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Spinally-Mediated Hyperalgesia in Experimental Diabetes

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in

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by

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Abstracts

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ABSTRACT OF THE DISSERTATION

Spinally-Mediated Hyperalgesia in Experimental Diabetes

by

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A proportion of diabetic patients experience chronic pain that is frequently refractory to treatment with conventional analgesics. Experimentally diabetic animals exhibit a range of behavioral abnormalities indicative of pain, and can be used to model human painful diabetic neuropathy. Studies suggest that pain during diabetes is not associated with structural damage to the peripheral nerves, and biochemical amplification of nociceptive processing may be of importance.

The formalin test is used to measure spinal sensitization in animals. Diabetic rats exhibit exaggerated flinching during this test, in addition to increased constitutive spinal expression of cyclooxygenase-2 (COX-2) protein.

Studies were designed to establish causality between increased spinal COX-2 activity and formalin hyperalgesia in diabetic rats. Rats with one, two or four weeks of streptozotocin-induced diabetes exhibited significantly increased levels of spinal COX-2 protein and activity, along with exaggerated paw flinching in response to 0.5% paw formalin injection. Increased flinching of diabetic rats was attenuated by intrathecal pre-treatment with a selective COX-2 inhibitor immediately prior to formalin injection, confirming the involvement of COX-2 activity in formalin hyperalgesia observed in diabetic rats.

We hypothesized that glucose flux through the polyol pathway in the central nervous system (CNS) causes elevated spinal COX-2 expression and formalin hyperalgesia in diabetic rats. Chronic treatment with insulin or ICI222155, an aldose reductase inhibitor (ARI) previously shown to cross the blood-brain barrier (BBB) and prevent formalin-evoked hyperalgesia in diabetic rats, prevented elevated spinal COX-2 protein and activity in diabetic rats. In contrast, the ARI IDD676 showed efficacy against AR activity only in the peripheral nervous system, and had no effect on elevated spinal COX-2 or hyperalgesia to paw formalin injection, despite demonstrating efficacy against indices of peripheral nerve dysfunction. In the spinal cord, aldose reductase (AR) expression was observed solely in oligodendrocytes, which also expressed COX-2. These data suggest that activity of AR in spinal oligodendrocytes may be a primary initiating factor linking hyperglycemia with hyperalgesia in diabetic rats. Further, CNS targeting of ARIs may be important in treating painful diabetic neuropathy in patients.

CHAPTER 1 – INTRODUCTION

1.1 Diabetes mellitus

1.1.1 Diabetes and secondary complications

'Diabetes mellitus' is the name given to a range of chronic metabolic diseases that afflict over 20 million people in the United States. All share the commonality of abnormal insulin functioning, which causes hyperglycemia. There are two major types of diabetes, type I or insulin-dependent, and type 2 or non-insulin-dependent. The first is a genetically-linked autoimmune disease in which the pancreatic beta cells are destroyed, thereby eliminating the body's natural source of insulin. The second is linked to obesity, a sedentary lifestyle, and genetic predisposition, and is typically characterized by an initial insulin resistance that, over time, transitions to include impaired insulin secretion. Traditionally, type I and type II diabetes have also been referred to as juvenile-onset and adult-onset, respectively. However, type II diabetes is occurring with increasing frequency in younger patients.

Because diabetes is a disease of chronic metabolic malfunctioning, much of the body can be affected, especially given that current health care makes it possible for patients to live for decades with the disease. Diabetes causes secondary complications affecting the kidneys (nephropathy), eyes (retinopathy), blood vessels (angiopathy), and nervous system (neuropathy), and it is the severity of these complications that ultimately impedes quality of life for diabetic patients.

1.1.2 Human diabetic neuropathy

Diabetic peripheral neuropathy encompasses a heterogeneous group of neuropathies that are typically subdivided into focal, multifocal, or symmetrical neuropathies (categorization by Thomas, 1973). Symmetric polyneuropathy comprises symmetric motor polyneuropathy, symmetric sensory polyneuropathy, symmetric sensorimotor polyneuropathy, and autonomic polyneuropathy. Of these, symmetric sensorimotor polyneuropathy is the most common neuropathy caused by diabetes and is the type modeled in this thesis. It is characterized as an axonal length-dependent, mostly distal ('stocking-glove') distribution of sensory and motor abnormalities that include nerve conduction velocity slowing, loss of normal sensation and presence of neuropathic pain. Symmetric motor polyneuropathy afflicts muscle groups of either proximal or distal location and is characterized by muscle weakness and atrophy. Autonomic polyneuropathy results in abnormal functioning of the autonomic nervous system, including the gastrointestinal, cardiovascular and genitourinary systems. The non-symmetric focal neuropathies include cranial neuropathies, truncal radiculopathies, and focal limb nerve lesions caused by entrapment or compression of a peripheral nerve or focal ischemia.

Diabetic symmetric sensorimotor polyneuropathy causes a pattern of structural damage to various parts of the peripheral nervous system. In the peripheral nerves of patients, large diameter fibers atrophy and eventually degenerate in a distal-to-proximal pattern referred to as "dying back" (Brown et al., 1976; Archer et al., 1983; Britland et al., 1992;), with evidence of

segmental demyelination and attempted remyelination (Behse et al., 1977). Small unmyelinated fibers are also observed to undergo regeneration and sprouting (Brown et al., 1976). Schwann cells undergo reactive, degenerative, and proliferative changes (Kalichman et al., 1998). Pathological changes in axons and Schwann cells are observed concomitantly and it is unclear which of these is the primary lesion site in diabetic peripheral nerves. In the autonomic nervous system, neuroaxonal dystrophy is evident as swelling of axon terminals, aggregation of phosphorylated neurofilaments, and abnormally large numbers of mitochondria and vacuoles (Schroer et al., 1992). In the skin, there is a loss of unmyelinated fibers that innervate the epidermis (Kennedy et al., 1996; Koskinen et al., 2005), but it has not been conclusively demonstrated that loss of epidermal innervation correlates with loss of sensation.

Diabetes is the most common cause of neuropathy in the developed world. Approximately half of all patients with diabetes will develop neuropathy of some sort during the course of their disease (Pirart et al., 1978), yet the relative susceptibility of patients with type I or type II diabetes to neuropathy is not certain. Some authors argue neuropathy is more common in type I diabetic patients (Dyck et al., 1999) while others contend that both types are equally susceptible to neuropathy (Vinik et al., 2000).

1.1.3 Human painful diabetic neuropathy

Pain can be classified as either nociceptive or neuropathic. Nociceptive pain involves activation of peripheral nociceptors in response to tissue

damage, and serves an important role in protecting the body from injury. In contrast, neuropathic pain occurs due to damage to the nervous system itself. Neuropathic pain is relatively common among patients with diabetic neuropathy, but its reported incidence varies widely between studies: from 10% in one study (Harati, 1996) to 46% in another (Ahroni et al., 1994). Nevertheless, even if 10% is a more accurate figure, that would translate into approximately 1.8 million people in the U.S. today suffering from painful diabetic neuropathy. Pain is the most disruptive symptom reported by patients with diabetic neuropathy, because it severely interferes with sleep, social interaction, and overall quality of life (Ahroni et al., 1994).

Diabetic pain presents as either a severe but short-lived experience, often following a period of uncontrolled hyperglycemia, or as a less severe but chronic problem. Pain may be spontaneous, or occur in response to stimuli that are normally innocuous (allodynia) or mildly painful (hyperalgesia). Allodynia can be particularly debilitating when, for example, the touch of bed sheets on the feet is perceived as painful. Pain can occur with or without simultaneous sensory loss.

1.1.4 Treatment of painful diabetic neuropathy in patients

Various types of drugs are prescribed to treat painful diabetic neuropathy. Opiates are commonly used, but because diabetic pain is largely refractory to these classic analgesics, patients require such high doses that general opiate side-effects including dependence, tolerance, and sedation are problematic. Treatment with tricyclic antidepressants offers pain relief that

marginally exceeds that seen with placebo treatment, but is limited by side-effects (Max et al., 1992). Treatment with antiepileptics including gabapentin and pregabalin has demonstrated some efficacy (Backonja et al., 1998; Eisenberg et al., 2001). However, their success is only marginally better than that seen with tricyclics (Morello et al., 1999; Gilron and Flatters, 2006). A recent review indicates that only one-third of patients with painful diabetic neuropathy are likely to receive more than 50% pain relief using this broad spectrum of drugs (Jensen et al., 2006). The lack of resounding success with any of these treatments may reflect the fact that none target the specific etiology of painful diabetic neuropathy. Thus the need to understand this mechanism is of the utmost importance.

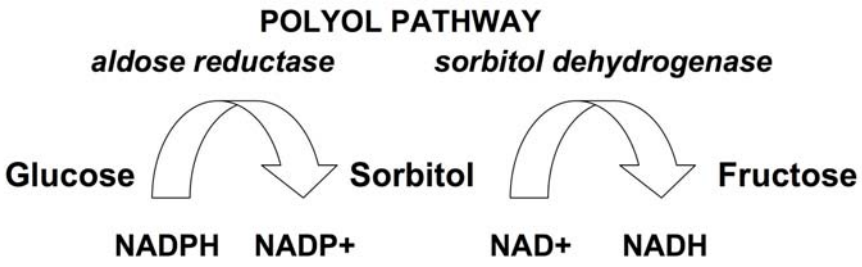
1.2 Rodent models of diabetic neuropathy

1.2.1 General classes of models

Diabetes can be modeled in rodents by chemical ablation of pancreatic β cells, by use of rats or mice bred to genetically exhibit impaired insulin secretion or insulin resistance, or by feeding of a galactose-rich diet. The model used most in this thesis is the streptozotocin-diabetic rat, which is the most thoroughly studied and documented model of type 1 diabetes. Injection of streptozotocin (STZ) in a rat or mouse chemically ablates pancreatic β cells, and induces extreme (20 – 40 mmol/L) hyperglycemia within a few days. These animals also rapidly develop measurable physiologic and behavioral abnormalities, as will be discussed below. One disadvantage of STZ-induced diabetes is that the animals can develop cachexia and die after just a few

weeks. However, body weight can be easily monitored, and animals losing too much weight can be given low doses of insulin to prevent death without affecting chronic hyperglycemia (see section 2.1).

Galactose intoxication in animals is used to produce excess metabolism of hexose sugar by aldose reductase. Aldose reductase is the first enzyme in the polyol pathway (see below), which is normally minimally active but under conditions of chronic hyperglycemia, excess glucose is shunted through this pathway.



Galactose intoxication provides galactose as an alternative substrate for aldose reductase, and thus galactose-intoxicated animals exhibit abnormally elevated aldose reductase activity. One advantage of this model is that galactose-intoxicated animals have normal insulin levels, which allows the contributions of aldose reductase activity and insulin deficiency to the pathogenesis of diabetic neuropathy to be separated. While it has been demonstrated that galactose-intoxicated rats show some indices of nerve and behavioral dysfunction seen in STZ-diabetic rats (Calcutt et al., 1994; Kalichman et al., 1998), this model has two major drawbacks. Firstly, excess glucose and not other hexose sugars may be uniquely responsible for some

pathogenic mechanisms in diabetes. Secondly, galactose reduced by aldose reductase becomes dulcitol, which cannot be acted on by the second enzyme of the polyol pathway, sorbitol dehydrogenase (see 1.3.1), and therefore there is no fructose accumulation in this model.

There are genetically diabetic rat and mouse strains available, including the db/db and ob/ob mouse and the BB Wistar rat, but these models share the disadvantage of requiring a relatively long period of time before showing signs of diabetes-related complications. Also, since diabetes appears spontaneously in these strains, the onset of diabetes cannot be as accurately controlled across a cohort as it can be with STZ-diabetic animals.

1.2.2 Nerve damage in diabetic rodents

Nerve damage can be observed in diabetic rodents, though it is not as robust as the damage present in human patients with diabetes. In the peripheral nervous system, diabetic rats and mice exhibit axonal dwindling (Jakobsen, 1976; Sima and Robertson, 1979; Ras and Nava, 1986) and shrinkage of dorsal root ganglia (DRG) neurons (Sidenius and Jakobsen, 1980; Jiang and Jakobsen, 2004). Also evident in the peripheral nerve are abnormal changes in Schwann cells (Sima and Robertson, 1979; Kalichman et al., 1998) and the loss of paranodal axo-glial junctional complexes (Sima et al., 1986). However, it has been observed that functional nerve abnormalities in diabetic rodents occur far earlier than structural changes (Sharma and Thomas, 1974; Walker et al., 1999), and therefore at least some aspects of

nerve dysfunction in these animals are likely due to metabolic, rather than structural problems.

In STZ-diabetic rodents, there is no overt Wallerian degeneration evident in peripheral nerve trunks, but there is a reduction of epidermal innervation as measured with protein gene product (PGP) 9.5 immunohistochemistry of the skin (Gibran et al., 2002; Christianson et al., 2003a; Toth et al., 2006; Leonelli et al., 2007). This reduction is evident as early as four weeks after induction of diabetes in mice, but takes longer to develop in rats (Gibran et al., 2002; Christianson et al., 2003a; Toth et al., 2006; Leonelli et al., 2007). This may explain why mice with 4 weeks of STZ-diabetes exhibit thermal hypoalgesia, whereas STZ-diabetic rats typically become hypoalgesic to thermal stimuli after 8 – 10 weeks of diabetes, as discussed in the next sub-section.

1.2.3 Behavioral abnormalities

Diabetic rats and mice exhibit a range of behavioral abnormalities, including depressed spontaneous motor activity. Altered sensory function is evident in altered responses to tactile testing with nylon von Frey filaments (Calcutt et al., 1996) and to thermal stimulation of the hindpaw (Calcutt et al., 2004) using a radiant heat source directed at the plantar paw surface as originally described by Hargreaves et al. (1988). Similarly, these animals have abnormal behavioral responses to the 'formalin test,' in which formalin is injected subcutaneously on the dorsal surface of the hindpaw. This is a commonly used variation of the test originally described by Dubuisson and

Dennis (1977) that evokes, in normal animals, a biphasic pattern of paw flinching (in rats) or licking (in mice) separated by a quiescent phase with little or no nocifensive behavior.

The formalin test plays a central role in this thesis, as it is well-suited to investigate biochemical spinal sensitization in diabetic rodents. Phase I of the formalin test reflects acute tissue injury caused by the formalin, while phase II reflects spinal sensitization that develops due to prolonged low-level activation of small diameter afferents, such that wide dynamic range (WDR) neurons in the dorsal horn become hyperexcitable (Haley et al., 1990; Puig and Sorkin, 1996; reviewed by Yaksh et al., 1999). Diabetic rats exhibit exaggerated flinching behavior during phase II of the formalin test, yet flinching during phase I is largely unchanged (Calcutt et al., 1995), suggesting that spinal or supraspinal processing of nociceptive information is exaggerated in diabetes.

1.3 Etiology

1.3.1 Pathogenesis of diabetic neuropathy

In 1993, the National Institute of Diabetes and Digestive and Kidney Diseases completed the Diabetes Control and Complications Trial (DCCT), a comprehensive clinical study of diabetes that involved tracking about 1,500 type 1 diabetic patients over 10 years, to determine how intensive glycemic control affected the onset and severity of diabetic complications. The DCCT showed that tight glycemic control is of crucial importance to diabetic patients, as it reduces the impact of complications. The onset of neuropathy after five years was reduced by 57% in patients practicing tight glycemic control

(Diabetes Control and Complications Trial Research Group, 1993), and more recently it has been shown that the severity of neuropathy in type 1 and type 2 diabetic patients is strongly correlated with glycemic control (Perkins et al., 2001). These epidemiological findings suggest that hyperglycemia itself is the dominant primary cause underlying diabetic neuropathy and underscore the importance of glycemic control in preventing complications associated with diabetes. However, good glycemic control is achieved in only a small portion of diabetic patients, due to reasons such as the complexity of the disease itself and poor patient compliance with a regimen of repeated self-testing of blood glucose and insulin injection (for review, see Del Prato, 2005, and Jacqueminet et al., 2005).

The mechanisms by which chronic hyperglycemia give rise to diabetic complications have been intensely studied for over 30 years, and the polyol pathway has consistently been a central focus of this research. First described in 1956 by Hers, the polyol pathway is an aldehyde metabolism pathway that comprises the enzymes aldose reductase (AR) and sorbitol dehydrogenase (SDH). Aldose reductase can act on a range of substrates, and its metabolism of glucose is of great significance in the diabetic condition. Glucose is converted to sorbitol by AR and SDH then oxidizes sorbitol to form fructose.

The polyol pathway enzymes are found in tissues throughout the body, including the inner medulla and cortex of the kidneys; Sertoli cells and mature spermatids of the testes; granulosa cells and oocytes of the ovaries; adrenal

glands; aorta; lungs; retina and lens of the eyes; perineuronal satellite cells of the DRG; myelinating Schwann cells of the peripheral nerves; brain; and spinal cord (Ludvigson and Sorenson, 1980a; Ludvigson and Sorenson, 1980b; Nishimura et al., 1988; Iwata et al., 1990; Kobayashi et al., 2002; Maekawa et al., 2002; Jiang et al., 2006). Under normal conditions, activity of the polyol pathway has only a minor role in glucose metabolism in most tissues, with a few exceptions. Polyol pathway production of sorbitol in the kidney balances locally high interstitial osmotic pressure caused by urine (Bagnasco et al., 1987; Oates and Goddu, 1987). Fructose produced via the polyol pathway nourishes sperm in the male reproductive tract (Ludvigson and Sorenson, 1980a). Polyol pathway activity is believed to facilitate lens fiber morphogenesis (Bondy and Lightman, 1989). Additionally, AR can act on substrates other than glucose and serves to metabolize steroids, norepinephrine, and reactive aldehydes (Grimshaw, 1992; Petrash et al., 1997; Kawamura et al., 1999; Pladzyk et al., 2006a).

Glucose metabolism via the polyol pathway is greatly increased under conditions of chronic hyperglycemia. The connection between aldose reductase activity and diabetic complications was first considered in the context of experimental sugar cataracts in rats. Studies examining cataracts in diabetic and galactose-intoxicated rats found similar lens abnormalities, including cortical vacuoles and nuclear opacity that ultimately led to opacification of the entire lens (Friedenwald et al., 1955; von Sallmann et al., 1958). Additionally, lens fibers from these animals were swollen and ruptured.

The similar pathology observed in these two types of sugar cataracts prompted the hypothesis that a common mechanism caused the lenticular damage. In 1959, van Heyningen showed that AR is present in the mammalian lens and converts glucose or galactose into sorbitol or galactitol (dulcitol), respectively. Further studies culminated in Kinoshita proposing the 'osmotic hypothesis' (1974). This hypothesis states that AR activity in the diabetic or galactosemic rat lens causes excess production of sorbitol or dulcitol, and since these polyols do not cross cell membranes very effectively, they accumulate within the lens fibers and create an osmotic imbalance that leads to swelling. The swelling then causes changes in membrane permeability, perturbations of ion and metabolite concentrations, and, ultimately, a complete breakdown of cellular integrity.

While the osmotic hypothesis has consistently been confirmed to successfully explain diabetic lenticular damage, an understanding of the pathogenesis of diabetic neuropathy has remained elusive. Following the proposed link between sorbitol accumulation and cellular damage in the diabetic lens, observed accumulations of sorbitol in the peripheral nerve prompted the hypothesis that a similar mechanism might initiate diabetic neuropathy (Gabbay, 1973). However, many studies have shown that there is not a linear correlation between nerve sorbitol accumulation and nerve dysfunction. For example, the peripheral nerves of diabetic mice have AR and polyol pathway flux (Calcutt et al., 1988), and nerve conduction velocity (NCV) slowing in these animals is preventable with aldose reductase inhibitor

treatment (Miwa et al., 1989). However, diabetic mice do not show accumulation of sorbitol in the peripheral nerves (Calcutt et al., 1988), suggesting that nerve dysfunction is caused by polyol pathway flux *per se* and not necessarily polyol accumulation.

Over the past two decades, numerous studies have nevertheless strengthened the causal connection between exaggerated polyol pathway flux and diabetes-induced complications. The polyol pathway enzymes are present in all tissues that are susceptible to development of diabetic complications, and these tissues share the property of insulin-independent extracellular glucose uptake. Thus in the diabetic condition, cells of these tissues are subject to extremely high levels of glucose diffusion into their cytosol, which causes both metabolic disturbances and exaggerated polyol pathway flux. There is now general agreement that polyol pathway flux itself causes a host of downstream secondary pathologic mechanisms, which probably act in concert to cause damage. These secondary mechanisms include increased oxidative stress, enhanced nonenzymatic glycosylation, ischemic hypoxia, and loss of neurotrophic support.

It is generally accepted that diabetes increases oxidative stress in the nervous system, largely by disrupting normal antioxidant defenses (for review, see Obrosova, 2005). The potent antioxidants taurine and ascorbate are reduced in diabetic peripheral nerve (Stevens et al., 1993; Obrosova et al., 2002). Additionally, several groups have demonstrated depletion of reduced glutathione (GSH) in the peripheral nerve in diabetes (Nagamatsu et al., 1995;

Cameron et al., 1999; Obrosova et al., 1999). Depletion of GSH interferes with the normal antioxidant mechanisms within cells, as GSH is required for glutathione peroxidase to reduce hydrogen peroxide and lipid hydroperoxides. It has further been shown that treatment with an aldose reductase inhibitor prevents the depletion of GSH in the diabetic lens (Lou et al., 1988), and that transgenic mice that do not express AR are protected from GSH depletion in the peripheral nerve in diabetes (Ho et al., 2006), thus implicating exaggerated polyol pathway flux in the depletion of GSH. Reduction of glucose to sorbitol by AR requires a hydride donor, with NADPH being preferentially used, and it has been observed that hyperglycemia in the lens causes a 3000% per hour turnover rate of NADPH to NADP (Cheng and Gonzalez, 1986). Thus it has been proposed that because AR competes with glutathione reductase for a potentially decreased pool of NADPH, the recycling of oxidized glutathione to GSH by glutathione reductase is impaired in diabetes (Cameron et al., 1999; Lee and Chung, 1999). However, NADPH deficiency has never been documented in the diabetic peripheral nerve, and it remains unclear how exactly exaggerated polyol pathway flux causes depletion of GSH.

Non-enzymatic glycosylation (glycation) of proteins is increased in the nervous system of diabetic humans and experimentally diabetic animals (Monnier and Cerami, 1982). This process occurs when monosaccharides bind to proteins via N-glycoside bonding, and fructose is ten times as potent a glycating agent as glucose (Suarez et al., 1989). This has clear implications for cells and tissues exposed to hyperglycemia and also exhibiting polyol

product accumulation in diabetes. Various proteins that are susceptible to glycation may indeed have their function compromised in diabetes, possibly contributing to nervous system damage. In the peripheral nervous system (PNS), glycated proteins include the myelin protein P_0, along with the axonal cytoskeletal proteins tubulin, neurofilaments, and actin (Vlassara et al., 1983; Vlassara et al., 1985; Ryle and Donaghy, 1995). Glycation of structural proteins within axons causes slow axonal transport, atrophy, and degeneration (Vlassara et al., 1985). In the CNS, the myelin proteins proteolipid protein (PLP) and myelin basic protein (MBP) are excessively glycated in diabetes (Vlassara et al., 1983; Weimbs and Stoffel, 1994). Spinal demyelination has been observed in tissue from diabetic humans but not diabetic rats (Reske-Nielsen and Lundbaek, 1968; DeJong, 1977), and it is possible that glycation of CNS myelin proteins causes a functional disturbance of myelination before overt demyelination is observed. This possibility has yet to be explored and is of particular interest in this dissertation.

Patients with chronic hypoxemia such as caused by chronic obstructive pulmonary disease can develop peripheral polyneuropathy (Narayan and Ferranti, 1978; Pfeiffer et al., 1990). Many studies have also implicated hypoxia in the dysfunction of diabetic peripheral nerves. In diabetic patients, measurement of endoneurial oxygen tensions indicate that the peripheral nerves are hypoxic compared with control subjects (Newrick et al., 1986). Further, reduced oxygen tensions correlate with slowed peripheral nerve conduction velocity (Young et al., 1992). Exercise increases nerve conduction

velocity in normal subjects but not in diabetic patients, and this is hypothesized to be due to impaired neural blood flow in the diabetic patients (Tesfaye et al., 1992). These data suggest that impaired blood flow to peripheral nerves may have an important role in causing nerve dysfunction in diabetes.

Impaired neurotrophic support has been implicated in the etiology of diabetic neuropathy, and the neurotrophic factors that have been studied in this context include nerve growth factor (NGF), neurotrophin-3 (NT-3), ciliary neurotrophic factor (CNTF), glial cell derived neurotrophic factor (GDNF), brain-dervied neurotrophic factor (BDNF), and insulin-like growth factors (IGFs). Of these, NGF, NT-3, CNTF, GDNF and BDNF are all synthesized by target cells and released to axons that innervate those cells. The neurotrophic factors are then retrogradely transported to neuronal cell bodies, where they regulate protein expression and can influence, among other things, cell survival (reviewed by Neet and Campenot, 2001). It has been shown that diabetes inhibits synthesis of these factors, and in combination with slowed axonal transport in diabetes, results in impaired neurotrophic support in the diabetic nervous system (Hellweg and Hartung, 1990; Calcutt et al., 1992; Rodriguez-Pena et al., 1995; Zhuang et al., 1997; Mizisin et al., 1999b). Correspondingly, treating diabetic rats with exogenous neurotrophic factors can ameliorate aspects of neuropathy. For example, exogenous NGF treatment of streptozotocin-diabetic rats reversed the depletion of substance P and calcitonin gene-related product (CGRP) in DRG neurons and prevented thermal hypoalgesia measured with the tailflick test (Apfel et al., 1994;

Tomlinson et al., 1997). Treatment with NT-3 prevented mitochondrial dysfunction in sensory neurons and reversed nerve conduction velocity slowing in streptozotocin-diabetic rats (Mizisin et al., 1999a; Huang et al., 2005). Treatment of streptozotocin-diabetic rats with CNTF prevented the development of thermal hypoalgesia and nerve conduction velocity slowing (Calcutt et al., 2004; Mizisin et al., 2004). Treatment with GDNF has been shown to prevent the loss of DRG neuron terminations in the skin and spinal cord of STZ-diabetic mice (Akkina et al., 2001; Christianson et al., 2003a). Finally, IGF-II infusion reversed hyperalgesic responses to deep paw pressure in diabetic rats (Zhuang et al., 1997). These studies indicate that loss of neurotrophic support contributes to the etiology of at least some aspects of diabetic neuropathy. Additionally, insulin acts as a neurotrophic factor in the nervous system via insulin and IGF receptors, and insulinopenia or insulin resistance may contribute to nervous system dysfunction during diabetes (Ishii, 1993). In support of this, it has been reported that treating STZ-diabetic rats with low-dose insulin therapy reversed tactile allodynia without affecting blood glucose levels (Hoybergs and Meert, 2007).

1.3.2 Pathogenesis of painful diabetic neuropathy

There is no accepted explanation for the mechanistic basis of painful diabetic neuropathy. Because both insulin- and noninsulin-dependent diabetic patients can experience pain, it likely does not occur directly due to lack of insulin *per se*, but rather due to chronic hyperglycemia and its associated metabolic consequences.

It has been generally accepted that the main diabetes-induced insult to the nervous system is found in the peripheral nerves. Extensive overt pathology is indeed evident in the peripheral nerves of diabetic patients, but nevertheless, diabetes' effect on the central nervous system is recognized and increasingly regarded as a potential contributor to the pathogenesis of painful diabetic neuropathy (Calcutt et al., 1995; Chen and Pan, 2002; Ciruela et al., 2003; Kimura et al., 2005). This is partly due to accumulating evidence suggesting that peripheral nerve abnormalities are not causally related to pain. In humans, early studies postulated that pain might be caused by ectopic activity in peripheral nerve axons undergoing degeneration and regeneration (Dyck et al., 1976; Archer et al., 1983). However, this has been disproved by studies comparing peripheral nerve pathology and incidence of diabetic pain. Llewelyn et al. (1991) found that the occurrence of pain in diabetic patients did not correlate with either degeneration of myelinated fibers or regeneration of myelinated or unmyelinated axons in the sural nerve. Indeed, patients with acute or chronic painful neuropathy had the least amount of axonal regeneration. Similarly, other studies have reported sural nerve fiber degeneration and regeneration common to all diabetic patients, regardless of whether or not they had pain (Britland et al., 1992; Malik et al., 2001).

In experimentally diabetic animals, the results of most studies are consistent with the idea that diabetes depresses peripheral nerve function, thus arguing against the importance of primary afferent activity in diabetic hyperalgesia. Slow axonal transport of structural proteins is reduced in

diabetes (Jakobsen and Sidenius, 1980), and large sensory fiber conduction is significantly slowed (Moore et al., 1980). Large myelinated fibers exhibit cell body shrinkage (Sidenius and Jakobsen, 1980) as well as axonal dwindling (Jakobsen, 1976). After nerve injury in diabetic rodents, regeneration is impaired (Bisby, 1980; Ekstrom and Tomlinson, 1989).

Despite the preponderance of evidence indicating impaired peripheral nerve function in diabetes, there is disagreement about whether diabetes exaggerates afferent input to the CNS from the periphery. There are five published electrophysiology studies that were performed on fine filaments of peripheral nerves (saphenous or tibial) from control and diabetic rats. Of these, the first study documented spontaneous activity reportedly emanating from the DRG itself, and found a diabetes-associated increase in the percentage of fibers exhibiting spontaneous activity (Burchiel et al., 1985). However, the actual percentage of fibers exhibiting spontaneous activity originating from the DRG in an average diabetic rat saphenous nerve was ~0.6%, or 28.45 ± 10.72 fibers, as compared with 14.36 ± 8.86 fibers in an average control saphenous nerve (Burchiel et al., 1985). The subsequent four electrophysiology studies comparing peripheral nerves of control and diabetic rats all reported activity of peripheral, non-DRG origin. Of these, one reported diabetes-associated increased spontaneous activity in Aβ, Aδ, and C fibers (Khan et al., 2002), while three reported no change in spontaneous activity in C fibers (Ahlgren et al., 1992; Russell and Burchiel, 1993; Chen and Levine, 2001). Ahlren et al. (1992), Chen and Levine (2001), and Khan et al. (2002)

reported no change in thermal or mechanical thresholds for activation of C fibers, but Khan et al. (2002) reported decreased activation thresholds for Aβ and Aδ fibers in diabetic rats.

Functional studies indicate that in response to paw formalin injection, diabetic rats exhibit decreased spinal release of glutamate (Malmberg et al., 2006) and substance P (Calcutt et al., 2000b), yet behaviorally they display exaggerated flinching. Glutamate and substance P are important components of the range of neurotransmitters used by primary afferents to communicate with dorsal horn neurons. However, spinal release of glutamate reflects release from not only primary afferents but also spinal interneurons (reviewed by Shapiro, 1997). In contrast, spinal substance P release predominantly reflects release from primary afferents, as indicated by the observation that spinal substance P content is depleted approximately 80% after dorsal rhizotomy or sciatic nerve transection (Jessell et al., 1979). Therefore, taken together, the *in vivo* measurements of paw formalin injection-evoked spinal release of glutamate and especially substance P are inconsistent with the possibility of increased peripheral drive underlying formalin hyperalgesia in diabetic rats, and instead suggest a role for spinal or supraspinal amplification of nociceptive processing.

There is evidence to suggest that the spinal cord and its sensory processing are indeed affected by diabetes. In humans, the spinal cord volume is reduced in neuropathic diabetics as compared with non-neuropathic diabetics or control subjects (Eaton et al., 2001), and spinal conduction

slowing has been observed in diabetic patients (Cracco et al., 1984). In diabetic rats, biochemical changes have been observed in the spinal cord. For example, spinal delivery of substance P induces a transient thermal hyperalgesia in normal rats, believed to be caused by an increased spinal release of prostaglandin E_2 (PGE_2; Hua et al., 1999; Yaksh et al., 2001). This hyperalgesia is protracted in diabetic rats (Calcutt et al., 2000a). This finding suggests two important conclusions: diabetes does cause biochemical changes either spinally or supraspinally that alter sensory processing, and given that spinal PGE_2 release is thought to cause intrathecal substance P-induced hyperalgesia, spinal prostaglandins could play a causal role in at least some forms of diabetic pain. These potential pathogenic mechanisms of painful diabetic neuropathy will be discussed in more detail below.

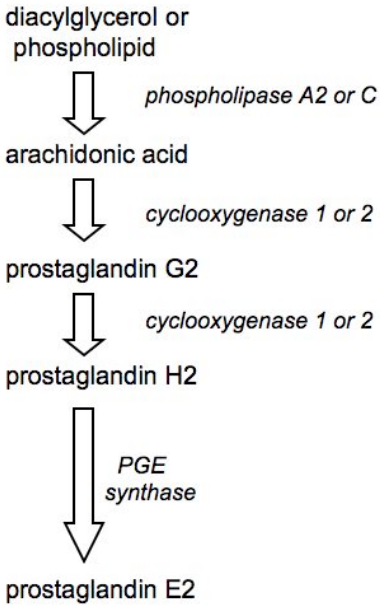
1.3.2.1 Hyperexcitability of spinal neurons

Diabetes causes spinal neurons to become hypersensitive to afferent input. In diabetic rats, dorsal horn low- and high-threshold and WDR neurons that comprise the spinothalamic tract show increased spontaneous activity, enlarged receptive fields, and lower thresholds and exaggerated responses to mechanical stimulation (Pertovaara et al., 2001; Chen and Pan, 2002). These changes in neuronal properties correlated with the onset of mechanical and thermal hyperalgesia and tactile allodynia, and the neurons were resistant to morphine-induced suppression of activity. These data indicate that diabetes in the rat augments normal spinal responses to sensory afferent information. This is a plausible explanation for perceptual amplification of pain or

generation of it when, normally, there would be none, and reinforces the rationale for studying the spinal cord as a site for augmentation of nociceptive processing in diabetes.

1.3.2.2 COX-2 in neuropathic and inflammatory pain states

PROSTAGLANDIN E2 SYNTHESIS



Cyclooxygenase (COX) is an enzyme that catalyzes the first two steps in the production of prostaglandins from arachidonic acid (AA). As a bifunctional enzyme, COX exhibits cyclooxygenase activity, which converts AA to the intermediary PGG_2, and peroxidase activity, which reduces PGG_2 to PGH_2. In turn, PGH_2 is then converted to active products by prostaglandin synthases (for review, see Smith, 1992). There are two known isoforms of COX, COX-1 and COX–2, in addition to a newly identified splice variant of COX-1 that is called COX-3 (Snipes et al., 2005; Kis et al., 2006). Of these, COX-1 is constitutively expressed in most tissues and plays important

physiologic roles throughout the body, including regulation of blood flow in the kidney and maintenance of normal platelet function (for review, see Crofford, 1997). In contrast, COX-2 is typically not expressed under normal conditions but is upregulated in response to injury and inflammation (for review, see Turini and DuBois, 2002). Importantly, the spinal cord is one site where COX-2 mRNA and protein are constitutively expressed (Beiche et al., 1996, 1998; Ebersberger et al., 1999).

Excessive spinal release of the prostaglandin PGE_2, particularly as catalyzed by COX-2, is believed to play a role in various neuropathic and inflammatory pain states (for review, see Svensson and Yaksh, 2002). In the chronic constriction injury (CCI) model, a model of nerve injury-induced pain, there is a significant upregulation of microglia-like cells with COX-2-like immunoreactivity forty days post-injury in the ipsilateral dorsal horn (Durrenberger et al., 2004). Tegeder et al., (2004) used the CCI model to show that treatment with an inhibitor of IκB kinase, a kinase that initiates a cascade that ultimately leads to COX-2 gene transcription, prevented CCI-induced development of tactile and cold allodynia. These experiments may suggest a role for COX-2 in neuropathic pain.

COX-2 is also implicated in post-inflammatory pain states. Six hours following unilateral rat hindpaw inflammation with carrageenan, COX-2 mRNA and PGE_2 are significantly upregulated throughout the CNS (Guay et al., 2004). The CNS COX-2 expression induced by paw inflammation with carrageenan has been localized to microglia, macrophages, and vascular

endothelial cells (Ichitani et al., 1997; Ibuki et al., 2003). Paw inflammation with Complete Freund's Adjuvant (CFA) increases the sural nerve stimulation-induced hamstring flexor reflex, an example of spinal sensitization. This effect is prevented by intrathecal delivery of ibuprofen (a nonselective inhibitor of COX-1 and COX-2), a treatment that has no effect on the reflex in rats without paw inflammation (Seybold et al., 2003). As another example, the nucleus pulposus of the intervertebral spinal disks, which is rich with an 'inflammatory soup' of cytokines and prostaglandins, can be applied directly to a spinal nerve root and causes upregulation of COX-2 immunoreactivity in neurons of the spinal cord. The allodynia that develops in this model is prevented by intrathecal treatment with a COX-2 antibody (Ohtori et al., 2004). The transcription factor for the COX-2 gene (NF-κB) can be pharmacologically inhibited, and doing so prevents CFA paw inflammation-induced tactile allodynia and thermal hyperalgesia (Lee et al., 2004). These experiments provide compelling evidence linking COX-2 and PGE_2 with pain in various facilitated pain states.

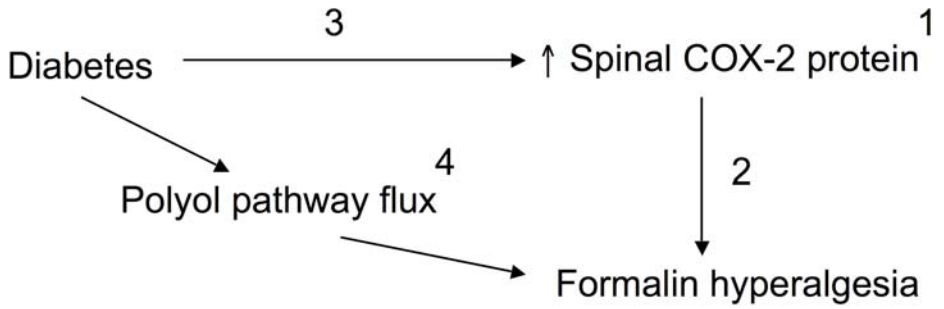
In addition to having a causal role in neuropathic and inflammatory pain, COX-2 and PGE_2 contribute to the spinal cord sensitization that underlies phase II of the formalin test in normal rats (Malmberg and Yaksh, 1995). Diabetic rats exhibit enhanced hyperalgesia during the formalin test, suggesting that COX-2 and PGE_2 might be linked to pain in diabetes.

1.3.2.3 COX-2 in painful diabetic neuropathy

An early report indicated efficacy of systemic non-steroidal anti-inflammatory drug (NSAID) treatment for painful diabetic neuropathy (Cohen and Harris, 1987), but the potential role of COX-2 and PGE_2 in painful diabetic neuropathy has received scant attention until recently. Freshwater et al. (2002) found that diabetic rats given a paw injection of formalin display, in addition to phase II hyperalgesia, a simultaneous prolongation of elevated spinal PGE_2 release. Formalin hyperalgesia in diabetic rats was dose-dependently ameliorated by intrathecal delivery of indomethacin, a nonselective COX inhibitor, or an antagonist of the PGE_2 receptor EP1. Additionally, diabetic rats had a three-fold increase in spinal COX-2 protein, versus control rats. These results implicate COX-2 and PGE_2 in at least some aspects of painful diabetic neuropathy, and constitute the founding impetus for this thesis's hypotheses.

1.4 Specific aims

Schematic illustrating prior results connecting experimental diabetes, formalin hyperalgesia and over-expression of spinal COX-2.



The following questions are addressed in this thesis:

1. Is the excess COX-2 protein present in the spinal cord of diabetic rats enzymatically active?
2. Is there a causal relationship between excess spinal COX-2 expression and formalin hyperalgesia in diabetic rats?
3. What is the mechanism by which diabetes causes elevated spinal COX-2 expression?
4. Where in the nervous system is polyol pathway flux relevant to the pathogenesis of formalin hyperalgesia and spinal sensitization in diabetic rats

CHAPTER 2 – GENERAL METHODS

2.1 Streptozotocin diabetic rodent model

Rats and mice were made diabetic by a single injection of streptozotocin (STZ), which is an antibiotic, antineoplastic agent belonging to a class of chemicals called nitrosoureas. When injected into a rat or mouse, it is taken up by GLUT2 glucose transporters expressed on pancreatic β cells (Rerup, 1970), where it induces apoptosis by releasing nitric oxide within the β cell causing DNA cleavage (Kaneto et al. 1995). The loss of β cells eliminates the natural source of secreted insulin, causing STZ-injected rats and mice to rapidly develop hyperglycemia, and their insulin deficiency makes these animals a model of type 1 diabetes. Physiologically, the animals show polydypsia, polyuria, polyphagia, diarrhea, and failure to gain weight or weight loss.

In these experiments, adult female Sprague-Dawley rats or adult male C57Bl/6 mice were used (Harlan, San Diego, CA). At the start of all experiments, rats were 9 – 12 weeks of age (225 – 250 g), and mice were 6 – 8 weeks of age (20 – 25 g). To induce diabetes, rodents were fasted overnight to minimize competition from circulating glucose for GLUT2 transport uptake into pancreatic beta cells, and injected intraperitoneally the following morning with freshly dissolved STZ using 55 mg/kg body weight for rats and 200 mg/kg body weight for mice (Sigma, St. Louis, MO). Three days after STZ injection, hyperglycemia was confirmed in blood taken via tail prick with a strip-operated reflectance meter (LifeScan, Milpitas, CA). Blood glucose levels

were also measured at death to confirm hyperglycemia. Blood glucose measurements can be affected by the conditions immediately before measurement, such as the extent to which the animal has been handled, whether the animal has been subjected to stress, and whether the blood is obtained before or after decapitation. In our experience, blood glucose levels can range between 4-10 mM in control rodents and between 15-50 mM in diabetic rodents. In all experiments in this thesis, a minimum blood glucose concentration of 15 mM (270 mg/dl) was required for an animal to be considered diabetic. Most diabetic animals exhibited a blood glucose of at least 30 mM (540 mg/dl) within a week of STZ injection.

With rare exception, each study was run with a group of STZ-diabetic rats (or mice) and a group of age-matched control animals for comparison of physiological, behavioral, and biochemical data. All animals were maintained 2-3 per cage (for rats) or 4 per cage (for mice) under standard vivarium conditions with 12:12 h light/dark cycle and free access to standard rodent chow (diet #7001, Harlan) and tap water. Animals were observed daily and weighed weekly. Due to polyuria, diabetic animals were kept on Tek-FRESH bedding, which is a paper bedding that absorbs up to three times its weight in liquids (Harlan). Prolonged diabetes can cause cachexia, and any diabetic rat with a body weight below 200 g or diabetic mouse below 20 g was treated with low doses of insulin to prevent further weight loss and fatal ketoacidosis. This was done by implanting subcutaneously 1/4 – 1/3 of a full-size slow-release insulin pellet (Linshin, Scarborough, Ontario, Canada), thus providing enough

insulin to maintain muscle mass without affecting hyperglycemia. Any animals showing lethargy and/or general poor health at the conclusion of a study were excluded from all analyses. All animal studies were approved by the UCSD Institutional Animal Care and Use Committee.

2.2 Biochemical assays

2.2.1 BCA protein assay

Protein concentrations were measured in homogenates of lumbar enlargements from rat spinal cords using a Bicinchoninic acid (BCA) protein assay kit (Pierce, Rockford, IL). The method, described by Smith et al. (1985) as a modification of the original work by Lowry et al. (1951), relies on the formation of a Cu^{2+}-protein complex under alkaline conditions, and a subsequent reduction of Cu^{2+} to Cu^{1+}. Each Cu^{1+} ion then reacts with BCA to produce a purple product, and absorbance in each sample is measured at 562 nm. Protein concentrations are then calculated based on a standard absorbance curve generated with standards containing known concentrations of bovine serum albumin (BSA).

2.2.2 Electrophoresis

To measure the expression of various proteins of interest, homogenates of spinal lumbar enlargements (segments L4-L6) were subjected to electrophoresis using the SDS-PAGE method, originally described by Laemmli (1970). The following procedure was applied to all Western blots performed for this thesis: Spinal cords were obtained from animals by hydraulic extrusion. From each animal, 1 cm of the lumbar

enlargement was homogenized in 400 μl of 50 mmol/l Tris buffer, pH 7.4, containing 0.5% Triton, 150 mmol/l NaCl, 1 mmol/l EDTA, and the protease inhibitors aprotonin (10 mg/ml), leupeptin (1 mg/ml), bacitracin (10 mg/ml), and benzamide (10 mg/ml). Homogenates were centrifuged at 10,000 x g for 15 min at 4°C, and the supernatant was separated into aliquots that were frozen at -20°C, with one aliquot saved at 4°C for subsequent measurement of protein concentration (described above). Equivalent amounts of protein were loaded from spinal cord homogenates onto NuPAGE Bis-Tris (4-12%) gels (Invitrogen, Carlsbad, CA), which were then run at 200 V for 1.5 hrs. Following electrophoresis, proteins were transferred to a nitrocellulose membrane (Amersham, Arlington Heights, IL) electrophoretically at 30 V for 1 hr. This method entails using current to move proteins out of the gel and onto the nitrocellulose membrane, which has an affinity for binding proteins (Towbin et al. 1979).

2.2.3 Immunoblotting

Proteins bound to a nitrocellulose membrane can be probed with antibodies raised against epitopes unique to proteins of interest. For these experiments, the following antibodies were used: a polyclonal anti-mouse COX-2 antibody raised in rabbit (Cayman Chemical, Ann Arbor, MI); a monoclonal anti-GFAP antibody raised in mouse (Chemicon, Temecula, CA); a monoclonal anti-NF-κB antibody raised in mouse that recognizes an epitope of the p65 subunit that is exposed only when the inhibitory protein IκBα is not bound to p65 (*i.e.*, when NF-κB is activated) (Chemicon); and a polyclonal

anti-EAAT1 antibody raised in rabbit (Abcam). Additionally, to provide loading controls, all gels were probed for the 'housekeeping' protein β-actin (Sigma, mouse monoclonal) or cyclophilin (Abcam, rabbit polyclonal).

Non-specific binding sites were blocked by immersing nitrocellulose membranes in a solution of either evaporated non-fat milk or BSA. After washing with a Tris-based buffer containing Tween-20 (TBS-T), membranes were incubated with the appropriate primary antibody either overnight at 4°C or for 1 hr at room temperature, depending on the affinity of the antibody for the protein. After washing with TBS-T, membranes were incubated for 1 hr with a secondary antibody raised in goat against rabbit or mouse IgG (Santa Cruz Biotechnology, Santa Cruz, CA), depending on the species in which the primary antibody was raised. These secondary antibodies are conjugated to horseradish peroxidase, which reacts with a chemiluminescent substrate (Pierce) to produce a fluorescent product wherever there is a protein-antibody complex on the membrane. Radiographic film was then exposed to the membrane, and exposure was quantified by densitometric scan using the Discovery Series Quantity One 1-D analysis software (Bio-Rad, Hercules, CA). Densities of protein bands of interest were then normalized to total β-actin or cyclophilin present per lane.

2.2.4 COX activity assay

To measure levels of enzymatically active COX in spinal cord homogenates, a COX activity assay (Cayman Chemical) was used. The COX enzyme exhibits cyclooxgenase and peroxidase activities, as it first converts

arachidonic acid to prostaglandin G_2 (PGG_2) and then reduces this endoperoxide to prostaglandin H_2 (PGH_2). The COX activity assay, based on work originally done by Kulmacz and Lands (1983), measures the peroxidase activity of COX. Spinal cords were obtained from animals by hydraulic extrusion. One centimeter of the lumbar enlargement (segments L4-L6) from each animal was gently scraped to remove the meningeal vessels, rinsed with 1 mmol/l Tris buffer, pH 7.4, containing 0.16 mg/ml heparin, and then homogenized in 400 μl of 0.1 mol/l Tris buffer, pH 7.8, containing 1 mmol/l EDTA. Homogenates were centrifuged at 10,000 x g for 15 min at 4°C, and supernatant collected and aliquoted. From each animal, separate aliquots were used to measure total COX activity and activity of COX-1 and COX-2 isozymes by adding selective inhibitors of COX-2 (DuP-697; Kargman et al., 1996) and COX-1 (SC-560; Smith et al., 1998), respectively. A separate aliquot was boiled to inactivate the COX enzyme and generate a background absorbance value. Heme and arachidonic acid were added to all samples, along with tetramethyl-*p*-phenylenediamine (TMPD), which is a reducing co-substrate for heme peroxidases. Any enzymatically active COX in each sample will act on arachidonic acid to produce PGG_2 and subsequently PGH_2, and the reduction of each molecule of PGG_2 will result in the appearance of two oxidized molecules of TMPD. Oxidized TMPD is highly colored and COX activity is calculated using the TMPD extinction coefficient of 0.00826 μM^{-1} and the measured absorbance in each sample at 590 nm after 5 min of incubation time.

2.2.5 Gas chromatography

In these experiments, sugars and polyols were measured by assay of their trimethylsilyl derivatives using a Hewlett Packard 5890 gas chromatograph fitted with a 25 m x 0.2 mm Hewlett Packard Ultra 1 capillary column and flame ionization detector. In this assay, a sample is injected at the head of the column into a stream of inert carrier gas that flows at a constant rate. The injected sample forms a stationary liquid phase adhered to the inner surface of the column, and a mobile gaseous phase that flows at the same rate as the carrier gas. The liquid and gaseous phases are in equilibrium, but this equilibrium is different for each component of the sample and determines the speed with which the component moves through the column. As individual components of the sample elute off the column, they are ionized and then quantified, and an integrator connected to the gas chromatograph draws and later integrates a peak representing that fraction. Thus each injected sample produces a print-out of integrated peaks, each of which can be compared to peaks produced by an injected standard containing known quantities of the compounds of interest.

To perform this assay, lumbar spinal cord samples and sciatic nerve samples taken at the mid-thigh level (for each, a 0.5 cm piece per rat) were weighed, lyophilized, re-weighed to calculate dry weight and water content, then boiled individually in distilled water with 30 μg of alpha methyl mannoside added to each. This provided an internal standard to determine efficiency of the sugar extraction. After boiling, zinc (II) sulfate and barium hydroxide were

added to each sample to precipitate proteins, and the samples were then boiled and centrifuged at 3,000 x g for 5 min at 10°C. Supernatants were collected and lyophilized overnight. 0.5 ml of a 10:2:1 mixture of pyridine, hexamethyldisilazane, and trimethylchlorosilane was added to each sample to produce trimethylsilyl (TMS) derivatives (Sweeley et al. 1963). The TMS derivatives were extracted by adding 2 ml of distilled water and 200 μl n-hexane, and 2 μl of the organic layer was injected into the gas chromatograph. External standards containing known quantities of alpha methyl mannoside, glucose, sorbitol, fructose, and myo-inositol were used to calculate quantities of these carbohydrates in each sample, which were then normalized to the dry weight of the original tissue.

2.2.6 Measurement of glycated hemoglobin

Measurement of glycated hemoglobin in blood (designated HbA1) is used clinically to monitor long-term blood glucose levels. During the 3 – 4 month period that a red blood cell is in circulation, glycated hemoglobin is formed continuously via adduction of glucose to the N-terminal of the hemoglobin protein's beta chain. Measurement of this non-enzymatic glycosylation of hemoglobin reflects the average exposure of hemoglobin to glucose during the lifespan of the red blood cell, and it has been shown that levels of glycated hemoglobin in diabetic patients are 2 – 3 fold higher than those in control subjects (Trivelli et al., 1971; Gabbay et al., 1979).

To measure the amount of glycated hemoglobin in blood, a reagent kit was used (Pointe Scientific, Inc., Canton, MI). Column chromatography of

whole blood using cation-exchange resins results in the separation of a fast-moving elute that is known to be the hemoglobin A1 fraction, containing hemoglobins $A1_{a-c}$ ($HbA1_a$, $HbA1_b$, and $HbA1_c$; Alperin at al., 1979; Gruber and Koets, 1979). The kit used to measure glycated hemoglobin in rat blood is based on the chromatographic separation of the fast-moving, glucose-bound hemoglobin from total hemoglobin. To perform the assay, a hemolyzed preparation of whole blood from each rat was individually mixed continuously for five minutes with a weak binding cation-exchange resin, providing time for the non-glycated hemoglobin ($HbA1_0$) to bind to the resin. After the 5 minutes of mixing, a filter was used for each sample to separate the resin-bound, non-glycated hemoglobin from the non-resin-bound glycated hemoglobin. A spectrophotometer was then used to measure the absorbance at 415 nm of the glycated hemoglobin fraction and the total hemoglobin fraction, and the ratio of these two absorbances gave the percentage glycated hemoglobin.

2.3 Immunohistochemistry

2.3.1 Double immunofluorescence

Rats were anesthetized via intraperitoneal injection of a cocktail (2 ml/kg) containing pentobarbital (12.5 mg/ml) and diazepam (1.25 mg/ml) in sterile saline and transcardially perfused with saline, followed by 4% paraformaldehyde in 0.1 M PBS, pH 7.4. Immediately following perfusion, the lumbar spinal cord was dissected and post-fixed in 4% paraformaldehyde at 4°C for 2 hours, cryoprotected by immersion in 0.1 M PBS (pH 7.4) containing 30% sucrose for 48 hours at 4°C, embedded in OCT medium, and stored at -

20°C. Tissue was cut into 10 µm-thick sections. Some sections were thaw-mounted on Superfrost Plus slides, for AR localization, while others to be examined for COX-2 localization were thaw-mounted on Superfrost slides for later removal in TBS-T for floating immunostaining. To localize COX-2 and AR protein expression, double immunofluorescence was then performed on these sections as follows: Sections were washed for 20 min in 0.05 M TBS buffer, pH 7.6, containing 0.3% Triton. To block non-specific antigen binding sites, sections were blocked with 10% goat serum in TBS-T for 1 hour, then incubated overnight at 4°C with one of the following primary mouse antibodies in TBS-T: neuron-specifc nuclear protein (NeuN; 1:2000, Chemicon MAB377) to visualize neurons (Mullen et al., 1992); glial fibrillary acidic protein (GFAP; 1:2000, Chemicon MAB360) to visualize astrocytes (Dahl and Bignami, 1973); *adenomatous polyposis coli* (APC-Ab7; 1:500, Calbiochem OP80, San Diego, CA) to visualize oligodendrocytes (Bhat et al., 1996); OX42/CD11b (1:50, Biosource ARS1122, Camarillo, CA) to visualize microglia (Robinson et al., 1986); or 40E-C (1:100, Developmental Studies Hybridoma Bank) to visualize radial glia (Alvarez-Buylla et al., 1987). The next day, sections were washed 3 x 8 min in TBS-T, and incubated with a secondary goat anti-mouse antibody conjugated to the fluorochrome Alexa-594 (1:500, Invitrogen) in TBS-T for 2 hours at room temperature. After 3 x 8 min washing in TBS-T, sections were incubated overnight with primary rabbit antibodies against COX-2 (1:400, Cayman Chemical) or AR (1:2000, a gift of Dr. Robert L. Sorenson) at 4°C in TBS-T with 5% goat serum. The next morning, after washing in TBS-T for 3 x

8 min, sections were incubated with a secondary goat anti-rabbit antibody conjugated to the fluorochrome Alexa-488 (1:500, Invitrogen) in TBS-T for 2 hours at room temperature. Finally, sections immunostained for COX-2 were placed flat on Superfrost slides, and cover-slips were mounted on slides with Prolong Gold anti-fade mounting medium (Invitrogen). Sections were then evaluated by confocal microscopy. Control staining was performed by omission of primary antibodies. To minimize differences in day to day staining intensity, spinal cord sections from control and diabetic rats were stained simultaneously under identical conditions.

2.3.2 Fluorescence microscopy

To visualize fluorescently labeled proteins, Olympus BX41 and BX51 epifluorescence microscopes were used. The microscopes each use a halogen lamp to illuminate the sample, along with excitation filters to selectively excite the sample at specified wavelengths and emission filters to selectively allow transmission of certain wavelengths from the excited fluorophores in the sample.

2.3.3 Confocal microscopy

To visualize fluorescently labeled proteins, a Zeiss LSM 510 Axiovert 100 (inverted stage) laser scanning confocal microscope was used. This microscope utilized two lasers: an Argon/Krypton laser, to excite the Alexa-488 fluorochrome (maximum emission at 520 nm, appearing green), and a Helium/Neon laser, to excite the Alexa-594 fluorochrome (maximum emission

at 620 nm, appearing red). Images were taken using a 40X or 60X oil-immersion objective.

2.4 *In vivo* methods

2.4.1 Intrathecal catheters and drug administration

In some studies, rats were treated with drugs delivered to the subarachnoid space surrounding the lumbar enlargement of the spinal cord. This was achieved using chronic indwelling catheters that were constructed using an 8.5 cm piece of PE-08 polyethylene tubing (0.008 inch inner diameter, 0.014 inch outer diameter) fused to a 3 cm piece of PE-10 polyethylene tubing (0.011 inch inner diameter, 0.025 inch outer diameter) so that the interiors of the two tubes were continuous. The point of fusion was covered with a small amount of dental acrylic, creating a bulge to hold the catheter in place at the base of the skull. Catheters were implanted as described by Yaksh and Rudy (1976). Rats were anesthetized with 5% isoflurane and maintained with 3% isoflurane. The back of the skull was shaved and the underlying skin sterilized with betadine. The head was immobilized in a stereotactic frame and an incision approximately 1 cm in length was made in the skin to allow the muscle at the base of the skull to be blunt dissected away, exposing the atlanto-occipital membrane overlying the cisterna magna. A small nick was made in the dura, and the PE-08 end of the catheter was inserted into the subarachnoid space and passed caudally until the dental acrylic bulge was just above the hole in the dura. The PE-10 section of the catheter was then passed through the skin of the forehead and

its end plugged with a small piece of metal wire. Animals were allowed to recover and any showing signs of neurologic dysfunction were killed. After catheter implantation, rats were housed singly to prevent cagemates from chewing implants and used for behavioral testing within 3-5 days.

To perform intrathecal injections, the metal wire plugging the PE-10 section of the catheter was removed, and a syringe was connected to a piece of silicon tubing that was placed onto the protruding end of the catheter, forming a tight seal. All drugs were given in a volume of 10 μL followed by a 10 μL flush of saline. Animals were lightly anesthetized with isoflurane during injections to prevent catheters being pulled out.

2.4.2 Measurement of thermal nociceptive thresholds

Thermal nociceptive testing was conducted with a modified version of the method originally described by Hargreaves et al. (1988). Rats were placed in a plexiglass enclosure on the glass floor of a Thermal Stimulation System (UARD, San Diego, CA), with a surface temperature maintained at 30°C. Rats were allowed to acclimate for 15 min, and a mobile radiant heat source was then positioned to apply heat to the plantar surface of the right hind paw. The latency from the initiation of heating to when the paw was deliberately withdrawn was recorded using an automated timer connected to movement sensors. The right hind paw of each rat was tested three times at 5 min intervals following an initial acclimation test, and the median of the three tests was used for statistical analysis.

The radiant heat source is calibrated to heat the glass above it at a rate of 1 °C per second, and automatically shuts off after 20 sec to prevent tissue injury. On each day that tests were done, the change in temperature of the glass floor over a 20 sec period was recorded to confirm that it increased at the rate of 1 °C per second. This slow rate of heating selectively actives C fibers (Yeomans and Proudfit, 1996).

2.4.3 Measurement of tactile thresholds

Rodents were transferred to a testing cage with a wire mesh bottom and allowed to acclimate for 15 min. Von Frey filaments (Stoelting, Wood Dale, IL) were used to determine the 50% probability mechanical threshold for foot withdrawal. A series of filaments were applied in sequence to the plantar surface of the right hind paw with a pressure that caused the filament to buckle. Lifting of the paw was recorded as a positive response and a lighter filament was chosen for the next measurement. If there was no response after 5 seconds, the next heaviest filament was used afterwards. This method was continued until four measurements had been made after an initial change in the behavior or until five consecutive negative or five consecutive positive responses had occurred. The resulting sequence of positive and negative scores was used to interpolate the 50% probability response threshold as described by Chaplan et al. (1994). Capsaicin depletion of C fibers does not diminish tactile allodynia in diabetic rats, suggesting that it is mediated by abnormal sensory input from Aβ and Aδ fiber afferents (Khan et al. 2002).

2.4.4 Formalin nociceptive assay

A modified version of the formalin test originally described by Dubuisson and Dennis (1977) was used in these studies. In order to maximize behavioral differences between control and diabetic animals, all studies used 0.5% formalin (Calcutt et al. 1995). For rats, 50 µl of 0.5% formalin solution was injected into the dorsum of the hind paw and defined flinches of the injected paw were counted per minute at 5 min intervals during the subsequent 60 min. For mice, 20 µl of 5% formalin solution was injected into the dorsum of the hind paw and nocifensive behavior was quantified as time spent attending to the injected paw (licking, biting, or shaking) per 2 min at 5 min intervals during the subsequent 60 min.

2.4.5 Measurement of sciatic nerve conduction velocities

Rats were anesthetized with 5% isoflurane and maintained with 3% isoflurane. The sciatic nerve was exposed via an incision in the flank followed by separation of underlying musculature by blunt dissection. A thermistor probe was placed adjacent to the nerve and the incision closed. Nerve temperature was maintained at 37°C by a heating lamp and thermal pad connected to a temperature regulator and the thermistor probe. The nerve was stimulated (single 5.0 V, 0.05 ms square wave pulse) by fine needle electrodes at the sciatic notch and then the Achilles tendon, and the evoked electromyogram recorded from the interosseus muscles via two fine needle electrodes. During each stimulation, activation of large motor fibers directly causes a response in the interosseus muscles (M wave), and activation of

large sensory fibers activates, in turn, a spinal monosynaptic reflex that then produces a response in the interosseus muscles (H wave). Because the two stimulation sites are separated by the distance between the sciatic notch and the Achilles tendon, the two M waves and the two H waves are slightly separated in time. The distance between the two sites of stimulation was measured using callipers and conduction velocity calculated as the latency between the A α/β wave peaks of the M wave for large motor fibers or H wave for large sensory fibers, divided by the distance between the two stimulation sites. Measurements were made in triplicate and the median measurement was used for statistical analysis.

2.5 *In vitro* methods

2.5.1 Generation of primary oligodendrocyte cultures

This method, called immunopanning, was based on a protocol kindly provided by Dr. Ben A. Barres. Immunopanning was originally described as a method for fractionation of lymphocytes (Mage et al., 1977; Wysocki and Sato, 1978), and was later adapted for glial cell purification by Barres et al. (1992). This method is also reviewed in detail in Banker and Goslin (1998). The basic premise is to use primary tissue to generate a dissociated suspension of mixed cell types. These dissociated cells are then sequentially passed over a series of “panning dishes,” each of which is coated with a particular antibody. In each dish, cells expressing surface antigens for the antibody coating that dish will stick to the dish and be left behind when the remaining cell suspension is removed from the dish. This allows negative selection to

remove unwanted cells, and a final positive selection step to purify the desired cell type.

To generate primary oligodendrocyte cultures, immunopanning was used to purify O-2A progenitor cells from adult rat spinal cord. O-2A progenitor cells are CNS precursor cells with restricted lineage potential that give rise *in vitro* to either oligodendrocytes or type-2 astrocytes (Raff et al, 1983). Differentiation into oligodendrocytes is the default fate of these O-2A progenitor cells, whereas differentiation into type-2 astrocytes requires the presence of inducing factors found in fetal sera of several species (Raff et al., 1983). O-2A progenitor cells are characterized as having a bipolar or unipolar morphology in culture, and they express a number of unique surface antigens that have been used to generate antibodies which bind specifically to the surface of O-2A progenitor cells, including O4 (Sommer and Schachner, 1981).

To obtain purified populations of O-2A progenitor cells, immunopanning was performed using dishes coated with Ran-2 and O4 antibodies. For each rat to be dissected, 1 Ran-2 dish and 1 O4 dish were prepared. The Ran-2-coated dish was used for negative selection, as the Ran-2 antibody binds to an antigen expressed on the surface of type-1 astrocytes and meningeal cells (Bartlett et al., 1980). The O4 dish was used for positive selection to purify O-2A progenitors. 100 mm Petri dishes were incubated overnight at 4°C with secondary antibodies in 10 ml of 50 mM Tris buffer (pH 9.5). For the Ran-2 dish, the secondary antibody used was affinity-purified polyclonal goat anti-

mouse IgG (heavy + light chain) at 40 μg/ml (Pierce). For the O4 dish, the secondary antibody used was affinity-purified polyclonal goat anti-mouse IgM (μ chain specific) at 10 μg/ml (Pierce). The next morning, each dish was washed 3 times with 10 ml of PBS before primary antibodies were added and allowed to incubate for at least 1 hour at 37°C. The O4 antibody used was a mouse monoclonal antibody (Sigma) prepared at a concentration of 1.25 μg/ml in 10 ml of PBS containing 0.2% BSA (Cohn fraction V, Sigma). The Ran-2 antibody was generated from a hybridoma cell line (American Type Culture Collection). This cell line was originally created by Bartlett et al. (1980) as a hybrid of mouse myeloma cells and spleen cells from mice immunized with enriched preparations of astrocytes. Each Ran-2 dish used for immunopanning contained 3 ml of Ran-2 hybridoma supernatant and 2 ml of PBS containing 1% BSA (Cohn fraction V, Sigma). The BSA was included in primary antibody solutions in order to block nonspecific adherence of dissociated cells to the panning dishes. After incubation with primary antibodies, each dish was rinsed three times with 10 ml PBS.

Spinal cords were removed from rats by hydraulic extrusion, immersed in Dulbecco's phosphate buffered saline without calcium or magnesium (DPBS-CMF) and minced with a scalpel. To begin digestion of the tissue, sterilely prepared collagenase (Sigma) was added at a concentration of 333 collagenase digestion units/ml in DPBS with calcium and magnesium. Minced tissue was incubated with the collagenase for 30 min at 37°C. Papain (Worthington Biochemical Corporation, Lakewood, NJ) was used as a second

digestive enzyme, at a concentration of 30 units per ml of DPBS with calcium and magnesium, and also containing 0.004% DNAse (Sigma). The papain solution was dissolved at 37°C, then activated just before use by adding L-cysteine (Sigma) at a concentration of 0.4 mg/ml papain solution in order to acidify the solution. The pH was then adjusted to 7.4 using 1N NaOH and the solution filtered through a 0.22 μm filter to sterilize. The collagenase solution was drained off the tissue, which was then incubated with the papain solution for 1 hr at 37°C.

After the papain incubation, the tissue was centrifuged for 5 min at 2,000 x g and the supernatant discarded. The pellet was resuspended in 10 ml of a solution consisting of 1.5 mg/ml BSA and 1.5 mg/ml ovomucoid. Ovomucoid is an egg white protein that is produced in chicken oviduct. The ovomucoid solution acts to inhibit activity of the digestive enzymes collagenase and papain and to wash away these enzymes. The tissue was gently serially triturated using #21 and #23 gauge needles, while successfully separated cells were regularly transferred to a 15 ml tube. The accumulated cell suspension was centrifuged for 5 min at 2,000 x g and the supernatant was discarded. The pellet was resuspended in a solution containing 10 mg/ml BSA and 10 mg/ml ovomucoid. The cell suspension was again centrifuged for 5 min at 2,000 x g and the supernatant discarded. The pellet was then resuspended in Dulbecco's Modified Eagle's Medium (DMEM) containing 4.5 g/L D-glucose, 4 mM L-glutamine, and 110 mg/L sodium pyruvate (Invitrogen) plus bovine pancreatic insulin (Sigma) at a concentration of 0.5 μg/ml.

The cell suspension was incubated on the Ran-2 dish for 30 min in an incubator set to 37°C and 10% CO_2. The high concentration of CO_2 was necessary because DMEM is buffered by sodium bicarbonate, and since sodium bicarbonate is weakly basic in solution, CO_2 dissolves into the medium and generates free hydrogen ions that neutralize the pH of the medium. The non-adherent cells were then transferred to the O4 dish and incubated for 45 min at 37°C and 10% CO_2. The non-adherent cells were discarded, and the dish washed 5 times with 10 ml of Leibovitz 15 (L15) medium (Invitrogen), which is a medium that is buffered by phosphates and free-base amino acids and therefore maintains a neutral pH in atmospheric conditions. Adherent O-2A progenitor cells were then removed from the dish by incubating with 10 ml of a trypsin solution (Invitrogen) containing 2.5 g/l of trypsin and 0.4 g/l of EDTA in Hanks' Balanced Salt Solution at 37°C and 2% CO_2 for 15 min. To stop the activity of trypsin, 10 ml of L15 containing 10% fetal calf serum (Invitrogen) was added to the dish, and the solution centrifuged for 5 min at 2,000 x g. The supernatant was discarded and the pellet resuspended in 10 ml DMEM containing 0.5% BSA (Cohn fraction V, Sigma). The cell suspension was centrifuged a final time for 5 min at 2,000 x g, and the pellet resuspended in 2 ml DMEM containing 0.5% BSA (Cohn fraction V, Sigma). A 50 μl aliquot of the suspension was removed and combined with 50 μl of Trypan blue (Invitrogen) for a cell count. Trypan blue was used to distinguish viable from non-viable cells, as only viable cells with intact membranes exclude the Trypan blue dye. Cells were counted using a hemacytometer.

The immunopanning method described here generated approximately 20,000 O-2A progenitor cells from a single adult rat spinal cord. A previous study reported isolation of approximately 15,000 O-2A progenitor cells per individual adult rat spinal cord (Engel and Wolswijk, 1996). The discrepancy in progenitor cell numbers may be due to use of different rat strains, or it may be because the prior study did not use immunopanning to isolate O-2A progenitor cells. Instead, Engel and Wolswijk (1996) studied spinal cord cells that adhered to coverslips, which included O-2A cells, microglia, type-1 astrocytes, Schwann cells, and other unidentified cell types.

Purified O-2A progenitor cells were cultured on 12 mm diameter round glass coverslips (Fisher Scientific) at a density of approximately 5,000 viable cells per coverslip, and each coverslip was placed in a well of a 24 well microplate. To provide a substrate for attachment, the coverslips were pre-coated with poly-d-lysine (PDL) by soaking them in a 10 μg/ml PDL solution prepared in water. The PDL (Sigma) was cell culture grade and 70 – 150 kDa in size. O-2A progenitor cells were allowed to settle on the coverslips for 10 min before adding 500 μl of culture medium and transferring to an incubator set to 37°C and 10% CO_2. The culture medium used was a chemically defined serum-free medium originally described by Bottenstein and Sato (Bottenstein and Sato, 1979). This medium is specially formulated for growing primary cultures of CNS cells in the absence of animal serum, which is advantageous because serum is a potential vector for many pathogens and its ability to support cell growth can vary widely depending on the original animal

source. The Bottenstein-Sato medium consisted of the following dissolved in DMEM: transferrin (100 μg/ml, Sigma); crystalline BSA (Cohn fraction V, 100 μg/ml, Sigma); progesterone (60 ng/ml, Sigma); putrescine (16 μg/ml, Sigma); sodium selenite (40 ng/ml, Sigma); thyroxine (40 ng/ml, Sigma); tri-iodothyronine (30 ng/ml, Sigma); and a cocktail of penicillin, streptomycin, and neomycin (50 μg/ml, 50 μg/ml, and 100 μg/ml, respectively, Invitrogen). Additionally, the medium contained NT-3 (1 ng/ml), N-acetyl cysteine (NAC; 63 μg/ml, Sigma), and insulin (5 μg/ml, bovine insulin from Sigma). Insulin and NT-3 are important growth factors necessary for survival of cultured oligodendrocytes (Barres et al., 1993), and NAC is necessary for the cells to maintain high intracellular glutathione levels (Almazan et al., 2000).

Cells were checked daily and a 50% medium change was performed every other day. Daily, fresh insulin (5 μg/ml) and NT-3 (1 ng/ml) were added to the culture medium.

2.5.2 Immunostaining of primary oligodendrocyte cultures

To immunostain oligodendrocytes growing on PDL-coated glass coverslips, the cells were first fixed for 10 min in 4% paraformaldehyde then washed 3 times in PBS. Cells were then incubated for 2 hr at room temperature with 50% normal goat serum diluted in an antibody buffer consisting of 150 mM NaCl, 50 mM Tris base, 1% BSA, and 100 mM L-lysine, diluted in distilled water and pH adjusted to 7.4. This blocking buffer was used to block non-specific binding sites on the cells. The blocking buffer also contained 0.4% Triton X-100 to permeabilize the cell membranes for

subsequent antibody penetration. The cells were then rinsed 3 times with PBS and incubated overnight at room temperature with a primary antibody diluted in the antibody buffer containing 10% normal goat serum and 0.08% Triton X-100. The following primary antibodies were used: rat monoclonal to myelin basic protein (MBP; 1:100, Abcam); rabbit polyclonal to AR (1:1000, gift of Dr. R. Sorenson); and rabbit polyclonal to COX-2 (1:200, Cayman Chemical). The next morning, cells were washed 3 times with PBS and incubated for 2 hours at room temperature with an appropriate secondary antibody diluted at 1:500 in the antibody buffer. The secondary antibodies used were goat anti-rabbit, conjugated with an Alexa 488 fluorophore (Invitrogen); goat anti-rat, conjugated with an Alexa 488 fluorophore (Invitrogen); or goat anti-rat, conjugated with an Alexa 594 fluorophore (Invitrogen). The Alexa 488 fluorophore emits fluorescence that appears green, while the Alexa 594 fluorophore emits fluorescence that appears red. Cells were then washed 3 times with PBS and incubated with the nuclear stain DAPI (Invitrogen) diluted in the antibody buffer (1:1000) for 30 min at room temperature. Cells were washed 3 times in PBS and post-fixed for 10 min at room temperature in ice-cold acid-alcohol, containing 5% glacial acetic acid and 95% ethanol. This post-fix decreases the chance that cells will detach from the coverslip when mounting the coverslip on a glass microscope slide. The cells were then washed 3 times in PBS and finally mounted on a glass microscope slide in Prolong Gold Antifade mounting medium (Invitrogen).

For double-staining, cells were first incubated (as described above) with antibodies against MBP overnight, then with goat anti-rat the next morning, followed by either COX-2 or AR antibodies for a second night, then goat anti-rabbit the next morning, along with DAPI staining, post-fixing, and mounting. Control staining was performed by omitting the primary antibodies and it was confirmed that incubating cells only with secondary antibodies produced no fluorescent labeling.

2.6 Statistical methods

Tactile 50% probability paw withdrawal thresholds obtained from rats are presented as group medians because these data do not have normal distributions. The 50% probability paw withdrawal threshold has an upper limit of 15 g, and many control rats did not respond to any of the Von Frey filaments used for testing. Any such rat was assigned a 50% probability withdrawal threshold of 15 g for statistical analysis, which was performed using a Kruskal-Wallis test with Dunn's *post-hoc* test.

All other data are presented as group mean ± SEM and were analyzed using parametric statistics. For studies including only two groups, a Student's unpaired t-test was used. A one-way ANOVA was used for comparing three or more groups, with Dunnett's or Student Newman-Keul's *post-hoc* tests, depending on whether comparisons were made against a single group or between all pairs of groups.

CHAPTER 3 – COMPARISON OF EXPERIMENTAL RAT AND MOUSE MODELS OF DIABETIC NEUROPATHY

3.1 Introduction

3.1.1 Experimentally diabetic rodents: relevance to human diabetic neuropathy

Beginning with early observations of structural and functional nervous system abnormalities in experimentally diabetic rats and mice, studies over the last 50 years have generated numerous hypotheses concerning how chronic hyperglycemia affects the nervous system. Understanding the pathogenesis of diabetic neuropathy is a central goal of this research, and many studies therefore focus on sensory abnormalities caused by diabetes, including pain. Modeling painful diabetic neuropathy with experimentally diabetic rodents is constrained by the question of how to assess pain in animals. In patients with diabetic neuropathy, spontaneous pain is particularly problematic but there is no way to reliably measure the presence of spontaneous pain in animals. Many studies therefore focus on measuring behavioral responses to mechanical, thermal, or chemical stimuli. Results with these tests suggest that pain perception is altered in diabetic rodents, which may make them useful models of painful diabetic neuropathy in humans (for review, see Calcutt, 2002).

3.1.2 Behavioral changes in STZ-diabetic rats and mice

Rats with a short duration (four weeks) of diabetes tend to be hyper-responsive to stimulation of the paw. Using von Frey filaments to measure tactile paw withdrawal thresholds, control rats generally do not respond to the

range of filaments presented (2 – 15 g), while diabetic rats respond at a force of approximately 3-5 grams (Calcutt and Chaplan, 1997). Thermal paw withdrawal latencies of diabetic rats reveal an early (2-3 weeks post-STZ) transient hyperalgesia that progresses to hypoalgesia by eight to twelve weeks post-STZ (Calcutt et al. 2000a; Calcutt et al. 2004). Thus these acute nociceptive tests reveal behavior in short-term diabetic rats that is suggestive of pain.

The formalin test is a model of chemical nociception that involves two phases of paw flinching, separated by a quiescent phase (Dubuisson and Dennis, 1977). This test is distinct from tactile or thermal testing in that its first phase reflects acute nociception, while the second phase reflects changes in excitability of the central nervous system. The spinal electrophysiology and pharmacology that underlie the flinching behavior are well-characterized. Flinching correlates with activity in spinal dorsal horn neurons (Dickenson and Sullivan, 1987), and the first phase of neuronal activity is directly caused by an initial period of rapid action potential firing in primary afferent fibers (Puig and Sorkin, 1996). This persistent activity causes a sustained release of substance P and the excitatory amino acids glutamate and aspartate, which produce a lasting depolarization of dorsal horn neurons (Urban and Randic, 1984; Malmberg and Yaksh, 1995). This depolarization allows for release of the magnesium block on NMDA receptors, resulting in calcium influx into dorsal horn neurons that in turn lowers thresholds for action potential firing (Haley et al., 1990; Woolf and Thompson, 1991; Yaksh et al., 1999). The

second phase of flinching results because an ongoing low level of activity in small afferent fibers is sufficient to cause the sensitized dorsal horn neurons to fire action potentials (Puig and Sorkin, 1996).

The flinching response of rats to paw formalin injection depends on the concentration of formalin. Use of 5% formalin elicits maximal flinching from control rats during both phases I and II, at a level that is matched by diabetic rats. Consequently, at this concentration of formalin the only behavioral difference between control and diabetic rats is that diabetic rats continue to flinch during the normally quiet quiescent period (Malmberg et al. 1993). If 0.5% formalin is used instead, diabetic rats exhibit increased flinching as compared with controls during the quiescent phase and phase II (Calcutt et al. 1995). Using 0.5% formalin is therefore advantageous because hyperalgesia in the diabetic rat is not obscured by maximal flinching from control and diabetic rats.

There is some disagreement in the literature regarding how diabetes alters responses to behavioral sensory tests in mice, with some reports indicating hyper-sensitivity of the paw and some indicating a general reduction of sensation (Christianson et al. 2003b; Rashid et al., 2003). This may be due to differences in genetic strains of mice and testing methods. In light of this disagreement, experiments were begun to first characterize how diabetes alters behavioral responses in C57Bl/6 mice using our testing methods. It has been reported that, in contrast to diabetic rats, diabetic mice do not exhibit hyperalgesia in response to paw formalin injection (Kamei et al., 1993).

Studies in diabetic rats have suggested a possible link between exaggerated spinal prostanoid signalling and formalin hyperalgesia (Freshwater et al., 2002). The present experiments were designed to confirm that diabetic mice lack formalin hyperalgesia and to study whether this might be due to normal spinal COX-2 expression. Finally, we also used the ARI Statil to assess whether exaggerated polyol pathway flux has a causal role in any of the behavioral abnormalities observed in diabetic mice.

3.2 Methods

3.2.1 Experimental organization

In a preliminary study, it was first determined how to effectively induce diabetes in a mouse using STZ. Published studies typically use one of two methods: a single, high-dose injection of STZ (Rerup and Tarding, 1969), or a series of low-dose injections of STZ given over 5 days (Like and Rossini, 1976; Cossel et al., 1985). The first of these offers the advantage of quicker induction of diabetes, but carries a greater risk of animals dying during the first few days after STZ injection (for review, see Wilson and Leiter, 1990). A group of C57Bl/6 mice was used to test the first, high-dose (200 mg/kg STZ given by intraperitoneal injection), method, and monitored for four weeks after injection of STZ.

A separate second study was designed to measure behavioral abnormalities in STZ-diabetic mice and to test whether these would be prevented by treatment with the ARI Statil. Male C57Bl/6 mice were randomly assigned to one of four groups: control, untreated diabetic, and control or

diabetic treated daily by oral gavage with Statil. Mice were made diabetic with a single intraperitoneal injection of 200 mg STZ per kg body weight, and hyperglycemia was confirmed three days later using blood taken by tail-prick and a strip-operated reflectance meter (see section 2.1). Statil treatment began on the same day that hyperglycemia was confirmed, at 50 mg Statil per kg body weight. The Statil was suspended in water and Tween-20. All mice were maintained for four weeks. At the end of the study, all mice underwent tactile and thermal testing, and half from each group were then killed for measurement of COX-2 protein in the lumbar spinal cord. The other half of mice in each group underwent 5% formalin testing before being killed.

3.2.2 Measurement of tactile thresholds

Von Frey filaments ranging from 0.6 - 5 g were used to assess 50% probability tactile withdrawal thresholds using the 'up-down' method as described by Chaplan et al. (1994; see section 2.4.3).

3.2.3 Measurement of thermal nociceptive thresholds

Thermal paw withdrawal latencies were measured by stimulating the center of the plantar surface of the paw with a radiant heat source (light) applied through a glass surface (Hargreaves et al., 1988), and measuring how much time elapsed before the mouse lifted its paw (see section 2.4.2).

3.2.4 Formalin nociceptive testing

Formalin testing was performed on mice by injecting 20 µl of 5% formalin into the dorsal surface of the hindpaw, and nocifensive behavior was quantified as time spent attending to the injected paw (licking, biting, or

shaking) per 2 min at 5 min intervals during the subsequent 60 min (see section 2.4.4).

3.3 Results

3.3.1 Effect of Statil treatment on behavioral abnormalities and elevated spinal COX-2 in STZ-diabetic mice

In the first study, we observed that mice injected with a single dose of STZ rapidly became hyperglycemic, exhibiting plasma glucose levels of at least 15 mmol/L. Over the course of four weeks it was observed that many mice lost enough weight to need low-dose insulin therapy to prevent cachexia. Because the single, high-dose injection of STZ successfully induced significant hyperglycemia in the majority of injected mice, this method was used for the second study. Because of the prevalence of rapid weight loss in these mice, mice in the second study were weighed daily to ensure low-dose insulin therapy would be started before any severe weight-loss or muscle wasting occurred.

At the start of the second study, the average weight of the mice was 24 ± 0.3 g. At the conclusion of the study, diabetic mice were hyperglycemic and exhibited weight loss in comparison with control mice (Table 3.1). Neither hyperglycemia nor weight loss were affected by Statil treatment (Table 3.1). In contrast to the tactile allodynia and thermal hyperalgesia observed in rats with four weeks of diabetes (Calcutt and Chaplan, 1997; Calcutt et al., 2004), mice with four weeks of diabetes had elevated tactile response thresholds and thermal withdrawal latencies, indicating sensory loss (Figures 3.1 and 3.2).

Statil treatment significantly reduced tactile response thresholds in diabetic mice, effectively restoring normal sensation in this test (Figure 3.1). The mean thermal paw withdrawal latency of statil-treated diabetic mice was not significantly different from either that of control mice or untreated diabetic mice, suggesting a tendency toward treatment efficacy (Figure 3.2).

Control mice subjected to 5% formalin testing exhibited a normal biphasic response curve, but diabetic mice displayed nocifensive behavior solely during phase I and no behavioral response at any later time point (Figure 3.3). This suggests that diabetic mice had a normal acute pain response to the formalin injection, but lacked the spinal sensitization necessary for the second behavioral phase. This was not affected by Statil treatment (Figure 3.3).

Measurement of spinal COX-2 protein revealed a threefold-increased level of expression in diabetic mouse spinal cord as compared to control mouse spinal cord, which was unaffected by Statil treatment (Figure 3.4).

3.4 Discussion

Behavioral testing of C57Bl/6 mice with four weeks of diabetes revealed a general trend of sensory loss, as these animals exhibited elevated tactile 50% probability paw withdrawal thresholds, prolonged thermal paw withdrawal latencies, and no response during phase II of the formalin test. These findings are in agreement with recently published studies involving STZ-diabetic C57Bl/6 mice, as it has been published that in this strain of mice, diabetes reduces responsiveness to tactile von Frey stimulation and formalin injection

(Christianson et al. 2003b). These data suggest that STZ-induced neuropathy progresses differently in mice than in rats, because rats with four weeks of STZ-induced diabetes show enhanced behavioral responses to tactile, thermal and formalin testing (Calcutt et al., 1995; Calcutt and Chaplan, 1997; Calcutt et al., 2004). As it has been observed that rats with longer durations of diabetes transition from thermal hyperalgesia to thermal hypoalgesia (reviewed by Calcutt, 2004), it may appear that neuropathy simply progresses more rapidly in diabetic mice than in diabetic rats. However, our group has observed that STZ-diabetic mice exhibit thermal hypoalgesia as early as one week after the induction of diabetes (Beiswenger KK, personal communication), which may suggest that sensory neuropathy is mechanistically different between diabetic rats and mice.

The major purpose for conducting these studies was to determine if, as observed with diabetic rats, diabetic mice exhibit co-incident hyperalgesia to paw formalin injection and elevated spinal COX-2 protein. Surprisingly, diabetic mice exhibited significantly elevated spinal COX-2 protein, yet completely lacked phase II of the formalin test. A lack of response during phase II despite a normal phase I response indicates that these mice lacked the spinal sensitization necessary to cause the second behavioral phase. This could be due to impaired spinal release of the sensitizing compounds PGE_2 and nitric oxide (Haley et al., 1992; Malmberg and Yaksh, 1995), or possibly to reduced C fiber input into the spinal cord during phase I. Measurement of intra-epidermal nerve fiber density in these diabetic mice indicated a

significant loss of innervation (Beiswenger KK, personal communication) – a loss that is not seen in rats with a similarly short duration (five weeks) of STZ-induced diabetes (Bianchi et al., 2004). However, the observation that phase I behavior in diabetic mice was normal indicates that primary afferents were sufficiently functional to transmit information about peripheral tissue injury to the CNS. The dying back of the primary afferents may nevertheless indicate general cellular dysfunction, which could render these DRG neurons incapable of the persistent, high level of action potential firing necessary to induce spinal sensitization. Whether due to reduced primary afferent function or impaired spinal release of sensitizing molecules, it is clear that diabetic mice do not develop spinal sensitization in response to paw formalin injection. Four weeks of diabetes in C57Bl/6 mice is sufficient to induce significant loss of intraepidermal innervation and corresponding sensory loss, and therefore C57Bl/6 mice with four weeks of STZ-induced diabetes are not an appropriate model of painful diabetic neuropathy.

Results from diabetic mice treated with Statil indicate that ARI treatment prevented the loss of normal tactile thresholds and tended to decrease thermal paw withdrawal latencies, but did not affect the lack of nocifensive behavior during phase II of the formalin test or elevated spinal COX-2 protein. These data suggest that blocking exaggerated AR activity had a selective effect on Aβ fibers, which did not affect spinal upregulation of COX-2 or the lack of spinal sensitization in response to paw formalin injection. A selective protective effect of ARI treatment on Aβ fibers is in agreement with the

observation that in the peripheral nerves, AR is most abundantly expressed in myelinating Schwann cells (Ludvigson and Sorenson; Powell et al., 1991; Jiang et al., 2006).

Limited or lack of efficacy of ARI treatment on thermal and formalin testing may indicate that the dose of Statil given (50 mg/kg) was insufficient. Diabetic mice have exaggerated polyol pathway flux in their peripheral nerves despite a lack of accumulation of polyol end-products (Calcutt et al., 1988). Pharmacological studies have suggested that achieving a full block of exaggerated polyol pathway flux may require a ten-fold higher dose of any particular ARI as would be needed to prevent polyol product accumulation (Oates et al., 2006). Treating diabetic mice with a higher dose of Statil might effectively prevent loss of thermal sensation and loss of the second phase of flinching in the formalin test. An alternative possibility is that limited or lack of efficacy of ARI treatment on thermal and formalin testing may reflect the inability of Statil to access the appropriate site of action. For example, the loss of phase II behavior during the formalin test in diabetic mice may reflect changes in the biochemistry of the spinal cord. If this were the case, a systemically delivered ARI might have to cross the blood brain barrier in order to prevent the loss of phase II, and it is unknown whether Statil can cross the blood brain barrier.

In conclusion, STZ-diabetic C57Bl/6 mice are useful as a model of the sensory loss that is often seen in diabetic patients, but are not a good model for using formalin testing to investigate spinal sensitization in diabetes. In light

of this finding, all subsequent studies in this thesis used STZ-diabetic rats instead of mice.

Figure 3.1.

Tactile 50% probability paw withdrawal thresholds (PWT) in control, untreated diabetic, and Statil-treated diabetic mice.

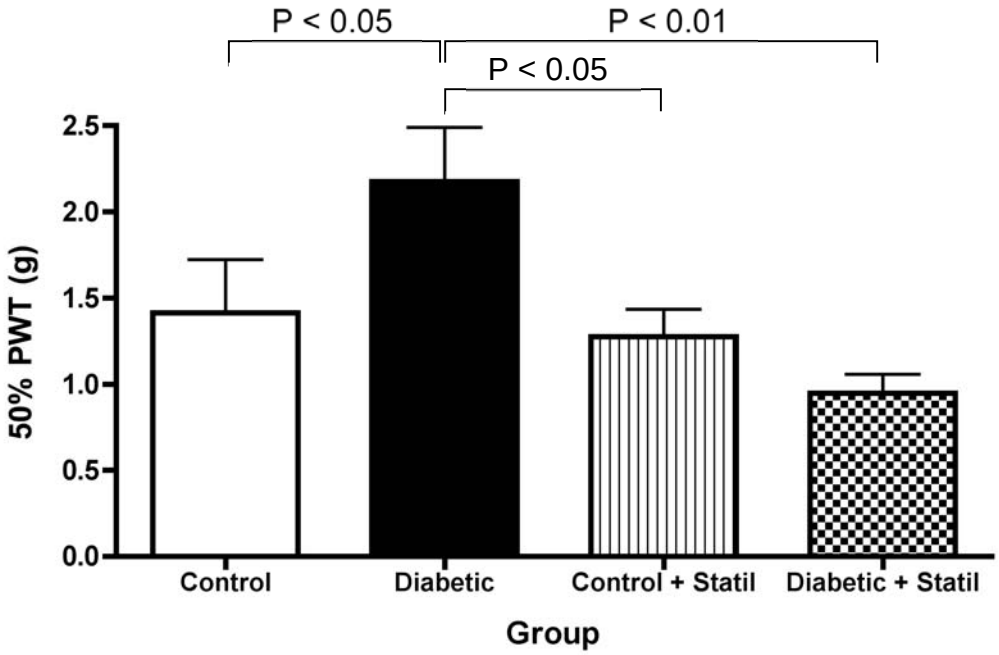


Figure 3.1. Diabetic mice exhibited tactile sensory loss, which was prevented by 4 weeks of treatment with Statil. Data are presented as group mean ± SEM, N = 7 - 8 per group, statistical comparison by one-way ANOVA with Student-Neuman-Keuls' *post-hoc* test.

Figure 3.2.

Thermal paw withdrawal latency (PWL) in control, untreated diabetic, and Statil-treated diabetic mice.

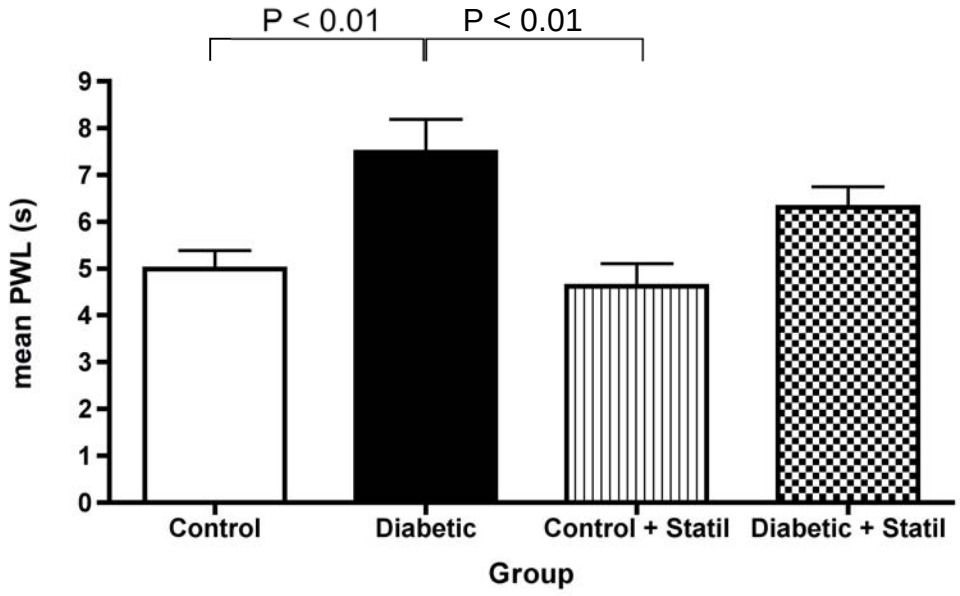


Figure 3.2. Diabetic mice exhibited thermal sensory loss. There was a trend toward restoration of normal sensation in Statil-treated diabetic mice, as the mean paw withdrawal latency for these mice was not significantly different from control mice or untreated diabetic mice. Data are presented as group mean ± SEM, N = 7 - 8 per group, statistical comparison by one-way ANOVA with Student-Neuman-Keuls' *post-hoc* test.

Figure 3.3.

Flinching response to 5% paw formalin injection in control, untreated diabetic, and Statil-treated diabetic mice.

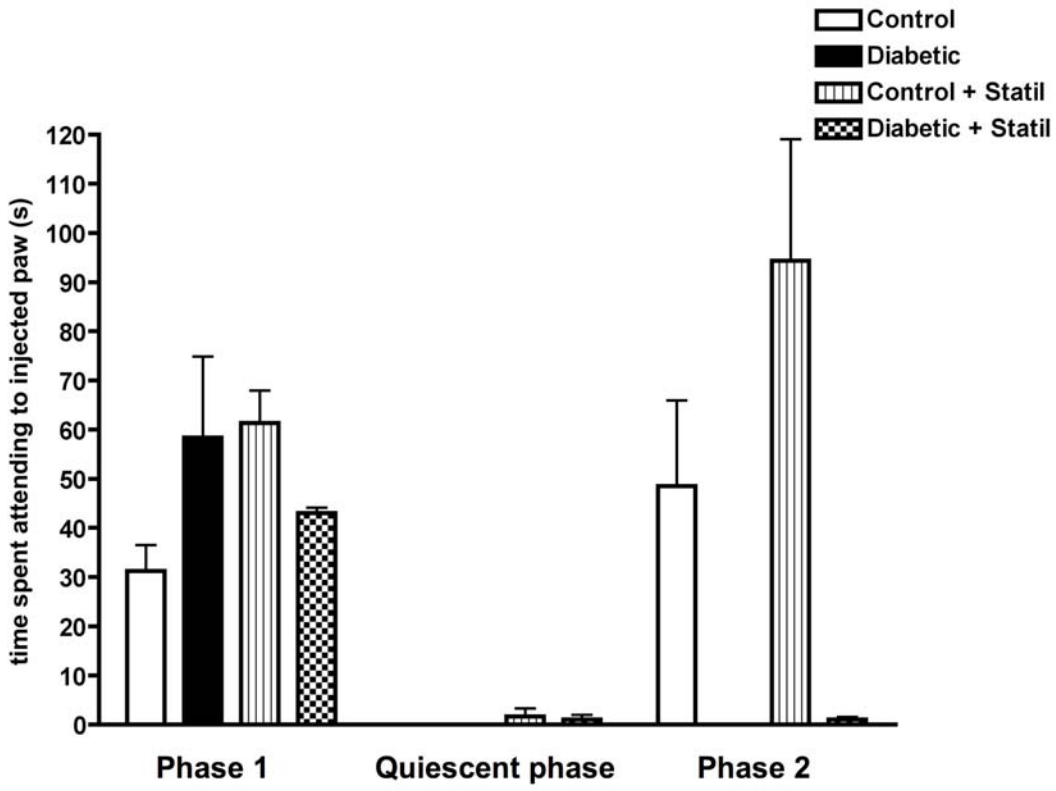


Figure 3.3. Diabetic mice exhibited no flinching during phase II of testing with 5% formalin. This was not prevented by 4 weeks of treatment with Statil. Data are presented as group mean ± SEM, N = 3 - 4 per group.

Figure 3.4.

Spinal COX-2 protein measured in control, untreated diabetic, and Statil-treated diabetic mice.

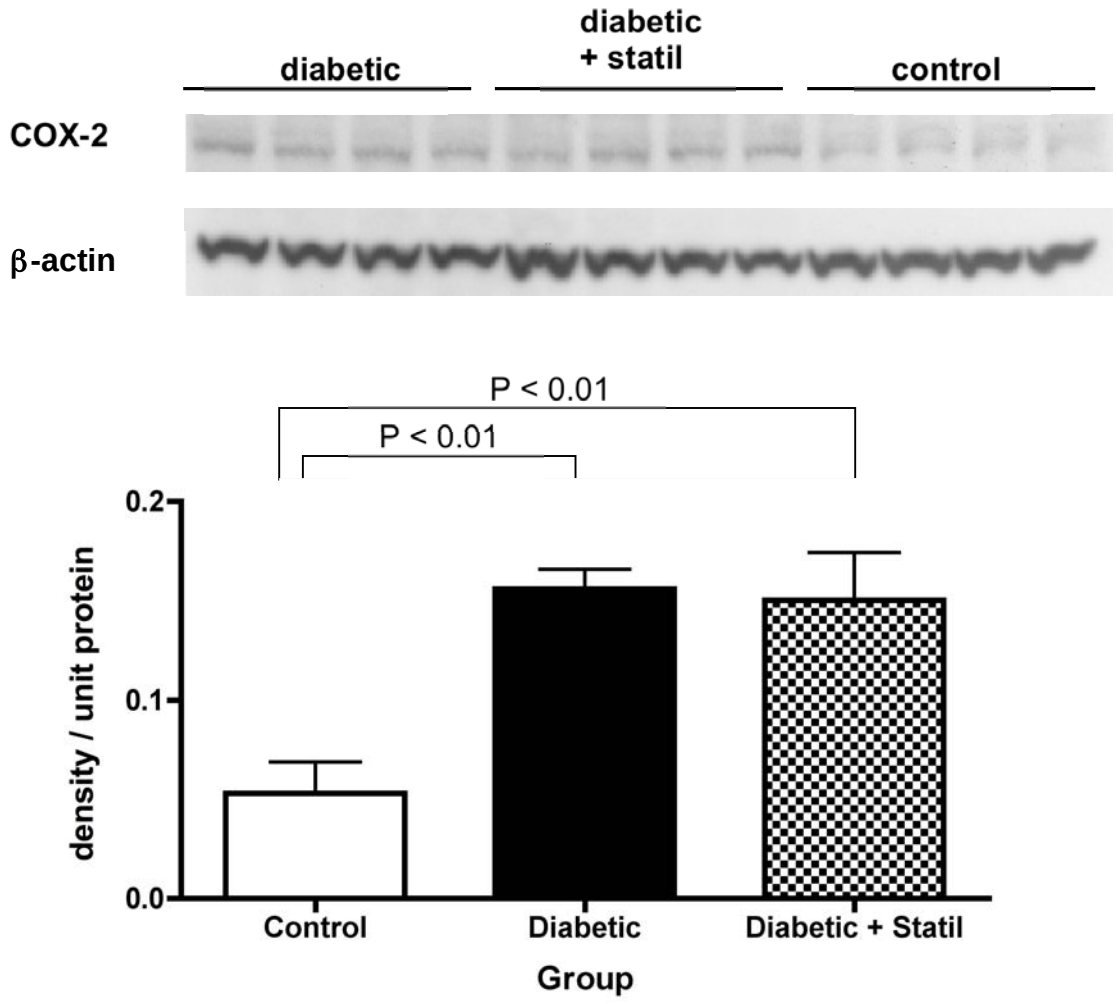


Figure 3.4. Diabetic mice showed a threefold increase in spinal COX-2 protein. This was not prevented by 4 weeks of treatment with Statil. Data are presented as group mean ± SEM, N = 4 per group, statistical comparison by one-way ANOVA with Student-Neuman-Keuls' *post-hoc* test. Western Blot values are normalized to β-actin.

Table 3.1.

Body weights and blood glucose levels for mice used to study behavior and spinal COX-2 levels.

Group	Body weight (g)	Blood glucose (mM)
Control (N = 8)	31 ± 1 [a]	All < 15
Diabetic (N = 7)	19 ± 1 [b]	All > 15
Control + Statil (N = 8)	29 ± 1 [a]	All < 15
Diabetic + Statil (N = 7)	19 ± 1 [b]	All > 15

Data are group mean ± SEM, except blood glucose levels. Statistical analysis of body weights was performed using a one-way ANOVA with Newman-Keuls' *post-hoc* test. [a] vs. [b], $P < 0.001$.

CHAPTER 4 – ROLE OF ELEVATED SPINAL CYCLOOXYGENASE-2 IN EXPERIMENTAL PAINFUL DIABETIC NEUROPATHY

4.1 Introduction

4.1.1 Exaggerated peripheral activity or CNS amplification in diabetic rats

A possible explanation of hyperalgesia and allodynia in experimental diabetes is the presence of exaggerated peripheral nociceptive input to the spinal cord. As discussed in section 1.3.2, electrophysiological studies of diabetes-induced changes in PNS activity have reported mixed results. Additionally, one study reported that compared with controls, DRG neurons from diabetic rats express higher levels of mRNA and protein for several types of sodium channels (Craner et al., 2002). The relevance of excessive sodium currents to pain in diabetes is supported by the finding that systemic lidocaine alleviates allodynia in diabetic rats (Calcutt et al., 1996). However, a separate study reported that mRNA for tetrodotoxin-resistant sodium channels is decreased in DRG neurons from diabetic rats (Okuse et al., 1997). To date, there is still no firm answer to whether diabetes increases input from the peripheral nerves to the spinal cord.

To some extent, this question has been superseded by studies directly measuring spinal release of excitatory neurotransmitters in response to paw formalin injection, as discussed in section 1.3.2, which suggest a role for spinal or supraspinal amplification of nociceptive processing in diabetic formalin hyperalgesia. Supraspinal amplification of nociceptive signals is beyond the scope of this dissertation. We have chosen to focus on spinal

amplification of nociceptive information because of evidence suggesting a biochemical sensitivity in the diabetic spinal cord. Diabetic rats have an increased number of binding sites for substance P in the dorsal spinal cord (Kamei et al., 1990), and spinal delivery of substance P produces a transient thermal hyperalgesia that is prolonged in diabetic rats relative to controls (Calcutt et al., 2000a). These data suggest that diabetes causes a spinal sensitivity to substance P.

The major goal of this dissertation has been to study how diabetes changes the biochemistry of the spinal cord to produce sensitization that leads to exaggerated pain behavior in the formalin test. The validity of using this paradigm to model painful diabetic neuropathy in human patients is supported by the observations that gabapentin, morphine, and benfotiamine alleviate painful diabetic neuropathy in humans (Backonja et al., 1998; Gilron et al., 2005; Haupt et al., 2005) and formalin hyperalgesia in diabetic rats (Courteix at al., 1998; Cesena and Calcutt, 1999; Sanchez-Ramirez et al., 2006).

4.1.2 The spinal cord as a site of amplified nociceptive processing

Previous work has shown that rats with four weeks of diabetes have a three-fold increase in spinal COX-2 protein (Freshwater et al., 2002). COX-2 catalyzes the production of PGE_2, an inflammatory lipid that is secreted in the spinal parenchyma and has a crucial role in the induction of spinal sensitization following injection of formalin into a rat's paw (Malmberg and Yaksh, 1995). This spinal sensitization, which develops following sustained C fiber activity during phase I and the quiescent phase of the formalin test,

primes the central nervous system to become hyper-responsive to nociceptive input, producing a large amount of flinching during phase II of the test despite the observation that C fiber activity during phase II is significantly lower than during phase I (Puig and Sorkin, 1996). The importance of spinal prostanoid signalling in causing the second phase of flinching, taken together with the observation that diabetic rats exhibit both elevated spinal COX-2 and hyperalgesia to paw formalin injection (Malmberg et al., 1993; Calcutt et al., 1995; Freshwater et al., 2002), suggests that excess COX-2 in the diabetic spinal cord may play a causal role in the hyperalgesia to paw formalin injection observed in diabetic rats. To test this possibility, we first determined whether hyperalgesia to paw formalin injection and elevated spinal COX-2 expression are co-incident in rats with different durations of diabetes. A second experiment was designed to test whether spinal COX-2 activity has a causal role in the hyperalgesia to paw formalin injection observed in diabetic rats.

4.2 Methods

4.2.1 Experimental organization

In a first study, eight groups of rats were used in parallel to separately measure the duration of diabetes necessary to induce changes in spinal COX-2 expression or changes in behavioral responses to paw formalin injection. COX-2 is an enzyme that undergoes use-inactivation (Smith and Lands, 1972; Kulmacz and Lands, 1983). It is not known how inactivated COX-2 protein is targeted for intracellular degradation, or whether there is intracellular accumulation of inactivated COX-2 protein (for review, see Fitzpatrick and

Soberman, 2001), so that COX-2 protein measured by Western blot may not necessarily be enzymatically active. We therefore planned to measure both spinal COX-2 protein and activity levels at different durations of diabetes. This required that formalin testing be performed on separate rats in order to avoid a potentially confounding induction of spinal COX-2 activity by paw injection of formalin. Six of the eight groups of rats were made diabetic with a single intraperitoneal injection of STZ (see section 2.1). One, two, and four weeks after the induction of diabetes, one group of diabetic rats was killed for measurement of spinal COX-2 protein and activity levels and one group of diabetic rats underwent 0.5% formalin testing (see sections 2.2.3, 2.2.4, and 2.4.4). The two groups of non-diabetic rats were used as controls for 0.5% formalin testing and spinal COX-2 expression (see sections 2.2.3, 2.2.4, and 2.4.4).

In a second study, we tested whether intrathecal treatment with the selective COX-2 inhibitor SC-58125 (Seibert et al., 1994; Cayman Chemical) can inhibit 5% formalin flinching in control rats. There is no published data documenting the ability of this inhibitor to affect behavioral responses to formalin in naïve rats. Six control rats were implanted with intrathecal catheters (see section 2.4.1), and allowed to recover for 3 – 5 days before undergoing 5% formalin testing (section 2.4.4). We ued 5% formalin for these rats because control rats exhibit only a minor second phase of flinching in response to paw injection of 0.5% formalin (Cesena and Calcutt, 1999). The rats were randomly assigned to receive by intrathecal injection (10 min before

paw formalin injection) either 20 μg of the selective COX-2 inhibitor SC-58125, dissolved in 10 μl of 10% dimethylformamide and 5% Tween-80 in saline, or 10 μl of the SC-58125 vehicle, consisting of 10% dimethylformamide and 5% Tween-80 in saline. After intrathecal injection of drug or vehicle, 10 μl of saline was injected to flush the drug or vehicle out of the catheter and into the subarachnoid space (section 2.4.1). Paw formalin injection was given 10 min after the intrathecal drug delivery. The dose for SC-58125 was chosen based on a previous study in which intrathecal treatment of control rats with SC-58125 alleviated intrathecal substance P-induced thermal hyperalgesia (Yaksh et al., 2001). For purposes of data analysis, phase II was divided into phases IIA and IIB because of previous data indicating that COX inhibition in control rats subjected to 5% formalin testing selectively reduced flinching during the first 30 min of phase II (Malmberg and Yaksh, 1994). Comparisons of behavior during each phase were made by summing the flinches recorded at measurement points within the phase. For each phase, the sum of flinches for vehicle-treated and SC-58125-treated rats were compared with an unpaired t-test.

In a third study, five groups of rats were made diabetic with a single intraperitoneal injection of STZ (see section 2.1). Diabetic rats, along with a group of age-matched control rats, were maintained for four weeks, at which point four of the groups of diabetic rats were implanted with intrathecal catheters (see section 2.4.1). Catheterized rats were allowed to recover for 3 – 5 days before use. All rats underwent 0.5% formalin testing (section 2.4.4).

The control rats and the non-catheterized diabetic rats were untreated before formalin testing. The catheterized diabetic rats were randomly assigned to receive one of the following by intrathecal injection, 10 min before paw formalin injection (see section 2.4.1): 65 μg of the NSAID indomethacin (Cayman Chemical, Ann Arbor, MI), a non-selective inhibitor of COX-1 and COX-2, dissolved in 10 μl of 20 mmol/l NaOH and 280 mmol/l D-glucose in distilled water; 10 μl of the indomethacin vehicle, consisting of 20 mmol/l NaOH and 280 mmol/l D-glucose in distilled water; 20 μg of the selective COX-2 inhibitor SC-58125 (Cayman Chemical), dissolved in 10 μl of 10% dimethylformamide and 5% Tween-80 in saline; or 10 μl of the SC-58125 vehicle, consisting of 10% dimethylformamide and 5% Tween-80 in saline. After intrathecal injection of drug or vehicle, 10 μl of saline was injected to flush the drug or vehicle out of the catheter and into the subarachnoid space (section 2.4.1). The dose and vehicle for indomethacin were chosen based on a previous study in which intrathecal treatment of diabetic rats with indomethacin alleviated hyperalgesia to paw injection of 0.5% formalin (Freshwater et al., 2002). The dose for SC-58125 was chosen based a previous study in which intrathecal treatment of control rats with SC-58125 alleviated intrathecal substance P-induced thermal hyperalgesia (Yaksh et al., 2001). Comparisons of behavior during each phase of the formalin test were made by summing the flinches recorded at measurement points within the phase.

4.2.2 Formalin testing

Rats were injected with 50 μl of 0.5% or 5% formalin in the dorsum of the hindpaw. For purposes of data analysis, the phases of flinching were defined as follows: phase I, 1-2 min and 5-6 min post-formalin injection; quiescent phase,10-11 min and 15-16 min post-formalin injection; phase IIA, 20-21 min, 25-26 min, 30-31 min, 35-36 min, 40-41 min, and 45-46 min post-formalin injection; and phase IIB, 50-51 min, 55-56 min, and 60-61 min post-formalin injection.

4.3 Results

4.3.1 Changes in spinal COX-2 protein and enzymatic activity and in formalin-evoked behavior after one, two, or four weeks of diabetes

COX-2 protein levels are elevated in the spinal cord of rats with four weeks of diabetes (Freshwater et al., 2002). We found that one week of diabetes induced a significant increase in spinal COX-2 protein that was still evident after two or four weeks of diabetes (Figure 4.1). Similarly, rats with one week of diabetes exhibited significantly elevated spinal COX-2 activity compared with control rats, and this was maintained through four weeks of diabetes (Figure 4.2). All diabetic rats used in these studies exhibited hyperglycemia, but no significant difference in weight compared with control rats (Table 4.1).

We next wanted to determine if elevated spinal COX-2 and the development of formalin hyperalgesia are co-incident in rats with one, two, or four weeks of diabetes. One week of diabetes was sufficient to cause a

hyperalgesic response to paw injection of 0.5% formalin, as indicated by a significant ($P < 0.05$ vs. controls) increase in the sum of flinches counted during the 60 min after paw formalin injection (Figure 4.3). The magnitude of hyperalgesia increased with duration of diabetes (Figure 4.3). Diabetic rats used in these studies exhibited hyperglycemia, but no significant difference in weight compared with control rats (Table 4.2).

4.3.2 Formalin-evoked behavior and spinal cyclooxygenase inhibition in control rats

In this study, we observed that intrathecal treatment with 20 μg of the COX-2 selective inhibitor SC-58125 10 min before paw injection of 5% formalin selectively attenuated flinching during phase IIA ($P < 0.05$ vs. vehicle-treated control rats; Figure 4.4). Flinching levels during phase I, the quiescent phase, and phase IIB were not significantly different between vehicle-treated and SC-58125-treated control rats.

4.3.3 Formalin-evoked behavior and spinal cyclooxygenase inhibition in diabetic rats

The cohort of diabetic rats used for this experiment exhibited weight loss (211 ± 3 g vs. 240 ± 8 g for controls; $P < 0.0001$) and blood glucose levels above 15 mmol/l after four weeks of diabetes. There was no significant difference in weights between the various sub-groups of diabetic rats (Table 4.3). Untreated rats with four weeks of diabetes exhibited significant hyperalgesia to paw formalin injection during phase II of the formalin test ($P < 0.001$ vs. control rats; Figure 4.5A). Intrathecal treatment with the non-

selective COX inhibitor indomethacin or the selective COX-2 inhibitor SC-58125 attenuated hyperalgesia of diabetic rats during phase II of the formalin test (for both, $P < 0.05$ vs. respective vehicle-treated diabetic rats; Figure 4.5B and C). There was no statistically significant difference in the number of flinches counted during phase I or the quiescent phase between any of the groups, indicating that diabetes selectively potentiated the spinally-mediated phase II and that none of the treatments affected acute pain behavior.

4.4 Discussion

In the first study of this chapter, we found that elevated spinal COX-2 and hyperalgesia to paw formalin injection are co-incident in rats with one, two, or four weeks of diabetes, potentially suggesting a causal relationship. To test this possibility, we acutely inhibited COX-2 activity in the spinal cord of diabetic rats immediately before formalin testing, and found that phase II flinching was selectively attenuated. This indicates that spinal COX-2 activity at least partly causes the hyperalgesia to paw formalin injection observed in diabetic rats, and supports our general hypothesis that spinal prostanoid signaling contributes to the spinal sensitization present in diabetic rats.

Because COX-2 is an enzyme that undergoes use-inactivation (Smith and Lands, 1972; Kulmacz and Lands, 1983), COX-2 protein measured by Western blot may not be enzymatically active. We therefore measured both protein and activity levels in spinal cord tissue from the rats in these studies. In all instances, we observed that relative changes in COX-2 protein expression were mirrored by changes in COX-2 activity levels (current results).

This suggests that excess COX-2 protein measured in spinal cord tissue from diabetic rats was enzymatically active.

The finding that acute COX-2 inhibition selectively suppressed flinching during phase II of the formalin test is in accordance with the known role of PGE_2 in sensitizing the spinal cord during formalin testing (Malmberg and Yaksh, 1995). Acute COX-2 inhibition did not completely prevent the second phase of flinching in diabetic rats, which is likely partly due to the observation that phase IIA of the formalin test is uniquely sensitive to NSAID treatment (Malmberg and Yaksh, 1992). Additionally, other factors independent of spinal PGE_2 release may contribute to spinal cord sensitivity in diabetic rats.

The observation that elevated spinal COX-2 expression is constitutive in diabetic rats raises the question of whether there is a constant release of excess PGE_2 into the spinal parenchyma. Microdialysis studies have shown that basal levels of PGE_2 in spinal cerebrospinal fluid are not significantly different between control and diabetic rats (Freshwater et al., 2002), but this may not reflect local PGE_2 concentrations immediately in the vicinity of dorsal horn neurons. Electrophysiological studies have revealed that in spinal cords of diabetic rats, low- and high-threshold and WDR neurons that comprise the spinothalamic tract show increased spontaneous activity, enlarged receptive fields, and lower activation thresholds and exaggerated responses to mechanical stimulation (Pertovaara et al., 2001; Chen and Pan, 2002). These data suggest a basal sensitization of dorsal horn neurons in diabetic rats that may or may not be caused by PGE_2. If dorsal horn neurons in diabetic rats

are hyperexcitable, this in turn raises the question of why diabetic rats do not exhibit exaggerated rates of flinching during phase I of testing with 0.5% formalin (Calcutt et al., 1995). This may simply be because control and diabetic rats are already flinching maximally during phase I, or it may be that although dorsal horn neurons in diabetic rats are constitutively sensitized, the depressed spinal release of substance P and glutamate in response to paw formalin injection (Calcutt et al., 2000b; Malmberg et al., 2006) results in apparently normal phase I flinching (Calcutt et al., 1995).

It is interesting to note that at all durations of diabetes tested, the relative increase in spinal COX-2 protein and activity remains constant, yet the magnitude of exaggerated flinching in response to paw formalin injection increased over time. This may reflect changes in substrate availability (arachidonic acid) for COX-2 due to changes in activity of the enzyme phospholipase A_2 (PLA_2). To date, there are no published studies of changes in spinal PLA_2 activity in diabetes, and this represents an important unexplored area of painful diabetic neuropathy research. Alternatively, the increase in magnitude of exaggerated flinching in response to paw formalin injection over time may reflect changes in peripheral input to the spinal cord during the first few weeks of STZ-induced diabetes. This possibility has not been addressed to date, as published studies of primary afferent fiber electrophysiology in diabetic rats have used rats with at least three to four weeks' duration of diabetes. Electrophysiological recordings of primary afferent fiber activity in

rats with very short durations of diabetes (one or two weeks) could address this issue.

The present results using 0.5% formalin to induce biphasic flinching agree closely with the onset of a monophasic allodynic flinching response to paw injection of 0.2% formalin in diabetic rats, with the exaggerated flinching response being observed after one week of diabetes and increasing in magnitude over time, to reach a maximal level after four weeks of diabetes (Freshwater and Calcutt, 2005; current results). In diabetic rats subjected to 0.5% formalin testing, intrathecal indomethacin pre-treatment attenuated flinching during phase II without affecting phase I (Freshwater et al., 2002, and current results). This reflects the importance of spinal COX activity in inducing spinal sensitization secondary to formalin-induced tissue damage, and suggests that spinal COX activity is not necessary for a normal acute pain response to tissue injury. The monophasic flinching induced by paw injection of 0.2% formalin in diabetic rats was also attenuated by intrathecal indomethacin injection. This may suggest that 0.2% formalin-induced flinching in diabetic rats is not an acute nociceptive response but rather arises due to spinal sensitization.

In summary, it can be concluded that diabetes causes spinal sensitization that is at least partly due to elevated activity of the enzyme COX-2. Elevated spinal COX-2 protein and activity levels may be considered a biochemical marker of this spinal sensitization.

Figure 4.1.

Spinal COX-2 protein levels in control rats, and rats with one, two or four weeks of diabetes.

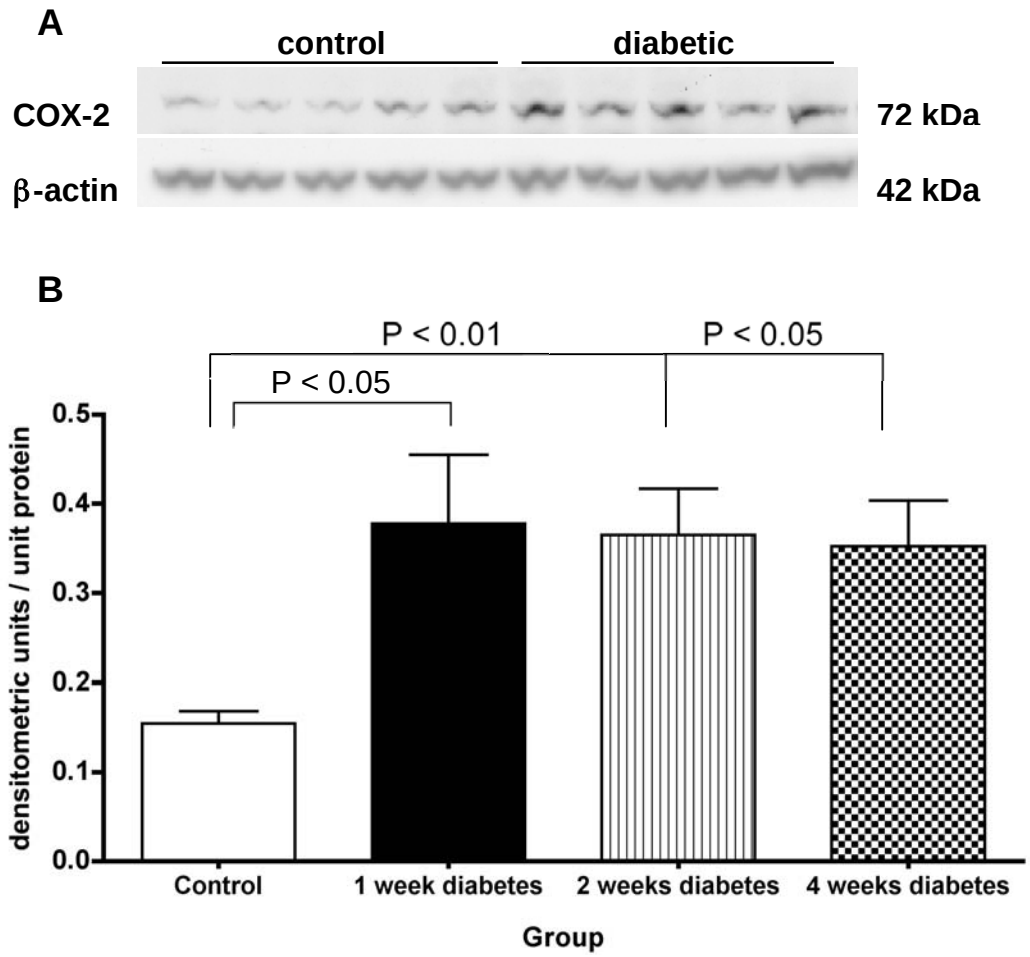


Figure 4.1. (A) Representative Western blots showing levels of COX-2 and β-actin in lumbar spinal cord from control rats or rats with four weeks of diabetes. (B) Densitometric quantification of COX-2-immunostained Western blots showing that rats with one, two, or four weeks of diabetes have a two-fold increase in spinal COX-2 protein levels, as compared with control rats. Data are presented as group mean ± SEM, N = 5 - 6 per group, statistical comparison by one-way ANOVA with Student-Neuman-Keuls' *post-hoc* test.

Figure 4.2.

Spinal COX-2 activity levels in control rats, and rats with one or four weeks of diabetes.

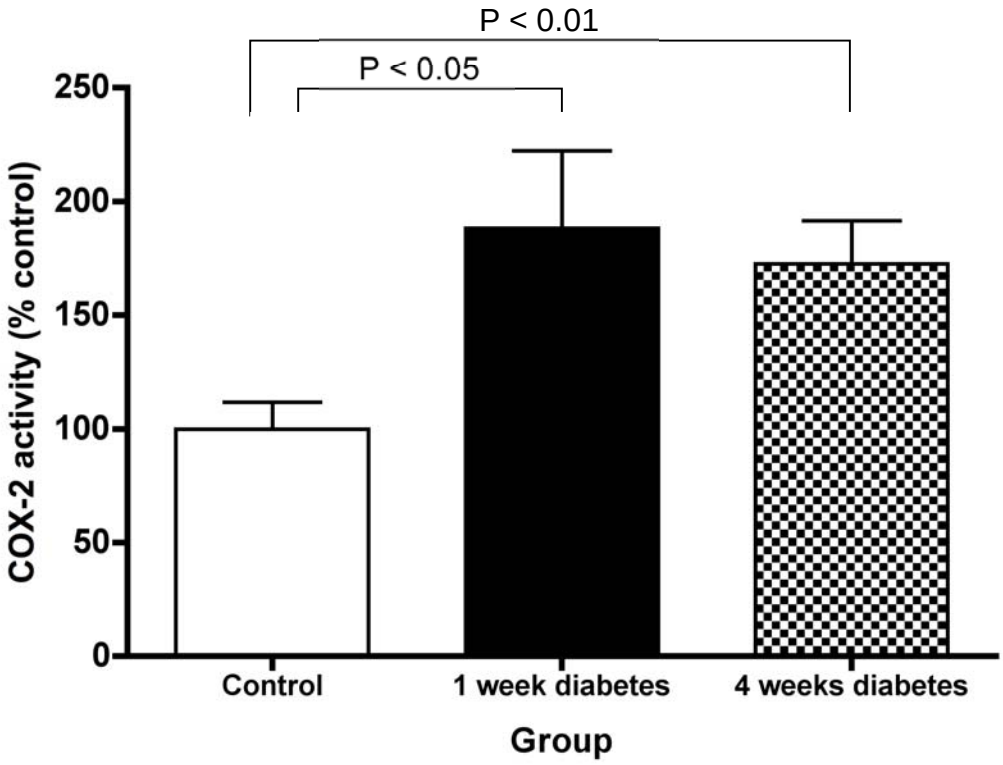


Figure 4.2. Rats with one or four weeks of diabetes exhibit a two-fold increase in spinal COX-2 activity, as compared with control rats. Data are presented as group mean ± SEM, N = 5 - 6 per group, statistical comparison performed using a one-way ANOVA with Dunnett's *post-hoc* test.

Figure 4.3 A

0.5% formalin flinching curves for control rats and rats with one, two, or four weeks of diabetes.

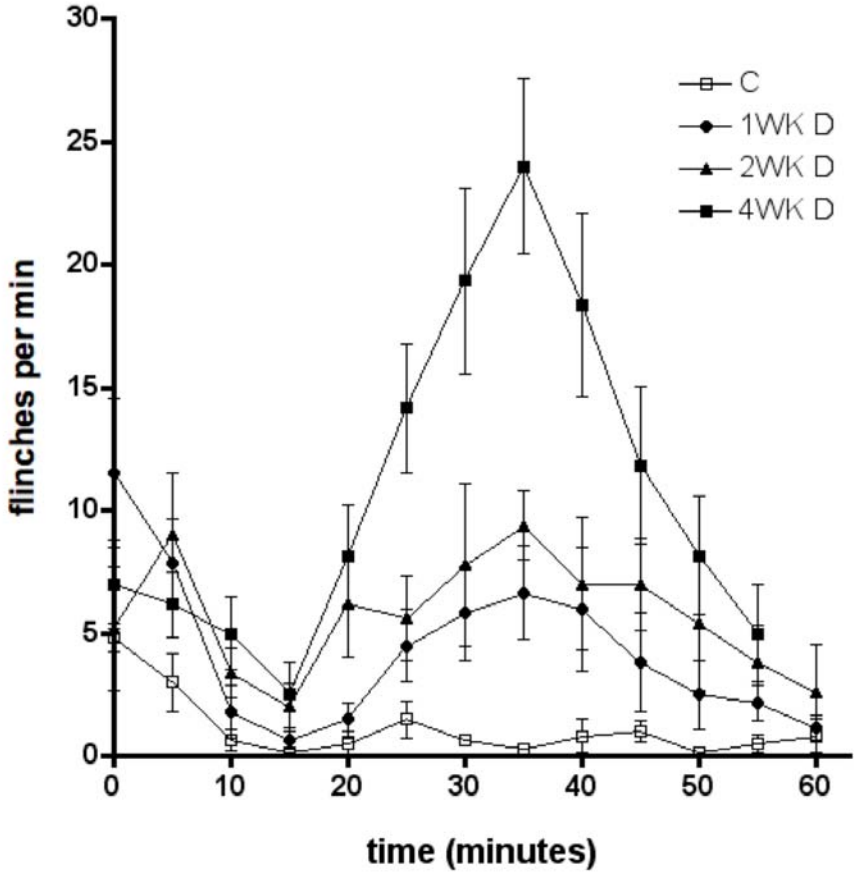


Figure 4.3 (A) Formalin-evoked flinching in control rats (C), and rats with one (1WK D), two (2WK D), or four (4WK D) weeks of diabetes. Data are presented as group mean ± SEM, N = 5 - 6 per group.

Figure 4.3 B

60 minute sum of flinches in response to paw injection of 0.5% formalin in control rats and rats with one, two, or four weeks of diabetes.

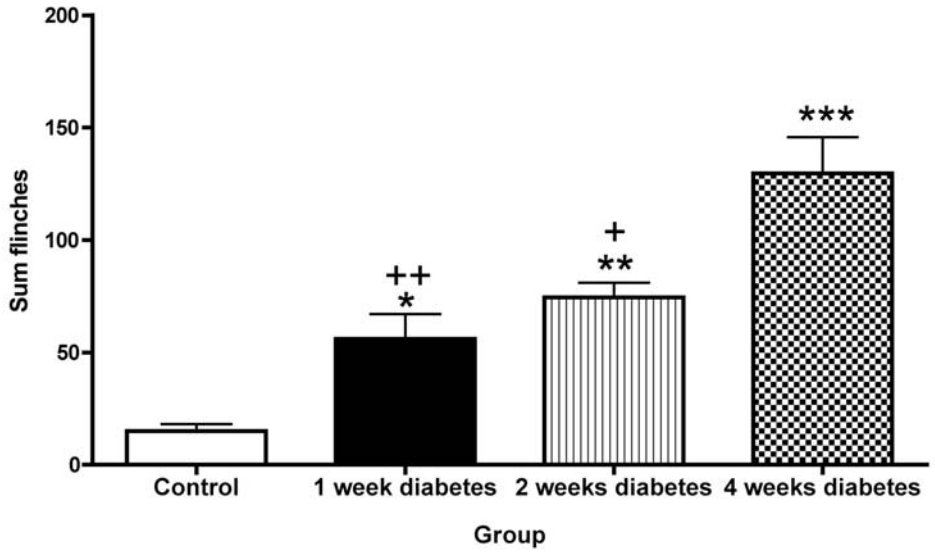
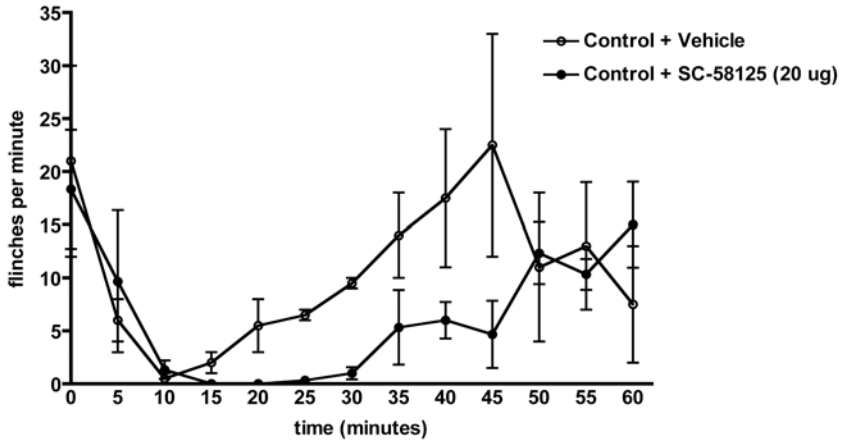


Figure 4.3 (B) Sum of flinches counted in the 60-min period after paw formalin injection in control rats, and rats with one, two, or four weeks of diabetes. * $P < 0.05$ vs. control; ** $P < 0.01$ vs. control; *** $P < 0.001$ vs. control; $^{+}$ $P < 0.01$ vs. 4 weeks diabetes; $^{++}$ $P < 0.001$ vs. 4 weeks diabetes. Data are presented as mean ± SEM, N = 5 - 6 per group, statistical comparison performed using a one-way ANOVA with Student-Newman-Keuls' *post-hoc* test.

Figure 4.4

Flinching response to paw injection of 5% formalin in untreated control rats and control rats pre-treated intrathecally with the COX-2 inhibitor SC-58125.

A



B

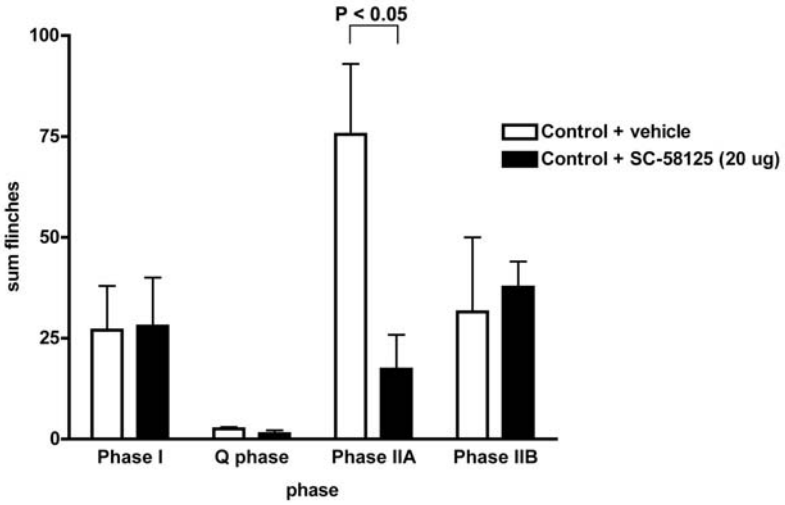


Figure 4.4. (A) Time course of formalin-evoked flinching in vehicle-treated control rats and control rats pre-treated intrathecally with 20 μg of SC-58125. (B) Sums of flinches counted in the 60-min period after paw formalin injection in vehicle-treated control rats and control rats pre-treated intrathecally with 20 μg of SC-58125, binned into phase I, the quiescent (Q) phase, phase IIA, and phase IIB. Data are presented as group mean ± SEM, N = 3 per group, statistical comparison performed using an unpaired t-test (one for each phase).

Figure 4.5

Flinching sums in response to paw injection of 0.5% formalin in control rats, untreated diabetic rats, and diabetic rats pre-treated with intrathecal injection of indomethacin or SC-58125.

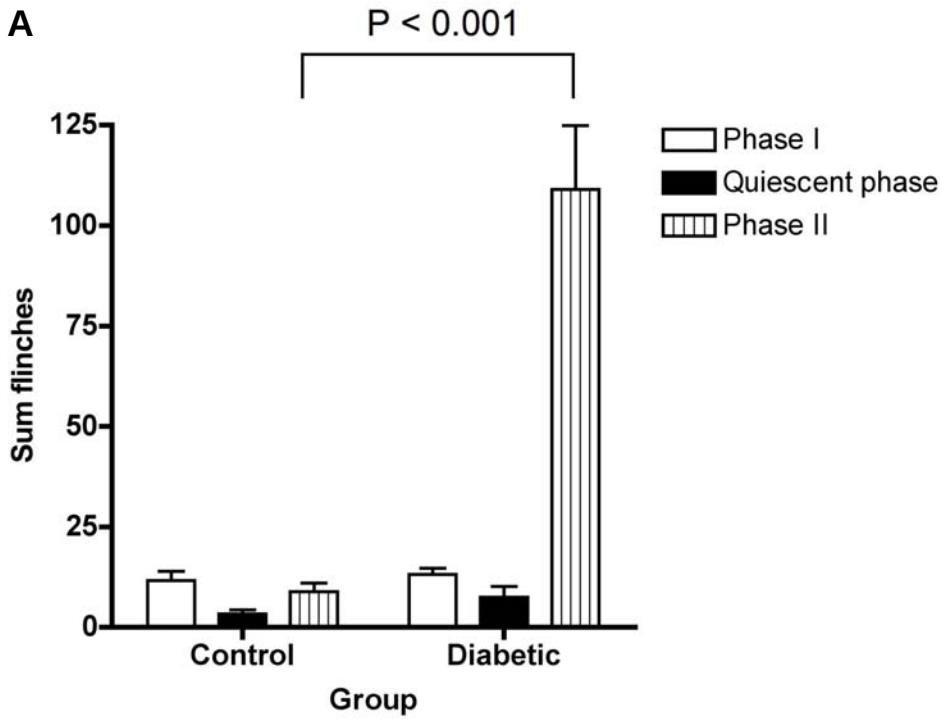


Figure 4.5. Flinches counted during phase I, the quiescent phase, and phase II of the formalin test. (A) Untreated control rats and untreated diabetic rats. (B) Diabetic rats treated intrathecally with 10 ul indomethacin vehicle (D + INDO V) or 10 ul vehicle containing 65 ug indomethacin (D + INDO). (C) Diabetic rats treated intrathecally with 10 ul SC-58125 vehicle (D + SC-58125 V) or 10 ul vehicle containing 20 ug SC-58125 (D + SC-58125). Data are presented as group mean ± SEM, N = 5 - 6 per group. For each panel, an unpaired t-test was used for each phase to compare the matched groups.

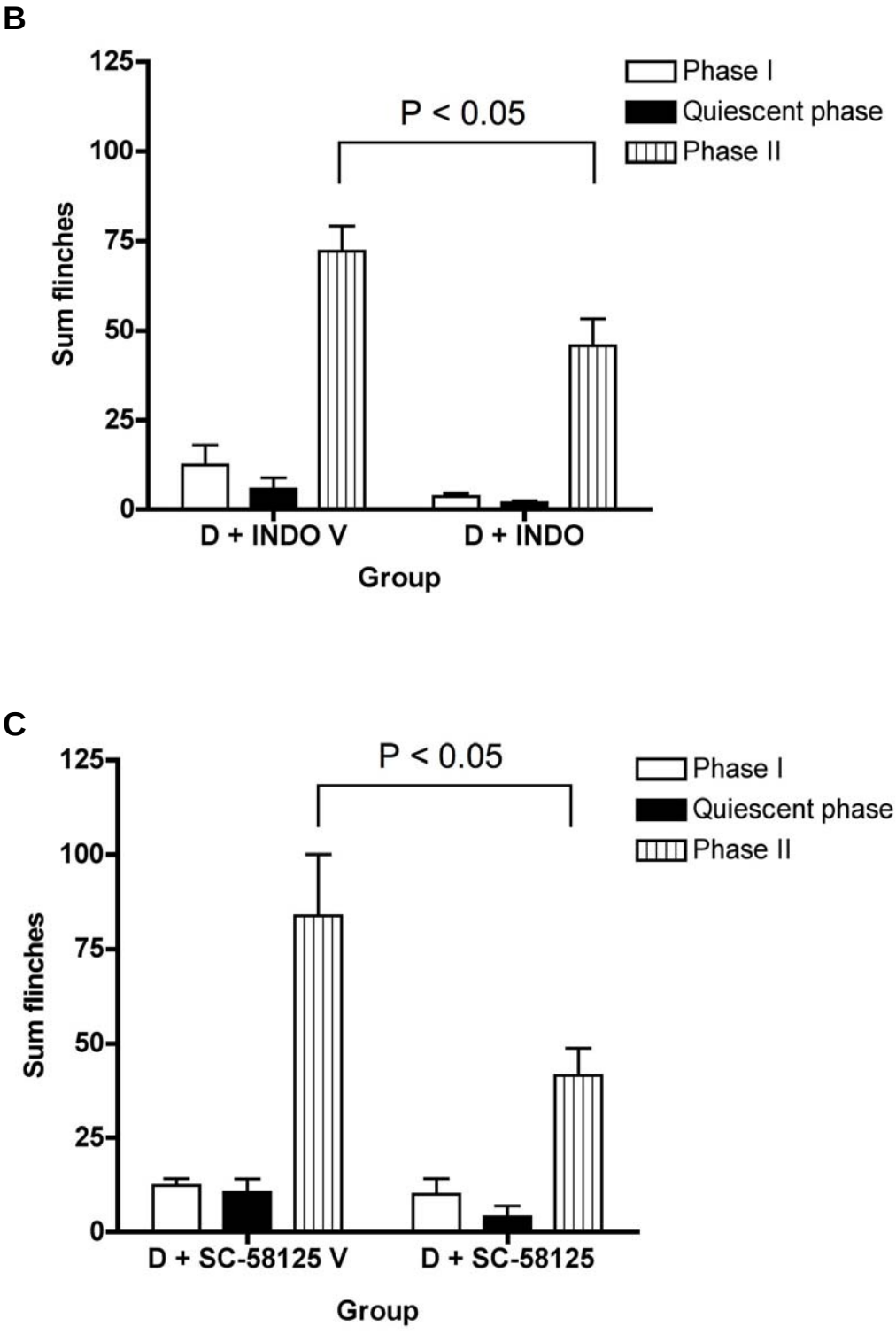


Figure 4.5. continued

Table 4.1.

Body weights and blood glucose levels for rats used to measure spinal COX-2 protein and activity levels.

Group	Body weight (g)	Blood glucose (mM)
Control (N = 5)	261 ± 6	All < 15
1 week of STZ-diabetes (N = 6)	228 ± 4	All > 15
2 weeks of STZ-diabetes (N = 6)	237 ± 11	All > 15
4 weeks of STZ-diabetes (N = 5)	231 ± 11	All > 15

Data are group mean ± SEM, except blood glucose levels. By one-way ANOVA analysis, there is no significant difference in body weight between any of the groups. At the start of this study, the average weight of all rats was 233 ± 2 g.

Table 4.2.

Body weights and blood glucose levels for rats injected with 0.5% formalin for behavioral testing.

Group	Body weight (g)	Blood glucose (mM)
Control (N = 6)	255 ± 4	All < 15
1 week of STZ-diabetes (N = 6)	238 ± 5	All > 15
2 weeks of STZ-diabetes (N = 6)	242 ± 10	All > 15
4 weeks of STZ-diabetes (N = 5)	240 ± 11	All > 15

Data are group mean ± SEM, except blood glucose levels. By one-way ANOVA analysis, there is no significant difference in body weight between any of the groups. At the start of this study, the average weight of all rats was 233 ± 2 g.

Table 4.3.

Body weights and plasma glucose levels for control and diabetic rats injected with 0.5% formalin to study effects of intrathecal COX inhibition.

Group	Body weight (g)	Blood glucose (mM)
Control (N = 6)	240 ± 8 [a]	All < 15
Diabetic (N = 6)	213 ± 6 [b]	All > 15
Diabetic + indomethacin (N = 6)	209 ± 3 [c]	All > 15
Diabetic + indomethacin vehicle (N = 5)	210 ± 6 [c]	All > 15
Diabetic + SC-58125 (N = 5)	214 ± 3 [c]	All > 15
Diabetic + SC-58125 vehicle (N = 5)	213 ± 7 [c]	All > 15

Data are group mean ± SEM, except blood glucose levels. Statistical analysis of body weights was performed using a one-way ANOVA with the Newman-Keuls' *post-hoc* test. [a] vs. [b], $P < 0.01$; [a] vs. [c], $P < 0.05$. At the start of this study, the average weight of all rats was 225 ± 4 g.

CHAPTER 5 – PATHOGENESIS OF SPINAL SENSITIZATION IN DIABETIC RATS

5.1 Introduction

The experiments in chapter 4 of this thesis provided evidence that spinal COX-2 activity has an important causal role in the hyperalgesia to paw formalin injection observed in diabetic rats. To extend this finding, experiments were designed to study the pathogenesis of elevated spinal COX-2 and formalin hyperalgesia in diabetic rats. A previous study demonstrated that four weeks of preventative systemic treatment with the ARI ICI222155 attenuated exaggerated flinching in diabetic rats in response to paw injection of 0.5% formalin (Calcutt et al., 1995). For the present experiments we used the ARI ICI222155 in order to determine if it would also prevent the upregulation of spinal COX-2 protein and activity in diabetic rats. Importantly, systemic treatment of diabetic rats with ICI222155 was shown to block polyol product accumulation both in the peripheral nerve and in the spinal cord (Calcutt et al., 1995). It is therefore unclear whether exaggerated polyol pathway flux in the PNS or CNS is causal to development of formalin hyperalgesia in diabetic rats. In view of the evidence against exaggerated peripheral activity as the source of formalin hyperalgesia in diabetic rats (as discussed in the general introduction to this thesis), we hypothesized that the efficacy of ICI222155 in preventing formalin hyperalgesia derived from a CNS action, possibly in the spinal cord. To provide evidence for this, we sought to compare how formalin hyperalgesia and exaggerated spinal COX-2 in diabetic

rats would be affected by systemic treatment with an ARI that does not cross the BBB and therefore only blocks AR activity in the PNS. Because it is not routinely known whether any particular ARI crosses the BBB, we chose the ARI IDD676 based on its inability to prevent an allodynic response to 0.2% formalin injection in the paw in diabetic rats (Freshwater and Calcutt, 2005). This lack of efficacy possibly suggested that, at the dose used, IDD676 did not sufficiently cross the BBB to block exaggerated polyol pathway flux in the CNS. A second experiment was therefore designed with two aims. We tested whether systemically treating diabetic rats with IDD676 would block polyol accumulation in the sciatic nerve and in the spinal cord, to determine if this ARI crossed the BBB, and examined the ability of systemic IDD676 treatment to affect the development of upregulated spinal COX-2 and formalin hyperalgesia in diabetic rats.

5.2 Methods

5.2.1 Experimental design

Two studies were performed, the first using the ARI ICI222155 and the second using the ARI IDD676, to determine how preventative treatment with these drugs would affect the development of elevated spinal COX-2 and behavioral abnormalities in diabetic rats. We hypothesized that because preventative treatment with ICI222155 blocked development of formalin hyperalgesia in diabetic rats (Calcutt et al., 1995), ICI222155 treatment would also prevent development of exaggerated spinal COX-2 protein and activity levels in diabetic rats. In contrast, having previously observed that treatment

with IDD676 did not prevent the development of an allodynic response to paw injection of 0.2% formalin in diabetic rats (Freshwater and Calcutt, 2005), we hypothesized that treatment this ARI would not sufficiently cross the blood brain barrier to inhibit spinal polyol pathway flux at the dose used (10 mg/kg), and therefore would not prevent the development of elevated spinal COX-2 or hyperalgesia to 0.5% formalin injection in the paw using this same dose. Additionally, the first study included a group of rats that were made diabetic by STZ and then immediately implanted with slow-release insulin pellets to reverse hyperglycemia for the duration of ARI treatment. This group of insulin-treated STZ-injected rats was included to confirm that elevated spinal COX-2 levels observed in diabetic rats were not due to STZ toxicity but rather to hyperglycemia and its associated metabolic consequences. It has already been documented that insulin therapy corrects hyperalgesia to paw formalin injection in STZ-diabetic rats, indicating that this hyperalgesia is not due to direct STZ toxicity (Calcutt et al., 1996).

The first study was designed to test whether treatment with insulin or systemic ICI222155 would prevent the upregulation of spinal COX-2 protein and activity in rats with 4 weeks of diabetes. From an initial cohort of rats, three subgroups were made diabetic by a single i.p. injection of STZ (see section 2.1). Three days later, hyperglycemia was confirmed with blood taken by tail-prick and a strip-operated reflectance meter (section 2.1). The day that hyperglycemia was confirmed, one group of diabetic rats received sub-cutaneous implants of slow-dissolving insulin pellets that deliver approximately

2-4 U insulin per day (Linshin, Scarborough, Ontario, Canada), to reverse hyperglycemia. Blood glucose levels in these animals were checked daily and any rat with a blood glucose level above 15 mmol/l received a second pellet of insulin. This group of rats was included in order to confirm that abnormalities observed in diabetic rats were not due to STZ toxicity but rather to hyperglycemia and its associated metabolic consequences. Also on the day that hyperglycemia was confirmed in the STZ-injected rats, one group of diabetic rats and half of the control rats began treatment with the ARI ICI222155 (4-amino-2,6-dimethylphenyl-sulphonyl nitromethane, Zeneca Pharmaceuticals, Macclesfield, UK), suspended in water and Tween-20, once daily by oral gavage at 20 mg ICI222155 per kg body weight. ICI222155, and its dose and treatment regimen, were chosen because of published observations that it penetrates the spinal cord and prevents the development of formalin hyperalgesia in diabetic rats (Calcutt et al., 1995). After four weeks, tactile and thermal paw withdrawal thresholds were measured in all rats (sections 2.4.3 and 2.4.2). Twenty-four hours after the last treatment with ICI222155, the rats were killed and spinal cords removed for measurement of spinal COX-2 protein and activity (sections 2.2.3 and 2.2.4). Additionally, blood was collected for measurement of HbA1c levels (section 2.2.6), and portions of spinal cord and sciatic nerve were harvested for measurement of glucose, fructose, sorbitol, and myo-inositol levels using gas chromatography (section 2.2.5). 0.5% formalin testing was not performed on these rats because we wished to measure spinal COX-2 activity levels, which can be

confounded by paw formalin-injection-induced upregulation of spinal COX-2 activity. Furthermore, it has already been shown that four weeks of treatment with ICI222155 at the same dose used in the present studies prevented the development of formalin hyperalgesia in diabetic rats (Calcutt et al., 1995).

The second study used the ARI IDD676 (3-[(4,5,7-trifluorobenzothiazol-2-yl)methyl]indole-N-acetic acid, Institute for Diabetes Discovery, Branford, CT). From an initial cohort of rats, two subgroups were made diabetic with a single i.p. injection of STZ (section 2.1). Three days later, hyperglycemia was confirmed with blood taken by tail-prick and a strip-operated reflectance meter (section 2.1). The day that hyperglycemia was confirmed, one group of diabetic rats began treatment with IDD676 suspended in water and Tween-20, once daily by oral gavage at 10 mg IDD676 per kg body weight. After four weeks of diabetes, thermal paw withdrawal latencies were measured in all rats (section 2.4.2), along with motor and sensory nerve conduction velocities (section 2.4.5). Twenty-four hours after the last IDD676 treatment, rats were subjected to 0.5% formalin testing (section 2.4.4). Immediately following the last time-point during formalin testing (one hour after formalin injection), spinal cords were removed for measurement of COX-2 protein levels (section 2.2.3). Spinal cord tissue was processed in this manner because although COX-2 mRNA is significantly upregulated in the spinal cord as early as 30 min after paw inflammation (Guay et al., 2004), upregulation of spinal COX-2 protein after paw inflammation is not observed for at least 2 – 3 hours (Ikubi et al., 2003; Guay et al., 2004; Zhang et al., 2007). Portions of spinal cord and

sciatic nerve were also harvested from these animals for measurement of glucose, fructose, sorbitol, and myo-inositol levels using gas chromatography (section 2.2.5).

5.3 Results

5.3.1 Effect of ICI222155 or insulin treatment on development of elevated spinal COX-2 and behavioral hyperalgesia

At the start of this study, the average weight of the rats was 240 ± 2 g. At the conclusion of the study, untreated diabetic rats exhibited weight loss and hyperglycemia that was unaffected by treatment with ICI222155 (Table 5.1). ICI222155 treatment also had no effect on body weights in control rats (Table 5.1). Diabetic rats treated with insulin had normal body weights and blood glucose levels below 15 mM (Table 5.1). Measurement of glycated hemoglobin levels in whole blood indicated a significantly elevated percentage in untreated diabetic rats that was unaffected by ICI222155 treatment (Table 5.1). Insulin-treated diabetic rats tended to have slightly lower levels of glycated hemoglobin, but there was no statistically significant difference between insulin-treated diabetic rats and control rats (Table 5.1).

Spinal cord and sciatic nerve tissue was removed from these animals for measurement of glucose, sorbitol, fructose, and myo-inositol with gas chromatography (section 2.2.5). This was done in order to measure whether polyol product accumulation was blocked in the CNS and/or PNS of ICI222155- and insulin-treated diabetic rats. The data obtained from spinal cord tissue revealed incomplete silylation of glucose, sorbitol, fructose, and

myo-inositol, presumably due to a mistake during the tissue processing. This resulted in a lack of a clear, single peak for each sugar in any given sample. Rather, each sugar burned off the column in a series of peaks, each reflecting a different degree of silylation. This prevented analysis of the data, because it was impossible to distinguish true peaks from noise, and because the pattern of silylation for each sugar was not consistent between samples. Therefore we do not have proof that polyol product accumulation was blocked in the CNS of ICI222155- and insulin-treated diabetic rats. However, it is documented that the same dose of ICI222155 blocks spinal polyol product accumulation in diabetic rats (Calcutt et al., 1995). Furthermore, measurement of glucose, sorbitol, fructose, and myo-inositol in the sciatic nerve revealed that ICI222155 and insulin treatment prevented the accumulation of fructose and the associated depletion of myo-inositol, indicating efficacy in the PNS (Table 5.2), which illustrates that the ICI222155 compound was functional.

ICI222155 treatment did not significantly affect levels of spinal COX-2 protein in control rats (Figure 5.1). Untreated rats with four weeks of diabetes showed significantly elevated levels of spinal COX-2 protein ($P < 0.001$ vs. untreated controls and ICI222155-treated controls; Figure 5.1). Four weeks of insulin treatment or treatment with ICI222155 prevented the increase in spinal COX-2 protein in diabetic rats ($P < 0.001$ and $P < 0.01$, respectively, vs. untreated diabetic rats; Figure 5.1). Measurement of spinal COX-2 activity levels revealed a parallel pattern. Untreated rats with four weeks of diabetes

showed significantly elevated levels of spinal COX-2 activity ($P < 0.05$ vs. controls; Figure 5.2), which was prevented by treatment with insulin or ICI222155 ($P < 0.05$ for both, vs. untreated diabetic rats; Figure 5.2). Behavioral tests revealed tactile allodynia in the untreated diabetic rats that was not significantly affected by treatment with ICI222155 or insulin (Figure 5.3). Thermal paw withdrawal latencies were not significantly different between any of the groups (Figure 5.4).

5.3.2 Effect of IDD676 treatment on development of elevated spinal COX-2 and behavioral changes

At the start of this study, the average weight of the rats was 244 ± 3 g. At the conclusion of the study, untreated diabetic rats exhibited weight loss and hyperglycemia that was not affected by treatment with IDD676 (Table 5.3). In the sciatic nerve, untreated diabetic rats accumulated significant levels of glucose, sorbitol, and fructose, and displayed a trend toward depletion of myo-inositol (Table 5.4). IDD676 treatment prevented the accumulation of polyol pathway products, caused a significant increase in glucose levels as compared with untreated diabetic rats, and tended to normalize myo-inositol levels in the sciatic nerve (Table 5.4). In the spinal cord, untreated diabetic rats accumulated glucose and fructose, but showed no detectable sorbitol (Table 5.4). The limit of detection of the gas chromatograph is 1.8 nmol per sample (Calcutt et al., 2004). Spinal myo-inositol levels were not different between control and diabetic rats (Table 5.4). IDD676 treatment had no effect on spinal levels of glucose, sorbitol, fructose,

or myo-inositol (Table 5.4). These data indicate that IDD676 was effective in the PNS, but did not cross the BBB sufficiently to block exaggerated polyol pathway flux in the spinal cord.

Measurement of spinal COX-2 protein indicated that IDD676 treatment did not prevent the increase in spinal COX-2 protein that was present in untreated diabetic rats (Figure 5.5). IDD676 treatment also had no effect on the hyperalgesia to 0.5% formalin observed in untreated diabetic rats (Figure 5.6). However, the inability of IDD676 to prevent elevated spinal COX-2 protein and formalin hyperalgesia was not due to a general failure of efficacy of the drug, as IDD676 treatment alleviated thermal hyperalgesia and nerve conduction velocity slowing in diabetic rats (Figures 5.7 and 5.8).

5.4 Discussion

These studies were performed to investigate the pathogenesis of spinal sensitization in diabetic rats. We were specifically interested in testing whether exaggerated polyol pathway flux causes the development of hyperalgesia and elevated spinal COX-2 in diabetic rats, and if so, where in the nervous system the activity of AR is relevant. The first study used the ARI ICI222155, known to cross the blood brain barrier when given systemically and prevent polyol formation in both the peripheral nerves and the spinal cord (Calcutt et al., 1995). ICI222155 treatment has also been shown to prevent hyperalgesia to paw formalin injection in diabetic rats (Calcutt et al., 1995). Having demonstrated the importance of elevated spinal COX-2 in causing hyperalgesia to paw formalin injection (chapter 4), we hypothesized that

ICI222155 treatment would also prevent the upregulation of spinal COX-2 in diabetic rats. This was what was observed in the present studies, as ICI222155-treated diabetic rats had normal levels of spinal COX-2 protein and activity. Thus, preventative treatment with an ARI that crosses the BBB blocks the development of hyperalgesia and elevated spinal COX-2 in diabetic rats. Treating diabetic rats from the onset of hyperglycemia with sufficient insulin to reverse hyperglycemia also prevented the development of elevated spinal COX-2 and hyperalgesia to paw formalin injection. This is an important control experiment illustrating that spinal sensitization and hyperalgesia in diabetic rats are not due to direct STZ-induced toxicity to the nervous system.

Tactile testing of these animals revealed allodynia in the untreated diabetic rats that was not significantly affected by treatment with ICI222155. This is in agreement with previously published results (Calcutt et al., 1996). Tissue injury-induced tactile allodynia is believed to reflect central sensitization (reviewed by Yaksh et al., 1999), which may suggest that spinal sensitization-mediated phase II formalin hyperalgesia and tactile allodynia in diabetic rats should be similarly affected by any given chronic treatment. However, the current data indicate that the tactile allodynia present in diabetic rats has an etiology that is distinct from that of formalin hyperalgesia. This dissociation has been reported previously (Calcutt et al., 2004). The only drug documented to affect tactile allodynia in diabetic rats when given chronically is nitecapone, a catechol-*O*-methyltransferase inhibitor with antioxidant properties (Pertovaara et al., 2001). The study design utilized systemic

nitecapone treatment, and as nitecapone exhibits poor penetration of the blood brain barrier (Nissinen et al., 1988), this suggests a peripheral site of action in its efficacy against diabetic tactile allodynia. This observation is inconsistent with the hypothesis that tactile allodynia in diabetic rats is caused by central sensitization.

Thermal paw withdrawal latencies were not significantly different between any of the groups. In the untreated diabetic rats, this may reflect a state of transition from early hyperalgesia to later hypoalgesia (reviewed by Calcutt, 2004). It is unclear whether the ICI222155- and insulin-treated diabetic rats were similarly in a state of transition or whether these treatments prevented any disorder in thermal sensation. In our group's experience, the timing of transition from thermal hyperalgesia to hypoalgesia in diabetic rats is somewhat variable, with hypoalgesia appearing 8-12 weeks after the onset of diabetes (Calcutt, 2004). This variability may explain why the untreated diabetic rats from the IDD676 treatment study exhibited thermal hyperalgesia, whereas the untreated diabetic rats from the ICI222155 treatment study did not.

The data obtained from rats treated with ICI222155 demonstrate an association between AR activity and upregulation of spinal COX-2 expression, but the site of action is unclear because ICI222155 blocks polyol product accumulation in the PNS and CNS. Based on our results with ICI222155, we next hypothesized that an ARI must sufficiently cross the BBB for access to CNS sites of action in order to prevent spinal sensitization in diabetic rats. To

test this, the ARI IDD676 was used as a control for comparison with ICI222155. Systemic treatment with IDD676 at a dose of 10 mg per kg body weight has been shown to be ineffective in preventing an allodynic flinching response to 0.2% paw formalin injection in diabetic rats, despite showing efficacy against both motor nerve conduction velocity slowing and accumulation of polyol products in the peripheral nerve (Freshwater and Calcutt, 2005). Although IDD66 treatment prevented indices of dysfunction in the PNS, it is possible that allodynia in response to 0.2% formalin is due to polyol pathway flux in the CNS and that at the given dose, IDD676 did not sufficiently cross the BBB to impede CNS polyol accumulation. Alternatively, if the etiology of allodynia induced by 0.2% formalin injection is different from that of 0.5% formalin hyperalgesia, this could explain why AR inhibition prevents hyperalgesia in response to 0.5% formalin but not allodynia in response to 0.2% formalin in diabetic rats. We decided to test the first of these two possibilities, and our second study was designed to determine whether IDD676 given systemically blocks polyol product accumulation in the PNS and the spinal cord, as well as to test the ability of IDD676 to protect against PNS and spinal disorders in diabetic rats. We found that IDD676 blocked formation of polyol products in the peripheral nerve but not in the spinal cord, confirming that, at least at this dose, IDD676 was unable to cross the BBB sufficiently to block polyol product accumulation in the spinal cord. IDD676 treatment had no effect on elevated spinal COX-2 or hyperalgesia to paw formalin injection in diabetic rats. However, IDD676 treatment did prevent

thermal hyperalgesia and nerve conduction velocity slowing in diabetic rats. These data indicate that blocking exaggerated polyol pathway flux in the PNS alone is insufficient to prevent spinal sensitization.

These data are significant because they are the first to suggest that the CNS action of an ARI is relevant to the pathogenesis of painful diabetic neuropathy. This represents a significant advancement of the understanding of how chronic hyperglycemia damages the nervous system to cause neuropathic pain. However, with these data alone, it is uncertain where in the CNS exaggerated polyol pathway flux causes neuropathic pain. We have chosen to focus on the spinal cord for several reasons. One difference between the two ARIs ICI222155 and IDD676 was that only the former blocked both polyol product accumulation and exaggerated COX-2 expression in the spinal cord at the doses used, emphasizing the spinal cord as a potentially important site of biochemical abnormalities in diabetes. There is a significant literature supporting the hypothesis that diabetes alters the biochemistry of the spinal cord, as discussed in the introduction of this thesis, and the formalin test used for these experiments provides a direct behavioral correlate of spinal sensitization. However, the possibility that exaggerated polyol pathway flux affects sensory processing in the brainstem and/or brain in diabetes cannot be discounted. An important follow-up experiment would be to use siRNA to knock-down spinal AR expression in rats immediately prior to inducing diabetes with STZ. If diabetic rats lacking spinal AR and concurrent polyol pathway flux did not develop elevated spinal COX-2 and hyperalgesia

to paw formalin injection, this would provide direct evidence for the hypothesis that exaggerated polyol pathway flux in the spinal cord causes neuropathic pain. It is possible to use siRNA to effectively ablate AR expression, as has been demonstrated *in vitro* using cultured rat vascular smooth muscle cells, murine macrophages, human lens epithelial cells, and human colon cancer cells (Ramana et al., 2004; Pladzyk et al., 2006b; Ramana et al., 2006a; Tammali et al., 2006). Additionally, Tammali et al. (2006) used siRNA *in vivo* to demonstrate that ablation of AR prevented tumor growth from ectopic SW480 human colon cancer cells. Thus siRNA should be effective in ablating spinal AR expression to determine if this prevents diabetes-induced hyperalgesia and upregulation of spinal COX-2.

Figure 5.1.

Spinal COX-2 protein levels in untreated control rats, untreated diabetic rats, control rats treated with ICI222155, and diabetic rats treated with ICI222155 or insulin.

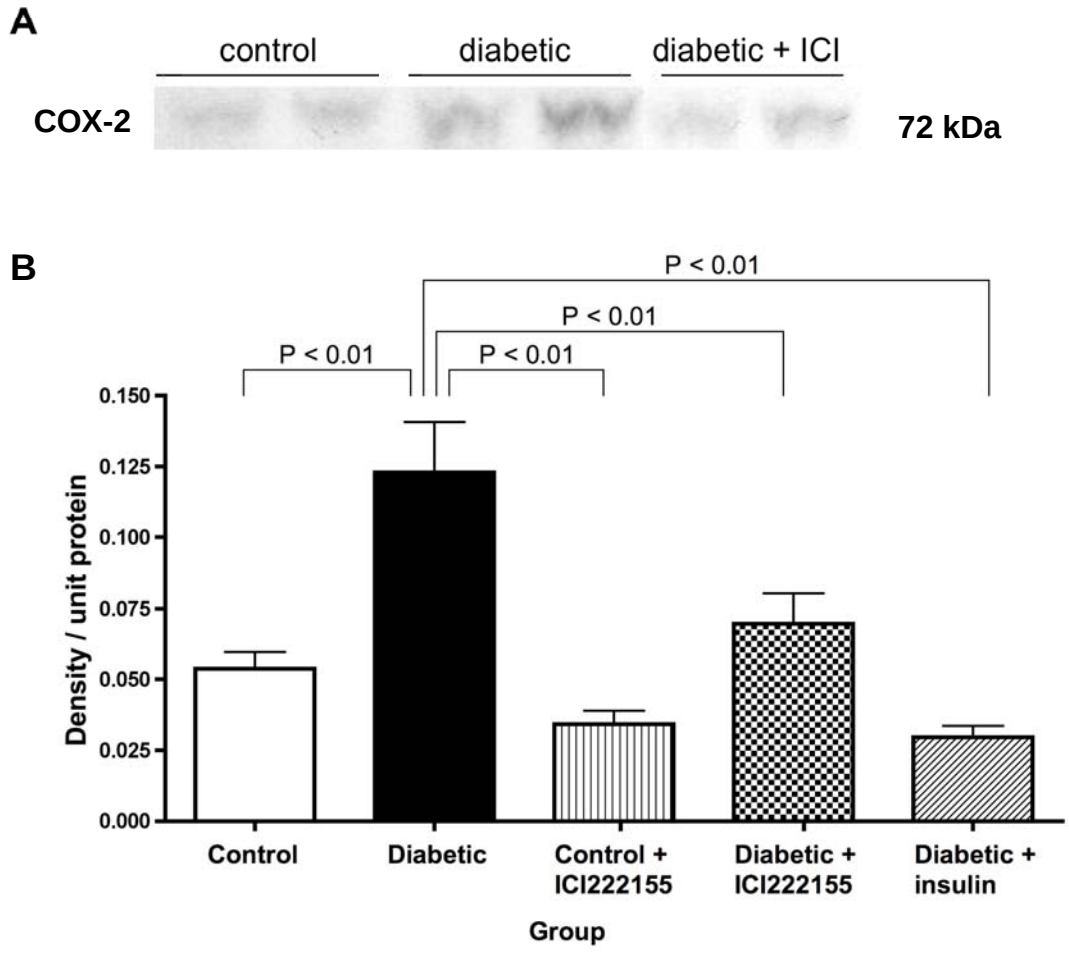


Figure 5.1. (A) Representative Western blot probed for COX-2, using lumbar spinal cord from control rats, untreated diabetic rats, and diabetic rats treated with ICI222155. (B) Densitometric quantification of COX-2-immunostained Western blots indicated that ICI222155 or insulin treatment prevented the increase in spinal COX-2 protein levels observed in untreated diabetic rats. Data are presented as group mean ± SEM, N = 9 - 10 per group, statistical comparison performed using a one-way ANOVA with the Student-Neuman-Keuls' *post-hoc* test. Western Blot values are normalized to β-actin.

Figure 5.2.

Spinal COX-2 activity levels in untreated control rats, untreated diabetic rats, and diabetic rats treated with ICI222155 or insulin.

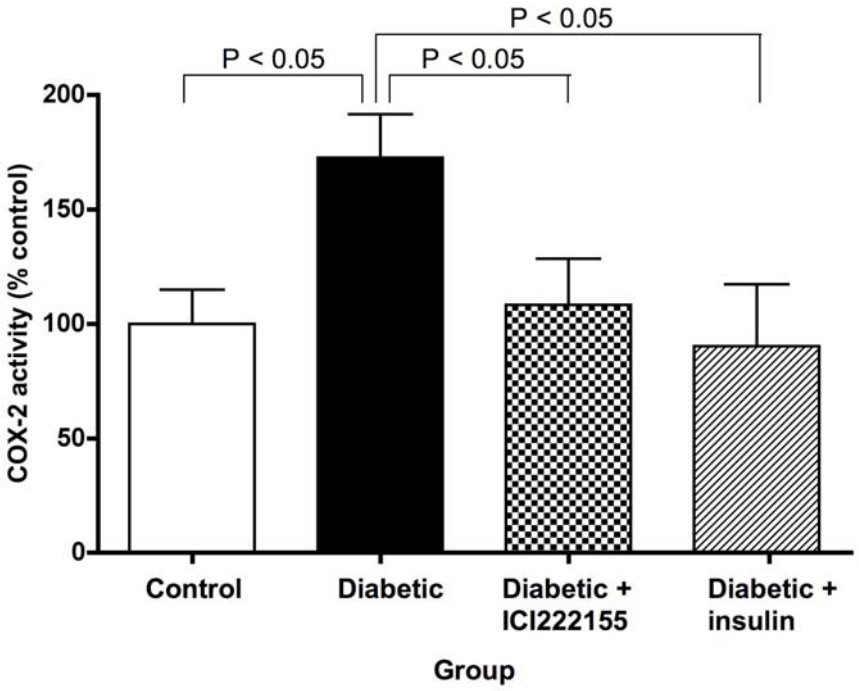


Figure 5.2. Quantification of spinal COX-2 activity revealed that ICI222155 or insulin treatment prevented the increase in spinal COX-2 activity levels observed in untreated diabetic rats. Data are presented as group mean ± SEM, N = 9 - 10 per group, statistical comparison by a one-way ANOVA with Dunnett's *post-hoc* test.

Figure 5.3.

Tactile 50% probability paw withdrawal thresholds in untreated control rats, untreated diabetic rats, control rats treated with ICI222155, and diabetic rats treated with ICI222155 or insulin.

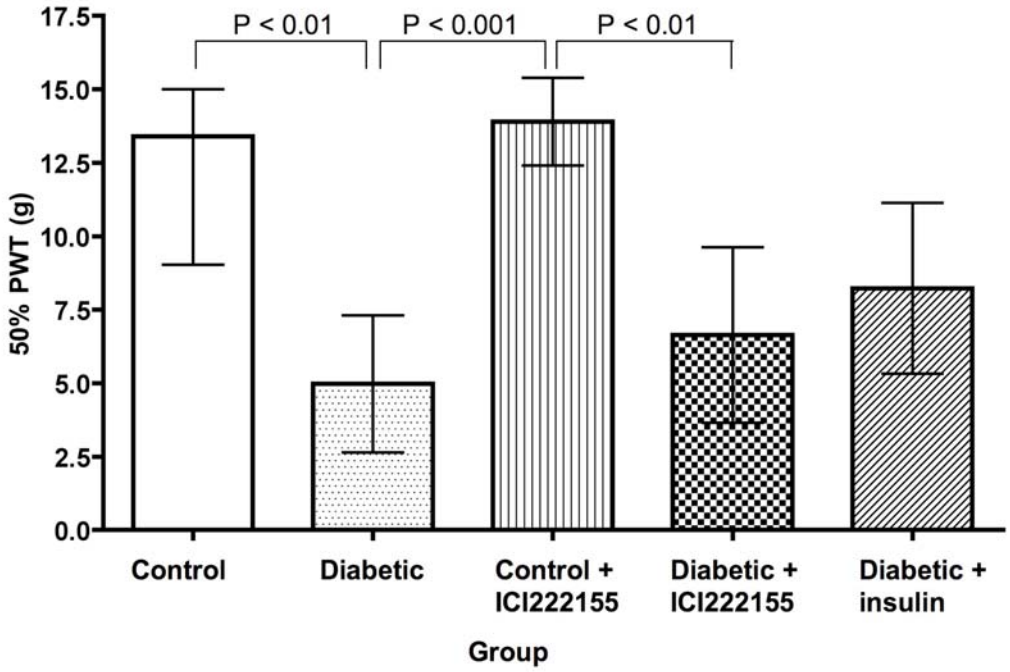


Figure 5.3. Measurement of tactile 50% probability paw withdrawal thresholds revealed tactile allodynia in untreated diabetic rats. ICI222155-treated and insulin-treated diabetic rats had withdrawal thresholds that were not significantly different from untreated control or diabetic rats, suggesting a tendency towards a protective effect of treatment. Data are presented as group median and interquartile range, N = 9 - 10 per group, statistical comparison performed using a Kruskal-Wallis test with Dunn's *post-hoc* test.

Figure 5.4.

Thermal paw withdrawal latencies in untreated control rats, untreated diabetic rats, control rats treated with ICI222155, and diabetic rats treated with ICI222155 or insulin.

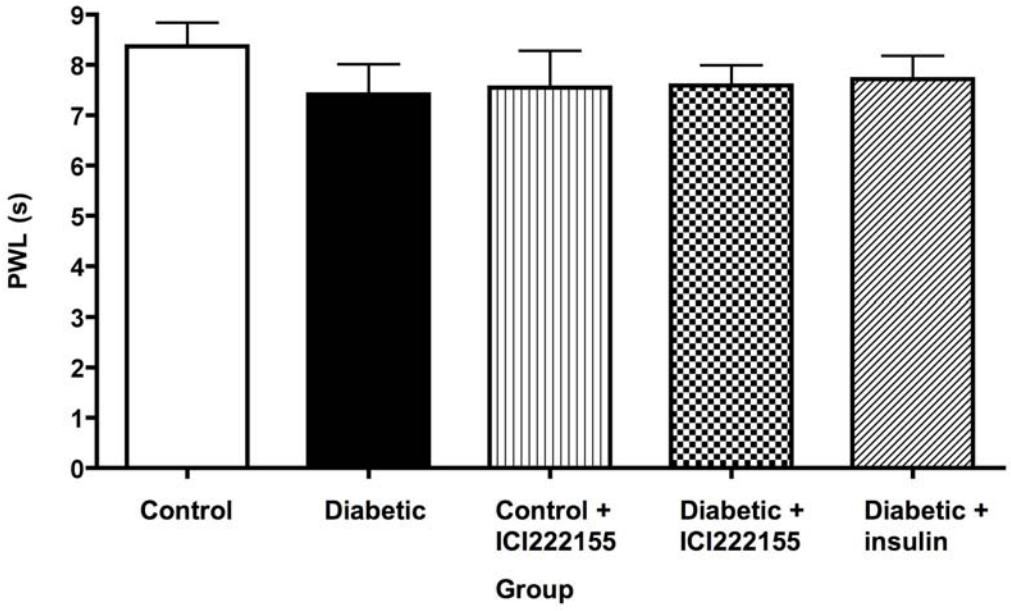


Figure 5.4. Measurement of thermal paw withdrawal latencies revealed no significant differences between any of the groups. Data are presented as group mean ± SEM, N = 9 - 10 per group, statistical comparison performed using a one-way ANOVA.

Figure 5.5.

Spinal COX-2 protein levels in untreated control rats, untreated diabetic rats, and diabetic rats treated with IDD676.

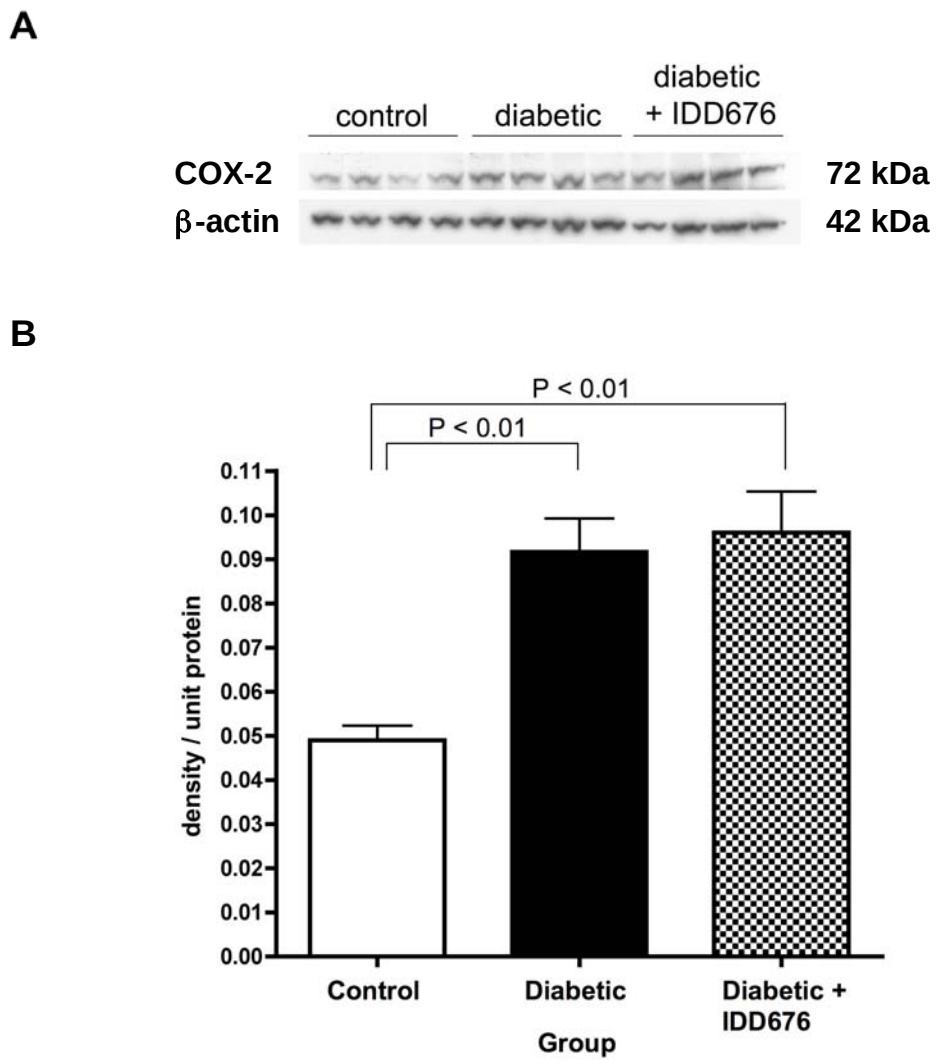


Figure 5.5. (A) Representative Western blots showing levels of COX-2 in lumbar spinal cord from control rats, untreated diabetic rats, and diabetic rats treated with IDD676. (B) Densitometric quantification of COX-2-immunostained Western blots indicated that IDD676 treatment did not prevent the increase in spinal COX-2 protein levels observed in untreated diabetic rats. Data are presented as group mean ± SEM, N = 8 per group, statistical comparison performed using a one-way ANOVA with Dunnett's *post-hoc* test.

Figure 5.6.

Flinching response to 0.5% formalin injected in the hindpaw of control rats, untreated diabetic rats, and diabetic rats treated with IDD676.

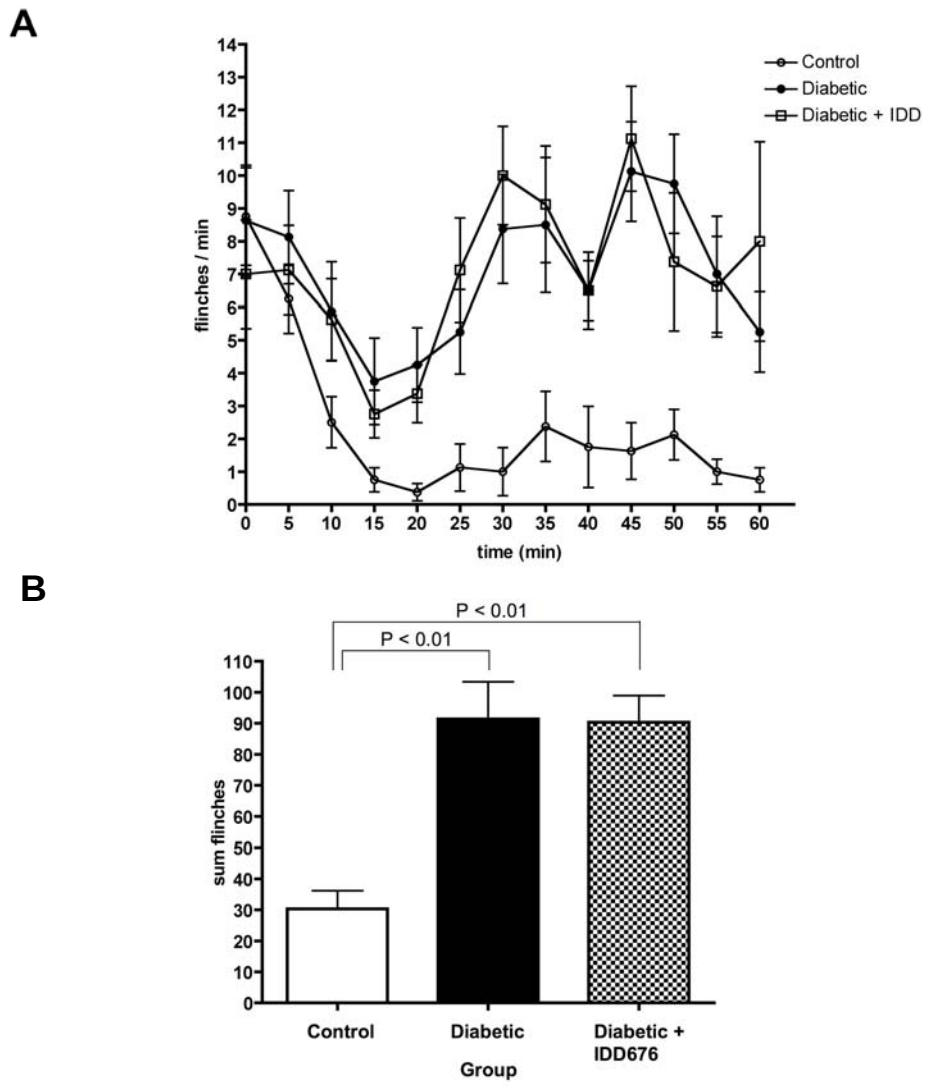


Figure 5.6. (A) Time course of formalin-evoked flinching in control rats, untreated diabetic rats, and diabetic rats treated with IDD676. (B) Total flinches counted in the 60 min after paw formalin injection in control rats, untreated diabetic rats, and diabetic rats treated with IDD676. IDD676 treatment did not prevent the development of hyperalgesia to paw formalin injection in diabetic rats. Data are presented as group mean ± SEM, N = 8 per group, statistical comparison performed using a one-way ANOVA with Dunnett's *post-hoc* test.

Figure 5.7.

Thermal paw withdrawal latencies (PWLs) measured in untreated control rats, untreated diabetic rats, and diabetic rats treated with IDD676.

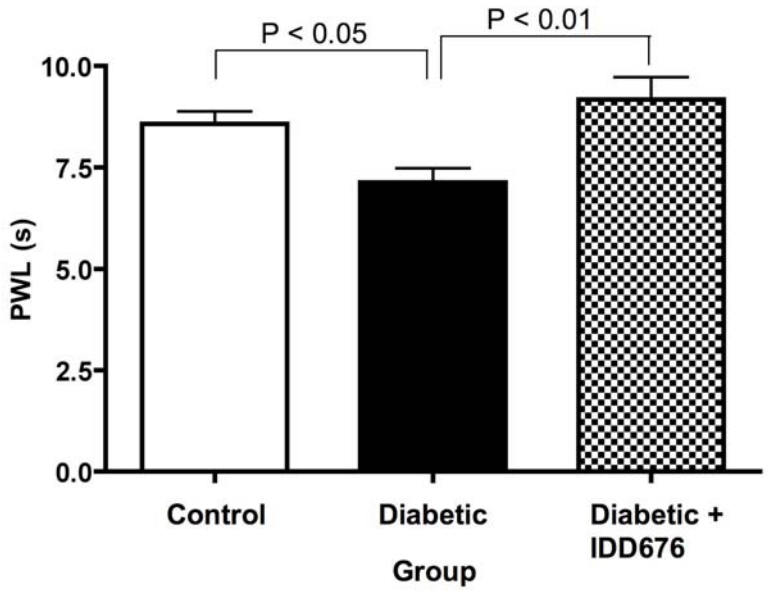


Figure 5.7. Measurement of thermal paw withdrawal latencies indicated that treating diabetic rats with IDD676 prevented the thermal hyperalgesia observed in untreated diabetic rats. Data are presented as group mean ± SEM, N = 8 per group, statistical comparison performed using a one-way ANOVA with the Student-Neuman-Keuls' *post-hoc* test.

Figure 5.8.

Motor and sensory nerve conduction velocities in untreated control rats, untreated diabetic rats, and diabetic rats treated with IDD676.

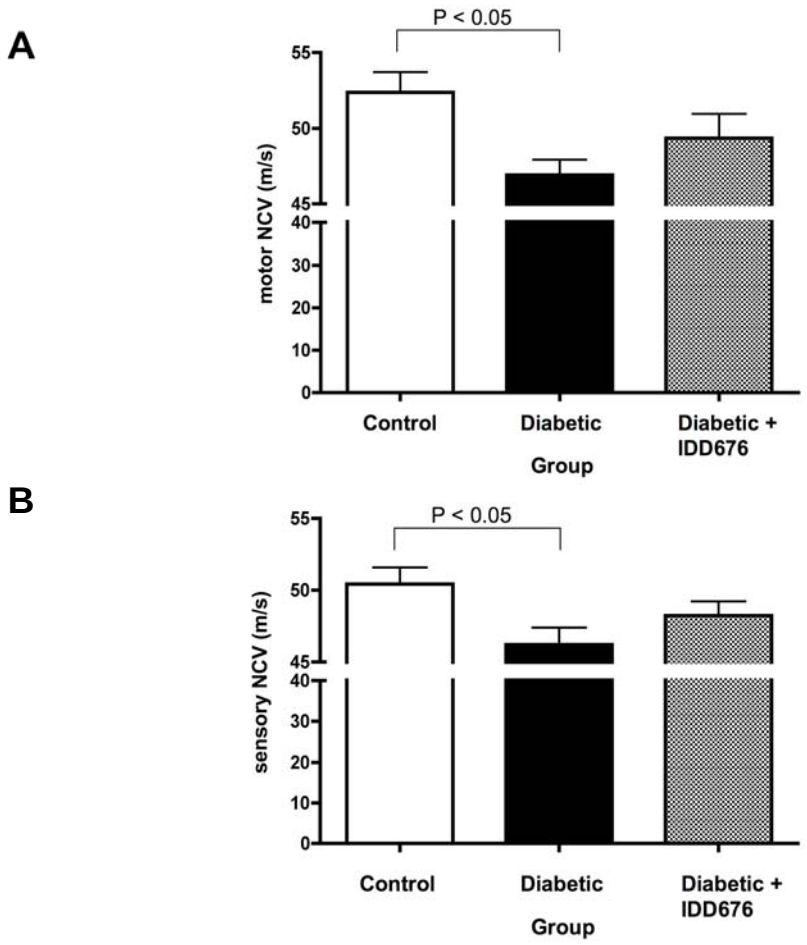


Figure 5.8. (A) Untreated diabetic rats exhibited slowing of motor nerve conduction velocity, whereas the motor nerve conduction velocity of diabetic rats treated with IDD676 was not significantly different from that of control rats or untreated diabetic rats. (B) Untreated diabetic rats exhibited slowing of sensory nerve conduction velocity, whereas the sensory nerve conduction velocity of diabetic rats treated with IDD676 was not significantly different from that of control rats or untreated diabetic rats. Data are presented as group mean ± SEM, N = 8 per group, statistical comparison performed using a one-way ANOVA with the Student-Neuman-Keuls' *post-hoc* test.

Table 5.1.

Body weights, blood glucose levels, and glycated hemoglobin levels for rats treated with ICI222155 or insulin.

Group	**Body weight (g)**	**Blood glucose (mM)**	**HbA1 (%)**
Control (N = 10)	260 ± 4 [a]	All < 15	4.0 ± 0.6 [a]
Control + ICI222155 (N = 10)	258 ± 5 [a]	All < 15	----
Diabetic (N = 10)	226 ± 8 [b]	All > 15	9.1 ± 1.3 [c]
Diabetic + ICI222155 (N = 9)	218 ± 6 [b]	All > 15	8.8 ± 1.1 [c]
Diabetic + insulin (N = 9)	270 ± 5 [a]	All < 15	3.1 ± 0.2 [a]

Data are group mean ± SEM, except blood glucose levels. Statistical analyses of body weights and HbA1 levels were performed using a one-way ANOVA with Newman-Keuls' *post-hoc* test. [a] vs. [b], $P < 0.001$; [a] vs. [c], $P < 0.01$.

Table 5.2.

Sugar and polyol levels measured in sciatic nerve, in control rats, untreated diabetic rats, and diabetic rats treated with ICI222155 or insulin.

	Control (N = 10)	**Diabetic (N = 10)**	**Diabetic + ICI222155 (N = 9)**	**Diabetic + insulin (N = 9)**
Nerve glucose	6.0 ± 0.4 [a]	55.0 ± 3.6 [b]	45.7 ± 3.2 [b]	5.2 ± 1.1 [a]
Nerve sorbitol	nd	0.3 ± 0.2	0.01 ± 0.01	0.2 ± 0.2
Nerve fructose	0.6 ± 0.1 [a]	15.2 ± 0.8 [b]	1.6 ± 0.3 [a]	0.4 ± 0.3 [a]
Nerve myo-inositol	11.1 ± 1.2 [c]	8.1 ± 0.6 [d]	12.1 ± 1.2 [c]	9.9 ± 0.5 [c]

Data are group mean ± SEM. Sugar and polyol levels are presented as nmol/mg dry weight. [a] vs. [b] $P < 0.001$; [c] vs. [d] $P < 0.05$. Statistical analysis performed using a one-way ANOVA with the Student-Newman-Keuls' *post-hoc* test. nd = not detected.

Table 5.3.

Body weights and blood glucose levels for rats treated with IDD676.

Group	Body weight (g)	Blood glucose (mM)
Control (N = 8)	261 ± 4 [a]	All < 15
Diabetic (N = 8)	223 ± 8 [b]	All > 15
Diabetic + IDD676 (N = 8)	234 ± 11 [c]	All > 15

Data are group mean ± SEM, except blood glucose levels. Statistical analysis of body weights was performed using a one-way ANOVA with the Newman-Keuls' *post-hoc* test. [a] vs. [b], $P < 0.01$; [a] vs. [c], $P < 0.05$.

Table 5.4.

Sugar and polyol levels measured in sciatic nerve and spinal cord, in control rats, untreated diabetic rats, and diabetic rats treated with IDD676.

	Control (N = 8)	**Diabetic (N = 8)**	**Diabetic + IDD676 (N = 8)**
Nerve glucose	4.2 ± 0.7 [a]	35.4 ± 2.8 [b]	47.1 ± 2.6 [c]
Nerve sorbitol	nd	3.3 ± 1.3	1.1 ± 0.6
Nerve fructose	0.4 ± 0.1 [a]	14.5 ± 1.2 [b]	1.1 ± 0.5 [a]
Nerve myo-inositol	13.6 ± 1.2	9.4 ± 0.8	14.0 ± 2.3
Spinal cord glucose	1.7 ± 0.3 [a]	12.5 ± 1.4 [b]	12.1 ± 1.3 [b]
Spinal cord sorbitol	nd	nd	nd
Spinal cord fructose	1.3 ± 0.2 [a]	3.1 ± 0.3 [b]	2.4 ± 0.2 [b]
Spinal cord myo-inositol	28.8 ± 0.7	27.6 ± 1.0	29.4 ± 0.5

Data are group mean ± SEM. Sugar and polyol levels are presented as nmol/mg dry weight. [a] vs. [b, c] $P < 0.001$; [b] vs. [c] $P < 0.01$. Statistical analysis was performed using a one-way ANOVA with the Student-Newman-Keuls' *post-hoc* test. nd = not detected.

CHAPTER 6 – REVERSAL OF ESTABLISHED HYPERALGESIA AND SPINAL SENSITIZATION IN EXPERIMENTAL DIABETIC NEUROPATHY

6.1 Introduction

Having studied the mechanism by which hyperalgesia and spinal sensitization develop in diabetic rats, we next wanted to investigate whether these phenomena can be reversed in diabetic rats. Reversal of painful symptoms associated with diabetes is significant because this mimics the situation experienced by patients with diabetes, who are treated to alleviate pain once the symptoms have begun. These experiments were therefore designed to test whether established hyperalgesia to paw formalin injection and elevated spinal COX-2 protein and activity levels can be pharmacologically reversed in rats with one or four weeks of diabetes. Because the data presented in chapter 4 indicated an important role for exaggerated polyol pathway flux in the CNS in driving the development of hyperalgesia and elevated spinal COX-2, the reversal studies presented in this chapter utilized treatment with the BBB-crossing ARI ICI222155. Insulin-treated diabetic rats were included as controls to prove that abnormalities in diabetic rats resulted from hyperglycemia and its metabolic consequences.

6.2 Methods

6.2.1 General experimental design

The initial goal of these studies was to determine if, after four weeks of untreated diabetes, interventional treatment with the ARI ICI222155 for four further weeks could reverse elevated spinal COX-2 protein and activity levels

along with hyperalgesia to paw formalin injection. We chose to intervene with ARI treatment after four weeks of untreated diabetes because this duration of diabetes was used in the prevention studies described in chapter 5. A group of insulin-treated diabetic rats was included to show that defects observed in untreated diabetic rats were not due to STZ toxicity. The results from this initial reversal study indicated that treatment with ICI222155 was unable to reverse established formalin hyperalgesia and exaggerated spinal COX-2 expression. Because four weeks of treatment with ICI222155 was able to prevent the development of formalin hyperalgesia and exaggerated spinal COX-2 expression (Calcutt et al., 1995; chapter 5), this suggested that at durations of diabetes greater than four weeks, exaggerated polyol pathway flux may not be the only factor driving spinal sensitization and hyperalgesia, and conversely, that at durations of diabetes less than four weeks, exaggerated polyol pathway flux is the primary factor driving spinal sensitization and hyperalgesia. This latter hypothesis was tested with a second, short-term reversal study to determine whether hyperalgesia and elevated spinal COX-2 expression could be reversed with ICI222155 treatment in rats with only one week of diabetes. A group of insulin-treated diabetic rats was again included to exclude the possibility that direct STZ toxicity caused the abnormalities observed in diabetic rats.

6.2.2 Experimental methods

Two studies were performed to determine if treatment with ICI222155 or insulin could reverse established formalin hyperalgesia and elevated spinal

COX-2 expression in rats with four weeks or one week of diabetes. In the first study, an initial cohort of rats was divided into one group of control rats and three groups of rats that were made diabetic by a single i.p. injection of STZ. Hyperglycemia was confirmed three days later using blood taken by tail prick and a strip-operated reflectance meter (section 2.1). After four weeks of untreated diabetes we assumed that all of the diabetic rats had exaggerated levels of spinal COX-2 protein and activity, and would display a hyperalgesic response to paw injection of 0.5% formalin, based on the data in chapters 4, 5, and published data (Malmberg et al., 1993; Calcutt et al., 1995; Freshwater et al., 2002). At this point in the study, one group of the diabetic rats began receiving ICI222155 treatment daily by oral gavage at 20 mg ICI22155 per kg body weight, and a second group of diabetic rats received sub-cutaneous implants of slow-dissolving insulin pellets that deliver approximately 2-4 U insulin per day (Linshin, Scarborough, Ontario, Canada), to reverse hyperalgesia. Blood glucose levels in the insulin-treated rats were checked daily and any rat with a blood glucose level above 15 mmol/l received a second pellet of insulin. ICI222155 and insulin treatment continued for four weeks, during which time the third group of diabetic rats and the control rats remained untreated. At the end of the study, each group of rats was sub-divided such that some underwent 0.5% formalin testing (section 2.4.4), while some were used solely for measurement of spinal COX-2 protein and activity levels (sections 2.2.3 and 2.2.4). This was done to avoid the possible upregulation of spinal COX-2 activity in response to paw formalin injection,

which would confound analysis of diabetes- and treatment-induced changes in COX-2 activity levels. Additionally, portions of spinal cord and sciatic nerve were frozen for later measurement of glucose, fructose, sorbitol, and myo-inositol levels using gas chromatography (section 2.2.5). All behavioral testing and tissue harvesting was done 24 hours after the last ICI222155 treatment.

The second experiment was designed to test whether insulin or ICI222155 treatment could reverse hyperalgesia to paw formalin injection and elevated spinal COX-2 protein in rats with one week of diabetes. Because we had previously always observed that COX-2 protein and activity levels co-vary (chapters 4 and 5), we restricted our measurements to spinal COX-2 protein levels in these animals. This allowed us to use only one cohort of animals for this short-term reversal study, because paw formalin injection could induce confounding increases in spinal COX-2 activity. Three groups of rats were made diabetic with a single i.p. injection of STZ, and hyperglycemia was confirmed three days later using blood taken by tail prick and a strip-operated reflectance meter (section 2.1). After one week of untreated diabetes we assumed that all of the rats had abnormally high levels of spinal COX-2 protein and activity, and that they all would display a hyperalgesic response to paw injection of 0.5% formalin, based on the data in chapter 4. At this point in the study, one group of diabetic rats remained untreated for a second week, one group began daily treatment by oral gavage with ICI222155 at 20 mg ICI222155 per kg body weight for one week, and the third group was implanted with slow-release insulin pellets to reverse hyperglycemia for one

week. All diabetic rats, along with a group of age-matched control rats, then underwent 0.5% formalin testing (section 2.4.4) before being killed, and spinal cords were removed to measure COX-2 protein levels (section 2.2.3). Additionally, portions of spinal cord and sciatic nerve were frozen for later measurement of glucose, fructose, sorbitol, and myo-inositol levels using gas chromatography (section 2.2.5). For the ICI222155-treated diabetic rats, formalin testing and subsequent tissue removal were performed 24 hours after the last ICI222155 treatment.

The results from the short-term reversal study presented an apparent discrepancy in that ICI222155 treatment and insulin treatment reversed formalin hyperalgesia but did not reverse elevated spinal COX-2 protein levels. This study was therefore repeated to determine if spinal COX-2 activity levels would be reversed by insulin treatment. ICI222155 treatment was not included because of a lack of availability of this drug. This study began with three groups of rats, two of which were made diabetic with a single i.p. injection of STZ. Hyperglycemia was confirmed three days later using blood taken by tail prick and a strip-operated reflectance meter (section 2.1). After one week of untreated diabetes, one group of diabetic rats remained untreated for a second week, and the second group was implanted with slow-release insulin pellets to reverse hyperglycemia for one week. All diabetic rats, along with the group of age-matched control rats, were then killed, and spinal cords were removed to measure COX-2 activity levels (section 2.2.4).

6.3 Results

6.3.1 Reversal of long-term established hyperalgesia and spinal sensitization

We first tested whether ICI222155 treatment could reverse established formalin hyperalgesia and elevated spinal COX-2 protein and activity levels in rats with four weeks of diabetes. At the start of this study, the average weight of the rats was 233 ± 1 g. At the conclusion of the study, the untreated diabetic rats exhibited hyperglycemia and little weight gain, which were not affected by ICI222155 treatment (Table 6.1). Insulin-treated diabetic rats had normalized body weights and blood glucose levels (Table 6.1). Gas chromatography of sciatic nerve samples indicated that ICI222155 treatment significantly increased glucose and myo-inositol levels, and blocked accumulation of sorbitol and fructose, demonstrating efficacy of this ARI in the PNS (Table 6.2). In the spinal cord, untreated diabetic rats showed significantly elevated glucose levels, and this was reversed by treatment with insulin but not with ICI222155 (Table 6.2). Sorbitol was not detected in any control rat spinal tissue but was detected at low levels in spinal tissue from untreated diabetic rats and ICI222155- or insulin-treated diabetic rats (Table 6.2). There was a statistically significant accumulation of fructose in the spinal cord of untreated diabetic rats, and this was reversed by treatment with insulin but not with ICI222155 (Table 6.2). There was no significant difference in spinal levels of myo-inositol between any of the groups (Table 6.2).

Untreated diabetic rats had significantly increased levels of spinal COX-2 protein as compared with control rats (Figure 6.1). Elevated spinal COX-2

protein expression was reversed by four weeks of insulin treatment, but not by four weeks of treatment with ICI222155 (Figure 6.1). To confirm that the excess COX-2 protein measured in spinal cord tissue from ICI222155-treated diabetic rats was enzymatically active, COX-2 activity levels were measured in untreated diabetic rats and ICI222155-treated diabetic rats. There was no difference in spinal COX-2 activity between these two groups (Figure 6.2). In response to paw injection of 0.5% formalin, untreated diabetic rats exhibited exaggerated flinching, and this was reversed in insulin-treated diabetic rats but not in ICI222155-treated diabetic rats (Figure 6.3).

6.3.2 Reversal of short-term established hyperalgesia and spinal sensitization

In the second study of this chapter, we tested whether formalin hyperalgesia and elevated spinal COX-2 protein in rats with one week of diabetes could be reversed by one further week of treatment with ICI222155 or insulin. At the start of this study, the average weight of the rats was 241 ± 1 g. At the conclusion of the study, untreated diabetic rats exhibited weight loss and hyperglycemia that were not affected by treatment with ICI222155 (Table 6.3). Insulin-treated diabetic rats did not exhibit weight loss as compared with control rats, and they were not hyperglycemic (Table 6.3).

In the sciatic nerve, untreated diabetic rats had significantly elevated levels of glucose, sorbitol and fructose, as compared with control rats (Table 6.4). ICI222155 treatment of diabetic rats had no effect on nerve glucose levels, but significantly blocked accumulation of fructose and sorbitol (Table 6.4). Levels of glucose, sorbitol, fructose and myo-inositol in the sciatic nerve

were not significantly different between control rats and insulin-treated diabetic rats (Table 6.4). In the spinal cord, levels of glucose and fructose were significantly elevated in untreated and ICI222155-treated diabetic rats, as compared with control rats or insulin-treated diabetic rats (Table 6.4). Sorbitol was not detected in spinal tissue from control rats, but spinal sorbitol accumulation was measured in untreated diabetic rats (Table 6.4). Treatment of diabetic rats with ICI222155 or insulin significantly blocked accumulation of sorbitol in the spinal cord (Table 6.4). Spinal levels of myo-inositol were not different among the groups (Table 6.4).

Measurement of spinal COX-2 protein levels revealed a two-fold increase in untreated diabetic rats versus control rats (Figure 6.4). ICI222155- and insulin-treated diabetic rats exhibited a two-fold increase in spinal COX-2 protein levels as compared with untreated diabetic rats (Figure 6.4). Untreated diabetic rats showed hyperalgesia to paw injection of 0.5% formalin, and this was completely reversed in diabetic rats treated with ICI222155 or insulin (Figure 6.5).

Because insulin and ICI222155 treatment reversed formalin hyperalgesia but did not reverse elevated spinal COX-2 protein levels, the short-term reversal study was repeated to test whether insulin treatment in this study design would reverse elevated spinal COX-2 activity levels. We were unable to test the ability of ICI222155 treatment to reverse elevated spinal COX-2 activity levels due to lack of this drug. At the start of this study, the average weight of the rats was 238 ± 2 g. At the end of the study, diabetic rats

exhibited hyperglycemia, which was reversed in insulin-treated diabetic rats, and there was no significant difference in weight between the groups (Table 6.5). Measurement of spinal COX-2 activity levels indicated a 50% increase in diabetic rats, as compared with control rats, and this difference was statistically significant by unpaired t-test ($P < 0.05$; Figure 6.6). In insulin-treated diabetic rats, the mean spinal COX-2 activity level was 90% of that observed in control rat tissue, indicating that insulin treatment reversed elevated spinal COX-2 activity.

6.4 Discussion

These studies were designed to assess whether established hyperalgesia and spinal sensitization in diabetic rats could be pharmacologically reversed. Reversal of established sensory and biochemical abnormalities in diabetic rats is clinically relevant because, although patients with diabetes are encouraged to practice glycemic control in order to prevent diabetic complications, patients who have developed painful diabetic neuropathy are treated symptomatically (Flint and Clements, 1988; DCCT, 1993; Boulton, 2003; Argoff et al., 2006; Jensen et al., 2006). In these studies, we found that after four weeks of untreated diabetes, four further weeks of treatment with ICI222155 did not reverse established hyperalgesia or elevated spinal COX-2 expression. In this same long-term reversal study design, insulin treatment was able to reverse established hyperalgesia and elevated spinal COX-2 expression. In rats with one week of diabetes, a subsequent week of treatment with insulin or ICI222155 reversed hyperalgesia

to paw formalin injection. In these treated diabetic rats, there was a paradoxical increase in spinal COX-2 protein as compared with untreated diabetic rats, but this excess COX-2 protein was likely enzymatically inactive, as measurement of spinal COX-2 activity levels indicated that increased activity in untreated diabetic rats was normalized by insulin treatment.

The results from the long-term reversal study prompt several questions, and the gas chromatography (GC) data set obtained from the rats used in this study is an important element to consider when interpreting the behavioral and biochemical data. In the sciatic nerve, the GC data (Table 6.2) are in agreement with published data (Calcutt et al., 1995). The nerve glucose levels were significantly elevated in ICI222155-treated diabetic rats as compared with untreated diabetic rats (Table 6.2). This effect is presumably due to the chronic blockade of the polyol pathway's ability to metabolize glucose, and has been observed in other studies involving ARI treatment of diabetic rats (Gillon et al., 1983; Willars et al., 1987; Calcutt et al., 1995). ICI222155 treatment also completely blocked nerve sorbitol accumulation, significantly reduced nerve fructose accumulation, and significantly increased nerve levels of myo-inositol (Table 6.2). Taken together, these data indicate clear efficacy of ICI222155 treatment in the peripheral nerve. However, the spinal GC data indicate that treatment of diabetic rats with ICI222155 did not produce a successful block of sorbitol or fructose accumulation in the spinal cord. In contrast, insulin treatment of diabetic rats normalized spinal sorbitol and fructose levels.

The differing abilities of insulin and ICI222155 treatment to block spinal polyol product accumulation may explain the results of ICI222155 and insulin treatment in this long-term reversal study. It is plausible that elevated spinal COX-2 and formalin hyperalgesia were reversed by insulin treatment but not by treatment with ICI222155 because only the former successfully blocked polyol product accumulation in the spinal cord. Alternatively, it is possible that at longer durations of diabetes, spinal sensitization is maintained by a pathogenic mechanism that is due to hyperglycemia and its metabolic consequences but is not directly maintained by ongoing exaggerated polyol pathway flux. Repeating the experiment with a higher dose of ICI222155, in an effort to successfully block spinal polyol product accumulation, could test this latter hypothesis. If spinal polyol product accumulation as well as formalin hyperalgesia and elevated spinal COX-2 were normalized by ARI treatment, this would suggest that through eight weeks of diabetes, exaggerated polyol pathway flux drives formalin hyperalgesia and elevated spinal COX-2. If, however, ARI treatment normalized spinal polyol product accumulation but did not normalize formalin hyperalgesia and elevated spinal COX-2, this would suggest that at durations of diabetes greater than four weeks, exaggerated polyol pathway flux is not the only factor driving spinal sensitization and hyperalgesia. This might occur if the initial four weeks of exaggerated polyol pathway flux in diabetic rats were to activate a downstream mechanism that, once activated, could not be turned off by subsequent intervention with an ARI. It is believed that exaggerated polyol pathway flux in diabetes causes a

range of secondary pathologic mechanisms that all contribute to nervous system damage (as discussed in section 1.3.1). These secondary pathologic mechanisms include increased oxidative stress, enhanced nonenzymatic glycation, and loss of neurotrophic support. It is plausible that after four weeks of exaggerated polyol pathway flux in diabetic rats, there is sufficient oxidative stress-associated damage, nonenzymatic glycation of proteins and loss of neurotrophic support in the CNS such that interventional treatment to reverse sensory abnormalities would require targeting of these secondary mechanisms in addition to blockade of AR activity.

It is unclear why the dose of ICI222155 used in the long-term reversal study was insufficient to block polyol product accumulation in the spinal cord, as this dose has demonstrated CNS efficacy previously (Caclutt et al., 1995) and in the present short-term reversal study (as discussed below). Recent data suggest that in order to achieve full block of exaggerated polyol pathway activity in diabetes, it may be necessary to use doses of ARIs ten-fold higher than what would be needed to achieve blockade of polyol product accumulation (Oates et al., 2006). It may be that blockade of polyol product accumulation is sufficient to prevent both development of sensory processing abnormalities in diabetic rats and reversal of such abnormalities if only a short duration of diabetes has elapsed, whereas reversal of long-term established sensory abnormalities may require a more complete block of the elevated activity of AR. To date, such high doses of ARIs have not been used for

patients, and there are no published reports of toxicity associated with such treatment in animals.

The duration of treatment with ICI222155 (four weeks) may not have been long enough to sufficiently inhibit polyol pathway activity and the associated polyol product accumulation. However, the results from the short-term reversal study indicate that after one week of diabetes, one week of treatment with ICI222155 was sufficient to inhibit spinal sorbitol accumulation. This suggests that in the long-term reversal study, four weeks of treatment with ICI222155 should have been sufficient to block spinal polyol product accumulation. While there are no published studies documenting efforts to reverse formalin hyperalgesia in diabetic rats using ARI treatment, results from clinical trials with the ARIs epalrestat, tolrestat, and fidarestat have demonstrated improvements in self-reported spontaneous pain only after at least three months of treatment (Macleod et al., 1992; Uchida et al., 1995; Hotta et al., 1996; Hotta et al., 2001). The mean duration of diabetes was at least 10 years in each study, indicating the patients in these trials had suffered from diabetes for a relatively long time. These clinical data may suggest that in rats with a longer duration of diabetes (four weeks, versus one week), a longer duration of ICI222155 treatment is necessary to successfully block spinal polyol product accumulation and to reverse formalin hyperalgesia and spinal sensitization. Alternatively, long-term inhibition of AR may cause an upregulation of AR expression, to compensate for chronic inhibition of the enzyme. This has been observed to occur with other systems. For example,

chronic treatment with lithium, which acutely inhibits adenylyl cyclase, causes an upregulation of adenylyl cyclase expression in the brain (Colin et al., 1991). This autoregulatory mechanism could explain why short-term treatment with 20 mg ICI222155 per kg body weight successfully affected spinal disorders in diabetic rats, while long-term treatment with the same dose did not.

The results from the short-term reversal study suggest that between one and two weeks of diabetes, exaggerated polyol pathway flux drives hyperalgesia to paw formalin injection, because treatment with the ARI ICI222155 during this time was sufficient to reverse hyperalgesia. In this study, spinal sorbitol accumulation was significantly reduced by ICI222155 treatment, which may indicate that inhibition of polyol product accumulation in the CNS is necessary to reverse spinal sensitization in diabetic rats.

The finding that both insulin and ICI222155 treatment reversed hyperalgesia but did not reverse elevated spinal COX-2 protein levels is seemingly contradictory. COX-2 is known to undergo use-inactivation (Smith and Lands, 1972; Kulmacz and Lands, 1983) but little is known about how inactivated COX-2 protein is recognized intracellularly and targeted for degradation, which suggests that inactivated COX-2 protein may accumulate within cells for an unknown length of time (for review, see Fitzpatrick and Soberman, 2001). It is therefore plausible that after one week of untreated diabetes, one week of treatment with ICI222155 or insulin normalized elevated spinal COX-2 activity, yet the duration of treatment was not long enough to allow degradation of the enzymatically inactive COX-2 protein. To test this,

the experiment was repeated to determine if insulin treatment would normalize spinal COX-2 activity levels, and we confirmed this to be the case. Having no ICI222155 left available to use prevented us from confirming that ICI222155 treatment would normalize spinal COX-2 activity levels in this study design, but it is possible that this would be the case, given that both insulin and ICI222155 treatment reversed formalin hyperalgesia.

A more puzzling question prompted by the short-term reversal study's results is why there was significantly more spinal COX-2 protein in ICI222155-treated and insulin-treated diabetic rats as compared with untreated diabetic rats. While we do not as yet have an explanation for this result, one possibility is that both ICI222155 and insulin treatment inactivated the entire PLA_2/COX-2-prostaglandin synthesis pathway and this interfered with the normal clearance of inactivated COX-2 protein, which allowed an excess accumulation of COX-2 protein.

In conclusion, at short durations of diabetes, hyperalgesia to paw formalin injection in diabetic rats is driven by exaggerated polyol pathway flux. At longer durations of diabetes, spinal sensitization is maintained by a pathogenic mechanism that arises from hyperglycemia and its metabolic consequences. Because a significant blockade of spinal polyol product accumulation was not achieved with ICI222155 treatment in the long-term reversal study, we cannot presently determine whether spinal sensitization at longer durations of diabetes is directly maintained by exaggerated polyol pathway flux.

Figure 6.1.

Spinal COX-2 protein levels in control rats, untreated diabetic rats, and diabetic rats treated with ICI222155 or insulin for four weeks after a prior four-week period of untreated diabetes.

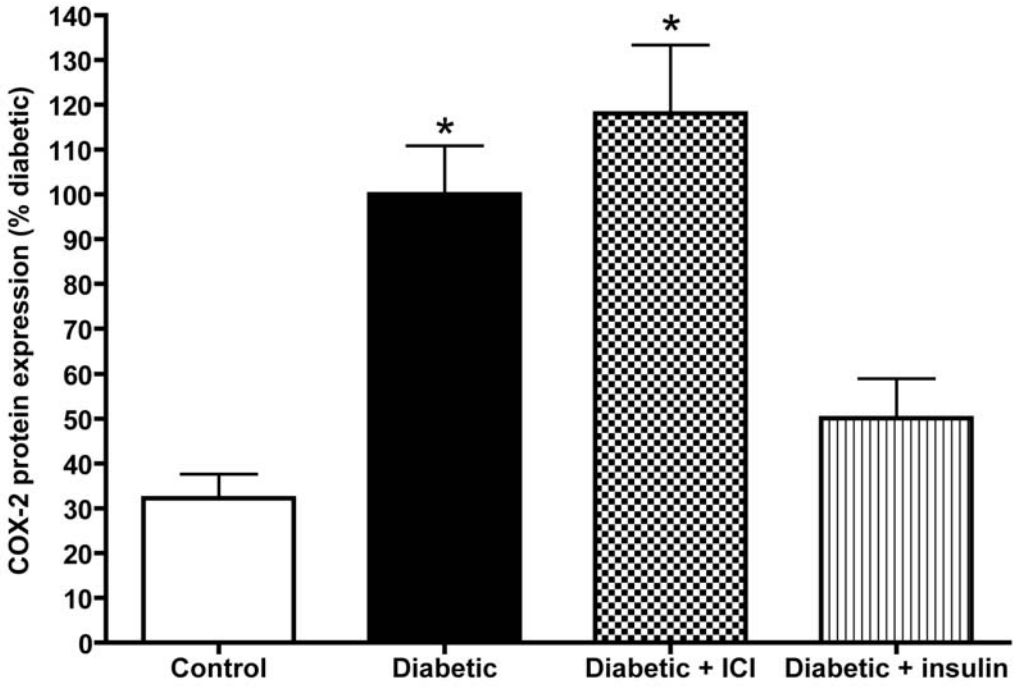


Figure 6.1. Densitometric quantification of COX-2-immunostained Western blots showing that insulin treatment reversed the increase in spinal COX-2 protein levels observed in untreated diabetic rats, but ICI222155 treatment did not. Western blot values were normalized to β-actin, and normalized levels of COX-2 protein expression are expressed as percent of untreated diabetic rats. Data are presented as group mean ± SEM, N = 7 - 8 per group, statistical comparison performed using a one-way ANOVA with the Student-Neuman-Keuls' *post-hoc* test. * $P < 0.01$ vs. control rats or insulin-treated diabetic rats.

Figure 6.2.

Spinal COX-2 activity levels in untreated diabetic rats and diabetic rats treated with ICI222155 for four weeks after a prior four-week period of untreated diabetes.

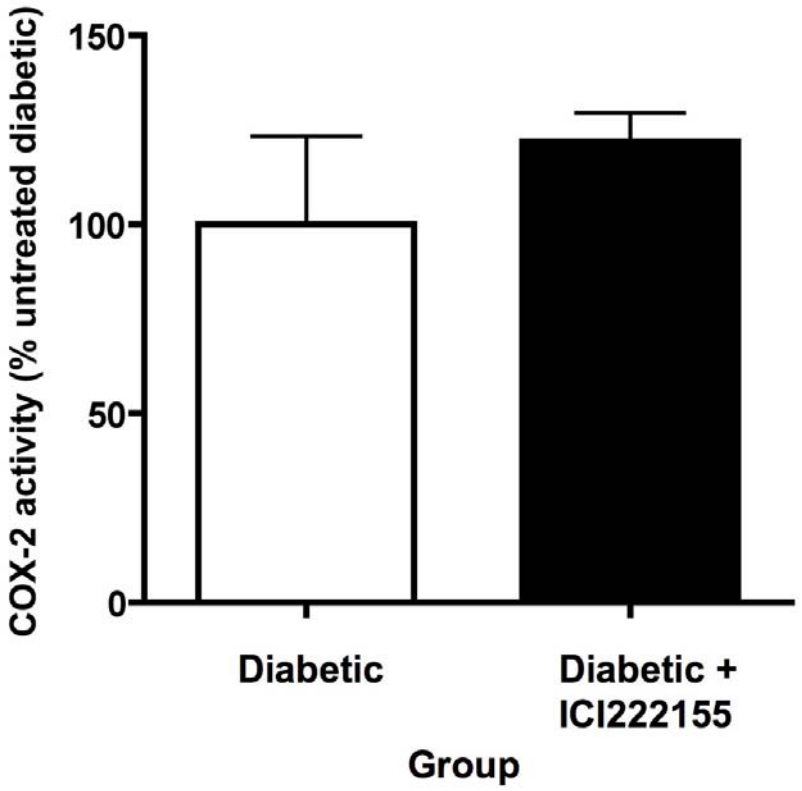


Figure 6.2. Measurement of spinal COX-2 activity levels revealed no significant difference between untreated diabetic rats and ICI222155-treated diabetic rats. Data are presented as group mean ± SEM, N = 8 per group, statistical comparison performed using an unpaired t-test.

Figure 6.3 A

Flinching response to 0.5% formalin injected in the hindpaw of control rats, untreated diabetic rats, and diabetic rats treated with ICI222155 or insulin for four weeks after a prior four-week period of untreated diabetes.

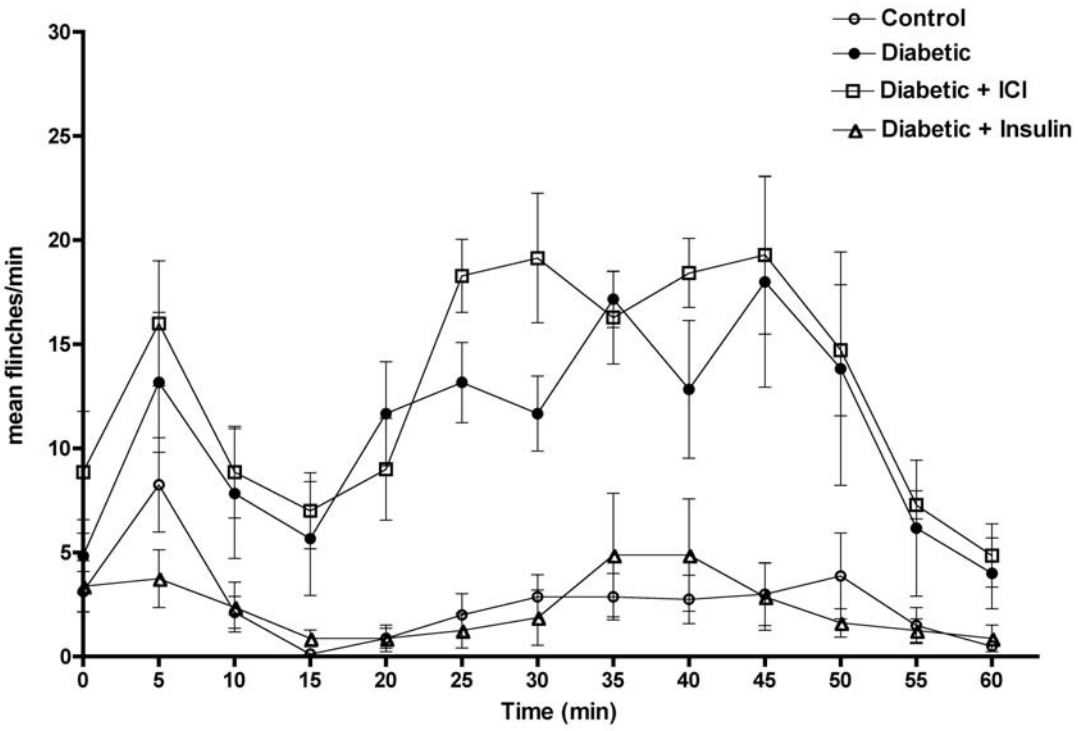


Figure 6.3. (A) Time course of formalin-evoked flinching in control rats, untreated diabetic rats, and diabetic rats treated with ICI222155 or insulin. Data are presented as group mean ± SEM, N = 6 - 8 per group.

Figure 6.3 B

Flinching response to 0.5% formalin injected in the hindpaw of control rats, untreated diabetic rats, and diabetic rats treated with ICI222155 or insulin for four weeks after a prior four-week period of untreated diabetes.

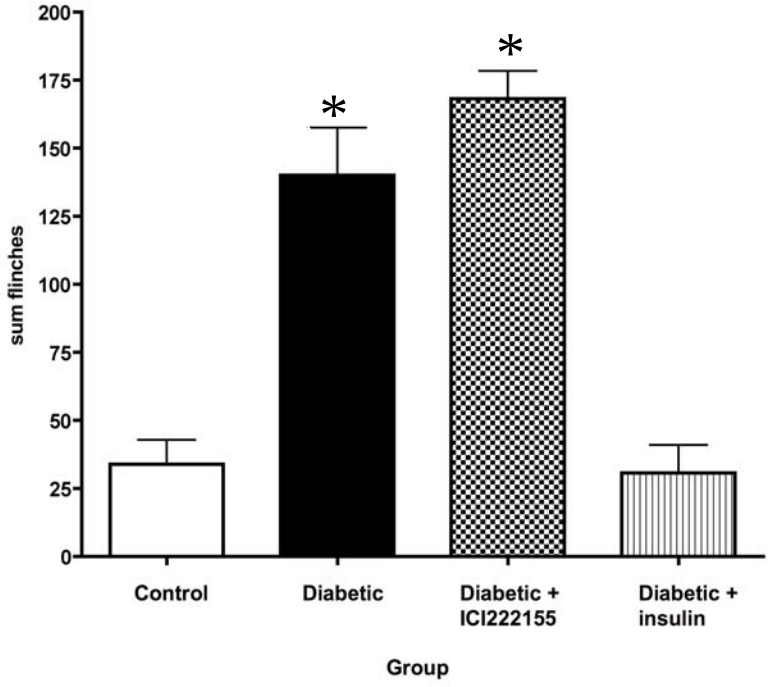


Figure 6.3. (B) Total flinches counted in the 60 min after paw formalin injection in control rats, untreated diabetic rats, and diabetic rats treated with ICI222155 or insulin. ICI222155 treatment did not reverse hyperalgesia to paw formalin injection whereas insulin treatment did. * $P < 0.001$ vs. control rats or insulin-treated diabetic rats. Data are presented as group mean ± SEM, N = 6 - 8 per group, statistical comparison performed using a one-way ANOVA with Dunnett's *post-hoc* test.

Figure 6.4.

Spinal COX-2 protein levels in control rats, untreated diabetic rats, and diabetic rats treated with ICI222155 or insulin for a period of one week after a prior one-week period of untreated diabetes.

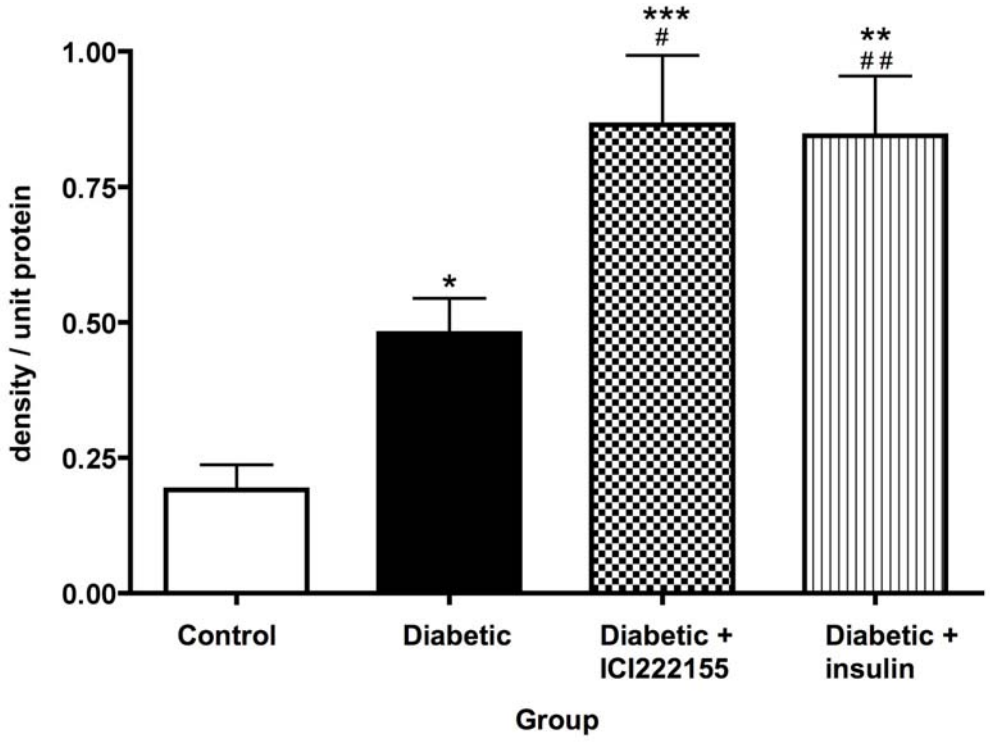


Figure 6.4. Densitometric quantification of COX-2-immunostained Western blots showing that untreated diabetic rats had twice as much spinal COX-2 protein as control rats. Diabetic rats treated with ICI222155 or insulin had nearly twice as much spinal COX-2 protein as untreated diabetic rats. Western blot values are normalized to β-actin. * $P < 0.05$ vs. control rats; ** $P < 0.01$ vs. control rats; *** $P < 0.001$ vs. control rats; $^{\#}$ $P < 0.05$ vs. untreated diabetic rats; $^{\#\#}$ $P < 0.01$ vs. untreated diabetic rats. Data are presented as group mean ± SEM, N = 6 - 8 per group, statistical comparison performed using a one-way ANOVA with the Student-Neuman-Keuls' *post-hoc* test.

Figure 6.5 A

Flinching response to 0.5% formalin injected in the hindpaw of untreated control rats, untreated diabetic rats, and diabetic rats treated with ICI222155 or insulin for one week after a prior one-week period of untreated diabetes.

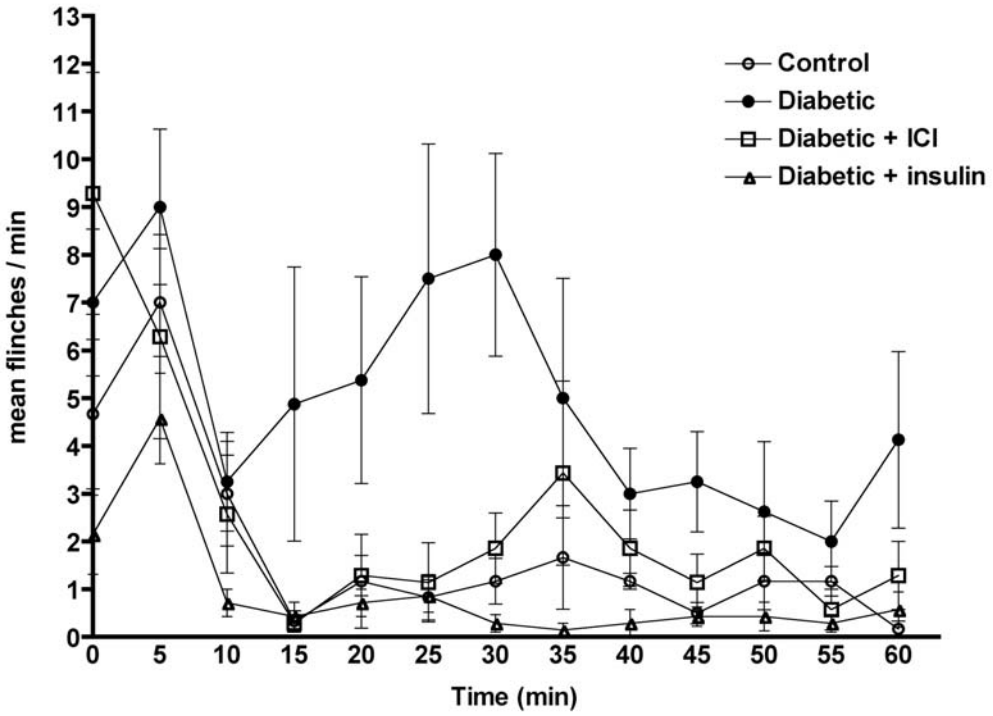


Figure 6.5. (A) Time course of formalin-evoked flinching in control rats, untreated diabetic rats, and diabetic rats treated with ICI222155 or insulin. Data are presented as group mean ± SEM, N = 6 - 8 per group.

Figure 6.5 B

Flinching response to 0.5% formalin injected in the hindpaw of untreated control rats, untreated diabetic rats, and diabetic rats treated with ICI222155 or insulin for one week after a prior one-week period of untreated diabetes.

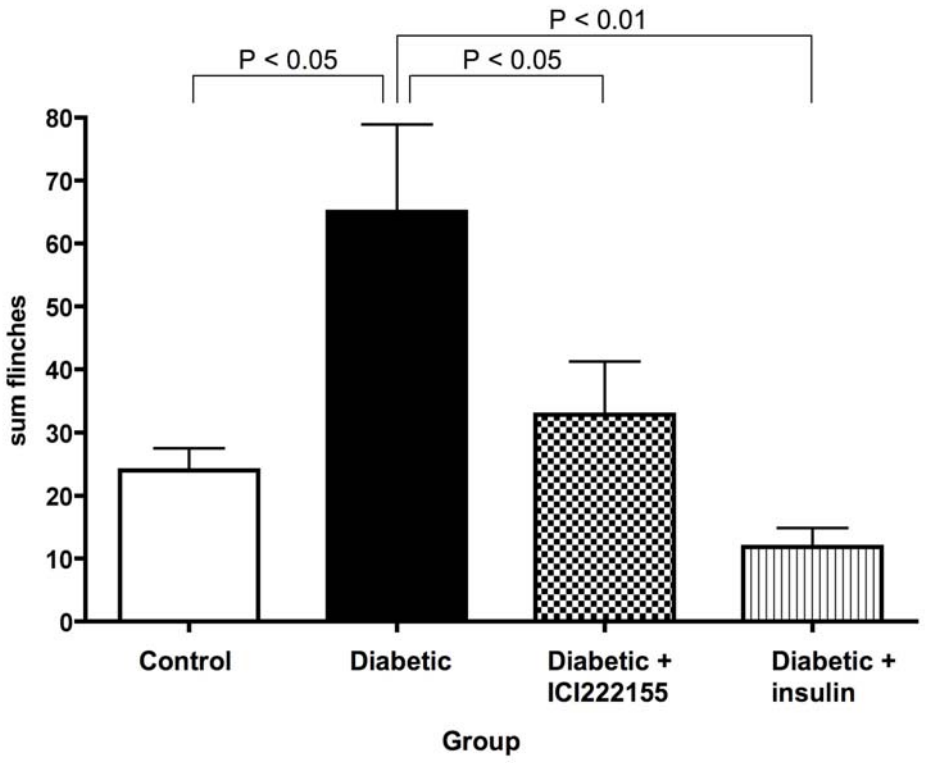


Figure 6.5. (B) Total flinches counted in the 60 min after paw formalin injection in control rats, untreated diabetic rats, and diabetic rats treated with ICI222155 or insulin, indicating that ICI222155 or insulin treatment reversed hyperalgesia to paw formalin injection. Data are presented as group mean ± SEM, N = 6 - 8 per group, statistical comparison performed using a one-way ANOVA with Student-Newman-Keuls' *post-hoc* test.

Figure 6.6.

Spinal COX-2 activity levels in control rats, untreated diabetic rats, and diabetic rats treated with insulin for a period of one week after a prior one-week period of untreated diabetes.

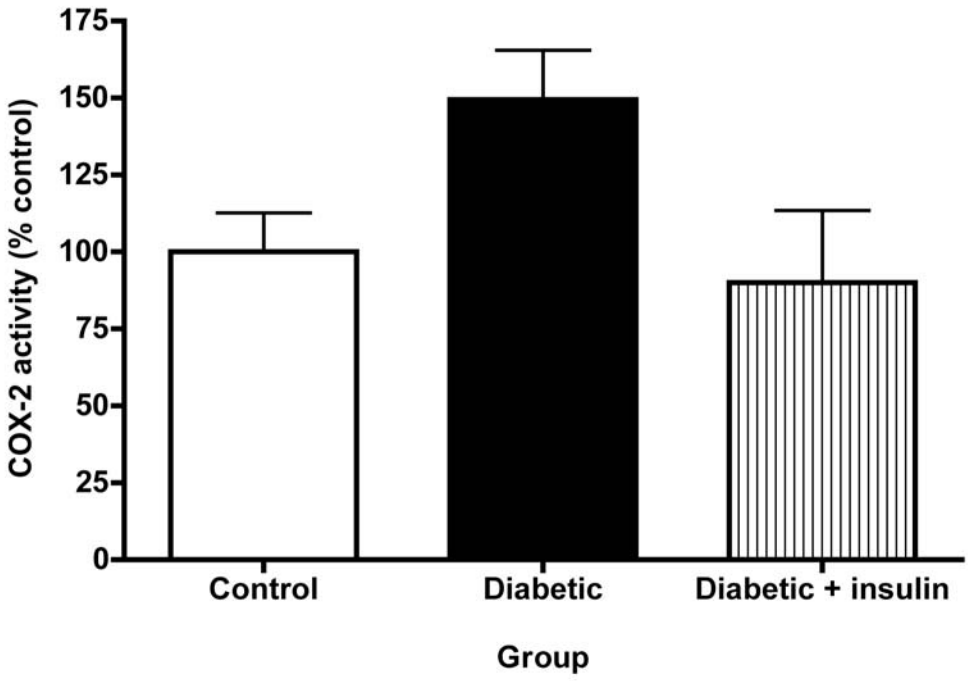


Figure 6.6. By one-way ANOVA analysis, there is a significant difference between the group means. However, *post-hoc* paired comparisons of the groups did not indicate statistically significant differences. Data are presented as group mean ± SEM, N = 7 - 8 per group.

Table 6.1.

Body weights and blood glucose levels of control rats, untreated diabetic rats and diabetic rats treated with ICI222155 or insulin for four weeks after a prior four-week period of untreated diabetes. Animals were subsequently used to measure behavioral response to 0.5% formalin or spinal COX-2 protein and activity.

Group	**Body weight (g)**	**Blood glucose (mM)**
Control (N = 7)	273 ± 7 [a]	All < 15
Diabetic (N = 16)	236 ± 7 [b]	All > 15
Diabetic + ICI222155 (N = 16)	242 ± 8 [b]	All > 15
Diabetic + insulin (N = 8)	283 ± 7 [a]	All < 15

Data are group mean ± SEM, except blood glucose levels. Statistical analysis of body weights was performed using a one-way ANOVA with the Newman-Keuls' *post-hoc* test. [a] vs. [b], $P < 0.01$.

Table 6.2.

Sugar and polyol levels measured in sciatic nerve and spinal cord, in control rats, untreated diabetic rats, and diabetic rats treated with ICI222155 or insulin for four weeks after a prior four-week period of untreated diabetes. Animals were subsequently used to measure behavioral response to 0.5% formalin or spinal COX-2 protein and activity.

	Control (N = 7)	**Diabetic (N = 16)**	**Diabetic + ICI222155 (N = 8)**	**Diabetic + insulin (N = 8)**
Nerve glucose	-	42.7 ± 3.9 [a]	56.3 ± 3.9 [b]	-
Nerve sorbitol	-	3.6 ± 0.5 [a]	0.1 ± 0.06 [c]	-
Nerve fructose	-	20.0 ± 1.6 [a]	3.2 ± 0.4 [c]	-
Nerve myo-inositol	-	9.6 ± 0.5 [a]	11.9 ± 0.7 [b]	-
Spinal cord glucose	1.6 ± 0.1 [a]	11.1 ± 0.9 [d]	9.5 ± 0.9 [d]	1.0 ± 0.1 [a]
Spinal cord sorbitol	nd	0.05 ± 0.03	0.08 ± 0.04	0.01 ± 0.01
Spinal cord fructose	0.7 ± 0.1 [a]	2.2 ± 0.3 [d]	1.8 ± 0.3 [e]	0.5 ± 0.1 [a]
Spinal cord myo-inositol	22.3 ± 2.3	25.4 ± 1.6	26.1 ± 1.3	24.0 ± 2.0

Data are group mean ± SEM. Sugar and polyol levels are presented as nmol/mg dry weight. For nerve values, each statistical comparison was made via an unpaired t-test. For each sugar or polyol measured in the spinal cord, a statistical comparison was performed using a one-way ANOVA and the Student-Newman-Keuls' *post-hoc* test. [a] vs. [b] $P < 0.05$; [a] vs. [c] $P < 0.0001$; [a] vs. [d] $P < 0.001$; [a] vs. [e] $P < 0.01$.

Table 6.3.

Body weights and blood glucose levels for rats treated with ICI222155 or insulin for one week after a prior one week period of untreated diabetes, and subsequently used for 0.5% formalin testing and measurement of spinal COX-2 protein levels.

Group	**Body weight (g)**	**Blood glucose (mM)**
Control (N = 6)	258 ± 2 [a]	All < 15
Diabetic (N = 8)	229 ± 4 [b]	All > 15
Diabetic + ICI222155 (N = 7)	221 ± 6 [b]	All > 15
Diabetic + insulin (N = 7)	267 ± 2 [a]	All < 15

Data are group mean ± SEM, except blood glucose levels. Statistical analysis of body weights was performed using a one-way ANOVA with Newman-Keuls' *post-hoc* test. [a] vs. [b], $P < 0.001$.

Table 6.4.

Sugar and polyol levels measured in sciatic nerve and spinal cord, in control rats, untreated diabetic rats, and diabetic rats treated with ICI222155 or insulin for one week after a prior one-week period of untreated diabetes.

	Control (N = 6)	**Diabetic (N = 8)**	**Diabetic + ICI222155 (N = 7)**	**Diabetic + insulin (N = 7)**
Nerve glucose	4.1 ± 0.6 [a]	46.4 ± 5.2 [b]	46.0 ± 2.1 [b]	1.3 ± 0.4 [a]
Nerve sorbitol	0.6 ± 0.4 [c]	2.7 ± 0.9 [d]	0.3 ± 0.2 [c]	0.3 ± 0.3 [c]
Nerve fructose	0.6 ± 0.3 [a]	14.7 ± 1.6 [b]	1.0 ± 0.1 [a]	0.1 ± 0.07 [a]
Nerve myo-inositol	12.7 ± 1.5	9.5 ± 0.7	9.8 ± 1.5	11.4 ± 1.1
Spinal cord glucose	1.5 ± 0.1 [a]	11.9 ± 2.0 [b]	12.4 ± 1.7 [b]	1.7 ± 0.1 [a]
Spinal cord sorbitol	nd	0.05 ± 0.02 [c]	0.01 ± 0.0 [d]	0.01 ± 0.01 [d]
Spinal cord fructose	1.2 ± 0.2 [a]	2.6 ± 0.3 [b]	2.6 ± 0.3 [b]	1.2 ± 0.1 [a]
Spinal cord myo-inositol	26.3 ± 1.5	27.7 ± 2.4	24.7 ± 1.1	28.0 ± 0.7

Data are group mean ± SEM. Sugar and polyol levels are presented as nmol/mg dry weight. A one-way ANOVA with the Student-Newman-Keuls' *post-hoc* test was used for all analyses, except for spinal sorbitol levels, for which a Kruskal-Wallis test with Dunn's *post-hoc* test was used to compare spinal sorbitol levels in untreated diabetic rats and ICI222155- or insulin-treated diabetic rats. [a] vs. [b] $P < 0.001$; [c] vs. [d] $P < 0.05$. nd = not detected.

Table 6.5.

Body weights and blood glucose levels for control rats, untreated diabetic rats, and diabetic rats treated with insulin for one week after a prior one week period of untreated diabetes, and subsequently used for measurement of spinal COX-2 activity levels.

Group	**Body weight (g)**	**Blood glucose (mM)**
Control (N = 8)	254 ± 6	All < 15
Diabetic (N = 7)	253 ± 4	All > 15
Diabetic + insulin (N = 8)	258 ± 1	All < 15

Data are group mean ± SEM, except blood glucose levels. By one-way ANOVA analysis, there is no significant difference in body weight between any of the groups.

CHAPTER 7 – DIABETES AND SPINAL OLIGODENDROCYTES

7.1 Introduction

7.1.1 Rationale for present experiments

The results from chapter 4 suggested that spinal activity of the COX-2 enzyme is at least partly responsible for formalin hyperalgesia observed in diabetic rats. The results from chapter 5 suggested that exaggerated polyol pathway flux in the CNS caused both formalin hyperalgesia and elevated spinal COX-2 in rats with four weeks of diabetes. We therefore investigated the cellular localization of COX-2 and AR in the spinal cords of control and diabetic rats, in order to construct a model for understanding how exaggerated polyol pathway flux in the CNS might ultimately disturb sensory processing in diabetes. Our results prompted us to perform a series of *in vitro* experiments, in which COX-2 and AR were studied in cultured oligodendrocytes.

Our cellular localization studies revealed that spinal AR expression is limited to oligodendrocytes. This suggested that oligodendrocytes are a site of injury in diabetes and have the potential to contribute to the pathogenesis of diabetic neuropathy, which prompted us to consider ways in which oligodendrocytes might be affected by diabetes. In addition to myelinating axons, oligodendrocytes perform a number of important functions including clearance of glutamate released during synaptic transmission (Pitt et al., 2003). We hypothesized that diabetes might disturb glutamate uptake by oligodendrocytes in the spinal cord, and the resulting insufficient glutamate clearance could cause excess excitation of adjacent sensory neurons,

possibly contributing to hyperalgesia. To study this, we chose to test whether rats with four weeks of diabetes exhibit decreased spinal levels of the excitatory amino acid transporter 1 (EAAT1; also known as GLAST), a glutamate and aspartate transporter constitutively expressed by oligodendrocytes and astrocytes (Kondo et al., 1995; Domercq and Matute, 1999; Pitt et al., 2003).

Oligodendrocytes are known to closely associate with astrocytes via gap junction coupling (Massa and Mugnaini, 1982; reviewed by Nagy and Rash, 2000). We therefore speculated that if oligodendrocytes are injured in diabetes due to exaggerated polyol pathway flux, this might adversely affect astrocytes, and this led us to measure spinal levels of GFAP protein in tissue from control rats and rats with four weeks of diabetes. It is well-documented that injury to the CNS causes astrocytes to become reactive and rapidly synthesize excess GFAP (reviewed in Eng et al., 2000), and increases in GFAP protein expression can therefore indicate abnormal astrocytic activation.

7.1.2 COX-2 expression in the spinal cord

Though COX-2 is not expressed in most tissues under normal conditions, it is constitutively expressed in the spinal cord. It is well-documented that COX-2 protein is found in dorsal horn neurons and motor neurons of the spinal cord in naïve rats, as demonstrated by immunohistochemistry (Willingale et al., 1997; Beiche et al., 1998; Resnick et al., 1998; Samad et al., 2001). The staining patterns indicate that COX-2 is

found in both the nuclear envelope and the cytoplasm of these neurons. In the dorsal horn, basal COX-2 expression is strongest in superficial laminae (Willingale et al., 1997; Beiche et al., 1998). COX-2 protein expression has also been reported in non-neuronal cells in the white matter of rat spinal cord, though there is disagreement as to the identity of these cells. One study reported COX-2 to be in white matter astrocytes of naïve rat spinal cord, although this result was presented as immunostaining for COX-2 with a GFAP counterstain that did not appear to overlap (Beiche et al., 1998). Another study reported constitutive COX-2 expression in radial glia of rat spinal cord white matter (Ghilardi et al., 2004).

Only recently have studies been published documenting COX-2 expression in the human spinal cord. There are a few reports of constitutive COX-2 expression in neurons (Koki et al., 2002; Maihofner et al., 2003) and one report of constitutive COX-2 expression in microglia or macrophages (Yiangou et al., 2006). One study did not find any constitutive COX-2 expression in human spinal cord (Kiaei et al., 2005).

7.1.3 AR expression in the spinal cord

Accumulation of sorbitol and fructose has been documented in the spinal cord of diabetic rats, indicating that the spinal cord must express both AR and sorbitol dehydrogenase (Gabbay et al., 1966; Gabbay and O'Sullivan, 1968; Calcutt et al., 1994; Calcutt et al., 1995). Additionally, activity of AR is significantly increased in the spinal cord of diabetic rats as compared with control rats (Ghahary et al., 1991). However, the cellular localization of AR in

rat spinal cord is unknown. In diabetic patients there is a significant accumulation of sorbitol in cerebrospinal fluid (Aloia and Nilakantan, 1973; Servo and Pitkanen, 1975; Servo et al., 1977), suggesting that AR is expressed in the human CNS, but there are no studies documenting AR expression in human spinal cord.

Any of the cell types in the spinal cord could potentially express AR, including neurons, astrocytes, oligodendrocytes, microglia, or endothelial cells. Given that in the peripheral nerves AR is predominantly expressed in myelinating Schwann cells (Ludvigson and Sorenson, 1980a), it is plausible that the equivalent cells of the CNS, spinal oligodendrocytes, may express AR. A few studies have documented low levels of AR expression in cultured astrocytes but these have all used primary cultures isolated from newborn rat brains that were astrocyte-rich but not pure (Wiesinger et al., 1990; Jacquin-Becker and Labourdette, 1997). It therefore cannot be concluded from these studies that AR is expressed in adult astrocytes. Although there are no published studies documenting AR expression in microglia, some studies have reported AR expression in peripheral macrophages (Brown et al., 2005; Ramana et al., 2006). There is one report of AR expression in neurons in human brain (Picklo et al., 2001), but a separate group found that AR was not expressed in neurons in naïve rat brain (Maallem et al., 2006).

7.2 Methods

To determine the cellular localization of COX-2 and AR in the spinal cord, double immunofluorescence studies were performed using spinal cord

tissue from rats with four weeks of diabetes and age-matched controls (4 per group). As described in section 2.3.1, the rats were killed and lumbar spinal cord tissue was removed for immunostaining. Stained sections were evaluated by fluorescence microscopy (section 2.3.2) and confocal microscopy (section 2.3.3).

To study the spinal expression of EAAT1 and GFAP, Western blotting was performed on lumbar spinal cord tissue. Western blots were probed with antibodies against EAAT1, cyclophilin, GFAP, and β-actin (section 2.2.3).

Experiments involving cultured oligodendrocytes used naïve rats that ranged in age from 3 months – 1 year. As described in section 2.5.1, the rats were killed and the entire spinal cord from each rat used to generate purified suspensions of O-2A progenitor cells that were then grown under conditions to cause differentiation into oligodendrocytes. Cultured oligodendrocytes were immunostained after 4 – 7 days in culture (section 2.5.2), using antibodies against myelin basic protein, COX-2, and AR. Stained cells were evaluated by fluorescence microscopy (section 2.3.2) or confocal microscopy (section 2.3.3).

7.3 Results

7.3.1 Spinal localization of COX-2 and AR protein

Immunohistochemistry performed on spinal cord tissue from control and diabetic rats revealed COX-2-IR in dorsal horn cells, ventral horn cells, and white matter. The overall pattern of COX-2 expression was not different between spinal cord tissue from control and diabetic rats, with no obvious

differences in staining intensity or the number of cells stained. Double immunofluorescence exhibited co-localization of COX-2-IR with NeuN-IR in the dorsal and ventral horn, revealing these COX-2-IR cells to be dorsal horn neurons and motor neurons, respectively (Figures 7.1 and 7.2). In the white matter, COX-2-IR was observed in the fine processes of cells that were immunoreactive for the oligodendrocyte marker APC (Figure 7.3). Diabetes did not induce COX-2 expression in any cell types that did not express COX-2 under normal conditions.

Immunohistochemistry performed on spinal cord tissue from control and diabetic rats revealed AR-IR in cells scattered throughout the white matter. As was observed with COX-2 immunostaining, diabetes did not change the overall pattern of AR expression in the spinal cord. To identify the AR-IR cells, double immunofluorescence was performed with antibody markers for astrocytes, oligodendrocytes, microglia and radial glia (GFAP, APC, CD11b, and 40E-C, respectively). AR-IR did not co-localize with GFAP-IR, CD11b-IR, or 40E-C-IR, indicating that AR is not expressed in astrocytes, microglia, or radial glia (Figure 7.4). AR-IR showed strong co-localization with the oligodendrocyte marker APC (Figure 7.5).

7.3.2 Diabetes-induced changes in glial cells

In analyzing spinal cord tissue from control and diabetic rats, we found that spinal expression of EAAT1 protein was significantly decreased in rats with 4 weeks of diabetes (Figure 7.6). Expression of GFAP protein was significantly increased in spinal cord tissue from rats with 4 weeks of diabetes

as compared with control rats (Figure 7.7), which is suggestive of reactive astrogliosis. We therefore immunostained spinal cord tissue from control and diabetic rats using the GFAP antibody, and observed that astrocytes in the diabetic spinal cord exhibited evidence of hypertrophy (Figure 7.8).

7.3.3 Primary cultures of oligodendrocytes express COX-2 and AR

To confirm *in vitro* that oligodendrocytes express COX-2 and AR, primary cultures of oligodendrocytes were obtained from dissociated rat spinal O-2A progenitor cells (section 2.5.1). Observation by light microscopy revealed that O-2A progenitor cells obtained from adult rat spinal cords rapidly differentiated to exhibit morphology characteristic of oligodendrocytes grown in culture (Mirsky et al., 1980; Kachar et al., 1986; Barres et al., 1992). The cells had large numbers of long, branching processes that ended in very fine processes, and in some cells, this highly differentiated morphology was observed within 24 hours of plating on poly-d-lysine-coated coverslips. The large numbers of processes observed on these cultured oligodendrocytes is consistent with their being grown on poly-d-lysine-coated coverslips, which surpasses laminin as an appropriate substrate for growth (Kachar et al., 1986).

To confirm that these cells were oligodendrocytes, they were immunostained with an antibody against myelin basic protein (MBP; section 2.5.2). It has been previously shown that after plating O-2A progenitor cells on poly-l-lysine-coated coverslips in conditions to cause differentiation into oligodendrocytes, MBP is expressed by these cells within 4-7 days (Cohen et al., 1991). In the present experiments, MBP-immunoreactivity (MBP-IR) was

strongest in the somata of cultured cells but also clearly present in the thickest proximal processes, as well as fine terminal processes and what appeared to be sheets of membrane at the ends of some of the thicker processes (Fig 7.9). These membranous expansions at the ends of processes have been described previously (Mirsky et al., 1980).

To determine whether oligodendrocytes express COX-2 and AR *in vitro*, cultured oligodendrocytes were double-stained for MBP and COX-2 or AR (section 2.5.2). Cells exhibiting MBP-IR also exhibited COX-2-IR, with the most intense COX-2-IR present in the cell body and fainter COX-2-IR visible in proximal and distal processes (Figure 7.10). Cells exhibiting MBP-IR exhibited strong AR-IR throughout the entire cell (Figure 7.11).

7.4 Discussion

Immunohistochemistry performed on spinal cord tissue from control and diabetic rats showed that diabetes does not induce COX-2 expression in cell types that do not express COX-2 under normal conditions. In spinal cord tissue from control and diabetic rats, COX-2-IR was observed in dorsal horn neurons and motor neurons of the ventral horn, in agreement with the literature (Willingale et al., 1997; Beiche et al., 1998; Resnick et al., 1998; Samad et al., 2001). COX-2-IR was also observed in oligodendrocytes in rat spinal cord tissue, and we confirmed that cultured oligodendrocytes obtained from rat spinal cord also express COX-2. Because diabetes did not alter the cellular localization of spinal COX-2, the excess COX-2 enzyme induced by diabetes must be present in neurons, oligodendrocytes, or both.

Constitutive COX-2 expression in oligodendrocytes *in vivo* has not been previously documented. However, one study has demonstrated that cultured oligodendrocytes isolated from adult pig brain can synthesize and release E- and F-series prostaglandins, including PGE_2 (Althaus and Siepl, 1997). In this study, basal release of PGE_2 was low but stimulation of the oligodendrocytes with LPS induced a dramatic upregulation of extracellular PGE_2 release. A separate study had shown that, in response to sublethal injury with terminal complement complexes, cultured oligodendrocytes synthesized and released a range of inflammatory lipid signaling molecules, including PGE_2 (Shirazi et al., 1987). These studies are important because they suggest oligodendrocytes must express all of the enzymes necessary to generate eicosanoids, including COX, and that oligodendrocytes can be a source of PGE_2 secretion. However, neither of these two studies indicated whether the cultured oligodendrocytes expressed COX-1, COX-2, or both. COX-2 expression has been reported in oligodendrocytes in ischemic neonatal human brain and in active multiple sclerosis spinal lesions (Toti et al., 2001; Carlson et al., 2006), suggesting that human oligodendrocytes at least have the capacity to express COX-2. There is also one report of constitutive $cPLA_2$ expression in oligodendrocytes of adult rat spinal cord (Liu et al., 2006). $cPLA_2$ is a PLA_2 isoform that is calcium dependent and membrane-bound, and phosphorylation of $cPLA_2$ *in vivo* results in enhanced arachidonic acid release (for review, see Dennis, 1994). As this $cPLA_2$-induced cleavage of arachidonic acid from lipid membranes is necessary to provide substrate for

COX activity, it would perhaps be expected that any cell type expressing COX-2 must also express $cPLA_2$. The present finding that COX-2 is expressed in rat spinal oligodendrocytes is therefore consistent with the finding of $cPLA_2$ expression in rat spinal oligodendrocytes (Liu et al., 2006).

Immunohistochemistry performed on spinal cord tissue from control and diabetic rats showed that diabetes does not induce AR expression in cell types that do not express AR under normal conditions. In spinal cord tissue from control and diabetic rats, AR-IR was observed solely in oligodendrocytes. As oligodendrocytes are the myelinating cells of the CNS, this result parallels AR expression in the peripheral nerve, where it is principally located in myelinating Schwann cells (Ludvigson and Sorenson, 1980a; Jiang et al., 2006). In culture, we confirmed that oligodendrocytes express AR. This result suggests that in diabetes, spinal oligodendrocytes may be a primary lesion site due to exaggerated polyol pathway flux.

Our immunohistochemistry results indicate that spinal oligodendrocytes express both COX-2 and AR, and this was also confirmed *in vitro*. Western blots performed on spinal cord tissue from control and diabetic rats indicate that diabetes induces a significant increase in spinal COX-2 protein expression (Freshwater et al., 2002; chapters 4 and 5). Based on the immunohistochemistry presented in this chapter, the increase of spinal COX-2 expression in diabetes must occur in neurons or oligodendrocytes. Having identified oligodendrocytes as a primary injury site in diabetes due to their expression of AR, it is possible that the diabetes-induced increase in spinal

COX-2 protein occurs in oligodendrocytes. To address this possibility, we plan to culture oligodendrocytes in normal or high glucose levels to determine whether a hyperglycemic environment causes oligodendrocytes to upregulate COX-2 protein and activity levels. Alternatively, confirmation that diabetes induces COX-2 upregulation in spinal oligodendrocytes could be obtained *in vivo* by use of a technique such as laser capture microdissection, which would allow quantification of COX-2 protein expression specifically in oligodendrocytes of the spinal cord (for review of laser capture microdissection, see Standaert, 2005).

We hypothesized that diabetes might disturb glutamate uptake by oligodendrocytes in the spinal cord, by reducing spinal levels of EAAT1. We found that four weeks of diabetes indeed induced a significant decrease in spinal EAAT1 protein levels. It is therefore possible that insufficient glutamate clearance by oligodendrocytes in the spinal cord contributes to spinal sensitization and hyperalgesia.

We speculated that if oligodendrocytes are injured in diabetes due to exaggerated polyol pathway flux, this might adversely affect astrocytes. Measurement of GFAP protein in lumbar spinal cord via Western blot revealed approximately 40% more GFAP protein in tissue from diabetic rats, compared with control rats. Consistent with our observation of increased spinal GFAP expression in diabetic rats, we also observed evidence for astrocytic hypertrophy in spinal cord tissue from diabetic rats. These observations suggest that astrocytes are adversely affected by chronic hyperglycemia. This

result adds to a growing body of evidence that diabetes damages the CNS, and highlights the potentially important role that CNS glial cells may have in diabetic neuropathy.

In summary, the results from this chapter along with the animal studies from chapters 4 – 6 prompt a novel and exciting possibility: that spinal oligodendrocytes are a primary site of injury in diabetes, and that these injured oligodendrocytes cause a chronic spinal sensitization that underlies the exaggerated pain behavior observed during the formalin test in diabetic rats. This possibility and its implications will be discussed in chapter 8.

Figure 7.1.

Spinal cord tissue immunostained for COX-2 and NeuN.

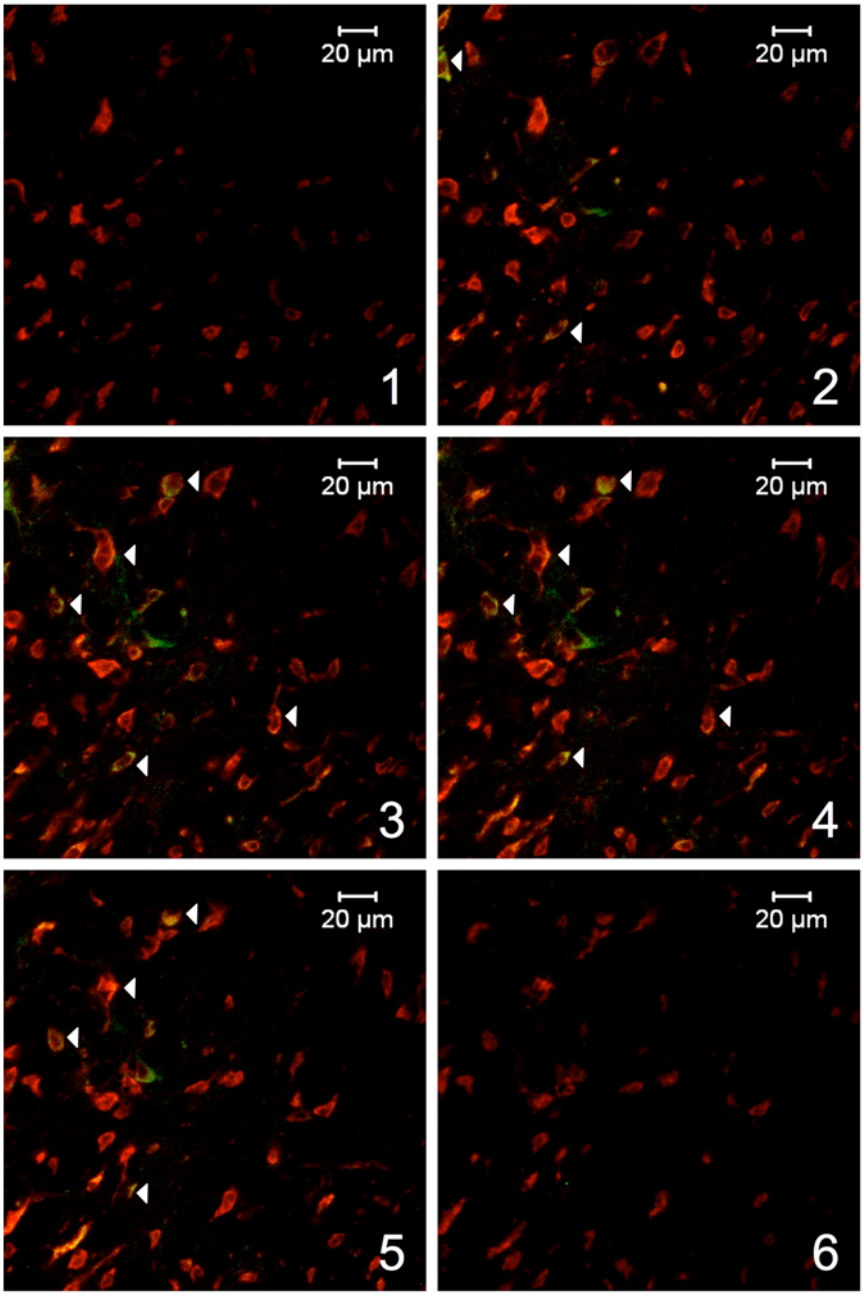


Figure 7.1. Spinal cord tissue from a control rat immunostained for NeuN (red) and COX-2 (green; overlap appears yellow), with the dorsal horn visible in the field of view. Arrowheads indicate cells exhibiting NeuN-IR and COX-2-IR. The images are from a Z-stack sequence acquired by confocal microscopy, with a spacing of 2.9 µm between each image.

Figure 7.2.

Spinal cord tissue immunostained for COX-2 and NeuN.

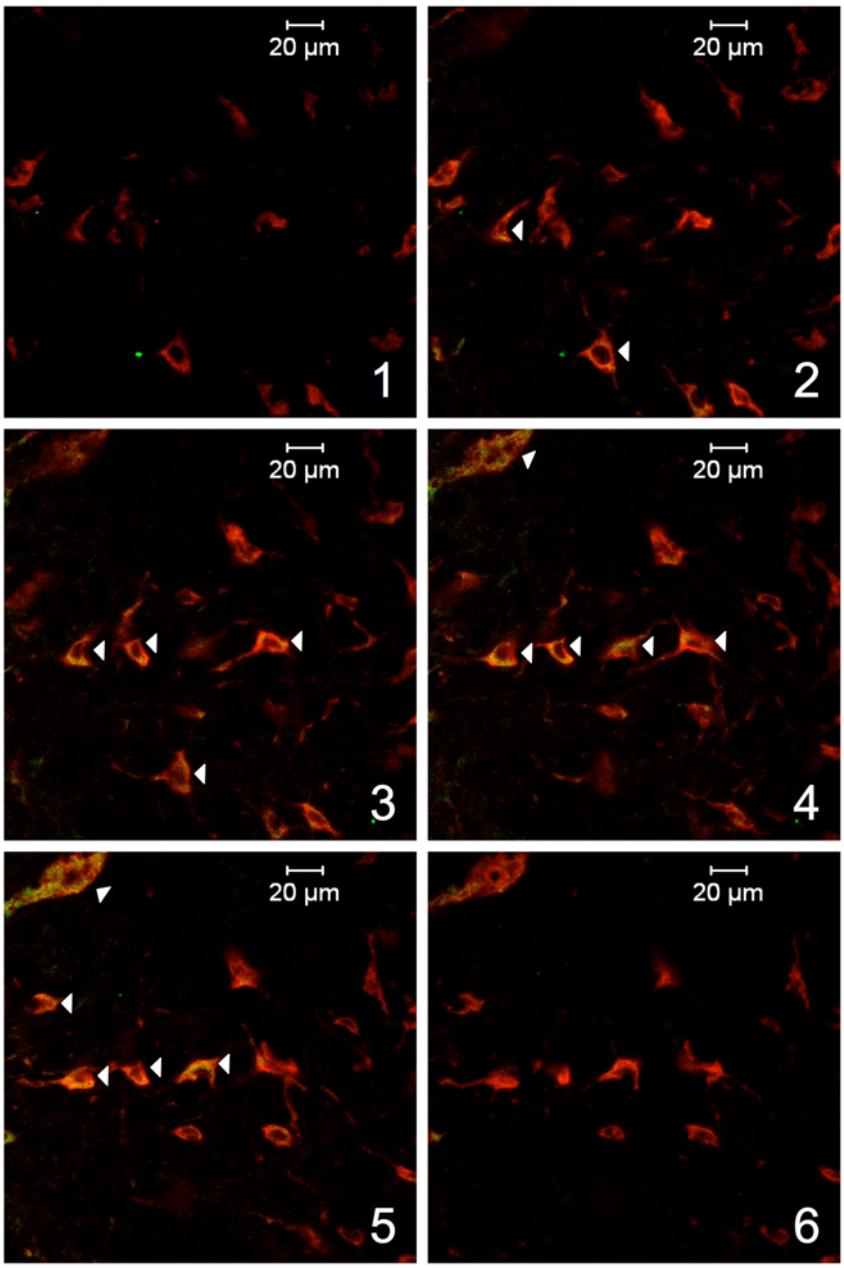


Figure 7.2. Spinal cord tissue from a control rat immunostained for NeuN (red) and COX-2 (green; overlap appears yellow), with the ventral horn visible in the field of view. Arrowheads indicate cells exhibiting NeuN-IR and COX-2-IR. The images are from a Z-stack sequence acquired by confocal microscopy, with a spacing of 2.84 μm between each image.

Figure 7.3.

Spinal cord tissue immunostained for COX-2 and APC.

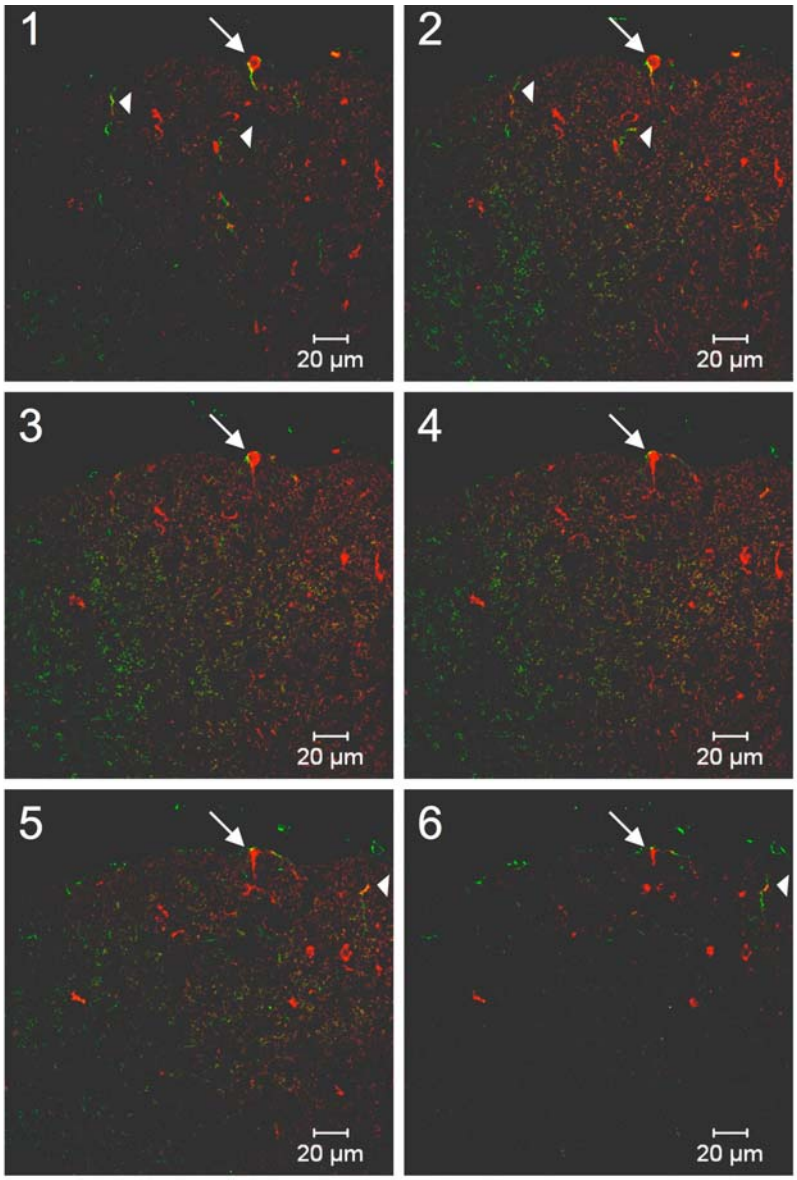


Figure 7.3. Spinal cord tissue from a diabetic rat immunostained for APC (red) and COX-2 (green; overlap appears yellow), with the white matter between the dorsal horns visible in the field of view. The arrow points to an APC-IR oligodendrocyte cell body with a COX-2-IR process attached. Arrowheads indicate fine processes exhibiting AR-IR and COX-2-IR. The images are from a Z-stack sequence acquired by confocal microscopy, with a spacing of 1.7 µm between each image.

Figure 7.4.

Spinal cord tissue immunostained for AR and 40E-C, GFAP, or CD11b.

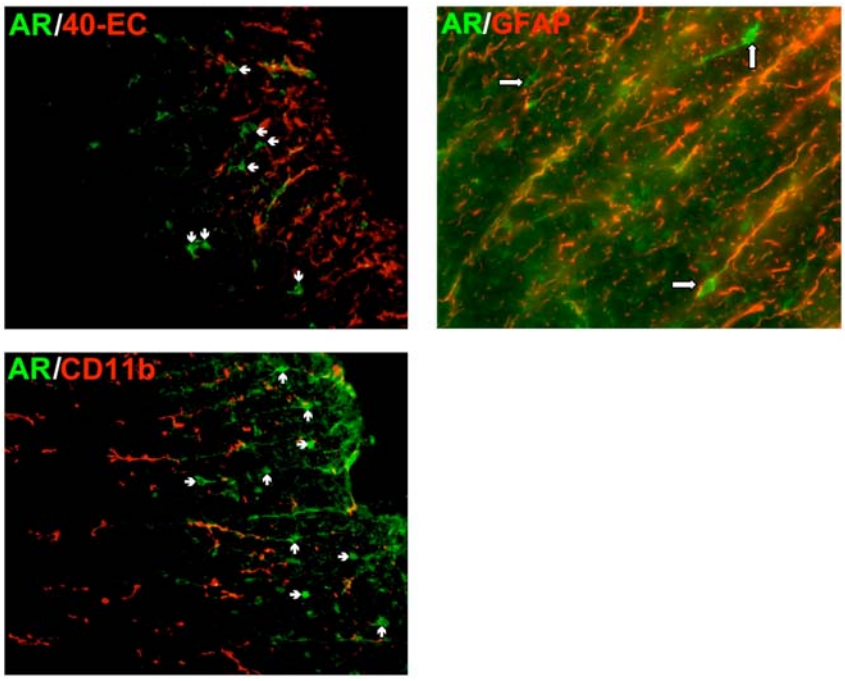


Figure 7.4. Spinal cord tissue from control rats immunostained for AR (green) and 40E-C (red), GFAP (red), or CD11b (red), indicating that AR is not expressed in radial glia, astrocytes, or microglia, respectively. Arrows point to AR-IR cell bodies. The images were obtained with a fluorescence microscope.

Figure 7.5.

Spinal cord tissue immunostained for AR and APC.

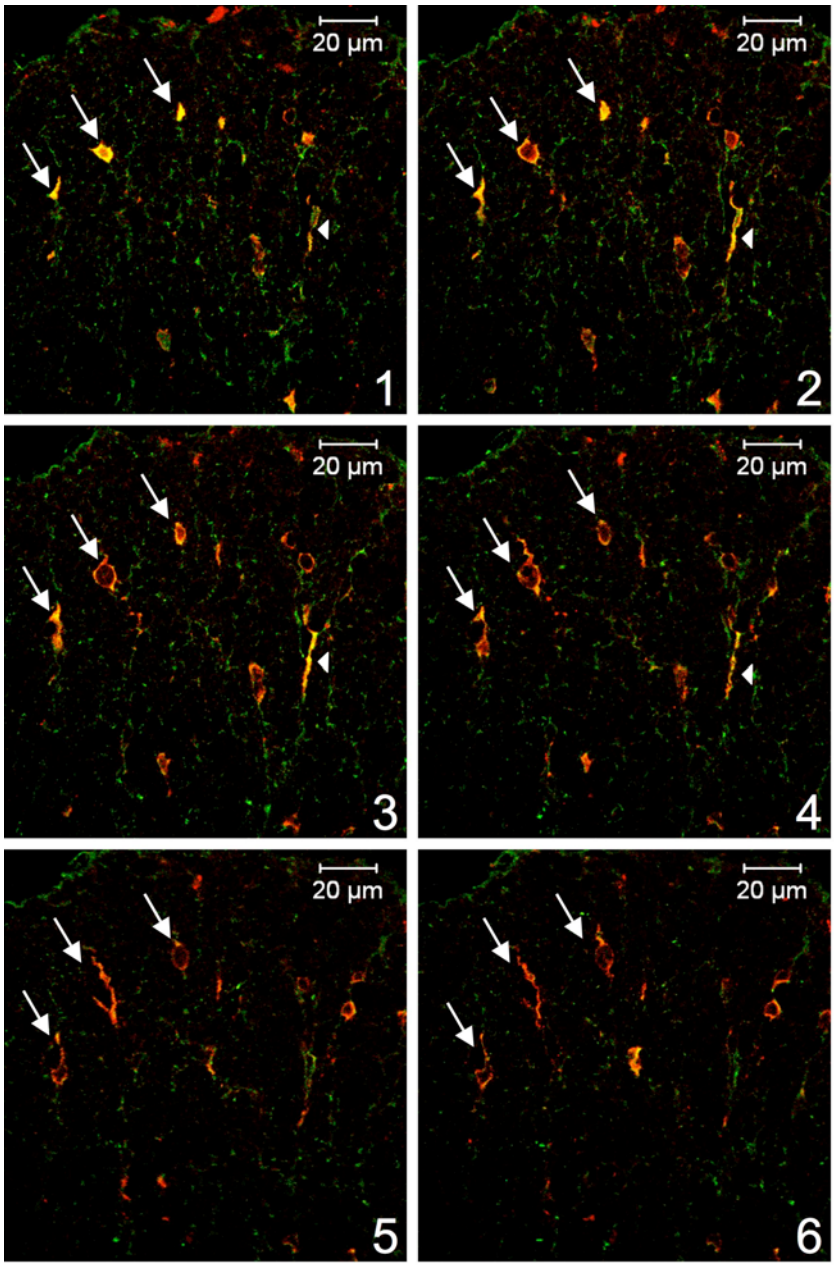


Figure 7.5. Spinal cord tissue from a diabetic rat immunostained for APC (red) and AR (green; overlap appears yellow), with white matter visible in the field of view. Cell bodies (arrows) and a thick process (arrowheads) exhibit AR-IR and APC-IR. The images are from a Z-stack sequence acquired by confocal microscopy, with a spacing of 1.38 µm between each image.

Figure 7.6.

Spinal EAAT1 protein levels in control rats and rats with 4 weeks of diabetes.

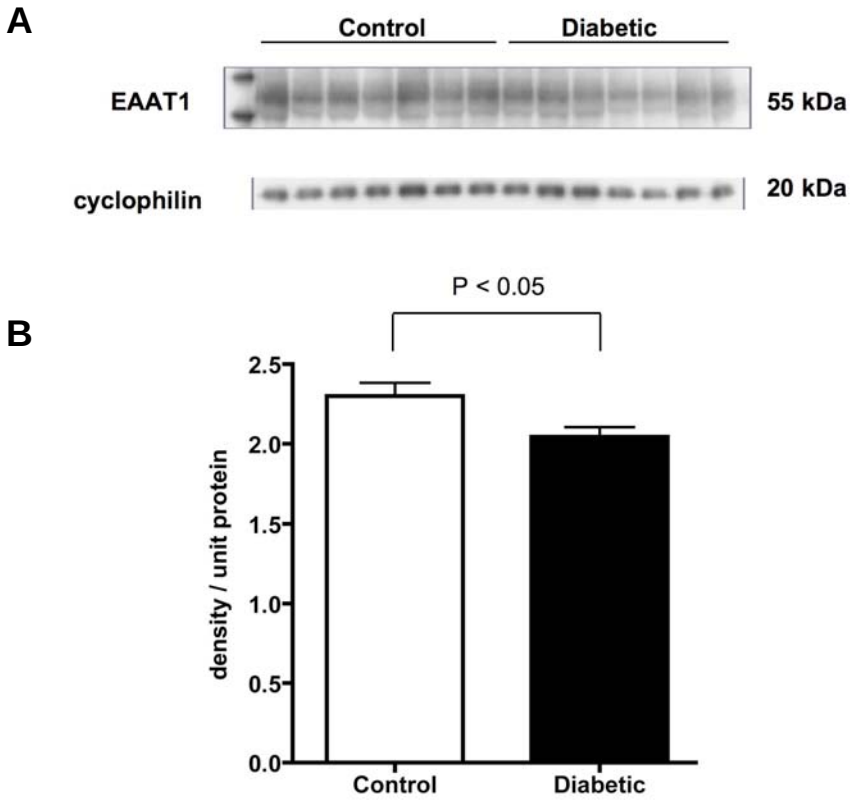


Figure 7.6. (A) Representitive Western blots probed for EAAT1 or cyclophilin, using spinal cord tissue from control rats and rats with four weeks of diabetes. Cyclophilin was used as a loading control. (B) Densitometric quantification of EAAT1-immunostained Western blots indicated a significant difference in spinal EAAT1 expression between control and untreated diabetic rats. Data are presented as group mean ± SEM, N = 8 per group, statistical comparison performed using an unpaired t-test.

Figure 7.7.

Spinal GFAP protein levels in control rats and rats with 4 weeks of diabetes.

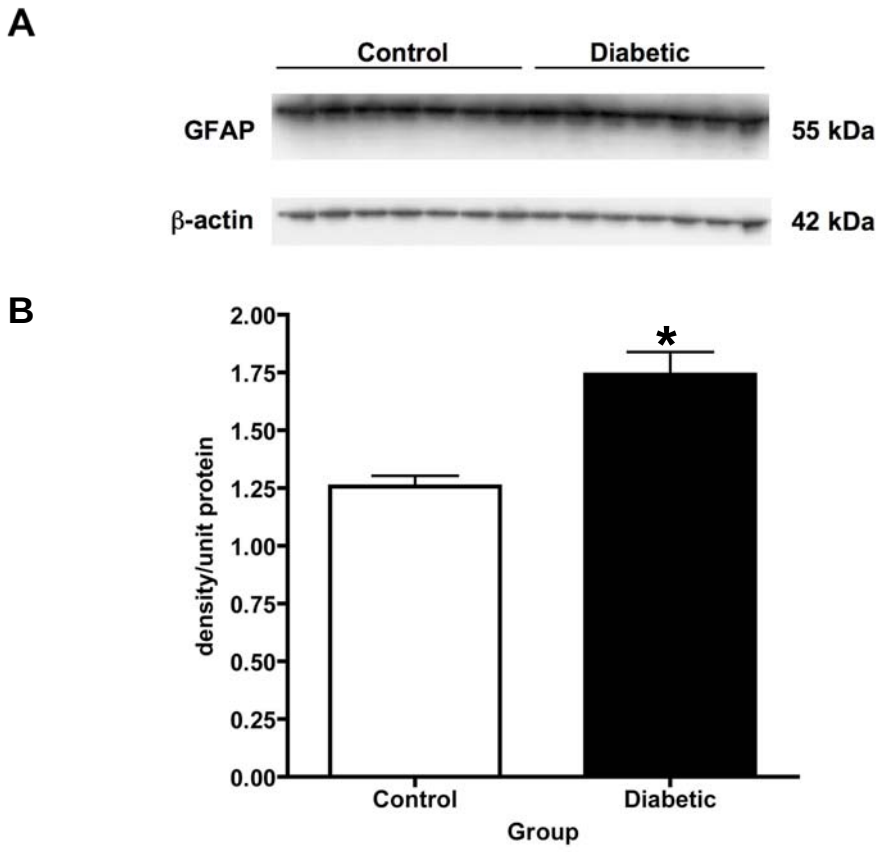


Figure 7.7. (A) Representative Western blot showing levels of GFAP in lumbar spinal cord from control rats and rats with 4 weeks of diabetes. β-actin protein levels were measured and used as a loading control. (B) Densitometric quantification of GFAP-immunostained Western blot indicated that 4 weeks of diabetes caused a significant ($P < 0.001$) increase in spinal GFAP expression. Data are presented as group mean ± SEM, N = 7 per group, statistical comparison performed using an unpaired t-test.

Figure 7.8

Morphology of astrocytes in lumbar spinal cord tissue from control rats and rats with four weeks of diabetes.

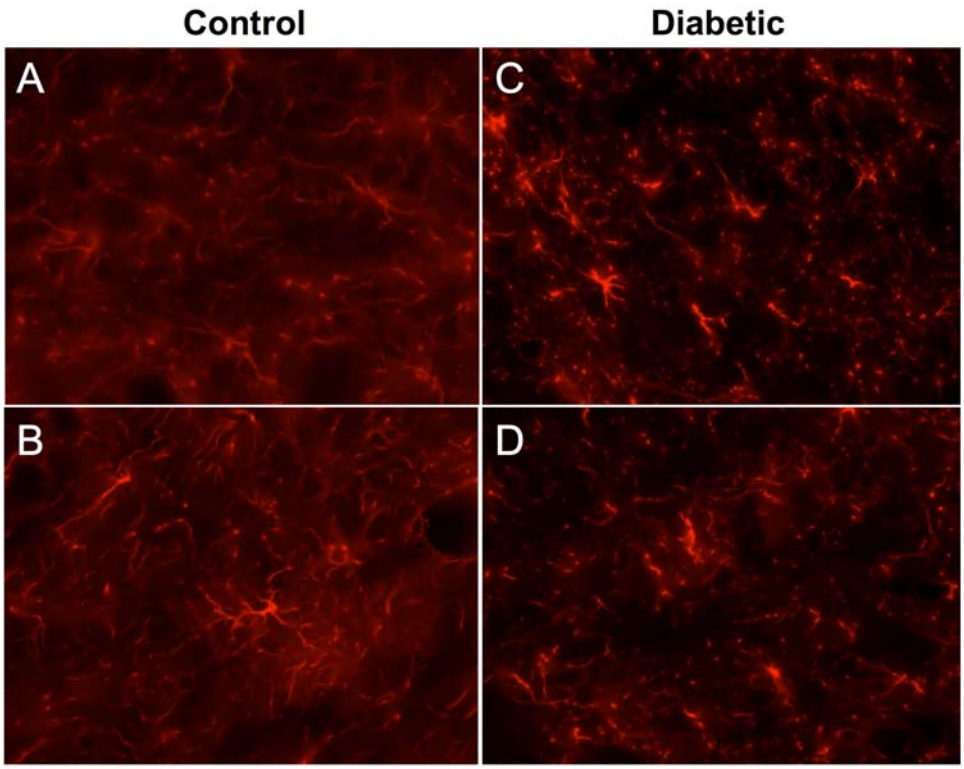


Figure 7.8. Lumbar spinal cord tissue immunostained for GFAP reveals evidence of astrocytic hypertrophy in the diabetic spinal cord. In (A) and (B), the astrocytes have long, thin processes, and cell bodies that appear normal. In contrast, in (C) and (D) the astrocytes exhibit enlarged cell bodies and shortened, thickened processes. The images were obtained with a fluorescence microscope.

Figure 7.9.

A cultured oligodendrocyte immunostained for myelin basic protein.

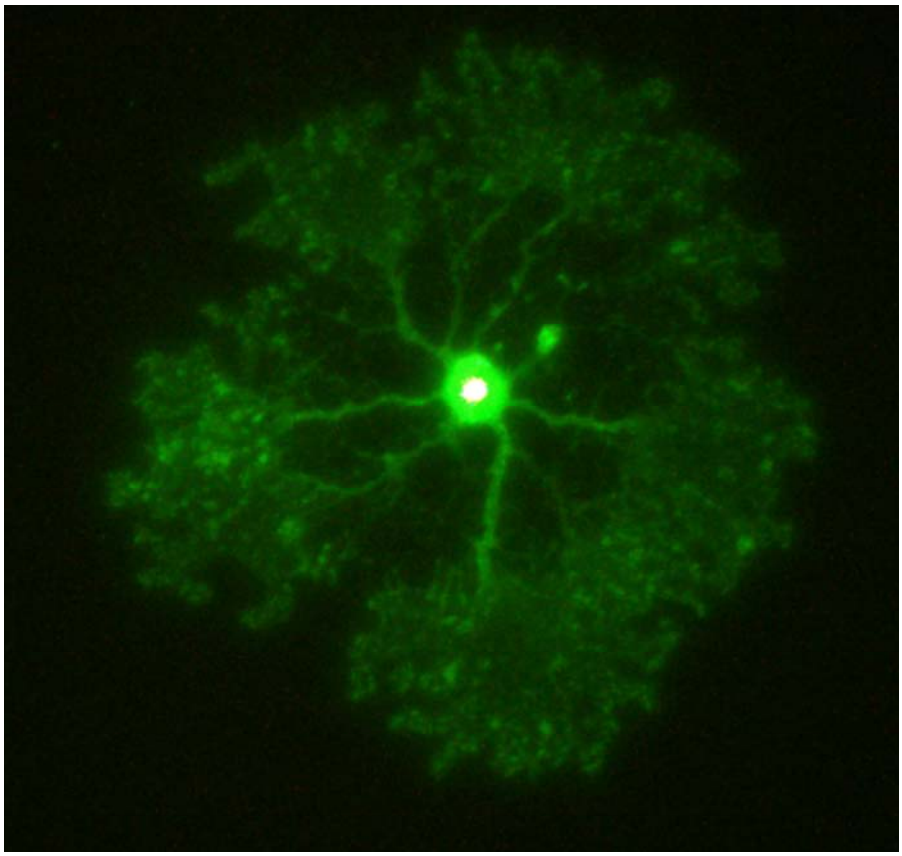


Figure 7.9. A cultured oligodendrocyte exhibits MBP-IR in the cell body, thick and fine processes, and sheets of membranous expansions at the ends of many thick processes. The image was obtained with a fluorescence microscope.

Figure 7.10.

A cultured oligodendrocyte immunostained for myelin basic protein and COX-2.

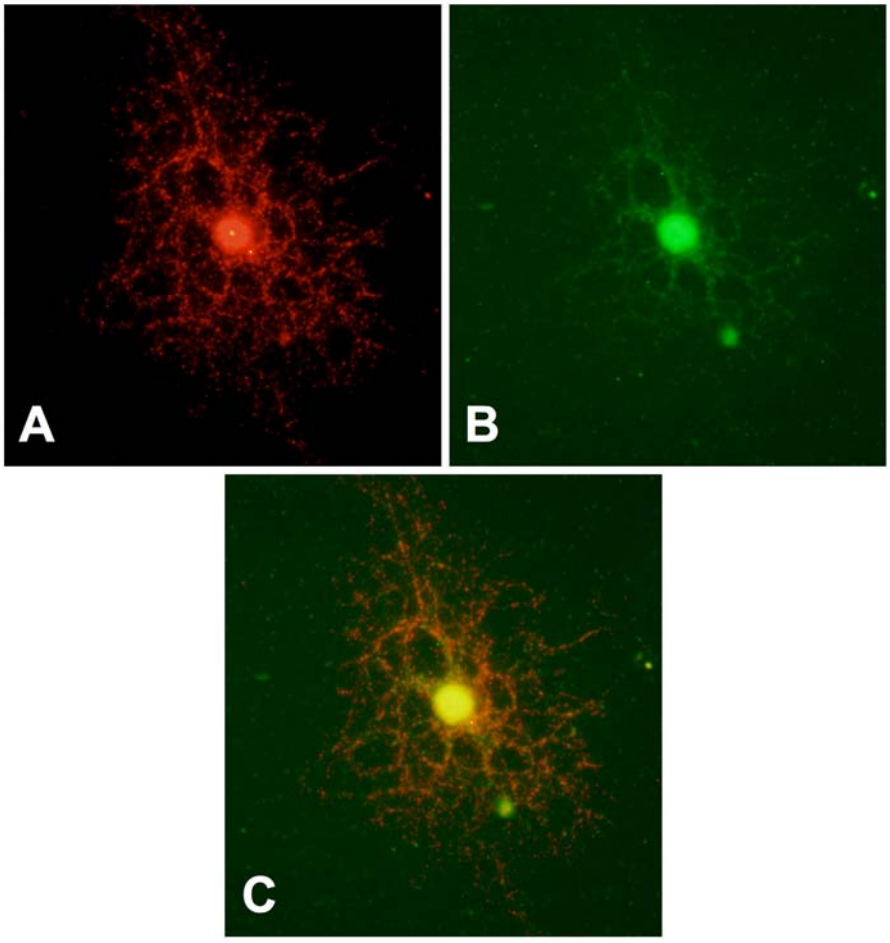


Figure 7.10. (A) A cultured oligodendrocyte exhibits MBP-IR (red) in the cell body, along with thick and fine processes. (B) COX-2-IR (green) is present in the cell body and both thick and fine processes. (C) In the merged image, colocalization of MBP-IR and COX-2-IR appears yellow. The images were obtained with a fluorescence microscope.

Figure 7.11.

A cultured oligodendrocyte immunostained for myelin basic protein and aldose reductase.

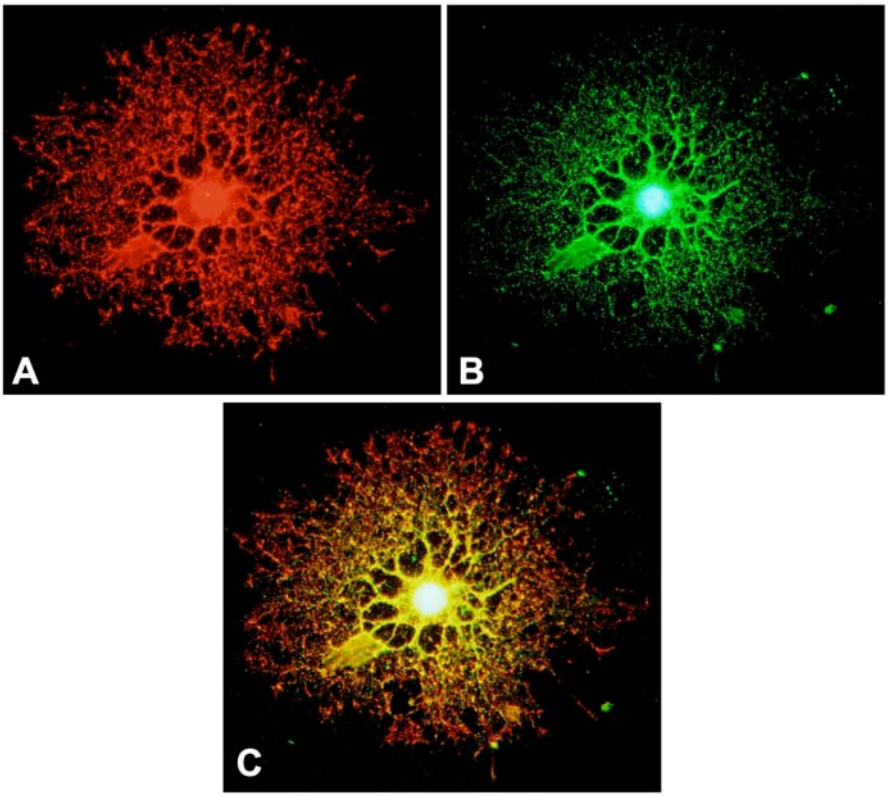


Figure 7.11. (A) A cultured oligodendrocyte exhibits strong MBP-IR (red) in the cell body, along with both thick and fine processes. (B) The cell also exhibits strong AR-IR (green) in the cell body and most processes, except for the most distal fine processes. DAPI staining of the nucleus appears blue in this image. (C) In the merged image, colocalization of MBP-IR and AR-IR appears yellow. The images were obtained with a fluorescence microscope.

CHAPTER 8 – DISCUSSION

8.1 Summary of results

In view of the potential link between exaggerated spinal prostanoid signaling and formalin hyperalgesia in diabetic rats (Freshwater et al., 2002), the first aim was to determine whether another rodent model of diabetes also exhibited elevated spinal COX-2 expression and hyperalgesia to paw formalin injection. In contrast with STZ-diabetic rats, which develop exaggerated pain responses to thermal, tactile, and formalin testing within the first four weeks of diabetes (Malmberg et al., 1993; Calcutt et al., 1995; Calcutt and Chaplan, 1997; Calcutt et al. 2000a), C57Bl/6 mice with four weeks of diabetes exhibited decreased sensitivity to thermal or tactile stimulation of the paw. Additionally, diabetic mice lacked a second phase of nocifensive behavior in response to paw formalin injection. It was observed that in C57Bl/6 mice, STZ-induced diabetes causes exaggerated spinal COX-2 protein expression, just as in STZ-diabetic rats. This suggests that exaggerated spinal COX-2 expression in diabetes is alone not sufficient to cause hyperalgesia as measured by the formalin test. Because diabetic mice exhibit a general trend of sensory loss, and because it was observed that these mice rapidly lost epidermal innervation, diabetic rats were used for all subsequent studies.

The next aim of this research was to investigate the role of the COX-2 isozyme in diabetes-induced spinal sensitization and hyperalgesia in rats. We found that rats with one, two, or four weeks of diabetes exhibit both formalin hyperalgesia and elevated spinal COX-2 protein and activity levels. To

test any potential causal link between elevated spinal COX-2 activity and formalin hyperalgesia, spinal COX-2 activity was acutely inhibited in diabetic rats subjected to 0.5% formalin testing, and it was observed that this significantly alleviated phase II flinching. These data suggest that spinal COX-2 activity at least partly causes the hyperalgesia to paw formalin injection observed in diabetic rats.

Having established that increased spinal COX-2 activity contributes to hyperalgesia, we next investigated the pathogenesis of elevated spinal COX-2 expression and formalin hyperalgesia in diabetic rats. Previous data had shown that systemic treatment with the ARI ICI222155 prevented development of hyperalgesia to paw injection of 0.5% formalin in diabetic rats (Calcutt et al., 1995), suggesting that polyol pathway flux causes this hyperalgesia. However, a separate study had demonstrated that treatment with the ARI IDD676 did not prevent development of exaggerated flinching in response to paw injection of 0.2% formalin in diabetic rats (Freshwater and Calcutt, 2005). We speculated that the apparent discrepancy between these two studies arose because ICI222155 and IDD676 have different sites of action within the nervous system. We hypothesized that AR activity in the CNS drives both elevated spinal COX-2 expression and formalin hyperalgesia in diabetes, that blocking spinal AR activity is necessary to inhibit elevated spinal COX-2 expression and formalin hyperalgesia in diabetes, and that while ICI222155 crosses the BBB to inhibit spinal AR activity (Calcutt et al., 1995), IDD676 does not. Our data confirmed these hypotheses. Treatment with IDD676 was

effective against polyol product accumulation in the PNS as well as against indices of peripheral nerve dysfunction, but had no effect on polyol product accumulation in the spinal cord, elevated spinal COX-2, or formalin hyperalgesia in diabetic rats. Treatment with ICI222155, previously shown to block spinal polyol product accumulation and prevent the development of formalin hyperalgesia (Calcutt et al., 1995), prevented the upregulation of spinal COX-2 expression in diabetic rats. These data indicate that AR activity in the CNS causes formalin hyperalgesia and exaggerated spinal COX-2 expression in diabetes. To localize spinal AR activity as the primary pathogenic lesion site, it will be necessary to ablate spinal AR expression using siRNA to determine whether rats selectively lacking spinal AR activity develop formalin hyperalgesia and elevated spinal COX-2 expression in diabetes.

The prevention studies of chapter 5 provide an important conceptual understanding of how diabetes causes spinal sensitization. The ultimate goal of this research is to model painful diabetic neuropathy in patients with diabetes, who are treated to reverse rather than prevent painful symptoms. We therefore studied whether formalin hyperalgesia and elevated spinal COX-2 expression could be pharmacologically reversed in diabetic rats. In rats with four weeks of diabetes, treatment with ICI222155 did not reverse formalin hyperalgesia, while insulin treatment did. The inability of ICI222155 treatment to reverse hyperalgesia in rats with four weeks of diabetes may be due to an unexpectedly ineffective block of spinal polyol product accumulation.

This possibility is supported by the observation that ICI222155 treatment reversed formalin hyperalgesia in rats with one week of diabetes, and also blocked spinal accumulation of sorbitol in these rats. Alternatively, the reversal study results may suggest that at longer durations of diabetes, hyperalgesia is maintained by a mechanism that arises from hyperglycemia and its associated metabolic consequences, but is not directly due to AR activity.

Having implicated spinal AR activity in the pathogenesis of exaggerated COX-2 expression and formalin hyperalgesia in diabetic rats, we next studied the spinal expression of AR and COX-2 protein in control and diabetic rats. Diabetes did not induce expression of AR or COX-2 in cell types that do not express these proteins under normal conditions. COX-2 was observed in dorsal horn neurons, motor neurons, and oligodendrocytes, while AR was observed solely in oligodendrocytes. The expression of COX-2 and AR in oligodendrocytes was confirmed in cultured oligodendrocytes. These data, taken together with the results from the ARI treatment studies, suggest that AR activity in spinal oligodendrocytes may be the initiating factor that links hyperglycemia with hyperalgesia in diabetic rats.

8.2 STZ-diabetic mice are not a useful model of painful diabetic neuropathy

We found that mice with four weeks of STZ-induced diabetes displayed a general trend of sensory loss. During formalin testing, these mice exhibited a normal first phase of nocifensive response, yet completely lacked a second

phase. This observation is in agreement with previous results documenting a lack of phase II behavior in STZ-diabetic mice (Kamei et al., 1993, 1997), with one difference being that the prior studies also reported a prolonged first phase of response. Interestingly, inhibition of substance P via intrathecal injection of spantide, given 10 min before paw formalin injection, reduced the duration of phase I behavior and caused a second phase of nocifensive behavior to appear in diabetic mice (Kamei et al., 1993). Based on these results, the authors hypothesized that a prolonged phase I in diabetic mice activates the endogenous spinal δ-opioid receptor-mediated antinociceptive system. This hypothesis was supported by the finding that intrathecal injection of a δ_1-opioid receptor antagonist selectively and dose-dependently increased the phase II behavioral response to paw formalin injection in diabetic mice (Kamei et al., 1997).

In the present studies, diabetes did not significantly affect the phase I response to paw formalin injection in mice, yet there was no phase II response. This suggests that even after a normal phase I response in diabetic mice, the spinal sensitization necessary to cause a second response phase can be absent. Formalin testing of diabetic mice is therefore conceptually interesting because of the disconnect between phase I and phase II observed in these animals. The impaired formalin-evoked spinal sensitization is not due to a lack of spinal COX-2 expression, as diabetic mice exhibited a significant upregulation of spinal COX-2 protein as compared with control mice. Instead, diabetic mice may have impaired spinal expression of any of the other

enzymes comprising the prostaglandin synthesis pathway, including PLA_2 or the prostaglandin synthases that convert the COX product PGH2 to various prostaglandin end-products (see section 1.3.2.2). To date, diabetes-induced changes in the spinal expression of these enzymes have not been documented in rats or mice, and therefore would be interesting to measure. Alternatively, it is possible that following paw formalin injection, diabetic mice have impaired spinal release of nitric oxide (NO), which has been shown to contribute to formalin-induced spinal sensitization (Haley et al., 1992). There is one report of a significant increase in brain nitrite levels in STZ-diabetic mice (Sharma et al., 2006), which may argue against the possibility of impaired spinal NO release in response to paw formalin injection in diabetic mice. However, the Laka strain of mice was used in this study and STZ-diabetes caused thermal hyperalgesia in these mice (Sharma et al., 2006). Given the difference in mouse strain and behavioral changes induced by diabetes in this study, the observed upregulation of nitrite may or may not be paralleled in the spinal cord of STZ-diabetic C57Bl/6 mice.

In summary, while formalin testing of STZ-diabetic mice is of conceptual interest, STZ-diabetic mice are not useful as a model of painful diabetic neuropathy because they do not exhibit exaggerated pain responses to thermal or tactile stimulation of the paw, or to paw formalin injection.

8.3 The contribution of abnormal spinal electrophysiology and biochemistry to painful diabetic neuropathy

Data from patients with diabetic neuropathy and experimentally diabetic rats argue against a structural abnormality in the PNS that correlates with the incidence of pain. In patients, the presence of pain does not correlate with degeneration of myelinated fibers or regeneration of myelinated or unmyelinated axons in the sural nerve (Llewelyn et al., 1991; Britland et al., 1992; Malik et al., 2001). Further, recent clinical results from patients with pre-diabetic neuropathy indicated that diet and exercise intervention increased intraepidermal nerve fiber density and decreased neuropathic pain (Smith et al., 2006). These data argue against the previously postulated hypothesis that pain is caused by ectopic activity in peripheral nerve axons undergoing degeneration and regeneration (Dyck et al., 1976; Archer et al., 1983). In experimentally diabetic rodents, functional nerve abnormalities occur far earlier than structural changes (Sharma and Thomas, 1974; Walker et al., 1999), and therefore at least some aspects of nerve dysfunction in these animals likely arise from metabolic disturbances. In the present experiments, an exaggerated painful behavioral response to paw injection of 0.5% formalin was observed as early as one week after the induction of diabetes. It has been previously reported that one week of STZ-induced diabetes is sufficient to cause an allodynic response to paw injection of 0.2% formalin (Freshwater and Calcutt, 2005). Tactile allodynia and thermal hyperalgesia also appear after only one to two weeks of STZ-induced diabetes (Calcutt et al., 1996;

reviewed by Calcutt, 2004). These data further suggest a biochemical cause of enhanced pain in diabetic rats, as structural changes in the peripheral nerve of diabetic rats are not observed until at least four weeks after induction of diabetes (Jakobsen, 1976; Sima and Robertson, 1979; Ras and Nava, 1986).

A biochemical amplification of nociceptive transmission in diabetes could occur anywhere throughout the neuraxis, including the primary afferent nociceptors. If primary afferents were to exhibit enhanced sensitivity in diabetes, and corresponding enhanced input to the spinal cord, this could explain the presence of exaggerated pain in diabetes. There is disagreement as to whether diabetes increases peripheral drive into the spinal cord. Systemic lidocaine alleviates tactile allodynia in diabetic rats (Calcutt et al., 1996), and it has been reported that DRG neurons from diabetic rats express higher levels of mRNA and protein for several types of sodium channels (Craner et al., 2002). However, a separate study found that mRNA for tetrodotoxin-resistant sodium channels is decreased in DRG neurons from diabetic rats (Okuse et al., 1997). Recording electrical activity in primary afferent fibers has also produced conflicting results. It has been reported that diabetes lowers thresholds for activation of individual C, Aβ and Aδ fibers (Chen and Levine, 2001; Khan et al., 2002), yet others have reported that mechanical and thermal thresholds of activation for individual C fibers are not significantly different between diabetic and control rats (Ahlgren et al., 1992).

A number of studies suggest that spinal dorsal horn neurons are sensitized in diabetes. Electrophysiological studies have revealed enhanced

spontaneous activity in WDR neurons in diabetic rat spinal cord (Pertovaara et al., 2001). This finding was replicated by Chen and Pan (2002), who found significantly enhanced spontaneous activity in neurons comprising the spinothalamic tract (STT) in diabetic rats. In this study, it was also observed that the average receptive field size of the recorded neurons was approximately three times larger in diabetic rats as compared with control rats, possibly suggesting a loss of tonic inhibition. Additionally, the population of STT neurons that was recorded from exhibited a shift in the distribution among low-threshold, high-threshold, and WDR neurons, such that diabetic rats had relatively fewer high-threshold neurons and more WDR neurons, again suggesting disinhibition in the neurons comprising the STT (Chen and Pan, 2002). These electrophysiological data strongly suggest that projection neurons in the diabetic spinal cord exhibit enhanced basal activity and augmented sensitivity to peripheral input.

Beyond electrophysiological data, several studies have suggested a specific sensitivity to substance P in the spinal cord of diabetic rats. Kamei et al. (1990) observed twice as much binding of substance P to membrane preparations from diabetic rat spinal cord, as compared with controls, indicating an upregulation of spinal substance P binding sites in STZ-diabetic rats. Behavioral confirmation of spinal sensitivity to substance P has also been documented. Intrathecal delivery of substance P to the spinal cord induces a transient thermal hyperalgesia in control rats (Malmberg and Yaksh, 1992), and this substance P-induced hyperalgesia is prolonged in diabetic rats

(Calcutt et al., 2000a). These data, taken together with the results of the electrophysiological studies described above, demonstrate the presence of neurochemical abnormalities in the spinal cord of diabetic rats.

The molecular basis of diabetes-induced spinal sensitization has received some attention. Diabetic rats exhibit hyperalgesic flinching behavior specifically during phase II of testing with 0.5% formalin, and as spinal PGE_2 release contributes to the induction of spinal sensitization following formalin injection (Malmberg and Yaksh, 1995), a logical question is whether there is enhanced spinal prostanoid signaling in diabetes. Freshwater et al. (2002) found that diabetic rats had a three-fold increase in constitutive spinal COX-2 protein, and a prolonged elevated spinal release of PGE_2 in response to paw formalin injection. Formalin hyperalgesia in diabetic rats was dose-dependently ameliorated by intrathecal delivery of indomethacin, a nonselective COX inhibitor, or of ONO 8711, an antagonist of the PGE_2 receptor EP1 (Freshwater et al., 2002). These data suggest an abnormal spinal upregulation of the enzyme COX-2 in diabetes, which may cause formalin hyperalgesia by catalyzing the production of excess PGE_2 in response to paw formalin injection.

The data presented in this thesis extend the evidence for spinal sensitivity in diabetic rats. We have shown that the excess COX-2 protein present in the spinal cord in diabetes is enzymatically active (chapters 4, 5, and 6). We have also demonstrated that normalizing spinal COX-2 activity in diabetic rats by pharmacological intervention normalizes the behavioral

response to paw formalin injection (chapters 5 and 6). In conclusion, many studies support the hypothesis that the spinal cord is sensitized in diabetes. This spinal sensitization contributes at least in part to exaggerated pain behaviors in diabetic rats.

8.4 Polyol pathway flux in the CNS contributes to painful diabetic neuropathy

An early observation that treatment with the ARI ICI222155 prevented the development of formalin hyperalgesia in diabetic rats implicated polyol pathway flux in the pathogenesis of this disorder (Calcutt et al., 1995), but the mechanism was not known. Previous work (Freshwater et al., 2002) and the data in chapter 4 indicate that elevated spinal COX-2 activity in the diabetic spinal cord is a significant contributor to spinal sensitivity underlying formalin-evoked hyperalgesia. We subsequently demonstrated that treatment with ICI222155, using the same dose and treatment regimen previously observed to prevent formalin hyperalgesia in diabetic rats (Calcutt et al., 1995), also prevented the upregulation of spinal COX-2 protein and activity in diabetic rats. This allowed us to propose that ARI treatment is able to prevent formalin hyperalgesia in diabetic rats by blocking the upregulation of spinal COX-2 protein and diabetes.

Having provided an explanation of how AR inhibition prevents the development of an exaggerated pain behavior in diabetic rats, it remained unclear where in the nervous system AR activity is important in causing hyperalgesia. When given systemically, the ARI ICI222155 blocks polyol

product accumulation in the PNS and in the spinal cord, suggesting that AR activity in the PNS, the spinal cord, or both could cause hyperalgesia. To address this, we treated diabetic rats with the ARI IDD676, which blocked polyol product accumulation in the PNS but not in the spinal cord (chapter 5). IDD676 treatment prevented small and large fiber dysfunction in the sciatic nerve, but had no effect on formalin hyperalgesia or elevated spinal COX-2. These data indicate that maintenance of normal PNS function is not sufficient to prevent spinal sensitization and suggest that polyol pathway flux in the CNS contributes at least in part to the pathogenesis of painful diabetic neuropathy.

8.5 Oligodendrocytes

Identification of oligodendrocytes as AR-expressing cells is a novel finding, and may suggest that oligodendrocytes are an important factor in diabetic neuropathy. Consideration of the normal biology and function of oligodendrocytes is crucial to understanding how oligodendrocyte dysfunction in diabetes could contribute to neuropathy and pain. Oligodendrocytes, along with astrocytes, comprise the macroglia found in the CNS. The term *oligodendroglia* was established by Del Rio Hortega in 1921 to describe macroglia exhibiting few processes when stained by metallic impregnation techniques. A single oligodendrocyte can myelinate a variable number of axonal segments depending on the species and location within the CNS, ranging from just one in the cat spinal cord (Bunge et al., 1961) to 20-30 in the rat optic nerve (Butt and Ransom, 1989). This variation reflects the morphological heterogeneity of oligodendrocytes that was originally described

by Del Rio Hortega (1922, 1924). He proposed a classification system defining four types of oligodendrocytes based on their number of processes and branching pattern, such that type I and II oligodendrocytes myelinate many internodes on multiple small diameter axons, whereas type III and IV oligodendrocytes myelinate only one or two internodes on large diameter axons (Del Rio Hortega, 1922, 1924). Subsequent studies using electron microscopy have confirmed Del Rio Hortega's original description of oligodendrocyte morphological heterogeneity (Stensaas and Stensaas, 1968; Remahl and Hildebrand, 1990; Bjartmar et al., 1994). However, transplantation studies have demonstrated that oligodendrocytes are not inherently committed to myelinating a particular size axon, but rather take cues from the local environment to control myelination (Fanarraga et al., 1998). Modern studies of oligodendrocytes typically do not distinguish among the four morphologically distinct types.

Oligodendrocytes develop from oligodendrocyte progenitor cells (OPCs), which are actively migratory cells (LeVine and Goldman, 1988; Kirby et al., 2006) that proliferate in response to axonal electrical activity (Barres and Raff, 1993). During development, OPCs migrate to distribute themselves uniformly throughout gray and white matter of the CNS (for review, see Miller, 2002). Most of the OPCs then transform into pre-oligodendrocytes, which are multiprocessed cells with little or no ability to migrate (Orentas and Miller, 1996). Pre-oligodendrocytes then progress through a series of developmental stages, including immature oligodendrocyte, mature non-myelinating

oligodendrocyte, and finally myelinating mature oligodendrocyte (for review, see Baumann and Pham-Dinh, 2001).

The earliest myelin-specific protein expressed is 2',3'-cyclic nucleotide-3'-phosphohydrolase (CNP), which is found on immature oligodendrocytes. Mature, non-myelinating oligodendrocytes express, in addition to CNP, myelin basic protein (MBP), proteolipid protein (PLP), and myelin-associated glycoprotein (MAG). Mature myelinating oligodendrocytes express CNP, MBP, PLP, MAG, and myelin oligodendrocyte glycoprotein (MOG). The timing of expression of these various myelin proteins reflects the status of oligodendrocyte development. The protein CNP is believed to associate with the submembrane cytoskeleton of oligodendrocytes in order to enable membrane expansion (Braun et al., 1988; Trapp et al., 1988; De Angelis and Braun, 1996a and 1996b). Mature, non-myelinating oligodendrocytes express the major myelin proteins MBP and PLP in preparation to myelinate axons, along with MAG, which facilitates glial-axonal cell adhesion (McIntyre et al., 1978). Expression of MOG in mature, myelinating oligodendrocytes may reflect a role for MOG in the maintenance of myelin sheaths (Matthieu and Amiguet, 1990; Amiguet et al., 1992), but to date the physiological role of MOG is not completely understood (Montague et al., 2006). Many studies have confirmed that development of mature oligodendrocytes from OPCs *in vivo* is precisely mimicked in culture. When OPCs are cultured alone in the absence of neurons, they progress through the normal oligodendrocyte developmental stages and ultimately express MBP, PLP, and MAG (Dubois-

Dalcq et al., 1986; Kim, 1990; See et al., 2004). In the presence of neurons and their associated axons, cultured oligodendrocytes will also express MOG in myelin sheaths (Solly et al., 1996). These *in vitro* observations support the hypothesis that OPCs are endowed with a 'developmental clock' that is intrinsic to the cell type (Temple and Raff, 1986).

Oligodendrocytes are the myelin-forming cells of the CNS and have been studied primarily in the context of this function. Rather than passively providing myelin sheaths as axonal insulation, oligodendrocytes have an active role in the development and maintenance of properly myelinated axons. During neurodevelopment, sodium channels are initially contiuously distributed along axons. The clustering of sodium channels at nodes of Ranvier is dependent on a protein secreted by oligodendrocytes (Kaplan et al., 1997), and can occur in the absence of direct oligodendrocyte-axon contact. Oligodendrocytes also regulate axonal caliber independently of myelin formation (Sanchez et al., 1996). After development, oligodendrocytes have a significant role in the maintenance of axons. Many of the myelin proteins expressed by oligodendrocytes have been demonstrated to support normal axonal survival (for review, see Edgar and Garbern, 2004). Patients with a null mutation in the gene encoding the myelin protein PLP exhibit axonal degeneration in the CNS in the absence of demyelination, and this axonal degeneration was also observed in PLP null mice (Garbern et al., 2002). Similarly, mice lacking the myelin protein CNP develop CNS myelin with normal ultrastructure, yet these mice develop motor deficits that coincide with

axonal swelling and neurodegeneration, which is ultimately fatal (Lappe-Siefke et al., 2003). Clearly, the myelin proteins expressed by oligodendrocytes are critical for the long-term maintenance of normal axonal integrity, through mechanisms that are not yet understood. In STZ-diabetic rats, there is one report of reduced expression of PLP and MAG in the brain, both at the protein and mRNA levels (Kawashima et al., 2007). The importance of PLP and MAG in normal axonal maintenance and glial-axonal adhesion, respectively, suggests that decreased CNS expression of these proteins during diabetes could impair their normal functions. Spinal expression of CNP, MBP, PLP, MAG, and MOG should be measured in tissue from control and diabetic rats, to determine if diabetes alters expression of these proteins.

8.6 Oligodendrocytes as a site of injury in diabetes

Identification of oligodendrocytes as a site of AR expression indicates that these cells are likely to be injured during diabetes as a result of exaggerated polyol pathway flux. A few studies provide evidence that diabetes damages CNS white matter in both patients and in experimentally diabetic rats. Spinal demyelination has been observed in tissue from diabetic humans (Reske-Nielsen and Lundbaek, 1968; DeJong, 1977) but not as yet in diabetic rats. Spinal conduction velocity slowing has been reported in diabetic rats (Carsten et al., 1989) and in patients with diabetes (Suzuki et al., 2000), suggesting that diabetes may cause a functional disturbance of myelin physiology in the CNS even in the absence of overt demyelination. In patients, diabetes has been shown to reduce white matter volume in the brain

(Anstey et al., 2006) and cause atrophy of the spinal cord (Eaton et al., 2001). In diabetic rats, there is impaired CNS remyelination after ethidium bromide-induced demyelination (Bondan et al., 2006). These data suggest that CNS myelin may be damaged by diabetes.

Any of the etiologic mechanisms that arise secondary to polyol pathway flux could cause damage to oligodendrocytes (see introduction section 1.3.1). Oligodendrocytes may be especially susceptible to damage via oxidative stress as oligodendrocytes express very low levels of reduced glutathione (GSH; Juurlink et al., 1998). Because GSH is required for glutathione peroxidase to reduce hydrogen peroxide and lipid hydroperoxides, this suggests that under normal conditions, oligodendrocytes have a limited ability to scavenge peroxides. As discussed in chapter 1, diabetes is associated with depletion of GSH in the peripheral nerves (Nagamatsu et al., 1995; Cameron et al., 1999; Obrosova et al., 1999). Recent work has documented a 30% reduction in levels of GSH in the spinal cord of STZ-diabetic rats (Ates et al., 2006). If the reduced expression of GSH extends specifically to oligodendrocytes, their inherently low levels of GSH expression might be easily overwhelmed, resulting in little defense within this cell type against free radicals. It has been observed that cultured rat brain oligodendrocytes subjected to oxidative stress exhibit upregulation of the heat shock protein alpha B-crystallin within 24 hours (Goldbaum and Richter-Landsberg, 2001). Oligodendrocytes in the brains of diabetic mice exhibit enhanced expression of alpha B-crystallin (Yaguchi et al., 2003), which could be evidence that

oligodendrocytes are subjected to oxidative stress in diabetes. At present, diabetes-induced changes in spinal alpha B-crystallin expression have not been reported, and this could readily be measured, using immunohistochemistry to determine if diabetes causes upregulation of alpha B-crystallin expression in spinal oligodendrocytes. It would also be interesting to test whether preventative treatment with an antioxidant can block the development of formalin hyperalgesia and exaggerated spinal COX-2 expression in diabetic rats. While such an experiment has not been performed as yet, several studies report that antioxidant treatment can reverse established allodynia and hyperalgesia in diabetic rats (Cameron et al., 2001; Anjaneyulu and Chopra, 2004; Li et al., 2005; Sayyed et al., 2006). These data may suggest that oxidative stress is necessary to maintain neuropathic pain in diabetes.

Another mechanism by which oligodendrocytes might be damaged in diabetes is via non-enzymatic glycation of constitutive myelin proteins. Glycation of axonal cytoskeletal proteins is believed to contribute to impaired axonal transport, as well as axonal atrophy and degeneration in diabetic peripheral nerve (reviewed by McLean, 1997). Myelin proteins reported to be glycated during diabetes include proteolipid protein (PLP) and myelin basic protein (MBP; Vlassara et al., 1983; Weimbs and Stoffel, 1994), but the effect of glycation on myelin is unknown at this time. Glycation of proteins induces conformational changes, so that glycation of PLP or MBP could presumably interfere with the function or maintenance of myelin.

Glycation of proteins ultimately leads to the formation of molecular complexes called advanced glycation end-products (AGEs), which are formed irreversibly. Expression of AGEs is increased in peripheral nerves from both diabetic patients and rats (reviewed by Karachalias et al., 2003, and Wada and Yagihashi, 2005). Macrophages express the receptor for AGEs, and one study reported that macrophages recognize and endocytose AGE-modified myelin, prompting the hypothesis that macrophage uptake of AGE-modified myelin may contribute to the segmental demyelination associated with diabetes (Vlassara et al., 1984). In this study, significant accumulation of AGEs sufficient to induce macrophage endocytosis of peripheral nerve myelin was present only after at least 1.5 years of diabetes, and not after only 4-5 weeks of diabetes (Vlassara, et al., 1984). This time-dependent property of macrophage recruitment to PNS myelin agrees with the observations that accumulation of AGEs in peripheral nerves of diabetic rats occurs after relatively long durations of diabetes (6 months; Karachalias et al., 2003) and that segmental demyelination is generally not observed in rats with relatively short durations of diabetes.

In the spinal cord of rats with four months of diabetes, a mild increase in expression of AGEs is present, and this increase is significantly greater after eight months of diabetes (Sensi et al., 1998). Because both PLP and MBP are reported to be glycated during diabetes (Vlassara et al., 1983; Weimbs and Stoffel, 1994), presumably CNS myelin undergoes AGE-modification after relatively long durations of diabetes. It is possible that after long durations of

diabetes, macrophages and/or microglia consume CNS myelin, leading to the spinal demyelination observed in patients with diabetes (Reske-Nielsen and Lundbaek, 1968; DeJong, 1977). To date, there are no reports of electron microscopy performed on spinal cord tissue from diabetic rats, and this would constitute a worthwhile study. Importantly, because accumulation of AGEs during diabetes requires at least several months duration of diabetes, macrophage/microglial endocytosis of AGE-modified CNS myelin is unlikely to explain the hyperalgesia to paw formalin injection observed in rats after only one week of diabetes. Nevertheless, the possibility that AGE-modification of CNS myelin induces demyelination is clinically important.

8.7 Polyol pathway flux in oligodendrocytes: a link between hyperglycemia and hyperalgesia?

In experimental painful diabetic neuropathy, we have observed that hyperalgesia is dependent on spinal sensitization mediated by overexpression of COX-2. Inhibition of AR activity in the CNS prevents hyperalgesia and spinal sensitization, and because these are spinal disorders, we examined spinal expression of AR and COX-2. Spinal AR is exclusively localized to oligodendrocytes, suggesting that injured oligodendrocytes might be capable of causing or at least initiating a state of sensitized nociceptive processing in diabetes. The possibility that oligodendrocytes modulate neuronal communication is a novel concept but perhaps not unexpected in view of the relatively recent discovery that astrocytes and microglia can modulate neurotransmission in various ways (reviewed by Kim and de Vellis, 2005;

Araque, 2006; Haydon and Carmignoto, 2006). Because oligodendrocytes themselves express COX-2 in addition to AR, it is plausible that the abnormal activity of AR in oligodendrocytes arising due to diabetes could trigger the upregulation of COX-2 in oligodendrocytes themselves. There is one report of high glucose enhancement of IL-1β-induced COX-2 expression, albeit in cultured rat vascular smooth muscle cells (Lee et al., 2000). Importantly, this effect was blocked by treatment with an ARI (Lee et al., 2000), suggesting that AR activity in hyperglycemic conditions can drive the expression of COX-2. Additionally, there are a number of studies indicating that AR activity regulates inflammatory responses induced by TNF-α, lipopolysaccharide (LPS), endotoxins, and growth factors in various culture systems and *in vivo* (Tammali et al, 2006; Ramana et al., 2006a; Ramana et al., 2006b; Pladzyk et al., 2006b; Tammali et al., 2007). At present, any diabetes-induced changes in pro-inflammatory cytokine expression in the spinal cord have not been reported.

If diabetes were to cause an upregulation of COX-2 in spinal oligodendrocytes, this may result in PGE_2 release from the oligodendrocytes that could then diffuse to dorsal horn neurons and sensitize them to nociceptive input from primary afferents (Pitchford and Levine, 1991), providing a mechanistic explanation for how hyperglycemia might cause hyperalgesia. Cultured oligodendrocytes are capable of synthesizing and releasing prostaglandins (Shirazi et al., 1987; Althaus and Siepl, 1997). The possibility that polyol pathway flux in oligodendrocytes causes spinal

sensitization in diabetes via PGE_2 release from oligodendrocytes fits well with the data presented in this thesis, and with the ability of oligodendrocytes to secrete inflammatory lipids in response to injury (Shirazi et al., 1987; Althaus and Siepl, 1997). There are also alternative speculative explanations. For example, recent data suggests that oligodendrocytes have a critical role in providing a 'potassium sink' that can buffer high levels of potassium ions that are present extracellularly after neuronal activity (Menichella et al., 2006). This potassium buffering is crucial to allow normal repolarization of neuronal membranes after action potential firing, as a low extracellular potassium concentration creates a concentration gradient that allows potassium ions to flow out of neurons after action potential firing. The ability of oligodendrocytes to buffer potassium is dependent on the connexin proteins Cx32 and Cx47 (Menichella et al., 2006). To date, diabetes-induced changes in connexin expression in the central nervous system have not been reported. There is one report of decreased Cx32 expression in the dorsal penile nerve of STZ-diabetic rats (Pitre et al., 2001). If Cx32 expression is similarly decreased in the spinal cord during diabetes, this may interfere with the ability of oligodendrocytes to buffer potassium, which could result in impaired membrane repolarization of adjacent sensory neurons. This could contribute to the enhanced basal activity and sensitivity to peripheral input documented in STT neurons of diabetic rats (as discussed in section 8.3). It would be interesting to measure Cx32 expression by Western blot in spinal cord tissue to determine whether diabetes causes downregulation of spinal Cx32

expression. If so, it would be valuable to study whether pharmacological interventions shown to prevent the development of hyperalgesia in diabetic rats also prevent the downregulation of spinal Cx32 expression.

Another way in which oligodendrocyte damage in diabetes might cause spinal sensitization is via abnormal neuronal sprouting. Some proteins that comprise myelin, including MAG and oligodendrocyte myelin glycoprotein (OMgp), have been shown play a critical role in restricting axonal sprouting in the mature CNS (reviewed by Hu and Strittmatter, 2004). This is believed to occur by direct binding of MAG or OMgp to the Nogo receptor, which then inhibits sprouting through an interaction with the transmembrane receptor 75 (Fournier et al., 2001; Wang et al., 2002a and 2002b; Liu et al., 2002). As CNS myelin component proteins are known to be glycated in diabetes (Vlassara et al., 1983), it is possible that this glycation interferes with the ability of MAG and/or OMgp to bind the Nogo receptor and perform normal inhibition of axonal outgrowth at nodes of Ranvier in the spinal dorsal horn. This could result in sprouting that might cause the terminations of primary afferents to be augmented, resulting in hyperalgesia and/or allodynia.

8.8 Clinical implications

The data presented in this thesis indicate that inhibiting AR activity in the spinal cord is crucial to effectively prevent or reverse formalin hyperalgesia in diabetic rats. This has clinical implications for treating patients with painful diabetic neuropathy, as design of ARIs to date has not focused on the ability of these compounds to penetrate the BBB. The relatively ineffective treatment

of painful diabetic neuropathy by orally delivered ARIs in clinical trials (Lewin et al., 1984; Boulton et al., 1990; Florkowski et al., 1991; Macleod et al., 1992) may be due to insufficient quantity of drug crossing the BBB to gain access to CNS sites of action. It may therefore be a worthwhile strategy to develop ARIs that can act both in the PNS and the CNS. Treating diabetic patients with such compounds could effectively prevent the development of painful symptoms. In patients who have already developed pain associated with diabetic neuropathy, treatment with BBB-crossing ARIs could reverse these symptoms. Alternatively, these patients could be treated to block spinal COX-2 activity. This would require either direct administration of current COX-2 selective inhibitors to the spinal cord, or development of COX-2 selective inhibitors that can readily penetrate the BBB when given systemically. Treatment with COX-2 selective inhibitors would be preferential to treatment with non-selective COX inhibitors, as inhibition of COX-1 is known to contribute to unwanted side effects in the periphery such as gastric ulcers (reviewed by Flower, 1996), and COX-1 exhibits physiologic functions in the CNS, including a role in pericyte-mediated regulation of vascular homeostasis (Dore-Duffy et al., 2005).

8.9 Future experimental directions

A number of experiments have been proposed above, which would begin investigating diabetes-induced changes in spinal oligodendrocytes. In addition to these experiments, two specific studies will be necessary to advance the hypothesis that AR activity in spinal oligodendrocytes causes

spinal sensitization via PGE_2 release. First, it will be important to determine whether blocking AR activity in the spinal cord is sufficient to protect against spinal sensitization. This cannot be achieved with a systemically delivered ARI, because such treatment carries the confound of acting in the PNS in addition to the CNS, and because a BBB-penetrating ARI will presumably block AR activity throughout the CNS and not just in the spinal cord. Therefore, to prove that blocking spinal AR activity is sufficient to protect against spinal sensitization, it will be necessary to selectively ablate AR expression in the spinal cord. This could be achieved by using siRNA to knock-down AR expression in adult rat spinal cord before inducing diabetes, to determine if diabetic rats lacking spinal AR do not develop hyperalgesia and elevated spinal COX-2 within four weeks.

Second, if diabetes does cause spinal sensitization by inducing an upregulation of COX-2 in oligodendrocytes, it must be demonstrated that oligodendrocytes are capable of upregulating COX-2 expression and secreting PGE_2 in response to hyperglycemia. This could be tested *in vitro* by culturing oligodendrocytes in normal or high glucose medium, to determine the effect on COX-2 expression and PGE_2 synthesis.

The data presented in this thesis suggest the general conclusion that oligodendrocytes have the potential to affect neurotransmission, possibly via prostaglandin signaling. Although the present data support this possibility specifically in the context of diabetic neuropathy, spinal prostaglandin signaling is an important component of a variety of neuropathic and

inflammatory chronic pain states (section 1.3.2.2). It is plausible that oligodendrocyte injury and subsequent upregulation of COX-2 is common to these other chronic pain states. This possibility merits further investigation, because it could suggest important new therapeutic targets for controlling chronic pain in patients.

Figure 8.1

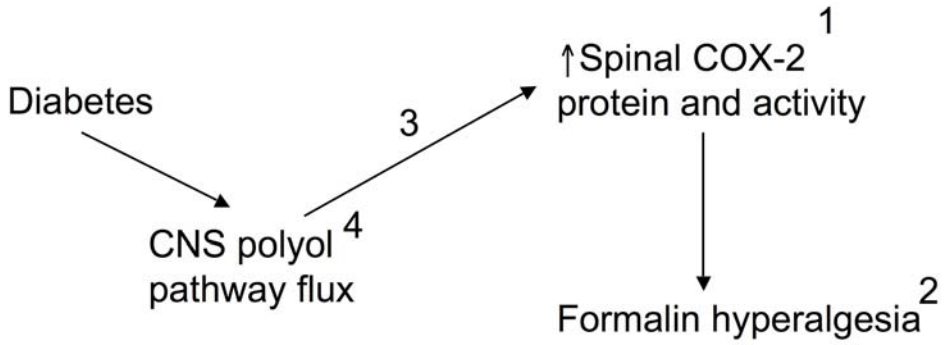


Figure 8.1. The main findings of this thesis are presented schematically above:

1. The excess COX-2 protein present in the spinal cord of diabetic rats is enzymatically active.
2. Formalin hyperalgesia in diabetic rats is at least partly caused by elevated spinal COX-2 activity.
3. Diabetes causes elevated spinal COX-2 expression and formalin hyperalgesia in diabetic rats via polyol pathway flux in the CNS.
4. Aldose reductase activity in spinal oligodendrocytes may be a mechanistic link between hyperglycemia and hyperalgesia.

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