Cell. Mol. Life Sci. (2010) 67:3017–3034
DOI 10.1007/s00018-010-0372-0

Cellular and Molecular Life Sciences

RESEARCH ARTICLE

Cellular and functional evidence for a protective action of neurosteroids against vincristine chemotherapy-induced painful neuropathy

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Received: 5 January 2010 / Revised: 1 April 2010 / Accepted: 6 April 2010 / Published online: 30 April 2010

Abstract Painful neuropathy is a major side-effect limiting cancer chemotherapy. Therefore, novel strategies are required to suppress the neuropathic effects of anticancer drugs without altering their chemotherapeutic effectiveness. By combining biochemical, neuroanatomical/neurochemical, electrophysiological and behavioral methods, we demonstrated that progesterone-derived neurosteroids including 5α-dihydroprogesterone and 3α,5α-tetrahydroprogesterone suppressed neuropathic symptoms evoked in naive rats by vincristine. Neurosteroids counteracted vincristine-induced alterations in peripheral nerves including 2′,3′-cyclic nucleotide 3′-phosphodiesterase, neurofilament-200 kDa and intraepidermal nerve fiber repression, nerve conduction velocity, and pain transmission abnormalities (allodynia/hyperalgesia). In skin-tumor rats generated with carcinosarcoma-cells, vincristine, which suppressed the skin tumor and restored normal blood concentration of vascular endothelial growth factor (VEGF), reproduced neuropathic side-effects. Administered alone, neurosteroids did not affect the tumor and VEGF level. Combined with vincristine, neurosteroids preserved vincristine anti-tumor action but counteracted vincristine-induced neural side-effects. Together, these results provide valuable insight into the cellular and functional mechanisms underlying anticancer drug-induced neuropathy and suggest a neurosteroid-based strategy to eradicate painful neuropathy.

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Keywords Anticancer drug-induced neuropathy · Biochemical mechanisms in peripheral nerves · CNPase · Cell culture · Immunohistochemistry · Neurofilament-200 kDa · Protein gene product PGP9.5 · Sandwich enzyme immunoassay

Introduction

Antineoplastic drugs remain the most effective and commonly used molecules in human cancer chemotherapy [1–3]. However, their therapeutical effectiveness is seriously limited by a major dose-dependent side-effect, which is painful peripheral neuropathy [4–6]. The anti-tumor effect of antineoplastic drugs is based on their ability to disturb tubulin monomer aggregation during microtubule formation in mitosis with subsequent inhibition of cell division [7]. It has been suggested that painful neuropathic effects of antineoplastic compounds may result from their toxic action on microtubules in peripheral nerves, leading to axonal transport impairment and axonal degeneration [8, 9]. Controversial investigations, which showed the occurrence of neuropathic pain symptoms in the absence of axonal degeneration, indicated that antineoplastic drug-evoked painful neuropathy is due to mitochondrial dysfunctions, loss of epidermal innervation, and neuroimmune interactions [10–12]. Therefore, additional data are necessary to clarify the mechanisms underlying painful neuropathy evoked by anti-tumor drugs. Most importantly, it becomes crucial to develop new strategies that may counteract the dose-limiting painful neuropathic effect of antineoplastic drugs without altering their main anti-tumor action, in order to improve treatments against cancer. Among the antineoplastic drugs is vincristine, which has been used for several decades and remains one of the most important molecules for the

treatment of childhood and adult malignancies [1–3, 13, 14]. A clear relationship between the dosage of vincristine and the severity of neuropathic symptoms has been demonstrated [15, 16]. Therefore, identification of neuroactive compounds capable of suppressing vincristine-evoked neuropathy may raise a hope for the improvement of antineoplastic drug-based therapy. It is well demonstrated that progesterone and its 5α-reduced metabolites, 5α-dihydroprogesterone (5α-DHP) and 3α,5α-tetrahydroprogesterone (3α,5α-THP), also called allopregnanolone, exert potent neuroprotective and neurotrophic effects [17–19]. In addition, we have recently observed that progesterone-derived neurosteroids also crucially control nociception [20–22]. Consequently, we hypothesized that progesterone, 5α-DHP, and 3α,5α-THP may counteract vincristine-induced neuropathic pain symptoms including allodynia and hyperalgesia. To check our hypothesis and gain insight into the mechanisms underlying anticancer drug-evoked neuropathy, we have used a multidisciplinary approach to investigate the effects of progesterone-derived neurosteroids on several biochemical, neuroanatomical/neurochemical, and functional parameters dysregulated by vincristine in peripheral nerves such as the non-compact myelin protein 2′,3′-cyclic nucleotide 3′-phosphodiesterase (CNPase), which is pivotal for axonal survival [23, 24], the axonal marker NF200, the density of intraepidermal nerve fibers (IENF) and the nerve conduction velocity (CV). Finally, we developed a rat experimental model of skin-cancer to check whether a treatment with progesterone, 5α-DHP, or 3α,5α-THP may suppress vincristine-evoked neuropathic symptoms and peripheral nerve alterations without reducing vincristine-induced anti-tumor action.

Materials and methods

Animals

Adult male Sprague-Dawley rats weighing 250–300 g were used. The experiments were performed with male animals in order to avoid fluctuations of results intervening in females due to endogenous circulated progesterone. Animal care and manipulations were performed according to the European Community Council Directives (86/609/EC) and under the supervision of authorized investigators. All experiments were performed minimizing the number of animals used and their suffering in accordance with the Alsace Department of Veterinary Public Health Guide for the Care and Use of Laboratory Animals (Agreement number 67-186). The animals were obtained from a commercial source (Janvier, Le Genest St Isle, France) and housed under standard laboratory conditions in a 12-h light/dark cycle with food and water ad libitum. Animals were allowed a 1-week acclimatization period before being used in experiments.

Drugs and treatments

Vincristine sulphate (VINC; Sigma-Aldrich, St. Louis, MO) was dissolved in physiological saline (NaCl 0.9%), used as vehicle, at 0.1 mg/ml and stored at 4°C. VINC was intraperitoneally (i.p.) injected daily, in two 5-day cycles with 2 days pause between cycles, at a concentration of 0.1 mg/kg per day depending on the daily body weight [25]. Control rats were injected with the vehicle NaCl (1 ml/kg) according to a similar schedule. Before the onset of vincristine (vs. vehicle) treatment, behavioral tests were performed in all rats in order to determine the pre-injection values of the mechanical (D0) and thermal (D1) nociceptive thresholds. After VINC or vehicle treatment onset, behavioral analyses were realized everyday to assess alternately the mechanical or thermal sensitivity.

Progesterone, 5α-DHP (Steraloids, Newport, RI), 3α,5α-THP or finasteride (Apin Chemicals, Abingdon, UK) were diluted in 2-hydroxypropyl-β-cyclodextrin or CDEX 15% in water (Sigma-Aldrich, St. Louis, MO) used as vehicle. Control rats were injected with the vehicle alone (NaCl 0.9% + CDEX 15%). Consequently, it should be noted that three different groups of control animals were used for the whole study: naive non-treated rats, NaCl-treated rats, and (NaCl + CDEX)-treated rats. Several investigations have shown that the dose 4 mg/kg of progesterone is an effective neuroprotective dose [17]. However, to determine the effective and optimal neurosteroid treatment, we have tested in the present study the dose- and injection frequency-dependent effects of progesterone (2 or 4 mg/kg every 2 or 4 days) on the mechanical nociceptive thresholds of vehicle- and vincristine-treated rats. Neurosteroids (NS) were i.p. administered immediately after the behavioral test session. Two types of treatments were performed using neurosteroids. Prophylactic neurosteroid treatments, which aimed at preventing the development of VINC-induced neuropathic pain, consisted in neurosteroid administration before the onset and during the treatment with VINC (neurosteroid administration started 8 days before VINC cycles). Corrective neurosteroid treatments, which aimed at suppressing painful neuropathic symptoms persisting after the end of VINC treatment, consisted in starting neurosteroid administration after the two VINC cycles (neurosteroid injections started 2 days after VINC cycles and lasted 1 week). Behavioral tests were performed every 2 days to determine the effects of progesterone-derived neurosteroids on nociceptive thresholds. Experimenters were completely blind to the experimental conditions of the animals.

The effects of finasteride (25 mg/kg) alone or associated with progesterone (4 mg/kg) were tested according to the

same procedure and schedule described above. Briefly, a first dose (25 mg/kg) of finasteride (Fin) or CDEX was injected at D1 after the assessment of pre-injection thresholds values at D0. Then, from D2 to D32 co-administrations of Fin (25 mg/kg) + progesterone (4 mg/kg) or Fin + CDEX were repeated every 2 days as the behavioral assessment of the mechanical nociceptive thresholds in all animal groups.

Experimental skin cancer model

Rat Walker 256 carcinosarcoma cells (W256; ECACC, Salisbury, UK) were cultured at 37°C in RPMI 1640 supplemented with 10% fetal bovine serum (FBS), 1% penicillin/streptomycin and 1% glutamine. At 80–90% of confluence, W256 cells were harvested and washed in phosphate buffered saline (PBS; pH 7.4). The cells were pelleted by brief centrifugation 900 rpm at 25°C. The supernatant was aspirated and the cells were resuspended in PBS at a density of 2×10^7 cells/200 µl after assessment of cell viability with the method of Trypan Blue exclusion in a Neubauer chamber. After shaving the right posterior flank of the animals and sterilizing the skin with 70% ethanol, W256 cells suspension was subcutaneously injected into the dorsal tissue of each rat (tumor-bearing group) with a 25-gauge needle. The day of inoculation was defined as day 0 (D0). Control animals were subcutaneously injected with PBS. At day 4, when the tumor was well implanted, animals were treated with NS and/or vincristine as described earlier. Tumor size was measured using a caliper and calculated according to the formula: tumor volume (mm^3) = $0.5 \times L \times w^2$, where L = length (mm), w = width (mm).

Assessment of VEGF serum level

Venous blood was collected individually from the tail vein before W256 cells or PBS inoculation. Blood collection was repeated in all rat groups every 4 days until the end of the 34-day period covered by the experience. After centrifugation (1,500 rpm, 20 min, 4°C), serum samples were aliquoted and stored at −20°C in order to perform assays in all groups at the same time. A commercially available quantitative sandwich enzyme immunoassay (Quantikine, R&D Systems Inc., Minneapolis, MN) was used to assess the serum level of vascular endothelial growth factor (VEGF) according to the manufacturer's instructions.

Nociceptive behavioral tests

Thermal hyperalgesia was assessed by using a Plantar test apparatus (Ugo Basile, Comerio, Italy) that measures the paw withdrawal latency in response to radiant heat [26]. The rats were first allowed to habituate to the apparatus for 10 min before testing. Each rat was placed individually in clear Plexiglas® boxes (23 × 18 × 14 cm) positioned on a clear plastic surface. The heat source was then positioned under the plantar surface of the hind paw and activated with an infra-red light beam. The heat source is connected to a timer that automatically switched off the heat when the paw was withdrawn. A cut-off time of 20 s was used to prevent tissue damage in absence of response. The mean paw withdrawal latency (in seconds) of hind paws was determined from an average of six separate measures (three per paw) at a given time point. The testing box was thoroughly cleaned between each test session.

The mechanical nociceptive sensitivity threshold was evaluated in individual rats placed on Plexiglas® boxes (30 × 30 × 25 cm) upon an elevated metal grid allowing access to the plantar surface of the hind paws. The presence of mechanical allodynia and hyperalgesia were assessed using a series of calibrated von Frey hairs (1, 2, 4, 6, 8, 10, 15, 26, 60, 100, 180, and 300 g; Stoelting, Wood Dale, IL), which were applied to the plantar surface of the hind paw with increasing force until the individual filament used just started to bend. The filament was applied for a period of 1–2 s and the procedure was repeated five times at 4- to 5-s intervals. The threshold for paw withdrawal was calculated by taking the average of ten (five per paw) repeated stimuli (in g) which induced a reflex paw withdrawal. Only robust and immediate withdrawal responses followed by a licking of the paw were considered as positive. Naive untreated rats never withdraw from stimulations less than 6 g but respond 15–20% of the time for 15 g stimulus and more than 70% of the time for 80 g considered as an indisputable nociceptive stimulation. Observation of responses for stimulations <6 g after drug administrations is indicative of mechanical allodynia. Increased level of responding for 15 g after treatment is indicative of mechano-hyperalgesia.

Electrophysiological studies

The sciatic nerves were rapidly dissected and ligatures were placed proximally near the exit point from the spinal canal and distally above the knee. The nerve was then cut externally to the ligatures. The preparation was immerged in bath medium (NaCl 133, KCl 2, $CaCl_2$ 1, $MgCl_2$ 2, HEPES 10 and glucose 10 pH 7.4) 5–10 min before recording. The sciatic nerve was put on chlorinated silver electrodes mounted in a classical homemade electrodes holder chamber. Square-shaped stimulating pulses of 0.1-ms duration generated by Clampex software (Axon Instruments, CA, USA) were applied to the distal end of the nerve through Digidata 1322A interface (Axon Instruments, CA, USA). Bipolar sciatic nerve action potentials (NAP) were recorded at the proximal end of the nerve

($\Delta x = 20$ mm distance from the stimulus point) using an ISO-DAM8A differential amplifier (World Precision Instruments, UK) with a bandwidth of 10–10,000 Hz. The signal was digitized (500 kHz) with the Digidata 1322A. Artifact of stimulation was obtained in isolation using a double pulse protocol allowing the second stimulation to occur in refractory period. This stimulus artifact was subtracted from recorded NAPs before analysis with Pclamp software (Axon Instruments, CA, USA).

Nerve CV was calculated using the latency between the beginning of stimulus artifact and the NAP onset ($\Delta t_{latency}$) or the NAP peak (Δt_{peak}) for the fastest and relatively slower fibers group, respectively, according to the equations:

$$CV_{latency} = \Delta x / \Delta t_{latency}$$

$$CV_{peak} = \Delta x / \Delta t_{peak}$$

Immunohistochemical studies

At the end of the last behavioral test session, sciatic nerves and hind paw plantar skins were removed to allow immunohistochemical studies. Thus, animals were deeply anesthetized with 25% urethane (0.5 ml/100 g, i.p.) and perfused transcardially with 100 ml of 0.1 M phosphate buffered (PB; pH 7.4). The perfusion was carried out with 450 ml of fixative solution (4% formaldehyde and 0.2% picric acid in PB). The nerves and plantar skins were rapidly dissected and postfixed in the same fixative solution for 24 h. Sciatic nerves and hind paw plantar skins were immersed in PB containing 15% sucrose for 24 h and then transferred into 30% sucrose PB for 24 h. Sciatic nerves and intraplantar skins were then placed in embedding medium (OCT, Tissue-Tek, Reichert-Jung, Nussloch, Germany) and immediately frozen at −80°C. Sagittal sections (10 µm thick) were cut in a cryostat HM 560 (Microm, Francheville, France) and mounted on glass slides coated with gelatin and chromium potassium sulfate. Tissue sections were pre-incubated for 1 h with 5% non-immune donkey or goat serum in PB containing 0.3% Triton X-100 (PBT). Afterwards, the sciatic nerve sections were incubated overnight at 4°C with a mouse monoclonal antibody against 2′,3′-cyclic nucleotide 3′-phosphodiesterase (CNPase; Sigma-Aldrich, St. Louis, MO) diluted at 1:100 in PBT or a mouse monoclonal antibody against the neurofilament 200 kDa (NF200; Clone N52; Sigma-Aldrich, St. Louis, MO) diluted at 1:250 in PBT. The sections of hind paw plantar skins were incubated overnight at 4°C with the rabbit polyclonal antibody against the protein gene product PGP9.5 (Cedarlane Laboratories, Ontario, Canada) diluted at 1:500 in PBT. The procedure was carried on by rinsing the sections three times in PB (15 min/rinse) and transferring them for 2 h into Alexa-488-conjugated donkey anti-mouse or FITC-conjugated goat anti-rabbit (Chemicon, Temecula, CA) diluted at 1:300 in PBT. Finally, the sections were rinsed for 1 h in PB and mounted in Vectashield (Vector Laboratories, Burlingame, CA). Although specificity of the antibodies has previously been demonstrated [8, 12, 27–29], internal control experiments were performed in the present work as follows: (1) substitution of CNPase, NF200 or PGP9.5 antiserum with PBT, (2) replacement of CNPase, NF200 or PGP9.5 antibody by non-immune mouse or rabbit serum and (3) preincubation of CNPase, NF200 or PGP9.5 antibody with purified CNPase, NF200 or PGP9.5, respectively. The preparations were examined under a multichannel confocal laser-scanning microscope (Leica Confocal Systems, Paris, France) assisted by a Pentium IV PC (Leica Microsystems). The number of CNPase immunoreactive cells was determined in a counting square of 200×200 µm^2 on sagittal sections of sciatic nerves isolated from each experimental group. Quantification of NF200 immunoreactivity was performed on sciatic nerve sagittal sections by using ImageJ software. Results were expressed as % of control (+SEM). The number of PGP9.5 positive-IENF was quantified as previously described [8, 12]: all ascending nerve fibers that cross into the epidermis were counted, no minimum length was required and secondary branching into the epidermis was excluded from quantification. IENF counts are expressed as the number per millimeters of epidermal border.

Statistical analyses

ANOVAs followed by Newman–Keuls post hoc comparisons were used. The data, which did not exhibit a Gaussian distribution, were analyzed by the non-parametric Mann–Whitney U test. The statistical significance of differences between sciatic nerve conduction velocities or VEGF serum levels were assessed by Student's t test. Data were analyzed with Statistica Software 5.1 (Statsoft, Maisons-Alfort, France). A p value of less than 0.05 was considered significant.

Results

Effects of vincristine on the mechanical nociceptive threshold

Before the onset of VINC or vehicle (NaCl 0.9%) treatment, the percentages of paw withdrawal for mechanical stimulations were 0% for von Frey filaments < 6 g, 15–20% for 15 g and more than 70% for 80 g. In animals receiving the vehicle, the percentages of withdrawal responses remained unchanged all treatment days (Fig. 1a–c). In contrast, after

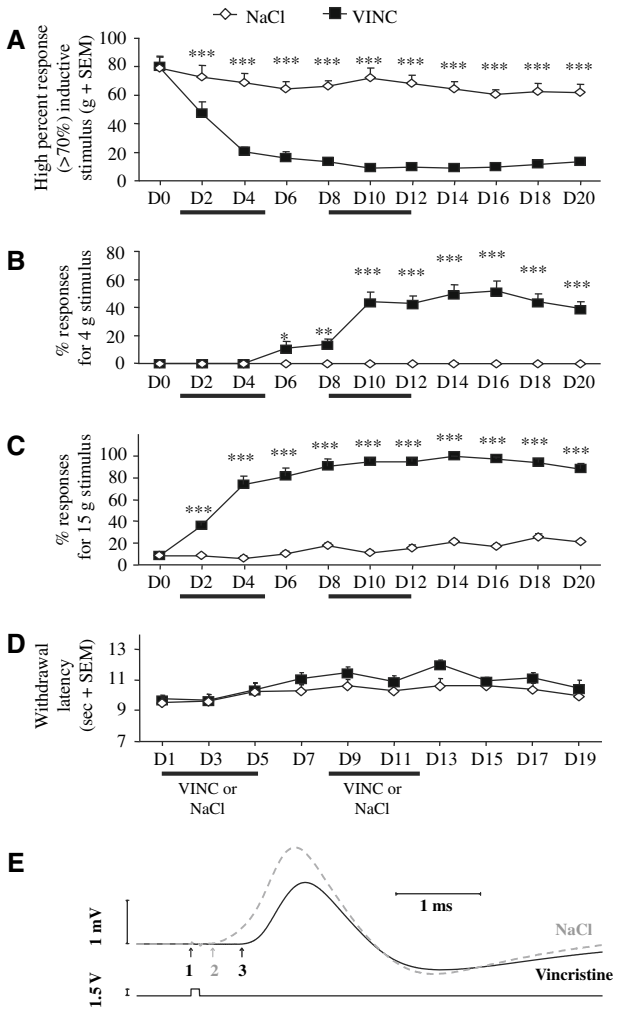


Fig. 1 **a** Effect of vincristine treatment on the mechanical nociceptive thresholds of naive rats. The *curves* (mean + SEM) were obtained with mechanical stimulations (g), which induced more than 70% withdrawal responses in each animal before or after drug administrations ($n = 10$ per group). **b**, **c** Time-course of mechanical allodynia (**b**) and hyperalgesia (**c**) induced by vincristine treatment. *Graphs* show the mean + SEM of the percentages of paw withdrawal responses to mechanical stimulation by Von Frey filament 4 g (**b**) or 15 g (**c**). **d** Effect of vincristine treatment on the thermal nociceptive thresholds of naive rats assessed by the Plantar test. Each point represents the mean + SEM of six observations in each of ten rats. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.005$. **e** Vincristine effect on nerve conduction velocity. Typical traces of rat sciatic NAP recorded from rats treated with NaCl (*dashed curve*) or vincristine (*filled curve*). The nerve was stimulated with a supra-maximal pulse potential as illustrated by the protocol trace (*lower trace*). The *arrows 1–3* indicate the beginning of the stimulation artifact (*1*) and the onset of NAP in control (*2*) and vincristine (*3*) conditions giving a calculated NAP $CV_{latency}$ in this case of 77.5 and 33.2 m/s for control and vincristine, respectively. Each NAP trace was obtained by averaging 50 consecutive original NAPs

VINC treatment onset, paw withdrawal responses were observed (10–50%; $p < 0.005$) for von Frey filaments < 6 g (mechanical allodynia) (Fig. 1b) and increased level (40–100%; $p < 0.005$) of responding was seen for 15 g (mechano-hyperalgesia) (Fig. 1c). VINC-induced mechanical allodynia and hyperalgesia, which started rapidly from the first 5 day-cycle of VINC treatment, was prolonged by the second cycle and persisted, at least 8 days, after withdrawal of VINC treatment (Fig. 1b, c).

Effects of vincristine on the thermal nociceptive threshold

The baseline withdrawal latency characterizing the thermal pain threshold was around 9.6 ± 0.2 s on each paw of naive rats before treatment onset. No significant changes of the thermal thresholds were observed in animals receiving VINC or the vehicle ($p = 0.59$), indicating that VINC treatment did not affect the heat thermal nociceptive threshold in naive rats (Fig. 1d).

Effects of vincristine on nerve conduction velocity

Figure 1e illustrates the effect of VINC [0.1 mg/kg per day] on sciatic nerve CV. The latency of the NAP onset was significantly increased (0.26–0.60 ms) giving in this case a $CV_{latency}$ value of 76.9 and 33.3 m/s for the control and VINC-treated rats, respectively. Mean values of 79.6 ± 1.5 and 47.8 ± 3.0 m/s ($n = 8$) were respectively obtained for control and VINC rats leading to a mean CV reduction of 40% for the fastest sciatic nerve fibers.

Effects of vincristine on CNPase and NF200 expression in sciatic nerves

Immunohistochemical experiments using a specific antibody against CNPase were combined with cell counts to determine the effects of VINC treatment on CNPase expression in Schwann cells surrounding axonal processes of the rat sciatic nerve. Intense immunoreactivity for CNPase was visualized on sagittal sections of naive rat sciatic nerves (Fig. 2a). Comparative analysis of the numbers of CNPase-positive Schwann cell bodies detected in counting squares (200 × 200 μm^2) showed that VINC treatment caused a 49% decrease of CNPase expression in sciatic nerves ($p < 0.001$) (Fig. 2a, b, g). Moreover, we assessed the effects of VINC on axonal degeneration in sciatic nerves thanks to comparative quantifications of the immunoreactivity for the specific axon marker NF200. A 44% decrease of NF200-immunostaining was detected in sciatic nerves of VINC-treated rats compared to controls ($p < 0.001$) (Fig. 2c, d, h).

Effects of vincristine on intraepidermal nerve fibers

Intraepidermal nerve fibers were firstly immunolabeled with a specific antibody against PGP9.5. Then, IENF

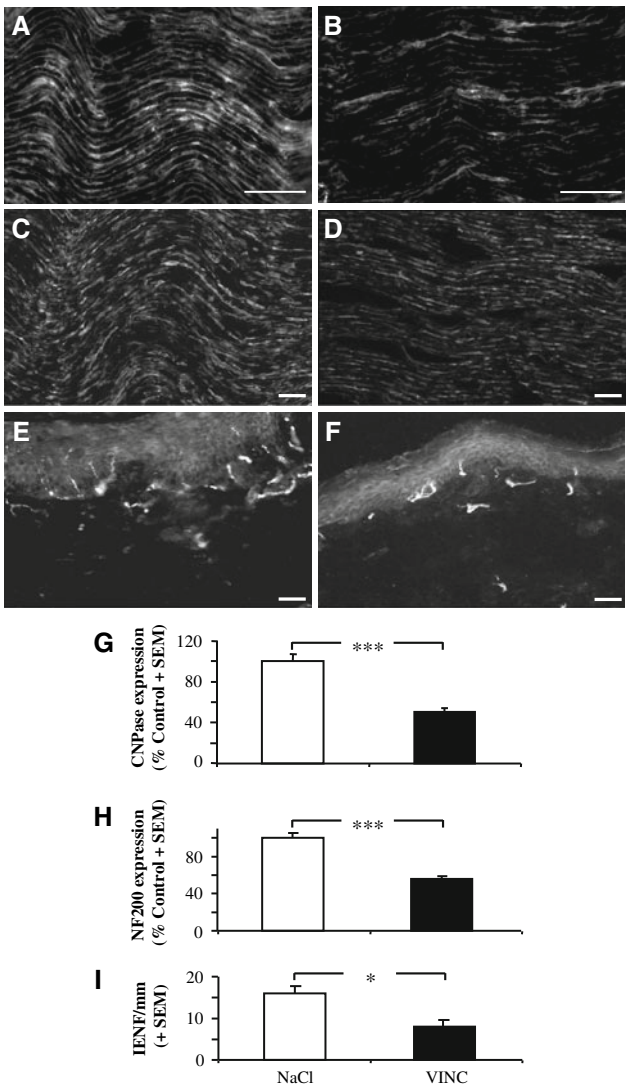


Fig. 2 **a**–**f** Photomicrographs of sagittal sections of sciatic nerves (**a**–**d**) and hind paw skins (**e**, **f**) dissected from NaCl- (**a**, **c**, **e**) and vincristine-treated (**b**, **d**, **f**) rats. The nerve sections were labeled with the monoclonal anti-CNPase revealed with Alexa-488-conjugated donkey anti-mouse. The skin sections were labeled with the polyclonal anti-PGP9.5 revealed with FITC-conjugated goat anti-rabbit. *Scale bar* 100 µm. Comparative analysis of the numbers of CNPase-positive Schwann cell bodies detected (**g**) and the density of NF200-immunolabeling (**h**) in sciatic nerve sections dissected from NaCl- and vincristine-treated rats ($n = 6$ per group). Each value is expressed as percent (+SEM) of CNPase-positive cells bodies (**g**) or NF200-immunolabeling density (**h**) detected in sciatic nerve sections of control (NaCl-treated) rats. **i** Comparative analysis of IENF density measured in hind paw skin sections dissected from NaCl- and vincristine-treated rats ($n = 4$ per group). *$p < 0.05$, ***$p < 0.001$

density was quantified in vehicle- and VINC-treated hind paw skin sections. VINC treatment decreased by 50% PGP9.5-immunoreactivity or IENF density in hind paw intraplantar skins ($p < 0.05$) (Fig. 2e, f, i).

Action of progesterone on vincristine-induced mechanical hyperalgesia and allodynia

Although 4 mg/kg was shown as an effective therapeutical dose of progesterone [17], we have tested the dose- and injection frequency-dependent effects of progesterone (2 or 4 mg/kg every 2 or 4 days) on the mechanical nociceptive thresholds of vehicle- and VINC-treated rats (Table 1). We observed that the optimal treatment was provided by 4 mg/kg of progesterone injected every 2 days (Table 1). Progesterone (4 mg/kg), which increased the mechanical pain threshold ($p < 0.05$), exerted a potent antinociceptive action in naive rats (Fig. 3a). Prophylactic administration of progesterone (4 mg/kg every 2 days), which started 8 days before VINC injections and was maintained during VINC cycles, completely prevented VINC-induced mechanical allodynia and hyperalgesia ($p < 0.005$) (Fig. 3a, c, e). Neuropathic pain symptoms did not reappear in VINC-treated rats after the end of progesterone treatment (D30 on Fig. 3a, c, e). Moreover, when neuropathic pain symptoms are already installed in VINC-treated rats, corrective progesterone treatment, which started 2 days after the end of VINC cycles, was able to eradicate allodynic and hyperalgesic symptoms ($p < 0.005$) and to restore normal mechanical threshold values (Fig. 3b, d, f). Interestingly, painful neuropathic symptoms existing in VINC-treated rats before the onset of progesterone-corrective treatments disappeared after 1 week of progesterone administration and the symptoms did not reappear after withdrawal of progesterone treatment (D22 on Fig. 3b, d, f).

Effects of progesterone on vincristine-induced nerve conduction velocity alterations

In accordance with its action on the mechanical sensitivity threshold assessed with behavioral methods (Fig. 3a–f), progesterone treatment (4 mg/kg every 2 days) also affected sciatic nerve CV measured with electrophysiological approach. Indeed, the CV of the fastest fibers was significantly increased by progesterone as shown in Fig. 3g ($CV_{latency} = 84.1 \pm 1.6$). More importantly, progesterone reversed VINC-induced $CV_{latency}$ decrease (Fig. 3g) and the mean value obtained in this case (80.5 ± 2.4) was similar to that of control rats (79.6 ± 1.5).

Effect of progesterone on CNPase expression in sciatic nerve

A strong increase of CNPase-immunoreactivity was visualized in sciatic nerves of progesterone compared to vehicle-treated rats (Fig. 4a, c). In addition, the decrease

Table 1 Dose- and injection frequency-dependent effects of progesterone on the mechanical nociceptive thresholds of control- and vincristine-treated rats

Injection frequency of PROG	Drug and dose	D0	D2	D4	D6	D8	D10	D12	D14
Every 2 days	NaCl + CDEX	116 ± 2.1	119.2 ± 2.6	116 ± 2.1	114.4 ± 2.4	114.4 ± 2.4	132 ± 9.9	152.8 ± 17.1	158.4 ± 17.3
	NaCl + PROG (2 mg/kg)	116 ± 2.1 ns1	118.7 ± 3.4 ns1	114.7 ± 2.5 ns1	116 ± 2.9 ns1	112 ± 2.7 ns1	117.3 ± 1.3 ns1	122.7 ± 1.3 ns1	135.3 ± 11.3 ns1
	NaCl + PROG (4 mg/kg)	118.7 ± 1.7 ns1	117.3 ± 3.2 ns1	114.7 ± 3.8 ns1	113.3 ± 3.4 ns1	142.7 ± 15.65 ns1	164.7 ± 19.5 ns1	226 ± 15.3**	220 ± 16.3*
	VINC + CDEX	110.7 ± 8.9 ns1	114.7 ± 4.3 ns1	120 ± 2.7 ns1	124.7 ± 17.72 ns1	134 ± 16.84 ns1	111.3 ± 8.29 ns1	75.33 ± 6.65***	41.5 ± 9.6***
	VINC + PROG (2 mg/kg)	117.3 ± 3.2 ns1, 2	117.3 ± 2.5 ns1, 2	114.7 ± 1.3 ns1, 2	116 ± 2.1 ns1, 2	110.7 ± 3.96 ns1, 2	112 ± 3.43 ns1, 2	101.3 ± 9.5**, #	90.67 ± 9.04***, #
	VINC + PROG (4 mg/kg)	107.3 ± 7.2 ns1, 2	116 ± 2.1 ns1, 2	127.3 ± 13 ns1, 2	109.3 ± 3.2 ns1, 2	160.7 ± 21.3 ns1, 2	164.7 ± 19.5 ns1, 2	126.7 ± 19.5 ns1, #	114.7 ± 3.82*,###
Every 4 days	NaCl + CDEX	116 ± 2.1	116 ± 2.1	113.3 ± 2.7	117.3 ± 2.5	118.7 ± 1.7	114.7 ± 2.5	118.7 ± 1.7	130 ± 12.6
	NaCl + PROG (2 mg/kg)	117.3 ± 2.5 ns1	116 ± 2.1 ns1	116 ± 2.9 ns1	117.3 ± 2.5 ns1	118.7 ± 1.7 ns1	117.3 ± 2.5 ns1	118.7 ± 1.7 ns1	131.3 ± 12.4 ns1
	NaCl + PROG (4 mg/kg)	116 ± 2.1 ns1	118.7 ± 3.4 ns1	114.7 ± 2.5 ns1	116 ± 2.9 ns1	112 ± 2.7 ns1	117.3 ± 1.3 ns1	122.7 ± 1.3 ns1	135.3 ± 11.3 ns1
	VINC + CDEX	120 ± 1.8 ns1	117.3 ± 1.3 ns1	120 ± 2.7 ns1	118.7 ± 1.7 ns1	120 ± 1.8 ns1	107.3 ± 7.5 ns1	86 ± 9.5*	57.5 ± 7.3***
	VINC + PROG (2 mg/kg)	121.3 ± 1.7 ns1, 2	118.7 ± 2.7 ns1, 2	121.3 ± 2.7 ns1, 2	118.7 ± 2.7 ns1, 2	122.7 ± 1.3 ns1, 2	117.3 ± 3.2 ns1, 2	84.67 ± 8.73**, ns2	49.22 ± 9.34***, ns2
	VINC + PROG (4 mg/kg)	114.7 ± 3.2 ns1, 2	117.3 ± 2.5 ns1, 2	116 ± 2.1 ns1, 2	117.3 ± 2.5 ns1, 2	112 ± 4 ns1, 2	112 ± 3.4 ns1, 2	102.7 ± 9.9 ns1, 2	92.67 ± 9.32*, ##

Injection frequency of PROG	Drug and dose	D16	D18	D20	D22	D24	D26	D28	D30
Every 2 days	NaCl + CDEX	169.6 ± 19.3	135.2 ± 14.1	133.6 ± 14.5	114.4 ± 1.31	134.4 ± 16.8	136.8 ± 13.95	133.6 ± 14.66	147.2 ± 17.3
	NaCl + PROG (2 mg/kg)	134.7 ± 14.1 ns1	145.3 ± 15.1 ns1	144 ± 15.6 ns1	155.3 ± 16.7*	171.3 ± 15.1 ns1	171.3 ± 15.1 ns1	196 ± 2.5*	200 ± 2.5*
	NaCl + PROG (4 mg/kg)	252 ± 21.47*	258 ± 18.8***	272 ± 17.7***	286 ± 14***	286 ± 14***	272 ± 17.1***	300 ± 0***	286 ± 14***
	VINC + CDEX	34.32 ± 8.16***	25.13 ± 10.27***	23.88 ± 10.63***	22.73 ± 10.95***	29.4 ± 13.48***	28.88 ± 13.03***	28.82 ± 13.06***	29.98 ± 12.16***
	VINC + PROG (2 mg/kg)	82 ± 8.25***, #	63.37 ± 5.47***, #	52.87 ± 7.99***, ns2	67.07 ± 12.31***, #	76 ± 8.07***, ns2	75.33 ± 6.65***, ns2	76.67 ± 6.32***, #	84 ± 7.59***, ##
	VINC + PROG (4 mg/kg)	120 ± 12.52 ns1, ###	117.3 ± 2.5 ns1, ###	124 ± 18.1 ns1, ##	122 ± 16.39 ns1, ##	121.3 ± 2.67 ns1, ###	121.3 ± 1.69 ns1, ###	120 ± 1.79 ns1, ###	120 ± 1.79 ns1,###
Every 4 days	NaCl + CDEX	131.3 ± 12.4	131.3 ± 12.2	118.7 ± 2.7	120 ± 1.8	117.3 ± 1.3	132.7 ± 12	133.3 ± 12.2	142 ± 14.1
	NaCl + PROG (2 mg/kg)	130 ± 12.6 ns1	131.3 ± 12.2 ns1	130.7 ± 10.2 ns1	132.7 ± 12 ns1	139.3 ± 14.8 ns1	153.3 ± 15.6 ns1	160.7 ± 17.7 ns1	168 ± 15.5 ns1
	NaCl + PROG (4 mg/kg)	161.3 ± 19.2 ns1	160 ± 19.7 ns1	175.3 ± 16.9*	185.3 ± 14.7*	192.7 ± 14.2***	192.7 ± 14.2*	218.7 ± 28.2*	219.3 ± 28.8*
	VINC + CDEX	44.3 ± 8.7***	28.4 ± 4.6***	17.7 ± 3.4***	17.8 ± 3.4***	16.5 ± 4.3***	15.05 ± 4.5***	15.15 ± 4.4***	15.6 ± 4.3***
	VINC + PROG (2 mg/kg)	41.13 ± 9.78***, ns2	33.28 ± 9.4***, ns2	26.82 ± 9.93***, ns2	27.3 ± 9.76***, ns2	32.15 ± 12.67***, ns2	33.95 ± 11.96***, ns2	35.97 ± 11.24***, ns2	36.35 ± 10.62***, ns2
	VINC + PROG (4 mg/kg)	90.67 ± 9.04*, #	70 ± 0.89***, ###	70 ± 1.37***, ###	79 ± 7.83*, ###	77.33 ± 7.84**, ###	84 ± 9.47*, ###	84.67 ± 9.2*, ###	100 ± 9.63*, ###

Threshold values in the table represent stimuli or Von Frey filaments (g ± SEM) evoking indisputable nociceptive responses (more than 70% of the time). $n = 6$ per group; *$p < 0.05$, **$p < 0.01$, ***$p < 0.005$. * compared to NaCl + CDEX; # compared to VINC + CDEX; *ns1*, not significant compared to NaCl + CDEX; *ns2*, not significant compared to VINC + CDEX. Control (NaCl + CDEX-treated) and vincristine-treated rats were obtained as described in "Materials and methods"

CDEX 2-hydroxypropyl-β-cyclodextrin, *PROG* progesterone, *VINC* vincristine

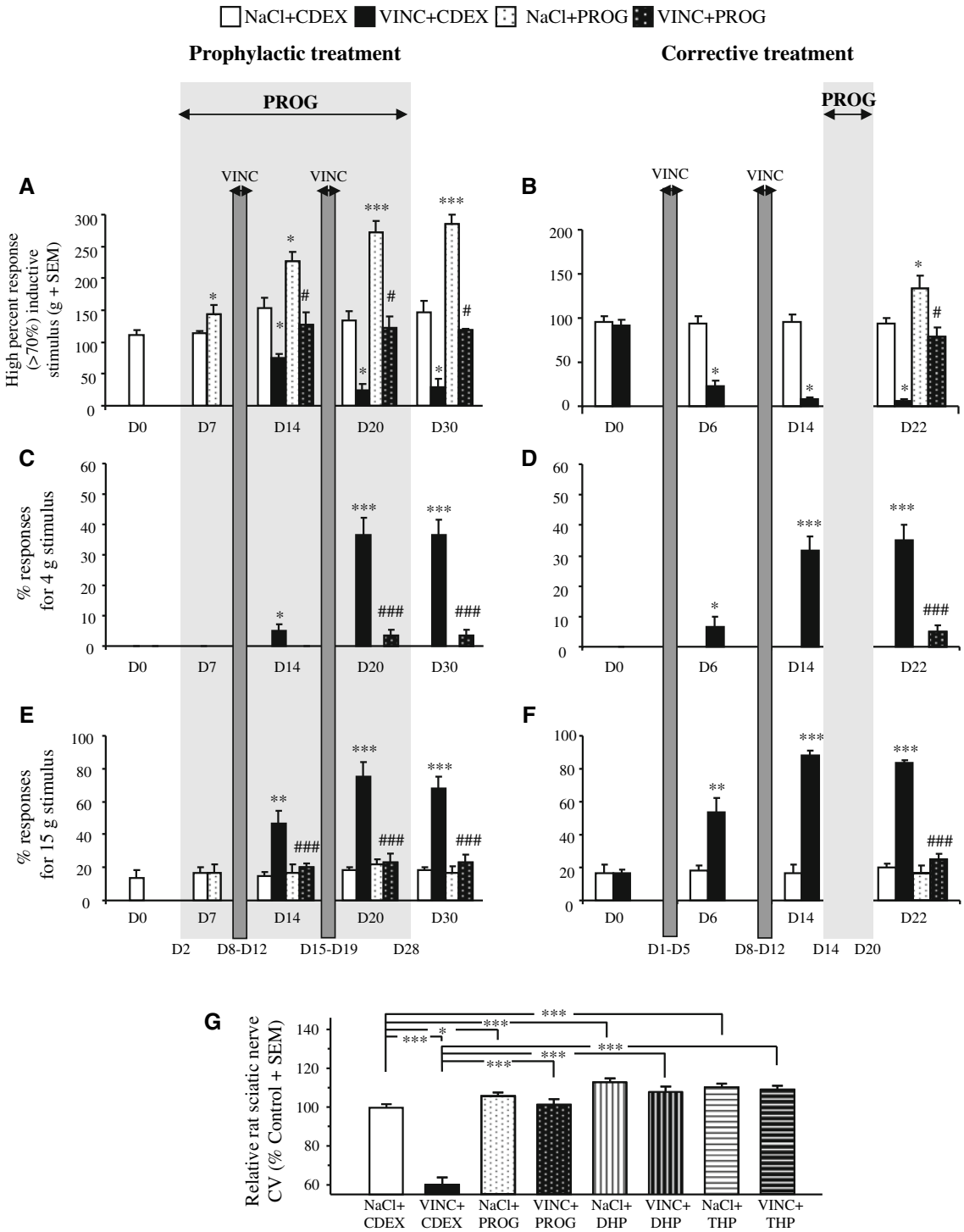


Fig. 3 Effect of progesterone (4 mg/kg per 2 days) prophylactic (**a**, **c**, **e**) or corrective (**b**, **d**, **f**) treatment on vincristine-induced neuropathic pain symptoms. **a**, **b** *Chart bars* (mean + SEM) were obtained with mechanical stimulations (g), which induced more than 70% withdrawal responses in each animal before or after drug administrations ($n = 6$ per group). **c**–**f** Antagonistic effect of progesterone against vincristine-induced mechanical allodynia (**c**, **d**) and hyperalgesia (**e**, **f**). *Chart bars* show the mean + SEM of the percentages of paw withdrawal responses to mechanical stimulation by Von Frey filament 4 g (**c**, **d**) or 15 g (**e**, **f**). $*p < 0.05$, $**p < 0.01$, $***p < 0.005$. *Asterisk* versus NaCl + CDEX, *Hash* versus VINC + CDEX. **g** Effects of progesterone, 5α-DHP and 3α,5α-THP on vincristine-induced conduction velocity (CV) decrease in sciatic nerves. All mean CV and SEM values obtained from rats treated as indicated in the histogram were calculated as % of the mean CV obtained from vehicle treated rats. $*p < 0.05$, $**p < 0.001$, $***p < 0.0001$

of CNPase expression caused by VINC treatment (Fig. 4b, m) was either prevented by prophylactic progesterone administration (Fig. 4d, m) or restored to CNPase basal expression level by corrective progesterone treatment (Fig. 4d, n). In particular, counting of CNPase-positive cells revealed that progesterone induced a 25% increase ($p < 0.05$) while VINC caused a 48% decrease of CNPase expression compared to controls (vehicle-treated rats) ($p < 0.001$). Prophylactic progesterone administration maintained the same level of CNPase expression in sciatic nerves of VINC as in vehicle-treated animals (Fig. 4m). Moreover, the decreased level of CNPase expression (−48%) detected in VINC-treated rats at the end of the two VINC cycles were restored to normal (100%) level after 1 week of corrective progesterone treatment (Fig. 4n).

Effects of progesterone on vincristine-induced axonal degeneration and loss of intraepidermal nerve fibers

We observed that VINC treatment induced 44% decrease of NF200-immunoreactivity in the sciatic nerves of VINC-treated rats ($p < 0.001$) (Fig. 4e, f, q, r). Similarly, we detected 50% decrease of PGP9.5-immunoreactivity or IENF density in hind paw intraplantar skins of VINC-treated rats compared to controls ($p < 0.05$) (Fig. 4i, j, s, t). Prophylactic progesterone administration totally prevented NF200, PGP9.5 or IENF repression in VINC-treated animals ($p < 0.001$ and $p < 0.05$, respectively) (Fig. 4q, s). In addition, decreased level of NF200-immunostaining (−44%) and reduced IENF density (−50%) detected in VINC-treated rats at the end of VINC cycles, were restored to baseline values (100%) after 1 week of progesterone corrective treatment ($p < 0.001$ and $p < 0.05$, respectively) (Fig. 4r, t).

Mechanism of action of progesterone against vincristine-induced painful neuropathy

It is well known that progesterone controls several biological processes through interactions with its classical or nuclear receptor (PR) but it has also been demonstrated that many effects of progesterone on the nervous system require its conversion into neuroactive metabolites such as 5α-DHP and 3α,5α-THP [18]. Therefore, to determine whether antinociceptive and neuroprotective effects exerted by progesterone against VINC-induced painful neuropathy depend on progesterone itself or require its conversion into neuroactive metabolites, we have tested the action of progesterone in the presence of finasteride, a potent inhibitor of 5α-reductase [30, 31]. Indeed, progesterone metabolism into 5α- or 3α,5α-reduced neurosteroids crucially depend on 5α-reductase, which converts progesterone into 5α-DHP prior to the reductive activity of 3α-hydroxysteroid oxido-reductase that transforms 5α-DHP into 3α,5α-THP. We observed that finasteride (25 mg/kg), which did not modify by itself the basal nociceptive thresholds in vehicle- and VINC-treated animals, completely blocked the ability of progesterone to exert antinociceptive or analgesic effect in control or VINC-induced neuropathic pain rats, respectively ($p < 0.005$) (Fig. 5a–c). In addition, the stimulatory effect of progesterone on CNPase expression in control rat nerves dramatically decreased in the presence of finasteride (25 vs. 3%, $p < 0.05$). Therefore, the co-administration of progesterone and finasteride failed to counteract CNPase repression in peripheral nerves of VINC-treated rats ($p < 0.001$) (Fig. 5d). Taken together, these results clearly show that inhibition (with finasteride) of progesterone 5α-reduction completely suppresses antinociceptive and neuroprotective actions exerted by progesterone. To confirm our observations with finasteride, we have also tested the effects of two major 5α-reduced metabolites of progesterone, 5α-DHP and 3α,5α-THP, on VINC-evoked painful neuropathy.

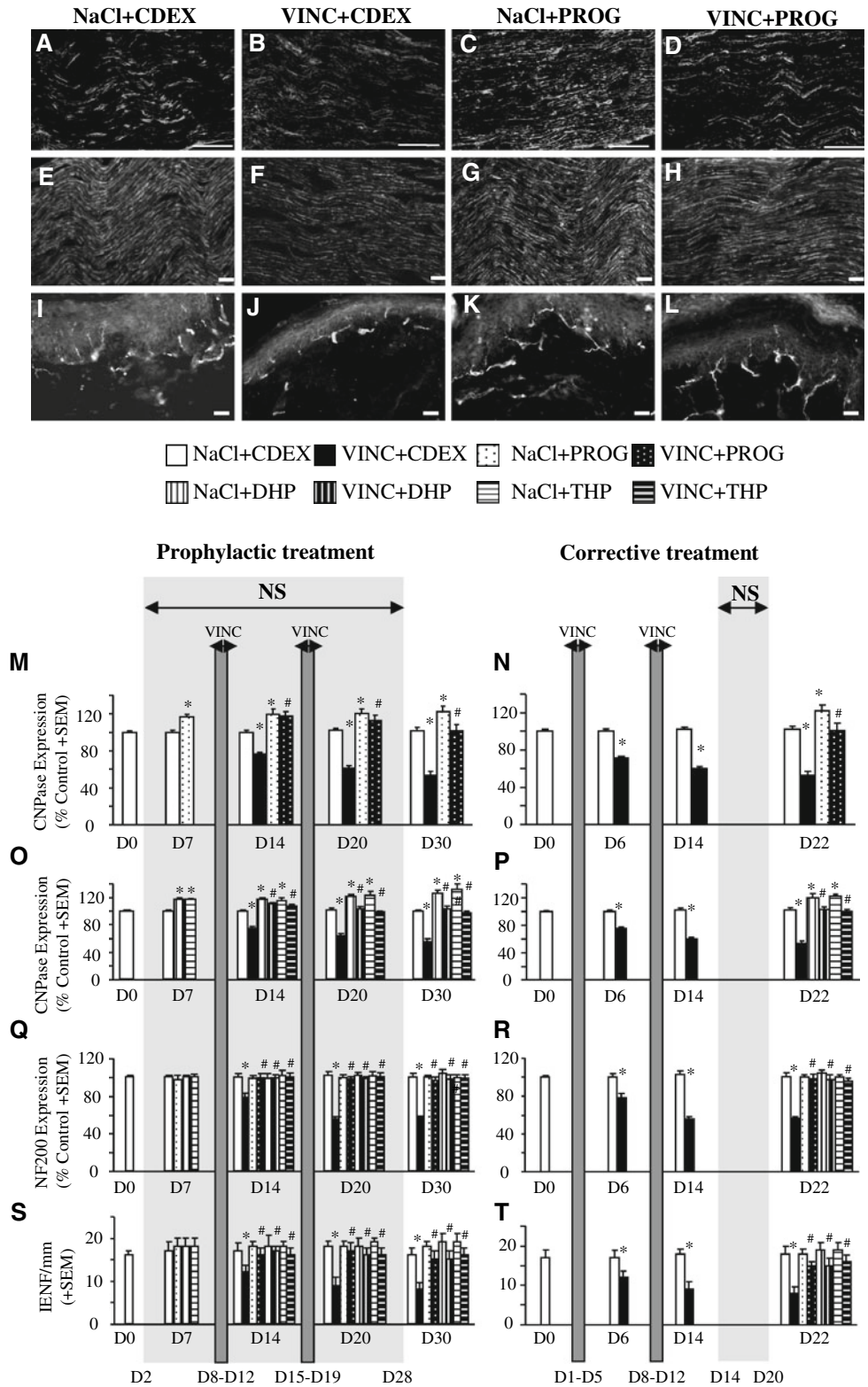
Effects of 5α-reduced neurosteroids (5α-DHP and 3α,5α-THP) on behavioral, electrophysiological, biochemical, and neurochemical parameters altered by vincristine

Action of 5α-DHP or 3α,5α-THP against vincristine-induced mechanical hyperalgesia and allodynia

Prophylactic administration of 5α-DHP or 3α,5α-THP (4 mg/kg every 2 days) efficiently prevented VINC-induced mechanical allodynia and hyperalgesia ($p < 0.01$) (Fig. 6a, c, e). When painful symptoms are already installed in VINC-treated rats, corrective treatment with 5α-DHP or 3α,5α-THP suppressed allodynic and hyperalgesic symptoms by restoring normal mechanical threshold values ($p < 0.01$) (Fig. 6b, d, f). As previously shown for progesterone, prophylactic or corrective treatments with 5α-DHP or 3α,5α-THP definitively suppressed neuropathic pain symptoms which did not reappear after withdrawal of neurosteroid injections (D22 and D30 on Figs. 3, 6).

Effects of 5α-DHP or 3α,5α-THP against vincristine-induced nerve CV alteration

As shown in Fig. 3g, 5α-DHP and 3α,5α-THP treatments were both capable of reproducing similar effects as progesterone including the reverse action on VINC-induced $CV_{latency}$ decrease.



Effects of 5α-DHP or 3α,5α-THP on CNPase expression

The neurosteroids 5α-DHP and 3α,5α-THP were both capable of stimulating CNPase-immunostaining in naive rat sciatic nerves ($p < 0.05$) (Fig. 4o, p). VINC-induced decrease of CNPase expression was prevented by prophylactic administration of 5α-DHP or 3α,5α-THP ($p < 0.01$) (Fig. 4o). Moreover, corrective treatments with 5α-DHP or

◀ **Fig. 4** **a–l** Photomicrographs of sagittal sections of sciatic nerves (**a–h**) or hind paw skins (**i–l**) dissected from NaCl + CDEX (**a**, **e**, **i**)-, NaCl + PROG (**c**, **g**, **k**)-, VINC + CDEX (**b**, **f**, **j**)- or VINC + PROG (**d**, **h**, **l**)-treated rats. *Scale bar* 100 μm. **m–t** Neuroprotective effects of prophylactic (**m**, **o**, **q**, **s**) or corrective (**n**, **p**, **r**, **t**) neurosteroid treatment against vincristine-induced alterations in sciatic nerves and intraplantar skin. **m–p** *Chart bars* show the numbers of CNPase-immunoreactive Schwann cell bodies detected in sciatic nerve sections dissected from NaCl + CDEX-, VINC + CDEX-, NaCl + PROG (**m**, **n**)-, VINC + PROG (**m**, **n**)-, NaCl + DHP (**o**, **p**)-, VINC + DHP (**o**, **p**)-, NaCl + THP (**o**, **p**)- or VINC + THP (**o**, **p**)-treated rats ($n = 6$ per group). Each value is expressed as percent (+SEM) of CNPase-positive cell bodies counted in sciatic nerve sections of control (NaCl + CDEX-treated) rats. $^{*}p < 0.05$ versus NaCl + CDEX, $^{\#}p < 0.01$ versus VINC + CDEX. **q–t** *Graphs* show NF200 expression in sciatic nerve sections (**q**, **r**) or IENF density in intraplantar skin sections (**s**, **t**) dissected from NaCl + CDEX-, VINC + CDEX-, NaCl + PROG-, VINC + PROG-, NaCl + DHP-, VINC + DHP-, NaCl + THP- or VINC + THP-treated rats. **q**, **r** Each value is expressed as percent (+SEM) of NF200-immunolabeling density detected in sciatic nerve sections of NaCl + CDEX-treated rats ($n = 6$ per group). $^{*}p < 0.01$ versus NaCl + CDEX, $^{\#}p < 0.001$ versus VINC + CDEX. **s**, **t** Each value is expressed as mean (+SEM) of IENF density counted in hind paw skin sections ($n = 4$ per group). $^{*}p < 0.05$ versus NaCl + CDEX, $^{\#}p < 0.05$ versus VINC + CDEX

3α,5α-THP were capable of reversing to control values decreased level of CNPase-immunoreactivity in the sciatic nerves of VINC-treated animals ($p < 0.01$) (Fig. 4p).

Effects of 5α-DHP or 3α,5α-THP on axonal degeneration and IENF density

In a similar manner to progesterone, prophylactic or corrective treatments with 5α-DHP and 3α,5α-THP were respectively able to prevent VINC-induced NF200 and IENF repression ($p < 0.001$ and $p < 0.05$) or to reverse to normal values decreased levels of NF200-immunoreactivity and IENF in sciatic nerves or intraplantar skin of VINC-treated rats ($p < 0.001$ and $p < 0.05$) (Fig. 4q–t).

Preservation of the potent antitumor action of vincristine in the presence of neurosteroids

To verify whether progesterone-derived 5α-neurosteroids do not interfere with the essential anti-tumor action of VINC, we have tested the effects of VINC combined with progesterone, 5α-DHP or 3α,5α-THP on the tumor volume and serum level of VEGF in a rat experimental model of skin cancer [32]. Firstly, we found that the treatment with VINC alone led to a complete disappearance of the tumor located on the right flank of skin cancer rats ($p < 0.001$) while progesterone, 5α-DHP or 3α,5α-THP administered alone was unable to reduce the tumor volume (Fig. 7a–g). When VINC was combined with progesterone, 5α-DHP or 3α,5α-THP, the potent anti-tumor action of VINC was totally preserved ($p < 0.001$) (Fig. 7g). Similarly, we observed that the increased VEGF serum concentration detected in tumor-bearing rats significantly decreased after the treatment with VINC alone or the co-treatment with VINC and progesterone, 5α-DHP or 3α,5α-THP ($p < 0.001$) (Fig. 7h). When administered alone, progesterone and its 5α-reduced metabolites were unable to modify VEGF serum level in tumor-bearing and control rats (Fig. 7h). Moreover we found that besides its potent anti-tumor effect (Fig. 7), VINC reproduced painful peripheral neuropathy (mechanical allodynia, hyperalgesia, $CV_{latency}$ decrease, CNPase and NF200 repression in sciatic nerves as well as IENF density reduction) in skin cancer rats (Fig. 8). Combination of VINC therapy with progesterone, 5α-DHP, or 3α,5α-THP preserved the potent anti-tumor action of VINC (Fig. 7) but completely suppressed VINC-evoked painful neuropathic side effects (Fig. 8). Indeed, progesterone-derived neurosteroids, which antagonized VINC-induced mechanical allodynia ($p < 0.01$) (Fig. 8b) and hyperalgesia ($p < 0.01$) (Fig. 8c), also counteracted CNPase, NF200, and IENF repression ($p < 0.005$, $p < 0.005$, and $p < 0.01$, respectively) (Fig. 8d–f) caused by VINC therapy in peripheral nerves and hind paw skins of tumor-bearing rats.

Discussion

By treating naive or skin cancer rats with VINC we observed that this commonly used antineoplastic drug caused neuropathic pain symptoms and several peripheral nerve alterations including the repression of the myelin protein CNPase (which is crucial for axon survival), a decrease of IENF density, and a down-regulation of NF200 (the specific axonal marker) [23, 24, 29, 33]. In agreement with our observations, axonal degeneration, IENF decrease or sensory terminal arbor dysregulation have previously been described in antineoplastic-treated animals or patients [12, 34, 35]. However, VINC-induced down-regulation of CNPase expression in peripheral nerves in vivo has never been demonstrated, and the present report constitutes the first one providing this crucial information. Therefore, when the specific contribution provided by our work is taken together with the previously published data mentioned above, it clearly appears that VINC generates painful neuropathy by causing in peripheral nerves biochemical and neuroanatomical/neurochemical abnormalities such as the repression of CNPase, the dysregulation of interactions between primary afferents, and epidermal tissues as well as the disorganization of axonal constitutive elements including NF200. Spontaneous discharges of primary sensory afferents are known to be capable of triggering central sensitization at the origin of abnormal stimulation-induced pain sensations [36–38]. Therefore, VINC-evoked biochemical and neuroanatomical/neurochemical alterations in

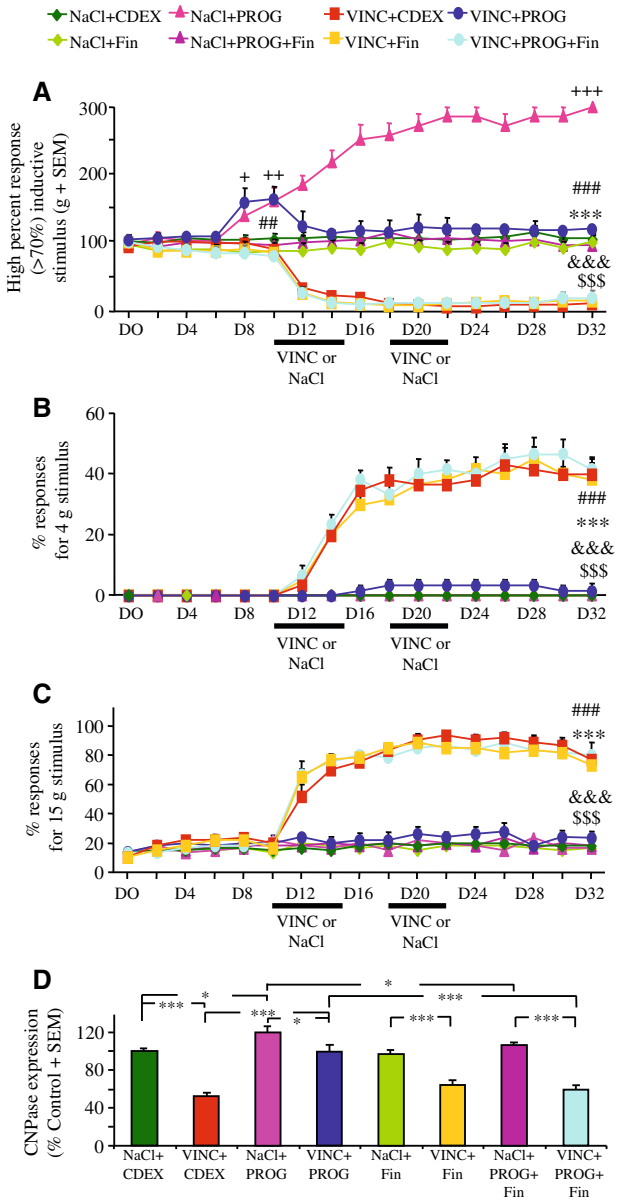


Fig. 5 Effects of finasteride (inhibitor of progesterone conversion into 5α-metabolites) or progesterone + finasteride on vincristine-induced painful neuropathy in naive rats. **a** The *curves* (mean + SEM) were obtained with mechanical stimulations (g), which induced more than 70% withdrawal responses in each animal before or after drug administrations ($n = 6$ per group). **b**, **c** Absence of action of finasteride (Fin) or progesterone + finasteride on vincristine-induced mechanical allodynia (**b**) and hyperalgesia (**c**). *Graphs* show the mean + SEM of the percentages of paw withdrawal responses to mechanical stimulation by Von Frey filament 4 g (**b**) or 15 g (**c**). $*p < 0.05$, $**p < 0.01$, $***p < 0.005$ at each time point from D12 to D32 (**a**, **c**) or from D14 to D32 (**b**). *Asterisk* NaCl + CDEX versus VINC + CDEX; *Plus* NaCl + CDEX versus NaCl + PROG; *Hash* VINC + CDEX versus VINC + PROG; *Dollar* NaCl + Fin versus VINC + Fin; *Ampersand* NaCl + PROG + Fin versus VINC + PROG + Fin. **d** Effects of Fin (25 mg/kg) alone or progesterone (4 mg/kg) + Fin on the numbers of CNPase-positive Schwann cell bodies detected in sciatic nerve sections dissected from NaCl + CDEX-, VINC + CDEX-, NaCl + PROG-, VINC + PROG-, NaCl + Fin-, VINC + Fin-, NaCl + PROG + Fin-, or VINC + PROG + Fin-treated rats ($n = 6$ per group). Each value is expressed as percent (+SEM) of CNPase-positive cell bodies counted in sciatic nerve sections of control (NaCl + CDEX-treated) rats. $*p < 0.05$, $***p < 0.001$

peripheral nerves may result in ectopic discharges leading to painful sensations. Consistently, it has been demonstrated that VINC-induced allodynia and hyperalgesia are associated with decreased peripheral nerve CV and ectopic discharges [5, 39]. In perfect concordance with these data, our electrophysiological and behavioral studies have clearly evidenced a significant $CV_{latency}$ reduction and a marked mechanical allodynia and hyperalgesia in VINC-treated rats. Interestingly, we observed that, contrary to the mechanical threshold, the heat thermal sensitivity was not modified by VINC, suggesting that anticancer drugs may selectively differentiate between mechanical and thermal components of pain.

Progesterone, which exerts trophic and neuroprotective effects, also promotes myelination in the central and peripheral nervous systems [18, 19, 40]. Several effects exerted by progesterone require its conversion into 5α-DHP (which interacts with PR) and 3α,5α-THP (which is a potent positive allosteric modulator of $GABA_A$ receptors) [18, 19, 41]. Because we found that endogenous regulation of progesterone metabolism in the spinal cord by substance P determines pain sensations [21, 22] and other evidence showed that progesterone and/or its metabolites may be safe and effective treatments for traumatic brain injury in humans [17], we hypothesized that progesterone and its derived neurosteroids may counteract VINC-induced painful neuropathy. Our results clearly show that progesterone (4 mg/kg per 2 days), which suppressed the mechanical hyperalgesia and allodynia evoked by VINC treatment, also counteracted all biochemical, neuroanatomical/neurochemical, electrophysiological, and functional alterations induced in peripheral nerves by VINC. Taken together, these observations clearly support our hypothesis stipulating the existence of a protective action of progesterone against VINC-induced painful neuropathy. In addition, our results demonstrate that the inhibition of progesterone conversion into 5α-reduced metabolites (5α-DHP and 3α,5α-THP) by finasteride [30, 31] completely blocked antinociceptive, analgesic, and neuroprotective effects exerted by progesterone against VINC-induced painful neuropathy. In fact, in the absence of finasteride, progesterone may be converted to the potent PR agonist 5α-DHP [42] and to 3α,5α-THP, which is also neuroprotective [19], allowing a strong stimulation or an optimal functioning of biochemical and neuroanatomical/neurochemical components in peripheral nerves such as CNPase, axonal constitutive proteins/transporters and IENF. In the presence

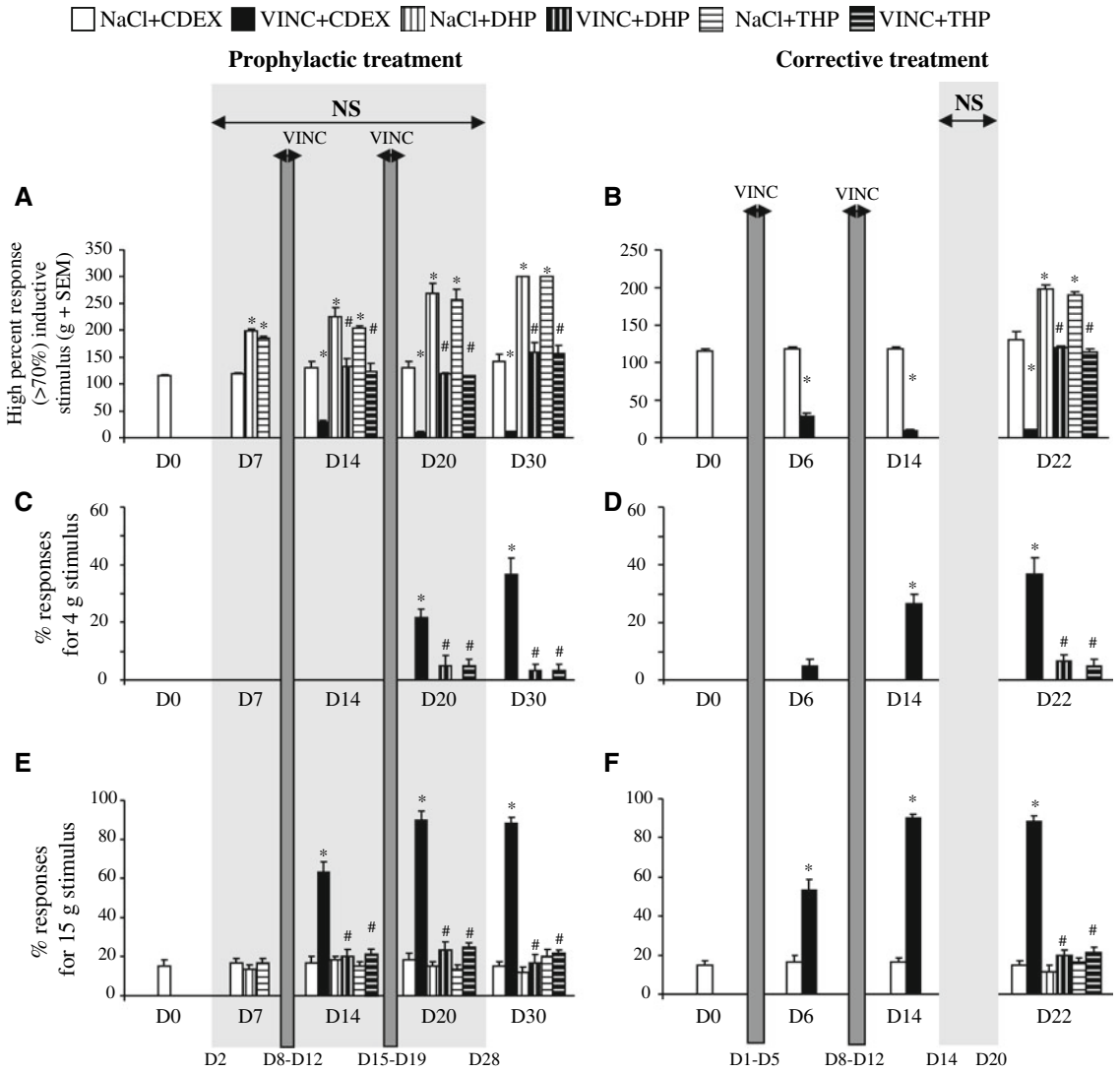


Fig. 6 Effects of prophylactic (**a**, **c**, **e**) or corrective (**b**, **d**, **f**) treatment with 5α-DHP or 3α,5α-THP (4 mg/kg per 2 days) on vincristine-induced painful neuropathy. **a**, **b** *Chart bars* (mean + SEM) were obtained with mechanical stimulations (g), which induced more than 70% withdrawal responses in each animal before or after drug administrations ($n = 6$ per group). **c**–**f** Antagonistic effect of 5α-DHP or 3α,5α-THP against vincristine-induced mechanical allodynia (**c**, **d**) and hyperalgesia (**e**, **f**). *Chart bars* show the mean + SEM of the percentages of paw withdrawal responses to mechanical stimulation by Von Frey filament 4 g (**c**, **d**) or 15 g (**e**, **f**). $^{*}p < 0.01$ versus NaCl + CDEX, $^{\#}p < 0.01$ versus VINC + CDEX

of finasteride, progesterone (or its metabolites that are not 5α-reduced), exerted only 3% increase on CNPase expression in control rat nerves and this slight stimulatory action failed to reverse the severe CNPase repression evoked by VINC treatment. In agreement with our observations, 5α-DHP also restored to normal values decreased levels of myelin-associated glycoprotein, peripheral myelin protein 22, and myelin basic protein in sciatic nerves of docetaxel-treated rats while progesterone rescue effect was weak or undetectable [43]. Altogether, these results show that the conversion into 5α-reduced metabolites is crucial for the expression of progesterone analgesic and neuroprotective effects. Indeed, progesterone-derived 5α-steroids such as 5α-DHP and 3α,5α-THP were both capable of counteracting painful symptoms and peripheral nerve disorganization or alterations evoked by VINC. Interestingly, it is well known that 5α-DHP may be interconverted into 3α,5α-THP by 3α-hydroxysteroid oxido-reductase abundantly expressed in the nervous system [21, 44]. Therefore, each one of the following situations, (1) progesterone administered alone, (2) 5α-DHP alone or (3) 3α,5α-THP alone, may generate substantial endogenous concentrations of 5α-DHP and 3α,5α-THP, which can exert neuroprotective and/or analgesic effects through PR (5α-DHP) and/or $GABA_A$ receptors (3α,5α-THP) [18, 19, 22, 41, 45]. In agreement with this suggestion, recent investigations have shown

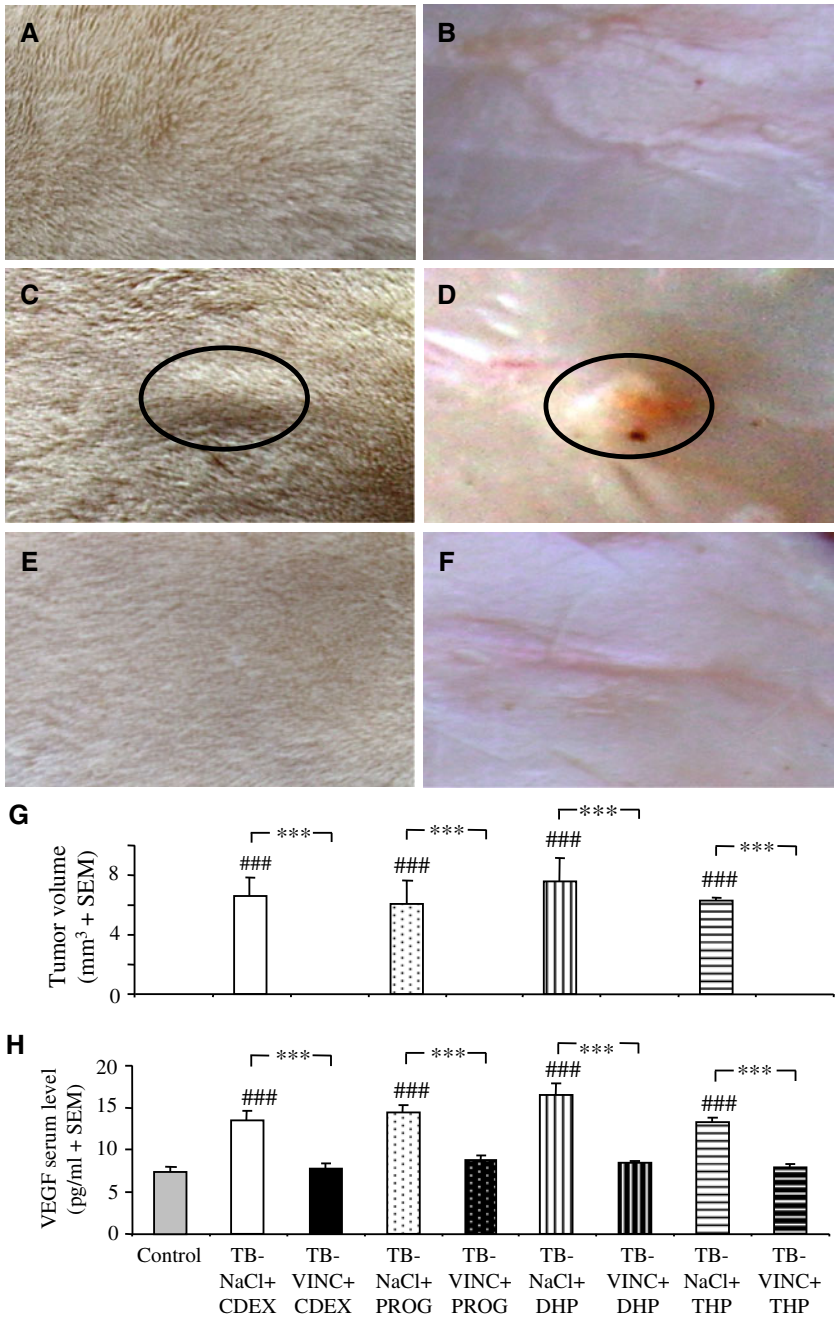
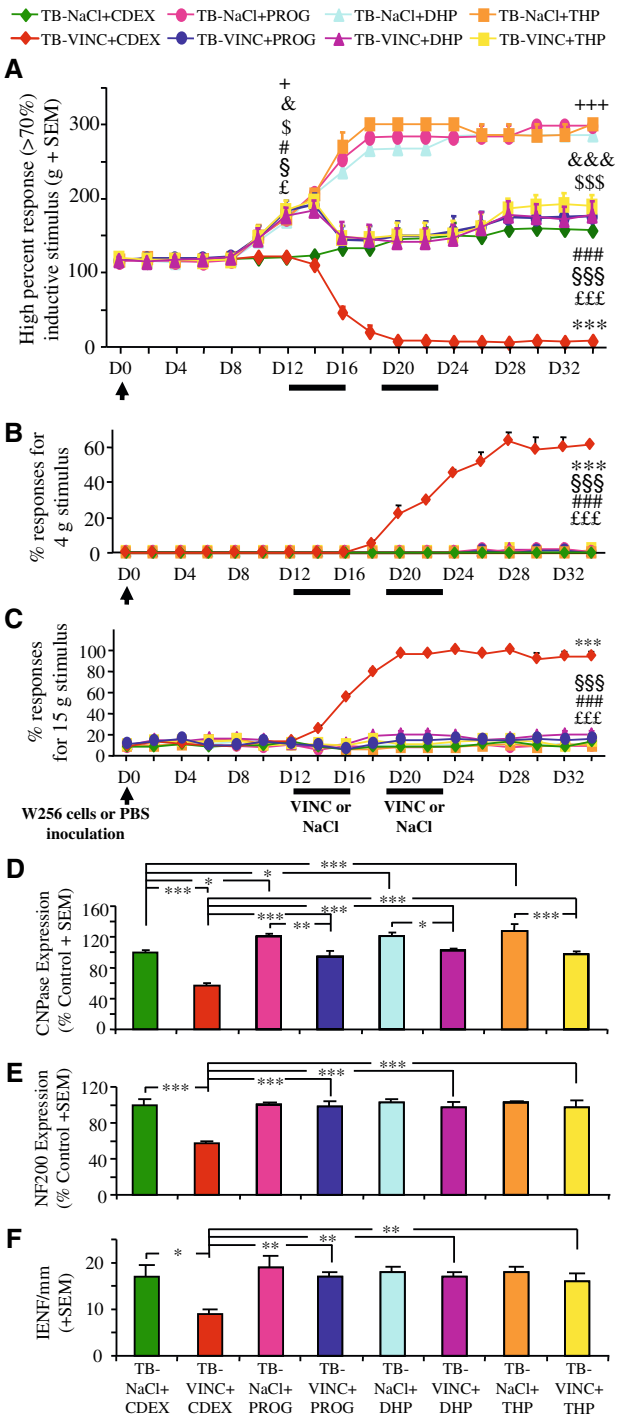


Fig. 7 Effects of vincristine, progesterone, 5α-DHP or 3α,5α-THP on tumor volume and VEGF serum level in tumor-bearing (TB) rats. **a**–**f** External (**a**, **c**, **e**) and internal (**b**, **d**, **f**) views of the posterior right flanks of TB and control rats 34 days after W256 carcinosarcoma cells or PBS inoculation. **a**, **b** Photomicrographs of the external (**a**) or internal (**b**) skin in the right flank of control animals (injected with PBS) showing the absence of tumor. **c**, **d** Photomicrographs of the external (**c**) or internal (**d**) skin in the right flank of tumor-bearing animals injected with W256 cells. The *black circle* shows the tumor location. **e**, **f** Treatment of skin cancer rats with vincristine completely suppressed the tumor, which disappeared on the external (**e**) and internal (**f**) sides. **g** Vincristine treatment alone or associated with progesterone, 5α-DHP or 3α,5α-THP (4 mg/kg) completely suppressed the tumor while the administration of progesterone, 5α-DHP, or 3α,5α-THP alone did not affect the tumor volume. Each *bar* represents the mean (+SEM) of six tumor volumes. ***$p < 0.001$; *Hash* a significant difference compared to control rats inoculated with PBS. **h** Vincristine treatment alone or associated with progesterone, 5α-DHP or 3α,5α-THP (4 mg/kg) restored baseline/physiological values of VEGF serum levels, which increased significantly in TB rats. Injections of progesterone, 5α-DHP, or 3α,5α-THP alone did not modify increased or baseline levels of VEGF detected in TB or control rats, respectively. Each *bar* represents the mean level (+SEM) of four dosages performed in duplicate. ***$p < 0.001$; *Hash* a significant difference compared to control rats inoculated with PBS



◀ **Fig. 8** Effects of neurosteroids on vincristine-induced pain symptoms and cellular alterations (CNPase and NF200 repression as well as IENF density decrease) in peripheral nerves of tumor-bearing (TB) rats. Progesterone, 5α-DHP, 3α,5α-THP (4 mg/kg) or the vehicle CDEX was administered every 2 days to vincristine- and NaCl-treated TB rats. **a** The *curves* (mean + SEM) were obtained with mechanical stimulations (g), which induced more than 70% withdrawal responses in each animal before or after drug administrations ($n = 6$ per group). **b**, **c** Antagonistic effects of progesterone, 5α-DHP, or 3α,5α-THP against vincristine-induced mechanical allodynia (**b**) and hyperalgesia (**c**) in TB rats. *Graphs* show the mean + SEM of the percentages of paw withdrawal responses to mechanical stimulation by Von Frey filament 4 g (**b**) or 15 g (**c**). $*p < 0.05$, $***p < 0.01$ at each time point from D14 to D34 (**a**, **c**) or from D20 to D34 (**b**). *Asterisk* TB-NaCl + CDEX versus TB-VINC + CDEX; *Plus* TB-NaCl + CDEX versus TB-NaCl + PROG; *Ampersand* TB-NaCl + CDEX versus TB-NaCl + DHP; *Dollar* TB-NaCl + CDEX versus TB-NaCl + THP; *Hash* TB-VINC + CDEX versus TB-VINC + PROG; *Sect* TB-VINC + CDEX versus TB-VINC + DHP; *Pound* TB-VINC + CDEX versus TB-VINC + THP. **d**, **e** Effect of progesterone, 5α-DHP, or 3α,5α-THP (4 mg/kg) on the numbers of CNPase-positive Schwann cell bodies (**d**) or on NF200 expression (**e**) detected in sciatic nerve sections dissected from NaCl + CDEX-, VINC + CDEX-, NaCl + PROG-, VINC + PROG-, NaCl + DHP-, VINC + DHP-, NaCl + THP- or VINC + THP-treated TB rats ($n = 6$ per group). Each value is expressed as percent (+SEM) of CNPase-positive cell bodies counted (**d**) or of NF200-immunolabeling density detected (**e**) in sciatic nerve sections of control (NaCl + CDEX-treated) TB rats. $*p < 0.05$, $**p < 0.01$, $***p < 0.005$. **f** Comparative analysis of IENF density measured in hind paw skin sections dissected from NaCl + CDEX-, VINC + CDEX-, NaCl + PROG-, VINC + PROG-, NaCl + DHP-, VINC + DHP-, NaCl + THP- or VINC + THP-treated TB rats ($n = 4$ per group). Each value is expressed as mean (+SEM) of IENF density counted in hind paw skin sections. $*p < 0.05$, $**p < 0.01$

beneficial actions of $GABA_A$ receptor agonists against experimental neuropathic symptoms [22, 46–48]. Because we used two different strategies of treatments (prophylactic and corrective) and investigated also time- and dose-dependent effects of progesterone, 5α-DHP and 3α,5α-THP, we are able to suggest the following five ideas to clarify the possible relationship between the neuroprotective and analgesic actions of progesterone-derived neurosteroids. (1) Administration of progesterone, 5α-DHP or 3α,5α-THP generated increased endogenous level of 3α,5α-THP, which strongly potentiated the GABAergic system and induced antinociception and analgesia. (2) The analgesic properties of progesterone-derived neurosteroids may explain their ability to prevent painful symptoms (allodynia and hyperalgesia) in animals that received subsequently VINC treatment (prophylactic neurosteroid injections). These potent analgesic properties can also explain the capacity of corrective neurosteroid administrations to counteract allodynic and hyperalgesic symptoms persisting in VINC-treated animals several days after the end of VINC treatment. (3) If progesterone, 5α-DHP, or 3α,5α-THP merely exerted analgesic (not neuroprotective) action, these neurosteroids can only induce a transient (not definitive) prevention or suppression of VINC-induced painful neuropathic symptoms. Indeed, after the catabolism and clearance of injected progesterone-derived neurosteroids, the GABA-related inhibition evoked by these neurosteroids will decrease, and painful neuropathic symptoms may reappear in VINC-treated rats in which neuroanatomical/neurochemical and functional alterations of peripheral nerve remained present. (4) Because prophylactic neurosteroid injections prevented neuroanatomical/neurochemical

and functional alterations (neuroprotective action) in rats receiving subsequently VINC, these animals did not exhibit painful neuropathic symptoms even after withdrawal of neurosteroid treatment (see D30 on Figs. 3, 4, 6). (5) More importantly, because corrective treatments with progesterone, 5α-DHP, and 3α,5α-THP reversed to normal values decreased levels of CNPase, NF200, and IENF in VINC-treated rat peripheral nerves (neuroprotective effects and/or repairing of neuroanatomical/neurochemical alterations), painful neuropathic symptoms existing in these animals before the onset of neurosteroid corrective treatments disappeared after 1 week of neurosteroid administration and the symptoms did not reappear after withdrawal of neurosteroid treatment (see D22 on Figs. 3, 4, 6). Altogether, these data indicate that both analgesic and neuroprotective actions of neurosteroids contributed to suppress definitively painful neuropathic symptoms in VINC-treated animals. Since VINC treatment selectively affected the mechanical and not the heat thermal sensitivity, beneficial effects of progesterone-derived neurosteroids described herein should mainly be related to the suppression of mechanical allodynia and hyperalgesia. However, it is noteworthy that analgesic effects of progesterone on thermal nociception have been reported in other experimental models [49–51]. Moreover, we observed that in neuropathic or VINC-treated rats, progesterone, 5α-DHP, or 3α,5α-THP significantly stimulated CNPase, NF200, and IENF levels in order to reverse these levels to normal values, but in naive animals, only CNPase expression was increased by neurosteroids while NF200 and IENF levels did not change. These results suggest that the expression of CNPase gene may be highly sensitive to the action or cellular signaling evoked by progesterone-derived neurosteroids in nerve cells. The sensitivity of NF200 and PGP9.5 (IENF) to neurosteroids may be enhanced in pathophysiological condition by various transcription or intracellular factors activated by VINC treatment [52].

We have also checked whether the beneficial neuroprotective and analgesic effects of progesterone, 5α-DHP, and 3α,5α-THP against VINC-evoked painful neuropathy may interfere with the essential anti-tumor action of vincristine. Therefore, we developed an experimental model of skin cancer in rats by using Walker 256 carcinosarcoma cells [32, 53]. Since VEGF is well known as an important biomarker of tumor-angiogenesis [54, 55], we focused on VEGF serum level when analyzing the tumor volume regression driven by vincristine in skin cancer rats. We observed that vincristine, which suppressed completely the tumor, also restored baseline/physiological values of VEGF serum levels which were elevated in tumor-bearing rats before the treatment. However, vincristine-based chemotherapy reproduced in skin cancer rats painful neuropathic symptoms such as mechanical allodynia, hyperalgesia, biochemical, neuroanatomical/neurochemical and functional alterations of peripheral nerves. When progesterone, 5α-DHP, or 3α,5α-THP alone was administered to skin cancer rats, no change of the tumor volume or VEGF serum concentration was observed, indicating that progesterone and its 5α-reduced metabolites are completely devoid of anti-tumor or pro-tumor activity, at least in the experimental model for intradermal carcinosarcoma. Therefore, we combined vincristine therapy with progesterone-derived neurosteroids and we observed that these neurosteroids counteracted completely vincristine-induced painful neuropathic symptoms without altering the potent anti-tumor action of vincristine, which led to the tumor suppression in skin cancer rats.

Microtubule disorientation may also be induced in unmyelinated sensory axons by long-term vincristine treatments but microtubule alterations remained discrete in peripheral nerves after a short treatment with moderate doses as used herein [56]. Therefore, it is difficult to predict whether progesterone-derived neurosteroids may also be effective against microtubule disorganization-evoked peripheral neuropathy. Since elegant studies have shown that pregnenolone (the precursor of progesterone) binds to microtubule-associated protein 2 and stimulates microtubule assembly [57, 58], it appears reasonable to suggest that pregnenolone or its analogs may also offer alternative opportunities against microtubule alteration-evoked peripheral neuropathies caused by repetitive/high doses or long-term antineoplastic treatments [16, 56, 59].

Taken together, our results open interesting possibilities for the improvement of cancer chemotherapy with novel strategies combining antineoplastic drugs and neuroprotective or analgesic neurosteroids such as progesterone, 5α-DHP, and 3α,5α-THP. The combined or adjunctive therapy associating neurosteroids and anticancer drugs may safely be used as a prophylactic strategy to prevent chemotherapy-induced neuropathic side-effects in patients developing non-hormone-dependent cancers. For precautionary measures, this prophylactic strategy should not be extended to patients with hormone-sensitive cancers, even though progesterone-derived 5α-neurosteroids did not affect the tumor volume and VEGF blood level in skin-cancer rats. Contrary to the prophylactic approach, which must be restricted to specific groups of patients, corrective therapy based on 5α-reduced neurosteroids may be used in a wide variety of patients to suppress painful neuropathic symptoms that persist several months after the end of chemotherapeutic treatments and complete eradication of tumors.

Acknowledgments This work was supported by grants from Association Ti'toine (Normandie, France) and Université de Strasbourg (France). L.M. was a postdoctoral fellow supported by Association Ti'toine (Normandie, France).

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