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Role of Liver X Receptor activation on diabetic peripheral neuropathy

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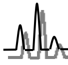

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1. PUBLICATIONS

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2. INTRODUCTION

2.1 DIABETES

Diabetes mellitus, is a major epidemic of this century (Shaw et al., 2010), which has increased in incidence by 50% over the past 10 years (Danaei et al., 2011). The World Health Organization states that 347 million people worldwide were suffering from diabetes in 2008, which equates to 9.5% of the adult population (Danaei et al., 2011). The incidence of diabetes is increasing rapidly with estimations and this number will almost double by 2030. Diabetes mellitus is more common in developed countries but occurs throughout the world. In the near future there will be the greatest increase but a large spread of pathology, however, is expected to occur in developing countries where there will be ~50% increase in diabetes by 2030 (Shaw et al., 2010).

Diabetes is clinically characterized by hyperglycemia due to chronic and/or relative insulin insufficiency (Mathis et al., 2001). In Diabetes Mellitus, also known as type 1 diabetes, hyperglycemia occurs as a result of a complex pathological process where the pancreatic beta-cells within the islets of Langerhans are destroyed due to genetic and environmental factors that lead to an autoimmune response (Davies et al., 1994). This process results in individuals relying essentially on exogenous insulin administration for survival. The genetic basis of this disease is not yet fully understood. Indeed, a number of major genetic determinants of type 1 diabetes such as alleles of the major histocompatibility locus (HLA) at the HLA-DRB1 and DQB1 loci (Nejentsev et al., 2007) and more recently the HLA-B*39 locus (Hyttinen et al., 2003) only account for some 40–50% of the familial clustering of this disorder. This suggests that there are other genetic loci involved in susceptibility to type 1 diabetes. The rising incidence of this pathology may also be influenced by insulin resistance, which has been reported as a risk factor for type 1 diabetes (Fourlanos et al., 2004). Although type 1 diabetes is an insulin-deficient state, features of insulin

resistance are increasingly common, with the high prevalence of obesity in Westernized populations.

COMPLICATIONS OF DIABETES

Diabetes is associated with many complications. Acute metabolic complications associated with mortality include diabetic ketoacidosis from exceptionally high blood glucose concentrations (hyperglycemia) and coma as the result of low blood glucose (hypoglycemia). In diabetes, the resulting complications are grouped under “microvascular disease” (due to damage to small blood vessels) and “macrovascular disease” (due to damage to the arteries). Microvascular complications include eye disease or “retinopathy,” kidney disease termed “nephropathy,” and neural damage or “neuropathy”. The major macrovascular complications include accelerated cardiovascular disease resulting in myocardial infarction and cerebrovascular disease manifesting as strokes. Other chronic complications of diabetes include depression, (Nouwen et al., 2011), dementia (Cukierman et al., 2005), and sexual dysfunction (Thorve et al., 2011).

DIABETIC NEUROPATHY

Peripheral nervous system (PNS) damage (i.e., diabetic neuropathy) is the most frequent symptomatic complication characterized by a spectrum of functional and structural changes in peripheral nerves including axonal degeneration, paranodal demyelination and loss of myelinated fibers and decreased nerve conduction velocity (Sugimoto et al., 2000, Vinik et al., 2000). More than 50% of diabetic subjects eventually develop neuropathy (Abbott et al., 2011), with a lifetime risk of extremity amputations. Diabetic neuropathy is a syndrome that hits both the somatic and autonomic divisions of the peripheral nervous system. There is, however, a growing appreciation that damage to the spinal cord (Selvarajah et al., 2006) and

the higher central nervous system (Wessels et al., 2006) can also occur and that neuropathy is a major factor in the impaired wound healing, erectile dysfunction, and cardiovascular dysfunction seen in diabetes. Disease progression in neuropathy was traditionally clinically characterized by the development of vascular abnormalities with subsequent hypoxia. Inhibitors of the renin-angiotensin system and α1-antagonists improve nerve conduction velocities in the clinical context, which is postulated to be a result of increases in neuronal blood flow. Advanced neuropathy due to nerve fiber deterioration in diabetes is characterized by altered sensitivities to vibrations and thermal thresholds, which bring to loss of peripheral sensitivity. Hyperalgesia, paresthesias, and allodynia also occur in a proportion of patients, with pain evident in 40–50% of those with diabetic neuropathy. Recently, however, there has been some controversy as to the inclusion of neuropathy as a "microvascular" complication, given that changes in neuronal blood vessels are considered by some investigators to be a secondary effect of an underlying neuronal and glial disorder associated with neuropathy rather than the vasculopathy being implicated as the cause of this group of complications. Indeed, recently there is some evidence suggesting that diabetic neuropathy selectively targets sensory and autonomic neurons over motor neurons, with little vascular involvement. (Forbes and Cooper, 2013)

The size of neurons is also important. It appears that in diabetes, longer nerve fibers show an earlier loss of nerve conduction velocity with loss of their nerve terminals. This is the reason why tingling and loss of sensation and reflexes are often first observed in the feet and then ascend to affect other areas, in particular the hands. This syndrome includes numbness, dysesthesia (pins and needles), sensory loss, and nighttime pain; early in the disease progression, also spatial awareness of limb location is affected. This includes a loss of sensation in response to injury leading to a high risk of

developing foot and leg ulcers, which can ultimately result in amputation. Some diabetic subjects can also incurr in multiple fractures and they can develop a Charcot joint, a degenerative condition characterized by loss in bone volume and eventually deformity. Progressive motor dysfunction is also common in diabetic neuropathy, which can lead to dorsiflexion of the digits of the hands and toes. In addition to motor neuron dysfunction, the autonomic nervous system is also influenced by diabetes. Common diabetes related abnormalities in autonomic function are: orthostatic hypotension, due to an inability to adjust heart rate and vascular tone to maintain blood flow to the brain and gastroparesis, nausea and bloating, that affect autonomic nerves innervating the gastrointestinal tract leading to altered efficacy of oral medications. In particular, delayed gastric emptying can dramatically affect glycemic control by delaying the absorption of key nutrients, as well as antidiabetic agents leading to imbalances in glucose homeostasis. In diabetes the duration of pathology and lack of glycemic control, consistent with other complications, are the major risk factors for neuropathy (1993). Other than optimization of glycemic control and management of neuropathic pain, there are no major therapies approved in either Europe or the United States for the treatment of diabetic neuropathy. In addition, as is seen with other complications, the mechanisms leading to diabetic neuropathy are poorly understood. At present, treatment generally focuses on alleviation of pain, but the process is generally progressive.

A possible clue to identify putative targets for a more effective treatment would be the identification of relevant molecular events occurring in the peripheral nerves under physiologic and pathologic conditions.

Experimental diabetic neuropathy and experimental diabetic encephalopathy show similar features to human complications (Alvarez et al., 2009, Beauquis et al., 2006). Recent findings from experimental models

of diabetic neuropathy and diabetic encephalopathy indicate that, at least in male rats, neuroactive steroids are protective agents (Roglio et al., 2007). The term neuroactive steroids defines steroids acting in the nervous system and includes molecules produced by the nervous system (neurosteroids) and hormonal compounds deriving from classical steroidogenic tissues (i.e., gonads and adrenal glands). Moreover, peripheral nerves express receptors for neuroactive steroids and consequently represent a target for them. For instance, treatment with progesterone (PROG), or its 5alpha-reduced metabolite, dihydroprogesterone (DHP), counteracts the increase in the number of fibers with myelin infoldings observed in the sciatic nerve of streptozotocin(STZ)-treated rat (Veiga et al., 2006b). Moreover, neuroactive steroids, such as PROG, testosterone (T) and their derivatives (e.g., DHP and tetrahydroprogesterone, THP, in the case of PROG; dihydrotestosterone, DHT, and 3α-diol, in the case of T), or dehydroepiandrosterone (DHEA) influence a variety of biochemical and functional parameters, including nerve conduction velocity, thermal threshold, skin innervation density, Na^+,K^+-ATPase activity and expression of myelin proteins, which are affected in STZ-treated rat (Leonelli et al., 2007, Roglio et al., 2007, Yorek et al., 2002).

Reduced nerve conduction velocity and increased thermal nociceptive response in diabetic peripheral neuropathy is associated with myelin alterations, (Herman, 2007). Similar structural abnormalities and neurophysiological changes have been observed in the peripheral nerves of streptozotocin (STZ)-treated rats, an experimental model that captures many features of type 1 diabetes (Biessels et al., 1999, Dyck et al., 1988). As it is seen in diabetic subjects, STZ-treated rats show increased morphological alterations in the myelinated fibers of the sciatic nerve (Veiga et al., 2006a).

Recently, it has been reported that in BB/Wor diabetic rats, type 1 diabetes reduces the expression of the lipogenic transcription factor sterol-regulatory element binding factor-1c (SREBP-1c) in Schwann cells (de Preux et al., 2007). It has also been shown that the acute phase of myelin lipid synthesis is regulated by SREBP cleavage activating protein (SCAP), an activator of SREBPs (Verheijen et al., 2009). These observations suggest that lack of insulin and/or of transcriptionally active SREBP-1c in peripheral nerves leads to decreased lipogenesis. Diminished or abnormal FA synthesis may play an important role in altered myelin lipid and protein composition. Such changes in myelin could affect membrane fluidity and function, ultimately contributing to the pathogenesis of DPN (Verheijen et al., 2009, Zuvic-Butorac et al., 2001). In support of this notion, studies in animal models have revealed a protective effect of administration of FAs on DPN (Coste et al., 2004).

2.2 MYELIN

Structural abnormalities and neurophysiological changes have been observed in the peripheral nerves of STZ-treated rats, an experimental model that captures many features of type 1 diabetes (Dyck et al., 1988). As described before the diabetic peripheral neuropathy induced by diabetes is associated with deleterious changes in peripheral nerves, such as decrease in nerve conduction velocity and myelin damage (Sugimoto et al., 2000, Valls-Canals et al., 2002).

The principal role of the myelin sheath is to allow the faster transmission of the nervous impulse along the axons, which it surrounds. It accomplishes this function in the same way as the insulation around an electrical wire lets electricity flow through the wire, by preventing its dissipation into the surrounding medium. The membrane of the myelinated axon expresses numerous physiologically active molecules, which intervene directly in the transmission of the nervous impulse. These include the voltage-sensitive sodium channels, that are directly responsible for the propagation of the action potential along the axon and that are concentrated at the non-insulated axonal segments, known as the nodes of Ranvier, as well as the fast and slow potassium channels responsible for various secondary electrophysiological activities involved in the recovery to resting-state conditions after the passage of a nervous impulse. The distribution of these molecules along the axonal membrane is closely associated with the discontinuous segmental structure of the electrically insulating myelin sheath, and it is proposed that the latter participates actively in the establishment of this distribution (Waxman and Ritchie, 1993, Salzer, 1997). The myelin sheath, through its electrical resistance and low capacitance, allows the depolarization of the internodal axonal membrane with a minimal consumption of energy. Indeed, the highly concentrated sodium channels located at the nodes of Ranvier are activated by the

depolarisation of the axonal membrane induced by the arrival of a wave of positively charged sodium ions, thus allowing the massive influx of more sodium ions into the axon and causing a new wave of depolarisation. The latter, following the sodium concentration gradient, is passively transmitted along the insulated internodal segment of the axon, to the next node of Ranvier, where a new action potential is generated. Due to the segmental disposition of the electrically insulating myelin membrane, the action potential appears to jump from one node of Ranvier to the next: hence the term "saltatory conduction" (Ritchie, 1984). Exactly the same process is involved in the conduction along non-myelinated fibers, except that the sodium channels are distributed along the whole length of the axon. The smaller density of the channels leads to a smaller amplitude of the depolarisation wave, whose effective range is therefore shorter, leading in turn to a slower rate of transmission of the nervous impulse along the axon. The insulating role played by the myelin sheath is crucial for the correct and efficient functioning of the nervous system, as evidenced by the devastating effects of myelin loss from both the PNS, in genetic diseases such as the Guillain-Barrè syndrome and Charcot-Marie-Tooth disease or in autoimmune diseases such as Diabetes Mellitus, and the CNS, in diseases such as multiple sclerosis. Next we will focus our attention on organization and composition in myelin sheath of PNS tryng to underline the importance of this phisiological structure, and to point out the risk of its loss after the appearance of myelin abnormalities caused by diabetes mellitus (Veiga et al., 2006a).

MYELIN SHEATH ORGANISATION IN THE PERIPHERAL NERVOUS SYSTEM

In PNS, myelin is formed by the differentiation of the plasma membrane of Schwann cells. The latter, named after Schwann who first described them 160 years ago, are flattened cells with an elongated nucleus orientated

longitudinally along the nerve fiber. The elaboration of the electrically insulating myelin sheath by the Schwann cells requires concentrating large amounts of non-conducting material around segments of the axon and excluding as much aqueous, cytosolic material from the structure as possible. At the same time, access to all levels of the sheath must be ensured in order to allow its maintenance, and regularly spaced, non-insulated segments have to be left to allow the generation of the action potentials by the sodium channels of the axon. The solution found by the Schwann cell was to elaborate a highly lipid-rich plasma membrane with high contents of galactosphingolipids and saturated very long-chain fatty acids, and to wrap this membrane tightly around the axon many times (Mugnaini, 1982).

PNS MYELIN COMPOSITION

The myelin sheath is a morphologically complex entity composed of both non-compacted structures and a significantly large proportion of compacted membranes, each of which plays important roles in the integrated function of the sheath. This structural complexity presents a substantial problem for the biochemical characterization of myelin, till today the most common method to analyse the isolated myelin composition was described by Norton and Poduslo (Norton and Poduslo, 1973) and allows the isolation of a fraction highly enriched in large fragments of the compacted membrane components of the sheath. Therefore, as underlined by Mugnaini over 20 years ago (Mugnaini, 1982) one should be aware that many of the properties relating to the biochemical composition of “myelin” presented in the literature, even recently, are representative of only one part of the myelin sheath, and should not be extrapolated to englobe the numerous other structures, about which we still know relatively little. Bearing this in mind, one of the major biochemical characteristics that distinguishes myelin from other

biological membranes is its high lipid-to-protein ratio: isolated myelin contains 70±80% lipids and 20±30% proteins. This relatively high lipid content and the particular characteristics of the lipids present in the sheath, provide the electrically insulating property required for the saltatory propagation of the nervous influx.

LIPIDS

Fatty Acids Biosynthesis

In eukaryotes, fatty acid synthesis depends on a large soluble multienzyme complex that carries out the sequential enzymatic reactions required to build fatty acids. Structural determination of the fatty acid synthase has provided insights into how its multiple enzymatic activities work together to perform reiterative cycles of fatty acid synthesis (Leibundgut et al., 2007, Jenni et al., 2006). Fatty acid synthesis begins with the transfer of acetyl and malonyl groups from their CoA derivatives to a sulfhydryl group on the acyl carrier protein (ACP) domain of fatty acid synthase. An acetyl group from the malonyl-ACP is transferred to the acetyl-ACP to form an acetoacyl-ACP, which is reduced, dehydrated, and reduced again to saturate the growing acyl chains inside a cavity of the fatty acid synthase complex. This process is repeated until the fatty acids reach a length of 16 carbons. The growing hydrophobic acyl chains remain soluble because they are bound to the ACP domain, which delivers the substrate to the four different enzymatic domains of the reaction cycle.

Long-Chain Saturated Fatty Acids Are Synthesized from Palmitate

Palmitate, the principal product of the fatty acid synthase system in animal cells, is the precursor of other long-chain fatty acids. It may be lengthened

to form stearate (18:0) or even longer saturated fatty acids by further additions of acetyl groups, through the action of fatty acid elongation systems present in the smooth endoplasmic reticulum and in mitochondria. The more active elongation system of the ER extends the 16-carbon chain of palmitoyl-CoA by two carbons, forming stearoyl-CoA. Although different enzyme systems are involved, and coenzyme A rather than ACP is the acyl carrier in the reaction, the mechanism of elongation in the ER is otherwise identical to that in palmitate synthesis: donation of two carbons by malonyl-CoA, followed by reduction, dehydration, and reduction to the saturated 18-carbon product, stearoyl-CoA.

Desaturation of Fatty Acids Requires a Mixed-Function Oxidase

Palmitate and stearate serve as precursors of the two most common monounsaturated fatty acids of animal tissues: palmitoleate, 16:1(Δ^9), and oleate, 18:1(Δ^9); both of these fatty acids have a single cis double bond between C-9 and C-10. The double bond is introduced into the fatty acid chain by an oxidative reaction catalyzed by fatty acyl–CoA desaturase, a mixed-function oxidase. Two different substrates, the fatty acid and NADH or NADPH, simultaneously undergo two-electron oxidations. The path of electron flow includes a cytochrome (cytochrome b5) and a flavoprotein (cytochrome b5 reductase), both of which, like fatty acyl–CoA desaturase, are in the smooth ER. Bacteria have two cytochrome b5 reductases, one NADH-dependent and the other NADPH-dependent; which of these is the main electron donor in vivo is unclear. In plants, oleate is produced by a stearoyl-ACP desaturase in the chloroplast stroma that uses reduced ferredoxin as the electron donor. Mammalian hepatocytes can readily introduce double bonds at the Δ9 position of fatty acids but cannot introduce additional double bonds between C-10 and the methyl-terminal

end. Thus mammals cannot synthesize linoleate, 18:2($\Delta^{9,12}$), or α-linolenate, 18:3($\Delta^{9,12,15}$). Plants, however, can synthesize both; the desaturases that introduce double bonds at the Δ^{12} and Δ^{15} positions are located in the ER and the chloroplast. The ER enzymes act not on free fatty acids but on a phospholipid, phosphatidylcholine, that contains at least one oleate linked to the glycerol. Both plants and bacteria must synthesize polyunsaturated fatty acids to ensure membrane fluidity at reduced temperatures. Because they are necessary precursors for the synthesis of other products, linoleate and linolenate are essential fatty acids for mammals; they must be obtained from dietary plant material. Once ingested, linoleate may be converted to certain other polyunsaturated acids, particularly γ-linolenate, eicosatrienoate, and arachidonate (eicosatetraenoate), all of which can be made only from linoleate. Arachidonate, 20:4($\Delta^{5,8,11,14}$), is an essential precursor of regulatory lipids, the eicosanoids. The 20-carbon fatty acids are synthesized from linoleate (and linolenate) by fatty acid elongation reactions.

Fatty Acids Composition of Myelin Sheath

All the major lipid classes (neutral lipids, phosphoglycerides and sphingolipids) and their respective subclasses encountered in other membranes, are also represented in the myelin membrane and thus, strictly speaking, there are no myelin-specific lipids. This absence of myelin-specific lipids contrasts sharply with the situation for the proteins, of which there are myelin-specific components. In both mammalian and non-mammalian species, lipids account for 72-78% of the dry mass of PNS myelin, and there appear to be significant differences between the relative quantitative distributions of the various major lipid subclasses from one species to another. In mice, the variability in the absolute amount of each polar lipid/cm of whole nerve from one individual to another seems to increase with age. However, the relative proportions of each lipid present in the

nerves of different mice at a given age is subject to less variation and, thus, seems to be more closely controlled (Heape et al., 1986). Cholesterol accounts for 20-30% of the total lipids in the PNS. In mouse and rabbit sciatic nerves, cholesterol accumulates continuously throughout the period of neo-myelinogenesis and during the subsequent period of myelin maturation (Yates and Wherrett, 1974, Juguelin et al., 1986). This accumulation pattern is consistent with the role proposed for this lipid in the stabilization and the compaction of the multilamellar myelin membrane (Nussbaum et al., 1969, Detering and Wells, 1976). There are more ethanolamine phosphoglycerides (28-39%) than those with choline, and plasmalogens (phosphatidalcholine and phosphatidalethanolamine) are reported to be significantly abundant in the PNS. Sphingomyelin is more enriched in peripheral nerve myelin, where it represents 10-35% of the total lipids, than in brain myelin, where it accounts for only 3-8% of the lipids (Norton and Cammer, 1984). Phosphatidylserine is not particularly abundant in the myelinated peripheral nerves of mice. Perhaps the most striking feature of the myelin lipid composition when compared to that of other biological membranes is the high content in monogalactosylsphingolipids, with cerebrosides (Gal-C) and sulfatides (SGal-C) accounting for 14-26% and 2-7%, respectively, of the total PNS myelin lipid mass in adults. These levels are nevertheless lower than those encountered in the CNS. PNS myelin also contains gangliosides (sphingolipids with an oligosaccharide head group including one or more sialic acid residues), but here again, the amounts are lower than those encountered in the CNS (Fong et al., 1976). The fatty acyl components of the myelin lipids present a distinctive distribution when compared with those observed in non-myelin membranes. Oleic acid [C18:1(n-9)] is the major fatty acid in the PNS myelin, comprising between 30 and 40% of the fatty acids of sciatic nerves (Pratt et al., 1969, Fressinaud et al., 1986, Bourre et al., 1993). The fatty acid composition appears to be similar in rats and

mice. For example, the proportions of C18:0, C18:1 and C18:2 measured in the 25-day-old mouse sciatic nerves are 9%, 33% and 3%, respectively (Garbay et al., 1998), while those in 21-day-old rat sciatic nerves are 6.5% for C18:0, 35% for C18:1 and 5% for C18:2 (Bourre et al., 1993, Bourre et al., 1997). Besides the high levels of fatty aldehydes associated with the plasmalogens, most of which are palmitoyl (C16:0), stearoyl (C18:0) and oleoyl (C18:1) derivatives, myelin is also characterized by its relatively high content in very long-chain fatty acids (>18 carbon atoms). The fatty acid composition has been determined for individual lipids (Klein and Mandel, 1978, Yao, 1982), for both the endoneurium and the perineurium of adult rats (Fressinaud et al., 1986), and for the whole mouse sciatic nerve during postnatal development (Heape et al., 1987). While the results of these studies are often difficult to compare due to differences in the approaches employed, taken together, they indicate that there is a high enrichment in saturated very long-chain fatty acids (20±24 carbon atoms) in myelinated peripheral nerve tissue, and that the large majority of these are present in the sphingolipids. The very long-chain fatty acids are mostly saturated and a-hydroxylated, or non-hydroxylated, and are amidified to the primary amine function of the lipid's sphingosyl moiety. The sphingosyl-fatty acyl couple comprises the ceramide “module” common to all sphingolipids and whose diversity is determined by the nature of the fatty acyl component. As in the CNS myelin, the most abundant of the very long-chain fatty acyl groups in adult mouse sciatic nerves is lignoceric acid (C24:0) (Heape et al., 1987).

PROTEINS

Proteins in PNS myelin represent between 20 and 30% of the myelin dry mass. One of the characteristics of PNS myelin proteins is that at least 60% are glycoproteins (glycoproteins are minor constituents of CNS myelin).

GLYCOPROTEINS

After separation of rat PNS myelin proteins by sodium dodecylsulfate-polyacrylamide gel electrophoresis, followed by nitrocellulose transfer and overlay with various radiolabeled lectins, Shuman (Shuman et al., 1983), detected 26 distinct glycoproteins. Only four glycoproteins appeared to be quite abundant. The most abundant is a 28 kDa protein, named protein-zero, or P0 (Kitamura et al., 1976, Roomi et al., 1978). A 22 kDa glycoprotein, called PAS II, was purified and characterized by Kitamura (Kitamura et al., 1976); this protein is now known as Peripheral Myelin Protein 22, or PMP22. Two high molecular mass glycoproteins are present in relatively small amounts: a 170-kDa protein (Shuman et al., 1983), now referred to as Periaxin, and the 100 kDa Myelin-Associated Glycoprotein, or MAG (Sternberger et al., 1979), which was first described in CNS myelin (Quarles et al., 1973). Epithelial cadherin (E-cadherin) was also recently shown to be present in small amounts in PNS myelin (Fannon et al., 1995).

Protein zero (P0)

P0 is the major protein of PNS myelin and represents between 50-70% of the total protein (Greenfield et al., 1973, Wiggins et al., 1975). It is a 28 kDa integral membrane glycoprotein, which, in mammals, appears to be expressed by myelinating Schwann cells, but neither by CNS glia, nor by the non-myelinating Schwann cells that populate many peripheral nerves (Brockes et al., 1980, Trapp et al., 1981, Martini and Schachner, 1988, Lemke and Chao, 1988). P0 protein is highly conserved among species (Sakamoto et al., 1987). The putative role for the P0 protein is to function as a membrane adhesion molecule and to promote and maintain the very tight compaction of the myelin structure by homophilic interactions ((Kirschner and Ganser, 1980, Lemke and Axel, 1985) X- ray data suggested that the

extracellular domain of P0 forms tetramers, with four molecules arranged around a central hole (Shapiro et al., 1996). These tetramers, also more recently observed with the full-length P0 protein (Inouye et al., 1999), may interact with their counterparts on the apposing cell membrane, holding the two extracellular surfaces together.

Peripheral myelin protein 22 kDa (PMP22)

Peripheral Myelin Protein 22, or PMP22, is a small, 22 kDa glycoprotein that was first purified and characterized from bovine PNS myelin (Kitamura et al., 1976). It represents 2-5% of PNS myelin proteins (Pareek et al., 1993). The PMP22 mRNA was cloned by differential colony hybridization screening of cDNA libraries from regenerating rat sciatic nerve (De Leon et al., 1991, Spreyer et al., 1991, Welcher et al., 1991). Analysis of the corresponding cDNA clones revealed a mature protein of 18 kDa, containing four putative transmembrane domains (Manfioletti et al., 1990). This glycoprotein carries an N-linked glycosylation (at asparagine 41) in its first putative extracellular loop, which is consistent with the apparent molecular mass of 22 kDa of the PMP22 protein purified from sciatic nerves (Snipes et al., 1993). The carbohydrate residue may be involved in mediating adhesive processes (Snipes et al., 1993), suggesting that its function in myelin may be similar to that of the P0 glycoprotein (Pareek et al., 1993). The expression of PMP22 is not restricted to the PNS, since the corresponding mRNA has been detected, albeit in low quantities, in lung, gut, heart, brain and NIH3T3 fibroblasts (Manfioletti et al., 1990, Spreyer et al., 1991, Welcher et al., 1991).

2.3 NEUROSTEROIDS

SYNTHESIS, METABOLISM AND RECEPTORS OF NEUROACTIVE STEROIDS IN NERVOUS SYSTEM

Several observations have demonstrated that Schwann cells and glial cells express Peripheral Benzodiazepine Receptor (PBR), now renamed as TSPO (Papadopoulos et al., 2006), its endogenous ligands are octadecaneuropeptide (Lacor et al., 1996, Lacor et al., 1999, Schumacher et al., 2001) and StAR (Benmessahel et al., 2004). These molecules participate in the cholesterol transport from intracellular stores to the inner mitochondrial membrane where the P450scc (i.e., the enzyme that converts cholesterol to pregnenolone, PREG) is located. Both P450scc and 3β-HSD (i.e., the enzyme converting pregnenolone into progesterone, PROG) are present in Schwann cells (Mensah-Nyagan et al., 1999; Mellon et al., 2001). Moreover, metabolism of native steroids into their 5α- and 3α-hydroxy-5α reduced derivatives via the enzymatic complex formed by the 5α-Reductase (5α-R) and the 3α-HSD (Melcangi et al., 2004) also occurs in peripheral nerves and in central nervous system. In particular, PROG can be converted into Dihydroprogesterone (DHP) and subsequently into Tetrahydroprogesterone (3α,5α-THP) or into the isomer Isoallopregnanolone (3β,5α-THP), Testosterone (T) into Dihydrotestosterone (DHT) and then into 5α-androstane-3α,17β-Diol (3α-Diol) or into isomer 5α-androstane-3α,17β-Diol (3β-Diol) (Melcangi et al., 2001b). Estrogens (E) can be aromatized directly from T or can be synthesized from androstenedione passing to the intermediate estrone (Melcangi et al. 2008); the synthesis via peripheral nerves in Schwann cells and central nervous system in glial cells, not only synthesizes and metabolizes neuroactive steroids, but also expresses classical and non-classical steroid receptors. Namely, classical steroid receptors for PROG (PR), estrogens (ER), androgens (AR), glucocorticoids

and mineralocorticoids (Groyer et al., 2006) have been demonstrated in rat in Schwann cells and in brain (Melcangi et al. 2008). In particular, AR expression has been demonstrated in rat sciatic nerve, but it seems to be located in the endoneurial compartment and not in Schwann cells (Jordan et al., 2002; Magnaghi et al., 1999). In central nervous system, neuroactive steroids have been reported to modulate also neurotransmitter receptors, like for instance γ-Amino Butyric Acid type A and B (GABA-A and GABA-B receptors), serotonin type 3 (5-HT3), N-Methyl-D-Aspartate (NMDA), α-Amino-3-hydroxy-5-Methyl-4-isoxazole Propionic Acid (AMPA), kainate receptor and an atypical intracellular receptor like the sigma 1 (Lambert et al., 2001, 2003; Maurice et al., 2001; Romieu et al., 2003). Some of these receptors, such as GABA-A (i.e., α2, α3, β1, β2 and β3 subunits) and GABA-B (i.e., GABA-B1 and GABA-B2) receptors have been identified (Melcangi et al., 1999; Magnaghi et al., 2004a) also in peripheral nerves and Schwann cells. Moreover, rat nerve expresses NMDA receptor 1 subunit, Glutamate Receptor 1 (GluR 1) AMPA subunit and GluR 5, 6 and 7 kainate subunits (Coggeshall and Carlton, 1998; Verkhratsky and Steinhauser, 2000), and Schwann cells of mammalian peripheral vestibular system express GluR 2, 3 and 4 (Dememes et al., 1995; Verkhratsky and Steinhauser, 2000). Finally, the presence of sigma 1 receptor has been recently confirmed at the level of Schwann cells of rat sciatic nerve (Palacios et al., 2004).

PERIPHERAL NERVOUS SYSTEM RESPONSES TO NEUROACTIVE STEROIDS

It is now clear that neuroactive steroids are able to modulate the expression of myelin proteins of PNS, such as P0 and the Peripheral Myelin Protein 22 (PMP22). Both in vivo (i.e., in the rat sciatic nerve) and in vitro (i.e., in cultures of rat Schwann cells), the synthesis of these two important myelin proteins is modulated by the treatment with PROG and its derivatives. Namely, the expression of P0 in sciatic nerve of adult male rats, as well as

that in rat Schwann cell culture, is increased by the treatment with PROG, 5α-DHP or 3α,5α-THP, while in case of PMP22, only 3α,5α-THP is effective (Melcangi et al., 1998, 1999, 2001a,b, 2005). Not only PROG and its neuroactive derivatives are able to influence the synthesis of myelin proteins, but also neuroactive derivatives of T are effective. For instance, in adult male rats, castration decreases the expression of P0 in the sciatic nerve (Magnaghi et al., 1999, 2004b) and the subsequent treatment with DHT or 3α-Diol is able to restore the levels of the messenger of this myelin protein (Magnaghi et al., 1999, 2004b). Castration also decreases the mRNA levels of PMP22 in sciatic nerve, but in this case only 3α-Diol is able to counteract this effect (Magnaghi et al., 2004b). A very similar pattern of effects is also evident in cultures of rat Schwann cells. In this experimental model, DHT increases P0 mRNA levels (Magnaghi et al., 1999), while the treatment with 3α-Diol increases PMP22 mRNA levels (Melcangi et al., 2000a). Several mechanisms seem to be involved in the effects of neuroactive steroids on the expression of myelin proteins. Thus, the expression of P0 seems to be under the control of classical receptors, such as PR and AR, while a role for a non-classical steroid receptor, like GABA-A receptor may be hypothesized in case of PMP22 (Melcangi et al., 2005). PR involvement on the expression of P0 may be confirmed by the finding that in cultured rat Schwann cells an antagonist of this steroid receptor, such as mifepristone, is able to block the stimulatory effect exerted by PROG or DHP (i.e., classical ligands of PR). Interestingly, this antagonist is also effective in blocking the effect of 3α,5α-THP (i.e., a neuroactive steroid which is able to interact with GABA-A receptor) on P0 (Magnaghi et al., 2001). Indeed, the activity of the 3α-HSD is bi-directional (Melcangi et al., 2001b) and consequently 3α,5α-THP might be retroconverted into DHP exerting its effect on P0 via an activation of PR. A role for the classical PR is also supported by in vivo observations. For instance, treatment with mifepristone since the first day of life decreases 20 days later the expression of P0 (Melcangi et al.,

2003a). Activation of a classical steroid receptor, such as PR, clearly suggests that the effect of PROG derivatives on P0 expression is due to a classical steroid genomic effect. This hypothesis is supported by the finding that a coactivator, such as Steroid Receptor Coactivator-1 (SRC-1) participates in the regulation of P0 gene expression by DHP. Indeed, as demonstrated in an immortalized cell line of Schwann cell (i.e., MSC80 cells) stably transfected to over- or down-express SRC-1, the effect of DHP on P0 expression was increased or completely lost respectively (Cavarretta et al., 2004). Moreover, a further support to the hypothesis that P0 is under the control of a classical steroid genomic mechanism is that putative progesterone responsive elements are present on P0 gene (Magnaghi et al., 1999). A role for AR in controlling expression of P0 may be also hypothesized. Indeed, in vivo treatment with an antagonist of this steroid receptor (i.e., flutamide) decreases the synthesis of P0 in rat sciatic nerve (Magnaghi et al., 2004b). Interestingly, inhibition of AR influences P0 synthesis in adult age only. This age-linked effect is different from what we have observed after the in vivo treatment with mifepristone, where PR antagonist is only able to decrease the synthesis of P0 at postnatal day 20 (Melcangi et al., 2003a). A possible hypothesis could be that PROG derivatives may be necessary for inducing P0 synthesis during the first steps of the myelination process, while the subsequent intervention of T derivatives will participate in the maintenance of this process. As mentioned above, the expression of PMP22 seems to be under the control of GABA-A receptor. In fact, experiments performed in Schwann cell cultures utilizing agonists or antagonists of GABA-A receptor have indicated that bicuculline (i.e., a specific antagonist of this receptor) completely abolishes the stimulatory effect exerted by 3α,5α-THP on PMP22, while muscimol (i.e., an agonist of GABA-A receptor) exerts a stimulatory effect on PMP22, which is comparable to that exerted by 3α,5α-THP (Magnaghi et al., 2001). The specificity of the effect of 3α,5α-THP on the GABA-A receptor

is also supported by the finding that isopregnanolone, which does not interact with GABA-A receptor, is unable to modify the expression of this myelin protein. Moreover, the finding that among T derivatives so far considered, only 3α-Diol (i.e., a neuroactive steroid which is able to interact with GABA-A receptor) significantly increases PMP22 mRNA levels (Magnaghi et al., 2004b; Melcangi et al., 2000a) gives further support to the role of GABA-A receptor in controlling PMP22 expression. Recent data, however, indicate that P0 and PMP22 expression is not only merely under the control of classical (i.e., PR and AR) and non-classical steroid (GABA-A receptor) receptors respectively, but sex is another variable. As recently demonstrated, at least in culture of rat Schwann cells, the effects of PROG and its derivatives on the expression of myelin proteins are sexually dimorphic (Magnaghi et al., 2006). Interestingly, neuroactive steroids not only regulate the expression of myelin proteins but also that of transcription factors (TFs) with a key role in Schwann cell physiology and in their myelinating program. For instance, data obtained in culture of rat Schwann cells (Guennoun et al., 2001; Mercier et al., 2001) have indicated that PROG stimulates the gene expression of Krox-20, Krox-24, Egr-3 and FosB. Moreover, we have recently demonstrated that not only PROG, but also its derivatives affect the synthesis of TFs, such as Krox-20 and Sox-10. Indeed, in culture of rat Schwann cells the expression of Krox-20 is stimulated by the treatment with DHP or 3α,5α-THP, while that of Sox-10 is only stimulated by DHP (Magnaghi et al., 2007). These observations, together with the concept that PROG derivatives are also able to influence directly the expression of myelin proteins suggest that they might coordinate Schwann cell myelinating program utilizing different intracellular pathways. Neuroactive steroids not only influence the expression of myelin proteins by Schwann cells but they also affect their proliferation. A stimulatory effect of PROG on proliferation of Schwann cells has been detected in vitro (Svenningsen and Kanje, 1999; Bartolami et al., 2003). An effect of androgens on Schwann cell

proliferation is also evident. Namely, the number of terminal Schwann cells unsheathing the synaptic junction between motor nerve endings and muscles decreases after castration and this effect is counteracted by T replacement (Lubischer and Bebinger, 1999). In addition, not only neuroactive steroids themselves, but also steroid coactivators, which, as previously mentioned participate in the effects exerted by neuroactive steroids on myelin proteins, are able to affect cell proliferation. It has been demonstrated that cell proliferation in immortalized Schwann cell lines (i.e., MSC80 cells) overexpressing SRC-1 is slower than in cells in which the coactivator expression is down regulated (Melcangi et al., 2005). In contrast, overexpression of another coactivator, such as Steroid Receptor RNA Activator (SRA), induces an increase in the proliferation of MSC80 cells (Melcangi et al., 2005).

The effects of neuroactive steroids mentioned above on the expression of myelin proteins and Schwann cell proliferation have important repercussions for myelin formation and the maintenance of myelin structure. Effects of neuroactive steroids on the expression of myelin proteins such as P0 and PMP22 are particularly relevant since these two proteins play an important role for the maintenance of the multilamellar structure of PNS myelin (D'Urso et al., 1990). P0, a member of the immunoglobulin gene superfamily (IgCAM), accounts for more than half of the total peripheral myelin proteins (Ishaque et al., 1980), and it is predominantly confined to the compact portion of the mature myelin. The importance of P0 for stabilizing compact myelin is illustrated by the severe phenotype of P0-negative mice generated by homologous recombination (Giese et al., 1992; Martini et al., 1995; Zielasek et al., 1996), with pathological alterations affecting both myelin and axonal compartments, similar to those occurring in some dominantly inherited human peripheral neuropathies (e.g., Charcot-Marie-Tooth type 1b, CMT1B and Déjérine-Sottas syndrome, DSS).

In agreement with the effect exerted on the proteins of peripheral myelin, PROG is also able to stimulate the myelin synthesis itself. For instance, PROG is able to accelerate the time of initiation and to enhance the rate of myelin synthesis in Schwann cells co-cultured with Dorsal Root Ganglia (DRG) neurons (Chan et al., 1998, 2000). Moreover, recent observations have indicated that also axonal compartment of PNS neurons may be considered a target for the action of neuroactive steroids. Thus, PROG affects the expression of neuronal genes that may promote myelination process by Schwann cells. For instance, in co-culture of Schwann cells and DRG neurons two genes, like a small Ras-like GTP binding protein (Rap 1b) and phosphoribosyl diphosphate synthase-associated protein, which are induced in co-cultures during myelin synthesis, are also induced by PROG treatment (Chan et al., 2000; Rodriguez-Waitkus et al., 2003). Moreover, it has been observed that the blockade of PR results in axonal impairment in the sciatic nerve of male rats. Indeed, morphological analysis of sciatic nerves of animals treated with mifepristone during development indicates a reduced axon diameter compared to myelin thickness and an increased neurofilament density (Melcangi et al., 2003a).

Neuroactive steroids act in peripheral nerves as physiological regulators and as protective agents for acquired and inherited peripheral neuropathy (Melcangi and Garcia- Segura, 2006; Schumacher et al., 2007; Roglio et al., 2008b; Melcangi and Panzica, 2009). Previous studies have shown that the levels of neuroactive steroids are reduced in the peripheral nerves of STZ diabetic rats (Caruso et al., 2008; Pesaresi et al., 2010). In turn, the administration of neuroactive steroids improves the neuropathic phenotype induced by diabetes (Veiga et al., 2006; Leonelli et al., 2007; Roglio et al., 2007; Cermenati et al., 2010). This suggests that increasing the levels of neuroactive steroids directly in the nervous system could be a therapeutic approach that may avoid the potential endocrine side effects of systemic

administration of neuroactive steroids. Indeed, we recently reported that a ligand of TSPO (Ro5-4864) that increases the local concentration of neuroactive steroids is effective at reducing the severity of diabetic neuropathy (Giatti et al., 2009).

2.4 LIVER X RECEPTORS

Steroidogenesis occurs in peripheral nervous system (Garcia-Segura and Melcangi, 2006), and this nervous fibers express proteins involved in the initiation of steroidogenesis such as StAR and TSPO as well as the enzymes involved in the synthesis and metabolism of neuroactive steroids (Garcia-Segura and Melcangi, 2006, Melcangi et al., 2008). Recently it was reported that StAR can also be modulate in the adrenal gland directly by Liver X Receptors (LXRs) (Cummins and Mangelsdorf, 2006).

LXRs are members of the nuclear receptors superfamily. LXRs have the classical structure of a nuclear receptor: a DNA binding domain, a ligand binding domain and a ligand independent activation function 2 (AF2) that, through the recruitment of coactivators and corepressors, regulates the activity of the receptor.

More than ten years ago these proteins were discovered, cloned and termed “orphan nuclear receptors” (Chawla et al., 2001). Today, since the physiological ligands are known, they are classified as “adopted orphans”.

Two different isoforms, LXRα (NR1H3) and LXRβ (NR1H2) are known. LXRα is predominantly expressed in the liver and at lower levels also in the intestine, macrophages, adipose tissue, lungs, kidneys and the adrenal gland, while LXRβ is broadly expressed (Li and Glass, 2004) including neurons, microglia, astrocytes (Gilardi et al., 2009) oligodendrocytes (Nelissen et al., 2012), and Schwann cells (Makoukji et al., 2011).

LXRs are ligand activated transcription factors that form an obligate heterodimers with the Retinoic X Receptor (RXR). The LXR/RXR complex, activated by ligands, binds a specific sequence, called LXR Responsive Element (LXRE) in the promoter of the target genes modulating their expressions. Usually the DNA sequence recognized by LXRs is a direct

repeat of the core G/AGGTCA separated by four nucleotides DR-4 (Willy et al., 1995). However, it has been reported that LXR can also bind an inverted repeat (IR) sequence without spacing nucleotides (IR0) (Uppal et al., 2007).

LXRs natural ligands are represented by oxysterols, an oxidized form of cholesterol produced from the cells as intermediates in steroid hormones or bile acids biosynthesis (Janowski et al., 1996). Accordingly with the nature of the physiological ligands, LXRs play an important role in cholesterol, and lipid metabolism.

The role of LXRs as an intracellular cholesterol sensor is primarily due to the activation of key genes in cholesterol efflux such as the ATP Binding Cassette (ABC) family and in particular ABCA1, ABCG1, ABCG5 and ABCG8, Apolipoprotein E (ApoE) and Cholesterol Ester Transfer Protein (CETP) (Cummins and Mangelsdorf, 2006). This activation, mediated by high affinity ligands, increases HDL level and induces cholesterol efflux (Beaven and Tontonoz, 2006). LXRs also play a role in lipogenesis and triglyceride synthesis primarily due to the upregulation of Sterol Regulatory Element Binding Protein-1c (SREBP-1c) and Fatty Acid Synthase (FAS) (Repa et al., 2000). All these genes are regulated by LXRs in a direct fashion due to the presence of one or multiple LXRE in the promoter region of the target genes.

The literature on LXRs during the last two decades is mainly focused on the role of these nuclear receptors in the liver, adipose tissue, pancreas and skeletal muscle. During the last ten years several evidences have addressed the fundamental role of LXRs also in the nervous systems.

LXR IN OBESITY AND DIABETES

With the advent of genome-wide association studies, several groups have reported potential connections between human LXR genes and obesity

and diabetes. To date, however, the data are somewhat conflicting, and mechanistic links between specific genetic changes and human physiology remain to be established. As key metabolic tissues express both LXRα and LXRβ and the vast majority of the known LXR target genes respond to both receptors, it seems unlikely that even a complete loss of function in only one of the four LXR alleles would cause a dramatic phenotype. A recent study in individuals with type 2 diabetes identified a Single Nucleotide Polymorphism (SNP) in LXRβ, with the minor allele being associated with reduced pancreatic β-cell function. As pancreatic β-cells express LXRβ but not LXRα, it is understandable how an LXRβ polymorphism might have a functional effect in this cell type (Ketterer et al., 2011). Some studies have suggested that SNPs in both LXRα and LXRβ are linked to obesity phenotypes (Solaas et al., 2010), but others have not observed these associations (Auboeuf et al., 1997). In murine models of obesity and diabetes, pharmacologic LXR activation has both beneficial and detrimental effects on pathways related to glucose metabolism and insulin sensitivity. It has been demonstrated that 1 week of treatment with the LXR agonist GW3965 was associated with a substantial improvement in glucose tolerance in mice fed a high-fat diet (Laffitte et al., 2003). These effects were correlated with reduced hepatic glucose output and enhanced glucose uptake in adipose tissue (Laffitte et al., 2003). Similar effects were observed in leptin-deficient ob/ob mice, with no change in hepatic glucose output but increased Glucose Transporter Type 4 (GLUT4) expression in fat (Grefhorst et al., 2005). GW3965 administration to rats on a high-fat diet improved insulin suppression of free fatty acids, increased glucose infusion rate and reduced insulin suppression of glucose production; however, glucose uptake was not affected in this model (Commerford et al., 2007). Thus, short-term treatment with an LXR agonist seems to increase peripheral glucose disposal and improve glycemic control despite hepatic steatosis. Studies in adipose tissue have shown that

LXR protects against Tumour Necrosis Factor-α (TNFα)-induced insulin resistance, improving insulin signalling at the level of Insulin Receptor Substrate 1 (IRS1) in human adipocytes (Fernández-Veledo et al., 2009). Similar effects were observed in mouse primary brown adipocytes and were associated with improved translocation of GLUT4 to the plasma membrane (Joseph et al., 2004). Longer-term studies with LXR agonists and LXR-deficient mice will be required to more precisely define the tissue-specific contributions of LXR signalling to lipid and glucose homeostasis.

LXRS AS MASTER REGULATORS OF FAT METABOLISM

In addition to their roles in cholesterol metabolism, the LXRs have profound effects on hepatic fat metabolism. These effects are mediated in part through the transcriptional regulation of SREBP-1c, the master regulator of fatty acid and triglyceride synthesis (Horton et al., 2002, Repa et al., 2000). LXRs bind two functional LXREs in the promoter region of the SREBP-1c gene, which are required for both the basal and inducible expression of SREBP-1c protein (Repa et al., 2000, Yoshikawa et al., 2001). In wild-type mice, cholesterol feeding or administration of a synthetic ligand for LXR and its heterodimeric partner RXR resulted in increased SREBP-1c expression, nuclear SREBP-1c protein levels, and fatty acid synthesis. These effects were abrogated in LXR-null mice, which are refractory to both cholesterol- and insulin-induced expression of SREBP-1c (Repa et al., 2000). In addition to SREBP-1c, other lipogenic genes, such as fatty acid synthase (FAS), have been reported as direct LXR targets (Joseph et al., 2002). In addition to the regulation of lipogenic gene expression, LXRs are involved in the regulation of fat metabolism, particularly as it relates to the diet. For example, a wild-type mouse requires LXRs to process and store dietary fat properly (Kalaany et al., 2005). When fed a high-fat, high-cholesterol "Western"-style diet, LXR-null mice are resistant to obesity and show better glucose tolerance. LXR-null mice, in fact, did not store dietary fat but they burn it off in their

peripheral tissues and display significantly increased metabolic rates and markedly suppressed hepatic fatty acid synthesis. Interestingly, resistance to diet-induced obesity depends on the presence of dietary cholesterol; this finding underlined an essential role for LXRs in regulating the balance between fat storage and oxidation (Kalaany et al., 2005). This study and others revealed the existence of an SREBP-1c-independent regulatory pathway to regulate hepatic fat metabolism and showed that LXRs are required for both the basal (Peet et al., 1998, Repa et al., 2000) and insulin-induced expression of SREBP-1c (Chen et al., 2004). From a pharmacological point of view, the finding that LXR agonists can regulate fat metabolism has been a thorn in the development of drugs such as those for reducing body cholesterol levels, enhancing insulin sensitivity, and protecting against atherosclerosis. Indeed, oral administration of the potent synthetic agonist T0901317 in mice and hamsters leads to increased hepatic fatty acid synthesis, hepatic steatosis, and increased VLDL triglycerides (Schultz et al., 2000, Grefhorst et al., 2002). When administered to hypercholesterolemic ApoE−/− or LDL receptor−/− mice, the LXR ligand also causes a 12-fold increase in plasma triglycerides (Grefhorst et al., 2002). Importantly, a majority of these detrimental lipid responses are due to activation of the SREBP-1c pathway (Kalaany et al., 2005, Liang et al., 2002), whereas activation of ABC transporters results in the beneficial therapeutic effects of lowering cholesterol. Interestingly SREBP-1c and the ABCA1 genes exhibit a striking difference in the ways in which LXRs regulate their promoters (Wagner et al., 2003): the absence of LXRs in mice causes derepression of basal ABCA1 expression in macrophages and the intestine; by contrast, basal SREBP-1c levels are decreased significantly. This is thought to result from the LXR-dependent, differential recruitment of the corepressors NCoR and SMRT to the ABCA1 and SREBP-1c promoters. Therefore, the development of an LXR partial agonist able to enhance basal expression of ABCA1 without affecting SREBP-1c expression would

have potential therapeutic value. The rationale for such pharmacological agents has been established with other nuclear receptor targets, such as the estrogen receptor (Osborne et al., 2000). From a physiological point of view, LXRs appear to function as upsteam master regulators of SREBP-1c-dependent and -independent lipogenic pathways that are induced in response to increased sterol as well as insulin levels. Hence, the physiological function of LXRs points to a survival response by the body to efficiently store carbohydrate- and high-fat diet–derived energy in the form of body fat.

LXRS AND CHOLESTEROL METABOLISM IN PERIPHERAL NERVOUS SYSTEM

Peripheral neuropathies are common disorders affecting PNS. Peripheral neuropathies may arise during the aging process, after mechanical injury, by metabolic disorders (e.g., diabetes mellitus), by infections and autoimmune diseases, or after exposure to such type of therapeutics or toxic compounds. Damages to the PNS can also be inherited as in the case of the Charcot–Marie–Tooth (CMT) disease (Melcangi and Panzica, 2006).

In the PNS also cholesterol plays a crucial role. In the PNS the majority of cholesterol is found in myelin and in particular in peripheral myelin producing cells such as the Schwann cells. To investigate the role of cholesterol in Schwann cells, two different mouse models have been generated and characterized. The first is a mouse model where the Squalene Synthase, a key enzyme to produce cholesterol, has been inactivated (SQS mice). The second model is a Schwann cells specific knock-out of the SREBP Cleavage Activation Protein (SCAP). Both models developed peripheral neuropathy due to a reduced myelination of the sciatic nerve. Moreover, in the SQS mutant Schwann cells it has been demonstrated that the export from the endoplasmic reticulum of the major

myelin protein P0 to growing myelin is dependent on cholesterol. These data undoubtedly prove the important role of cholesterol and fatty acids in myelin generation (Saher et al., 2005, Verheijen et al., 2009). Given the key role of lipids in the PNS and that LXRs are central in the regulation of cholesterol homeostasis and fatty acid metabolism, it is important to address their biology in this tissue either in physiological or in pathological conditions. Makoukji and colleagues highlighted the importance of LXRs in regulating myelin peripheral nerves. Indeed, this team demonstrated that LXRDKO mice displayed thinner myelin compared with the age matched in wild type control animals (Makoukji et al., 2011). Thus, the lack of LXRs in the sciatic nerve alters the myelin structure (Makoukji et al., 2011).

Recently, it was reported that LXRs directly modulate Steroidogenic Acute Regulatory Protein (StAR) expression in the adrenal gland, a transfer protein regulating cholesterol shuttling into mitochondria, a key step in the initiation of steroid hormone synthesis (Cummins and Mangelsdorf, 2006). Steroidogenesis also occurs in peripheral nerves where it results in the formation of neuroactive steroids (Garcia-Segura and Melcangi, 2006, Melcangi et al., 2008). Peripheral nerves express proteins involved in the initiation of steroidogenesis, such as StAR and the Translocator Protein-18 kDa (TSPO), as well as the enzymes involved in the synthesis and metabolism of neuroactive steroids, such as Cytochrome P450 side chain cleavage (P450scc), 3-Hydroxysteroid Dehydrogenase (3-HSD), and 5-Reductase (5-R) (Garcia-Segura and Melcangi, 2006, Melcangi et al., 2008). Neuroactive steroids act in peripheral nerves as physiological regulators and as protective agents for acquired and inherited peripheral neuropathy (Garcia-Segura and Melcangi, 2006, Melcangi and Panzica, 2009). This suggests that increasing the levels of neuroactive steroids directly in the nervous system could be a therapeutic approach that may avoid the potential endocrine side effects of systemic administration of neuroactive

steroids. Indeed, we recently reported that a ligand of TSPO (Ro5-4864) that increases the local concentration of neuroactive steroids is effective at reducing the severity of diabetic neuropathy (Giatti et al., 2009).

Given that neuroactive steroids are cholesterol-derived molecules, we hypothesized that LXRs may have protective effects on peripheral neuropathy by modulating the levels of these hormones. Supporting our hypotesis are some results obtained previously in a study by using a synthetic activator of the TSPO (Giatti et al., 2009).

Moreover, Verheijen and colleagues, by using the SCAP Schwann cells-specific knock-out demonstrated that not only cholesterol is important for myelin formation but also fatty acids (Verheijen et al., 2009). In fact, the LXRs directly regulate expression of the lipogenic transcription factor SREBP-1c, and of key enzymes involved in FA biosynthesis, such as FAS and stearoyl-CoA desaturase-1 (SCD-1) (Kalaany and Mangelsdorf, 2006, Schultz et al., 2000). By the ability of LXRs to modulate lipid levels, an evaluation of the potential of a synthetic LXR activator to reverse diabetes-induced lipid abnormalities in sciatic nerve myelin could be hypotized.

3. AIM OF THE STUDY

Diabetic peripheral neuropathy (DPN) appears frequently in patients diagnosed with type 1 or type 2 diabetes (Association, 2007), this side effect is associated with deleterious changes in peripheral nerves, such as myelin damage and decrease in nerve conduction velocity (Sugimoto et al., 2000, Valls-Canals et al., 2002). The myelin sheath is a multilayered membrane produced in the peripheral nervous system by differentiation of the plasmatic membrane of Schwann cells (Garbay et al., 2000). The main role of this membrane is to allow efficient transmission of nerve impulses along the axons that it surrounds (Waxman and Ritchie, 1993). Several studies demonstrated that in the peripheral nerves of streptozotocin (STZ)-treated rats, an experimental model that captures many features of type 1 diabetes (Herman, 2007) are present structural abnormalities and neurophysiological changes affecting this protective and functional structure (Veiga et al., 2006a).

It is of interest to note that a role of neuroactive steroids as protective agents has been proposed for neurodegenerative diseases (Leonelli et al., 2007).

Neuroactive steroids have been shown to exert neuroprotective effects on central nervous system (CNS) and peripheral nervous system (PNS) in experimental models of diabetic neuropathy (Giatti et al., 2009, Pesaresi et al., 2010). It has been observed that nervous system is able to synthesise neuroactive steroids, and also to express different receptors that can bind neuroactive steroids achieving to exert protective effects. However, increasing the levels of neuroactive steroids directly in the nervous system could be a therapeutic approach that may avoid the potential endocrine side effects of systemic administration of neuroactive steroids.

Recently, it was reported that Liver X Receptors directly modulate steroidogenic acute regulatory protein (StAR) expression in the adrenal

gland, a transfer protein regulating cholesterol shuttling into mitochondria, a key step in the initiation of steroid hormone synthesis (Cummins and Mangelsdorf, 2006).

LXRs α and β (NR1H3 and NR1H2 respectively) are ligand activated transcription factors that belong to the nuclear receptor superfamily and they, upon activation, principally act preventing excessive cholesterol intracellular accumulation.

In addition to their role in the regulation of cholesterol homeostasis, the LXRs also modulate fatty acid metabolism. In fact, the LXRs directly regulate expression of the lipogenic transcription factor SREBP-1c, and of key enzymes involved in fatty acid biosynthesis such as fatty acid synthase (FAS) and stearoyl-CoA desaturase-1 (SCD-1) (Schultz et al., 2000). Recently, it has been reported that in BB/Wor diabetic rats, type 1 diabetes reduces the expression of the lipogenic transcription factor SREBP-1c in Schwann cells (de Preux et al., 2007). It has also been shown that the acute phase of myelin lipid synthesis is regulated by SREBP cleavage activation protein (SCAP), an activator of SREBPs (Verheijen et al., 2009). These observations suggest that lack of insulin and/or of transcriptionally active SREBP-1c in peripheral nerves leads to decreased lipogenesis. Diminished or abnormal fatty acid synthesis may play an important role on altered myelin lipid and protein composition. Such changes in myelin could affect membrane fluidity and function, ultimately contributing to the pathogenesis of DPN (Verheijen et al., 2009). In support of this notion, studies in animal models have revealed a protective effect of administration of fatty acids on DPN (Coste et al., 2004). However, the specific changes in myelin lipid and protein composition induced by diabetes, as well as the molecular mechanism responsible for these defects, remain to be identified.

In this panorama, the aim of our research was to understand the role of LXR activation on peripheral neuropathy induced by diabetes.

Our first objective is to understand whether LXR activation, due to is effect on StAR, drives neuroactive steroid synthesis in sciatic nerve, and if treatment with LXR ligands ameliorates diabetes induced neuropathy, as evaluated by a variety of functional and biochemical parameters. We try to demonstrate if LXR activation promotes steroidogenesis, cholesterol disposal, and raises the local levels of neuroactive steroids. An increase of these levels can exert neuroprotective effects against peripheral neuropathy induced by diabetes.

Then we will focus our experiments on the LXRs ability to modulate fatty acids metabolism. The ability of LXRs to modulate lipid levels suggests the evaluation of LXR activation by a synthetic to reverse diabetes-induced lipid abnormalities in sciatic nerve myelin. Here, we would understand if LXR activation, by its ability to modulate fatty acids biosynthesis, is able to normalize myelin's lipid profile, restores expression of genes involved in Fatty Acids (FA) biosynthesis, reestablishes nuclear levels of SREBP-1c and absolute levels of P0, and corrects the major myelin abnormalities induced by diabetes in peripheral nerves.

At last we will investigate the possible crosstalk between these two different neuroprotective mechanisms involving steroids and fatty acids. In particular we will focus our attention on the neuroactive steroids that will be restored by LXR activation in diabetic setting as describe in the first objective. Once established which neuroactive steroids are brought-back to control levels we will administrate these molecules to diabetic animals to prove their role in regulating myelin structure, function and lipid composition.

The analyses necessary to achieve these three objectives will be conducted on sciatic nerves or purified myelin of rats, using different

analytical techniques. Lipidomic profile analyses will use liquid chromatography tandem mass spectrometry (LC-MS/MS) and gas chromatography mass spectrometry (GC-MS). The lipidomic profile will be corroborated by gene expression analysis of key genes in fatty acid biosynthesis and will be complemented by a morphometric analysis of myelinated fibers.

These studies will be carry out trying to extend the neuroprotective actions of LXR so far observed in multiple neuronal injury models, such as ischemic brain injury, Alzheimer's disease, and Niemann-Pick C disease (Repa et al., 2007, Zelcer et al., 2007, Morales et al., 2008, Sironi et al., 2008, Cheng et al., 2010). Finally, we are trying to suggest that LXR may be a promising therapeutic target for diabetic neuropathy.

4. MATERIALS AND METHODS

4.1 MATERIALS AND METHODS

ANIMALS

Two-month-old male Sprague Dawley rats, Crl:CD BR (Charles River) were used. The animals were maintained in quarters with controlled temperature and humidity. The light schedule was 14 h light and 10 h dark (lights on at 6:30 AM). The animals were handled following the European Union Normative (Council Directive 86/609/EEC), with the approval of the Institutional Animal Use and Care Committees. Special care was taken to minimize animal suffering and to reduce the number of animals used to the minimum required for statistical accuracy. Induction of diabetes and experimental treatments. Diabetes was induced by a single i.p. injection of freshly prepared streptozotocin (65 mg/kg; Sigma) in 0.09 M citrate buffer, pH 4.8. Control animals were injected with 0.09 M citrate buffer at pH 4.8. Hyperglycemia was confirmed 48 h after streptozotocin injection by measuring tail vein blood glucose levels using a glucometer OneTouch Ultra2 (LifeScan). Only animals with plasma glucose levels >300 mg/ml were classified as diabetic. Glycemia was also assessed before and at the end of treatment with GW3965 (2 months after streptozotocin injection, see below), 3 months after streptozotocin administration. At 2 months, diabetic animals were treated once a week with GW3965 50 mg/kg (i.e., they received 4 subcutaneous injections) (STZ+GW3965 group) or every other day and for a month with a subcutaneous injection of DHP (STZ+DHP group) or 3α-diol (STZ+3α-diol group) (3.3 mg/kg body weight; total of sixteen treatments). The rationale for the once a week treatment with GW3965 is that daily systemic activation of LXRs is thought to result in hypertriglyceridemia (due to increased hepatic lipogenesis), an

undesirable side effect (Schultz et al., 2000). Thus, we sought to use the minimal dose of GW3965 that would show benefit while minimizing the potential for side effects. Nondiabetic and diabetic control rats were treated with vehicle. GW3965 was synthesized as previously described (Marino Jr et al., 2009). Non-diabetic and diabetic control rats were treated with vehicle (sesame oil). Special care was taken to minimize animal suffering and to reduce the number of animals used to the minimum required for statistical accuracy.

REAGENTS

Steroids analyses: 5-Pregnen-3β-ol-20-one (PREG), progesterone (PROG), 5α-pregnane-3, 20-dione (DHP), 3α-hydroxy-5α-pregnen-20-one (THP), 3β-hydroxy-5α-pregnen-20-one (isopregnanolone), testosterone (T), 5α-androstane-17β-ol-3-one (DHT), 5α-androstane-3α,17β-diol (3α- diol), and dehydroepiandrosterone (DHEA) were purchased from Sigma-Aldrich. 2,2,4,6,6 –17α,21,21,21-D9-PROG (D9-PROG) was purchased from Medical Isotopes; 2,4,16,16-D4-17β-estradiol (D4- 17β-E) was obtained from CDN Isotopes. 17,21,21,21-D4-PREG (D4- PREG) was kindly synthesized by Prof. P. Ferraboschi (Department of Medical Chemistry, Biochemistry and Biotechnology, Universitá di Milano, Milano, Italy). Solid phase extraction (SPE) cartridges (Discovery DS-C18 500 mg) were from Supelco. All solvents and reagents were HPLC grade (Sigma-Aldrich). GW3965 was synthesized in house as previously described (Marino Jr et al., 2009).

*Fatty acids analyses***:** The internal standards (ISs) heneicosanoic acid (C21:0) and 5α-cholestane, and all other standards for FAs and cholesterol analyses were purchased from Sigma-Aldrich.

ASSESSMENT OF NEUROACTIVE STEROIDS BY LIQUID CHROMATOGRAPHY TANDEM MASS SPECTROMETRY

Tissue and plasma samples were extracted and purified according to Caruso (Caruso et al., 2008). Briefly, samples (100 mg of tissue or 0.5 ml of plasma) were added with internal standards (D4-17b-E, 1 ng/sample; D9-PROG, 0.2 ng/sample; D4-PREG, 5 ng/sample) and homogenized in 2 ml of MeOH/acetic acid (99:1, v/v) using an ultrasonic homogenizer (Bransonic Ultrasonics). After an overnight incubation at 4°C, samples were centrifuged at 12,000 rpm for 5 min and the pellet was extracted twice with 1 ml of MeOH/acetic acid (99:1, v/v). The organic phases were combined and dried with a gentle stream of nitrogen in a 40°C water bath. Samples were resuspended with 3 ml of MeOH/H2O (10:90, v/v) and passed through an SPE cartridge. Steroids were eluted in MeOH, concentrated and transferred into auto-sampler vials before liquid chromatography–tandem mass spectrometry (LC–MS/MS) analysis. Quantitative analysis was performed on the basis of calibration curves prepared and analyzed using deuterated internal standards. Calibration curves were extracted as described above for samples. Positive atmospheric pressure chemical ionization (APCI) experiments were performed using a linear ion trap-mass spectrometer (LTQ, Thermo Electron) equipped with a Surveyor liquid chromatography Pump Plus and a Surveyor Autosampler Plus (Thermo Electron). The LC mobile phases were (A) H2O/0.1% formic acid and (B) methanol (MeOH)/0.1% formic acid. The gradient (flow rate 0.5 ml/min) was as follows:

- T0.0 70% A
- T1.5 70% A
- T2.0 55% A
- T3.0 55% A
- T35.0 36% A

- T40.0 25% A
- T41.0 1% A
- T45.0 1% A
- T45.2 70% A
- T55.0 70% A

The split valve was set at 0–6.99 min to waste, 6.99–43.93 min to source and 43.93–55 to waste. The Hypersil Gold column (100 x 3 mm, 3 μm; Thermo Electron, USA) was maintained at 40°C. The injection volume was 25 μl and the injector needle was washed with MeOH/ water 1/1 (v/v). Peaks of the LC–MS/MS were evaluated using a Dell workstation by means of the software Excalibur release 2.0 SR2 (Thermo Electron). The mass spectrometer was operated in the positive ion mode with the APCI source using nitrogen as sheath, auxiliary and sweep gas at flow rates of 23, 8, 2 (arbitrary units), respectively. Other ion-source parameters: vaporizer temperature 450°C, ion-source collision-energy (SID) 20 V, capillary temperature 275°C. The mass spectrometer was used in MS/MS mode using helium as collision gas. The relative collision-energy was set at 35% for D4-17β-E, 3α-diol and at 35% using the Wide Band Activation mode (Thermo Electron) for all the other steroids. Samples were analyzed using the transitions previously reported by (Pesaresi et al., 2010).

LIPIDOMIC ANALYSES ON PURIFIED MYELIN

Myelin was purified from sciatic nerve of control, STZ, and STZ-treated with GW3965 rats as described by Norton and Poduslo in 1973 (Norton and Poduslo, 1973).

Phospholipid, FA, and cholesterol extraction methods: Briefly their method was based on separation of different myelin structures using high speed

centifugation on sucrose gradient. Internal standards (Heneicosanoic acid, 1500 ng/sample; ^{13}C18-Linoleic acid, 500 ng/sample; 5α-Cholestane, 50 μg/sample) were added to myelin samples, and lipid extraction was performed using two different extraction methods. The first separates lipidic molecules using Folch method [chloroform/MeOH 2:1, v/v], while the second extract lipids just adding methanol to the purified myelin. After extraction, in both these methods, samples were left overnight at 4°C. The organic residue was divided in two fractions: one for the analysis of free cholesterol and phospholipids (fraction A, 60% of the total sample), and the other for analysis of total FAs and total cholesterol (fraction B, 40% of the total sample). Total FAs and cholesterol were obtained from samples by acid hydrolysis (Taguchi and Ishikawa, 2010). Fraction B was resuspended in chloroform-MeOH 1:1, v/v. 1M HCl:MeOH (1:1, v/v) was added to the total lipid extract and shaken for 2 h. Chloroform-water (1:1, v/v) was added, and the lower organic phase was collected, transferred into tubes and dried under nitrogen flow. The residue was resuspended in 1ml of MeOH and splitted 60/40 for total FA and total cholesterol analysis, respectively. These two methods were proved to be efficient in lipidic molecules extraction from purified myelin. In fact the analyses performed showed no significant differences between samples prepared differently.

Lipidomic profile by LC-MS/MS: Also lipidomic analyses has been performed using two different methods developed for two different instruments that has been used during both experiments. Method I has been optimized for analyses on a linear ion trap-mass spectrometer LTQ, (Thermo Electron, USA) while Method II has been developed on a different system that uses an API 4000 triple quadrupole instrument (AB Sciex, USA). Even in this case the methods showed no differences between quantification of absolute values of myelin components.

Method I: The aliquot for FA quantification transferred into auto-sampler vials for LC-MS/MS analysis. Quantitative analysis was performed on the basis of calibration curves prepared and analyzed daily. Electrospray ionization (ESI) experiments were performed using a linear ion trap-mass spectrometer equipped with a Surveyor liquid chromatography Pump Plus and a Surveyor Autosampler Plus. The LC mobile phases were water and MeOH/2-propanol (50:50, v/v). The gradient (flow rate 0.2 ml/min) was as follows:

- T 0 : 100% A
- T 5 : 100% A
- T 5.1 : 50% A
- T 25 : 50% A
- T 25.1 : 100% A
- T 40 : 100% A

The Hypersil GOLD TM pentafluorophenyl column (100mm × 2.1mm, 3μm) was maintained at 40°C and he injection volume was 20μl. Peaks area were evaluated using a Dell workstation by means of the software Excalibur, release 2.0 SR2. The mass spectrometer was operated in negative-ion mode with the ESI source using nitrogen as sheath, auxiliary, and sweeps gas, respectively. The mass spectrometer was operated in MS/MS mode using helium as a collision gas. The relative collision energy was set at 35% for all analyzed molecules.

Method II: For FA quantification, aliquots of each sample (10 μl) were diluted 1:10 in MeOH/water (50:50 v/v), transferred into a 96 well plate and placed in an auto-sampler for LC-MS/MS analysis. Quantitative analysis was performed with calibration curves prepared and analyzed daily by electrospray ionization (ESI) using a triple quadrupole analyzer (API 4000, AB Sciex, USA). The LC mobile phases were: water/10mM

isopropylethylamine/15 mM acetic acid (phase A) and MeOH (phase B). The gradient (flow rate 0.5 ml/min) was as follows:

- T 0: 20% A
- T 20: 1% A
- T 25: 1% A
- T 25.1: 20% A
- T 30: 20% A.

The Hypersil GOLD C8 column (100 mm × 3 mm, 3 μm) was maintained at 40°C. The mass spectrometer was operated in selective ion monitoring (SIM)/SIM mode. Peaks area were evaluated using a Dell workstation by means of the software Analyst release 4.1. The mass spectrometer was operated in negative-ion mode.

Cholesterol quantification: Fractions for the quantitative analysis of free and total cholesterol, were first derivatized with a mixture of bis-trimethylsilyltrifluoroacetamide-pyridine (4:1 v/v) for 30 min at 60°C, and then injected into a gas chromatograph-MS (GC-MS, Varian Saturn 2100). The MS was operated in the electron impact ionization mode. GC-MS analyses were performed as follows: 1μl sample was injected in splitless mode (inlet was kept at 270°C with the helium flow at 1.0 ml/min) at the initial 180°C. The oven was fi rst kept at 180°C for 1 min, ramped at 50°C/min to 240°C, then at 5°C/min to 300°C for 6 min. The ions used for the quantification of cholesterol were at m/z 368 for cholesterol and m/z 357 for 5α-cholestane, the IS. The selection of ions for selective ion monitoring (SIM) analysis was based on mass spectra of pure compounds, and the quantification was based on calibration curves freshly prepared using a fixed concentration of the IS, and different increasing of cholesterol, in a range from 0 to 10 μg/μl. The amount of cholesteryl esters was calculated

by subtracting the amount of free cholesterol from total cholesterol. Cholesteryl esters were evaluated to verify the myelin quality and purity.

Phospholipid analysis: ESI analysis of the major phospholipid classes was accomplished by utilization of either positive or negative ionization modes. Samples were directly infused for acquisition of MS/MS spectra. Changes between detected phospholipid families were calculated as percent of single phospholipid species normalized to total phospholipid analyzed.

QUANTITATIVE REAL TIME PCR (RT-QPCR)

RNA was prepared using the Nucleospin® RNA II kit (Macherey-Nagel; Milano, Italy). RNA was analyzed by TaqMan CFX384 RTqPCR using the iScriptTM one-step RT-PCR kit for probes (Bio-Rad; Milano, Italy). Samples were arrayed in 384-well format in triplicate as multiplexed reactions of target gene with 36B4 as reference gene. Probe and primer sequences were purchased from Eurofins MWG-Operon (Milano, Italy) and are available on request.

TRANSFECTION AND PROTEIN ANALYSIS

Expression constructs flag tagged for the mature forms of SREBP-1a, SREBP-1c, and SREBP-2 were a gift of Dr. T. Osborne (Addgene plasmid #26801, #26802, and #26807, respectively; Addgene, Cambridge, MA). Five micrograms of each SREBP expression construct and 5 µg of pcDNA3 (the backbone plasmid of the SREBPs) were transfected individually in Hek293T cells using Fu- GENE 6 (Promega; Milano, Italia) according to manufacturing instructions in 6-well tissue culture plates. Six hours after transfection, the cells were cultured in DMEM with 10% FBS, and 48 h later, total protein

extracts were prepared using modifi ed RIPA buffer [50 mM Tris-HCl (pH 7.4), 1% Nonidet P-40, 0.25% sodium deoxycholate, 150 mM NaCl, 1mM EDTA, and protease inhibitor mixture (Roche; Monza, Italy)]. Western blotting was performed with a mouse monoclonal anti-SREBP-1 (Santa Cruz Biotechnology; Milano, Italy) at a dilution of 1:200, or a mouse polyclonal anti-Flag 1:1,000 (Sigma-Aldrich). Sciatic nerves from three different animals per experimental group were lysed using modified RIPA buffer. Western blotting was performed with a mouse monoclonal anti-SREBP-1 (Santa Cruz Biotechnology) at a dilution of 1:200, a rabbit polyclonal anti-P0 (Sigma-Genosys; Milano, Italy) at a dilution of 1:500, or a rabbit polyclonal anti-peripheral myelin protein 22 (PMP22) (Inbios; Napoli, Italy) at a dilution of 1:5,000. As a loading control, membranes were probed with a mouse monoclonal anti-β-actin (Sigma-Aldrich) at a dilution of 1:1,000. All secondary antibodies were from Sigma-Aldrich. Quantification of protein levels was performed using Quantity One software (Bio-Rad). Analyses of protein levels were repeated at least three times with different sets of animals.

MORPHOMETRIC ANALISYS

Morphometric analyses were performed by Luis Miguel Garcìa Segura from Cajal Institute of Madrid. Sciatic nerves were removed, cut in segments (1–2 mm length) and fixed by immersion in 1% paraformaldehyde and 1% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4, for 24 h at 4°C. Tissue samples were washed in phosphate buffer, postfi xed for 2 h in 1% buffered OsO 4 , dehydrated, and embedded in araldite. Nerves were sectioned transversely using a Reichert Ultra-cut microtome. Semi-thin sections (0.5 μm) were stained with toluidine blue and analyzed by light microscopy. Thin sections were examined using a JEOL 1200 EXII electron microscope. For

morphometric analysis of myelinated fi bers, areas of semithin sections, covering at least 25% of the total cross-sectional profile of the nerve, were chosen by systematic random sampling of squares (Mayhew et al., 1984). The size of axons, the *g* ratio, the percentage of normal and abnormal fibers, the percentage of fibers with infoldings and with alteration in myelin compaction, were analyzed using a JEOL 1200 EXII electron microscope. The *g* ratio was calculated as the quotient between the axon size and the fiber size (Arbuthnott et al., 1980). A minimum of 100 myelinated fibers were assessed from at least eight animals per group.

ASSESSMENT OF PHYSIOLOGICAL PARAMETERS

Thermal nociceptive threshold: Nociceptive threshold to radiant heat was quantified using the hot plate paw withdrawal test as previously described (Bianchi et al., 2004). Briefly, a 40 cm high Plexiglas cylinder was suspended over the hot plate and the temperature was maintained at 50±0.2°C. Paw withdrawal latency was defined as the time between placing the rat on the hot plate and the time of withdrawal, or licking of hindpaw, or discomfort manifested by the animal. The test was done every 2 weeks starting from the second week after STZ injection. Animals were tested twice, with a 30 min interval between tests.

Nerve conduction velocity: At the end of treatment, antidromic tail NCV was assessed using a Myto EBNeuro electromyography apparatus as previously described (Meregalli et al., 2010). Briefly, recording ring electrodes were placed distally in the tail of Unanesthetized animals. The stimulating ring electrodes were placed 5 and 10 cm proximally with respect to the recording point. Latency of the potentials recorded at the two sites after nerve stimulation was determined (peak-to-peak, stimulus duration 100 ms, filter 1 Hz–5 MHz) and NCV calculated. All

neurophysiological studies were done under standard conditions in a temperature-controlled room adjacent to the animal housing room. Body temperature and vital conditions of the animals were monitored during the neurophysiological examination.

Na+,K+-ATPase activity: Tibial stumps were dissected out, desheathed and homogenized in a chilled solution containing 0.25 M sucrose, 1.25 mM EGTA and 10 mM Tris, pH 7.5, at 1:20 (w/v) in a glass-glass Elvehjem–Potter homogenizer (DISA), and stored at -80°C for ATPase determinations. Na+,K+-ATPase activity was determined spectrophotometrically as previously described (Bianchi et al., 2004). Protein content in homogenates was determined by Lowry's method with bovine serum albumin as standard.

STATISTICAL ANALYSES

Statistical analyses were performed via one-way ANOVA followed by Tukey-Kramer posttest. All statistical analyses were performed with GraphPad PRISM version 5 (San Diego, CA).

Principal component analysis was performed using Unscrambler, version 10.1 (Camo Software; Oslo, Norway).

5. RESULTS

LXRS ARE ACTIVE IN SCIATIC NERVE

To investigate the potential role of Liver X Receptors (LXRs) in diabetes-induced peripheral neuropathy, we first assessed whether the two isoforms of LXR are expressed in peripheral nerve, such as the sciatic nerve, and whether their levels change in diabetic rats. LXRα and LXRβ are both expressed in rat sciatic nerve and their mRNA levels are unaffected by diabetes (Fig. 1).

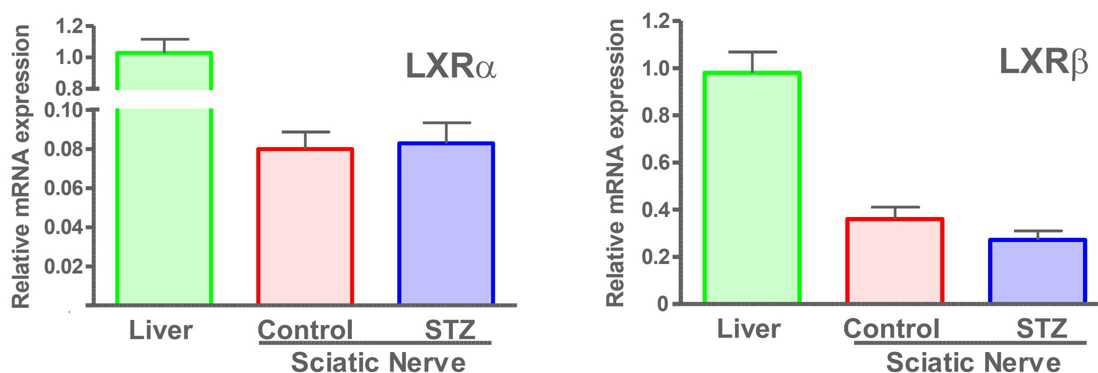


Figure 1: LXRα and LXRβ are expressed in sciatic nerve and their levels are unaffected by diabetes. The bars represent the relative mRNA expression of shown genes normalized to the housekeeping gene 36B4. Data are presented as mean ±SEM (n=9).

LXRβ is the dominant isoform present in sciatic nerve. The expression of LXRα and LXRβ in sciatic nerve is substantially lower than that in liver, a tissue with considerable LXR activity (Fig. 1).

To establish whether the levels of LXR present in sciatic nerve have functional significance, we measured LXR target gene expression after administration of a synthetic LXR ligand. GW3965 was given once a week for a month to STZ-treated rats 2 months after the induction of diabetes.

Expression of two *bona fide* LXR target genes involved in cholesterol efflux such as ATP Binding Cassette A1(ABCA1) and ATP Binding Cassette G1 (ABCG1), which are not regulated in sciatic nerve in this model of diabetes, was significantly increased after treatment with GW3965 (Fig. 2a), indicating that LXRs are active transcriptional regulators in sciatic nerve. Notably, diabetes did not regulate the expression of both ABCA1 and ABCG1. In contrast, GW3965 treatment did not affect expression of key regulators of cholesterol synthesis, such as HydroxyMetylGlutaryl Coenzime A (HMGCoA) reductase and Sterol Regulatory Element Binding protein 2 (SREBP-2) (Fig. 2b).

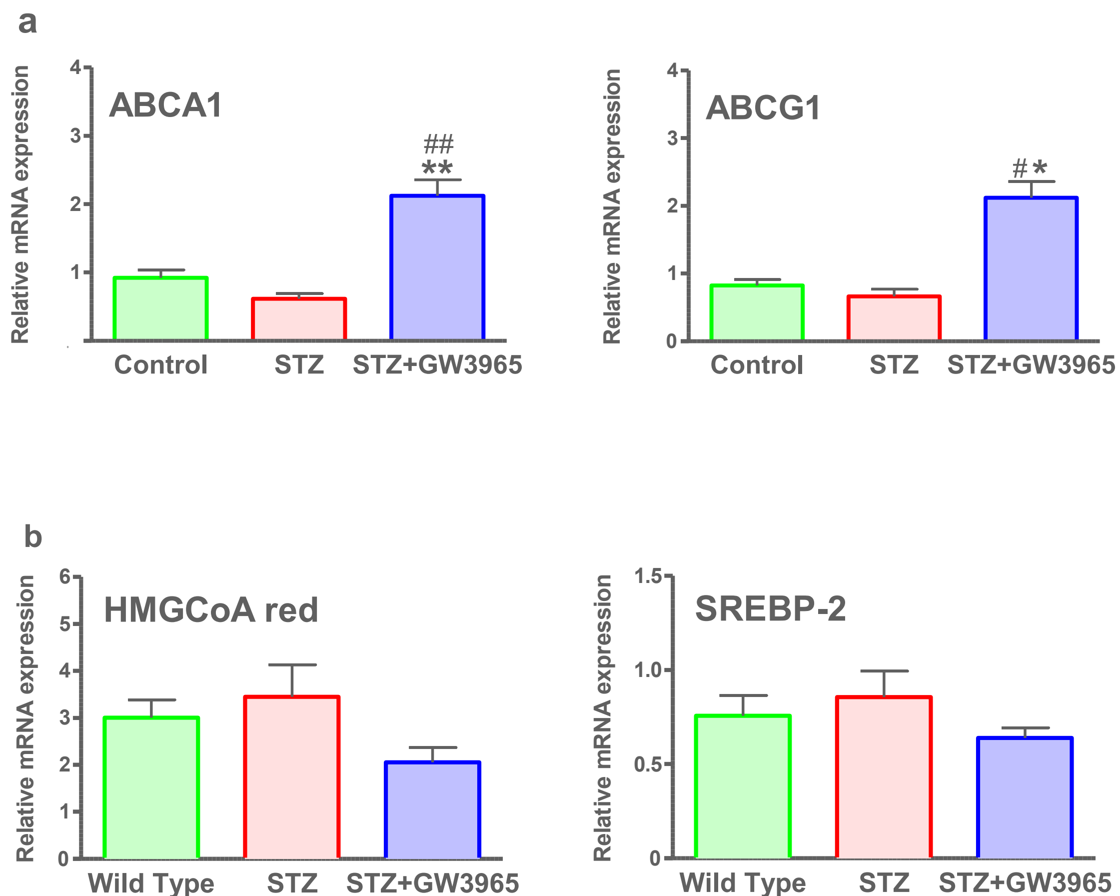


Figure 2: a) LXR activation by GW3965 treatment induces mRNA levels of ABCA1 and ABCG1, classical LXR target genes involved in cholesterol efflux in the sciatic

nerve. These data indicate that the ligand reaches the sciatic nerve and that the LXRs are activated. b) Expression levels of HMGCoA reductase and SREBP-2, two genes involved in cholesterol synthesis. The mRNA levels of these genes are unchanged by diabetes and/or by GW3965 treatment. The bars represent the relative mRNA expression of shown genes normalized to the housekeeping gene 36B4. Data are presented as mean ±SEM (n=9). Statistical analysis is performed by one-way ANOVA followed by Tukey–Kramer posttest. *p<0.05, **p<0.001 vs control; #p<0.05, ##p<0.001 vs STZ-treated rats.

ACTIVATION OF LXR MODULATES NEUROACTIVE STEROID LEVELS

Compared with normoglycemic controls, diabetic rats showed notable differences in the expression of several important genes involved in steroidogenesis and neuroactive steroids metabolism. In particular, mRNA levels of Steroidogenic Acute Regulatory Protein (StAR) and Translocator Protein 18KDa (TSPO) (proteins involved in cholesterol shuttling into the mitochondria), Cytochrome P450 Side Chain Cleavage (P450scc) (the enzyme responsible of the conversion of cholesterol into pregnenolone), and 5α Reductase (5α-R) (which converts progesterone and testosterone into their 5α-reduced metabolites, dihydroprogesterone and dihydrotestosterone respectively) were significantly decreased in the diabetic state (Fig. 3). Treatment of diabetic animals with an LXR activator restored mRNA levels of StAR, P450scc and 5α-R to the level observed in normoglycemic controls, but had no effect on TSPO expression (Fig. 3).

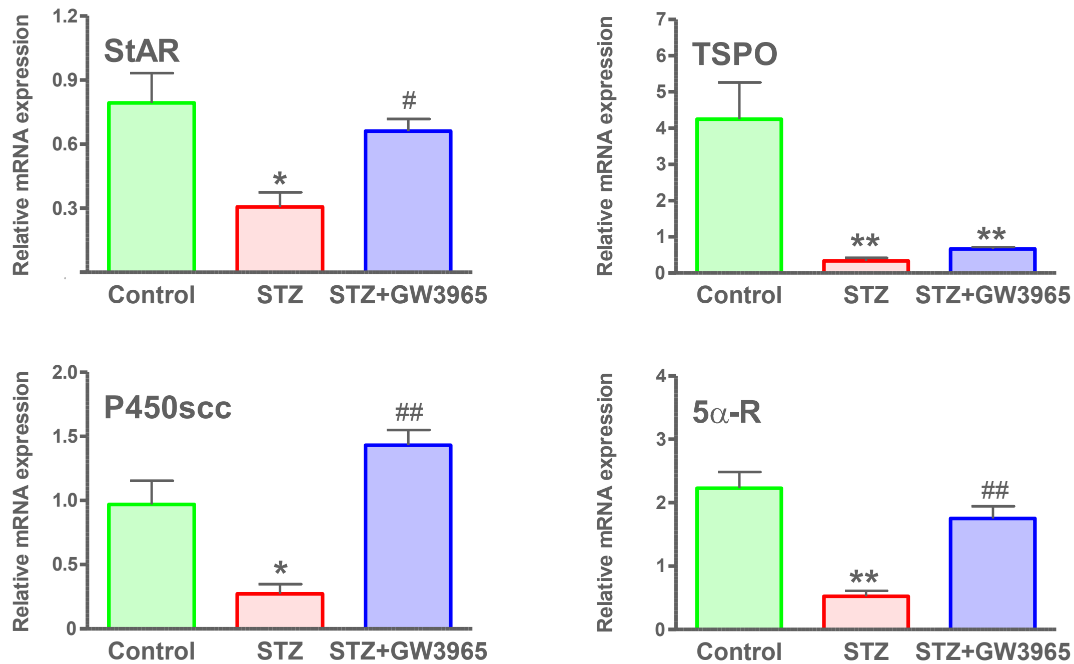


Figure 3: Gene expression of steroidogenic acute regulatory protein (StAR), translocator protein-18 kDa (TSPO), cytochrome P450 side chain cleavage (P450scc) and 5a reductase (5α-R), in sciatic nerve. As shown, LXR activation by GW3965 treatment in diabetic rats restores to normal levels the expression of StAR, P450scc and 5α-R but it does not affect TSPO levels. The bars represent the relative mRNA expression of shown genes normalized to the housekeeping gene 36B4. Data are presented as mean ±SEM (n=9). Statistical analysis is performed by one-way ANOVA followed by Tukey–Kramer posttest. *p<0.05, **p<0.001 vs control rats; #p<0.05, ##p<0.001 vs STZ-treated rats.

In agreement with these gene expression patterns, we showed that diabetes decreased neuroactive steroid levels in sciatic nerve, and that treatment with an LXR ligand counteracted these effects. Indeed, LC–MS/MS analysis showed that the levels of PREG, PROG, DHP, isopregnanolone, T and its derivatives, DHT and 5α-androstane-3α,17β-diol (3α-diol) were significantly decreased in the sciatic nerve of diabetic rats (Table 1).

	PREG	PROG	DHP	THP	ISOPREG	DHEA	T	DHT	3α-diol
CTRL	1.52±0.23	1.58±0.22	8.86±0.59	1.00±0.16	2.19±0.38	0.24±0.04	1.52±0.23	0.80±0.11	1.01±0.13
STZ	0.63±0.05*	0.62±0.09*	5.89±0.24**	0.65±0.03	0.61±0.07**	0.17±0.02	0.24±0.05**	0.18±0. 03**	0.57±0.10*
STZ +GW	1.45±0.33#	1.71±0.24##	9.17±0.59##	0.92±0.05	1.07±0.06*	0.29±0.04	0.27±0.13**	0.20±0.04**	1.09±0.13#

Table 1: Analysis of neuroactive steroids levels by LC-MS/MS in sciatic nerves of control, STZ, and STZ rats treated with GW3965. Data are expressed as pg/mg of tissue and are represented by mean ± SEM. The n is 8 for control, 8 for STZ and 6 for STZ+GW3965. Statistical analysis was performed by one-way analysis of variance (ANOVA) followed by Tukey–Kramer post-test. *P<0.05, **P<0.001 vs. control rats #P<0.05, ##P<0.001 vs. STZ treated rats.

LXR activation completely reversed the diabetes induced decrease in PREG, PROG, DHP and 3α-diol levels. Interestingly, the levels of these neuroactive steroids also fell in the plasma of diabetic rats, but treatment with the LXR agonist had no effect on them (Table 2).

	PREG	PROG	DHP	THP	ISOPREG	DHEA	T	DHT	3α-diol
CTRL	0.64±0.07	0.82±0.12	1.22±0.11	0.49±0.10	0.33±0.10	0.059±0.007	4.12±0.57	0.065±0.006	1.63±0.27
STZ	0.32±0.09*	0.28±0.05**	0.83±0.04*	0.36±0.12	0.28±0.09	0.058±0.009	0.70±0.021**	0.064±0. 01	0.54±0.13**
STZ +GW	0.29±0.03*	0.68±0.19	0.78±0.16*	0.75±0.17	0.68±0.19	0.065±0.008	0.25±0.08**	0.055±0. 005	0.71±0.21*

Table 2: Analysis of neuroactive steroids levels by LC-MS/MS in plasma of control, STZ, and STZ rats treated with GW3965. Data are expressed as pg/mg of tissue and are represented by mean ± SEM. The n is 8 for control, 8 for STZ and 6 for STZ+GW3965. Statistical analysis was performed by one-way analysis of variance (ANOVA) followed by Tukey–Kramer post-test. *P<0.05, **P<0.001 vs. control rats.

LXR ACTIVATION REDUCE DIABETES-INDUCED NEUROPATHY

Since activation of LXRs in diabetic sciatic nerves restored neuroactive steroid levels to a near-normal state, we examined whether this LXR-mediated increase could be associated with a neuroprotective effect. Table 3 shows that 3 months after the induction of diabetes, STZ-treated rats had higher blood glucose and significantly lower weight than non-diabetic control rats.

	CTRL	STZ	STZ + GW
Weight (grams)	524.1±60.4	270.4±46.4**	288.8±50.4**
Glycemia (mg/dl)	84.2±19.2	953.1±238.1**	949.7±207.9**
Triglycerides (mg/dl)	68.9±19.2	83.6±38.6	89.5±47
NEFA (meq/l)	0.70±0.24	0.67±0.31	0.72±0.28

Table 3: Body weight and blood chemistry of control, STZ and STZ rats treated with GW3965. Legend: Data are expressed as the mean ±SEM, n=14. Statistical analysis is performed by one-way analysis of variance (ANOVA) followed by Tukey–Kramer post-test. *P<0.05, **P<0.001 vs. control rats. NEFA are non-esterified fatty acids.

Plasma levels of triglycerides and nonesterified fatty acids were unaffected by diabetes. Treatment with GW3965, an LXR agonist, had no effect on these parameters. However, LXR activation did result in significant neuroprotective effects as measured by functional and biochemical tests. Treatment with GW3965 was able to significantly reduce the increase in thermal sensitivity brought about by diabetes (Fig. 4).

In addition to this decrease in thermal nociceptive threshold in LXR ligand-treated animals, we also observed that antidromic tail nerve conduction velocity (NCV), which is significantly reduced by diabetes, was enhanced

by LXR ligand treatment. Moreover, treatment with the LXR agonist reverted the reduction in Na+, K+-ATPase activity in sciatic nerve brought about by diabetes (Fig. 4).

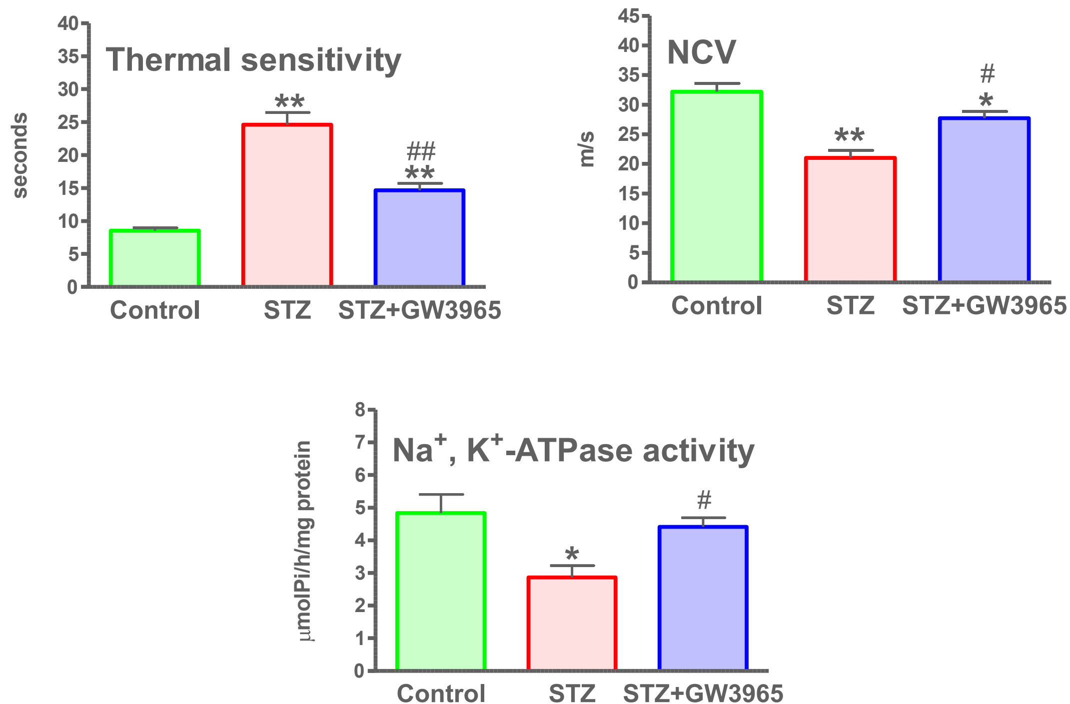


Figure 4: Thermal sensitivity, nerve conduction velocity, and Na+, K+-ATPase activity in control, STZ and STZ treated with GW3965 rats. Data are expressed as withdrawal latency in seconds for heat sensitivity threshold (control, n=10, STZ, n=12; STZ+GW3965, n=14), as m/s for NCV (control, n=14; STZ, n=12; STZ+GW3965, n=14) and as µmol Pi/h per mg protein for Na+, K+-ATPase (control, n=6; STZ n=7; STZ + GW3965, n=6), and are mean ±SEM. Statistical analysis is performed by one-way ANOVA followed by Tukey–Kramer posttest. *p<0.05, **p<0.001 vs control rats; #p<0.05, ##p<0.001 vs STZ-treated rats.

LXR ACTIVATION REVERT CHANGES IN MYELIN LIPID COMPOSITION INDUCED BY DPN

We have shown that treatment of STZ-treated rats with a synthetic liver X receptor (LXR) ligand promotes cholesterol utilization and increases neuroactive steroid levels in sciatic nerve, resulting in significant improvements in physiological parameters brought down by diabetes. Moreover LXR is able to regulate the expression of Sterol Regulatory Element Binding Factor 1C (SREBP-1C). This transcription factor is one of the most important regulator in lipid biosynthesis. Here, we report that rats with DPN display an altered myelin lipid composition pattern, blunted expression of key genes in the Fatty Acids (FA) biosynthetic pathway, and decreased levels of the active form of the lipogenic transcription factor SREBP-1c and the chief myelin protein zero (P0). These defects are associated with increased myelin infoldings in the sciatic nerve of diabetic rats. Activation of LXR with a synthetic ligand normalizes myelin's lipid profile, restores expression of genes involved in FA biosynthesis, reestablishes nuclear levels of SREBP-1c and absolute levels of P0, and corrects the major myelin abnormalities induced by diabetes in peripheral nerves. These changes are associated with improved performance in functional tests.

Diabetes was induced in adult Sprague-Dawley rats by a single STZ injection. To explore the role of myelin lipids in the pathogenesis of DPN, and to evaluate whether LXR activation (through its ability to induce the master regulator of lipogenesis, SREBP-1c) could play a role, we compared age-matched nondiabetic and STZ-treated rats dosed with either vehicle or the synthetic LXR ligand GW3965 once a week for a month, starting two months after the induction of diabetes. Three months after the induction of diabetes, both groups of STZ-treated rats showed decreased body weight and increased glycemia relative to nondiabetic controls (Table 3). As previously demonstrated, diabetes affected functional parameters such as

thermal nociceptive threshold and nerve conduction velocity, indicating that the animals developed peripheral neuropathy. LXR activation reversed these diabetes-induced peripheral nerve deficits (Fig. 4).

To test the hypothesis that diabetes induces an altered lipid composition profile in peripheral nerves, we performed detailed lipidomic analyses on sciatic nerve myelin purified from control, STZ, and STZ-GW3965-treated rats. The phospholipid families profile of purified myelin revealed that diabetes primarily decreases the levels of glycosphingolipids (GSLs), whereas phosphatidyl serine (PS) and phosphatidyl choline (PC) were mildly increased (Fig. 6). Levels of phosphatidyl ethanolamine (PE), phosphatidyl inositol (PI), sphingomyelin (SM), and sulfatides (Sulf) were unchanged (Fig. 6).

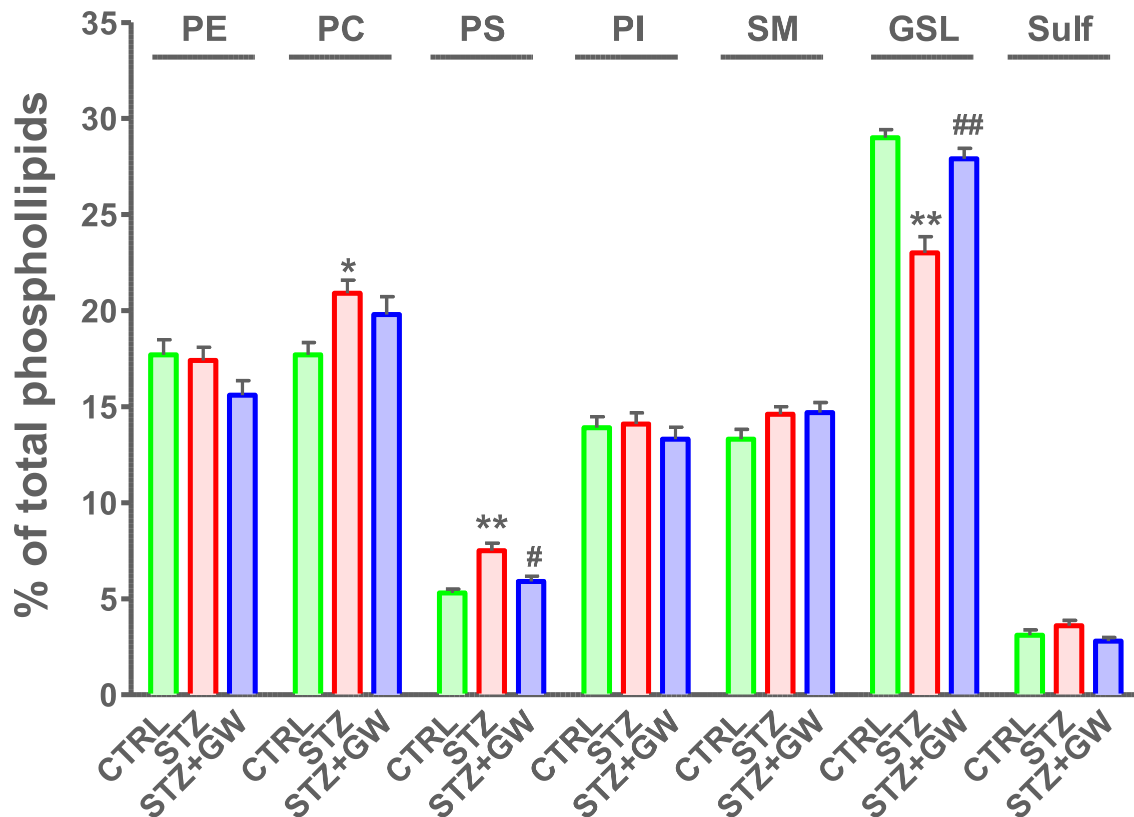
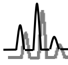


Figure 6: Percent of detected phospholipid families of sciatic nerve purified myelin from control, STZ and STZ-treated with GW3965 animals. Statistical analysis was performed by one-way ANOVA followed by Tukey-Kramer posttest. Data are expressed as mean ±SEM. n=11 animals per group. *P<0.05, **P<0.001 vs. control rats; #P<0.05, ##P<0.001 vs. STZ-treated rats. PE, phosphatidyl ethanolamine; PC, phosphatidyl coline; PS, phosphatidyl serine; PI, phosphatydil inositol; SM, sphingomyelin; GSL, glycosphingolipid; Sulf, sulfatide.

We also detected a 60% decrease in sciatic nerve myelin cholesterol content (Fig. 7). Remarkably, LXR activation in this diabetic setting restored the levels of all these lipids to those found in the nondiabetic control group (Fig. 6, 7).



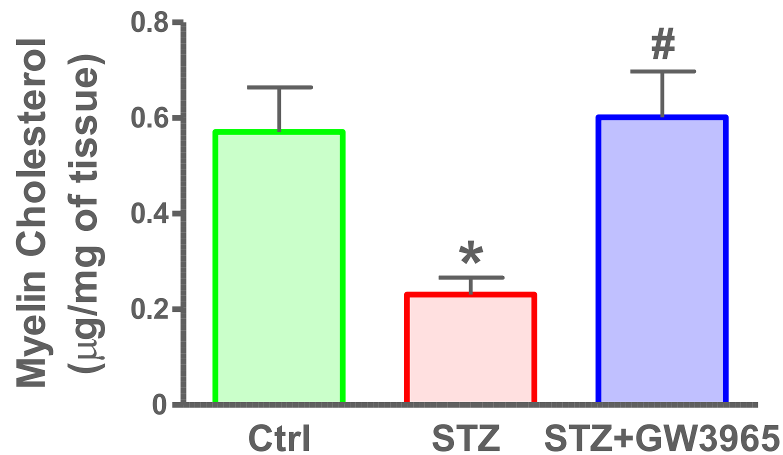


Figure 7: Cholesterol levels of sciatic nerve purified myelin from control, STZ and STZ-treated with GW3965 animals. Statistical analysis was performed by one-way ANOVA followed by Tukey-Kramer posttest. Data are expressed as mean ±SEM. n=11 animals per group. *P<0.05, **P<0.001 vs. control rats; #P<0.05, ##P<0.001 vs. STZ-treated rats.

Next, we evaluated the FA composition of the analyzed phospholipids families in purified myelin. Principal component analysis of myelin FAs revealed two separate clusters: the first includes control and STZ-GW3965-treated rats and the second the STZ diabetic animals (Fig. 8a, 8b).

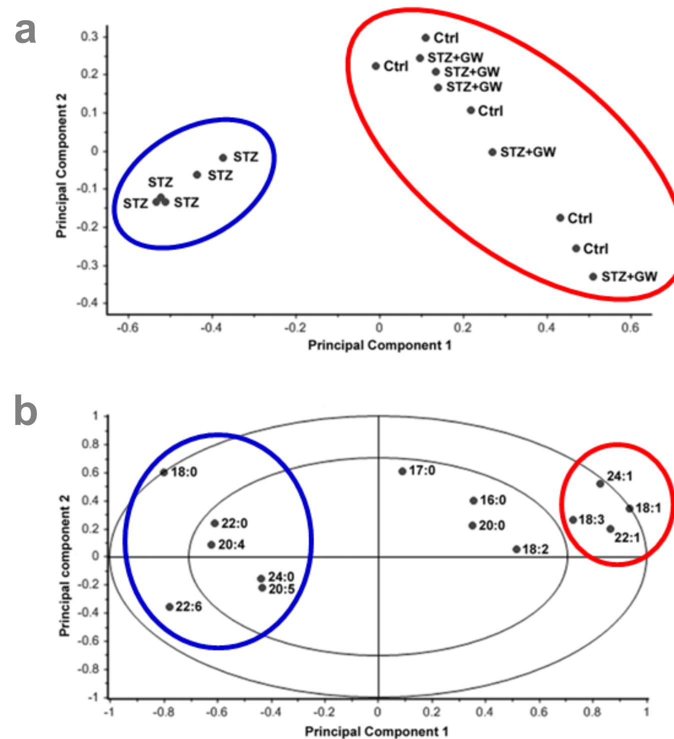


Figure 8: Principal component analysis of sciatic nerve purified myelin from control, STZ, and STZ-treated with GW3965 animals. a): Score plot of the lipid composition analysis of five animals per group. The two coordinates (Principal Component 1 and Principal Component 2) represent together >80% of the variance of these 15 samples. Principal Component 1 accounts for 73% of this variance and clearly identifies two clusters. The first includes the control and STZ-GW3965 animals (red circle), whereas the diabetic rats (blue circle) belong to the second cluster, indicating that the lipid profile is different between the two clusters. b): Correlation loadings plot computed for each FA analyzed for the displayed principal components. The red circle (control and STZGW3965-treated groups) and the blue circle (STZ-vehicle treated group) represent the FAs enriched in the considered cluster. For instance, 18:0 may be considered a marker for the STZ group, whereas 18:1 is a marker for control and STZ-GW3965-treated animals. The two ellipses indicate how much variance is taken into account. The outer ellipse accounts for 100% of explained variance. The inner ellipse shows 50% of explained variance.

Moreover, the correlation graph showed each FA analyzed associated to the two identified clusters (Fig. 8b). These plots and the myelin FA quantifications (Fig. 9) indicate that diabetes led to increased levels of saturated stearic (C18:0), behenic (C22:0), and lignoceric (C24:0) acids, whereas monounsaturated oleic acid (C18:1), nervonic acid (C22:1), and erucic (C24:1) acid decreased (Figs. 9, 8a, 8b), indicating the inability of vehicle-treated diabetic rats to desaturate FAs. In addition, γ-linolenic acid (C18:3) was decreased in STZ rats, but arachidonic (C20:4), eicosapentaenoic (C20:5), and docosahexaenoic (C22:6) acids were increased (Figs. 9, 8a, 8b). All changes in FA levels induced by diabetes were restored to normal levels by LXR activation (Figs. 9, 8a, 8b).

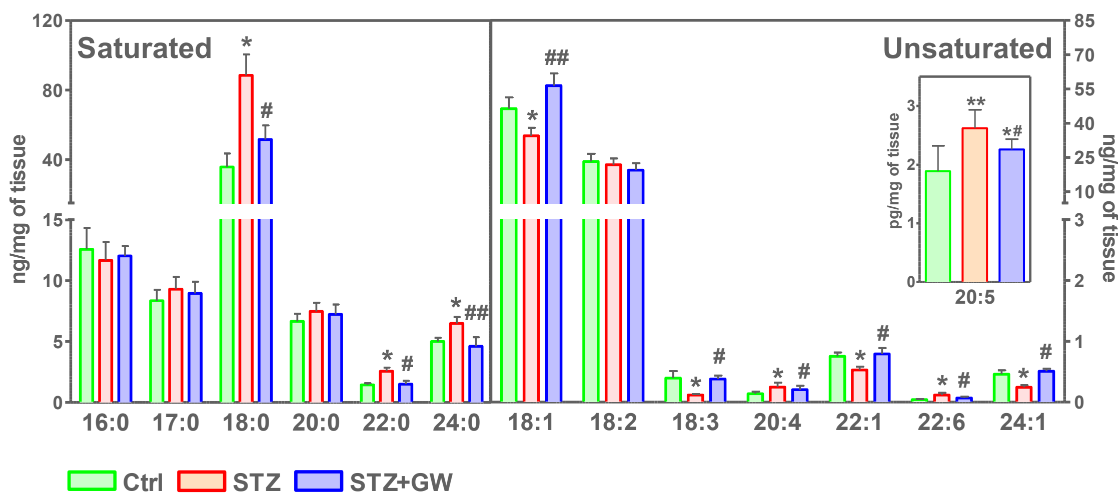


Figure 9: FA profile of sciatic nerve purified myelin from control, STZ and STZ-treated with GW3965 animals. Statistical analysis was performed by one-way ANOVA followed by Tukey-Kramer posttest. Data are expressed as mean ±SEM. n=11 animals per group. *P<0.05, **P<0.001 vs. control rats; #P<0.05, ##P<0.001 vs. STZ-treated rats.

Furthermore, in diabetic animals, we detected a significant shift in the levels of monounsaturated FAs toward PUFAs (Fig. 10), along with an

increased ratio of C18:0/C18:1 corresponding to a decreased desaturation index (Fig. 11).

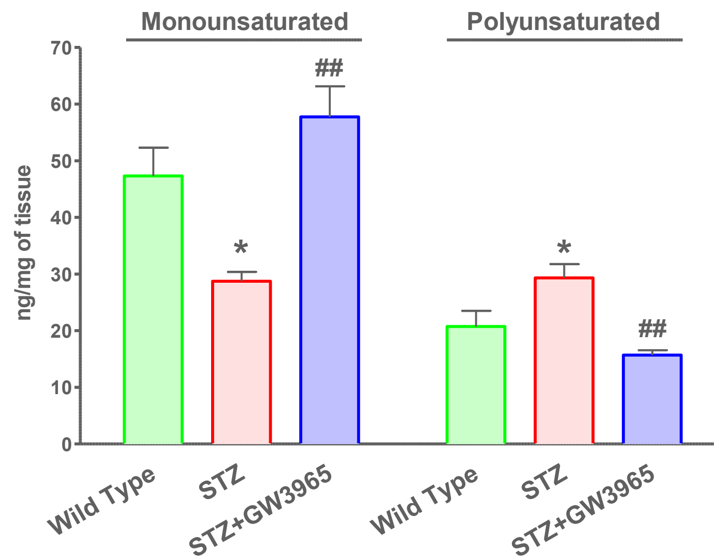


Figure 10: Levels of monounsaturated and polyunsaturated FAs of sciatic nerve purifyed myelin from control, STZ and STZ-treated with GW3965 animals. Statistical analysis was performed by one-way ANOVA followed by Tukey-Kramer posttest. Data are expressed as mean ±SEM. n=11 animals per group. *P<0.05, **P<0.001 vs. control rats; #P<0.05, ##P<0.001 vs. STZ-treated rats.

In addition, diabetes also decreased the C18:1/ C18:2 ratio, an index correlating with altered membrane fluidity (Fig. 11) (Chrast et al., 2011). LXR activation once again brought the levels of these FAs to those of control rats and normalized both desaturation and membrane fluidity indexes (Fig. 10, 11).

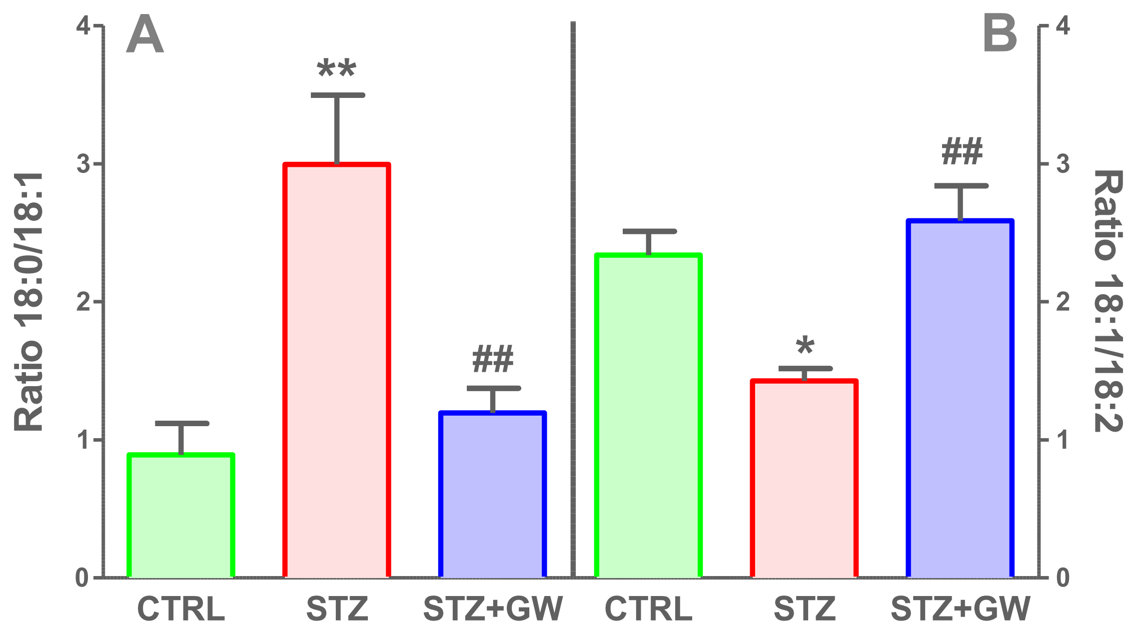
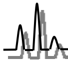


Figure 11: A) Desaturation index as ratio of C18:0 (stearic acid)/C18:1 (oleic acid) and B) membrane fluidity as ratio of C18:1 (oleic acid)/C18:2 (linoleic acid) of sciatic nerve purified myelin from control, STZ and STZ-treated with GW3965 animals. Statistical analysis was performed by one-way ANOVA followed by Tukey-Kramer posttest. Data are expressed as mean ±SEM. n=11 animals per group. *P<0.05, **P<0.001 vs. control rats; #P<0.05, ##P<0.001 vs. STZ-treated rats.

We also identified FA species such as C18:1 and C18:3 that are primarily associated with the most abundant glycosphingolipids (PC and GSL, Fig. 6), as being less represented in the diabetic group relative to normal and diabetic GW3965-treated animals (Fig. 12).



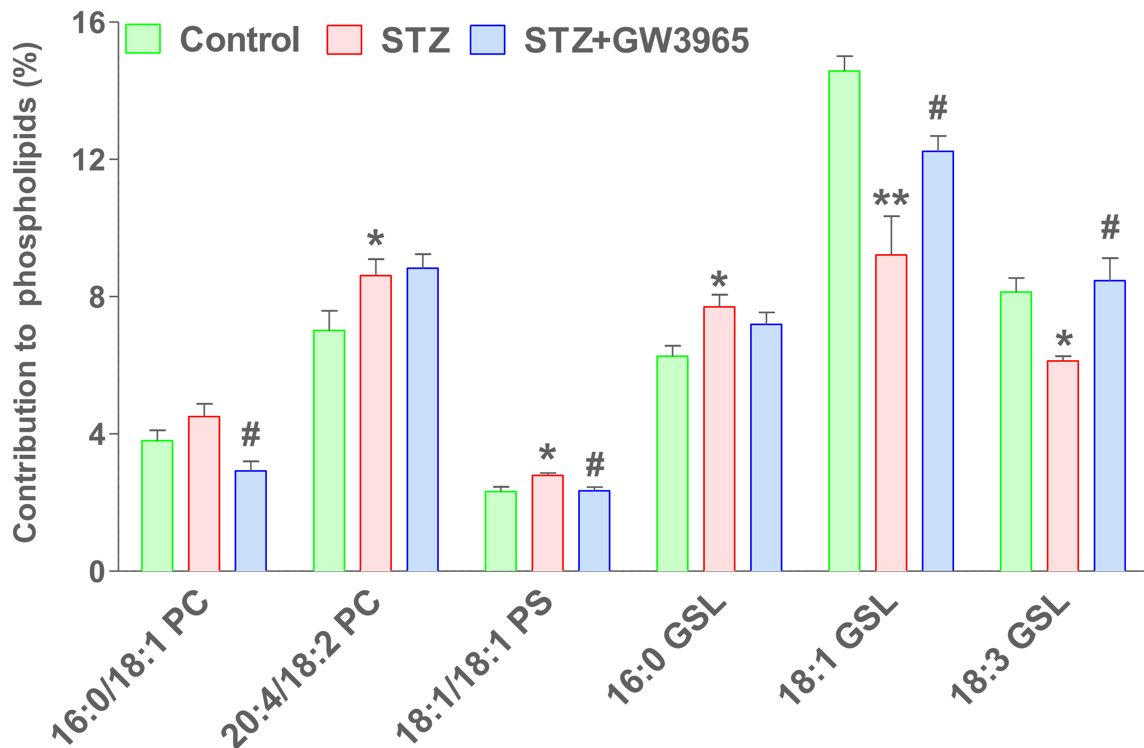


Figure 12: FAs associated to the significantly different phospholipids of sciatic nerve purified myelin from control, STZ and STZ-treated with GW3965 animals. Statistical analysis was performed by one-way ANOVA followed by Tukey-Kramer posttest. Data are expressed as mean ±SEM. n=11 animals per group. *P<0.05, **P<0.001 vs. control rats; #P<0.05, ##P<0.001 vs. STZ-treated rats.

Importantly, the lipid profile changes observed in diabetic rats were not detected in plasma, indicating that the effects of diabetes on lipogenesis occur locally in the sciatic nerve (data not shown). Taken together, these results indicate that myelin from diabetic animals has a different lipid composition pattern and that treatment with an LXR ligand, a lipogenic stimulus, restores the lipid profile to that of non-diabetic controls.

LXR ACTIVATION RESTORES EXPRESSION OF KEY GENES IN FA BIOSYNTHESIS SUPPRESSED BY DIABETES

To better understand the observed changes in myelin lipid profiles, the mRNA level of genes involved in lipid biosynthesis were analyzed in the sciatic nerve of control, STZ, and STZ-GW3965-treated rats. We found that lipogenic genes such as SREBP-1c, acetyl-CoA carboxylase a (ACACA), FAS, SCD-1, and -2 [SCD-2, the main isoform expressed in peripheral nerves (Garbay et al., 1998), and FA desaturase-1 and -2, were all downregulated in diabetic sciatic nerve, and that their expression was brought back to nondiabetic levels by pharmacologic LXR activation (Fig. 13). Expression of enzymes involved in FA elongation, such as elongase of very long chain FAs 5 and 6, (Elovl 5 and 6) were unchanged in all experimental groups (Fig. 13).

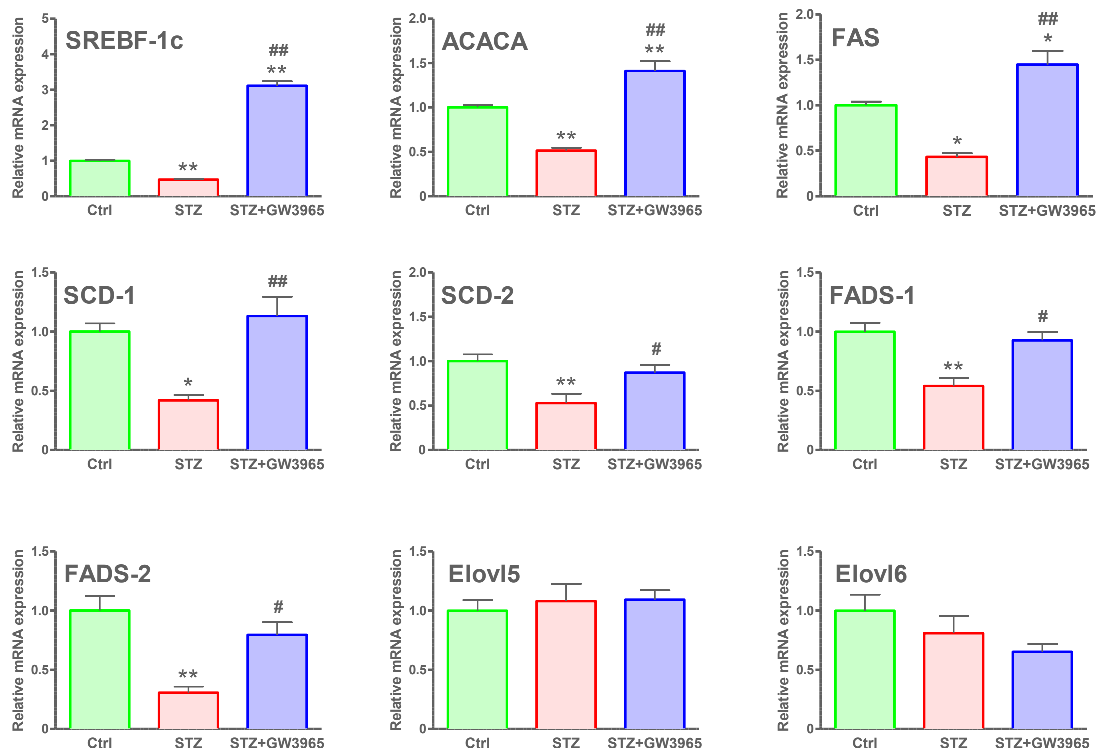


Figure 13: Expression profile of FA biosynthesis genes. Statistical analysis was performed by one-way ANOVA followed by Tukey-Kramer posttest. The bars

represent the relative mRNA expression of shown genes normalized to the housekeeping gene 36B4. Data are expressed as mean ±SEM. n=8 animals per group. *P<0.05, **P<0.001 vs. control rats; #P<0.05, ##P<0.001 vs. STZ-treated rats.

As expected, non-diabetic rats treated with GW3965 also showed increased expression of SREBP-1c, FAS, and SCD-1 and -2 compared with nondiabetic rats receiving vehicle (data not shown). However, in this nonpathological setting, these changes in gene expression are not sufficient to significantly affect myelin lipid composition (data not shown). These findings show that pharmacological LXR activation can restore the levels of key genes involved in lipid synthesis whose expression is suppressed in the sciatic nerve of diabetic animals.

ACTIVATION OF LXR RESTORES TRANSCRIPTIONALLY ACTIVE SREBP1-C

Because SREBP-1c is a classic LXR target gene and all FA biosynthesis genes affected by diabetes are known to be regulated by this lipogenic transcription factor (Shimomura et al., 1999), we measured the levels of the cytoplasmic precursor and the mature nuclear form of SREBP-1c. The precursor form of SREBP-1c is anchored in endoplasmic reticulum membranes. Upon stimulation (e.g., insulin, in the case of SREBP-1c), the precursor is cleaved into a mature form that shuttles to the nucleus to activate expression of enzymes involved in FA biosynthesis (Wang et al., 1994). We found that the sciatic nerve of diabetic animals contains less transcriptionally active SREBP-1 (mature form), a decrease that is rescued by LXR activation (Fig. 14).

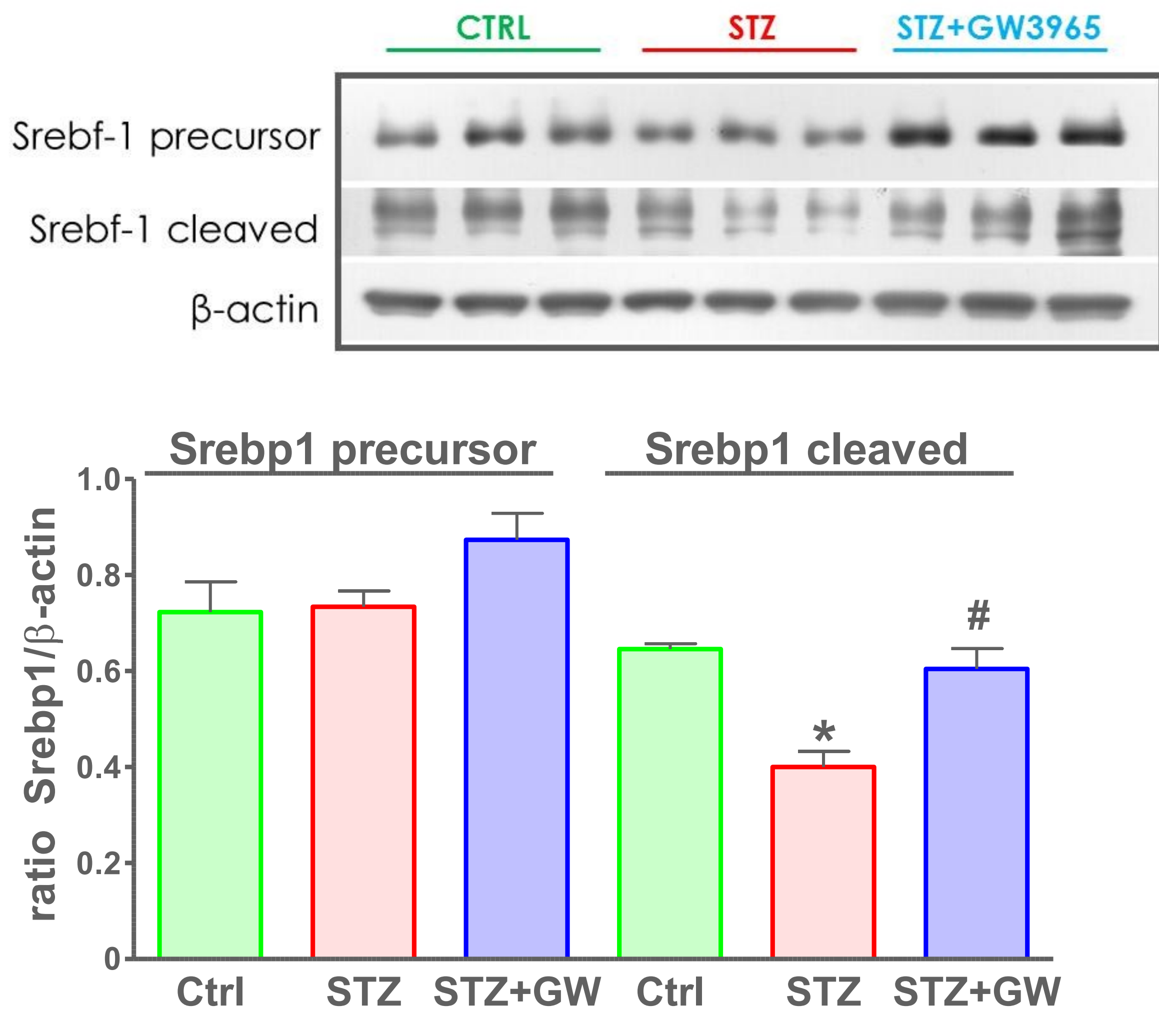


Figura 14: Representative Western blot analysis and quantification of SREBP-1c precursor (125 kDa) and transcriptionally active form (cleaved, 68 kDa) in control, STZ, and STZ-treated with GW3965 rat sciatic nerve.

The SREBP-1 antibody used was validated using extracts from HEK293T cells transfected with Flag-tagged expression constructs for the mature forms of SREBP-1a (Toth et al., 2004), SREBP-1c (Toth et al., 2004), and SREBP-2 (Jeon et al., 2008). The SREBP-1 antibody detected the mature forms of SREBP-1a and SREBP-1c, but it did not cross-react with SREBP-2 (data not shown). To exclude a role for SREBP-1a, we measured the mRNA levels of this transcription factor. Expression of SREBP-1a was not affected by diabetes or

treatment with the LXR agonist GW3965 (data not shown). These results indicate that SREBP-1c is the isoform most affected by DPN.

LXR ACTIVATION IMPROVES MYELIN PROTEIN COMPOSITION

In addition to lipids, myelin is composed of about 20–30% protein. We found that the levels of P0 protein, which represent between 50% and 70% of total myelin protein in peripheral nerves (Garbay et al., 2000), were decreased by diabetes (Fig. 15), confirming data already present in the literature (Mayhew ey al., 1984) Pharmacological LXR activation restored P0 levels to those found in nondiabetic control rats; levels of PMP22 were unchanged among experimental groups (Fig. 15).

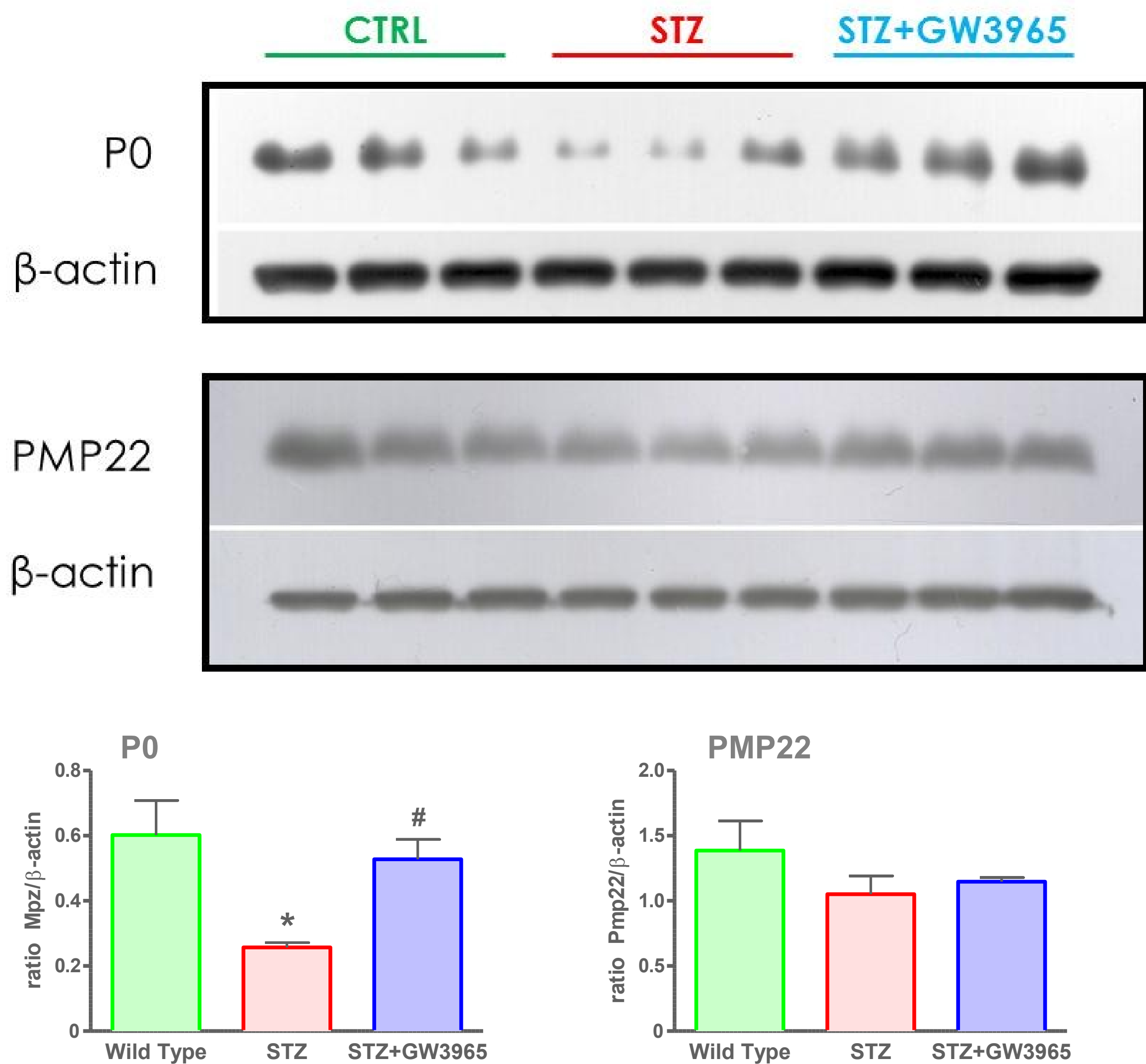


Figura 15: Myelin protein analysis. Representative P0 and PMP22 Western blot analyses and relative quantifications. Statistical analysis was performed by one-way ANOVA followed by Tukey-Kramer posttest. Data are expressed as mean ±SEM. n=3 animals per group. *P<0.05 vs. control rats; #P<0.05 vs. STZ-treated rats.

THE SCIATIC NERVE OF STZ-TREATED DIABETIC RATS SHOWS MYELIN ABNORMALITIES THAT CAN BE REVERSED BY LXR ACTIVATION

To investigate whether the observed effects on myelin lipid and protein composition induced by diabetes are associated with myelin abnormalities,

we performed morphometric and morphological analyses. We observed that myelin fibers from diabetic animals have a reduction in normal fibers and show increased myelin abnormalities (Fig. 16). These pathological changes were reversed to what is seen in nondiabetic animals by treatment with the LXR ligand (Fig. 16).

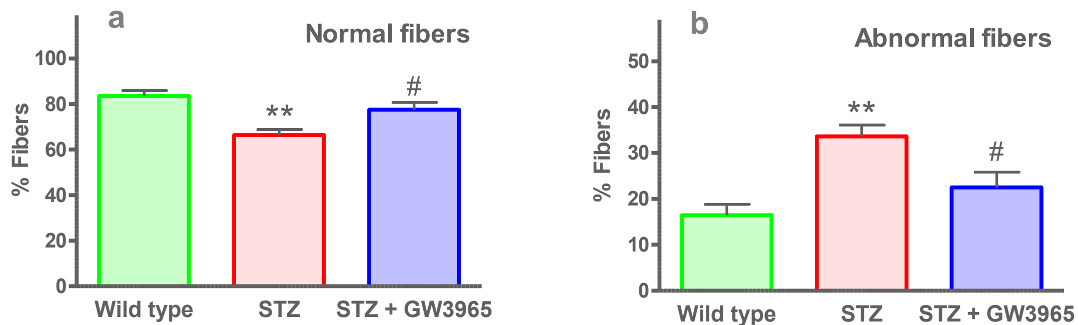
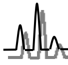


Figura 16: Morphometric and TEM analysis of myelinated fibers in sciatic nerve of control, STZ, and STZ-treated with GW3965 rats. a): Percentage of normal fibers. b): Percentage of abnormal fibers. Statistical analysis was performed by one-way ANOVA followed by Tukey-Kramer posttest. Data are expressed as mean ±SEM. n=8 animals per group. **P<0.001 vs. control rats; #P<0.05 and ##P<0.001 vs. STZ-treated rats.

In particular, the diabetic state increased myelin infoldings, and these were restored to normal levels by LXR ligand treatment (Fig. 17).



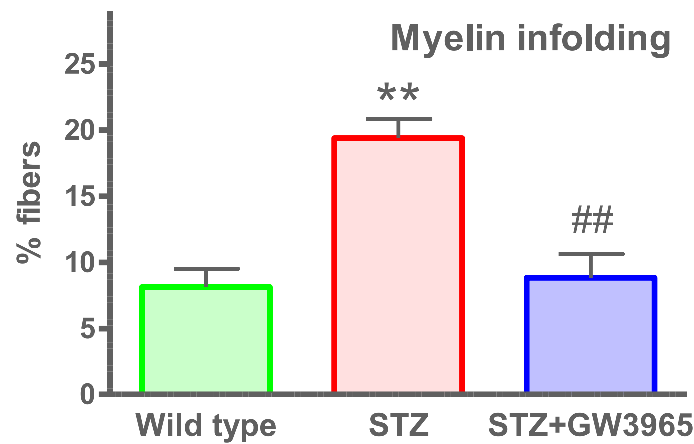


Figura 17: Morphometric and TEM analysis of myelinated fibers in sciatic nerve of control, STZ, and STZ-treated with GW3965 rats. Percentage of fibers with myelin infoldings. Statistical analysis was performed by one-way ANOVA followed by Tukey-Kramer posttest. Data are expressed as mean ±SEM. n=8 animals per group. **P<0.001 vs. control rats; #P<0.05 and ##P<0.001 vs. STZ-treated rats.

Percentage of alterations in myelin irregular fiber shape and compaction (Fig. 18a, 18b), as well as *g*-ratio and axonal diameter (Fig. 18c, 18d), were similar among experimental groups

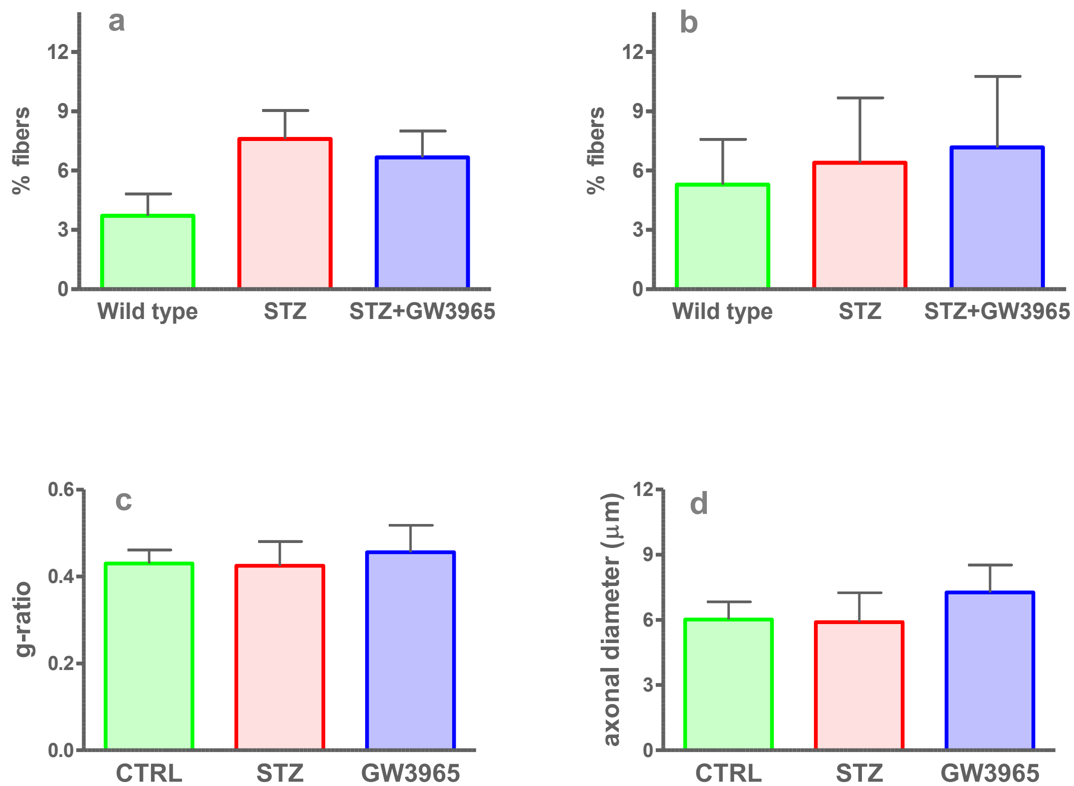


Figura 18: Morphometric and TEM analysis of myelinated fibers in sciatic nerve of control, STZ, and STZ-treated with GW3965 rats. a): Percentage of fibers with myelin irregular shapes. b): Percentage of fibers with alterations in myelin compaction c): g-ratio of myelinated fibers d): Axonal diameter. Statistical analysis was performed by one-way ANOVA followed by Tukey-Kramer posttest. Data are expressed as mean ±SEM. n=8 animals per group. **P<0.001 vs. control rats; #P<0.05 and ##P<0.001 vs. STZ-treated rats.

Myelin ultrastructure studies using transmission electron microscopy (TEM) confirmed observations from myelin fiber analysis (Fig. 19).

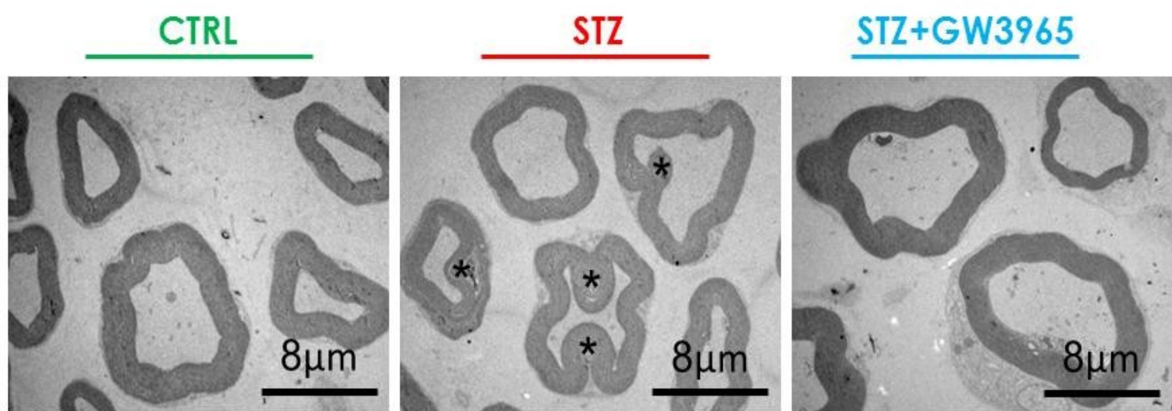


Figura 19: Morphometric and TEM analysis of myelinated fibers in sciatic nerve of control, STZ, and STZ-treated with GW3965 rats. H: Detail of myelinated nerves analyzed by TEM. Myelin infoldings are observed in diabetic rats (asterisks). Scale bars: 8 µm.

These data show that STZ-induced peripheral neuropathy is characterized by myelin abnormalities such as infoldings, which are reversed to control by activation of LXR.

DIABETES INDUCED AN ALTERED MYELIN LIPID PROFILE IN THE SCIATIC NERVE

Till now has been underlined the protective effects of neuroactive steroids (Melcangi and Panzica, 2009) on peripheral nerves and these results allowed us to associate these observations with effects obtained after LXR activation. Alltoghether this led us to study whether neuroactive steroids may exert their protective effects on DPN by regulating myelin lipid profile, through the regulation of fatty acid biosynthetic pathway and ultimately protecting myelin structure and function. In this study we focus our attention on DHP or 3α-diol, two neuroactive steroids restored to control levels by LXR activation (Tab. 1).

As reported in Table 4, three months after the induction of diabetes, all the experimental groups of STZ-treated rats showed decreased body weight and increased glycemia relative to non-diabetic controls. In agreement with our previous findings (Fig. 4), diabetes affected functional parameters such as thermal nociceptive threshold (Table 4) and the treatment with DHP or 3α-diol partially reverted this diabetes-induced peripheral nerve deficit.

	CTRL	STZ	STZ + DHP	STZ +3α-diol
Weight (grams)	532.5±11.3	317.5±31.3**	291.4±13.8**	293.3±19.9**
Glycemia (mg/dl)	180±14.7	701.5±17.4**	677±36.7**	700.6±22.3**
Thermal nociceptive threshold (sec.)	11.2±0.88	34.6±2.3**	19.8±1.3*##	19.4±3.4*##

Table 4: Body weight, glycemia (fed) and thermal nociceptive threshold of control and STZ rats treated with vehicle, DHP or 3α-diol. Data are expressed as the mean ±SEM, n=7-10 rats per experimental group. Statistical analysis is performed by one-way analysis of variance (ANOVA) followed by Tukey–Kramer post-test. *P<0.05, **P<0.001 vs. control group; ##P<0.001 vs. STZ group.

To test whether DHP and/or 3α-diol may reduce the altered lipid pattern induced by diabetes in peripheral myelin, we performed detailed lipidomic analyses on sciatic nerve myelin purified from control, STZ, STZ-DHP and STZ-3α-diol treated rats. We first focused our attention on total fatty acids, confirming our previous observations on the effects exerted by diabetes (Fig. 20). These data were obtained with the different method of lipid extraction, however both the experiments showed comparable fatty acids quantifications and for this reason they can be used indistinctly.

Diabetic animals showed increased levels of saturated stearic acid (C18:0) and this was associated with decreased levels of the corresponding monounsaturated oleic acid (C18:1) (Fig. 20). In addition, γ-linolenic acid

(C18:3) levels were decreased in STZ rats, while arachidonic acid (C20:4) levels, a metabolite of C18:3, were increased (Fig. 20).

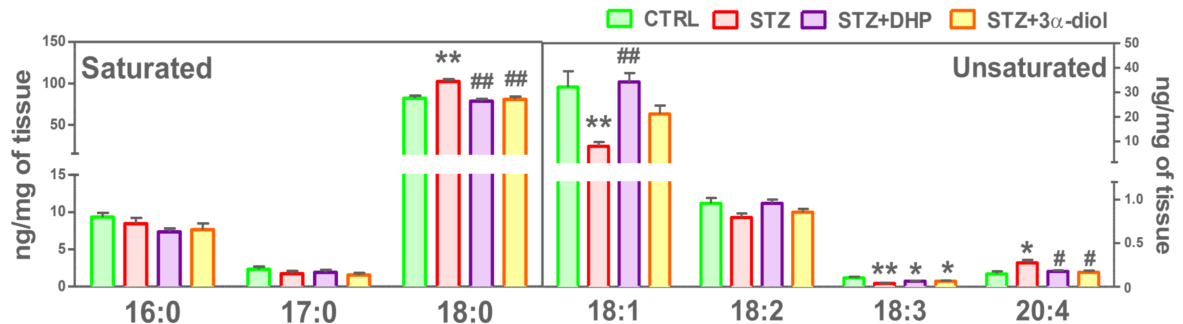


Figure 10: Lipidomic profile of sciatic nerve purified myelin from non-diabetic control, STZ and STZ-treated DHP or 3α-diol. Total fatty acid profile of myelin purified from the three experimental group. Statistical analysis was performed by one-way ANOVA followed by Tukey-Kramer post test. Data are expressed as mean ±SEM. n=7-10 animals per group. *P<0.05, **P<0.001 vs. control rats; #P<0.05, ##P<0.001 vs. STZ-treated rats.

These changes were suggestive of the inability to properly desaturate fatty acids in vehicle-treated diabetic rats. Indeed, diabetes induced an increased ratio of C18:0/C18:1, corresponding to an altered desaturation index (Fig. 21) as well as a decreased ratio of the C18:1/C18:2, an index correlating with changes in membrane fluidity (Fig. 21) (Chrast et al., 2011). In addition, diabetes reduced cholesterol levels in myelin. (Fig. 21).

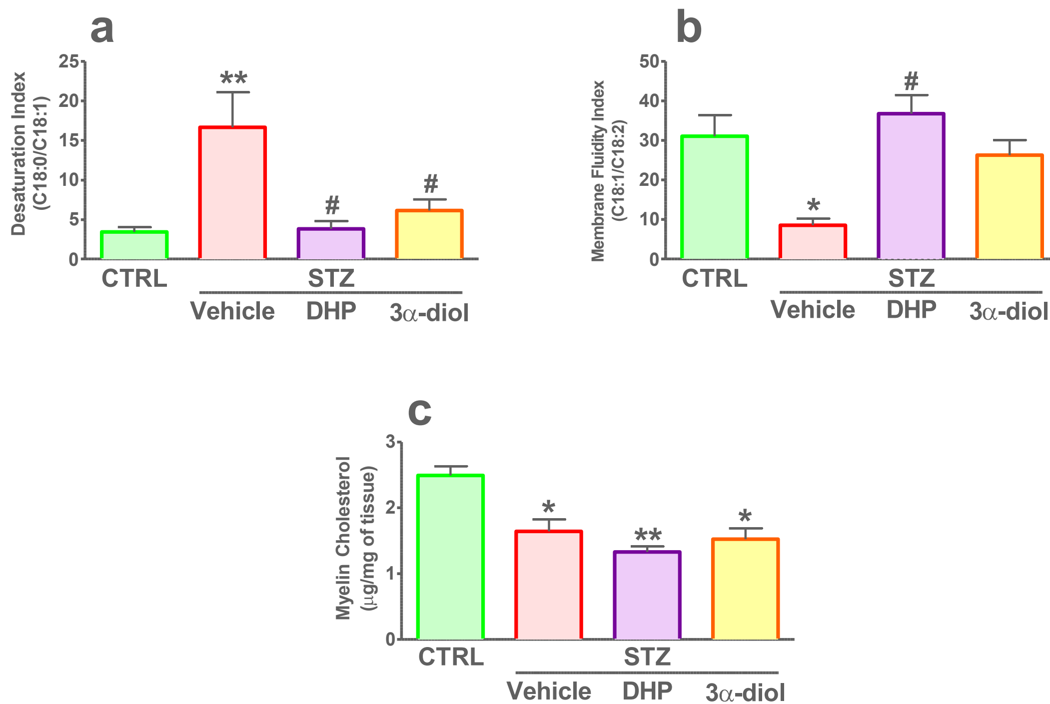


Figure 21: Lipidomic profile of sciatic nerve purified myelin from non-diabetic control, STZ and STZ-treated DHP or 3a-diol. a): Desaturation index as ratio of C18:0 (stearic acid)/C18:1 (oleic acid) and b): membrane fluidity index as ratio of C18:1 (oleic acid)/C18:2 (linoleic acid). c): Myelin cholesterol levels. Statistical analysis was performed by one-way ANOVA followed by Tukey-Kramer post test. Data are expressed as mean ±SEM. n=7-10 animals per group. *P<0.05, **P<0.001 vs. control rats; #P<0.05, ##P<0.001 vs. STZ-treated rats.

TREATMENTS WITH DHP OR 3α-DIOL PARTIALLY NORMALIZED TO CONTROL VALUES THE MYELIN LIPID PROFILE IN THE SCIATIC NERVE OF DIABETIC RATS

The altered lipid pattern induced by diabetes in the myelin of the sciatic nerve was partially restored to non-diabetic control values by the treatment with DHP or 3α-diol. DHP and 3α-diol treated rats showed normal levels of stearic acid (C18:0), oleic acid (C18:1) (Fig. 20) and normalized values of the C18:0/C18:1 and C18:1/C18:2 ratios (Fig. 21). In contrast, neither DHP nor 3α-diol were able to restore the cholesterol levels (Fig. 21) and γ-linolenic acid (C18:3) while its metabolite arachidonic acid (C20:4) was normalized to control values (Fig. 20).

DIABETES DECREASED THE EXPRESSION OF KEY GENES INVOLVED IN FATTY ACID BIOSYNTHESIS AND THIS EFFECT WAS COUNTERACTED BY THE TREATMENTS WITH DHP OR 3α-DIOL

To substantiate the detected changes in myelin lipid profiles, and to provide evidence that the two neuroactive steroids may modulate fatty acid biosynthesis we measured the mRNA level of genes encoding molecules or enzymes involved in the *de novo* lipogenic pathway. We observed that the expression of a major lipogenic transcription factor gene, such as the sterol regulatory element binding protein-1c (SREBP-1c), as well as the expression of enzymes, such as acetyl-CoA carboxylase a (ACC), fatty acid synthase (FAS), stearoyl-CoA desaturase-1 (SCD-1) and fatty acid desaturase 1 and 2 (FADS1 and FADS2), were down-regulated in diabetic sciatic nerve, and were brought back to non-diabetic levels by treatment with DHP or 3α-diol (Fig. 22).

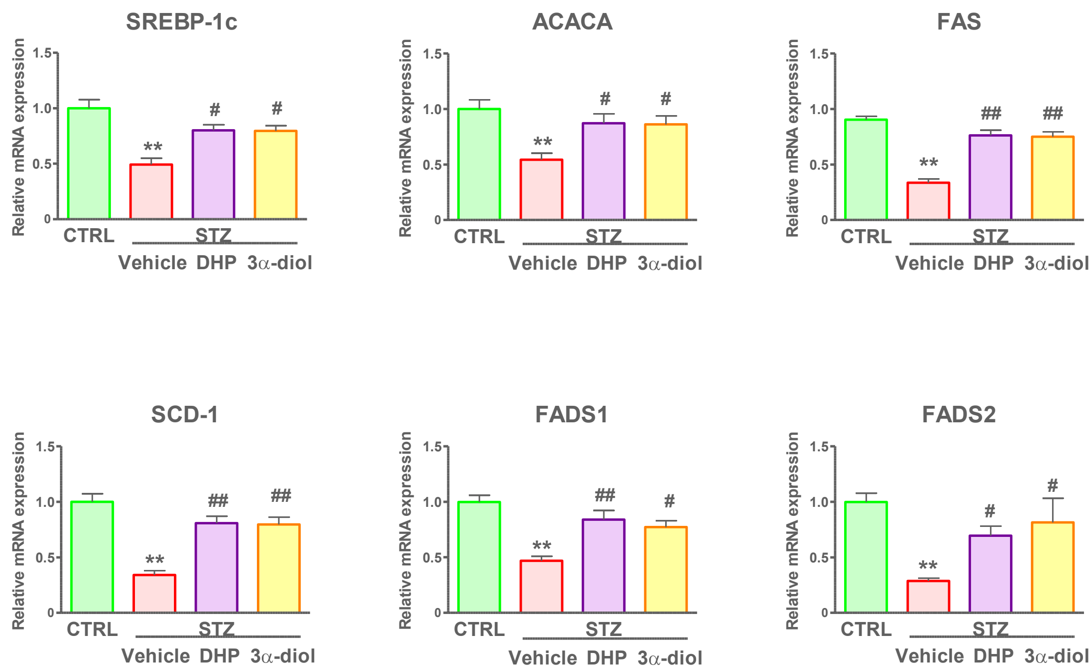


Figura 22: Expression analyses of fatty acid biosynthesis genes. Statistical analysis was performed by one-way ANOVA followed by Tukey-Kramer post-test. Data are expressed as mean ±SEM. n=7-10 animals per group. **P<0.001 vs. control rats; #P<0.05, ##P<0.001 vs. STZ-treated rats.

On the contrary, expression of enzymes involved in fatty acid elongation, such as elongase of very long chain fatty acids 5 and 6 (Elovl 5 and Elovl 6), were unchanged in all experimental groups (data not shown).

TREATMENT WITH DHP OR 3α-DIOL REDUCED MYELIN ALTERATIONS INDUCED BY DIABETES

The diameter and the total number of myelinated fibers were not affected neither by diabetes nor by treatments (data not shown). However, we observed that myelinated fibers from diabetic animals had increased numbers of myelin infoldings (Fig. 23), a deleterious effect restored to

normal levels by DHP or 3α-diol administration (Fig. 23). In addition, the g-ratio was similar among experimental groups (Fig. 23), indicating that diabetes does not influence myelination *per se* but only the lipid composition of the sheath.

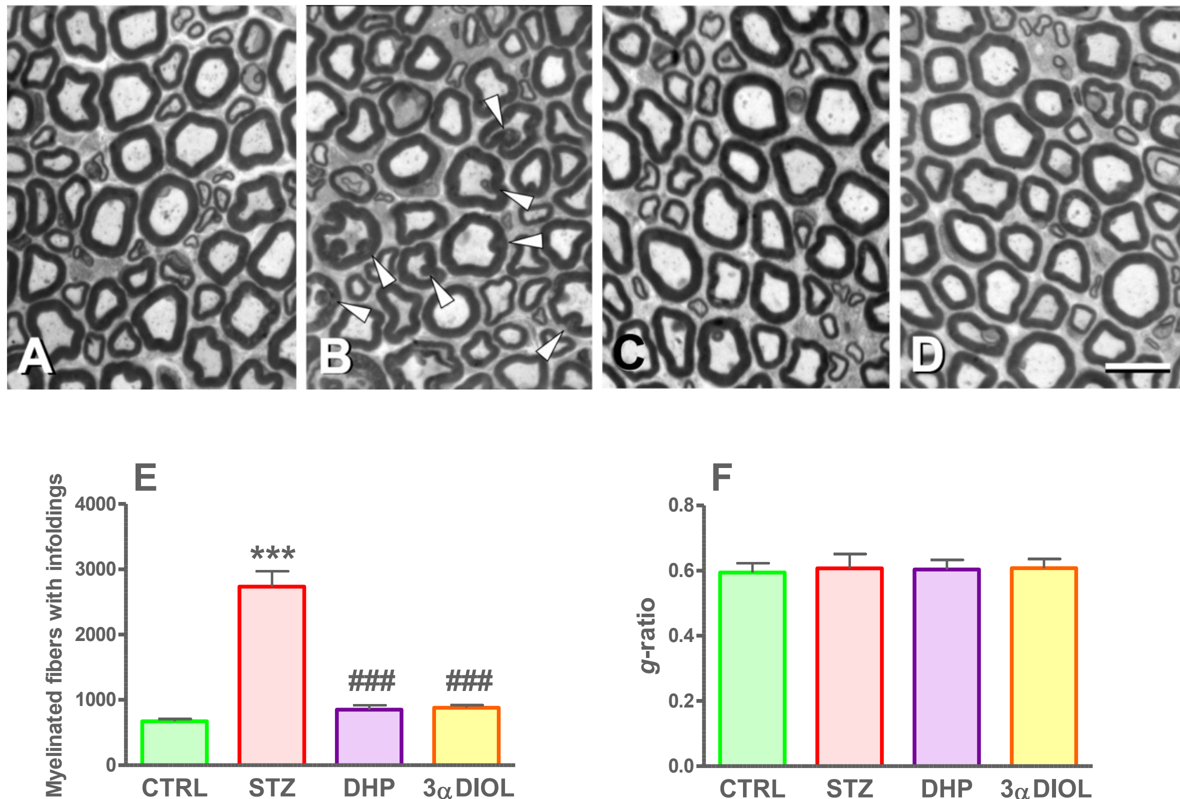


Figure 23: Morphometric and morphological analyses of myelinated fibers in the sciatic nerve of control, STZ, and STZ-treated with DHP or 3α-diol. Detail of myelinated nerves (A, control; B, STZ; C, STZ treated with DHP; D, STZ treated with 3α-diol). Myelin infoldings are indicated in diabetic rats (arrowheads in panel B). Scale bar: 12 µm. E): Percentage of fibers with myelin infoldings. F): g ratio of myelinated fibers. Statistical analysis was performed by one-way ANOVA followed by Tukey-Kramer post-test. Data are expressed as mean ±SEM, n=6 animals per experimental group. **P<0.001 vs. control rats; ##P<0.001 vs. STZ-treated rats.

6. DISCUSSION

Diabetes is one of the leading cause of mortality worldwide. Chronic hyperglycemia usually results in complications such as high blood pressure, blindness, kidney malfunction, and nervous system disease (American Diabetes, 2007, Herman, 2007). Diabetic neuropathy appears frequently in patients diagnosed with either type 1 or type 2 diabetes; in fact, 60% to 70% of diabetics develop nerve injuries. Nerve malfunction and damage is primarily due to decreased blood flow and high blood glucose levels; the extent of abnormalities is more pronounced if the hyperglycemia is not controlled properly. Peripheral nerve injuries may affect cranial nerves or nerves from the spinal column and their branches (Sugimoto et al., 2000, Vinik et al., 2000). Neuroactive steroids have been shown to exert neuroprotective effects in experimental models of diabetic neuropathy (Leonelli et al., 2007). Has been recently shown that increasing cholesterol shuttling into the mitochondria using a TSPO ligand resulted in an increase in the low levels of neuroactive steroids present in the sciatic nerve of diabetic animals that was accompanied by nerve protective effects (Giatti et al., 2009). In this research, we report similar findings using synthetic activator of the LXR nuclear receptors. The low levels of PREG (the first steroid hormone formed from cholesterol) measured in sciatic nerve of STZ-treated diabetic animals (Pesaresi et al., 2010) were increased upon LXR ligand treatment. LXR activation is known to play a role in steroidogenesis in the adrenal gland, directly regulating StAR expression, an important molecule involved in the initial process of steroidogenesis (Cummins and Mangelsdorf, 2006). In agreement with these results, we found that in peripheral nerves, such as the sciatic nerve, activation of LXR in the context of diabetes restores normal StAR mRNA levels. Moreover, we also found that the mRNA levels of P450scc, the enzyme converting cholesterol to PREG, were completely restored to physiological levels in STZ treated rats treated with GW3965. In contrast, TSPO expression was not influenced by treatment with GW3965.

Whether simultaneous activation of StAR and TSPO is necessary to activate steroidogenesis is a subject of debate (Bogan et al., 2007; Rone et al., 2009). In our study, the normalization of StAR and P450scc mRNA levels may be sufficient to account for the increased levels of PREG measured in sciatic nerve. We also found restored levels of PROG and its metabolite DHP, as well as metabolites of T, such as 3α-diol, in the sciatic nerve of diabetic rats treated with the LXR ligand. At least in the case of PROG and DHP, the increased levels observed in LXR-treated diabetic rats may be due to greater availability of their precursors (e.g., PREG), as well as to increased expression of the enzyme that generates DHP (e.g., 5α-reductase). Interestingly, LXR activation did not affect plasma levels of PREG, PROG, DHP and 3α-diol demonstrating that the observed increase of neuroactive steroids levels was due to enhanced local production and not to uptake from the periphery. This observation is quite significant because increasing levels of neuroactive steroids directly in the nervous system and not in plasma, may avoid possible endocrine side effects exerted by these molecules.

Because LXR activation restored local production of neuroactive steroids, we tested whether these effects resulted in neuroprotection in diabetic STZ-treated rats. Treatment with an LXR ligand ameliorated the impairment in NCV, thermal threshold, and Na+, K+-ATPase activity brought about by the diabetic state.

LXR activation may impact neuroactive steroid levels through its ability to regulate transcription of genes involved in cholesterol homeostasis. We demonstrated that the expressions of HMGCoA reductase and SREBP-2, key regulators of cholesterol synthesis, were unchanged among experimental groups, suggesting that the increased neuroactive steroid levels seen with LXR activation in the sciatic nerve is not related to the enhanced cholesterol synthesis. Thus, we hypothesize that the benefits of LXR

activation in this setting are due to a promotion of cholesterol utilization, similar to what showed in the adrenal gland (Cummins and Mangelsdorf, 2006). In support of this idea, we observed an upregulation of cholesterol efflux genes, such as ABCA1 and ABCG1, in the sciatic nerve of animals treated with GW3965. These genes are direct LXR targets, and their induction would be expected to maintain the level of free cholesterol at a safe limit by promoting cholesterol efflux. In summary, as shown in Figure 24, we demonstrated that LXR activation in a diabetic setting that result in peripheral neuropathy can have a beneficial effect.

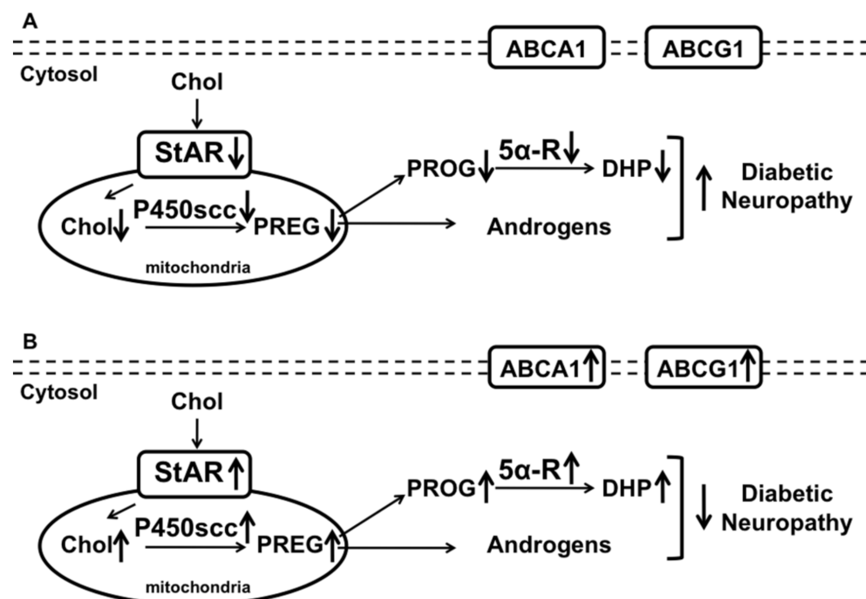


Figure 24: Proposed model of LXRs activation in diabetic neuropathy. The entrance of cholesterol into mitochondria is accomplished by the steroidogenic acute regulatory protein (StAR), a transport protein that regulates cholesterol transfer from the outer mitochondrial membrane to the inner membrane. Here, cholesterol is the substrate of P450scc enzyme, the first enzymatic step in the neuroactive steroid synthesis. A) In the diabetic state, we observed a reduced neuroactive steroid synthesis in the sciatic nerve due to decreased expression of StAR, P450scc and 5α-reductase (5α-R). B) The treatment of diabetic rats with the GW3965, a LXR synthetic ligand, restored the expression of the steroidogenic enzymes, and the neuroactive steroid levels affected by diabetic neuropathy. Moreover, LXR activation also induced the expression of the cholesterol efflux genes such as

ABCA1 and ABCG1. In conclusion, the activation of LXRs promotes cholesterol utilization and finally protects from peripheral neuropathy-induced diabetes.

With these experiments on neuroactive steroids induction via LXR activation we have shown that treatment of diabetic STZ-treated rats with a synthetic LXR ligand, promotes steroidogenesis, cholesterol disposal, and raises the local levels of neuroactive steroids, resulting in significant improvements in thermal nociceptive activity, nerve conduction velocity, and Na+,K+-ATPase activity. Alltogether these effects are associated with neuroprotection against peripheral neuropathy induced by diabetes.

In addition to their role in the regulation of cholesterol homeostasis, the LXRs also modulate FA metabolism. In fact, the LXRs directly regulate expression of the lipogenic transcription factor SREBP-1c, and of key enzymes involved in FA biosynthesis, such as fatty acid synthase (FAS) and stearoyl-CoA desaturase-1 (SCD-1) (Kalaany and Mangelsdorf, 2006, Schultz et al., 2000). Due to the ability of LXRs to modulate lipid levels we evaluated the potential of a synthetic LXR activator to reverse diabetes-induced lipid abnormalities in sciatic nerve myelin.

Alterations of myelin lipid and protein composition in peripheral nerves are often associated with metabolic derangements and neurodegenerative disorders, and can result in reduced saltatory conduction of nerve impulses throughout the axons (Chrast et al., 2011). Peripheral neuropathy comprises functional and structural changes in peripheral nerves, such as a reduction in nerve conduction velocity, axonal degeneration, paranodal demyelination, and loss of myelinated fibers (Sugimoto et al., 2000, Vinik et al., 2000). Some of the morphological alterations in myelinated fibers of peripheral nerves associated with hyperglycemia are also seen in rat models of STZ-induced diabetic neuropathy (Veiga et al., 2006a). These alterations include myelin invaginations in the axoplasm (infoldings) and

myelin evaginations in the Schwann cell cytoplasm (outfoldings), as well as alterations in myelin compaction such as abnormally wide incisures and aberrant separation of myelin lamellae. Similar to those seen in aged rodents (Azcoitia et al., 2003), the predominant myelin abnormalities found in STZ-treated rats are myelin infoldings (Veiga et al., 2006a).

We have found that diabetes indeed induces an altered lipid profile in myelin. Diabetes results in a concomitant decrease in myelin of cholesterol and monounsaturated FAs, and an increase in polyunsaturated FAs, which together with changes in phospholipid species and in the desaturation (C18:0/C18:1 ratio) and membrane fluidity (C18:1/C18:2 ratio) indexes, may be responsible for altered myelin lipid-protein interactions. We also observed in the hyperglycemic state a reduction in myelin P0. It appears that diabetes modifies myelin's lipid profile and protein composition, changes that result in altered membrane fluidity and myelin abnormalities (Verheijen et al., 2009). These data are consistent with papers showing that changes in myelin lipid composition in STZ-treated animals correlate with altered biophysical properties of the myelin sheath, as assessed by electron spin resonance spectroscopy (Zuvic-Butorac et al., 2001). Our principal component analysis shows that control and diabetic rats differ in the lipid composition of myelin. The differences may be the result of decreased expression of genes involved in FA biosynthesis, as most of them are downregulated by DPN. Furthermore, these changes were associated with increased myelin abnormalities and reduced performance of diabetic animals in functional tests. While STZ-diabetic animals show profound differences in body weight and nutritional status compared with control rats, plasma lipids do not show the altered profile seen in myelin lipids, suggesting that DPN has a specific impact on peripheral nerve lipid synthesis. Because insulin is a known activator of SREBP-1c transcription, the lack of insulin in STZ-treated rats may account for the observed reduction of

nuclear, active SREBP-1c (Chen et al., 2004). It has been reported that insulin activates SREBP-1c and FAS promoter activity directly in rat-isolated Schwann cells, indicating that this hormone regulates lipid metabolism in peripheral nerves (de Preux et al., 2007). The role of this regulatory pathway in peripheral nerves is still unclear; nevertheless, its downregulation contributes to the pathogenesis of DPN. Thus, it may be possible that insulin administration, by activating SREBP-1c and FA synthesis, could ameliorate the altered myelin lipid and protein pattern induced by diabetes. The response to insulin requires two LXR binding sites in the promoter of SREBP-1c; disruption of these LXR binding sites does not lower basal transcription but severely reduces the transcriptional induction induced by insulin (Chen et al., 2004). In our diabetic model, to reactivate SREBP-1c, we decided to use a selective LXR ligand rather than insulin itself because in addition to its ability to induce SREBP-1c expression, insulin can also reduce systemic glucose levels, and thus obscure the specific effects due to exclusive activation of SREBP-1c. LXR activation in diabetic rats restored expression of key genes of FAS synthesis and brought myelin lipid content back to control levels. These beneficial effects of LXR activation are probably due to LXR's ability to restore the levels of nuclear-active SREBP-1c. Treatment with synthetic LXR ligands also brought back to normal the levels of myelin P0. Whether this is due to SREBP-1c activation, or to changes in myelin lipid content that influence protein packaging in this membrane, remains an open question. Because neuroactive steroids enhance P0 expression (Melcangi et al., 2005) and LXR activation also increases neuroactive steroid levels in sciatic nerve, it is possible that the beneficial effects of LXR activation on P0 levels are due to greater concentration of neurosteroids. LXR activation also restored myelin structure to that seen in nondiabetic rats and improved performance of treated animals in functional tests. These improvements were seen in the absence of side effects that have been associated with LXR-SREBP-1c activation, such as hepatic steatosis (data

not shown). This is probably the result of our very mild, but effective, dosing regimen. It is interesting to note that LXR double knockout mice exhibit thinner myelin sheaths in peripheral nerves (Makoukji et al., 2011). These observations highlight the significance of the LXR-SREBP-1c axis in the control of myelin lipid content, and suggest that synthetic LXR activators may be useful to reverse myelin abnormalities brought about by diabetes.

Our first study demonstrated that DPN causes a decrease in the levels of dihydroprogesterone (DHP) and 3α-diol in the peripheral nerves. DHP and 3a-diol exert protective actions in a rat model of DPN, by mechanisms that still remain not fully characterized. Then, we demonstrated that LXR activation is able to ameliorate the peripheral neuropathic phenotype in diabetic rats acting on lipid metabolism and the protective effects has also been proved to be coupled with the restoration to non-diabetic control values of DHP and 3α-diol levels in peripheral nerves.

The experience gained with LXR activation on DPN together with the protective effects of neuroactive steroids so far ascertained (Melcangi and Panzica, 2009) led us to evaluate whether DHP or 3α-diol may act on DPN by influencing myelin lipid profile, through the regulation of fatty acid biosynthetic pathway and ultimately protecting myelin structure and function.

Our data show that DHP and 3α-diol, in the context of DPN, modulate directly the de novo lipogenic pathway. Thus, both neuroactive steroids are able to almost fully restore the altered myelin lipid profile to the levels of non-diabetic controls consequently improving myelin morphology.

Defects in desaturation are considered responsible for alterations in myelin structure and function (Garbay et al., 2000, Verheijen et al., 2009). Stearic acid (C18:0) and oleic acid (C18:1) are the most abundant fatty acids associated to phospholipids in the peripheral myelin of rats and mice. The

levels of these two most representative fatty acids in the myelin of the sciatic nerve were altered by diabetes and restored to control values by DHP or 3α-diol treatment. Moreover, oleic acid (C18:1) is produced by desaturation of the saturated stearic acid (C18:0), a biochemical reaction catalyzed by the stearoyl-CoA desaturase-1 (SCD-1). Stearic acid (C18:0) is either synthesized in the body from palmitic acid (C16:0) by elongation or ingested directly with the diet. In our study, Palmitic acid (C16:0) levels in myelin were not affected, indicating that the observed accumulation of stearic acid (C18:0) detected in the myelin of diabetic rats was due to an impaired desaturation of stearic acid (C18:0). In this regard, the most important outcome of the DHP or 3α-diol treatment is their ability to bring back the expression levels of SCD-1 to control values, thus restoring the desaturation of stearic acid (C18:0) to oleic acid (C18:1). Consequently, steroids treatment also normalizes the desaturation and possibly the membrane fluidity indexes.

Cholesterol is a molecule required for myelin stabilization and compaction (Nussbaum et al., 1969, Detering and Wells, 1976) and plays an essential role in coordinating myelin membrane assembly. Indeed, the inactivation of cholesterol biosynthesis in Schwann cells led to substantial hypomyelination (Saher et al., 2005). In the present study in diabetic animals, we did not detect hypomyelination (see *g*-ratio data in Fig. 19) but myelin infoldings, most probably associated to changes in membrane fluidity (as suggested by the ratio of C18:1/C18:2). This is likely the result that diabetes was induced in adult animals and not as in the conditional inactivation of squalene synthase in mice occurring from birth or before (Saher et al., 2005). On this basis, it seems that the restoration of fatty acid desaturation by the DHP or 3α-diol treatments, which were unable to restore cholesterol levels, is one the main phenomenon required to protect myelin lipid profile from alterations induced by diabetes.

In conclusion, as summarized in Figure 25, our findings indicate that treatments with DHP and 3α-diol are able to modulate the lipogenic pathway and to normalize the lipid species in the peripheral myelin of diabetic rats, resulting in protection against DPN.

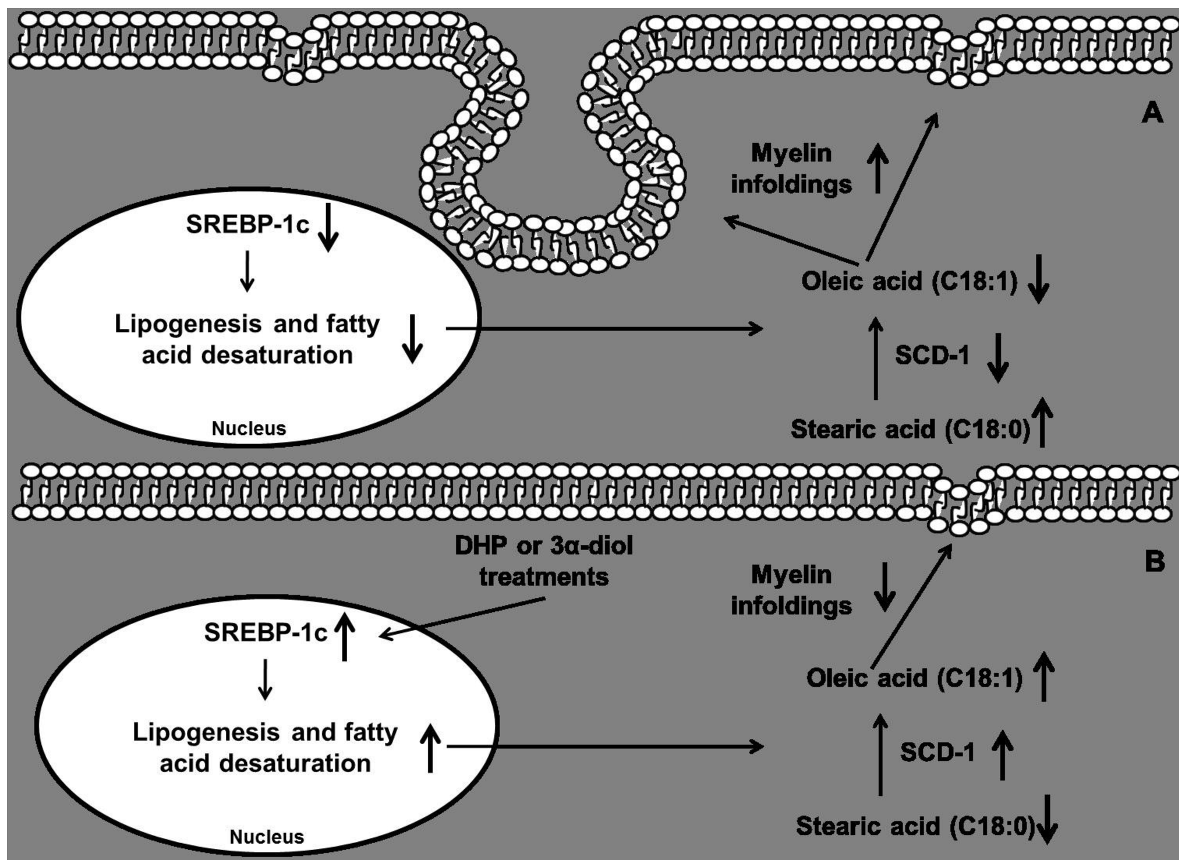


Figure 25: Proposed model of the protective effects exert by DHP and 3a-diol in diabetic peripheral neuropathy. A) In diabetic conditions de novo lipogenesis is reduced and stearic acid (C18:0) is accumulated while its corresponding desaturated fatty acid such as the oleic (C18:1) is reduced. These situations favor the myelin infoldings. B) The treatments with DHP or 3a-diol normalized lipogenesis and improved the desaturation of stearic acid (C18:0) into oleic acid (C18:1), the two most abundant fatty acids present in purified myelin.

Finally, the regulation of myelin proteins by neuroactive steroids (Pesaresi et al., 2011) and the modulation of myelin fatty acid species by the same molecules suggest that both pathways are strictly interconnected and required for proper myelin structure and function.

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