ-diabetic rats.

Therefore, we investigated the effects of saline intake on (i) blood pressure, (ii) baroreflex sensitivity and (iii) inflammation in the cardiovascular centres in STZ diabetic rats. In addition, we investigated whether inhibition of microglia in the PVN can reduce or prevent hypertension in STZ diabetic rats treated with saline.

4.2 Results

4.2.1 Effect of 2 weeks 1% NaCl intake on general features of diabetic rats

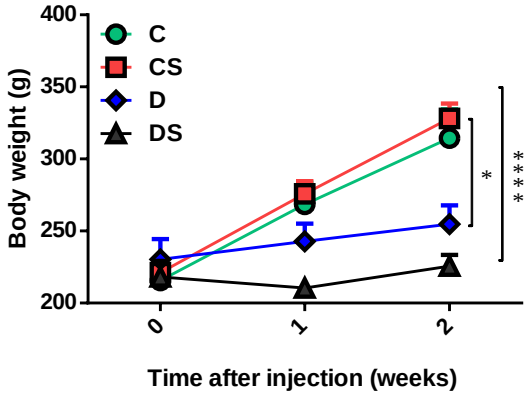
When Male Sprague Dawley rats were injected with either STZ or citrate buffer and maintained for 2 weeks with free access to either tap water or saline to drink, the non-diabetic control animals given water (C) and non-diabetic animals given saline (CS) exhibited a gradual increase in body weight, but diabetic animals given water (D) and diabetic animals given saline (DS) showed no significant change in body weight (Figure 4-1A). As expected, the body weights of the D group were significantly lower ($P<0.01$) compared with the C group animals, which is consistent with previous studies (chapter 3). Also, the body weights of the DS group were significantly lower ($P<0.001$) when compared with the CS group. Saline treatment did not significantly affect body weight in either the diabetic or control animals. Blood glucose levels were lower than 8 mmol/L in C and CS groups and higher than 25 mmol/L in D and DS groups, indicating extreme hyperglycaemia (Figure 4-1B). The D group exhibited significantly higher ($P < 0.0001$) blood glucose levels compared with the C group, as expected. This was also the case for blood glucose levels in the DS group when compared to the CS group ($P < 0.0001$). Saline treatment had no effect on blood glucose in diabetic or control animals. The results of body weight and blood glucose measurements indicated that STZ-treated diabetic rats exhibited changes characteristic of this model (Schaan *et al.*, 1997; Maeda *et al.*, 2007).

Regarding fluid intake, drinking both 1% saline and tap water increased progressively over 2 weeks in the D and DS groups, but this was not the case in the C and CS groups, suggesting that diabetic animals were compensating for substantial fluid loss (Figure 4-1C). Daily water intake was significantly greater ($P<0.001$) in the D group when compared to the C group, and the DS group drank a significantly greater ($P < 0.0001$) volume of saline than the CS group.

In diabetic animals, the daily saline intake was significantly higher ($P < 0.0001$) compared to the daily water intake. This was not the case in the control animals.

Because increased plasma osmolarity and dehydration are implicated in neuronal activation in STZ diabetic rats (Rana *et al.*, 2014), we measured the plasma osmolarity and haemoglobin concentration in all groups at 2 weeks. No significant differences were found in the level of plasma osmolarity between C and CS or between the D and DS groups (Figure 4-2A). As expected, plasma osmolarity was significantly increased ($P<0.01$) in the D group when compared with the C group; however, interestingly, saline treatment significantly decreased ($P<0.05$) the plasma osmolarity to normal levels in diabetic animals. As an indicator of blood volume status, the haemoglobin concentration was also similar between the C and CS groups but not between the D and DS groups (Figure 4-2B). In the D group, the Haemoglobin concentration were significantly higher ($P<0.05$) than in the C group; however, saline intake in diabetic animals significantly reduced ($P<0.05$) the levels of haemoglobin compared to the D group. The results of the plasma osmolarity and haemoglobin measurements suggested that STZ diabetic rats were dehydrated and that drinking saline restored blood parameters to normal levels.

A.



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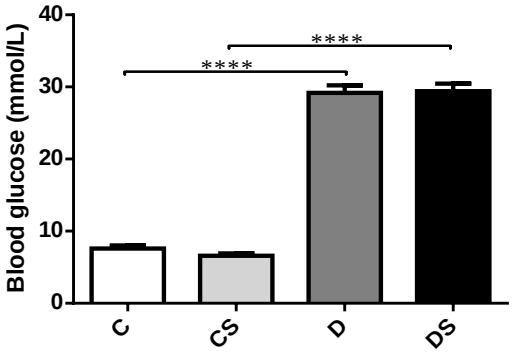

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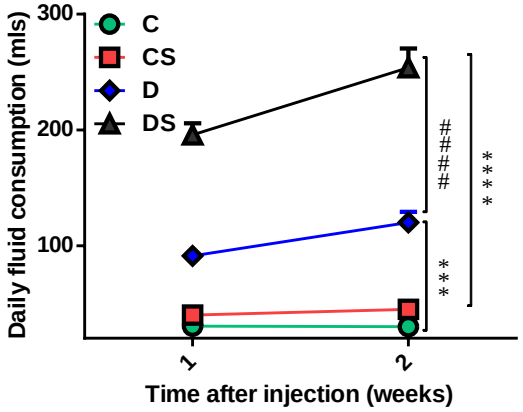


Figure 4-1. Effect of 2 weeks saline treatment on body weight, blood glucose and fluid intake of diabetic rats. A: Body weight measured at weekly intervals in control animals (C) and diabetic animals (D) given tap water to drink and control animals (CS) and diabetic animals (DS) given 1% NaCl solution. B: Blood glucose levels measured at 2 weeks after the injection of rats with either STZ or citrate buffer. C: The amount of tap water or 1% NaCl solution ingested was monitored daily by weighting water bottles and then averaged for each week. Number of rats = 6 in each group. Data are expressed as the mean ± SEM. Significance was evaluated using a one way ANOVA followed by Tukey's post hoc test for all comparisons (C vs D, CS vs DS and D vs DS) in this and all subsequent figures; * indicates $P<0.05$; *** indicates $P<0.001$; **** indicates $P < 0.0001$ for comparisons between diabetic groups and their respective controls; #### indicates $P <0.0001$ for comparisons between diabetic groups.

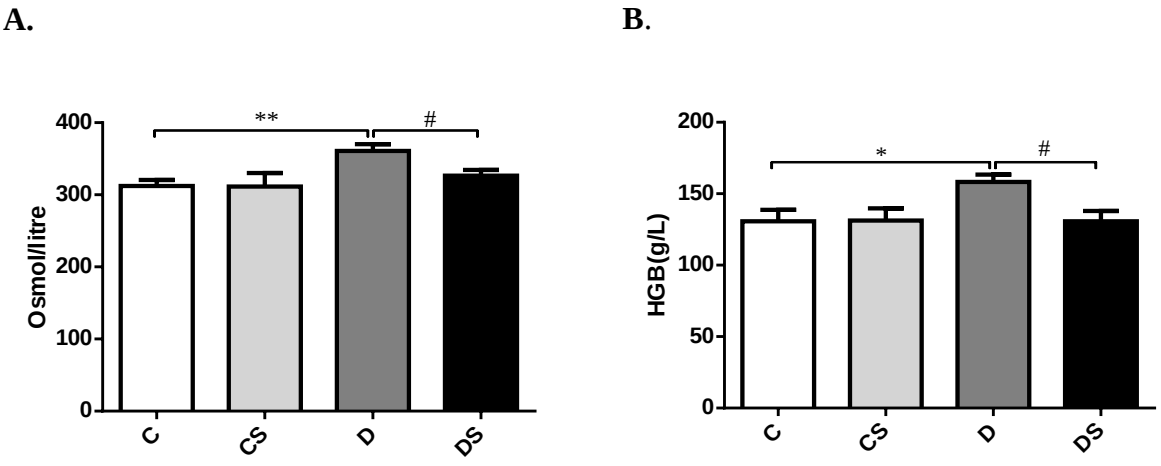


Figure 4-2. Effect of 2 weeks saline treatment on plasma osmolarity and haemoglobin in diabetic rats. A: Plasma osmolarity (Osmol/litre); and B: Haemoglobin concentration (g/litre) measured at the end of the experimental period in control animals (C) and diabetic animals (D) given tap water and control animals (CS) and diabetic animals (DS) given 1% NaCl solution. Number of rats = 8,4,10 and 12 respectively. Data are expressed as the mean ± SEM. * indicates $P<0.05$; ** indicates $P<0.01$ for comparisons between diabetic groups and their respective controls; ♯ indicates $P<0.05$ for comparisons between diabetic groups.

4.2.2 Measurement of baroreflex-mediated bradycardia in diabetic rats

In experiments to investigate the baroreflex-mediated bradycardia at two weeks after STZ or citrate injection, the mean arterial blood pressure under anaesthesia was not significantly different between the C and D or between the CS and DS groups (Figure 4-3A). Also, no significant differences were found in the heart rate among the groups (Figure 4-3B). A progressive increase in the maximal mean arterial blood pressure and a decrease in the maximal heart rate in response to the infusion of increasing amounts of phenylephrine was seen in all groups. The maximal mean arterial blood pressure response to phenylephrine at all doses was not significantly different between the D and C groups (Figure 4-4) (Figure 4-5A). The same pattern was also observed in the DS group compared to the CS group. Saline intake had no effect on blood pressure changes in response to phenylephrine in the diabetic or control animals. The maximal heart rate response to phenylephrine was also similar between the D and C groups (Figure 4-5B). In the DS group, the heart rate response to phenylephrine appeared smaller compared with the CS and D groups but was not significantly different, although the variability was high, meaning our tests were underpowered for detecting small differences; however, when baroreflex sensitivity was quantified as the ratio of maximal change in heart rate divided by the maximal change in mean arterial blood pressure, there was less variability. The baroreflex-mediated bradycardia in response to increasing doses of phenylephrine was similar between the D and C groups (Figure 4-5C); however, there was a significant reduction in baroreflex sensitivity in the DS group compared to the CS ($P<0.05$) and D ($P<0.01$) groups.

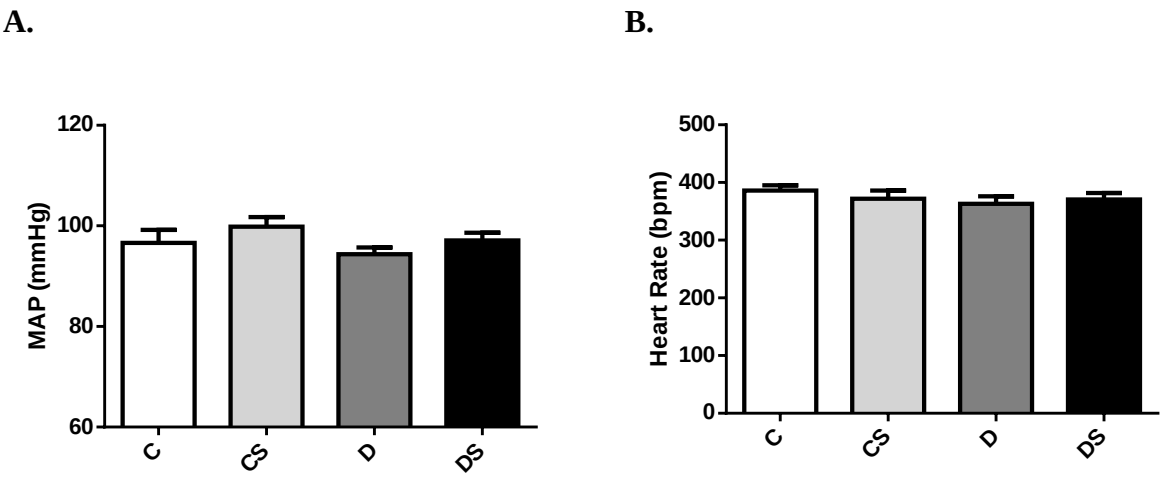


Figure 4-3. Effect of 2 weeks saline treatment on cardiovascular parameters in diabetic rats under anaesthesia. A: The mean arterial blood pressure (MAP); and B: The heart rate of unconscious control animals (C) and diabetic animals (D) given tap water and control animals (CS) and diabetic animals (DS) given 1% NaCl solution. Number of rats = 6, 6, 6 and 7 respectively. Data are expressed as the mean ± SEM. No significant differences were observed.

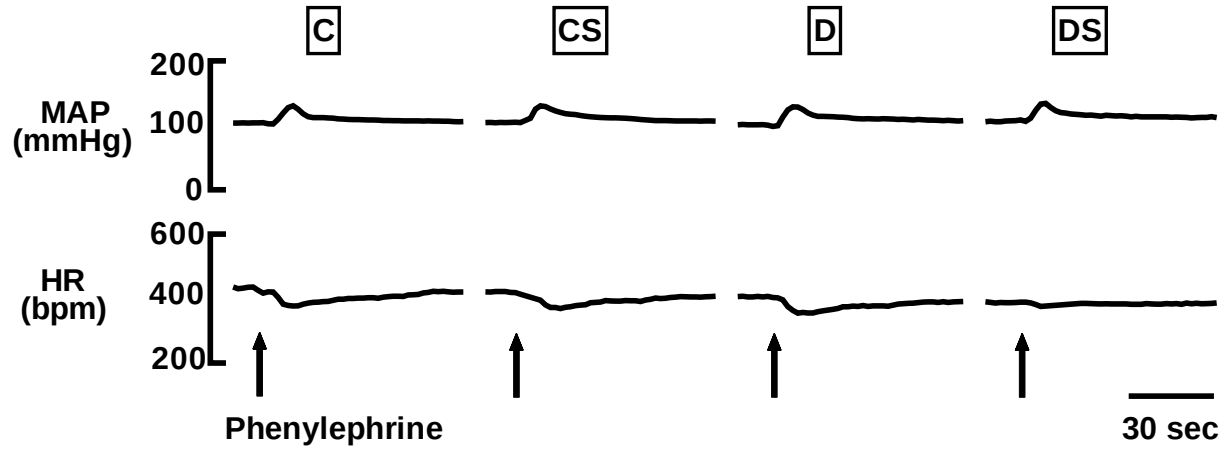


Figure 4-4. Measurement of baroreflex-mediated bradycardia in diabetic rats. Representative recordings showing changes in arterial blood pressure and heart rate in response to phenylephrine (10 µg/kg at the arrow) in control animals (C) and diabetic animals (D) given tap water and control animals (CS) and diabetic animals (DS) given 1% NaCl solution.

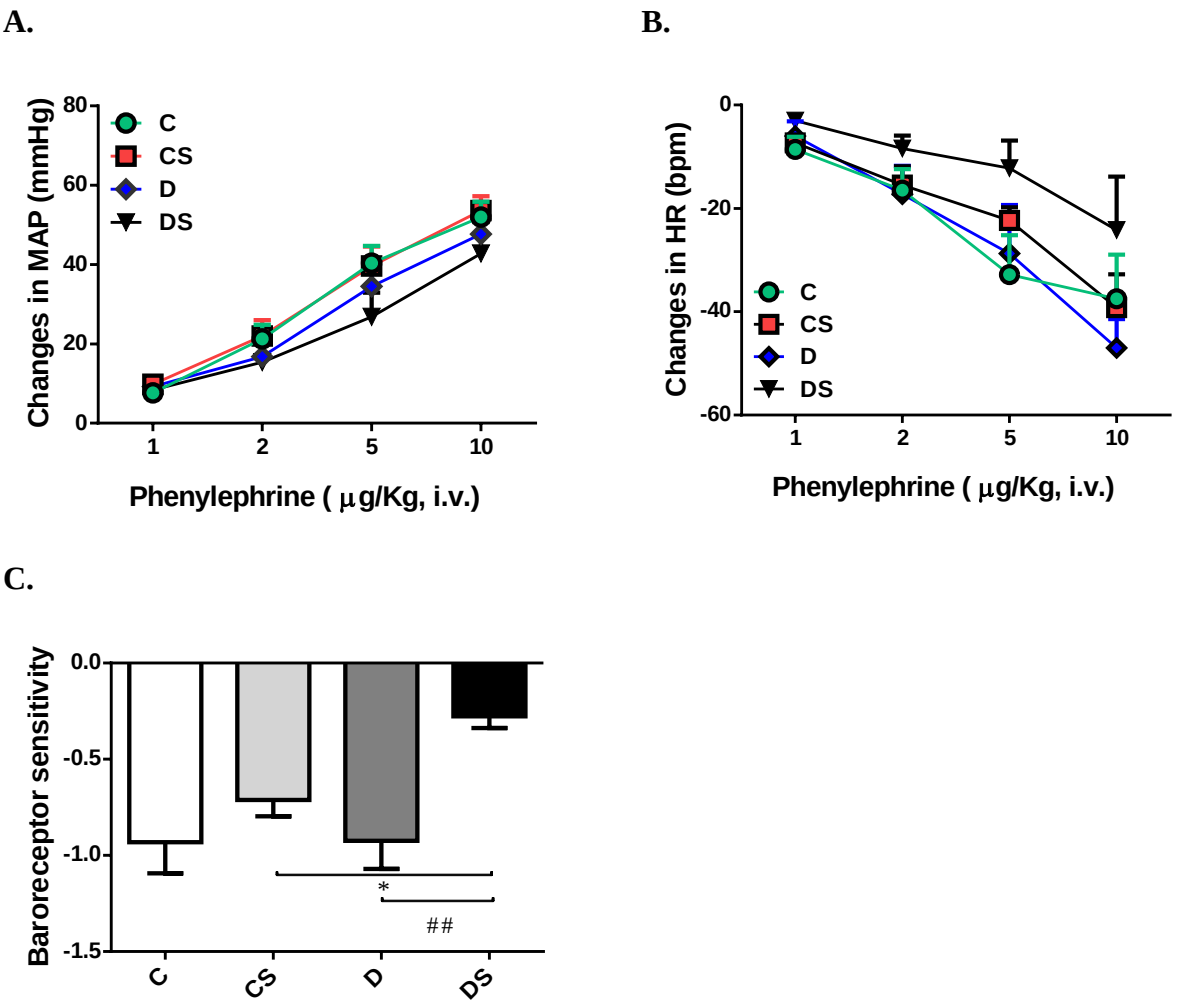


Figure 4-5. Cardiovascular responses to phenylephrine in diabetic rats. A: Changes in mean arterial pressure (MAP); and B: changes in heart rate (HR) in response to graded doses of intravenous phenylephrine (1, 2, 5 and 10 µg/kg) in control (C) and diabetic animals (D) given tap water and control (CS) and diabetic animals (DS) given saline. C: Baroreflex sensitivity (bpm/mmHg) of bradycardic responses represented as the average of the maximal change in heart rate divided by the maximal change in mean arterial blood pressure for each phenylephrine dose and then averaged for each animal. Baroreflex-mediated bradycardia was significantly attenuated in the salt-treated diabetic group compared with the diabetic group and the salt-treated control group. Number of rats = 6, 6, 6 and 7 respectively. Data are expressed as the mean ± SEM. * indicates $P<0.05$ for comparisons between diabetic groups and their respective controls; ## indicates $P<0.01$ for comparisons between diabetic groups.

4.2.3 OX-42 Immunohistochemistry in cardiovascular centres in diabetic rats

The OX-42 antibody was used to identify morphological changes in microglia cells in the cardiovascular centres in STZ rats. At two weeks following STZ or vehicle injection, microglia displayed a normal morphology with small cell bodies and long, fine-branched processes throughout the PVN region in the D and C groups, which is consistent with our previous study (Rana *et al.*, 2014) (Figure 4-6). While no microglial activation was seen in the PVN in the CS group, saline intake in the DS groups caused a significant increase in the percentage of activated microglia with large cell bodies and short, thick processes compared to the PVN microglia in the CS ($P<0.0001$) and D ($P<0.0001$) groups. Saline intake in diabetic animals, though not in the control group, resulted in approximately a 5-fold elevation in the percentage of microglia that were activated compared with other groups (Figure 4-9A). Interestingly, a significant increase in the percentage of activated microglial was restricted to the parvocellular PVN (Figure 4-12A).

In the NTS region in which baroreceptive afferent nerves terminate, microglial cells showed normal morphology with small cell bodies and long, fine-branched processes in the D and C groups, which is in agreement with our previous study (Rana *et al.*, 2014) (Figure 4-7). In contrast, darkly stained microglia with shorter, thicker processes were clearly observed in the NTS in the DS group. The quantification of the percentage of activated microglia in the NTS in the DS group showed a significantly greater activation compared to the CS ($P<0.001$) and D ($P<0.001$) groups (Figure 4-9B).

Microglial cells in the RVLM region exhibited normal morphology in the D and C groups (Figure 4-8). In contrast, in the DS group, microglia showed markedly shortened, thicker processes and stronger staining for OX-42 compared with other groups. The quantitative analysis of microglial activation in the RVLM region indicated that saline intake caused a

significant increase in the percentage of activated microglia in the DS group compared to the CS ($P<0.01$) and D ($P<0.05$) groups (Figure 4-9C).

To confirm our observation, we measured the microglial process length in the brain areas examined. We found that in the DS group, the microglia in the PVN, NTS and RVLM regions had significantly shorter processes compared to the microglia in the CS ($P<0.0001$) and D ($P<0.0001$) groups (Figure 4-10 A-B-C).

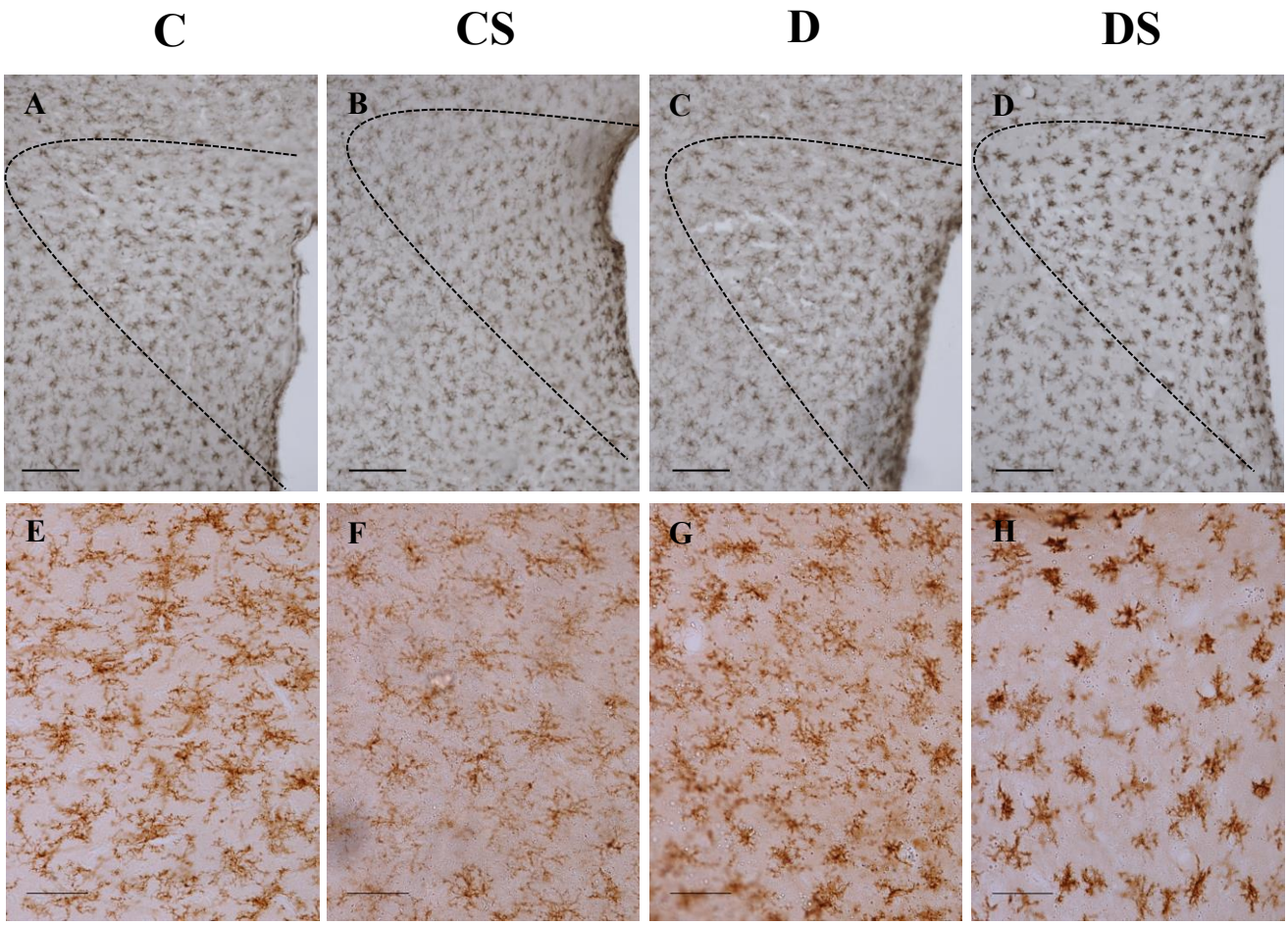


Figure 4-1. OX-42 Immunohistochemistry in the PVN in diabetic rats. Photomicrographs showing CD11b (OX-42 clone) immunoreactive microglia in the paraventricular nucleus (PVN) in control (C) and diabetic animals (D) given tap water and control animals (CS) and diabetic animals (DS) given 1% NaCl solution. Dotted lines in low-power images (A-D) outline the PVN. High-power images (E-H) show the morphology of microglia in the parvocellular PVN region in detail. OX-42 antibody staining shows microglia with normal morphology with small cell bodies and long, fine-branched processes throughout the PVN region in the C, CS and D groups, while activated microglia showing larger cell bodies and shorter, thicker processes are seen in the DS group. Bar = 100 μm in A–D, 50 μm in E–H.

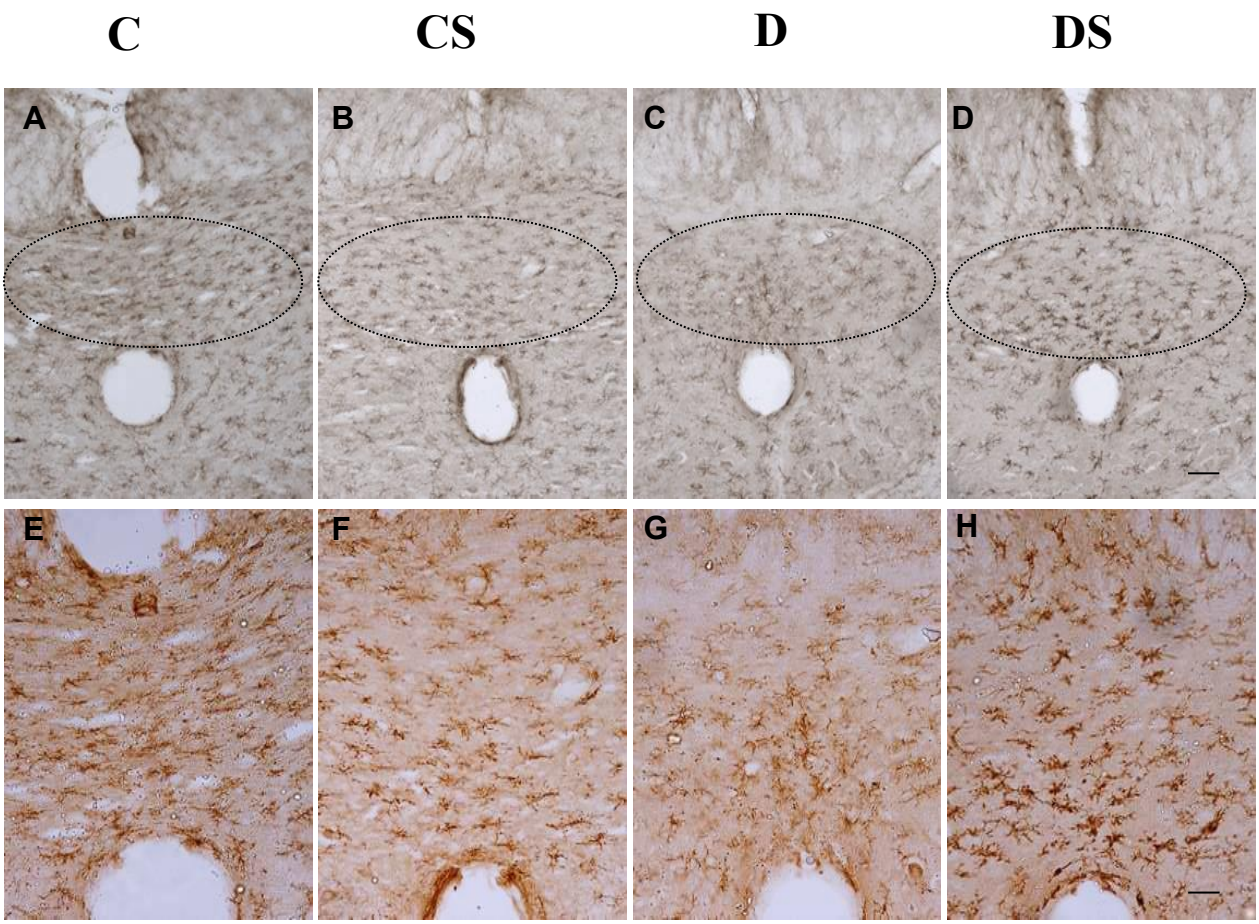


Figure 4-2. OX-42 Immunohistochemistry in the NTS in diabetic rats. Photomicrographs showing CD11b (OX-42 clone) immunoreactive microglia in the nuclear tractus solitarius (NTS)in control (C) and diabetic animals (D) given tap water and control (CS) and diabetic animals (DS) given 1% NaCl solution. Dotted lines in low-power images (A-D) outline the NTS. High-power images (E-H) show the detailed morphology of microglia in NTS region. OX-42 antibody staining showing microglia with normal morphology was seen throughout the NTS region in the C, C-S and D groups, while activated microglia showing intense immunolabelling, larger cell bodies and thicker processes were common in the NTS in the D-S group. Bar = 200 μm.

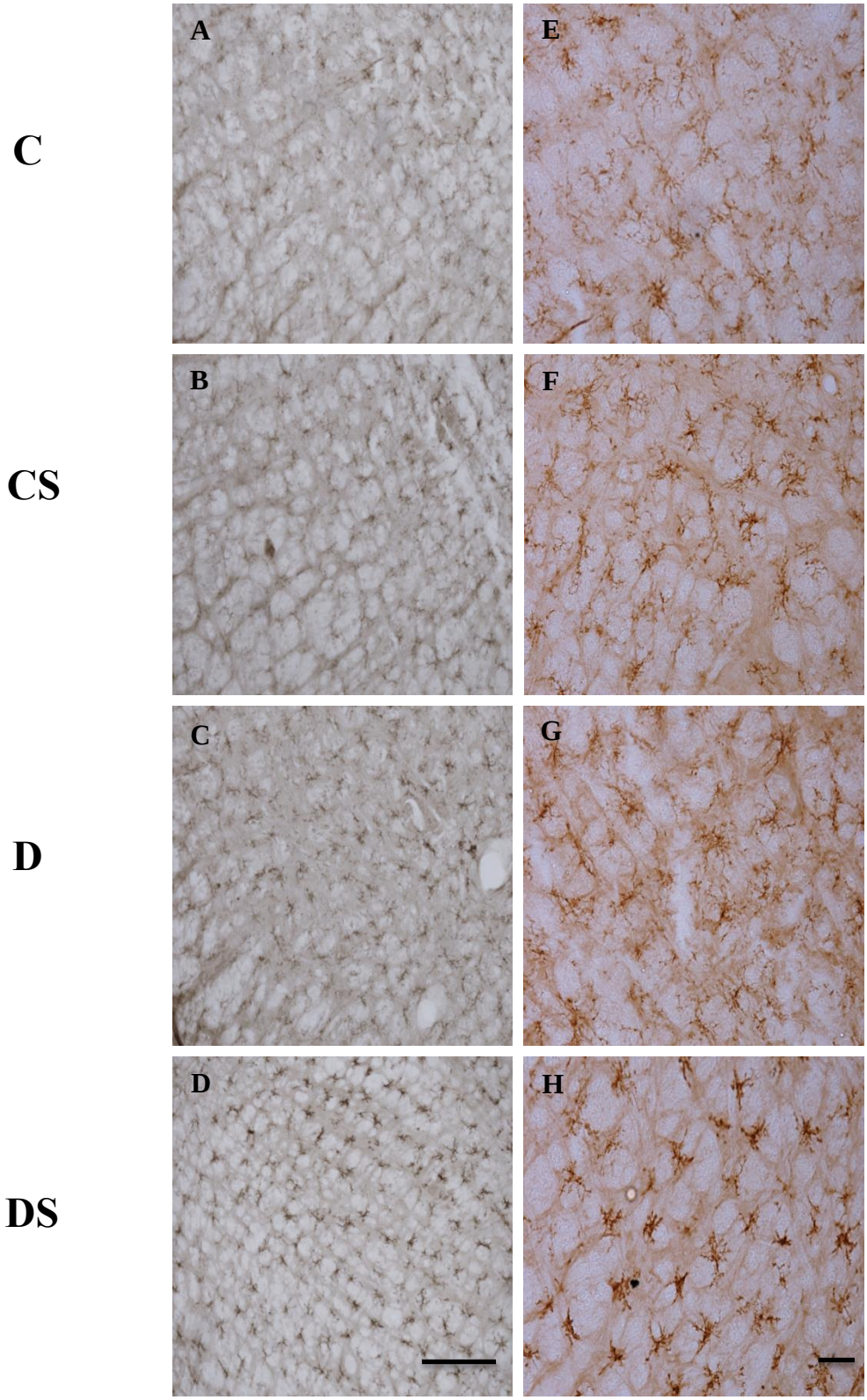


Figure 4-3. OX-42 Immunohistochemistry in the RVLM in diabetic rats. Photomicrographs showing CD11b (OX-42 clone) immunoreactive microglia in the rostral ventrolateral medulla (RVLM) in control (C) and diabetic animals (D) given tap water and control (C-S) and diabetic animals (D-S) given saline. Low-power images (left panel) and high-power images (right panel) show the morphology of microglia in the RVLM region. a normal microglia morphology was seen throughout the RVLM region in the C, C-S and D groups, while activated microglia showing intense immunolabelling, larger cell bodies and shorter, thicker processes were again common in the D-S group. Bar = 200 μm left panel, 100 μm in right panel.

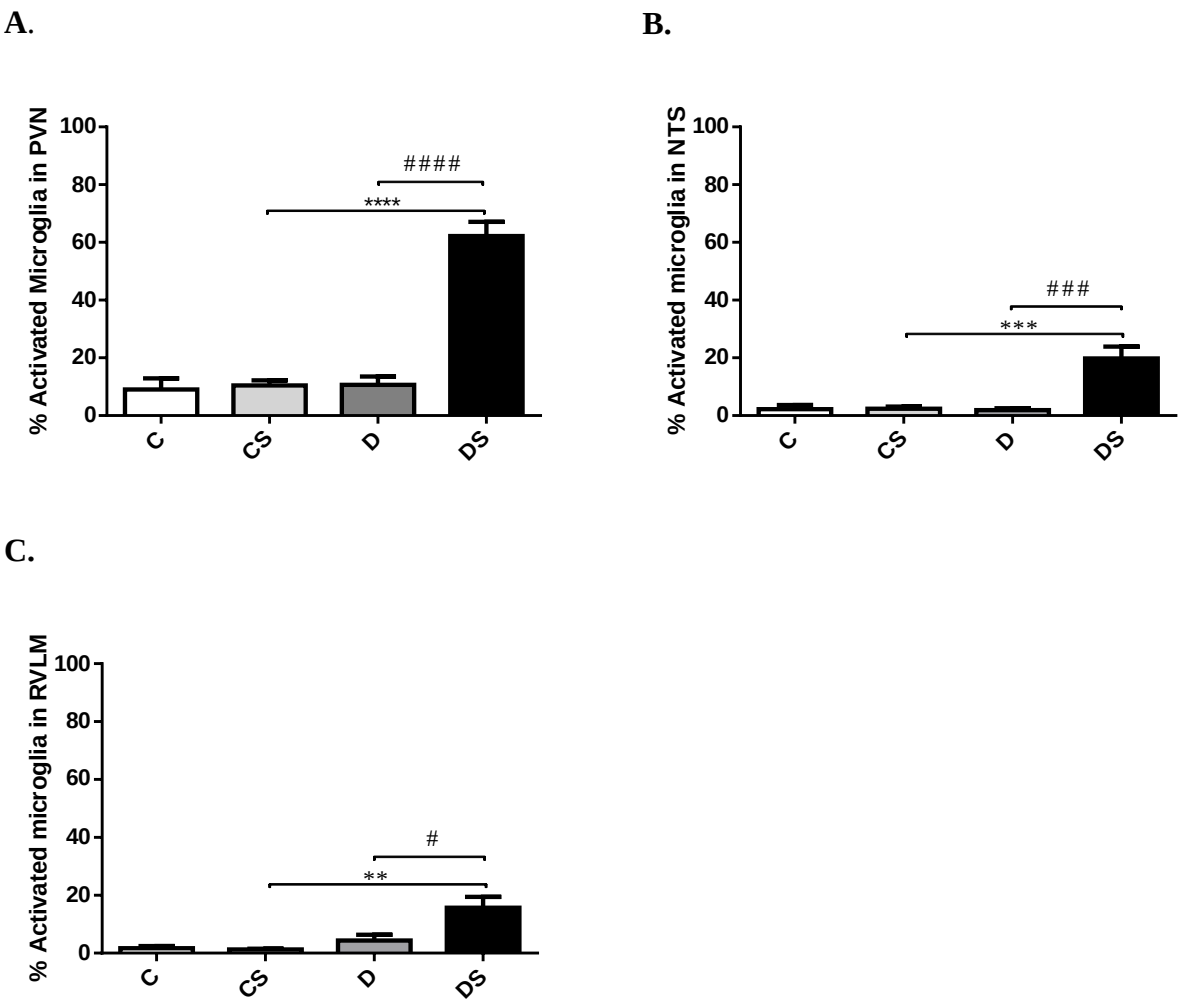


Figure 4-9. Microglial activation in cardiovascular centres of the brain of 2 week saline-treated diabetic rats. A: The percentage of microglia showing activated morphology in the paraventricular nucleus (PVN); B: in the nucleus tractus solitarius (NTS); and C: in the rostral ventrolateral medulla (RVLM) of control (C) and diabetic animals (D) given tap water and control (CS) and diabetic animals (DS) given saline. After 2 weeks, the percentage of activated microglia was significantly increased in the saline-treated diabetic group when compared with the diabetic group and the saline-treated control group. Number of rats = 4, 4, 4 and 7 respectively. Data are expressed as the mean ± SEM. ** indicates P<0.01; *** indicates P<0.001; **** indicates P < 0.0001 for comparisons between diabetic groups and their respective controls; # indicates P<0.05; ### indicates P<0.001; #### indicates P <0.0001 for comparisons between diabetic groups.

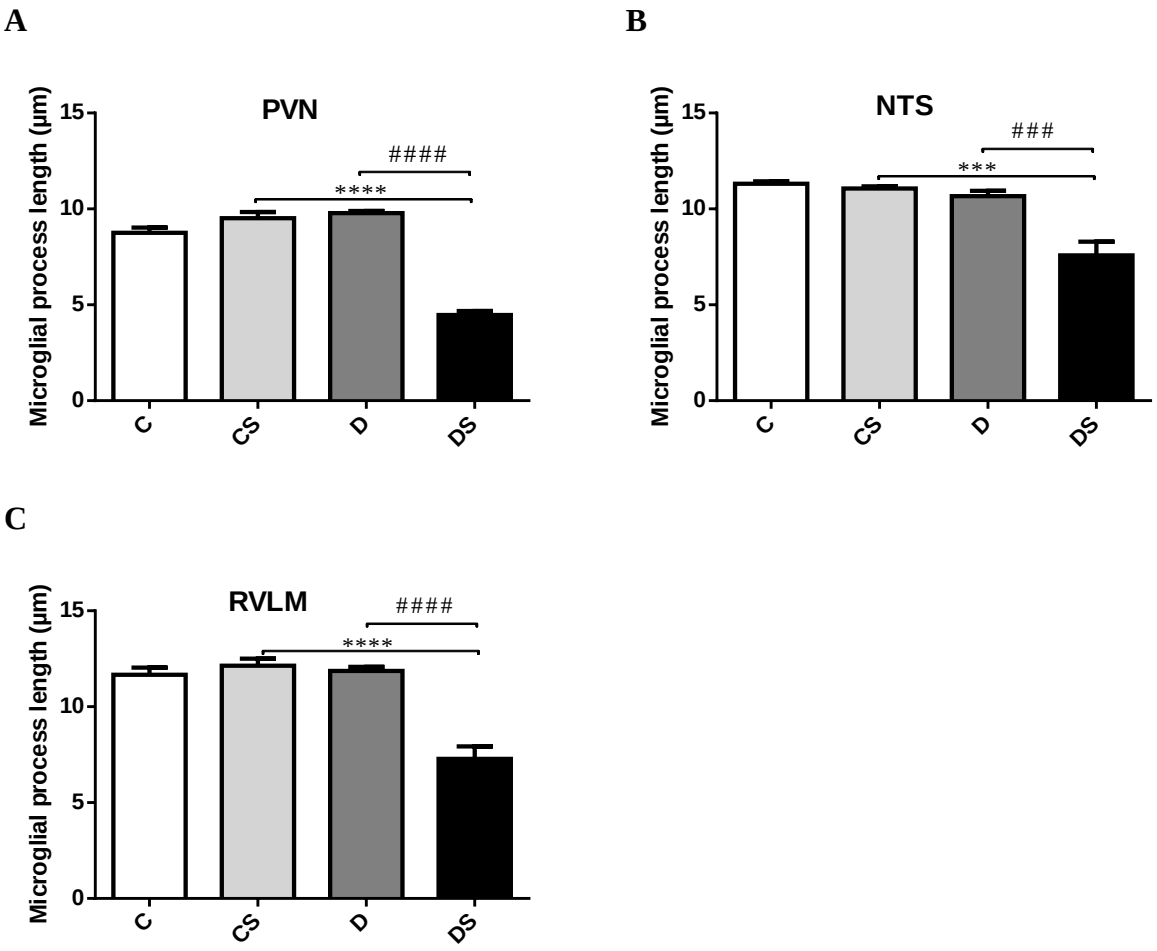


Figure 4-10. Microglia exhibit shorter processes in cardiovascular centres of the brain of 2 week saline-treated diabetic rats. A: Average length of microglial processes in the paraventricular nucleus (PVN); B: in the nucleus tractus solitarius (NTS); and C: in the rostral ventrolateral medulla (RVLM) of control (C) and diabetic animals (D) given tap water (D) and control (CS) and diabetic animals (DS) given saline. Number of rats = 4, 4, 4 and 7 respectively. Data are expressed as the mean ± SEM. *** indicates P<0.001; **** indicates P < 0.0001 for comparisons between diabetic groups and their respective controls; ### indicates P<0.001; #### indicates P <0.0001 for comparisons between diabetic groups.

4.2.4 C-fos immunoreactivity in cardiovascular centres in diabetic rats

In this study, an antibody to c-fos was used to quantify neuronal activation in the regions where microglia were activated. In the PVN in the C and CS groups, the number of fos-immunoreactive neurons was smaller compared to the D and DS groups (Figure 4-11 A). On quantification, we observed a significant increase in the number of fos-immunoreactive neurons in the D group compared to ($P<0.01$) the C group. The DS group also exhibited a greater level of fos activity than seen in ($P<0.05$) the CS group. In the D group, a significant increase in the number of fos-immunoreactive neurons was seen in both the magnocellular and parvocellular subdivisions of PVN (Figure 4-12 B). Interestingly, in the DS group, there was a significant increase in the number of fos-immunoreactive neurons only in the parvocellular PVN. In contrast to the PVN, the counts of fos-immunoreactive neurons in the NTS and RVLM regions showed no significant difference among all groups (Figure 4-11 B-C).

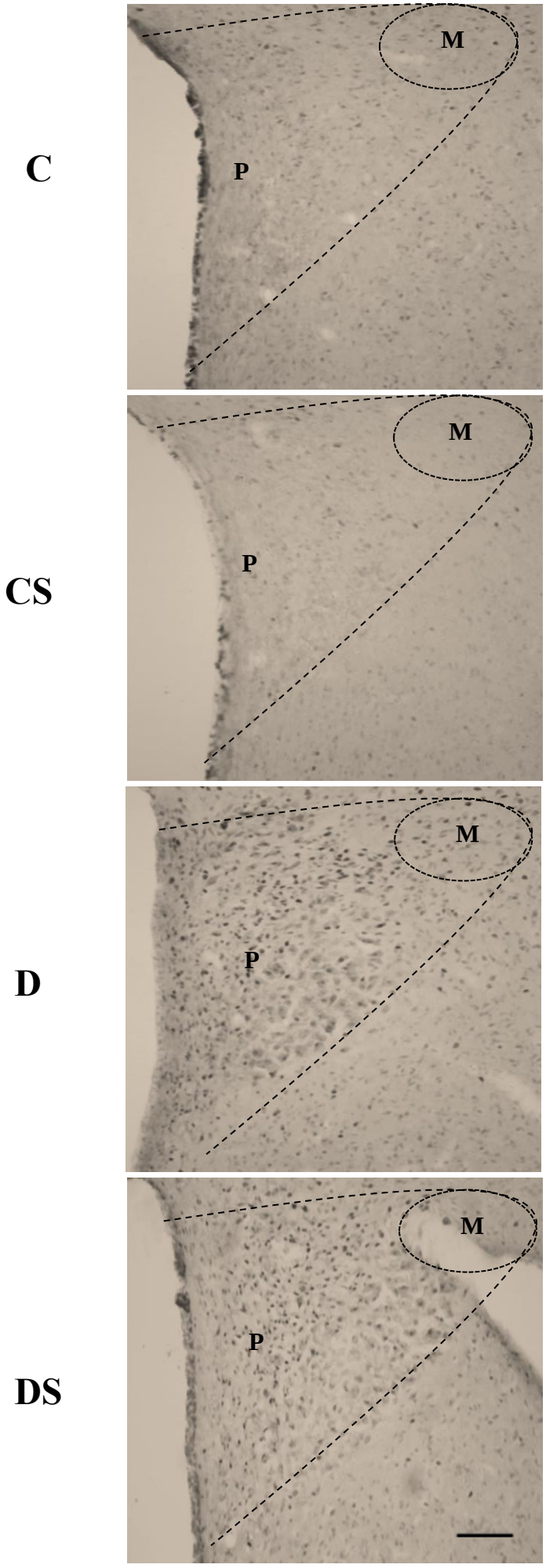

C
M
P
CS
M
P
D
M
P
DS
M
P

Figure 4-11. Fos activity in the PVN in diabetic rats. Photomicrographs showing neuronal nuclei stained with anti-Fos antibody in the paraventricular nucleus (PVN) in control (C) and diabetic animals (D) given tap water and control animals (CS) and diabetic animals (DS) given 1% NaCl solution.Dotted lines the PVN. Parvocellular (P) and magnocellular (M regions of the paraventricular nucleus are delineated. Bar = 100 μm.

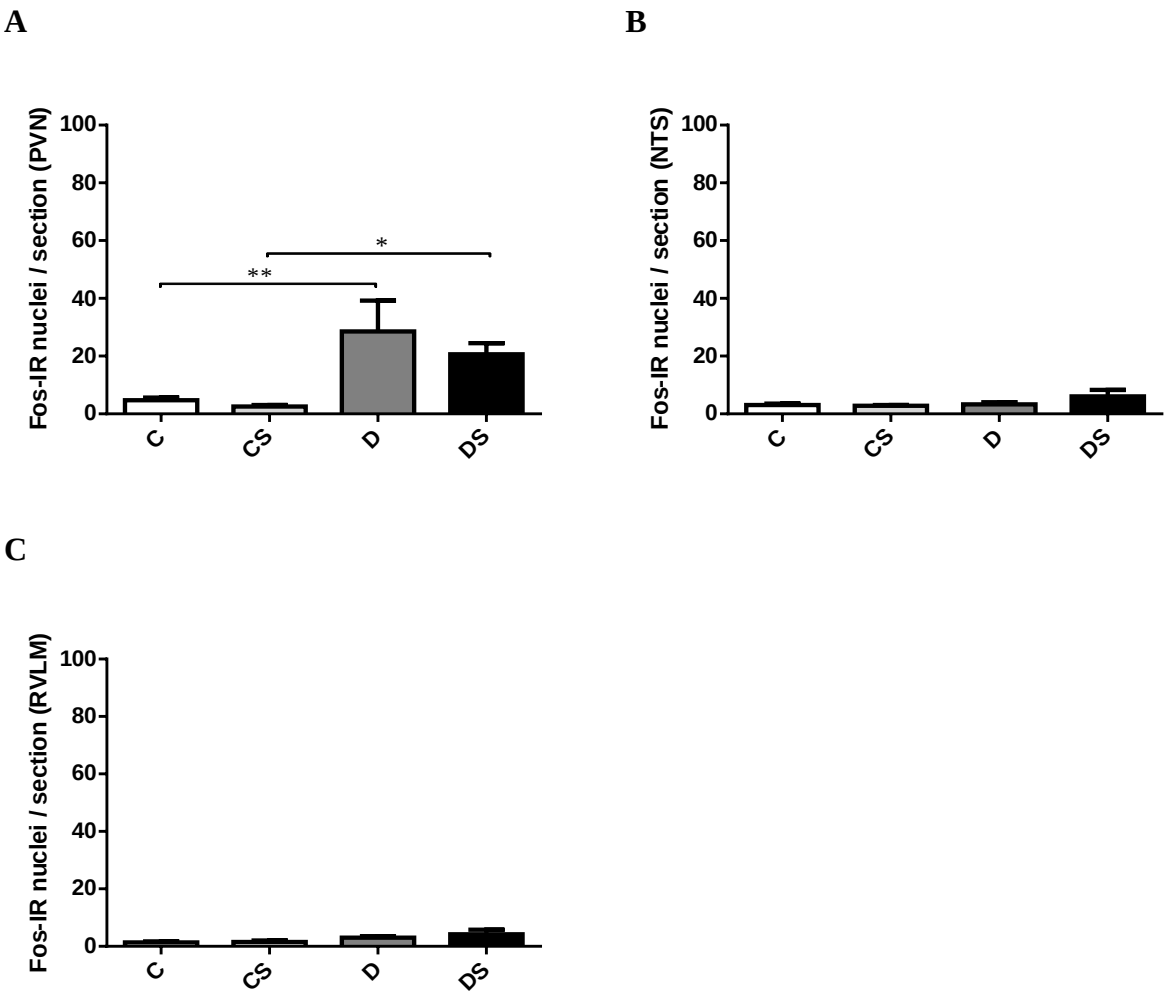


Figure 4-12. Fos-IR immunoreactivity indicating activated neurons in cardiovascular centres of the brain in diabetic rats. A: Quantification of Fos-IR positive cell nuclei in the paraventricular nucleus (PVN); B: in the nucleus tractus solitarius (NTS); and C: in the rostral ventrolateral medulla (RVLM) of control (C) and diabetic animals (D) given tap water and control (CS) and diabetic animals(DS) given saline. Number of rats = 7, 6, 4 and 5 respectively. Data are expressed as the mean ± SEM. * indicates $P<0.05$; ** indicates $P<0.01$ for comparisons between diabetic groups and their respective controls.

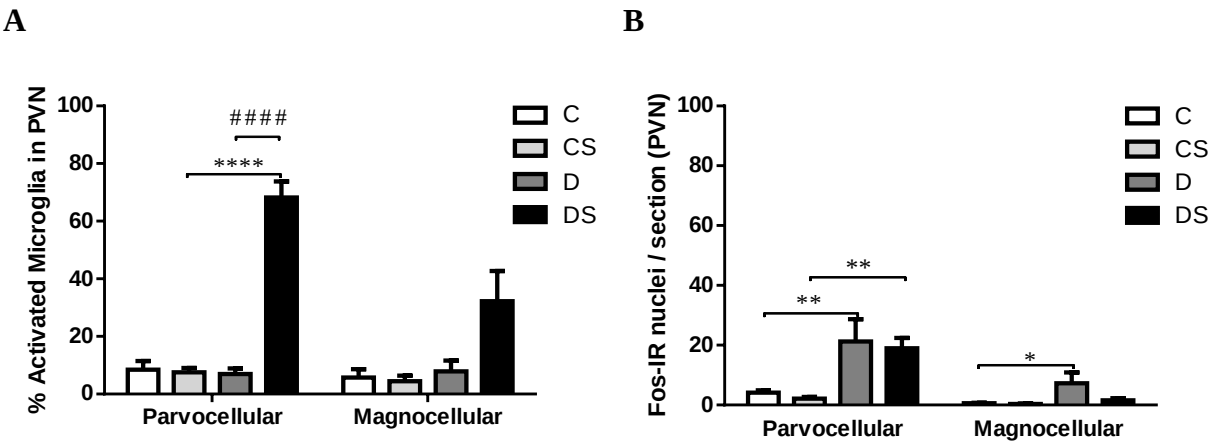


Figure 4-13. Microglial and neuronal activation in the parvocellular and magnocellular portions of the paraventricular nucleus of diabetic rats. A: The percentage of microglia showing activated morphology in the parvocellular and magnocellular of the paraventricular nucleus (PVN) of control (C) and diabetic animals (D) given tap water and control (CS) and diabetic animals (DS) given saline. B: Quantification of Fos-IR positive cell nuclei in the same regions. Number of rats = 5, 5, 4 and 7 respectively. Data are expressed as the mean ± SEM. * indicates P<0.05; ** indicates P<0.01; **** indicates P < 0.0001 for comparisons between diabetic groups and their respective controls; #### indicates P <0.0001 for comparisons between diabetic groups.

4.2.5 Effect of saline and minocycline treatment on blood pressure in awake diabetic rats.

Because we observed no change in the blood pressure in anaesthetized STZ diabetic rats treated with saline and microglia become activated in the PVN following saline intake in STZ diabetic rats, we therefore investigated the effect of the inhibition of microglial activation in the PVN in another cohort of STZ diabetic rats by direct ICV infusion into the lateral ventricle of either saline OR minocycline. At two weeks after treatment with STZ or citrate, there was no significant difference in systolic blood pressure between the control (CWS) and diabetic animals (DWS) infused with saline and given tap water to drink, which is in agreement with previous studies (Kawashima *et al.*, 1978; Hayashi *et al.*, 1983) (Figure 4-13). Minocycline infusion in diabetic animals given tap water (DWM) had no effect on systolic blood pressure compared to the DWS group. In contrast, diabetic animals infused with saline and given 1% NaCl (DSS) exhibited a greater increase in systolic blood pressure than those seen in the CWS and DWS ($P<0.0001$) groups, which is consistent with a previous study, suggesting saline causes hypertension in these rats (Maeda *et al.*, 2007). The increase in systolic blood pressure in the DSS group was prevented by minocycline infusion ($P<0.0001$) (Figure 4-13). These results suggest that central inflammation contributes to the development of hypertension in STZ diabetic rats treated with saline.

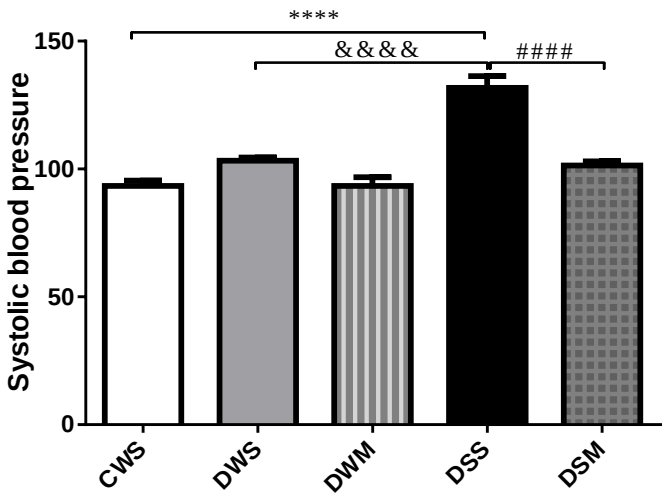


Figure 4-14. Effect of minocycline treatment on the development of hypertension in diabetic rats treated with saline for 2 weeks. Systolic blood pressure of conscious control animals (CWS) and diabetic animals (DWS) given tap water to drink for 2 weeks and diabetic animals (DSS) given 1% NaCl solution with an ICV infusion of saline and diabetic animals given tap water (DWM) or 1% NaCl (DSM) with an ICV infusion of minocycline. Number of rats = 5, 7, 9, 9 and 8 respectively. Data are expressed as the mean ± SEM. Significance was evaluated using a one way ANOVA followed by Tukey's post hoc test for all comparisons (CWS vs DWS , CWS vs DSS, DWS vs DWM , DWS vs DSS and DSS vs DSM).**** indicates <0.0001 for comparisons between diabetic groups and their respective controls; &&&& indicates P <0.0001 for comparisons between diabetic groups; #### indicates P<0.0001; for comparisons between diabetic groups and their respective groups of diabetic animals with minocycline treatment.

4.3 Discussion

There is evidence that drinking saline causes hypertension in diabetic animals, but how this occurs and whether CNS pathological changes contribute to it has not been investigated. Thus, in this study, we aimed to investigate the effects of 1% NaCl on cardiovascular parameters and whether saline intake influences inflammation in CNS cardiovascular centres in STZ diabetic rats. The main findings of the present study were that 1% NaCl (i) increased the mean arterial blood pressure of conscious STZ-diabetic rats, (ii) accelerated the dysfunction of bradycardic baroreflex and (iii) accelerated the activation of microglia in the PVN, NTS and RVLM and that (iv) ICV minocycline prevented the development of hypertension in diabetic rats treated with saline.

In the present study, there was no change in the blood pressure of awake diabetic animals when compared to the control, which is consistent with other studies at this early stage of diabetes (Kawashima *et al.*, 1978; Hayashi *et al.*, 1983). In contrast, when given to diabetic animals, 1% NaCl caused a significant increase in systolic blood pressure in awake animals, confirming a previous study by Maeda et al. (2007). Some authors have suggested that reduced blood volume in STZ rats due to osmotic diuresis is the mechanism of normotension or hypotension in STZ-diabetic rats (Hebden *et al.*, 1986a; De Angelis *et al.*, 2000). Thus, hypertension in diabetic animals given saline may be explained in part by increased blood volume. Interestingly, in the current study, saline intake also reduced the haemoglobin concentration and plasma osmolality to normal levels in diabetic rats, which indicates a normalization of blood volume. The effect of drinking 1% NaCl on the fluid balance of diabetic rats has not been studied previously. Our data provide strong support for the hypothesis that saline treatment normalises blood volume and may be responsible for the development of hypertension in STZ diabetic rats; however, it is possible that there are other

mechanisms involved in the development of hypertension in these animals besides blood volume.

Reduction in baroreflex sensitivity has been implicated in the pathogenesis of hypertension (Grassi *et al.*, 1998; Gao *et al.*, 2002; Salgado *et al.*, 2007; Maliszewska-Scislo *et al.*, 2008). While baroreflex sensitivity in STZ diabetic animals have been widely investigated, few studies on the effects of saline intake on baroreflex sensitivity have been conducted in STZ diabetic rats. In the present study, we observed no changes in baroreflex sensitivity in diabetic animals compared to the control, which is in agreement with previous studies that have shown that the baroreflex-mediated bradycardia was unaltered in short-term (5 to 14 days) STZ diabetic rats (Maeda *et al.*, 1995; Van Buren *et al.*, 1998). In contrast, 1% NaCl caused an impairment of baroreflex sensitivity in diabetic animals at 2 weeks. Interestingly, several studies have reported that baroreflex sensitivity is reduced in long-term (4 to 42 weeks) STZ-diabetic rats (Van Buren *et al.*, 1998; Dall'Ago *et al.*, 2002; Harthmann *et al.*, 2007; Souza *et al.*, 2007). Therefore, our results suggest that 1% NaCl accelerates the development of baroreflex dysfunction in diabetic animals at early stages, and this dysfunction may contribute to the development of hypertension seen in awake diabetic animals.

Because we did not observe a significant difference in the blood pressure among diabetic groups under anaesthesia, the decreased baroreflex sensitivity is not attributable to a simple blood pressure change. Urethane may interrupt the mechanism that generates high blood pressure in salt-treated diabetic rats, but it is the anaesthetic of choice for studying baroreceptor reflexes. In support of this, Barringer et al. demonstrated that urethane had no effect on the baroreflex-mediated bradycardia in the normotensive rat (1990). The only other published study to have investigated baroreflex sensitivity in diabetic animals treated with saline was inconclusive (Maeda *et al.*, 2007). We suspect that the difference between their

results and ours is likely due to their use of ketamine/xylazine anaesthesia. Considered collectively, these data indicate that 1% NaCl treatment for 14 days impaired the baroreflex-mediated bradycardia response selectively in diabetic rats without affecting blood pressure or heart rate under anaesthesia.

To understand the potential mechanism of these changes on cardiovascular parameters, we examined whether microglia are activated in CNS cardiovascular centres. In the PVN, we found no activation of microglia in diabetic animals at two weeks following STZ injection, which is in agreement with our previous study that reported that microglia become activated at later stages (Rana *et al.*, 2014). In contrast, we show here that 1% NaCl accelerated microglial activation in the PVN in STZ-induced diabetic rats. This activation was selectively in the PVN and not seen in surrounding areas. To clarify the pathological consequences of microglial activation in the PVN of diabetic rats treated with 1% NaCl, we measured blood pressure via the tail cuff and investigated the effect of the inhibition of microglial activation in another cohort of animals. There was a significant increase in the systolic blood pressure only in diabetic rats treated with saline. By administering minocycline, which acts as an anti-inflammatory drug, into the lateral brain ventricle, we observed a marked reduction in blood pressure. It has been shown previously that microglial activation in the PVN contributes to the development of neurogenic hypertension (Shi *et al.*, 2010a). In this context, our results strongly suggest that hypertension seen in diabetic rats given saline is neurogenic and that increased microglial activation in the PVN may cause the increased blood pressure in these rats. Because saline treatment had no effect on blood pressure in the control rats, it appears that 1% NaCl intake accelerates and increases the inflammatory response seen in the PVN in response to diabetes.

The data from this study do not outline the mechanisms by which saline intake accelerates microglial activation in diabetic animals, thereby impairing baroreflex sensitivity and causing

hypertension; however, it is clear from other studies that high salt intake contributes to increased blood pressure. In fact, the only current information about the mechanisms by which saline may influence brain inflammation is based on studies using highly concentrated sodium diets (Koga *et al.*, 2008; Braga, 2010). In this study, diabetic rats given saline consumed a larger amount of 1% NaCl compared to the control rats, but it is not clear if diabetic rats drank more 1% NaCl than they needed because their excretion was also high. Therefore, we must consider the possibility that the brain inflammation seen in our study could be in part due to increased NaCl intake. Thus, despite the fact that saline normalises haemoglobin concentration and plasma osmolality, it may also contribute to brain inflammation in diabetic rats.

A high salt intake (8% NaCl in food or 2% NaCl to drink) increases reactive oxygen species formation in brain regions that are involved in autonomic control (Koga *et al.*, 2008; Braga, 2010). The increase in the generation of reactive oxygen species can enhance the influx of Ca^{2+} in neurons, which in turn leads to neuronal activation (Wang *et al.*, 2003; Zimmerman & Davisson, 2004). It is well-known that microglia become activated following neuronal death, damage or over excitation (Hathway *et al.*, 2009; Lu *et al.*, 2009). In addition, we previously reported increased neuronal activity in the PVN of diabetic rats at 6 weeks following STZ injection; however, increased microglial activation was not observed until 8 weeks following STZ injections. Rana et al. (2014) suggested that microglial activation in the PVN may be secondary to prolonged intense activation of PVN neurons in diabetic rats.

In the parvocellular PVN, we observed increased neuronal activation in diabetic rats at 6 weeks following STZ injection (Rana *et al.*, 2014). These neurons play a role in the regulation of autonomic function, including sympathetic nerve activity and blood pressure, as well as endocrine functions. Since microglial activation was not seen in this region in diabetic rats given tap water at 2 weeks, our data support the hypothesis that neuronal activation

precedes microglial activation. The reason that parvocellular PVN neurons were activated in diabetic rats is not clear; however, parvocellular PVN neurons have been shown to be activated by haemorrhage (Badoer *et al.*, 1993; Badoer & Merolli, 1998), suggesting that those neurons may be activated by a decrease in blood volume seen in diabetic animals. Also, infusion of hypertonic saline has been shown to increase neuronal activation in this region (Bealer & Metcalf, 2005). Thus, this activation may be also a result of increased plasma osmolarity in diabetic rats. Therefore, it appears that the parvocellular division of the PVN responds to disturbances in blood volume and plasma osmolarity in diabetic rats. In diabetic rats treated with saline, we observed neuronal and microglial activation in the parvocellular division of the PVN, although the haemoglobin concentration and plasma osmolarity were normalised, suggesting that the mechanisms by which PVN neurons become activated in these rats are different.

Activated microglia have also been shown to cause neuronal over excitation via the release of proinflammatory molecules and reactive oxygen species (Shi *et al.*, 2011; Wu *et al.*, 2012). Interestingly, Shi et al. (2010a) reported that microinjection of IL-1β into the PVN increased PVN neuronal activation and blood pressure, suggesting IL-1β secretion from activated microglia enhances PVN neuronal activation to increase blood pressure. This suggests that PVN microglial activation may be a cause of over excitation of PVN neurons, which in turn elevated blood pressure seen in diabetic rats treated with saline.

Interestingly, in the magnocellular division of the PVN, we also observed increased neuronal activation in diabetic rats but not in saline-treated diabetic rats. This result is in agreement with previous studies that reported increases in neuronal activity in the magnocellular of the PVN at 2 weeks following STZ injection (Krukoff & Patel, 1990). The magnocellular part of the PVN is the main site involved in secreting oxytocin and vasopressin in response to osmotic and other stimuli to control body fluid balance and blood volume. It has been

suggested that elevated levels of plasma osmolality (Charlton *et al.*, 1988; Brooks *et al.*, 1989) and dehydration (Zheng *et al.*, 2002) in diabetic animals may be responsible for neuronal activation in the magnocellular PVN and consequently increased vasopressin secretion (Zerbe *et al.*, 1979; Brooks *et al.*, 1989). In the present study, diabetic rats displayed increased plasma osmolality as well as increased haemoglobin concentration, which indicates depletion in blood plasma volume and may explain the activation of magnocellular neurons seen in diabetic rats. Interestingly, in the magnocellular PVN, there was no neuronal activation in diabetic rats treated with saline, potentially because blood volume was normalised.

In the present study, we also show microglial activation in the NTS and RVLM in diabetic rats treated with saline. These autonomic brain areas are also involved in cardiovascular regulation. The NTS plays a pivotal role in mediating the baroreceptor reflex. In addition, the NTS also receives inputs from the PVN. The activation of microglia and neurons was not observed in the NTS in diabetic rats at 2 weeks after STZ injection, which is in agreement with our previous study (Rana *et al.*, 2014). In contrast, there was an increase in the activation of microglia in diabetic rats treated with saline. Another study reported that microinjection of proinflammatory cytokine (IL-6) into the NTS cause a marked decrease in the baroreceptor sensitivity (Takagishi *et al.*, 2010). It is well-known that activated microglia are capable of secreting proinflammatory cytokines, such as IL-6. Thus, it is possible that microglial activation may explain the decreased baroreceptor sensitivity in diabetic rats treated with saline.

The RVLM also showed an increase in microglial activation in diabetic rats treated with saline. RVLM is an important autonomic region that is involved in blood pressure regulation through modulating sympathetic nerve activity. It also receives inputs from other cardiovascular centres, including PVN and NTS. An increase in reactive oxygen species in

the RVLM has been shown to precede the development of hypertension (Koga *et al.*, 2008; Braga, 2010; Nunes & Braga, 2011). Interestingly, a recent study on LPS-hypertensive animals reported that microglial activation was associated with increased reactive oxygen species in the RVLM (Wu *et al.*, 2012). Along with our results, these studies suggest that activated microglia contribute to an increased production of reactive oxygen species and may contribute to hypertension seen in diabetic rats treated with saline.

Interestingly, there was no increase in neuronal fos expression in the NTS and RVLM in diabetic rats treated with saline, suggesting that the mechanisms by which microglia become activated in those areas are different from those in the PVN. Still, it could also be argued that c-fos expression is a very binary classification for each cell type, so it is not capable of showing an increase in the intensity of the activation of a group of neurons if the change does not lead to an increase in the number of neurons activated. On the other hand, there was no appreciable increased expression of c-fos in any condition; thus, c-fos may not be expressed in all activated neurons in the NTS and RVLM. Alternatively, microglial activation in those regions could be mediated by other mechanisms. For example, a study on STZ diabetic rats reported microglial activation in the dorsal horn of the spinal cord (Tsuda *et al.*, 2008). This study suggested that damage to peripheral sensory neurones that terminate in the dorsal horn was responsible for microglial activation. Therefore, while we do not know the effect of saline intake on the terminals of autonomic sensory neurons, damage of those neurons may be responsible for microglial activation in the NTS and RVLM.

4.4 Conclusion

In conclusion, our data indicated that 1% NaCl intake increases blood pressure in STZ diabetic rats. Saline treatment also accelerates microglial activation in the PVN in diabetic rats. Microglial inhibition in the PVN prevents the development of hypertension in diabetic rats, suggesting that microglial activation is involved in the mechanisms of hypertension in

these animals. Microglial activation in the PVN may be either a cause or an effect of neuronal activation or both. In addition, saline intake can significantly effect baroreflex sensitivity in diabetic rats, and this is associated with microglial activation in the NTS and RVLM; however, the role of microglia in the NTS and RVLM is not clear, and further research is required to demonstrate the effect of microglia inhibition in those regions. Overall, the attenuation of inflammation in cardiovascular centres may be a novel strategy for lowering the risk of cardiovascular diseases in diabetes. In addition, salt intake may have profound and complex effects on brain inflammation and blood pressure in diabetic humans.

Chapter Five: Drinking 1% NaCl for 6 Weeks Increases Baroreflex Sensitivity in STZ Diabetic Rats

5.1 Introduction

Salt intake has been associated with the risk of the development of hypertension and cardiovascular disease in both humans and animals (Campese, 1994; Sacks *et al.*, 2001; Jones, 2004; Brooks *et al.*, 2005). Dehydration is common in diabetes, and low salt intake may cause deleterious effects. Still, the mechanisms underlying the effect of salt intake on hypertension and its effects in diabetes has not been fully understood. It has been suggested that salt intake contributes to the development of hypertension via several potential mechanisms, including dysregulation of autonomic nervous system or increased blood volume, but which of these mechanisms are relatively important in diabetes is not known (Brooks *et al.*, 2005).

Autonomic dysfunction in relation to the cardiovascular system has been reported in humans with type 1 diabetes and animals with chronic hyperglycaemia. This includes an abnormal increase in the renal sympathetic activity (Perin *et al.*, 2001; Patel *et al.*, 2011). Evidence demonstrates that abnormal activation of the sympathetic nerve is crucial in the development of hypertension (Dampney *et al.*, 2005; Esler, 2011; Takahashi, 2012). The cause of the abnormal activation of sympathetic nerves in diabetes is not well understood; however, there is increasing evidence that an impairment of baroreflex sensitivity, which is important in controlling both sympathetic activity and blood pressure levels, may play an important role. Interestingly, although impairment of baroreflex sensitivity is reported in long-term STZ-induced diabetic rats, we have previously reported that drinking 1% NaCl accelerates the dysfunction of bradycardic baroreflex in STZ-induced diabetic rats at early stages (chapter 4). This dysfunction was also associated with an increase in the mean arterial blood pressure, suggesting that impairment of baroreflex sensitivity may be the potential mechanism that contributes to the development of hypertension in STZ-induced diabetic rats.

However, the central mechanism by which saline intake triggers baroreflex dysfunction in STZ diabetic rats is not completely understood. Interestingly, we have previously reported that saline intake in STZ diabetic rats not only accelerates baroreflex dysfunction but also increases microglial activation in the PVN, RVLM and NTS (chapter 4). Microglial inhibition in the PVN prevents the development of hypertension in these animals, suggesting that microglial activation is involved in the mechanisms of hypertension (chapter 4). Another study has shown that microinjection of proinflammatory cytokine (IL-6) into the NTS caused a marked decrease in the baroreceptor sensitivity (Takagishi *et al.*, 2010). Activated microglia are known to release proinflammatory molecules, such as IL-6. Thus, this inflammation seen in STZ diabetic rats treated with saline at early stages may be responsible for baroreflex dysfunction; however, there is a lack of data on the effect saline intake has on CNS inflammation in the long term after STZ treatment.

While it is clear from our study and other previous studies that saline intake can accelerate and intensify diabetic cardiovascular complications in short-term STZ diabetic rats, there is evidence that drinking saline improved cardiac function in the long term (Dai *et al.*, 1994). Therefore, it appears that saline intake has paradoxical effects on the cardiovascular system, and this effect may be time dependent. It is not known whether STZ diabetic rats treated with 1% NaCl for longer periods show similar baroreflex alterations to the ones seen during early stages or whether 1% NaCl causes further depression in the baroreflex function.

At early stages in STZ diabetic rats, drinking saline accelerates microglial activation in the brain nuclei that are involved in cardiovascular regulatory functions. While this activation may explain the baroreflex dysfunction that is seen in these animals, it is not consistent with improving cardiac function in long-term STZ diabetic rats treated with saline. Whether saline intake reduces or increases CNS inflammation in STZ diabetic rats given saline for long periods is not known.

Therefore, we investigated the effects of saline intake on blood pressure and baroreflex sensitivity in long-term STZ diabetic rats. In addition, we investigated whether saline intake influences inflammation in the cardiovascular centres in long-term STZ diabetic rats.

5.2 Results

5.2.1 Effect of 6 weeks saline intake on general features of diabetic rats

When Male Sprague Dawley rats were injected with either STZ or citrate buffer and maintained for 6 weeks with free access to either tap water or saline to drink, the non-diabetic control animals given water (C) and non-diabetic animals given saline (CS) exhibited a gradual increase in body weight. The same pattern was also observed in diabetic animals given water (D) and diabetic animals given saline (DS), but the weight gained in these diabetic groups was lower when compared with the control groups (Figure 5-1A). As expected, the body weights of the D group were significantly lower ($P<0.01$) compared with the C group animals, which is consistent with previous studies (chapter 3). Also, the body weights of the DS group were significantly lower ($P<0.001$) when compared with the CS group. Saline treatment in long-term STZ diabetic rats did not significantly affect body weight in either diabetic or control animals, which is in agreement with our previous study, in the short term (chapter 4). Blood glucose levels were lower than 8 mmol/L in the C and CS groups and higher than 25 mmol/L in the D and DS groups, indicating extreme hyperglycaemia (Figure 5-1B). The D group exhibited significantly higher ($P < 0.0001$) blood glucose levels compared with the C group, as expected. This was also the case for the blood glucose levels in the DS group compared to the CS group ($P < 0.0001$). Interestingly, saline treatment significantly reduced ($P<0. 0001$) blood glucose levels in the diabetic group, though not to normal levels, and it had no effect on blood glucose in the control animals. The results of body weight and blood glucose measurements indicated that STZ-treated diabetic rats exhibited changes that are characteristic of this model.

Regarding fluid intake, drinking both 1% saline and tap water increased progressively over 6 weeks in the D and DS groups, but this was not the case in C and CS groups, suggesting that diabetic animals were compensating for substantial fluid loss (Figure 5-1C). Daily water

intake was significantly greater ($P<0.0001$) in the D group compared to the C group, as expected. Also, the daily saline intake was significantly greater ($P<0.0001$) in diabetic animals when compared with control animals. In diabetic animals, the daily saline intake was significantly higher ($P < 0.01$) compared to the daily water intake, which is consistent with data observed in early stages (chapter 4). This was not the case in the control animals.

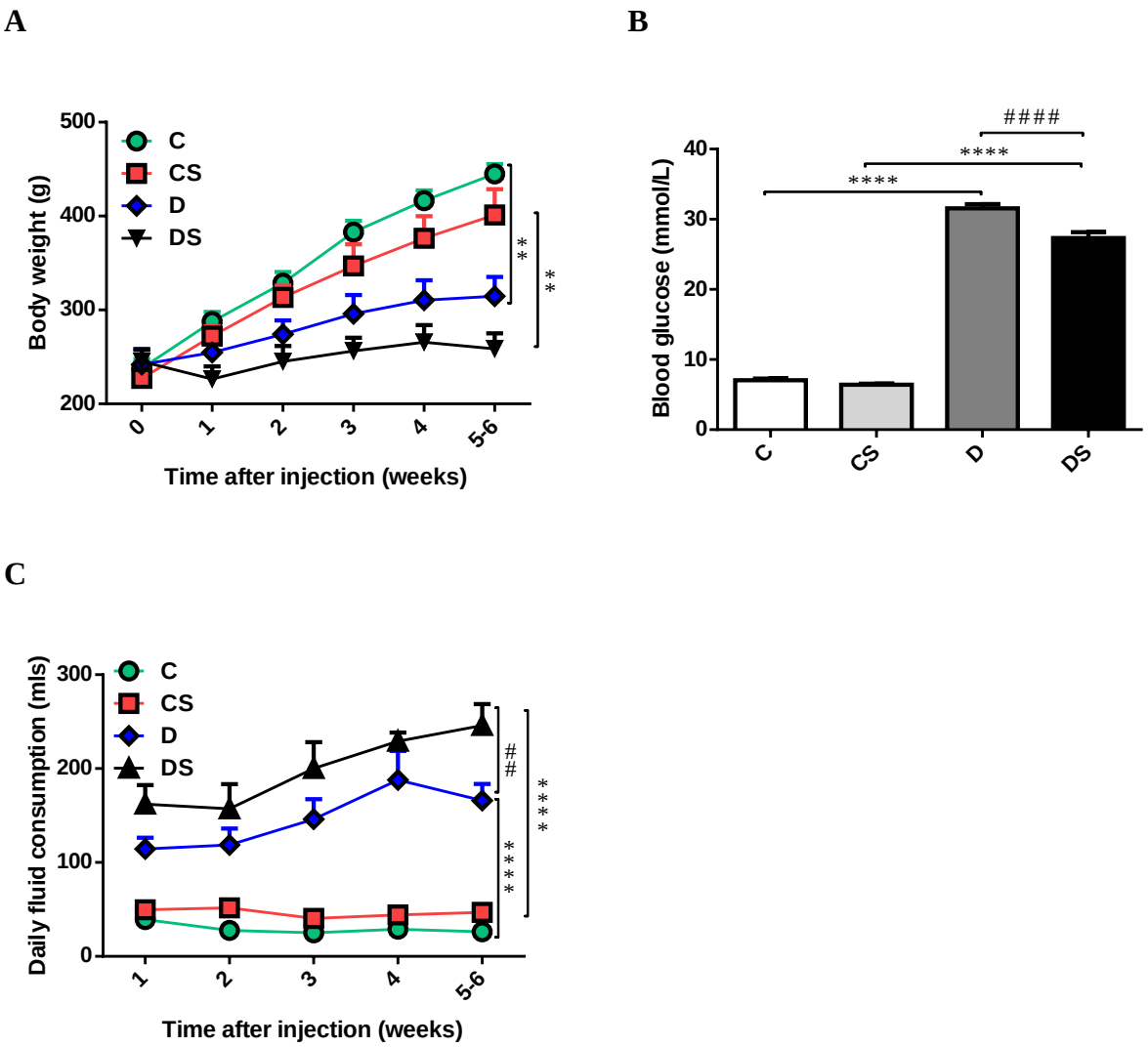


Figure 5-1. Effect of 6 weeks saline treatment on body weight, blood glucose and fluid intake of diabetic rats. A: Body weight measured at weekly intervals in control animals (C) and diabetic animals (D) given tap water to drink and control animals (CS) and diabetic animals (DS) given 1% NaCl solution. B: Blood glucose levels measured at 6 weeks after injection of rats with either STZ or citrate buffer. C: The amount of tap water or 1% NaCl solution ingested was monitored daily by weighting water bottles and then averaged for each week. Number of rats = 4 in each group. Data are expressed as the mean ± SEM. Significance was evaluated using a one way ANOVA followed by Tukey's post hoc test for all comparisons (C vs D, C vs CS, CS vs DS and D vs DS) in this and all subsequent figures; ** indicates P<0.01; **** indicates P < 0.0001 for comparisons between diabetic groups and their respective controls; ## indicates P<0.01; #### indicates P <0.0001 for comparisons between diabetic groups.

5.2.2 Measurement of baroreflex-mediated bradycardia in diabetic rats

In experiments to investigate the baroreflex-mediated bradycardia in the long term (6 weeks) after STZ or citrate injection, the mean arterial blood pressure under anaesthesia was not significantly different between the C and D or between the CS and DS groups (Figure 5-2A). Also, no significant differences were found in heart rate among the groups (Figure 5-2B). These results are consistent with a previous study at early stages (chapter 4). A progressive increase in the maximal mean arterial blood and decrease in the maximal heart rate in response to the infusion of increasing amounts of phenylephrine was seen in all groups. The maximal mean arterial blood pressure response to phenylephrine at all doses was not significantly different between the D and C groups (Figure 5-3A). The same pattern was also observed in the DS group compared to the CS group. Saline intake had no effect on blood pressure changes in response to phenylephrine in the diabetic or control animals. The maximal heart rate response to phenylephrine was also similar between the DS and CS group (Figure 5-3B); however, in the D group, the heart rate response to phenylephrine was significantly lower at 1, 2 and 5 µg doses but not 10 µg compared with the C groups. When baroreflex sensitivity was quantified as the ratio of maximal change in heart rate divided by the maximal change in mean arterial blood pressure, the baroreflex-mediated bradycardia in response to increasing doses of phenylephrine was similar between the DS and CS groups (Figure 5-3C); however, there was a significant reduction in baroreflex sensitivity in the D group when compared to the CW group ($P<0.05$), which is in agreement with the findings of others in long-term STZ diabetic rats. Interestingly, in late stages, saline treatment significantly increased ($P<0.05$) baroreflex sensitivity to normal levels in diabetic animals, which is opposed to our data at early stages of diabetes. These data suggested that saline has paradoxical effects on baroreflex sensitivity in STZ diabetic rats and that this effect is time dependent.

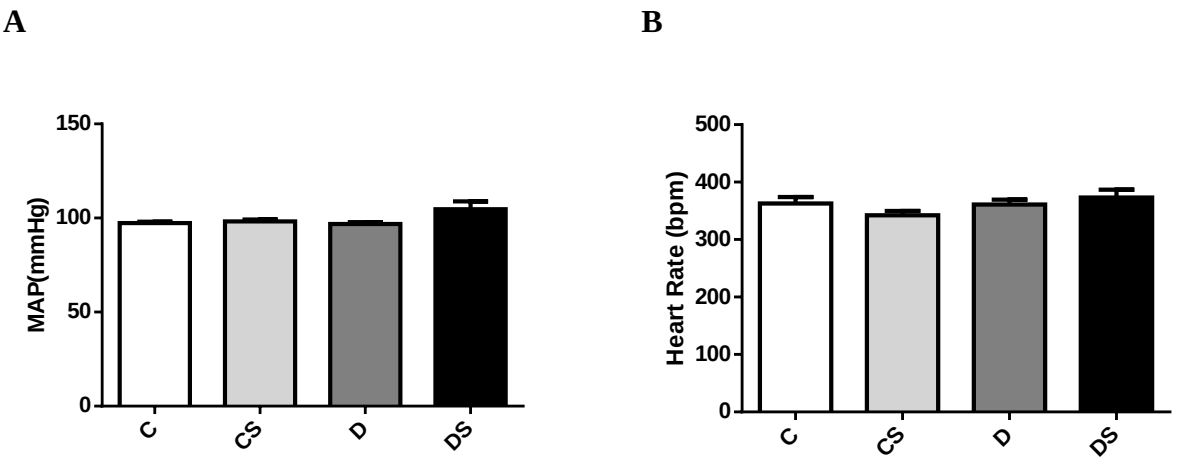


Figure 5-2. Effect of 6 weeks saline treatment on cardiovascular parameters in diabetic rats under anaesthesia. A: The mean arterial blood pressure (MAP); and B: The heart rate of unconscious control animals (C) and diabetic animals (D) given tap water and control animals (CS) and diabetic animals (DS) given 1% NaCl solution. Number of rats = 4 in each group. Data are expressed as the mean ± SEM. No significant differences were observed.

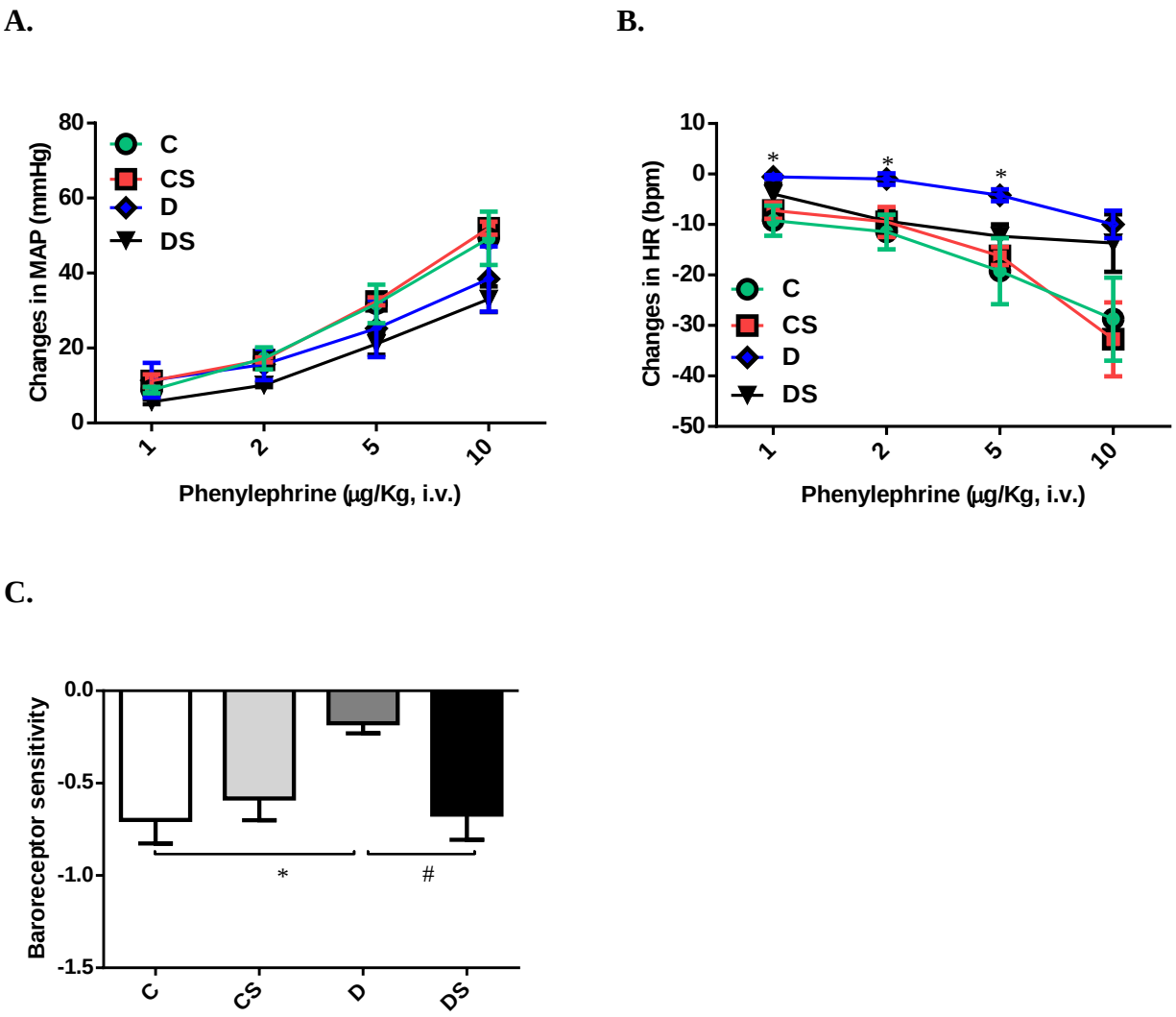


Figure 5-3. Cardiovascular responses to phenylephrine in diabetic rats. A: Changes in mean arterial pressure (MAP); and B: changes in heart rate (HR) in response to graded doses of intravenous phenylephrine (1, 2, 5 and 10 µg/kg) in control (C) and diabetic animals (D) given tap water and control (CS) and diabetic animals (DS) given saline. C: Baroreflex sensitivity (bpm/mmHg) of bradycardic responses represented as the average of the maximal change in heart rate divided by the maximal change in mean arterial blood pressure for each phenylephrine dose and then averaged for each animal. Baroreflex-mediated bradycardia was significantly attenuated in the diabetic group compared with the diabetic group treated with saline and the control group. Number of rats = 4, 4, 5 and 3 respectively. Data are expressed as the mean ± SEM. * indicates $P<0.05$ for comparisons between diabetic groups and their respective controls; ♯ indicates $P<0.05$ for comparisons between diabetic groups.

5.2.3 OX-42 Immunohistochemistry in cardiovascular centres in diabetic rats

The OX-42 antibody was used to identify morphological changes in microglia cells in the cardiovascular centres in STZ rats. At 6 weeks following STZ or vehicle injection, microglia displayed normal morphology with small cell bodies and long, fine-branched processes throughout the PVN region in the C and CS groups, which is consistent with our previous study at early stages (chapter 4) (Figure 5-4). In contrast to our data at 2 weeks, there was a significant increase in the percentage of activated microglia with large cell bodies and short, thick processes in the PVN in the D group ($P<0.01$) compared to PVN microglia in the C group. Also, the percentage of activated microglia was significantly increased in the PVN in the DS group compared to PVN microglia in the CS group (Figure 5-6A). Saline treatment had no effect on the percentage of activated microglial in the diabetic group or control group. A significant increase in the percentage of activated microglial in diabetic groups was seen in both the parvocellular and magnocellular PVN (Figure 5-9A).

In the NTS region in which baroreceptive afferent nerves terminate, microglial cells showed normal morphology with small cell bodies and long, fine-branched processes in the C, CS and DS groups (Figure 5-5). In contrast, darkly stained microglia with shorter, thicker processes were clearly observed in the NTS in the D group. The quantification of the percentage of activated microglia in the NTS in the D group showed a significantly greater activation compared to the C ($P<0.01$) and DS ($P<0.001$) groups (Figure 5-6B). Saline treatment in long-term STZ diabetic rats reduced the increase in the percentage of activated microglia in the NTS seen at 2 weeks following STZ treatment. This reduction in microglial activation was associated with improving baroreflex sensitivity, suggesting that NTS inflammation contributes to baroreflex dysfunction in short-term STZ diabetic rats treated with saline.

Microglial cells in the RVLM region exhibited normal morphology in the D and C groups. The same pattern was also observed in the DS and CS groups. The quantitative analysis of microglial activation in the RVLM region indicated that no significant differences were found among any of the groups (Figure 5-6C).

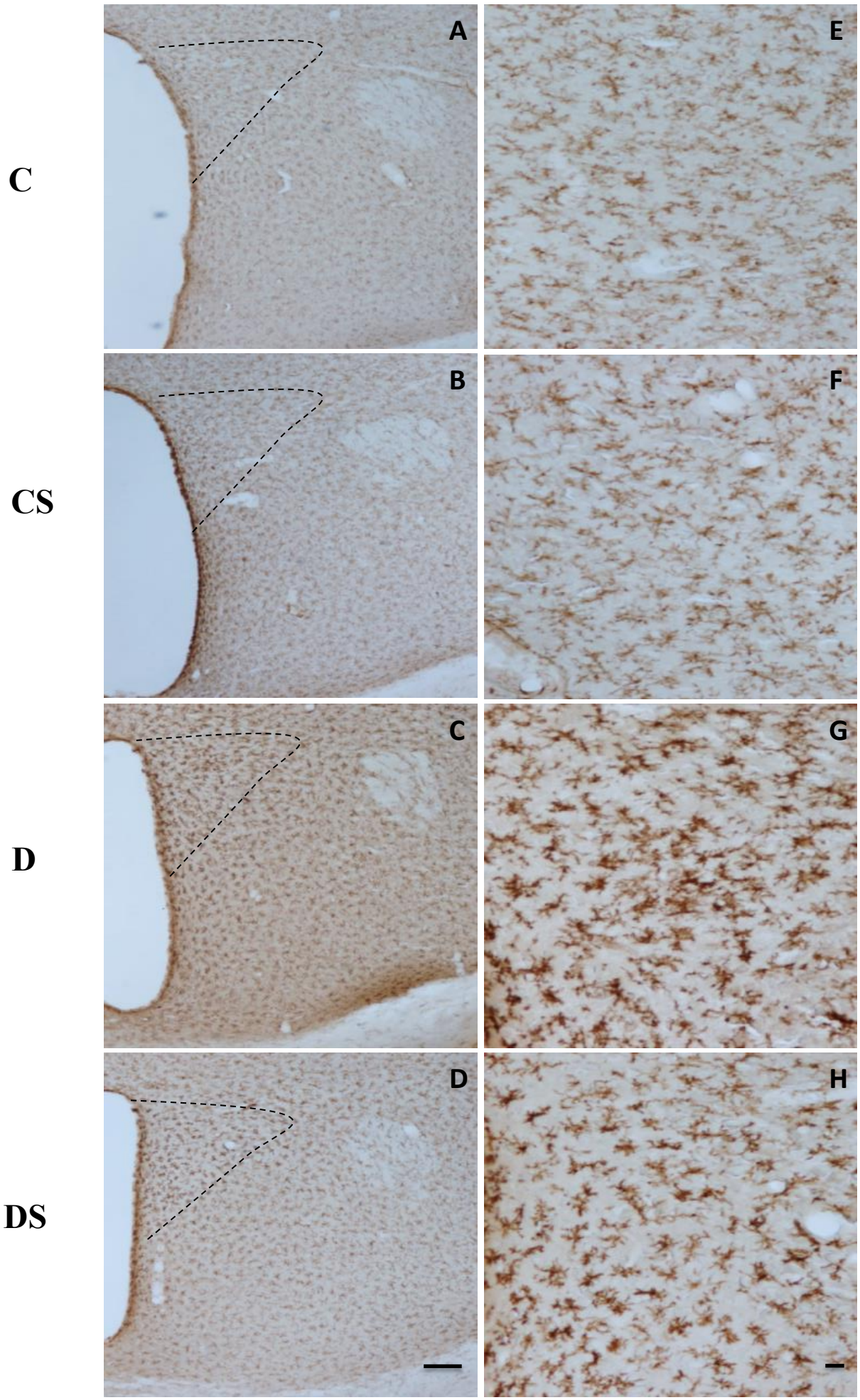

C
CS
D
DS
A
E
B
F
C
G
D
H

Figure 5-4. OX-42 Immunohistochemistry in the PVN in diabetic rats. Photomicrographs showing CD11b (OX-42 clone) immunoreactive microglia in the paraventricular nucleus (PVN) in control (C) and diabetic animals (D) given tap water and control animals (CS) and diabetic animals (DS) given 1% NaCl solution. Dotted lines in low-power images (A-D) outline the PVN. High-power images (E-H) show the morphology of microglia in the parvocellular PVN region in detail. OX-42 antibody staining shows microglia with normal morphology with small cell bodies and long fine-branched processes throughout the PVN region in the C and CS groups, while activated microglia showing larger cell bodies and shorter, thicker processes are seen in the D and DS group. Bar = 100 μm in A–D, 50 μm in E–H.

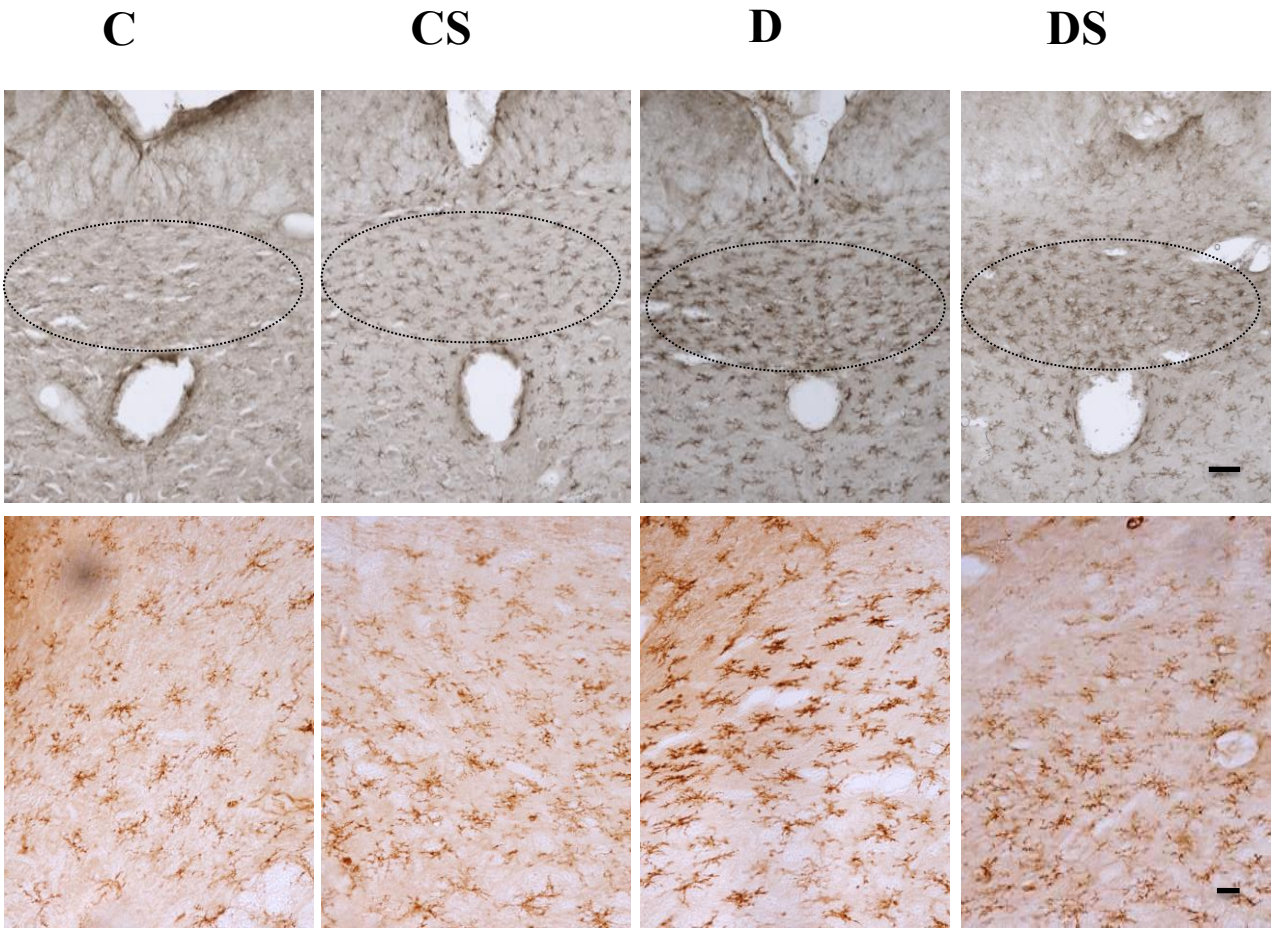


Figure 5-1. OX-42 Immunohistochemistry in the NTS in diabetic rats. Photomicrographs showing CD11b (OX-42 clone) immunoreactive microglia in the nuclear tractus solitarius (NTS) in control (C) and diabetic animals (D) given tap water and control (CS) and diabetic animals (DS) given 1% NaCl solution. Dotted lines in low-power images (A-D) outline the NTS. High-power images (E-H) show the detailed morphology of microglia in NTS region. OX-42 antibody staining showing microglia with normal morphology was seen throughout the NTS region in the C, CS and DS groups, while activated microglia showing intense immunolabelling, larger cell bodies and thicker processes were common in the NTS in the D group. Bar = 200 μm in A–D, 100 μm in E–H.

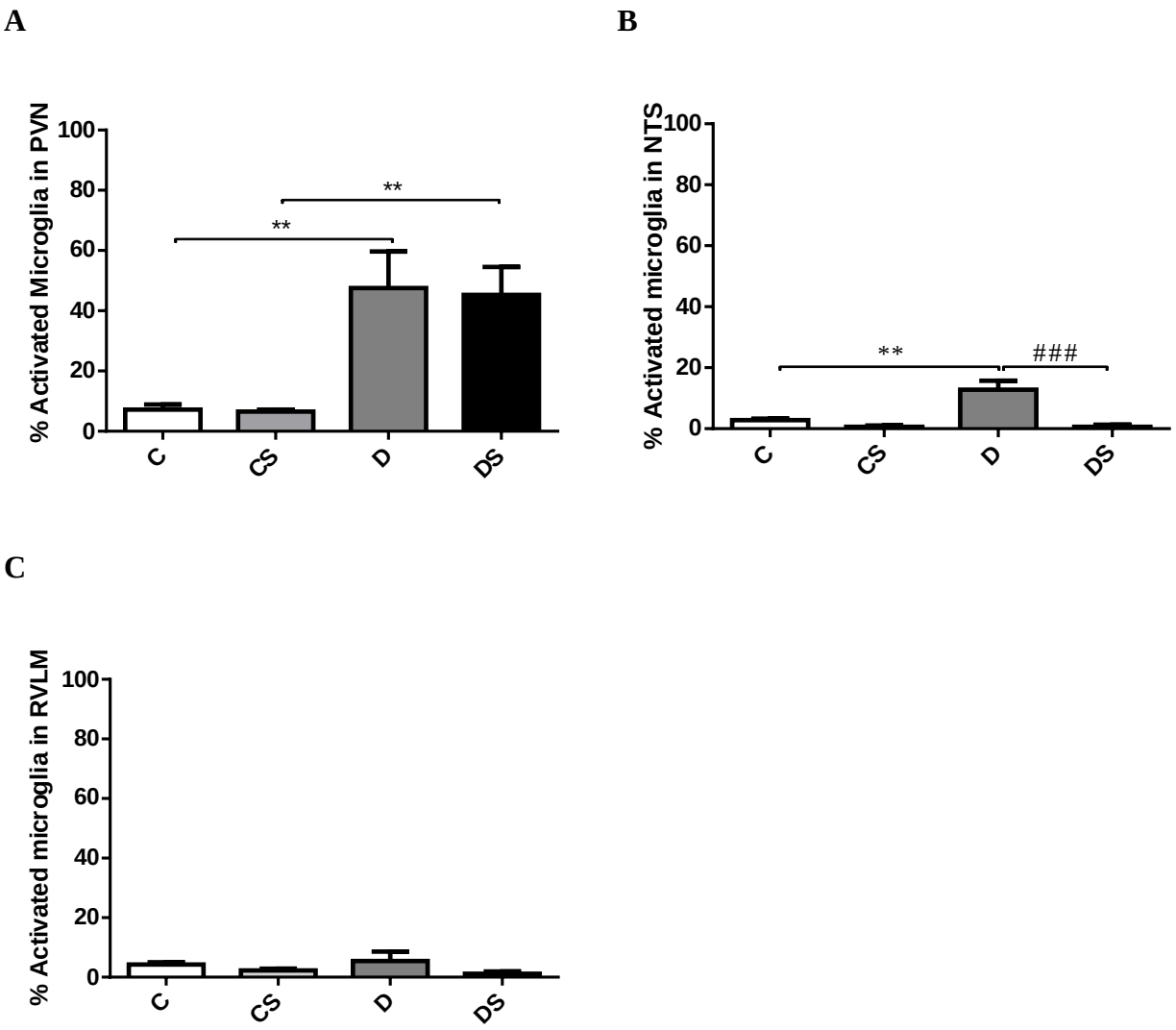


Figure 5-6. Microglial activation in cardiovascular centres of the brain of 6 weeks saline-treated diabetic rats. A: The percentage of microglia showing activated morphology in the paraventricular nucleus (PVN); B: in the nucleus tractus solitarius (NTS); and C: in the rostral ventrolateral medulla (RVLM) of the control (C) and diabetic animals (D) given tap water and control (CS) and diabetic animals (DS) given saline. After 6 weeks, the percentage of activated microglia was significantly increased in the PVN in the diabetic groups compared with the control groups. Also, the percentage of activated microglia was significantly increased in the NTS in the diabetic group compared with other groups. Number of rats = 4 in each group. Data are expressed as the mean ± SEM. ** indicates $P<0.01$ for comparisons between diabetic groups and their respective controls; ### indicates $P<0.001$ for comparisons between diabetic groups**.**

5.2.4 C-fos immunoreactivity in cardiovascular centres in diabetic rats

In this study, an antibody to c-fos was used to quantify neuronal activation in the regions in which microglia were activated. In the PVN in the C and CS groups, the number of fos-immunoreactive neurons was smaller compared to the D and DS groups (Figure 5-7). On quantification, we observed a significant increase in the number of fos-immunoreactive neurons in the D group compared to ($P<0.05$) the C group (Figure 5-8A). No significant differences were observed in the number of fos-immunoreactive neurons between the DS and CS groups. In the D group, a significant increase in the number of fos-immunoreactive neurons was restricted to parvocellular subdivisions of the PVN (5-9B). The number of fos-immunoreactive neurons in the NTS was not significantly different between the C and D or between the CS and DS groups (Figure 5-8B); however, saline treatment significantly reduced ($P<0.05$) fos activity in the NTS in the diabetic animals. This was not case in the control animals. The counts of fos-immunoreactive neurons in the NTS and RVLM regions showed no significant difference among any of the groups (Figure 5-8C).

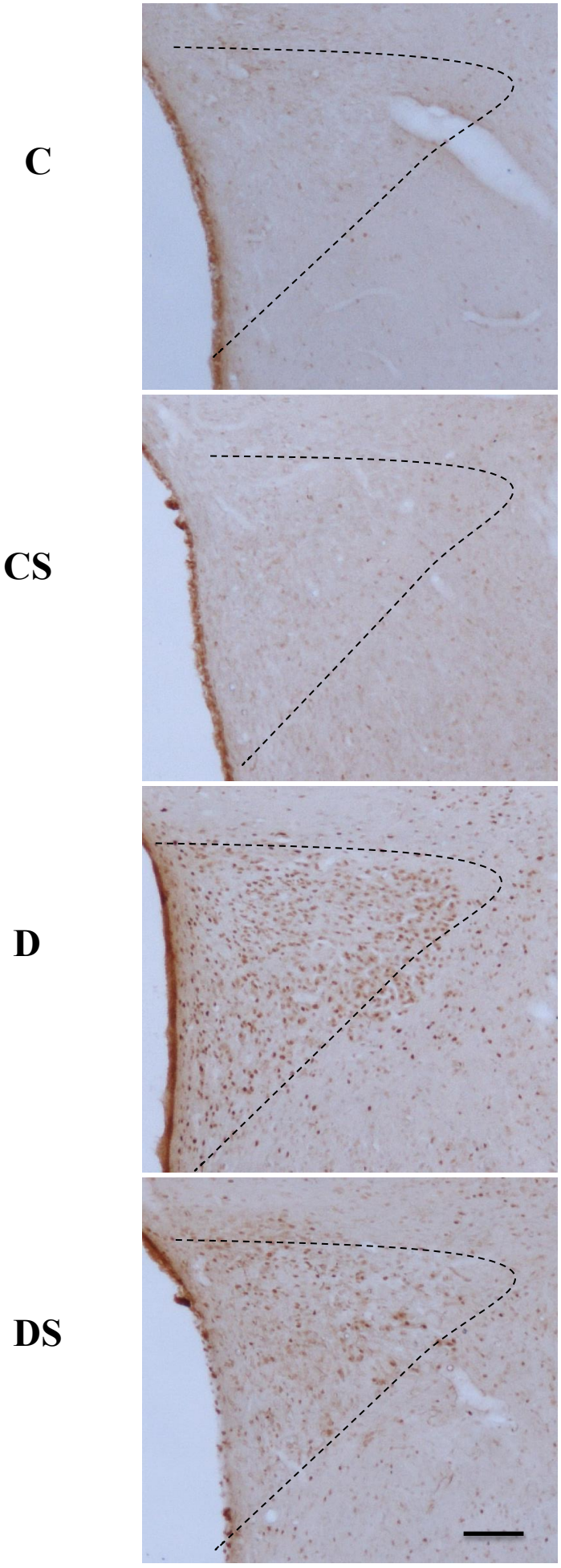

C
CS
D
DS

Figure 5-7. Fos activity in the PVN in diabetic rats. Photomicrographs showing neuronal nuclei stained with anti-Fos antibody in the paraventricular nucleus (PVN) in control (C) and diabetic animals (D) given tap water and control animals (CS) and diabetic animals (DS) given 1% NaCl solution.Dotted lines the PVN. Bar = 100 μm.

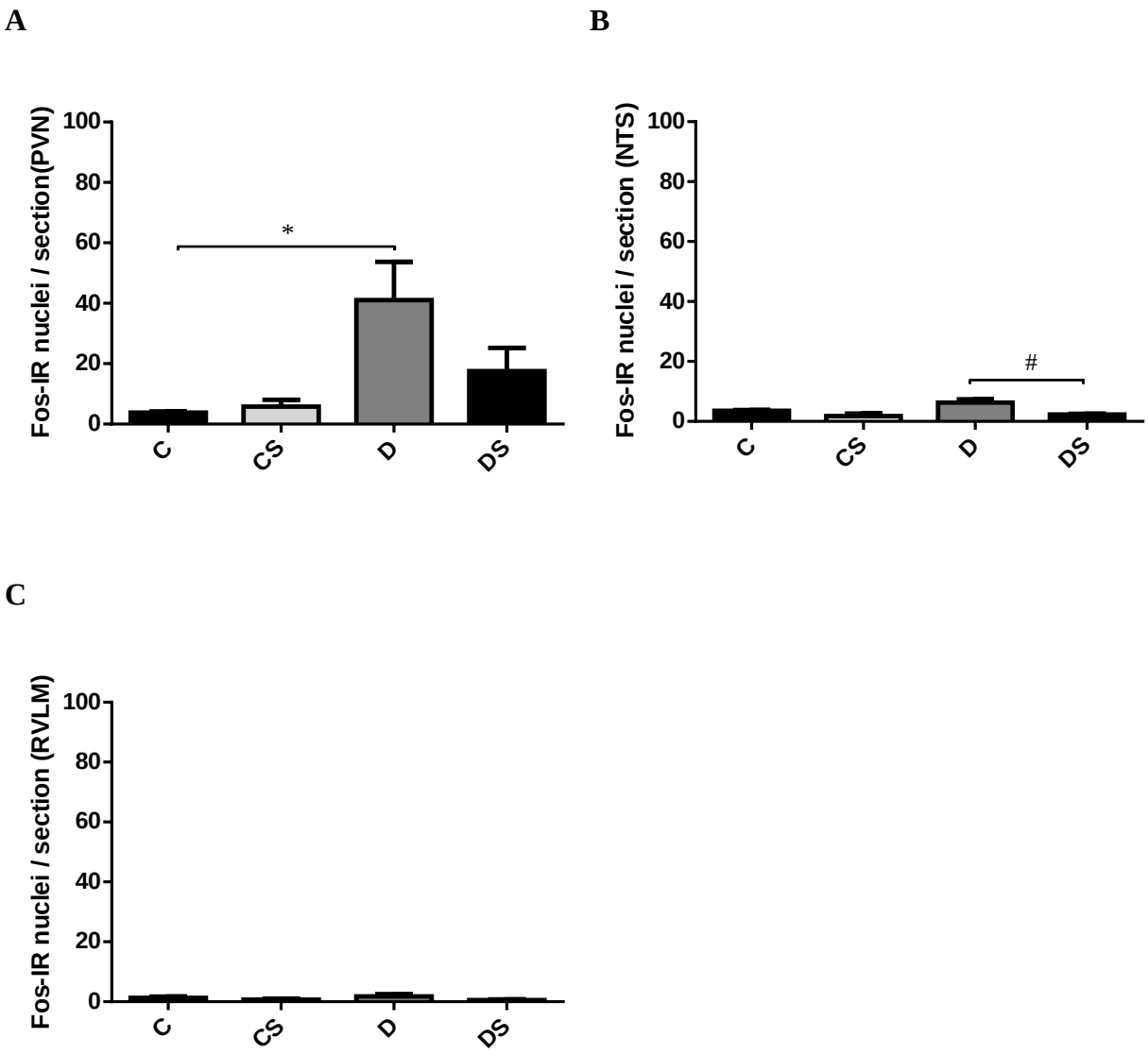


Figure 5-8. Fos-IR immunoreactivity indicating activated neurons in cardiovascular centres of the brain in diabetic rats. A: Quantification of Fos-IR positive cell nuclei in the paraventricular nucleus (PVN); B: in the nucleus tractus solitarius (NTS); and C: in the rostral ventrolateral medulla (RVLM) of control (C) and diabetic animals (D) given tap water and control (CS) and diabetic animals (DS) given saline. Number of rats = 4 in each group. Data are expressed as the mean ± SEM. * indicates $P<0.05$ for comparisons between diabetic groups and their respective controls; # indicates $P<0.05$ for comparisons between diabetic groups.

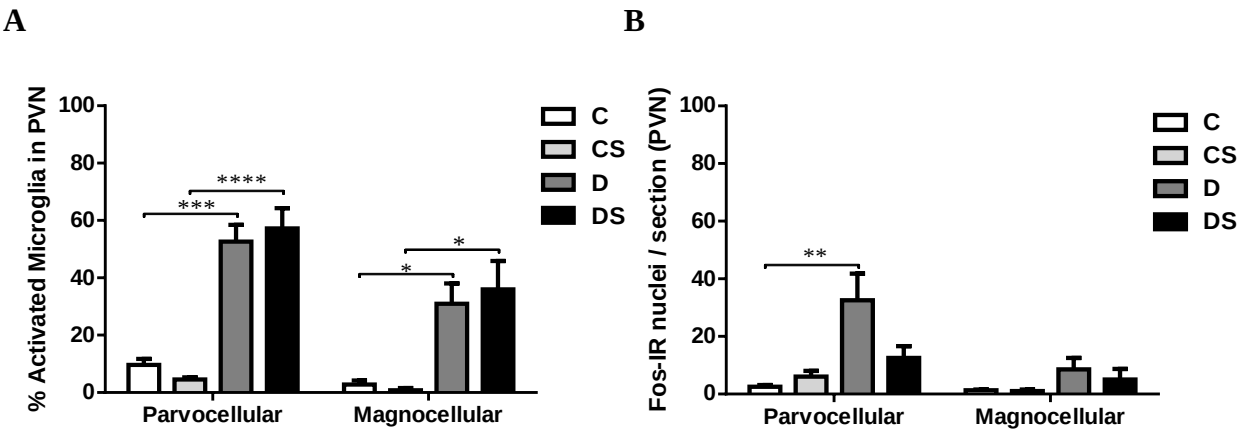


Figure 5-9. Microglial and neuronal activation in the parvocellular and magnocellular portions of the paraventricular nucleus of diabetic rats. A: The percentage of microglia showing activated morphology in the parvocellular and magnocellular of the paraventricular nucleus (PVN) of control (C) and diabetic animals (D) given tap water and control (CS) and diabetic animals (DS) given saline. B: Quantification of Fos-IR positive cell nuclei in the same regions. Number of rats = 4 in each group. Data are expressed as the mean ± SEM. * indicates $P<0.05$; ** indicates $P<0.01$; *** indicates $P<0.001$; **** indicates $P < 0.0001$ for comparisons between diabetic groups and their respective controls.

5.3 Discussion

While drinking saline causes improvement in some cardiovascular changes in long-term STZ- induced diabetic rats, there is a lack of data on the effect of saline intake on baroreflex sensitivity and CNS inflammation. Thus, in this study, we aimed to investigate the effects of 1% NaCl on baroreflex sensitivity and whether saline intake influences inflammation in CNS cardiovascular centres in long-term STZ diabetic rats. The main findings of the present study were that 6 weeks of treatment with 1% NaCl (i) had no effect on the mean arterial blood pressure under anaesthesia but (ii) restored the function of bradycardic baroreflex and (iii) reduced the activation of microglia and neurons in the NTS in STZ diabetic rats.

We previously reported no changes in baroreflex sensitivity at 2 weeks in STZ diabetic animals compared to the control. In contrast, in the current study, there was dysfunction in the baroreflex-mediated bradycardia in the longer term, which is in agreement with previous studies (Van Buren *et al.*, 1998; Dall'Ago *et al.*, 2002; Harthmann *et al.*, 2007; Souza *et al.*, 2007). This suggests that a reduction in baroreflex sensitivity is dependent on the time course of diabetes. The detailed mechanism of bradycardic baroreflex dysfunction in STZ diabetic rats is not known. There is evidence that suggests CNS inflammation can alter baroreflex function. For example, in a previous study, we have shown that the NTS exhibited microglial activation that was associated with baroreflex dysfunction in short-term STZ diabetic rats treated with saline. In addition, Takagishi et al. reported that microinjection of IL-6 into the NTS decreased baroreceptor sensitivity (Takagishi *et al.*, 2010). It is well documented that activated microglia can contribute to inflammation by producing proinflammatory cytokines, such as IL-6. Therefore, we investigated whether microglia are activated in autonomic brain areas that are involved in cardiovascular regulation, including the baroreceptor reflex. In the NTS but not in the RVLM, we found activation of microglia in diabetic animals at 6 weeks following STZ injection, which is in agreement with our previous study that reported that

microglia become activated at later stages (Rana *et al.*, 2014). Consistent with this result, it is well-known that over time after STZ treatment, autonomic dysfunction occurs. This includes abnormal increases in the renal sympathetic activity and impairment of baroreflex sensitivity. It is believed that these pathological changes in the autonomic nervous system may contribute to the development of cardiovascular complications (Grassi *et al.*, 1998; Gao *et al.*, 2002; Salgado *et al.*, 2007; Maliszewska-Scislo *et al.*, 2008). In the present study, microglial activation in the NTS in diabetic rats was observed around the time that autonomic system dysfunction occurred. These data suggest that microglial activation in the NTS may be responsible for baroreflex dysfunction seen at 6 weeks in STZ diabetic animals.

Interestingly, prolonged saline intake in STZ diabetic rats caused improvement of baroreflex sensitivity, which is not consistent with the effect of saline seen at 2 weeks. The detailed mechanism by which saline intake restores the baroreflex dysfunction is not known. A likely mechanism would be involvement of neuroinflammation in autonomic centres in the brain. In contrast to early stages of diabetes, saline intake reduced microglial activation in the NTS in STZ diabetic rats compared to STZ diabetic rats given tap water to drink. Also, there was no increase in microglial activation in the RVLM at 6 weeks in STZ diabetic rats treated with saline, suggesting that drinking saline reduces microglial activation over time. This reduction in microglial activation was accompanied with an enhancement of baroreceptor sensitivity. These findings also provide support for the effect of NTS microglial activation on baroreflex function.

The mechanisms by which microglia become activated in diabetic animals and saline intake reduces this activation are not completely understood. As discussed previously, neuronal over excitation can enhance microglial activation (Hathway *et al.*, 2009; Lu *et al.*, 2009). Therefore, we examined whether neurons were activated in CNS cardiovascular centres. In the NTS, we observed no significant increase in neuronal activation in diabetic rats compared

to non-diabetic control rats. Thus, the present finding is consistent with our earlier observations, supporting our interpretation that microglial activation was not mediated by neurons activation in the NTS and that damage to sensory neurons could be the potential mechanism; however, interestingly, prolonged saline treatment was able to reduce NTS neuron activation in diabetic rats. Studies on naïve animals have shown that salt intake potentiates the depressor responses to excitation of the NTS with a microinjection of glutamate (Ito *et al.*, 1999; Isogai *et al.*, 2005), but the exact mechanism is not clearly understood.

A change in sodium balance is a possible mechanism that may be involved in altering the NTS neuron activity and baroreflex function. A sodium imbalance is characteristic of STZ diabetic rats because their excretion of sodium is high (Wald & Popovtzer, 1984; Schaan *et al.*, 2005). Animal studies have shown that sodium depletion can cause impairment of the baroreceptor sensitivity (Kunze & Brown, 1978; Echtenkamp & Anderson, 1988; Huang & Leenen, 1994). Moreover, sodium depletion has been shown to activate the NTS neurons (Geerling & Loewy, 2007). We speculate that the dysfunction of baroreflex may be related to sodium depletion in STZ diabetic rats; therefore, drinking saline may restore sodium balance, thereby improving baroreflex sensitivity.

Another possibility is that hormonal factors, such as renin angiotensin aldosterone, may influence the NTS neurons and baroreflex sensitivity. There have been studies that have demonstrated that an increase in angiotensin II is associated with diminished baroreflex sensitivity in dahl salt-sensitive rats (Ferrari *et al.*, 1984) and that the inhibition of renin angiotensin aldosterone can improve baroreflex sensitivity in hypertensive animals (Gordon *et al.*, 1981). In addition, microinjection of angiotensin II into the NTS decreased baroreceptor sensitivity (Casto & Phillips, 1986). An increase in the level of circulating angiotensin II can also activate neurons in brain regions that are involved in autonomic

control (Li *et al.*, 1997). In early stages of diabetes, Kalinyak et al. found no change in the levels of angiotensin II in STZ diabetic rats (Kalinyak *et al.*, 1993). In contrast, increased internal angiotensin II has been found at 6-8 weeks following STZ injection (Anderson *et al.*, 1993). Interestingly, salt intake has been shown to reduce angiotensin II in Zucker diabetic fatty rats (Takenaka *et al.*, 2011). Thus, the decreased neuronal activity in diabetic rats treated with saline may be a result of decreased angiotensin II. Consequently, we presume that elevated angiotensin II may be responsible, at least in part, for the dysfunction of baroreflex seen in diabetic animals and that saline intake reduces angiotensin II, which in turn enhances baroreflex sensitivity.

In addition to the role of angiotensin II in baroreflex dysfunction, it has also been shown that systemic infusion of angiotensin II increases blood pressure and activates microglia in the PVN (Shi *et al.*, 2010a). In the present study, there was no change or reduction in the blood pressure of diabetic animals under anaesthesia compared to the control, which is in agreement with other studies at this later stage (Fazan *et al.*, 1997; Van Buren *et al.*, 1998; Fazan *et al.*, 1999; Schaan *et al.*, 2004). In addition, we observed no changes in the blood pressure of anaesthetized diabetic animals when given 1% NaCl to drink, which is consistent with our previous study at early stages of diabetes (chapter 4), although we and others have provided evidence that drinking 1% NaCl caused a significant increase in blood pressure in awake diabetic animals in the short and longer terms (Santos *et al.*, 1995; Maeda *et al.*, 2007). Because of the potential effects of anaesthesia on the mechanism that generates hypertension, recording actual blood pressure under anaesthesia is not a valid method. Therefore, it is difficult to compare the effect of saline on blood pressure seen in our study and other previous studies in awake animals (chapter 4). Although saline was able to restore baroreflex dysfunction in diabetic rats in this study, another study reported that saline intake increases blood pressure at 8 weeks after STZ injection in awake animals. This suggests that prolonged

saline intake contributes to the development of hypertension in diabetic rats independently of baroreflex sensitivity, but how this occurs is not known.

We previously provided evidence that saline intake promotes the development of hypertension and induces microglial activation in the PVN at 2 weeks in STZ diabetic rats and that the inhibition of microglial activation via a minocycline drug prevents hypertension. This suggests that microglial activation in the PVN contributes to this neurogenic hypertension. In the present study, we observed increased microglial activation in the PVN in diabetic rats compared with control rats, which is in agreement with our previous study that reported that microglia become activated at later stages. Also, we found microglial activation in the PVN of diabetic rats treated with saline, similar to our observations at earlier stages of diabetes. Microglial activation was seen in the parvocellular and magnocellular PVN; however, the functional consequences of microglial activation in the PVN are not known, and further studies are needed.

The mechanisms by which microglia become activated in the PVN in diabetic groups remain to be determined. We previously provided evidence that microglial activation in the PVN may be secondary to prolonged intense activation of PVN neurons in diabetic rats. Interestingly, neuronal activation was observed in the parvocellular PVN but not in the magnocellular. The parvocellular PVN is involved in the regulation of autonomic function. We previously reported increased neuronal activity in this region at 2 weeks following STZ injection, but the microglia were not activated. In the present study at 6 weeks, both neuronal and microglial activation were seen in this region. Thus, it appears that microglial activation is related to neuronal activation. In diabetic rats treated with saline, no increase in neuronal activation was observed in the parvocellular or magnocellular division of the PVN, although microglia were activated. These findings indicate that microglial activation can occur without

neuronal activation, suggesting that saline may trigger microglial activation directly or via another mechanism.

5.4 Conclusion

In conclusion, our data indicated that 1% NaCl intake enhances baroreflex sensitivity in long-term STZ diabetic rats. This enhancement was accompanied by a reduction in microglial activation in the NTS in diabetic rats treated with saline. Microglial activation in the NTS may contribute to the attenuated arterial baroreflex function observed in diabetic rats through the production of proinflammatory cytokines; however, further research is required to determine the full effect of microglia inhibition in the NTS. Saline intake had no effect on blood pressure in long-term STZ diabetic rats under anaesthesia. We conclude that prolonged saline intake may have beneficial effects on the cardiovascular system through a reduction of brain inflammation in diabetes.

Chapter Six: Inhibition of Microglial Activation Improves Cardiac Dysfunction in STZ Diabetic Rats

6.1 Introduction

Type I diabetes mellitus is an independent risk factor for the development of cardiovascular complications, which are the leading cause for 80% of deaths in diabetic patients (Kannel & McGee, 1979; Bell, 2003; Hayat *et al.*, 2004). Diabetic cardiomyopathy is a common cause of heart failure that may occur independently of arterial hypertension and coronary artery disease (Rubler *et al.*, 1972; Francis, 2001; Boudina & Abel, 2007). Diabetic cardiomyopathy is characterised by an impaired ventricular function of the heart. This includes left ventricular diastolic dysfunction (Joffe *et al.*, 1999; Schannwell *et al.*, 2001), and the progression of this dysfunction accelerates systolic dysfunction, which in turn contributes to the development of heart failure (Fonseca, 2003). While several mechanisms have been implicated in the pathogenesis of diabetic cardiomyopathy, including endothelial dysfunction and myocardial fibrosis (Tschöpe *et al.*, 2005; Asbun & Villarreal, 2006), the exact mechanism is still unclear.

Activity in the sympathetic nervous system that controls the heart and blood vessels has been shown to increase in heart failure diseases (Packer, 1988). Such activation may exacerbate and cause some of the aforementioned cardiovascular problems since interventions such as 'beta-blocker' drugs can improve patient prognosis following myocardial infarction, which is another common complication of diabetes (Butler *et al.*, 2006); however, whether this approach is useful in diabetes has not been investigated. There is growing evidence that abnormal cardiac function is associated with over-activity of sympathetic nerves measured by norepinephrine in both humans (Langer *et al.*, 1995; Stevens *et al.*, 1997; Perin *et al.*, 2001) and animals with type 1 diabetes (Paulson & Light, 1981; Schmid *et al.*, 1999). In addition, an abnormal activation of the sympathetic nervous system has also been reported in animals with type 1 diabetes via a direct measurement (Patel *et al.*, 2011). This abnormal activation of

the sympathetic nervous system has been suggested to contribute to the development of heart failure in diabetes, but the cause is not known.

Sympathetic nerve activity in the cardiovascular system is controlled via several regions in the brain called cardiovascular centres. The PVN is a key site within the brain that regulates sympathetic nerve activation. Our study and other previous studies on STZ-induced diabetic animals have reported abnormally increased neuronal activity in the PVN (Krukoff & Patel, 1990; Zheng *et al.*, 2002; Rana *et al.*, 2014), but the central mechanisms behind this neuronal activation are not well understood. Interestingly, inflammation in the PVN has been shown to activate PVN neurons in other diseases (Shi *et al.*, 2010a; Shi *et al.*, 2011).

Previously, we have shown that the resident immune cells within CNS, called microglia, become activated in the PVN in diabetic rats (Rana *et al.*, 2014). Microglia have been implicated in the pathogenesis of several neurodegenerative diseases, including neuropathic pain. Once they become activated, they release proinflammatory molecules, which have been shown to cause neuronal hyperexcitability by increasing excitation and decreasing inhibition in the spinal cord (Sweitzer *et al.*, 1999; Tsuda *et al.*, 2008; Milligan & Watkins, 2009).

Therefore, we hypothesize that activated microglia may be the cause for PVN neurons activation, which in turn activates sympathetic drive and contributes to cardiac dysfunction. The use of drugs that inhibit microglial activation in diabetic rats will help to understand whether microglial activation contributes to the development of diabetic complications. It has been suggested that dehydration may be responsible for the PVN neuron activation seen in diabetic rats.

Accordingly, we investigated whether the inhibition of microglia in the PVN via minocycline can reduce or prevent PVN neuron activation and thereby result in an improved cardiac

function in STZ-induced diabetic rats. In addition, we investigated whether plasma osmolarity and/or dehydration contributes to PVN neuron activation.

6.2 Results

6.2.1 Effect of minocycline on general features of diabetic rats

At 6 weeks following injection with either STZ or citrate buffer, the body weights of diabetic animals with an ICV infusion of saline (DS) were significantly lower ($P<0.05$) compared with control animals with an ICV infusion of saline (CS), as expected (Figure 6-1A). The body weights of diabetic animals with an ICV infusion of minocycline (DM) were also significantly lower ($P<0.001$) compared with the CS group. Minocycline did not significantly affect the body weight of diabetic animals. Blood glucose levels were around the normal levels (6-7 mmol/L) in the CS group, but the diabetic groups showed high blood glucose (higher than 25 mmol/L) (Figure 6-1B). As expected, the diabetic groups exhibited significantly higher ($P < 0.0001$) blood glucose levels compared with the CS group, and minocycline treatment had no effect on blood glucose. Daily water intake was significantly greater ($P<0.0001$) in the DS group compared to the CS group (Figure 6-1C), which is consistent with previous studies (chapter 5). The DM group drank a significantly greater ($P < 0.0001$) volume of water than the CS group. Interestingly, minocycline treatment in diabetic animals significantly reduced the daily water intake. Heart weight (heart weight/body weight) was not significantly different between the CS and DS or between the DS and DM groups (Figure 6-2A); however, kidney weight (kidney weight/body weight) was significantly greater ($P<0.0001$) in the DS group compared to the CS group (Figure 6-2B). This was also the case for kidney weight in the DM group compared to the CS group ($P < 0.05$). Despite this, minocycline treatment significantly decreased the kidney weight of diabetic animals.

To assess whether STZ diabetic rats were dehydrated, we measured plasma osmolarity, haemoglobin and haematocrit at the end of the experimental period. As expected, the plasma osmolarity was significantly elevated ($P<0.001$) in the DS group compared with the CS group, which is consistent with our previous study (Rana *et al.*, 2014) (Figure 6-3A);

however, no significant differences were found in plasma osmolarity between the DM group and CS group. When compared to the DS group, minocycline treatment significantly reduced ($P<0.05$) plasma osmolarity, suggesting it had restored plasma osmolarity to normal levels in diabetic animals. As indicators of blood volume status, the haematocrit and Haemoglobin concentration were significantly elevated ($P<0.01$) in the DS group compared to the CS group (Figure 6-3B-C). The haematocrit ($P= 0.13$) and Haemoglobin concentration ($P= 0.30$) were similar between the DM and CS groups, suggesting that minocycline-treated diabetic rats were not dehydrated; however, there was no significant difference in the haematocrit ($P= 0.18$) and Haemoglobin concentration ($P= 0.21$) between the diabetic animals.

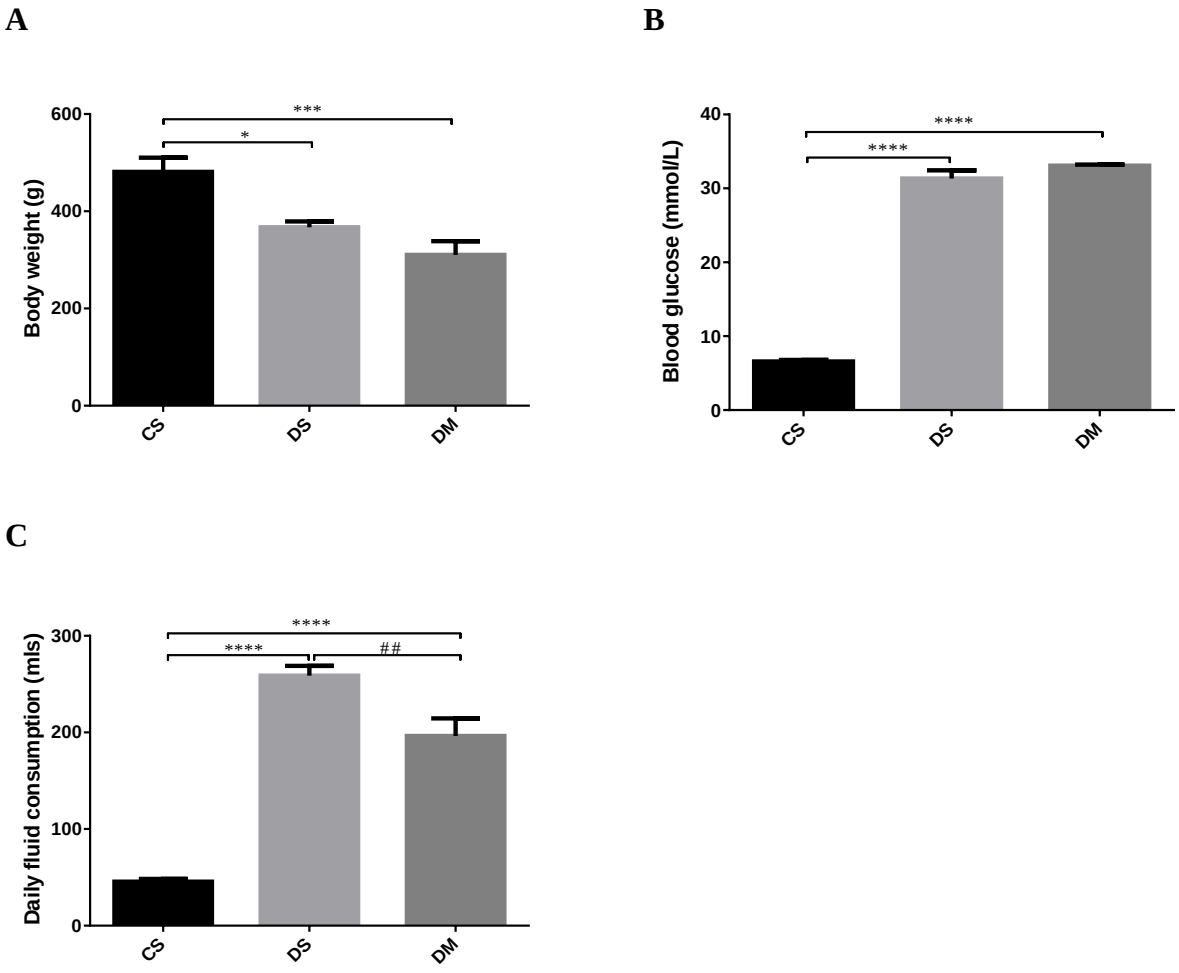


Figure 6-1. Effect of minocycline treatment on body weight, blood glucose and fluid intake of diabetic rats. A: Body weight measured at 6 weeks after injection of rats with either STZ or citrate buffer in control animals and diabetic animals with an ICV infusion of saline (CS, DS, respectively) and diabetic animals with an ICV infusion of minocycline (DM). B: Blood glucose levels measured at 6 weeks. C: The amount of tap water ingested was monitored daily at week 6 by weighting water bottles. Number of rats = 6, 8, and 8 respectively. Data are expressed as the mean ± SEM. Significance was evaluated using a one way ANOVA followed by Tukey's post hoc test for all comparisons (CS vs DS, CS vs DM, and DS vs DM) in this and all subsequent figures; * indicates $P<0.05$; ** indicates $P<0.01$; *** indicates $P<0.001$; **** indicates $P < 0.0001$ for comparisons between diabetic groups and controls; ♯ indicates $P<0.05$; ♯♯ indicates $P<0.0$; ♯♯♯ indicates $P<0.001$; ♯♯♯♯ indicates $P < 0.0001$ for comparisons between diabetic groups.

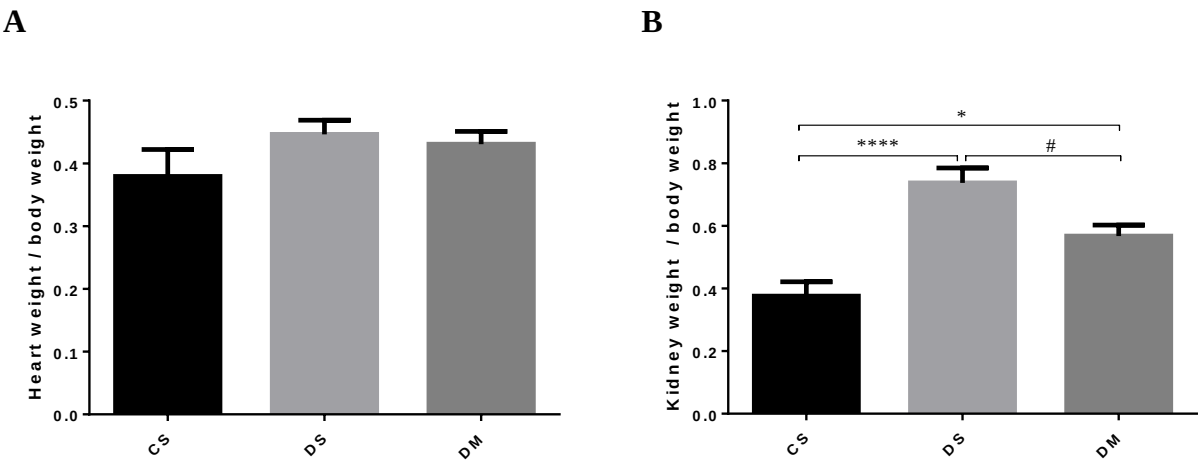


Figure 6-2. Effect of minocycline treatment on heart weight and kidney weight in diabetic rats. A: Heart weight (heart weight / body weight); and B: kidney weight (kidney weight / body weight) as a proportion of the body weight of control animals and diabetic animals with an ICV infusion of saline (CS, DS respectively) and diabetic animals with an ICV infusion of minocycline (DM). Number of rats = 6, 8, and 8 respectively. Data are expressed as the mean ± SEM.

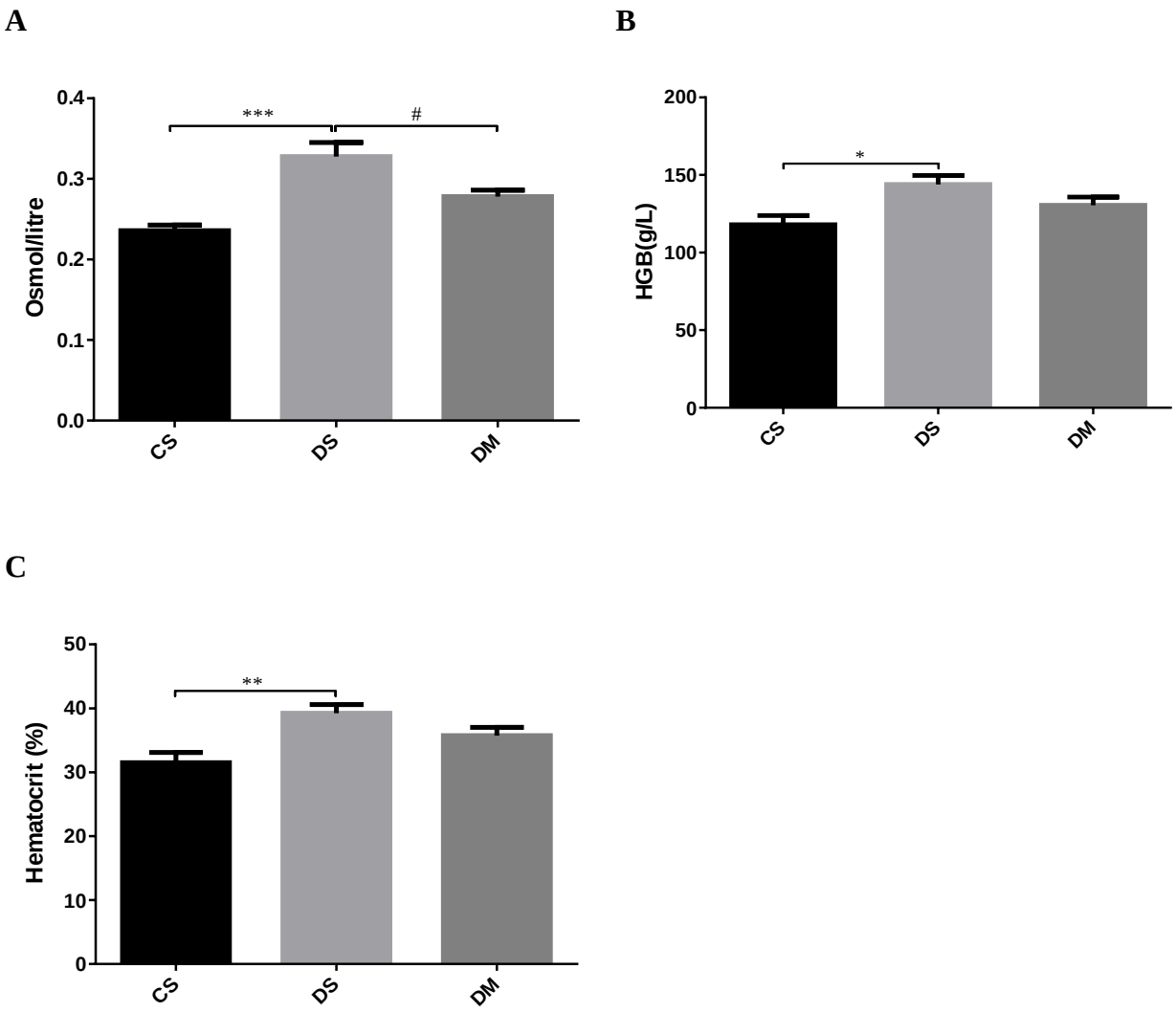


Figure 6-3. Effect of minocycline treatment on plasma osmolarity and haemoglobin in diabetic rats. A: Plasma osmolarity (Osmol/litre); B: Haemoglobin concentration (g/L); and C: Haematocrit (%) measured at the end of the experimental period in control animals and diabetic animals with an ICV infusion of saline (CS, DS respectively) and diabetic animals with an ICV infusion of minocycline (DM). Number of rats = 6, 8, and 8 respectively. Data are expressed as the mean ± SEM.

6.2.2 Echocardiographic measurements in diabetic rats

The left ventricle of diabetic animals displayed a significant increase in the internal diameter in systole and diastole compared to the CS group, ($P<0.01$) and ($P<0.0001$), respectively (Figure 6-4A-B). The treatment of diabetic animals with ICV minocycline significantly reduced the left ventricle internal diameter in systole and diastole compared with the DS group, ($P<0.05$) and ($P<0.01$), respectively. No significant difference was seen in the internal diameter in the systole between the DM and CS groups, but there was a significant difference in the diastole. Diabetic animals demonstrated a significant reduction ($P<0.0001$) in the E/A ratio compared with the CS group (Figure 6-4C). No differences were observed in the E/A ratio between the DM group and CS group. Interestingly, minocycline treatment significantly improved ($P<0.0001$) the E/A ratio in diabetic animals. Heart rate did not differ among any of the groups (Figure 6-4D).

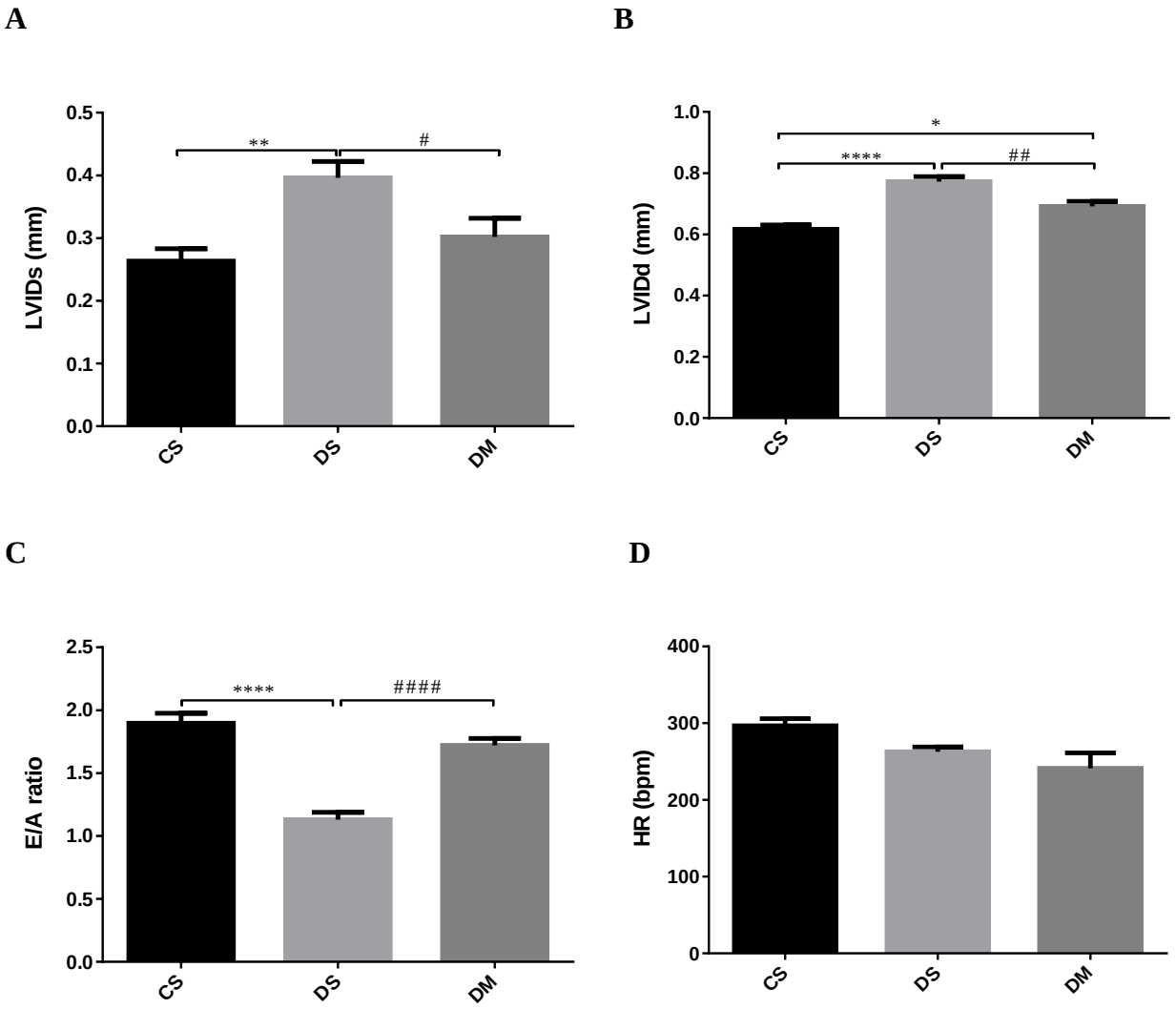


Figure 6-4. Effect of minocycline treatment on echocardiographic measurements in diabetic rats. A: Left ventricular internal diameter in systole — LVIDs (mm); B: left ventricular internal diameter in diastole — LVIDd (mm); C: E/A ratio (the ratio of the early (E) to late (A) ventricular filling velocities); and D: the heart rate of control animals and diabetic animals with an ICV infusion of saline (CS, DS respectively); and diabetic animals with an ICV infusion of minocycline (DM). Number of rats = 6, 8, and 8 respectively. Data are expressed as the mean ± SEM.

6.2.3 Measurement of haemodynamic parameters in diabetic rats

Immediately after the echocardiography procedure, cardiac catheterization was performed to assess the left ventricular end diastolic pressure, dP/dt max and heart rate. Compared to control rats, the DS group exhibited significantly higher ($P<0.0001$) left ventricular end diastolic pressure at 6 weeks following STZ injection, indicating clear left ventricular dysfunction (Figure 6-5A). No difference was observed in the left ventricular end diastolic pressure between the DM group and CS group. The treatment of diabetic animals with minocycline significantly restored ($P<0.0001$) the elevated end diastolic pressure to levels comparable to the CS group. The maximal rate of pressure change (dP/dt max) was significantly lower ($P<0.05$) in the DS group compared to the level observed in the CS group (Figure 6-5B); however, there was no significant difference in the maximal rate of pressure change between the DM group and CS group. Minocycline did not significantly affect the maximal rate of pressure change in diabetic animals ($P= 0.07$). The heart rate under deep anaesthesia was similar among all groups (Figure 6-5C).

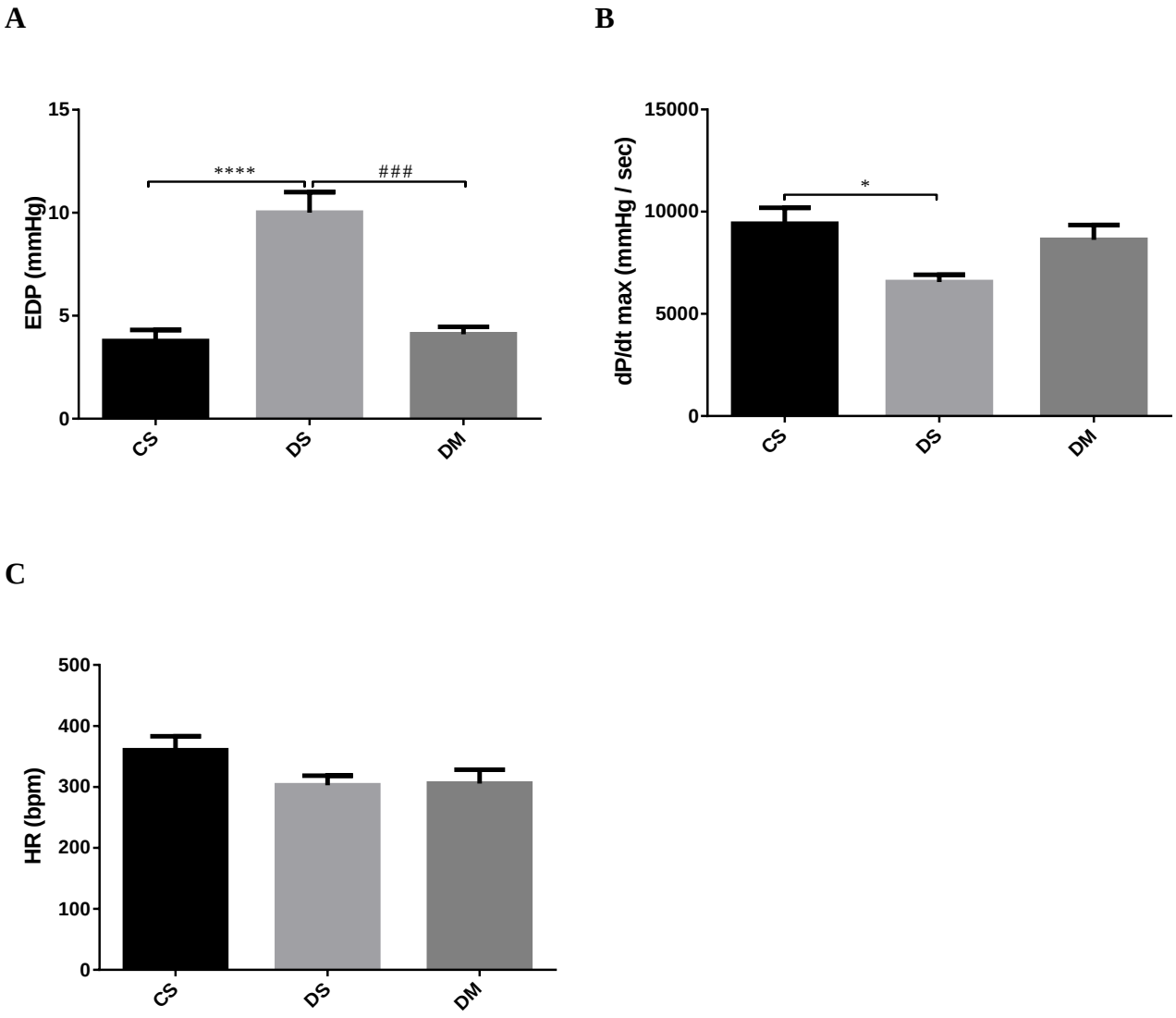


Figure 6-5. Effect of minocycline treatment on hemodynamic parameters in diabetic rats. A: End-diastolic pressure; (EDP); B: the maximal rate of pressure change (dP/dt max); and C: the heart rate of control animals and diabetic animals with an ICV infusion of saline (CS, DS, respectively); and diabetic animals with an ICV infusion of minocycline (DM). Number of rats = 6, 8, and 6 respectively. Data are expressed as the mean ± SEM.

6.2.4 OX-42 and C-fos Immunohistochemistry in the paraventricular nucleus in diabetic rats

At 6 weeks following STZ or vehicle injection, the microglia displayed normal morphology with small cell bodies and long, fine-branched processes throughout the PVN region in the CS group, which is consistent with our previous study (Rana *et al.*, 2014); however, microglial cells in the PVN in the DS group appeared to be present in an activated form with larger cell bodies and shorter, thicker processes (Figure 6-6). The quantification of the percentage of activated microglia in the PVN showed that it was lower than 7% in the CS group and higher than 60% in the DS group (Figure 6-8 A). Thus, the DS group exhibited a significant increase ($P<0.0001$) in the percentage of activated microglia compared with the CS group. There was no significant difference in the percentage of activated microglia in the PVN between the DM group and CS group. Minocycline treatment in diabetic animals caused a significant reduction ($P<0.0001$) in the percentage of activated microglia in the PVN.

The number of fos-immunoreactive neurons in the PVN in the CS group appeared smaller when compared to the DS group (Figure 6-7). On quantification, we observed a significant increase ($P<0.01$) in the number of fos-immunoreactive neurons in the DS group compared to the CS group (Figure 6-8 B). Fos-immunoreactive neurons in the PVN showed no significant difference between the DM group and CS group. When the diabetic groups were compared, the increased number of fos-immunoreactive neurons seen in the PVN in the DS was significantly reduced ($P<0.05$) by the minocycline treatment.

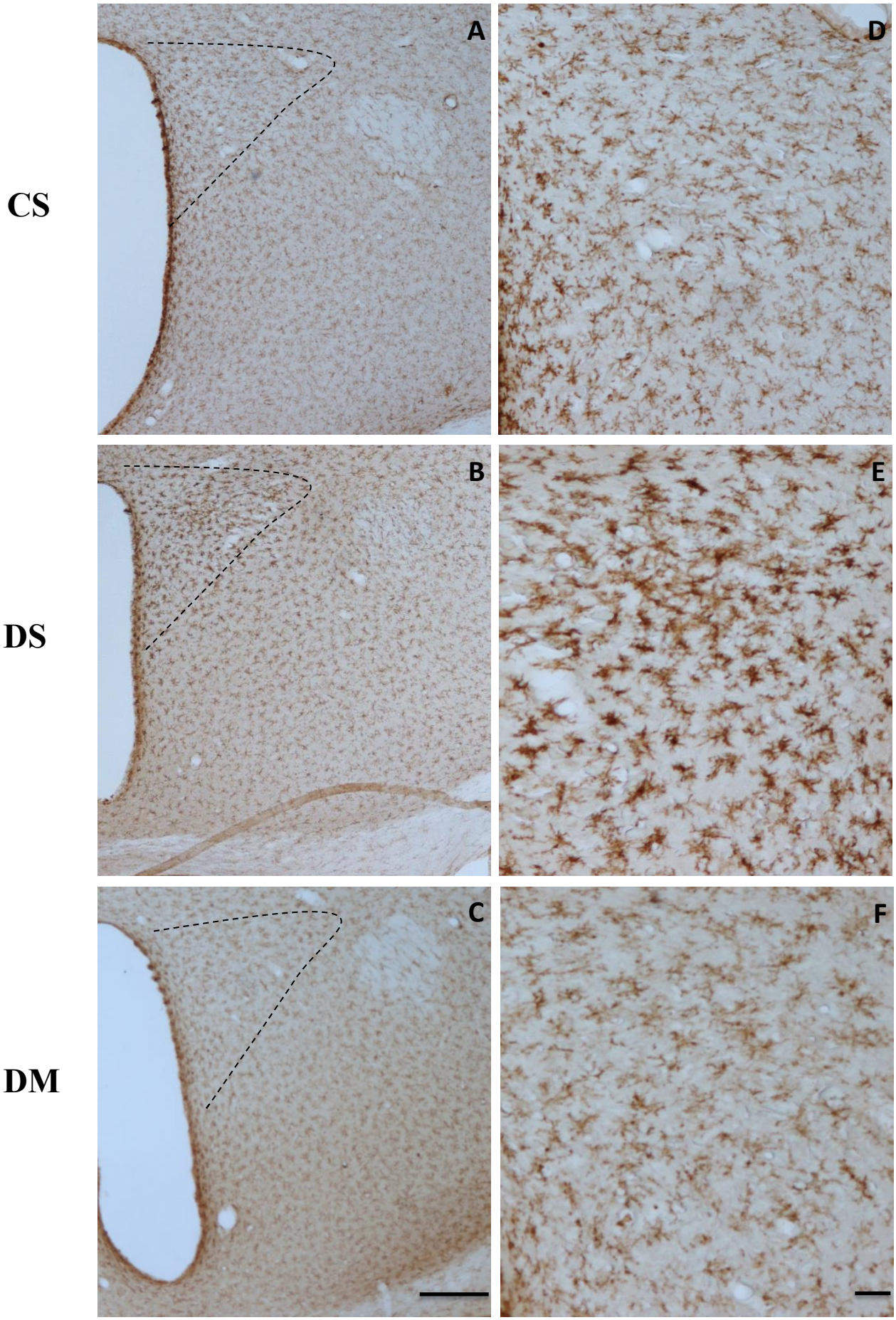
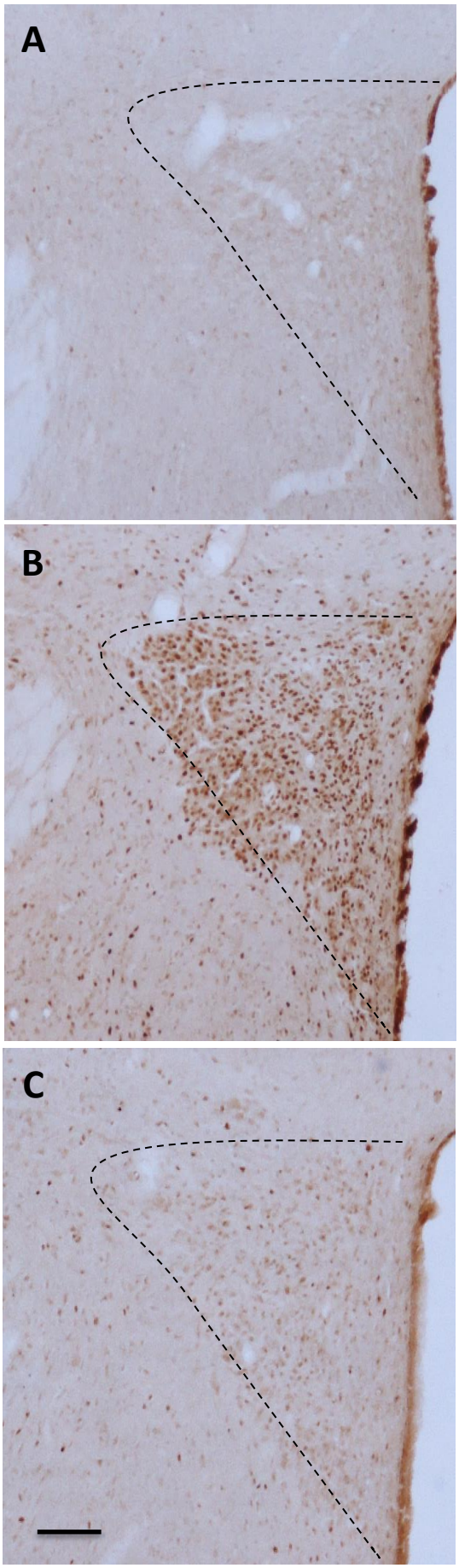

CS
DS
DM
A
B
C
D
E
F

Figure 6-6. Morphology of microglia in STZ diabetic rats. Photomicrographs showing CD11b (OX-42 clone) immunoreactive microglia in the paraventricular nucleus (PVN) of control animals and diabetic animals with an ICV infusion of saline (CS, DS respectively) and diabetic animals with an ICV infusion of minocycline (DM). Dotted lines in low-power images (A-C) outline the PVN. High-power images (D-F) show the morphology of microglia in the PVN region in detail. OX-42 antibody staining shows microglia with normal morphology with small cell bodies and long, fine-branched processes throughout the PVN region in the CS and DM groups, while activated microglia showing larger cell bodies and shorter, thicker processes are seen in the DS group. Bar = 100 μm in A–D, 50 μm in E–H.



CS

DS

DM

Figure 6-7. Fos activity in the PVN in diabetic rats. Photomicrographs showing neuronal nuclei stained with anti-Fos antibody in the paraventricular nucleus (PVN) of control animals and diabetic animals with ICV infusion of saline (CS, DS respectively); and diabetic animals with ICV infusion of minocycline (DM). Dotted lines outline the PVN. Scale bar = 100 μm.

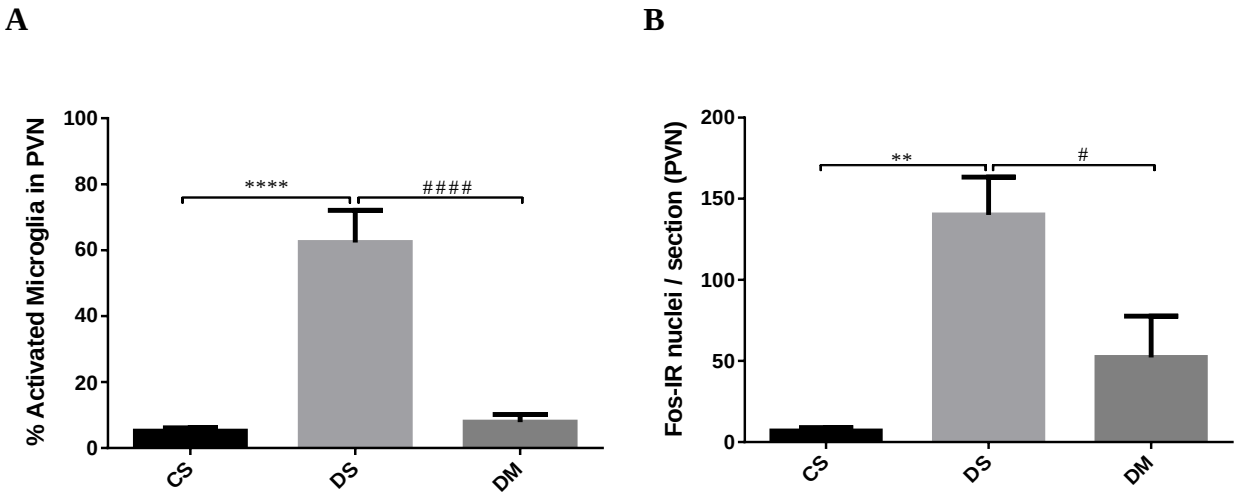


Figure 6-8. Effect of minocycline treatment on microglial and neuronal activation in the paraventricular nucleus of diabetic rats. A: The percentage of microglia showing activated morphology in the paraventricular nucleus (PVN) of control animals and diabetic animals with an ICV infusion of saline (CS, DS respectively) and diabetic animals with an ICV infusion of minocycline (DM). B: Quantification of Fos-IR positive cell nuclei in the same region. Number of rats = 6 in each group. Data are expressed as the mean ± SEM.

6.3 Discussion

There is evidence that an over-activity of the sympathetic nervous system is a potential contributing factor to the development of diabetic cardiomyopathy, which is characterized by left ventricular dysfunction; however, whether PVN inflammation contributes to this sympathetic over-activity has not been investigated. Therefore, in this study, we aimed to investigate cardiovascular function in diabetic rats at 6 weeks following STZ injection and whether the inhibition of microglial activation in the PVN can reverse any of the changes seen. The main findings of the present study were that diabetic rats exhibited (i) left ventricular dysfunction, including elevated end diastolic pressure, an increased internal diameter in systole and diastole and a decreased E/A ratio; (ii) renal hypertrophy and elevated plasma osmolarity; (iii) microglial and neuronal activation in the PVN and that (iv) ICV minocycline inhibited microglial and neuronal activation and that (v) this inhibition was accompanied by improved left ventricular and renal changes without affecting hyperglycaemia or the body weight in STZ diabetic rats.

In the present study, we observed increased microglial activation in the PVN in diabetic rats compared with control rats, which is similar to our observations in an earlier study (chapter 5). This activation of microglia was accompanied by left ventricle impairment and renal hypertrophy. Increased microglial activation in the PVN and left ventricle and renal changes were significantly attenuated via administering ICV minocycline. Minocycline treatment not only attenuated microglial activation but also neuronal activation in the PVN in diabetic rats; however, minocycline did not alter the glycaemia level in diabetic rats, indicating that it improved cardiovascular and renal function via another mechanism.

There is evidence that suggests that an abnormal activation of sympathetic nerves can result in ventricular dysfunction in human and animals with heart failure (Aronson & Burger, 2002; Guggilam *et al.*, 2008). In the current study, echocardiographic measurements of the left

ventricle function and structure demonstrated diastolic dysfunction in STZ diabetic rats when compared with control rats, which is consistent with other previous studies at the same or earlier stages (Hoit *et al.*, 1999; Wichi *et al.*, 2007; Arozal *et al.*, 2009). Similarly, a direct evaluation of left ventricle function demonstrated increased end diastolic pressure and decreased dP/dt max in diabetic rats, confirming diastolic dysfunction, which is in agreement with previous studies (Riad *et al.*, 2007; Wichi *et al.*, 2007; Arozal *et al.*, 2009); however, treating diabetic animals with ICV minocycline caused a significant reduction in left ventricular end diastolic pressure. The increase in internal diameter in the systole and diastole in diabetic rats was also significantly decreased by minocycline, and the decrease in the E/A ratio was also significantly reversed by minocycline. In almost all cases, there was no longer any difference from the control. These data indicate that ICV minocycline improved left ventricle diastolic function in STZ diabetic rats. Although we did not measure sympathetic activity directly in the present study, other studies have reported that over-activity of the sympathetic nervous system occurs in both humans and animals with type 1 diabetes (Perin *et al.*, 2001; Patel *et al.*, 2011; Salman *et al.*, 2011). Thus, the cardiovascular dysfunction seen in diabetic animals may be related to autonomic dysfunction.

PVN neuronal activation has been implicated in driving sympathetic overactivity in several diseases, including hypertension (Allen, 2002), heart failure (Li *et al.*, 2003), water deprivation (Stocker *et al.*, 2005) and chronic hypoxia (Sharpe *et al.*, 2013). In the present study, we observed increased neuronal and microglial activation in the PVN in diabetic rats at 6 weeks after STZ injection, which is consistent with our previous observation (chapter 5). Minocycline treatment significantly reduced both microglial and neuronal activation in the PVN. This drug has multiple anti-inflammatory actions (Yrjänheikki *et al.*, 1999; Du *et al.*, 2001; Sapadin & Fleischmajer, 2006). Proinflammatory cytokines in the PVN have been shown to contribute to the activation of PVN neurons in cardiovascular disease models (Shi *et al.*, 2010a; Shi *et al.*, 2011). In addition, a study on rats with chronic heart failure, which is

associated with increased sympathetic activity, reported evaluated levels of proinflammatory cytokines, such as TNF-α and IL-1β, in the PVN (Felder *et al.*, 2003; Francis *et al.*, 2004a; Francis *et al.*, 2004b). Inhibition of these cytokines in the PVN has been shown to reduce sympathetic over-activity, resulting in improvement in left ventricle function, including left ventricle end diastolic pressure in these animals (Guggilam *et al.*, 2008). In addition, Yu et al. reported that the microinjection of anti-inflammatory cytokines (IL-10) into the PVN in rats with heart failure decreased left ventricle end diastolic pressure (Yu *et al.*, 2007). One possible source of these cytokines is microglia. Once microglia become activated in response to infection, injury or inflammation of the CNS, they release proinflammatory molecules and cytotoxic factors, such as nitric oxide (NO) and tumor necrosis factor (TNF-α), that induce neuronal over excitation. We have previously provided evidence that activated microglia in the PVN contribute to hypertension in STZ diabetic rats (chapter 4). Along with these studies, our data suggest that activated microglia in the PVN may release proinflammatory cytokines that lead to the observed over-activation of PVN neurons, which activates the sympathetic nerves and thereby contributes to cardiac dysfunction in diabetic rats.

As discussed previously, abnormal sympathetic over-activation is reported in diabetic humans and animals. This abnormality has also been shown to be involved in the pathogenesis of renal dysfunction in diabetes, including glomerular hyper-filtration and increased renal plasma flow. For instance, an elevated glomerular filtration rate has been shown to occur in STZ diabetic animals, and renal denervation reversed glomerular hyper filtration (Luippold *et al.*, 2004; Salman *et al.*, 2011). Although we did not measure the renal haemodynamic properties directly in the present study, diabetic rats exhibited an increased kidney weight/body weight ratio, indicating that STZ diabetic rats display renal hypertrophy. Several studies have reported that increased kidney size is associated with elevated glomerular filtration rate as seen in diabetic humans (Christiansen *et al.*, 1981; Gundersen & Mogensen, 1981) and animals (Luippold *et al.*, 2004; Malatiali *et al.*, 2008). It was believed

that an increase in kidney size precedes glomerular hyperfiltration, which would lead to subsequent albuminuria and diabetic nephropathy (Christiansen, 1984; Bak *et al.*, 2000; Zerbini *et al.*, 2006). Thus, the enlarged kidney size observed in STZ diabetic rats may be indicative of renal dysfunction. Interestingly, this hypertrophy was significantly reversed by ICV minocycline treatment. The precise mechanism is not clear, but inhibition of abnormal PVN activation via minocycline would be expected to reduce sympathetic activity and may therefore attenuate renal hypertrophy in diabetic rats via this mechanism.

In addition to sympathetic nerve over-activity, other mechanisms may be involved in the development of renal hypertrophy in diabetic rats. In the present study, we found a significant increase in plasma osmolality in diabetic rats compared with the control. The haematocrit and Haemoglobin concentration were also increased in diabetic rats, indicating depletion in blood plasma volume. These results suggest that our diabetic animals were dehydrated, which is in agreement with our previous observation at an earlier stage (chapter 4). The PVN neurons would cause secretion of vasopressin in response to osmotic stimuli to regulate body fluid balance and blood volume in diabetic animals (Zerbe *et al.*, 1979; Charlton *et al.*, 1988; Brooks *et al.*, 1989; Zheng *et al.*, 2002). Elevated vasopressin secretion is well documented in diabetic humans and animals (Zerbe *et al.*, 1985). This elevation has been implicated in the development of diabetic renal complications, including renal hypertrophy, glomerular hyperfiltration and albuminuria (Bouby *et al.*, 1999; Lamarche *et al.*, 1999; Donnelly *et al.*, 2000). Normalization vasopressin secretion in vasopressin-deficient rats injected with STZ to induce diabetes reversed these structural and physiological changes (Bardoux *et al.*, 1999; Bouby *et al.*, 1999). Therefore, we speculate that renal hypertrophy may be related to an increased vasopressin concentration in STZ diabetic rats, and minocycline treatment attenuated PVN neuronal activation and consequently normalised vasopressin secretion, preventing renal hypertrophy.

Our previous study on STZ diabetic rats showed that neuronal activation in the PVN occurs at an earlier stage (2 weeks) than the onset of microglial activation in the PVN (6-8weeks), suggesting that neuronal activation precedes microglial activation in this nucleus; however, the result obtained here that minocycline treatment attenuated both microglial and neuronal activation suggests that the opposite may be true. It has been reported that minocycline inhibits microglial activation without affecting neurons in the spinal cord of neuropathic rats (Raghavendra *et al.*, 2003; Ledeboer *et al.*, 2005). Thus, a possible scenario is that firstly, neuronal activation at early stages induces microglial activation. Consequently, via a positive feedback mechanism, microglial activation induces the secondary and sustained neuronal activity that appears to cause the diabetic cardiovascular and renal complication observed in this study.

Although several studies have reported activated neurons in the PVN in STZ diabetic animals (Krukoff & Patel, 1990; Zheng *et al.*, 2002; Morgado *et al.*, 2011; Rana *et al.*, 2014), the mechanism by which diabetes induces this activation in the hypothalamic PVN or other cardiovascular regions is unclear. A large body of evidence shows that hyperglycaemia induces the production of reactive oxygen species in the PVN (Patel *et al.*, 2011) and other brain areas in STZ diabetic rats (Acar *et al.*, 2012; Ola *et al.*, 2014). It has been reported that the increase in the generation of reactive oxygen species can enhance the influx of Ca^{2+} in neurons, which in turn leads to neuronal activation (Wang *et al.*, 2003; Zimmerman & Davisson, 2004). We have preliminary evidence that an antioxidant can significantly attenuate the increase in neuronal activation in the PVN in STZ diabetic mice at 5 weeks following the induction of diabetes. Reactive oxygen species in dorsal horn neurons have been shown to contribute to neuronal hyperexcitability and the development of neuropathic pain, since the administration of antioxidants prevents neuropathic pain-like behaviours (Stevens *et al.*, 2000; Ho *et al.*, 2006). These findings suggest that PVN neuron activity may be mediated by increased reactive oxygen species in diabetic animals, but it is also clear that

microglia are a major source of reactive oxygen species in the brain and that reactive oxygen species are required for microglial activation and cytokine release.

Elevated levels of plasma osmolality in diabetic animals has also been implicated in PVN neuronal activation (Charlton *et al.*, 1988; Brooks *et al.*, 1989); however, it seems unlikely that elevated plasma osmolality seen in diabetic rats is responsible for the neuronal activation because the inhibition of PVN via minocycline reduced the plasma osmolality to normal levels. Despite this, if a positive feedback mechanism is involved, then breaking the cycle at any point may prevent multiple detrimental outcomes.

Interestingly, ICV minocycline was able to restore cardiac and renal dysfunction seen in our diabetic rats without affecting high blood glucose. Whatever the mechanisms by which high blood glucose can cause central inflammation may be, it is clear from our animals at 6 weeks following STZ injection that the changes in the brain are a major cause of the development of the cardiovascular and renal complications seen. Indeed, at this stage, our study provides little evidence of a direct effect of high blood glucose in cardiac function. Our data suggest that these pathological changes are mediated by CNS inflammation as a second mechanism or pathway to contribute to these diabetic complications.

6.4 Conclusion

This study suggests that microglial activation in the PVN in STZ-induced diabetic rats leads to PVN neuronal excitation. We speculate that this increase in the neuronal activity could explain increased sympathetic activity seen in humans and animals with type 1 diabetes. Consequently, this abnormal activity may be responsible for altered cardiac and renal function in diabetes. We provide evidence here that the inhibition of central inflammation may lead to preventing cardiac and renal diseases associated with diabetes. Thus, targeting

microglial activation in the PVN may be an effective therapeutic approach through preventing neuronal activity and multiple diabetic complications.

Chapter Seven: Drinking 1% NaCl for 6 Weeks Improves Cardiac Dysfunction in STZ Diabetic Rats: The Role of Neuroinflammation

7.1 Introduction

Cardiovascular complications are the likely cause of morbidity and mortality in approximately 80% of diabetes mellitus patients (O'Keefe *et al.*, 1999). As previously discussed, diabetic cardiomyopathy occurs frequently in humans and animals with type 1 diabetes, and this includes left ventricular diastolic dysfunction (Joffe *et al.*, 1999; Schannwell *et al.*, 2001). Over-activity in the sympathetic nervous system that controls the cardiovascular system is associated with diabetes. Such activity may contribute to left ventricular dysfunction and consequently to the development of heart failure in diabetes (refer to chapter 6).

The paraventricular nucleus (PVN) in the hypothalamus is a major focus for studies on cardiovascular complications because it regulates the autonomic nervous system and the endocrine system, including their cardiovascular functions (van den Pol, 1982; Badoer, 2001, 2010). Previously, we have reported that PVN neurons are activated in STZ diabetic rats, potentially due to dehydration in these animals (refer to chapter 6). Therefore, increased sympathetic nerve activity and diabetic complications may be caused by dehydration and its effects on the PVN. In support of this, Stocker and Keith have reported that renal sympathetic nerve activity is increased in water-deprived rats, PVN neurons are activated by this stimulus and renal sympathetic nerve activity is decreased via inhibiting PVN neurons in these animals (Stocker *et al.*, 2004). Treatment with normal saline may be expected to reverse this dehydration in diabetes, but the effect of long-term saline intake on dehydration and cardiovascular changes in STZ diabetic rats has not been investigated.

In contrast, our previous data suggest that the activation of PVN neurons is also caused by local microglial activation in STZ diabetic rats and that inhibiting microglia prevents dehydration (refer to chapter 6). Microglia are the primary immune cells that mediate inflammation in the CNS. They have been implicated in the pathogenesis of several

neurodegenerative diseases, including neuropathic pain. When these cells become activated, they can further induce inflammatory processes via secreting cytokines, such as tumour necrosis factor alpha (TNF-α), interleukins (IL), interferon (INF), reactive oxygen species and superoxides. These cytokines have been found to increase neuronal excitability in the CNS in many neurological disorders (refer to chapter 6). We have previously provided evidence that the pharmacological inhibition of PVN microglia can prevent not only PVN neuronal activity but also the cardiac dysfunction seen in diabetic animals, suggesting that inflammation in the CNS may be a mechanism contributing to the development of cardiovascular complication in diabetes (refer to chapter 6). The interactions between dehydration and CNS inflammation are therefore complex and require further investigation.

Our previous study found that drinking 1% NaCl influenced CNS inflammation in STZ diabetic rats (refer to chapters 4-5). Oral intake of 1% NaCl caused the activation of microglia in the PVN and increases in blood pressure in diabetic rats at 2 weeks following STZ injection. The inhibition of microglial activation prevented the hypertension seen in these animals (refer to chapter 4); however, drinking 1% NaCl for 6 weeks in STZ diabetic rats reduced the activation of microglia and neurons in the NTS, a baroreceptor reflex regulator region. This reduction was accompanied by a restoration of the function of the bradycardic baroreflex in STZ diabetic rats (refer to chapter 5). Thus, it appears that drinking 1% NaCl for a longer period in STZ diabetic animals produces beneficial effects on the cardiovascular system.

Our previous study showed that microglial activation in the PVN may cause the cardiac dysfunction seen in diabetic rats (refer to chapter 6). Dai et al. (1994) reported that saline intake for 6 weeks improved *ex vivo* cardiac function in STZ diabetic rats. Despite this, in our study, prolonged saline intake did not reduce microglial activation in the PVN in STZ

diabetic rats, even though the number of rats tested was small (refer to chapter 5), suggesting the effect of long-term saline on the cardiac function may not occur via the PVN.

Therefore, in this study, we investigated the effects of prolonged saline intake on (i) the *in vivo* cardiac function, (ii) dehydration and (iii) microglial activation in the PVN in STZ diabetic rats. In addition, we investigated whether the microglial inhibitor minocycline provided any additional effect to that of prolonged saline treatment in STZ diabetic rats.

7.2 Results

7.2.1 Effect of saline plus or minus minocycline on general features of diabetic rats

At 6 weeks following injection with either STZ or citrate buffer, the body weights of diabetic animals (DWS) given tap water to drink and diabetic animals (DSS) given 1% NaCl solution with an ICV infusion of saline and diabetic animals given 1% NaCl with an ICV infusion of minocycline (DSM) were significantly lower ($P<0.01$, <0.001 and < 0.01, respectively) compared with control animals with an ICV infusion of saline (CWS), as expected (Figure 7-1A). Saline and saline plus minocycline treatments did not significantly affect the body weight of diabetic animals. As expected, all diabetic groups exhibited significantly higher ($P < 0.0001$) blood glucose levels compared with the CWS group, and saline ± minocycline treatment had no effect on blood glucose (Figure 7-1B). Daily water intake was significantly greater ($P<0.0001$) in all diabetic groups when compared to the CWS group (Figure 7-1C). Saline ± minocycline treatment in diabetic animals showed no significant effect on daily fluid intake. Heart weight (heart weight/body weight) was not significantly different between any of the groups (Figure 7-2A); however, kidney weight (kidney weight / body weight) was significantly greater ($P<0.0001$) in the DWS group compared to the CWS group (Figure 7-2B). This was also the case for kidney weight in the DSS and DSM groups compared to the CWS group ($P < 0.05$), but the saline + minocycline treatment tended to decrease kidney weight compared to the DWS group ($P = 0.07$).

To assess whether STZ diabetic rats were dehydrated and whether saline ± minocycline prevented dehydration, we measured plasma osmolarity, haemoglobin and haematocrit at the end of the experimental period. As expected, plasma osmolarity was significantly elevated in the DWS group as well as in the DSS group ($P<0.001$ and <0.05, respectively) compared with the CWS group (Figure 7-3A); however, no significant differences were found in plasma

osmolarity between the DSM group and CWS group. When compared to the DWS group, the combination of saline and minocycline treatment significantly reduced ($P<0.05$) plasma osmolarity, suggesting that plasma osmolarity was restored to normal levels in these animals. As an indicator of blood volume status, the haematocrit and Haemoglobin concentration were significantly elevated ($P<0.01$) only in the DWS group compared to the CWS group (Figure 7-3B-C). There was no significant difference between the DSS and CWS groups, suggesting that diabetic rats treated with saline did not have a significantly reduced blood volume. This also was the case between the DSM and CWS groups; however, there was also no significant effect of saline ± minocycline on haematocrit and Haemoglobin concentration when compared to diabetic animals. The results of plasma the osmolarity, haemoglobin and haematocrit measurements indicated that STZ-treated diabetic rats were dehydrated, and saline ± minocycline tended to prevent this characteristic of this model.

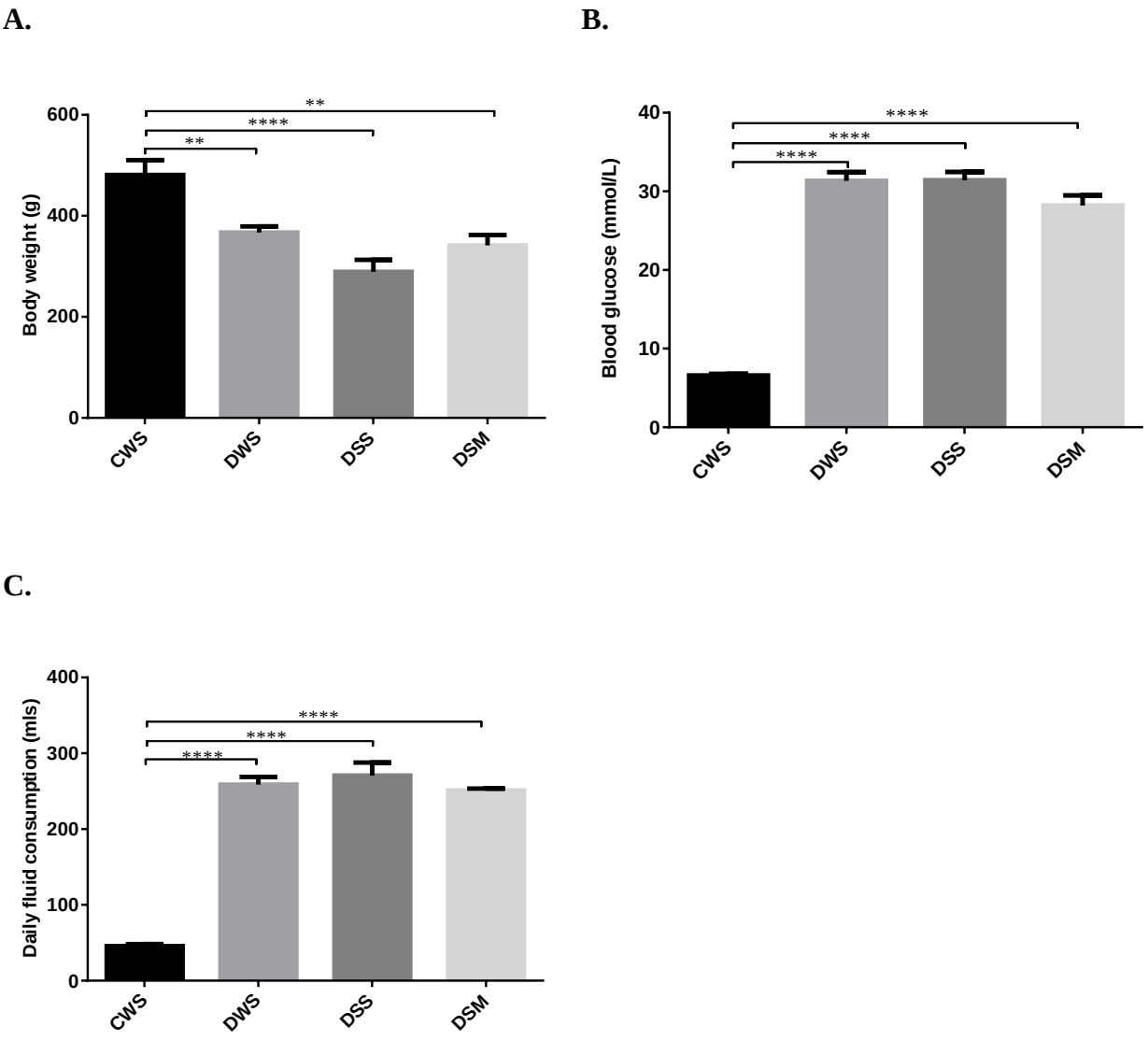


Figure 7-1. Effect of saline plus or minus minocycline treatment on body weight, blood glucose and fluid intake of diabetic rats. A: Body weight measured at 6 weeks after injection of rats with either STZ or citrate buffer in the following groups: control animals (CWS), diabetic animals (DWS) given tap water to drink with an ICV infusion of saline and diabetic animals (DSS) given 1% NaCl solution with an ICV infusion of saline and diabetic animals (DSM) given 1% NaCl with an ICV infusion of minocycline. B: Blood glucose levels measured at 6 weeks in these groups. C: The amount of tap water or 1% NaCl ingested was monitored daily at week 6 by weighting water bottles. Number of rats = 6, 8, 8 and 8 respectively. Data are expressed as the mean ± SEM. Significance was evaluated using a one way ANOVA followed by Tukey's post hoc test for all comparisons (CWS vs DWS , CWS vs DSS, CWS vs DSM, DWS vs DSS, DWS vs DSM and DSS vs DSM) in this and all subsequent figures; * indicates P<0.05; ** indicates P<0.01; *** indicates P<0.001; **** indicates P < 0.0001 for comparisons between diabetic groups and controls; ♯ indicates P<0.05; ♯♯ indicates P<0.0; ♯♯♯ indicates P<0.001; ♯♯♯♯ indicates P < 0.0001 for comparisons between diabetic groups; & indicates P<0.05; && indicates P<0.01; &&& indicates P<0.001 for comparisons between diabetic groups and diabetic animals with minocycline treatment.

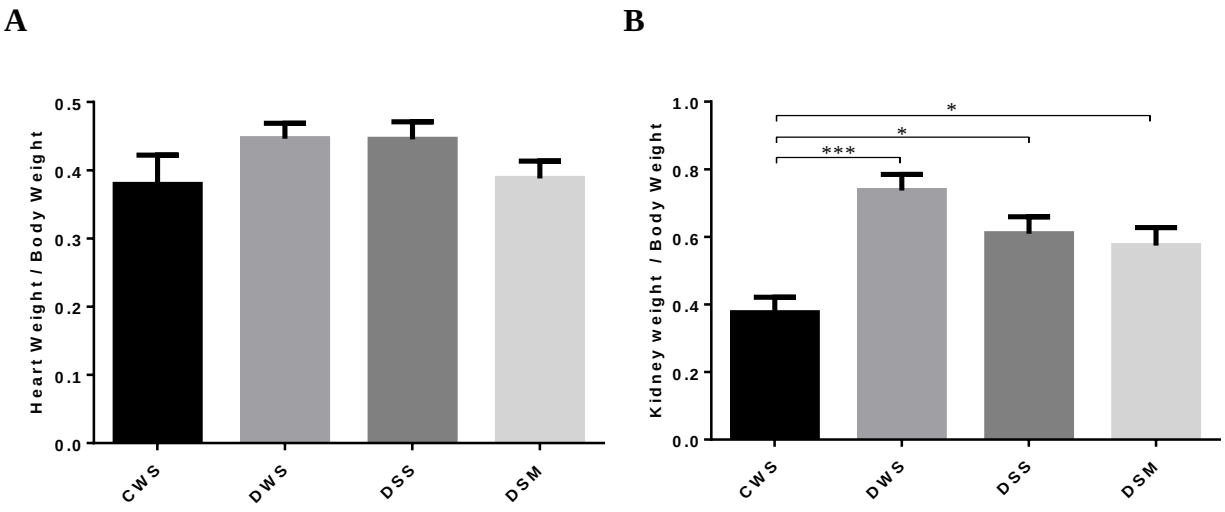


Figure 7-2. Effect of saline plus or minus minocycline treatment on heart weight and kidney weight in diabetic rats. A: Heart weight (heart weight / body weight); and B: kidney weight (kidney weight / body weight) as a proportion of control animals (CWS), diabetic animals (DWS) given tap water to drink with an ICV infusion of saline and diabetic animals (DSS) given 1% NaCl solution with an ICV infusion of saline and diabetic animals (DSM) given 1% NaCl with an ICV infusion of minocycline. Number of rats = 6, 7, 7 and 7 respectively in each group. Data are expressed as the mean ± SEM.

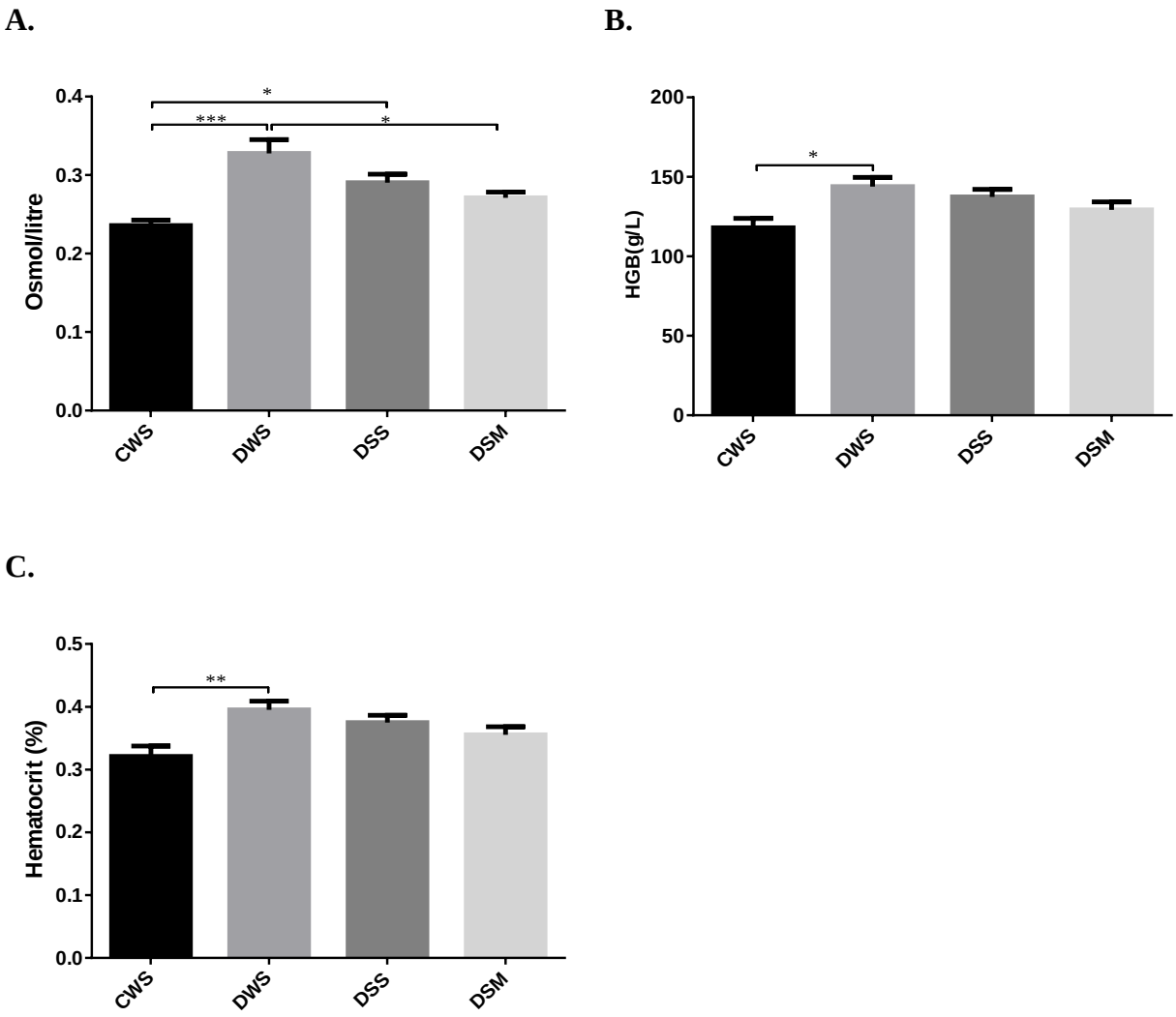


Figure 7-3. Effect of saline plus or minus minocycline treatment on plasma osmolarity and haemoglobin in diabetic rats. A: Plasma osmolarity (Osmol/litre); B: Haemoglobin concentration (g/L); and C: Haematocrit (%) measured at the end of the experimental period in control animals (CWS), diabetic animals (DWS) given tap water to drink with an ICV infusion of saline and diabetic animals (DSS) given 1% NaCl solution with an ICV infusion of saline and diabetic animals (DSM) given 1% NaCl with an ICV infusion of minocycline. Number of rats = 6, 8, 8 and 7 respectively in each group. Data are expressed as the mean ± SEM.

7.2.2 Echocardiographic measurements in diabetic rats

The left ventricle of diabetic animals given water to drink displayed a significant increase in the internal diameter in systole and diastole compared to the CWS group ($P<0.001$ and $P<0.01$, respectively) (Figure 7-4A-B). In contrast, no significant difference was seen in the internal diameter in the systole and diastole between the DSS and CWS groups or the DMS and CWS groups. Treatment of diabetic animals with a combination of saline and minocycline significantly reduced the left ventricle internal diameter in systole and diastole compared with the DWS group ($P<0.0001$ and $P<0.01$, respectively), suggesting saline ± minocycline reversed structural changes in the heart. When compared to the DSS group, minocycline + saline treatment had no significant effect on the internal diameter in the diastole, but it significantly ($P<0.001$) reduced the internal diameter in the systole, suggesting that the effect of saline was intermediate. The E/A ratio of the DWS and DSS groups demonstrated a significant reduction ($P<0.001$ and <0.01, respectively) compared with the CWS group, suggesting diastolic dysfunction (Figure 7-4C); however, no differences were observed in the E/A ratio between the DSM group and CWS group, and the combination of saline and minocycline significantly improved ($P<0.01$) the E/A ratio in the DWS and DSS groups, suggesting no effect of saline on this parameter in diabetes. The heart rate under anaesthesia did not differ among any of the groups (Figure 7-4D).

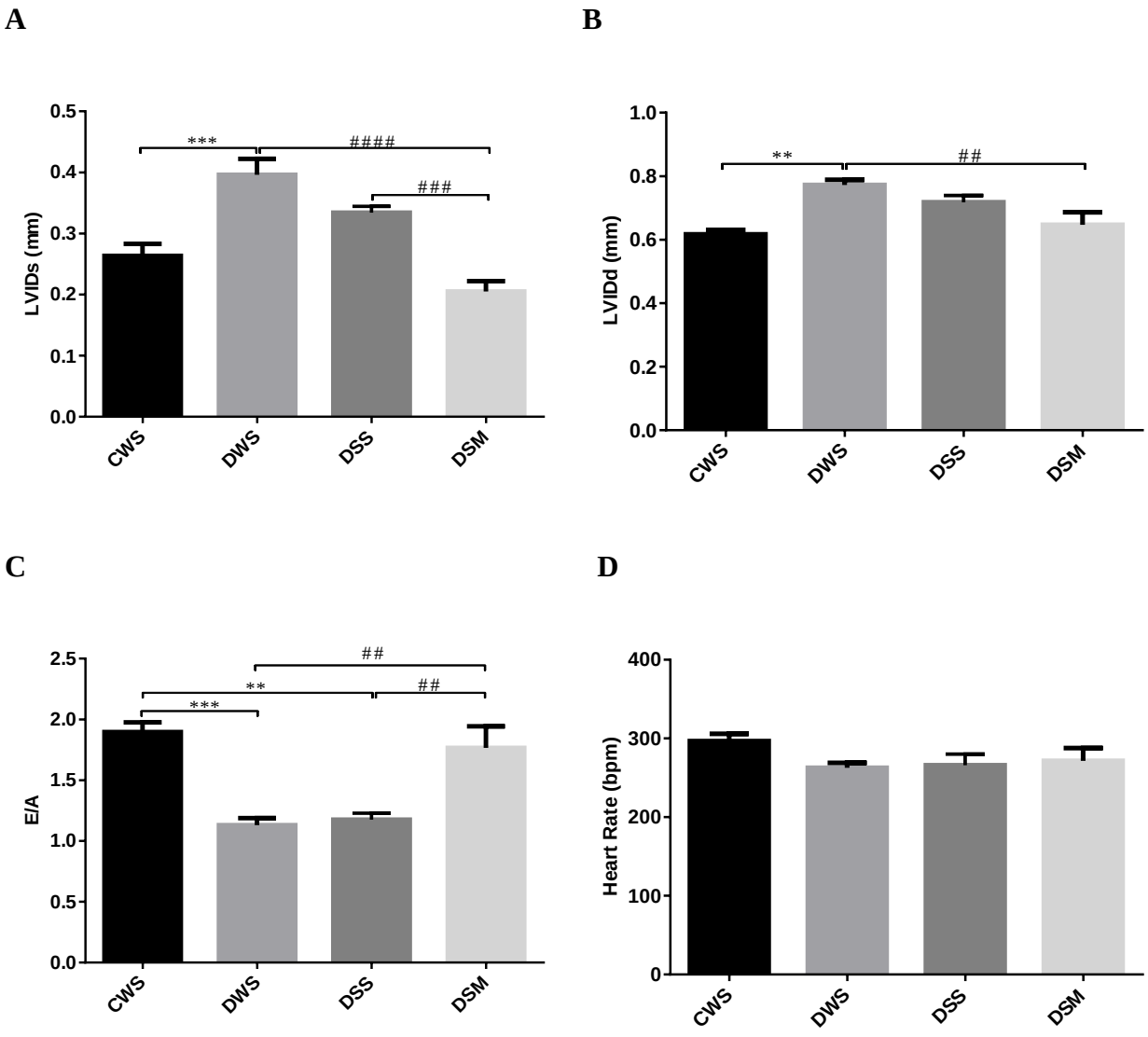


Figure 7-4. Effect of saline plus or minus minocycline treatment on echocardiographic measurements in diabetic rats. A: Left ventricular internal diameter in systole — LVIDs (mm); B: left ventricular internal diameter in diastole — LVIDd (mm); C: E/A ratio (the ratio of the early (E) to late (A) ventricular filling velocities); and D: the heart rate of control animals (CWS), diabetic animals (DWS) given tap water to drink with an ICV infusion of saline and diabetic animals (DSS) given 1% NaCl solution with an ICV infusion of saline and diabetic animals (DSM) given 1% NaCl with an ICV infusion of minocycline. Number of rats = 6, 8, 6 and 8 respectively. Data are expressed as the mean ± SEM.

7.2.3 Measurement of haemodynamic parameters in diabetic rats

Immediately after echocardiography, cardiac catheterization was performed to assess the left ventricular end diastolic pressure and the maximal rate of pressure change (dP/dt max). Heart rate was again measured during this procedure. Compared to control rats, the DWS group exhibited significantly higher ($P<0.0001$) left ventricular end diastolic pressure at 6 weeks following STZ injection, indicating clear left ventricular dysfunction (Figure 7-5A). Treatment of diabetic animals with saline significantly restored ($P<0.001$) the elevated end diastolic pressure to levels comparable to the CWS group. This also was the case in diabetic rats treated with minocycline compared with the DWS group ($P<0.0001$). No difference was observed in left ventricular end diastolic pressure in the DSS and DSM groups compared with the CWS group. When dP/dt max was quantified, it was significantly lower ($P<0.05$) in the DWS and DSS groups compared to the level observed in the CWS group (Figure 7-5B); however, there was no significant difference in the maximal rate of pressure change between the DSM group and CWS group. Despite this, a combination of minocycline and saline did not significantly increase the maximal rate of pressure change in diabetic animals. The heart rate under deep anaesthesia was similar among all groups (Figure 7-5C). These data indicated that saline ± minocycline treatment was able to prevent diastolic dysfunction seen in diabetic animals given water to drink.

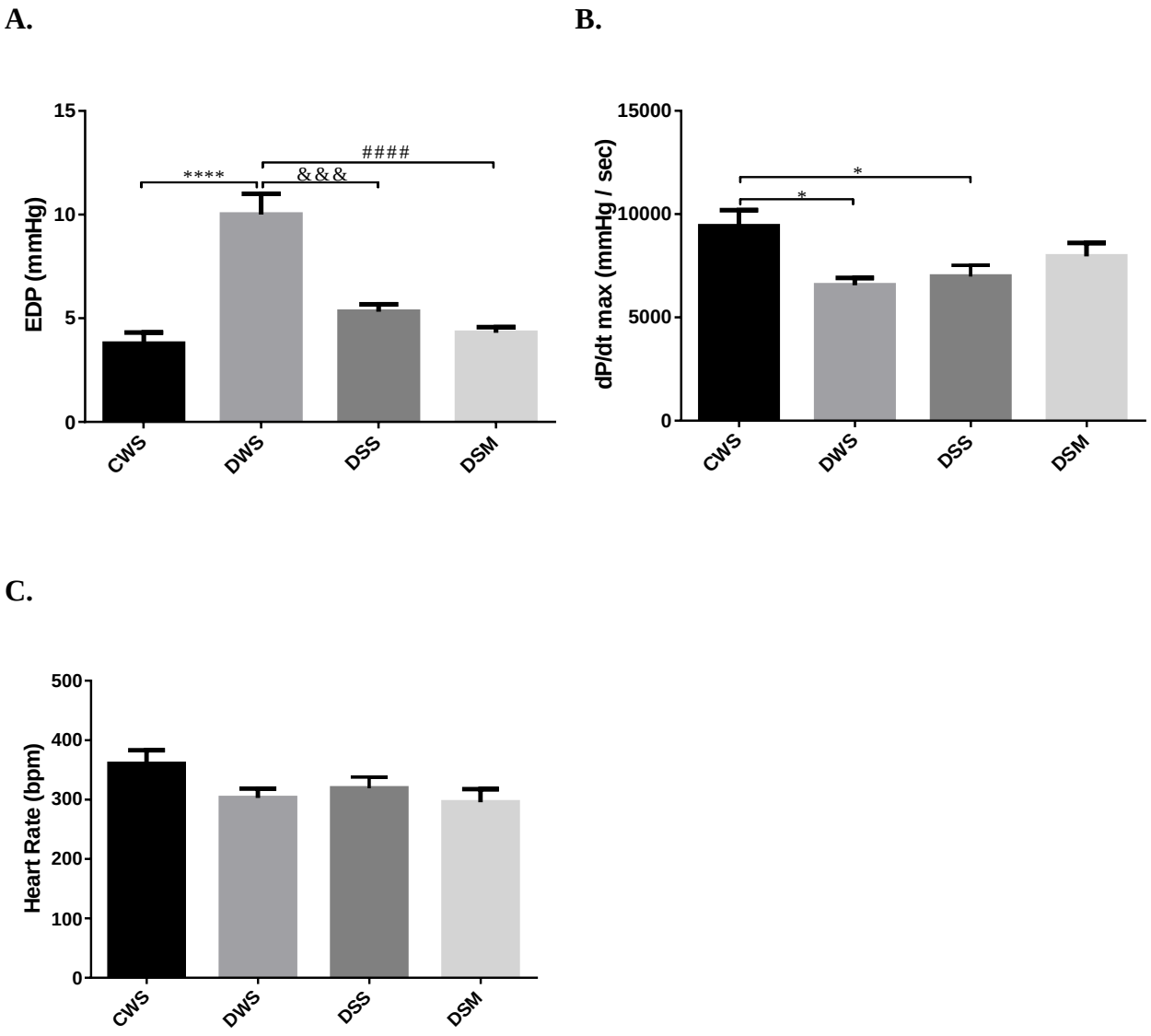


Figure 7-5. Effect of saline plus or minus minocycline treatment on hemodynamic parameters in diabetic rats. A: end-diastolic pressure; (EDP); B: the maximal rate of pressure change (dP/dt max); and C: the heart rate of in control animals (CWS), diabetic animals (DWS) given tap water to drink with an ICV infusion of saline and diabetic animals (DSS) given 1% NaCl solution with an ICV infusion of saline and diabetic animals (DSM) given 1% NaCl with an ICV infusion of minocycline. Number of rats = 6, 8, 7 and 7 respectively. Data are expressed as the mean ± SEM.

7.2.4 OX-42 and C-fos Immunohistochemistry in the paraventricular nucleus in diabetic rats

At 6 weeks following vehicle injection, microglia in the CWS group displayed normal morphology with small cell bodies and long, fine-branched processes throughout the PVN region, which is consistent with our previous study; however, microglial cells in the PVN in the DWS group appeared to be present in an activated form with larger cell bodies and shorter, thicker processes (Figure 7-6). The quantification of the percentage of activated microglia in the PVN showed that it was lower than 7% in the CWS group and higher than 60% in the DWS group (Figure 7-8A). Thus, the DWS group exhibited a significant increase ($P<0.0001$) in the percentage of activated microglia compared with the CWS group. Saline treatment in long-term STZ diabetic rats significantly reduced ($P<0.01$) the percentage of activated microglia in the PVN compared to the DWS group, but it was still significantly higher ($P<0.0001$) compared with the CWS group, suggesting that saline treatment was not able to inhibit microglial activation completely. There was no significant difference in the percentage of activated microglia in the PVN between the DSM group and CWS group. Minocycline treatment in diabetic animals given saline caused a significant reduction ($P<0.0001$) in the percentage of activated microglia in the PVN compared to diabetic animals to a level comparable to that in the CWS group.

The number of fos-immunoreactive neurons in the PVN in the DWS group appeared larger compared to the CWS group (Figure 7-7). On quantification, we observed a significant increase ($P<0.001$) in the number of fos-immunoreactive neurons in the DWS group compared to the CWS group (Figure 7-8B). Although the DSS groups also exhibited a significant increase ($P<0.05$) in the number of fos-immunoreactive neurons compared to the CWS group, saline treatment significantly reduced ($P<0.05$) fos activity in the PVN in the diabetic animals. The number of fos-immunoreactive neurons in the PVN showed no

significant difference between the DSM group and CWS group. When diabetic groups were compared, the increased number of fos-immunoreactive neurons seen in the PVN in the DWS and DSS groups was significantly reduced ($P<0.0001$ and <0.05) by the minocycline treatment. These results suggested that saline ± minocycline treatment prevented the activation of PVN neurons in STZ diabetic rats.

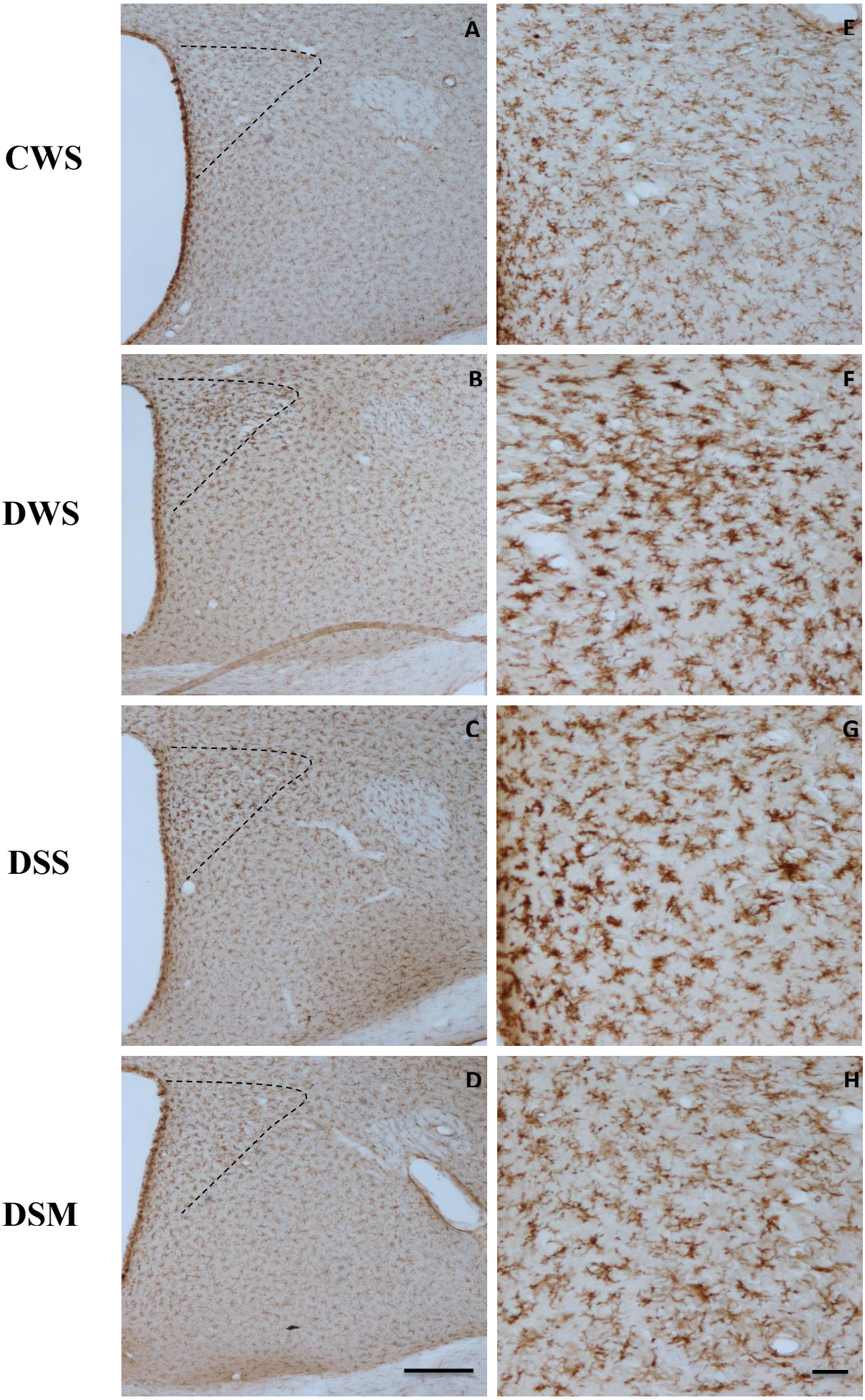

CWS
DWS
DSS
DSM
A
B
C
D
E
F
G
H

Figure 7-6. Morphology of microglia in STZ diabetic rats. Photomicrographs showing CD11b (OX-42 clone) immunoreactive microglia in the paraventricular nucleus (PVN) of control animals (CWS), diabetic animals (DWS) given tap water to drink with ICV infusion of saline and diabetic animals (DSS) given 1% NaCl solution with ICV infusion of saline; and diabetic animals (DSM) given 1% NaCl with ICV infusion of minocyclineDotted lines in low-power images (A-C) outline the PVN. High-power images (D-F) show the morphology of microglia in the parvocellular PVN region in detail. OX-42 antibody staining shows microglia with normal morphology with small cell bodies and long fine-branched processes throughout the PVN region in the CS and DM groups, while activated microglia showing larger cell bodies and shorter, thicker processes are seen in the DS group. Bar = 100 μm in A–D, 50 μm in E–H.

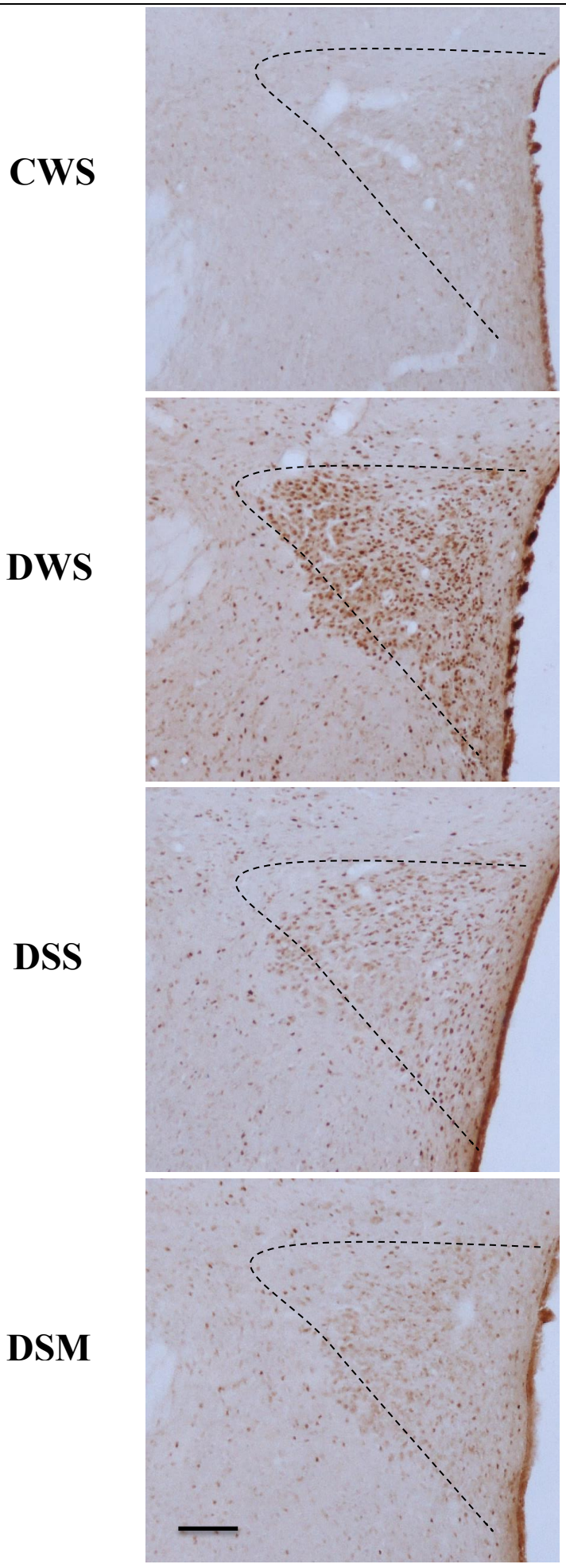

CWS
DWS
DSS
DSM

Figure 7-7. Fos activity in the PVN in diabetic rats. Photomicrographs showing neuronal nuclei stained with anti-Fos antibody in the paraventricular nucleus (PVN) of control animals (CWS), diabetic animals (DWS) given tap water to drink with ICV infusion of saline and diabetic animals (DSS) given 1% NaCl solution with ICV infusion of saline; and diabetic animals (DSM) given 1% NaCl with ICV infusion of minocycline.Dotted lines the PVN. Bar = 100 μm.

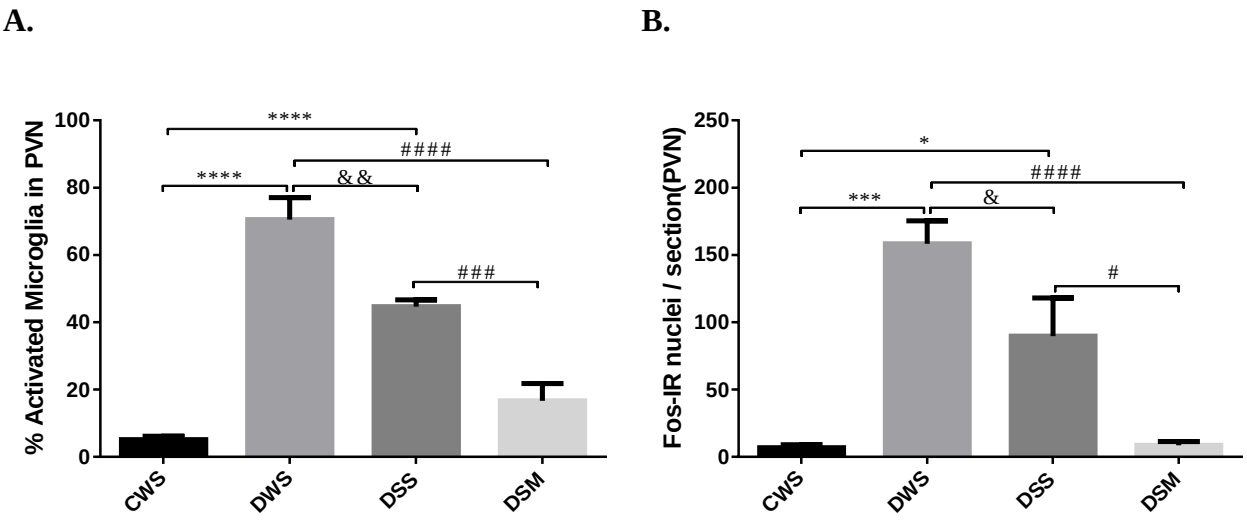


Figure 7-8. Effect of saline plus or minus minocycline treatment on microglial and neuronal activation in the paraventricular nucleus of diabetic rats. A: The percentage of microglia showing activated morphology in the paraventricular nucleus (PVN) of control animals (CWS), diabetic animals (DWS) given tap water to drink with an ICV infusion of saline and diabetic animals (DSS) given 1% NaCl solution with an ICV infusion of saline and diabetic animals (DSM) given 1% NaCl with an ICV infusion of minocycline. B: Quantification of Fos-IR positive cell nuclei in the same region. Number of rats = 6 in each group. Data are expressed as the mean ± SEM.

7.3 Discussion

There are increasing indications from previous studies that prolonged drinking of normal saline reduced cardiovascular complications in STZ diabetic animals, including cardiac dysfunction (Dai *et al.*, 1994) (chapter 5), but the exact mechanism is still unclear. We previously provided evidence that inflammation in CNS cardiovascular centres plays a key role in the pathogenesis of diabetic cardiovascular complications; however, whether the effect of saline intake on cardiovascular complications is mediated by PVN inflammation has not been investigated. Therefore, in this study, we aimed to investigate the effects of 1% NaCl on the cardiovascular function *in vivo*, whether saline intake influences PVN inflammation in longer term (6 weeks) STZ-diabetic rats and whether the inhibition of microglial activation in the PVN can reverse any of the changes seen. The main findings of the present study were that prolonged treatment with 1% NaCl (i) restored elevated end diastolic pressure and (ii) reduced the activation of microglia and neurons in the PVN and that (iii) ICV minocycline further inhibited microglial and neuronal activation and that (iv) this inhibition was accompanied by a further improvement in left ventricular function in STZ diabetic rats.

In the present study, the structure of the left ventricle in diabetic rats treated with saline tended to be normalised when assessed by an echocardiographic approach, although there was no significant difference when functional parameter was measured (E/A ratio). Similarly, a direct evaluation of the left ventricle function demonstrated that saline treatment significantly restores elevated end diastolic pressure in diabetic rats to the level seen in control rats but not dP/dt max. Thus, although 1% NaCl intake did not significantly improve all aspects of left ventricle functions and structure, these data provide evidence confirming an improvement in cardiac function in STZ diabetic rats treated with saline, which is in agreement with previous *ex vivo* studies (Dai *et al.*, 1994); however, how this effect of saline occurs is not known. Interestingly, the improvement observed in cardiac function in diabetic

rats treated with saline was associated with a significant reduction in microglial activation in the PVN when compared to diabetic rats. We previously provided evidence that microglial activation in the PVN contributes to the development of left ventricle dysfunction in STZ diabetic rats. Inhibition of this activation via minocycline improved cardiac function. In addition, in a previous study, we have shown that another CNS cardiovascular centre, the NTS, exhibited microglial activation that was associated with baroreflex dysfunction in STZ diabetic rats at the same stages examined here. Prolonged saline intake reduced the NTS microglial activation and restored the baroreflex dysfunction seen in STZ diabetic rats. Therefore, this current study also supports the hypothesis that drinking saline in long-term STZ diabetic rats can prevent cardiovascular complications through a reduction in PVN inflammation.

To understand the effect of microglial activation in the PVN in diabetic rats treated with saline, we inhibited this activation via ICV minocycline in these animals. We observed that minocycline treatment significantly reduced microglial activation in the PVN when compared to both of the other diabetic groups to the level comparable to control rats, which is consistent with our previous study (chapter 6). As a consequence of this inhibition, diabetic rats treated with saline plus minocycline exhibited a significant improvement in almost all aspects of cardiac performance compared to diabetic groups, and there was no significant difference from the control rats. Because the effect of the combination of saline and minocycline treatment on cardiac function and PVN inflammation was greater when compared to the effect of saline treatment alone in the present study, collectively, these data suggest that changes in the left ventricle function in diabetic groups were paralleled by the levels of microglial activation in the PVN seen in these rats.

Several lines of evidence have suggested that inflammatory changes within the PVN mediate the over-activity of the sympathetic nervous system, which is linked to the pathogenesis of

the development of cardiovascular-related diseases, including hypertension, myocardial infarction and heart failure, which are major consequences of diabetes. These inflammatory changes include abnormally increased proinflammatory cytokines. In rats with acute myocardial infarction that is associated with increased sympathetic activity, elevated TNF-α and IL-1β in the PVN has been reported (Felder *et al.*, 2003; Francis *et al.*, 2004a; Francis *et al.*, 2004b). Microinjection of a TNF-α blocker into the PVN of rats with heart failure has been shown to decrease sympathetic hyperactivity (Guggilam *et al.*, 2008; Kang *et al.*, 2010). This reduction in sympathetic activity normalised left ventricle end diastolic pressure in these animals (Guggilam *et al.*, 2008). In addition, Yu et al. (2007) reported that the microinjection of anti-inflammatory cytokines (IL-10) into the PVN in rats with heart failure decreased left ventricle end diastolic pressure. Our laboratory demonstrated that microglial activation in the PVN also occurs in animal models of heart failure. As discussed previously, once microglia become activated in response to infection, injury or inflammation of the CNS, they release pro-inflammatory molecules and cytotoxic factors, such as nitric oxide (NO) and TNF-α. Therefore, one possible source of these cytokines is activated microglia. Along with these studies, our data suggest that activated microglia observed in the PVN in diabetic rats may secrete proinflammatory cytokines that activate the sympathetic nerves, and thereby contributes to the cardiac dysfunction seen here.

In addition to the beneficial effect of drinking saline on the cardiac function of STZ diabetic rats, emerging evidence confirms a previous result that kidney function is also improved by saline intake (McDonald *et al.*, 1969). Vallon et al. reported that drinking 1% NaCl for 6 weeks following STZ injection caused a reduction in glomerular hyperfiltration (Vallon *et al.*, 1997). Glomerular hyper-filtration is a risk factor for the development of diabetic nephropathy and renal failure (Mogensen & Christensen, 1984; Mogensen, 1986). In the present study, we did not measure the glomerular filtration rate, but we did measure kidney size. Saline intake tended to reduce kidney hypertrophy in diabetic rats, although there was

no significant difference, which is consistent with a previous study (Vallon *et al.*, 1997). This was also the case when diabetic rats were treated with saline + minocycline. In fact, several studies have reported that increased kidney size is associated with an elevated glomerular filtration rate, as seen in diabetic humans (Christiansen *et al.*, 1981; Gundersen & Mogensen, 1981) and animals (Luippold *et al.*, 2004; Malatiali *et al.*, 2008). It has been suggested that increased kidney size contributes to the glomerular hyperfiltration seen in diabetes (Christiansen, 1984; Bak *et al.*, 2000; Zerbini *et al.*, 2006). Therefore, these data suggest that saline ± minocycline treatment in long-term STZ diabetic rats may blunt renal changes. Our data on plasma osmolality also support this view.

Abnormally high renal sympathetic activation has been implicated in causing glomerular hyper filtration because renal denervation reversed glomerular hyper filtration in STZ diabetic rats (Luippold *et al.*, 2004; Salman *et al.*, 2011). It is well documented that PVN neuronal activation contributes to increased sympathetic nerve discharge observed in several diseases, including hypertension (Allen, 2002), heart failure (Li *et al.*, 2003), water deprivation (Stocker *et al.*, 2005) and chronic hypoxia (Sharpe *et al.*, 2013). Thus, the decreased PVN neuronal activity in diabetic rats treated with saline ± minocycline may explain improved renal changes in diabetic rats.

Elevated vasopressin secretion is another possible mechanism that may induce renal changes seen in diabetic rats (Bouby *et al.*, 1999; Lamarche *et al.*, 1999; Donnelly *et al.*, 2000). Normalization vasopressin secretion has been shown to prevent renal hypertrophy and glomerular hyperfiltration in vasopressin-deficient rats injected with STZ to induce diabetes (Bardoux *et al.*, 1999; Bouby *et al.*, 1999). In the present study, we found a significant increase in plasma osmolarity in diabetic rats compared with the control. Haematocrit and Haemoglobin concentration were also increased in diabetic rats, indicating depletion in blood plasma volume. These results suggest that our diabetic animals were dehydrated. In response

to osmotic stimuli to regulate body fluid balance and blood volume in diabetes, the PVN neurons would cause secretion of vasopressin (Zerbe *et al.*, 1979; Charlton *et al.*, 1988; Brooks *et al.*, 1989; Zheng *et al.*, 2002). In this study, treatment with saline prevented dehydration seen in diabetic animals, and the effects were found to be significant with the combination of saline and minocycline. Therefore, we speculate that decreased PVN neuronal activity by saline ± minocycline would normalise vasopressin secretion, which may in turn prevent renal hypertrophy.

We previously provided evidence that minocycline treatment not only attenuated microglial activation but also neuronal activation in the PVN in diabetic rats, suggesting that microglial activation induces the secondary and sustained neuronal activity via a positive feedback mechanism. In this study, saline treatment reduced both PVN microglia and neuron activation in diabetic rats, though not completely. Also, the increase in microglial and neuronal activation within the PVN was significantly inhibited by minocycline, which is consistent with our earlier observations. Thus, the present finding supports our interpretation that neuronal activation may be mediated by microglial activation in the PVN.

The mechanisms by which saline intake reduces microglial activation in the PVN are not clearly understood. Increased internal angiotensin II has been found at 6-8 weeks following STZ injection (Anderson *et al.*, 1993). Several reports have established that an increase in the renin-angiotensin system is associated with diabetic cardiomyopathy in experimental and clinical studies (Kumar *et al.*, 2012; Mandavia *et al.*, 2013) and that the inhibition of the renin angiotensin/aldosterone system can improve cardiac dysfunction in STZ diabetic rats (Zheng *et al.*, 2015). In addition, the involvement of the renin-angiotensin system in PVN inflammation has previously been reported. For example, systemic infusion of angiotensin II in naïve rats has been shown to cause hypertension and activate microglial cells in the PVN (Shi *et al.*, 2010a). Interestingly, salt intake has been shown to reduce angiotensin II in

Zucker diabetic fatty rats (Takenaka *et al.,* 2011). Therefore, prolonged drinking of saline may reduce microglial activation indirectly through a reduction in the angiotensin II levels in STZ diabetic rats. Consequently, we presume that elevated angiotensin II may be responsible, at least in part, for cardiac dysfunction seen in diabetic animals and that saline intake reduces angiotensin II, which in turn improves this dysfunction.

7.4 Conclusion

The present study demonstrated that the cardiac and renal complications seen in STZ-induced diabetic rats were prevented by drinking 1% NaCl. Saline treatment also reduces microglial activation in the PVN in diabetic rats. Microglial inhibition in the PVN via ICV minocycline prevents the development of diabetic complications. This strongly suggests that microglial activation within the PVN is involved in the mechanisms of these complications. The beneficial effect of saline intake in diabetic rats was mediated by a reduction in central brain inflammation induced by diabetes; however, the exact mechanism is not clear, and further studies are needed.

Chapter Eight: Neuronal and Microglial Activation in the Cardiovascular Centers of Diabetic Mice: Effect of a High-fat diet and an Antioxidant

8.1 Introduction

As discussed in previous chapters, sympathetic nerve over-activity is widely accepted as a risk factor for several cardiovascular complications related diseases, including hypertension, cardiomyopathy, atherosclerosis, myocardial infarction and stroke. Blocking the increase in nerve activity with beta-blockers has been shown to decrease the mortality rate and improve outcomes for patients following a heart attack (Butler *et al.*, 2006). Elevated sympathetic nerve activity has been characterized in humans and animals with type 1 diabetes mellitus (Refer to chapter 1); however, the cause of this abnormal activation in diabetes is not clear.

Sympathetic nerve activity to the cardiovascular system is controlled via several regions in the brain, including the paraventricular nucleus (PVN). Previously, we have reported that the resident immune cells within CNS, called microglia, become activated in the PVN in STZ diabetic rats. These cells have been implicated in the development of many neurological disorders, including neuropathic pain. Once they become activated, they release proinflammatory molecules, which have been shown to induce the death of neurons as well as cause neuronal hyperexcitability by increasing excitation and decreasing inhibition in the spinal cord (Sweitzer *et al.*, 1999; Tsuda *et al.*, 2008; Milligan & Watkins, 2009). Moreover, we provided evidence that activated microglia contribute to PVN neuronal hyperexcitability, hypertension and cardiomyopathy in STZ diabetic rats and that the inhibition of microglia in the PVN via minocycline prevents the hypertension and cardiac dysfunction seen in these animals (Refer to chapter 4, 6 and 7). Therefore, we hypothesize that activated microglia may be the cause of PVN neuronal activity, which in turn activates sympathetic drive and contributes to cardiovascular complications; however, whether microglial activation occurs in other species and other models of diabetes is not known. The mouse is the most suitable animal model for genetic manipulations and can also be used in cellular and molecular studies. Therefore, for several reasons, we determined that it was important to examine

whether microglia are activated in cardiovascular centres in STZ diabetic mice and the time course of any activation. This would also provide an opportunity to further investigate mechanisms and test the effects of other interventions in CNS inflammation in diabetes.

Hydrogen sulphide (H_2S) donor has been reported to play beneficial roles in cardiovascular diseases, such as hypertension (Zheng *et al.*, 2011) and atherosclerosis (Beltowski *et al.*, 2010), but deficiency in the levels of H_2S in the blood is reported in diabetic humans and STZ diabetic animals (Jain *et al.*, 2010). Several studies have documented that lower levels of H_2S contribute to the development of diabetic complications, including diabetic cardiomyopathy and nephropathy (Lefer, 2008; Szabo, 2012). Interestingly, H_2S has been shown to act as an anti-inflammatory in activated microglia in *vitro* experiments (Hu *et al.*, 2007); however, the effect of the systemic infusion of H_2S on PVN inflammation in STZ mice is not known.

Type II diabetes is the most common form of diabetes in humans, and it has become a serious health problem worldwide. It is characterized by hyperglycemia due to insulin resistance and often associated with obesity in humans. Therefore, whether PVN inflammation contributes to complications of type II diabetes deserves investigation. A high fat diet is a major contributing factor in the pathogenesis of type II diabetes; however, a high fat diet only causes insulin resistance but not hyperglycemia in rodents. Thus, in this study, we combined a high-fat feeding with a low dose of STZ to produce an animal model that is more closely related to human type II diabetes (Reed *et al.*, 2000; Sugano *et al.*, 2006; Zhang *et al.*, 2009). Abnormal sympathetic nerve activity has also been implicated in the development of cardiovascular complications in type II diabetic animals (Carlson *et al.*, 2000). Whether similar pathophysiological changes in the PVN as seen in type I diabetic animals may cause this increase in sympathetic nerve activity in models of type II diabetes has not been investigated.

Therefore, in the present study, we examined (i) whether microglia and neurons are activated in the PVN in STZ diabetic mice, (ii) the time course of microglial and neuronal activation and (iii) the effects of hydrogen sulphide and high-fat feeding on the PVN in these mice.

8.2 Results

8.2.1 Body weights and blood glucose of STZ diabetic mice

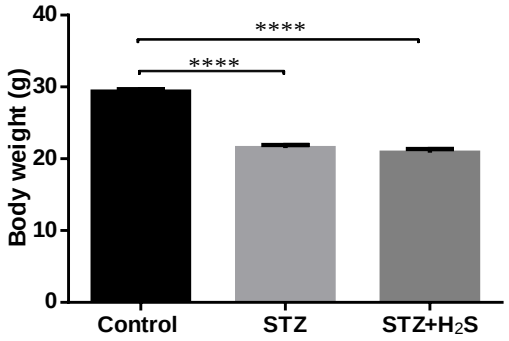
At 7 weeks following injection of C57BL6/J mice with STZ (50 mg/kg/day, 5 days), the body weights of diabetic mice (STZ) were found to be significantly lower ($P < 0.0001$) than the control mice (Figure 8-1A). The body weights of STZ mice treated with Na H_2S (STZ+H_2S) were also significantly lower ($P<0.0001$) compared with the control mice. There was no significant difference in the body weights between the STZ and STZ+ H_2S groups. Blood glucose levels in STZ and STZ+ H_2S groups were significantly higher ($P<0.0001$) in comparison to control mice (Figure 8-1B). Hydrogen sulphide had no effect on blood glucose in STZ mice.

In a second study, male FVB/N mice were injected with STZ (50mg/kg/day, 5 days) and maintained for 16 weeks. The body weights of the control mice were significantly greater than in diabetic mice (STZ) (Figure 8-1C). Blood glucose levels in STZ mice were significantly higher ($P < 0.0001$) than in the control mice (Figure 8-1D). The STZ treatment had less of an effect on body weight in FVB/N mice than in C57BL6/J mice, but the blood glucose levels were similar.

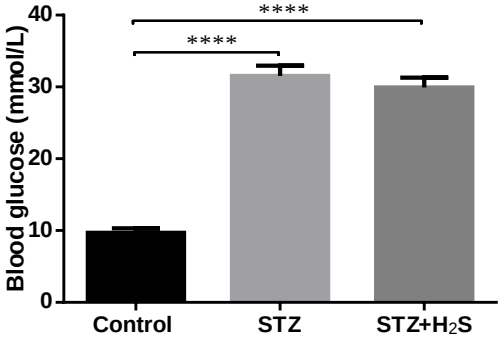
In the final study, at 10 weeks after C57BL6/J mice were injected with STZ (40 mg/kg/day, 5 days), the body weights were not significantly different when compared to the control mice (Figure 8-1E). This was also the case between the control mice and STZ mice with fat feeding (STZ+FF). Fat feeding did not significantly affect the body weight of STZ mice; however, blood glucose levels in STZ mice were significantly higher ($P<0.01$) compared with control mice, as expected (Figure 8-1F). This was also the case for blood glucose levels in the STZ+FF group ($P < 0.0001$) compared to the control mice. Fat feeding in STZ mice caused a significant increase in blood glucose levels ($P<0.01$) compared with STZ-alone

mice. The blood glucose in mice injected with a low dose of STZ alone was lower compared with mice treated with a high dose.

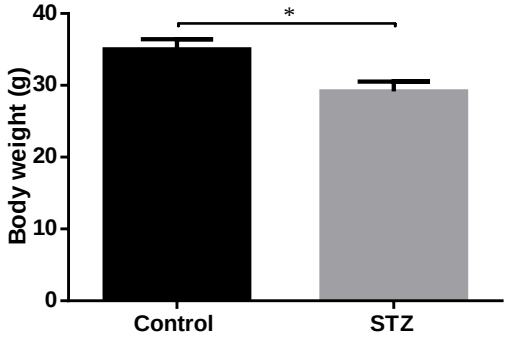
A.


Body weight (g)
40
30
20
10
0
****
****
Control
STZ
STZ+H2S

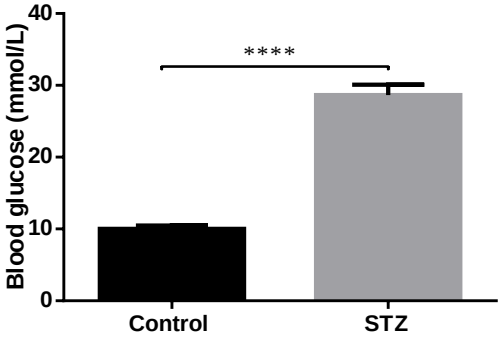
B.


Blood glucose (mmol/L)
40
30
20
10
0
****
****
Control
STZ
STZ+H2S

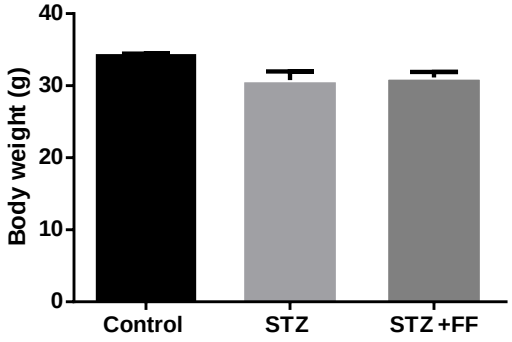
C.


Body weight (g)
40
30
20
10
0
*
Control
STZ

D.


Blood glucose (mmol/L)
40
30
20
10
0
****
Control
STZ

E.


Body weight (g)
40
30
20
10
0
Control
STZ
STZ+FF

F.

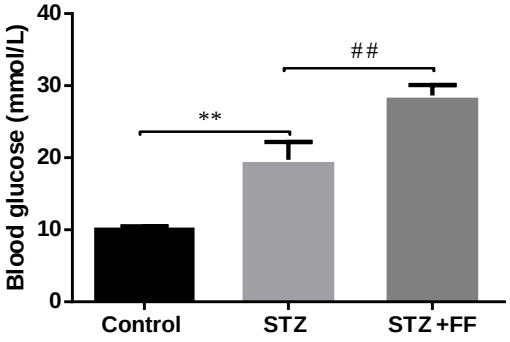

Blood glucose (mmol/L)
40
30
20
10
0
**
##
Control
STZ
STZ+FF

Figure 8-1. Body weights and blood glucose of STZ diabetic mice. Top panel: Body weights (A); and blood glucose levels (B) measured at 7 weeks after injection of mice with either STZ or citrate buffer in control animals, diabetic animals (STZ) or diabetic animals treated with hydrogen sulphide (STZ+ H_2S). Number of mice = 4 in each group. Middle panel: Body weights (C); and blood glucose levels (D) of mice at 16 weeks following treatment with either citrate buffer (control) or STZ. Number of mice = 4 in each group. Bottom panel: Body weights (E); and blood glucose levels (F) measured at 10 weeks after injection of mice with either citrate buffer in control animals or STZ in diabetic animals (STZ) and diabetic animals treated with fat feeding (STZ+FF). Number of mice = 12 in each group. Data are expressed as the mean ± SEM. Significance was evaluated using a one way ANOVA followed by Tukey's post hoc test for all comparisons in the top and bottom panels or an unpaired t test in the middle panel. In this and all subsequent figures; * indicates $P<0.05$; ** indicates $P<0.01$; *** indicates $P<0.001$; **** indicates $P < 0.0001$ for comparisons between diabetic groups and control; ♯ indicates $P<0.05$; ♯♯ indicates $P<0.0$; ♯♯♯ indicates $P<0.001$; ♯♯♯♯ indicates $P < 0.0001$ for comparisons between diabetic groups.

8.2.2 Plasma osmolarity and haematocrit in STZ diabetic mice

In this study, we measured plasma osmolarity and haematocrit at 7 and 16 weeks after STZ treatment because increased plasma osmolarity and dehydration are implicated in neuronal activation in STZ diabetic rats. We observed a significant increase in plasma osmolarity in STZ mice at 8 weeks ($P<0.001$) compared to the control mice (Figure 8-2A); however, no significant differences were found in plasma osmolarity between STZ mice treated with H_2S and the control mice. When compared to STZ mice, Na H_2S treatment significantly reduced ($P<0.01$) plasma osmolarity. At 16 weeks, plasma osmolarity was also significantly higher ($P < 0.01$) in STZ mice compared to the control mice (Figure 8-2B).

Regarding haematocrit , we found no significant difference in haematocrit between STZ mice and control mice at 7 weeks (Figure 8-2C). Also, the haematocrit was similar between the STZ mice and control mice at later stages (Figure 8-2D). H_2S had no effect on haematocrit in STZ mice.

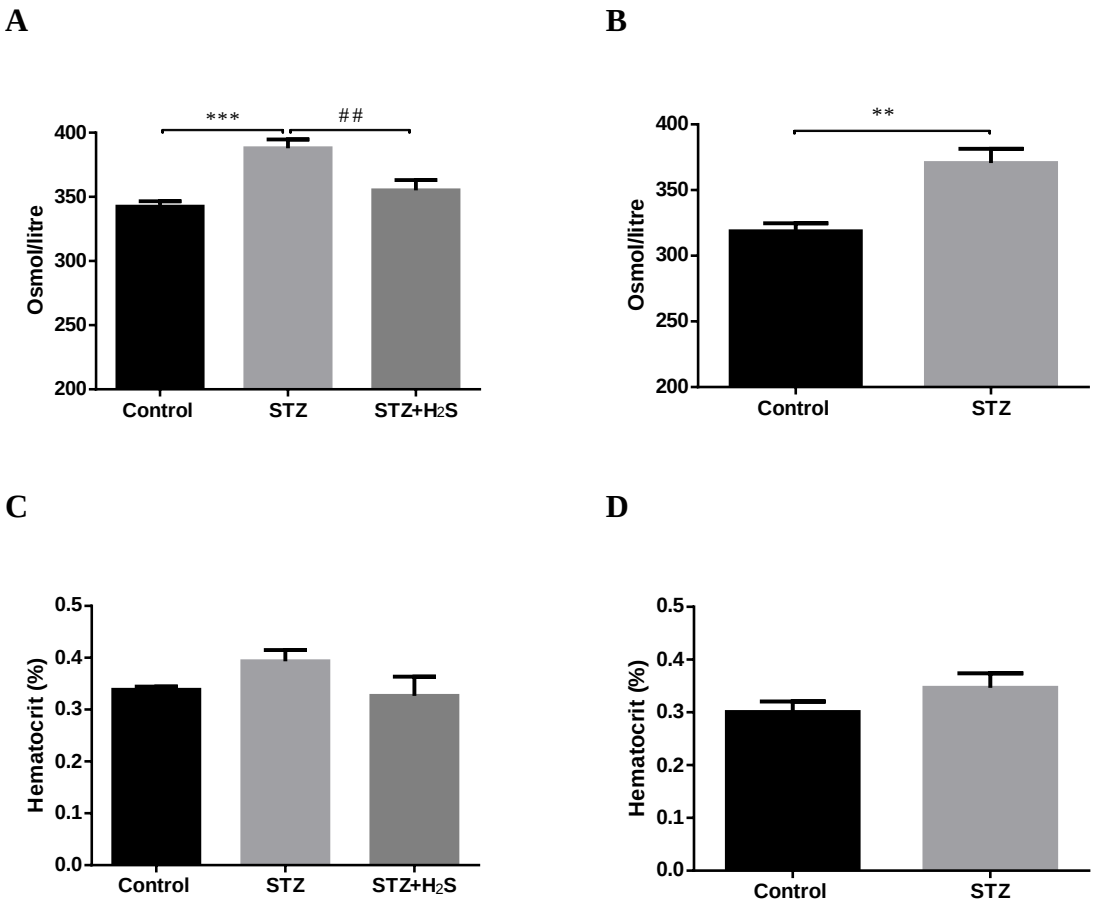


Figure 8-2. Plasma osmolarity and haematocrit in STZ diabetic mice. Left panel: Plasma osmolarity (A); and Haematocrit (C) measured at 7 weeks after injection of mice with either citrate buffer in control animals or STZ in diabetic animals (STZ) and diabetic animals treated with Na H_2S (STZ+ H_2S). Right panel: Plasma osmolarity (B); and Haematocrit (D) of mice at 16 weeks following treatment with either citrate buffer or STZ. Number of mice = 4 in each group. Data are expressed as the mean ± SEM.

8.2.3 CD11b and C-fos Immunohistochemistry in the paraventricular nucleus in diabetic mice

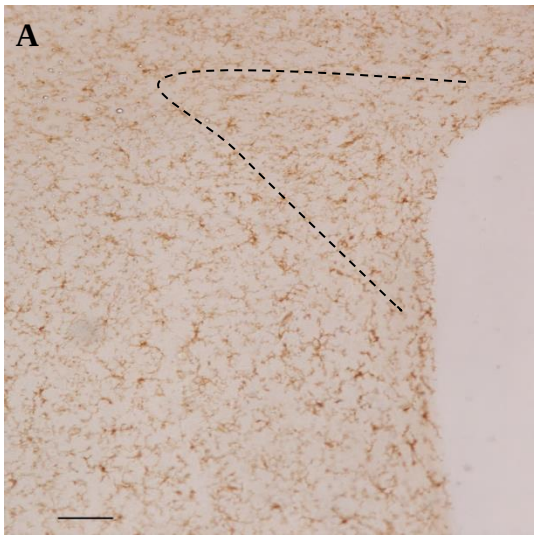
A CD11b antibody was used to identify morphological changes in microglia cells in the PVN in STZ mice. The microglia displayed normal morphology with small cell bodies and long, fine-branched processes throughout the PVN region in the control mice examined at 7 weeks (Figure 8-3). A similar observation was seen in the PVN in STZ mice with and without H_2S treatment at same stages, although STZ-alone mice appeared to show a more subtle change in microglial morphology. The quantitative analysis of the percentage of microglial activation in the PVN region indicated that no significant differences were found at 7 weeks when comparing STZ mice and control mice (Figure 8-5A). The percentage of activated microglia in the PVN was not significantly affected by H_2S treatment in STZ mice. In the PVN in control mice, the number of fos-immunoreactive neurons was smaller compared to STZ mice at 7 weeks (Figure 8-4). On quantification, we observed a significant increase ($P<0.0001$) in the number of fos-immunoreactive neurons in STZ mice compared to control mice (Figure 8-5B). Interestingly, H_2S treatment was able to be significantly reduce ($P<0.0001$) fos activity in the PVN in STZ mice.

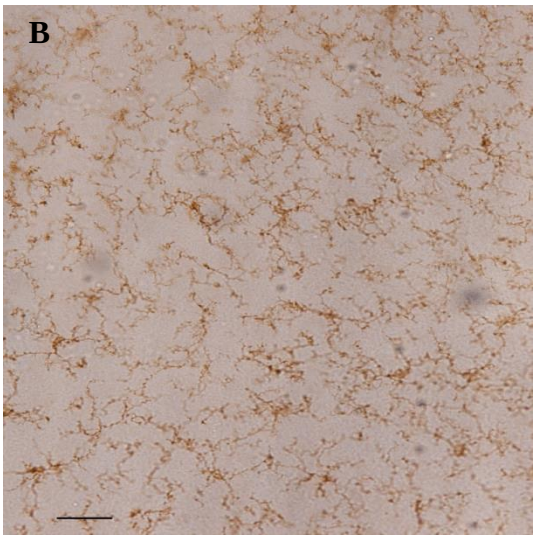
At later stages (16 weeks), the microglia in the PVN in STZ mice exhibited an activation form with larger cell bodies and thicker processes when compared to control mice (Figure 8-6). The percentage of activated microglia was significantly greater ($P<0.0001$) in the PVN in STZ mice compared with the control mice (Figure 8-8A). Compared with control mice, the counts of fos-immunoreactive neurons in the PVN showed a greater ($P<0.01$) level of fos activity in the PVN in STZ mice at this stage (Figure 8-7) (Figure 8-8B).

In the STZ + fat feeding study, microglial cells in the PVN region exhibited normal morphology in control mice at 10 weeks (Figure 8-9). This was also the case in low-dose STZ mice. There was no significant difference in the percentage of activated microglia

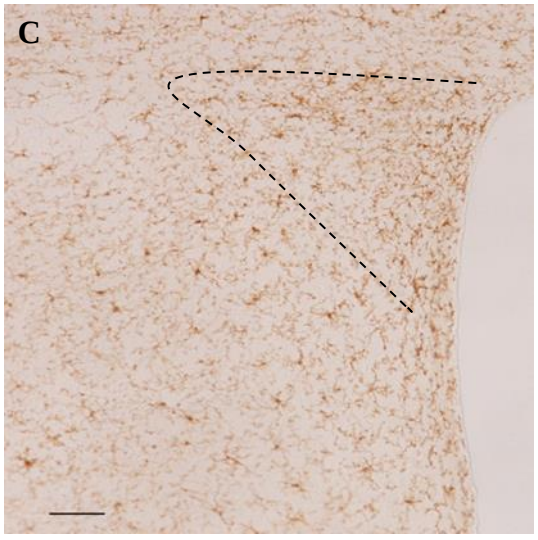
between the low-dose STZ and control mice (Figure 8-11A). Fat feeding had no effect on the percentage of microglial activation in low-dose STZ mice. In diabetic mice, a significant increase ($P<0.05$) in number of fos-immunoreactive neurons was seen in the PVN compared with the control, despite the lower blood glucose in these animals (Figure 8-10) (Figure 8-11B). No significant differences were observed in the number of fos-immunoreactive neurons between the low-dose STZ mice treated with fat feeding and control mice. Low-dose STZ mice treated with fat feeding displayed a significant reduction ($P<0.05$) in the levels of fos activity when compared to STZ mice on a normal diet.

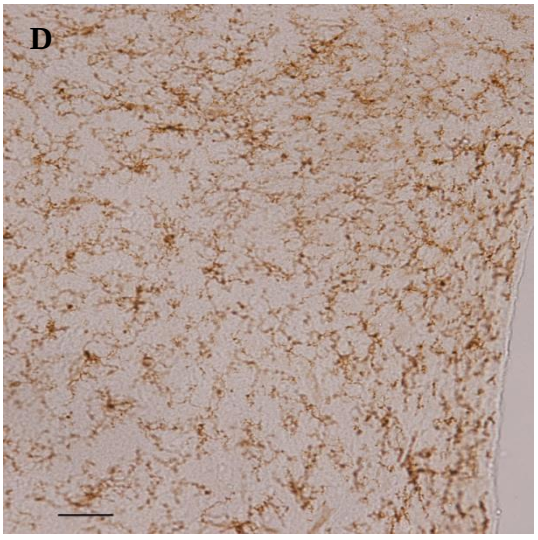
Control



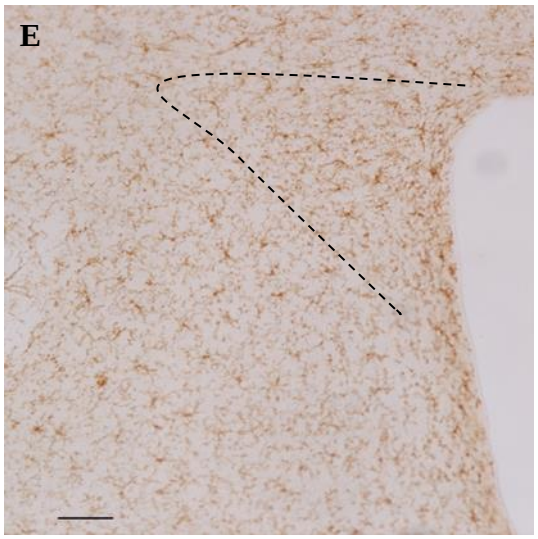


STZ





STZ+ H_2S



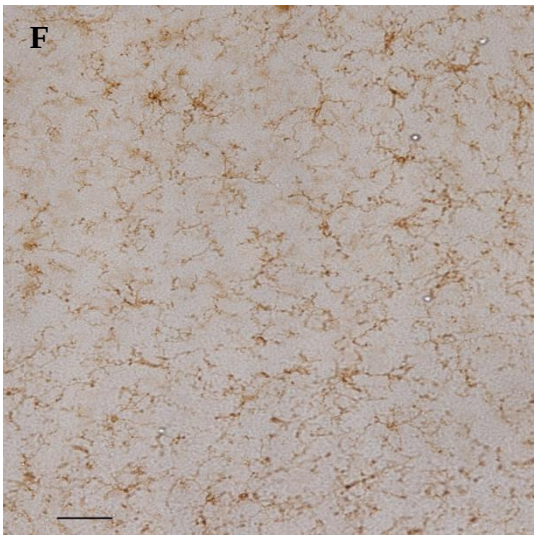
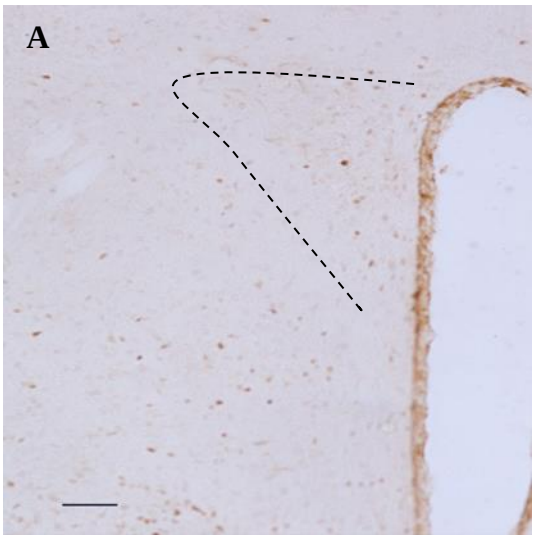
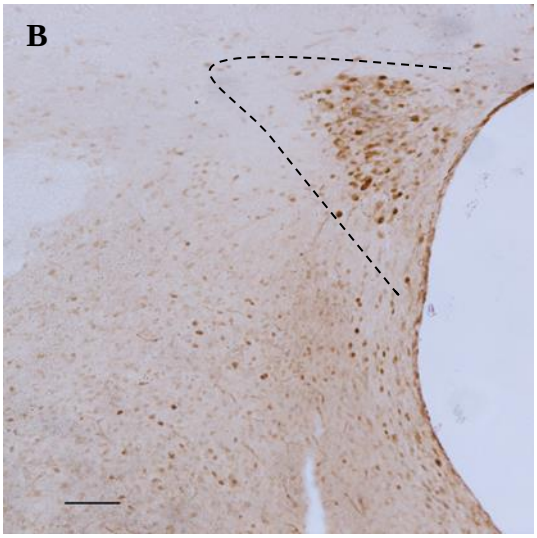


Figure 8-3. Morphology of microglia in STZ mice at 7 weeks after STZ treatment. Photomicrographs showing CD11b immunoreactive microglia in the paraventricular nucleus (PVN) in control, diabetic (STZ) and diabetic animals treated with Na H_2S (STZ+ H_2S) at 7 weeks. Dotted lines in low-power images (A, C and E) outline the PVN. High-power images (B, D and F) show the morphology of microglia in the PVN region in detail. Scale bar = 100 μm.

Control



STZ



STZ+ H_2S

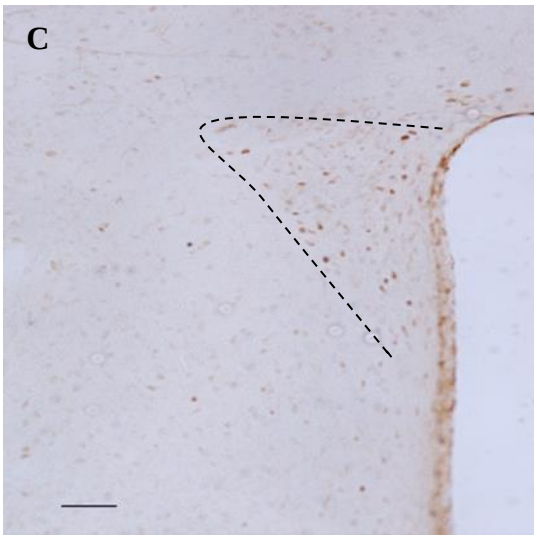


Figure 8-4. Fos activity in STZ mice at 7 weeks after STZ treatment. Photomicrographs showing neuronal nuclei stained with anti-Fos antibody in the paraventricular nucleus (PVN) in control, diabetic (STZ) and diabetic animals treated with Na H_2S (STZ+ H_2S) at 7 weeks. Dotted lines outline the PVN. Scale bar = 100 μm.

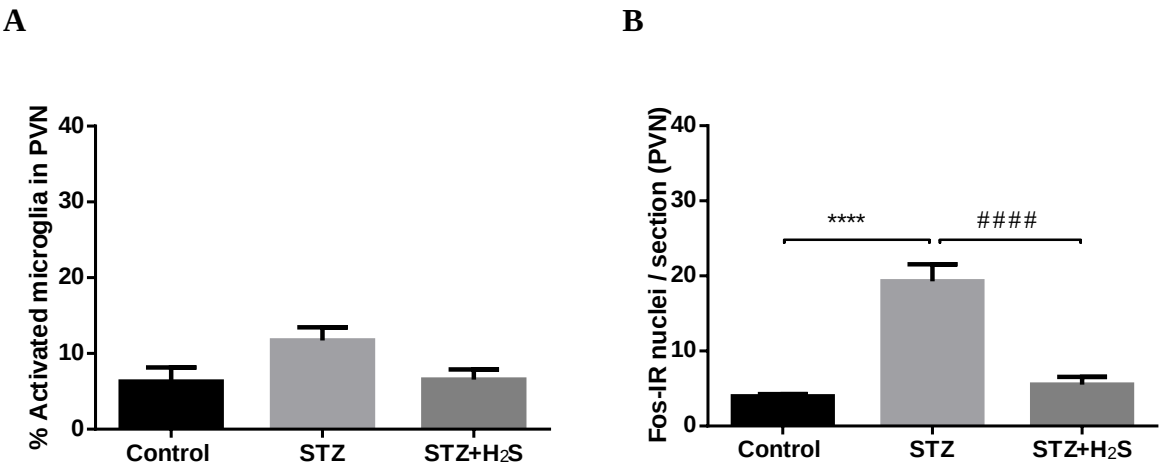


Figure 8-5. Microglial and neuronal activation in the paraventricular nucleus of STZ mice at 7 weeks after STZ treatment. A: The percentage of microglia showing activated morphology in the paraventricular nucleus (PVN) of control, diabetic (STZ) and diabetic animals treated with Na H_2S (STZ+ H_2S). B: Quantification of Fos-IR cell nuclei in the same region. Number of mice = 4 in each group. Data are expressed as the mean ± SEM.

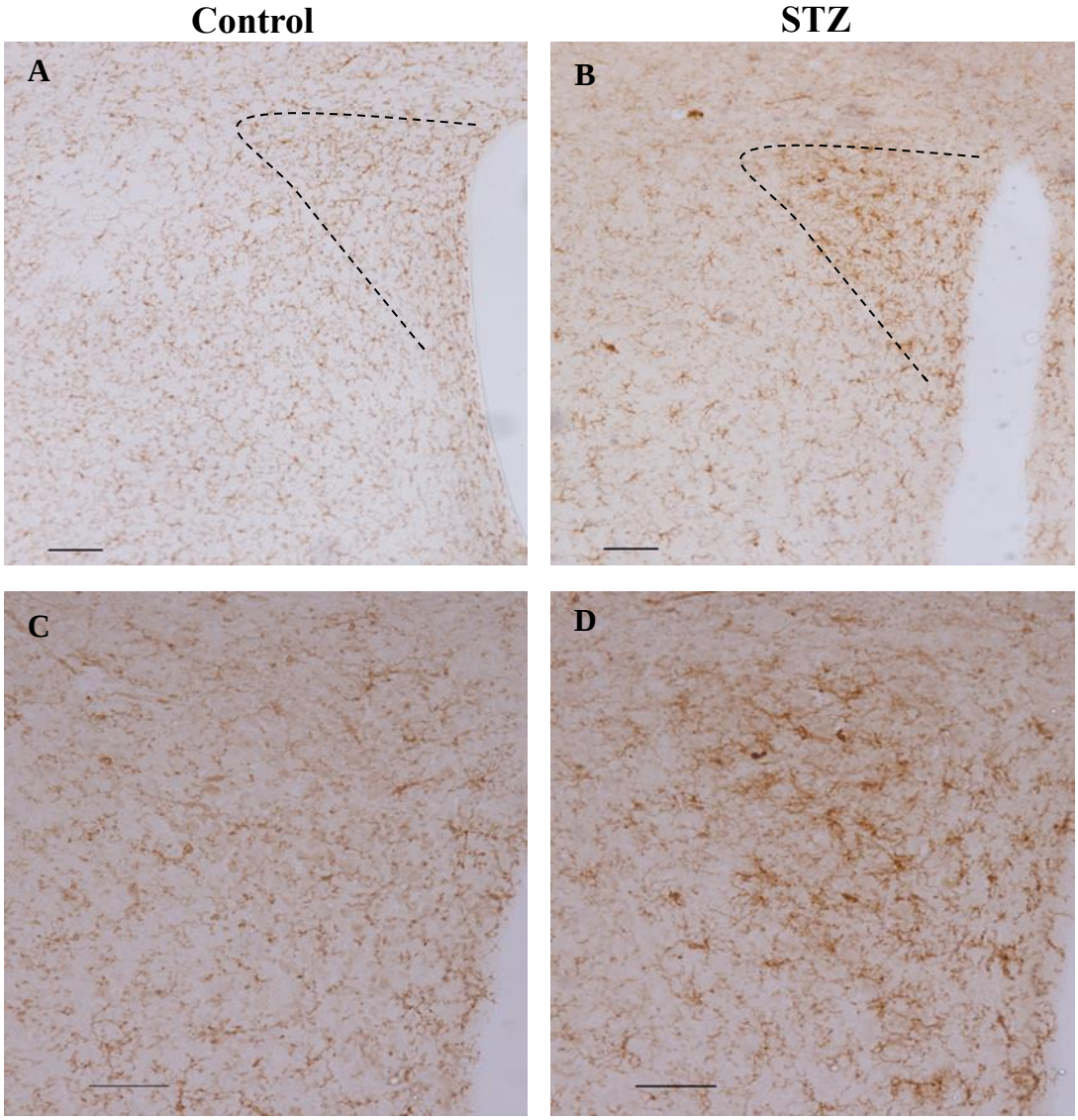


Figure 8-1. Morphology of microglia in STZ mice at 16 weeks after STZ treatment. Photomicrographs showing CD11b immunoreactive microglia in the paraventricular nucleus (PVN) in control and diabetic animals (STZ) at 16 weeks. Dotted lines in low-power images (A-B) outline the PVN. High-power images (C-D) show the morphology of microglia in the PVN region in detail. CD11b antibody staining shows microglia with normal morphology with small cell bodies and long, fine-branched processes throughout the PVN region in the control, while activated microglia showing larger cell bodies and thicker processes are seen in the STZ group. Scale bar = 100 μm in (A-B), 50 μm in (C-D).

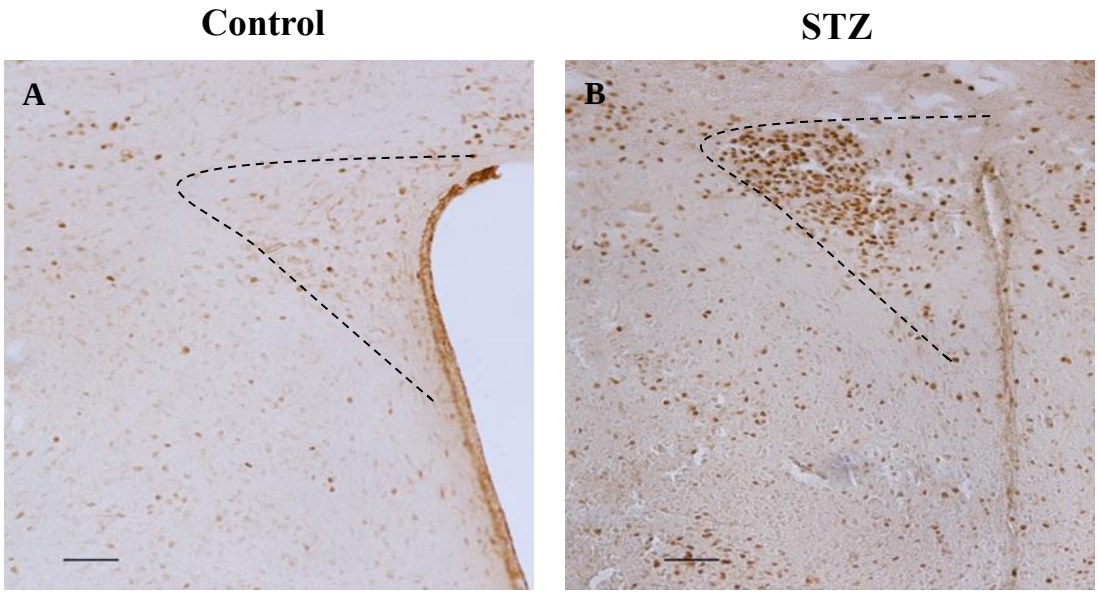


Figure 8-2. Fos activity in STZ mice at 16 weeks after STZ treatment. Photomicrographs showing neuronal nuclei stained with anti-Fos antibody in the paraventricular nucleus (PVN) in control and diabetic animals (STZ) at 16 weeks. Dotted lines outline the PVN. Bar = 100 μm.

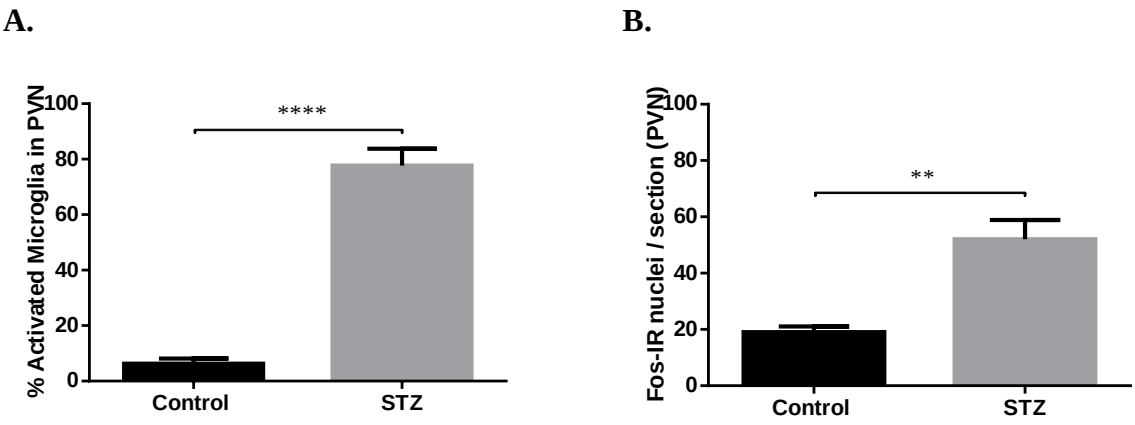
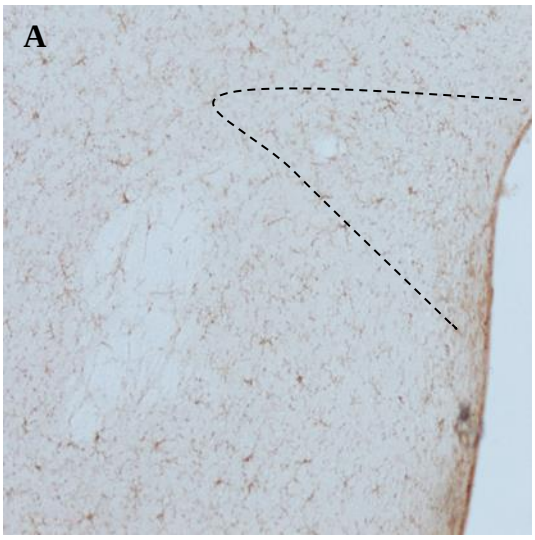
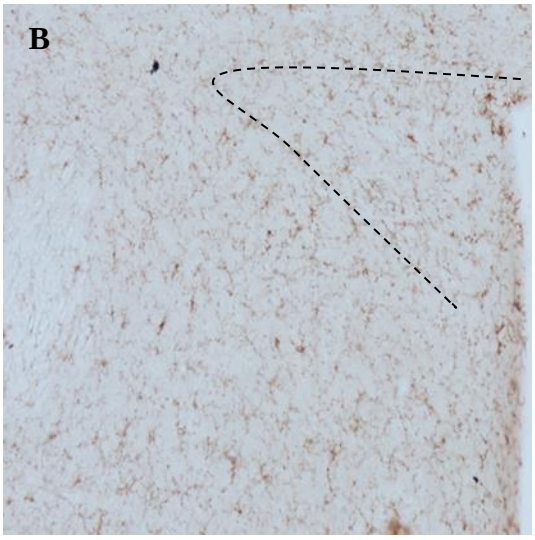


Figure 8-8. Microglial and neuronal activation in the paraventricular nucleus of STZ mice at 16 weeks after STZ treatment. A: The percentage of microglia showing activated morphology in the paraventricular nucleus (PVN) of control and diabetic animals (STZ). B: Quantification of Fos-IR cell nuclei in the same region. Number of mice = 4 in each group. Data are expressed as the mean ± SEM.

Control



STZ



STZ + Fat feeding

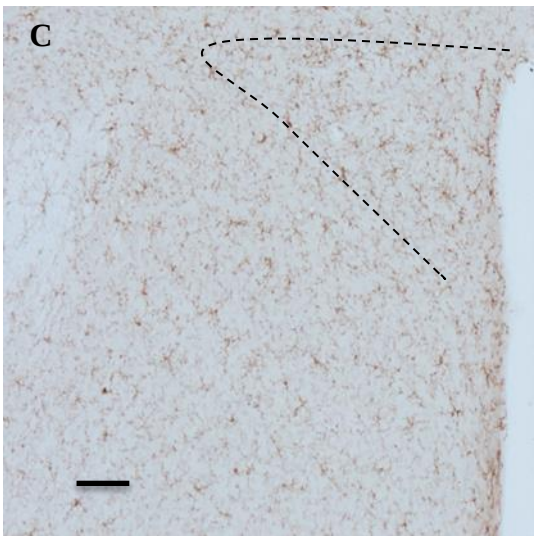
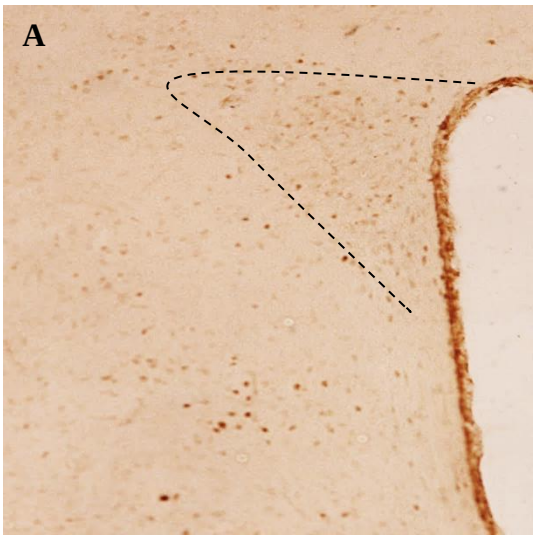
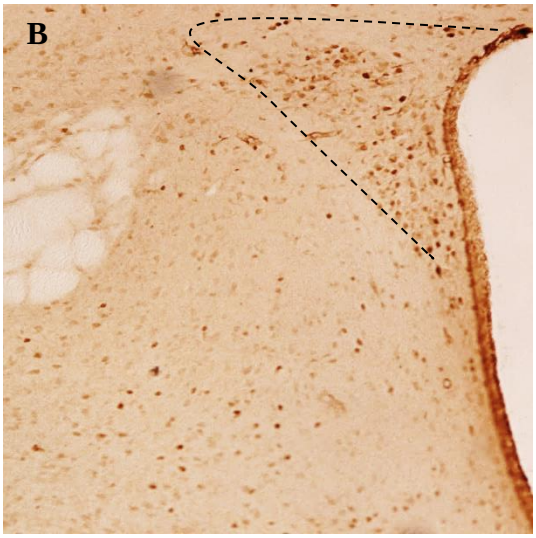


Figure 8-9. Morphology of microglia in STZ mice and STZ + Fat Fed mice at 10 weeks following STZ treatment. Photomicrographs showing CD11b immunoreactive microglia in the paraventricular nucleus (PVN) in control, diabetic (STZ) and diabetic + fat feeding animals (STZ + Fat feeding) at 10 weeks. Dotted lines outline the PVN. Images (A-C) show the morphology of microglia in the PVN. CD11b antibody staining in all cases shows microglia with normal morphology with small cell bodies and long, fine-branched processes throughout the PVN region. Scale bar = 100 μm.

Control



STZ



STZ+Fat feeding

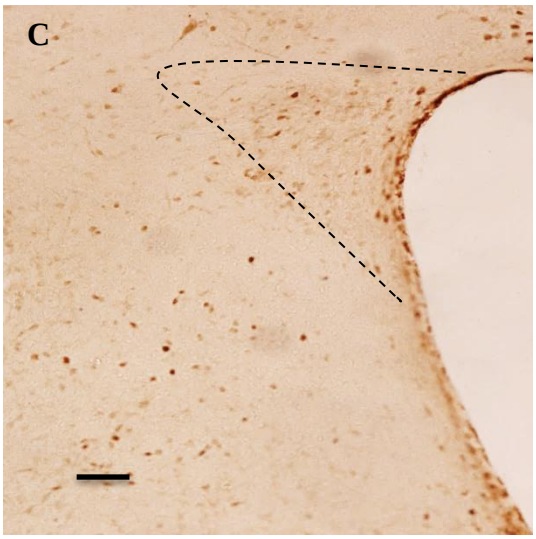


Figure 8-10. Fos activity in STZ mice and STZ + Fat Fed mice at 10 weeks following STZ treatment. Photomicrographs showing neuronal nuclei stained with an anti-Fos antibody in the paraventricular nucleus (PVN) in control (A), diabetic (B) and diabetic + fat feeding animals (C) at 10 weeks. Dotted lines outline the PVN. Scale bar = 100 μm.

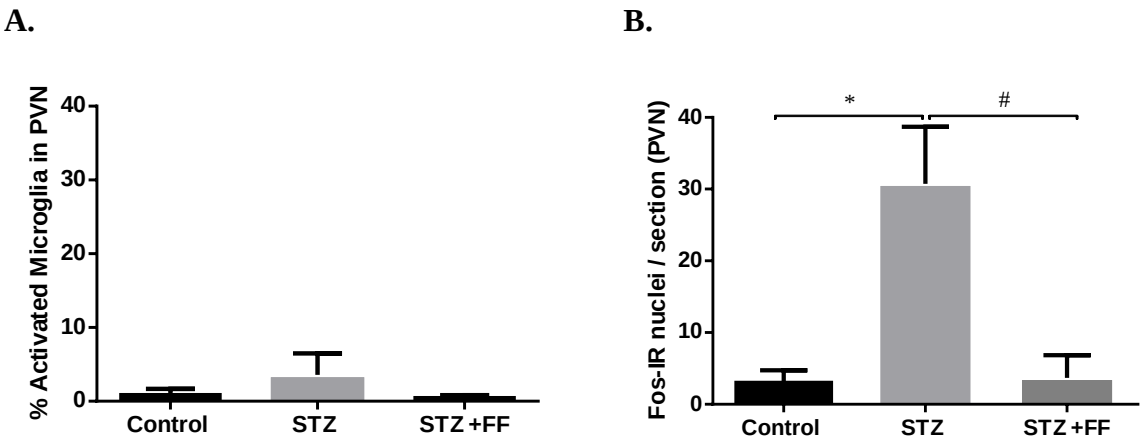


Figure 8-11. Microglial and neuronal activation in the paraventricular nucleus of STZ mice and STZ + Fat Fed mice at 10 weeks following STZ treatment. A: The percentage of microglia showing activated morphology in the paraventricular nucleus (PVN) of control, diabetic (STZ) and diabetic + fat feeding animals (STZ+FF). B: Quantification of Fos-IR cell nuclei in the same region. Number of mice = 4 in each group. Data are expressed as the mean ± SEM.

8.3 Discussion

We previously provided evidence that PVN microglia become activated in STZ diabetic rats at 6 and 8 weeks following STZ treatment. Despite this, whether PVN inflammation also occurs in other species and other models of diabetes is not known. Therefore, in this study, we aimed to investigate whether microglia are activated in the PVN in diabetic mice at 7 and 16 weeks after STZ injection and whether hydrogen sulphide and high-fat feeding influence PVN inflammation in STZ diabetic mice at 7 and 10 weeks, respectively. The main findings of the present study were that (i) microglia are activated in the PVN in STZ diabetic mice at 16 weeks after STZ injection but not at 7 weeks or 10 weeks (low-dose STZ), (ii) PVN neuron activation was observed in STZ diabetic mice at all stages examined, suggesting that neuronal activation precedes microglial activity (iii) and both Na H_2S and high-fat feeding treatments inhibited PVN neuron activation in STZ diabetic mice.

It is well-known that microglia are the resident immune inflammatory cells in the central nervous system. Once microglia become activated in response to infection, injury or inflammation of the CNS, they undergo morphological changes and can release proinflammatory molecules and cytotoxic factors, such as nitric oxide and tumor necrosis factor (TNF)-α, which induces neuronal activity (Li *et al.*, 2005b; Shi *et al.*, 2010a). Activated microglia have been implicated in the pathologies of the CNS that are associated with brain inflammation, such as Alzheimer's disease, Parkinson's disease, multiple sclerosis and neuropathic pain (chapter 1). We previously reported microglial activation in the PVN in STZ diabetic rats. From our previous studies, we have provided evidence that activated microglia in the PVN and other cardiovascular centres in the brain of STZ diabetic rats are involved in the development of diabetic cardiovascular and renal complications (chapter 4, 5, 6 and 7). This observation complements the findings of other studies on hypertensive animal models (Shi *et al.*, 2011). In the present study in mice, we found that microglial activation in

the PVN was present at 16 weeks but not at 7 weeks after STZ injection. Some STZ diabetic mice (3 out of 4) at the 7 week stage exhibited an increase in the percentage of activated microglia, but the variability was high, suggesting our tests may be underpowered for detecting small differences. At 10 weeks (low-dose STZ), there was no indication of microglial activation. STZ diabetic mice (at 10 weeks) did not show extreme hyperglycaemia, as seen with higher STZ doses at other stages. These findings suggest that microglial activation in the PVN of STZ diabetic mice is dependent on the time course and severity of diabetes.

To clarify the mechanisms of changes in the PVN in STZ diabetic mice, we examined whether PVN neurons were activated in these animals. We found activation of neurons in the nucleus in diabetic mice at all stages tested (7 and 16 weeks). Therefore, the data support our suggestion that microglial activation in the PVN may be secondary to the prolonged intense activation of PVN neurons (Wu & Ling, 1998). Despite this, how diabetes induces this activation in the hypothalamic PVN is not known.

Previously, we and other researchers provided evidence that PVN neuronal activation contributes to the development of cardiovascular diseases, including hypertension and cardiac dysfunction in STZ diabetic rats (chapter 6 and 7). Interestingly, in the present study, systemic treatment with a Na H_2S donor in STZ-diabetic mice (7 weeks) caused the inhibition of PVN neuron activation, which is consistent with other studies, showing that H_2S has a neuroprotective role (Kimura & Kimura, 2004; Kimura *et al.*, 2006; Umemura & Kimura, 2007). No significant difference was seen in the percentage of activated microglia between the STZ and control mice, but we cannot rule out that H_2S also has an inhibition effect on microglia functions. Deficiency in the levels of H_2S has been shown to occur in diabetic humans as well as in STZ-induced diabetic rats (Jain *et al.*, 2010), and this deficiency contributes to the development of diabetic complications (Lefer, 2008; Szabo, 2012).

Although we did not measure cardiovascular changes in the present study, another study has reported that exogenous H_2S reduced blood pressure in diabetic hypertensive rats (Ahmad *et al.*, 2012). This study also reported lower levels of H_2S in these animals compared to the control. In fact, H_2S has anti-inflammatory actions, and several studies have shown that H_2S reduces microglial and neuronal activation *in vitro* (Hu *et al.*, 2007; Umemura & Kimura, 2007; Hu *et al.*, 2009). Combining our results with these studies, we speculate that the effect of H_2S on diabetic cardiovascular complications may be mediated, at least in part, by the inhibition of central inflammation in the PVN; however, the mechanisms through which H_2S reverses the PVN neuronal activity require further investigation.

As discussed previously, hyperglycaemia has been implicated in the increase of the generation of reactive oxygen species in the PVN (Patel *et al.*, 2011) and other brain areas in STZ diabetic rats (Acar *et al.*, 2012; Ola *et al.*, 2014). It is well documented that such an increase in the generation of reactive oxygen species can lead to neuronal activation through an enhanced influx of Ca^{2+} in neurons (Wang *et al.*, 2003; Zimmerman & Davisson, 2004). Reactive oxygen species in dorsal horn neurons have been shown to contribute to neuronal hyperexcitability and the development of neuropathic pain since the administration of antioxidants prevents neuropathic pain-like behaviours (Stevens *et al.*, 2000; Ho *et al.*, 2006). It is well documented that H_2S can protect neurons via its anti-oxidant effects (Kimura & Kimura, 2004; Kimura *et al.*, 2006). In the present study, Na H_2S significantly attenuated the increase in neuronal activation in the PVN without affecting blood glucose levels, as seen in STZ diabetic mice. Therefore, H_2S may reduce neuronal activation indirectly through a reduction in the reactive oxygen species levels in the PVN in STZ diabetic mice.

Another possible mechanism of PVN neuronal activation is the elevation levels of plasma osmolality in diabetic animals (Charlton *et al.*, 1988; Brooks *et al.*, 1989). In the current study, elevated plasma osmolality seen in diabetic mice was reduced to normal levels

following treatment with Na H_2S. Interestingly, a recent study on diabetic hypertensive animals reported that H_2S improved renal function in these animals (Ahmad *et al.*, 2012). Therefore, taken together, these findings suggest that H_2S can prevent hyperglycaemia-induced changes in the function of the kidneys and the PVN. Despite this, whether the H_2S has a direct effect on neurons in the PVN or an indirect effect through preventing kidney damage and increasing plasma osmolarity remains to be determined.

Like type 1 diabetes, type 2 diabetes in animals is also associated with cardiovascular disease and over-activity of the sympathetic nerves that control the cardiovascular system. As discussed previously, PVN inflammation contributes to the development of cardiovascular complications seen in STZ-induced hyperglycaemia in rats (type 1 diabetes), but whether similar changes in the PVN occur in models of type II diabetes is not clear. Therefore, we examined whether microglia and neurons were activated in the CNS cardiovascular centres. In the PVN, there was no significant difference in the percentage of microglia activated in mice treated with a low dose of STZ and high fat feeding, although their blood glucose levels were high compared to the control. Also, we observed no microglial activation in the arcuate nucleus in either low-dose STZ mice or mice treated with a low dose of STZ and high fat feeding, which is in agreement with a previous study from our lab in fat-fed rats and in STZ-induced diabetic rats; however, these data are inconsistent with studies that reported that high-fat feeding caused microglial activation in the PVN in mice (de Kloet *et al.*, 2014) and in the arcuate nucleus in rats (Thaler *et al.*, 2012). In these studies, high-fat feeding was accompanied by obesity, but our mice did not become obese following treatment with fat feeding. This may be due to a lower percentage of fat in food used here compared to earlier studies. The differences in the percentage of fat in the diet associated with differences in body weight may explain our data. Therefore, microglial activation in fat-fed animals may be a consequence of obesity that was absent in our model.

Interestingly, the addition of a high-fat diet to the low-dose STZ treatment significantly attenuated the increase in neuronal activation seen in the PVN in low-dose STZ diabetic mice. We have no explanation for this phenomenon, but the systemic infusion of glucose has been shown to excite PVN neurons (Dunn-Meynell *et al.*, 1997). This excitation in the PVN was reversed in diet-induced obese animals (Levin *et al.*, 1998). Collectively, these data suggest that a high-fat diet may reduce central glucose sensitivity; however, how this occurs is not known, and further research is needed.

8.4 Conclusion

The present study demonstrated that neuronal and microglial activation occur in the PVN in STZ-induced diabetic mice. Hydrogen sulphide treatment reduces neuronal activation in the PVN and prevents dehydration in these animals, but it does not significantly affect blood glucose. These findings suggest that the dehydration seen in these animals was not a direct effect of high blood glucose. They also suggest that using hydrogen sulphide can prevent diabetic changes in the function of the PVN. No microglial activation was observed in the cardiovascular centres of the brain in mice treated with a low-dose of STZ and high-fat feeding. These findings suggest that the activation of microglia in the hypothalamus does not occur in all animal models of diabetes. High-fat feeding blocks the neuronal activation seen in the PVN of STZ-treated mice; however, the exact mechanism is not clear, and further studies are needed.

Chapter Nine: General Discussion

Prior to this thesis, it was known that diabetes contributes to defects in the autonomic nervous system (Tesfaye *et al.*, 2010). Impairment of the baroreflex control of heart rate and abnormal increases in the renal sympathetic activity are important candidate mechanisms for the development of diabetic complications, and they contribute to morbidity and mortality in diabetic patients (Ewing *et al.*, 1980; Kempler *et al.*, 2002; El-Menyar, 2006). These abnormalities in the autonomic neurons system are also reported in diabetic animals. Neuroinflammation, including oxidative stress and proinflammatory cytokine release in central autonomic centres, emerged as potentially important contributing factors in the pathogenesis of autonomic dysfunction and its consequences in other diseases. Resident immune cells (microglia and astrocytes) were recognized to play crucial roles in the pathogenesis of several CNS disorders and activated microglia, and astrocytes had been observed in central autonomic centres in diabetic animals; however, little was known about the influence of 1% NaCl, antioxidants and high-fat feeding on microglial activation in central autonomic centres in diabetic animals of different species. More importantly, whether inhibition of activated microglia in the PVN could reduce or prevent cardiovascular changes in STZ diabetic rats was not clear. Therefore, the overall aim of this thesis was to advance our understanding of the mechanisms of deleterious cardiovascular complications in diabetes, and in particular, to address the role of brain inflammation in these complications and the mechanisms of microglial activation in this condition.

The main findings of the present thesis were that (1) microglia and astrocytes are activated in the PVN in STZ diabetic rats at 6-8 weeks after STZ injection but not at earlier stages; (2) drinking 1% NaCl causes increased microglial activation in the cardiovascular centres, hypertension and dysfunction of bradycardic baroreflex at 2 weeks post STZ treatment in diabetic rats; (3) prolonged drinking of 1% NaCl reduces the activation of microglia in the cardiovascular centres, restores the function of the bradycardic baroreflex and normalises elevated end diastolic pressure at 6 weeks post STZ treatment in diabetic rats; (4) inhibition

of microglial activation in the PVN via ICV minocycline infusion prevented the development of diabetic complications in STZ diabetic rats with or without 1% NaCl treatment; (5) microglial activation does not appear to mediate astrocyte activation in the PVN and astrocyte activation may not be associated with the development of diabetic complications in STZ diabetic rats; (6) microglial activation also occurs in the PVN in STZ diabetic mice at later stages than neuronal activation, and at early stages, hydrogen sulphide as well as high-fat feeding treatment attenuates PVN neuronal activity.

9.1 Role of neuroinflammation in deleterious cardiovascular complications in diabetes

In chapters 5 and 6, we have shown that both baroreflex sensitively and cardiac function are impaired in STZ diabetic rats compared to control rats. This finding confirms previously published studies on rats (Fazan *et al.*, 1999; Gouty *et al.*, 2001; Schaan *et al.*, 2004; Wichi *et al.*, 2007; Arozal *et al.*, 2009). Baroreflex dysfunction along with sympathetic over-activity are important cardiovascular risk factors in diabetes (Ewing *et al.*, 1980; Kempler *et al.*, 2002; El-Menyar, 2006). In addition, a positive correlation has been shown between diabetic autonomic dysfunction and diabetic cardiac dysfunction (Fang *et al.*, 2004). Thus, an improvement of autonomic function may be a novel strategy for lowering cardiovascular disease risk in diabetic patients. The mechanisms behind the dysregulation of the autonomic nervous system in diabetic rats are not well understood but may include inflammation and changes in central autonomic centres.

We have confirmed the previously published data that microglia are activated in the PVN and NTS in STZ diabetic rats (Rana *et al.*, 2014). Moreover, we have observed that microglia become activated in the PVN in STZ diabetic mice, indicating that a similar mechanism may be responsible for the activation of PVN microglia in both species in diabetes. To understand the role of microglia in the development of diabetic complications, we have inhibited

activated microglia via ICV infusion of minocycline. Our data from **chapters 4, 6 and 7** revealed that minocycline ameliorates diabetic complications of STZ diabetic rats; it reduces the PVN neuronal activity, prevents hypertension, improves cardiac function and protects kidney tissue. The results strongly suggest that cardiovascular and renal pathological processes in diabetes are largely mediated, at least in this time frame, by inflammation in cardiovascular centres of the brain. This finding is consistent with published data about the role of activated microglia in increased neuronal excitability in the CNS (Tsuda *et al.*, 2005) as well as in the development of cardiovascular complications in other diseases (Shi *et al.*, 2010a). Thus, attenuation of microglial activation in the PVN may be a novel strategy for lowering cardiovascular and renal disease risks in diabetes.

Astrocytes are another important cell for mediating inflammation in the CNS. Activated astrocytes release proinflammatory cytokines and have been implicated in spinal neuronal hyperexcitability and consequently the development of diabetic neuropathic pain (Sweitzer *et al.*, 1999; Milligan *et al.*, 2001; Wei *et al.*, 2008). **In chapter 3**, we have confirmed the previous data that astrocytes are activated in the PVN in STZ diabetic rats (Luo *et al.*, 2002); however, our results indicate that astrocyte activation does not play a crucial role in the development of diabetic complications since the inhibition of microglial activation via ICV minocycline was able to prevent diabetic complications but was not able to inhibit astrocyte activation. Hence, it appears that the functional consequences of astrocyte activation in the PVN may be different from those in the spinal cord (Okada-Ogawa *et al.*, 2009).

Our observations that ICV infused minocycline inhibits PVN inflammation and that this inhibition improves left ventricular and renal changes in STZ diabetic rats beg the question: is the effect of minocycline a result of crossing the blood-brain barrier and acting peripherally rather than exerting its influence in the CNS? The latter view is an unlikely possibility because Bhatt et al. reported that the oral administration of minocycline did not significantly

improve cardiac function in STZ diabetic rats as it did in our study, even though the dose of minocycline used was much greater (50mg/kg) and for the same period compared with our study (Bhatt & Veeranjaneyulu, 2012). Despite this, since minocycline was given orally, it was potentially metabolised in the gut or liver before reaching the rest of the body. Consequently, the concentration of minocycline may not have been sufficient to act on the brain to reverse cardiac function when compared with our study; however, when minocycline was combined with aspirin, which also has anti-inflammatory actions and can enter the CNS, the cardiac function in STZ diabetic rats was improved (Bhatt & Veeranjaneyulu, 2012). This suggests that the beneficial effects of anti-inflammatory drugs in both studies are centrally mediated.

Along with neuroinflammation, there is also evidence that increased oxidative stress mediates neuronal activity in cardiovascular autonomic centres and contributes to the pathogenesis of autonomic dysfunction. Reactive oxygen species have been shown to contribute to neuronal hyperexcitability in the CNS (Stevens *et al.*, 2000; Ho *et al.*, 2006) and to abnormal cardiac reflex function in STZ diabetic rats (Ustinova *et al.*, 2000). **In chapter 8**, we have shown that the systemic infusion of H_2S reduces neuronal activation in the PVN in STZ diabetic mice. We also provided evidence that PVN neuronal activation contributes to the development of cardiovascular diseases in STZ diabetic rats **(chapters 6 and 7)**. Deficiency in the levels of H_2S is reported in diabetic humans (Jain *et al.*, 2010), and this deficiency contributes to the development of diabetic complications (Lefer, 2008; Szabo, 2012). Spontaneously, when hypertensive rats were injected with STZ, they showed low levels of H_2S, and when these animals were treated with exogenous H_2S, blood pressure was reduced (Ahmad *et al.*, 2012). It is well documented that H_2S can protect neurons via its anti-oxidant effects (Kimura & Kimura, 2004; Kimura *et al.*, 2006). Therefore, we proposed that H_2S may mediate its beneficial effect on cardiovascular complications through attenuating oxidative stress as well as PVN neuronal activation.

A high-fat diet is a major contributing factor in the pathogenesis of type 2 diabetic complications, but whether PVN inflammation contributes to these complications is not clear. **In chapter 8**, we have shown that no microglial activation was observed in the PVN in mice treated with a low dose of STZ and high-fat feeding. These findings suggest that the activation of microglia in the hypothalamus does not occur in all animal models of diabetes. Interestingly, high-fat feeding blocks neuronal activation seen in the PVN of STZ treated mice; however, the exact mechanism is not clear, and further studies are needed.

9.2 The effect of drinking 1% NaCl on cardiovascular parameters and CNS inflammation

Previous data have shown different effects of 1 % NaCl intake on diabetic complications in short and long-term STZ diabetic rats (Dai *et al.*, 1994; Santos *et al.*, 1995; Maeda *et al.*, 2007); however, no previous studies have investigated the effect of drinking 1% NaCl on CNS inflammation and fluid balance in diabetic rats and the contribution of these mechanisms to diabetic complications. Our results from **chapters 4, 5 and 7** confirm previous studies, indicating that diabetic complications were differentially affected by short (2 weeks) and longer term (6 weeks) treatment of drinking 1% NaCl in STZ diabetic rats (Dai *et al.*, 1994; Santos *et al.*, 1995; Maeda *et al.*, 2007).

Diabetes in patients is commonly associated with hypertension, which is considered the likely cause of developing cardiovascular disease in around 75% of persons with diabetes (Bild & Teutsch, 1987). One major known contributor to the development of hypertension and its consequences in diabetic humans is salt intake (He & MacGregor, 2003; Franz, 2008). Our observation that 2 weeks of treatment with 1% NaCl causes increased blood pressure in STZ diabetic rats **(chapter 4)** is consistent with another study (Maeda *et al.*, 2007). Two weeks of 1% NaCl intake was able to normalise blood volume in these animals. Some evidence suggests that a lack of hypertension in STZ diabetic rats is due to decreased blood volume,

which may explain our findings (Hebden *et al.*, 1986a; De Angelis *et al.*, 2000). Dysfunction in baroreflex sensitivity is also believed to precede hypertension (Grassi *et al.*, 1998; Gao *et al.*, 2002; Salgado *et al.*, 2007; Maliszewska-Scislo *et al.*, 2008). We have shown that 2 weeks of 1% NaCl intake causes dysfunction of the bradycardic baroreflex in STZ diabetic rats compared STZ diabetic rats given tap water. Based on this data, we cannot rule out baroreflex dysfunction as the mechanism for blood pressure changes seen in diabetic rats given 1% NaCl; however, these changes are accompanied by increased microglial activation within the cardiovascular autonomic centres of the brain. The inhibition of activated microglia in the PVN via ICV infusion of the drug minocycline prevents the hypertension seen when 1% NaCl is given. While 1% NaCl seems to act on blood volume increases and/or baroreflex dysfunction to develop hypertension, our data provide strong support for the hypothesis that activation of microglia in the PVN is required for the development of neurogenic hypertension in these diabetic rats, as concluded by Shi in another model of hypertension (Shi *et al.*, 2010a).

In contrast, 6 weeks of treatment with 1% NaCl restored baroreflex sensitivity and improved cardiac function compared to STZ diabetic rats given tap water, confirming the results of a previous study on cardiac function (Dai *et al.*, 1994). While our findings appear to be contradictory to the established dogma, some researchers have demonstrated that very low salt diets have the potential for adverse effects on human patients with diabetes (Ekinci *et al.*, 2011; Thomas *et al.*, 2011) and heart failure (Paterna *et al.*, 2008; Paterna *et al.*, 2009). In fact, the effect of low-salt intake in reducing blood pressure is well documented in humans, but this intervention also increased renin–angiotensin–aldosterone system activity and increased sympathetic nerve over-activity (Graudal *et al.*, 1998; Grassi *et al.*, 2002), both of which can contribute to the development of cardiovascular complications. At 6-8 weeks after STZ treatment, diabetic rats exhibited elevated levels of circulating angiotensin II (Anderson *et al.*, 1993) as well as abnormal sympathetic over-activity (Patel *et al.*, 2011). In our study,

STZ diabetic rats showed dehydration, which is characterised by both water and salt loss, and in the longer term, 1% NaCl intake prevents this dehydration, as seen in shorter term experiments. Therefore, we speculate that cardiovascular changes seen in STZ diabetic rats in this study may be related to salt deficiency and that longer term treatment with 1% NaCl normalises salt levels and consequently prevents diabetic complications in these animals. Interestingly, improved cardiovascular outcomes were associated with reduction in the activation of microglia and neurons in cardiovascular autonomic centres. Therefore, it appears that in both the short and longer term, neuroinflammation is the mechanism that links salt and cardiovascular complications in STZ diabetic rats.

This conclusion is controversial because hypertension is considered a major cause of cardiovascular disease in humans. We have shown that STZ diabetic rats given 1% NaCl develop hypertension in the short term, but the same pattern was also observed in the longer term in another study (Santos *et al.*, 1995). Despite this, in the longer term, drinking 1% NaCl prevents cardiovascular complications of STZ diabetic rats in ours and other studies (McDonald *et al.*, 1969; Dai *et al.*, 1994). These data suggest that 1% NaCl intake provides beneficial effects on the cardiovascular system, even though blood pressure is high in STZ diabetic animals. They also suggest there is no causal link relation between changes in blood pressure and cardiovascular disease in these diabetic animals, as concluded in some studies on diabetic humans (Bild & Teutsch, 1987; Epstein & Sowers, 1992).

9.3 Mechanism of microglial activation in diabetes

This thesis has also shed light on the potential mechanisms by which microglia become activated in the PVN in STZ diabetic animals. It is well-known that microglia become activated following neuronal over excitation (Hathway *et al.*, 2009; Lu *et al.*, 2009). Our results from **chapters 4, 5, 6 and 8** indicated that neuronal activity occurs before microglial activation in the PVN in STZ diabetic animals, suggesting that neuronal activation causes

microglial activation (Rana *et al.*, 2014). Our studies also provided further insight into the mechanisms that initiate the PVN neuronal activity in diabetic animals. Both STZ diabetic rats and mice showed signs of dehydration **(chapters 4, 5, 6 and 8)**. This stimulus has been shown to activate PVN neurons in another animal model (Stocker *et al.*, 2004). We have shown that longer term 1% NaCl intake prevents dehydration and also reduces the activation of neurons and microglia in the PVN **(Chapter 7)**. Thus, one possible mechanism underling microglial activation is the intense neuronal activity elicited in the PVN by dehydration in STZ diabetic rats.

Another potential mechanism behind PVN neuronal activation may be the increased production of reactive oxygen species in diabetes. Hyperglycaemia has been shown to induce the production of reactive oxygen species in STZ diabetic rats (Nishikawa *et al.*, 2000; Brownlee, 2005). These chemicals are known to increase cytokine production and neuronal hyperactivity in the brain (Stevens *et al.*, 2000; Ho *et al.*, 2006). Our results **(chapter 8)** show that an antioxidant can significantly attenuate the increase in neuronal activation in the PVN in STZ diabetic mice, suggesting that reactive oxygen species play an important role in the PVN neuronal activation seen in STZ diabetic animals; however, the detailed mechanism by which reactive oxygen species elicit PVN neuronal activation in diabetes requires further investigation.

Despite these possible mechanisms, activated microglia have also been shown to cause neuronal over excitation via the release of proinflammatory molecules and reactive oxygen species (Shi *et al.*, 2011; Wu *et al.*, 2012). Our results obtained from **chapters 6 and 7** reveal that ICV minocycline treatment significantly reduces both microglial and neuronal activation in the PVN in STZ diabetic rats. This suggests that neuronal activation may be responsible for initiating microglial activation, but activated microglia may also increase the secondary and sustained neuronal activity via a positive feedback mechanism.

One potential large range positive feedback may involve angiotensin II, which is a hormonal factor that regulates blood pressure and fluid balance during the dehydrated state through stimulating sympathetic nervous activity. This hormone acts on different tissues, including the brain, and has also been shown to be an important mediator of inflammation and oxidative stress in the PVN (Sriramula *et al.,* 2013). In early stages of diabetes, Kalinyak et al. found no change in the levels of angiotensin II in STZ diabetic rats (Kalinyak *et al.*, 1993). In contrast, increased plasma angiotensin II has been found at 6-8 weeks following STZ injection (Anderson *et al.*, 1993). It was reported that the systemic infusion of angiotensin II in naïve rats activates microglial cells in the PVN (Shi *et al.,* 2010a). Our results from **chapters 3, 4, 5, 6 and 7** show no significant increase in microglial activation in diabetic rats until 6-8 weeks after STZ injection. Therefore, increased angiotensin II levels in STZ diabetic rats may be responsible for microglial activation. Interestingly, drinking 1% NaCl reduced the activation of microglia in the NTS and PVN in STZ diabetic rats **(chapters 5 and 7)**. In fact, salt intake has been shown to reduce angiotensin II in Zucker diabetic fatty rats (Takenaka *et al.,* 2011). Thus, it is likely that 6 weeks of 1% NaCl intake may reduce microglial activation indirectly through a reduction in the angiotensin II levels in STZ diabetic rats.

Another possibility is that peripheral proinflammatory cytokines induce microglial activation in the brain. Previous studies have provided evidence that peripheral proinflammatory cytokines, such as IL-β and TNF-α, lead to microglial activation in the CNS (Wu *et al.*, 2012). Elevated levels of these cytokines in the blood stream are reported in STZ diabetic rats (Ugochukwu & Figgers, 2007). Thus, increased peripheral cytokines, which are also believed to initiate the inflammatory process in the PVN in other models, may also play a role here (Felder *et al.*, 2009; Felder, 2010); however, how this occurs in diabetic animals is not clear, and further studies are needed.

Therefore, based on all of these potential mechanisms, we propose the following hypothesis as a mechanism for the development of diabetic complications. Dehydration stimulates PVN neuronal excitation, resulting in the activation of microglia. Alternatively, increased peripheral cytokines and/or peripheral angiotensin II in diabetes lead to an increase in the production of reactive oxygen species. This can directly activate PVN microglial activation, which in turn increases the production of proinflammatory cytokines and reactive oxygen species. These chemicals would further stimulate neuronal activity in the PVN via a positive feedback mechanism. This increase in the PVN neuronal activity would result in abnormal activation of the sympathetic nerve system, and thereby contributes to the development of diabetic complications.

9.4 Clinical implications

Our observations in this thesis illustrate that STZ-induced hyperglycaemia in a rat model produces several symptoms that mimic those in humans with diabetes, especially the cardiovascular changes. STZ rats exhibit changes in the baroreflex sensitivity and cardiac dysfunction, and these changes are well documented in diabetic humans **(chapters 5 and 6)**; however, STZ rats do not show hypertension as seen in humans with diabetes **(chapter 3)**. Thus, an STZ rat is an adequate model to investigate the mechanisms of hyperglycaemia induced cardiovascular complications of diabetes, which are independent of hypertension, and to identify possible therapeutic targets.

Because of its consequences for many organ systems, diabetic autonomic neuropathy could be regarded as the most serious complication of diabetes associated with an increased risk of the development of cardiovascular complications. Although several trials have reported that controlling blood glucose levels reduces diabetes related complications, this approach does not completely prevent the development and progression of these complications. Indeed, our finding that inhibiting or reducing microglial activation in cardiovascular autonomic centres

prevents the development of cardiovascular complications in STZ diabetic rats are potentially of substantial clinical importance. While in type 1 diabetic patients, neuronal abnormalities have been found at brain autopsy (Reske-Nielsen *et al.*, 1966), no definitive study of neuroinflammation in diabetic humans has been conducted. Thus, in the clinical setting, future research is needed to investigate whether inflammation in cardiovascular centres of the brain occurs in humans with diabetes. If neuroinflammation is proven, the inhibition of microglial activation will provide an exciting new strategy for treating and preventing diabetic complications and therefore be beneficial for future generations.

This thesis not only provides insights into the mechanisms underlying the development of diabetic complications, it also provides potential therapeutic interventions that differ from the current therapies. We have shown that 1% NaCl intake in the short term increases blood pressure in STZ diabetic rats. It is possible that there is no extreme salt depletion at 2 weeks in STZ diabetic rats, and these rats obtain more salt than they need. Consequently, a high salt intake contributes to an increase in blood pressure. In contrast, drinking 1% NaCl for a longer period produces beneficial effects on the cardiovascular system of STZ diabetic rats. It would be expected that at 6 weeks, STZ diabetic rats excrete larger amounts of salt in their urine than at earlier stages and that drinking 1% NaCl will normalise salt balance and thereby inhibit neuroinflammation and improve the function of the cardiovascular system in these diabetic animals. Such data suggest that both high and low salt intake may be associated with adverse outcomes in diabetes, supporting the complex effects of salt that are evident from epidemiological studies. For example, several studies have shown convincing evidence of the beneficial effect of low salt intake in terms of the reduction in blood pressure and cardiovascular diseases. In the context of type 1 diabetes, dietary salt restriction has also been recommended via many international guidelines (Franz, 2008). This recommendation has been obtained from a clinical study showing that dietary salt restriction reduced hypertension in some but not all patients with type 1 diabetes (Gerdts *et al.*, 1996); however, other clinical

studies have demonstrated that low salt intake enhanced the risk of the development of cardiovascular disease in diabetic patients (Ekinci *et al.*, 2011; Thomas *et al.*, 2011). Consequently, consistent with this data, the beneficial effects of drinking 1% NaCl obtained here and in other studies suggest that dietary sodium restriction, which is usually recommended in the clinical setting, may not be the best treatment strategy for diabetic patients. Therefore, the current recommendation may put diabetic patients at risk for the development of cardiovascular disease and other diabetic complications associated with increased sympathetic nerve activity. More studies are needed to evaluate the most efficacious range of salt intake in these patients. In addition, whether the correction of salt intake may also be effective in preventing cardiovascular complications and CNS inflammation in individual diabetic humans awaits further investigation.

9.5 Future directions

In this thesis, we have demonstrated that microglial activation in the PVN is a major contributing factor in the development of cardiovascular complications in STZ diabetic rats. The data from **chapters 6 and 7** indicated that activated microglia may be in part responsible for increased activity of PVN neurons. Studies have shown that when microglia become activated, they can release many factors, including proinflammatory cytokines, reactive oxygen species, ATP and BDNF, all of which can induce neuronal activity; however, determining which specific stimulus from microglia is generating the results presented in this study requires future experiments.

The data from **chapters 4 and 6** indicated that microglial activation also occurs in the NTS and RVLM, which are involved in cardiovascular regulation and particularly in baroreflex receptor responses. There is evidence that inflammation in the RVLM participates in the development of hypertension in other diseases (Koga *et al.*, 2008; Braga, 2010; Nunes & Braga, 2011; Wu *et al.*, 2012). Furthermore, the microinjection of proinflammatory cytokines

(IL-6) into the NTS caused a marked decrease in the baroreceptor sensitivity, which is believed to precede hypertension (Takagishi *et al.*, 2010); however, the role of activated microglia in the NTS and RVLM in the pathology of the cardiovascular complications in diabetic animals is not clear, and further research is required to demonstrate the effect of microglial inhibition in these regions.

As discussed previously, a deficiency in the levels of H_2S is reported in diabetic humans (Jain *et al.*, 2010), and this deficiency contributes to the development of diabetic complications, including diabetic cardiomyopathy and nephropathy (Lefer, 2008; Szabo, 2012). The data from **chapter 8** provided evidence that the systemic infusion of H_2S reduces neuronal activation in the PVN in STZ diabetic animals; however, in this study, the consequences of H_2S on PVN inflammation were not tested due to the lack of microglial activation in short-term STZ diabetic mice. Thus, future experiments should investigate the effect of H_2S treatment on microglial activation as well as cardiovascular changes in long-term STZ diabetic animals.

The data from this thesis has provided evidence that an imbalance of salt intake and excretion in diabetes can have a negative impact on the cardiovascular system via causing CNS inflammation. Due to enhanced urinary excretion in diabetic humans and animals, deficiency in other minerals, such as magnesium, may also occur. Magnesium has received substantial attention for its roles in the pathogenesis of cardiovascular diseases (Kolte *et al.*, 2014). The depletion of magnesium has been reported in patients with type I diabetes (Mooradian & Morley, 1987) and STZ diabetic animals (Soltani *et al.*, 2005). Several studies on STZ diabetic rats have documented that diabetic complications, including hypertension and nephropathy, are improved by treatment with magnesium (Soltani *et al.*, 2005; Parvizi *et al.*, 2014); however, the possibility that increased dietary magnesium could improve cardiac functions in STZ diabetic rats has not been previously investigated. Hence, future

experiments could explore whether dietary magnesium can reduce cardiac dysfunction and help prevent neuroinflammation in animal models of diabetes.

9.6 Conclusion

The impact of diabetic complications, such as cardiovascular disease and diabetic kidney disease, on the health and survival of human populations is considerable and is growing worldwide, as the number of people with diabetes is increasing at an alarming rate; however, protecting diabetic patients from these complications remains a challenge in the field, as the exact nature and mechanisms contributing to diabetic complications have not been well defined. This thesis provided convincing evidence that neuroinflammation is a potential mechanism underling the development of diabetic complications. This thesis also improved our understanding of the complex role of salt intake in diabetic complications, providing novel knowledge about the mechanisms by which salt intake contributes to the pathogenesis of cardiovascular complications in diabetes. Indeed, the data presented here may lead to the development of new and better approaches to treating diabetes and its complications.

Chapter Ten: References

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