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Sex differences in neuroactive steroid levels in the nervous system of diabetic and non-diabetic rats

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ABSTRACT

Neuropathy and encephalopathy represent important complications of diabetes. Recent observations obtained in experimental models have suggested that, in male rats, neuroactive steroids are protective agents and that their levels in peripheral (PNS) and central (CNS) nervous system are strongly affected by the disease.
It is interesting to highlight that incidence, progression and severity of diabetic neuropathy and diabetic encephalopathy are different in the two sexes. Consequently, it is important to determine the changes in neuroactive steroid levels in the PNS and the CNS of both males and females. To this aim, we have evaluated the levels of neuroactive steroids such as, pregnenolone, progesterone and its metabolites, testosterone and its metabolites, and dehydroepiandrosterone in different CNS regions (i.e., cerebral cortex, cerebellum and spinal cord) and in the sciatic nerve of control and diabetic (i.e., induced by streptozotocin) male and female rats. Data obtained by liquid chromatography–tandem mass spectrometry indicate that the levels of neuroactive steroids show sex and regional differences in control animals. Streptozotocin-induced diabetes resulted in a strong general decrease in neuroactive steroid levels, in both the PNS and the CNS. In addition, the effects of diabetes on neuroactive steroid levels also show sex and regional differences.
These findings may have strong implications for the development of new sex-oriented therapies for the treatment of diabetic neuropathy and diabetic encephalopathy, based on the use of neuroactive steroids.

Introduction

Damage at the levels of peripheral nervous system (PNS) (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). Neurophysiological and structural changes at the level of central nervous system (CNS) areas have been reported (i.e., diabetic encephalopathy) (McCall, 2002) and are associated with cognitive deficits and increased risk of dementia, stroke, cerebrovascular and Alzheimer disease and psychiatric disorders, such as depression and eating disorders (Biessels et al., 2008, 2002; Gispen and Biessels, 2000; Jacobson et al., 2002; Kodl and Seaquist, 2008). Experimental diabetic neuropathy and experimental diabetic encephalopathy show similar features to human complications (Alvarez et al., 2009; Beauquis et al., 2006; Bianchi et al., 2004; Biessels et al., 1999; Biessels and Gispen, 2005; Franzon et al., 2005; Kamboj et al., 2008; Kawashima et al., 2007; Lauria et al., 2005; Leonelli et al., 2007; Mastrocola et al., 2005; Nitta et al., 2002; Stranahan et al., 2008; Yagihashi, 1997; Zhang et al., 2008).

Recent findings from experimental models of diabetic neuropathy and diabetic encephalopathy indicate that, at least in male rats, neuroactive steroids are protective agents (Aragno et al., 2002; Beauquis et al., 2008; De Nicola et al., 2009; Leonelli et al., 2007; Roglio et al., 2007; Saravia et al., 2004, 2006; Veiga et al., 2006; Yorek et al., 2002). 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., 2006). 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). In the CNS, DHEA protects

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the hippocampus from diabetic damage reducing nuclear factor-kappa B nuclear translocation (Aragno et al., 2002). 17β-estradiol counteracts the strong reduction of cell proliferation rate in the dentate gyrus of STZ-treated rats, as well as the abnormal expression of astrocytic markers in the hippocampus (Saravia et al., 2004, 2006).

Another important aspect that has recently emerged is that diabetes does not only influence the plasma level of sex steroids due to dysfunction in the reproductive axis (Babichev et al., 1998; Durant et al., 1998; Leonelli et al., 2007; Roglio et al., 2007; Salonia et al., 2006; Tanaka et al., 2001; van Dam et al., 2003) but also affects the local levels of neuroactive steroids in the PNS and CNS (Caruso et al., 2008a; Leonelli et al., 2007; Roglio et al., 2007). In particular, we recently demonstrated that a decrease of pregnenolone (PREG), PROG and T, as well as of their derivatives, occurs both in a peripheral nerve, such as the brachial nerve, and in CNS structures of male STZ-treated rats (Caruso et al., 2008a).

Interestingly, diabetic neuropathy is more frequent in men than in women (ratio male/female 2.9) (Basit et al., 2004; Booya et al., 2005). Moreover, males develop neuropathy earlier than females (Aaberg et al., 2008). Neuropathic pain and negative sensory symptoms are more frequent in females, whereas muscle weakness and atrophy is more frequent in male patients (Kiziltan and Benbir, 2008). Motor nerve conduction abnormalities and ulnar nerve involvement are also more frequent and severe in males (Kiziltan and Benbir, 2008; Kiziltan et al., 2007). In particular, nerve conduction studies indicated that men have lower amplitudes and conduction velocities and longer latencies than female patients (Albers et al., 1996). In the STZ-treated rats, the paw withdrawal threshold is reduced more in females than in males, suggesting that nociceptive threshold is differently affected in the two sexes (Joseph and Levine, 2003).

Moreover, diabetic encephalopathy is associated with a variety of disorders, such as cognitive deficits and increased risk of dementia, stroke, cerebrovascular and Alzheimer disease and psychiatric disorders, clearly showing sex differences in incidence, progression and severity (Andersen et al., 1999; Farace and Alves, 2000; Fratiglioni et al., 1997; Kaye, 2008; Marcus et al., 2008; Niemeier et al., 2007; Simonds and Whiffen, 2003).

On this basis, to verify whether changes in neuroactive steroid levels occur also in females could be extremely important. To this aim, we have used liquid chromatography–tandem mass spectrometry to measure the levels of neuroactive steroids such as, PREG, PROG and its metabolites (DHP, THP and isopregnanolone), T and its metabolites (DHT and 3α-diol), and DHEA in several CNS regions (cerebral cortex, cerebellum and spinal cord), in the sciatic nerve and in plasma of control and diabetic male and female rats.

Materials and methods

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-3α17b-diol (3α-diol), and dehydroepiandrosterone (DHEA), were purchased from Sigma Aldrich. 17,21,21,21-D_4-PREG (D4-PREG) was kindly synthesized by Dr. P. Ferraboschi (Dept. of Medical Chemistry, Biochemistry and Biotechnology, University of Milano, Italy); 2,2,4,6,6-17α,21,21,21-D_9-PROG (D_9-PROG) was obtained from Medical Isotopes, (Pelham, NH, USA); 2,4,16,16-D_4-17β-estradiol (D_4-17β-E) was obtained from CDN Isotope Pointe-Claire (Quebec-Canada). SPE cartridges (Discovery DS-C18 500 mg) were from Supelco, Italy. All solvents and reagents were HPLC grade (Sigma Aldrich, Italy).

LC–MS/MS analysis

Positive atmospheric pressure chemical ionization (APCI+) experiments were performed with a linear ion trap - mass spectrometer (LTQ, ThermoElectron Co, San Jose, CA, USA) using nitrogen as sheath, auxiliary and sweep gas. The instrument was equipped with a Surveyor liquid chromatography (LC) Pump Plus and a Surveyor Autosampler Plus (ThermoElectron Co, San Jose, CA, USA). The mass spectrometer was employed in MS/MS mode using helium as collision gas.

The LC mobile phases were (A) H_2O/0.1% formic acid and (B) methanol (MeOH)/0.1% formic acid. The gradient (flow rate 0.5 ml/min) was as follows: T_0 70%A, $T_{1.5}$ 70%A, T_2 55%A, T_3 55%A, T_{35} 36%A, T_{40} 25%A, T_{41} 1%A, T_{45} 1%A, $T_{45.2}$ 70%A, and T_{55} 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 (100×3 mm, 3 μm; ThermoElectron Co, San Jose, CA, 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 (ThermoElectron Co, San Jose, CA, USA). Samples were analyzed using the transitions reported in Table 1.

Study design and sample preparation

Two-month-old male and female Sprague–Dawley rats, Crl:CD BR (Charles River, Italy) were housed in the animal care facility of the Department of Endocrinology Pathophysiology, and Applied Biology at the University of Milan with controlled temperature and humidity. The light schedule was 14 h light and 10 h dark (lights on at 6.30 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. For each sex, rats were randomly divided into two groups (control and diabetes).

Diabetes was induced by a single intravenous injection of freshly prepared STZ (65 mg/kg; Sigma, Italy) in citrate buffer 0.09 M 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/dl were classified as diabetic. Three months after the diabetes induction, rats were sacrificed and the cerebral cortex, the cerebellum, the spinal cord, the sciatic nerve and the plasma were collected and stored at −80 °C until analyzed.

To determine the different phases of the estrous cycle, rats were monitored by daily vaginal smears and only those demonstrating at least two consecutive 4-day cycles were used in the study. Animals were sacrificed on proestrous and samples extracted and purified according to Caruso et al. (2008a). Briefly, samples were added with internals standards, homogenized in 2 ml of MeOH/acetic acid (99:1 v/v) using a tissue lyser (Qiagen, Italy). After an overnight extraction

Table 1
Analytical parameters.

	Precursor ions	Transitions monitored	RRT	IS	Segment
D_4-17β-E	259	135, 161	1	–	1
T	289	97, 109	1,20	D_4-17β-E	1
DHEA	271	197, 213	1,31	D_4-17β-E	1
D_9-PROG	324	100	1	–	2
PROG	315	97, 109	1,01	D_9-PROG	2
DHT	291	255	0,88	D_9-PROG	2
3α-diol	257	121, 135, 147, 161, 175	1,02	D_9-PROG	2
D_4-PREG	303	159, 175, 185, 199, 211, 229, 241	1	–	3
PREG	299	159, 199	1,01	D_4-PREG	3
DHP	299	189	1,03	D_4-PREG	3
Isopregnanolone	301	159, 173, 213, 227	1,11	D_4-PREG	3
THP	301	159, 173, 213, 227	1,17	D_4-PREG	3

RRT: relative retention time (calculated against the IS monitored in the corresponding segment); IS: internal standard.

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. The samples were resuspended with 3 ml of MeOH/H_20 (10:90 v/v) and passed through a SPE cartridges, previously activated with MeOH (5 ml) and MeOH:H_2O 1:9 v/v (5 ml), the steroids were eluted in MeOH, concentrated and transferred in autosampler vials before the 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 and analyzed as described above for samples.

Statistical analysis

The linearity of the standard curve (r^2), the accuracy (%) and the precision (CV%) inter-series were judged by GraphPad4 PRISM (version 4). Student's *t*-test was used to determine significant differences between control and diabetic tissues.

Results

Fig. 1 shows the levels of neuroactive steroids in male and female sciatic nerve of control and STZ-treated rats. As reported, the levels of PREG, PROG and its derivatives (DHP, THP and isopregnanolone) were significantly higher in female than in male controls. On the contrary, the levels of T and DHT were significantly higher in male than in female controls. A significant impact of diabetes on neuroactive steroid levels was detected both in males and in females, but with some sex differences. In particular, PREG, T, DHT and 3α-diol were significantly decreased in males but not in females, while PROG, THP and isopregnanolone were decreased only in females.

Neuroactive steroid levels were also different in the CNS of male and female animals. In agreement with findings from the sciatic nerve of control animals (Fig. 1), PROG and THP levels were significantly higher in the cerebral cortex of control females compared to males, while T and DHT were significantly higher in control males than in control females (Fig. 2). However, in contrast to the results from the sciatic nerve of control animals (Fig. 1), the levels of PREG, as well as of DHP and isopregnanolone in the cerebral cortex were similar in males and females (Fig. 2). Moreover, DHEA and 3α-diol were significantly higher in control males than in control females (Fig. 2). Diabetes induced a decrease in the levels of PREG, PROG and its derivatives (DHP, THP and isopregnanolone) in the male and female cerebral cortex. In contrast, the levels of T and its derivatives (DHT and 3α-diol) were decreased in diabetic males but not in diabetic females (Fig. 2).

In control animals, the pattern of neuroactive steroids observed in the cerebellum (Fig. 3) was very similar to that of the cerebral cortex (Fig. 2). Indeed, PROG was higher in control females than in control males, while T, DHT, 3α-diol and DHEA levels were higher in control males (Fig. 3). However, in contrast to cerebral cortex, DHP levels were significantly higher in the cerebellum of control females. Diabetes induced a decrease in PREG levels in the cerebellum of both sexes. In addition, a decrease of DHP, T and DHT levels was detected in diabetic males, while PROG and isopregnanolone were decreased only in diabetic females (Fig. 3).

Fig. 4, shows the levels of neuroactive steroids in the lumbar portion of the spinal cord of control and STZ-treated rats. As in the cerebellum, PROG and DHP were higher in the spinal cord of control females, while T and its derivatives (DHT and 3α-diol) were higher in the spinal cord of control males. Moreover, similar to the cerebral cortex, the spinal cord of control females had higher levels of THP (Fig. 4). Diabetes induced a significant decrease of PREG, PROG and DHP in the spinal cord of both male and females. THP, T, DHT and 3α-diol were decreased by diabetes in the male, but not female, spinal cord (Fig. 4).

Discussion

The present analysis obtained by liquid chromatography–tandem mass spectrometry indicates that the levels of neuroactive steroids are sexually dimorphic in PNS and CNS structures of control animals. In particular, we found that in a peripheral nerve, such as the sciatic nerve, the levels of PREG, PROG and its derivatives (i.e., DHP, THP and isopregnanolone) are significantly higher in females, while those of T and its reduced metabolite, DHT, are significantly higher in males. In the case of PREG and isopregnanolone, this sex difference was not observed in the CNS and thus seems to be specific to the PNS. In contrast to PREG and isopregnanolone, PROG, THP, T and DHT show a sex difference in all the CNS areas that were analyzed (i.e., cerebral cortex, cerebellum and spinal cord). Interestingly, 3α-diol and DHEA levels are significantly higher in the CNS of males compared to females. This sex difference seems to be specific to the CNS, since it was not detected in the sciatic nerve.

These results confirm and extend previous observations by our lab and others (Caruso et al., 2008a; Meffre et al., 2007b), indicating that the levels of neuroactive steroids in control animals are different: i) between PNS and CNS; ii) among CNS areas analyzed here and iii) between the two sexes.

Both *in situ* synthesis and the uptake from periphery may contribute to determine the levels of neuroactive steroids in the PNS and the CNS. Namely, *in situ* formation in the nervous system has been supported by various molecular and biochemical studies demonstrating the presence of molecules involved in this process, including translocator protein-18 kDa and steroidogenic acute regulatory protein. In further support of *in situ* formation, several key steroid synthesizing enzymes are present in the nervous system, including cytochrome P450 side chain cleavage (P450scc, the enzyme producing PREG), 3β-hydroxysteroid dehydrogenase (3β-HSD, the enzyme producing PROG), cytochrome P450c17 (P450c17, the enzyme producing DHEA) and 5α-reductase (5α-R) and 3α-hydroxysteroid oxidoreductase (3α-HSOR) (i.e., the enzymes converting PROG and T into their metabolites) (Melcangi et al., 2008).

Very few studies have been so far performed to evaluate possible sex differences in the expression of steroid synthesizing enzymes in the nervous system. P450scc expression has been reported to be higher in the cerebral cortex of women than in men (Watzka et al., 1999), but no sex differences were reported in the rodent brain (Kohchi et al., 1998; Mellon and Deschepper, 1993). In agreement with these findings and others obtained by gas chromatography/mass spectrometry (GC/MS) (Meffre et al., 2007b), we observed that in the CNS areas analyzed here, PREG levels are not different in male and female rats. Unfortunately, P450scc expression has been not so far evaluated in male and female sciatic nerve, where we have detected a sex difference.

It is well known that PROG originating in the periphery is taken up by the nervous system in considerable amounts (Billiar et al., 1975), while production of its neuroactive derivatives takes place directly in the CNS and the PNS (Melcangi and Panzica, 2006). In the present study we observed that in the PNS and CNS, females have higher levels of PROG than males, which reflect their plasma levels. Indeed, our analyses were performed in female rats on proestrous day, when higher levels of PROG in plasma are observed in comparison to males [female ($n=8$) 14.26 ± 2.7 pg/μl vs. male ($n=8$) 1.0 ± 0.28 pg/μl; $p<0.001$]. These findings are also in agreement with previous observations obtained in pseudopregnant rats, where PROG plasma levels are also very high (Meffre et al., 2007b). On the other hand, high PROG levels in the nervous system of females might also be due to an increase of substrate (i.e., PREG) and/or 3β-HSD, the key enzyme in PROG synthesis. However, this might be only the case of PNS, where high levels of PREG are observed in females. In CNS structures, we observed that PREG levels are not different in male and female animals. As mentioned above, our observations are in agreement with

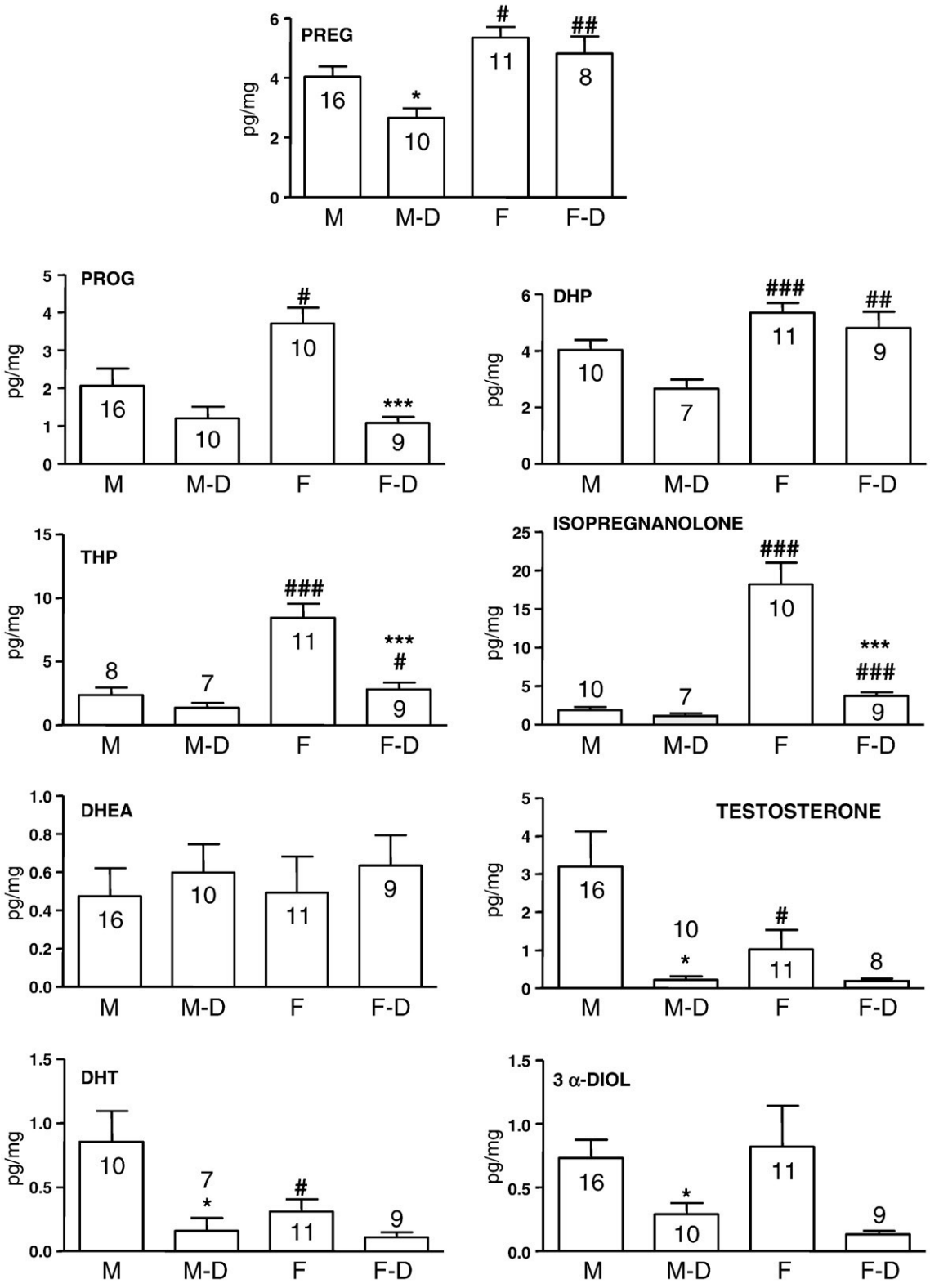

Fig. 1. Neuroactive steroid levels in the sciatic nerve of male (M) and female (F) control and diabetic (MD, FD) rats. Neuroactive steroid levels were evaluated by LC–MS/MS. Data are expressed as pg/mg tissue ± SEM (number of determinations are indicated by each column). $^{\#}p<0.05$; $^{\#\#}p<0.01$ and $^{\#\#\#}p<0.005$ vs. male and $^{*}p<0.05$; $^{**}p<0.01$ and $^{***}p<0.005$ vs. control.

analysis performed by GC/MS and with the similar expression of 3β-HSD observed in male and pseudopregnant females (Meffre et al., 2007a,b).

Among PROG metabolites, the case of isopregnanolone seems to be extremely interesting. Indeed, as we have previously observed (Caruso et al., 2008b), we confirmed here that the levels of this

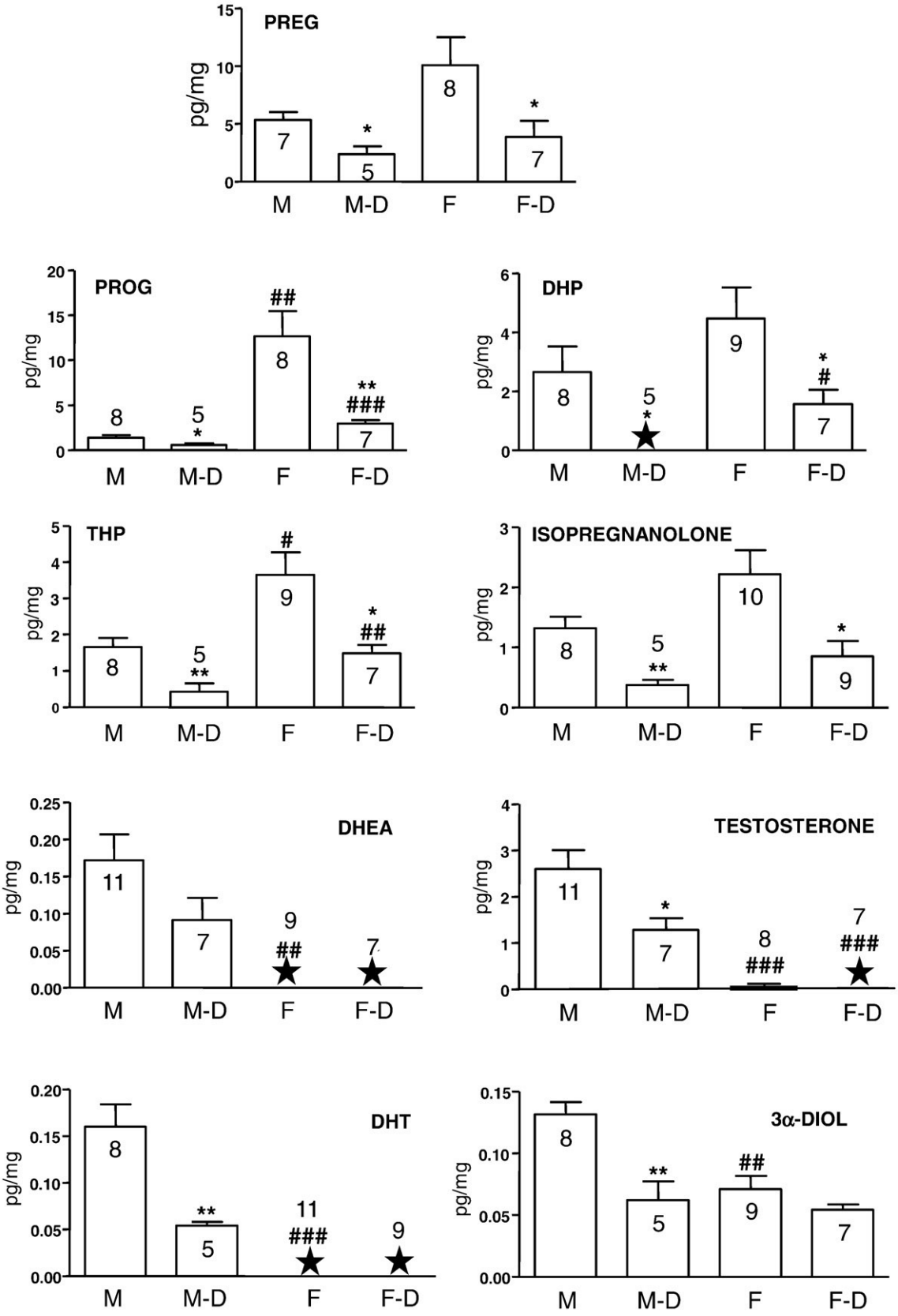

Fig. 2. Neuroactive steroid levels in cerebral cortex of male (M) and female (F) control and diabetic (MD, FD) rats. Neuroactive steroid levels were evaluated by LC–MS/MS. Data are expressed as pg/mg tissue ± SEM (number of determinations are indicated by each column). $^{\#}p<0.05$; $^{\#\#}p<0.01$ and $^{\#\#\#}p<0.005$ vs. male and $^{*}p<0.05$; $^{**}p<0.01$ and $^{***}p<0.005$ vs. control. Stars, values under the detection limit for quantification (0.25 for DHP, 0.02 for T and 0.05 pg/mg tissue for DHEA and DHT).

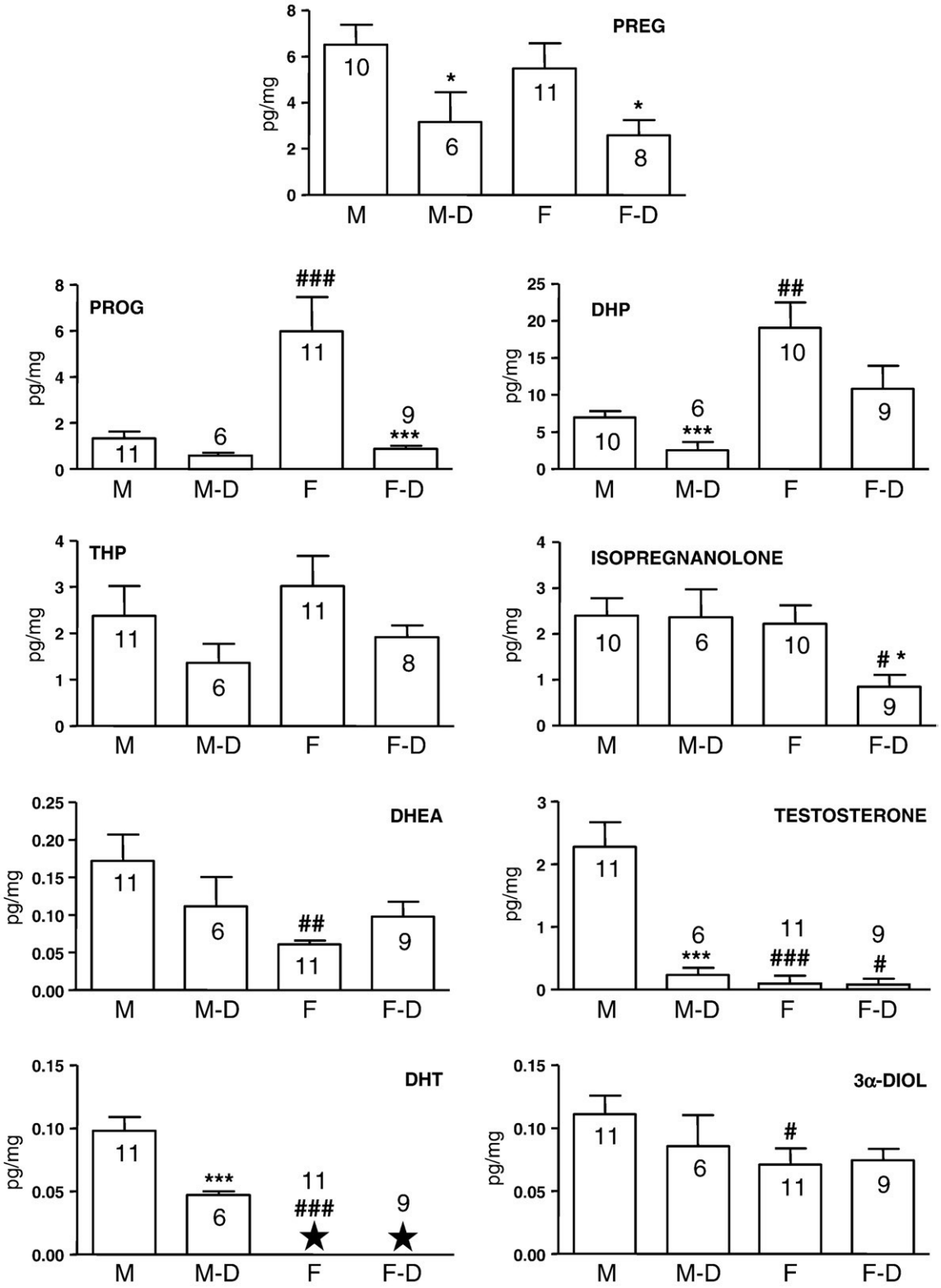

Fig. 3. Neuroactive steroid levels in cerebellum of male (M) and female (F) control and diabetic (MD, FD) rats. Neuroactive steroid levels were evaluated by LC–MS/MS. Data are expressed as pg/mg tissue ± SEM (number of determinations are indicated by each column). $^{\#}p<0.05$; $^{\#\#}p<0.01$ and $^{\#\#\#}p<0.005$ vs. male and $^{*}p<0.05$; $^{**}p<0.01$ and $^{***}p<0.005$ vs. control. Stars, values under the detection limit for quantification (0.05 pg/mg tissue for DHT).

neuroactive steroids are much higher in peripheral nerves of females than males. In contrast to the PNS, this sex difference in isopregnanolone levels seems not to be present in the CNS. Isopregnanolone is produced directly from DHP or through epimerization of THP (Huang and Luu-The, 2000). Moreover, as recently observed in healthy women, treatment with isopregnanolone induces an increase of THP

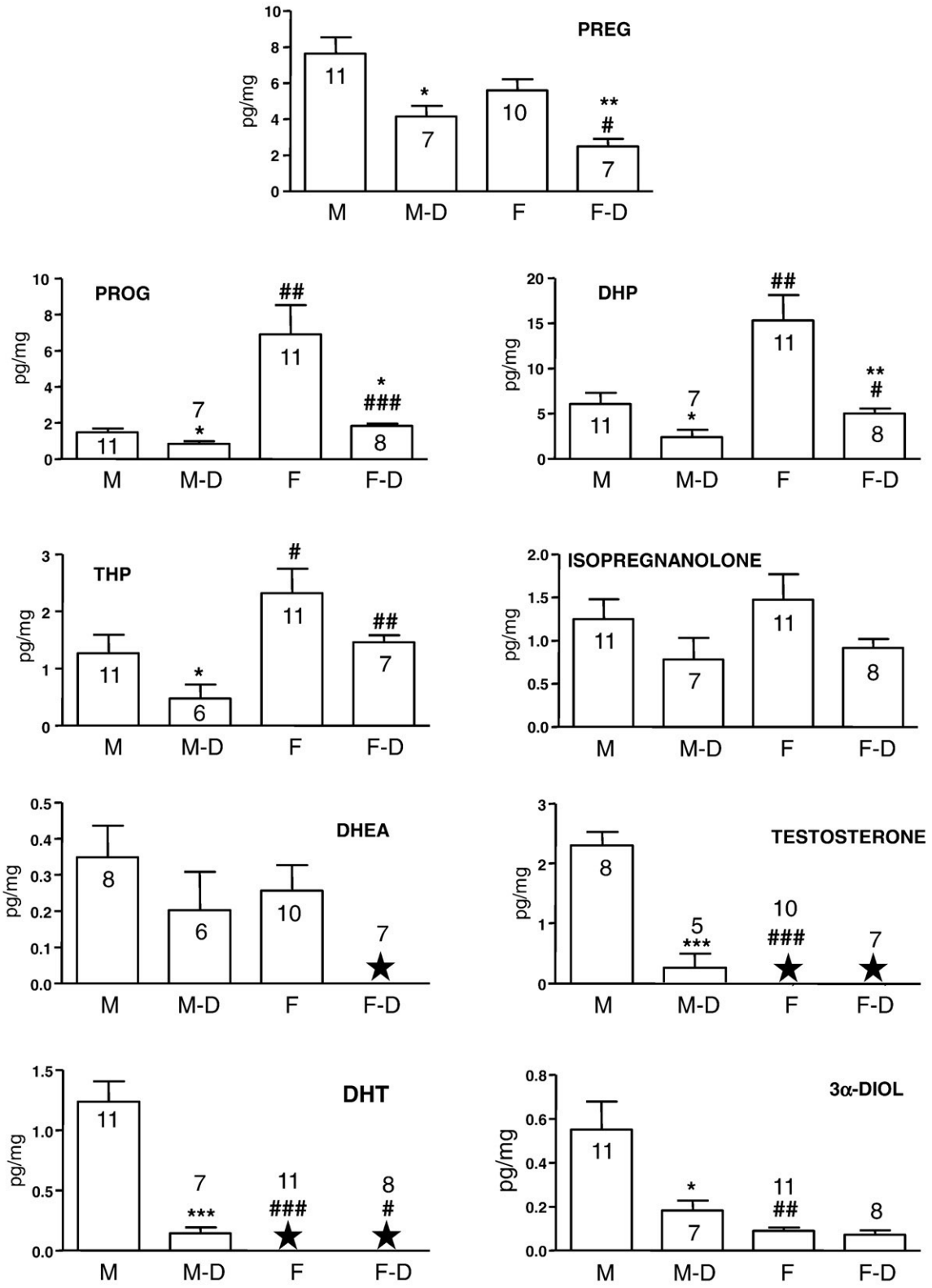

Fig. 4. Neuroactive steroid levels in spinal cord of male (M) and female (F) control and diabetic (MD, FD) rats. Neuroactive steroid levels were evaluated by LC–MS/MS. Data are expressed as pg/mg tissue ± SEM (number of determinations are indicated by each column). $^{\#}p<0.05$; $^{\#\#}p<0.01$ and $^{\#\#\#}p<0.005$ vs. male and $^{*}p<0.05$; $^{**}p<0.01$ and $^{***}p<0.005$ vs. control. Stars, values under the detection limit for quantification (0.02 for T and 0.05 pg/mg tissue for DHEA and DHT).

plasma levels (Hedstrom et al., 2009), suggesting, at least in plasma, a bi-directional metabolism of these two neuroactive steroids. In our analyses, we observed that both DHP and THP are higher in female sciatic nerve, however, with few exceptions these sex differences are also observed in CNS areas, suggesting that the high levels of isopregnanolone observed in sciatic nerve probably are not only due to an increase of its substrates.

In agreement with previous observations (Caruso et al., 2008a,b; Meffre et al., 2007b), we observed here that T and its derivatives are significantly higher in the male, than in female, nervous system. These

elevated levels in males are observed both in the PNS and the CNS and likely due to the higher T levels observed in male plasma compared to female plasma [male ($n=8$) 3.79 ± 0.8 pg/μl vs. female ($n=8$) <0.02 pg/μl, which is below the detection limit of quantification]. However, *in situ* synthesis may also contribute to the sex difference. Indeed, in the cerebral cortex and cerebellum, levels of the androgen precursor, DHEA, show a sex difference with higher levels in males than in females.

In agreement with previous observations indicating that diabetes influence plasma levels of sex steroids (Babichev et al., 1998; Durant et al., 1998; Leonelli et al., 2007; Roglio et al., 2007; Salonia et al., 2006; Tanaka et al., 2001; van Dam et al., 2003) we here observed that PROG levels in plasma are significantly decreased both in female [control ($n=8$) 14.26 ± 2.7 pg/μl vs. diabetic ($n=8$) 0.49 ± 0.028 pg/μl; $p<0.001$] and in male animals [control ($n=8$) 1.0 ± 0.28 pg/μl vs. diabetic ($n=8$) 0.12 ± 0.02 pg/μl; $p<0.05$]. Moreover, T levels in plasma of male animals are significantly decreased by diabetes [control ($n=8$) 3.79 ± 0.8 pg/μl vs. diabetic ($n=8$) 0.31 ± 0.11 pg/μl; $p<0.01$] and are below the detection limit of quantification both in control and diabetic female animals (<0.02 pg/μl). Our data also indicate that both in the PNS and the CNS, diabetes results in a strong general decrease in the levels of neuroactive steroids. These findings confirm and extend our previous observations obtained in male animals (Caruso et al., 2008a) and are in line with the observed protective effects of neuroactive steroids in diabetic neuropathy and diabetic encephalopathy (Aragno et al., 2002; Beauquis et al., 2008; De Nicola et al., 2009; Leonelli et al., 2007; Roglio et al., 2007; Saravia et al., 2004, 2006; Veiga et al., 2006; Yorek et al., 2002). As mentioned in the Introduction, diabetic complications in the PNS show a sex difference in terms of impact, progression and severity, with a general worse course in males (Aaberg et al., 2008; Albers et al., 1996; Basit et al., 2004; Booya et al., 2005; Kiziltan and Benbir, 2008; Kiziltan et al., 2007). Interestingly, we observed here that the levels of PREG, T and its derivatives are significantly decreased by diabetes in male sciatic nerve. PROG levels, as well as its metabolites, THP and isopregnanolone are decreased in females. The finding that these two metabolites, which are related to GABA-A receptor function, are affected only in females could be highly relevant. Indeed, neuropathic pain and negative sensory symptoms are more frequent in female diabetic patients (Kiziltan and Benbir, 2008). Moreover, in STZ-treated rats, nociceptive threshold is differently affected in both sexes, with the paw withdrawal threshold being more reduced in females than in males (Joseph and Levine, 2003). A role for GABA-A receptor and THP in neuropathic pain has been recently proposed (Meyer et al., 2008; Naik et al., 2008; Pathirathna et al., 2005a,b). In this context, it is important to note that THP is a well known positive modulator of GABA-A receptor (Belelli and Lambert, 2005; Gee et al., 1988). While, isopregnanolone does not bind directly to the GABA-A receptor (Bitran et al., 1991), it can antagonize the effect of THP on the GABA-A receptor *in vitro* and *in vivo* (Backstrom et al., 2005; Lundgren et al., 2003; Wang et al., 2002). Thus, sex differences in the involvement of GABA-A receptors in diabetic neuropathy might be hypothesized.

Diabetes also induces damage in CNS (i.e., diabetic encephalopathy). However in this case no specific observations have been so far performed to evaluate possible sex differences, which however are very clear in disorders associated to diabetic encephalopathy (e.g., cognitive deficits and increased risk of dementia, stroke, cerebrovascular and Alzheimer disease and psychiatric disorders) (Andersen et al., 1999; Farace and Alves, 2000; Fratiglioni et al., 1997; Kaye, 2008; Marcus et al., 2008; Niemeier et al., 2007; Simonds and Whiffen, 2003). We here observed sex differences in the levels of T, PROG and their derivatives in the brains of diabetic animals. In particular, with the exception of 3α-diol in cerebellum, T and its derivative levels are decreased with diabetes in male, but not in female, CNS areas. PROG levels show a sex difference in the cerebellum of diabetic rats, with levels decreased only in females. The levels of PROG metabolites are impaired in a different way. Thus, DHP and THP levels are only decreased in male cerebellum and spinal cord respectively, while isopregnanolone levels are only decreased in female cerebellum. These findings indicate that the diabetes-induced decrease of neuroactive steroid levels in the CNS is sexually dimorphic in a brain region specific manner.

Interestingly, levels of neuroactive steroids are also decreased in cerebral areas of Alzheimer's disease patients. In particular, a significant decrease of PREG sulfate and DHEA sulfate levels (Weill-Engerer et al., 2002), DHEA (Brown et al., 2003) and THP (Marx et al., 2006) have been reported in male patients. It is interesting to note that a close association between diabetes mellitus and Alzheimer's disease has been proposed. Indeed, extensive abnormalities of insulin and insulin-like growth factor signaling pathways also occur in this neurodegenerative disorder (Steen et al., 2005). Thus, alterations in neuroactive steroid levels might be interpreted as a common feature of the cognitive impairment occurring in diabetes mellitus and Alzheimer's disease.

In conclusion, the observations reported here indicate that the levels of neuroactive steroids in the PNS and the CNS show sex differences under basal conditions and are differently affected by diabetes in male and female rats. These sex differences may affect the result of therapies based on neuroactive steroids and may suggest new therapeutic strategies. Thus, these findings may represent a relevant background for new sex-specific therapies for diabetic neuropathy and diabetic encephalopathy, based on the use of neuroactive steroids.

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