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Neuroactive Steroid Levels in a Transgenic Rat Model of CMT1A Neuropathy

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Abstract Charcot–Marie–Tooth type 1A (CMT1A) represents 80% of all the demyelinating hereditary motor and sensory neuropathies. As recently suggested, neuroactive steroids may have a role in a therapeutic strategy for peripheral neuropathies, including CMT1A. To this aim, an accurate qualitative and quantitative analysis of neuroactive steroid levels in this disease could be extremely important to define effective pharmacological strategies. We here analyzed by liquid chromatography-tandem mass spectrometry the levels of neuroactive steroids present in the sciatic nerve of male and female peripheral myelin protein 22 transgenic rats ($PMP22_{tg}$ rats; i.e., an experimental model of CMT1A) and of the corresponding wild-type littermates. We observed that, both in $PMP22_{tg}$ rats and in the wild types, the levels of neuroactive steroids, such as progesterone, tetrahydroprogesterone (THP), isopregnanolone (3β,5α-THP), testosterone, dihydrotestosterone, and 5α-androstane-3α, 17β-diol (3α-diol) are sexually dimorphic. It is interesting to note that the levels of 3β,5α-THP and of 3α-diol, which are exclusively detectable in sciatic nerve of female and male rats, respectively, are strongly decreased in $PMP22_{tg}$ rats. 3β,5α-THP and 3α-diol are modulators of gamma-amino butyric acid A receptor. Thus, the present findings may be considered an interesting background for experiments aimed to evaluate the possible therapeutic effects of modulators of this neurotransmitter receptor in male and female $PMP22_{tg}$ rats.

Keywords Sciatic nerve · GABA-A receptor · Gender · Liquid chromatography tandem mass spectrometry · Peripheral neuropathy

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Introduction

Inherited peripheral neuropathies are a group of disorders collectively referred to Charcot–Marie–Tooth (CMT) disease. Among these, CMT1A is due to a 1.5-Mb duplication of chromosome 17p11.2, containing the gene coding for peripheral myelin protein (PMP22; Lupski et al. 1991). As CMT1A patients carry three copies of the gene, an overexpression of PMP22 is the causal factor of this disease (Hanemann and Muller 1998). Several animal models able to reproduce CMT1A disease are available. Among these, a rat overexpressing PMP22 (i.e., $PMP22_{tg}$ rats) mimics most of the human CMT1A symptoms, including peripheral demyelination, onion bulb formation, secondary axonal loss, and progressive muscle atrophy (Sereda et al. 1996). A pharmacological therapy for CMT1A would be particularly important because this disease, representing 80% of all the demyelinating hered-

itary motor and sensory neuropathies, with a prevalence of one case in 2,500, is a frequent and sometimes disabling disorder. It is interesting to note that a role of neuroactive steroids as protective agents has been proposed not only for acquired (Koenig et al. 1995; Melcangi et al. 1998, 2000; Azcoitia et al. 2003; Huppenbauer et al. 2005; Leonelli et al. 2007; Roglio et al. 2007; Schumacher et al. 2007; Veiga et al. 2006) but also for inherited peripheral neuropathies, like for instance the CMT1A. Thus, the treatment with an antagonist of progesterone (PROG) receptor (i.e., onapristone) in $PMP22_{tg}$ rats improved several performances of this animal, being also able to reduce PMP22 expression (Sereda et al. 2003), to prevent axonal loss, to ameliorate the compound muscle action potential, and to increase muscle strength and mass (Meyer zu Horste et al. 2007). These observations are relevant because a specific treatment for human CMT1A is not yet available. However, to build the best effective pharmacological and functional strategy, it would be extremely important to evaluate whether the tissue levels of neuroactive steroids are affected in $PMP22_{tg}$ rats and whether differences linked to the gender exist. To this aim, by liquid chromatography–tandem mass spectrometry (LC-MS/MS), we evaluated in sciatic nerve of male and female $PMP22_{tg}$ rats and of the corresponding wild-type littermates, the levels of pregnenolone (PREG), PROG and its derivatives, dihydroprogesterone (DHP) and tetrahydroprogesterone (3α,5α-THP), isopregnanolone (3β, 5α-THP) testosterone (T) and its derivatives, dihydrotestosterone (DHT) and 5α-androstane-3α, 17β-diol (3α-diol). We here demonstrate that the levels of PROG, 3α,5α-THP, 3β,5α-THP, T, DHT, and 3α-diol in the sciatic nerve are sexually dimorphic and that among these, the levels of 3β,5α-THP and 3α-diol are decreased in $PMP22_{tg}$ rats.

Materials and Methods

Materials

5-Pregnen-3β-ol-20-one (PREG), PROG, 5α-pregnane-3, 20-dione (DHP), 3α-hydroxy-5α-pregnen-20 one (3α,5α-THP), 3β-hydroxy-5α-pregnen-20 one (3β,5α-THP), T, 5α-androstane-17β-ol-3-one (DHT), 5α-androstane-3α,17β-diol (3α-diol), were purchased from Sigma Aldrich. 17,21,21,21-D_4-PREG (D_4-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) and 2,4,16,16-D_4-17β-estradiol (D_4-17β-E) from CDN Isotope Pointe-Claire (Quebec, Canada). SPE cartridges (Discovery DS-C18 500 mg) were from Supelco, Italy. All solvents and reagents were high-performance liquid chromatography grade (Sigma-Aldrich, Italy).

Animals

The study was performed on male and female 60-day-old rats overexpressing PMP22 (Sereda et al. 1996). As the hemizygous condition is a more appropriate model of CMT1A, we compared hemizygous rats with their wild-type littermates. Rearing conditions were consistent with the guidelines of the Italian Health Ministry relating to the use and storage of transgenic organisms. After killing the animals, sciatic nerves were dissected and immediately frozen in liquid nitrogen.

Liquid Chromatography–Tandem Mass Spectrometry Analysis

The samples were processed and purified as previously described (Caruso et al. 2007). Briefly, samples (100 mg/tissue) were added with internal standards and homogenized in 2 ml of MeOH/acetic acid (99:1 v/v) using an ultrasound homogenizer (Bransonic Ultrasonic, USA). After an overnight 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 SPE cartridge, previously activated with MeOH (5 ml) and MeOH/H_2O 10:90 v/v (5 ml). The steroids were eluted in MeOH, concentrated, and transferred in autosampler vials before the LC-MS/MS analysis.

Positive atmospheric pressure chemical ionization experiments were performed using a linear ion trap–mass spectrometer (LTQ, ThermoElectron, San Jose, CA) equipped with a Surveyor liquid chromatography Pump Plus and a Surveyor Autosampler Plus (ThermoElectron), as previously described (Caruso et al. 2007). Quantitative analysis was performed on the basis of calibration curves daily prepared and analyzed: Blank samples (6% albumin in phosphate-buffered saline) were spiked with D_4-17β-E (1 ng/sample), D_9-PROG (0.2 ng/sample), and D_4-PREG (5 ng/sample), as internal standards. Increasing amounts (0.05–5 ng/sample) of each steroid were added. Calibration curves were extracted and analyzed as already described for samples.

Statistical Analysis

Calibration curve parameters were judged by GraphPad4 PRISM (version 4). Student's t-test was used to determine significant differences between control and CMT tissues.

Results and Discussion

As shown in Fig. 1, the levels of PROG (panel b), 3α,5α-THP (panel d), and 3β,5α-THP (panel e) present in the sciatic nerve of female wild type are significantly higher than those present in male wild type. A very similar pattern is also evident in case of $PMP22_{tg}$ rats. Namely, the levels of PROG and 3α,5α-THP (panel b and d) detected in the sciatic nerve of female $PMP22_{tg}$ rats are significantly higher than those observed in the corresponding male siblings. It is interesting to note that the levels of 3β,5α-THP (panel e) in the sciatic nerve of female $PMP22_{tg}$ rats are significantly lower than those present in the corresponding wild-type animals.

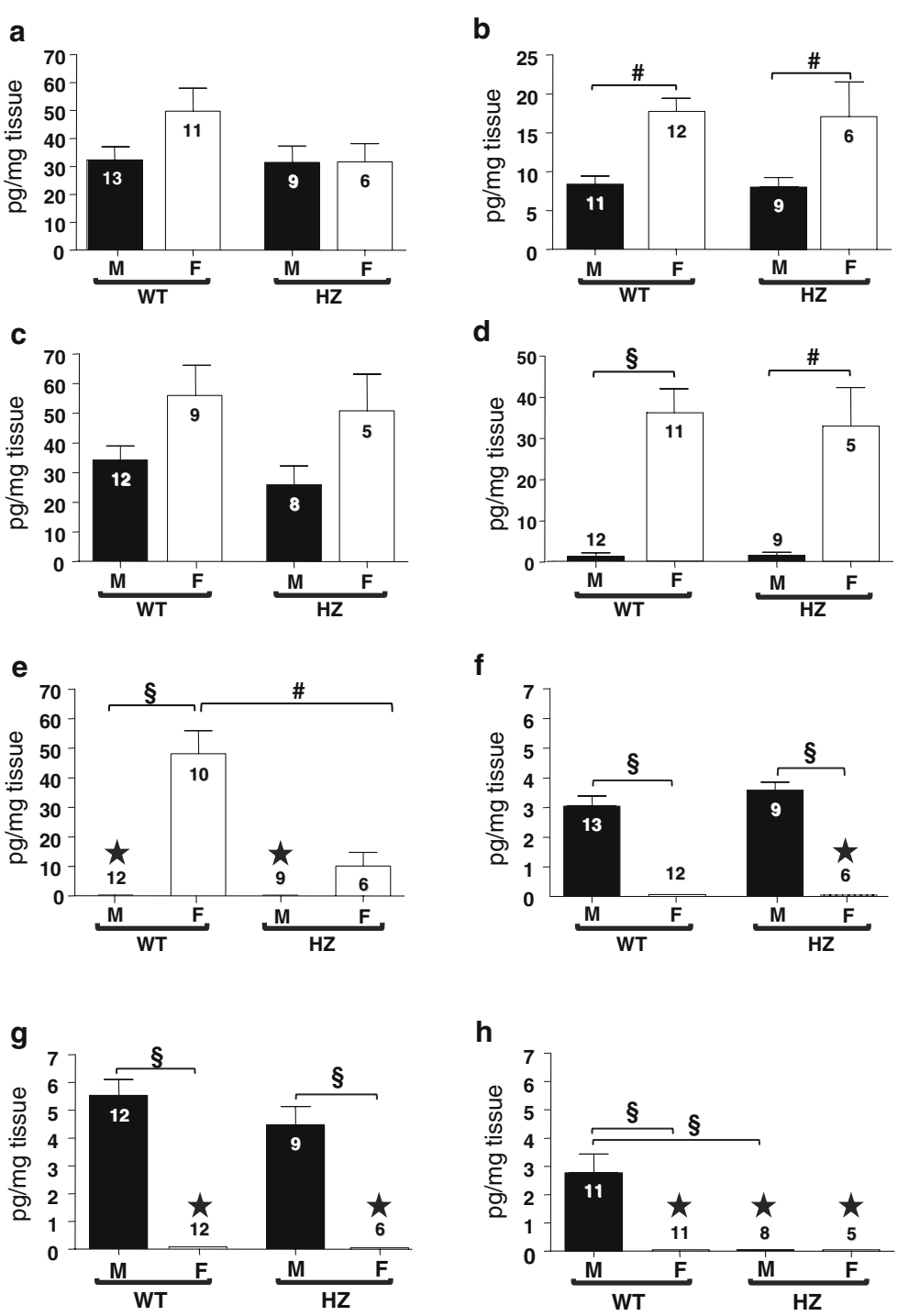

Figure 1 Neuroactive steroid levels in the sciatic nerve of 60-day-old male (*M*) and female (*F*) wild-type (*WT*) and hemizygous (*HZ*) $PMP22_{tg}$ rats. Neuroactive steroid levels evaluated by LC-MS/MS are: PREG (**a**), PROG (**b**), DHP (**c**), 3α,5α-THP (**d**), 3β,5α-THP (**e**), T (**f**), DHT (**g**), and 3α-diol (**h**). Data are expressed as pg/mg tissue± SEM (number of determinations are indicated by each column). # $P<0.01$ and § $P<0.001$. *Stars*, values under limit of quantification (0.1 for 3β,5α-THP, 0.02 for T and 0.05 pg/mg tissue for DHT and 3α-diol)

Furthermore, the levels of T (panel f) and its derivatives (i.e., DHT and 3α-diol, panel g and h) in the sciatic nerve are sexually dimorphic. However, at variance to what is observed in case of PROG and its derivatives, the levels of these neuroactive steroids are significantly higher in male wild-type than in corresponding female wild-type rats. A similar pattern is also evident in case of $PMP22_{tg}$ rats. In case of 3α-diol (panel h), the levels of this neuroactive steroid observed in the sciatic nerve of male $PMP22_{tg}$ rats are significantly lower than those present in the corresponding wild-type littermates.

On the contrary, the levels of PREG (panel a) and DHP (panel c) present in sciatic nerve of male and female wild types and in the corresponding $PMP22_{tg}$ rats are not significantly different (Fig. 1).

Altogether, data here reported indicate for the first time that in the sciatic nerve of this experimental model of CMT1A and in the corresponding wild-type littermates, the levels of some of the neuroactive steroids analyzed (i.e., PROG, 3α,5α-THP, 3β,5α-THP, T, DHT and 3α-diol) are sexually dimorphic. It is interesting to note that among these, the levels of 3β,5α-THP and 3α-diol, which are only present in sciatic nerve of female and male rats, respectively, are strongly affected by the pathology.

3β,5α-THP and 3α-diol are two metabolites of DHP and DHT, respectively. Consequently, the low levels of these two neuroactive steroids observed in the sciatic nerve of $PMP22_{tg}$ animals could be explained by a decrease of the levels of their substrates. Alternatively, an impairment of their synthesis, like for instance alteration of expression or activity of the enzyme 3β-hydroxysteroid oxidoreductase in case of 3β,5α-THP and the enzyme 3α-hydroxysteroid oxidoreductase in case of 3α-diol, could be hypothesized. As here reported, our data seem to deny a decrease in substrates because both DHP and DHT levels are unmodified in the sciatic nerve of transgenic animals.

The finding that peripheral nerve degeneration may be associated with decreased levels of neuroactive steroids is also supported by our recent observations obtained in an experimental model of diabetic neuropathy (i.e., male rat raised diabetic by injection with streptozotocin; Caruso et al. 2007; Roglio et al. 2007). Namely, in this experimental model of acquired neuropathy, a decrease in the levels of PREG, DHP, THP, T, DHT, and 3α-diol in peripheral nerve occurred (Caruso et al. 2007; Roglio et al. 2007).

3α-diol and 3β,5α-THP, at variance to their precursors (i.e., DHT and DHP, respectively), are not able to bind the classical steroid receptors, such as androgen and PROG receptors but modulate gamma-amino butyric acid (GABA)-A receptors. However, the way by which these two neuroactive steroids affect the neurotransmitter receptor is different. In fact, 3α-diol and 3α,5α-THP are considered as positive modulators of this neurotransmitter receptor (Belelli and Lambert 2005; Frye et al. 1996; Gee et al. 1988). On the contrary, 3β,5α-THP is not able to directly interact with this neurotransmitter receptor (Bitran et al. 1991, Gee et al. 1987), but it seems to antagonize the effect of 3α,5α-THP on GABA-A receptor (Backström et al. 2005; Lundgren et al. 2003; Wang et al. 2002). As we have previously demonstrated, GABA-A receptor is expressed both in sciatic nerve and in Schwann cells (Melcangi et al. 1999), and its activation, by neuroactive steroids, such as 3α,5α-THP and 3α-diol, increases PMP22 expression (Melcangi et al. 1999, 2000). It is interesting to note that, as we recently observed, this effect is sexually dimorphic. Namely, in primary rat Schwann cells, the expression of PMP22 is stimulated by 3α,5α-THP only in culture obtained from female rat sciatic nerves (Magnaghi et al. 2006).

Differences depending on the gender in peripheral nerves (e.g., rat pudendal nerve) have been also observed in terms of number, caliber, and density of myelinated and unmyelinated axons and Schwann cells (Moore and White 1996). Moreover, different experimental models of acquired peripheral neuropathies have shown gender-related differences on function and recovery of nociception (Joseph and Levine 2003a,b; Kovacic et al. 2004). Finally, as recently observed, CMT women show higher disability (especially in measurements related to symptoms and pain) than men (Padua et al. 2006).

In conclusion, our results seem to suggest an involvement of GABA-A receptor in CMT1A pathology, which could be different depending on the gender. We believe that the present findings represent an important background for future experiments aimed to explore the possible effects of modulators of GABA-A receptors in the male and female CMT1A experimental model. Moreover, to compare the outcome of these treatments with what was previously observed with the antagonist of PROG receptor (Sereda et al. 2003; Meyer zu Horste et al. 2007) and to explore the possibility of addictive effects of the two therapeutic approaches will be extremely important.

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