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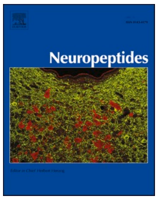

Increased expression of dendrin in the dorsal horn of the spinal cord during stress is regulated by sex hormones

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ABSTRACT

Chronic stress has various effects on organisms and is sex-specific. The aim of the study was to describe the expression of synapse strengthening protein, dendrin, in the spinal cord (SC) and the dependence of its expression on chronic stress and sex hormones. Thirteen-month-old female and male rats were castrated (ovariectomy [F-OVX] or orchidectomy [M-ORX]) or sham-operated (F-SH or M-SH), respectively. At age 15 months, three 10-day-sessions of sham stress (control, C) or chronic stress (S) were conducted. Dendrin expression was present in the thoracic SC segments and the dorsal root ganglia (DRG). In the SC, dendrin expression was prominent in superficial laminae of the dorsal horn and lamina X (central canal). The M-ORX-S group had the highest dendrin expression in the dorsal horn, being significantly higher than the M-ORX-C or M-SH-S groups ($P < 0.05$). Dendrin expression was significantly higher in the F-SH-S group than the F-SH-C group ($P < 0.05$), as well as in the F-SH-S than the M-SH-S ($P < 0.05$). Co-localization with the α-d-galactosyl-specific isolectin B4 (IB4) in central projections of the DRG neurons in the dorsal horn of the SC was 7.43 ± 3.36%, while with the calcitonin gene-related peptide (CGRP) was 8.47 ± 4.45%. Localization of dendrin was observed in soma and nuclei of neurons in the dorsal horn. Dendrin expression in pain-processing areas of the SC, the DRG neurons and their peripheral projections suggest possible roles in pain perception and modulation. Stress-induced increase in dendrin expression and its dependence on sex hormones may partially explain sex-specific pain hypersensitivity induced by stress.

1. Introduction

Chronic stress is a situation in which an individual continually experiences a challenging emotional and physiological event, over a long period of time (Myslivecek and Kvetnansky, 2006). Responses to stress, and to pain, play protective roles for the purpose of maintaining the internal environment of the body (Lunde and Sieberg, 2020). However, when those two responses become chronically challenged, due to prolonged activation of afferent pathways, maladaptive neuroplasticity in the areas linked to the nociceptive processing occurs (Abdallah and

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Abbreviations: AA, aromatase activity; AMPARs, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors; CGRP, calcitonin gene-related peptide; DRG, dorsal root ganglia; F-OVX-C, female ovariectomized control (sham stress) group; F-OVX-S, female ovariectomized chronic stress group; F-SH-C, female sham-operated control (sham stress) group; F-SH-S, female sham-operated chronic stress group; IB4, α-d-galactosyl-specific isolectin B4; KIBRA, kidney and brain expressed protein; LTP, long-term potentiation; M-ORX-C, male orchidectomized control (sham stress) group; M-ORX-S, male orchidectomized chronic stress group; M-SH-C, male sham-operated control (sham stress) group; M-SH-S, male sham-operated chronic stress group; NF200, neurofilament 200; PGP, protein gene product 9.5, also known as ubiquitin carboxyl-terminal hydrolase-1 (UCH-L1); SC, spinal cord.

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Geha, 2017; Li et al., 2020; Liu et al., 2019; Woda et al., 2016). The maladaptive chronic stress-induced neuroplasticity could explain chronic pain conditions such as irritable bowel syndrome, fibromyalgia, migraine (Greenwood-Van Meerveld and Johnson, 2017; Woda et al., 2016), and other pain involving syndromes often associated with anxiety or depressive disorders (Ji et al., 2018). A considerable body of evidence indicates the presence of gender differences in responses to stress and pain (Elzahaf et al., 2016; Mills et al., 2019). Basic and clinical studies of the past decade have given attention to the fluctuation of gonadal hormones as a major reason for gender differences in nociceptive sensitivity to acute, inflammatory and neuropathic pain (Aloisi, 2017; Melchior et al., 2016). Although many studies have reported a pro-nociceptive role of estradiol in somatic and visceral pain models (Ji et al., 2011; Spooner et al., 2007), other studies suggested the opposite (Coulombe et al., 2011; Craft, 2007). For example, in the colorectal distension model of visceral pain, the nociceptive role of estradiol depends on which receptor is activated (alpha or beta) (Cao et al., 2012; Ji et al., 2011).

In contrast, findings of the testosterone role in nociception are consistent – its action is protective (antinociceptive) (Aloisi et al., 2003; Aloisi et al., 2004; Fanton et al., 2017; White and Robinson, 2015). The pro-nociceptive role of estradiol and antinociceptive role of testosterone are the results of different changes in spinal excitatory or inhibitory glutamatergic receptors under stress (Ji et al., 2018). All this considered, the prevalence of chronic pain conditions is significantly higher in women and also depends on the female reproductive cycle (Puri et al., 2005; Mills et al., 2019; Woda et al., 2016).

Dendrin is a protein kinase substrate with multiple sites available for phosphorylation assumed to be an important protein for synaptic plasticity (Neuner-Jehle et al., 1996). The role in synaptic plasticity is related to its dendritic localisation in neocortical forebrain neurons (Herb et al., 1997). Dendrin-coding mRNA is transported into the dendrites where the protein synthesis takes place, presumably to ensure rapid and economical delivery of dendrin to where it is needed. This process enables focused switches in synaptic properties (Steward, 1995). To the best of our knowledge, the presence of dendrin protein in the nervous system has been described only in the rat forebrain (Rhyner et al., 1990). As yet, there are no publications of its distribution or potential function in the spinal cord (SC).

Chronic pain is a widespread condition that affects one-quarter of the adult population with serious impacts on quality of life (Abdallah and Geha, 2017; Mills et al., 2019). For most patients, there are still no adequate means for pain management (Abdallah and Geha, 2017). Therefore, a better understanding of the way in which chronic stress leads to maladaptive pain responses that manifest as some form of a chronic pain condition is needed. We hypothesized that dendrin plays a role in maladaptive plasticity and pain perception during chronic stress. Hence, we examined its expression in the SC and the dorsal root ganglia (DRG) and their peripheral projections. Furthermore, we proposed that sex hormones are involved in dendrin expression and sex-specific sensitivity to pain during exposure to chronic stress.

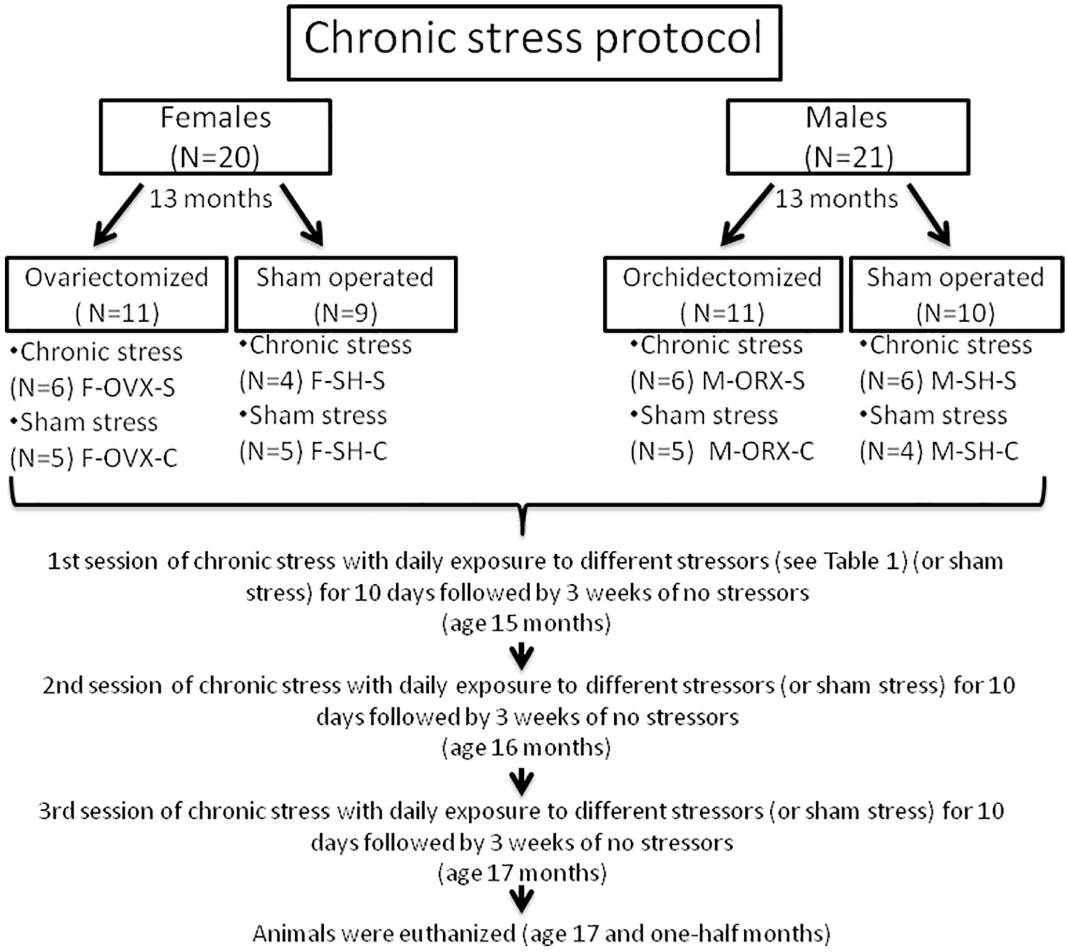

Fig. 1. Study design. F-OVX-S – female ovariectomized chronic stress group; F-SH-S – female sham-operated chronic stress group; F-OVX-C – female ovariectomized control (sham-stress) group; F-SH-C – female sham-operated control (sham stress) group; M-ORX-S – male orchidectomized chronic stress group; M-SH-S – male sham-operated chronic stress group; M-ORX-C – male orchidectomized control (sham-stress) group; M-SH-C – male sham-operated control (sham-stress) group.

2. Materials and methods

2.1. Animal experiment

Twenty female and twenty-one male Sprague Dawley-Charles River rats (Charles River, Wilmington, MA, USA) were used in the study (Fig. 1). Animals were housed in standard cages with two animals per cage at room temperature (21 ± 2 °C), with humidity of 40–60%. Standard laboratory conditions, including rat chow and water ad libitum, and 12 h light/12 h dark cycle, were applied at all times except during some stressors of the chronic stress protocol (see below). Experiments were carried out at the Animal Facility of the Faculty of Pharmacy, University of Szeged, and approved by the National Scientific Ethical Committee on Animal Experimentation in Hungary (approval number: IV./3796/2015).

2.2. Ovariectomy and orchidectomy

At the age of 13 months, the rats were anesthetized with isoflurane (Florane®, Abbott Laboratories Ltd., Queenborough, UK). One group of female rats ($N = 11$) was ovariectomized and one group of male rats ($N = 11$) was orchidectomized, while the remaining of the animals were sham-operated (females: $N = 9$; males: $N = 10$). Ovariectomy of female rats was made after midline abdominal incision, under general anesthesia with isoflurane (Forane® isofluranum, Abbott Laboratories Ltd., Queenborough, UK) (Li et al., 2014; Rodriguez-Landa et al., 2019). Male rats were castrated according to Idris (Idris, 2012). After surgery, food and water were available ad libitum. The animals were closely monitored for 72 h. The stress experienced by the animals during surgery was considered irrelevant because the surgeries were performed at least 4 weeks before the chronic stress protocol, as previously described (Balog et al., 2015a).

2.3. Chronic stress protocol

Two months after the surgeries (age 15 months), the ovariectomized, orchidectomized and sham-operated groups of animals were divided into two groups as follows (Fig. 1): a chronic stress group with 22 rats (6 ovariectomized [F-OVX-S] and 4 sham-operated females [F-SH-S]; 6 orchidectomized [M-ORX-S] and 6 sham-operated males [M-SH-S]) and a sham stress (control) group with 19 rats (5 ovariectomized [F-OVX-C] and 5 sham-operated females [F-SH-C]; 5 orchidectomized [M-ORX-C] and 4 sham-operated males [M-SH-C]).

The chronic stress protocol consisted of three separate sessions, each lasting 10 days (Balog et al., 2015a; Balog et al., 2015b; Ivic et al., 2016). The rats were subjected to a different stressor (see below and Table 1) or to sham stress each day. Rats in the sham stress groups were exposed to the same environment as the chronic stress groups, but without the stressors (except for the food deprivation and glucose tolerance test [GTT] stressors). After each session, there was a period of three weeks with no stressors.

Table 1
Daily stressors that were applied to rats in the chronic stress groups[†] during the three sessions of the chronic stress protocol.

Day	Stressors during 1st Session	Stressors during 2nd and 3rd Sessions
1.	Food deprivation (12*h*)	Cage rotation (40 min)
2.	GTT[‡]	Swim test
3.	Cold restraint (+4 °C, 60 min) + food deprivation	Cold restraint (+4 °C, 60 min) + food deprivation
4.	GTT	Noise overnight (9:00 pm - 9:00 am)
5.	Light overnight (9:00 pm - 9:00 am)	Light overnight (9:00 pm - 9:00 am)
6.	Cage rotation (40 min)	Cage rotation (40 min)
7.	Swim test	Swim test
8.	Noise overnight (9:00 pm - 9:00 am)	Noise overnight (9:00 pm - 9:00 am)
9.	Cold restraint (+4 °C, 60 min) + food deprivation	Cold restraint (+4 °C, 60 min) + food deprivation
10.	GTT	GTT

[†] Chronic stress groups ($N = 22$) included 6 ovariectomized female rats, 4 sham-operated female rats, 6 orchidectomized male rats and 6 sham-operated male rats.

[‡] Glucose tolerance test.

The stressors included (Table 1): (i) *Food deprivation* for 12 h in the chronic stress groups, but no comparable stressor in the sham stress groups; (ii) *GTT* in the chronic stress groups, but no comparable stressor in the sham stress groups; (iii) *Cold restraint*, in which the chronic stress groups were restrained in metal tubes for 60 min in a cold room at +4 °C, whereas the sham stress groups was just exposed to tubes in the same room at ambient temperature; (iv) *Light overnight,* in which the lights were on from 9:00 pm – 9:00 am for the chronic stress groups, whereas the sham stress groups were also under the lights but with half of the cage covered; (v) *Noise overnight,* in which alarms sounded at irregular intervals from 9:00 pm–9:00 am for the chronic stress groups, whereas radio music was played at low volume in the background during same time period for the sham stress groups; (vi) *Swim test*, in which each rat in the chronic stress groups swam for 3 min in a bucket filled with cold water, whereas each rat in the sham stress groups was placed in a bucket without water for 3 min; and (vii) *Cage rotation,* in which each cage was rotated for 40 min after placing it on a laboratory shaker, while the cages for the sham groups were put on a shaker that was turned off for 40 min.

After completion of the chronic stress protocol, the rats were euthanized using isoflurane anesthesia at the age of 17 and one-half months. The cranial thoracic SC tissues (Th1-Th3 levels) were collected for analysis.

2.4. Western blot

2.4.1. Protein isolation

After a 2-month-old male, Sprague Dawley rat was euthanized with isoflurane anesthesia, whole brain tissue and the SC were removed and snap-frozen. The tissue was homogenized using a Minilys (Bertin Technologies SAS, Montigny-le-Bretonneux, France) in ice-cold RIPA buffer (50 mM Tris, 1 mM EDTA, 150 mM NaOH, 1% Triton, 0.5% DOC, 0.1% SDS, pH 7.4) with added protease and phosphatase inhibitors (1:1000 aprotinin, 1:1000 Na-vanadate, 1:200 phenylmethylsulfonyl fluoride [PMSF], 1:1000 leupeptin). Homogenized tissues were incubated for 2 h at +4 °C with constant agitation, followed by centrifugation for 20 min at 12000 rpm at +4 °C. The supernatants containing protein lysate were collected into new tubes. Protein concentration was measured using Pierce™ BCA Protein Assay Kit (Cat. No. 23225, Thermo Scientific, Waltham, MA, USA), according to the manufacturer's instructions.

2.4.2. Antibodies and Western blot analysis

The proteins were subjected to standard protocols for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting (Henderson and Wolf, 1992). Then the proteins were transferred onto nitrocellulose membranes (GE Healthcare, Chicago, Illinois, USA) and immunostained with anti-dendrin antibody (1:500, AB15299-I, EMD Millipore, Temecula, USA) and β-actin antibody (1:5000, A5316, Merck KGaA, Darmstadt, Germany). The bands were detected using HRP-conjugated secondary antibodies (1:6000, P0448, DakoCytomation, Carpinteria, CA, USA) and ECL Plus substrate (Thermo Fisher Scientific Inc., Waltham, MA, USA).

2.5. Immunohistochemical staining

SC tissue samples were dissected and fixated by immersion in 4% buffered paraformaldehyde. All the spinal cord specimens that we compared, including control and treated groups were treated the same way. After washing in phosphate buffer saline (PBS) and dehydration in rising concentrations of ethanol, dehydrated samples were embedded in paraffin before serial sectioning (5 μm thick transverse sections) and mounted on glass slides. Sections were deparaffinized in xylene and rehydrated in descending concentrations of ethanol in water as previously described (Agnic et al., 2018; Filipovic et al., 2017; Poljicanin et al., 2015).

Next, the sections were washed in PBS and the protein block (ab64226, Abcam, Cambridge, UK) was applied for 30 min. Then the sections were incubated overnight in a humid chamber with rabbit anti-dendrin polyclonal primary antibody (1:100, AB15299-I, EMD Millipore, Temecula, USA). After incubation, the sections were washed in PBS three times for 10 min and Alexa Fluor 488-conjugated donkey anti-rabbit IgG H&L secondary antibody (1:400, ab150073, Abcam, Cambridge, UK) was applied for 1 h. After washing in PBS, nuclei were stained with 4′6′-diamidino-2-phenylindole (DAPI) solution for 2 min, and cover-slipped. Control staining, with the exclusion of the primary antibody, resulted in no staining of tissue.

We also performed dual immunohistochemistry with rabbit anti-dendrin and FITC-conjugated α-d-galactosyl-specific isolectin B4 (IB4) (1:200, ALX-650-001F, Enzo, Farmingdale, NY, USA), as well as rabbit anti-dendrin and goat polyclonal to calcitonin gene-related peptide (CGRP) primary antibody (1:1000, ab36001, Abcam, Cambridge, UK) with the application of appropriate secondary antibodies (Rhodamine Red™-X (RRX) AffiniPure Donkey Anti-Goat IgG (H + L) 705–295-003; Rhodamine (TRITC) AffiniPure Donkey Anti-Rabbit IgG (H + L) 711–025-152; both from Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA).

Skin, heart and dorsal root ganglia (DRG) samples were obtained from the bank of animal tissues of the Laboratory for Neurocardiology (3-month-old male Sprague Dawley rats; tissue fixed in 4% paraformaldehyde in PBS and embedded in paraffin as described above). Skin and heart were stained with a mixture of primary antibodies: rabbit anti-dendrin polyclonal antibody (1:100, AB15299-I, EMD Millipore, Temecula, USA) and mouse monoclonal antibody against protein gene product 9.5 (anti-PGP9.5, 1:500, 480,012, Invitrogen, Camarillo CA, USA), with an appropriate anti-rabbit secondary antibody, described above (Alexa Fluor 488-conjugated donkey anti-rabbit IgG H&L) and Rhodamine Red-X-AffiniPure Donkey Anti-Mouse IgG (H + L) antibodies (715–295-151; Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA). Dual immunohistochemistry of DRG neurons was carried out with anti-dendrin and following primary antibodies: FITC-conjugated IB4, goat anti-CGRP and mouse anti-neurofilament 200kD (NF200) monoclonal antibody (1:100, MAB5266, EMD Millipore, Temecula, USA) with appropriate rhodamine X-conjugated donkey anti-mouse secondary antibody.

2.5.1. Data acquisition and quantification immunohistochemical analysis

The sections were observed under an epifluorescent microscope (Olympus BX61, Tokyo, Japan). Images were captured using a cooled digital camera DP71 (Olympus, Tokyo, Japan) and Olympus CellA software (Olympus Life Sciences Microscopy, Tokyo, Japan). The photomicrographs were taken at objective magnification factors of ×4 (to capture the entire SC segment); ×20 (to capture only the dorsal horn of the SC); and ×40 and ×100 (for capturing the details) (final magnification ×40, ×200, ×400 and ×1000; respectively). The photomicrographs were processed and subsequently analyzed by using ImageJ software (National Institutes of Health, Bethesda, MD, USA) and processing protocol was adjusted from already published (Luetić et al., 2020; Milardović et al., 2020). First, the fluorescence leakage reduction was performed by subtraction of red counter-signal from green fluorescence and then the median filter was used with a radius of 5.0 pixels. Subsequently, each picture was adjusted to the threshold method (triangle thresholding algorithm ~~method~~) and analyzed by measuring the fluorescence percentage area.

Analysis of overlapping regions obtained by dual immunohistochemistry in the dorsal horn of the SC and neurons of the DRG, after image processing as described above, was carried out using Adobe Photoshop (Adobe Inc., San Jose, California, USA) by merging them and calculating the fluorescence percentage area for every protein by itself and the overlapping part.

2.6. Statistical analyses

Mead's resource equation was used for sample size estimation, where the degree of freedom is well above 20. Hence, the study power was above 80%.

PAST 3.22 Software (Øyvind Hammer, Natural History Museum, University of Oslo, Norway) was used for statistical analyses (Hammer, Harper, & Ryan, 2001). To determine significant differences among groups, ANOVA with Welch correction for unequal variances was used. The difference between particular groups was tested by the *t*-test with Welch correction for unequal variances. Statistical significance was considered at $P < 0.05$. We did not use multiple comparisons correction because all of the comparisons were preplanned. Normality and homogeneity of data were tested by Anderson-Darling and Levene's tests, respectively. Differences between groups were expressed as a fold change calculated as the relative difference to sham stressed animals.

2.7. Tissue preparation for electron microscopy

Tissues were prepared for electron microscopy by using an immunogold immunohistochemistry protocol (Filipovic et al., 2020; Kosovic et al., 2020; Vitlov Uljevic et al., 2019). Samples were fixed in 4% paraformaldehyde in PBS, washed in PBS and then cut into 20 μm thick sections on a vibratome (Vibratome Series 1000, Pelco 101, Ted Pella, Inc., Redding, CA, USA). Following the wash in PBS, sections were incubated first in 50% ethanol for permeabilization and then in primary rabbit anti-dendrin polyclonal antibody (1:100, AB15299-I, EMD Millipore, Temecula, USA) for 48 h at +4 °C. After incubation, sections were rinsed in PBS and then incubated overnight with gold-conjugated donkey anti-rabbit secondary antibody (1:1000, 711-205-152, Jackson Immuno Research Laboratories, Inc., West Grove, PA, USA). Following another wash in PBS, sections were post-fixed in 1% osmium tetroxide (1 h), then dehydrated in ethanol and embedded in Durcupan ACM resin (Sigma-Aldrich Inc. St. Louis, Missouri, USA). The size of gold particles used in this study was 12 nm. The sections were observed with a transmission electron microscope (JEM JEOL 1400, Jeol Ltd., Tokyo, Japan).

3. Results

3.1. Western immunoblot specificity analysis of dendrin in brain and SC

We tested the specificity of the dendrin antibody using Western blots of the brain and SC homogenates from a 2-month-old male rat. There was a specific strong band at 72 kDa in the brain. In SC, the same band but with weaker intensity was found (Fig. 2a).

3.2. Specific localization pattern of dendrin in SC

Localization and quantification of dendrin expression were analyzed in thoracic segments (Th1-Th3 levels) of the SC from female and male rats in the experimental groups. As expected, dendrin expression was found in neurons and all parts of the gray matter. The expression was strong in large lower motor neurons of the ventral horn. In addition, a distinct expression of dendrin was seen in the dorsal horn. The most prominent staining with the highest intensity was found in laminae I and II, and in the area around the central canal (lamina X) (Fig. 2b).

Intensity profiles for dendrin expression in the SC were plotted. In most of the groups, there was a decreasing intensity of dendrin immunofluorescence from dorsal to the ventral area in sections of the SC. The highest intensity was found in the F-SH-S, F-OVX-C and F-OVX-S groups (Fig. 2c) and the M-ORX-S group (Fig. 2d). The lowest intensity was

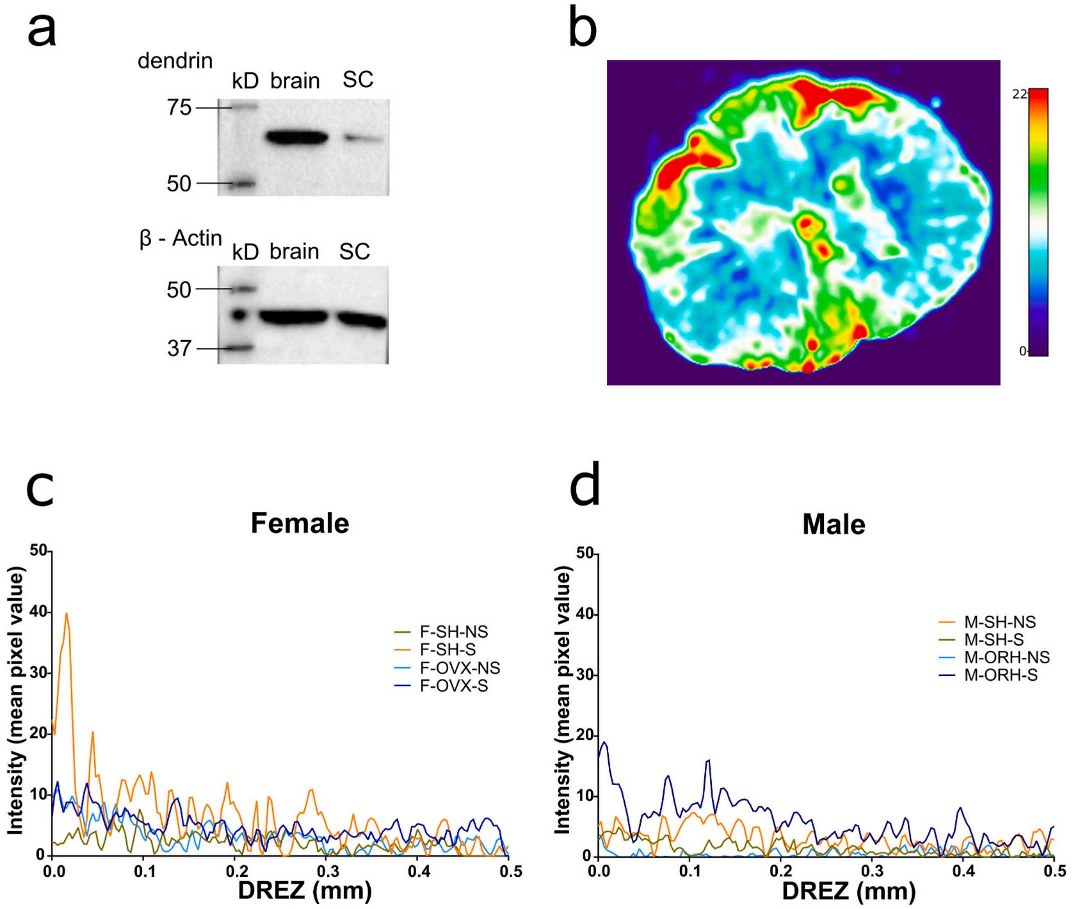

Fig. 2. (a) Western blot analysis characterization of the dendrin antibody. There was a strong band at 72 kDa in the brain and a weaker band at the same position in the spinal cord (SC). The β-actin protein was used as a loading control at 42 kDa. (b) Intensity distribution heatmap for dendrin expression in the SC of rats in the F-SH-S group. Intensity range (pixel value 0–255): 0–6 (dark blue), 6–10 (bright blue), 10–12 (white), 12–16 (bright green), 16–20 (yellow), and 20–22 (bright red). (c, d) Intensity plot profiles for dendrin expression along the dorsoventral axis through the superficial laminae of the dorsal horn. Individual intensity plots for each of the female groups (c) and male groups (d) are shown. The distance from the dorsal root entering zone (DREZ) to the central canal is presented on the X-axis. The intensity of dendrin expression is expressed as the mean pixel value on the Y-axis.

observed in the F-SH-C group (Fig. 2c) and the M-SH-C, M-SH-S and M-ORX-C groups (Fig. 2d).

3.3. Overlapping of dendrin immunofluorescence signal with markers for primary afferent nociceptive fibers and DRG neurons

In the dorsal horn, we found dendrin immunoreactivity overlapping with IB4 and CGRP in central projections of DRG primary afferent nociceptive fibers (Fig. 3a and b). Overlapping of the threshold percentage area for IB4 was 7.43 ± 3.36% of the total area while overlapping for CGRP was 8.47 ± 4.45% of the total area, with no significant difference between groups. Because of these data, we explored the possible expression of dendrin in DRG neurons. Surprisingly, we found strong dendrin immunoreactivity in the soma of DRG neurons that were immunoreactive for IB4, CGRP and NF200 (Fig. 3c-e). We also found the co-localization of dendrin with PGP 9.5 (Protein gene product 9.5, UCH-L1, PARK5) in peripheral neuronal projections in the skin and heart (Fig. 3f and g). PGP 9.5 is a pan-neuronal marker, present in neurons, as well as in the nerve fibers in the central and peripheral nervous system (Lee et al., 2012).

3.4. Dendrin expression differences between groups based on chronic stress, castration, gender, and sex hormones

We investigated the effects of chronic stress, castration (orchidectomy or ovariectomy), gender and the combination of these factors on dendrin expression in the SC. To assess the effect of chronic stress on dendrin expression we compared the two male sham-operated groups: M-SH-S and M-SH-C. Chronic stress did not increase dendrin expression (relative difference = 0.35-fold; 95% confidence interval [CI] -0.426 to 1.127; P = 0.4534) (Figs. 4, 5). In contrast, when the female sham-operated groups were compared, dendrin expression nearly quadrupled in F-SH-S rats compared to F-SH-C rats, and the difference was statistically significant (relative difference = 3.669-fold; 95% CI 1.261 to 6.079; P = 0.0445) (Figs. 4, 6).

To evaluate the effect of castration alone we compared the two male control groups (i.e., sham stress): M-SH-C vs. M-ORX-C (relative difference = 0.376-fold; 95% CI -0.731 to 1.483; P = 0.4709), and the two female control groups: F-SH-C vs. F-OVX-C group (relative difference = 1.387-fold; 95% CI -0.083 to 2.857; P = 0.0809). Neither comparison showed a significant difference (Fig. 4).

The effect of gender by itself did not influence dendrin expression (M-SH-C vs. F-SH-C; relative difference = 0.577-fold; 95% CI -0.673 to 1.827; P = 0.3170) (Fig. 4). However, when exposed to chronic stress female rats had a significant 10-fold increase in dendrin expression compared to male rats (F-SH-S vs. M-SH-S relative difference = 10.358-fold; 95% CI 5.351 to 15.353; P = 0.0322). Moreover, when sham-stressed animals were castrated there was also a significant 5-fold increase in dendrin expression for female rats compared to male rats (F-OVX-C vs. M-ORX-C relative difference = 5.034-fold; 95% CI 1.407 to 8.662; P = 0.0266).

To examine how stress might influence the expression of dendrin in

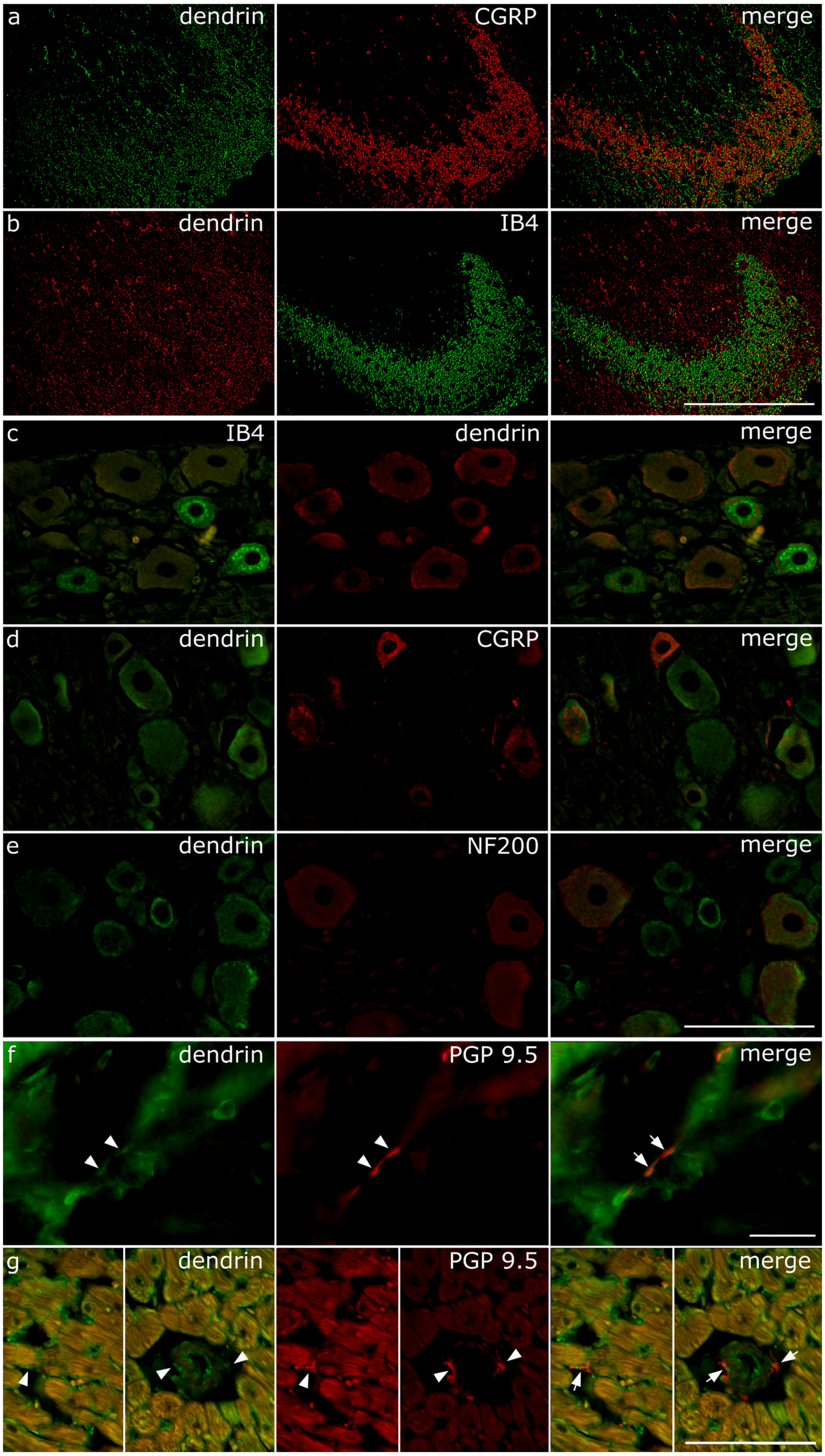

Fig. 3. Dendrin expression in the dorsal horn of spinal cord, dorsal root ganglia (DRG) neurons and peripheral nerves. (a, b) Expression of dendrin in the dorsal horn. Representative photomicrographs after thresholding the images of immunostained sections. (a) dendrin (green), calcitonin gene-related peptide (CGRP, red) and co-localization of these proteins ("merge"). (b) dendrin (red), α-d-galactosyl-specific isolectin B4 (IB4, green) and co-localization (merge). Magnification factor x200, scale bar: 200 μm. (c, d, e) Representative photomicrographs of DRG neurons after immunostaining. Dendrin expression in DRG neurons and co-localization with three neuronal markers: (c) IB4, (d) CGRP and (e) neurofilament 200 (NF200). Magnification factor x400, scale bar: 100 μm. (f, g) Expression of dendrin in peripheral nerves of rat skin and heart. Representative photomicrographs of (f) skin and (g) heart made by immunostaining for dendrin (green) and co-localization with neuronal marker protein gene product 9.5 (PGP 9.5, red). In the heart, dendrin immunoreactive neuronal fibers were found between cardiomyocytes, sub-endo/epicardial areas (not shown) and around blood vessels. Magnification factor x1000 in (f), x400 in (g), scale bar: 100 μm (both). Arrowheads indicate dendrin immunoreactive nerve fibers (green) recognized by co-localization with PGP 9.5 (red). In rightmost panels, arrows indicate co-localization (orange). (a, b – M-SH-C; c-g - 3-month-old male Sprague Dawley rats).

castrated animals, we compared the two male orchidectomized groups: M-ORX-S and M-ORX-C. In castrated male rats, chronic stress dramatically and significantly increased dendrin expression by almost 12-fold (relative difference = 11.653-fold; 95% CI 7.807 to 15.5; *P* = 0.0003) (Figs. 4, 5). In contrast, the combination of castration and chronic stress did not significantly affect dendrin expression in female rats (F-OVX-S vs. F-OVX-C relative difference = 1.015-fold; 95% CI -0.813 to 2.843; *P* = 0.2204) (Figs. 4, 6).

These findings led us to evaluate the influence of sex hormones in chronically stressed animals (M-SH-S vs. M-ORX-S; F-SH-S vs. F-OVX-S).

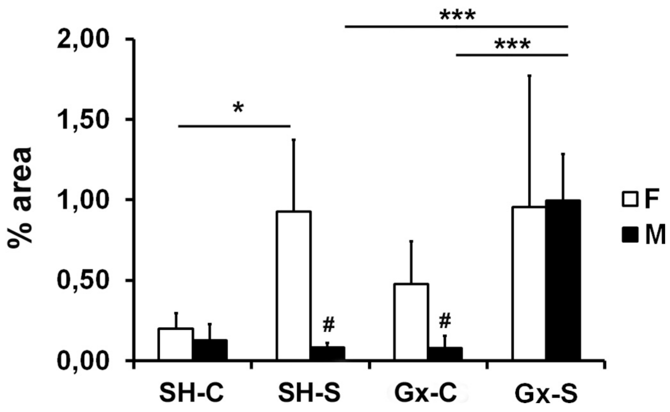

Fig. 4. Expression of dendrin in the dorsal horn of the rat spinal cord in the experimental groups. Dendrin expression was quantified by measuring fluorescence percentage area (% area); * $P < 0.05$;*** $P < 0.001$ - statistically significant difference between indicated groups; # $P < 0.05$ - statistically significant difference between genders. Abbreviations: SH-C, sham-operated control group (sham stress); SH-S, sham-operated chronic stress group; Gx-C, orchidectomized/ovariectomized control group (sham stress); Gx-S, orchidectomized/ovariectomized chronic stress group.

In male rats, castration significantly augmented the effect of chronic stress on dendrin expression by 11-fold (relative difference = 11.166-fold; 95% CI 7.945 to 14.388; $P = 0.0005$) (Figs. 4, 5). Whereas castration in females did not show a synergistic effect with chronic stress (relative difference = 0.029-fold; 95% CI -1.093 to 1.153; $P = 0.9469$) (Figs. 4, 6).

3.5. *Ultrastructural characterization of dendrin in the dorsal horn of the SC*

Transmission electron microscopy confirmed dendrin immunoreactivity in the dorsal horn neurons of the SC from M-SH-C rats, by using immunogold staining. As expected, gold particles were found in the postsynaptic area as well as in the neuronal soma and nucleus. It seems that the immunoreaction was distributed randomly throughout the neuronal soma and nucleus. Although less numerous, the gold particles were found in the presynaptic area and axon terminals confirming the immunoreactivity for dendrin in these areas as well. The immunoreaction in these areas usually was distributed near the presynaptic membrane close to synaptic vesicles and mitochondria (Fig. 7).

4. Discussion

Gonadal hormones greatly influence the perception and modulation of how stressors impact an organism (Heck and Handa, 2019). In this study, our main focus was to explore the potential interaction between the effects of chronic stress and gonadal hormones in the SC. A prominent difference in the amount of dendrin expression was found.

To the best of our knowledge, there is no data available about dendrin expression in the SC before our study. Besides the expected presence of dendrin in neurons, we found dendrin expression concentrated in the SC areas associated with pain processing, namely the superficial laminae of the dorsal horn and lamina X (encircling the central canal). We also found overlapping areas of dendrin with IB4- and CGRP-immunoreactive central projections of the primary sensory neurons in superficial laminae of the dorsal horn. Furthermore, we found strong dendrin immunoreactivity in the soma of DRG neurons, as well as in their peripheral projections in the skin and viscera (heart). Taken together, these findings may suggest that the possible alterations in dendrin expression are related to the refinement of sensory information, in particular the processing of pain.

Previous studies have described dendrin as a protein bound to the dendritic spines, which means it belongs to the group of proteins with dendritically localized neuronal mRNAs (Pinkstaff et al., 2001). Although the expression is associated with synapses, there is also a characterization of dendrin in neuronal soma with accumulation on polyribosomes (Neuner-Jehle et al., 1996). By using transmission electron microscopy in combination with immunogold labeling, we found similar results in soma but also with a presence in the neuronal nucleus. A mechanism underlying the accumulation of dendrin in the nucleus might be possible through modulation of its interaction with the synaptic scaffolding protein MAGI/S-SCAM. Change in interaction with MAGI/S-SCAM may be responsible for the regulation of dendrin release from the synapse and its movement into the nucleus where it may then act as a retrograde messenger (Kremerskothen et al., 2006). The presynaptic presence of dendrin in the central projections of DRG neurons is not clear. On the presynaptic terminal, dendrin may also contribute to the LTP modulation, through increasing or decreasing the release of neurotransmitters through some other interaction yet to be discovered, important for the effective reorganization of neural circuits (Yang and Calakos, 2013).

Despite the above-mentioned findings, the exact role of dendrin in neurons is still unclear. It is believed that the role of dendrin in the brain is closely related to plastic events at the synaptic level, mainly because of its connection with the kidney and brain expressed protein (KIBRA), a synaptic scaffold protein responsible for regulating spatial learning and memory (Ji et al., 2019; Kremerskothen et al., 2003). It was found that the nature of the interaction between KIBRA and dendrin is important for learning and memory (Ji et al., 2019). After application of inhibitory peptide and causing disruption in KIBRA-dendrin interaction, synaptic AMPAR (α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor) expression is reduced with consequences of attenuation of the excitatory synaptic transmission, long-term potentiation (LTP) (Ji et al., 2019), synaptic strength and plasticity (Diering and Huganir, 2018; Herb et al., 1997; Kawata et al., 2006; Kremerskothen et al., 2006; Makuch et al., 2011; Schochet et al., 2008). Previous studies have shown that chronic stress is linked to maladaptive pain responses because of its impact on pain processing areas via influence on glutamate receptor expression (Yuen et al., 2016). Taking all of the above into consideration, it can be assumed that exposure to chronic stress can cause changes in dendrin expression and through that, alteration of the SC synaptic plasticity. We found a significant increase in dendrin expression in the dorsal horn of rats exposed to chronic stress. However, this response was sex-dependent, observed only in female rats. Moreover, it seems that male sex hormones have a suppressive effect because an increase in dendrin expression induced by chronic stress appeared in male rats only after orchidectomy (i.e. after steep down-regulation of testosterone).

These findings may be a possible explanation for the different effects of gonadal hormones upon dendritic spine density and branching. It was previously found that testosterone in males induces dendritic branching but has no effect on spine density. In contrast, it was found that estradiol increases dendritic spine density (Todd et al., 2007). Both of these effects were related to AMPAR trafficking. In addition, there is an indication that females have more potential for forming fear memory by acute stressors than males, which is mediated through an increase in LTP induction and AMPARs (GluR1) in the lateral nucleus of the amygdala (Chen et al., 2014). On the other hand, the down-regulation of testosterone after a gonadectomy resulted in the excitability and plasticity enhancement via LTP in the CA3 area as a counterbalance to the decreased amount of spine synapses (Hojo et al., 2009; Skucas et al., 2013). The state of excitability of the spinal neurons is therefore critical to proper discrimination of noxious stimuli and it could be related to changes in dendrin expression. On the other hand, sex-dependent differences of dendrin expression, especially after sex hormones depletion, may depend on aromatase activity (AA). In the study, Roselli et al. described the dependence of AA and castration in the hypothalamus, while in females the estrous cycle and castration do not affect AA, in

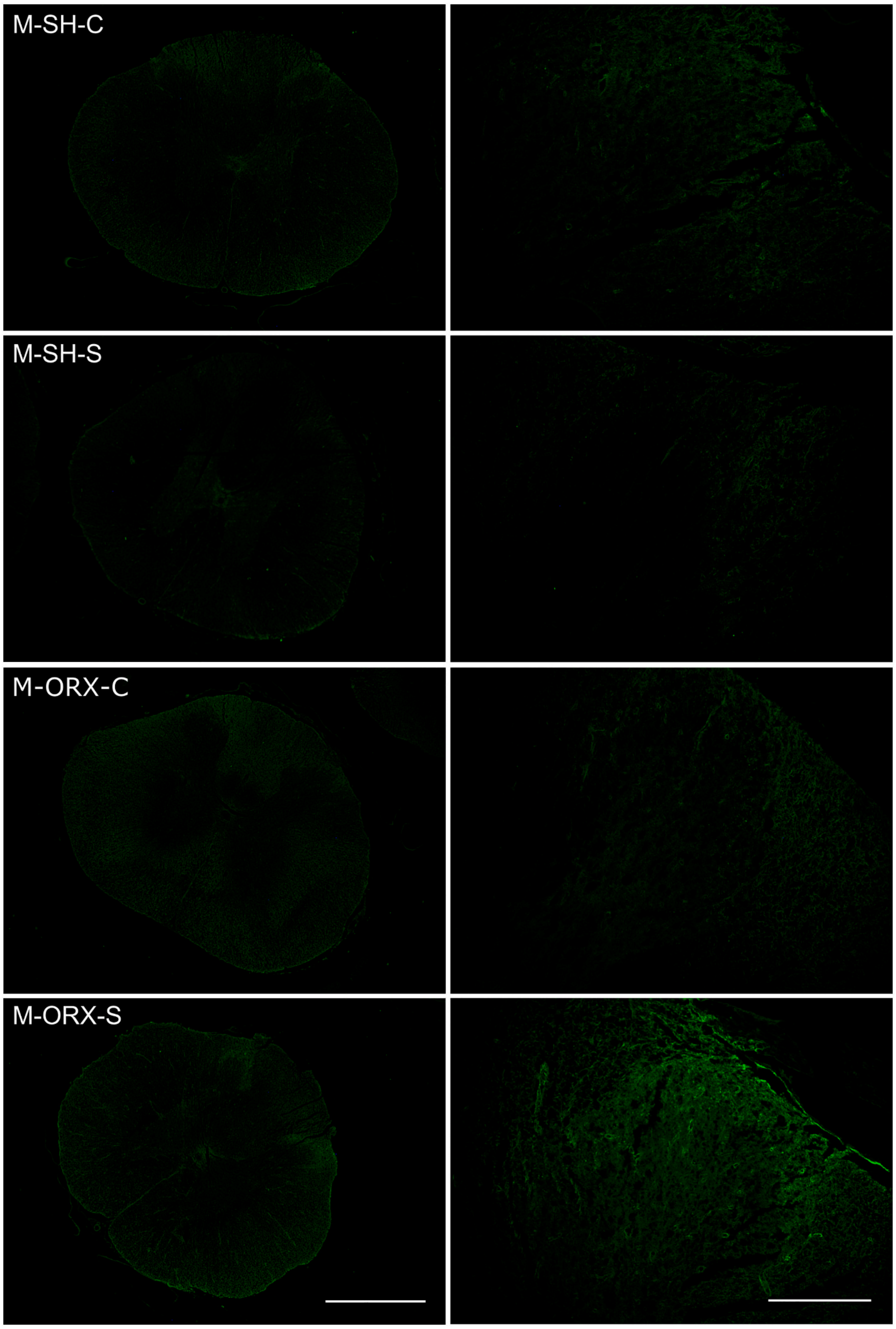

Fig. 5. Representative photomicrographs showing dendrin expression in the dorsal horn of spinal cords of different groups of male rats. Original magnification: x40, scale bar: 2 mm; and x200, scale bar: 100 μm. Abbreviations: M-SH-C, male sham-operated control (sham stress) group; M-SH-S, male sham-operated chronic stress group; M-ORX-C, male orchidectomized control (sham stress) group; M-ORX-S, male orchidectomized chronic stress group.

male rats castration drastically decreases AA (Roselli and Resko, 1993). Furthermore, Dickens et al. reported changes in AA due to acute stress exposure depending on the sex and brain region of Japanese quail (Dickens et al., 2011).

In conclusion, a better understanding of the neurobiological background of the connection between chronic stress and pain can help us elucidate the numerous pathological changes that underlie the onset of stress-related conditions. The presence of dendrin in nociceptive areas of the dorsal horn of the SC, and its expression in the DRG and peripheral neurons, suggest its possible role in nociceptive pathway modulation as a possible linkage in which the influence of stress and pain overlap. Further studies are needed to explain the role of the stress-induced increase in dendrin expression that was found only in non-ovariectomized female and orchidectomized male rats, in sex-specific pain hypersensitivity induced by stress.

Author contributions

All authors have full access to all data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis. N.F., R.G., M.H. and S.G.V. - conceptualization; N.F., M.J., R. G., M.H. and S.G.V. - methodology; M.J., N.F., M.Ba., V.I., M.Bo., A.R., I. B., N.K., I.R., K.F.S., K.V., R.G., M.H. and S.G.V. - investigation; N.F., M. J., B.B. - formal analysis; N.F., K.V., R.G., M.H. and S.G.V. - resources; M.

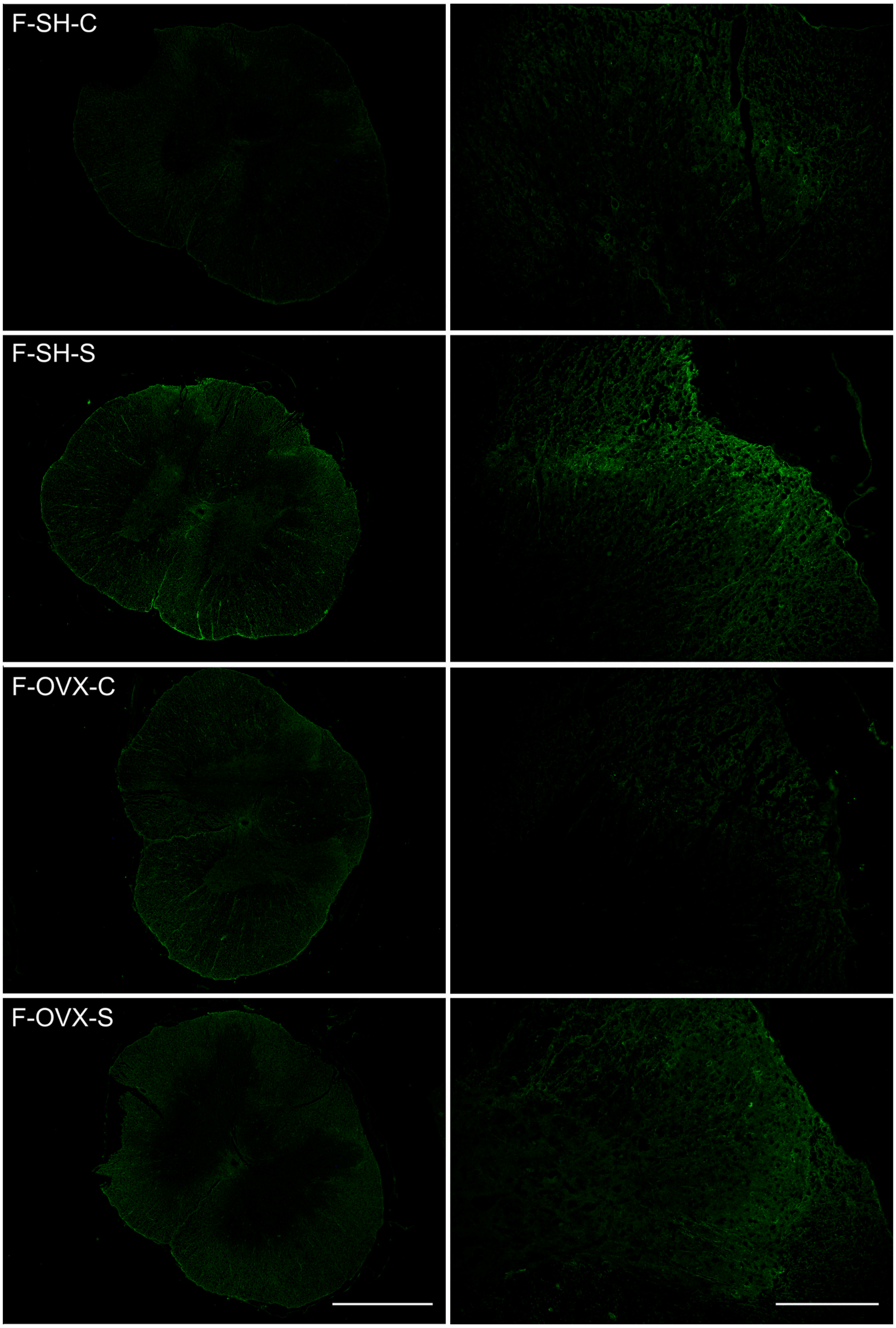

Fig. 6. Representative photomicrographs showing dendrin expression in the dorsal horn of spinal cords of different groups of female rats. Original magnification: x40, scale bar: 2 mm; and x200, scale bar: 100 μm. Abbreviations: F-SH-C, female sham-operated control (sham stress) group; F-SH-S, female sham-operated chronic stress group; F-OVX-C, female ovariectomized control (sham stress) group; F-OVX-S, female ovariectomized chronic stress group.

J. and N.F. writing-original draft; M.J., M.Ba., V.I., M.Bo., B.B., A.R., I. B., N.K., I.R., R.G., K.F.S., M.H., K.V., S.G.V. and N.F. - writing-review and editing; M.J., I.B., N.F. - visualization; N.F. - supervision; N.F. - project administration; N.F., K.V., R.G., M.H. and S.G.V. - funding acquisition.

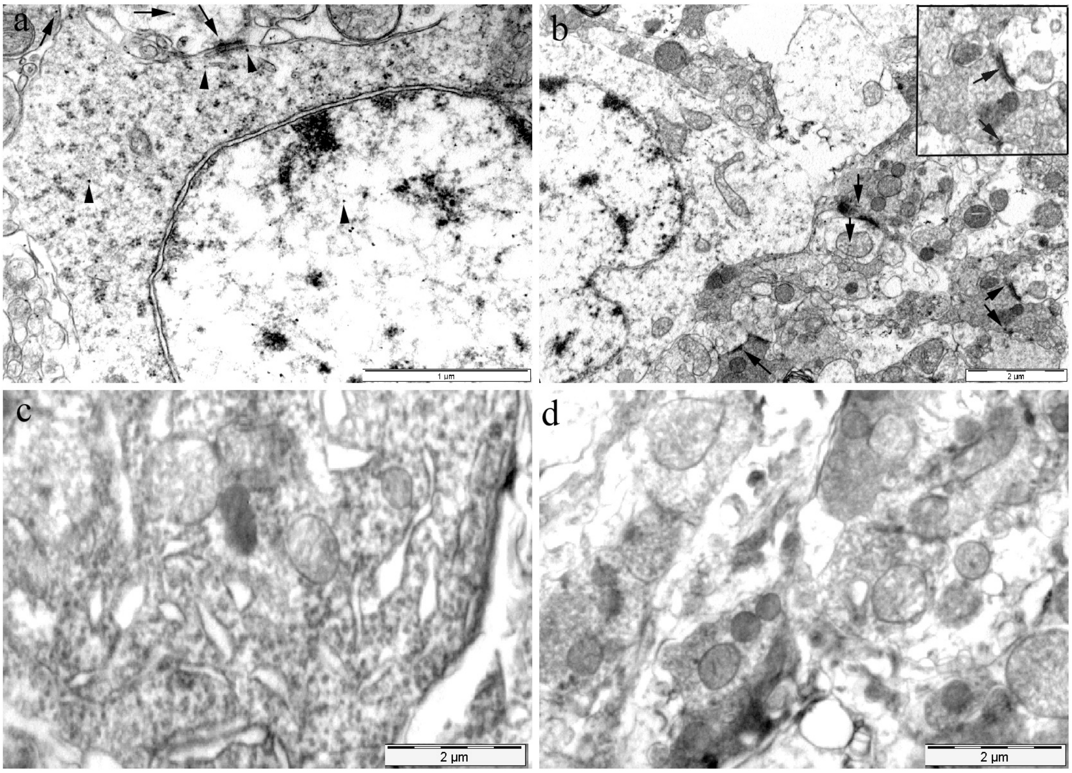

Fig. 7. Representative transmission electron microscopy photomicrographs of dendrin immunoreactivity in dorsal horn neurons of the spinal cord. (a) Immunoglod staining for dendrin in axon terminals and presynaptic area (arrows) as well as in neuronal soma, postsynaptic area and nucleus (arrowheads). Scale bar: 1 μm. (b) Immunogold staining for dendrin in the axon terminals and presynaptic area (arrows). Scale bar: 2 μm. (c) Negative control in the area of neuronal soma. Scale bar: 2 μm. (d) Negative control in the area of axon terminals. Scale bar: 2 μm.

Ethical committee approval

Experiments were carried out at the Animal Facility of Faculty of Pharmacy, University of Szeged, approval number: IV./3796/2015.

Declaration of Competing Interest

The authors declare that they have no conflicts of interest.

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