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Sciatic Nerve Injury Induces Apoptosis of Dorsal Root Ganglion Satellite Glial Cells and Selectively Modifies Neurosteroidogenesis in Sensory Neurons

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KEY WORDS

neurosteroid; neuropathy; cell death; neuroprotection; pain; nerve cell viability

ABSTRACT

Neurosteroids are synthesized either by glial cells, by neurons, or within the context of neuron-glia cross-talk. Various studies suggested neurosteroid involvement in the control of neurodegeneration but there is no evidence showing that the natural protection of nerve cells against apoptosis directly depends on their own capacity to produce neuroprotective neurosteroids. Here, we investigated the interactions between neurosteroidogenesis and apoptosis occurring in sensory structures of rats subjected to neuropathic pain generated by sciatic nerve chronic constriction injury (CCI). Using the terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL), we observed no apoptotic cells in the spinal cord up to 30 days after CCI although pain symptoms such as mechano-allodynia, thermal and mechanical hyperalgesia were evidenced with the Hargreaves's behavioral and von Frey filament tests. In contrast, double-labeling experiments combining TUNEL and immunostaining with antibodies against glutamine synthetase or neuronal nuclei protein revealed apoptosis occurrence in satellite glial cells (SGC) (not in neurons) of CCI rat ipsilateral dorsal root ganglia (DRG) at day 30 after injury. Pulse-chase experiments coupled with high performance liquid chromatography and flow scintillation detection showed that, among numerous biosynthetic pathways converting [^{3}H]pregnenolone into various [^{3}H]neurosteroids, only [^{3}H]estradiol formation was selectively modified and upregulated in DRG of CCI rats. Consistently, immunohistochemical investigations localized aromatase (estradiol-synthesizing enzyme) in DRG neurons but not in SGC. Pharmacological inhibition of aromatase caused apoptosis of CCI rat DRG neurons. Altogether, our results suggest that endogenously produced neurosteroids such as estradiol may be pivotal for the protection of DRG sensory neurons against sciatic nerve CCI-induced apoptosis.

INTRODUCTION

Several diseases of the nervous system result from the dysregulation of endogenous mechanisms involved in the protection of nerve cells against death. Therefore, identification of endogenous molecules, which play a pivotal role in the regulation of nerve cell viability and neurodegenerative processes, may certainly offer interesting possibilities to develop effective neuroprotective therapies. The past three decades were marked by a major finding indicating that neurons and glial cells are capable of synthesizing autonomously bioactive steroids also called neurosteroids (Baulieu et al., 1999; Mensah-Nyagan et al., 1999; Patte-Mensah and Mensah-Nyagan, 2008; Patte-Mensah et al., 2006). Various studies have suggested that neurosteroids may be involved in the control of neurodegenerative mechanisms (Brinton and Wang, 2006 a,b; Griffin et al., 2004; Leonelli et al., 2007; Schumacher et al., 2004; Thomas et al., 1999; Wang et al., 2005). However, there is no evidence showing that nerve cell viability or natural protection of neurons and glial cells against apoptosis directly depends on their own ability to produce endogenous neuroprotective neurosteroids. Degeneration or damage to nerve cells in the peripheral or central nervous system leads to various pathological consequences including neuropathic pain which constitutes a major health concern. Indeed, neuropathic pain, which can have delayed onset after initial neural tissue injuries, may be present in the absence of detectable lesions, may spread beyond cutaneous distribution of damaged nerves or may exist bilaterally in mirror image sites (Woolf and Mannion, 1999; Zimmermann, 2001). These features make extremely difficult the diagnosis and treatment of neuropathic pain but also complicate the characterization of appropriate experimental or animal models to investigate this disease. Most neuropathic pain models are made by injuring the spinal cord (SC) or peripheral nerves with total transection, crush, tight or loose ligatures (Bennett, 1998; Brinkhus and Zimmermann, 1983; Campbell and

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Meyer, 2006; Kawakami et al., 1994; Lee et al., 2000; Lombard et al., 1979; Mills et al., 2001). Because the pathological impact of each type of injury is different, animal models generate distinct or controversial results which need to be specifically analyzed within the context of each experimental condition. For instance, Maione et al. (2002) reported apoptotic genes expression in the SC dorsal horn (DH) of the rat model of sciatic nerve constriction (CCI) obtained by loose ligation whereas Polgár et al., (2003, 2005) who have used both spared nerve injury and CCI models, detected no apoptotic neurons in DH, indicating that the loss of spinal neurons is not required for the development of tactile allodynia and thermal hyperalgesia. Moreover, neuronal and glial cell apoptosis has been evidenced in rat dorsal root ganglia (DRG) after sciatic nerve axotomy (Whiteside et al., 1998) or L5 spinal nerve crush (Campana and Myers, 2003) while the survival of primary afferent neurons has been suggested by the absence of degeneration in the nerve 1 cm proximal to the constriction in CCI rats (Bennett and Xie, 1988; Munger et al., 1992). The survival of primary afferent neuronal cell bodies in CCI rat DRG remains however a matter of speculation because there is no evidence showing the absence of apoptotic neurons in CCI rat DRG. Therefore, we decided to use the TUNEL method in order to investigate whether or not apoptosis actually occurs in DRG and SC of CCI rats within a period of 30 days following the nerve injury. Because at day 30 after CCI we detected apoptosis occurrence in DRG satellite glial cells (SGC), but not in neurons, we sought to determine whether this phenomenon interferes with neurosteroidogenic ability of DRG sensory neurons surrounded by apoptotic SGC. To reach this goal, we combined pulse-chase experiments, HPLC analyses and flow-scintillation detection (Kibaly et al., 2005, 2008; Mensah-Nyagan et al., 1994, 1996 a,b; Patte-Mensah et al., 2003, 2005; Schaeffer et al., 2006) to investigate the conversion of tritiated pregnenolone ([^{3}H]PREG) into various [^{3}H]neurosteroids in DRG isolated from the lumbosacral region of naive, sham-operated and CCI rats 30 days after the nerve constriction. Because estradiol was the main neurosteroid the production of which was upregulated in CCI rat DRG, we have also studied the presence and cellular distribution of aromatase (estradiol-synthesizing enzyme) in DRG of control and neuropathic animals. Finally, the direct involvement of endogenously produced estradiol in the protection of DRG neurons against CCI-induced apoptosis was investigated by using letrozole, the pharmacological inhibitor of aromatase activity.

MATERIALS AND METHODS

Animals

Adult male Sprague-Dawley rats weighing 225–249 g were used in this study. Animal care and manipulations were performed according to the European Community Council directives (86/609/EC) and under the supervision of authorized investigators. 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*. Surgical operations were made under ketamine (75 mg/kg)/xylasine (5 mg/kg) anesthesia. Neuropathic pain was induced by sciatic nerve chronic constriction injury (CCI) according to the protocol described by Bennett and Xie (1988). All experiments followed the International Association for the Study of Pain ethical guidelines (Zimmermann, 1983). Briefly, after dissection at the middle of the thigh, 4–5 mm of the common sciatic nerve was tied loosely with four ligatures spaced by 1 mm. Various groups of control rats were used: some were not operated upon (naive rats) and others were sham-operated (exposure of the right or left sciatic nerve without ligature). The animals were inspected every day to observe their recovery from the surgical operation and parameters indicating the occurrence of neuropathic pain including the gait, posture of the affected hind paw, and condition of claws (Bennett and Xie, 1988). Animals were euthanized 5, 10, 15, 20, 25, or 30 days after induction of the sciatic nerve CCI in order to perform biochemical and immunohistochemical experiments. Before the sacrifice, nociceptive behavioral analyses were performed to check the presence or absence of neuropathic pain symptoms such as thermal and/or mechanical hyperalgesia and allodynia.

Chemicals and Reagents

Synthetic steroids including progesterone (PROG), pregnenolone (PREG), 17α-hydroxyprogesterone (17OHP ROG), testosterone, 3α-androstanediol (3αDIOL) and 17β-estradiol were purchased from Steraloids (Newport, RI). Dichloromethane (DCM) was from Acros Organics (Noisy Le Grand, France). Hexane and isopropanol were obtained from Fischer Bioblock Scientific (Illkirch, France). Propylene Glycol, Dulbecco's modified Eagle's medium (DMEM) and hydroxypropyl cellulose were supplied from Sigma (St Louis, MO). Tritiated steroids such as 7-^{3}H(N)-pregnenolone ([^{3}H]PREG), 1,2,6,7-^{3}H(N)-progesterone ([^{3}H]PROG), 9,11,12-^{3}H(N)-testosterone and 9,11-^{3}H(N)-3α-androstanediol ([^{3}H]3αDIOL) were obtained from PerkinElmer (Boston, MA). The 17α-hydroxy-[1,2,6,7-^{3}H]-progesterone ([^{3}H]17OHPROG) was purchased from Amersham (Piscataway, NJ). Letrozole and the anti-aromatase monoclonal antibody (#677, Sasano et al., 2003, 2005) were kindly provided by Dr. D. Evans of Novartis (Basel, Switzerland).

Nociceptive Behavioral Tests

Thermal hyperalgesia was assessed by using a Plantar test apparatus (Ugo Basile, Comerio, Italy) which measures the paw withdrawal latency in response to radiant heat (Hargreaves et al., 1988). The rats were first allowed to habituate to the experimental room for at least 2 h and then 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 infrared light beam. The heat source is connected to a timer which 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 latencies (in seconds) for the ipsilateral and contralateral hind paws were determined from an average of three separate measures on each hind paw (a total of six measures per animal) 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/or hyperalgesia was assessed using a series of calibrated von Frey hairs (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 4–5 s intervals. The threshold for paw withdrawal was calculated by taking the average of five repeated stimuli (in g), which induced a reflex paw withdrawal. Thus, at a given time point, five measures were obtained from each hind paw (a total of 10 measures per animal). Only robust and immediate withdrawal responses followed by a licking of the paw were considered as positive. Naive rats never withdraw from stimulations less than 6 g but respond 15%–20% of the time for 15 g stimulus and more than 80% for 100 g considered as an indisputable nociceptive stimulation. Observation of responses for stimulations <6 g after sciatic nerve injury is indicative of mechanical allodynia. Increased level of responding for 15 g after surgery is indicative of mechano-hyperalgesia.

Terminal Deoxynucleotidyl Transferase-Mediated dUTP Nick End Labeling (TUNEL) Assay and Immunohistochemistry Procedure

Animals were deeply anaesthetized with 25% urethane (0.5 mL/100 g intraperitoneal) and perfused transcardially with 100 mL of 0.1 M phosphate buffer (PB; pH 7.4). The perfusion was carried out with 450 mL of fixative solution (4% paraformaldehyde in PB). The spinal cord and DRG from lumbosacral region were rapidly dissected and post fixed in the same fixative solution for 24 h. The tissues were immersed in PB containing 15% sucrose for 12 h and then transferred into 30% sucrose in PB for 24 h. After that, the tissues were placed in embedding medium (OCT, Tissue-Tek, Reichert-Jung, Nussloch, Germany) and immediately frozen at −80°C. Coronal 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.

Apoptotic cell death was detected using in situ Cell Death detection kit (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instructions. Briefly, after permeabilisation with PB containing 0.5% triton X-100, sections were incubated with the TUNEL reaction mixture containing terminal deoxynucleotidyl transferase (TdT) and dUTP coupled to fluorescein for 60 min at 37°C. Negative controls were performed by omitting TdT. After the TUNEL assay, DRG sections were divided into four groups that were incubated overnight at 4°C with one of the following solution. Group 1 was incubated overnight with a mouse monoclonal antibody directed against glutamine synthetase (Chemicon, Temecula, CA) diluted at 1:200 in PB containing 0.3% triton X-100 (PBT); the procedure was carried out by rinsing Group 1 DRG sections three times in PB (10 min per rinse) and incubating them with a Cy3-conjugated donkey anti-mouse IgG (DAM/Cy3) diluted at 1:800 in PBT for 2 h at room temperature. Groups 2 and 3 DRG sections were incubated overnight with the mouse monoclonal antibody directed against neuronal nuclei protein or NeuN (Chemicon) diluted at 1:1,000 in PBT; after rinsing three times in PB, DRG sections were incubated with a DAM/Alexa-488 diluted at 1:300 in PBT (Group 2) or with a DAM/Cy3 (1:800 in PBT, Group 3) for 2 h at room temperature. Group 4 DRG sections were incubated overnight with PBT; after rinsing three times in PB, Group 4 DRG sections were incubated either with DAM/Cy3 or DAM/Alexa-488 as described above.

To investigate the presence and distribution of aromatase, DRG sections were pre-incubated for 1 h with PBT containing 5% non-immune donkey serum. Afterwards, the sections were incubated with the anti-aromatase mouse monoclonal antibody #677 (1:100 in PBT) for 72 h at 4°C. After three rinses in PB, DRG sections were incubated with DAM/Cy3 (1:800 in PBT) for 2 h at room temperature. Adjacent DRG sections were incubated overnight with the mouse monoclonal anti-NeuN and, after rinsing with PB, the revelation was performed with DAM/Alexa-488 secondary antibody (1:300 in PBT for 2 h).

At the end of TUNEL and immunohistochemical procedures, all DRG sections were rinsed three times in PB and mounted in vectashield. The preparations were examined under a fluorescence DMR microscope equipped with a digital camera assisted by a pentium IV PC computer (Leica, Wetzlar, Germany) or under a multichannel confocal laser-scanning microscope (Leica Confocal Systems, Paris, France).

The number of TUNEL-immunoreactive cells, NeuN-containing cells or double labeled was determined in a counting square of 100 × 100 μm^2 in DRG of vehicle (VEH) or letrozole-treated CCI rats. Letrozole was intrathecally injected at day 20, 24, and 28 after sciatic nerve CCI in a volume of 20 μL/250 g in hydroxypropyl cellulose (0.3% in water) used as VEH. Direct transcutaneous intrathecal injections (i.t.) were performed as described by Mestre et al. (1994). Briefly, animals were first

anesthetized no more than 2 min with a mixture of 4% of halothane in O_2/N_2O (30:70 v/v). Then, a 26G° × 1/2-in. needle connected to a 50 μL Hamilton syringe was inserted through the vertebral column into the subarachnoid space between vertebrae L4 and L5. Successful placement was indicated by a stereotypical tail flick reflex: when the needle entered the subarachnoid space, a sudden lateral movement of the tail was observed.

Pulse Chase Experiments

For each experiment, animals were euthanized by decapitation. Lumbo-sacral DRG were rapidly dissected and pre-incubated for 15 min in 2 mL 0.9% NaCl at 37°C. Afterwards, the DRG were incubated at 37°C for 3 h in 1.5 mL of DMEM (pH 7.4) containing 100 nM [^{3}H]PREG supplemented with 1% propylene glycol. The incubation with [^{3}H]PREG was made in a water-saturated atmosphere (95% air, 5% CO_2) which made it possible to maintain the pH at 7.4. At the end of the incubation period, the reaction was stopped by adding 0.5 mL of ice-cold DMEM and transferring the tubes into a cold water bath (0°C). Newly synthesized neurosteroids released by the DRG were extracted from the incubation medium three times with 2 mL of DCM and the organic phase was evaporated on ice under a stream of nitrogen. The dry extracts were redissolved in 2 mL of hexane and prepurified on Sep-Pak C_{18} cartridges (Waters Associates, Milford, MA). Steroids were eluted with a solution made of 50% isopropanol and 50% hexane. The solvent was evaporated in a RC-10-10 Speed Vac Concentrator and the dry extracts were kept at −20°C until HPLC analysis. The extraction efficiency was 89 ± 7%.

HPLC-Flo/One Characterization of Steroids

The newly synthesized steroids extracted from the incubation medium already purified on Sep-Pak cartridges were characterized using a previously validated method which combines HPLC analysis and flow-scintillation detection (Kibaly et al., 2005, 2008; Mensah-Nyagan et al., 1994, 1996 a,b; Patte-Mensah et al., 2003, 2004, 2005; Schaeffer et al., 2006). Briefly, the prepurified extracts were analyzed by reversed-phase HPLC on a Gilson liquid chromatograph (322 pump, UV/VIS 156 detector, Unipoint system, Gilson, Middleton, WI) equipped with a 4.6 × 250 mm SymetryShield C_{18} column (Waters Associates) equilibrated with 100% hexane. The radioactive steroids were eluted at a flow rate of 0.5 mL/min using a gradient of isopropanol (0%–60% over 65 min) including five isocratic steps at 0% (0–10 min), 1% (30–35 min), 2% (40–45 min), 30% (50–55 min) and 60% (60–65 min). The tritiated steroids eluted from the HPLC column were directly quantified with a flow-scintillation analyzer (Radiomatic Flo/One-Beta A 500, Packard Instruments, Meriden, CT) equipped with a Pentium IV PC computer for measurement of the percentage of total radioactivity contained in each peak. Synthetic steroids used as reference standards were chromatographed under the same conditions as the extracts obtained from the cell incubation media and their elution positions were determined by ultraviolet absorption using a UV/VIS 156 detector (Gilson).

The elution positions of steroids change on analytic columns after the purification of a certain number of tissue extracts. Therefore, to optimize the characterization of newly synthesized neurosteroids, synthetic tritiated neuroactive steroids including [^{3}H]PREG, [^{3}H]PROG, [^{3}H]testosterone, [^{3}H]estradiol, and [^{3}H]3αDIOL were also used as reference standards, chromatographed under the same conditions as the extracts, and identified by their elution times with the Flo/One computer system before and after each extract analytic run.

Quantification of Steroid Biosynthesis

The amount of radioactive steroids formed by the conversion of [^{3}H]PREG was calculated as a percentage of the total radioactivity contained in all peaks resolved by the HPLC-Flo/One system, including [^{3}H]PREG itself.

Statistical Analyses

Statistical analyses were performed with the 5.1. version of Statistica software (Statsoft, Maison Alfort, France). Student's *t*-test or ANOVAs followed by Tuckey *post hoc* comparisons were used.

RESULTS

Behavioral Assessment of Neuropathic Pain Symptoms

Prior to animal sacrifice and biochemical investigations, nociceptive behavioral tests were performed to check the presence or absence of neuropathic pain symptoms in CCI rats and control animals used in the study. Thermal hyperalgesia ($P < 0.001$), mechanical allodynia ($P < 0.001$), and mechano-hyperalgesia ($P < 0.001$) were detected on the ipsilateral paw during 30 days following the sciatic nerve constriction (see Fig. 1). The withdrawal latency characterizing the thermal pain threshold was generally around 11.5 + 0.5 s on each paw of sham-operated rats and on the contralateral paw of neuropathic animals. On the ipsilateral paw of sciatic neuropathic rats, the withdrawal latency to the thermal stimulations decreased to 6.5 + 0.5 s (Fig. 1A). Similarly, we observed that the inductive mechanical stimulus capable of evoking high percent withdrawal responses (≥80%) strongly decreased for the ipsilateral paw of neuropathic rats after sciatic nerve CCI (Fig. 1B). In particular, the von Frey filaments <6 g, which are normally non-noxious stimuli, become able to induce withdrawal responses (10%–20%) of the ipsilateral paw of neuropathic rats (mechanical allodynia) from

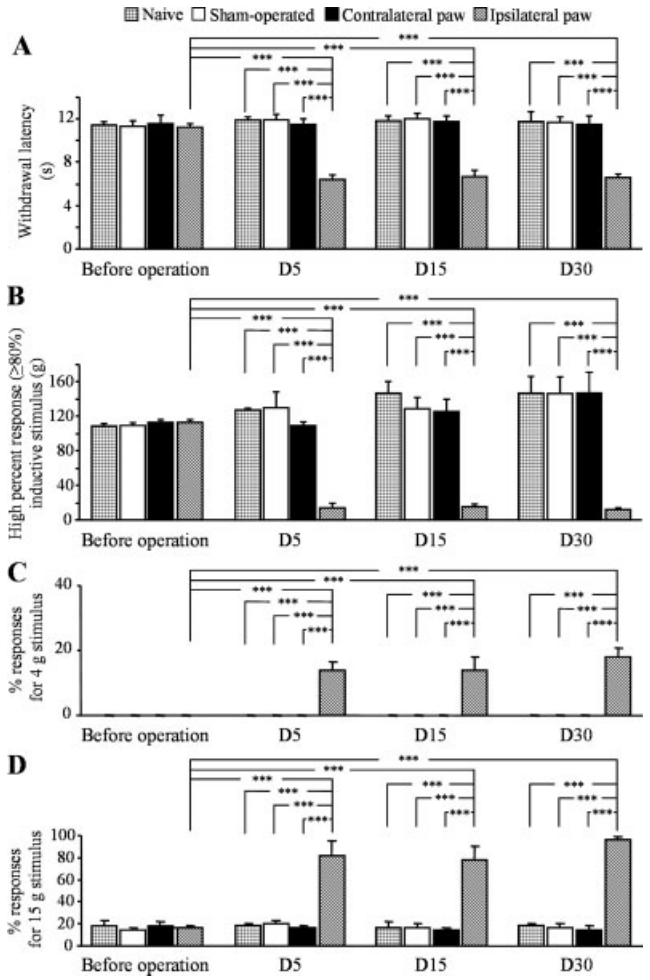

Fig. 1. Thermal (**A**) and mechanical (**B–D**) nociceptive thresholds of the ipsilateral and contralateral paws of naive, sham-operated, and CCI rats at day 5 (D5), 15 (D15), or 30 (D30) after sciatic nerve injury. For the thermal nociceptive thresholds (A) each point represents the mean + SEM of three observations on each paw of five naive, five sham-operated, or five CCI rats. For the mechanical nociceptive thresholds (B–D) each point represents the mean + SEM of five observations on each paw of five naive, five sham-operated, or five CCI rats. (B) Chartbars obtained with mechanical stimulations (g) which induced more than 80% withdrawal responses on each paw of five naive, five sham-operated, or five CCI rats before or after sciatic nerve injury. (C, D) Time-course of mechanical allodynia (C) and hyperalgesia (D) induced by sciatic nerve injury. Graphs show the mean + SEM of the percentages of paw withdrawal responses to mechanical stimulation by Von Frey filament 4 g (C) or 15 g (D). ***$P < 0.001$.

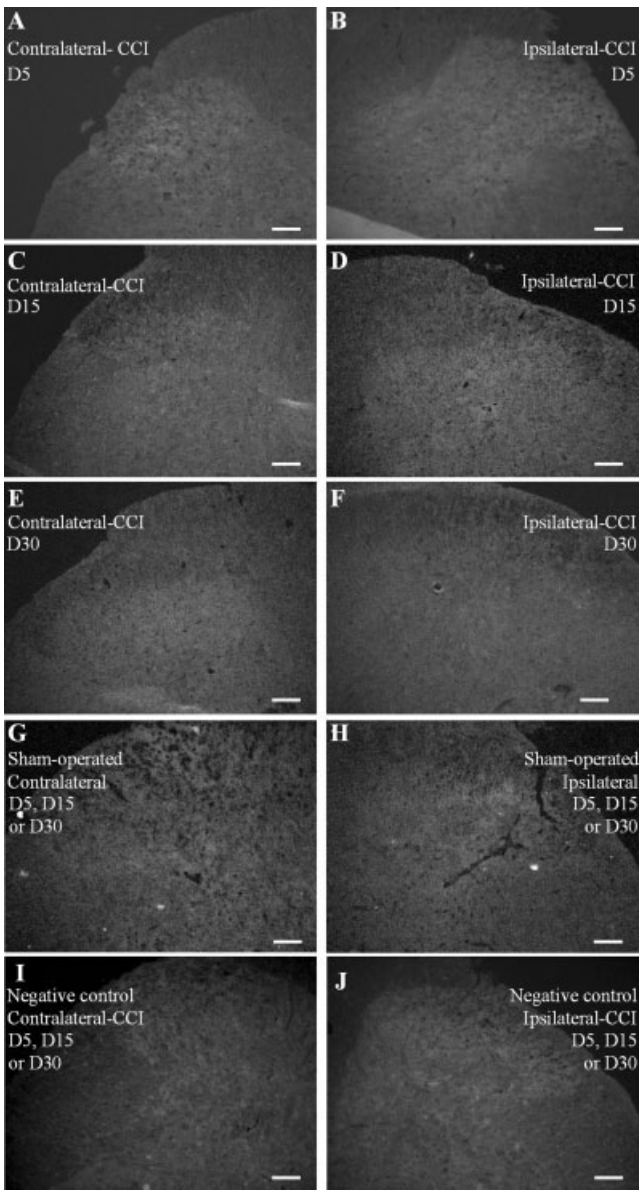

Fig. 2. TUNEL assays on DH sections of CCI rats at day 5 (**A**, **B**), 15 (**C**, **D**), or 30 (**E**, **F**) after sciatic nerve injury. Controls were performed on DH sections of sham-operated rats (**G**, **H**) or by omitting terminal deoxynucleotidyl transferase in the incubation medium (negative control) of DH sections from CCI rats (**I**, **J**). No apoptotic cell was detected at any given time point investigated. DH, spinal cord dorsal horn. Scale bar: 100 μm.

days 5 to 30 after sciatic nerve CCI (Fig. 1C). Moreover, an increased level (from 15%–20% to 80%–100%) of responding on the ipsilateral (neuropathic) paw was seen for 15 g stimulus indicating the presence of mechanical hyperalgesia (Fig. 1D).

Detection of Apoptosis in the Spinal Cord and DRG of Neuropathic Rats

As shown in Fig. 2, no TUNEL-positive cell was detected in the SC of CCI rats and control animals at any given time point investigated within the 30 day-period following the sciatic nerve injury. In contrast, TUNEL-staining indicating the occurrence of apoptosis was detected in ipsilateral DRG of CCI rats 30 days after sciatic nerve ligature (see Fig. 3). The comparison of NeuN-immunoreactivity (Fig. 4A,B) to the distribution of TUNEL-positive elements (Fig. 4C–E) in ipsilateral DRG of CCI rats at day 30 after nerve injury revealed that the apoptotic material was not localized in sensory neurons but was expressed in glial cells surrounding these neurons (Fig. 4D,E). Indeed, double-labeling experiments showed that TUNEL-positive or apoptotic cells also contained immunoreactivity for

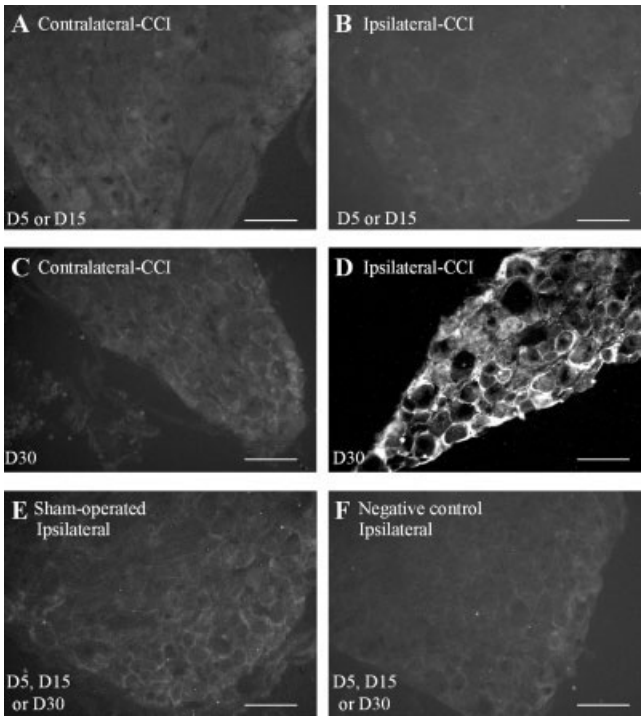

Fig. 3. TUNEL assays on DRG sections of CCI rats at day 5 or 15 (**A**, **B**) and at day 30 (**C**, **D**) after sciatic nerve injury. Controls were performed on DRG sections of sham-operated rats (**E**) or by omitting terminal deoxynucleotidyl transferase in the incubation medium (negative control) of ipsilateral DRG sections from CCI rats (**F**). TUNEL-positive or apoptotic cells were detected in ipsilateral DRG of CCI rats at day 30 after sciatic nerve injury (**D**). Scale bar: 100 μm.

glutamine synthetase (Fig. 4C–K), a well known marker of satellite glial cells (Hanani, 2005). No TUNEL-immunoreactive cells were visualized in contralateral DRG isolated from CCI rats as well as in DRG obtained from sham-operated or naive rats (Fig. 3A,C,E). Control experiments performed by incubating DH or DRG sections with the TUNEL reaction mixture without TdT also showed the absence of labeling at all given time points investigated (Figs. 2I,J, and 3F).

Neurosteroidogenesis in DRG

The biosynthesis of neurosteroids was investigated in DRG of naive, sham-operated and CCI rats at day 30 after sciatic nerve injury because apoptosis was evidenced in SGC of CCI rat ipsilateral DRG at this time point (Figs. 3 and 4). Qualitative analysis performed with HPLC-Flo/One system revealed that, in all animal groups, a 3 h incubation of DRG with the precursor [^{3}H]PREG yielded the formation of various radioactive metabolites including [^{3}H]PROG, [^{3}H]testosterone, [^{3}H]3αDIOL, [^{3}H]17OHPROG and [^{3}H]estradiol (Fig. 5A–D). Quantitative assessments were performed in order to compare the amounts of each [^{3}H]metabolite generated from the precursor [^{3}H]PREG in DRG of naive, sham-operated and CCI rats (see Fig. 6). The amount of newly-synthesized [^{3}H]PROG was higher in ipsilateral DRG of CCI rats compared to sham-operated and naive rats (Fig. 6A) but no significant difference was detected when the comparison was made between ipsilateral and contralateral DRG of CCI rats (Fig. 6A). The levels of [^{3}H]17OHPROG, [^{3}H]testosterone and [^{3}H]3αDIOL produced from [^{3}H]PREG were not modified in DRG under CCI-induced neuropathic pain situation (Fig. 6B–D). In contrast, sciatic nerve CCI-evoked neuropathic pain significantly increased the amount of newly-synthesized [^{3}H]estradiol in ipsilateral DRG containing apoptotic SGC compared to contralateral (+76%), sham-operated (+67%) and naive DRG (+118%) (Fig. 6E).

Presence and Cellular Distribution of Aromatase in DRG

Immunohistochemical experiments revealed the presence of aromatase in several cell bodies of control and CCI rat DRG (see Fig. 7). Aromatase-like immunoreactivity was found in small, medium and large size cell bodies of control and neuropathic animals (Fig. 7A,D). Cell type identification using anti-NeuN, the specific neuronal marker, showed that all aromatase-positive perykarya in control (Fig. 7A–F) and CCI rat (Fig. 7G–L) DRG corresponded to neuronal cell bodies. No immunoreactivity for aromatase was detected in glutamine synthetase-positive cells (data not shown).

Effect of Letrozole on DRG Cell Survival

Endogenous production of estradiol in DRG cells was blocked by i.t. injection of letrozole (1 mg/kg), the pharmacological inhibitor of aromatase activity. In the presence of letrozole, apoptosis occurred in CCI rat DRG neurons and SGC whereas in VEH-treated CCI animals only SGC expressed the TUNEL-labeling (Fig. 8A–C). Furthermore, comparative analyses and counting of double-labeled NeuN-TUNEL-positive cells showed that letrozole treatment induced apoptosis in 62% of CCI rat DRG neurons compared to controls ($P < 0.001$, Fig. 8D).

DISCUSSION

By combining TUNEL assays with double-labeling experiments using the neuronal marker anti-NeuN (Lind et al., 2005; Mullen et al., 1992; Wolf et al., 1996) or the SGC marker anti-glutamine synthetase (Hanani, 2005), we found no neuronal death in CCI rat lumbar SC and DRG up to 30 days after injury, although pain symptoms were evidenced by behavioral analyses. Apoptosis was identified only in SGC surrounding sensory neurons in lumbosacral DRG ipsilateral to the CCI at day 30 after sciatic nerve injury. These results contribute to the clarification of mechanisms involved in the generation of neuropathic pain symptoms such as mechano-allodynia, thermal and mechanical hyperalgesia in CCI rats. Indeed, the data revealed that, in CCI

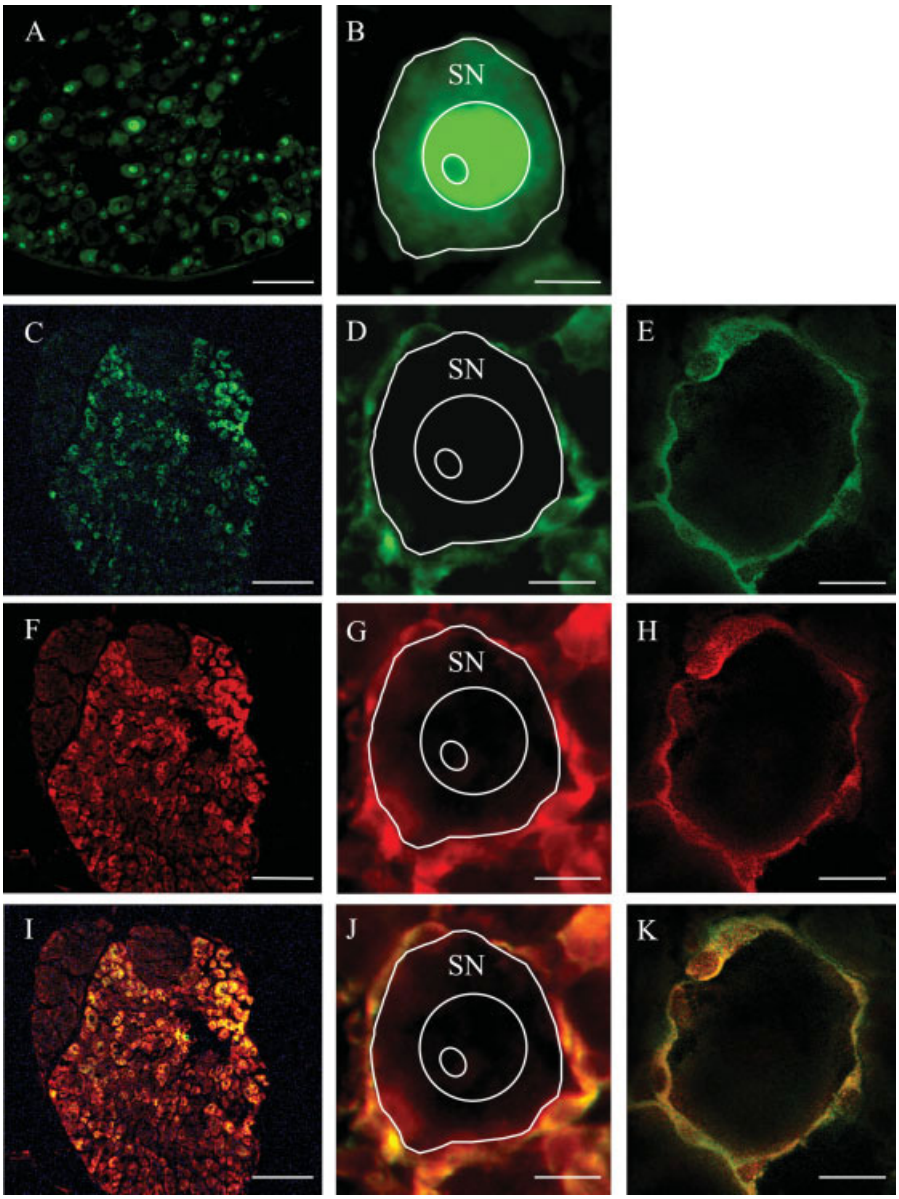

Fig. 4. Identification of TUNEL-positive cells in CCI rat ipsilateral DRG at day 30 after sciatic nerve injury. (**A**, **B**) Photomicrographs showing CCI rat ipsilateral DRG sensory neurons (SN) labeled with a monoclonal anti-NeuN and revealed in green by DAM/Alexa-488. (**C–E**) Fluorescence DMR microscope (C, D) or dual-channel confocal laser-scanning microscope (E) photomicrographs showing TUNEL-positive cells (green) in ipsilateral CCI rat DRG at day 30 after sciatic nerve injury. As shown in (D) by the schematic drawing of the sensory neuron (SN) observed in (B), no TUNEL-positive element was visualized in neuronal cell bodies. (**F–H**) Labeling of SGC [same DRG sections shown in (C), (D), and (E)] with a monoclonal antiglutamine synthetase revealed in red by DAM/Cy3. SNs were devoid of glutamine synthetase-immunoreactivity. (F, G) DMR microscope; (H) dual-channel confocal laser-scanning microscope. (**I–K**) Merged images of (C) and (F) (I), (D) and (G) (J) or (E) and (H) (K) showing (in yellow) the co-localization of TUNEL-positive material and glutamine synthetase-immunoreactivity in SGC surrounding SNs. Scale bars: (A), (C), (F), (I), 100 μm; (B), (D), (E), (G), (H), (J), (K), 10 μm. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

rat model, neuronal death in the SC or DRG is not required for the generation of neuropathic pain which results from abnormal excitability of sensory afferents induced by sciatic nerve loose ligature and from damages to myelinated as well as unmyelinated fibers (Basbaum et al., 1991; Bennett and Xie, 1988; Munger et al., 1992; Nuytten et al., 1992). A controversy was introduced in the literature by various papers: (i) a series of studies evoking neuronal and glial death in the SC and DRG have concluded that the generation of neuropathic pain depends on cell death after nerve injury (Azkue et al., 1998; de Novellis et al., 2004; Maione et al., 2002; Whiteside and Munglani, 2001); (ii) other articles reporting the absence of apoptotic neurons in the SC have shown that the loss of neurons is not required for the development of neuropathic pain symptoms (Polgár et al., 2003, 2004, 2005). In fact, most authors who observed neuronal death in the SC and DRG after nerve injury have used total transection, axotomy, or nerve crush inducing a severe nerve trauma that may explain

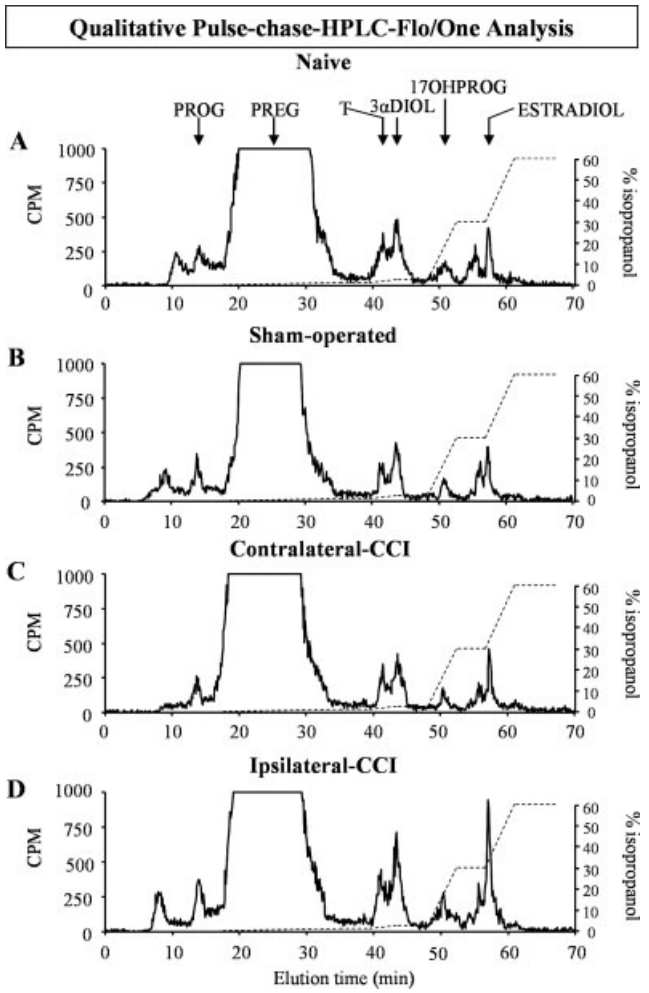

Fig. 5. HPLC-Flo/One characterization of [^{3}H]-neurosteroids produced, after a 3-h incorporation of [^{3}H]PREG into DRG isolated from naive (**A**) and sham-operated (**B**) rats or from the contralateral (**C**) and ipsilateral (**D**) sides of CCI rats at day 30 after sciatic nerve injury. The ordinate indicates the radioactivity measured in the HPLC eluent. The dashed line represents the gradient of secondary solvent (% isopropanol). The arrows indicate elution position of standard steroids. PROG, progesterone; PREG, pregnenolone; T, testosterone; 3αDIOL, 3α-androstanediol; 17OHPROG, 17α-hydroxyprogesterone and estradiol.

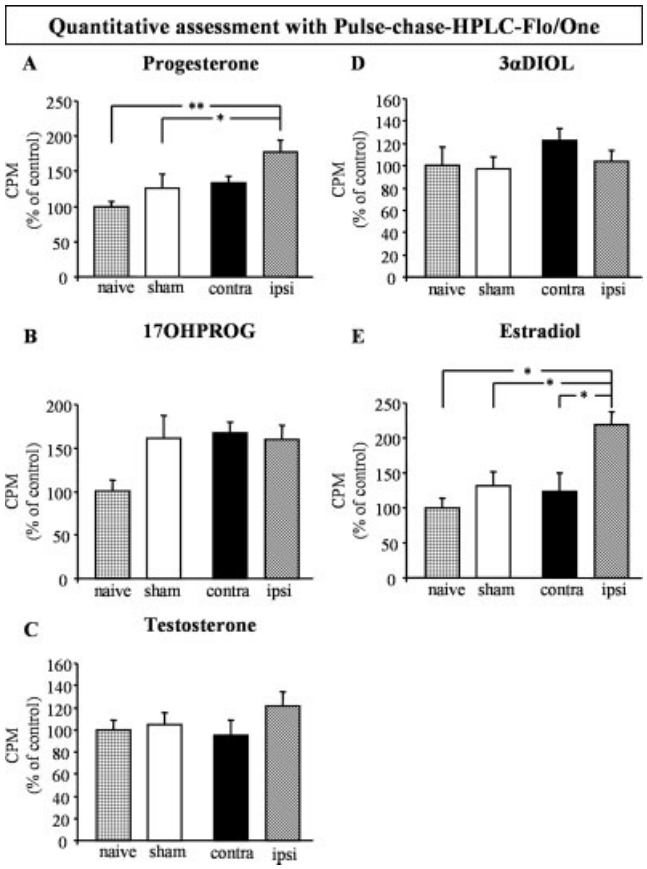

Fig. 6. Quantitative analysis of the amounts of [^{3}H]progesterone (**A**), [^{3}H]17α-hydroxyprogesterone (**B**), [^{3}H]testosterone (**C**), [^{3}H]3αDIOL (**D**), and [^{3}H]estradiol (**E**) produced, after a 3-h incubation period, from [^{3}H]PREG in DRG isolated from naive, sham-operated, and CCI (ipsilateral and contralateral DRG) rats at day 30 after sciatic nerve ligature. The values were obtained from experiments similar to that presented in Fig. 5. Each value was calculated as the relative amount of [^{3}H]-neurosteroid compared with the total [^{3}H]-labeled compounds resolved by HPLC-Flo/One characterization (×100). Each value was then expressed as percentage of the amount of each steroid formed in DRG of naive rats. *$P < 0.05$; **$P < 0.01$.

the cascade of events leading to neuronal and glial cell death (for review, Scholz and Woolf, 2007). The absence of neuronal apoptosis was mainly described by Polgár and coworkers who have used both spared nerve injury and CCI models but investigated the impact of nerve injury only on the SC and not on DRG (Polgár et al., 2003, 2004, 2005). Therefore, the present work makes it possible to specify that, when the nerve injury is induced with a moderate approach like a loose ligation (CCI rats), the subsequent slight trauma is not enough serious to trigger neuronal death in sensory structures, but is sufficient to generate functional disturbances leading to neuropathic pain symptoms.

Apoptosis occurrence in ipsilateral DRG SGC at day 30 after sciatic nerve CCI may result from the action of proinflammatory cytokines including interleukins (1 and 6) and tumor necrosis factor α which are generally produced at the nerve injury site and are axonally transported to DRG (Muñoz-Fernández and Fresno, 1998; Myers et al., 1999; Ohtori et al., 2004; Watkins and Maier, 2002). However, before considering this hypothesis as credible, we found important to determine the reason why pro-apoptotic cytokines, which reached ipsilateral DRG at day 30 after peripheral nerve injury, induced the expression of TUNEL-positive elements only in SGC but not in DRG sensory neurons. Therefore, we investigated whether or not DRG neurons surrounded by apoptotic SGC may develop a mechanism to cope, as long as possible, with the aggressive extracellular environment. Thus, we studied the ability of these DRG neurons to convert [^{3}H]pregnenolone into various neuroprotective neurosteroids thanks to pulse-chase experiments combined with HPLC analysis and flow scintillation detection (Kibaly et al., 2005, 2008; Mensah-Nyagan et al., 1994, 1996 a,b; Meyer et al., 2008; Patte-Mensah et al., 2003, 2004, 2005, 2006; Schaeffer et al., 2006,

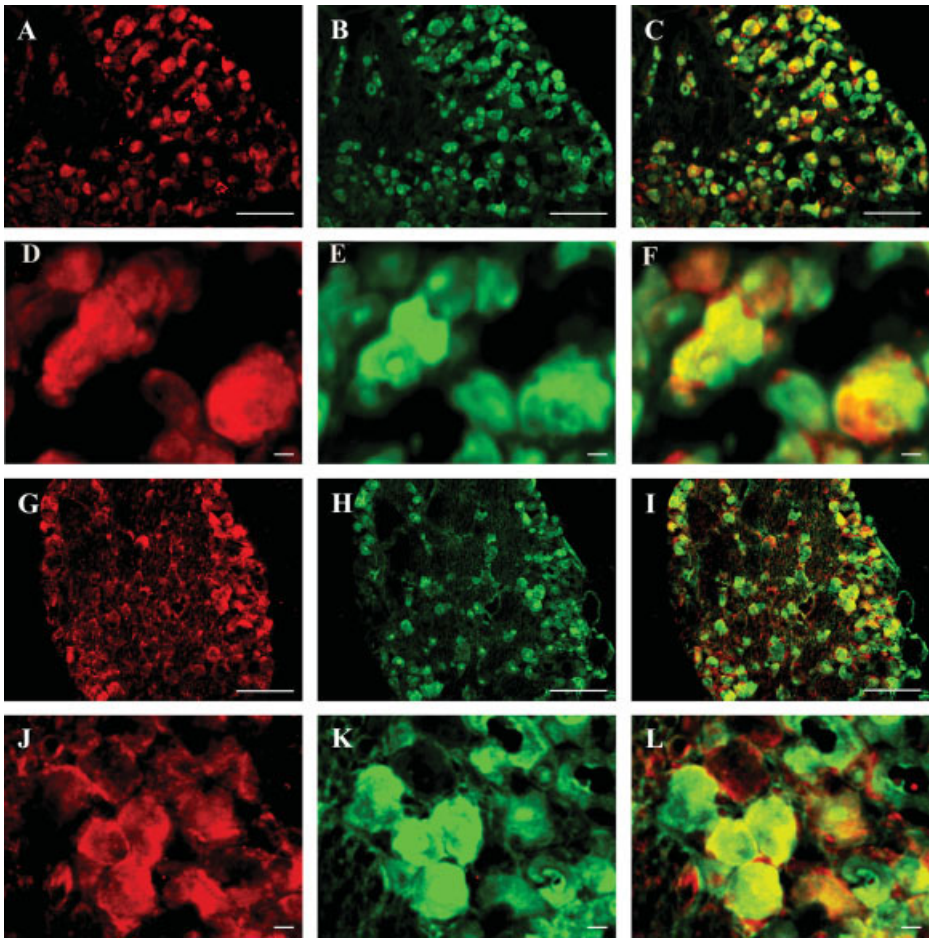

Fig. 7. Cell type identification of aromatase expression in control (**A–F**) and CCI (**G–L**) rat DRG. (A, D, G, J) Aromatase immunoreactivity revealed in red in control (A, D) and CCI (G, J) rat DRG. (B, E, H, K) NeuN-positive cells revealed in green in control (B, E) and CCI (H, K) rat DRG. (C, F, I, L) Merged images of (A) and (B) (C), (D) and (E) (F), (G) and (H) (I), (J) and (K) (L) showing (in yellow) the co-localization of aromatase positive cells with NeuN-immunoreactive neurons. Scale bars: (A), (B), (C), (G), (H), (I), 100 μm; (D), (E), (F), (J), (K), (L), 10 μm. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

2008 a,b; Venard et al., 2008). Along the neurosteroid biosynthetic pathway converting [^{3}H]pregnenolone into several metabolites, only the neosynthesis of [^{3}H]estradiol was significantly increased in DRG neurons surrounded by apoptotic SGC. This result suggests that, through a paracrine or a cross-talk mechanism, pro-apoptotic factors induced in DRG SGC by sciatic nerve CCI have triggered in DRG sensory neurons a selective increase of the activity of aromatase, the key estradiol-synthesizing enzyme. In agreement with this hypothesis, our immunohistochemical investigations revealed that aromatase is indeed expressed in DRG neurons but not in SGC. Neuroprotective actions of endogenous estradiol have been well-demonstrated in various experimental models but not in CCI rat DRG (Azcoitia et al., 2001; Fester et al., 2006; Garcia-Segura et al., 1999 a,b, 2001, 2003; McCullough et al., 2003; Veiga et al., 2005). Therefore, we performed i.t. injections of letrozole, the conventional inhibitor of aromatase activity (Buzdar, 2001; Lamb and Adkins, 1998), to block endogenous estradiol synthesis in CCI rat DRG. In the presence of letrozole, apoptosis occurred in both SGC and neurons of CCI rat DRG while in the absence of letrozole only SGC were apoptotic at day 30 after sciatic nerve injury. The fact that letrozole-induced blockade of aromatase (exclusively expressed in DRG neurons) causes apoptosis in CCI rat DRG neurons clearly demonstrates that aromatase activity (synthesizing endogenous estradiol) is pivotal for DRG neuron survival. Altogether, our observations suggest that the up-regulation of estradiol production in CCI rat DRG neurons surrounded by apoptotic SGC may be an adaptive mechanism triggered by these neurons to protect themselves against death. Thus, it appears that, even if the excitability and functions of these neurons are permanently disturbed by the hostile environment generated by sciatic nerve injury and apoptosis occurrence in SGC, they may survive, as long as possible, thanks to endogenous hyper-production of neuroprotective neurosteroid-estradiol. These data may reflect the situation occurring in patients subjected to neuropathic pain induced by slight nerve irritations or partial nerve entrapment which disturb primary afferent functions but do not necessarily cause DRG neuronal death.

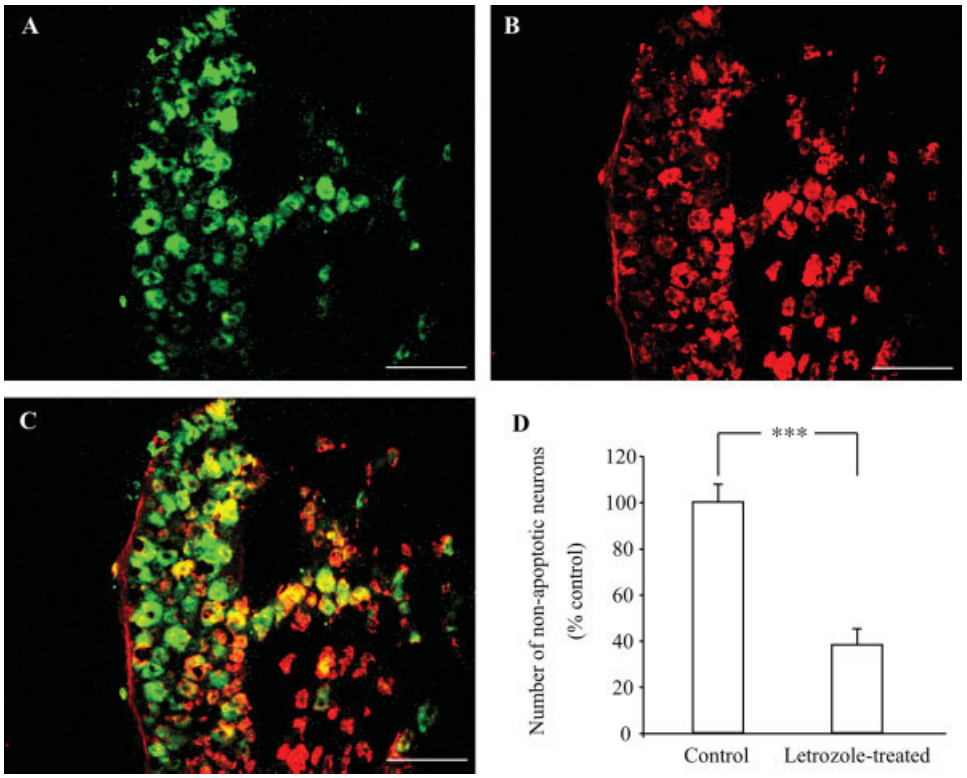

Fig. 8. Effect of letrozole on CCI rat DRG neuron viability. (**A**) TUNEL-positive cells (green) in ipsilateral CCI rat DRG treated with letrozole. (**B**) CCI rat ipsilateral DRG neurons labeled with anti-NeuN and revealed in red. (**C**) Merged photomicrographs of (A) and (B) showing in yellow the colocalisation of TUNEL-positive material and NeuN-immunoreactivity in sensory neurons. Scale bars: 100 μm. (**D**) Comparative analysis of the number of non-apoptotic DRG neurons (TUNEL-negative and NeuN-positive) detected in letrozole- or vehicle (control)-treated CCI rats. Each chartbar represents the mean + SEM of three different series of cell count performed in each of four vehicle- or letrozole-treated CCI rats. The cell counts were performed in a well-defined DRG surface: 100 × 100 μm^2 (10^4 μm^2). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Our results also showed that progesterone synthesis was stimulated in DRG of CCI rats compared to sham-operated animals. But, we did not detect a statistically significant difference between the amounts of [^{3}H]progesterone newly-synthesized from [^{3}H]pregnenolone in ipsilateral and contralateral DRG of CCI rats. Whether or not this lack of difference between CCI rat ipsilateral and contralateral DRG may be related to a statistical bias is a matter of speculation. However, it is noteworthy that progesterone was also identified as a neuroprotective neurosteroid in various models and the possibility that progesterone may also contribute to adaptive mechanisms triggered in DRG neurons surrounded by apoptotic SGC cannot be excluded (Ghoumari et al., 2003; Koenig et al., 1995; Leonelli et al., 2007; Schumacher et al., 2007).

In conclusion, the present report demonstrates the occurrence of a selective regulation of neurosteroidogenic pathways in CCI rat DRG neurons surrounded by apoptotic SGC. The results also suggest the involvement of locally-synthesized neurosteroids in the control of neurodegenerative processes in DRG.

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