Cell. Mol. Life Sci. 64 (2007) 1158 – 1168
1420-682X/07/091158-11
DOI 10.1007/s00018-007-7002-5

Cellular and Molecular Life Sciences

Research Article

Testosterone derivatives are neuroprotective agents in experimental diabetic neuropathy

I. Roglio[a, †], R. Bianchi[b, †], S. Giatti[a], G. Cavaletti[c], D. Caruso[d], S. Scurati[d], D. Crippa[e], L. M. Garcia-Segura[f], F. Camozzi[g], G. Lauria[g] and R. C. Melcangi[a, *]

[a] Department of Endocrinology and Center of Excellence on Neurodegenerative Diseases, University of Milan, Via Balzaretti 9, 20133 Milano (Italy), Fax: +39 02 50318204, e-mail: roberto.melcangi@unimi.it
[b] Department of Molecular Biochemistry and Pharmacology, "Mario Negri" Institute for Pharmacological Research, Milano (Italy)
[c] Department of Neurosciences and Biomedical Technologies, University of Milan "Bicocca", Monza (Italy)
[d] Department of Pharmacological Sciences, University of Milan, Milano (Italy)
[e] Schering SpA, Segrate (Milano) (Italy)
[f] Instituto Cajal, C.S.I.C., Madrid (Spain)
[g] Neuromuscular Diseases Unit, National Neurological Institute "Carlo Besta", Milano (Italy)

Received 4 January 2007; received after revision 13 February 2007; accepted 27 March 2007
Online First 9 April 2007

Abstract. In this study we have assessed the effect of testosterone (T), dihydrotestosterone (DHT) and 5α-androstan-3α, 17β-diol (3α-diol) therapies on diabetic neuropathy. Diabetes was induced in adult male rats by the injection of streptozotocin and resulted in decreased T and increased 3α-diol levels in plasma and in decreased levels of pregnenolone and DHT in the sciatic nerve. Moreover, a reduced expression of the enzyme converting T into DHT (*i.e.*, the 5α-reductase) also occurs at the level of sciatic nerve, suggesting that the decrease of DHT levels could be due to an impairment of this enzyme. Chronic treatment for 1 month with DHT or 3α-diol increased tail nerve conduction velocity and partially counteracted the increase of thermal threshold induced by diabetes. Treatment with DHT increased tibial Na^+,K^+-ATPase activity and the expression of myelin protein P0 in the sciatic nerve. DHT, 3α-diol and T reversed the reduction of intra-epidermal nerve fiber density induced by diabetes. These observations indicate that T metabolites can reverse behavioral, neurophysiological, morphological and biochemical alterations induced by peripheral diabetic neuropathy.

Keywords. Neuroactive steroids, rat, sciatic nerve, streptozotocin, dihydrotestosterone, 3α-diol, 5α-reductase, androgen receptor.

Introduction

Progressive damage of peripheral nerves represents one of the most important complications of diabetes mellitus occurring in more than 50% of patients. Axonal degeneration and slowing in nerve conduction velocity (NCV) reflect loss of myelinated fibers and paranodal demyelination [1, 2]. Moreover, we have recently demonstrated that the gene expression of myelin proteins, such as glycoprotein zero (P0) and peripheral myelin protein 22 (PMP22), which play a crucial role in maintaining the multilamellar structure of peripheral nerve myelin [3, 4], is affected by

† These authors contributed equally to this study.
* Corresponding author.

diabetes [5]. Decreased Na^+,K^+-ATPase activity in peripheral nerves and reduced intra-epidermal nerve fiber density associated with impaired nociceptive threshold are well described both in humans and streptozotocin (STZ)-induced neuropathy [6–14].

Current treatment of diabetic neuropathy relies on the control of glycemic and vascular risk factors [15], which does not totally prevent its occurrence or progression. Recent studies showed that neuroactive steroids might provide new therapeutic options. Yorek et al. [16] observed that dehydroepiandrosterone prevents vascular and neuronal dysfunction in the sciatic nerve of STZ-treated rats. In the same model, we recently observed that progesterone (P) and its derivatives, dihydroprogesterone (DHP) and tetrahydroprogesterone (THP), reversed the impairment of NCV and thermal threshold, restored intra-epidermal nerve fiber density, improved Na^+,K^+-ATPase activity, and counteracted the decrease of gene expression of myelin proteins, such as P0 and PMP22 [5]. We also observed that P administration results in a significant reduction in the number of fibers with myelin abnormalities in the sciatic nerve of STZ-treated rats [17].

Overall, the observations from experimental peripheral neuropathy models (*i.e.*, physical trauma, aging, or genetic diseases) indicate that P and its derivatives are neuroprotective agents [18–23].

Other neuroactive steroids exert promising neuroprotective effects. In several peripheral neuropathy models, testosterone (T) and its derivative, dihydrotestosterone (DHT), accelerate regeneration and functional recovery of nerves [24–29]. These steroids can also exert a control on the expression of some myelin proteins. For instance, in adult male rats castration decreases the expression of P0 and PMP22 in the sciatic nerve [30, 31]. The subsequent treatment with DHT or its metabolite, 5α-androstan-3α, 17β-diol (3α-diol) was able to restore the levels of P0 messenger, whereas 3α-diol induced an increase of the synthesis of PMP22 [30, 31]. A role of androgen receptor (AR) in controlling the expression of P0 was confirmed by the finding that *in vivo* treatment with an antagonist of this steroid receptor (*i.e.*, flutamide) decreases the synthesis of P0 in rat sciatic nerve [31]. On this basis we hypothesized that T and its metabolites, DHT and 3α-diol, might protect peripheral nerves from degeneration induced by diabetic neuropathy. Therefore, our study was designed to assess whether plasma and sciatic nerve levels of neuroactive steroids were affected by STZ-induced neuropathy and whether in this experimental model T, DHT and 3α-diol had neuroprotective effects at neurophysiological, biochemical and neuropathological levels.

Materials and methods

Animals and treatments

Animals. Two-month-old male Sprague-Dawley rats, Crl:CD BR (Charles River, Italy) were utilized. The animals were maintained in the department animal quarters with controlled temperature and humidity. The light schedule was 14 h light and 10 h dark (lights on at 06.30 h). The animals were handled following the European Union Normative (Council Directive 86/609/EEC), with the approval of our Institutional Animal Use and Care Committees.

Induction of diabetes and experimental treatments. Diabetes was induced by a single intravenous injection of freshly prepared STZ (65 mg/kg; Sigma, Italy) 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 STZ injection by measuring tail vein blood glucose levels using a Glucomen tester (Menarini, Italy). Only animals with mean plasma glucose levels above 300 mg/100 ml were classified as diabetic. Glycemia was also assessed before the treatment with steroids (2 months after STZ injection, see below) and tested at scheduled death, 3 months after STZ.

At 2 months after the STZ injection, rats received 16 subcutaneous injections (every other day) of 1 mg T, DHT or 3α-diol (Sigma, Italy) dissolved in 200 µl sesame oil. Control rats received 200 µl vehicle (sesame oil). Rats were killed 24 h after the last treatment.

Steroids concentration in plasma and sciatic nerves

Steroids in plasma and sciatic nerves were extracted according to Vallée et al. [32] with minor modification. Briefly, the internal standard deuterium-labeled 17,21,21,21-D4 pregnenolone (5 ng/sample; D4-PREG; prepared by Dr. P. Ferraboschi, Department of Medical Chemistry, Biochemistry and Biotechnology, University of Milan, Italy) was added to the samples. The plasma sample (0.5 ml) of each animal was independently analyzed, while sciatic nerves samples from different animals were pooled to obtain a 100 mg tissue/sample. Acetic acid (1%) in methanol was added to the samples before homogenization by sonication and loading to C18 cartridges (Discovery DSC-18, 500 mg cartridge, Supelco, Italy). The steroid fraction was eluted with methanol (5 ml) and the organic phase was reconstituted with methanol:water (1:1) before the injection in a RP-C_{18} analytical column (XTerra, Waters, Ireland; 3.5 µm, 100 mm×4.6 mm inner diameter).

The high-performance liquid chromatograph (Surveyor LC Pump Plus, Thermo Electron Co) was coupled to an LTQ (Thermo-Electron Co, USA) linear ion trap mass spectrometer (LC/MS) equipped with an atmospheric pressure chemical ionization (APCI) source operating in the positive ion mode. Each steroid was identified on the basis of the retention time and the tandem MS (MS/MS) spectrum of reference compounds. The quantitative analyses were done monitoring specific ions (selected ion chromatogram mode, SIC) in the MS/MS spectrum obtained by collision of precursor ion in MS spectrum as reported in Table 1. Samples were quantified by means of calibration curves using a deuterium-labeled internal standard.

Real-time PCR

Following total RNA extraction, the samples were processed for real-time PCR to assess the influence of diabetes on 5α-reductase and androgen receptor (AR) gene expression. A 1-µg aliquot of each sample was treated with DNase, to avoid DNA contamination, then reverse transcribed using a High-Capacity cDNA Archive Commercial Kit (Applera, Italy). The real-time PCR reaction was performed using the TaqMan Universal PCR Master Mix (Applera) with the specific TaqMan MGB probe purchased from Applera. Thermal cycling conditions included a pre-run of 2 min at 50°C and 10 min at 95°C. Cycle conditions were 40 cycles at 95°C for 15 s and 60°C for 1 min according to the TaqMan Universal PCR Protocol. We utilized the ABI Prism 7000 Sequence Detection System (Applera). Each sample was run in triplicate for

Table 1. Precursor ion in mass spectroscopy (MS) and the selected ion in tandem MS (MS/MS) spectrum for testosterone (T), dihydrotestosterone (DHT), 5α-androstan-3α, 17β-diol (3α-diol), pregnenolone (PREG) and 17,21,21,21-D4 pregnenolone (D4-PREG).

Analites	T	DHT	3α-diol	PREG	D4-PREG
Precursor ions (*m/z*)	289	291	257	299	303
MS/MS selected ions (*m/z*)	253	255	175	225	175

the quantification of the 5α-reductase or AR gene expression as compared to the internal 18S control gene.

A comparative cycle threshold (Ct) method was used to calculate the relative target gene expression. To use this method, we performed a validation experiment to demonstrate that the efficiencies of target genes amplifications and 18S rRNA were approximately equal and close to 100%, as suggested by the manufacturer (user bulletin, No. 2; Applera). The Ct, which is the PCR cycle at which the fluorescent signal is first detectable, was then determined for all PCR reactions. For analysis, the Ct value for the internal standard (*i.e.*, 18S RNA) was first subtracted from the Ct value for the cDNA of interest (*i.e.*, 5α-reductase or AR). This value is denoted as the ΔCt. Next, the ΔCt value generated from control samples was subtracted from the ΔCt for experimental samples. This equation sets the control sample to a reference value of 0 and generates a ΔΔCt for experimental samples. These values were averaged for each group; these mean values were used to generate the N-fold difference in RNA expression relative to the control using the equation: $2^{-\Delta\Delta Ct}$; with this equation the control = 1. Although this data transformation accurately illustrates the logarithmic amplification following each PCR cycle, statistical evaluation was performed using the ΔΔCt value. Due to the logarithmic transformation of the data, it is visually misleading to depict a standard deviation or standard error of the mean; thus real-time PCR data for 5α-reductase, do not include such error bars.

Functional analyses

Thermal nociceptive threshold. The nociceptive threshold to radiant heat was quantified using the hot plate paw withdrawal test as previously described [7]. 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, in each test section.

Nerve conduction velocity. At the end of the treatments antidromic tail NCV was assessed using a Myto EBNeuro electromyograph (EBNeuro, Italy) as previously described [33]. Briefly, recording ring electrodes were placed distally in the tail. The stimulating ring electrodes were placed 5 and 10 cm proximally with respect to the recording point. The latencies 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 the neurophysiological studies were done under standard conditions in a temperature-controlled room adjacent to the animal housing room.

Skin biopsies. Peripheral nerve damage was assessed by pathological examination of the skin in the hindpaw footpad by the quantification of intra-epidermal nerve fiber density [12]. Briefly, hindpaws were collected at death. After separating the plantar glabrous skin, which included epidermis and dermis, from the underlying metatarsal bones, 3-mm round samples were taken and immediately fixed in 2% paraformaldehyde-lysine periodate for 24 h at 4°C, cryoprotected overnight, and serially cut with a cryostat to obtain 20-μm sections. Two sections from each footpad were randomly selected and immunostained with rabbit polyclonal anti-protein gene product 9.5 (PGP 9.5; Biogenesis, Poole, UK) using a free-floating protocol. Two observers blinded to the healthy or neuropathic status of rats, independently counted the total number of PGP 9.5-positive intra-epidermal nerve fibers in each section under a light microscope at high magnification, with the assistance of a microscope-mounted video camera. Individual fibers were counted as they crossed the dermal–epidermal junction, and secondary branching within the epidermis was excluded. The length of the epidermis was measured using a computerized system (Microscience Inc., Seattle, WA) and the linear density of intra-epidermal nerve fibers (IENF/mm) was obtained.

Biochemical analyses

Na^+,K^+-ATPase activity. Tibial stumps were dissected out, de-sheathed and homogenized in 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, Italy), and stored at –80°C for ATPase determinations. Na^+,K^+-ATPase activity was determined spectrophotometrically as previously described [34]. Protein content in homogenates was determined by Lowry's method [35] with bovine serum albumin as standard.

cRNA probes. The specific pCR®II-TOP0® (Invitrogen, Italy) plasmids contain the following inserts: 387 bp for P0, 414 bp for PMP22, and 290 bp for18 s. The cRNA antisense probes were generated by *in vitro* transcription of different specific pCR®II-TOP0® plasmids in presence of [^{32}P]CTP (Amersham, Italy) as the labeled nucleotide. All the cRNA probes were obtained with specific activity $>10^8$ cpm/μg.

RNase protection assay. Total RNA from snap-frozen sciatic nerves was isolated by phenol-chloroform extraction according to the method of Chomczynski and Sacchi [36]. Samples of total RNA (10–12 μg) were utilized in the RNase protection assay, as previously described [5]. Briefly, after ethanol precipitation samples were dissolved in 20 μl hybridization solution (80% formamide, 40 mM PIPES pH 6.4, 400 mM sodium acetate pH 6.4 and 1 mM EDTA) containing 150 000 cpm of each ^{32}P-labeled cRNA probe and 50 000 cpm of ^{32}P-labeled cRNA 18 s probe. After heating at 85°C for 10 min, the probes were allowed to hybridize the endogenous RNAs at 45°C overnight. Subsequently, the samples were diluted with 200 μl RNase digestion buffer (300 mM NaCl, 10 mM Tris-HCl pH 7.4, 5 mM EDTA pH 7.4) containing a 1:400 dilution of an RNase cocktail (1 μg/μl RNase A and 20 U/μl RNase T1) and incubated for 30 min at 30°C. Then, 10 μg proteinase K and sodium dodecyl sulfate (10 ml of 20% stock solution) were added to the samples and the mixture was incubated at 37°C for 15 min. The samples were then extracted with phenol-chloroform and precipitated with ethanol. The pellet was dried and resuspended in loading buffer (80% formamide, 0.1% xylene cyanol, 0.1% bromophenol blue, 2 mM EDTA) boiled at 95°C for 5 min and separated on a 5% polyacrylamide gel, under denaturing conditions (7 M urea). The protected fragments were visualized by autoradiography and their sizes were determined using ^{32}P-end labeled (T4 polynucleotide kinase) *Msp*I-digested pBR322 fragments.

RNA calculation. The levels of mRNA for P0, PMP22, and 18 s rRNA were calculated by measuring the peak densitometric area of the autoradiography analyzed with a Kodak Snap Scanner. To ensure that the autoradiographic bands were in the linear range of intensity, different exposure times were used. The mean control value within a single experiment was set to 100 and all the other values were expressed as a percentage of this. Values of controls from different experiments were all within 10%.

Table 2. Body weight and glucose levels of control, diabetic and diabetic rats treated with T, DHT or 3α-diol.

Animal	Body weight (g)	Body weight (g)	Blood glucose (mg/100 ml)
	Before STZ injection	At sacrifice	At sacrifice
Control	246.5±2.2	536.5±13.6	96±5
Diabetic	247.0±2.8	289.1±15.2***	534±31***
Diabetic + T	246.3±1.8	305.8±15.9***	588±16***
Diabetic + DHT	245.7±2.9	284.4±18.4***	552±22***
Diabetic + 3α-diol	234.3±6.3	320.1±29.3***	540±15***

Mean ± SEM (n=8). *** $p<0.001$ vs control.

Statistical analysis

The quantitative data obtained by the experiments have been analyzed through inferential statistical analysis according with the experimental protocols and the nature of the data. Unpaired Student's t-test was applied to couples of independent variables. Data from experiments with more than two independent variables have been analyzed by one-way analysis of variance (ANOVA) followed by Tukey-Kramer post-test. A p value less than or equal to α, set to 0.05, indicates a statistically significant effect. All these statistical analyses were performed by GraphPad PRISM (version 4).

Results

As shown in Table 2, diabetic rats had high blood glucose at the end of the experiment and, as expected, significantly less weight gain than non-diabetic control rats. Treatment with T, or its reduced derivatives, DHT and 3α-diol, did not significantly modify body weight and blood glucose levels compared to diabetic vehicle-treated rats (Table 2).

Levels of pregnenolone (PREG), T and its metabolites, DHT and 3α-diol, present in plasma and in sciatic nerves of control and diabetic animals untreated with neuroactive steroids were assessed by LC-MS/MS. Figure 1 shows the selected ion chromatogram corresponding to each steroid analyzed. After 3 months of diabetes, PREG levels were unchanged in plasma (Fig. 2a) but were significantly decreased in sciatic nerves (Fig. 3a). In addition, T plasma levels were significantly lowered (Fig. 2b). A similar tendency, which however did not reach the level of significance, was also observed for the level of T measured in the sciatic nerve (Fig. 3b). DHT levels did not change in plasma (Fig. 2c), but a significant decrease occurred in sciatic nerves (Fig. 3c). Furthermore, 3α-diol levels were significantly increased by diabetes in plasma (Fig. 2d), but not in sciatic nerves (Fig. 3d). On the basis of these observations we then evaluated whether the decrease of DHT levels in the sciatic nerve of diabetic animals might be due to an impairment of 5α-reductase (*i.e.*, the enzyme converting T into DHT). As shown in Figure 4, the levels of this enzyme evaluated by real-time PCR were significantly decreased in the sciatic nerve of diabetic animals untreated with neuroactive steroids. In contrast, AR levels were unmodified in sciatic nerves, *i.e.*, data expressed as $2^{-\Delta\Delta Ct}$ values did not show significant difference in sciatic nerve of diabetic (0.842, n=5) vs control (1.000, n=6) animals.

In agreement with our previous observations, diabetes reduced tail nerve NCV by 30% (Fig. 5). Chronic treatment with DHT or 3α-diol significantly improved NCV, while treatment with T was ineffective. Data in Figure 6 show that diabetes induced an increase of about 80% in thermal threshold and that DHT and 3α-diol partially counteracted this effect, significantly decreasing the thermal latency by 38% and 24%, respectively. Treatment with T was ineffective.

As shown in Figure 7, diabetic rats showed a 50% reduction in tibial Na^+,K^+-ATPase activity that was partially but significantly restored by treatment with DHT (40% increase compared to diabetic rats treated with vehicle). In diabetic rats treated with T or 3α-diol, Na^+,K^+-ATPase activity showed a similar tendency without reaching a statistical significance (Fig. 7).

Skin biopsy confirmed the neuroprotective effect of T metabolites. Diabetes significantly reduced intraepidermal nerve fiber density and T, DHT and 3α-diol were able to totally restore this decrease (Fig. 8).

In agreement with our previous observations, diabetes reduced the expression of myelin proteins, such as P0 and PMP22 (Figs 9 and 10). As shown in Figure 9, treatment with DHT increased P0 mRNA levels in sciatic nerve of diabetic rat, while both T and 3α-diol were ineffective. In contrast, none of these neuroactive steroids were effective in stimulating the low PMP22 mRNA levels present in sciatic nerve of diabetic rat (Fig. 10).

Discussion

The treatment of diabetic neuropathy represents an important challenge for biomedical research. Indeed, despite multiple attempts, no satisfactory manage-

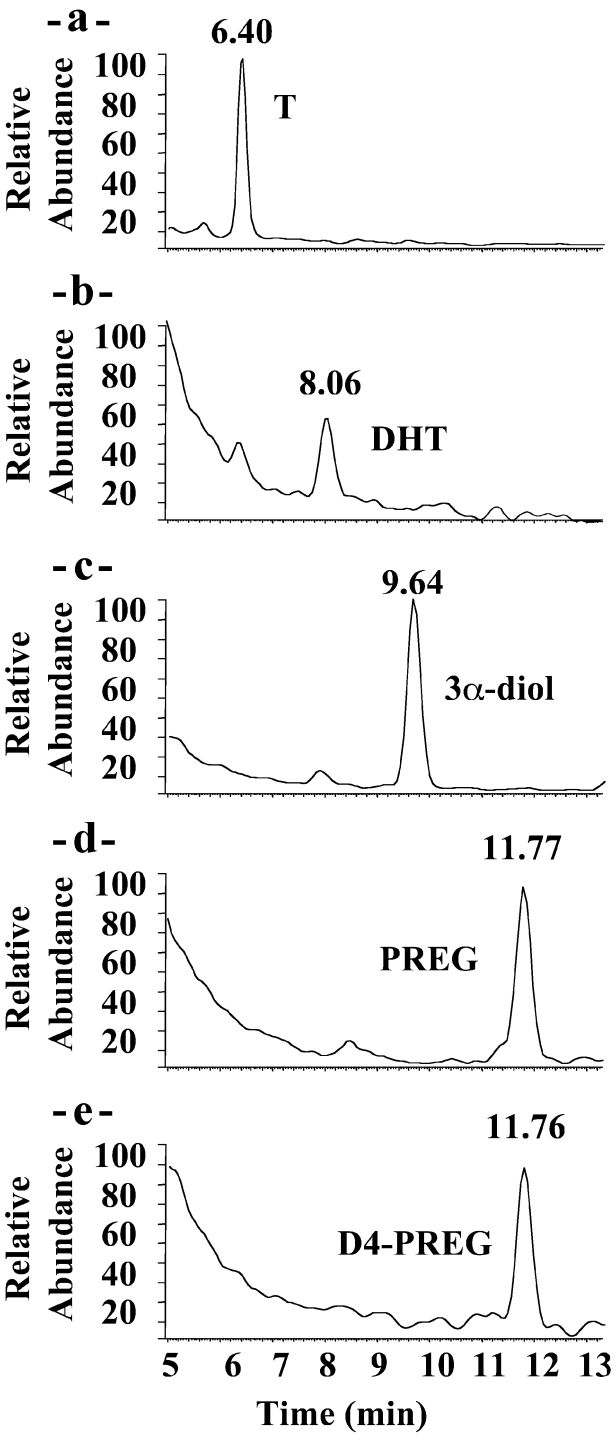

Figure 1. Representative liquid chromatography-tandem mass spectrometry (LC-MS/MS) selected ion chromatograms (SIC) of a sciatic nerve sample. The identity of each peak is based on the retention time and MS/MS spectra of authentic compounds. (*a–e*) SIC traces corresponding to testosterone (T), dihydrotestosterone (DHT), 5α-androstan-3α, 17β-diol (3α-diol), pregnenolone (PREG) and to deuterium-labeled pregnenolone (D4-PREG), respectively.

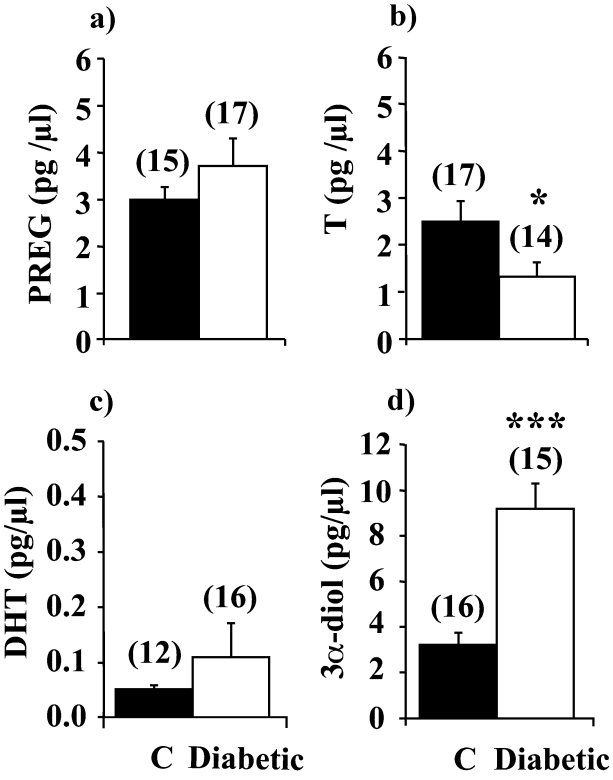

Figure 2. PREG (*a*), T (*b*), DHT (*c*) and 3α-diol (*d*) levels in plasma of control (C) and diabetic animals untreated with neuroactive steroids. Data are expressed as pg/µl and are the mean ± SEM (number of rats in each group in parentheses). Unpaired Student's *t*-test; * $p<0.05$ vs control; *** $p<0.001$ vs. control.

ment is yet available. We have very recently observed that STZ-induced diabetes causes P plasma levels to drop steeply in male rats and that treatment with this neuroactive steroid and its metabolites, DHP and THP, exert important protective effects on peripheral nerve damage [5]. Indeed, chronic treatment with these neuroactive steroids significantly improved NCV, thermal threshold, intra-epidermal nerve fiber density, Na^+,K^+-ATPase activity, and gene expression of important peripheral myelin proteins, such as P0 and PMP22 [5]. Moreover, P administration also resulted in a significant reduction of myelin abnormalities observed in the sciatic nerve of STZ treated rats [17].

In agreement with observations showing dysfunction in reproductive axis associated with diabetes [37–45], we here demonstrate that T plasma levels are also significantly decreased in STZ-treated rats. Moreover, our data indicate that diabetes causes alterations in the levels of steroids in sciatic nerves that do not completely reflect the changes observed in plasma. Indeed, the decrease in T levels observed in the plasma of diabetic rats was not detected in the sciatic nerves. In addition, diabetes significantly decreases PREG and DHT levels in sciatic nerves but does not

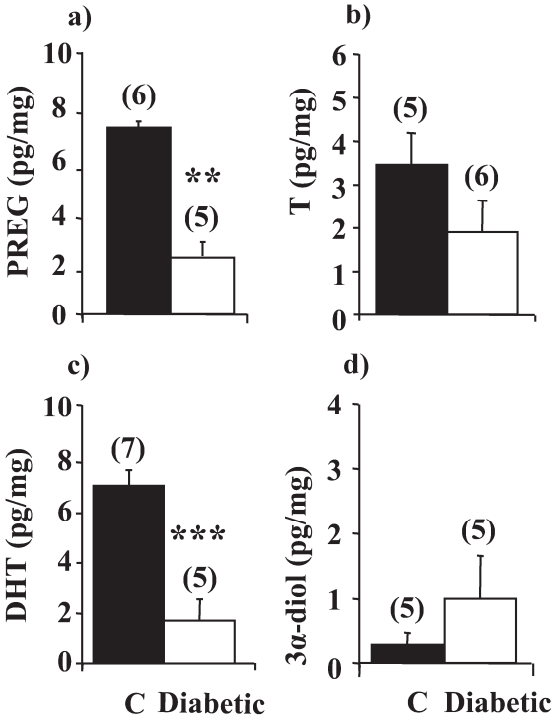

Figure 3. PREG (*a*), T (*b*), DHT (*c*) and 3α-diol (*d*) levels in sciatic nerve of control (C) and diabetic animals untreated with neuroactive steroids. Data are expressed as pg/mg and are the mean ± SEM (number of determinations in each group in parentheses). Unpaired Student's *t*-test; ** $p<0.01$ vs control; *** $p<0.001$ vs control.

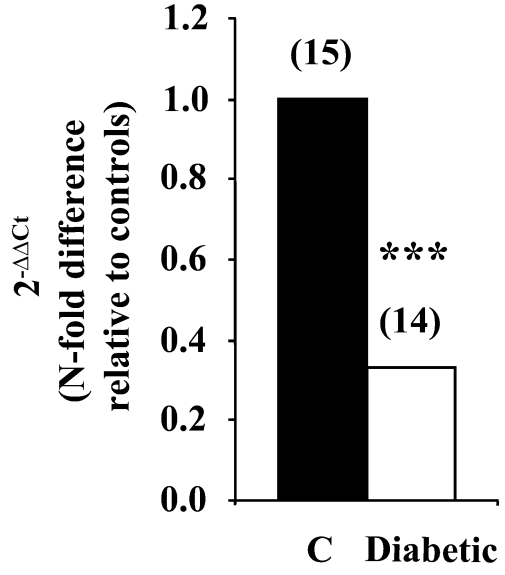

Figure 4. 5α-reductase mRNA levels in sciatic nerve of control (C) and diabetic animals untreated with neuroactive steroids. Data, after normalization with 18 s rRNA (ΔCt), are expressed as difference (ΔΔCt) vs ΔCt in controls and averaged for each experimental group (number of rats in parentheses). The columns represent the $2^{-\Delta\Delta Ct}$ values (see Materials and methods). Statistical evaluation was performed using the ΔΔCt value. Unpaired Student's *t*-test; *** $p<0.001$ vs control.

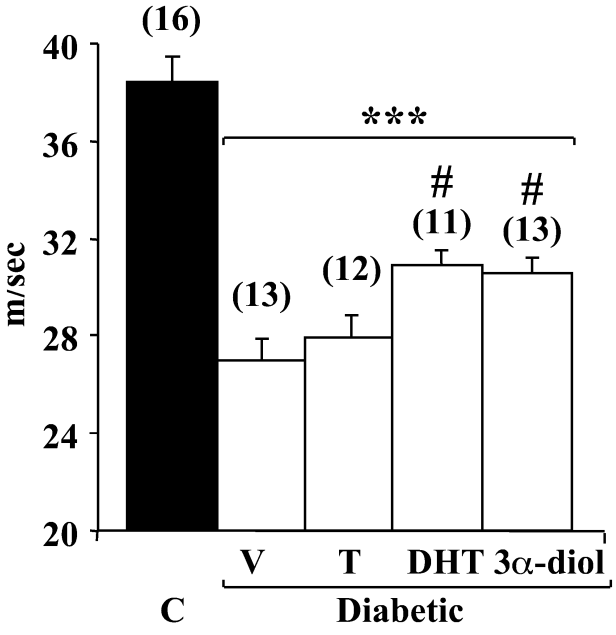

Figure 5. DHT and 3α-diol partially restore the decrease in NCV in diabetic rats. Tail NCV was measured at the end of the experiment (12 weeks) in control (C) and diabetic rats treated with vehicle only (V), or with T, DHT, or 3α-diol. Data are expressed as m/s and are the mean ± SEM (number of rats in each group in parentheses). ANOVA followed by Tukey-Kramer post-test; *** $p<0.001$ vs control; # $p<0.05$ vs diabetic injected with vehicle.

affect the levels of these steroids in plasma. Conversely, the levels of 3α-diol were significantly increased in the plasma of diabetic rats, while they remained unchanged in the sciatic nerves.

These findings suggest that diabetes differentially alters steroid synthesis in endocrine glands and in sciatic nerves. Formation of PREG and other steroids in sciatic nerve is not surprising. It has been clearly established that Schwann cells express molecules able to participate in the transport of cholesterol to the inner mitochondrial membrane where cytochrome P450scc (*i.e.*, the enzyme forming PREG) is located [46]. Indeed, the expression of the peripheral benzodiazepine receptor (PBR, now re-named translocator protein-18kDa [47]), of its endogenous ligand octadecaneuropeptide [48–50], and of the steroidogenic acute regulatory (StAR) protein has been demonstrated [51].

Altered levels of neuroactive steroids in peripheral nerves have been also reported in other experimental models. For instance, the expression of PBR is increased in Schwann cells after nerve lesion, and returns to normal levels when regeneration is completed [49]. This induction has been interpreted as an endogenous neuroprotective response to the damage. The changes are in keeping with the evidence that P biosynthesis is up-regulated in the spinal cord of rats

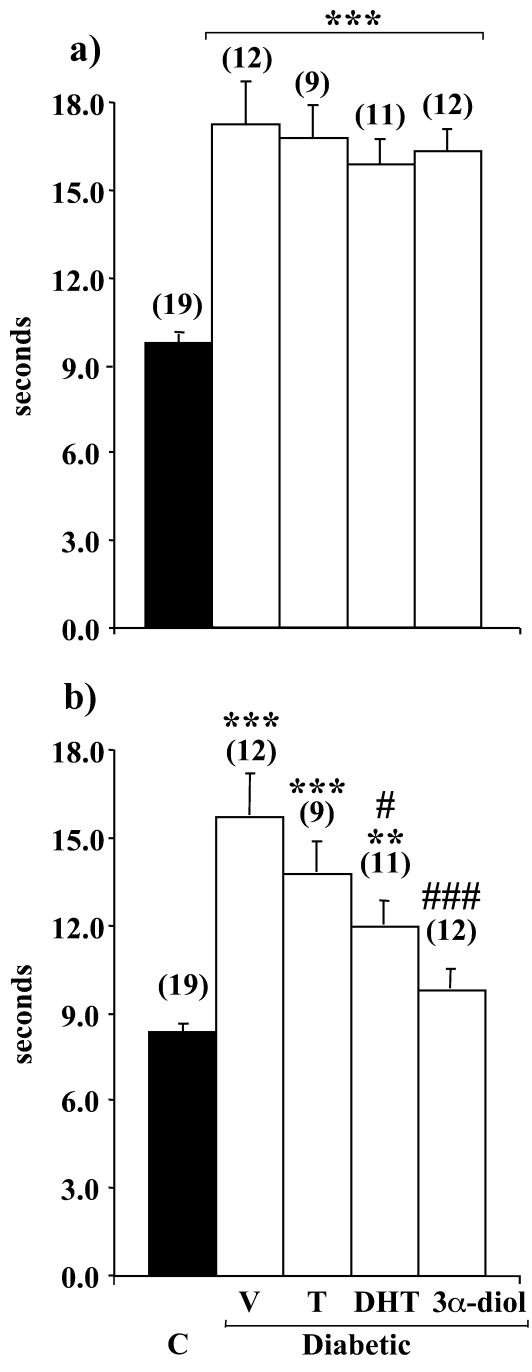

Figure 6. DHT and 3α-diol significantly improve the impaired sensitivity in diabetic rats. The heat sensitivity threshold is expressed as withdrawal latency in seconds and was evaluated at the beginning (*a*) and end of treatment (*b*) in control (C) and in diabetic rats treated with vehicle only (V), or with T, DHT, or 3α-diol. Data are mean ± SEM (number of rats in each group in parentheses). ANOVA followed by Tukey-Kramer post-test; ** $p<0.01$ vs control; *** $p<0.001$ vs control; # $p<0.05$ vs diabetic injected with vehicle; ### $p<0.001$ vs diabetic injected with vehicle.

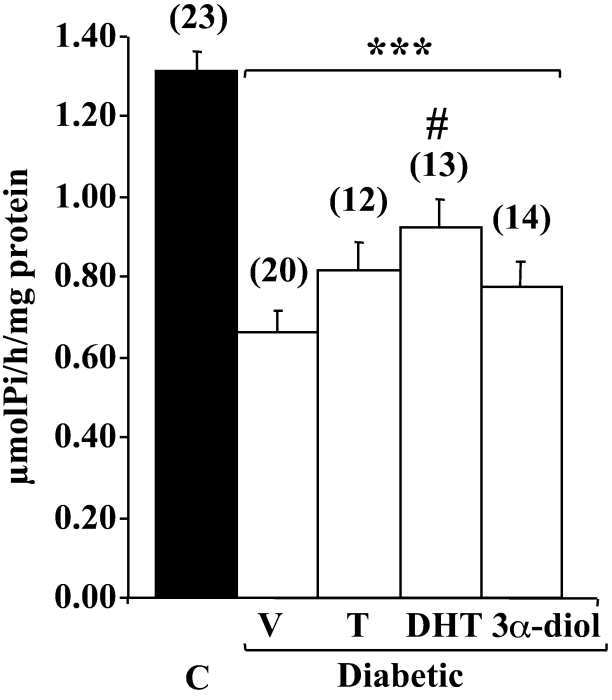

Figure 7. DHT partially restores the decrease in tibial Na^+,K^+-ATPase activity in diabetic rats. Na^+,K^+-ATPase activity was measured at the end of the experiment (12 weeks) in control (C) and diabetic rats treated with vehicle only (V), or with T, DHT, or 3α-diol. Data are expressed as μmol Pi/h per mg protein and are the mean ± SEM (number of rats in each group in parentheses). ANOVA followed by Tukey-Kramer post-test; *** $p<0.001$ vs control; # $p<0.05$ vs diabetic injected with vehicle.

with STZ-induced diabetes [52]. Furthermore, it has been observed that neurosteroidogenesis is altered in an animal model of neuropathic pain. Indeed, sciatic nerve ligature increased the expression of cytochrome P450-scc in spinal and supra-spinal networks and raised the concentrations of PREG and THP in the spinal cord [53]. In the context of diabetes, this finding could be very interesting because neuropathic pain is an important component of diabetic neuropathy.

The decrease of DHT in the sciatic nerves of diabetic rats reported here does not seem to be the consequence of the reduced levels of the general precursor PREG, since the levels of T, the direct precursor of DHT, were not significantly affected by diabetes. Consequently, the decreased levels of DHT probably reflect an impairment of the enzyme devoted to its production (*i.e.*, the enzyme 5α-reductase that converts T into DHT). In agreement with this hypothesis, we have observed that the mRNA levels of 5α-reductase are significantly decreased in the sciatic nerve after 3 months of diabetes. On the other hand, the decrease of DHT in diabetic sciatic nerve could be also due to an increase of DHT metabolism. Indeed, it has been demonstrated that DHT may be further converted into 3α-diol, by the action of the enzyme 3α-hydroxysteroid dehydrogenase (3α-HSD), as well as in androstanedione, by the action of the enzyme

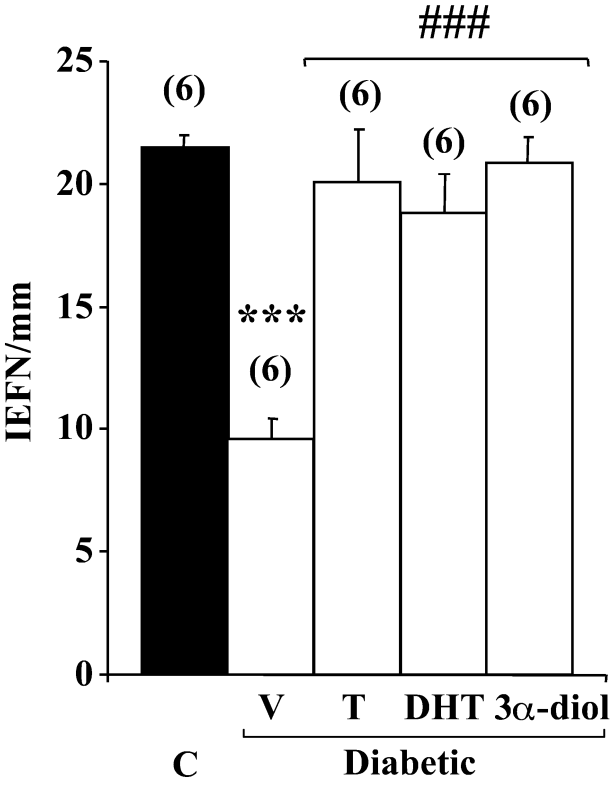

Figure 8. Treatment with T, DHT or 3α-diol restores skin innervation in STZ-induced diabetic neuropathy. Quantification of intra-epidermal nerve fiber (IENF) density in the hindpaw footpad at the end of the experiment (12 weeks) in control (C) and diabetic rats treated with vehicle only (V), or with T, DHT or 3α-diol. Data are expressed as the linear density of IENF and are the mean ± SEM (number of rats in each group in parentheses). ANOVA followed by Tukey-Kramer post-test; *** $p<0.001$ vs control; ### $p<0.001$ vs diabetic injected with vehicle.

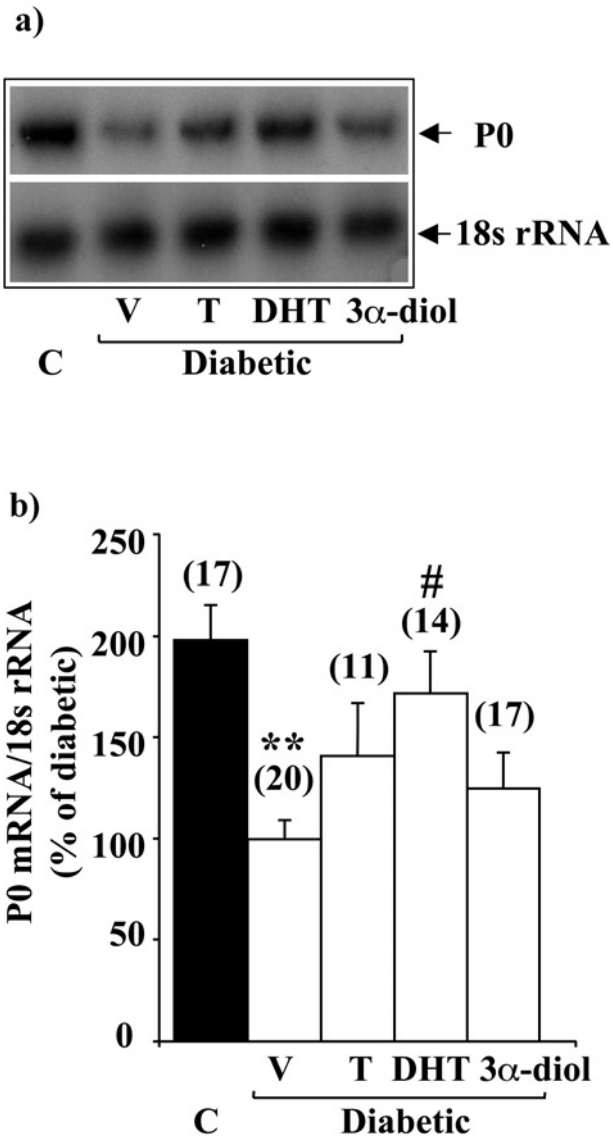

Figure 9. DHT counteracts the decrease of P0 mRNA in sciatic nerve of diabetic male rats. (*a*) Representative RNase protection assay blot of one of the experiments performed. (*b*) P0 mRNA levels at the end of the experiment (12 weeks) in control (C) and in diabetic rats treated with vehicle only (V), or with T, DHT, or 3α-diol. The data are percentages of the levels in diabetic rats. The columns represent the means ± SEM after normalization with 18S rRNA (number of rats in each group in parentheses). ANOVA followed by Tukey-Kramer post-test; ** $p<0.01$ vs control; # $p<0.05$ vs diabetic injected with vehicle.

17β-hydroxysteroid dehydrogenase. These metabolic pathways have been extensively described in peripheral tissues [54], but scarce observations are available for peripheral nerves. We previously demonstrated that *in vitro* rat sciatic nerve actively converts T into DHT and subsequently into 3α-diol [55]. In agreement with this previous observation, here we detected 3α-diol levels in sciatic nerve. However, the levels of this metabolite were not affected by diabetes, suggesting that a modification in the conversion of DHT into 3α-diol is not contributing to the decreased DHT levels in the sciatic nerve of diabetic rats. However, an effect of diabetes on the conversion of DHT into androstanedione cannot be excluded.

Based on the finding reported here that both steroid plasma and tissue levels are affected by diabetes, and that P and its derivatives can reverse peripheral nerve degeneration [5], we investigated if T and its derivatives, DHT and 3α-diol, have similar effect in STZ-induced neuropathy. We found that DHT and 3α-diol exert a neuroprotective effect at biochemical, neuropathological and neurophysiological level. In particular, DHT was able to reverse the alterations induced by diabetes on NCV, thermal threshold, intra-epidermal nerve fiber density, Na^+,K^+-ATPase activity and expression of myelin protein P0. This latter effect is in agreement with what we previously observed in non-diabetic animals [30, 31]. The neuroprotective activity of DHT could be mediated by the interaction with

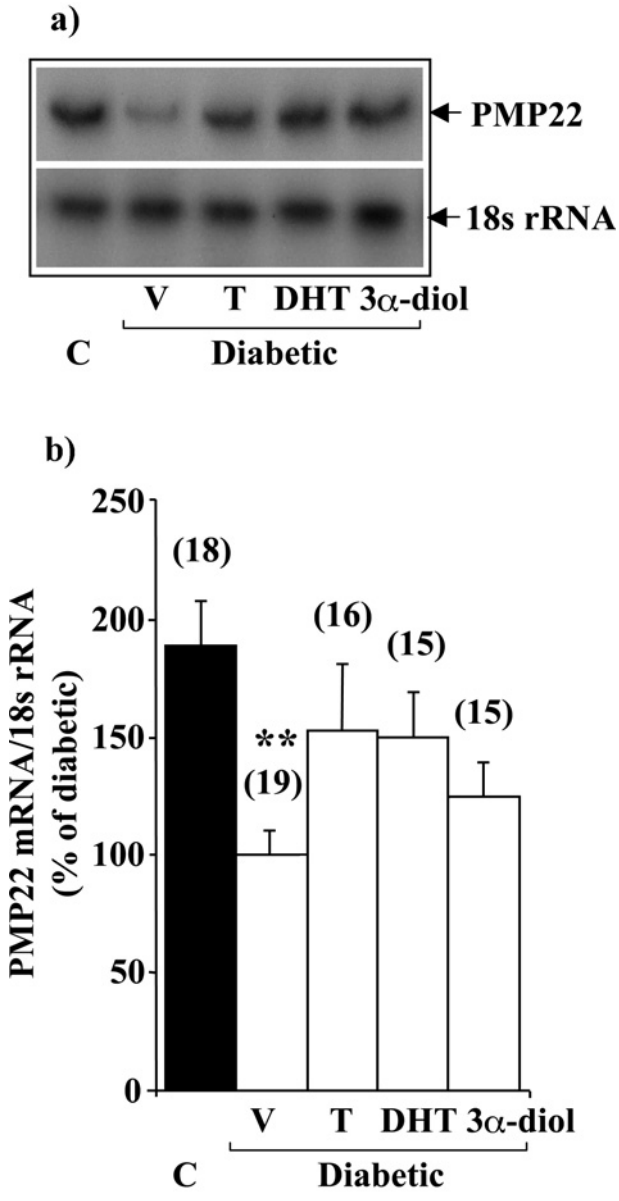

Figure 10. Treatment with T, DHT or 3α-diol was ineffective in counteracting the decrease of PMP22 mRNA in sciatic nerve of diabetic male rats. (*a*) Representative RNase protection assay blot of one of the experiments. (*b*) PMP22 mRNA levels at the end of the experiment (12 weeks) in control (C) and in diabetic rats treated with vehicle only (V), or with T, DHT, or 3α-diol. The data are percentages of levels detected in diabetic rats. The columns represent the means ± SEM after normalization with 18S rRNA (number of rats in each group in parentheses). ** $p<0.01$ vs control.

AR. DHT has a much higher affinity for AR than T [56], and, as demonstrated here by real-time PCR, the expression of this steroid receptor is not modified in the sciatic nerve of diabetic male rat.

Treatment with T significantly restored intra-epidermal nerve fiber density, although for the other parameters considered here only a tendency to restoration was observed, which, however, did not reach a statistical significance. This partial neuroprotective effect of T compared with its derivatives might be explained by the reduced expression of 5α-reductase in the sciatic nerve of diabetic rats. Indeed, very low levels of DHT could be locally formed in diabetic male rats treated with T.

Our findings indicate that the metabolite of DHT, 3α-diol, is also effective in counteracting the effects of diabetes on NCV, thermal threshold and intra-epidermal nerve fiber density. Although 3α-diol is unable to directly bind AR [56], it can be back-converted to DHT and consequently exert its effects through activation of AR. This can occur since the conversion of DHT into 3α-diol by the enzyme 3α-hydroxysteroid dehydrogenase is a reversible process [57]. On the other hand, 3α-diol may potentially interact with the estrogen receptor β (ERβ), another classical steroid receptor. Observations obtained by Pak et al. [58] indicate that 3α-diol is able to mediate the transcription preferentially through a splice variant of ERβ (*i.e.*, ERβ 2), which has been identified in several rat tissues, including the brain [59]. In addition, recent data suggest that some of the effects of 3α-diol to enhance learning could be mediated through ERβ localized in the hippocampus [60]. Although ERβ has not been directly assessed in the sciatic nerves of adult animals, its expression is very low in Schwann cells cultured from sciatic nerves of neonatal rats [61]. However, the expression of ERβ is increased in motor neurons of the spinal cord after sciatic nerve injury [62], and may therefore be involved in the mechanisms of nerve regeneration.

Another possible pathway mediating the effect of 3α-diol could be the interaction with GABA-A receptor [63, 64], which is present both in sciatic nerve and in Schwann cells [65]. Diabetes seems to affect the ability of neuroactive steroids to interact with this receptor, at least in Schwann cells. For instance, at variance to that observed in non-diabetic animals [30, 31], as demonstrated here, 3α-diol was ineffective in stimulating the expression of P0 and PMP22 in diabetic animals. This is in agreement with our previous observations for another neuroactive steroid, THP. Indeed, THP, which is well know as a potent ligand of GABA-A receptor [66], is able to increase the expression of P0 and PMP22 [65] in non-diabetic animals, but was ineffective in diabetic animals [5].

In conclusion, our findings indicate that DHT and 3α-diol exert neuroprotective actions against the pathological changes induced by experimental diabetes in peripheral nerves. These findings suggest that T metabolites may represent a promising therapeutic strategy for the treatment of diabetic neuropathy.

Acknowledgments. The financial supports of PRIN (2005060584_004) and FIRST from University of Milan, Italy to R. C. M., Ministerio de Educacion y Ciencia, Spain (SAF2005-00272) to L. M. G-S., "Fondazione Banca del Monte di Lombardia" to G. C. and "Ricerca Finalizzata Ministero della Salute n. 2005/51" to G. L., G. C., R. C. M. are gratefully acknowledged.

1 Sugimoto, K., Murakawa, Y. and Sima, A. A. F. (2000) Diabetic neuropathy – a continuing enigma. Diabetes Metab. Res. Rev. 16: 408 – 433.
2 Vinik, A. I., Park, T. S., Stansberry, K. B. and Pittenger, G. L. (2000) Diabetic neuropathies. Diabetologia 43: 957 – 973.
3 Bronstein, J. M. (2000) Function of tetraspan proteins in the myelin sheath. Curr. Opin. Neurobiol. 10: 552 – 557.
4 Suter, U. and Scherer, S. S. (2003) Disease mechanisms in inherited neuropathies. Nat. Rev. Neurosci. 4: 714 – 726.
5 Leonelli, E., Bianchi, R., Cavaletti, G., Caruso, D., Crippa, D., Garcia-Segura, L. M., Lauria, G., Magnaghi, V., Roglio, I. and Melcangi, R. C. (2007) Progesterone and its derivatives are neuroprotective agents in experimental diabetic neuropathy: a multimodal analysis. Neuroscience 144: 1293 – 1304.
6 Berry, E. M. (1997) Dietary fatty acids in the management of diabetes mellitus. Am. J. Clin. Nutr. 66: 991S-997S.
7 Bianchi, R., Buyukakilli, B., Brines, M., Savino, C., Cavaletti, G., Oggioni, N., Lauria, G., Borgna, M., Lombardi, R., Cimen, B., Comelekoglu, U., Kanik, A., Tataroglu, C., Cerami, A. and Ghezzi, P. (2004) Erythropoietin both protects from and reverses experimental diabetic neuropathy. Proc. Natl. Acad. Sci. USA 101: 823 – 828.
8 Biessels, G-J., Cristino, N. A., Rutten, G-J., Hamers, F. P. T., Erkelens, D. W. and Gispen, W. H. (1999) Neurophysiological changes in the central and peripheral nervous system of streptozotocin-diabetic rats. Brain 122: 757 – 768.
9 Eckersley, L. (2002) Role of the Schwann cell in diabetic neuropathy. Int. Rev. Neurobiol. 50: 293 – 321.
10 Greene, D. A., Lattimer-Greene, S. and Sima, A. A. (1989) Pathogenesis of diabetic neuropathy: role of altered phosphoinositide metabolism. Crit. Rev. Neurobiol. 5: 143 – 219.
11 Lauria, G., Morbin, M., Lombardi, R., Borgna, M., Mazzoleni, G., Sghirlanzoni, A. and Pareyson, D. (2003) Axonal swellings predict the degeneration of epidermal nerve fibers in painful neuropathies. Neurology 61: 631 – 636.
12 Lauria, G., Lombardi, R., Borgna, M., Penza, P., Bianchi, R., Savino, C., Canta, A., Nicolini, G., Marmiroli, P. and Cavaletti, G. (2005) Intraepidermal nerve fiber density in rat footpad: neuropathologic–neurophysiologic correlation. J. Peripher. Nerv. Syst. 10: 199 – 205.
13 Periquet, M. I., Novak, V., Collins, M. P., Nagaraja, H. N., Erdem, S., Nash, S. M., Freimer, M. L., Sahenk, Z., Kissel, J. T. and Mendell, J. R. (1999) Painful sensory neuropathy: prospective evaluation using skin biopsy. Neurology 53: 1641 – 1647.
14 Yagihashi, S. (1997) Nerve structural defects in diabetic neuropathy: do animals exhibit similar changes? Neurosci. Res. Commun. 21: 25 – 32.
15 Tesfaye, S., Chaturve,di N., Eaton, S. E. M., Ward, J. D., Manes, C., Ionescu-Tirgoviste, C., Witte, D. R. and Fuller, J. H. (2005) Vascular risk factors and diabetic neuropathy. N. Engl. J. Med. 352: 341 – 350.
16 Yorek, M. A., Coppey, L. J., Gellett, J. S., Davidson, E. P., Bing, X., Lund, D. D. and Dillon, J. S. (2002) Effect of treatment of diabetic rats with dehydroepiandrosterone on vascular and neural function. Am. J. Physiol. Endocrinol. Metab. 283: E1067-E1075.
17 Veiga, S., Leonelli, E., Beelke, M., Garcia-Segura, L. M. and Melcangi, R. C. (2006) Neuroactive steroids prevent peripheral myelin alterations induced by diabetes. Neurosci. Lett. 402: 150 – 153.
18 Azcoitia, I., Leonelli, E., Magnaghi, V., Veiga, S., Garcia-Segura, L. M. and Melcangi, R. C. (2003) Progesterone and its derivatives dihydroprogesterone and tetrahydroprogesterone reduce myelin fiber morphological abnormalities and myelin fiber loss in the sciatic nerve of aged rats. Neurobiol. Aging 24: 853 – 860.
19 Koenig, H. L., Schumacher, M., Ferzaz, B., Do Thi, A. N., Ressouches, A., Guennoun, R., Jung-Testas, I., Robel, P., Akwa, Y. and Baulieu, E. E. (1995) Progesterone synthesis and myelin formation by Schwann cells. Science 268: 1500 – 1503.
20 Leonelli, E., Ballabio, M., Consoli, A., Roglio, I., Magnaghi, V. and Melcangi, R. C. (2006) Neuroactive steroids: a therapeutic approach to maintain peripheral nerve integrity during neurodegenerative events. J. Mol. Neurosci. 28: 65 – 76.
21 Melcangi, R. C., Magnaghi, V., Galbiati, M., Ghelarducci, B., Sebastiani, L. and Martini, L. (2000) The action of steroid hormones on peripheral myelin proteins: a possible new tool for the rebuilding of myelin? J. Neurocytol. 29: 327 – 339.
22 Melcangi, R. C., Cavarretta, I. T. R., Ballabio, M., Leonelli, E., Schenone, A., Azcoitia, I., Garcia-Segura, L. M. and Magnaghi, V. (2005) Peripheral nerves: a target for the action of neuroactive steroids. Brain Res. Rev. 48: 328 – 338.
23 Sereda, M. W., Meyer zu Horste, G., Suter, U., Uzma, N. and Nave, K.-A. (2003) Therapeutic administration of progesterone antagonist in a model of Charcot-Marie-Tooth disease (CMT-1A). Nat. Neurosci. 9: 1533 – 1537.
24 Huppenbauer, C. B., Tanzer, L., DonCarlos, L. L. and Jones, K. J. (2005) Gonadal steroid attenuation of developing hamster facial motoneuron loss by axotomy: equal efficacy of testosterone, dihydrotestosterone, and 17-beta estradiol. J. Neurosci. 25: 4004 – 4013.
25 Jones, K. J., Brown, T. J. and Damaser, M. (2001) Neuroprotective effects of gonadal steroids on regenerating peripheral motoneurons. Brain Res. Rev. 37: 372 – 382.
26 Tanzer, L. and Jones, K. J. (1997) Gonadal steroid regulation of hamster facial nerve regeneration: effects of dihydrotestosterone and estradiol. Exp. Neurol. 146: 258 – 264.
27 Tanzer, L. and Jones, K. J. (2004) Neurotherapeutic action of testosterone on hamster facial nerve regeneration: temporal window of effects. Horm. Behav. 45: 339 – 344.
28 Yu, W. H. (1982) Effect of testosterone on the regeneration of the hypoglossal nerve in rats. Exp. Neurol. 77: 129 – 141.
29 Vita, G., Dattola, R., Girlanda, P., Oteri, G., Lo Presti, F. and Messina, C. (1983) Effects of steroid hormones on muscle reinnervation after nerve crush in rabbit. Exp. Neurol. 80: 279 – 287.
30 Magnaghi, V., Cavarretta, I., Zucchi, I., Susani, L., Rupprecht, R., Hermann, B., Martini, L. and Melcangi, R. C. (1999) P0 gene expression is modulated by androgens in sciatic nerve of adult male rats. Mol. Brain Res. 70: 36 – 44.
31 Magnaghi, V., Ballabio, M., Gonzalez, L. C., Leonelli, E., Motta, M. and Melcangi, R. C. (2004) The synthesis of glycoprotein P0 and peripheral myelin protein 22 in sciatic nerve of male rats is modulated by testosterone metabolites. Mol. Brain Res. 126: 67 – 73.
32 Vallée, M., Rivera, J. D., Koob, G. F., Purdy, R. H. and Fitzgerald, R. L. (2000) Quantification of neurosteroids in rat plasma and brain following swim stress and allopregnanolone administration using negative chemical ionization gas chromatography/mass spectrometry. Anal. Biochem. 287: 153 – 166.
33 Tredici, G., Tredici, S., Fabbrica, D., Minoia, C. and Cavaletti, G. (1998) Experimental cisplatin neuronopathy in rats and the effect of retinoic acid administration. J. Neurooncol. 36: 31 – 40.
34 Bianchi, R., Marini, P., Merlini, S., Fabris, M., Triban, C., Mussini, E. and Fiori, M. G. (1988) ATPase activity defects in alloxan-induced diabetic sciatic nerve recovered by ganglioside treatment. Diabetes 37: 1340 – 1345.
35 Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193: 265 – 275.
36 Chomczynski, P. and Sacchi, N. (1987) Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal. Biochem. 162: 156 – 159.

37 Babichev, V. N., Adamskaya, E. I., Kuznetsova, T. A. and Shishkina, I. V. (1998) Neuroendocrine control of the gonadotropic function of the hypophysis in experimental diabetes. Neurosci. Behav. Physiol. 28: 1 – 7.
38 Castellano, J. M., Navarro, V. M., Fernandez-Fernandez, R., Roa, J., Vigo, E., Pineda, R., Dieguez, C., Aguilar, E., Pinilla, L. and Tena-Sempere, M. (2006) Expression of hypothalamic KiSS-1 system and rescue of defective gonadotropic responses by kisspeptin in streptozotocin-induced diabetic male rats. Diabetes 55: 2602 – 2610.
39 Durant, S., Christeff, N., Coulaud, J., Nunez, E. A., Dardenne, M. and Homo-Delarche, F. (1998) Basal concentrations of various steroids in the nonobese diabetic (NOD) mouse and effect of immobilization stress. Autoimmunity 28: 249 – 258.
40 El'tseva, T. V., Adamskaya, E. I., Peryshkova, T. A. and Babichev, V. N. (1993) Disturbance of neuroendocrine regulation of sexual behavior of male rats with streptozotocin diabetes. Neurosci. Behav. Physiol. 23: 538 – 544.
41 Hassan, A. A., Hassouna, M. M., Taketo, T., Gagnon, C. and Elhilali, M. M. (1993) The effects of diabetes on sexual behavior and reproductive tract function in male rats. J. Urol. 149: 148 – 154.
42 Salonia, A., Lanzi, R., Scavini, M., Pontillo, M., Gatti, E., Petrella, G., Licata, G., Nappi, R. E., Bosi, E., Briganti, A., Rigatti, P. and Montorsi, F. (2006) Sexual function and endocrine profile in fertile women with type 1 diabetes. Diabetes Care 29: 312 – 316.
43 Sudha, S., Valli, G., Julie, P. M., Arunakaran, J., Govindarajulu, P. and Balasubramanian, K. (2000) Influence of streptozotocin-induced diabetes and insulin treatment on the pituitary-testicular axis during sexual maturation in rats. Exp. Clin. Endocrinol. Diabetes 108: 14 – 20.
44 Tanaka, M., Nakaya, S., Kumai, T., Watanabe, M., Matsumoto, N. and Kobayashi, S. (2001) Impaired testicular function in rats with diet-induced hypercholesterolemia and/or streptozotocin-induced diabetes mellitus. Endocr. Res. 27: 109 – 117.
45 van Dam, E. W., Dekker, J. M., Lentjes, E. G., Romijn, F. P., Smulders, Y. M., Post, W. J., Romijn, J. A. and Krans, H. M. (2003) Steroids in adult men with type 1 diabetes: a tendency to hypogonadism. Diabetes Care 26: 1812 – 1818.
46 Garcia-Segura, L. M. and Melcangi, R. C. (2006) Steroids and glial cell function. Glia 54: 485 – 498.
47 Papadopoulos, V., Baraldi, M., Guilarte, T. R., Knudsen, T. B., Lacapere, J. J., Lindemann, P., Norenberg, M. D., Nutt, D., Weizman, A., Zhang, M. R. and Gavish, M. (2006) Translocator protein (18 kDa): new nomenclature for the peripheral-type benzodiazepine receptor based on its structure and molecular function. Trends Pharmacol. Sci. 27: 402 – 409.
48 Lacor, P., Benavides, J. and Ferzaz, B. (1996) Enhanced expression of the peripheral benzodiazepine receptors and its endogenous ligand octadecaneuropeptide (ODN) in the regenerating adult rat sciatic nerve. Neurosci. Lett. 220: 61 – 65.
49 Lacor, P., Gandolfo, P., Tonon, M. C., Brault, E., Dalibert, I., Schumacher, M., Benavides, J. and Ferzaz, B. (1999) Regulation of the expression of peripheral benzodiazepine receptors and their endogenous ligands during rat sciatic nerve degeneration and regeneration: a role for PBR in neurosteroidogenesis. Brain Res. 815: 70 – 80.
50 Schumacher, M., Guennoun, R., Mercier, G., Désarnaud, F., Lacor, P., Bénavides, J., Ferzaz, B., Robert, F. and Baulieu, E. E. (2001) Progesterone synthesis and myelin formation in peripheral nerves. Brain Res. Rev. 37: 343 – 359.
51 Benmessahel, Y., Troadec, J.-D., Cadepond, F., Guennoun, R., Buchanan Hales, D., Schumacher, M. and Groyer, G. (2004) Downregulation of steroidogenic acute regulatory protein (StAR) gene expression by cyclic AMP in cultured Schwann cells. Glia 45: 213 – 228.
52 Saredi, S., Patte-Mensah, C., Melcangi, R. C. and Mensah-Nyagan, A. G. (2005) Effect of streptozotocin-induced diabetes on the gene expression and biological activity of 3 beta-hydroxysteroid dehydrogenase in the rat spinal cord. Neuroscience 135: 869 – 877.
53 Patte-Mensah, C., Li, S. and Mensah-Nyagan, A. G. (2004) Impact of neuropathic pain on the gene expression and activity of cytochrome P450side-chain cleavage in sensory neural networks. Cell. Mol. Life Sci. 61: 2274 – 2284.
54 Bélanger, A., Pelletier, G., Labrie, F., Barbier, O. and Chouinard, S. (2003) Inactivation of androgens by UDP-glucuronosyltransferase enzymes in humans. Trends Endocrinol. Metab. 14: 473 – 479.
55 Melcangi, R. C., Celotti, F., Ballabio, M., Poletti, A. and Martini, L. (1990) Testosterone metabolism in peripheral nerves: presence of the 5alpha-reductase-3alpha-hydroxysteroid-dehydrogenase enzymatic system in the sciatic nerve of adult and aged rats. J. Steroid Biochem. 35: 145 – 148.
56 Roselli, C. E., Horton, L. E. and Resko, J. A. (1987) Time-course and steroid specificity of aromatase induction in rat hypothalamus-preoptic area. Biol. Reprod. 37: 628 – 633.
57 Melcangi, R. C., Magnaghi, V., Galbiati, M. and Martini, L. (2001) Formation and effects of neuroactive steroids in the central and peripheral nervous system. Int. Rev. Neurobiol. 46: 145 – 176.
58 Pak, T. R., Chung, W. C. J., Lund ,T. D., Hinds, L. R., Clay, C. M. and Handa, R. J. (2005) The androgen metabolite, 5α-androstane-3β, 17β-diol, is a potent modulator of estrogen receptor-β1-mediated gene transcription in neuronal cells. Endocrinology 146: 141 – 155.
59 Petersen, D. N., Tkalcevic, G. T., Koza-Taylor, P. H., Turi, T. G. and Brown, T. A. (1998) Identification of estrogen receptor β2, a functional variant of estrogen receptor β expressed in normal rat tissues. Endocrinology 139: 1082 – 1092.
60 Edinger, K. L. and Fye, C. A. (2007) Androgens' effects to enhance learning may be mediated in part through actions at estrogen receptor-β in the hippocampus. Neurobiol. Learn. Mem. 87: 78 – 85.
61 Groyer, G., Eychenne, B., Girard, C., Rajkowski, K., Schumacher, M. and Cadepond, F. (2006) Expression and functional state of the corticosteroid receptors and 11 beta-hydroxysteroid dehydrogenase type 2 in Schwann cells. Endocrinology 147: 4339 – 4350.
62 Islamov, R. R., Hendricks, W. A., Katwa, L. C., McMurray, R. J., Pak, E. S., Spanier, N. S. and Murashov, A. K. (2003) Effect of 17 beta-estradiol on gene expression in lumbar spinal cord following sciatic nerve crush injury in ovariectomized mice. Brain Res. 966: 65 – 75.
63 Frye, C. A., Van Keuren, K. R. and Erkine, M. S. (1996) Behavioral effects of 3alpha-androstanediol. I: modulation of sexual receptivity and promotion of GABA-stimulated chloride flux. Behav. Brain Res. 79: 109 – 118.
64 Gee, K. W., Bolger, M. B., Brinton, R. E., Coirini, H. and McEwen, B. S. (1988) Steroid modulation of the chloride ionophore in rat brain: structure-activity requirements, regional dependence and mechanism of action. J. Pharmacol. Exp. Ther. 246: 803 – 812.
65 Melcangi, R. C., Magnaghi, V., Cavarretta, I., Zucchi, I., Bovolin, P., D'Urso, D. and Martini, L. (1999) Progesterone derivatives are able to influence peripheral myelin protein 22 and P0 gene expression: possible mechanisms of action. J. Neurosci. Res. 56: 349 – 357.
66 Lambert, J. J., Belelli, D., Peden, D. R., Vardy, A. W. and Peters, J. A. (2003) Neurosteroid modulation of $GABA_A$ receptors. Prog. Neurobiol. 71: 67 – 80.