Diabetic neuropathic pain: a role for testosterone metabolites

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Abstract

Diabetic neuropathy is associated with neuropathic pain in about 50% of diabetic subjects. Clinical management of neuropathic pain is complex and so far unsatisfactory. In this study, we analyzed the effects of the testosterone metabolites, dihydrotestosterone (DHT), and 3α-diol, on nociceptive and allodynia thresholds and on molecular and functional parameters related to pain modulation in the dorsal horns of the spinal cord and in the dorsal root ganglia of rats rendered diabetic by streptozotocin injection. Furthermore, the levels of DHT and 3α-diol were analyzed in the spinal cord. Diabetes resulted in a significant decrease in DHT levels in the spinal cord that was reverted by DHT or 3α-diol treatments. In addition, 3α-diol treatment resulted in a significant increase in 3α-diol in the spinal cord compared with control values. Both steroids showed analgesic properties on diabetic neuropathic pain, affecting different pain parameters and possibly by different mechanisms of action. Indeed, DHT counteracted the effect of diabetes on the mechanical nociceptive threshold, pre- and post-synaptic components, glutamate release, astrocyte immunoreactivity, and expression of interleukin-1β (IL1β), while 3α-diol was effective on tactile allodynia threshold, glutamate release, astrocyte immunoreactivity and the expression of substance P, toll-like receptor 4, tumor necrosis factor-α, transforming growth factor β-1, IL1β, and translocator protein. These results indicate that testosterone metabolites are potential agents for the treatment of diabetic neuropathic pain.

Key Words
- neuroactive steroids
- diabetic neuropathy
- cytokines
- astrocyte immunoreactivity
- substance P
- streptozotocin
- glutamate
- synaptic proteins

Introduction

Neuropathic pain is a common consequence of diabetes mellitus that strongly impairs quality of life (Smith & Argoff 2011, Tesfaye & Selvarajah 2012). Neuro-inflammation, altered neurotransmission mediated by excitatory amino acids and release of neuropeptides, may be involved in neuropathic pain. In particular, hyperexcitability and spontaneous hyperactivity of primary afferents (Chen & Levine 2003) and spinal dorsal horn neurons (Chen & Pan 2002) have been detected in the experimental model of streptozotocin (STZ)-induced diabetes. In this model, increased release of glutamate is associated with hyperactivity of the post-synaptic...
glutamate receptor (N-methyl-D-aspartate, (NMDA) and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid, (AMPA)) (Calcutt & Chaplan 1997, Malcangio & Tomlinson 1998, Tomiyama et al. 2005). Consistent with this, the frequency of glutamatergic excitatory post-synaptic currents (EPSCs) and the amplitude of evoked monosynaptic and polysynaptic EPSCs in the dorsal horn are significantly higher in diabetic rats than in controls (Li et al. 2010). Moreover, higher glutamatergic excitation is also supported by pharmacologic studies showing that in diabetic animals hyperalgesia and allodynia are counteracted by NMDA and non-NMDA receptors antagonists (Calcutt & Chaplan 1997, Malcangio et al. 1998). Hyperexcitability of the dorsal horn neurons caused by the enhanced glutamate release may contribute to the maintenance of diabetic neuropathic pain (Chen & Pan 2002, Chen et al. 2009).

Substance P (SP) has also an important role in spinal nociceptive processing (Millan 1999, Ribeiro-da-Silva & Hokfelt 2000). It acts on neurons and glial cells, enhancing the transmission of nociceptive information in the spinal cord. In response to this stimulus, microglia and astrocytes produce and secrete proinflammatory cytokines that contribute to the development and maintenance of central sensitization and pain by amplifying the noxious neurotransmission (Watkins & Maier 2003, Milligan & Watkins 2009).

Clinical management of neuropathic pain is complex. The analgesic response to treatments, such as anti-inflammatory drugs associated with tricyclic antidepressants, calcium channel, α2-δ ligands, and opioids, is unsatisfactory and side effects are common. Furthermore, only 40% of patients obtain partial pain relief (Dworkin et al. 2007). Therefore, it is very important to search for new therapeutic alternatives, such as the use of neuroactive steroids, which are also neuroprotective agents (Melcangi et al. 2008, 2011, Giatti et al. 2012, Schumacher et al. 2012).

Glial cells express translocator protein-18 kDa (TSPO), a molecule involved in the control of steroidogenesis, by the facilitation of the transport of cholesterol to the inner mitochondrial membrane, where the first enzyme for steroidogenesis (i.e., P450scc) is located (Melcangi et al. 2008). A role for TSPO in diabetes (Giatti et al. 2009) and neuropathic pain (Wei et al. 2013) has been proposed, suggesting that neuroactive steroids may participate in the control of neuropathic pain. Indeed, some neuroactive steroids exert antinociceptive actions in different animal models (Goodchild et al. 2000, Winter et al. 2003, Todorovic et al. 2004, Mensah-Nyagan et al. 2009). Among these, two testosterone metabolites, dihydrotestosterone (DHT) and 5α-androstane-3α,17β-diol (3α-diol), have been reported to induce, in gonadectomized rats, analgesia in tail flick and in the paw lick test (Edinger & Frye 2004). Interestingly, these two neuroactive steroids exert neuroprotective actions in the STZ-model (Roglio et al. 2007). However, their effects on diabetic neuropathic pain have not been assessed. In this study, we have evaluated the effects of DHT and 3α-diol on nociceptive and allodynia thresholds in STZ-rats. Furthermore, we have assessed the levels of DHT and 3α-diol in the spinal cord and molecular and functional parameters in the dorsal horn and dorsal root ganglia related to pain modulation.

Materials and methods

Animals

Two-month-old male Sprague–Dawley rats, CD BR (Charles River, Lecco, Italy) were used. Animals were handled according to the European Union Normative (Council Directive 86/609/EEC), with the approval of our Institutional Animal Use and Care Committees.

Experimental design

The animals were randomly divided into four experimental groups: i) control animals (CTR); ii) diabetic animals treated with vehicle (V); iii) diabetic animals treated with DHT, and iv) diabetic animals treated with 3α-diol. Rats were rendered diabetic by a single i.p. injection of freshly prepared STZ (60 mg/kg; Sigma–Aldrich) in citrate buffer (0.09 M pH 4.8). Control animals received injections of citrate buffer alone. Diabetes was confirmed 48 h after STZ induction by measurement of tail vein blood glucose. Only animals with mean plasma glucose levels above 300 mg/dl were classified as diabetic. On the same day, the rats received the first of the 16 s.c. injections (every other day) of 1 mg of DHT (n=37) or 3α-diol (n=36) (Sigma–Aldrich) dissolved in 200 μl of sesame oil. CTR (n=35) and V-treated rats (n=32) received injections of 200 μl of vehicle (sesame oil). The total duration of the experiment was 1 month. The treatment schedule applied was the same as previously demonstrated by us to be effective in diabetic neuropathy and other experimental models of peripheral neuropathy (Leonelli et al. 2007, Roglio et al. 2007, 2008, 2009, Melcangi et al. 2011, Pesaresi et al. 2011). Animals were killed 24 h after the last treatment and the spinal cord dorsal horn and dorsal root ganglia were rapidly isolated and stored according to the protocol of analysis.
Quantitative analysis of neuroactive steroids by liquid chromatography tandem mass spectrometry

Neuroactive steroids in spinal cord were extracted as previously described (Pesaresi et al. 2010) and independently analyzed for the quantitative determination of DHT and 3α-diol. Briefly, 2,2,4,6,6,17,21,21-D9-PROG (D9-PROG) was added to the samples as internal standard. Acetic acid (1%) in methanol was added and the tissue sample was homogenized by sonication. After loading onto C18 cartridges, the steroid fraction was eluted with methanol (5 ml), and the organic phase was dissolved in methanol:water (1:1) before the injection in a RP-C18 analytical column (Hypersil Gold, ThermoFisher Co., San José, CA, USA; 3 mm, 100 mm × 3 mm inner diameter). The HPLC (Surveyor LC Pump Plus, ThermoFisher Co.) was coupled to an LTQ (ThermoFisher Co.) linear ion trap mass spectrometer (LC/MS) equipped with an atmospheric pressure chemical ionization source operating in the positive ion mode. DHT and 3α-diol were identified on the basis of the retention time and the tandem mass spectrometry (MS/MS) spectrum of reference compounds. The quantitative analyses were done by monitoring specific ions in the MS/MS spectrum obtained by collision of precursor ions in the MS spectrum, as previously described (Pesaresi et al. 2010). Samples were quantified by means of freshly prepared calibration curves. Samples from six animals for each experimental group were analyzed.

Randall–Selitto paw withdrawal test

The mechanical noxious threshold was quantified in 12 animals for each experimental group at the end of the experiment (1 month) using the Randall–Selitto paw withdrawal test with an analgesy meter (Ugo Basile, Comerio, Varese, Italy), which generates a linearly increasing mechanical force. The results represent the maximum pressure (expressed in grams) tolerated by the animals. Each animal was tested twice, the test was done first on the right and then on the left paw, with a 30-min interval, and the values were averaged.

von Frey test

Tactile allodynia was assessed in 12 animals for each experimental group at the end of the experiment (1 month) by placing the rats on an elevated metal mesh floor to provide access to the plantar surface of the right hind paws. An inverted, clear plastic cage was placed over each rat and the animals were allowed to acclimate, for 10–15 min, to the test environment before experiment initiation. Tactile sensitivity was evaluated using a series of calibrated Semmes–Weinstein (Stoelting, Wood Dale, IL, USA) von Frey filaments, with bending forces ranging from 0.6 to 15 g. The von Frey filaments were applied perpendicularly to the mid-plantar surface of the right hind paw and mechanical allodynia was determined by sequentially increasing and decreasing the stimulus strength (‘up-down’ paradigm of the filament presentation). Paw licking or vigorously shaking after stimulation were considered pain-like responses. The purpose of the up-down method is to cross the sensory threshold several times and successively titrate the stimulus strength in both sub- and supra-threshold ranges to reach the true sensory threshold. When paw withdrawal is absent, stronger stimuli are applied until withdrawal returns. Then, lighter stimuli are applied until the paw fails to withdraw. The perceptual threshold lies between the positive and negative responses. Fifty percent values were calculated. Therefore, the tactile sensory threshold equaled the weakest gram force to elicit paw withdrawal on at least 50% of its applications.

Tissue manipulation

The spinal cord dorsal horns were homogenized on ice in lysis buffer (PBS, pH 7.4, added with 1% Nonidet P-40) supplemented with protease cocktail inhibitor (Roche). Crude homogenates were centrifuged for 5 min at 400 g to remove particulate matter. The supernatants’ protein concentration of each sample was assayed relative to the BSA standard according to the method of Bradford (1976).

Preparation of postsynaptic fractions

The triton-insoluble postsynaptic fraction (TIF) was purified using a previously validated biochemical fractionating method (Gardoni et al. 2001). Briefly, whole spinal cord dorsal horn tissue was homogenized in 0.32 M ice-cold sucrose, containing 1 mM HEPES, 1 mM MgCl2, 1 mM EDTA, 1 mM NaHCO3, and 0.1 mM PMSF at pH 7.4 in presence of a complete set of protease (Complete) and phosphatase inhibitors (Roche Diagnostics). Homogenates were centrifuged at 13,000 g for 15 min to obtain a crude membrane fraction. The pellet was resuspended in 1 mM HEPES, KCl 75 mM, 1% Triton X-100 in presence of Complete in a glass-glass Potter-Elvehjem tissue grinder and centrifuged at 100,000 g for 1 h at 4 °C. The pellet was resuspended in buffer containing 20 mM HEPES and it was homogenized in a glass-glass Potter-Elvehjem...
tissue grinder. The TIF so obtained was stored at −80 °C until processing.

**Western blotting**

Proteins were separated by 10% SDS-PAGE and electro-phoretically transferred to nitrocellulose membranes (Trans-blot, Bio-Rad) at a constant voltage of 30 V overnight at 4 °C. The membranes were then blocked at room temperature with 10% non-fat dried milk in PBS-Tween 0.1% and successively incubated overnight at 4 °C with either mouse MABs that recognize α-tubulin (Sigma–Aldrich), synapsin-1 (Synaptic Systems, Gottingen, Germany), syntaxin-1 (Synaptic Systems), GluN2A subunit of the NMDAR (Life Technologies), GluN2B subunit of the NMDAR (Neuromab, Davis, CA, USA), GluN1 subunit of the NMDAR (Life Technologies) or PSD-95 (Neuromab) or a rabbit polyclonal antibody that recognizes rat GluN2B subunit phosphorylated at tyrosine 1472 (p-GluN2B; phospho Y1472, Calbiochem, Millipore, Billerica, MA, USA) or a rabbit polyclonal antibody that recognizes rat GluN1 subunit phosphorylated at serine 896 (p-GluN1; phospho S896). Primary antibodies were diluted in 3% non-fat dried milk–PBS–Tween 0.1%. After the incubation with the primary antibody, the membranes were washed for 1 h in PBS–Tween 0.1% and successively incubated for 2 h at room temperature with the respective HRP-conjugated secondary antibody: sheep anti-mouse IgG (Amersham Biosciences) or goat anti-rabbit IgG (Santa Cruz Biotechnology, Inc.). The membranes were processed for protein detection using the chemiluminescence ECL Plus western blotting system (Perkin Elmer, Monza, Italy) and autoradiographic films (Hyperfilm ECL, GE Healthcare, Milano, Italy). Digital quantification of bands intensity was performed using ImageJ 1.43u software (Wayne Rasband, NIH, Bethesda, MD, USA). The results from each membrane were normalized to the α-tubulin values. Samples from six animals for each experimental group were analyzed.

**Real-time PCR**

RNA from spinal cord dorsal horn or dorsal root ganglia was prepared using the Nucleospin RNA II kit (Macherey-Nagel, Duren, Germany) and analyzed by a TaqMan qRT-PCR (quantitative real-time) instrument (CFX96 real-time system, Bio-Rad Laboratories) using the iScript one-step RT-PCR kit for probes (Bio-Rad Laboratories). The specific TaqMan probes, purchased from Life Technologies, were interleukin-1β (IL1β) (Rn00580432_m1), tumor necrosis factor α (TNF-α) (Rn99999017_m1), transforming growth factor β1 (TGFβ1) (Rn00572010_m1), SP (Rn00562002_m1), and toll-like receptor 4 (TLR4) (Rn00569848_m1). The probe for the TSPO was 18 kDa and was purchased from Eurofins MWG-Operon (Ebersberg, Germany). Samples were run in 96-well formats in triplicate as multiplexer reactions with a normalizing internal control (18s rRNA). Samples from six to seven animals for each experimental group were analyzed.

**Purification of synaptosomes**

Synaptosomes were prepared essentially as described previously (Milanese et al. 2011). Dorsal spinal cord segments were homogenized at 4 °C, utilizing a Teflon/glass homogenizer (clearance 0.25 mm), in ten volumes of ice-cold sucrose 0.32 M, buffered with Tris–HCl at pH 7.4. The homogenized tissue was centrifuged (5 min, 1000 g at 4°C) in order to remove nuclei and cellular debris. Then, the supernatant was gently stratified on a four interfaces were collected, washed by centrifugation (15 min, 20 200 g at 4 °C), and then resuspended in physiological medium (NaCl 140 mM; KCl 3 mM; MgCl2 1.2 mM; CaCl2 1.2 mM; NaH2PO4 1.2 mM; HEPES 10 mM; glucose 10 mM; pH 7.4). Protein content was measured as reported by Bradford (1976) using BSA as a standard.

**Synaptosomes stimulation**

Aliquots of synaptosomal suspension were layered on microporous filters placed at the bottom of a set of parallel superfusion chambers maintained at 37 °C for 15 min (Superfusion System, Ugo Basile) (Milanese et al. 2011). Superfusion was then started with a standard medium at a rate of 0.5 ml/min and continued for 48 min. After 36 min of superfusion to equilibrate the system, samples were collected according to the following scheme: two 3-min samples (t=36–39 min and t=45–48 min; basal outflow) before and after one 6-min sample (t=39–45 min; stimulus-evoked release). A 90-s period of stimulation was applied at t=39 min, after the collection of the first sample. Stimulation of synaptosomes was performed with 15 mM KCl, substituting for equimolar concentration of NaCl. Collected samples were analyzed for endogenous glutamate content. The stimulus-evoked overflow was estimated by subtracting the transmitter content of the two 3-min samples (basal outflow) from the release evoked in the 6-min sample collected during and after the
depolarization pulse (stimulus-evoked release). Amino acid release was calculated as pmol/mg of protein.

Glutamate release determination

Endogenous glutamate content was measured by HPLC analysis, following pre-column derivatization with o-phthalaldehyde and gradient separation on a C18 reverse-phase chromatographic column (10×4.6 mm, 3 μm; at 30 °C; Chrompack, Middleburg, The Netherlands) coupled with fluorometric detection (excitation wavelength 350 nm; emission wavelength 450 nm). Homoserine was used as an internal standard.

The following buffers were utilized: solvent A, 0.1 M sodium acetate (pH 5.8)/methanol, 80:20; solvent B, 0.1 M sodium acetate (pH 5.8)/methanol, 20:80; solvent C, sodium acetate (pH 6.0)/methanol, 80:20. The gradient program was as follows: 100% C in 4 min from the initiation of the program; 90% A and 10% B in 1 min; 42% A and 58% B in 14 min; 100% B in 1 min; isocratic flow 2 min; 100% C in 3 min; flow rate 0.9 ml/min. Samples from six to nine animals for each experimental group were analyzed.

Immunohistochemistry

The lumbar enlargement of the spinal cord was fixed for 24 h at 4 °C in 4% paraformaldehyde in 0.1 M PBS pH 7.4. Tissue blocks were washed in buffer and cut into horizontal sections (50 μm thick) using a vibratome.

Immunohistochemistry was carried out on free-floating sections under moderate shaking. All washes and incubations were done in 0.1 M PBS pH 7.4, containing 0.3% BSA and 0.3% Triton X-100. The endogenous peroxidase activity was quenched in a solution of 3% H2O2 in 30% methanol for 10 min at room temperature. After washing, sections were incubated overnight at 4 °C with a MAB for glial fibrillary acidic protein (GFAP, diluted 1:500; Clone GA5, Sigma–Aldrich), a marker of astrocytes. The sections were then rinsed in buffer and incubated with biotinylated goat anti-mouse IgG (diluted 1:300; Pierce, Rockford, IL, USA) for 2 h at room temperature. After washing, sections were incubated for 90 min at room temperature with avidin–biotin–peroxidase complex (diluted 1:250; Immunopure ABC peroxidase staining kit, Pierce). The reaction product was revealed using 2 μg/ml 3,3’-diaminobenzidine (Sigma–Aldrich) and 0.01% H2O2 in 0.1 M PBS. The sections were dehydrated, and mounted on gelatinized slides, coverslips were added, and the sections were examined with a Leica DMRB-E microscope. Some sections were processed for Nissl staining in order to identify Rexed laminae.

Morphometric analysis

GFAP-immunoreactive cells were counted in the dorsal portion of the dorsal horn, within the limits of Rexed laminae I–III. Rexed laminae I–III were identified in consecutive Nissl stained sections, according to the description of Molander et al. (1984). The number of GFAP-immunoreactive cells was estimated by the optical dissector method (Howard & Redd 1998), using total thickness for dissector height (Hatton & von Bartheld 1999) and a counting frame of 55×55 μm. A total of ten counting frames were assessed for each animal. The results were averaged for each animal and the value for each animal was used for the statistical analysis. Section thickness was measured using a digital length gauge device (Heidenhain-Metro MT 12/ND221, Traunreut, Germany) attached to the stage of a Leitz microscope. All GFAP-immunoreactive cell somas that came into focus while focusing down through the dissector height inside the dissector frame were counted. All counts were performed on coded sections. Sections from seven to ten animals for each experimental group were analyzed.

Statistical analysis

Data were analyzed by one-way ANOVA, followed by Tukey’s Multiple Comparison as post-test or by Student’s t-test as indicated. Data from von Frey tests were analyzed with a Dixon non-parametric test (Chaplan et al. 1994). The number of animals in each experimental group is indicated in the figure legends.

Results

Analysis of DHT and 3α-diol in the spinal cord

DHT levels were significantly decreased in the spinal cord 1 month after STZ injection (Fig. 1A). In contrast, STZ did not significantly affect the levels of 3α-diol (Fig. 1B). The administration of DHT resulted in a significant increase in the levels of DHT in the spinal cord, to values that were significantly higher than values after STZ treatment and similar to control levels (Fig. 1A). The same treatment did not affect 3α-diol levels (Fig. 1B). The administration of 3α-diol resulted in a significant increase in its levels, to values significantly higher than those observed in the control group (Fig. 1B) and in a significant increase in DHT levels,
to values that were significantly higher than values observed after STZ treatment and similar to control levels (Fig. 1A).

**Pain assessment in diabetic rats**

Nociceptive threshold in the Randall Selitto test was significantly decreased after 1 month of diabetes (Fig. 2A). Treatment with DHT completely counteracted this decrease (Fig. 2A) while 3α-diol was ineffective (data not shown). Tactile allodynia threshold in the von Frey test was also decreased after 1 month of diabetes (Fig. 2B). In this case, only 3α-diol treatment was able to exert a significant effect, even if it did not counteract completely the effect of diabetes (Fig. 2B) while DHT was ineffective (data not shown).

**Levels of pre- and post-synaptic protein and glutamate release in diabetic rats**

Diabetes induced a significant increase in synapsin-1 and syntaxin-1 in the dorsal horn. DHT (Fig. 3A, B and C), but not 3α-diol (data not shown), counteracted this increase. Endogenous basal (Fig. 4A) and KCl-stimulated (Fig. 4B) glutamate release was increased in synaptosomes obtained from the dorsal horn of STZ rats compared with control rats. Both DHT and 3α-diol completely counteracted the effect of STZ (Fig. 4).

In contrast to the changes observed in the presynaptic compartment, we did not detect an effect of diabetes on the expression of main postsynaptic density proteins, such PSD-95, GluN1, GluN2A and GluN2B, analyzed in a TIF obtained from dorsal horn tissue (Fig. 5).

**NMDAR phosphorylation as a consequence of diabetes**

NMDA channel activity is potentiated by protein kinase C (PKC) and src-mediated NMDAR phosphorylation.
Diabetes increased the src-dependent Tyr1472 phosphorylation of GluN2B (pGluN2B Y1472), an effect that was counteracted by DHT (Fig. 6A and B) but not by 3α-diol (data not shown).

We also analyzed the phosphorylation of GluN1 at serine 896 (pGluN1 S896, Fig. 6 C and D), since it was previously reported that the levels of pGluN1 are increased in the spinal cord of STZ animals (Daulhac et al. 2011). However, we did not detect a significant change in the levels of pGluN1 under our experimental conditions. The apparent discrepancy may be due to the fact that we used lower dose of STZ. In addition, our analysis was restricted to the postsynaptic fraction of the dorsal horn. Therefore, possible modifications in pGluN1 levels in other regions of the spinal cord may explain the different results.

**SP expression, glial activation, and TSPO in diabetic rats**

SP gene expression in dorsal root ganglia was upregulated by diabetes and this was completely counteracted by 3α-diol (Fig. 7) but not DHT (data not shown).

Diabetes increased the number of GFAP-immunoreactive cells in the dorsal horn. Both DHT and 3α-diol significantly reduced the effect of diabetes on the number of GFAP-immunoreactive cells in the dorsal horn. Both DHT and 3α-diol significantly reduced the effect of diabetes on the number of GFAP-immunoreactive cells in the dorsal horn.
of GFAP-immunoreactive cells (Fig. 8). Diabetes also induced an increase in the mRNA levels of Tlr4, Tnf-α, Tgfβ1, Tspo (Fig. 9), and Il1b (Fig. 10). This effect was counteracted by 3α-diol (Figs 9 and 10). Treatment with DHT was effective at reducing Il1b gene expression (Fig. 10) but had no effect on Tlr4, Tnf-α, Tgfβ1, and Tspo expression (data not shown).

**Discussion**

Neuroactive steroids exert a variety of neuroprotective effects in several animal models, including STZ diabetic neuropathy (Roglio et al. 2007, Pesaresi et al. 2010, Melcangi et al. 2011). The efficacy of these molecules has been also explored with success on neuropathic pain (Goodchild et al. 2000, Winter et al. 2003, Todorovic et al. 2004, Mensah-Nyagan et al. 2009). However, with few exceptions (Edinger & Frye 2004, Kibaly et al. 2008, Mensah-Nyagan et al. 2008), the effect of testosterone and particularly of its metabolites DHT and 3α-diol on neuropathic pain has not been assessed. In this study, we have observed that DHT and 3α-diol had analgesic properties on diabetic neuropathic pain, indicating that these molecules may be useful therapeutic candidates for the clinical management of neuropathic pain in humans.

**Figure 5**
Effect of diabetes on the levels of PSD-95, GluN1, GluN2A, and GluN2B in the dorsal horn. Postsynaptic proteins were assessed by western blotting on triton-insoluble postsynaptic fractions from the dorsal horn of the spinal cord, using antibodies that recognize PSD-95 and GluN1, GluN2A and GluN2B. (A) Representative western blots. (B) Levels of PSD-95, GluN1, GluN2A, and GluN2B expressed as percentages of control (CTR) values. Data are means ± S.E.M. Statistical analysis was performed by Student's t-test (n = 6 for each group).

**Figure 6**
Effects of diabetes in the presence or the absence of DHT treatment on the levels of pGluN1 S896 and pGluN2B Y1472 GluN2B in the dorsal horn. The levels of GluN1 phosphorylated at serine 896 (pGluN1 S896) and of GluN2B phosphorylated at tyrosine 1472 (pGluN2B Y1472) were assessed by western blotting of Triton-insoluble postsynaptic fractions from the dorsal horn of the spinal cord. (A) Representative western blots showing pGluN2B Y1472 and total GluN2B levels. (B) Levels of pGluN2B Y1472 normalized to total GluN2B levels and expressed as percentages of control (CTR) values. (C) Representative western blots showing pGluN1 S896 and total GluN1 levels. (D) Levels of pGluN1 S896 normalized to total GluN1 levels and expressed as percentages of control (CTR) values. V, diabetic animals that received injections of vehicle. Data are means ± S.E.M. Statistical analysis was performed by one-way ANOVA followed by Tukey’s multiple comparison as post-test (B) or by Student’s t-test (D). *P < 0.05 vs CTR and #P < 0.05 vs V (n = 6 for each group).
DHT levels were significantly reduced in the spinal cord of diabetic rats, in agreement with a previous study showing a reduction in the levels of DHT and 3α-diol in the spinal cord of male rats, 3 months after STZ injection (Pesaresi et al. 2010). However, our results show that by 1 month after induction of diabetes, the concentration of 3α-diol in the spinal cord has only a tendency to decrease that does not reach statistical significance. The levels of DHT in the spinal cord of diabetic rats were recovered to control values after treatment with DHT or 3α-diol. In contrast, the levels of 3α-diol in the spinal cord were not affected by DHT. However, treatment with 3α-diol resulted in levels of this steroid that were significantly higher than control values.

Interestingly, DHT and 3α-diol exerted their analgesic effects by affecting different pain parameters. Indeed, DHT counteracted the effect exerted by diabetes on the mechanical nociceptive threshold, while 3α-diol was effective on the tactile allodynia threshold. A possible hypothesis to explain the different analgesic effects of DHT and 3α-diol could be that the two neuroactive steroids act on different nervous fibers. Indeed, as demonstrated by Treede & Cole (1993), A-δ or C-fibers are involved in hyperalgesia, while A-β fibers are involved in allodynia. In addition, DHT and 3α-diol had different effects on the parameters related to neuropathic pain, with the exception of the STZ-induced increase in basal and stimulated glutamate release, the number of GFAP-immunoreactive cells, and the expression of Il1β in the dorsal horn, all of which were counteracted by both DHT and 3α-diol. In contrast, DHT, but not 3α-diol, counteracted the STZ-induced increase in the expression of synapsin-1 and...
neuroactive steroid. The mRNA levels of Tlr4 (A), Tnf-α (B), Tgf-β1 (C), and Tspo (D) were measured in the dorsal horn of non-diabetic (CTR), STZ-diabetic (V), and STZ-diabetic rats treated with 3α-diol. Data are means ± S.E.M. and are expressed as percentages of control values. Statistical analysis was performed by one-way ANOVA followed by Tukey’s multiple comparison as post-test. *P<0.05 and **P<0.01 vs CTR and *P<0.05 vs V (n = 6 for each group).

Figure 10
Effects of diabetes and of treatment with DHT or 3α-diol on interleukin-1β (IL1β) mRNA levels in the dorsal horn. The mRNA levels of IL1β were measured in the dorsal horn of non-diabetic (CTR), STZ-diabetic (V), STZ-diabetic treated with DHT (DHT), and STZ-diabetic rats treated with 3α-diol. Data are means ± S.E.M. and are expressed as percentages of the control value. Statistical analysis was performed by one-way ANOVA followed by Tukey’s multiple comparison as post-test. **P<0.01 vs CTR and *P<0.05 vs V (n = 6 for each group).

syntaxin-1 in the dorsal horn and the STZ-induced increase in src-dependent phosphorylation of GluN2B at tyrosine 1472 (pGluN2B) in the synaptic fraction of the dorsal horn. These results are consistent with the reduction in neuropathic pain after the genetic deletion of synapsin proteins in a model of nerve injury (Bogen et al. 2006, Schmidtke et al. 2008). In addition, it is known that src-dependent phosphorylation enhances NMDAR function by increasing channel open time and open probability upon binding of glutamate and glycine, leading to augmented synaptic GluN2B-containing NMDAR currents (Nakazawa et al. 2001, Lavezzari et al. 2003, Przybylowski et al. 2005). Furthermore, enhanced levels of pGluN2B are associated with inflammatory and neuropathic pain (Liu et al. 2008, Qiu et al. 2011, Yang et al. 2011). Therefore, the increased levels of pGluN2B in the dorsal horn of STZ animals may be related to the decrease in the threshold of mechanical nociception and tactile allodynia and the increase in glutamatergic transmission observed in this model (Calcutt & Chaplin 1997, Malcangio & Tomlinson 1998, Tomiyama et al. 2005). Therefore, the DHT-induced decrease in synapsin-1 and pGluN2B levels in the dorsal horn may contribute to the analgesic effect of this neuroactive steroid.

Unlike DHT, 3α-diol decreased the expression of SP in the dorsal root ganglia of diabetic animals. Activation of NMDA receptor induces the release of SP in spinal cord (Malcangio et al. 1998). In agreement with this, we demonstrate herein that gene expression of SP is increased in the dorsal root ganglia after 1 month of diabetes. It is known that SP upregulates the TLR4 pathway (Tamizhselvi et al. 2011). Indeed, our present findings indicate that TLR4 is upregulated in the dorsal horn of diabetic rats in association with the increase in the mRNA levels of SP in the dorsal root ganglia. Interestingly 3α-diol, but not DHT, counteracted the increase induced by diabetes in the expression of TLR4. This may contribute to the analgesic action of 3α-diol, since the activation and upregulation of TLR4 is correlated with neuropathic pain (Yan et al. 2012, Wei et al. 2013).

Activation of TLR4 is one of the triggers involved in the production of cytokines (Akira et al. 2006). Moreover, in an experimental model of neuropathic pain, increased expression of SP is associated with enhanced astrocyte reactivity and cytokine release (Watkins & Maier 2003, Milligan & Watkins 2009). Interestingly, enhanced expression of SP was also associated with astrocyte activation in type 2 diabetes db/db mouse model of neuropathic pain (Dauch et al. 2012). In agreement with this, here we have detected an increased number of GFAP-immunoreactive astrocytes and an increased gene expression of pro- (IIβ and Tnf-α) and anti- (TGFβ-1) inflammatory cytokines in the dorsal horn of diabetic rats. All these effects of diabetes were counteracted by 3α-diol,
which decreased the expression of IL1β, TNF-α, and TGFβ-1, and reduced the number of GFAP-immunoreactive astrocytes in the dorsal horn of diabetic rats.

The upregulation of Tlr4 expression by diabetes and its decrease by 3α-diol may also have consequences for local steroidogenesis, since upregulation of Tspo has been demonstrated in another experimental model of neuropathic pain (i.e., spinal nerve ligation) and this upregulation was prevented by pharmacological blockage of TLR4 (Wei et al. 2013). Tspo gene expression was also upregulated in the dorsal horn in our diabetic model, an effect that was counteracted by 3α-diol, but not by DHT. The consequences of the regulation of Tspo by 3α-diol are unknown. However, previous observations of STZ-rats have demonstrated that the activation of Tspo by a synthetic ligand such as Ro5-4864, exerts neuroprotective effects on peripheral nerves (Giatti et al. 2009), and increases the levels of neuroactive steroids, such as DHT, in the peripheral nerves and the spinal cord (Mitro et al. 2012).

Altogether, these findings indicate that DHT and 3α-diol utilize different signaling pathways to exert their analgesic effect in diabetic neuropathic pain. In this context, it is important to mention that 3α-diol, unlike DHT, is unable to interact with androgen receptor (AR), but it may potentially interact with estrogen receptor (ER) β or with a non-classical steroid receptor, such as GABA-A receptor (Melcangi et al. 2008). All these receptors are expressed in neurons and astrocytes (Garcia-Segura & Melcangi 2006). Therefore, the effects observed here may be due to interaction with AR in the case of DHT and with ERβ and/or GABA-A receptor in the case of 3α-diol. For instance, the effect of DHT on astrocytes could represent a direct action on these cells and/or an indirect effect on neurons. Indeed, astrocytes express AR (Garcia-Segura & Melcangi 2006) and react as well to increases in glutamate (Watkins & Maier 2003, Milligan & Watkins 2009). Moreover, it is interesting to note that DHT, via interaction with AR, is able to decrease the expression of IL1β in other tissues (Khalkhali-Ellis et al. 2002, Beauregard & Brandt 2004). The role of GABA-A receptor in the effects of 3α-diol may be supported by the finding that impaired spinal GABAergic inhibitory function is involved in neuropathic pain, including that of diabetes (Jolivalt et al. 2008, Takazawa & MacDermott 2010, Cao et al. 2011). In addition, since 3α-diol can be retro-converted into DHT (Melcangi et al. 2008) and since the administration of 3α-diol resulted in a significant increase in DHT levels in the spinal cord, the action of 3α-diol may be in part mediated by its precursor metabolite. However, although 3α-diol restored the levels of DHT in the spinal cord to physiological values, it was not able to fully imitate the effects of its metabolite. This indicates that the activation of ERβ and/or GABA-A receptors by high 3α-diol levels may result in the inhibition of some of the actions of DHT. A possible hypothesis could be a down-regulation of AR expression. Indeed, even if in a different cellular system, a down-regulation of AR occurred through ERβ activation (Bektic et al. 2004). Future experiments with ligands of ERβ or GABA-A receptors are necessary to clarify their mechanism of action on neuropathic pain.

In summary, our findings indicate that DHT and 3α-diol have analgesic effects on diabetic neuropathic pain by affecting different mechanisms. These results indicate that testosterone metabolites are valuable candidates for the treatment of diabetic neuropathic pain, at least in male individuals. However, since androgens may have adverse effects, including the induction of neuronal apoptosis (Yang et al. 2002, 2005, Caruso et al. 2004, Estrada et al. 2006, Gatson & Singh 2007), further studies are necessary to exclude undesirable effects of the treatments before envisaging a possible clinical application of the present findings.

Declaration of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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