CMLS, Cell. Mol. Life Sci. 61 (2004) 2274–2284
1420-682X/04/172274-11
DOI 10.1007/s00018-004-4235-4

CMLS Cellular and Molecular Life Sciences

Research Article

Impact of neuropathic pain on the gene expression and activity of cytochrome P450side-chain-cleavage in sensory neural networks

C. Patte-Mensah, S. Li and A. G. Mensah-Nyagan *

Laboratoire de Neurophysiologie Cellulaire et Intégrée, Unité Mixte de Recherche 7519, Centre National de la Recherche Scientifique, Université Louis Pasteur, 21 rue René Descartes, 67084 Strasbourg Cedex (France), Fax: +33 388 61 3347, e-mail: gmensah@neurochem.u-strasbg.fr.

Received 8 June 2004; received after revision 2 July 2004; accepted 13 July 2004

Abstract. Development of efficient therapy against chronic and stubborn pains requires fundamental identification of adequate cellular and molecular targets. This study combined cellular, molecular and biochemical approaches to investigate the gene expression and enzymatic activity of cytochrome P450side-chain-cleavage (P450scc) in spinal neural networks under normal and neuropathic pain states. P450scc is the key onset enzyme for steroidogenesis in endocrine glands and for neurosteroid biosynthesis in nerve cells. The P450scc gene was over-expressed in spinal and supra-spinal networks during neuropathic pain provoked by sciatic nerve ligature. Plasticity was observed in P450scc cellular distribution in pain circuits and its activity also increased inducing in vivo, hyper-secretion of pregnenolone and allopregnanolone which strongly stimulates type A receptors for γ-aminobutyric acid, a pivotal neurotransmitter involved in pain modulation. These results, by establishing a direct link between neuropathic pain and neuroactive steroid formation in the nervous system, open new perspectives for chronic-pain modulation by endogenous neurosteroids.

Key words. Steroidogenic enzyme; P450scc gene; neuroactive steroid; neurosteroid; pain; sensory system; cellular and molecular components of neurosteroidogenesis.

The medical treatment of chronic neuropathic pains remains difficult since they are refractory or resistant to the currently available classical analgesics [1–4]. Stubborn pains provoke, all around the world, persistent suffering, disability, medical expenses and losts of jobs for millions of patients. Therefore, identification of cellular and molecular components which may serve as targets for the development of novel therapeutic agents is a crucial challenge for biomedical research. Progress has been made over the past two decades thanks to various studies which have allowed the characterization of classical neurotransmitters and neuropeptides involved in pain modulation [for reviews see refs 5, 6]. However, a variety of pathological pains, unfortunately, can still not be alleviated by medical treatment [4, 7–9]. We therefore need to identify other key cellular and molecular factors affected in sensory neural circuits during chronic pain in order to provide pivotal information which may complete data currently available for the development of efficient therapy against stubborn pains.

Another important finding during the two last decades was the discovery of the capacity of nerve cells to synthesize bioactive steroids or neurosteroids which regulate nervous system activity [10, 11]. Neurosteroids are produced by various steroidogenic enzymes localized in neurons and glial cells [12–15]. Among these enzymes, cy-

* Corresponding author.

tochrome P450side-chain cleavage (P450scc) plays a crucial role since it catalyzes cholesterol conversion into pregnenolone (PREG), the first pivotal step for neurosteroid biosynthesis [10, 16]. Neurosteroidogenesis is well conserved through the vertebrate phylum suggesting that it might be crucial for life and this observation has raised great hopes for the development of novel therapies for various neural disorders [10, 11, 17].

Recent pharmacological studies have suggested neurosteroid involvement in pain modulation [18–22]. However, a direct link between pain processing and neurosteroid biosynthesis has never been investigated and it was impossible to know whether or not cellular and molecular components of neurosteroidogenesis are affected during chronic pain. Having recently localized P450scc in pain pathways [23], we now decided to determine the impact of neuropathic pain provoked by sciatic nerve ligature [24] on P450scc gene expression and enzymatic activity in the spinal pain circuit in order to see whether cellular and molecular sites of neurosteroidogenesis may be considered as potential targets for the development of novel therapeutic agents. Modifications of P450scc gene expression in pain circuits were investigated in neuropathic-pain rats using a highly sensitive tool, the real-time polymerase chain reaction (rt-PCR) after reverse transcription (RT). Immunocytochemical studies combined with confocal microscopic analysis (CLSM) were performed to determine changes in the cellular distribution of P450scc in spinal sensory neural networks during painful states. Moreover, a well-validated method combining pulse-chase experiments, high-performance liquid chromatography analysis (HPLC) and flow scintillation detection [23, 25–27] allowed in vitro investigation of P450scc activity in sensory structures of normal and neuropathic-pain rats. Finally, the impact of neuropathic pain on neurosteroid production in pain neural pathways in vivo was assessed by radiommunoassays after HPLC purification of tissue extracts.

Materials and methods

Animals

Adult male (mainly) and female (one series of experiments) Sprague-Dawley rats weighing 300–350 g were used. 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 (Harlan, Le Malcourlet, 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. The neuropathic pain was provoked according to the protocol described by Bennett and Xie [24] and all experiments followed the International Association for the Study of Pain ethical guidelines [28]. Briefly, after dissection at the middle of the thigh, 4–5 mm of the common sciatic nerve were tied loosely with four ligatures spaced by 1 mm. Two categories of animals were constituted according to the ligated side (right or left). Various groups of control rats were used: some were not operated (naïve 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 good 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 [24]. Animals were sacrificed 10 days after the sciatic nerve ligature for all experiments except for time-course studies performed on days 0, 2, 6 and 10 after the ligatures.

RT and rt-PCR

The TRIzol reagent (Invitrogen, Paisley, UK) was used according to the manufacturer's instructions for the extraction of total RNA from gonads, lumbar spinal cord (SC) or brainstem areas containing well-known nociceptive supra-spinal nuclei (SSN) such as the parabrachial, raphe magnus and dorsal raphe nuclei [5, 6]. The quality of RNA was electrophoretically determined by ethidium bromide-stained agarose gels and by the optical density (OD) absorption ratio $OD_{260nm}/OD_{280nm} > 1.7$. RT of 20 µg total RNA was performed at 37 °C for 1 h and the reaction system for first-strand cDNA was as follows: 160 U Moloney murine leukemia virus (Amersham Biosciences Europe, Freiburg, Germany), 0.16 µg random hexamer [pd(N)6 random primer; Pharmacia, Milan, Italy], 2 mM dNTP mixture (Fermentas, Hanover, Md.) in a final volume of 40 µl. rt-PCRs were performed using a LightCycler (Roche Diagnostics, Mannheim, Germany). The specific primer sequences for P450scc were: forward, 5′-TCA AAG CCA GCA TCA AGG AG-3′ (nucleotides 1141–1160) and reverse, 5′-GCA GCC TGC AAT TCA TAC AG-3′ (nucleotides 1613–1594) [29]. The primers for the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were: forward, 5′-ACC ACA GTC CAT GCC ATC AC-3′ (nucleotides 3069–3088) and reverse, 5′-TCC ACC ACC CTG TTG CTG TA-3′ (nucleotides 3624–3605) (Clontech, Palo Alto, Calif.). PCR was performed in a total volume of 20 µl including 0.5 µg cDNA, 0.5 µM specific primers, 2 µl LightCycler-DNA Master SYBR Green I kit (Roche Diagnostics) containing Taq polymerase, dNTP, $MgCl_2$, and SYBR Green I dye. A negative control without cDNA template (called water) was run simultaneously with every assay. Standard curves were obtained by using serial dilution of testis cDNA. The amplification program consisted of one denaturing step of 8 min at 95 °C, followed by different amplification cycles of 5 s at 95 °C, 5 s at 62 °C and 18 s at

72 °C. Amplifications were followed by melting-curve analysis to ensure the specificity of PCR products and the absence of non-specific products. Based on analyses and calculation integrating adequate standard curves, levels of transcripts encoding P450scc and GAPDH were determined in samples from gonads, SC and SSN using the LightCycler Software available in the rt-PCR system. The concentration of P450scc mRNA in each sample was calculated after normalization of rt-PCR P450scc product to GAPDH. For each sample, the rt-PCR experiment was repeated four times. The results are expressed as the mean ± SE.

Immunocytochemistry

Two different antibodies against P450scc were used. The first antiserum (Abscc I; a generous gift from Dr Tsutsui, Hiroshima, Japan) was raised in rabbit against purified P450scc from bovine adrenocortical mitochondria and has previously been used successfully to localize P450scc in the central nervous system (CNS) and classical steroidogenic glands of rat [23, 30–32]. The second antiserum (Abscc II), purchased from Chemicon (Temecula, Calif.), was generated in rabbit against the carboxy-terminal amino acids 509–526 of rat P450scc. The specificity and effectiveness of this antiserum have previously been demonstrated by Western blot and immunocytochemical analysis [33]. The mouse anti-neuronal nuclei monoclonal antibody (anti-NeuN) and fluorescein isothiocyanate goat anti-rabbit (GAR/FITC) antibody were also purchased from Chemicon. Alexa-488-conjugated donkey anti-mouse (DAM/Alexa-488) and Alexa-555-conjugated goat anti-rabbit (GAR/Alexa-555) were supplied by Interchim Molecular Probes (Eugene, Ore.). The immunofluorescence procedure was conducted as previously described [23]. Briefly, fixed SC and SSN 20-µm-thick sections were pre-incubated for 1 h with 5% non-immune donkey serum in 0.1 M phosphate buffer (PB) containing 0.3% Triton-X100 (PBT). Afterwards, the sections were incubated overnight at room temperature in the P450scc antiserum (Abscc I or II) diluted at 1:500 in PBT. The procedure continued by rinsing the sections three times in PB and transferring them for 1.5 h into GAR/FITC diluted at 1:100 in PBT. Finally, the sections were rinsed for 1 h in PB and mounted in Vectashield (Vector Laboratories, Burlingame, Calif.). For neuronal cell body identification, double-labeling experiments were performed by incubating the sections with a mixture containing P450scc antiserum (Abscc I or II, 1:500) and the monoclonal anti-NeuN (1:1000). In these conditions, P450scc immunoreactivity was revealed with GAR/Alexa-555 and NeuN immunostaining with DAM/Alexa-488. 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 multi-channel laser CLSM, LSM510 (Zeiss, Göttingen, Germany).

The specificity of the immunoreaction was controlled by (i) substitution of P450scc antiserum with PB, (ii) replacement of P450scc antiserum by non-immune rabbit serum and (iii) pre-incubation of P450scc antibodies with purified bovine adrenal P450scc (10 µg/ml).

Pulse-chase-HPLC-Flo/One detection

Dichloromethane, hexane, isopropanol and propylene glycol were obtained from Merck (Darmstadt, Germany). Dulbecco's modified Eagle's medium (DMEM) was purchased from Invitrogen. Tritiated steroids such as 9,11,12-^{3}H(N)-allopregnanolone ([^{3}H]ALLOPREG), 1,2-^{3}H(N)-cholesterol ([^{3}H]CHOL) and 7-^{3}H(N)-pregnenolone ([^{3}H]-PREG) were obtained from PerkinElmer (Boston, Mass.). For each experiment, 100 mg SSN or SC (dorsal half of the lumbar segment) tissues were pre-incubated for 15 min in 2 ml 0.9% NaCl at 37 °C. The tissues were homogenized with a glass potter-elvehjem homogenizer in 1.5 ml DMEM (pH 7.4) containing 10^{-8} M [^{3}H]CHOL supplemented with 1% propylene glycol. The homogenates were incubated at 37 °C for 18 h in a water-saturated atmosphere (95% air, 5% CO_2) allowing maintenance of the pH at 7.4. At the end of the incubation period, the reaction was stopped by adding 500 µl ice-cold DMEM and transferring the tubes into a cold water bath (0 °C). Newly synthesized unconjugated neurosteroids were extracted three times with 2 ml dichloromethane and the organic phase was evaporated on ice under a stream of nitrogen. The dry extracts were redissolved in 2 ml hexane and pre-purified on Sep-Pak C_{18} cartridges (Waters Associates, Milford, Mass.). Steroids were eluted with a solution made of 60% isopropanol and 40% hexane. The solvent was evaporated in a RC-10-10 Speed Vac Concentrator (Jouan, St Herblain, France) and the dry extracts were kept at 4 °C until HPLC analysis. To determine the recovery of newly synthesized steroids during the extraction procedure, SC homogenates were incubated for 10 min with 10^{5} cpm of [^{3}H]PREG and extracted as described above. The extraction efficiency was 89 ± 7%. The newly synthesized steroids extracted from tissue homogenates already purified on Sep-Pak cartridges were characterized using a previously validated method which combines HPLC analysis and flow scintillation detection assisted by a computer system [23, 25–27]. The amount of radioactive steroids formed by the conversion of [^{3}H]CHOL was expressed as a percentage of the total radioactivity contained in all peaks resolved by the HPLC-Flo/One system, including [^{3}H]CHOL itself. Each value is the mean of four independent experiments.

HPLC-radioimmunoassays

Endogenous PREG or ALLOPREG were extracted from the lumbar SC, SSN tissues or blood with dichloromethane as described above. The concentrations of PREG or

ALLOPREG were determined by radioimmunoassays after HPLC analysis of the tissue extracts using antisera against PREG or ALLOPREG courteously provided by Dr Y. Akwa (Paris, France). The final dilutions of the anti-PREG and anti-ALLOPREG were 1:60,000 and 1:100,000, respectively.

Statistical analysis

All values presented are means ± SE of four different experiments. Student's t test was used for comparisons in pairs while ANOVA followed by Bonferroni's test was applied for multi-parameter analyses.

Results

Effects of neuropathic pain on P450scc gene expression in sensory neural pathways

The levels of transcripts encoding P450scc in the SC dorsal horn (DH) and SSN were examined by rt-PCR in control and neuropathic-pain rats. Figure 1 shows representative amplification curves for the P450scc gene, GAPDH used as a housekeeping gene and the negative control. The continuous fluorescence monitoring of DNA copy numbers within an ample dynamic range provided a highly sensitive detection of specific template signals for P450scc (fig. 1A, B) and GAPDH (fig. 1C) in the SC, SSN and testis, a classical steroidogenic tissue. Amplification reactions were followed by melting-curve analysis to ensure the specificity of PCR products (fig. 2A, B). The ranges of P450scc mRNA concentrations detected in the nervous tissue were 10^3- to 10^4-fold lower than that measured in testis (fig. 1A, B). Peripheral mononeuropathic pain significantly increased the level of P450scc mRNA in the SC and SSN (fig. 3). After normalization of rt-PCR P450scc product to GAPDH, P450scc mRNA concentrations in the lumbar SC and SSN of neuropathic-pain rats seemed to be 3- and 2.78-fold higher, respectively, than in controls (fig. 3).

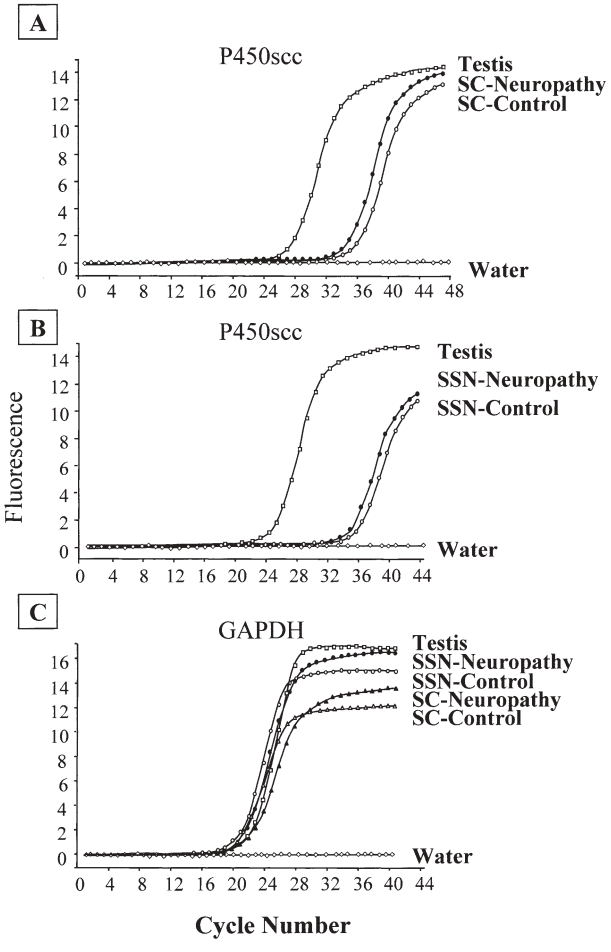

Figure 1. rt-PCR amplification of P450scc and GAPDH genes. rt-PCR obtained in the testis, SC and SSN of neuropathic-pain and control rats.

Impact of painful inputs on the cellular distribution of P450scc protein in DH sensory networks

Immunocytochemical experiments using specific antibodies against P450scc were combined with CLSM analysis for the identification of changes provoked by the peripheral neuropathy in the cellular distribution of P450scc in the SC (fig. 4). The CLSM used offered the opportunity to select the z-plane of focus by a computer-controlled fine-stepping motor which allowed adjustment of the focal plane in as little as 0.1-µm increments. Therefore, series of images (galleries) could be made with 0.5- to 1-µm optical thickness to thoroughly compare the P450scc distribution in spinal sensory neural networks of control (fig. $4A_{1-15}$) and neuropathic-pain (fig. $4B_{1-15}$) rats. Nociceptive inputs evoked by the sciatic nerve ligature induced a significant increase in the density of P450scc-positive nerve fibers in the ipsilateral DH laminae I–III of neuropathic rats as shown by comparison in pairs (A_1 versus B_1, A_2/B_2, …, A_{15}/B_{15}) of images in galleries A and B (fig. 4). Additional P450scc-containing fibers which appeared during the painful state can easily be visualized on the focal planes shown in fig. $4B_6$–B_{15} (neuropathic rats) compared, respectively, to fig. $4A_6$–A_{15} (controls). The region of the SC showing a high plasticity in P450scc distribution corresponded to sensory areas well known to contain neurons involved in pain transmission (fig. 4C). The fine CLSM 0.5- to 1-µm optical thickness allowed good observation of increases in the density of P450scc-positive nerve fibers but did not facilitate the determination of modifications in the number of sensory neuronal cell bodies containing the enzyme. Therefore, we performed double-labeling experiments on 20-µm-thick sections using antisera against P450scc and NeuN, the specific marker for neuronal cell bodies. The immunohistological preparations were examined with CLSM using 9-µm increment or optical thick

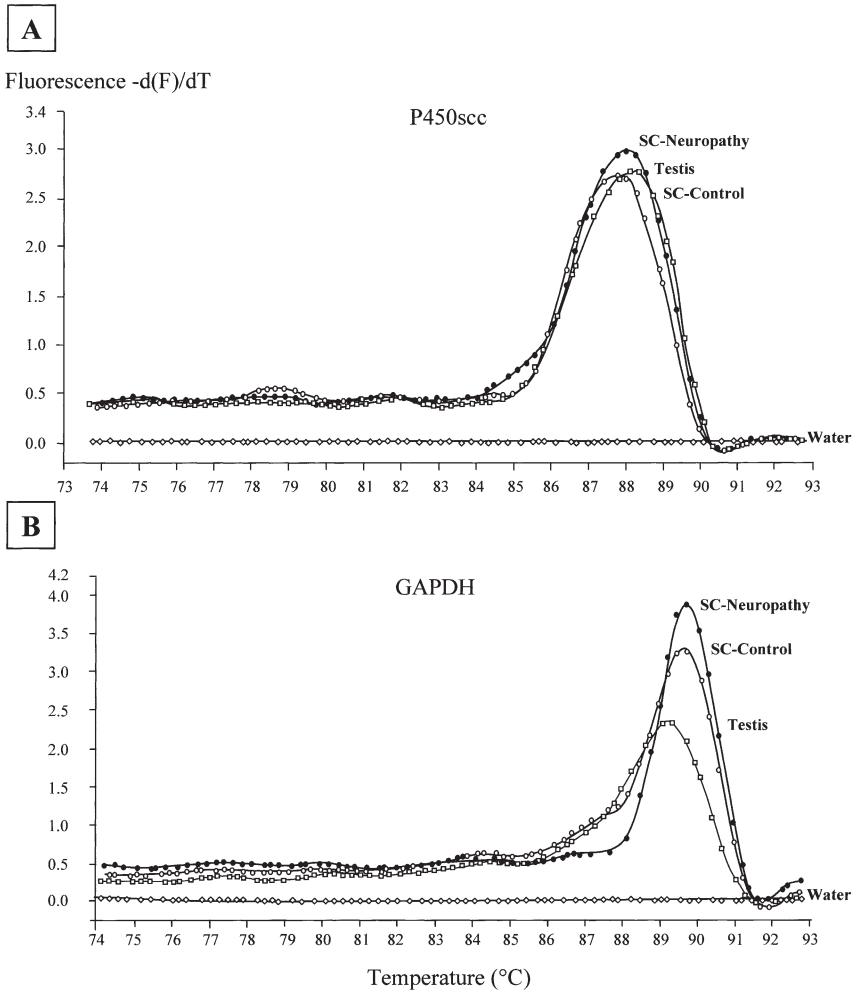

Figure 2. Specificity of P450scc and GAPDH PCR products. Melting-curve analysis of P450scc (*A*) and GAPDH (*B*) PCR products showing the very high degree of specificity of amplified dsDNA. Performance of melting analysis for each PCR product in the same closed capillary used for amplification meant that the risk of contamination could be avoided and enabled easy differentiation of specific fragments from non-specific products.

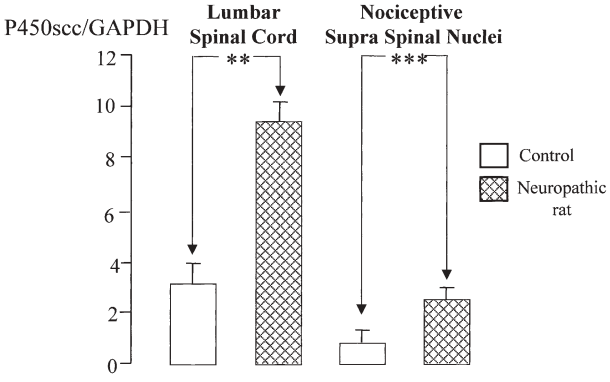

Figure 3. Effect of neuropathic pain on P450scc gene expression. The level of P450scc mRNA is expressed as a ratio of the amount of GAPDH mRNA in the SC and SSN of control and neuropathic-pain rats. Each value is the mean ± SE of four independent experiments. ** $p < 0.01$, *** $p < 0.001$.

sections, and numerous neuronal cell bodies containing both P450scc and NeuN could be detected (fig. 5). Thus, we counted perikarya expressing both P450scc and NeuN immunoreactivities (fig. 5D–F) in the lumbar DH of neuropathic-pain rats and controls. The cell counting revealed a 45% increase in P450scc-positive sensory neurons during the neuropathic-pain state. In addition, changes observed in P450scc expression during neuropathic pain were restricted to the DH and no significant modifications were detected in the ventral horn of the SC which contains motors areas (fig. 6).

Effects of neuropathic pain on P450scc enzymatic activity in vitro

Reversed-phase HPLC analysis was coupled with flow scintillation detection to compare the conversion of [^{3}H]CHOL into [^{3}H]PREG by tissue homogenates from the lumbar SC and SSN of control and neuropathic rats.

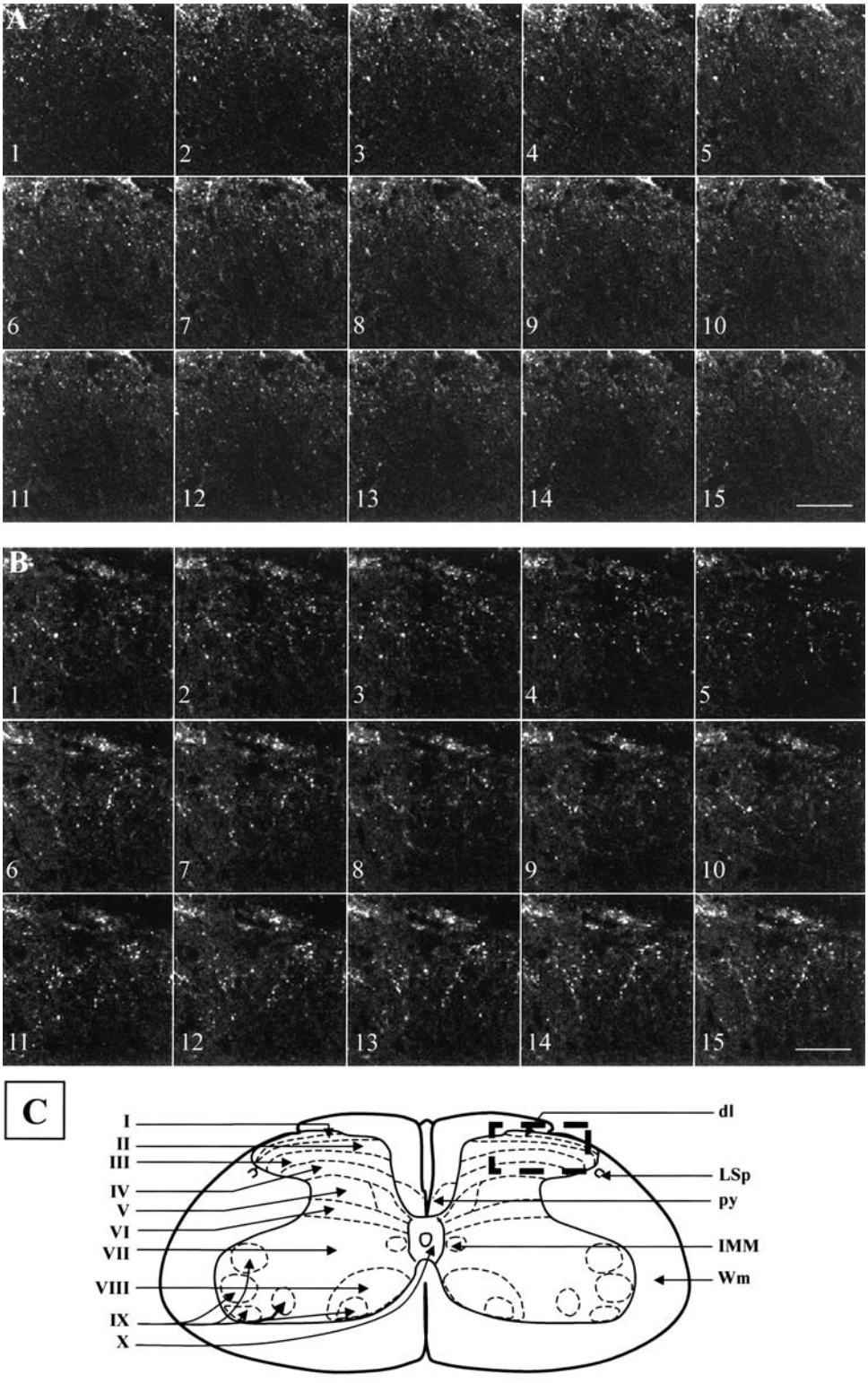

Figure 4. Effect of neuropathic pain on P450scc cellular distribution. Comparative CLSM analysis was performed between the distribution of P450scc in the lumbar spinal DH sensory networks of control (*A*) and neuropathic-pain (*B*) rats. Each gallery contains 15 images spaced by 1 µm. Comparison should be made between A_1 and B_1, A_2/B_2, …, A_{15}/B_{15}. Additional P450scc-positive fibers which appeared during the neuropathic painful state can be visualized mainly on focal planes shown in images B_6–B_{15} compared, respectively, to A_6–A_{15} (controls). The same results were obtained with AbsccI and AbsccII. Scale bars, A_{1-15} and B_{1-15}, 50 µm. (*C*) Schematic representation of a transversal section of the lumbar SC showing sub-divisions into laminae I–X. The dashed box indicates the region scanned in CLSM to obtain images A_{1-15} and B_{1-15}. dl, dorsolateral fasciculus; IMM, intermediomedial cell column; LSp, lateral spinal nucleus; py, pyramidal tract; Wm, white matter.

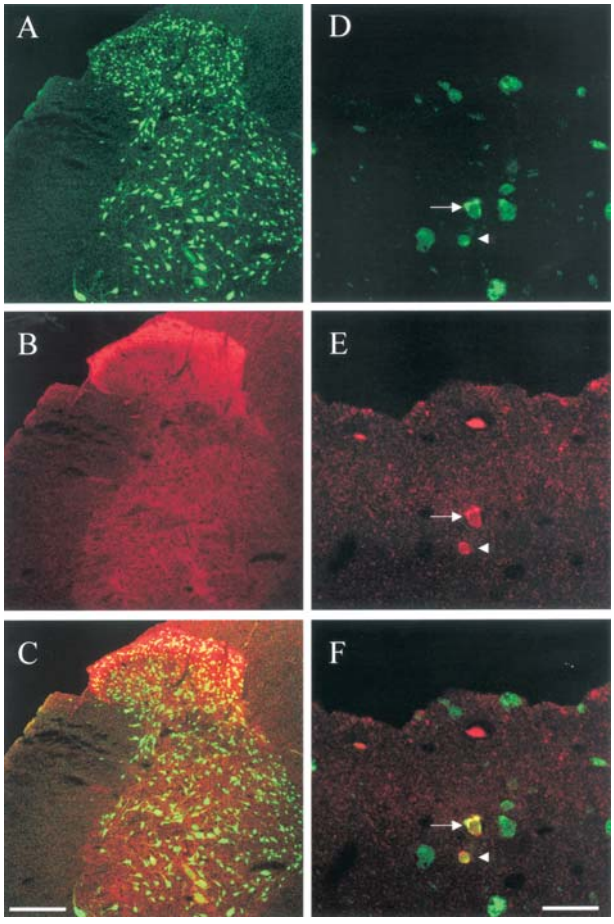

Figure 5. Double-labeling identification of P450scc-positive sensory neurons. The photomicrographs, obtained by dual-channel CLSM analysis of SC sections, show the co-localization of NeuN (a well-known specific marker for neuronal cell bodies) and P450scc in several perikarya of the DH. (*A*), General view of an SC section labeled with anti-NeuN revealed with DAM/Alexa-488. (*B*) View of the same section in *A* labeled with P450scc antiserum and revealed with GAR/Alexa-555. (*C*) Combination of the two images acquired in *A* and *B*. (*D–F*) High-magnification photomicrographs showing two different types of co-expression of NeuN and P450scc in neuronal cell bodies. When the immunoreactivity for NeuN was intense in the cytoplasm (arrow in *D*) as with P450scc-immunostaining (arrow in *E*), co-localization appeared in yellow (arrow in *F*). In contrast, when the immunoreactivity for NeuN was restricted to the nuclear compartment (arrowhead in *D*), the co-localized neuronal cell bodies exhibited a green nucleus (presence of NeuN) surrounded with a red cytoplasm (presence of P450scc) (arrowhead in *F*). These two types of co-localized neuronal cell bodies were counted and the number was 45% higher in the DH of neuropathic-pain rats compared to controls. Scale bars: *A–C* 200 µm; *D–F* 20 µm.

After 18 h incubation, the amount of newly synthesized [^{3}H]PREG from [^{3}H]CHOL was 80% and 115% higher, respectively, in tissue homogenates from the lumbar SC and SSN of neuropathic-pain rats compared to controls (fig. 7A).

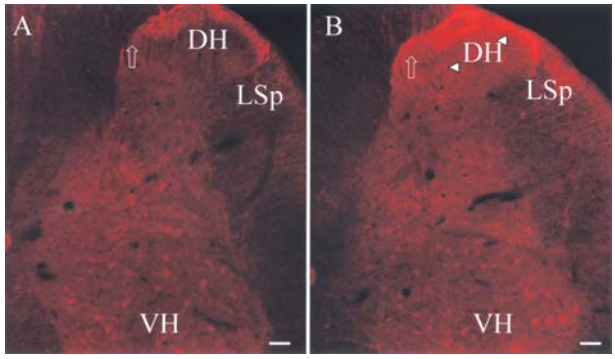

Figure 6. Distribution of P450scc immunoreactivity in the SC of control and neuropathic-pain rats. The photomicrographs show a general view of P450scc distribution in areas of lumbar SC located at the ipsilateral side of the sham-operated or ligated sciatic nerve, respectively, in control (*A*) and neuropathic-pain (*B*) rats. The intensity of P450scc immunostaining was very high in the DH of neuropathic-pain animals compared to controls. P450scc immunoreactivity was strongly expressed in the lateral spinal nucleus (LSp, an area involved in nociception) and in the left lateral side (open arrows) of the DH in neuropathic rats (*B*) while the staining was very low in the corresponding zones in controls (*A*). Additional P450scc-positive fibers extending from the DH toward the deep laminae can be observed in neuropathic rats (arrowheads). No significant difference was visualized in the ventral horn (VH). Scale bars, 100 µm.

Impact of painful inputs on neurosteroid production in pain neural pathways in vivo

HPLC purification of the lumbar SC, SSN and serum extracts combined with radioimmunological detection of PREG revealed that the endogenous concentration of this neurosteroid significantly increased in sensory neural structures during neuropathic painful states while plasma levels of PREG did not change (fig. 7B). The concentrations of PREG in blood, SC and SSN of control rats were 0.167 ± 0.003 ng/ml, 47.23 ± 1.02 ng/g and 6.8 ± 0.07 ng/g, respectively, and levels detected in neuropathic-pain animals were 0.209 ± 0.008 ng/ml (blood), 88.46 ± 1.02 ng/g (SC) and 9.56 ± 0.27 ng/g (SSN). Since PREG can serve as a precursor for the biosynthesis of other neurosteroids including ALLOPREG, a potent positive modulator of γ-aminobutyric acid type A receptors ($GABA_A$-R), time-course studies were performed to assess whether or not the hyper-production of PREG in the SC during neuropathic pain provoked an increase in the local concentration of ALLOPREG. As shown in figure 8 (filled symbols), endogenous levels of PREG and ALLOPREG, which increased markedly in the SC of neuropathic rats 2 days after the sciatic nerve ligature, reached a maximum by day 6 and declined moderately (PREG) or very slightly (ALLOPREG) at day 10. The concentrations of these two neurosteroids did not change from days 0 to 10 in the SC of control animals (fig. 8, other symbols).

Table 1. Levels of P450scc mRNA in the SC and SSN of male and female rats in normal and neuropathic-pain situations.

	SC				SSN			
	control		neuropathic rat		control		neuropathic rat	
	male	female	male	female	male	female	male	female
P450scc/ GAPDH	3.2 ± 0.8	2.8 ± 0.6	9.4 ± 0.8	8.6 ± 0.5	0.9 ± 0.4	1.1 ± 0.6	2.5 ± 0.5	3.1 ± 0.4

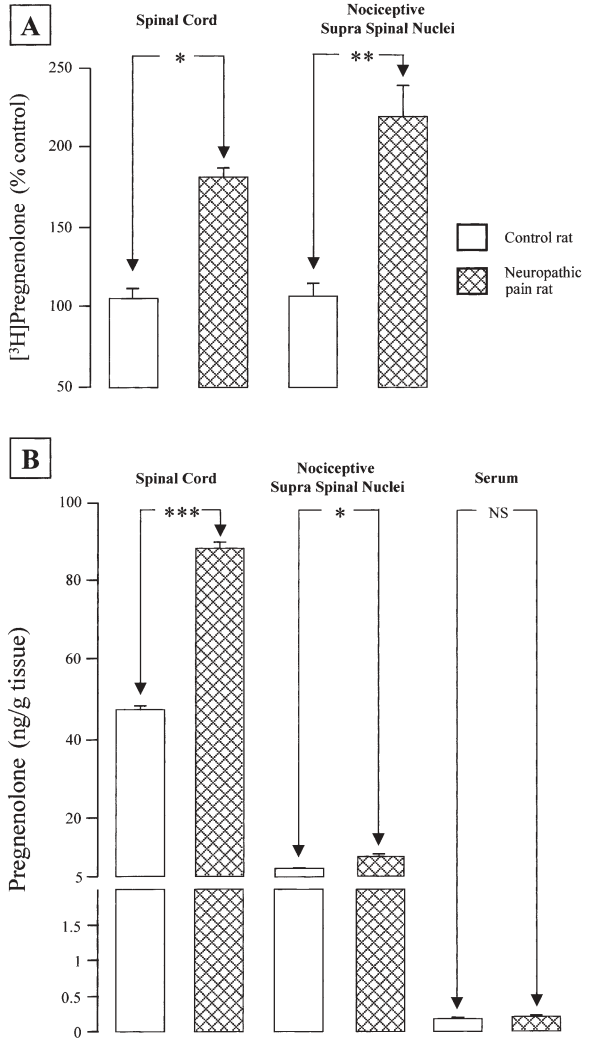

Figure 7. Impact of neuropathic pain on P450scc enzymatic activity in vitro and in vivo. (*A*) Effects of neuropathic painful inputs on P450scc enzymatic activity were determined in vitro by investigating the conversion of [^{3}H]CHOL into [^{3}H]PREG by SC and SSN homogenates of control and neuropathic-pain rats. Before expressing data as percentage of control, each value was calculated as the relative amount of [^{3}H]PREG compared with the total [^{3}H]-labeled compounds resolved by HPLC-Flo/One characterization (×100). (*B*) Endogenous concentrations of PREG produced in vivo were determined by radioimmunoassays after HPLC analysis of blood, SC or SSN tissue extracts of control and neuropathic-pain rats. Each value presented in *A* and *B* is the mean ± SE of four independent experiments. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. NS, not statistically different.

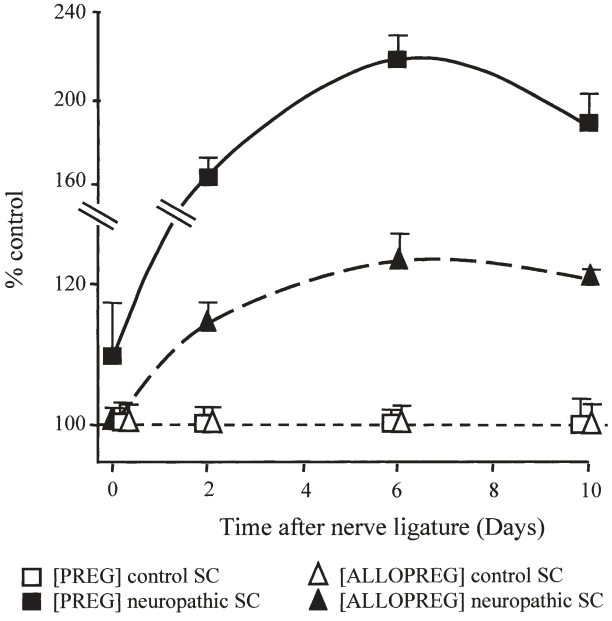

Figure 8. Kinetics of neurosteroid production in the painful state. Time-course measurement of endogenous concentrations of PREG and ALLOPREG in the SC of control and neuropathic-pain rats. The concentrations of PREG and ALLOPREG were determined by combining HPLC analysis of tissue extracts and radioimmunoassays as described in Materials and methods. The values are expressed as percentages of the amount of PREG or ALLOPREG detected in the SC of control animals sacrificed the same day and in exactly the same conditions as neuropathic rats. Each value represents the mean ± SE of four independent experiments.

Sex comparison

A series of studies was performed with adult female rats and similar modifications were observed in P450scc gene expression in sensory neural networks under painful states. Table 1 summarizes the quantitative distribution of P450scc mRNAs in the SC and SSN of female and male rats in normal and neuropathic-pain situations.

Discussion

Pharmacological and behavioral studies have suggested neurosteroid involvement in pain modulation but a direct impact of pain on the process of neurosteroidogenesis in

nerve cells has never been demonstrated [18–22]. By combining various cellular, molecular and biochemical approaches, this work is the first to show that the gene expression, cellular distribution and biological activity of P450scc, the key onset enzyme for neurosteroid biosynthesis, are significantly modified in sensory neural circuits during the neuropathic pain state. In animals submitted to chronic pain, the P450scc gene was up-regulated in pain neural networks of the SC and in nociceptive SSN including the parabrachial, raphe magnus and dorsal raphe nuclei [5, 6]. This up-regulation of the gene in pain circuits led to an increase in the cellular distribution of P450scc enzymatic protein and its biological activity in vitro as well as in vivo. Our results, which unambiguously demonstrate that cellular, molecular and biochemical components of neurosteroidogenesis are affected in pain neural pathways in the painful state, suggest that these components may be considered as potential targets for investigations aiming to develop new therapeutic strategies against chronic pain. The model of neuropathic pain used in this work (sciatic nerve tied loosely by ligatures) is that validated by Bennett and Xie in 1988 [24], which has served for several published studies on pain and is well-recognized by the International Association for the Study of Pain as reproducing in rat disorders of pain sensation like those seen in humans. We observed that the up-regulation of the P450scc gene during neuropathic pain was limited to the DH sensory areas and no significant changes were detected in the ventral horn which is mainly involved in motor functions [34]. However, because our observations were made for only 10 days following the sciatic nerve ligature, over longer periods, we cannot exclude the possibility that P450scc gene expression and its enzymatic activity may also be affected in motor areas of the nervous system due to postural disturbances associated with chronic sciatic pain [24].

The significant increase in P450scc gene expression during neuropathic pain, the cellular redistribution of P450scc enzymatic protein in the spinal DH and changes of P450scc biological activity strongly indicate that neurosteroidogenesis is an endogenous mechanism triggered or activated in appropriate neural circuits to facilitate adaptation of the body to the chronic pain state. In support of this hypothesis, recent studies have shown that the neurosteroid PREG (produced by P450scc and hyper-released in the painful state) binds to neural microtubule-associated protein and promotes cytoskeleton development by increasing tubulin polymerization and microtubule formation [35, 36]. As the modulation of neural plasticity described in the pathogenesis of chronic pain is important for the development of novel therapy [37], hyper-production of PREG, the cytoskeleton stimulator, in pain neural circuits constitutes a promising observation that deserves particular attention for future investigations. Furthermore, our time-course studies revealed that hyper-secretion of PREG provoked by neuropathic pain in the SC led to an increase in the endogenous concentration of ALLOPREG, a potent positive modulator of $GABA_A$-R which play an important role in the regulation of painful sensations [4, 8–10, 17, 37]. ALLOPREG is a neurosteroid derived from PREG thanks to complementary enzymatic activities of 3β-hydroxysteroid dehydrogenase, 5α-reductase and 3α-hydroxysteroid dehydrogenase, all of which have been localized in sensory neural networks [38, 39]. Since ALLOPREG is capable of significantly stimulating the GABAergic system (the main inhibitory neurotransmitter) [10, 17], increase in its production observed in sensory neural circuits during the painful state may be an endogenous mechanism activated by neuropathic animals to decrease their sensitivity to painful messages. Identification of effects of ALLOPREG injection into animals using diverse pain behavioral tests will certainly help in the future to confirm this suggestion. However, as the objective of this paper was not to focus on behavioral actions of injected steroids as already described [18–22], the new conceptual framework we would like to suggest to foster investigations of neurosteroid actions in pain modulation is the identification of psychotropic agents capable of interfering with transcriptional mechanisms, gene promoters and bioactivity of P450scc and other key steroidogenic enzymes in sensory neural circuits. Determination of the effects of such psychotropic drugs and identification of their interactions with classical neurotransmitter systems and proteins involved in neuronal plasticity increasing the gain in pain [37] may certainly be a good contribution to the exciting but very difficult research area on pathological pains. Moreover, it is important to recall that biochemical pathways leading to the formation of ALLOPREG from PREG can also generate progesterone which has previously been shown to affect sensitivity to pain by increasing levels of endomorphins and opiate receptors in the CNS [40, 41]. This observation also strongly supports the hypothesis suggesting an activation of neurosteroidogenic pathways by neuropathic rats to cope with the chronic pain state.

For the broad research domain on neuroactive steroids in general, our results indicate that a promising strategy to advance rapidly toward therapeutic exploitation of neurosteroids would be, in addition to membrane receptor actions of injected steroids, to target promoters, transcriptional pathways and catalytic sites of steroidogenic enzymes in adequate circuits controling neurobiological functions before correlating evoked responses with neurosteroidogenesis. In support of this suggestion, recent elegant studies revealed that transcription of P450scc and 17α-hydroxylase genes in the nervous system requires factors that are different to those involved in adrenals and gonads [42, 43]. It may be possible, therefore, to target selectively transcriptional mechanisms of neurosteroido-

genic key enzymes without altering classical steroidogenesis in endocrine glands.

The last decade was marked by active research on neurosteroidogenesis in the human brain [44, 45]. An increase in the cerebrospinal fluid content of neurosteroids has been reported in patients with unipolar major depression who are receiving fluoxetine or fluvoxamine, two selective serotonin reuptake inhibitors; normalization of cerebrospinal fluid ALLOPREG content in depressed patients was apparently sufficient to mediate, via the positive allosteric modulation of $GABA_A$-R, the anxiolytic and antidysphoric actions of fluoxetine or fluvoxamine [46]. Recently, high levels of proteins involved in the formation of plaques and neurofibrillary tangles in Alzheimer's disease were correlated with decreased cerebral concentrations of two neurosteroids, pregnenolone sulfate and dehydroepiandrosterone sulfate [47]. These data, together with the results presented here on cellular, molecular and biochemical components of neurosteroidogenesis under normal and painful states, open new perspectives for the understanding and treatment of various neural disorders.

Acknowledgments. This work was supported by grants from the Conseil Régional d'Alsace, Centre National de la Recherche Scientifique (CNRS, France), Université Louis Pasteur (Strasbourg, France) and Fondation pour la Recherche Médicale (FRM, France). Thanks to E. Jover for discussion about PCR.

1 Arner S. and Meyerson B. A. (1988) Lack of analgesic effect of opioids on neuropathic and idiopathic forms of pain. Pain **33:** 11–23
2 Cherny N. I., Thaler H. T., Friedlander-Klar H., Lapin J., Foley K. M., Houde R. et al. (1994) Opioid responsiveness of cancer pain syndromes caused by neuropathic or nociceptive mechanisms: a combined analysis of controlled, single-dose studies. Neurology **44:** 857–861
3 Benedetti F., Vighetti S., Amanzio M., Casadio C., Oliaro A., Bergamasco B. et al. (1998) Dose-response relationship of opioids in nociceptive and neuropathic postoperative pain. Pain **74:** 205–211
4 Bennett G. J. (2003) Neuropathic pain: a crisis of definition? Anesth. Analg. **97:** 619–620
5 Millan M. J. (1999) The induction of pain: an integrative review. Prog. Neurobiol. **57:** 1–164
6 Millan M. J. (2002) Descending control of pain. Prog. Neurobiol. **66:** 355–474
7 Kingery W. S. (1997) A critical review of controlled clinical trials for peripheral neuropathic pain and complex regional pain syndromes. Pain **73:** 123–139
8 Koltzenburg M. (1998) Painful neuropathies. Curr. Opin. Neurol. **11:** 515–521
9 Woolf C. J. and Mannion R. J. (1999) Neuropathic pain: aetiology, symptoms, mechanisms, and management. Lancet **353:** 1959–1964
10 Baulieu E. E., Robel P. and Schumacher M. (1999) Contemporary Endocrinology, Humana, Totowa
11 Mensah-Nyagan A. G., Do-Rego J. L., Beaujean D., Luu-The V., Pelletier G. and Vaudry H. (1999) Neurosteroids: expression of steroidogenic enzymes and regulation of steroid biosynthesis in the central nervous system. Pharmacol. Rev. **51:** 63–81
12 Compagnone N. A. and Mellon S. H. (2000) Neurosteroids: biosynthesis and function of these novel neuromodulators. Front. Neuroendocrinol. **21:** 1–56
13 Tsutsui K., Ukena K., Usui M., Sakamoto H. and Takase M. (2000) Novel brain function: biosynthesis and actions of neurosteroids in neurons. Neurosci. Res. **36:** 261–273
14 Mensah-Nyagan A. G., Beaujean D., Luu-The V., Pelletier G. and Vaudry H. (2001) Anatomical and biochemical evidence for the synthesis of unconjugated and sulfated neurosteroids in amphibians. Brain Res. Brain Res. Rev. **37:** 13–24
15 Mensah-Nyagan A. G., Do-Rego J. L., Beaujean D., Luu-The V., Pelletier G. and Vaudry H. (2001) Regulation of neurosteroid biosynthesis in the frog diencephalon by GABA and endozepines. Horm. Behav. **40:** 218–225
16 Le Goascogne C., Robel P., Gouezou M., Sananes N., Baulieu E. E. and Waterman M. (1987). Neurosteroids: cytochrome P-450scc in rat brain. Science **237:** 1212–1215
17 Mellon S. H. and Griffin L. D. (2002) Neurosteroids: biochemistry and clinical significance. Trends Endocrinol. Metab. **13:** 35–43
18 Goodchild C. S., Guo Z. and Nadeson R. (2000) Antinociceptive properties of neurosteroids. I. Spinally-mediated antinociceptive effects of water-soluble aminosteroids. Pain **88:** 23–29
19 Goodchild C. S., Robinson A. and Nadeson R. (2001) Antinociceptive properties of neurosteroids. IV. Pilot study demonstrating the analgesic effects of alphadolone administered orally to humans. Br. J. Anaesth. **86:** 528–534
20 Ueda H., Inoue M., Yoshida A., Mizuno K., Yamamoto H., Maruo J. et al. (2001) Metabotropic neurosteroid/sigma-receptor involved in stimulation of nociceptor endings of mice. J. Pharmacol. Exp. Ther. **298:** 703–710
21 Gambhir M., Mediratta P. K. and Sharma K. K. (2002) Evaluation of the analgesic effect of neurosteroids and their possible mechanism of action. Indian J. Physiol. Pharmacol. **46:** 202–208
22 Winter L., Nadeson R., Tucker A. P. and Goodchild C. S. (2003) Antinociceptive properties of neurosteroids: a comparison of alphadolone and alphaxalone in potentiation of opioid antinociception. Anesth. Analg. **97:** 798–805
23 Patte-Mensah C., Kappes V., Freund-Mercier M. J., Tsutsui K. and Mensah-Nyagan A. G. (2003) Cellular distribution and bioactivity of the key steroidogenic enzyme, cytochrome P450side chain cleavage, in sensory neural pathways. J. Neurochem. **86:** 1233–1246
24 Bennett G. J. and Xie Y. K. (1988) A peripheral mononeuropathy in rat that produces disorders of pain sensation like those seen in man. Pain **33:** 87–107
25 Mensah-Nyagan A. G., Feuilloley M., Dupont E., Do-Rego J. L., Leboulenger F., Pelletier G. et al. (1994) Immunocytochemical localization and biological activity of 3 beta-hydroxysteroid dehydrogenase in the central nervous system of the frog. J. Neurosci. **14:** 7306–7318
26 Mensah-Nyagan A. G., Do-Rego J. L., Feuilloley M., Marcual A., Lange C., Pelletier G. et al. (1996) In vivo and in vitro evidence for the biosynthesis of testosterone in the telencephalon of the female frog. J. Neurochem. **67:** 413–422
27 Mensah-Nyagan A. G., Feuilloley M., Do-Rego J. L., Marcual A., Lange C., Tonon M. C. et al. (1996) Localization of 17beta-hydroxysteroid dehydrogenase and characterization of testosterone in the brain of the male frog. Proc. Natl. Acad. Sci. USA **93:** 1423–1428
28 Zimmermann M. (1983) Ethical guidelines for investigations of experimental pain in conscious animals. Pain **16:** 109–110
29 Oonk R. B., Krasnow J. S., Beattie W. G. and Richards J. S. (1989) Cyclic AMP-dependent and -independent regulation of cholesterol side chain cleavage cytochrome P-450 (P-450scc) in rat ovarian granulosa cells and corpora lutea: cDNA and deduced amino acid sequence of rat P-450scc. J. Biol. Chem. **264:** 21934–21942

30 Suhara K., Gomi T., Sato H., Itagaki E., Takemori S. and Katagiri M. (1978) Purification and immunochemical characterization of the two adrenal cortex mitochondrial cytochrome P-450-proteins. Arch. Biochem. Biophys. **190:** 290–299
31 Ikushiro S., Kominami S. and Takemori S. (1992) Adrenal P-450scc modulates activity of P-45011 beta in liposomal and mitochondrial membranes: implication of P-450scc in zone specificity of aldosterone biosynthesis in bovine adrenal. J. Biol. Chem. **267:** 1464–1469
32 Ukena K., Usui M., Kohchi C. and Tsutsui K. (1998) Cytochrome P450 side-chain cleavage enzyme in the cerebellar Purkinje neuron and its neonatal change in rats. Endocrinology **139:** 137–147
33 Roby K. F., Larsen D., Deb S. and Soares M. J. (1991) Generation and characterization of antipeptide antibodies to rat cytochrome P-450 side-chain cleavage enzyme. Mol. Cell. Endocrinol. **79:** 13–20
34 Haines D. E., Mihailoff G. A. and Yezierski R. P. (1997) The spinal cord. In: Fundamental Neuroscience, pp. 129–141, Haines D. E. (ed.), Churchill Livingstone, New York
35 Murakami K., Fellous A., Baulieu E. E. and Robel P. (2000) Pregnenolone binds to microtubule-associated protein 2 and stimulates microtubule assembly. Proc. Natl. Acad. Sci. USA **97:** 3579–3584
36 Plassart-Schiess E. and Baulieu E. E. (2001) Neurosteroids: recent findings. Brain Res. Brain Res. Rev. **37:** 133–140
37 Woolf C. J. and Salter M. W. (2000) Neuronal plasticity: increasing the gain in pain. Science **288:** 1765–1769
38 Guennoun R., Schumacher M., Robert F., Delespierre B., Gouezou M., Eychenne B. at al. (1997) Neurosteroids: expression of functional 3beta-hydroxysteroid dehydrogenase by rat sensory neurons and Schwann cells. Eur. J. Neurosci. **9:** 2236–2247
39 Patte-Mensah C., Penning T. M. and Mensah-Nyagan A. G. (in press) Anatomical and cellular localization of neuroactive 5α/3α-reduced steroid synthesizing enzymes in the spinal cord. J. Comp. Neurol.
40 Dawson-Basoa M. and Gintzler A. R. (1997) Involvement of spinal δ opiate receptors in the antinociception of gestation and its hormonal simulation. Brain Res. **757:** 37–42
41 Dawson-Basoa M. and Gintzler A. R. (1998) Gestational and ovarian sex steroid antinociception: synergy between spinal k and δ opioid systems. Brain Res. **794:** 61–67
42 Compagnone N. A., Zhang P., Vigne J. L. and Mellon S. H. (2000) Novel role for the nuclear phosphoprotein SET in transcriptional activation of P450c17 and initiation of neurosteroidogenesis. Mol. Endocrinol. **14:** 875–888
43 Hammer F., Compagnone N. A., Vigne J. L., Bair S. R. and Mellon S. H. (2004) Transcriptional regulation of P450scc gene expression in the embryonic rodent nervous system. Endocrinology **145:** 901–912
44 Stoffel-Wagner B. (2001) Neurosteroid metabolism in the human brain. Eur. J. Endocrinol. **145:** 669–679
45 Schumacher M., Weill-Engerer S., Liere P., Robert F., Franklin R. J., Garcia-Segura L. M. et al. (2003) Steroid hormones and neurosteroids in normal and pathological aging of the nervous system. Prog. Neurobiol. **71:** 3–29
46 Uzunova V., Sheline Y., Davis J. M., Rasmusson A., Uzunov D. P., Costa E. et al. (1998) Increase in the cerebrospinal fluid content of neurosteroids in patients with unipolar major depression who are receiving fluoxetine or fluvoxamine. Proc. Natl. Acad. Sci. USA **95:** 3239–3244
47 Weill-Engerer S., David J. P., Sazdovitch V., Liere P., Eychenne B., Pianos A. et al. (2002) Neurosteroid quantification in human brain regions: comparison between Alzheimer's and nondemented patients. J. Clin. Endocrinol. Metab. **87:** 5138–5143