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FMUP FACULDADE DE MEDICINA
UNIVERSIDADE DO PORTO

O SISTEMA SOMATOSSENSITIVO NO RATO COM NEUROPATIA DIABÉTICA

THE SOMATOSENSORY SYSTEM DURING PAINFUL DIABETIC NEUROPATHY

Programa Doutoral em Neurociências

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To my supervisor Professor Isaura Tavares

To my son, Gonçalo

To my parents

To Paulo

Prologue

My interest for science begun in the high school during the microscopic observation of onions' cells in mitotic division. Early in the course of my Degree in Nutrition Sciences I realized that scientific research would be my future. My first contact with research was in the Department of Hygiene and Epidemiology of the Faculty of Medicine of Porto, as a researcher fellowship, but it was in the Department of Experimental Biology (former Institute of Histology and Embryology) that I found the ideal place to perform the work with which I dreamed for a couple of years. Therefore, it was in that place that, under the supervision of Prof. Isaura Tavares, I conducted the studies presented in this PhD dissertation.

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I thank my little son for his love, his kisses and his beautiful smile. He will be a piece of me forever. I thank my parents with all my heart…. I am who I am because of them. I thank the support and patience of my husband, Paulo, who has to deal with all my doubt, my anger, my disappointments, my fears and my tears.

To finish, a sentence that is not of my own (and who wrote it may remember) but that faithfully describes what I feel: “***with a son meanwhile, with many miles traveled and a husband not always present….you must always believe”.***

Ao abrigo do Art.º 8º do Decreto-Lei nº 388/70 fazem parte desta dissertação as seguintes publicações:

1. Morgado C, Tavares I. C-fos expression at the spinal dorsal horn of streptozotocin-induced diabetic rats. Diabetes Metab Res Rev. 2007; 23:644-652.
2. Morgado C, Pinto-Ribeiro F, Tavares I. Diabetes affects the expression of GABA and potassium chloride cotransporter in the spinal cord: a study in streptozotocin diabetic rats. Neurosci Lett. 2008; 438:102-106.
3. Morgado C, Pereira-Terra P, Cruz CD, Tavares I. Minocycline completely reverses mechanical hyperalgesia in diabetic rats through microglia-induced changes in the expression of the potassium chloride co-transporter 2 (KCC2) at the spinal cord. Diabetes Obes Metab. 2011; 13:150-159.
4. Morgado C, Pereira-Terra P, Tavares I. Alpha-lipoic acid normalizes nociceptive neuronal activity at the spinal cord of diabetic rats. Diabetes Obes Metab. 2011, 13: 736-741.
5. Morgado C, Terra PP, Tavares I. Neuronal hyperactivity at the spinal cord and periaqueductal grey during painful diabetic neuropathy: effects of gabapentin. Eur J Pain. 2010; 14:693-699.
6. Morgado C, Silva L, Pereira-Terra P, Tavares I. Changes in serotoninergic and noradrenergic descending pain pathways during painful diabetic neuropathy: the preventive action of IGF1. Neurobiol Dis. 2011, 43: 275-284.

Abbreviations

AGE	Advanced glycation end products
BDNF	Brain-derived neurotrophic factor
BLA	Basolateral amygdala
CCL2	Chemokine (C-C motif) ligand 2
CeA	Central nucleus of the amygdala
CGRP	Calcitonin gene-related peptide
COX-2	Ciclooxygenase 2
CNS	Central Nervous System
CREB	cAMP response element-binding
DN	Diabetic Neuropathy
Drt	Dorsal reticular nucleus
ELISA	Enzyme linked immunosorbent assay
ERK	Extracellular-signal regulated kinases
STZ	Streptozotocin
GABA	γ-aminobutyric acid
GAD	Glutamic acid desidrogenase
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
HT	Hydroxytryptamine
IGF1	Insulin growth factor 1
IL1	Interleukin 1
IL6	Interleukin 6
KCC2	Potassium chloride co-transporter 2
LA	Lateral amygdala
MAPK	Mitogen activated protein kinases
MODY	Maturity onset diabetes of the young
NFkβ	Nuclear factor kβ
NO	Nitric Oxide
PAG	Periaqueductal grey matter
PARP	Poly (ADP-Ribose) polymerase
PDN	Painful diabetic neuropathy
PKC	Protein kinase C
PW	Pathway
RAGE	Advanced glycation end products receptor
ROS	Reactive oxygen substances
RVM	Rostroventromedial medulla
T1DM	Type 1 Diabetes Mellitus
T2DM	Type 2 Diabetes Mellitus
TH	Tyrosine hydroxylase
TpH	Tryptophan hydroxylase
TrKB-Fc	Tyrosine kinase B- constant fragment
USA	United States of America
USRDS	United States Renal Data System
WHO	World Health Organization

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Resumo

A diabetes é uma doença metabólica cuja prevalência tem aumentado de forma alarmante. A neuropatia é uma complicação que ocorre em aproximadamente 50% dos doentes diabéticos, dos quais cerca de 25% manifestam queixas de dor espontânea, alodínia e hiperalgesia. O estudo dos mecanismos responsáveis pela dor neuropática diabética tem-se direccionado para as causas periféricas mas o efeito analgésico de fármacos com actuação central e as alterações observadas no sistema nervoso central de indivíduos diabéticos sugerem a ocorrência de causas centrais. A presente dissertação de Doutoramento tem como objectivo geral estudar os mecanismos centrais subjacentes à dor neuropática diabética de modo a desenvolver futuramente estratégias preventivas ou de tratamento deste problema.

Como modelo animal de diabetes utilizámos o rato injectado com estreptozotocina (STZ), uma toxina que destrói selectivamente as células β pancreáticas, induzindo diabetes com características de tipo 1, tais como hiperglicemia, insulinopenia e diminuição dos níveis de IGF1 (*insulin-growth factor 1*). Utilizámos animais com 4 semanas de diabetes, dado que a este tempo os animais manifestam sinais comportamentais estáveis de hiperalgesia e alodínia mecânicas.

O trabalho inclui 6 publicações. Na primeira, utilizámos o estudo da indução do proto-oncogene *c-fos* em neurónios da medula espinhal para demonstrar que a diabetes induz hiperactividade espontânea dos neurónios da medula espinhal e hiperexcitabilidade dos mesmos em resposta a estímulos inócuos e nóxicos. Estas alterações neuronais podem contribuir para a dor espontânea, hiperalgesia e alodínia na neuropatia diabética. Na segunda publicação, estudámos os possíveis mecanismos subjacentes às referidas alterações funcionais dos neurónios espinhais. Com base em estudos que apontavam para distúrbios no papel do GABA na medula espinhal de ratos diabéticos, avaliámos a neurotransmissão GABAérgica espinhal, tendo-se verificado aumento na expressão do GABA no corno dorsal da medula espinhal de ratos diabéticos. Estudámos a expressão do co-transportador iónico potássio-cloro do tipo 2 (KCC2) na medula espinhal dos ratos diabéticos, visto ter sido demonstrado que a actividade deste co-transportador contribui para a acção pós-sináptica do GABA. Os ratos diabéticos apresentaram menor expressão de KCC2 no corno dorsal da medula espinhal. O aumento da expressão de GABA em conjunto com a diminuição de expressão de KCC2 poderá inverter o papel inibitório do GABA na medula espinhal e contribuir para a hiperactividade neuronal espinhal.

As duas publicações seguintes destinaram-se a esclarecer os mecanismos indutores da diminuição da expressão espinhal do KCC2. Na terceira publicação, colocámos a hipótese da microglia e do BDNF (*brain-derived neurotrophic factor*) serem factores determinantes. A activação microglial aumentava significativamente no corno dorsal da medula espinhal dos ratos diabéticos, sem que ocorresse aumento de expressão de BDNF. A inibição da actividade microglial, por administração intratecal de minociclina, normalizou a expressão de KCC2 no corno dorsal da medula espinhal e reverteu totalmente a hiperactividade neuronal e hiperalgesia nos ratos diabéticos. O bloqueio da acção do BDNF, por administração intratecal de sequestrador de BDNF (TrKB-Fc), apresentou efeitos moderados. Este trabalho demonstrou que a microglia tem um papel-chave na modulação da actividade neuronal durante a diabetes, através dos

seus efeitos na expressão de KCC2. Na quarta publicação, a hipótese colocada foi a de que o stress oxidativo constitui outro mecanismo de regulação do KCC2. Os níveis de stress oxidativo na medula espinhal de ratos diabéticos estavam aumentados. O tratamento antioxidante com ácido alfa-lipóico reverteu o stress oxidativo e a hiperactividade neuronal na medula espinhal dos ratos diabéticos e aumentou moderadamente a expressão de KCC2. Estes resultados sugerem que o stress oxidativo contribui para a hiperactividade neuronal espinhal, através do seu efeito na expressão de KCC2.

As duas publicações finais da tese estudaram a modulação descendente da dor, direccionando-se para os efeitos da diabetes em centros-chave de controlo da dor. Na quinta publicação, verificámos que a diabetes induz hiperactividade neuronal na substância cinzenta periaqueductal (PAG), que era revertida pelo tratamento com gabapentina, um anticonvulsivante utilizado no tratamento da dor neuropática diabética. Adicionalmente, a gabapentina revertia a hiperalgesia mecânica e a hiperactividade neuronal na medula espinhal. Na sexta publicação, estudámos as regiões do tronco cerebral que servem de "*relay*" à PAG na modulação descendente da dor, nomeadamente a área serotoninérgica do bolbo rostroventromedial (RVM) e os grupos noradrenérgicos A_5, A_6 e A_7. A imunodetecção de enzimas envolvidas na síntese de serotonina e noradrenalina, a hidroxilase do triptofano (TpH) e a tirosina hidroxilase (TH), respectivamente, mostrou que os ratos diabéticos tinham números superiores de neurónios serotoninérgicos no RVM e noradrenérgicos no A_5, o que era acompanhado por aumento dos níveis espinhais de serotonina e noradrenalina, quantificados por ELISA. A actividade neuronal, avaliada pela expressão de formas fosforiladas de ERKs (*extracelullar-regulated kinases*) nos neurónios serotoninérgicos, e de CREB (*cAMP regulated binding protein*) nos neurónios noradrenérgicos, estava aumentada no RVM e no A_5 dos ratos diabéticos. Este trabalho demonstrou que a diabetes afecta o sistema descendente de controlo da dor através da modulação serotoninérgica e noradrenérgica. O tratamento dos ratos diabéticos com doses de IGF1 que não reverteram a hiperglicemia, normalizou a actividade dos neurónios da PAG, preveniu as alterações no sistema serotoninérgico e noradrenérgico, reverteu a hiperactividade espinhal e diminuiu os sinais comportamentais de hiperalgesia.

Colectivamente, os dados obtidos nesta dissertação de doutoramento demonstram que a etiologia da dor neuropática diabética não se resume apenas às alterações dos nervos periféricos, mas engloba alterações de áreas centrais envolvidas no controlo da dor. A diabetes induz hiperactividade e hiperexcitabilidade dos neurónios nociceptivos da medula espinhal, para as quais parece contribuir a perda de inibição GABAérgica mediada por redução na expressão de KCC2, a qual é provavelmente induzida por activação microglial e stress oxidativo na medula espinhal. A hiperactividade espinhal deverá aumentar a actividade dos neurónios da PAG e recrutar vias descendentes serotoninérgicas e noradrenérgicas envolvidas na modulação da dor, por activação dos neurónios do RVM e do A_5. Torna-se agora necessário avaliar o papel da serotonina e noradrenalina na modulação da transmissão nociceptiva na medula espinhal durante a diabetes o que poderá abrir novas linhas de investigação no tratamento da dor neuropática diabética.

Abstract

Diabetes is a metabolic disease with an alarmingly increasing prevalence. Neuropathy affects about 50% of diabetic patients and is associated with spontaneous pain, allodynia and hyperalgesia in 25% of the cases. The study of the mechanisms underlying diabetic neuropathic pain has been directed to the peripheral causes but considering the analgesic effect of central-acting drugs and the changes detected in the central nervous system of diabetic patients it is likely that central mechanisms are also involved. This PhD thesis aims at investigating the central mechanisms underlying diabetic neuropathic pain in order to develop new preventive or treatment strategies for this problem.

As animal model of diabetes, we used the rat injected with streptozotocin (STZ rats), a toxin that selectively destroys pancreatic β cells, inducing type 1 diabetes, characterized by hyperglycemia, insulin deficiency and decreased levels of insulin-growth factor 1 (IGF1). We used the animals at 4 weeks of diabetes since at this time the rats show stable behavioral signs of mechanical hyperalgesia and allodynia.

This dissertation includes six publications. In the first publication, we demonstrated that diabetes induces spontaneous hyperactivity and hyperexcitability of spinal nociceptive neurons in response to innocuous and noxious stimuli, using the *c-fos* method. These neuronal changes may contribute to spontaneous pain, allodynia and hyperalgesia during diabetic neuropathy. In the second publication, we studied the possible mechanisms underlying the functional changes occurring at spinal nociceptive neurons. Based on studies showing impairments in the role of GABA at the spinal cord of diabetic rats, we evaluated the spinal GABAergic neurotransmission and detected increased expression of GABA in the spinal dorsal horn of diabetic rats. We studied the expression of the ionic co-transporter potassium-chloride type 2 (KCC2) in the spinal cord of diabetic rats, as has been demonstrated that the activity of this co-transporter contributes to the postsynaptic action of GABA. Diabetic rats presented lower expression of KCC2 in the spinal dorsal horn. The increased expression of GABA along with the decreased expression of KCC2 may invert the inhibitory role of GABA at the spinal cord and contribute to spinal neuronal hyperactivity.

The following two publications aimed to clarify the mechanisms which induce the reduction in the expression of spinal KCC2. In the third publication, we considered the microglia and brain-derived neurotrophic factor (BDNF) as key factors. The microglial activation increased significantly in the spinal dorsal horn of diabetic rats, with no changes in the expression of BDNF. Inhibition of microglial activity by intrathecal administration of minocycline, normalized the expression of KCC2 in the spinal dorsal horn and reversed completely the neuronal hyperactivity and hyperalgesia in diabetic rats. Blocking the action of BDNF by intrathecal administrations of BDNF sequester (TrkB-Fc) showed moderate effects. This work demonstrated that microglia have a key role in the modulation of neuronal activity during diabetes through its effects on the expression of KCC2. In the fourth publication, we considered oxidative stress as another mechanism of regulation of KCC2. The levels of oxidative stress on the spinal cord of diabetic rats were increased. The antioxidant treatment with alpha-lipoic acid reversed the oxidative stress and neuronal hyperactivity in the spinal cord of diabetic rats and moderately increased the expression of KCC2. These results suggest that

oxidative stress contributes to the spinal neuronal hyperactivity through its effect on expression of KCC2.

The two final publications of the thesis studied the descending modulation of pain, focusing on the effects of diabetes in key centers of pain control. In the fifth publication, we observed that diabetes induces neuronal hyperactivity at the periaqueductal grey matter (PAG), which was reversed by treatment with gabapentin, an anticonvulsant used to treat diabetic neuropathic pain. Additionally, gabapentin reversed the mechanical hyperalgesia and neuronal hyperactivity at the spinal cord. In the sixth publication, we studied the regions of the brainstem that act as relay stations of the PAG descending pain modulation, including the serotoninergic rostroventromedial medulla (RVM) and the A_5, A_6 and A_7 nordarenergic groups. The immunodetection of enzymes involved in the synthesis of serotonin and noradrenaline, tryptophan hydroxylase (TpH) and tyrosine hydroxylase (TH), respectively, showed that diabetic rats presented higher numbers of serotonergic and noradrenergic neurons at the RVM and A_5. These changes were accompanied by increased levels of serotonin and noradrenaline at the spinal cord, as quantified by ELISA. The neuronal activity, as assessed by the expression of phosphorylated forms of extracelullar-regulated kinases (ERKs) and cAMP-regulated binding protein (CREB) in serotonergic and noradrenergic neurons, respectively, was increased in the RVM and A_5 in the diabetic rat. This study showed that diabetes affects the pain control descending system by modulating the serotoninergic and noradrenergic neurotransmission. The treatment of diabetic rats with IGF1, at doses that did not reverse the hyperglycemia, normalized the neuronal activity at the PAG, prevented the changes in the serotonergic and noradrenergic systems, reversed the spinal hyperactivity and ameliorated hyperalgesia detected in STZ rats.

Taken together, the results present in this PhD thesis demonstrate that the etiology of diabetic neuropathic pain is not confined to changes in peripheral nerves, but involves changes in central areas involved in pain control. Diabetes induces hyperactivity and hyperexcitability of spinal nociceptive neurons, probably due to the loss of GABAergic inhibition induced by decreased expression of KCC2, which is mediated by oxidative stress and microglial activation at the spinal cord. It is likely that spinal hyperactivity increases the activity of PAG neurons, recruiting the serotoninergic and noradrenergic descending pathways involved in pain modulation, via activation of neurons at the RVM and at the A_5. It is now necessary to evaluate the role of serotonin and noradrenaline in the modulation of spinal nociceptive transmission during diabetes, which could open new lines of research in the treatment of diabetic neuropathic pain.

*“Life is short, unpleasant and **painful**…”*

Excerpt from the Clinical description of diabetes by Aretaeus of Cappadocia (2nd century AD). Adapted from Papaspyros NS (1952). The History of Diabetes Mellitus.

Introdução

Diabetes is a major health problem worldwide with an alarming increasing prevalence and was considered a pandemia by the World Health Organization (WHO, 1999). Diabetes is one of the most important leading causes of mortality and morbidity in developing countries, due not only to the disease by itself, but also to its complications (Ali et al., 2010; Ma and Tong, 2010; Stene et al., 2010). Complications of diabetes, such as the cardiovascular diseases, sexual dysfunction, retinopathy, nephropathy and neuropathy, are responsible for increased disability and reduced quality of life as well as decreased life expectancy among diabetic patients (Ali et al. 2010; Giacco and Brownlee, 2010; Candido et al., 2010). They represent an enormous economic and social burden in most societies (Ali et al., 2010). As stated by the International Diabetes Federation *"Diabetes is certain to be one of the most challenging health problems in the 21st century*" (International Diabetes Federation, 2006).

1. Definition and classification of diabetes mellitus

Diabetes mellitus is a metabolic disease characterized by chronic hyperglycemia induced by defects on insulin secretion, insulin action or both (WHO, 1999). It is classified as type 1 (T1DM), type 2 (T2DM), gestational or other specific types of diabetes (WHO, 1999).

Type 1 Diabetes Mellitus

T1DM is caused by loss of pancreatic β-cells and comprises autoimmune (type 1A) and non-autoimmune or idiopathic (Type 1B) diabetes (WHO, 1999; Alberti, 2010). The former is accompanied by evidences of autoimmunity, with detection of antibodies against insulin, glutamine-acid decarboxilase and/or β-cells (Eisenbarth, 2007; Concannon et al., 2009; Alberti, 2010; Delli et al., 2010). The etiology of the type 1B diabetes remains unknown (McLarty et al, 1990; Alberti, 2010).

T1DM is characterized by lack of insulin secretion and has an abrupt onset. The affected patients require insulin replacement, with daily exogenous administrations, to maintain normoglycemia (Alberti, 2010; Delli et al., 2010).

Type 2 Diabetes Mellitus

T2DM is characterized by resistance to insulin action (WHO, 1999; Alberti, 2010, Yki-Jarvinen, 2010). Initially T2DM patients present hyperinsulinemia, a response to overcome the insulin resistance. Insulin deficiency develops with the progression of the disease as a consequence of β-cell dysfunction (Alberti, 2010; Alsahli and Gerich, 2010). The affected patients are commonly treated with oral antidiabetic drugs and do not require insulin replacement to maintain reasonable glycemia control. Treatment with insulin could be necessary within the course of the disease, due to the crescent deterioration of β-cell function (Alberti, 2010; Alsahli and Gerich, 2010). This type of diabetes is closely associated with unhealthy life styles, namely those related with food and physical activity habits.T2DM has an important hereditary influence, occurring within families and being more prevalent in some ethnic groups (Ma and Tong, 2010).

Gestational Diabetes Mellitus

Gestational diabetes mellitus is defined as hyperglycemia first detected during pregnancy (WHO, 1999; Alberti, 2010). In most cases, glycemic levels normalize after the delivery, but in high risk patients it can trigger a T2DM. There are several gestational complications associated with gestational diabetes mellitus, namely intrauterine fetal death, congenital malformations, neonatal hypoglycemia, jaundice, prematurity and macrosomia (Dornhorst and Banerje, 2010).

Other specific types of diabetes

The types of diabetes included in this category comprise diabetes induced by a wide range of conditions, but account for a small number of cases. Diabetes can occur as a result of specific genetic defects in insulin secretion and action, as is the case of maturity onset diabetes of the young (MODY) (WHO, 1999; Alberti, 2010, Jones and Hattersley, 2010). Additionally, diabetes may also be a consequence of several other diseases, such as pancreatitis, hemochromatosis, cystic fibrosis, Cushing syndrome, acromegaly, pheochromocytoma, glucagonoma and hyperthyroidism (WHO, 1999; Alberti, 2010; Hanley, 2010). Many drugs and chemicals can also cause diabetes (Gittoes et al, 2010), as well as some infection diseases, as mumps, congenital rubella, coxsackie B and cytomegalovirus (WHO, 1999; Alberti, 2010). There are also several genetic syndromes associated with increased risk of diabetes, namely the Down Syndrome, Turner Syndrome and Klinefelter Syndrome (WHO, 1999; Alberti, 2010).

2. The Prevalence and Costs of Diabetes

According to the International Diabetes Federation, diabetes affects more than 285 million people worldwide and can reach 435 million persons by 2030. It accounts now for about 6.8% of total global mortality (International Diabetes Federation, 2009) and the increasing prevalence rates worldwide point for a rise in morbidity and disability. Life expectancy of diabetic patients is estimated to be about 7-15 years less than that of general population (Morgan et al., 2000; Franco et al., 2007). Diabetes is believed to induce important loss of human and social resources in almost all societies, which is expected to worsen in the next few years (Ali et al, 2010).

T1DM accounts for approximately 5-10% of world diabetic population. Its incidence is increasing about 3-4% per year (Stene et al., 2010). T1DM is associated with an approximately 2 - to 10 fold excess risk of premature mortality (Dahlquist and Kallén, 2005; Soedamah-Muthu et al., 2006; Stene, 2010) and increased morbidity,

which are particularly related with acute and late-onset complications of the disease (Stene, 2010). In fact, after about 10 – 15 years of diabetes, microvascular and macrovascular chronic diabetes complications start to affect T1DM patients (Stene, 2010), which, considering that the incidence of this type of diabetes peaks at the puberty (DIAMOND Project group, 2006), afflict the patients at very young ages.

T2DM accounts for 90 – 95% of all cases worldwide and its prevalence has been growing rapidly, namely in lower socioeconomic groups (WHO, 1999; Ali et al., 2010; Ma and Tong, 2010). T2DM has, in most of the cases, a late diagnosis and about 30 – 50% of T2DM cases are as yet unrecognized (Aschner, 2002; International Diabetes Federation, 2009; Ma and Tong, 2010; Ali et al., 2010). The late diagnosis of T2DM is thought to be the reason for the high prevalence of diabetic complications in this type of diabetes. Most of the patients already present signs of diabetic complications at the time of the diagnosis and the majority dies from cardiovascular and renal disease (Morrish et al., 2001).

Overall, diabetes imposes important health, social and economic burdens worldwide. It results in an 1.5-5 times increase in health care expenses over the general population (Rubin et al., 1992; Henriksson and Jonsson, 1998; Shobhana et al., 2000; Ricordeau et al., 2003; Zhang et al., 2004; Koster et al., 2006), with a cumulatively total cost of about € 263 billion in 2010 (International Diabetes Federation, 2009). The elevated costs derive mostly from the treatment of its complications, such as stroke, coronary artery disease, renal failure, blindness, amputation and neuropathic pain (Ali et al., 2010).

In Portugal, diabetes affects 7,3% of the general population, with an estimated cost of about € 1500 M in 2009, which represents approximately 1% of the Portuguese Internal Product and about 9% of the expenses of Portuguese Health Department (Observatório Nacional de Diabetes, 2010).

3. Complications of diabetes

All forms of diabetes may develop complications (Giacco and Brownlee, 2010; Candido et al, 2010). Diabetes was shown to dramatically increase the risk for coronary artery disease, ischemic cerebrovascular disease, myocardial infarction and peripheral artery disease (Candido et al., 2010; Amory and Weinberger, 2010; Sillesen, 2010). In fact, death from stroke and myocardial infarction remain the major leading causes of mortality in T1DM and T2DM (Nathan et al., 2005; Dale et al., 2008; Ali et al., 2010). Diabetes also increases the risk of renal dysfunction and is the primary cause of dialysis or kidney transplantation in the USA (USRDS, 2008). About one quarter of diabetic patients present visual impairments (Ali et al., 2010; Scanlon, 2010). In fact, proliferative diabetic retinopathy was shown to affect approximately 67% and 33% of T1DM and T2DM patients, respectively (Klein, 1984, Scanlon, 2010). Diabetic retinopathy was considered to be the major leading cause of adult blindness in the USA and comprises 5% of all cases of blindness globally (Ali et al., 2010).

Neuropathy is a very common consequence of diabetes. More than 60% of diabetic patients develop neuropathy, which may include distal symmetrical polyneuropathy, mononeuropathies and autonomic neuropathies that, ultimately, cause erectile dysfunction, gastroparesis, foot disorders and altered pain sensations (Wheeler et al., 2007; Tesfaye, 2009; Obrosova, 2009; Ziegler, 2010; Tesfaye et al., 2010). Erectile dysfunction, which affects approximately 35-50% of men with diabetes, is triggered by failure of nitric oxide (NO) – mediated smooth muscle relaxation secondary to autonomic neuropathy and endothelial dysfunction (Ziegler, 2007; Price, 2010), while the gastroparesis is induced by sympathetic, parasympathetic and enteric neural dysfunction (Malagelada, 2007; Tesfaye et al., 2010; Ziegler, 2010). Foot disorders are triggered by the combined consequences of sensory neuropathy, peripheral vascular disease and infection and are the major cause of all non-traumatic amputations worldwide (Boulton, 2005; Lyons, 2007; Boulton, 2010). Altered pain sensation results

from the affectation of somatosensory system and will be addressed in more detail later in the text, since it is the focus of the present PhD dissertation.

4. Diabetic Neuropathies

The most recent definition and classification of diabetic neuropathy (DN) were updated by a panel of experts in the end of 2009 and DNs were separated as typical and atypical DNs (Tesfaye et al., 2010).

The typical DN comprises the sensorimotor polyneuropathy, which is the commonest form of diabetic neuropathy, affecting approximately 30% of diabetic patients (Shaw et al., 2003; Tesfaye, 2007; Ziegler, 2010). It is characterized by symmetrical and length-dependent affectation of peripheral nerves with reduction in sensory and motor nerves conduction velocities. Chronic sensory abnormalities are very often presented, with neuropathic pain co-existing with loss of sensation. Autonomic dysfunction may also be present (Tesfaye, 2007; Tesfaye, 2009; Obrosova 2009; Ziegler, 2010; Tesfaye et al., 2010).

Atypical DN comprises the acute painful DN, focal and multifocal neuropathies and autonomic neuropathy (Tesfaye et al., 2010). Acute painful DN is characterized by continuous burning pain that affects, predominantly the soles and presents nocturnal exacerbation. It is accompanied by allodynia, frequently described as unpleasant cutaneous contact with clothes, and hypersensitivity to painful stimuli (hyperalgesia) (Ziegler, 2010; Tesfaye et al., 2010). These manifestations have an abrupt onset, but subside rapidly and no recurrences are normally observed. It is mostly correlated with severe weight loss and insulin treatment onset (Ziegler, 2010). These acute syndromes are relatively uncommon when compared with the chronic painful neuropathy associated with the sensorimotor polyneuropathy (Ziegler, 2010). Focal and multifocal neuropathies comprise a wide variety of conditions, as cranial neuropathy, mononeuropathy of the limbs, diabetic truncal neuropathy and diabetic amyotrophy (Ziegler, 2010). The large majority of these conditions affects long-term diabetic

patients and is usually accompanied by pain symptoms. Almost all cases have partial or complete resolution of the manifestations when carefully diagnosed and treated (Ziegler, 2010; Tesfaye et al., 2010). Diabetic autonomic neuropathy is a neuropathic condition that affects predominantly the autonomic nervous system. It may present repercussions in cardiovascular, gastrointestinal, and genitourinary systems and sudomotor function (Stevens, 2007; Ziegler, 2007; Malagelada, 2007; Ziegler, 2010; Tesfaye et al., 2010).

Central nervous system dysfunction

Little attention has been given to the central nervous system (CNS) during DN. Nevertheless, degenerative lesions in the CNS were already reported in diabetic patients (Biessels, 2007). Previous studies demonstrated demyelination and loss of axon cylinders in the posterior columns (Reske-Nielsen and Lundbaek, 1968; Slager, 1978; Mizisin et al., 2007), known as myelopathy, degeneration and loss of cortical and hypocampal neurons (Reske-Nielsen et al., 1965; Reske-Nielsen and Lundbaek, 1968; Li et al, 2002) and abnormalities in the midbrain and cerebellum (Reske-Nielsen and Lundbaek, 1968), defined as encephalopathy (Biessels, 2007). Overall those central changes may contribute to the higher prevalence of cognitive and memory deficits detected in diabetic patients (Schmidt et al., 2004; Brands et al., 2005). Recent findings also show an increased occurrence of Alzheimer-like changes during diabetes (Li et al., 2007), which may also contribute to such deficits. Recently, there were described structural and functional impairments at the central pain control system, as the spinal cord (Eaton et al., 2001; Pertovaara et al., 2001; Chen and Pan, 2002; Selvarajah et al., 2006; Mizisin et al., 2007) and the thalamus (Selvarajah et al., 2008; Sorensen et al., 2008; Fischer et al., 2009; Fischer and Waxman, 2010; Selvarajah et al., 2011), which were suggested to contribute to the altered pain sensations observed in diabetic patients.

5. Painful diabetic neuropathy (PDN)

Pain is the most distressing symptom of DN (Tesfaye and Kempler, 2005; Backonja et al., 2007; Boulton, 2007; Tesfaye, 2009; Obrosova, 2009; Ziegler, 2010; Tesfaye et al., 2010). Painful diabetic neuropathy (PDN) is defined by IASP (International Association for the Study of Pain) as "pain arising as a direct consequence of abnormalities in the peripheral somatosensory system in people with diabetes." The pain is commonly distal, symmetrical and with nocturnal exacerbation (Tesfaye and Kempler, 2005; Tesfaye, 2007; Tesfaye, 2009; Ziegler, 2010; Tesfaye et al., 2010). It is perceived spontaneously and described as prickling, burning, lancinating and shooting "like electric shocks". It is often accompanied by hyperalgesia and allodynia (Tesfaye and Kempler, 2005; Tesfaye, 2007; 2009; Obrosova, 2009; Tesfaye et al., 2010; Ziegler, 2010). This pain condition may persist for several years (Tesfaye et al., 2010; Ziegler, 2010) and is resistant to conventional pain treatments, causing considerable disability and impairing the quality of life (Shaw et al., 2003; Daousi et al., 2006; Ziegler, 2010), namely by interfering with sleep (Galer et al., 2000). PDN may be accompanied by sensory loss and is commonly associated with autonomic neuropathy (Tesfaye et al., 2010; Ziegler, 2010).

The estimates of pain occurrence are quite variable, ranging from 3 to 25% of diabetic patients, according the diagnosis criteria used (Tesfaye et al., 2010). It is believed that neuropathic pain affects about one quarter of the population with diabetes (Shaw et al., 2003; Tesfaye and Kempler, 2005; Daousi et al., 2006; Tesfaye, 2009; Ziegler, 2010; Tesfaye et al., 2010) and is one of the major complains that leads diabetic patients to seek health care (Tesfaye, 2009).

The treatment of PDN has been almost symptomatic, since most of drugs directed to counteract the pathogenetic mechanisms were shown to present irrelevant clinical applicability (Tesfaye, 2009; Ziegler, 2010; Zilliox and Russel, 2011). Currently,

only the antioxidant alpha-lipoic acid and epalrestat, an aldose reductase inhibitor, are used for PDN treatment in several European countries and in Japan, respectively (Tesfaye, 2009; Ziegler, 2010; Zilliox and Russel, 2011). In what concern the pain relieving agents it is known that peripherically-acting drugs, as the topical capsaicin and the 5% lidocaine patches, have only moderate efficacy in this pain condition (Tesfaye, 2009; Ziegler, 2010; Zilliox and Russel, 2011). Central-acting drugs like antidepressants (including tryciclic antidepressants and dual serotonin and noradrenaline reuptake inhibitors) and anticonvulsants (pregabalin and gabapentin), were shown to be the agents with the greatest pain relief effects and have been used as first line treatments for PDN (Tesfaye, 2009; Ziegler, 2010; Zilliox and Russel, 2011). Neverthless, their effects are moderate, eliciting 50% pain reduction in about 30%-50% of treated diabetic patients (Tesfaye, 2009; Ziegler, 2010; Zilliox and Russel, 2011). Important concerns exist in the use of such agents during diabetes due to their several contra-indications, such as unstable angina, myocardial infarction, heart failure, ventricular arrhythmias, hepatic impairment and end-stage renal disease. These health complications normally co-exist in patients affected by diabetic neuropathic (Ziegler, 2010; Zilliox and Russel, 2011). Furthermore, these drugs can elicit important side-effects that compromise the treatment compliance and may worsen the diabetic condition, as dry mouth, sedation, tiredness, somnolence, dizziness, nausea, constipation, weight gain and increase in fast blood glucose (Tesfaye, 2009; Ziegler, 2010; Zilliox and Russel, 2011). These data show that the development of new and more efficacious agents is necessary, which can only be achieved upon a deeper knowledge of the etiological mechanisms involved in this neuropathic pain condition.

5.1. The pain mechanisms

The etiology of chronic pain associated to diabetic neuropathy remains unclear. Most of the studies on PDN have been focused on the peripheral aspects of pain

(Calcutt, 2002; Sima, 2003; Kles and Vinik, 2006; Calcutt and Backonja, 2007; Zochodne, 2007; Tavakoli et al., 2008; Obrosova, 2009; 2009a), with central mechanisms being scarcely studied (Calcutt and Chaplan, 1997; Calcutt et al., 2000; Pertovaara et al.,2001; Chen and Pan, 2002; Selvarajah et al., 2006; Selvarajah and Tesfaye, 2006; Malmberg et al., 2006; Ramos et al., 2007; Sorensen et al., 2008; Selvarajah et al., 2008; Jolivalt et al., 2008; Cauda et al., 2009; Fischer et al., 2009; Fischer and Waxman, 2010; Selvarajah et al., 2011). Pain perception involves complex and numerous neuronal networks and mechanisms, with intervention of peripheral nerve fibers, spinal cord, brainstem, subcortical and cortical brain areas (**Figure 1**) (Millan, 2002; Vanegas and Schaible, 2004; Heinricher et al., 2009; Ossipov et al., 2010).

Nociceptive information is conveyed from the periphery to the spinal dorsal horn by primary afferents. At the spinal dorsal horn, afferent terminals release excitatory neurotransmitters (such as glutamate, substance P and calcitonin gene related peptide- CGRP) which interact with interneurons and projection neurons (Renn and Dorsey, 2005; Todd, 2010). Subsequently, the projection fibers ascend through the contralateral spinothalamic tract targeting the thalamus and sending collateral projections to several areas as periaqueductal grey matter (PAG) and rostroventromedial medulla (RVM) (Renn and Dorsey, 2005; Ossipov et al., 2010).

Pain modulation involves complex neural supraspinal networks which are mainly activated by the thalamus. In fact, after targeted by spinal ascending projections, the thalamus communicates with cortical areas, as the somatosensory, pre-frontal, insular and cingulate cortices and subcortical areas, as the amygdala, triggering the activation of the descending pain pathways as well as pathways involved in the cognitive and emotional responses to pain (Millan, 2002; Heinricher et al., 2009; Ossipov et al., 2010). In fact, the amygdala projects to the cortex and sends feedback projections to the thalamus, which was shown to be involved in the integration of

cognitive, memory and emotional components of pain (Price, 2002; Neugebauer et al., 2004; Ossipov et al., 2010). Moreover, descending pathways from the cortex, the amygdala and the hypothalamus, reach the PAG, which uses relay stations as the RVM and pontine noradrenergic cell groups (the A_5, A_6 and A_7 groups), to indirectly modulate the nociceptive transmission at the spinal cord (Bajic and Proudfit, 1999; Millan et al., 2002; Vanegas and Schaible, 2004; Renn and Dorsey, 2005; Pertovaara, 2006; Yoshimura and Furue, 2006; Heinricher et al., 2009; Ossipov et al., 2010).

Descending projections from the RVM reach the spinal dorsal horn through the dorsal lateral funiculus and establish synaptic connections with primary afferent terminals, projection neurons and interneurons (Millan, 2002; Ossipov et al., 2010). Part of those descending fibers release serotonin, but a considerable proportion is glycinergic or GABAergic (Vanegas and Schaible, 2004; Ossipov et al., 2010). Although less studied, the glycinergic and GABAergic descending projections from the RVM were shown to induce antinociception (Kato et al., 2006). On the other hand, serotonin presents a bidirectional role, eliciting pain inhibition as well as facilitation, depending on the serotonin receptors at the spinal dorsal horn (Porreca et al., 2002; Drogul et al., 2009). Indeed, several studies reported inhibitory effects of serotonin by binding to 5-HT1 and 5-HT7 (Vanegas and Schaible, 2004; Lopez-Garcia, 2006; Drogul et al., 2009) receptors, while binding to 5-HT3 elicits neuronal excitation (Drogul et al., 2009).

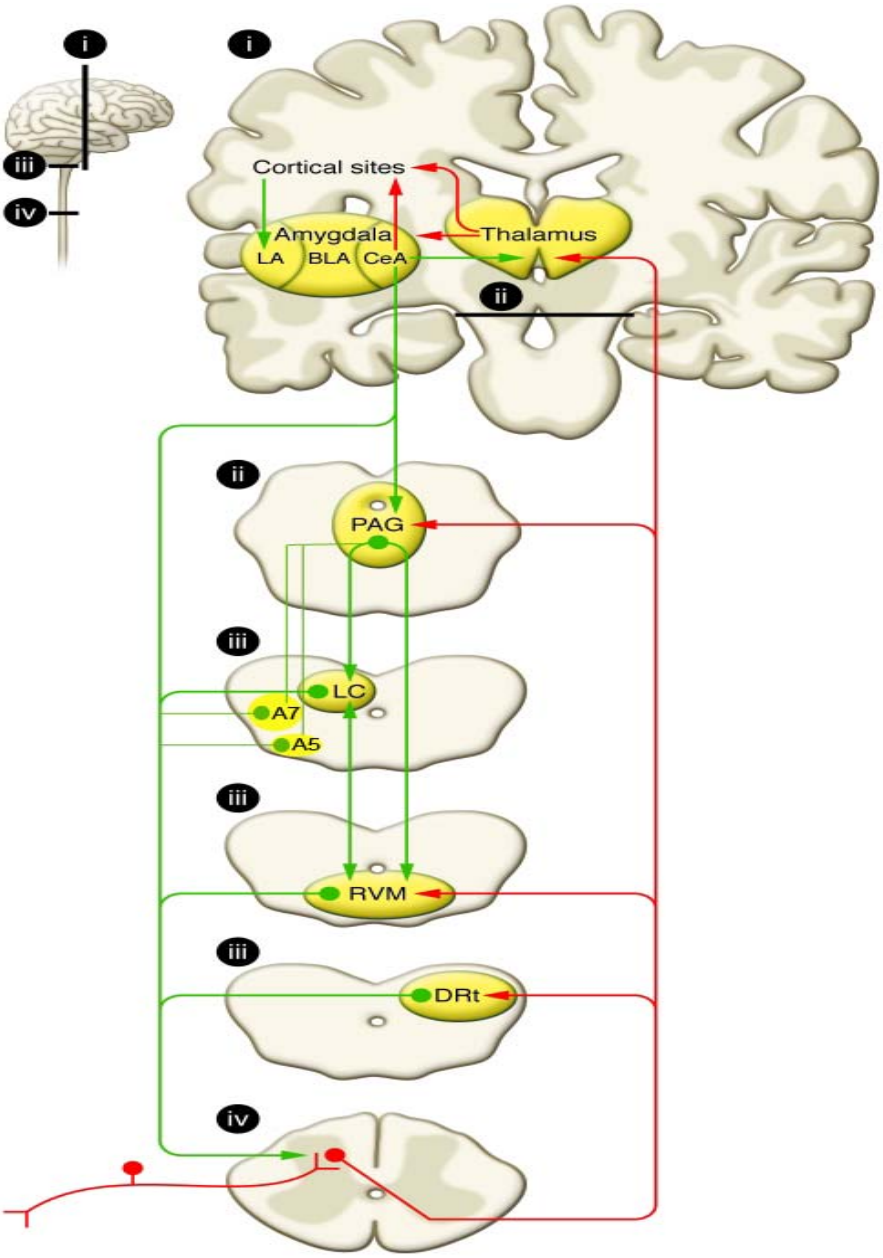

Figure 1 – Schematic representation of the pain pathways. Nociceptive information is brought from the periphery by primary afferents to the spinal dorsal horn. Then, nociceptive input ascends to the thalamus and sends collateral projections to the PAG, RVM and dorsal reticular nucleus (red lines). Interconnections between higher brain centers (thalamus, cortical areas and amygdala) trigger the descending modulation of pain. Descending pathways project to the PAG, which uses the RVM and pontine noradrenergic cell groups (such as A_5, A_6 and A_7) as relay stations to modulate pain transmission at the spinal cord. The areas "i–iv" in the small diagram correspond to the levels of neuroaxis presented in the larger diagram. BLA - basolateral amygdala; CeA - central nucleus of the amygdala; DRt - dorsal reticular nucleus; LA - lateral amygdala; LC - Locus coeruleus; PAG - periaqueductal grey matter; RVM - rostroventromedial medulla. Adapted from Ossipov et al., 2010.

Neurons located at the A_5, A_6 and A_7 cell groups send descending projections, which terminate in the superficial dorsal horn and release noradrenaline (Pertovaara, 2006; Yoshimura and Furue, 2006). Noradrenaline blocks pain transmission by inhibiting the activity of projection neurons and the release of excitatory neurotransmitters from primary afferents (Pertovaara, 2006; Yoshimura and Furue, 2006; Ossipov et al., 2010). These inhibitory actions were shown to be mediated by binding of noradrenaline to α2 adrenoreceptors located at the terminals of primary afferents, excitatory interneurons and projection neurons (Pertovaara, 2006; Yoshimura and Furue, 2006; Ossipov et al., 2010). In addition, noradrenaline was shown to induce depolarization of spinal GABAergic and, probably, glycinergic interneurons by binding to pre-synaptic α1 adrenoreceptors, enhancing the release of GABA and glycine, the major spinal inhibitory neurotransmitters (Pertovaara, 2006; Yoshimura and Furue, 2006; Gassner et al., 2009; Ossipov et al., 2010). Therefore, the net effect of noradrenaline at spinal cord seems to be inhibitory.

Dysfunctions on neurotransmitters-receptors systems at spinal cord compromise the final effect of descending pain modulation. Changes in the expression and activity of spinal serotoninergic and noradrenergic receptors impair the actions of serotonin and noradrenaline released by descending projections. Moreover, increased expression and binding affinity of glutamate, substance P or CGRP receptors at spinal dorsal horn enhance pain transmission, which can be potentiated by impairments in the expression and activity of GABA and glycine receptors (Kuner, 2010). Indeed, the inhibitory actions of GABA at spinal cord may be diminished by reduced expression or impaired activity of GABA receptors (Zeilhofer, 2008; Kuner, 2010; Gwak and Hulsebosh, 2011). GABA elicits inhibitory effects at the spinal dorsal horn by binding to $GABA_A$ and $GABA_B$ receptors (Malcangio and Bowery, 1996; Gwak and Hulsebosh, 2011). The $GABA_A$ receptor is ionotropic and when activated induces the opening of chloride ion (Cl^-) channels located at the membranes of excitatory interneurons and projection neurons (Malcangio and Bowery, 1996; Gwak and Hulsebosh, 2011). The

increase in the Cl^- conductance in those neurons can induce hyperpolarization and depolarization, depending on the intracellular Cl^- concentration (Payne et al, 2003; Coull et al., 2003; Price et al., 2009; Gwak and Hulsebosh, 2011). In the mature nervous systems, Cl^- concentration is lower in the intracellular compartment than in the extracellular space. In such condition, GABA binding to $GABA_A$ induces influx of Cl^-, which results in membrane hyperpolarization and neuronal inhibition. During the nervous system development and in some pathological conditions, Cl^- accumulates at the intracellular compartment due to the low expression and activity of potassium-chloride co-transporter 2 (KCC2) (Payne et al., 2003; Price et al., 2009). This co-transporter is markedly expressed at spinal cord and is the responsible for setting low neuronal intracellular Cl^- concentration (Payne et al., 2003; Coull et al., 2003). Reduced expression and activity of KCC2 were shown to induce increase intracellular Cl^- concentration, which attribute an excitatory role to GABA when binding to $GABA_A$ receptors, since the activation of those receptors will induce Cl^- efflux and, consequently, membrane depolarization (Coull et al., 2003; Wake et al, 2007). Therefore, a fine homeostasis of Cl^- at spinal dorsal horn, achieved by a normal activity of KCC2, is mandatory for the inhibitory action of GABA. KCC2 impairment was shown to invert the GABA role, which can, ultimately, compromise the effects of descending pain modulation. Similar changes are likely to affect the glycinergic neurotransmission at the spinal cord, since the activity of glycine receptors also depends on Cl^- equilibrium (Payne et al., 2003; Price et al., 2005; Zeilhofer, 2005). Moreover, reduced expression and activity of $GABA_B$ receptors have also an important impact on spinal pain transmission, mainly by reducing the inhibition of excitatory neurotransmitters release (Malcangio and Bowery, 1996; Enna and McCarson, 2006; Kuner, 2010).

Recent studies have been shown that neurons are not the only players in pain transmission and modulation, with increasing evidences pointing for an important role of glia. Neuron-glia crosstalk was shown to be crucial for initiation and maintenance of chronic pain conditions at spinal and supraspinal levels (Zhao et al., 2007; Wei et al.,

2008; Inoue and Tsuda, 2009; McMahon and Malcangio, 2009; Roberts et al., 2009; Ren and Dubner, 2010; Gosselin et al., 2010; O'Callaghan and Miller, 2010). In fact, spinal microglia was shown to be activated in neuropathic pain involving traumatic injuries of peripheral nerves, chemotherapy-induced neuropathies and in animal models of diabetic neuropathy (Zhang and De Koninck, 2006; Peters et al., 2007; Tsuda et al., 2008). Moreover, treatments with microglia inhibitors were shown to prevent and reverse the pain responses in animal models of neuropathic pain (Clark et al., 2007), reinforcing its role in pain processing. The events that lead to spinal microglia activation are not yet fully elucidated. It was demonstrated that microglia responds to the increased release of excitatory neurotransmitters (glutamate and substance P), neurotrophic factors (e.g. brain derived neurotrophic factor - BDNF), ATP and chemokines (e.g. CCL2 and fractalkine) that occur at the spinal cord of animal models of traumatic neuropathic pain (McMahon and Malcangio, 2009). Upon stimulation microglia was believed to activate the transcriptional activator nuclear factor kβ (NFkβ), which was shown to regulate the microglial production of pro-inflamatory cytokines (Rasley et al., 2002; McMahon and Malcangio, 2009). Furthermore, it was also observed increased phosphorilation of p38 mitogen activated protein kinase (MAPK), which was show to potentiate the synthesis and release of cytokines, to increase the transcription of NFkβ and to augment the expression of interleukynes 1 (IL-1) and 6 (IL-6) and of the cyclooxygenase 2 enzyme (COX 2). Consistent with the p38-mediated microglia effects on pain are the antiallodynic and antihyperalgesic effects of p38 MAPK inhibitors in animal models of neuropathic and inflammatory pain (Svensson et al., 2003; Ji and Suter, 2007; McMahon and Malcangio, 2009). Interistingly, some molecules that activate microglia were demonstrated to be also produced by microglia itself. In fact, ATP and BDNF were shown to activate microglial cells, but also to be released by them in neuropathic pain condition (Coull et al., 2003; 2005). Those molecules were shown to interfere with neuronal and astrocytes activities, inducing the release of several mediators that affect the microglia

responsiveness (McMahon and Malcangio, 2009). It is now consensual that exists a continuous crosstalk between migroglia and neurons, which may concur to perpetuate the pain condition. Nevertheless, it remains to be ascertained which are the cells and molecules that trigger that crosstalk. It is important to mention that microglia activation was also observed at supraspinal areas involved in pain control in animal models of traumatic neuropathic pain and that the response states of those cells seem to vary among the different pain-related brain regions. In fact, microglia was shown to be activated in the RVM and thalamus during neuropathic pain (Wei et al., 2008; Zhao et al., 2007), while no changes were observed at the PAG or anterior cingulate cortex (Zhang et al., 2008). The contribution of microglia activation at the RVM and thalamus to pain responses were demonstrated by the prevention or reversal of pain behaviors in animals receiving local infusions of p38 MAPK inhibitors (Wei et al., 2008; Zhao et al., 2007). The mechanisms that mediate the effects of supraspinal microglia activation on pain remain unknown.

5.2. Pathogenesis of painful diabetic neuropathy

Diabetes-induced hyperglycemia affects more dramatically the cells that present insulin-independent glucose uptake, such the endothelial and nerve cells. In these cells, the intracellular glucose concentrations rise in parallel with hyperglycemia, inducing a condition known as intracellular hyperglycemia. This phenomenon damages the cells and, consequently, the tissues using 6 major mechanisms: i) increased polyol pathway flux; ii) increased formation of advanced glycation end-products (AGEs); iii) increased expression and activation of AGEs receptors; iv) activation of protein kinase C (PKC); v) increased activity of hesoxamine pathways and vi) increased production of reactive oxygen species (ROS) by the mitochondria (Tesfaye, 2009; Obrosova 2009a; Giacco and Brownlee, 2010; Ziegler, 2010).

The increased polyol pathway flux damage the cell by sorbitol-induced osmotic stress, reduction in Na/K^+ - ATPase activity, increased $NADH/NAD^+$ and decreased

NADPH. The decreased in NADPH seems to impair the antioxidant endogenous defense of the cell by diminishing the regeneration of reduced glutathione (Obrosova, 2009a; Giacco and Brownlee, 2010). AGEs are formed by non-enzymatic reactions of glucose and AGE precursors (e.g. methylglyoxal and glyoxal) with cytosolic and extracellular proteins and lipids, altering their normal function. Furthermore, extracellular AGEs bind to their specific receptors (RAGE) presented in several cells and tissues, which induces ROS overproduction and activates the transcription of NFκB. NFκB transcription induces pathologic changes in gene expression, favouring pro-inflammatory conditions (Toth et al., 2007; Obrosova, 2009a; Giacco and Brownlee, 2010; Ziegler, 2010). Increased intracellular diacylglicerol, a result of increased glucose flux through the glycolytic pathway, along with the effects of RAGE-AGEs interaction enhances PKC activity (Giacco and Brownlee, 2010), which was shown to potentiate the abovementioned impairments (Obrosova, 2009a; Ziegler, 2010). Activation of the hexosamine pathway by hyperglycemia seems also to cause several changes in gene expression (e.g. increases in tumor growth factor α and β 1) and in protein function (Giacco and Brownlee, 2010).

All the above mentioned metabolic effects seem to be triggered by a single common process: the mitochondrial overproduction of ROS (**Figure 2**).

It is important to mention that n-6 essential fatty acids and prostaglandin metabolism is also impaired during diabetes, which was shown to alter nerve membrane structure and induce microvascular abnormalities (Obrosova, 2009a; Ziegler, 2010). Several other metabolic mechanisms were also shown to affect the peripheral nerves during diabetes, such as oxidative stress-independent overactivation of poly(ADP-ribose) polymerase (PARP), mitochondrial energy failure, activation of MAPKs, increased expression of COX-2 and 12/15-lipoxygenase, downregulation of superoxide dismutase activity, altered homeostasis and signaling of calcium (reviewed by Obrosova, 2009a).

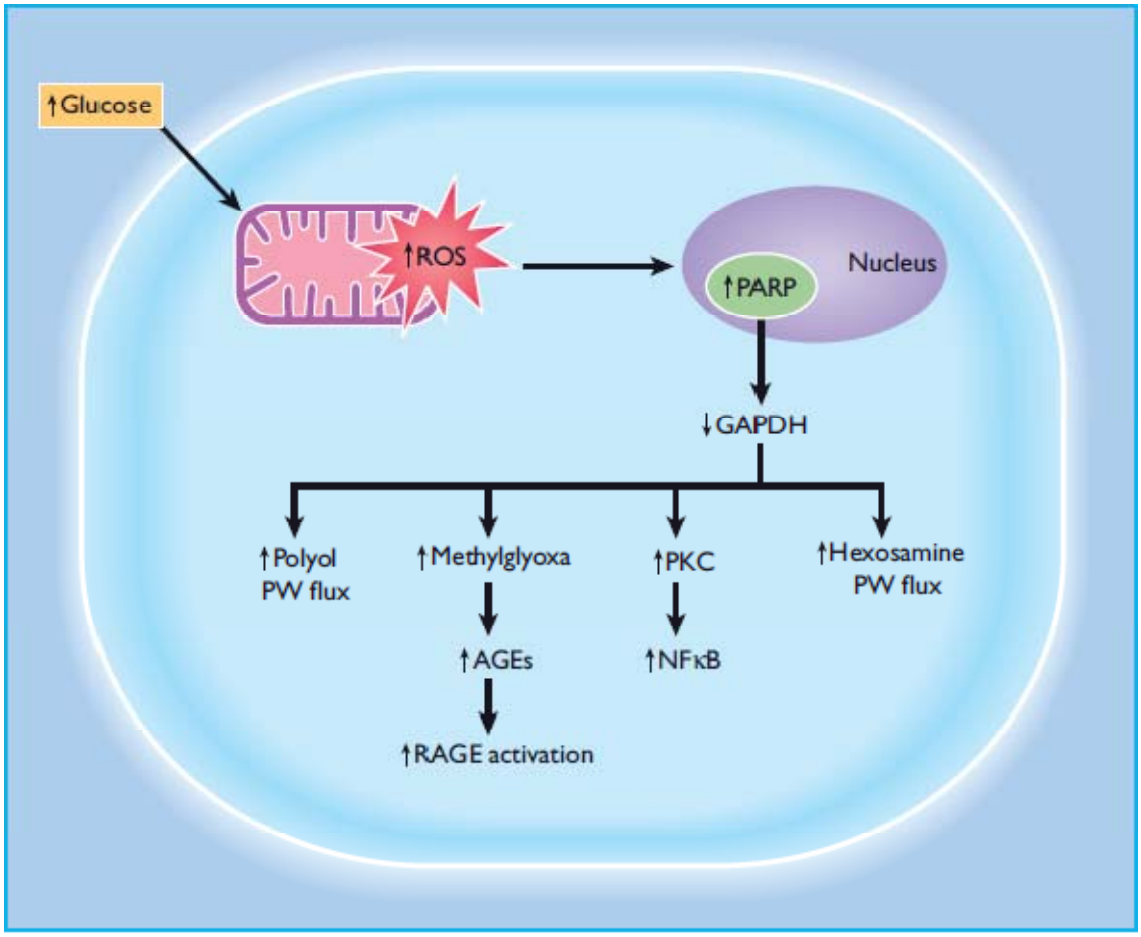

Figure 2 – Metabolic disarrangements induced by hyperglycemia. The unifying theory-overproduction of ROS by mitochondria triggers increased polyol pathway flux, AGEs formation, PKC activity and hexosamine pathway flux. From Giacco and Brownlee, (2010).

Importantly, all these metabolic and molecular abnormalities seem to be interrelated and were shown to affect the vascular and the neuronal elements of the peripheral nervous systems during diabetes.

The occurrence of these impairments at the CNS was less studied, with few reports showing spinal cord and brain affectation. Indeed, oxidative stress and cell death were shown to occur at the CNS of diabetic rats (Li et al., 2002), as well as increased PARP, PKC and p38 MAPK activities (Ramakrishnan et al., 2005; Honda et al., 2007; Lupachyck et al., 2011). Furthermore, AGEs-RAGE signaling seems also to be potentiated in CNS and contribute to its impairment (Toth et al., 2007).

It is important to mention that hyperglycemia was not considered the only culprit of diabetic neuropathy. Insulin, insulin-like growth factor I (IGF1) and C-peptide deficits were shown to elicit glycemia-independent neuroprotective effects (Ishii, 1995; Jianbo et al., 2002; Mohamed-Ali and Pinkney, 2002; Pierson et al., 2003; Brussee et al., 2004; Sima et al., 2008; Kamiya et al., 2009) counteracting the consequences of hyperglycemia. This suggests that these factors also exert an important role in the etiology of diabetic neuropathy. The plasma levels of IGF1 are significantly reduced in patients with diabetic neuropathy (Migdalis et al., 1995; Guo et al., 1999; Busiguina et al., 2000; Jianbo et al., 2002). In nerve tissues of diabetic rats the levels of IGF1 are also reduced (Pierson et al., 2003; Sima, 2003). The lower expression and activity of IGF1 in type 1 animal models seem to contribute to the higher severity of neuropathic abnormalities in these animals when compared with animals with type 2 diabetes (Ishii and Lupien, 1995; Zhuang et al., 1996; Schmidt et al., 1999; Pierson et al., 2003; Sima, 2006). Furthermore, IGF1 administrations, which do not alter the blood glucose concentrations, were shown to improve sensory deficits, nerve regeneration and autonomic neuropathy in diabetic rats (Ishii and Lupien, 1995; Zhuang et al., 1996; Schmidt et al., 1999; Russel and Feldman, 1999; Toth et al., 2006). IGF1 was also suggested to be involved in the neurodegenerative processes detected at the hippocampus of diabetic rats (Li et al., 2002; 2005). The abovementioned results suggest that the IGF1 deprivation may concur to diabetic neuropathy. The effects of IGF1 deficits on the spinal cord and brainstem areas involved in pain control were never evaluated. Taking into account that those regions present IGF1 receptors (Folli et al., 1994), IGF1-mediated effects are likely to occur, with possible repercussions on pain modulation mechanisms.

5.3. Structural, functional and neurochemical abnormalities at peripheral nervous system

Diabetes affects unmyelinated (C) and thinly myelinated (Aδ) fibers as well as large myelinated fibers (A β) (Obrosova, 2009, 2009a; Ziegler, 2010). The pattern of affectation is length-related, with the longer fibers of the extremities being damaged first ("glove and stocking" affectation) and develops as a "dying-back" neuropathic process (Ziegler, 2010). Morphologically, the affected nerves show Wallerian degeneration accompanied by axonal atrophy, axonal loss and defective nerve regeneration (Sima, 2003). Loss of intraepidermal nerve fibers is common and is negatively correlated with pain manifestations (Ziegler, 2010; Loseth et al., 2010). Reduced peripheral nerve conduction velocities, affecting both motor and sensory nerves, were observed both in diabetic patients (Nakamura et al., 1992; Tesfaye, 2009, Obrosova, 2009; 2009a; Ziegler, 2010) and in animal models of diabetic neuropathy (Carsten et al., 1989; Calcutt, 2004; 2004a; Loseth et al., 2010). In addition, several molecular abnormalities were found in the peripheral nerves of animal models of diabetes. Increased expression and activity of sodium and calcium channels were observed in the peripheral nerves of diabetic rats (Hirade et al., 1999; Craner et al., 2002; Hong et al., 2004; Umeda el al., 2010), which may be underlying the ectopic activity and hyperexcitability of C and Aδ fibers observed during diabetes (Burchiel et al., 1985; Alhgren et al., 1994; Khan et al., 2002; Chen and Levine, 2001; 2003). Neurotransmitter systems are also affected, with decreased expression of substance P, somatostatin, CGRP and vasoactive intestinal peptide detected at the peripheral nerves of diabetic rats, along with increased levels of neuropeptide Y, dynorphin and glutamate (Noda et al., 1990; Di Giulio et al., 1992; Diemel et al., 1992; 1994; Rittenhouse et al., 1996; Jolivalt et al., 2006). The expression and binding affinity of α1 adrenoreceptors were shown to be augmented in the dorsal root ganglia (DRGs) of diabetic rats (Lee et al., 2000; Teasell and Arnold, 2004). Similar increases were

detected in the expression of κ-opioid receptors at peripheral nerves (Jolivalt et al., 2006). Meanwhile, the expression of transient receptor vanniloid type 1 is decreased in skin nerve fibers (Facer et al., 2007). The contribution of these changes to PDN and the mechanisms of action remain unclear. Furthermore, a loss of neurotrophism was observed during PDN, with peripheral depletion of neurotrophic factors as the nerve growth factor, neurotrophin-3 and IGF1 (Calcutt, 2002; Sima, 2003; Calcutt and Backonja, 2007; Ziegler, 2010), which may explain the impaired capacity of nerve regeneration and the degeneration of peripheral nerves during diabetes (Ishii and Lupien, 1995; Calcutt, 2002; Sima, 2003; Calcutt and Backonja, 2007). Neurotrophism impairment seems also to contribute to the altered neurotransmitters expression (Sima, 2003). As a consequence of glycation and hyperphosphorilation of proteins from the cytoskeleton, axonal transport at peripheral nerves was shown to be reduced (Fink et al., 1987; Macione et a., 1989; Fernyhoug et al., 1999), which point for a less efficient communication and molecular transport (as neurotransmitters, receptors and neurotrophic factors) between axonal terminals and neuronal soma and, consequently, to inadequate responses to peripheral stimuli (Laduron and Janssen, 1986).

The large majority of functional and neurochemical changes summarized above were shown to be prevented or reverted by normoglycemia and by approaches that inhibit the metabolic effects of hyperglycemia (Sima, 2003; Calcutt and Backonja, 2007; Obrosova, 2009a; Ziegler, 2010). Recently, more attention has been directed to the glycemia-independent role of insulin, C-peptide and IGF1 (Ishii, 1995; Sima, 2003). Treatments with those agents were shown to reverse, prevent or ameliorate the peripheral nerve functional abnormalities as well as PDN symptoms (Ishii and Lupien, 1995; Ishii, 1995; Zhuang et al., 1996; Schmidt et al., 1999; Russel and Feldman, 1999; Mohamed-Ali and Pinkney, 2002; Jianbo et al., 2002; Toth et al., 2003; Pierson et al., 2003; Brussee et al., 2004; Sima et al., 2008; Kamiya et al., 2009).

The changes affecting the peripheral nerves during diabetes are due to direct effects on nerve components (neurons and Schwann cells), but are also potentiated by

the impaired blood flow observed at the *vasa nervorum*, which results from endothelial dysfunction (Obrosova 2009a; Ziegler, 2010). Vascular dysfunction of *vasa nervorum* was shown to induce hypoxia in peripheral nerves, contributing substantially to increase the oxidative stress burden (Tesfaye and Kempler, 2005; Calcutt and Backonja, 2007; Obrosova, 2009a; Tesfaye, 2009; Ziegler, 2010).

5.4. Impairments at central pain control system

Besides the effects on peripheral nervous system, PDN was also shown to be associated with impairments at central areas involved in pain transmission, namely the spinal cord (Eaton et al., 2001; Pertovaara et al., 2001; Chen and Pan, 2002; Selvarajah et al., 2006; Malmberg et al., 2006) and the thalamus (Selvarajah et al., 2008; Cauda et al., 2009; Fischer et al., 2009, Fischer and Waxman, 2010; Li et al., 2010; Selvarajah et al., 2011).

The spinal cord was shown to be affected during PDN in animal models of diabetes as well as in humans, showing structural and functional impairments (Carsten et al., 1989; Kamei et al., 1990; 1991; Field et al., 1998; Bitar et al., 1999; 2000; Li et al., 1999; Suzuki et al., 2000; Gallego et al., 2003; Chen and Pan, 2003; Malmberg et al., 2006, Tomiyama et al., 2006; Mizisin et al., 2007; Wang et al., 2007; 2010). Neurochemical changes at the CNS have been studied in animal models of diabetes. As in the periphery, spinal cord also presents increased expression or/and activity of voltage-dependent calcium and sodium channels (Voytenko et al., 2000; Luo et al., 2002; Craner et al., 2002), which probably concur for the spontaneous hyperactivity and hyperexcitability of spinal nociceptive neurons (Pertovaara et al., 2001; Chen and Pan, 2002) and support the analgesic effects of anticonvulsants during PDN (Marais et al., 2001; Jensen, 2002; Moore et al., 2002). Furthermore, changes in spinal excitatory mechanism were demonstrated (Kamei et al., 1990; Field et al., 1998; Li et al, 1999; Tomiyama et al., 2006). Despite the decreased spinal glutamate content and release (Malmberg et al., 2006), increased spinal glutamatergic neurotransmission was shown

to occur, probably mediated by increased expression and activation of glutamate receptors (Li et al, 1999; Tomiyama et al., 2006; Li et al., 2010; Daulhac et al., 2011). Moreover, pronociceptive actions of substance P are also augmented by increased expression of neurokinin 1 receptors (Kamei et al., 1990; 1991; Field et al., 1998). On the other hand, spinal inhibitory mechanisms seem to be reduced namely due to decreased expression and/or activity of $GABA_B$ (Wang et al., 2007; 2011) and *μ*-opoid receptors (Chen et al., 2002), along with impaired spinal release of endogenous opioids (Williams et al., 2008).

More recently, spinal glia was shown to play a role in PDN. Increased activation of spinal microglia was observed in diabetic rats during PDN (Daulhac et al., 2006; Tsuda et al., 2008; Wodarski et al., 2009) and was shown to increase the production of pro-inflammatory mediators (Talbot et al., 2010) and to contribute to pain (Daulhac et al., 2006; Tsuda et al., 2008; Daulhac et al., 2011). The effects of spinal microglia activation during PDN were not yet fully elucidated and its mechanisms of action are almost unknown. Considering that the effects and mediators of microglia activation seem to depend on pain condition (McMahon and Malcangio, 2009), the study of microglia contribution to the neuropathic pain condition associated to diabetic neuropathy should be considered.

More recently, studies using magnetic ressonance spectroscopy in humans and electrophysiological recordings in animals detected thalamic functional impairments (Selvarajah et al., 2008; Fischer et al., 2009) during PDN, demonstrating the involvement of supraspinal pain-related areas. As reviewed above (item **5.1. "The pain mechanisms"**), pain control involves several other brain areas, namely brainstem regions, which remain to be addressed during PDN.

Despite the high availability of drugs, the treatment of PDN remains a challenge for the clinicians, mainly due to the low efficacy of those agents and constraints associated with their contra-indications and side-effects. In the future, it will be

important to develop pharmacological agents based on the specific pathogenetic mechanisms occurring at the pain control system during PDN.

6. Objectives and study outline

Considering the poor knowlege of the central effects of diabetes and their contribution to PDN, it is necessary to perform a detailed study of the central pain control system during diabetes, in order to develop mechanistic-related pharmacological approaches to manage this distressing condition. In the present work, we used STZ rats to study the functional and neurochemical changes occurring at the spinal dorsal horn and at brainstem areas devoted to descending pain modulation.

This dissertation includes 6 publications, which are presented according the rationale of the overall study.

In the first study, we evaluated the spontaneous, noxious and innocuous-induced activity of the spinal cord neurons in STZ rats with behavioural signs of PDN. We used the expression of Fos protein, which is usually synthesized by spinal dorsal horn neurons upon peripheral nociceptive stimulation (Coggeshal, 2005), to assess the activation pattern of spinal nociceptive neurons.

In the second study, we aimed to evaluate the role of GABA-mediated pain modulation at spinal dorsal horn. We quantified the expression of GABA and its synthesizing enzyme (glutamic acid decarboxilase, GAD) at the spinal dorsal horn of STZ rats with PDN. We also assessed the expression of the potassium-chloride co-transporter 2 (KCC2) in order to infer about its contribution for GABA role in this chronic pain condition.

Based on the findings from the previous studies, we developed additional experiments aiming the study of possible mechanisms implicated in KCC2 impairment during PDN. We focused our study in the role of microglia activation (publication 3) and oxidative stress (publication 4). We evaluated the activation of spinal microglia and the levels of oxidative stress in the spinal dorsal horn of STZ rats and the effects of their reversal on KCC2 expression, neuronal activity and nociception.

The study of descending pain modulatory circuits during PDN was a core aim of the present work. The publication 5 included a time-course evaluation of spontaneous neuronal activity at the PAG and the effects of gabapentin on the activity of neurons from spinal dorsal horn and PAG. We further evaluated the changes occurring in the serotoninergic and noradrenergic pathways acting as relay stations of PAG descending modulation (publication 6) and quantified the serotonin and noradrenaline content at spinal cord. Then, we focused our study in the most important brainstem areas that receive inputs from PAG and modulate spinal neurotransmission by sending direct descending projections to spinal dorsal horn, as the rostroventromedial medulla (RVM) and the A_5 and A_7 noradrenergic cell groups, taking into account the features of the noradrenergic innervation of the spinal cord in the rat strain used in the present study (Tavares et al, 1997). In the RVM, we assessed the neuronal expression of tryptophan-hydroxylase (TpH), the rate limiting enzyme in serotonin synthesis, in order to identify the serotoninergic neurons. We also evaluated this activity by combining TpH expression with the expression of extracellular signal-regulated kinases (ERKs) phosphorilated form. The noradrenergic neurons of the A_5 and A_7 cell groups were identified by immunodectection of tyrosine hydroxylase (TH), an enzyme involved in noradrenaline synthesis. The activity of those neurons was quantified by double immunofluorescence for TH and the phosphorilated form of cAMP element-binding protein (CREB), which is suitable for the evaluation of neuronal activity.

Another aim of the present study was to evaluate other contributing factors for PDN, rather than the well-known hyperglycemia. Therefore, in the sixth study we evaluated the glycemia-independent role of IGF1 in the functional and neurochemical impairments detected in the previous studies.

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RESEARCH ARTICLE

C-*fos* expression at the spinal dorsal horn of streptozotocin-induced diabetic rats

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Abstract

Background Pain during diabetic neuropathy is associated with peripheral nerve damage but recent evidences suggest the occurrence of central effects. We used the activation of the c-*fos* protooncogene to study the activity of spinal dorsal horn neurons in streptozotocin (STZ)-induced diabetic rats in the absence of stimulation or in response to innocuous or noxious stimuli.

Methods Four weeks after saline or STZ (50 mg/kg) injection, rats were anaesthetized and either not further manipulated or submitted to innocuous (gentle touch), noxious mechanical (pinching) or noxious thermal (radiant heat) stimulation of the hindlimb skin. In each experimental situation, the numbers of Fos-immunoreactive (Fos-IR) neurons occurring in the superficial (laminae I–II) or deep (laminae III–V) dorsal horn were compared.

Results In the absence of stimulation, STZ-injected rats presented significantly higher numbers of Fos-IR neurons than controls, both in the superficial and deep dorsal horn (DDH). In comparison with the respective baseline levels, innocuous stimulation did not induce a significant increase in the numbers of Fos-IR neurons in controls or STZ-rats. Noxious mechanical and noxious thermal stimuli increased the numbers of Fos-IR neurons, both in control and STZ-rats, but in a more pronounced manner when diabetic rats were subjected to noxious mechanical stimulation.

Conclusions The present study demonstrates that the responses of spinal cord neurons are strongly affected during diabetes. The higher baseline neuronal activity probably underlies the spontaneous pain detected during diabetes since the spinal dorsal horn is the major relay station in the ascending transmission of nociceptive input to the brain. Copyright © 2007 John Wiley & Sons, Ltd.

Keywords diabetes; pain; spinal impairment; mechanical hyperalgesia; thermal responses; spontaneous pain

Introduction

Pain is an important complication of diabetes and can occur spontaneously or results from innocuous (allodynia) or noxious (hyperalgesia) stimuli [1–4]. The study of the mechanisms of painful diabetic neuropathy has been mainly focused at the peripheral nervous system, whereas the central effects of the disease need to be more deeply addressed [1,5]. Several studies suggested that the spinal dorsal horn, the first relay station of the nociceptive pathway, is affected by diabetes. Diabetic patients with distal symmetrical neuropathy present decreases in the spinal cord area [6]. Electrophysiological recordings

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in diabetic rats demonstrated higher activity of spinal dorsal horn neurons both spontaneously or in response to innocuous or noxious stimulation [7,8]. Neurochemical changes were also reported at the spinal dorsal horn in animal models of diabetes, affecting the SP/NK1 system and the expression of NMDA receptors [9–12].

C-*fos* expression has been used as an anatomical marker for neuronal activation by nociceptive-related stimuli at the spinal dorsal horn level [13,14]. Since these initial demonstrations of c-*fos* expression in the nucleus of neurons at the spinal dorsal horn, multiple studies have consistently demonstrated that most of the stimuli used to induce c-*fos* expression at the dorsal horn are noxious [15]. Innocuous stimulation can occasionally result in dorsal horn c-*fos* expression mainly in chronic pain conditions and due to central sensitization [16]. The study of c-*fos* expression allows the evaluation of the nociceptive activation of large numbers of spinal neurons and the determination of the pattern of neuronal activation, according to the stimulus nature and the laminae of location of c-*fos*-expressing neurons [15]. Studies with neuropathic pain models due to traumatic nerve injury reported a significant increase in the numbers of Fos-immunoreactive (Fos-IR) neurons in the spinal dorsal horn even in the absence of additional stimulation [17–19] and following the application of innocuous stimuli [20–25]. This demonstrates that the installation of chronic neuropathic pain is associated with plastic changes at the spinal dorsal horn, which need to be studied during diabetes, since this is associated with an important non-traumatic neuropathic condition. C-*fos* expression has been used to evaluate neuronal activity in the brain of streptozotocin (STZ)-induced diabetic animals. These studies demonstrated that c-*fos* expression increases in some brain regions [26,27] and decreases in others [27–29], showing that c-*fos* expression is not a direct result of STZ or a general reaction to the stress of malnutrition and dehydration during STZ-induced diabetes, but rather to the diabetic condition *per se*.

During diabetes, the distinct pain modalities are differentially affected. The thresholds to mechanical stimulation are considerably decreased both in patients and animal models [30–32]. Reports of thermal responses in diabetic animals are quite variable and include hypoalgesia [7,31–33], unaltered responses [34], and hyperalgesia [35, 36]. One possibility to explain this variability is the lack of correction of thermal responses to baseline skin temperatures in diabetic rats. In fact, changes in skin temperatures were shown to affect the behavioural responses to thermal stimuli and hypothermia due to blood flow impairment, which is a feature of diabetes [37–41].

In the present study, we evaluated the responses of spinal dorsal horn neurons of STZ-injected animals in baseline conditions and after innocuous, noxious mechanical or noxious thermal stimuli. In order to characterize the behavioural responses of diabetic animals according to the stimulus modality and to confirm the presence of behavioural signs of neuropathy, we also studied the mechanical and thermal responses using respectively the Randall–Selitto and the tail-flick behavioural tests. Furthermore, we evaluated the effects of the decreased basal skin temperature of diabetic rats in behavioural thermal evaluations by correcting these responses to the baseline skin temperatures.

Materials and methods

Male Wistar rats (Charles River; Barcelona, Spain), weighing 250–350 g at the beginning of the experiments, were used. Animals were housed two per cage, in a room with a constant temperature ($22 \pm 2\,^{\circ}C$) and humidity ($55 \pm 5\%$) and with a 12-h light/dark cycle, and received food and water *ad libitum*. Experiments were performed in accordance with the ethical guidelines for the study of pain in conscious animals [42] and the European Community Council Directive 86/609/EEC.

Induction of diabetes

Diabetes was induced as described previously [43]. Briefly, the animals received an intraperitoneal (i.p.) injection of STZ (50 mg/kg body weight; Sigma-aldrich, St Louis, USA) diluted in 0.9% saline, and were further provided with 10% sucrose in the drinking solution to prevent hypoglycaemia. Control rats were injected with equal volume of saline. Three days after the injection, glucose concentration was measured in blood samples collected from the tail vein using Accu Chek Sensor Comfort (Roche Diagnostics, Germany). Only rats with glucose concentration higher than 300 mg/dL were considered diabetic and were included in the STZ-injected group.

Behavioural assessment of nociception

A total of 12 animals (6 controls and 6 diabetic patients) were subjected to a time-course behavioural evaluation of mechanical and thermal nociception, at the day prior to STZ or saline injection and at 1, 2, 3, and 4 weeks post-injection. During the 8 days prior to the onset of behavioural evaluation, the animals were daily handled by the experimenter for habituation purposes.

Mechanical nociception was assessed using the Randall–Selitto test (Ugo Basile; Comerio VA, Italy). Ascending mechanical forces were applied to the right hindpaw and the mechanical force (in grams) that induces hindpaw withdrawal was recorded. The mechanical threshold of each animal was the average of three consecutive measurements, taken at 5-min intervals. Thermal nociception was assessed by recording the tail-flick latency using a tail-flick test apparatus with temperature set for $52\,^{\circ}C$ (Ugo Basile; Comerio VA, Italy). The response latency for

each animal was the average of three consecutive measurements, taken at 5-min intervals. The cut-off latency in the tail-flick test was settled to 24 s in order to avoid tissue damage. On the basis of previous observations that the skin temperature affects the outcome of tail-flick studies [37–41], we recorded the tail skin temperature of each animal just before thermal behavioural evaluation using a thermoprobe attached to the tail (Sensortek, USA; Greisinger Electronic, Germany). Tail-flick response latency data were adjusted for the tail temperatures, as described previously [37].

The results of the behavioural experiments were statistically analysed using one-way analysis of variance (ANOVA), followed by Tukey *post-hoc* test for multiple comparisons. Unpaired sample *t*-test was used to compare behavioural data between saline and STZ-injected rats.

C-*fos* evaluation

One day after the final behavioural assessment (4 weeks after STZ or saline injection), the animals were anaesthetized with an i.p. injection of 35% chloral hydrate (1mL/kg body weight). Five saline and five STZ-injected rats were not subjected to further manipulation and were used to establish the baseline Fos levels. The remaining rats ($n = 5$ per group and per stimulus) were submitted to innocuous, noxious mechanical or noxious thermal stimuli of the hindlimb skin at the internal aspect of the left thigh, according to previously published protocols [44–46]. Briefly, innocuous mechanical stimulation consisted of gentle touch applied manually with the experimenter's thumb to the hindlimb skin for 15 s, as described previously [20]. Noxious mechanical stimulation consisted of pinching the left hindlimb skin for 1–3 s with a rat-tooth forceps. Noxious thermal stimulation was performed using a radiant heat source (HLTC-200T, npi electronic GmbH) in pulses of 30 s, at 65 ± 2 °C [44–46]. The temperature was controlled by the thermoprobe, described in the preceding text, placed at the skin surface. All the stimuli were applied every 2 min during 2 h. Reinforcement of the anaesthesia during the stimulation period was done by an additional injection of 35% chloral hydrate (0.5 mL/kg body weight).

At the end of the stimulation period, the animals were transcardially perfused with 200 mL of phosphate-buffered saline (PBS), followed by 1000 mL of 4% paraformaldehyde in 0.1 M phosphate buffer (PB), pH 7.4. Spinal cord segments T13-L3 were removed, post-fixed for 4 h in the same fixative, and cryoprotected overnight in 30% sucrose in PB. Coronal sections, 40 μm thick, were obtained using a freezing microtome, and every fourth section was collected in 0.1 M PBS. Fos protein was immunohistochemically detected using the avidin–biotin–peroxidase method, as described previously [46]. Briefly, the sections were treated with 1% hydrogen peroxidase for 10 min to inhibit endogenous peroxidase activity followed by incubation for 2 h in a blocking solution of 10% normal swine serum in 0.3% Triton X 25% in PBS (PBST) with 0.1 M glycine. The sections were then incubated overnight at 4 °C in the rabbit anti-Fos antiserum (Ab5; Oncogene), diluted at 1 : 10 000 in PBST. After being washed in PBST, the sections were incubated in biotinylated swine anti-rabbit immunoglobulin (DakoCytomation, Denmark), diluted at 1 : 200, for 1 h, at room temperature. They were then washed in PBST, incubated in the avidin–biotin complex (Vectastain, Vector Laboratories, USA) for 1 h, and stained with diaminobenzidine (DAB; 10 mg DAB in 20 mL of tris-HCL 0.05 M, pH 7.6 solution with 5 μL of 30% hydrogen peroxide). Finally, the sections were washed in PBS and mounted on gelatine-coated slides, air dried and coverslipped.

In animals not subjected to further stimulation, the baseline Fos levels were determined by counting the numbers of Fos-IR neurons occurring bilaterally in the dorsal horn of 40 sections randomly taken from segments T_{13}–L_3 (10 sections from each segment), with the aid of a camera lucida. Fos-IR neurons were positioned in two dorsal horn regions: the superficial dorsal horn (SDH), encompassing laminae I–II and the deep dorsal horn (DDH), including laminae III–V [47]. According to several previous studies, Fos-IR neurons were counted when the immunoreaction for the Fos protein was expressed at the nucleus, independently of the staining intensity [15,44–46]. Independent sample *t*-test was used to compare the mean numbers of baseline Fos-IR neurons between non-stimulated saline and STZ-injected rats. In stimulated animals, the same analysis was performed but counting was performed in the ipsilateral dorsal horn using 20 sections from L_1–L_2 segments (10 sections from each segment). Counting was limited to these segments since previous studies using the same stimulation protocol [44,45] showed that they exhibit the higher Fos expression. Mean numbers of Fos-IR neurons expressed after innocuous and noxious stimulation in control and STZ-injected groups were compared by one-way analysis of variance (ANOVA), followed by

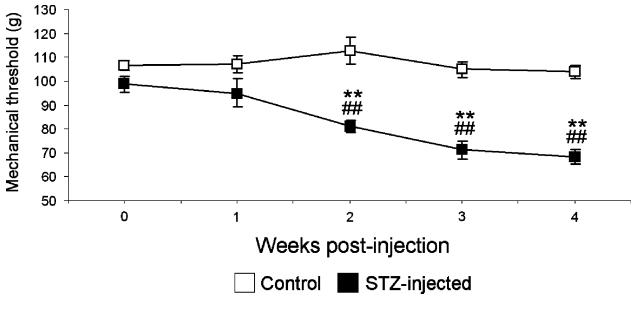

Figure 1. Time-course changes of mechanical response thresholds in control and diabetic rats. Diabetic animals exhibit mechanical hyperalgesia from 2 weeks after STZ injection until the end of the experiments. Significance of symbols: * – comparisons of STZ-injected animals with controls; # – comparisons with initial threshold values. One symbol: $p < 0.05$; two symbols: $p < 0.01$

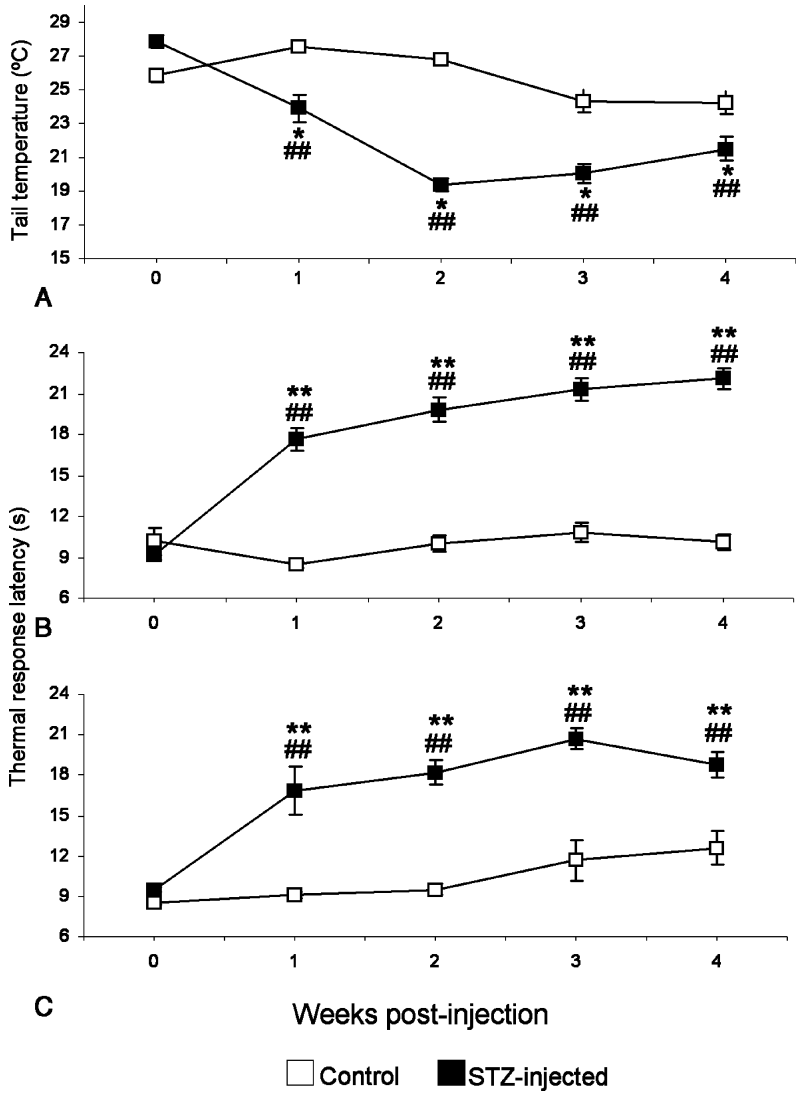

Figure 2. Time-course changes of thermal response thresholds in control and diabetic rats (tail-flick test temperature 52 °C). Panel A depicts changes in tail temperatures during the 4 weeks of the experiments. Panels B and C show thermal response latencies before (B) and after (C) adjustment to the baseline tail temperature. As soon as after 1 week after STZ injection, diabetic animals are slightly hypothermic (A) but this did not affect the thermal hypoalgesia exhibited by these animals (B–C). Significance of symbols: * – comparisons of STZ-injected animals with controls; # – comparisons with initial threshold values. One symbol: $p < 0.05$; two symbols: $p < 0.01$

Tukey *post-hoc* test for multiple comparisons. Results are expressed as mean ± standard error of the mean (SEM).

Results

Three days after STZ injection, the animals developed hyperglycaemia (blood glucose concentration >300 mg/dL) and remained hyperglycaemic during the course of the study. At 4 weeks post-injection, STZ-injected rats presented blood glucose concentrations higher than saline-injected rats (STZ-injected group: 484.1 ± 32.21 mg/dL; saline group: 136.9 ± 18.71 mg/dL) and body weights lower than their age-matched controls (STZ-injected group: 285 ± 13.4 g; saline group: 420 ± 5.6 g), which agrees with previous studies using this animal model [31,35,36,43]. STZ-injected animals presented the typical symptoms of diabetes, as previously described in reports of the model [31,35,36,43], but otherwise appeared normal.

Behavioural results

Before saline or STZ injections, no significant differences in baseline mechanical or thermal nociceptive responses were detected (Figures 1, 2).

Mechanical responses

The mechanical threshold of STZ-injected animals gradually decreased during the 4 weeks of study (Figure 1). Significant decreases in mechanical thresholds

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were detected from 2 weeks after STZ injection, with marked hyperalgesic responses at 4 weeks (about 31% change over initial values).

Thermal responses

The baseline tail temperatures of STZ-injected animals significantly decreased as soon as 1 week after STZ injection (Figure 2(a)) and remained lower during the 4 weeks of study. Contrarily, the thermal response thresholds in the tail-flick test of STZ-injected animals increased significantly 1 week after STZ injection and remained higher during the 4 weeks of the study. This was verified both before (Figure 2(b)) and after (Figure 2(c)) correcting the thermal response latencies to the tail temperature.

Fos study

Baseline Fos expression

In non-stimulated control rats, only a few Fos-IR neurons were detected in the spinal dorsal horn. Compared to the controls, non-stimulated STZ-injected rats presented more Fos-IR neurons, located bilaterally (Figure 3(b)). In STZ-injected rats, the numbers of Fos-IR neurons in the dorsal horn of spinal segments T13–L3 were higher than the respective values of the control group, mainly in the SDH (Figure 3(c)), but also in the DDH (Figure 3(d)).

Innocuous and noxious-evoked Fos expression

Innocuous mechanical stimulation

Innocuous mechanical stimulation did not induce a significant increase in the numbers of Fos-IR neurons in the dorsal horn of control and STZ-injected animals (Figures 4(a), (b), 5(a) and (b)). Although STZ-injected rats presented significantly higher numbers of Fos-IR neurons in the SDH than controls after innocuous stimulation, this was due to the higher baseline Fos expression observed in the former experimental group and not due to the effects of innocuous stimulation, since the numbers of Fos-IR neurons after innocuous stimulation were similar to those obtained in non-stimulated STZ-injected rats (Figure 5(a)).

Noxious mechanical stimulation

The numbers of Fos-IR neurons in the SDH and DDH of control and STZ-injected animals subjected to noxious mechanical stimulation (Figure 4(c), (d)) were higher than the values obtained in the respective experimental groups of non-stimulated animals (Figure 5(c), (d)). The increase in the numbers of Fos-IR neurons was more pronounced in the SDH of STZ-injected animals, and these animals presented significantly higher numbers of Fos-IR neurons at the SDH than controls.

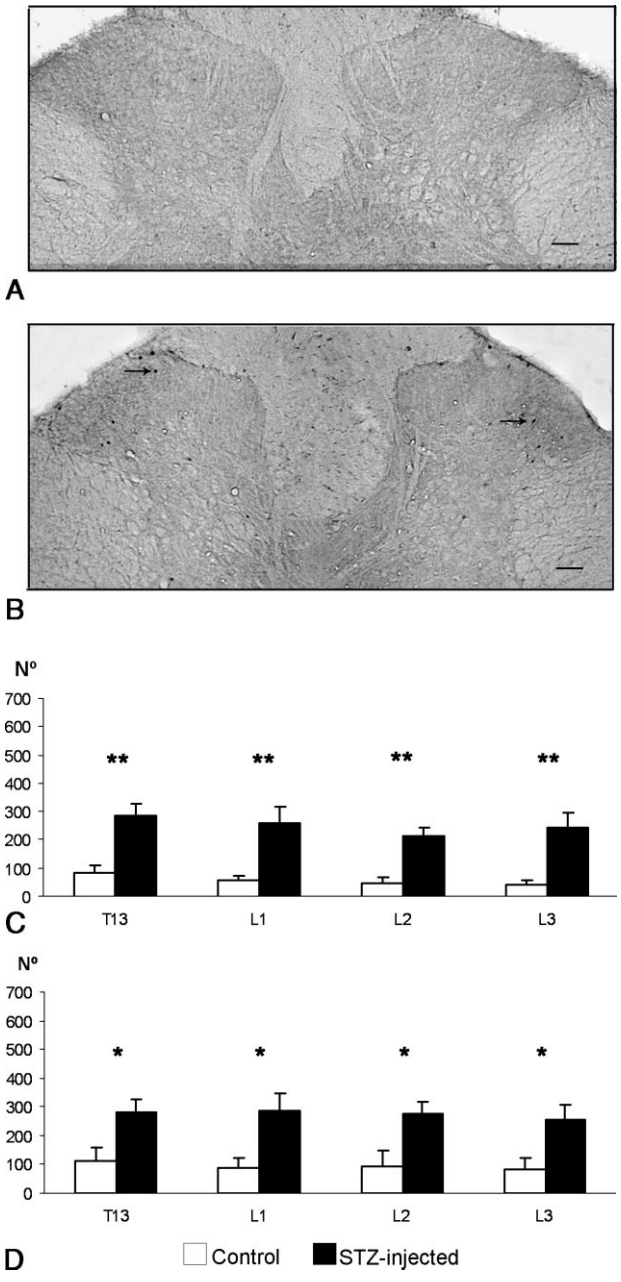

Figure 3. Baseline Fos expression at 4 weeks post-injection. The photomicrographs were taken at the L2 spinal segment of a control (A) and an STZ-injected animal (B). Panels C–D show the mean numbers of Fos-IR neurons (±SEM) counted bilaterally in laminae I–II (C) or III–V (D). Even in the absence of stimulation, diabetic animals presented significantly higher levels of Fos (arrows) than controls in all the analysed spinal segments. * – $p < 0.05$; ** – $p < 0.01$. Scale bar in: 100 μm

Noxious thermal stimulation

The numbers of Fos-IR neurons in the SDH of control and STZ-injected animals subjected to noxious thermal stimulation (Figure 4(e), (f)) were significantly higher than the values obtained in the respective experimental groups not subjected to stimulation (Figure 5(e), (f)), which did not occur at the DDH (Figure 5(f)). No differences were detected in thermal noxious–evoked Fos expression between control and STZ-injected rats (Figure 5(e), (f)).

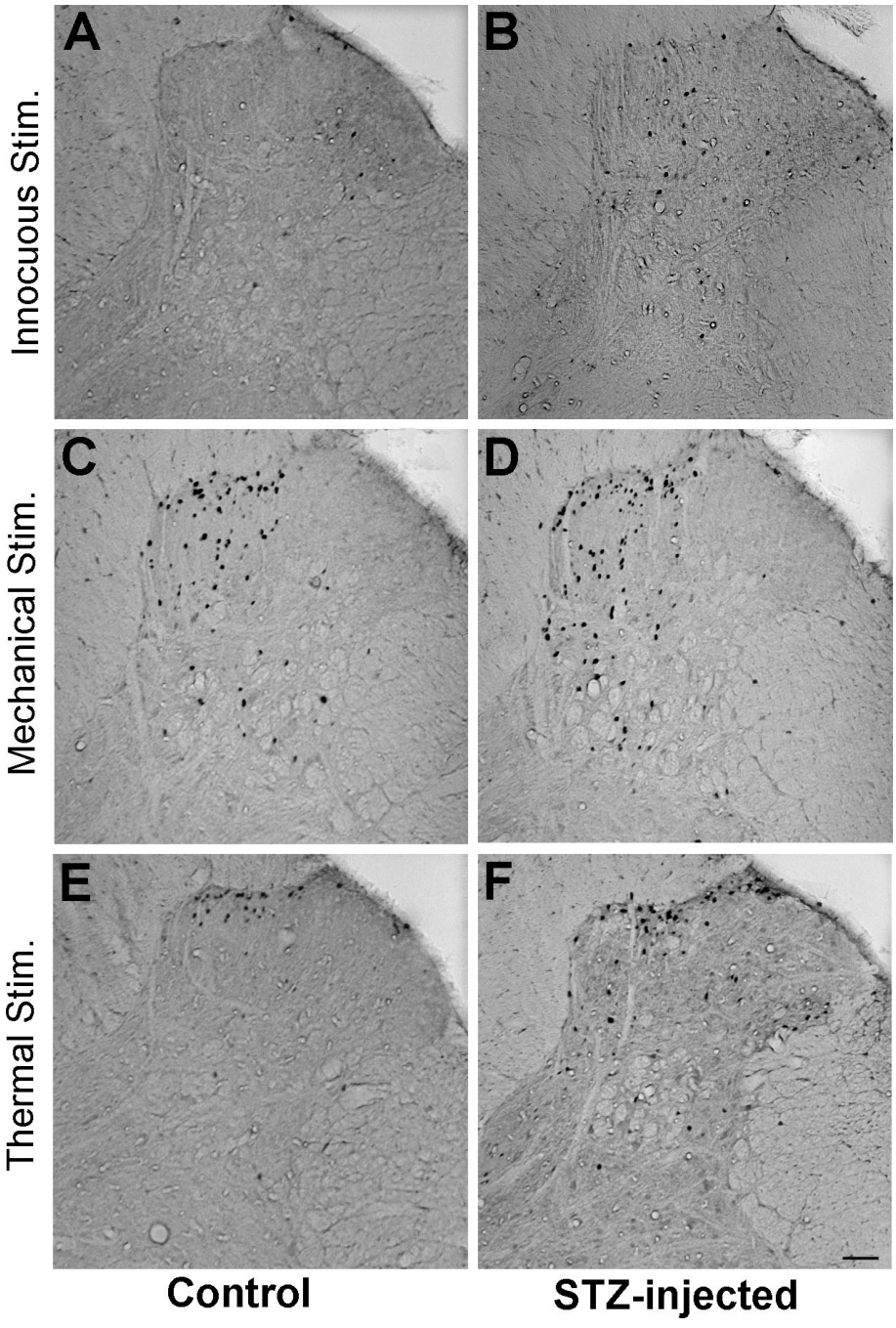

Figure 4. Fos expression at the L2 spinal segment evoked by innocuous (A, B), noxious mechanical (C, D) and noxious thermal (E, F) stimulation of control (A, C, E) and STZ-injected (B, D, F) animals, at 4 weeks post-injection. Significance of lettering: Innocuous Stim. – animals submitted to innocuous mechanical stimulation; Mechanical Stim. – animals submitted to noxious mechanical stimulation; Thermal Stim. – animals submitted to noxious thermal stimulation. Scale bar: 100 μm

Discussion

Using the activation of the c-*fos* protooncogene to monitor nociceptive activation of spinal dorsal horn neurons, the present study is the first to demonstrate spinal cord impairments during diabetes. The high activity at the dorsal horn of diabetic animals in the absence of stimulation is a major finding of the present study and probably accounts for the spontaneous pain detected in diabetes [2–4]. This increased spinal activity agrees with previous electrophysiological recordings in the same animal model [7,8]. However, due to the specific features of electrophysiological studies, it was neither possible to study large neuronal populations nor correlate neuronal responses with their location at the dorsal horn, an important issue due to the specific laminar organization of the spinal cord [48]. The present data show that both the superficial and the DDH are affected during diabetes. This generalized effect of diabetes at the dorsal horn probably affects nociceptive transmission to the brain, since both the superficial and DDH participate

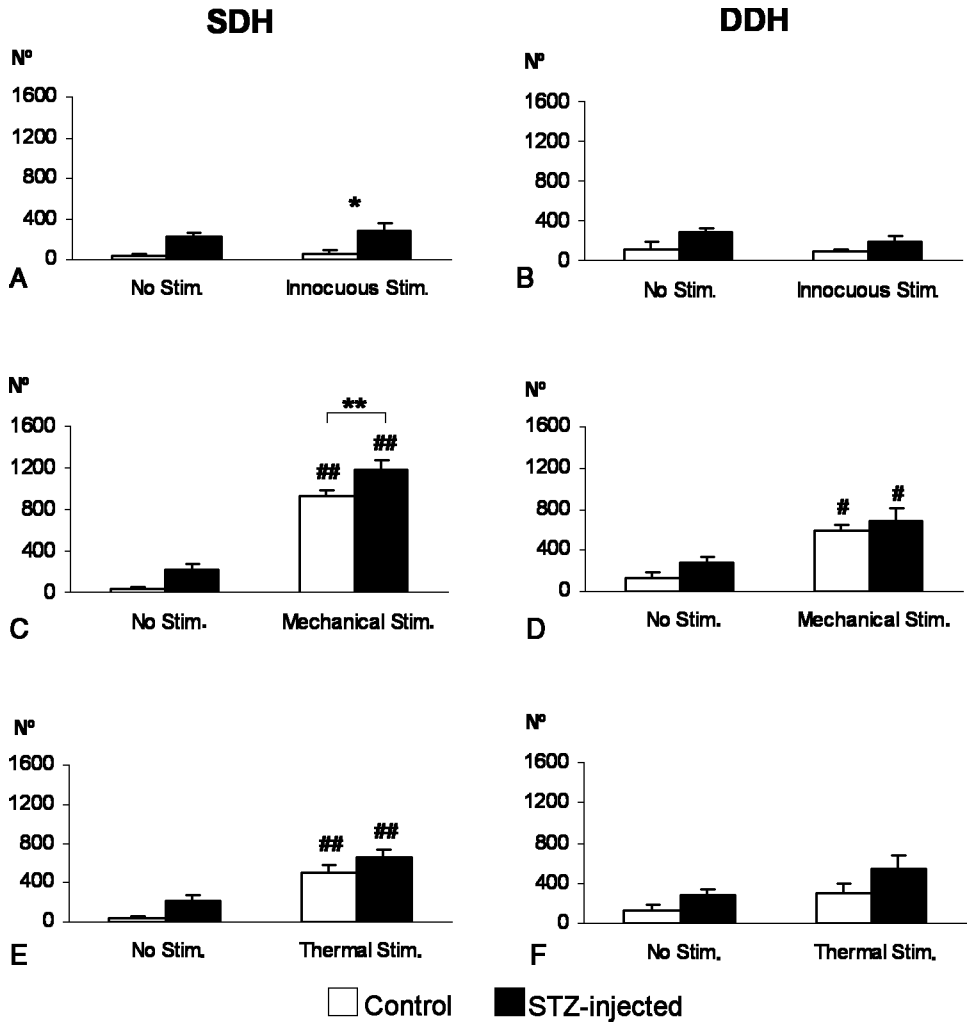

Figure 5. Effects of innocuous (A, B), noxious mechanical (C, D) and noxious thermal (E, F) stimulation in Fos expression at the L1–L2 spinal segments, at 4 weeks post-injection. The graphs show the mean numbers of Fos-IR neurons (±SEM) counted at the ipsilateral laminae I–II (A, C, E) or III–V (B, D, F). Innocuous stimulation did not increase the baseline levels of Fos in control or STZ-injected animals. The effects of noxious mechanical stimulation in increasing Fos expression were more pronounced than those of noxious thermal stimulation. Significance of symbols and lettering: No Stim. – non-stimulated animals; Innocuous Stim. – animals submitted to innocuous mechanical stimulation; Mechanical Stim. – animals submitted to noxious mechanical stimulation; Thermal Stim. – animals submitted to noxious thermal stimulation; * – comparisons of STZ-injected animals with controls; # – comparisons with baseline values. One symbol: $p < 0.05$; two symbols: $p < 0.01$

in the main spinofugal pathways [49,50]. Neurons with increased baseline responses during diabetes were, in fact, shown to belong to the spinothalamic tract [8]. The mechanisms underlying the increased baseline activity of spinal neurons probably relate to the constant barrage of peripheral input since peripheral fibres of diabetic rats show ectopic activity [51]. It may also reflect plastic changes at the spinal cord and, more interestingly, of brainstem centres devoted to descending pain modulation [52–54].

The high baseline activity during diabetes agrees with previous reports in traumatic neuropathic models [15]. However, diabetes appears to induce specific effects in spinal cord neurons in what concerns the responses to innocuous stimulation. In several traumatic neuropathic models, innocuous stimuli induce c-*fos* expression [15,20,23–25], whereas in our diabetic rats the innocuous mechanical stimulus failed to increase c-*fos* expression in relation to baseline levels, in spite of the long duration of stimulus delivery. Since mechanical allodynia is a feature of diabetic neuropathy [7,8,32], it is likely that the high baseline Fos levels in diabetic animals uncovered any innocuous-evoked increases in c-*fos* expression induced by innocuous mechanical stimulation.

Noxious mechanical stimulation significantly increased the numbers of Fos-IR neurons relative to non-stimulated diabetic rats, both at the SDH and DDH. This stimulus also increased c-*fos* expression in relation to control animals subjected to the same stimulation protocol. The effects of thermal noxious stimulation in c-*fos* expression at the dorsal horn were only seen at the SDH, both in control and STZ groups, with no significant differences between them. The higher effects of noxious stimulation in the superficial dorsal horn, which mimic the pattern of c-*fos* expression in normal conditions, appear to be a specific feature of this chronic pain model since, in inflammatory

pain and some neuropathic traumatic conditions, an important contingent of activated neurons are located at DDH laminae [15].

Several studies showed a positive correlation between the magnitude of behavioural pain responses and spinal c-*fos* expression evoked by those behavioural tests [55–57]. It would be interesting to search for similar correlations in our diabetic animals which were not done in the present study due to the fact that the tests used in behavioural assessment do not induce c-*fos* expression [13,46]. Incidentally, since the results of mechanical hyperalgesia match the noxious mechanical–evoked increases in c-*fos* expression, the thermal hypoalgesia is hard to conciliate with thermal-evoked increases in c-*fos* expression [7,31–33]. On the basis of reports showing that it is necessary to correct the thermal latency responses to the tail temperature [37–41], it was proposed that the decreased skin temperature of diabetic rats could account for the thermal hypoalgesia of diabetic animals [7]. By showing that, even after adjusting the thermal response latencies to the decreased baseline tail temperature, diabetic animals exhibited thermal hypoalgesia, we demonstrate that thermal hypoalgesia should be due to factors other than hypothermia. Since c-*fos* is expressed in neurochemically heterogeneous spinal neurons, most of which contain inhibitory neurotransmitters [58] or are under strong inhibitory actions [46], it is possible that thermal stimulation activates spinal c-*fos* expression mainly in inhibitory spinal neurons.

In conclusion, the present study demonstrates that the baseline activity of spinal dorsal horn neurons is strongly increased 4 weeks after induction of diabetes. In addition to the structural changes at the spinal cord of diabetic patients [6], the present and previous [7,8] functional evaluations demonstrate that neuronal activity at the spinal cord is strongly affected during diabetes. The present results are putatively relevant to the treatment of pain during diabetes, suggesting the importance of pharmacological approaches directed to the spinal dorsal horn and prevention of possible supraspinal alterations induced by the spinal functional impairment.

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Conflict of interest

None declared.

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Publication 2

Diabetes affects the expression of GABA and potassium chloride cotransporter in the spinal cord: a study in streptozotocin diabetic rats

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Diabetes affects the expression of GABA and potassium chloride cotransporter in the spinal cord: A study in streptozotocin diabetic rats

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Sensitization

ABSTRACT

Painful diabetic neuropathy is associated to hyperexcitability and spontaneous hyperactivity of spinal cord neurons. The underlying pathophysiological mechanisms are not clear. Increases in excitatory neurotransmission at the spinal cord, involving glutamate and SP, seem to account for the abnormal neuronal activity, but inhibitory influences were never evaluated. This study aims to analyse the expression of GABA, its synthesizing enzyme glutamic acid decarboxylase (GAD) and the potassium chloride cotransporter (KCC2), in the spinal dorsal horn of streptozotocin (STZ)-induced diabetic rats. Four weeks after saline or STZ (60 mg/kg) injection, animals were sacrificed and the spinal segments L2–L3 were removed and immunoreacted for GABA, GAD and KCC2, or processed for western blotting for KCC2. Densitometric quantification was performed in the superficial dorsal horn (laminae I, II and III) of immunoreacted sections and in the immunoblots. STZ rats presented a significant increase of GABA expression in laminae II and III when compared with control animals, while no differences were detected in GAD expression. A significant decrease in KCC2 expression was detected by immunohistochemistry in laminae I and II, which was confirmed by immunoblotting. Increased GABA levels, along with decrease in KCC2 expression, may underlie the abnormal neuronal activity detected in the spinal cord of diabetic rats. Reduction in KCC2 expression was shown to lead to increases in intracellular chloride concentration and, in such condition, GABA binding to $GABA_A$ receptor induces membrane depolarization, provoking neuronal excitation rather than inhibition. Based on these findings, we propose that a loss of GABA-mediated inhibitory tone at the spinal cord may result in neuronal hyperexcitability and spontaneous hyperactivity during diabetes.

Painful diabetic neuropathy is a frequent complication of diabetes. It is characterized by spontaneous pain and increased sensitivity to mechanical and chemical stimuli [11,29,31]. It has been proposed that hyperexcitability and spontaneous hyperactivity of primary afferents [1,6,7,13] and spinal dorsal horn neurons [5,24,27] detected in streptozotocin (STZ)-induced diabetes, might account for those signs of diabetic neuropathy. The neurobiological mechanisms associated with the spinal hyperactivity remain, however, unclear. Glutamate seems to be involved since an increase in binding affinity and density of NMDA and AMPA receptors were detected in the spinal cord of diabetic rats [15,33], in spite of the decrease in glutamate levels [19]. A higher glutamatergic excitation is also supported by pharmacologic studies showing that hyperalgesia and allodynia in diabetic animals is reversed by NMDA and non-NMDA receptors antagonists [2,17]. The increase in excitation is further reinforced by similar changes of the SP/NK1 system. The diminished release of SP from primary afferents in the spinal dorsal horn of diabetic rats appears to be counterbalanced by an increase in the expression of NK1 receptors [14]. Little is know about the inhibitory modulation of spinal nociceptive transmission during diabetic neuropathy. In the adult nervous system, GABA is the major inhibitory neurotransmitter involved in spinal modulation of pain. However, GABA can also have an excitatory role in immature neurons [8,25] and during pathological conditions like neuropathic pain [9,26]. GABA may exert its function on spinal cord neurons through activation of ionotropic post-synaptic $GABA_A$ receptors, which will promote input or output of chloride from the neuron depending on intracellular concentration of the anion. The influx of chloride into the neuron results in hyperpolarization, while the opposite occurs with chloride exit. In mature neurons, chloride homeostasis is mainly set by two cotransporters: Na^+–K^+ Cl^-cotransporter 1 (NKCC1) and K^+ Cl^-cotransporter 2 (KCC2). The former imports

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chloride into the cell and is present in dorsal root ganglion neurons and glia [26], but is scarce in spinal dorsal horn neurons [23]. KCC2 exports chloride out of the neuron and is expressed by nociceptive spinal neurons [9,26]. KCC2 has, therefore, a more important role in setting low chloride concentration in nociceptive neurons of the superficial dorsal horn, accounting for the inhibitory role of GABA. A reduction in the expression of KCC2 in the superficial spinal dorsal horn in chronic pain models induced by peripheral nerve injury [9] or inflammation [37] was recently proposed to induce a shift in the action of GABA from inhibitory to excitatory [9,20,37].

In order to study the role of GABA in the spinal cord during diabetes, the present study evaluates the expression of GABA, its synthesizing enzyme glutamic acid decarboxylase (GAD) and KCC2 in the superficial spinal dorsal horn of rats with diabetes induced by STZ injection.

A total of 18 male Wistar rats (Charles River, France), weighing 250–350 g, were used. The experiments were performed in accordance with the ethical guidelines for the study of pain in conscious animals [38] and the European Community Council Directive 86/609/EEC. Diabetes was induced by an intraperitoneal (i.p.) injection of STZ (60 mg/kg body weight; Sigma–Aldrich, St. Louis, USA) freshly diluted in 0.9% saline (STZ group; $n = 9$). Controls received equal volumes of saline (control group; $n = 9$). Three days after the injection, glucose concentration was measured in tail vein blood samples using a glucose oxidase-impregnated test strip (Accu Chek Sensor Comfort, Roche Diagnostics, Germany). Only rats with glucose concentration higher than 300 mg/dl were considered diabetic and included in the study. The animals were evaluated once a week to confirm the existence of behavioral signs of diabetic neuropathy, as described previously [24] with mechanical and thermal responses evaluated using, respectively, the Randall-Selitto and the tail-flick tests. Four weeks after STZ or saline injections, five animals from each group were sacrificed by perfusion to perform immunohistochemistry for GABA, GAD or KCC2 whereas the remaining four were decapitated for western blotting analysis of KCC2. The transcardiac perfusions were performed after anaesthesia with an i.p. injection of 35% chloral hydrate (1 ml/kg body weight), using phosphate-buffered saline (PBS), followed by 4% paraformaldehyde. Spinal cord segments L2–L3 were then removed, post-fixed for 4 h in the same fixative, and cryoprotected overnight in 30% sucrose. Coronal frozen sections, 40 μm thick, were obtained and serially collected in four series. Each series was immunohistochemically processed for GABA, GAD 65/67 or KCC2 using the avidin–biotin–peroxidase method, whereas the fourth was stained with formol–thionin for laminae delimitation, as described previously [28]. The sections processed for immunohistochemistry were treated with 1% hydrogen peroxidase for 10 min for inhibition of endogenous peroxidase activity before incubation in a blocking solution of 10% normal serum in 0.3% Triton X 25% in PBS (PBST) with 0.1 M glycine for 2 h. The sections were then incubated overnight at 4 °C in anti-GABA raised in guinea pig (1:4000; Abcam) or anti-GAD 65/67 (1:2000; Biotrend) or anti-KCC2 (1:20,000; Sigma–Aldrich), both raised in rabbit. All primary antibodies were diluted in PBST with 2% normal serum raised in the species were the respective secondary antibodies were produced. After being washed in PBST, the sections were incubated during 1 h in biotinylated secondary antibodies, namely goat anti-guinea pig (1:200; Vector Laboratories) for GABA or swine anti-rabbit (1:200; DaKo) for GAD 65/67 and KCC2. The sections were then washed in PBST, incubated in the avidin–biotin complex (Vectastain, Vector Laboratories) and stained with diaminobenzidine (DAB; 10 mg DAB in 20 ml of Tris–HCl 0.05 M, pH 7.6 solution with 5 μl of 30% hydrogen peroxide). Finally, the sections were washed in PBS, mounted on gelatine-coated slides, air-dried and coverslipped with xylol. Sections were observed under a light microscope and the images were acquired using a high-resolution digital camera coupled to a computer. Immunolabelling densitometric quantification was performed for spinal laminae I, II and III using the NIH Image 1.52 software (US National Institutes of Health). In the animals used for western blotting for KCC2 quantification, the spinal segments L2–L3 were immediately removed after decapitation and homogenized in buffer (50 mM Tris, pH 7.6, 100 mM NaCl, 0.1% Triton X 100, 1 mM EDTA). The protein concentration was measured using Bradford Assay (BioRad) and adjustments were performed to enable equal protein loading on the electrophoresis gel. Samples were heated at 65 °C for 15 min and 40 μg protein per lane were loaded, electrophoresed on 8% SDS-PAGE and electroblotted onto nitrocellulose membranes. The membranes were blocked in 5% nonfat milk in Tris-buffered saline with Tween 0.1 M, pH 7.2–7.4 (TBST:milk) for 1 h, and incubated overnight at 4 °C with the rabbit anti-KCC2 antibody (1:2000; Sigma–Aldrich) diluted in TBST:milk. The membranes were washed in TBST:milk, incubated in anti-rabbit secondary antibody conjugated to horseradish peroxidase (1:3000), rinsed in TBST, and the signal was detected using a sensitive chemiluminescence reagent (Pierce Biotechnology). Alfa-tubulin was used as an internal standard (mouse anti-α-tubulin, 1:5000; Sigma–Aldrich). Densitometric analysis of the bands was carried out on digitized images using NIH Image 1.52 software (US National Institutes of Health). All the statistical comparisons between values of controls and STZ rats were performed using non-parametric Mann–Whitney test (body weight, blood glucose, mechanical and thermal thresholds and optical densities). Results are expressed as mean ± standard error of the mean (S.E.M.).

Three days after STZ injection, the animals developed hyperglycemia and remained hyperglycemic until the end of the study (Table 1). At 4 weeks post-injection, STZ rats presented body weights significantly lower than their age-matched controls and developed mechanical hyperalgesia and thermal hypoalgesia (Table 1), which agrees with previous studies using this animal model [4,17,24,27]. The distribution of GABA and GAD immunoreaction at the spinal grey matter was mainly restricted to laminae I–III with much more pronounced labelling at lamina II [21,32]. Compared with the control group, the STZ group presented higher optical density values for GABA at laminae II and III (both with $p < 0.05$; Figs. 1A, B and 2A). However, increased GABA immunoreactivity in STZ group was not accompanied by differences in the expression of its synthesizing enzyme, since optical densities in the sections immunoreacted for GAD 65/67 were similar in STZ and control groups (Figs. 1C, D and 2B). The immunoreaction for KCC2 was confined to the grey matter and it was not seen at white matter, supporting the neuronal expression of KCC2 previously demonstrated [30]. KCC2 immunoreaction prevailed mainly in lam-

Table 1
Body weight, blood glucose concentration, mechanical and thermal nociceptive thresholds at 4 weeks after saline or STZ injection

	Body weight (g)	Blood glucose (mg/dl)	Mechanical threshold (g)	Thermal threshold (s)
Control	412 ± 5.6	131 ± 18.7	110 ± 6.7	9 ± 1.4
STZ	282 ± 13.4*	492 ± 32.2*	68 ± 13.8*	21 ± 3.7*

Data are presented as mean ± S.D.
* $p < 0.05$.

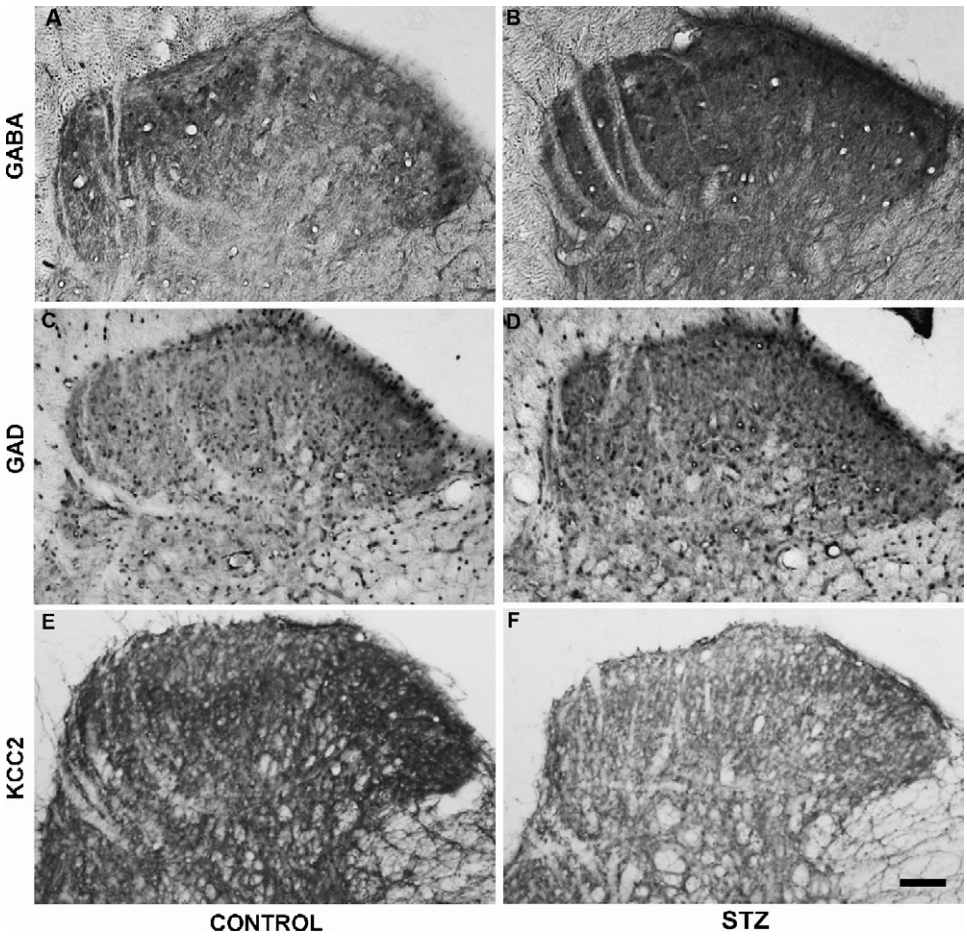

Fig. 1. GABA (A and B), GAD (C and D) and KCC2 (E and F) expression at the L2 spinal segment of control (A, C and E) and STZ (B, D and F) rats, at 4 weeks post-injection. Scale bar: 100 μm.

inae I–III accounting for about 73% of all the immunolabelling and was mainly concentrated in laminae I (33%) and II (31%). About 15% of KCC2 immunoreaction was located in the ventral horn. STZ rats showed a significant decrease in KCC2 expression when compared with the control group in laminae I and II (both with $p < 0.05$; Figs. 1E, F and 2C). Immunoblot analysis supports the immunohistochemistry results, showing an important decrease (about 33.8%; $p < 0.05$) in expression of KCC2 protein in STZ rats (Fig. 3).

The expression of GABA was here shown to be significantly increased in the superficial dorsal horn of diabetic rats when compared with control animals. This finding is in agreement with previous studies reporting increased GABA levels in spinal cord [19] of diabetic rats and may represent an attempt to counterbalance the increased excitatory input in spinal cord due to peripheral nerve fibres spontaneous hyperactivity. Increased GABA levels were not accompanied by increased GAD expression, which does not exclude the possible involvement of the enzyme in such deregulation, since its expression cannot be considered a surrogate for enzyme function [22]. In spite of normal expression, GAD may be overactive leading to increased synthesis of GABA during diabetes. High content of GABA may also be due to reduced removal of GABA from the synaptic cleft resulting from abnormal function of GABA transporters (GATs). These are unexplored mechanisms which must be studied in order to explain the aetiology of GABA overexpression. Another issue to ascertain is the effect of GABA overexpression on the function of $GABA_B$ receptors. A recent electrophysiological study showed that the function of $GABA_B$ receptors is reduced in the spinal cord during diabetic neuropathy [35], which may explain the reduced analgesic effect of the $GABA_B$ agonist blacofen in experimental models of diabetes [17]. A decrease in the inhibitory function of GABA in the spinal cord during diabetes is further indicated by the changes in the actions on $GABA_A$ receptors. Neuronal response induced by GABA binding to $GABA_A$ receptors is determined by intracellular chloride concentration, which mainly depends on KCC2 function [9,23,26]. In neuropathic pain models induced by nerve injury it was recently demonstrated that GABA can act as an excitatory neurotransmitter when binding to $GABA_A$ receptor in the spinal cord, due to a reduction of KCC2 expression. This affects transmembrane anion gradients in a way that the normally inhibitory mechanisms induced by GABA become excitatory [9]. The present study demonstrates a significant reduction in KCC2 expression in the superficial dorsal horn of diabetic rats which was confirmed by other research groups (Nigel Calcutt, personal communication). Based on the mechanism presented by Coull et al. [9], these results suggest that in superficial dorsal horn of diabetic rats, GABA may act as an excitatory rather than inhibitory neurotransmitter by binding to post-synaptic $GABA_A$ receptors, concurring to perpetuating of spinal neuronal hyperactivity. This, along with reduced $GABA_B$ receptor function, appears to induce a loss of GABA-mediated spinal inhibitory tone in painful diabetic neuropathy. The mechanisms that underlie this change in KCC2 expression might involve brain derived neurotrophic factor (BDNF) action, since BDNF was shown to cause a depolarizing shift in anion reversal potential in spinal lamina I neurons and blockade of endogenous BDNF reversed this depolarizing shift in animals with peripheral nerve injury [10]. A recent study in hippocampal neurons

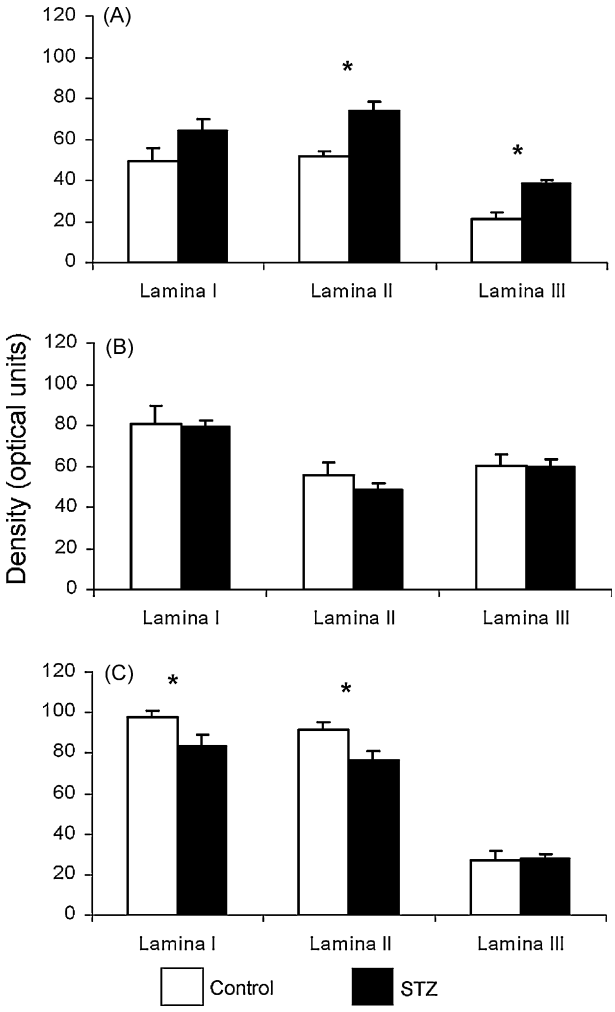

Fig. 2. Immunolabelling optical densities in laminae I–III for GABA (A), GAD (B) and KCC2 (C). STZ rats presented significantly increased expression of GABA in laminae II and III and decreased expression of KCC2 in laminae I and II. No changes were detected in GAD expression. $^{*}p < 0.05$.

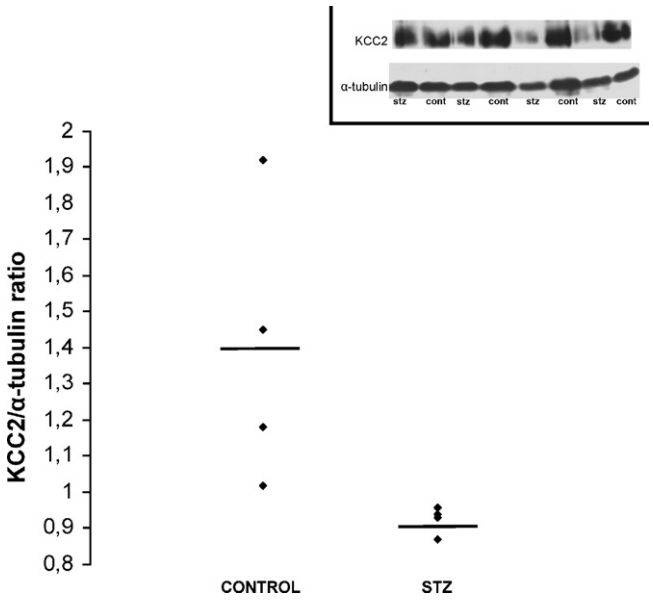

Fig. 3. KCC2 protein expression in the spinal cord segments L2–L3. Immunoblotting revealed that KCC2 levels were decreased in STZ rats (control *vs.* STZ: 1.4 ± 0.19 *vs.* 0.9 ± 0.02; $p < 0.05$). Lines present mean values. Insert presents images of the immunoblot bands of all the animals used in the present study.

confirmed a BDNF involvement in the decrease KCC2 expression and function and suggested that neuronal oxidative stress, one of the most important consequences of diabetes [31], may also lead to similar changes [34].

Other issue that deserves consideration is the effects of GABA and KCC2 changes during diabetes in other neurochemical systems at the spinal cord. Regarding GABA, it is known that when binding to $GABA_B$ receptors it inhibits the release of glutamate and neuropeptides from the primary afferent terminals and of glycine and GABA from interneurons [16,18,36]. The basal levels of glutamate and of noxious-evoked release of substance P (SP) and glutamate were shown to be lower in the spinal cord of diabetic rats, but basal SP content were unaltered [3,19]. Diabetic rats do not present either basal or noxious-evoked differences in the spinal cord glycine release [3,19]. The increased levels of GABA detected in the present study may contribute to the tonic depression of glutamatergic spinal input and to the reduced evoked glutamate and SP release detected in diabetic rats, but is unlikely to affect glycinergic function. Since the present study used animals not stimulated it was not expected any difference in spinal SP immunoreactivity between control and diabetic rats. Despite the decrease in excitatory input, spinal dorsal horn neurons remained hyperactivated [24], suggesting an impairment of spinal nociceptive processing involving mainly spinal neurons rather then primary afferents. As to KCC2, the underexpression detected in the present study is expected to impair glycine-mediated neuronal inhibition during diabetes, since glycine activates chloride channels [26,30]. Similarly to GABA, its function is highly dependent on intracellular chloride concentration and it was shown to function as an excitatory neurotransmitter in KCC2 knockout animals [12,26]. A detailed characterization of neurochemical changes at the spinal cord of diabetic rats is out of the scope of the present study but possible alterations of several neurotransmitters related to the here reported alterations in GABA and KCC2 need to be ascertained in future studies.

In summary, the present data show that diabetes induces a decrease in spinal KCC2 expression that probably alters neuronal chloride homeostasis and renders GABA excitatory, as occurs in neuropathic pain induced by peripheral nerve injury [9] and in inflammatory pain [37]. Therefore, the increase of GABA expression in the spinal cord of diabetic animals may account for spinal sensitization, spontaneous pain and hyperalgesia reported during diabetes.

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Publication 3

Minocycline completely reverses mechanical hyperalgesia in diabetic rats through microglia-induced changes in the expression of the potassium chloride co-transporter 2 (KCC2) at the spinal cord

Diabetes, Obesity and Metabolism 13: 150–159, 2011.

ORIGINAL ARTICLE

Minocycline completely reverses mechanical hyperalgesia in diabetic rats through microglia-induced changes in the expression of the potassium chloride co-transporter 2 (KCC2) at the spinal cord

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Aim: Neuronal hyperactivity at the spinal cord during mechanical hyperalgesia induced by diabetes may result from a decrease in the local expression of the potassium chloride co-transporter 2 (KCC2), which shifts the action of the neurotransmitter γ-amminobutiric acid (GABA) from inhibitory to excitatory. In this study, we evaluated the effects of spinal microglia inhibition or brain-derived neurotrophic factor (BDNF) blockade on KCC2 expression, spinal neuronal activity and mechanically induced pain responses of streptozotocin (STZ)-diabetic rats.

Methods: Four weeks after induction of diabetes, the STZ-diabetic rats received daily intrathecal injections, for 3 days, of minocycline (microglia inhibitor), TrkB/Fc (BDNF sequester) or saline. Behavioural responses to mechanical nociceptive stimulation of STZ-diabetic rats were evaluated by the Randall-Selitto test. The lumbar spinal cord was immunoreacted against the Fos protein (marker of neuronal activation) or KCC2, which was also quantified by western blotting. BDNF levels at the spinal cord were quantified by an enzyme-linked immunosorbent assay (ELISA).

Results: Minocycline treatment reversed the mechanical hyperalgesia, increased Fos expression and decreased the KCC2 expression detected in STZ-diabetic rats to control levels. Treatment with TrkB/Fc was less effective, inducing moderate effects in mechanical hyperalgesia and Fos expression and only a partial correction of KCC2 expression. BDNF levels were not increased in STZ-diabetic rats.

Conclusions: This study demonstrates that the microglial activation at the spinal cord contributes to mechanical hyperalgesia and spinal neuronal hyperactivity induced by diabetes, apparently by regulating the KCC2 expression. These effects do not seem to be mediated by BDNF, which is an important difference from other chronic pain conditions. New targets directed to prevent spinal microglia activation should be considered for the treatment of mechanical hyperalgesia induced by diabetes.

Keywords: BDNF, c-fos, diabetes, diabetes complications, diabetic neuropathy, experimental pharmacology, glia, minocycline, neuropharmacology, pain, TrkB/Fc, type 1 diabetes

Date submitted 20 July 2010; date of first decision 28 September 2010; date of final acceptance 1 November 2010

Introduction

Diabetic neuropathy (DN) is a major complication of diabetes, affecting 30–50% of patients. A major reduction of life quality is because of the spontaneous and mechanical evoked pain, which is reported as a major complaint by 25% of patients with DN [1,2]. Chronic pain induced by diabetes represents a health and economic burden in industrialized countries because of the increasing prevalence of diabetes and low efficacy of pharmacological treatments [3].

Pain induced by diabetes is characterized by spontaneous pain, mechanical hyperalgesia and tactile allodynia [2,4,5], which are associated with changes at the peripheral and central somatosensory systems. As to the latter, functional changes at the spinal cord were shown to be involved in this pain conditions [5,6]. In streptozotocin (STZ)-diabetic rats, nociceptive spinal neurons have higher spontaneous activity [4,5,7] which have been ascribed to local neurochemical changes. In the spinal cord of STZ-diabetic rats, a decrease in the expression of the potassium chloride co-transporter 2 (KCC2) accounts for a shift in the action of the γ-amminobutiric acid (GABA) from inhibitory to excitatory [8,9]. In normal conditions, KCC2 sets a low intracellular chloride concentration and GABA binding to $GABA_A$ receptors induces chloride influx, inhibiting neuronal activity [10]. During diabetes, the reduced expression of KCC2 in the spinal cord leads to intracellular chloride accumulation and GABA binding to $GABA_A$ receptors induces chloride output, which results in neuronal excitation and pain. In fact, intrathecal administration of bicuculline ($GABA_A$ receptor antagonist) in STZ-diabetic rats elicits antinociceptive effects, supporting the excitatory role of GABA at the spinal cord during diabetes [8,9].

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In traumatic neuropathic and inflammatory pain, the spinal expression of KCC2 was shown to be modulated by the brain-derived neurotrophic factor (BDNF). The levels of the BDNF increase at the spinal cord and the blockade of BDNF action normalizes the KCC2 expression and induces analgesia [11,12]. The increase in the levels of spinal BDNF during traumatic neuropathic pain seems to be caused by microglial activation at the spinal cord, with an involvement of p38 mitogen-activated protein kinase (p38 MAPK) [13,14]. As the activation of microglia was recently described in the spinal cord of STZ-diabetic rats [15–17] it is important to evaluate if microglia is involved in the reduction of KCC2 expression during diabetes through a BDNF-dependent action. These studies are crucial inasmuch that DN has specific aetiology and underlying mechanisms.

The present study used STZ-diabetic rats to evaluate the effects of inhibiting spinal microglia or blocking BDNF action on mechanical hyperalgesia, spinal neuronal activation and KCC2 expression at the spinal cord. Nociceptive activation of spinal neurons was evaluated analysing Fos expression, because of the value of this technique to monitor large nociceptive neuronal populations [18]. Microglia was inactivated by minocycline, a tetracycline-analogue antibiotic, which inhibits p38 MAPK [19] and presents analgesic effects in traumatic neuropathic pain models [20]. BDNF was blocked by intrathecal administration of TrkB/Fc in a dose that was previously shown to induce analgesia in traumatic neuropathic pain and normalize KCC2 expression at the spinal cord [11,21,22].

Materials and Methods

Animals

Adult male Wistar rats (12 weeks of age, Charles River; Barcelona, Spain), weighing 250–300 g at the beginning of the experiments, were used. The animals were housed two per cage in a room with a constant temperature ($22 \pm 2\ ^{\circ}C$) and humidity ($55 \pm 5\%$) and 12 h light/dark cycles and received food and water *ad libitum*. Experiments were performed in accordance with the European Community Council Directive 86/609/EEC and the ethical guidelines for the study of pain in conscious animals [23].

Induction of Diabetes

Type 1 diabetes was induced by an intraperitoneal (i.p) injection of STZ (60 mg/kg body weight; Sigma-Aldrich, Barcelona, Spain), dissolved in 0.1 M citrate buffer, pH 4.5. Control animals received an i.p injection of citrate buffer. The glucose concentration was measured 3 days after STZ or buffer injections and before sacrifice in a blood sample from the tail vein, using Accu Chek Sensor Comfort (Roche Diagnostics, Berlin, Germany). Only rats with glucose concentrations higher than 270 mg/dl were considered STZ-diabetic rats.

Treatment Protocols

Four weeks after STZ injection, the animals were injected with the following substances ($n = 10$ per experimental group): saline (STZ+saline group), 100 μg of minocycline (STZ+Mino group) or 5 μg of TrkB/Fc (STZ+TrkB/Fc group). All substances were purchased from Sigma-Aldrich. Minocycline and TrkB/Fc were intrathecally infused once a day, for 3 days, using treatment protocols adapted from previous studies [20,21]. Briefly, for intrathecal deliveries, the catheters were implanted into the lumbar subarachnoid space, under deep anaesthesia by an i.p. injection of a mixture of ketamine (6 mg/100 g) and medetomidine (0.025 mg/100 g). A laminectomy was performed at T_7–T_9 levels and a 3 cm silicone catheter with a diameter of 3.2 mm (SF1291; SF Medical, Hudson, MA, USA) was placed into the subarachnoid space, with the tip positioned at L_4–L_5 levels. A 25 μl Hamilton syringe was attached to the cannula and 25 μl of saline, minocycline solution or TrkB/Fc was slowly injected, followed by flushing with 25 μl of saline to assure that all the solution was injected into the subarachnoid space, as previously described [24,25]. After each administration, the top of the cannula was carefully sealed. The surgical procedures lasted approximately 20 min and all incisions were carefully closed and washed with the povidone–iodine antiseptic solution. A group of age-matched non-diabetic control rats was included in the study ($n = 10$). The position of the catheter tip was checked during the dissection procedure. Only the animals with the catheter correctly positioned at L_4–L_5 were included in the study.

Behavioural Evaluation of Mechanical Nociception

Mechanical nociception was evaluated by the Randall-Selitto test (Ugo Basile, Comerio, Italy) on the day prior to STZ or buffer injections, immediately before the onset of each treatment (pretreatment evaluation) and 90 min after the last drug delivery (posttreatment evaluation). All animals were handled daily by the experimenter for habituation purposes for a period of 8 days prior to the onset of behavioural evaluation. Ascending mechanical forces were applied to the dorsal surface of the right hindpaw. The mechanical force (in g) inducing hindpaw withdrawal was recorded. The paw pressure threshold (PPT) of each animal was the average of three consecutive measurements, taken with 5-min intervals. Tactile allodynia was also evaluated using the dynamic plantar aesthesiometer test (Ugo Basile, Comerio, Italy) in order to better characterize the STZ model. Additional animals were injected with saline or STZ as above ($n = 4$ each) and behavioural evaluation was performed at the time points referred by the Randall-Sellito test, as previously described [5]. Briefly, the animals were placed in clear acrylic boxes with a metal grid floor and stimulation was performed with a 0.5 mm filament, which delivered a linearly increasing force (2.5 g/s) to the plantar surface of the hindpaw. The force necessary to elicit a paw withdrawal was recorded and the paw withdrawal threshold (PWT) was calculated as the average, in g, of three consecutive tests with at least 5 min between each test.

Immunohistochemistry

Five rats from each experimental group were anaesthetized 3 h after the last treatment with an i.p. injection of 35% chloral hydrate (1 ml/kg body weight) and sacrificed by vascular perfusion with 200 ml of phosphate-buffered saline (PBS),

followed by 1000 ml of 4% paraformaldehyde in 0.1 M phosphate buffer (PB), pH 7.4. Spinal segments L_4–L_5 were removed, postfixed in the same fixative and cryoprotected overnight in 30% sucrose in PB. Coronal sections 40-µm thick, were obtained using a freezing microtome. Every fourth section was collected in 0.1 M PBS and each set was immunohistochemically reacted for KCC2, Fos protein and CD11b (microglial marker) [26]. Immunohistochemical detection using the avidin–biotin peroxidase method was performed as previously described [4,27]. Briefly, the sections were treated with 1% hydrogen peroxidase for 10 min to inhibit endogenous peroxidase activity before incubation for 2 h in a blocking solution containing 10% normal serum and 0.1 M glycine diluted in 0.3% Triton × 25% in PBS (PBST). The sections were then incubated 2 overnights at 4 °C in each primary antibody, namely rabbit anti-KCC2 (1 : 10 000; Sigma-Aldrich), rabbit anti-Fos (1 : 5000; Ab5; Calbiochem, Darmstadt, Germany) or mouse anti-CD11b (clone OX-42; 1 : 2000; Millipore, Schwalbach/Ts, Germany), all diluted in PBST. After being washed in PBST, the sections were incubated in biotinylated swine anti-rabbit (for KCC2 and Fos detections) or rabbit anti-mouse (for CD11b detection) immunoglobulins, from Dako (Glostrup, Denmark), diluted in 1 : 200, for 1 h. Sections were then washed in PBST, incubated for 1 h in the avidin–biotin complex (Vectastain, Vector Laboratories, Burlingame, CA, USA) and stained with diaminobenzidine (10 mg in 20 ml of 0.05 M Tris–HCl, pH 7.6 solution with 5 µl of 30% hydrogen peroxide). Sections were then washed in PBS, mounted on gelatine-coated slides, air-dried and coverslipped with xylol. After observation under a light microscope, the images were acquired using a high-resolution digital camera coupled to a computer. In 10 randomly taken sections, immunolabelling for KCC2 and CD11b was quantified by densitometric analysis at the spinal dorsal horn (laminae I–V), using the NIH Image 1.52 software (US National Institutes of Health), as previously described [9]. The numbers of Fos-immunoreactive (Fos-IR) neurons were also counted in laminae I–V, as previously described [28].

To confirm microglia activation, double immunofluorescence for CD11b and phosphorylated form of p38 MAPK (phospho p38) was performed in spinal sections of controls and STZ-diabetic rats (three animals per group). The sections were treated with 1% borohydrate for 20 min before incubation for 2 h in a blocking solution of 10% normal goat serum in PBST. The sections were then incubated 2 overnights at 4 °C in the primary antibodies, mouse anti-CD11b (1 : 1000) and rabbit anti-phospho p38 (1 : 500; Cell Signalling, Danvers, MA, USA). After being washed in PBST, the sections were incubated in fluorescent secondary antibodies, goat anti-mouse Alexa 488 and goat anti-rabbit Alexa 594 (1 : 500; Molecular Probes, Carlsbad, CA, USA) for 1 h. Sections were observed using an Apo Tome microscope (Zeiss, Göttingen, Germany) and the images were acquired using a high-resolution digital camera coupled to a computer. Serial optical planes separated by 0.3 µm were collected in the *z*-axis and merged images were obtained using the Apo Tome software.

Western Blotting

The expression of phospho p38 and KCC2 were quantified by western blotting. Five animals from the Control, STZ+saline and STZ+Mino experimental groups were used in the western blotting for phospho p38. For the KCC2 western blotting, five animals per experimental group were used and all the experimental groups were considered. After decapitation, the spinal segments L_4–L_5 were immediately removed and homogenized in buffer (50 mM Tris, pH 7.6, 100 mM NaCl, 0.1% Triton X 100, 1 mM EDTA) added with a proteinase inhibitor cocktail and a phosphatase inhibitor cocktail (both at 1 : 100; Sigma-Aldrich). The protein concentration was measured using Bradford Assay (BioRad, Amadora, Portugal) and adjustments were performed to equalize protein concentration. Samples were heated at 65 °C for 15 min and 40 µg protein per lane was loaded, electrophoresed on 10% SDS-PAGE and electroblotted onto the nitrocellulose membranes. The membranes were blocked in 5% non-fat milk in Tris-buffered saline with Tween 0.1 M, pH 7.2–7.4 (TBST) for KCC2 and in 5% bovine serum albumin in TBST for phospho p38 for 1 h and incubated overnight at 4 °C with the rabbit anti-KCC2 antibody (1 : 2000; Sigma-Aldrich) and rabbit anti-phospho p38 (1 : 1000, Cell Signalling). The membranes were washed and incubated in antirabbit secondary antibody conjugated to horseradish peroxidase (1 : 5000; Jackson Labs, Bar Harbor, ME, USA) and rinsed in TBST. The signal was detected using a sensitive chemiluminescence reagent (SuperSignal West Pico Chemiluminescent Substrate, Thermo Scientific, Waltham, MA, USA). Antibody against phospho p38 was then stripped incubating the membranes with 10% SDS for 15 min. Following blocking, the membranes were incubated 2 overnights with rabbit anti-p38 (1 : 1000, Cell Signalling) at 4 °C. The signal detection was performed as previously described. Alfa-tubulin was used as an internal standard, using mouse anti-α-tubulin (1: 10 000; Sigma-Aldrich) as the primary antibody. Digital images of western blots were analysed by densitometry using NIH image 1.52 software.

BDNF Quantification

The content of BDNF in the spinal cord of control and untreated STZ-diabetic rats was determined using the BDNF Emax ImmunoAssay System (Promega, Madison, WI, USA) in the supernatant of spinal homogenates from L_4–L_5 segments. Assays were performed according to the manufacturer's instructions, using an antibody sandwich format in 96-well plates. Plates were coated with anti-BDNF polyclonal antibodies. Samples (10 µl) or BDNF standards were added to each well and plates were incubated. After several washes, anti-BDNF monoclonal antibodies were added and plates were again incubated. After thorough washes, the amount of bound monoclonal antibody was detected using IgG-horseradish peroxidase-conjugated antibody. The unbound conjugate was removed by washing. The plate was then incubated with 100 µl of substrate solution for 10 min. Hydrochloric acid was added to stop the reactions. Colour change was measured with a Synergy HT Microplate Reader (Bio-Tek Instruments, Winooski, VT, USA) at 450 nm. All

samples were run in duplicate and values were averaged. BDNF levels were normalized to the protein concentration in each sample, quantified by Bradford Assay.

Specificity of Antibodies

The specificity of each primary antibody was previously demonstrated [9,26,27]. Control experiments were performed by omission of primary or secondary antibodies. Additionally for KCC2, p38 and phospho p38 the specificity of the primary antibodies was demonstrated by western blotting with bands detected at 140 kDa for KCC2 and at 40 kDa for p38 and phospho p38.

Statistical Analysis

SPSS statistics version 18 (IBM, Armonk, New York, NY, USA) was used for statistical analysis. Behavioural, imunohistochemistry and western blotting data were compared by ANOVA followed by Tukey's *post hoc* test for multiple comparisons. BDNF levels were compared by independent sample *t*-test. Pretreatment and posttreatment behavioural responses were compared by paired-sample *t*-test. Statistical significance was settled at $p < 0.05$. Results are expressed as mean ± standard error of the mean (s.e.m.).

Results

At 4 weeks postinjection, STZ-diabetic rats presented significantly lower body weights and higher blood glucose levels than age-matched non-diabetic control animals (Table 1). The treatments with Mino and TrkB/Fc had no effects on body weights or glycaemia.

Characterization of STZ-diabetic Rats

STZ-diabetic rats presented a significant increase in the expressions of CD11b and phospho p38 in microglial cells, when compared with control animals (figures 1, 2A–C, E).

Table 1. Body weights and glycaemia of experimental groups.

Experimental groups	Body weight (g)	Blood glucose concentration (mg/dl)
Control	409 ± 5.4	131 ± 18.7
STZ+saline	287 ± 3.8*	492 ± 32.2*
STZ+Mino	283 ± 7.3*	501 ± 28.4*
STZ+TrkB/Fc	285 ± 4.3*	498 ± 50.3*

Mean values ± standard error of the mean (s.e.m.). STZ, streptozotocin. *$p < 0.01$ vs. control.

Furthermore, the microglia of STZ-diabetic rats presented morphological changes that match the well-described signs of microglial activation, namely larger soma and thicker and shorter processes than control animals (*cf.* figures 1A, B, 2B, C). Untreated STZ-diabetic rats presented much lower PPTs than controls ($p = 0.003$; figure 3A). Tactile allodynia was also detected in untreated STZ-diabetic rats (PWT at 4 weeks postinjection: 19.90 ± 2.04 and 14.10 ± 0.98 in control animals and STZ-diabetic rats, respectively; $p = 0.019$), further confirming the existence of mechanical hypersensitivity in our STZ-diabetic rats. As the Randall-Selitto test is highly predictable of clinical applicability of analgesic drugs [29], the subsequent behavioural evaluations were performed using only this test. STZ-diabetic rats presented a higher Fos expression at the spinal dorsal horn than controls ($p = 0.025$; figure 3B–D). The expression of KCC2 decreased in STZ+saline rats in relation to controls ($p = 0.009$; figure 3F–H).

Effects of Minocycline

After minocycline treatment, the activation of microglia detected at the spinal cord of untreated STZ-diabetic rats was inhibited, with a significant decrease in the expression of CD11b and phospho p38 (figure 2A, E) and reversal of morphological signs of microglial activation (*cf.* figure 2C, D). The mechanical hyperalgesia detected in untreated STZ-diabetic

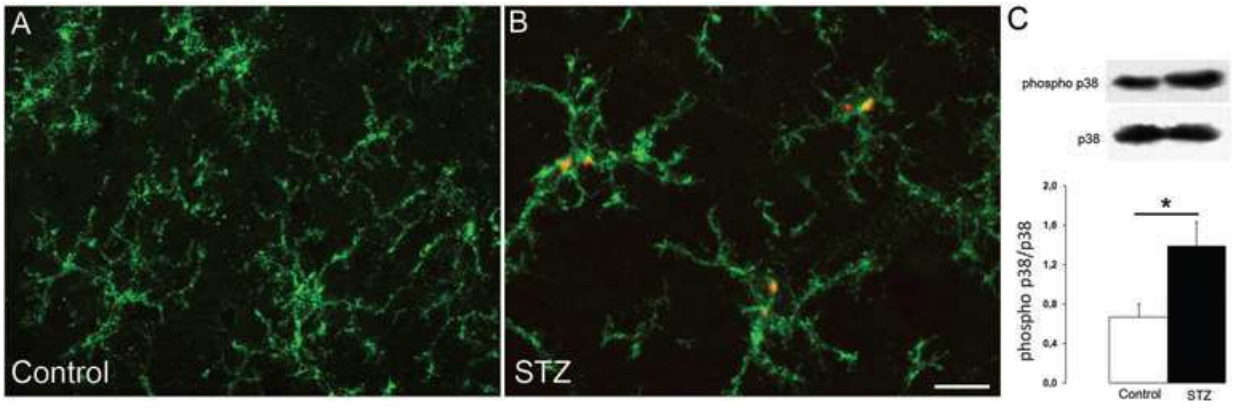

Figure 1. Microglia activation at the spinal cord of streptozotocin (STZ)-diabetic rats. Panels (A) and (B) show double immunoreactions for CD11b (marker of microglia; in green) and phospho p38 (marker of microglia activation; in red) in representative sections taken from the L_4 spinal segment in control (A) and STZ-diabetic (B) rats. Panel (C) shows representative blots and the quantification by western blotting of phospho p38 in spinal homogenates of control and STZ-diabetic rats. STZ-diabetic rats present microglia activation at the spinal cord, demonstrated by morphological changes of microglia (larger soma and thicker and shorter processes) and increased expression of phospho p38 (significant increase in the ratio between phospho p38 and total p38). Significance of symbol: *$p < 0.05$. Scale bar in (B): 50 µm (both photomicrographs are at the same magnification).

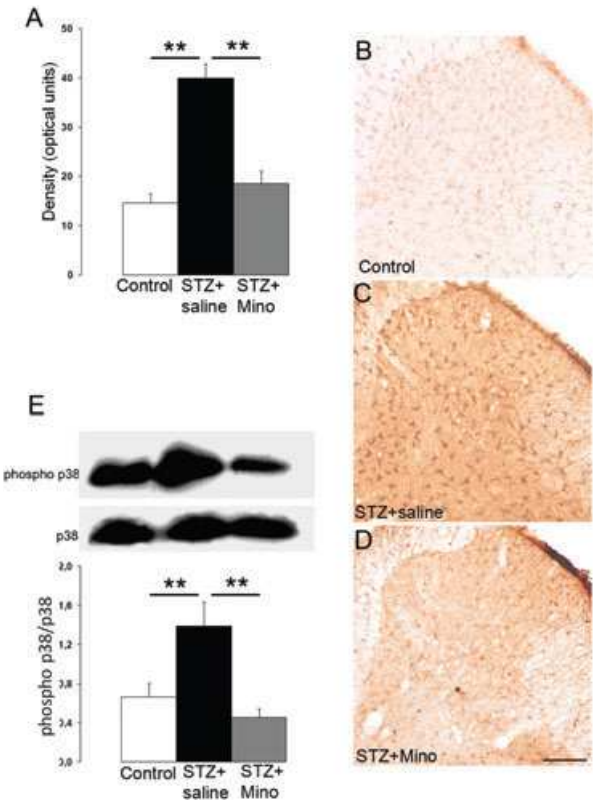

Figure 2. Effects of minocycline treatment on microglial activation. Panel (A) shows the expression of CD11b (marker of microglia) at the spinal dorsal horn of control and of streptozotocin (STZ)-diabetic rats injected with saline (STZ+saline) or treated with minocycline (STZ+Mino). Panels (B–D) show photomicrographs of the L_4 spinal sections labelled for CD11b in the three experimental groups. Panel (E) shows representative blots and the quantification by western blotting of phospho p38 expression in the L_4–L_5 spinal cord homogenates in the three experimental groups. Minocycline significantly reduced CD11b and phospho p38 expression, indicating that the treatment was effective in the reversion of microglia activation. Significance of symbol: $^{**}p < 0.01$. Scale bar in (D): 100 µm (all photomicrographs are at the same magnification).

rats was reversed to the levels of control rats in the STZ+Mino group with statistically significant differences in relation to untreated STZ rats ($p = 0.007$; figure 3A). The increased spinal Fos expression, detected in STZ+saline rats, was also reversed to control levels after minocycline treatment (figure 3B–E) with statistical significant differences only between the STZ+saline and STZ+Mino groups ($p = 0.017$; figure 3B, D, E). The expression of KCC2 was also reversed to control levels when STZ-diabetic rats were treated with minocycline (figure 3F–I), with statistical significant differences only between the STZ+saline and STZ+Mino groups ($p = 0.007$; figure 3F, H, I). The result of western blotting performed for KCC2 supported the quantitative immunohystochemical analysis (figure 3J).

Effects of TrkB/Fc

Blocking the BDNF action in STZ rats by TrkB/Fc ameliorated mechanical nociception (figure 4A), with statistical significant differences in relation to untreated STZ rats ($p = 0.037$). TrkB/Fc reduced the number of Fos-IR neurons in STZ-diabetic rats, with significant differences in relation to the STZ+saline group ($p = 0.018$; figure 4B–E). The TrkB/Fc treatment increased KCC2 expression in STZ-diabetic rats in relation to untreated STZ-diabetic rats ($p = 0.029$; figure 4F–H). However, this reversion did not reach control levels, as the statistical significant differences were detected between STZ+TrkB/Fc and control animals ($p = 0.047$; figure 4F–I). western blotting results for KCC2 confirmed the immunohystochemical data (figure 4J).

We also evaluated the effects of TrkB/Fc in the activation of microglia (figure 5). TrkB/Fc treatment induced a significant reduction of microglia activation in STZ-diabetic rat ($p = 0.019$), as demonstrated by the decrease of CD11b expression (figure 5), but did not reverse it to the levels of control animals ($p = 0.009$).

BDNF Levels

The results of the ELISA study are shown in Table 2. BDNF levels in untreated STZ-diabetic rats were not different from control rats. Treatment with minocycline or TrkB/Fc did not alter BDNF levels.

Discussion

The present study shows that the mechanical hyperalgesia and spinal neuronal hyperactivity detected during diabetes may be reversed by correcting the KCC2 levels at the spinal cord, through the inhibition of spinal microglia. BDNF levels at the spinal cord did not change in diabetic rats and the inhibition of BDNF did not reverse the KCC2 expression to control levels, indicating that mechanisms besides BDNF are probably involved in the correction of KCC2 expression by minocycline. This BDNF-independent effect of microglia on KCC2 expression differs from other chronic pain models, which suggests that the mechanical hyperalgesia induced by diabetes is caused by particular mechanisms and reinforces the need to develop specific treatments.

Diabetic rats presented marked microglia activation at the spinal cord, which agrees with recent results in diabetic rats with different genetic backgrounds [15–17] and demonstrates that this is a common feature of diabetes. Minocycline was effective in inhibiting microglial activity, as evidenced by the induction of resting-state morphology and by the reduction of the spinal levels of phospho p38 MAPK and CD11b. In neuropathic and inflammatory chronic models it was shown that activated microglia at the spinal cord is associated with increased BDNF levels, which leads to a decrease in neuronal KCC2 expression [11,12,30,31]. In our STZ-diabetic rats, the significant microglial activation in the spinal cord did not affect the spinal content of BDNF. Furthermore, BDNF inhibition by TrkB/Fc did not correct KCC2 expression in diabetic rats to control levels. Although it could be argued that higher doses or prolonged treatment with TrkB/Fc should have been used to increase the effects of BDNF sequestration, the current protocol was previously shown to significantly decrease BDNF expression at

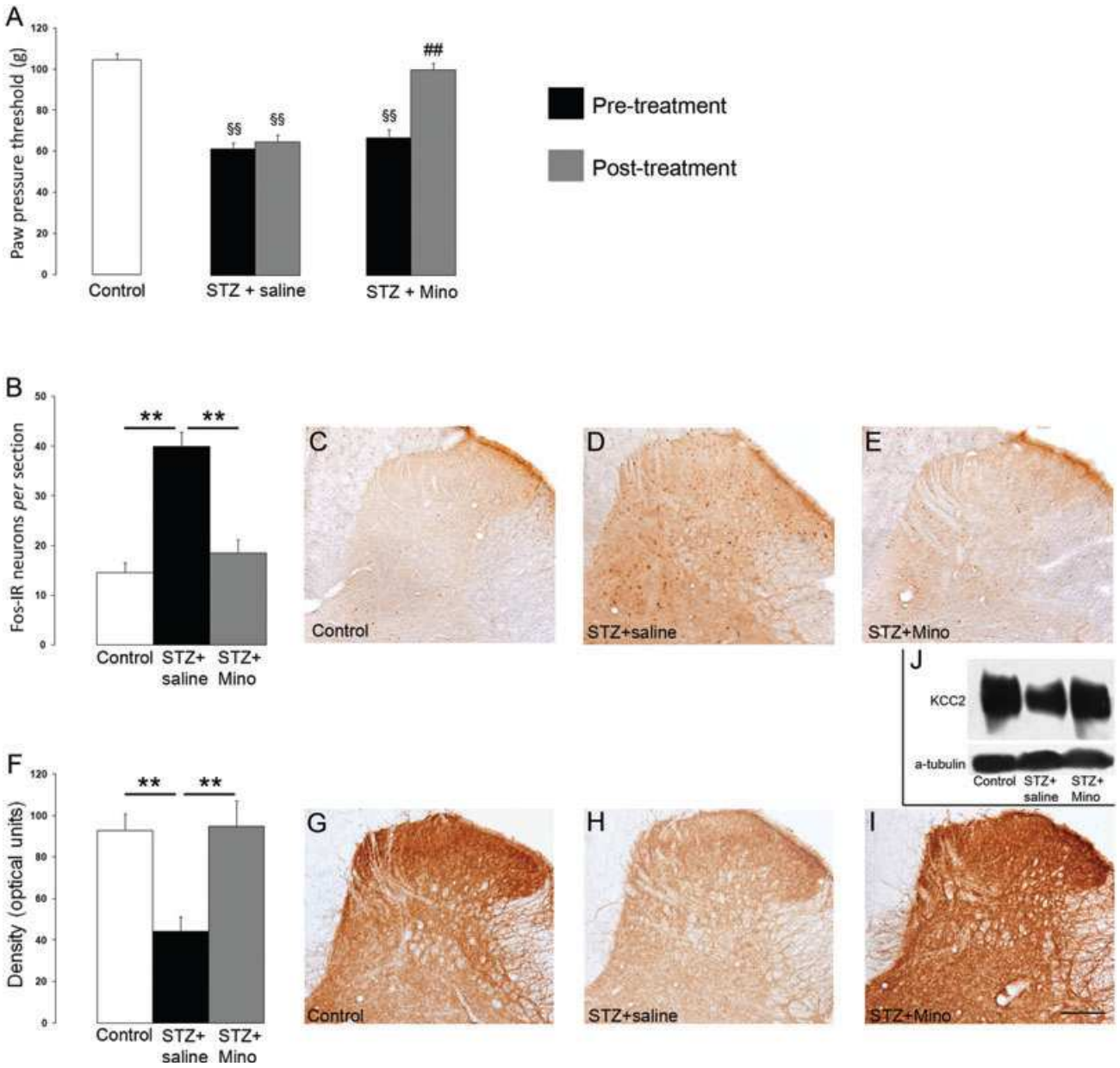

Figure 3. Effects of minocycline treatment on mechanical nociception (A), Fos expression (B–E) and KCC2 expression (F–J). Panel A shows the behavioural responses in the Randall-Selitto test in control and in streptozotocin (STZ)-diabetic rats, before (pretreatment) and after (posttreatment) injection of saline (STZ+saline) or minocycline (STZ+Mino). Panel (B) shows the mean numbers per section of Fos-immunoreactive (Fos-IR) neurons at the spinal dorsal horn of the L_4–L_5 segments in the three experimental groups (control, STZ+saline and STZ+Mino) and panels (C–E) show representative photomicrographs of the L_4 spinal cord sections. Panel (F) shows KCC2 expression at the spinal dorsal horn of the three experimental groups and panels (G–I) show representative photomicrographs of the L_4 spinal cord sections labelled for KCC2. Panel (J) shows representative blots of KCC2 in each experimental group. Minocycline treatment completely normalized mechanical nociception, totally reverted spinal hyperactivity and corrected KCC2 expression to control levels. Significance of symbols: #, comparison with pretreatment STZ+Mino animals; §, comparison with control animals; $^{**}p < 0.01$. Scale bar in (I): 100 µm (all photomicrographs are at the same magnification).

the spinal cord in traumatic neuropathic pain models [21]. In addition, increasing TrkB/Fc doses could affect motor functions and impair behavioural evaluation because BDNF is crucial for the function of motoneurons [32]. The suggestion that during diabetes the microglia-mediated effect on KCC2 expression is independent of BDNF represents a considerable difference of pain induced by diabetes from other chronic pain conditions and suggests that it is caused by specific mechanisms. Discarded a role for BDNF, several candidates could mediate the action of microglia in decreasing KCC2 expression, namely the cytokines interleukin (IL)-1β, tumour necrosis factor (TNF)-α, IL-6 and prostaglandin E2 (PGE2) [33,34]. In a recent study, an increase of IL-1β and TNF-α expressions in the spinal cords of STZ-diabetic rats was normalized after

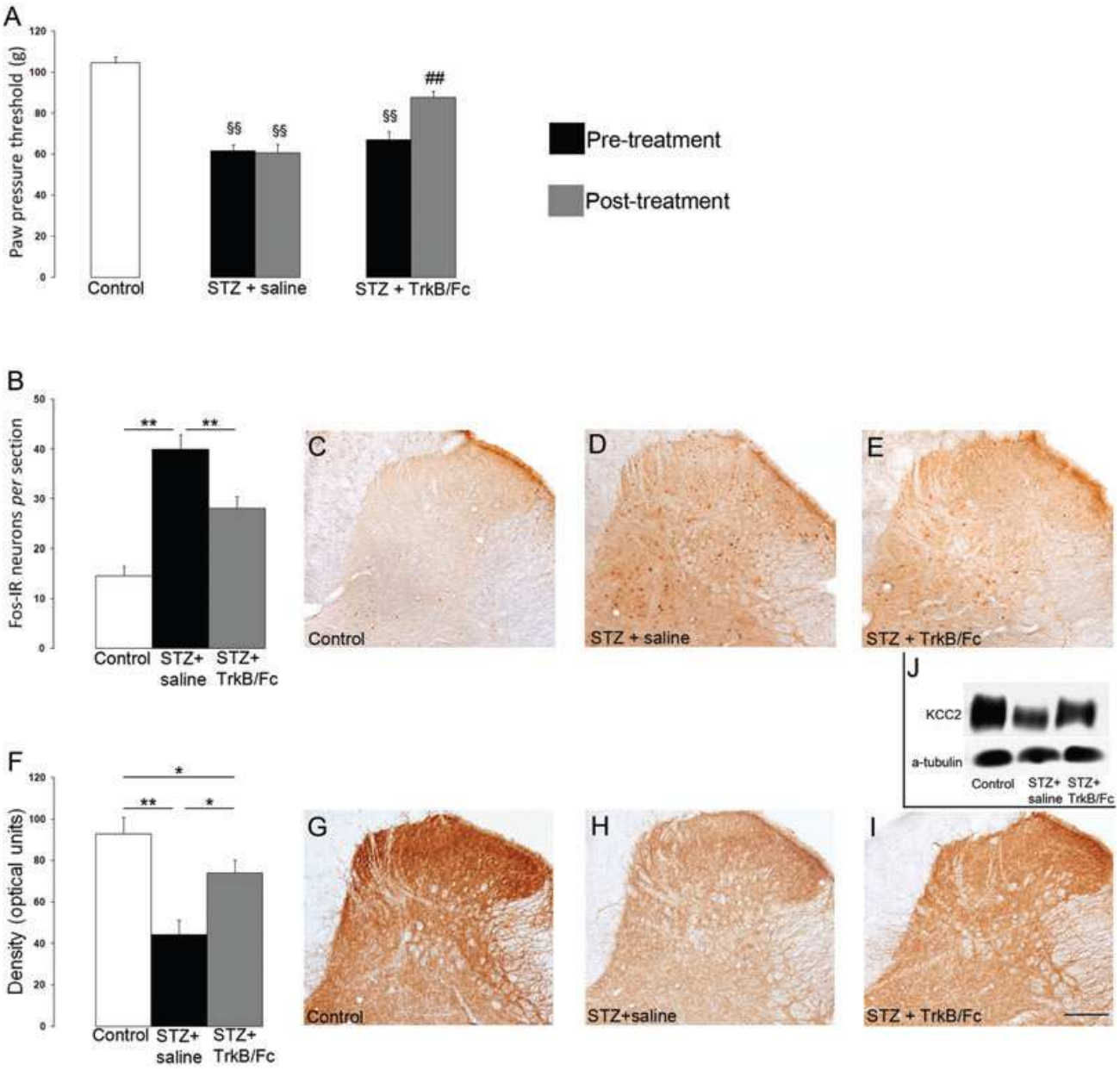

Figure 4. Effects of brain-derived neurotrophic factor (BDNF) action blockade on mechanical nociception (A), Fos expression (B–E) and KCC2 expression (F–J). Panel (A) shows behavioural responses in the Randall-Sellito test in control animals and in streptozotocin (STZ)-diabetic rats before (pretreatment) and after (posttreatment) injection of saline (STZ+saline) or TrkB/Fc (STZ+TrkB/Fc). Panel (B) shows the mean numbers per section of Fos-immunoreactive (Fos-IR) neurons at the spinal dorsal horn of control, STZ+saline and STZ+TrkB/Fc rats and panels (C–E) show photomicrographs of representative sections of L_4 segment immunoreacted for Fos in the three experimental groups. Panel (F) shows KCC2 expression at the spinal dorsal horn of control and STZ+saline or STZ+TrkB/Fc rats. Panels (G–I) show representative photomicrographs of the L_4 spinal sections labelled for KCC2. Panel (J) shows representative blots of KCC2 protein for each experimental group. Blocking BDNF action at the spinal cord of STZ rats, ameliorated mechanical nociception, reverted spinal hyperactivity and increased KCC2 expression but not to the levels of control animals. Significance of symbols: #, comparison with pretreatment STZ+TrkB/Fc animals; §, comparison with control animals; $^{*}p < 0.05$; $^{**}p < 0.01$. Scale bar in (I): 100 µm (all photomicrographs are at the same magnification).

minocycline treatment [17], indicating an involvement of these two cytokines. Future studies will be performed to evaluate the involvement of these cytokines, along with IL-6 and PGE2, in the changes of KCC2 expression during diabetes.

This study also shows that BDNF inhibition by TrkB/Fc has beneficial effects on behavioural pain responses and spinal neuronal hyperactivity during diabetes, despite the moderate effect on KCC2 expression. A direct effect of TrkB/Fc on spinal microglia is a possibility suggested by the reduction of microglial activation detected in the spinal cord of STZ-diabetic rats treated with TrkB/Fc. This is a new finding which may be explained by the recent demonstration that microglia

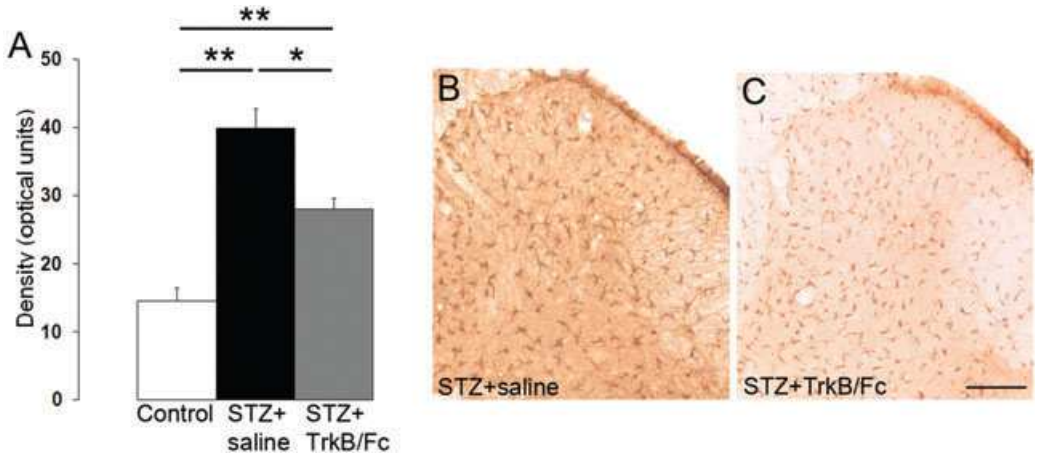

Figure 5. Effect of brain-derived neurotrophic factor (BDNF) blockade on microglial activation. Panel (A) presents the expression of CD11b (marker of microglia) at the spinal dorsal horn of control, streptozotocin (STZ)-diabetic rats injected with saline (STZ+saline) or treated with TrkB/Fc (STZ+TrkB/Fc). Panels (B) and (C) show photomicrographs of L_4 spinal sections labelled for CD11b in the STZ+saline and STZ+TrkB/Fc rats. TrkB/Fc reduced CD11b expression in STZ-diabetic rats, but it remained significantly higher than that from control rats. Significance of symbols: $^{*}p < 0.05$; $^{**}p < 0.01$. Scale bar in (C): 100 μm (all photomicrographs are at the same magnification).

Table 2. Brain-derived neurotrophic factor (BDNF) content in L_4–L_5 spinal segments.

Experimental groups	BDNF (ng/mg protein)
Control	0.47 ± 0.04
STZ+saline	0.56 ± 0.08
STZ+Mino	0.49 ± 0.04
STZ+TrkB/Fc	0.52 ± 0.05

Mean values ± standard error of the mean (s.e.m.). STZ, streptozotocin.

is activated by BDNF, through binding with a truncated tropomyosin-related kinase B (tTrkB) receptor expressed by microglial cells [35]. The antinociceptive effects of TrkB/Fc may also be derived from a BDNF action on spinal neurons. BDNF facilitates the release of GABA from spinal interneurons [36] and during diabetes the inhibitory action of GABA shifts to excitatory at the spinal cord [8,9]. It is, therefore, possible that BDNF blockade decreased GABA release in STZ-diabetic rats and, herein, reduced spinal neuronal excitability. Another possibility to explain the antinociception induced by TrkB/Fc is a blockade of excitatory neurotransmission at the spinal cord as BDNF induces the release of excitatory neurotransmitters and activates spinal glutamatergic receptors [37]. Finally, a direct action of BDNF on cAMP response element-binding protein phosphorylation is also possible, because this mechanism was previously shown to account for BDNF-induced increase in Fos expression in spinal neurons [37]. The mechanisms mediating the beneficial effects of BDNF in mechanical hyperalgesia and spinal Fos expression in STZ-diabetic rats needs further clarification.

In summary, this study shows for the first time that microglia inhibition corrects KCC2 expression during diabetes by mechanisms that are independent from BDNF action. As decreased expression of KCC2 affects spinal GABA neurotransmission, eliciting excitatory rather than inhibitory actions when binding to $GABA_A$ receptors [10,13] during diabetes [8], it is likely that the normalization of KCC2 expression at the spinal cord by minocycline treatment restored the inhibitory function of GABA at the spinal cord of diabetic rats. In fact, the relationship between KCC2 expression and GABA action was clearly demonstrated previously by showing that control animals intrathecally treated with a KCC2 inhibitor develop a hyperalgesic pattern of response similar to that of STZ-diabetic rats [8]. Those pain responses were totally reversed by $GABA_A$ receptor antagonist. In control animals, with normal expression of KCC2, neither the antagonist nor the agonist of $GABA_A$ receptor affected mechanical nociception [8]. As minocycline induced a complete normalization of KCC2 expression in our STZ-diabetic rats it is likely that treating the animals with antagonist or agonists of $GABA_A$ receptor will elicit the effects observed in control non-diabetic animals.

Translational consequences of the present data showing a complete reversal of mechanical hyperlagesia by intrathecal minocycline treatment should be considered in the future. The systemic or oral delivery of minocycline was shown to be effective in reversing DN [17,38]. Although these effects were interpreted only by peripheral actions of the drug and according to the present results, with intrathecal delivery of minocycline, the effects may be because of direct spinal actions. Minocycline is a second-generation antibiotic belonging to the tetracycline family, approved by the FDA for the treatment of acne vulgaris, some sexually transmitted diseases and rheumatoid arthritis [39]. In humans, long-term treatment with minocycline was shown to be generally safe and well tolerated. However, gastrointestinal and central nervous system reversible side effects (e.g. vestibular effects, benign intracranial hypertension) were reported, along with rare cases of autoimmune disorders, such as lupus and hepatitis [40]. Minocycline is being tested in several neurodegenerative diseases and other disorders, including spinal trauma and diabetes macular edema (http://www.clinicaltrials.gov). Clinical trials will be needed to

confirm the beneficial effects of minocycline on pain during DN, to evaluate the safety, efficacy and tolerability of this drug in comparison with the current first-line therapies for painful DN. These include tricyclic compounds, selective serotonin and noradrenaline reuptake inhibitors (such as duloxetine) and anticonvulsants (such as pregabalin or gabapentin), which present side effects and limited efficacy. New drugs that inhibit spinal microglia should be developed as a possible next step in the treatment of pain induced by diabetes.

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Conflict of Interest

The authors do not declare any conflict of interest relevant to this manuscript.

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Publication 4

Alpha-lipoic acid normalizes nociceptive neuronal activity at the spinal cord of diabetic rats

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ORIGINAL ARTICLE

α-Lipoic acid normalizes nociceptive neuronal activity at the spinal cord of diabetic rats

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Aim: To evaluate the effects of antioxidant treatment of streptozotocin (STZ)-diabetic rats with α-lipoic acid (α-LA) in neuronal and microglial activation at the spinal cord, an important relay station of nociceptive transmission. Because of the role of the potassium chloride co-transporter 2 (KCC2) in neuronal activation at the spinal cord and the influence of microglia in KCC2 expression, we also evaluated the effects of α-LA in KCC2 expression at the spinal cord.

Methods: Four weeks after STZ injection, the rats received daily intraperitoneal injections of α-LA (100 mg/kg), during 2 weeks. Mechanical nociception was evaluated before and after α-LA treatment. Spinal cords were immunoreacted against 8-OH-dG (marker of oxidative stress damage), Fos (marker of neuronal activation) and CD11b (marker of microglia). KCC2 expression was evaluated by immunohistochemistry and western blotting.

Results: Treatment with α-LA decreased the 8-OH-dG and Fos expressions to controls' levels, but did not affect CD11b. Treatment with α-LA alleviated mechanical hyperalgesia and partially corrected KCC2 expression.

Conclusions: This study shows that neuronal hyperactivity at the spinal cord of STZ-diabetic rats can be corrected by α-LA, which may account for alleviation of mechanical hyperalgesia. These effects are probably partially mediated by KCC2, but are independent from microglia.

Keywords: α-lipoic acid, animal pharmacology, c-fos, diabetes, diabetes complications, diabetic neuropathy, drug mechanism, KCC2, microglia, pain, spinal cord, type 1 diabetes

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Introduction

Oxidative stress damage has been implicated in a variety of pathological processes that underlie diabetic complications, as retinopathy [1], nephropathy [2] and neuropathy [3]. Diabetic neuropathy affects 30–50% of diabetic patients, with spontaneous and mechanical-evoked pain reported as major complaints by about one quarter of the patients [4]. Diabetes induces oxidative stress in peripheral nerves, which are probably to contribute to nerve dysfunction and altered pain sensations observed during diabetic neuropathy [3,5].

α-Lipoic acid (α-LA) is a powerful scavenger of free radicals and metal chelator [6]. In experimental models of diabetic neuropathy, antioxidant treatment with α-LA normalizes nerve blood flow, nerve conduction velocity and nociception [3,5]. In diabetic patients, α-LA significantly decreases oxidative stress [7], improves microcirculation [8] and reduces pain [9]. The mechanisms of action of α-LA on pain have only been studied at the periphery. Considering the ability of α-LA to cross the brain–blood barrier [10] and the observation that α-LA reverses the impairment in kinin (1) receptor expression at the spinal cord of insulin-resistant animal models [11], we hypothesized that α-LA has also central effects in painful diabetic neuropathy, namely at the spinal cord. This is important as it was previously showed that streptozotocin (STZ)-diabetic rats presented hyperactivity of spinal nociceptive neurons [12]. This increased neuronal activity was shown to be associated with a decreased expression of the potassium chloride co-transporter 2 (KCC2) at the spinal cord [13,14], which accounts for a shift in the action of γ-aminobutiric acid (GABA) from inhibitory to excitatory in the spinal dorsal horn of STZ-diabetic rats [13]. Spinal microglia activation was shown to account for those impairments [15]. Oxidative stress is an additional mechanism that may underlie changes in the expression and activity of KCC2 in the spinal cord during diabetes. In an oxidative stress model of hippocampal neuronal cultures, the expression and activity of KCC2 is decreased [16]. Antioxidants interfere with the inflammatory response of microglial cultures [17]. Oxidative stress may, therefore, trigger KCC2 impairments during diabetes, either by a direct neuronal action or by interfering with microglial activation.

This study evaluates the effects of an antioxidant treatment with α-LA in neuronal and microglial activation at the spinal cord of STZ-diabetic rats and investigates an involvement of KCC2.

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Materials and Methods

Animals

Male Wistar rats (aged 12 weeks; Charles River, Barcelona, Spain), weighing 250–300 g, were used. The animals were housed in a room with controlled temperature (22 ± 2° C) and humidity (55 ± 5%) and 12-h light/dark cycles. The animals received food and water *ad libitum*. Experiments were performed in accordance with the European Community Council Directive 86/609/EEC and the ethical guidelines for the study of pain in conscious animals [18].

Induction of Diabetes

Type 1 diabetes was induced by an intraperitoneal (i.p.) injection of STZ (60 mg/kg body weight; Sigma-Aldrich, Barcelona, Spain), dissolved in 0.1 M citrate buffer, pH 4.5. Control animals were injected with citrate buffer. Glucose concentration was measured in a blood sample from the tail vein, using Accu Chek Sensor Comfort (Roche Diagnostics, Berlin, Germany), 3 days after those i.p. injections and before sacrifice. Only rats with glucose concentrations higher than 270 mg/dl were included in the STZ-diabetic group.

Treatment Protocols

Four weeks after STZ injection, the rats received daily i.p. injections of α-LA (STZ + α-LA group) or saline (STZ + saline group) during the following 2 weeks. Both experimental groups included 10 rats. α-LA (Sigma-Aldrich) was used in a dose (100 mg/kg body weight) settled according to previous studies [3,5]. Groups of age-matched non-diabetic control rats and STZ-diabetic animals with 4 weeks of disease (STZ + 4 w group) were included in the study (n = 10 per group).

Behavioural Evaluation of Mechanical Nociception

All animals were handled daily by the experimenter for habituation purposes during 8 days prior to the onset of behavioural evaluation. Mechanical nociception was evaluated using the Randall-Selitto test (Ugo Basile, Comerio, Italy) at three timepoints: at the day prior to STZ or buffer injections, immediately before the treatment onset (pretreatment evaluation) and 90 min after the last α-LA or saline injections (post-treatment evaluation). The paw pressure threshold (PPT) was determined at the right hindpaw as described previously [15].

Immunohistochemistry

Five rats from each experimental group were deeply anaesthetized 3 h after the last treatment with an i.p. injection of 35% chloral hydrate (1 ml/kg body weight) and perfused with phosphate-buffered saline (PBS), followed by 4% paraformaldehyde in 0.1 M phosphate buffer (PB), pH 7.4 [12,15]. The spinal segments L4–L5 were removed, post-fixed and cryoprotected overnight in 30% sucrose in PB. Every fourth coronal frozen section, 40-µm thick, was collected in 0.1 M PBS. Each section set was immunohistochemically reacted for 8-OH-dG (marker of oxidative stress nucleic acid damage) [19], Fos protein (marker of nociceptive neuronal activation at the spinal cord) [20], CD11b (microglial marker) [21] or KCC2 [14], using the avidin–biotin–peroxidase method. The spinal cord sections immunoreacted for 8-OH-dG were pretreated with 2 N HCl during 10 min to denature nucleic acids, followed by 5 min immersion in Tris-base 1 M. Following treatment with 1% hydrogen peroxidase for 10 min to inhibit endogenous peroxidase activity, sections were incubated in a blocking solution of 10% normal serum in 0.3% Triton X 25% in PBS (PBST) with 0.1 M glycine for 2 h. The sections were then incubated two overnights at 4° C in each primary antibody, namely mouse anti-8-OH-dG (1 : 1600; Trevigen, Gaithersburg, MD, USA), rabbit anti-Fos (1 : 5000; Ab5; Calbiochem, Darmstadt, Germany), mouse anti-CD11b (clone OX-42; 1 : 2000, Millipore, Schwalbach/Ts, Germany) or rabbit anti-KCC2 (1 : 10000; Sigma-Aldrich), all diluted in PBST. After washings in PBST, the sections were incubated in biotinylated rabbit anti-mouse (for 8-OH-dG and CD11b detection) or swine anti-rabbit (for Fos and KCC2 detection), all from Dako (Glostrup, Denmark), diluted at 1 : 200, for 1 h. After washings in PBST, the sections were incubated for 1 h in the avidin–biotin complex (Vectastain, Vector Laboratories, Burlingame, CA, USA) and stained with diaminobenzidine [12,15]. Light microscope images were acquired by a blind-observer using a high-resolution digital camera coupled to a computer. In 10 randomly taken sections, immunolabelling for 8-OH-dG, CD11b and KCC2 was quantified by densitometric analysis at the spinal dorsal horn, using the NIH Image 1.52 software (US National Institutes of Health), as described previously [14]. The numbers of Fos-immunoreactive (Fos-IR) neurons were counted at spinal dorsal horn (laminae I–V), as described previously [22].

Western Blotting

The expression of KCC2 was quantified by western blotting in spinal homogenates of spinal segments L4–L5 of 5 animals per experimental group, as described previously [14]. Briefly, the membranes were incubated with the rabbit anti-KCC2 antibody used above (1 : 2000) and alfa-tubulin, used as an internal standard, was identified using a mouse anti-α-tubulin antibody (1 : 10000; Sigma-Aldrich, Spain). Digital images were analysed by densitometry using NIH Image 1.52 software, as described previously [14].

Specificity of Antibodies

Control experiments were performed by omission of primary or secondary antibodies. In the case of Fos experiments, the specificity of immunostaining was further confirmed by lack of immunoreactions in naive animals [22]. Specificity of KCC2 primary antibody was also showed by western blotting with band detected at 140 KDa [14]. The specificities of the anti-CD11b and anti-8-OH-dG antibodies were previously showed [19,21].

Statistical Analysis

SPSS Statistics version 18 (IBM, Armonk, NY, USA) was used for statistical analysis. Behavioural, imunohistochemistry and

Table 1. Body weights and glycaemia of experimental groups.

Experimental groups	Body weight (g)	Blood glucose concentration (mg/dl)
Control	409 ± 5.4	131 ± 18.7
STZ + 4 w	283 ± 7.3*	503 ± 28.4*
STZ + saline	287 ± 3.8*	492 ± 32.2*
STZ + α-LA	290 ± 2.4*	486 ± 61.2*

Mean values ± s.d. α-LA, α-lipoic acid; STZ, streptozotocin.
*$p < 0.01$ versus control.

western blotting data were compared by analysis of variance followed by Tukey *post hoc* test for multiple comparisons. Pretreatments and post-treatments behavioural responses were compared by paired sample *t*-test. Statistical significance was settled at $p < 0.05$. Results are expressed as mean ± standard error of the mean (s.e.m.).

Results

Characterization of Untreated STZ-Diabetic Rats

Untreated STZ-diabetic rats at 4 weeks post-injection and saline-injected STZ-diabetic rats presented significantly lower body weights and higher blood glucose levels than age-matched non-diabetic control animals (Table 1). STZ-diabetic rats presented increased responses to mechanical stimuli, showed by the lower PPTs in the Randall-Sellito test (figure 1).

STZ-diabetic rats also presented changes at the spinal dorsal horn, when compared to controls. The expression of 8-OH-dG was significantly higher in untreated and saline-treated STZ-diabetic rats in comparison with controls (figure 2), demonstrating increased oxidative stress nucleic acids damage during diabetes. STZ-diabetic rats presented higher numbers of Fos-IR neurons at the spinal dorsal horn than controls (figure 3A–E), indicating higher nociceptive activation of spinal neurons. The microglia of the former animals presented larger soma and thicker and shorter processes and increased expression of CD11b (figure 3F–J), which is considered an indication of microglial activation [15]. The expression of KCC2 was lower in STZ-diabetic rats (figure 4).

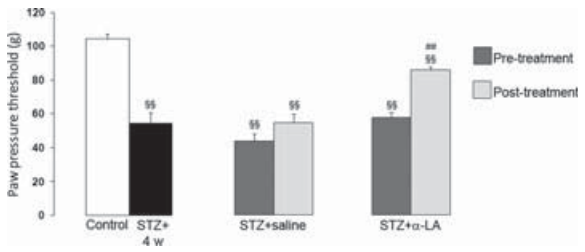

Figure 1. Effects of α-lipoic acid (α-LA) treatment on mechanical hyperalgesia. The graph represents the behavioural responses (paw pressure thresholds in the Randall-Sellitto test) in non-diabetic control animals, streptozotocin (STZ)-diabetic rats with 4 weeks of the disease (STZ + 4 w) and in STZ-diabetic rats before (pretreatment) and after (post-treatment) injections of α-LA or saline. The antioxidant treatment with α-LA ameliorated the mechanical nociception, but without normalization to the control levels. Significance of symbols: § comparison with non-diabetic control animals; # comparison with pretreatment STZ + α-LA group; two symbols: $p < 0.01$.

Effects of Treatment with α-LA

Treating STZ-diabetic rats with α-LA had no effects on body weights or blood glucose levels (Table 1), which is in accordance with previous studies using similar α-LA dose and even with higher treatment duration [3,5].

Behavioural Pain Responses

Treatment of STZ-diabetic rats with α-LA ameliorated mechanical nociception, with PPTs values intermediate between controls ($p = 0.009$) and STZ + saline rats ($p = 0.000$; figure 1).

Oxidative Stress Nucleic Acids Damage

Treatment of STZ-diabetic rats with α-LA significantly reduced the expression of 8-OH-dG to the levels of control animals (figure 2), showing that α-LA totally reversed the signs of oxidative stress nucleic acid damage at spinal dorsal horn during diabetes.

Spinal Neuronal and Microglia Activity

Treatment with α-LA decreased the number of Fos-IR neurons to the levels of control animals (figure 3A–E), indicating a reversal of nociceptive activation of spinal neurons after treatment. The α-LA had no effects on the CD11b microglial expression, with microglial cells maintaining the activated phenotype detected in untreated and saline-treated STZ-diabetic rats (figure 3F–J).

Spinal KCC2 Expression

Treatment with α-LA moderately increased the KCC2 immunolabelling to levels intermediate between controls ($p = 0.048$) and STZ + saline rats ($p = 0.031$) (figure 4A–F). These results were confirmed by western blotting (figure 4B).

Discussion

This study showed, for the first time, that α-LA blocks oxidative stress at the spinal cord and controls the activity of nociceptive spinal neurons during diabetes. In a manner similar to other pathologies, oxidative stress induced by diabetes is probably to decrease KCC2 expression at the spinal cord, which was blocked here by the antioxidant treatment with α-LA.

Antioxidant treatment with α-LA fully reversed both oxidative stress nucleic acids damage and neuronal hyperactivity at the spinal cord of STZ-diabetic rats. The mechanisms used by α-LA to reduce the neuronal hyperactivity at the spinal cord were investigated in the present study. Treatment with α-LA interferes with KCC2 expression at the spinal cord neurons, indicating that this co-transporter accounts for the α-LA-mediated reversion of neuronal hyperactivity during diabetes. Although microglia activation appears to account to the reduction of spinal KCC2 expression in STZ-diabetic rats [15], microglia does not appear to be involved in the effects of

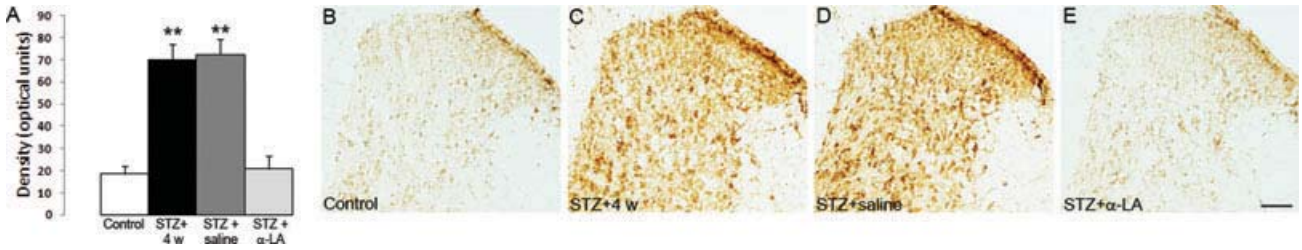

Figure 2. Effects of α-lipoic acid (α-LA) treatment on oxidative stress nucleic acids damage in the spinal dorsal horns. Panel (A) represents 8-OH-dG expression in control animals, streptozotocin (STZ)-diabetic rats with 4 weeks of the disease (STZ + 4 w) and STZ-diabetic rats treated with saline (STZ + saline) or α-LA (STZ + α-LA). Panels (B–E) are representative photomicrographs of L4 spinal cord sections labelled for 8-OH-dG. Damage induced in the nucleic acids by oxidative stress was significantly increased during diabetes and was reversed by α-LA. Significance of symbols: * comparison with non-diabetic control animals and STZ + α-LA rats, two symbols: $p < 0.01$. Scale bar: 100 μm. All photomicrographs are at the same magnification.

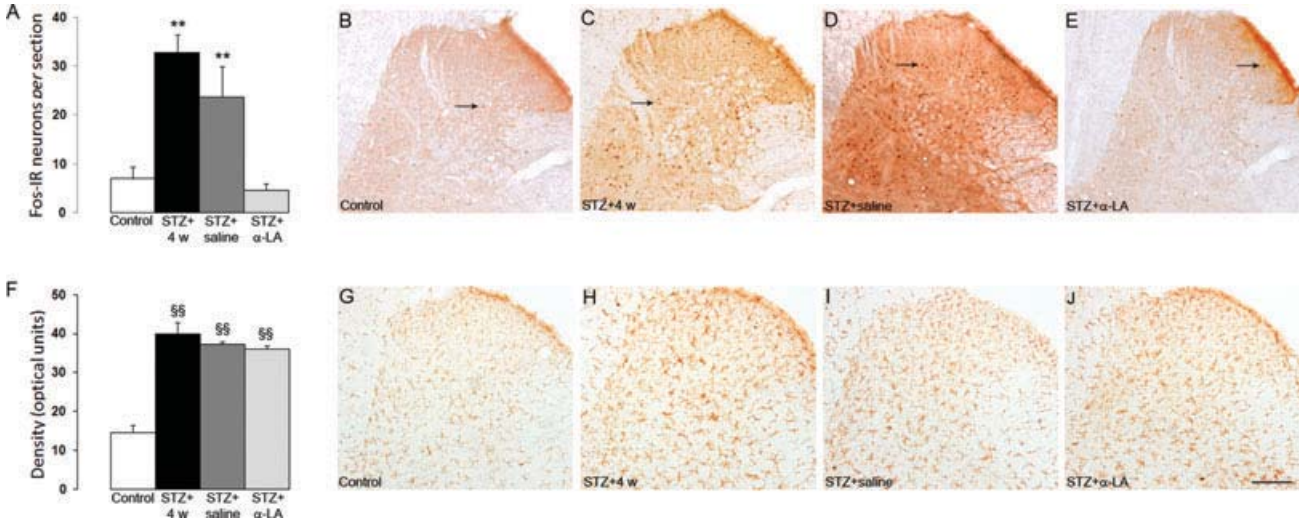

Figure 3. Effects of α-lipoic acid (α-LA) treatment on neuronal and microglial activity at the spinal dorsal horn. Panel (A) presents the mean numbers *per* section of Fos-IR neurons. Panels (B–E) are representative photomicrographs of L4 spinal cord section labelled for Fos (arrows) in control animals (B), streptozotocin (STZ) + 4 w (C), STZ + saline (D) and STZ + α-LA (E) rats. Panel (F) presents the CD11b expression and panels (G–J) are representative photomicrographs of L4 spinal cord sections labelled for CD11b in control animals (G), STZ + 4 w (H), STZ + saline (I) and STZ + α-LA (J) rats. α-LA reversed the neuronal hyperactivity observed in STZ + 4 w and STZ + saline rats to control levels, but had no effect on microglial activation. Significance of symbols: * comparison with non-diabetic control animals and STZ + α-LA rats; § comparison with non-diabetic control group; two symbols: $p < 0.01$. Scale bar: 100 μm. All photomicrographs are at the same magnification.

α-LA over KCC2 expression. As per the KCC2 role in neuronal function, this co-transporter sets the intracellular chloride concentration and influences the role of GABA when binding to $GABA_A$ ionotropic post-synaptic receptor. In normal conditions, GABA binding to $GABA_A$ receptors induces chloride influx inhibiting neuronal activity [23]. During diabetes, the reduced expression of KCC2 in the spinal cord leads to intracellular chloride accumulation and GABA binding to $GABA_A$ receptors induces chloride output, which results in neuronal excitation [13]. In agreement, intrathecal administration of bicuculline ($GABA_A$ receptor antagonist) in STZ-diabetic rats elicits antinociception [13]. The decrease in KCC2 expression has been shown to reduce KCC2 activity [16] and increase neuronal responses in pathological conditions, other than diabetes, namely in axotomy, seizures and traumatic neuropathic pain [23].

The beneficial effects of α-LA treatment on blocking behavioural signs of diabetic neuropathy have been ascribed to its actions on peripheral nerve function [3,5]. In our study, we cannot discard peripheral effects of α-LA treatment as we did not evaluate nerve function. We used a short α-LA treatment, which was implemented only after diabetic neuropathy was established. Although this treatment clearly reversed neuronal spinal hyperactivity, it was not so effective in mechanical nociception. This is probably because of the fact that peripheral nerve damage induced by diabetes is marked at the treatment onset and, therefore, could not be corrected by our protocol. Previous studies also reported an incomplete reversal of diabetes-induced peripheral nerve changes by α-LA, even with higher doses and longer treatment period [24]. Whether the higher effects of α-LA on the nociceptive activation of spinal neurons indicate a preferential site of action of α-LA at the spinal cord will be investigated by performing intrathecal deliveries of α-LA. KCC2 is unlikely to represent the sole mechanism of α-LA action, as the treatment completely reversed neuronal hyperactivity but elicited only a partial correction of KCC2

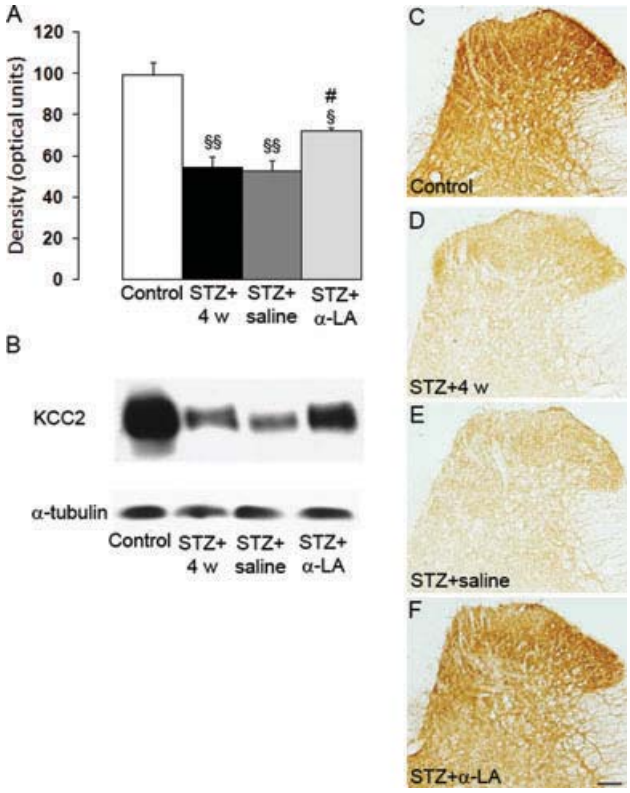

Figure 4. Effects of α-lipoic acid (α-LA) treatment on potassium chloride co-transporter 2 (KCC2) expression at spinal dorsal horn. Panel (A) presents KCC2 expression detected by immunohistochemistry. Panel (B) shows representative blots of KCC2 protein for each experimental group. Panels (C–F) are representative photomicrographs of L4 spinal cord sections immunoreacted for KCC2. Antioxidant treatment with α-LA increased KCC2 expression, but not to control levels. § comparison with non-diabetic control animals; # comparison with streptozotocin (STZ) + 4 w and STZ + saline groups; one symbol: $p < 0.05$; two symbols: $p < 0.01$. Scale bar: 100 µm. All photomicrographs are at the same magnification.

expression. The inhibitory effects of α-LA on spinal neuronal activity may also arise from its inhibitory actions on Ca(V)3.2 T-type calcium channels [25]. These channels are probably to be involved, as painful diabetic neuropathy is reversed by knockdown of Ca(V)3.2 T-type calcium channels through intrathecal administration of antisense oligonucleotides [26]. Future studies will evaluate the relative contribution of KCC2 and Ca(V)3.2 T-type channels in the α-LA effects at spinal cord during diabetes.

In summary, this study shows that α-LA presents central effects during diabetic neuropathy by reversing the neuronal hyperactivity at spinal dorsal horn, which could be partially mediated by its effect on KCC2 expression and seems to be independent from microglia activity. In a manner similar to other areas of the central nervous system, it is possible that oxidative stress in the spinal cord during diabetes decreases surface expression of KCC2 in spinal neurons, triggered by its dephosphorylation, which will induce functional changes of this co-transporter [16]. The beneficial effects of α-LA antioxidant treatment in reversing behavioural signs of diabetic neuropathy in STZ-diabetic rats fully match data with other antioxidants, namely the resveratrol [27] and vitamin E [28], pointing to the importance of increasing antioxidant intake by diabetic patients. As the diabetic neuropathy has common features in type 1 and type 2 diabetes, but α-LA increases insulin sensitivity and decreases glucose levels in the former, which does not occur in similar treatment protocol of STZ-diabetic rats [3], it is important to study if the neurobiological basis of the beneficial effects of α-LA in animal models of type 2 diabetes [29] are also based in corrections of KCC2 expression at the spinal cord. The effects of α-LA are not confined to pathological conditions as α-LA has also beneficial effects on healthy animals and humans, namely, by: (i) increasing the expression and activity of antioxidant enzymes [30]; (ii) reducing the oxidative stress damage [7]; and (iii) preventing neurodegenerative diseases [30]. These findings show that, besides its curative effects, α-LA is a health promoter by preventing the disease.

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Conflict of Interest

C. M. helped in the design, conduct/data collection, analysis and writing of the manuscript. P. P.-T. helped in the conduct/data collection. I. T. helped in design and writing the manuscript. All the authors have no conflict of interests.

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Publication 5

Neuronal hyperactivity at the spinal cord and periaqueductal grey during painful diabetic neuropathy: effects of gabapentin

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Neuronal hyperactivity at the spinal cord and periaqueductal grey during painful diabetic neuropathy: Effects of gabapentin

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ABSTRACT

Painful diabetic neuropathy may be due to impairments in descending modulation of nociceptive transmission at the spinal cord. In the present study, streptozotocin diabetic rats (STZ rats) with neuropathic symptoms (mechanical hypersensitivity) were used to perform a time-course evaluation of neuronal activity at the spinal dorsal horn and at the periaqueductal grey matter (PAG), a major brainstem area of pain modulation. The expression of Fos protein, a marker of nociceptive activation, progressively increased at the spinal dorsal horn at 4 and 10 weeks. At the PAG, increases in Fos expression were detected until the 4th week, with a reversal to baseline values at 10 weeks in all areas except the ventrolateral PAG. Co-localisation of Fos with NeuN ascertained the neuronal nature of Fos-expressing cells at the spinal cord and PAG. Four weeks after diabetes induction, the effects of gabapentin (i.p. injection of 50 mg/kg, daily during 3 days) were assessed. Gabapentin decreased Fos expression at the spinal cord and PAG and reversed mechanical hypersensitivity. The present study shows that diabetic neuropathy is accompanied by a progressive increase of the spontaneous neuronal activity at the spinal cord. Changes in descending modulation of nociceptive transmission from the PAG are likely to occur during diabetic neuropathy, probably with exacerbation of facilitatory actions. The effects of gabapentin in reversing the behavioural signs of diabetic neuropathy and neuronal hyperactivity in the spinal cord and PAG reinforce the central causes of diabetic neuropathy and point to the central targets of the drug.

1. Introduction

Diabetes is the most common cause of peripheral neuropathy (Mokdad et al., 2000). It is frequently associated with mechanical hyperalgesia, tactile allodynia and spontaneous pain (Courteix et al., 1993; Benbow et al., 1994; Pertovaara et al., 2001). The peripheral mechanisms of painful diabetic neuropathy are well established but the effects at the central nervous system remain poorly understood. The spinal cord is affected during diabetic neuropathy (Pertovaara et al., 2001; Eaton et al., 2001). Using the c-fos proto-oncogene as a marker of neuronal activation, an increased activity of spinal dorsal horn neurons was recently detected in streptozotocin-induced diabetic rats (STZ rats; Morgado and Tavares, 2007). This agrees with electrophysiological recordings showing increased activity of spinal neurons (Pertovaara et al., 2001), some of which belong to the spinothalamic tract (Chen and Pan, 2002). Our previous c-fos study was performed 4 weeks after diabetes induction. It is unclear if the increased c-fos expression is a transient reaction of spinal neurons to diabetes and if it reverses with the progression of the disease.

The causes of that spinal nociceptive neuronal hyperactivity during diabetic neuropathy remain unclear. It may derive from the constant barrage of peripheral input (Burchiel et al., 1985), but may also reflect impairments of descending modulation. The midbrain periaqueductal grey (PAG) plays a key role in descending pain control. It conveys to the spinal cord, pain modulation triggered at higher brain centres (Behbehani, 1995; Vanegas and Schaible, 2004). Increased neuronal activity at the PAG was proposed to account to traumatic neuropathic pain (Burgess et al., 2002). In STZ animals, imaging (Paulson et al., 2007) and pharmacology (Kamei et al., 1992) studies indicate that the PAG appears to be affected. Microinjection of higher doses of morphine in the PAG is necessary to induce analgesia in STZ rats when compared to other pain models, indicating an opioid-dependent dysfunction of the PAG during diabetic neuropathy (Kamei et al., 1992).

Gabapentin is a centrally-acting drug, frequently used as analgesic in diabetic patients and STZ rats (Cesenã and Calcutt, 1999; Vinik, 2005). Gabapentin inhibits glutamatergic excitatory neurotransmission at the spinal dorsal horn (Patel et al., 2000; Moore et al., 2002) and acts on brain regions involved in pain control, such as the locus coeruleus (LC; Hayashida et al., 2008). The effects of

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gabapentin in neuronal activity were never studied in diabetic neuropathy.

In the present study, we used STZ rats to: (i) perform a time-course study of neuronal activity at the spinal cord and PAG at 4 and 10 weeks, and (ii) evaluate the effects of gabapentin on behaviour and in neuronal activity in the spinal cord and PAG. Neuronal activity was monitored using the c-fos method since it is a reliable anatomical marker of activation by nociceptive-related stimuli (Hunt et al., 1987; Harris, 1998; Coggeshall, 2005). Contrarily to electrophysiological recordings, c-fos expression allows to evaluate large neuronal populations and to determine their precise anatomical locations (Coggeshall, 2005).

2. Methods

2.1. Animals

Adult male Wistar rats (12 weeks age, Charles River, Barcelona, Spain), weighing 250–350 g at the beginning of the experiments, were used. The animals were housed twice per cage in a room with a constant temperature (22 ± 2 °C) and humidity (55 ± 5%) and with 12 h light/dark cycle, and received food and water *ad libitum*. Experiments were performed in accordance with the European Community Council Directive 86/609/EEC and the ethical guidelines for the study of pain in conscious animals (Zimmermann, 1983).

2.2. Diabetic neuropathy

Type 1 diabetes was induced by an intraperitoneal (i.p.) injection of STZ (60 mg/kg body weight; Sigma–Aldrich, St. Louis, USA). Three days later, the glucose concentration was measured in a blood sample collected from the tail vein using Accu Chek Sensor Comfort (Roche Diagnostics, Germany). Only rats with glucose concentrations higher than 270 mg/dl were considered diabetic (Calcutt, 2004b), being included in the study as the "STZ" group.

Mechanical sensitivity is the pain modality more consistently changed in different models of diabetes (Courteix et al., 1993; Malcangio and Tomlinson, 1998; Fox et al., 1999; Pertovaara et al., 2001; Calcutt et al., 2004a; Morgado and Tavares, 2007; Morgado et al., 2008). Mechanical hyperalgesia and allodynia were here evaluated since they are typical in this rat strain (Malcangio and Tomlinson, 1998; Pertovaara et al., 2001; Morgado and Tavares, 2007; Morgado et al., 2008; Wodarski et al., 2009). Behavioural evaluation was performed before diabetes induction (time 0) and at 4 and 10 weeks using the paw pressure test (PPT, Ugo-Basile; Comerio, VA, Italy; $n = 5$) and the dynamic plantar aesthesiometer (DPA, Ugo-Basile; Comerio, VA, Italy; $n = 5$). During the 8 days prior to the tests, the animals were daily handled by the experimenter for habituation purposes. In the paw pressure test, ascending mechanical forces were applied to the dorsal surface of right hindpaw and the mechanical force (in grams) that induced hindpaw withdrawal was recorded. The paw withdrawal threshold (PWT) of each animal was the average of three consecutive measurements, taken with 5 min intervals. In the dynamic plantar aesthesiometer test, animals were placed in clear acrylic boxes with a metal grid floor and acclimatised for 15 min before testing. The stimulus was applied with a filament (0.5 mm), which applied a linearly increasing force (2.5 g/s) to the plantar surface of the hind paw. The force necessary to elicit a paw withdrawal was recorded. The PWT was calculated as the average of three consecutive tests with at least 5 min between each test. The statistical analysis was performed using One-Way Analysis of Variance (ANOVA) followed by Tukey *post hoc* test for multiple comparisons.

2.3. c-fos Evaluation

For the study of c-fos expression at the spinal cord and PAG, the STZ rats were deeply anaesthetised with an i.p. injection of 35% chloral hydrate (1 ml/kg body weight) and sacrificed at one of the following time-points ($n = 5$ *per* time-point): 0 (prior to STZ injection), 4 and 10 weeks. The anaesthesia was performed 30 min after completion of behaviour evaluation. Five addition STZ rats, naïve as to behavioural evaluation were perfused 4 weeks after STZ injection to evaluate Fos expression at the spinal cord and PAG. Vascular perfusion was performed with 200 ml of phosphate-buffered saline (PBS), followed by 1000 ml of 4% paraformaldehyde in 0.1 M phosphate buffer (PB), pH 7.4. In order to ascertain the glucose levels in STZ rats previously assessed by Accu Check Sensor Comfort, the colorimetric hexokinase assay (Olympus AU5400; Germany) was performed in 1 ml of blood collected immediately before vascular perfusion in non-fasting animals. Spinal segments L_4–L_5 and the brainstem were removed, post-fixed for 2–4 h in the same fixative, and cryoprotected overnight in 30% sucrose in PB. Coronal sections, 40 μm thick, were obtained using a freezing microtome, and every fourth section was collected in 0.1 M PBS. Fos protein was immunohistochemically detected using the avidin–biotin–peroxidase method, as described previously (Morgado and Tavares, 2007). Briefly, the sections were treated with 1% hydrogen peroxidase for 10 min to inhibit endogenous peroxidase activity before incubation in a blocking solution of 10% normal swine serum in 0.3% Triton X 100 in PBS (PBST) with 0.1 M glycine for 2 h. The sections were then incubated during two overnights at 4 °C in the primary antibody, rabbit anti-Fos (Ab5; Oncogene; Germany), diluted at 1:10,000 in PBST. After being washed in PBST, the sections were incubated in biotinylated swine anti-rabbit immunoglobulin (Dako, Denmark), diluted at 1:200, for 1 h, at room temperature. The sections were then washed in PBST, incubated for 1 h in the avidin–biotin complex (Vectastain, Vector Laboratories, USA) followed by a mixture of 10 mg diaminobenzidine, 5 μl of 30% hydrogen peroxide in 20 ml of Tris–HCl 0.05 M, pH 7.6. The numbers of Fos-immunoreactive (Fos-IR) cells occurring bilaterally in laminae I–II (superficial dorsal horn) and III–V (deep dorsal horn) were counted in 20 spinal sections in which laminae delimitation was performed according to Paxinos and Watson (Paxinos and Watson, 2005), as described previously (Morgado and Tavares, 2007). The numbers of Fos-IR cells occurring in 10 sections encompassing the PAG were counted. The PAG was subdivided in dorsal medial PAG (DMPAG), dorsal lateral PAG (DLPAG), lateral PAG (LPAG) and ventrolateral PAG (VLPAG), according to Paxinos and Watson (Paxinos and Watson, 2005). Mean numbers of Fos-IR cells *per* section were compared by ANOVA followed by Tukey *post hoc* test for multiple comparisons.

In order to determine the neuronal nature of Fos-IR cells, a double immunofluorescence reaction using NeuN as a neuronal marker was performed in one additional set of spinal and brainstem sections from animals with 4 and 10 weeks of diabetes. Briefly, the sections were treated with 1% borohydrate for 20 min before incubation in a blocking solution of 10% normal goat serum in PBST with 0.1 M glycine for 2 h. The sections were then incubated during two overnights at 4 °C in a mixture of the rabbit anti-Fos referred above (1:5000) and mouse anti-NeuN (1:300; Chemicon, Germany). After being washed in PBST, the sections were incubated, for 1 h at room temperature, in a mixture of two fluorescent secondary antibodies: goat anti-rabbit Alexa 488 and goat anti-mouse Alexa 594 (both at 1:1000; Molecular Probes, USA). Sections were observed using an Apo Tome microscope (Zeiss, Germany) and the images were acquired using a high-resolution digital camera coupled to a computer. For the acquisition of higher magnifications, serial optical planes separated by 0.5 μm were collected in the *z*-axis. Merged images were obtained using the Apo Tome software.

2.4. Gabapentin treatment

Four weeks after STZ injection, the rats received a daily i.p. injection of gabapentin ("GBP" group, 50 mg/kg; Merck, Portugal) or saline ("saline" group) during 3 days. The gabapentin dose was defined based on the analgesic effects in several pain models, including diabetic neuropathy (Cesenã and Calcutt, 1999; Wodarski et al., 2009). The behavioural responses were evaluated 30 min after the last gabapentin injection using PPT and DPA tests, as described above. An additional non-diabetic age-matched control animal group, not submitted to any treatment, was also evaluated ("control" group).

All animals were sacrificed by vascular perfusion about 1 h after the last gabapentin injection. The spinal segments L_4–L_5 and the brainstems were removed, and immunohistochemically processed for Fos detection. Fos-IR cells in the spinal cord and PAG were counted following the experimental protocols mentioned above (see c-fos *evaluation*). The mean numbers of Fos-IR cells *per* section were compared by independent sample *t* test. Behavioural data analysis was performed by ANOVA followed by Tukey *post hoc* test for multiple comparisons.

3. Results

3.1. Characterisation of experimental groups

STZ rats presented significant lower body weights and higher blood glucose concentrations at 4 and 10 weeks when compared with values obtained prior diabetes induction (Table 1). In the two behavioural tests (PPT and DPA), the PWTs of STZ rats were significantly decreased at 4 week post-injection and remained low until the 10th week, confirming the painful neuropathic condition. No differences in PWTs were detected from 4 to 10 weeks (Table 1).

3.2. c-fos Expression at spinal cord and PAG

Only a few Fos-IR cells were detected prior to STZ injection in the spinal dorsal horn (Fig. 1A, B and E). Statistically significant increases in the numbers of Fos-IR neurons were detected at 4 weeks (Fig. 1A, C and F), and even in a more pronounced manner at 10 weeks (Fig. 1A, D and G), with statistical differences between the two time-points. The increases in Fos expression affected similarly the superficial (laminae I–II) and deep dorsal horn (laminae III–V). No differences in Fos expression in the spinal cord were detected between STZ rats subjected to behavioural evaluation and STZ rats naïve as to behavioural evaluation (superficial dorsal horn: evaluated rats 46.2 ± 16.4 vs. 43.9 ± 16.2 in non-evaluated; deep dorsal horn: evaluated rats 158.0 ± 69.0 vs. 131.0 ± 69.4 in non-evaluated).

In all PAG sub-regions, the expression of Fos was very low before STZ injection (Fig. 2A, B and E). Significant increases in the numbers of Fos-IR cells were detected in all PAG sub-regions at 4 weeks (Fig. 2A, C and F), which persisted only at the VLPAG at the 10th week, whereas in other PAG areas a reversal to baseline levels was detected (Fig. 2A, D and G). No differences in Fos expression in the PAG were detected between STZ rats subjected to behavioural evaluation and STZ rats naïve as to behavioural evaluation (DMPAG: evaluated rats 43.0 ± 5.1 vs. 36.0 ± 15.2 in non-evaluated; DLPAG: evaluated rats 48.0 ± 7.0 vs. 45.5 ± 21.2 in non-evaluated; LPAG: evaluated rats 122.0 ± 9.7 vs. 92.2 ± 42.2 in non-evaluated; VLPAG: evaluated rats 65.8 ± 9.1 vs. 67.0 ± 23.5 in non-evaluated).

Double-immunostaining for Fos and NeuN at the spinal cord (Fig. 3A–C) and PAG (Fig. 3D–F) confirmed that the expression of Fos was confined to neurons, since a total co-localisation was detected both at 4 and 10 weeks post-injection.

Table 1
Body weights, glycemia and PWT of experimental groups.

STZ rats		Week 0	Week 4	Week 10
Weight (g)		303 ± 8.6	282 ± 13.4[a]	286 ± 10.4[a]
Glycemia (mg/dl)		118 ± 6.3	824 ± 98.7[a]	666 ± 66.3[a]
PWT (g)	PPT	110 ± 6.7	68 ± 13.8[a]	69 ± 10.9[a]
	DPA	24 ± 1.4	14 ± 1.1[a]	13 ± 1.1[a]

Time-course changes at weights, glycemia and mechanical nociceptive behavioural responses (PPT and DPA behavioural tests) of STZ rats along the time of diabetes. Mean values ± SEM.

[a] $p < 0.01$ vs. week 0.

3.3. Effects of gabapentin treatment

Gabapentin treatment improved mechanical nociception, restoring PWTs to values similar to age-matched non-diabetic control rats both in the PPT (Fig. 4A) and the DPA (Fig. 4B) behavioural tests. The glycemic values and body weights of gabapentin-treated rats were similar to STZ rats with the same time of diabetes (blood glucose concentration (mg/dl): STZ rats: 824 ± 98.7 vs. Gabapentin-treated STZ rats: 787 ± 86.2; body weights (g): STZ rats: 282 ± 13.4 vs. Gabapentin-treated STZ rats: 286 ± 10.6).

Gabapentin-treated rats presented significant decrease in spinal Fos expression when compared with saline-treated rats, both at the superficial and deep dorsal horn (Fig. 4C). The numbers of Fos-IR neurons in all PAG sub-regions were also reduced by gabapentin treatment (Fig. 4D).

4. Discussion

This study demonstrates that diabetes is associated with overtime increases in Fos expression at the spinal cord. In the PAG, the increases in Fos expression occurred in all regions at 4 weeks whereas at 10 weeks they were maintained only at the VLPAG. Gabapentin treatment at 4 weeks prevents the increases in Fos expression and reverses the behavioural signs of diabetic neuropathy.

Our study shows that Fos expression at the spinal cord and PAG of diabetic rats occurs exclusively in neurons. This is important since Fos expression may occur in glia (Reissig et al., 2008) and glial activation increases in diabetic rats (Wodarski et al., 2009; Tsuda et al., 2008). The increased neuronal activity at the spinal cord of STZ diabetic rats detected agrees with previous reports (Pertovaara et al., 2001; Chen and Pan, 2002; Morgado and Tavares, 2007) whereas the increased neuronal activity at the PAG of diabetic rats was here reported for the first time. The value of the analysis of Fos expression to monitor the nociceptive activity of large neuronal populations is solid (Coggeshall, 2005) and is reinforced by the present findings. Fos expression was previously demonstrated at the spinal cord in several chronic pain models in the absence of additional stimulation, which also occurred in STZ rats (Williams et al., 1991; Kajander et al., 1996; Catheline et al., 1999; Tokunaga et al., 1999; Bester et al., 2000; Dai et al., 2001; Kosai et al., 2001; Tsai et al., 2002; Coggeshall, 2005; Jergova and Cizkova, 2005; Morgado and Tavares, 2007). In the brain of STZ rats, Fos expression was shown to increase in some regions (Zheng et al., 2002; Revsin et al., 2005) and decrease in others (Zheng et al., 2002; Jang et al., 2003; Gouty et al., 2003), showing that it cannot be a direct result of STZ or indicate putative physiological disturbances of diabetic animals. That the gabapentin treatment decreased Fos expression at the spinal cord and PAG and reversed

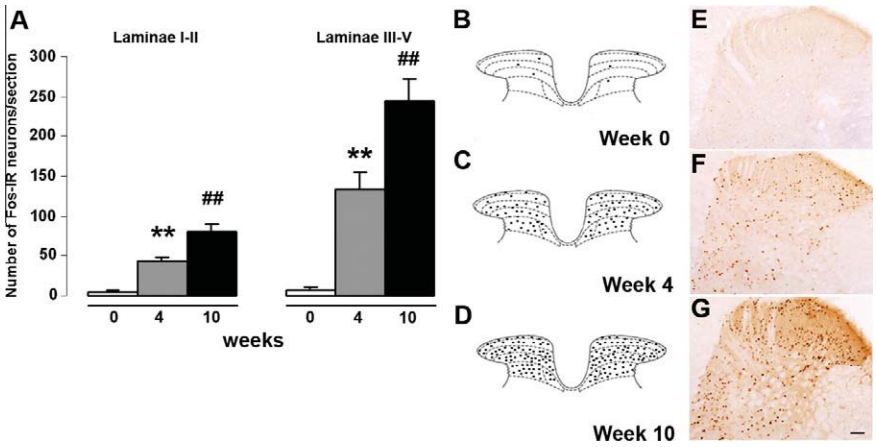

Fig. 1. Time-course changes in Fos expression in the spinal dorsal horn of segments L_4–L_5. Panel A depicts the mean numbers (SEM) of Fos-IR neurons *per* section and panels B–D show diagrams of L_4 illustrating Fos immunoreaction in rats prior (B) and at 4 (C) and 10 (D) weeks after induction of diabetes. Each dot represents two Fos-IR neurons. Panels E–F are photomicrographs of Fos labelled neurons in rats prior to diabetes induction (E) and at 4 (F) and 10 (G) weeks post-induction. The numbers of Fos-IR neurons increased significantly at 4 and 10 weeks after diabetes induction, affecting the superficial (laminae I–II) and deep (laminae III–V) dorsal horn. Significance of symbols: $^{**}p < 0.01$, week 4 vs. all other time-points, $^{\#\#}p < 0.01$, week 10 vs. the other time-points. Scale bars in G: 100 μm (all photomicrographs are at the same magnification).

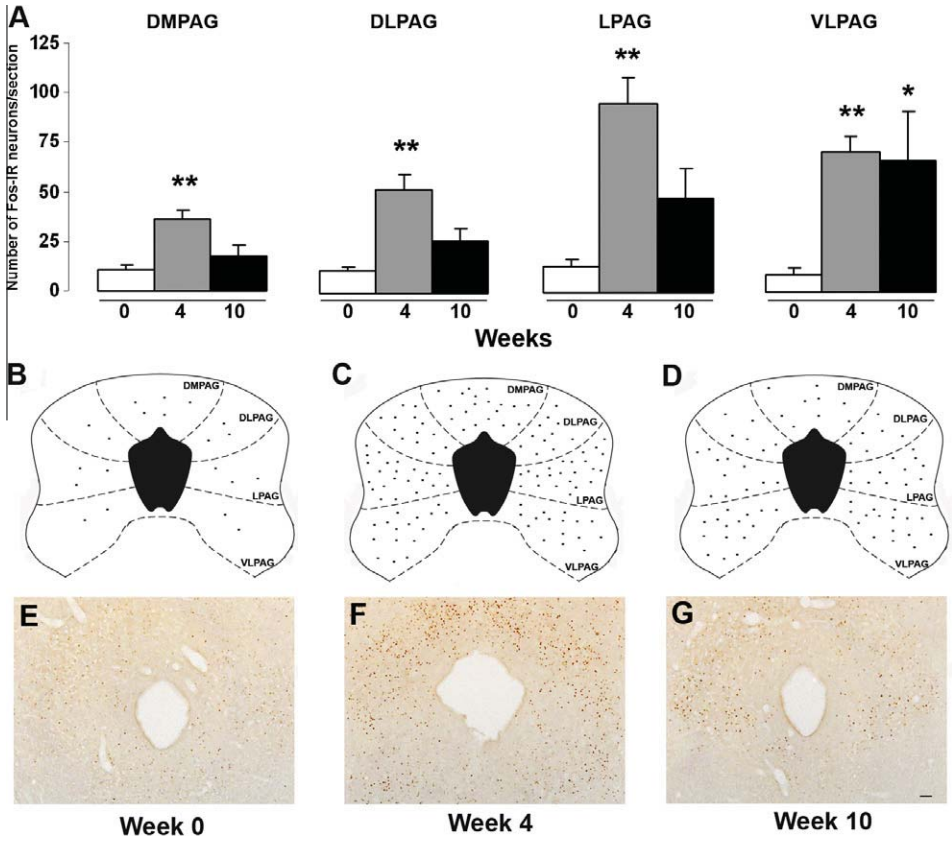

Fig. 2. Time-course changes in Fos expression at PAG. Panel A presents the mean numbers (SEM) of Fos-IR neurons *per* section in the DMPAG, DLPAG, LAPG and VLPAG. Panels B–D show diagrams of the PAG illustrating the distribution of Fos immunoreaction in STZ rats prior (B) and 4 (C) and 10 (D) weeks after diabetes induction. Each dot represents two Fos-IR neurons. Panels E and F are photomicrographs showing Fos labelled neurons in rats prior to diabetes induction (E) and at 4 (F) and 10 (G) weeks post-induction. A significant increase in Fos levels was observed at VLPAG in STZ rats at 4 and 10 weeks. *Comparison with time-point 0 weeks. One symbol: $p < 0.05$, two symbols: $p < 0.01$. Scale bars in G: 100 μm (all photomicrographs are at the same magnification).

mechanical hyperalgesia in STZ rats further reinforces that Fos expression is independent of the diabetic condition.

Previous studies showing increased activity of spinal neurons only evaluated one time-point and did not perform time-course analysis (Pertovaara et al., 2001; Chen and Pan, 2002; Morgado and Tavares, 2007;). By showing that Fos expression at the spinal cord increases along the time of diabetes (4 and 10 weeks), the present study indicates, for the first time, that spontaneous neuronal activity at the spinal cord increases along the time of the disease. Another new result of the present study is the higher increase in Fos at the deep dorsal horn of L_4–L_5 segments when compared with our previous reports at T_{13}–L_3 segments (Morgado

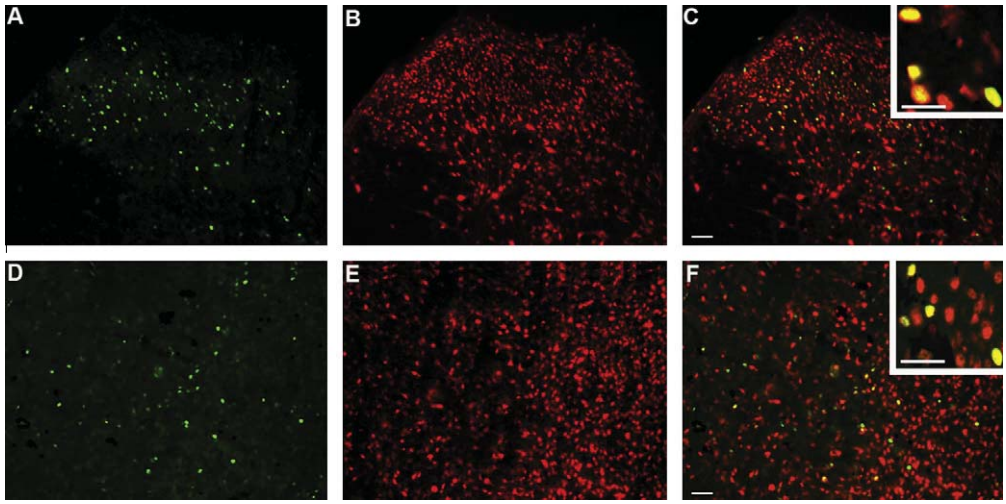

Fig. 3. Photomicrographs of sections with double immunodetection of Fos (green, A, D) and NeuN (red, B, E) at the spinal dorsal horn (A–C) or VLPAG (D–F) of rats 4 weeks after diabetes induction. In C and F the merged images demonstrate the total co-localisation of Fos and NeuN. The insets show details of the labelling after optical sectioning (18 optical sections). Scale bar in C and F: 50 μm; scale bar of insets: 25 μm.

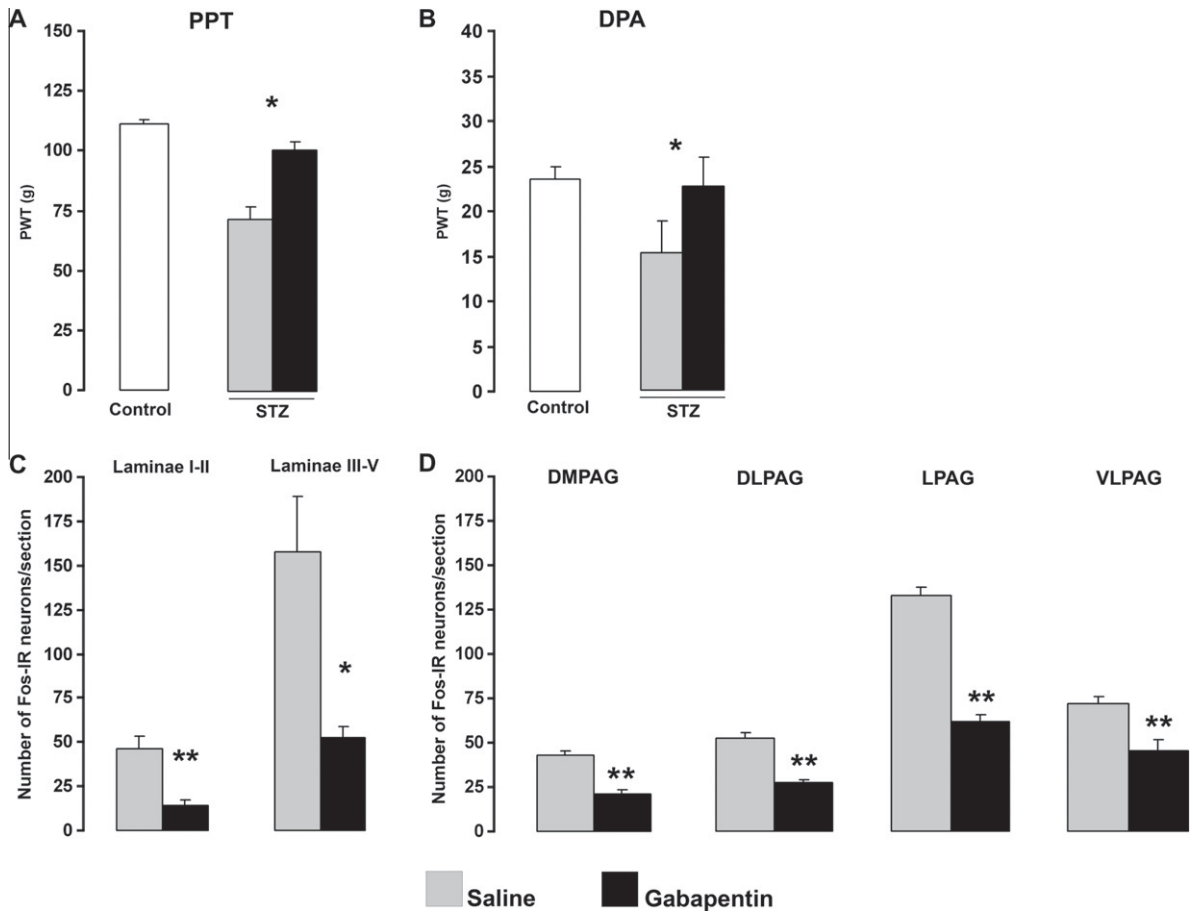

Fig. 4. Effects of gabapentin (GBP) treatment in paw withdrawal thresholds (A, B), and Fos expression at the spinal dorsal horn (C) and PAG (D). Gabapentin significantly increased the paw withdrawal threshold to levels similar to the non-diabetic "control" group both in PPT (A) and DPA (B) behavioural tests. Fos expression at the spinal dorsal horn and PAG was significantly lower in STZ rats treated with gabapentin than in saline-inject STZ rats. Significance of symbols: $^{*}/^{**}p < 0.05/p < 0.01$ – Gabapentin- vs. saline-injected rats.

and Tavares, 2007). It is possible that these differences are related to the fact that L_4–L_5 segments receive input from the hindlimb and a prevalence of a distal affectation occurs in diabetes (Calcutt, 2004b). The overtime increase in spinal c-fos expression was not accompanied by parallel increases in mechanical sensitivity. Several explanations may be raised for the dissociation between Fos

expression and behavioural analysis. A positive correlation between the magnitude of spinal Fos expression and behavioural responses is only achieved if Fos is directly evoked by the behavioural tests (Presley et al., 1990; Bullit et al., 1992; Herdegen and Leah, 1998). In the present study, Fos expression was not elicited by the behavioural tests since no differences were detected between diabetic rats naïve to behavioural evaluation and diabetic rats that underwent behavioural analysis. This agrees with our previous results showing that innocuous stimulation during 2 h does not increase Fos expression in the spinal cord (Morgado and Tavares, 2007). Diabetic neuropathy appears to differ from inflammatory pain, in which a brief innocuous stimulus is enough to trigger Fos expression at the spinal cord (Ma and Woolf, 1996; Pinto et al., 2007). The need to evaluate if spontaneous pain increases along the time of diabetes, as suggested by the present data, may be impaired by the lack of reliable methods to study spontaneous pain in animals, a major caveat of basic pain research (Chizh et al., 2009). Another issue to evaluate is if the increase in Fos expression in the spinal cord of STZ rats is accompanied by Fos increases at the dorsal root ganglion (DRG) neurons, which seems likely since other pain studies demonstrate parallel increases in the spinal cord and DRGs (e.g. Zhang et al., 2009). Since diabetic rats present increased spontaneous primary afferent activity (Burchiel et al., 1985) it is expected that DRGs neurons of these animals present higher Fos expression.

The data on increased neuronal activation at the PAG of STZ rats deserves a special analysis since it was recently suggested that neuronal activity at the PAG of STZ rats is reduced using a blood-flow dependent imaging technique (Paulson et al., 2007). Besides differences in animal strain, dose of STZ for diabetes induction and the time-point evaluated (6 weeks), the opposite findings may be due to some limitations of blood-flow dependent techniques to study the diabetic brain, inasmuch that impaired brain flow and vasoreactivity occur during diabetes (Last et al., 2007). The magnitude and time-dependent increases in Fos expression at the PAG exhibited regional changes that may be explained by the role of the PAG sub-regions. Only the VLPAG maintained higher Fos expression at 10 weeks. The VLPAG is the area that conveys the majority of descending modulatory input (Behbehani, 1995; Keay and Bandler, 2004). It is likely that increased facilitation of nociceptive transmission from the PAG occurs during diabetic neuropathy, in a manner similar to other chronic pain models (Vanegas and Schaible, 2004; Lovick, 2008). The PAG, long considered to inhibit pain transmission at the spinal cord, was recently shown to exert also facilitation (Vanegas and Schaible, 2004; Lovick, 2008). Besides the increase in facilitation, a decrease in the inhibitory effects of the PAG could also occur. This is supported by the fact that an important contingent of VLPAG neurons is made of GABAergic interneurons that express opioid receptors (Vaughan et al., 1997; Commons et al., 2000) and higher doses of morphine need to be injected in the PAG of STZ rats to induce analgesia (Kamei et al., 1992). Studies that assess the neurochemical nature of Fos-IR neurons at the VLPAG, along with pharmacological manipulations of the VLPAG will be performed to fully evaluate the implications of the present findings. Besides indicating that nociceptive modulation from the VLPAG is progressively impaired during diabetes, our results show increases in Fos expression in other PAG areas only at 4 weeks, namely at the LPAG, DMPAG and DLPAG. The LPAG is involved in responses to "escapable" physical stressors, such as acute noxious stimulation (Keay and Bandler, 2004) whereas the DMPAG and DLPAG are mainly activated in response to fear and anxiety (Behbehani, 1995; Keay and Bandler, 2004). These results suggest that it is important to evaluate if those physiological parameters (stress, fear and anxiety) are higher at 4 than at 10 weeks of diabetes or if the decreases at 10 weeks are due to adaptation of CNS to such conditions.

Gabapentin was shown to reduce neuronal activity at the spinal cord and PAG and strongly ameliorate mechanical nociception. The mechanisms of the analgesic action of gabapentin during diabetic neuropathy are unclear. Our study suggests that the inhibition of neuronal hyperactivity at the spinal cord and PAG could account for the analgesic efficacy of gabapentin in diabetic neuropathy. The present study does not clarify the local of action of gabapentin. It may act directly on the spinal cord, reducing ascending transmission of nociceptive input. Gabapentin may also reduce neuronal activity at the PAG activity and decrease neuronal activation at the spinal cord, by depressing descending facilitation. An action of gabapentin in other brain areas cannot be excluded inasmuch that is know to target brain areas devoted to pain control, such as the LC (Hayashida et al., 2007, 2008). Instillation of gabapentin in the PAG and intrathecal administration will be performed in STZ rats. Besides determining the central targets of gabapentin in diabetic neuropathy, we will also study the role of glia. A role for microglia in the increase of neuronal activity at the spinal cord was demonstrated in several neuropathic pain models (Cao and Zhang, 2008; Inoue and Tsuda, 2009; Owolabi and Saab, 2006), namely of diabetic neuropathy (Tsuda et al., 2008; Wodarski et al., 2009). Taking into account that gabapentin reverses microglia hyperactivity in the spinal cord of diabetic rats (Wodarski et al., 2009), it is important to evaluate if gabapentin-induced decrease in neuronal spinal activity is mediated by inhibition of the microglia.

In conclusion, diabetes induced long-term hyperactivity at the spinal dorsal horn and PAG, which seems to be due to central effects of the disease. It is now important to study more deeply the central mechanisms namely to establish if they are only caused by the increased bidirectional transmission between the spinal cord and the brain, as in several chronic pain conditions (Terayama et al., 2000; Pinto et al., 2006) or if specific features of diabetes, such as insulin deprivation, may also affect the function of spinal and PAG neurons (Freychet, 2000). The knowledge of the central mechanism involved in pain during diabetic neuropathy can provide the substrate for the development of more effective and selective pharmacological analgesics for painful diabetic neuropathy.

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Publication 6

Changes in serotoninergic and noradrenergic descending pain pathways during painful diabetic neuropathy: the preventive action of IGF1

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Changes in serotoninergic and noradrenergic descending pain pathways during painful diabetic neuropathy: The preventive action of IGF1

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ABSTRACT

Painful diabetic neuropathy (PDN) induces neuronal hyperactivity at the spinal cord and periaqueductal gray (PAG), a key area in descending nociceptive modulation. Since the PAG uses relay stations at serotoninergic and noradrenergic brainstem areas, we determined the serotonin and noradrenaline levels at the spinal cord of streptozotocin-diabetic rats and at those brainstem areas (serotoninergic rostroventromedial medulla and noradrenergic A_5 and A_7 cell groups). Since, during diabetes, the levels of insulin growth factor 1 (IGF1) decrease, reducing its neurotrophic effect in the brain, we also studied the effects of IGF1 treatment. One week after diabetes induction, subcutaneous injections of IGF1 (2.5 mg/kg) were performed during 3 weeks. Body weights, glycemia, and mechanical nociception were weekly evaluated until the end of the study, the time when the animals were subjected to a modified formalin test to study chemical allodynia. Serotonin and noradrenaline levels were quantified by ELISA at the spinal cord, whereas at the brainstem, the quantification was performed by immunohistochemistry against, respectively, tryptophan hydroxylase (TpH) or tyrosine hydroxylase (TH). STZ-diabetic rats exhibited mechanical hyperalgesia and chemical allodynia, along with higher spinal levels of serotonin and noradrenaline and higher numbers of neurons expressing TpH at the RVM and TH at the A_5 noradrenergic cell group. Treatment with IGF1 prevented the behavioral signs of PDN and reversed the neuronal hyperactivity at the spinal cord and ventrolateral PAG and the neurochemical changes at the spinal cord and at the brainstem. Based on the facilitatory role of serotoninergic and noradrenergic descending modulation during chronic pain, the increased serotonin and noradrenaline innervation of the dorsal horn in STZ-diabetic rats may probably account for enhanced pain during PDN. The benefits of IGF1 in PDN are probably due to blockade of the increased peripheral input to the somatosensory system, but direct central actions cannot be discarded. The value of IGF1 in PDN treatment deserves further evaluation.

Introduction

Neuropathy affects a large portion of diabetic patients. Their quality of life is further reduced due to pain complaints, which occurs in about one quarter of patients with diabetic neuropathy (Quattrini and Tesfaye, 2003; Tesfaye, 2009). Painful diabetic neuropathy (PDN) is characterized by spontaneous pain, mechanical hyperalgesia, and tactile allodynia (Tesfaye, 2009). It is accompanied by functional and neurochemical changes at peripheral nerves, spinal cord, and supraspinal pain control areas (Sima, 2003; Selvarajah et al., 2006, 2008; Morgado and Tavares, 2007; Morgado et al., 2010). Streptozotocin-diabetic rats (STZ-diabetic rats) present spontaneous activity of spinal dorsal horn neurons (Pertovaara et al., 2001; Chen and Pan, 2002; Morgado and Tavares, 2007; Morgado et al., 2010). Increased neuronal activity was recently demonstrated in a key brainstem center of pain modulation—the periaqueductal gray matter (PAG; Morgado et al., 2010). The PAG does not project directly to the spinal cord but uses relay stations located at the brainstem, with a well-known involvement of the rostroventromedial medulla (RVM; Millan, 2002; Heinricher et al., 2009). Descending nociceptive modulation is performed by the release of serotonin and noradrenaline at the spinal cord (Millan, 2002). Serotoninergic innervation of the spinal dorsal horn is mainly originated from the RVM. Noradrenergic innervation derives mainly from the A5, A6, and A7 cell groups of the brainstem, with variable contributions of each area in different rat strains (Proudfit, 2002). Whereas descending modulation is known to inhibit acute pain, during chronic pain situations, an imbalance of inhibitory and facilitatory actions occurs (Porreca et al., 2002). Impairment of descending inhibition and enhancement of facilitation are proposed to increase nociceptive transmission at the spinal cord during chronic pain (Tracey and Mantyh, 2007). The participation of the RVM and noradrenergic cell groups in potentiating chronic pain was demonstrated in inflammatory (Howorth et al., 2009) and traumatic neuropathic pain models (Ma and Eisenach, 2003;

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Hayashida et al., 2008), but with variable degrees depending on the pain condition (Porreca et al., 2002). During PDN, the role of serotoninergic and noradrenergic areas in descending modulation was never studied. Inconsistent results were obtained as to the levels of serotonin in the brainstem and spinal cord of STZ-diabetic rats (Sandrini et al., 1997; Padayatti and Paulose, 1999; Gallego et al., 2003). As to the noradrenergic system, diabetes-induced increases in the levels of noradrenaline were reported at the brainstem and spinal cord (Bitar et al., 1999; Bitar and Pilcher, 2000). However, the brainstem areas that are affected by diabetes and account for those changes were never evaluated. Supporting a role of descending serotoninergic and noradrenergic systems in PDN, antidepressant drugs that act on the reuptake of serotonin and noradrenaline are effective in PDN treatment (Tesfaye, 2009).

Hyperglycemia is no longer considered the exclusive etiological factor of PDN (Sima, 2003; Pierson et al., 2003). It has been suggested that insulin growth factor 1 (IGF1) deprivation, which occurs in type 1 diabetes, may account to the higher severity of PDN in this type of diabetes, in comparison to type 2 (Ishii, 1995; Schmidt et al., 1999; Sima and Kamiya, 2006; Zhuang et al., 1996). Further supporting a role of IGF1 in diabetic neuropathy, it was shown that the reduction of circulating levels of IGF1 is greater in patients with neuropathy than in those without neuropathic complaints (Migdalis et al., 1995; Guo et al., 1999). IGF1 exhibit glycemia-independent neuroprotective effects, preventing peripheral nerve damage and promoting nerve regeneration, which may account for the analgesic ability of IGF1 during PDN (Ishii, 1995; Schmidt et al., 1999; Zhuang et al., 1996; Piriz et al., 2009). Suppression of IGF1 actions and downregulation of IGF1 receptors was demonstrated in the brain of diabetic rats (Li et al., 2005; Zhang et al., 2008; Sima et al., 2009), but no studies were ever directed to the specific brainstem areas involved in descending pain control. Considering the aforementioned information and the occurrence of IGF1 receptors at the brainstem (Folli et al., 1994), we performed a double-goal study. First, we wanted to measure the levels of serotonin and noradrenaline in the spinal cord of STZ-diabetic rats and the respective contribution of the serotoninergic and noradrenergic brainstem centers referred above. Second, we aimed to evaluate if treatment with IGF1 doses that do not reverse hyperglycemia affects the behavioral signs of PDN, neuronal hyperactivity at the PAG and at the spinal cord, and serotoninergic and noradrenergic brainstem systems.

Materials and methods

Animals

A total of 40 male Wistar rats (Charles River Laboratories, Barcelona, Spain), weighing 280–300 g at the beginning of the experiments, were used. The experiments were performed in accordance with the ethical guidelines of the European Community Council Directive 86/609/EEC and of the International Association for the Study of Pain in conscious animals (Zimmermann, 1983).

Animals were housed 2 per cage, in a room with constant temperature (22 ± 2 °C) and humidity (55% ± 5%) and a 12-h light/dark cycle, and received food and water ad libitum. The animals were daily handled by the experimenter for habituation purposes and were used according to the general experimental procedures summarized in Fig. 1.

Induction of diabetes

Diabetes was induced in 20 rats by an intraperitoneal (i.p.) injection of STZ (60 mg/kg body weight) dissolved in 1 M citrate buffer (pH = 4.5). Twenty rats received equal volumes of the vehicle (control group). Three days after the injections, glucose concentration was measured in tail vein blood samples using a glucose oxidase impregnated test strip (Accu Chek Sensor Comfort, Roche Diagnostics, Germany). Only rats with glucose concentration higher than 270 mg/dl were considered diabetic and included in the STZ group. All the animals were weekly weighed and daily observed during the study.

Treatment protocol

One week after vehicle or STZ injection, the rats were injected subcutaneously with 2.5 mg/kg body weight of recombinant human IGF1 (STZ + IGF1 group; $n = 10$; control + IGF1 group; $n = 10$) or 300 µl of saline (STZ + saline group; $n = 10$; control + saline group; $n = 10$). All animals were injected 3 times per week during 3 weeks. The injections were performed at the same time of the day (10 h, a.m.) and were evenly distributed throughout the week (Mondays, Wednesdays, and Fridays). Immediately before the injections of IGF1 or saline, blood glucose concentrations were measured as above

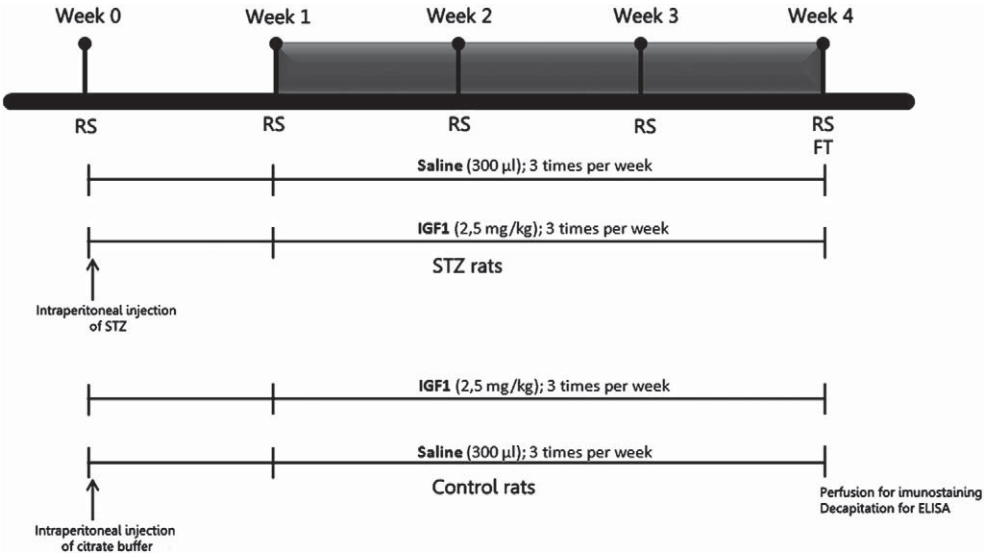

Fig. 1. Schematic overview of the experimental procedures. Each experimental group included 5 animals. Behavioral evaluation was performed using the Randall–Selitto (RS) and formalin (FT) tests. Animals were sacrificed at 4 weeks post-injection by vascular perfusion or decapitation. Spinal cord and brainstem sections were immunoreacted against Fos protein, TpH, or TH and noradrenaline and serotonin contents were quantified in lumbar spinal cord homogenates.

to confirm the maintenance of hyperglycemia in STZ-diabetic rats. Prior to the sacrifice, 1 ml of blood was collected by cardiac punction and non-fasting glucose levels were quantified by the colorimetric hexokinase assay (Olympus AU5400; Germany), as described previously (Wright et al., 1971).

Behavioral tests

Mechanical nociception was weekly evaluated using the Randall–Selitto test (Ugo-Basile, Comerio, Italy), as described previously (Morgado et al., 2010). Briefly, an increasing pressure was applied onto the dorsal surface of right hindpaw with a cone-shaped plunger. The paw withdrawal threshold (PWT) was defined as the force, in grams, at which the rat withdrew the paw. The PWT of each animal was the average of three consecutive measurements, taken at 5-min intervals.

At the day after the last injection of IGF1 or saline, a formalin test modified to evaluate chemical allodynia in STZ-diabetic rats was performed as described previously (Freshwater and Calcutt, 2005). Briefly, the animals were injected with 50 µl of 0.2% formalin into the right hindpaw dorsum, immediately placed in an individual open Plexiglas chamber (10×20×24 cm) and their behavior was recorded during the following 60 min. The total number of paw flinches of the injected paw or hindquarters was counted by an investigator unaware of the experimental design. Contrary to the original formalin test, this modified test with a low formalin concentration does not evoke the characteristic 3 phases of responses (Freshwater and Calcutt, 2005). The modified formalin test induces almost no behavioral effects in control rats whereas in STZ-diabetic rats it triggers a marked monophasic period of flinching that ceases within an hour of injection (Freshwater and Calcutt, 2005). The value of the test to evaluate chemical allodynia has been described (Calcutt, 2004; Freshwater and Calcutt, 2005).

Sacrifice of the animals

Two hours after formalin injection, the animals were deeply anesthetized with an i.p. injection of 35% chloral hydrate (1 ml/kg body weight) and sacrificed by transcardiac vascular perfusion or decapitation ($n=5$ from each experimental group and sacrifice method). Vascular perfusion was performed with 200 ml of phosphate-buffered saline (PBS), followed by 1000 ml of 4% paraformaldehyde (4% PFA) in 0.1 M phosphate buffer (PB), pH 7.4. The spinal segments L4–L5 and the brainstems were removed and post-fixed for 2–4 h in 4% PFA and cryoprotected overnight in 30% sucrose in 0.1 M PBS at 4 °C or immediately storage at −80 °C.

ELISA

Serotonin and noradrenaline contents were evaluated in lumbar spinal cord homogenates from all animals, using the following research ELISA kits from LDN, Germany: BA E-5900 for serotonin and BA 10-5200 for noradrenaline. ELISA assays were performed according manufacturer's instructions.

Immunohistochemistry

Coronal sections, 40 µm thick, were obtained using a freezing microtome. One of each 4 sections of spinal cord and brainstem levels comprising the PAG were immunoreacted against Fos protein, as previously described (Morgado et al., 2010). Brainstem sections comprising the RVM were processed for immunodetection of TpH and those encompassing the A5–A7 noradrenergic cell groups were immunoreacted for TH. Briefly, all sections were treated with 1% hydrogen peroxidase for 10 min to inhibit the activity of endogenous peroxidase before incubation in a blocking solution of 10% normal serum in 0.3% Triton X 25% in PBS (PBST) with 0.1 M glycine for 2 h. The sections were then incubated two overnights at 4 °C in rabbit anti-Fos (1:10,000; Ab5, ref. PC38; Calbiochem, Germany), sheep anti-TpH (1:1000; ref. AB1541; Millipore, Spain), or rabbit anti-TH (1:6000; ref. OPA1-04050; Affinity BioReagents, Germany) primary antibodies. After being washed in PBST, the sections were incubated in biotinylated swine anti-rabbit immunoglobulin for Fos and TH (ref. E0353; Dako, Denmark) and in donkey anti-sheep immunoglobulin for TpH (ref. B-7390; Sigma-Aldrich, Spain), all diluted at 1:200, for 1 h, at room temperature. Sections were then washed in PBST, incubated for 1 h in the avidin–biotin complex (Vectastain, Vector Laboratories, USA), and stained with diaminobenzidine (10 mg diaminobenzidine in 20 ml of Tris–HCl 0.05 M, pH 7.6 solution with 5 µl of 30% hydrogen peroxide). Sections were mounted on gelatin-coated slides, air-dried, and coverslipped with xylol. Fos-immunoreactive (Fos-IR) neurons occurring in the spinal dorsal horn (laminae I–VI) ipsilateral to the stimulated hindlimb were counted in 10 sections. For the PAG, countings were performed in 10 randomly selected sections. Only the ventrolateral part of the PAG (VLPAG) was counted since it is the PAG area with more pronounced increases in Fos expression during PDN (Morgado et al., 2010). Twenty brainstem sections comprising the RVM were processed for immunodetection of TpH and 20 sections that encompassed the A5–A7 noradrenergic cell groups were immunoreacted for TH. Neurons immunoreactive for TpH (TpH-IR) were counted in all immunoreacted sections encompassing the RVM. Neurons immunoreactive for TH (TH-IR) were quantified in all sections presenting A5 and A7 noradrenergic cell groups. The A6 noradrenergic cell group was not quantified since, in Wistar rats, this nucleus does not contribute strongly to the noradrenergic innervation of the spinal cord (Tavares et al., 1996, 1997) and a lower participation in such innervation implies a reduced contribution for pain modulation (West et al., 1993). Delimitation of spinal cord laminae, VLPAG, RVM, A5, and A7 noradrenergic cells groups was performed according to Paxinos and Watson (2005).

In order to ascertain the neuronal nature of the immunoreactive cells for Fos, TpH, or TH, each immunoreaction was combined with immunostaining for the neuronal marker NeuN. Double immunofluorescence reactions were performed in additional spinal and brainstem sections of STZ-diabetic rats. Briefly, the sections were treated with 1% borohydrate, for 20 min, before incubation in a blocking solution containing 10% normal horse serum in PBST with 0.1 M glycine, for 2 h. The sections were then incubated during 2 overnights at 4 °C in a mixture of a mouse anti-NeuN (1:300; ref. MAB377; Chemicon, Germany) and each primary antibody referred above for single immunoreactions, but in a higher concentration (anti-Fos at 1:5000; sheep anti-TpH at 1:500; rabbit anti-TH at 1:3000). After being washed in PBST, the sections were incubated, for 1 h at room temperature, in a mixture of two fluorescent secondary antibodies: goat anti-mouse Alexa 488 (ref. A-11001) for NeuN combined with donkey anti-rabbit Alexa 594 (ref. A-21207) for Fos or TH and donkey anti-sheep Alexa 568 (ref. A-21099) for TpH. All secondary antibodies were diluted at 1:500 (Molecular Probes, USA). Sections were examined using an ApoTome microscope (Zeiss, Germany) and the images were acquired using a high-resolution digital camera coupled to a computer. For the acquisition of higher magnifications, serial optical sections, separated by 1 µm, were collected in the *z* axis. Merged images were obtained using the ApoTome software.

Considering the role of extracellular signal-regulated kinases (ERKs) and cAMP response element-binding protein (CREB) as promoters of TpH and TH mRNA synthesis, respectively (Wood and Russo, 2001; Shimizu et al., 2004), and as markers of neuronal activation (Ma and Quirion, 2001; Pancaro et al., 2003; Obata and Noguchi, 2004), we quantified the expression of phosphorylated ERKs (pERKs) and phosphorylated CREB (pCREB) in the TpH and TH-labeling neurons. This was done to assess if the expression of TpH and TH during diabetes is also mediated by these promoters and to infer

about neuronal activation of serotoninergic and noradrenergic neurons. Double immunofluorescence reactions were performed to compare the expression of pERKs in TpH-IR neurons along with the expression of pCREB in TH-IR neurons between control and untreated STZ rats. Briefly, the sections were treated with 1% borohydrate for 20 min before incubation during 2 h in a blocking solution of 10% suitable normal serum in PBST. One set of sections were then incubated with rabbit anti-pERKs (1:200; ref. 4370; Cell Signaling, Germany) and sheep anti-TpH (1:500; Millipore, Germany) and other set was incubated with rabbit anti-pCREB (1:1000; Millipore, Germany) and mouse anti-TH (1:2000; ref. T1299; Sigma-Aldrich, Spain), during two overnights at 4 °C. After being washed in PBST, the sections were incubated in donkey anti-rabbit Alexa 488 (ref. A21206) for pERKs, donkey anti-sheep Alexa 568 for TpH, goat anti-rabbit Alexa 488 (ref. A11034) for pCREB, or goat anti-mouse Alexa 594 (ref. A-11005) for TH (Molecular Probes, USA) fluorescent immunoglobulins diluted at 1:500, for 1 h at room temperature. Sections were observed using an ApoTome microscope (Zeiss, Germany) and the images were acquired using a high-resolution digital camera coupled to a computer. Serial optical planes separated by 1 μm were collected in the *z*-axis and merged images were obtained using the ApoTome software. The numbers of TpH-IR neurons and cells double-labeled for TpH and pERKs (TpH^+pERKs^+ neurons) were counted at the RVM. Neurons immunoreactive for TH and those presenting double labeling (TH^+pCREB^+ neurons) were quantified at A5 and A7 noradrenergic cell groups. The percentage of double labeling in the respective neurochemical type was compared between control and untreated STZ animals. The percentages of control animals were considered as the basal levels (equal to 1) and the results for STZ group are presented as fold-increase over the control percentages.

Specificity of antibodies

The specificity of each primary antibody was previously demonstrated (Haycock, 1989; Pinto et al., 2007; Pinna et al., 2007; Riedel et al., 2008; Wu et al., 2009; Malleret et al., 2010). Negative control experiments included the omission of primary or secondary antibodies.

Drugs

Streptozotocin was purchased from Sigma-Aldrich (Barcelona, Spain) and recombinant human IGF1 (mecasermin; Increlex®) was kindly donated by Ipsen Portugal.

Statistical analysis

SPSS Statistics version 18 (IBM, Armonk, New York, USA) was used for statistical analysis. Data were compared by one-way analysis of variance (ANOVA) followed by Fisher LSD post-hoc test for multiple comparisons. Time-course analysis of behavioral data was compared by ANOVA repeated measures for each experimental group. Chi square test (χ^2 test) was used for analysis of proportions of double immunofluorescence results. Statistical significance was settled as $p<0.05$. Results are presented as mean ± SEM.

Results

All STZ-diabetic animals developed hyperglycemia at 3 days post-injection. Saline-treated STZ rats presented a progressive slight weight loss (±5 g per week) during the study (Fig. 2A). IGF1 treatment did not affect the blood glucose concentration in STZ rats, which remained above 270 mg/dl during all the study (Table 1) but attenuated the body weight decrease, without normalization to the levels of non-diabetic control rats (Fig. 2A). IGF1 treatment of non-diabetic rats had no effects in body weight (Fig. 2A).

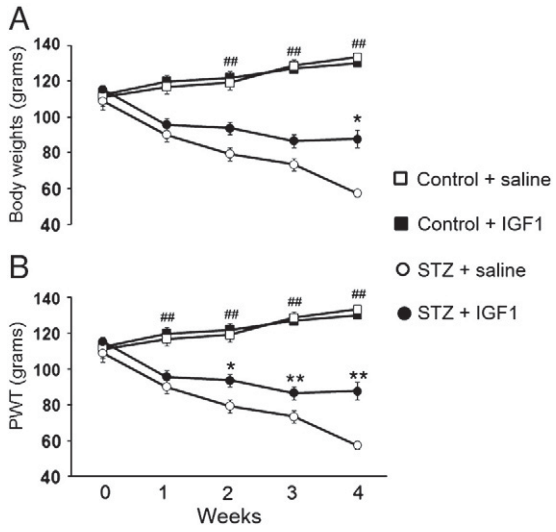

Fig. 2. Body weights and mechanical nociception of the experimental groups. Panel A presents the body weight changes of saline-treated (control + saline; white squares) and IGF1-treated (control + IGF1; black squares) control rats and saline-treated (STZ + saline; white circles) and IGF1-treated (STZ + IGF1; black circles) STZ-diabetic rats. Panel B presents the time-course changes in mechanical nociception in the 4 experimental groups. Treatment with IGF1 prevented the progressive weight loss and mechanical hyperalgesia observed in STZ + saline rats. Significance of symbols: # indicates comparison between controls and STZ groups; * indicates comparison between STZ + saline and STZ + IGF1. One symbol: $p<0.05$; two symbols: $p<0.01$.

Behavioral responses

STZ-diabetic rats presented a significant decrease in PWTs at 1 week post-injection of STZ (Fig. 2B). The PWTs of STZ + saline rats decreased progressively during the 4 weeks of study. The PWTs of STZ-diabetic rats treated with IGF1 remained unchanged after treatment onset. At 4 weeks post-injection, STZ + IGF1 rats presented significantly higher PWTs than STZ + saline rats, but significantly lower than non-diabetic control rats treated with IGF1 or injected with saline (Fig. 2B). IGF1 treatment had no effects on PWTs in non-diabetic control animals (Fig. 2B).

Paw flinches induced by formalin injections in STZ + saline rats were significantly higher than in non-diabetic control animals (Fig. 3A), indicating the existence of chemical allodynia, as described in previous studies (Freshwater and Calcutt, 2005). Treatment with IGF1 prevented the increase in the number of paw-flinches in STZ-diabetic rats since STZ + IGF1 rats presented responses similar to control animals (Fig. 3A). IGF1 treatment had no effects in the responses to formalin injections in non-diabetic control animals (Fig. 3A).

Table 1
Blood glucose concentrations of experimental groups.

Experimental group	Blood glucose concentration (mg/dl)
Control + saline	121 ± 7.1[a]
Control + IGF1	129 ± 9.1[a]
STZ + saline	785 ± 87.3
STZ + IGF1	777 ± 91.4

[a] $p<0.001$ vs. STZ groups.

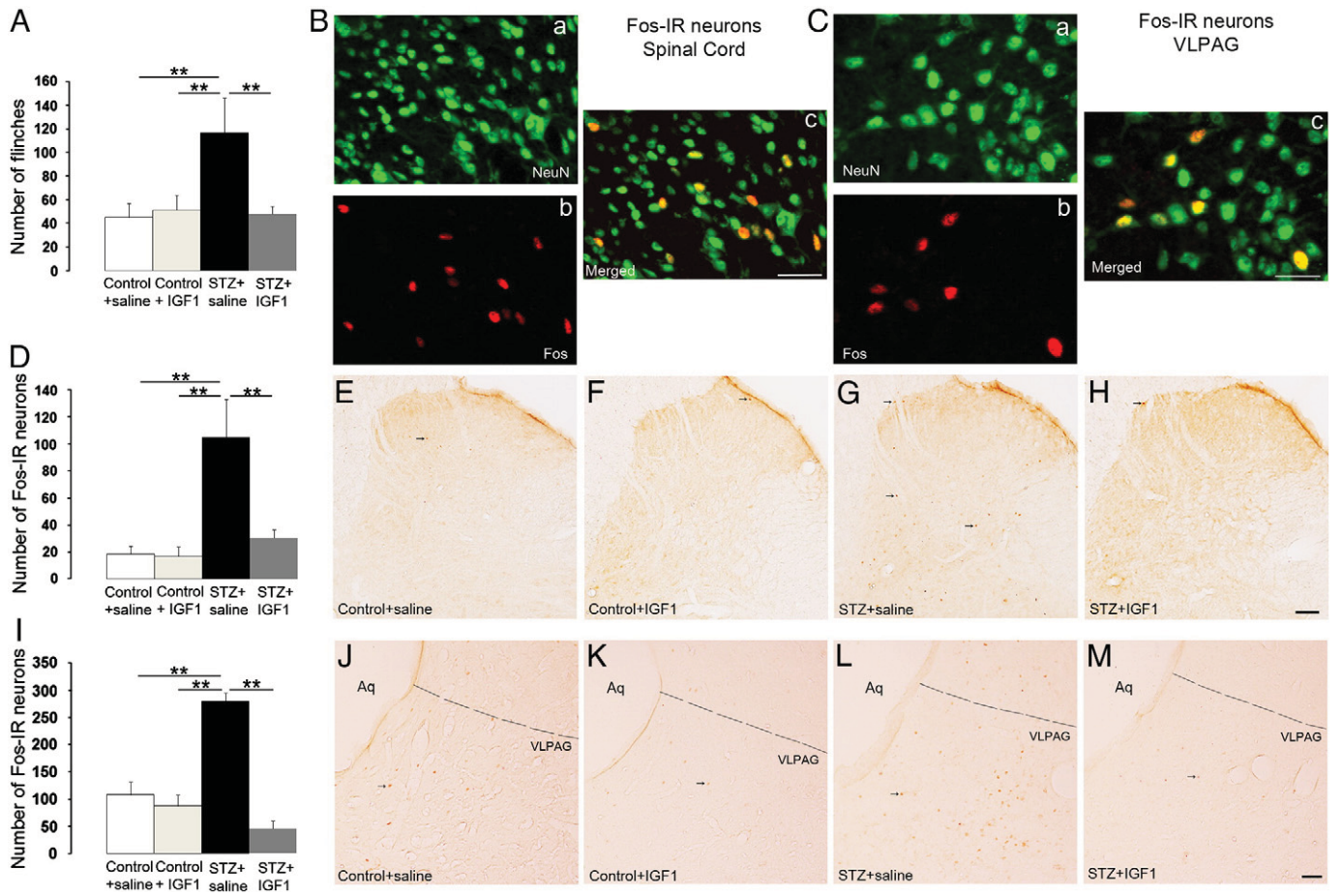

Fig. 3. Formalin-induced behavioral responses and neuronal activity at spinal cord and ventrolateral periaqueductal gray (VLPAG). Panel A presents total countings of paw flinches in saline-treated (control + saline) and IGF1-treated (control + IGF1) control rats and saline-treated (STZ + saline) and IGF1-treated (STZ + IGF1) STZ-diabetic rats. Panel B shows the co-localization (Bc) of NeuN (green; Ba) and Fos (red; Bb) at the spinal cord. Panel C shows the co-localization (Cc) of NeuN (green; Ca) and Fos (red; Cb) at VLPAG. Panels D and I show the total number of activated neurons (Fos-IR) at spinal dorsal horn of L4–L5 segments and VLPAG, respectively. Panels E–H and J–M are representative photomicrographs of L5 spinal segment (E–H) and VLPAG (J–M) immunoreactive for Fos. The modified formalin, used to evaluate chemical allodynia, induced significantly higher numbers of paw flinches and Fos-IR neurons (arrows) at spinal dorsal horn and VLPAG of STZ + saline rats. Those changes were prevented by IGF1 treatment. Significance of symbols: ** indicates $p<0.01$. Scale bar in B and C: 50 μm; scale bar in E–H and J–M: 100 μm.

Spinal cord and VLPAG neuronal activity

Double-immunostaining for Fos and NeuN at the spinal cord (Fig. 3B, a–c) and VLPAG (Fig. 3C, a–c) demonstrated that the expression of Fos was confined to neurons since Fos was exclusively expressed in cells with NeuN immunoreactivity. The numbers of Fos-IR neurons were significantly higher in STZ + saline rats than in all other experimental groups, both at the spinal dorsal horn (Fig. 3D–G) and at the VLPAG (Fig. 3I–L). Treatments with IGF1 reversed Fos expression to the control levels at the spinal dorsal horn (Fig. 3D–H) and VLPAG (Fig. 3I–M) in STZ-diabetic rats, but had no effects in non-diabetic control animals.

Serotoninergic and noradrenergic pain control system

In STZ + saline rats, serotonin content was higher at the lumbar spinal cord than in control animals (Fig. 4A). The neuronal nature of TpH-IR cells was evident by the morphology of these cells, which presented stained soma and dendrites (Fig. 4C). The neuronal nature of TpH-IR cells was further confirmed by double-immunostaining for TpH and NeuN. All TpH-IR cells were also labeled for NeuN (Fig. 4D). The numbers of TpH-IR neurons at the RVM were significantly higher in STZ + saline rats in comparison with non-diabetic controls (Fig. 4B, E–G). The proportion of TpH-IR neurons labeled for pERKs (TpH^+ $pERKs^+$ neurons) was higher in STZ + saline rats when compared with control animals (Fig. 5A and B). Treatments with IGF1 prevented the increased levels of serotonin at the spinal cord (Fig. 4A) and RVM (Fig. 4B) in STZ-diabetic rats (Fig. 4B, E–H), but had no effects on non-diabetic control animals (Fig. 4B and F).

The content of noradrenaline in the spinal cord of the STZ + saline group was significantly higher than in control rats (Fig. 4I). TH immunoreactivity was confined to neurons as demonstrated by the neuronal phenotype of the TH-IR cells (Fig. 4K) and by the exclusive expression of TH in NeuN-IR cells (Fig. 4L). The numbers of TH-IR neurons in the pontine A5 noradrenergic cell group were significantly higher in STZ + saline animals than in non-diabetic controls (Fig. 4J, M–O). No differences in the numbers of TH-IR neurons were detected at the A7 noradrenergic cell group between STZ + saline animals and non-diabetic controls (Fig. 4J). The proportion of TH-IR neurons that were also pCREB-IR (TH^+pCREB^+ neurons) was higher in the A5 noradrenergic cell group of STZ + saline injected rats (Fig. 5C and D). No differences in the proportions TH^+pCREB^+ at A7 noradrenergic cell group were detected between the experimental groups (Fig. 5E and F). Noradrenaline levels at spinal

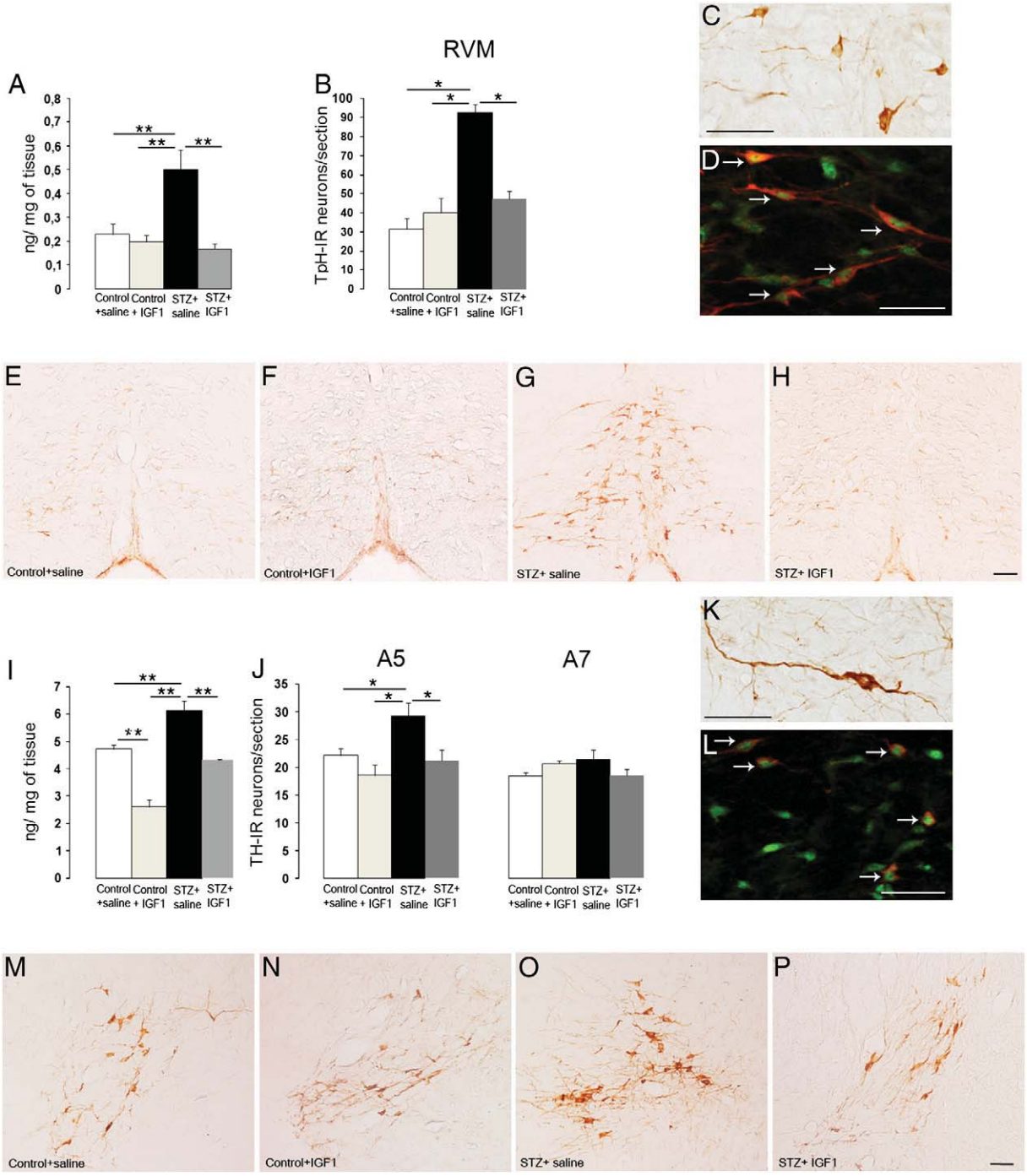

Fig. 4. Changes in the serotoninergic and noradrenergic pain control system. Panel A shows the content of serotonin in L4–L5 spinal cord segments for all experimental groups and panel B presents the number of TpH immunoreactive neurons (serotoninergic neurons) at the RVM of saline-treated (control + saline) and IGF1-treated (control + IGF1) control rats and saline-treated (STZ + saline) and IGF1-treated (STZ + IGF1) STZ-diabetic rats. Panel C is a representative photomicrograph of TpH-IR neuron at the RVM showing the neuronal morphology of the labeled cells (somata and dendrites). Panel D shows the co-localization of TpH (red) and NeuN (green) immunoreactions (arrows). Panels E–H are representative photomicrographs of RVM showing TpH immunoreactivity in each experimental group. Panel I presents the levels of noradrenaline in L4–L5 spinal cord segments of all experimental groups and Panel J presents the number of TH labeled neurons (noradrenergic neurons) at A5 and A7 noradrenergic cell groups. Panel K is a representative photomicrograph of TH-IR neuron at the A5 cell group showing the neuronal morphology of the labeled cell (soma and primary and secondary dendrites). Panel L shows the co-localization of TH (red) and NeuN (green) immunoreactions (arrows). Panels M–P are representative photomicrographs of A5 cell noradrenergic group labeled for TH. STZ + saline rats presented a significant increase in the spinal content of serotonin and noradrenaline (A and I), which was accompanied by increased numbers of TpH and TH immunoreactive neurons at RVM (B, G) and A5 (J, O), respectively. IGF1 fully prevented those changes. $^{*}p<0.05$; $^{**}p<0.01$. Scale bar in C, D, K, and L: 50 μm; scale bar in E–H and M–P: 100 μm.

cord were normalized to control levels by IGF1 administration (Fig. 4I). Treatment with IGF1 also prevented the increase in the numbers of TH-IR neurons at the A5 noradrenergic cell group in STZ-diabetic rats (Fig. 4J, M–P). In non-diabetic control rats, IGF1 administration induced a significant decrease in spinal noradrenaline content (Fig. 4I), along with a non-significant tendency for a reduction in the numbers of TH-IR neurons at the A5 noradrenergic cell group (Fig. 4J and N).

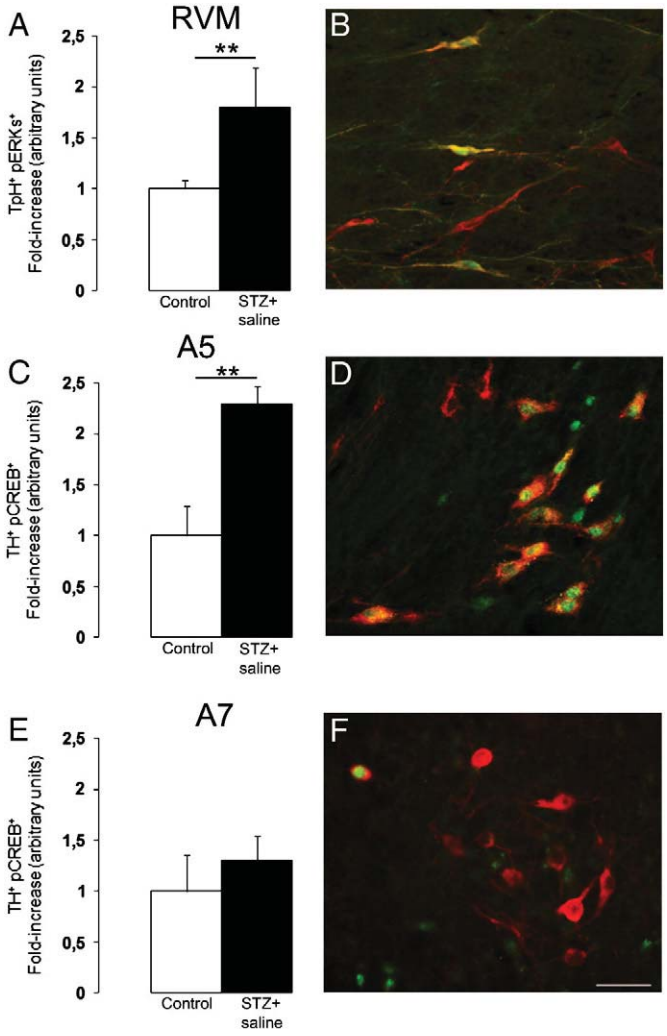

Fig. 5. Expression of pERKs or pCREB on TpH-IR or TH-IR neurons, respectively, in control + saline and STZ + saline rats. Panel A presents the fold-increase over the control percentage of double labeled neurons for TpH and pERKs (TpH^+pERKs^+) in saline-treated rats (STZ + saline) when compared with control animals at the RVM. Panel B is a representative photomicrograph of RVM neurons double labeled for TpH (red) and pERKs (green). Panels C and E present the fold-increase over the control percentage of double labeled neurons for TH and pCREB (TH^+pCREB^+) in STZ + saline rats when compared with control animals at the A5 (C) and A7 (E) noradrenergic cell groups. Panels D and F are representative photomicrographs of A5 (D) and A7 (F) neurons double labeled for TH (red) and pCREB (green), showing details of the labeling after optical sectioning (20 optical sections). Control percentages were considered as the basal levels (equal to 1) and results for STZ + saline group are presented as the fold-increase over control percentages. STZ + saline rats present higher proportions of double labeled neurons in RVM and A5 noradrenergic cell groups than control animals. Significance of symbols: $^{**}p<0.01$. Scale bar: 50 μm.

Discussion

The present study shows, for the first time, that, during diabetes, an increase in the numbers of serotoninergic and noradrenergic neurons in key brainstem centers for descending pain is associated with increased levels of serotonin and noradrenaline at the spinal cord. Based on the facilitatory role of serotonin and noradrenaline in other chronic pain conditions, our study suggests that PDN can probably be due to impairments in descending pain modulation. This study also shows that treatment with IGF1 normalizes the levels of serotonin and noradrenaline, reverses the behavioral signs of PDN, and decreases neuronal activation at the VLPAG and spinal dorsal horn.

Monoaminergic systems during PDN

Regarding the serotoninergic system, higher numbers of TpH-expressing neurons were detected in the RVM of STZ-diabetic rats. By performing a study directed to a specific brainstem serotoninergic area (the RVM), we demonstrated, for the first time, that the RVM can account for the increased levels of serotonin in the brainstem of diabetic rats (Padayatti and Paulose, 1999). This is important since the RVM is the main relay station used by the PAG in conveying pain modulation from higher brain centers, such as the amygdala and the anterior cingulate cortex (Heinricher et al., 2009; Tracey and Mantyh, 2007). The expression of TpH, the enzyme marker of serotoninergic neurons (Grahame-Smith, 1964), is known to be regulated by the activation of the ERK pathway (Wood and Russo, 2001). We demonstrated that the percentages of TpH-IR neurons that expressed pERKs were higher in the RVM of STZ-diabetic rats than in control animals. Since, at the cerebral cortex, serotonin levels were previously shown to decrease in STZ-diabetic rats (Sandrini et al., 1997), it will be important to study if there is suppression in the expression of pERKs in the cortex during diabetes. Such evaluation, in conjunction with the present data, could demonstrate a pERK-inducible increase in serotonin expression during diabetes.

As to the noradrenergic system, we detected higher numbers of TH-expressing neurons at the A5 noradrenergic cell group of STZ-diabetic rats, along with increased levels of noradrenaline at the spinal cord. These results nicely match the fact that the A5 noradrenergic cell group is the main contributor to the noradrenergic innervation of the spinal cord in the rat strain used in the present study (Tavares et al., 1996, 1997). The increase in noradrenaline levels could be a specific effect of diabetes in noradrenergic metabolism since the mRNA levels for TH were previously shown to increase in the brain of diabetic rats and to normalize after euglycemic insulin treatment (Figlewicz et al., 1996). Further supporting a specific effect of diabetes in noradrenergic metabolism, the levels of noradrenaline were previously shown to increase in other organs of diabetic rats, namely the heart (Ganguly et al., 1986). However, the increased expression of TH could also be a response to chronic pain installation since it was also reported in traumatic neuropathic models in the A6 noradrenergic cell group (Ma and Eisenach, 2003). The expression of the mRNA for TH is known to be regulated by the activation of the CREB pathway (Shimizu et al., 2004). Incidentally, it should be noted that the percentages of TH-IR neurons that were also labeled for pCREB were higher in the A5 noradrenergic cell group of STZ-diabetic rats than in control animals. This did not occur in the A7 noradrenergic cell group, where the numbers of TH-IR neurons also did not change in STZ-diabetic rats. These findings suggest a pCREB-dependent increase in TH expression during diabetes by molecular mechanisms that remain to be determined.

Since pERKs and pCREBs are markers of neuronal activation (Ma and Quirion, 2001; Pancaro et al., 2003; Obata and Noguchi, 2004), the increased expression of these factors in serotoninergic neurons of the RVM and in noradrenergic neurons of the A5 cell group, respectively, indicates that PDN involves a higher recruitment of descending modulation from these areas. The VLPAG, generally considered a key regulator in pain modulation from the brain, also showed higher neuronal activation in our STZ-diabetic rats, as shown by the higher c-fos expression. Since the VLPAG projects both to the RVM and the A5 noradrenergic cell group (Bajic and Proudfit, 1999), it remains to ascertain if the VLPAG triggers the putatively increased neuronal activation at those serotoninergic and noradrenergic neurons. Collectively, the data point to an increased activity in pain modulatory centers of the brain which is in agreement with the increased modulation in other chronic pain models (Danzinger et al., 1999). However, the net balance of increased descending modulation in PDN should be facilitation of nociceptive transmission. These results agree with other chronic pain models in which an imbalance of inhibition

and facilitation occurs, with a large prevalence of facilitation (Porreca et al., 2002). This has been extensively demonstrated for the RVM in traumatic neuropathic pain and in inflammatory pain models (Heinricher et al., 2009). Our STZ-diabetic rats exhibited mechanical hyperalgesia, chemical allodynia, and increased nociceptive responses of spinal neurons. This indicates that, during diabetes, the increased descending drive by serotonin and noradrenaline is eliciting pronociceptive effects which may be explained by 1) reduction of the release of serotonin and noradrenaline at the spinal cord due to accumulation in axonal terminals; 2) decrease in the expression and/or activity of serotonin and/or noradrenaline receptors; and 3) changes in the role of serotonin and noradrenaline in pain modulation due to the preferential action on receptors that induce pain facilitation. Favoring the first two hypotheses, during diabetes, the release of serotonin and noradrenaline at spinal cord was shown to be impaired (Suh et al., 1996; Bitar et al., 1999) and the expression and binding affinity of subtypes of serotonin and noradrenaline receptors is altered (Bitar et al., 1999; Padayatti and Paulose, 1999). Considering that descending pain modulation from the RVM exerts dual influences upon nociception, the third hypothesis should also be considered. Serotonin released at the spinal cord from the RVM inhibits pain transmission through the activation of spinal 5-HT7 receptors and enhances pain through spinal 5-HT3 receptors (Drogul et al., 2009). Pain facilitation from the RVM was also demonstrated by the analgesia detected after silencing serotoninergic RVM neurons (Wei et al., 2010). Together, the results suggest that the increased activation of serotoninergic neurons at the RVM, along with the increased serotonin content at the spinal cord observed in STZ-diabetic rats, may contribute to spinal neuronal hyperactivity and increased pain behavioral responses detected in this model of chronic pain. This hypothesis will be evaluated in a near future.

In what concerns descending noradrenergic modulation, it is known that noradrenaline acts upon post-synaptic α1 adrenergic receptors, inducing GABA and glycine release (Pertovaara, 2006; Gassner et al., 2009). The high noradrenaline levels at the spinal cord of STZ-diabetic rats are likely to increase GABA release at the spinal cord. During diabetic neuropathy, GABA is known to elicit excitatory effects rather than inhibitory (Jolivalt et al., 2008; Morgado et al., 2008). The release of GABA by α1 adrenoreceptor-mediated actions during diabetes will, therefore, enhance the activity of spinal neurons and account for an increase in pain behavioral responses in STZ-diabetic rats. This pain facilitation mediated by noradrenaline during diabetes can further be reinforced by noradrenaline actions upon α2 adrenergic receptors. During diabetes, the expression and function of spinal α2 adrenergic receptors are impaired (Bitar and Pilcher, 2000). Since, in normal conditions, noradrenaline acts upon spinal pre-synaptic α2 adrenergic receptors, inhibiting the release of excitatory neurotransmitters, any dysfunctions in these receptors will attenuate the antinociceptive effect of noradrenaline at the spinal cord. Therefore, the inhibitory effects of IGF1 on noradrenergic descending neurotransmission could probably concur for the reduction in spinal neuronal activity and hyperalgesia/allodynia in STZ-diabetic rats. Intrathecal administration of agonists and antagonists of serotonin and noradrenaline receptor subtypes will be performed to elucidate the role of these neurotransmitters during PDN. Such study could provide important data to design new drugs that are more directed to the relevant serotonin or noradrenaline receptors affected at the spinal cord during PDN, without the generalized effects of antidepressants that act upon serotonin and noradrenaline reuptake.

Effects of IGF1

A striking finding of the present study was the observation that IGF1 prevented the increases in serotonin and noradrenaline levels and normalized behavioral responses and neuronal hyperactivity at the VLPAG and spinal cord in STZ-diabetic rats. The improvement of behavioral signs of diabetic neuropathy after IGF1 treatment has been previously described, even with lower IGF1 doses and later onset of treatment (Ishii, 1995; Schmidt et al., 1999; Zhuang et al., 1996; Piriz et al., 2009). Since these studies demonstrated that IGF1 has a peripheral effect by reducing peripheral nerve damage, it is likely that a decrease in peripheral input was induced in our STZ-diabetic rats by the IGF1 treatment. In a previous study, this decrease in peripheral input failed to show central consequences at the somatosensory cortex (Piriz et al., 2009) whereas, in our IGF1-treated STZ-diabetic rats, the decrease in behavioral signs of PDN was accompanied by a reversion of neuronal hyperactivity at the VLPAG and by normalization of the levels of serotonin and noradrenaline in the brainstem. Besides blocking the peripheral input from the periphery, as proposed by the studies referred above, IGF1 may act directly at the brain since circulating IGF1 crosses the blood–brain barrier, reaches the brain (Carro et al., 2000), and IGF1 receptors occur in the analyzed brainstem areas (Folli et al., 1994). It is, however, unlikely that IGF1 had a direct action on the RVM and A5 neurons of STZ rats since IGF1 has been shown to increase monoamine levels (Hoshaw, 2008) and the reverse effect was never reported. This is further supported by the lack of effects of IGF1 in the serotoninergic system of control rats. Curiously, in these animals, IGF1 decreases spinal levels of noradrenaline without significantly reducing the numbers of TH-IR neurons at the A5 cell group. This does not support a direct action of IGF1 at the A5 and is rather explained by an IGF1-mediated enhancement of cathecolamine breakdown, which was already proposed in previous studies with PC12 rat pheochromocytoma cells (Bach and Leeding, 2002). We cannot exclude a direct effect of IGF1 at the VLPAG which would decrease the hyperactivation of local neurons and, herein, depress the recruitment of descending modulatory drive through the serotoninergic and noradrenergic relay brainstem stations. Supporting this possibility, IGF1 has been shown to elicit long-term depression in cultured neurons by downregulating AMPA receptor signaling (Wang and Linden, 2000). The methodological constraints of performing continuous infusions of IGF1 at the VLPAG in STZ-diabetic rats to test this hypothesis will be circumvented by blocking the peripheral effects of IGF1 using an anti-IGF1 antibody (Glasper et al., 2010) and studying the effects of IGF1 treatment. An interesting issue about IGF1 is its interaction with growth hormone (GH). IGF1 is mainly produced by the liver upon the GH stimulation. GH levels were shown to be decreased in STZ-diabetic rats, which may concur for IGF1 deficits in this animal model (Yang et al., 1990; Boujon et al., 1995). Therefore, it is likely that a treatment with GH would raise blood IGF1 concentrations in STZ-diabetic rats and could induce similar effects to those of the current IGF1 protocol. This cannot, however, be tested due to ethical constraints. STZ-diabetic rats have reduced liver GH receptor expression, which makes these animals less responsive to GH treatment (Menon et al., 1994). To effectively raise IGF1 levels, it will probably be necessary the administration of high doses of GH, which induces renal hypertrophy (Landau et al., 2003). On the contrary, the IGF1 treatment does not induce any impairment in animal welfare.

In summary, we demonstrated an increased recruitment of descending nociceptive modulation during PDN, which affects the serotoninergic and noradrenergic systems. The importance of IGF1 in mediating functional plasticity in the brain during normal conditions and in several pathologies (Torres-Aleman, 1999), such as diabetes, deserves further investigation. The benefits of an early treatment with IGF1 in preventing the signs of PDN may be considered in the future after clarifying the mechanisms of its putative actions in pain modulation from the brain. Since diabetic patients present spontaneous pain (Tesfaye, 2009), it will be important to evaluate if IGF1 relieves spontaneous pain in STZ-diabetic rats, in a manner similar to the improvement of evoked pain (allodynia and hyperalgesia). The lack of behavioral tests to evaluate spontaneous pain is a limitation of current experimental pain research (Mogil et al., 2010) that needs to be circumvented in the future.

Acknowledgments

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Final considerations

The studies included in this PhD Thesis provide evidences for the existence of central effects induced by diabetes at the somatossensory system, namely in the spinal cord and in pain control areas of the braintem. The spinal dorsal horn was shown to be affected, since our data suggest spontaneous hyperactivity of neurons located in laminae involved in nociceptive processing (**Publication 1**). This neuronal hyperactivity is probably due to a shift in the inhibitory role of GABA, since the expression of the KCC2 is reduced at the spinal dorsal horn and this impairs the post-synaptic action of GABA (**Publication 2**). The reduction in KCC2 expression is probably mediated by activation of microglia (**Publication 3**) and oxidative stress (**Publication 4**), since a correction of these factors increases KCC2 expression, reverses spinal neuronal activity and decreases behavioral signs of PDN. In addition, our results show that key pain control areas in the brain are affected during diabetes. Neurons at the PAG were shown to be activated during PDN (**Publication 5**), which was accompanied by an increase in the number and activity of serotoninergic and noradrenergic neurons, respectively, in the RVM and A_5 cell group, two important relay stations of PAG-mediated pain modulation (**Publication 6**). In addition, the spinal levels of serotonin and noradrenaline were shown to be increased (**Publication 6**). These findings show the affectation of the serotoninergic and noradrenergic descending pain modulatory systems during PDN, which may also account for the increased spinal cord activity detected in STZ rats.

1. Central neurobiological mechanisms of painful diabetic neuropathy

The studies included in this Thesis provided, for the first time, a simultaneous analysis of the spinal cord and supraspinal pain control areas, along with behavioral evaluation of signs of PDN. This comprehensive approach provides some data regarding the central mechanisms that may subserve PDN, which are centered at the spinal cord and pain control areas of the brainstem.

1.1. The spinal dorsal horn during diabetes

By studying the expression of the Fos protein, we showed that PDN is associated with spontaneous hyperactivity of nociceptive neurons, affecting all spinal dorsal horn laminae (**Publication 1**). This finding suggests a general change in spinal nociceptive neurotransmission, which probably enhances the responses to both noxious and innocuous peripheral stimuli. Our data corroborate the findings from electrophysiological recordings showing hyperactivity of wide-dynamic neurons of the spinal dorsal horn (Pertovaara et al., 2001), some of which are engaged in supraspinal transmission of nociceptive information since they belong to the spinothalamic tract (Chen and Pan, 2002). Besides the increased peripheral barrage due to primary afferent hyperexcitability (Burchiel et al., 1985; Ahlgren et al., 1994; Chen and Levine, 2001; Khan et al., 2002; Chen and Levine, 2003), the spinal neuronal hyperactivity may involve alterations in local spinal circuits and descending pain modulatory mechanisms (Millan, 2002; Vanegas and Schaible, 2004; Pertovaara, 2006; Heinricher et al., 2009; Ossipov et al., 2010). The supraspinal influences will be discussed in **item 1.2** ("**The pain modulatory system of the brainstem during diabetes"**). As to the spinal mechanisms, the increased spinal GABA levels along with the decreased expression of KCC2 (**Publication 2**), suggests an inversion of the post-synaptic role of GABA from inhibitory to excitatory. This has been previously demonstrated in other pain models (Payne et al., 2003; Coull et al., 2003; Mantyh and Hunt, 2004; Price et al., 2005). It was also demonstrated in STZ rats after our quantitative data about GABA/KCC2 were published (**Publication 2**). Jollivalt and collaborators (2008) showed that bicucculine, the antagonist of post-synaptic ionotropic $GABA_A$ receptor, elicits antinociceptive effects (Jolivalt et al., 2008). Altogether, the changes on the excitatory mechanisms and on the pre-synaptic action of GABA that affect the spinal cord of STZ rats (Kamei et al., 1990; Calcutt and Chaplan, 1997; Malcangio and Tomlinson, 1998; Li et al., 1999; Calcutt et al., 2000; Li et al., 2002; Tomiyama et al., 2005; Malmberg et al., 2006; Wang et al., 2007), in addition with the impairment on post-synaptic GABA role, are

likely to culminate in spinal hyperactivity and exacerbation of pain behavioral responses.

As to the mechanisms which may subserve the decreases in the expression of KCC2 in STZ rats, the results of this PhD Thesis indicate an involvement of microglia and oxidative stress. The cross-talk between microglia and neurons has been extensively addressed in the last decade. Studies in animal models of traumatic neuropathic pain and inflammatory pain showed that microglia is involved in neuronal function and affects the nociceptive processing (reviewed by Inoue and Tsuda, 2009; McMahon and Malcangio, 2009; O'Callaghan and Miller, 2010; Gosselin et al., 2010; Gao and Ji, 2010; Ren and Dubner, 2010). During diabetes, microglia activation was described at the spinal cord (Tsuda et al., 2008; Wodarski et al, 2009; Talbot et al, 2010). Microglia activation is probably involved in PDN, since gabapentin, an analgesic drug used in PDN treatment, reduces microglia activity at the spinal cord (Wordaski et al, 2009) and microglia inhibition ameliorates behavioral signs of PDN (Tsuda et al., 2008). The results of the studies included in **Publication 3** also support the involvement of microglia in the hyperalgesia detected in the STZ rats, probably through its effect on KCC2 expression at the spinal cord. It will be important to elucidate the molecular mechanisms underlying the effects of microglia in KCC2 expression during diabetes, since, in our study, microglia-mediated reduction of KCC2 expression was BDNF-independent. This is a specific feature of PDN inasmuch that BDNF-mediated effect of microglia was demonstrated in other chronic pain models (Yajima et al., 2002; Coull et al, 2005; Ramer et al., 2007; Miletic and Miletic, 2008; Zhou et al., 2011).

As to oxidative stress, a frequent consequence of diabetes that affects several tissues, namely peripheral nerves (Stevens et al., 2000; van Dam, 2002; Obrosova et al., 2003; Pop- Busui et al., 2006; Lin et al., 2006), we showed that it also occurs centrally, affecting the spinal dorsal horn. Oxidative stress influences the activity of nociceptive neurons at the spinal cord of STZ rats, which seems to be partially mediated by its effect on KCC2 expression (**Publication 4**). Since the antioxidant

treatment with α-lipoic acid was performed systemically, we can not exclude that it also acts in areas of the somatossensory system, other than the spinal cord. However, since oxidative stress affects the phosphorylation and expression of KCC2 (Wake et al., 2007), this probably accounted for the decreases in KCC2 expression at the spinal cord (**Publication 4**). Collectively, the studies addressing the changes at the spinal cord during diabetes indicate neuronal hyperactivity which is likely to affect spinofugal transmission of nociceptive information. The increased arrival of nociceptive input from the spinal cord to the brain is likely to have important impacts on spinal neurotransmission since several supraspinal pain control areas form reciprocal loops with the spinal cord (Almeida et al., 2006; Ossipov et al., 2010).

1.2. The pain modulatory system of the brainstem during diabetes

The studies included in the last 2 publications of this Thesis indicate impairments in key pain control centres of the brain during diabetes. PAG neurons were shown to be hyperactivated in STZ rats (**Publication 5**) and increased neuronal activity was detected in the thalamus in the same animal model of diabetes (Fischer et al., 2009; Fischer and Waxman, 2010). The increased thalamic neuronal activity appears to be independent from the peripheral barrage since blocking the peripheral input does not block thalamic activity. This has been used to propose that during PDN the thalamus becomes a “pain generator/amplifier” (Fischer et al., 2009; Fischer and Waxman, 2010). It will be important to perform similar studies at the PAG by blocking peripheral input when the PAG has increased neuronal activity to ascertain the moment when PAG hyperactivity is independent from the peripheral barrage. After this timepoint, PDN treatment exclusively directed to peripheral causes is unlikely to be effective.

The activation of descending pain modulation pathways at PAG relay stations was also demonstrated in the present PhD Thesis (**Publication 6**). Neurons at the RVM and A_5 noradrenergic group, two important brainstem areas targeted by PAG

projections and involved in descending modulation of spinal nociceptive transmission (Behbehani, 1995; Bajic and Proudfit, 1999; Vanegas and Schaible, 2004; Ossipov et al., 2010), were shown to be activated in STZ rats. Neurons at the RVM and A_5 project to the spinal dorsal horn, where they release serotonin and noradrenaline, respectively (Millan, 2002; Pertovaara, 2006; Heinricher et al., 2009; Ossipov et al., 2010). The increase of serotonin and noradrenaline levels in the spinal cord of STZ rats confirms the activation of serotoninergic and noradrenergic descending pathways (**Publication 6**). Serotonin may elicit inhibitory and facilitatory effects, depending on the targeted serotonin receptors at the spinal cord (Drogul et al., 2009). Noradrenaline was shown to be exclusively inhibitory by binding to α2 adrenoreceptors and inhibiting the release of excitatory neurotransmitters and to α1 adrenoreceptors but increasing the release of GABA and glycine at the spinal cord (Pertovaara, 2006; Gassner et al., 2009). It is important to ascertain the net pain modulatory effect of each neurotransmitter at the spinal cord during PDN. Considering the spinal hyperactivity detected in STZ rats, the increased behavioral responses and the changes in spinal GABA neurotransmission (item **1.1. "The spinal dorsal horn during diabetes"**), pain facilitation is likely to prevail over inhibition. It is important to mention that disturbances at 5-HT receptors were reported in STZ rats (Padayatti and Paulose, 1999), which may alter serotonin actions, probably by reducing its inhibitory effects. Additionally, in STZ rats, noradrenergic α2 adrenoreceptors showed reduced expression and binding affinity (Bittar et al., 1999; Bittar and Pilcher, 2000) while α1 adrenoreceptors are hyperresponsive (Teasell and Arnold, 2004). This could imply a reduction in the pre-synaptic inhibition mediated by noradrenaline. Moreover, the higher responsiveness of α1 adrenoreceptors can increase GABA release, which, considering the shift in the inhibitory role of GABA at spinal dorsal horn of STZ rats (Jolivalt et al., 2008), may concur for neuronal hyperactivity and pain. A detailed study of the function and integrity of serotonin and noradrenaline receptors at spinal cord of STZ rats should be performed in order to study the relative contribution of pain facilitation and loss of pain

inhibition during the PDN. Moreover, that study could support the development of more specific pharmacological approaches, directed to the altered receptor(s) and not affecting the whole neurotransmitter system.

Since diabetes is a systemic condition, some of the changes detected at the spinal cord during diabetes can occur also at the brain. As to oxidative stress, our recent data show the occurrence of oxidative stress damage in neurons of the RVM and A_5 (Morgado et al., 2010). We also showed that oxidative stress occurs in serotoninergic RVM neurons and noradrenergic A_5 neurons (Morgado et al., 2011a). We are currently evaluating if antioxidant treatments of STZ rats can reverse the oxidative stress in those brainstem areas. In the case of the antioxidant α- lipoic acid, it was previously reported that it may affect noradrenaline levels in the brain after systemic administrations (Santos et al., 2010). Based on the results of our **publication 6**, it will be important to study the effects of that antioxidant treatment in the brainstem, namely in the A_5 noradrenergic cell group. Another issue to consider is microglia activation. It was previously shown that microglia is activated in the RVM in other models of chronic pain and seems to concur for descending pain facilitation, since pain responses normalize upon local administration of microglial inhibitors (Wei et al., 2008; Roberts et al., 2009). Furthermore, microglia activation was also observed in the thalamus and its inhibition was shown to elicit analgesia (Zhao et al., 2007).

Collectively, the findings obtained in the spinal cord and brainstem of STZ rats in this PhD Thesis are summarized in **Figure 1**.

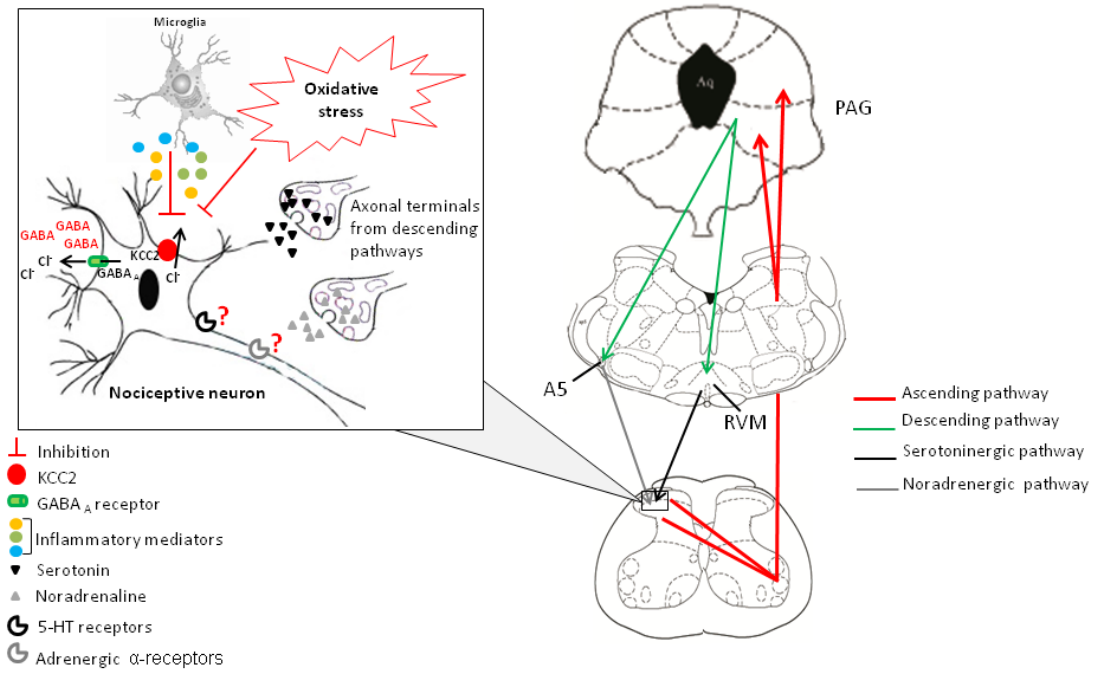

Figure 1 – Schematic representation of the effects of diabetes in areas of the somatossensory system involved in pain transmission/modulation. The diagram takes into account the data analyzed in item 1 of this Discussion.

The results obtained show that during PDN the hyperactivity of spinal nociceptive neurons probably occurs due to changes in spinal GABAergic neurotransmission, caused by the reduction in local expression of KCC2. This might be triggered by microglia activation and oxidative stress. The data also suggest that descending pain modulation circuits are affected including *i)* activation of neurons at the PAG; *ii)* increased activity and number of serotoninergic and noradrenergic neurons of the RVM and A_5 cell group, respectively and *iii)* increased serotonin and noradrenaline content at spinal cord. However, despite the recruitment of descending modulation pathways, allodynia and hyperalgesia persisted in STZ rats, which point for disturbances in the spinal responses to descending pain modulation. The loss of inhibition mediated by serotonin and noradrenaline is probably related with impairments on neurotransmitter release or/and changes in the respective receptor activities. The first hypothesis is supported by our recent data showing that the release of serotonin is

reduced at the spinal cord of STZ-rats with 10 weeks of diabetes when compared with age-matched control animals (Morgado et al., 2011b). Curiously, our recent data comparing STZ rats with 4 and 10 weeks of diabetes suggest that during the progression of diabetes, descending pain modulation is affected in a time-dependent manner. Long-term diabetes is associated with decreased numbers of serotoninergic RVM neurons and noradrenergic A_5 neurons. Curiously, the spinal serotonin content remained increased while the opposite occurs with noradrenaline levels (Morgado et al., 2011b). The spinal release of serotonin seems to be diminished, considering the lower content of serotonin in the cerebrospinal fluid from the spinal lumbar enlargement in STZ-rats when compared with control animals (Morgado et al., 2011b). Future studies will address the neurobiological mechanisms subserving these changes in serotoninergic and noradrenergic neurotransmission during diabetes.

2. New perspectives in the treatment of PDN

The management of PDN remains a challenge for the clinicians, since it is often only partially responsive or even unresponsive to the existing pharmacological treatments. Most of therapeutic approaches are symptomatic, with α-lipoic acid and epalrestat being the only pathogenetic-related existing drugs (Ziegler et al., 2009, Tesfaye, 2009; Obrosova, 2009; Zilliox and Russel, 2011). Besides α-lipoic acid, the present PhD Thesis used several compounds that may be valuable for PDN treatment, namely gabapentin, minocycline and IGF1. Regarding α-lipoic acid, our study supports a role of this antioxidant in neuronal function, considering that it ameliorated KCC2 expression in the spinal cord of STZ rats, indicating that the benefits of α-lipoic acid extend to the central nervous system and not only to the periphery (**Publication 4**). Similar studies of the effects of α-lipoic acid in higher pain-related areas should be considered in the future since, as discussed above, we recently detected oxidative stress in serotoninergic neurons of the RVM and noradrenergic neurons of the A_5 cell

group (Morgado et al., 2010). The present findings reinforce the use of α-lipoic acid for treat or, eventually, prevent PDN.

Gabapentin or its structurally related compound pregabalin are commonly used in neuropathic pain treatment (Hwang and Yaksh, 1997; Abdi et al., 1998; Field et al., 1999; Vinik, 2005). Their predominant mechanism of action is the inhibition of calcium currents via high-voltage-activated channels containing the α2δ-1 subunit (Marais et al., 2001; Dooley et al., 2007), which was shown to be elevated in the spinal cord of STZ rats (Luo et al., 2002). Antinociceptive actions of gabapentin were mainly studied at the spinal cord. There are studies showing that spinal administration of gabapentin *i)* regulates α2δ-1 subunit neuronal expression (Xiao et al., 2007), *ii)* interacts indirectly with NMDA receptors, reducing the glutamate excitatory action (Partridge et al., 1998; Shimoyama et al., 2000); *iii)* reduces the electrical-evoked and mechanical-evoked responses of wide-dynamic neurons (Corderre et al., 2005; Donovan-Rodriguez et al., 2005; Omori et al., 2009) and *iv)* reduces the release of excitatory amino acids (Patel at al., 2000; Moore at al., 2002; Maneuf et al., 2004). When given systemically, gabapentin reduces oxidative stress (Baydas et al., 2005) and spinal microglia activation (Wodarsky et al., 2009). Our study corroborates the spinal effects of gabapentin in PDN, showing the normalization of nociceptive spinal cord neurons activity upon gabapentin treatment (**Publication 5**). In addition, we show for the first time that gabapentin reversed the neuronal hyperactivity at the PAG of STZ rats, supporting the effects of gabapentin on supraspinal pain-related areas (Hayashida et al., 2008). This effect could result from a direct action on PAG neurons or/and reduced spinal activity and, thereafter, diminished nociceptive ascending input. The expression of α2δ-1 subunit of high-voltage-activated calcium channels at the PAG supports a direct effect of gabapentin on PAG neurons (Cole et al., 2005). In addition, α2δ-1 subunit is also expressed in other areas involved in nociceptive processing, as the thalamus (Cole et al., 2005), which if inhibited by gabapentin will reduce the ascending input to higher centers and, consequently, diminish the activation of the descending

pathways. It will be interesting to ascertain the relative contribution of the PAG and the thalamus to the gabapentin-induced analgesia by performing local inoculations in each brain area. In addition, and based on the effects on PAG activity, it will be important to assess the effects of gabapentin in serotoninergic and noradrenergic descending pathways recruitment during PDN.

Considering the increasing amount of data showing that microglia activation is involved in the installation of chronic pain (reviewed by Inoue and Tsuda, 2009; McMahon and Malcangio, 2009; O'Callaghan and Miller, 2010; Gosselin et al., 2010; Gao and Ji, 2010; Ren and Dubner, 2010), it will be important to ascertain the effect of microglia inhibition in PDN in humans. Minocycline, a microglia inhibitor, was here shown to reverse the hyperalgesia in STZ rats, by inhibiting neuronal hyperactivity and normalizing KCC2 expression at the spinal cord. This suggests that minocycline, or other agents that inhibit microglia activity (such as inhibitors of SFKs or p38 MAPK; Zhou et al., 2011), could be promising therapeutic compounds to treat PDN, alone or in combination with other treatments. Indeed, recent studies demonstrated that minocycline attenuates morphine tolerance, contributing to increase its analgesic efficacy in post-herpetic neuralgia (Chen et al., 2010). Encouraging the use of minocycline during PDN, there are studies showing that this drug inhibits intraepidermal fiber loss, an important clinical feature of PDN (Boyette-Davis and Dougherty, 2011; Loseth et al., 2010). Since minocycline crosses the blood-brain barrier (Colovic and Caccia, 2003) systemic administrations could provide dual benefits in PDN, by preventing peripheral nerve damage and spinal cord impairments. Besides its inhibitory effects on microglia activity, minocycline was shown to reduce oxidative stress (Padi and Kulkarni, 2008), inhibit Na+ channels in the primary afferent neurons (Kim et al., 2011) and decrease spinal glutamate neurotransmission (Nie et al., 2010). It is also likely that minocycline affects supraspinal areas devoted to pain control. In fact, minocycline was shown to inhibit microglia in the thalamus (Zhao et al., 2007) and RVM (Wei et al, 2008; Roberts et al., 2009) and this may contribute for its analgesic

effect. It is important to mention that minocycline exerts neuroprotective effect in various experimental models of neurodegenerative diseases (Blum et al., 2004; Kim and Suh, 2009) and is currently being tested in humans for the treatment of diabetes macular edema and several other neurological conditions (http://www.clinicaltrials.gov).

Diabetic neuropathy is no longer exclusively attributed to hyperglycemia. Lack of insulin and IGF1 play an important role in the ethiopathogeny and severity of this complication of diabetes (Ishii, 1995; Sima, 2003; Pierson et al., 2003; Sima, 2006). Our study also supports the involvement of IGF1 deficits in PDN, since the administration of low doses of IGF1 ameliorated pain responses in STZ rats, prevented the functional impairments at the spinal cord and PAG and the neurochemical changes at the serotoninergic and noradrenergic pain control areas (**Publication 6**). The mechanisms involved in the antinociceptive action of IGF1 should be evaluated in future studies namely by performing direct IGF1 administration at pain control centers of the brain. Based in the positive effects of IGF1 on experimental models of PDN, it will be important to evaluate if IGF1 is useful to treat diabetic patients. Clinical trials show that IGF1 is well tolerated, which point for high compliance rates (Rosenbloom, 2009). Caution should, however, be taken with monitoring blood glucose levels, particularly in diabetic patients under treatment with insulin or antidiabetic drugs, since IGF1 increases insulin sensitivity (Mohamed-Ali and Pinkney, 2002) and may induce hypoglycemic episodes.

In conclusion, we show that PDN is associated with several and complex changes in the central pain system. In order to achieve complete pain relief in this chronic pain condition, combined treatment approaches, addressing the multiple changes in several areas of the somatossensory system, should be considered.

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