Substance P inhibits progesterone conversion to neuroactive metabolites in spinal sensory circuit: A potential component of nociception

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Communicated by Etienne-Emile Baulieu, Collège de France, Le Kremlin-Bicêtre, France, April 11, 2005 (received for review October 4, 2004)

A crucial biochemical reaction in vertebrates is progesterone conversion into neuroactive metabolites such as dihydroprogesterone (5α-DHP) and tetrahydroprogesterone (3α,5α-THP), which regulate several neurobiological processes, including stress, depression, neuroprotection, and analgesia. 3α,5α-THP is a potent stimulator of type A receptors of GABA, the main inhibitory neurotransmitter. Here, we show that in the spinal sensory circuit progesterone conversion into 5α-DHP and 3α,5α-THP is inhibited dose-dependently by substance P (SP), a major mediator of painful signals. We developed a triple-labeling approach coupled with multichannel confocal microscope analysis, which revealed that, in the spinal cord (SC), SP-releasing afferents project on sensory neurons expressing simultaneously neurokinin 1 receptors (rNK1) and key enzymes catalyzing progesterone metabolism. Evidence for a potent inhibitory effect of SP on 5α-DHP and 3α,5α-THP formation in the SC was provided by combining pulse–chase experiments using [^{3}H]progesterone as precursor, HPLC, recrystallization of [^{3}H]metabolites to constant specific activity, and continuous flow detection of radioactive steroids. The action of SP on progesterone metabolism was mimicked by the rNK1-specific agonist [Sar-9,Met(O$_2$)^{11}]-SP. The selective rNK1 antagonist SR140333 totally reversed the effect of SP on progesterone conversion into 5α-DHP and 3α,5α-THP. These results provide direct evidence for the occurrence of anatomical and functional interactions between the SP-rNK1 system and neuroactive steroid-producing cells in the SC. The data suggest that, through the local control of 3α,5α-THP concentration in spinal sensory circuit, the SP-rNK1 system may indirectly interfere with GABA$_A$ receptor activity in the modulation of nociceptive transmission.

neurosteroid | pain | spinal cord | steroids | nervous system

The spinal cord (SC) is a pivotal structure controlling many neurophysiological activities, including somatosensory transmission, locomotion, reflexes, and neurovegetative functions. Primary sensory inputs arising from receptors in various areas of the body are integrated in multisynaptic relays in the SC that convey these inputs toward the brain (1, 2). A variety of molecules, including glutamate, substance P (SP), neurotrophins, calcitonin-gene related peptide, and adenosine triphosphate, are thought to be involved in the central transmission of sensory messages (2, 3). Among these neuromodulators, SP, the role of which is clearly established and well documented, is considered as a key neuropeptide mediating nociceptive message transmission from peripheral afferents to sensory neurons located in the SC dorsal horn (DH) (4–6). In particular, it has been shown that projection neurons in lamina I of rat DH expressing neurokinin 1 receptors (rNK1s) are selectively innervated by SP-containing afferents and respond to noxious stimulation (7). Direct structural-functional evidence for SP-mediated nociceptive transmission has also been provided in cats by multidisciplinary studies that combined various approaches, including intracellular recording from DH neurons *in vivo*, characterization of these neurons on the basis of their responses to peripheral noxious stimulation, cellular labeling by horseradish peroxidase, and electron microscopic identification of synaptic contacts (6–9). Moreover, intrathecal injection of SP conjugated to the cytotoxin saporin selectively destroyed DH neurons bearing rNK1 and strongly reduced hyperalgesia after capsaicin treatment, indicating that spinal rNK1 neurons are important for the development of hyperalgesia (4, 5). However, intracellular changes or neurochemical modifications that spinal rNK1 neurons undergo during nociception are poorly characterized. This situation hampers the understanding of spinal mechanisms involved in pathological pain and, consequently, the development of adequate therapeutic strategies.

In a recent series of studies, we observed that the rat DH is an active neurosteroidogenic center that contains several sensory neurons expressing the isoenzyme type 2 of 5α-reductase (5α-R) and 3α-hydroxysteroid oxydo-reductase (3α-HSOR), also called 3α-hydroxysteroid dehydrogenase (10, 11). 5α-R and 3α-HSOR are the two key enzymes catalyzing progesterone conversion into dihydroprogesterone (5α-DHP) and tetrahydroprogesterone (3α,5α-THP) or allopregnanolone, which control important functions such as sexual behaviors, neuroprotection, stress, anxiety, analgesia, sleep, and locomotion (12–15). Investigations of rats submitted to neuropathic pain provoked by sciatic nerve ligature allowed the observation of hyperproduction of 3α,5α-THP in spinal sensory networks under the painful state (16). Because 3α,5α-THP is a potent stimulator of GABA$_A$ receptors, which play a pivotal role in the modulation of pain sensation (12, 13, 17–19), increase of 3α,5α-THP formation in the DH appeared to be an endogenous mechanism triggered by neuropathic animals to cope with the chronic pain (16). Therefore, we found it important to determine neuroanatomical and functional interactions between the spinal SP-rNK1 system controlling pain transmission and the process of progesterone conversion into 5α-DHP and 3α,5α-THP occurring in DH neurons, to clarify neurochemical pathways involved in nociception. To investigate the possible existence of an appropriate anatomical organization that can allow, *in vivo*, interactions between SP-releasing afferents, rNK1-expressing neurons, and progesterone-metabolizing cells in the DH, a triple-immunolabeling study was performed and combined with multichannel analysis using a confocal laser scanning microscope. Determination of the action of SP on progesterone conversion into 5α-DHP and 3α,5α-THP in the SC was achieved with a well validated method combining pulse–chase experiments, HPLC, continuous flow scintillation detection, and recrystallization of radioactive steroids (10, 16, 20–22).

Abbreviations: DH, dorsal horn; 5α-DHP, dihydroprogesterone; 3α-HSOR, 3α-hydroxysteroid oxydo-reductase; 5α-R, 5α-reductase; SC, spinal cord; 3α,5α-THP, tetrahydroprogesterone; rNK1, neurokinin 1 receptor; SP, substance P.

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www.pnas.org/cgi/doi/10.1073/pnas.0502968102

Methods

Animals. Adult female and male Sprague–Dawley rats weighing 300 g were used. Animal care and manipulations were performed according to 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.

Antisera, Chemicals, and Reagents. The rabbit antiserum against the human carboxyl-terminal 25 aa of 5α-R type 2 (amino acid residues 227–251) and the lyophilized peptide used as immunogen were kindly provided by D. W. Russell (Southwestern Medical Center, Dallas). The polyclonal antibody against rat 3α-HSOR and the purified immunogen were generous gifts from T. M. Penning (University of Pennsylvania School of Medicine, Philadelphia). The guinea pig antiserum against rNK1 and SP receptor control peptide (rNK1 control peptide) were supplied by Chemicon International (Temecula, CA). The mouse mAb against SP was provided by Immundiagnostik (Bensheim, Germany). Alexa 555-conjugated goat anti-rabbit and Alexa 488-conjugated goat anti-guinea pig were purchased from Molecular Probes. Cyanine5-conjugated goat anti-mouse was from Jackson ImmunoResearch. SP and the selective rNK1 agonist [Sar-9,Met(O_2)^{11}]-SP were from Sigma. The specific rNK1 antagonist SR140333 was a generous gift from Sanofi-Aventis (Chilly-Mazarin, France). Synthetic steroids including progesterone, 5α-DHP, and 3α,5α-THP were provided by Steraloids (Newport, RI). Dichloromethane, hexane, isopropanol, and propylene glycol were obtained from Merck. DMEM was purchased from Invitrogen. The radioactive progesterone [1,2,6,7-^{3}H(N)-progesterone] with a specific activity equivalent to 82 Ci/mmol was supplied by Amersham Pharmacia Biosciences. Synthetic 9,11,12-^{3}H(N)-tetrahydroprogesterone or [^{3}H]3α,5α-THP (specific activity, 56 Ci/mmol) was purchased from PerkinElmer.

Immunohistochemistry. Animals were deeply anaesthetized with 25% urethane (0.5 ml/100 g, i.p.) 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% formaldehyde and 0.2% picric acid in PB). The SC was rapidly dissected and postfixed in the same fixative solution for 24 h. The tissues were embedded in 5% agar after the postfixation period, cut in transverse 20-μm-thick sections with a vibratome VT 1000 S (Leica, Wetzlar, Germany), and collected in PB. All tissue sections were preincubated for 1 h with 5% nonimmune goat serum in PB containing 0.3% Triton X-100 (PBT). Afterward, the sections were incubated for 48 h at 4°C with a mixture containing PBT and three antisera directed, respectively, against SP (1:500), rNK1 (1:1,500), 5α-R type 2 (1:500), or 3α-HSOR (1:500). The procedure was carried out by rinsing the sections three times in PB (15 min per rinse) and transferring them into a mixture made of Alexa 555-conjugated goat anti-rabbit, Alexa 488-conjugated goat anti-guinea pig, and cyanine5-conjugated goat anti-mouse diluted at 1:300 in PBT for 1.5 h. Finally, the sections were rinsed for 1 h in PB and mounted in Vectashield (Vector Laboratories). The preparations were examined under a multichannel confocal laser scanning microscope, LSM510 (Zeiss).

The specificity of the immunoreaction was controlled by (*i*) substitution of each primary antiserum with PB and (*ii*) preincubation of each antibody with the corresponding immunogen (purified SP, rNK1 control peptide, lyophilized 5α-R-immunogen-peptide, or 3α-HSOR-immunogen-peptide).

Pulse–Chase and HPLC-Flo/One Characterization of Neuroactive Steroids. Conversion of [^{3}H]progesterone into [^{3}H]5α-DHP and [^{3}H]3α,5α-THP was studied by using a previously validated approach that combines the pulse–chase technique, HPLC purification of tissue extracts, and continuous flow detection of newly synthesized ^{3}H steroids (10, 16, 21, 22). Briefly, 100 mg of lumbar SC slices (dorsal half) was incubated at 37°C for 3 h in 1.5 ml of DMEM containing 10^{-8} M [^{3}H]progesterone supplemented with 1% propylene glycol in the absence or presence of test substances. At the end of the incubation period, newly synthesized ^{3}H steroids released by the SC slices in the medium and ^{3}H-neuroactive steroids remaining in the slices were extracted with dichloromethane and prepurified on Sep-Pack C_{18} cartridges (Waters) by using a solution made of 60% isopropanol and 40% hexane. The solvent was evaporated in a RC-10^{-10} Speed Vac Concentrator (Jouan, Saint Herblain, France), and the dry prepurified extracts were reconstituted in 400 μl of hexane before analysis on a RP-HPLC system (322 pump, UV/VIS 156 detector, Unipoint system, Gilson). The HPLC column was a 4.6 $\times$ 250 mm SymetryShield C_{18} (Waters) equilibrated with 100% hexane. The radioactive steroids were eluted at a flow rate of 0.5 ml per min by 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) equipped with a Pentium IV 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 tissue extracts, and their elution positions were determined by UV absorption with a UV/VIS 156 detector (Gilson).

To optimize the characterization of newly synthesized neuroactive steroids, synthetic tritiated steroids including [^{3}H]progesterone and [^{3}H]3α,5α-THP were also used as reference standards, chromatrographed under the same conditions as tissue extracts, and identified by their elution times with the Flo/One computer system.

Recrystallization. In a series of HPLC analyzes of biological extracts, the [^{3}H] steroids eluted from the column were collected at 0.5-min intervals. HPLC fractions suspected as containing [^{3}H]5α-DHP and [^{3}H]3α,5α-THP (according to their elution times) were submitted to four consecutive crystallization procedures (20). Briefly, an aliquot of ^{3}H-labeled HPLC eluate was evaporated in a glass test tube, and 20 mg of authentic unlabeled steroid (5α-DHP or 3α,5α-THP) was added to it. Crystallization occurred in a mixture of ethanol and water, and formation of crystals incorporated authentic [^{3}H] steroids into forming a crystal matrix. Nonauthentic compounds were left in mother liquor and removed. The ratio of the specific activity of the crystals to that of the mother liquor was determined, and recrystallization was considered successful when the product recovery exceeded 70% and the percentage of error was $<5\%$.

Quantification of Neuroactive Steroid Formation and Statistical Analysis. The amount of radioactive neuroactive steroids synthesized from [^{3}H]progesterone was expressed as a percentage of the total radioactivity contained in all peaks resolved by the HPLC-Flo/One system, including [^{3}H]progesterone itself. Each value is the mean of four independent experiments. Student's *t* test was used for comparison in pairs, and ANOVA followed by Bonferroni's test was applied for multiparameter analysis.

Results

Anatomical Relationship Between the SP-rNK1 System and Progesterone-Metabolizing Neurons in the Spinal DH. As we have reported (11), several neurons of the rat spinal DH contain both 5α-R and

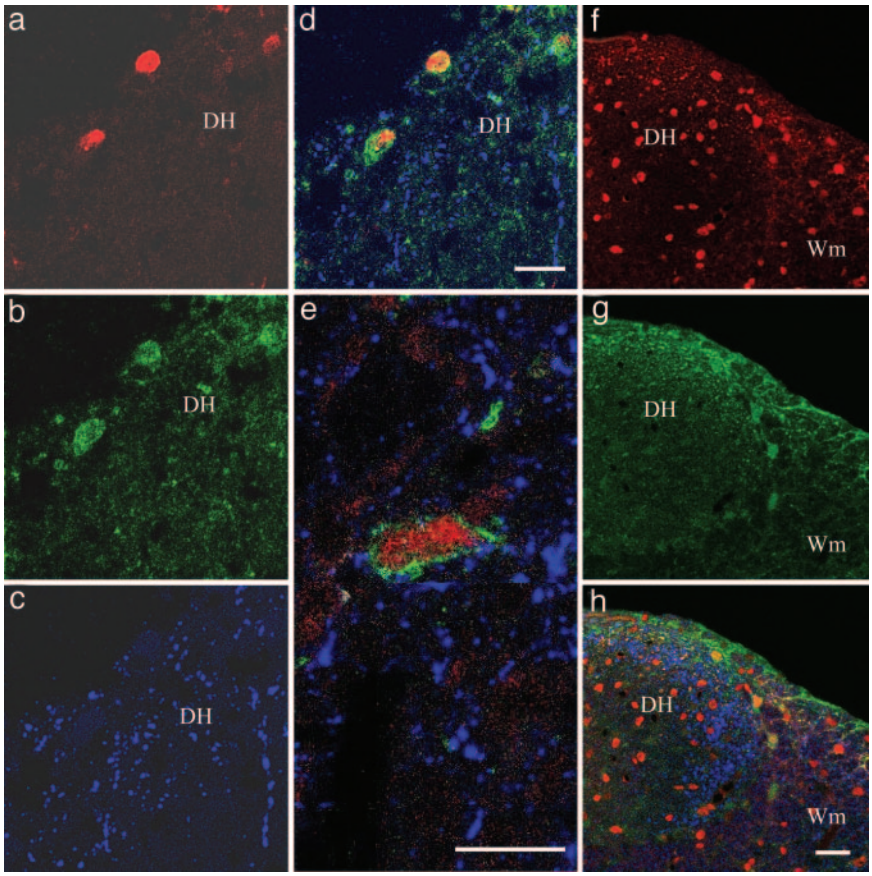

Fig. 1. Anatomical relationship between SP-releasing terminals, rNK1 neurons, and progesterone-metabolizing cells in the SC. (*a*) Confocal laser scanning microscope photomicrograph of a lumbar SC section labeled with anti-5α-R and revealed by Alexa 555-conjugated goat anti-rabbit. (*b*) The same section as in *a* labeled with anti-rNK1 and revealed by Alexa 488-conjugated goat anti-guinea pig. (*c*) The same section as in *a* labeled with anti-SP and revealed by cyanine5-conjugated goat anti-mouse. (*d*) Merged photomicrograph of *a–c* showing SP-positive fibers projecting in the close vicinity of 5α-R-NK1-immunoreactive neurons. (*e*) Highly magnified view of a DH lamina I neuron that contains both 5α-R and rNK1 immunoreactivities and is innervated by SP-positive terminals. (*f*) Photomicrograph of a SC section labeled with anti-3α-HSOR and revealed by Alexa 555-conjugated goat anti-rabbit. (*g*) The same section as in *f* labeled with anti-rNK1 and revealed by Alexa 488-conjugated goat anti-guinea pig. (*h*) Merged image showing the triple labeling of the SC section (*f*), which was also incubated with anti-SP and revealed by cyanine5-conjugated goat anti-mouse. SP-immunoreactive fibers are in the close vicinity of 3α-HSOR-rNK1-positive neurons in the DH lamina I. Wm, white matter. (Scale bars: 20 μm.)

3α-HSOR, the two key enzymes that convert progesterone into neuroactive metabolites such as 5α-DHP and 3α,5α-THP (Fig. 1). To determine whether afferent terminals releasing SP project on DH neurons expressing both 5αR and rNK1 immunoreactivities, SC slices were incubated with a mixture of three different antibodies directed against SP, rNK1, and 5αR. The triple-labeling studies revealed the presence of numerous SP-positive beaded nerve fibers in the close vicinity of neurons expressing simultaneous immunostaining for rNK1 and 5αR in the DH lamina I (Fig. 1 *a–e*). At high magnification with the confocal laser scanning microscope, it appeared that several SP-immunoreactive fibers were connected to lamina I rNK1-positive neurons that contained 5α-R immunostaining (Fig. 1*e*). The triple-labeling experiments were also performed to check the existence of contacts between SP-containing terminals and lamina I neurons expressing both 3α-HSOR and rNK1 immunoreactivities. As shown in Fig. 1 *f–h*, DH neurons containing 3α-HSOR and rNK1 were also closely surrounded by SP-positive terminals. Although we have previously demonstrated the specificity of the 5α-R and 3α-HSOR immunoreactions in the rat SC (11), internal control experiments were performed in this study. Preincubation of the 5α-R antiserum, 3α-HSOR antibody, anti-SP, or anti-rNK1 with their respective immunogen peptides resulted in a complete disappearance of immunoreactivity (data not shown).

Effects of SP on the Conversion of [^{3}H]Progesterone into [^{3}H]5α-DHP and [^{3}H]3α,5α-THP by DH Sensory Neurons. After a 3-h incubation of dorsal SC slices with [^{3}H]progesterone, RP-HPLC analysis of the tissue homogenates and incubation media made it possible to resolve two major radioactive metabolites coeluting with [^{3}H]5α-DHP and [^{3}H]3α,5α-THP (Fig. 2*a*). To further verify the authenticity of [^{3}H]5α-DHP and [^{3}H]3α,5α-THP newly synthesized from [^{3}H]progesterone, radioactive metabolites eluted from the HPLC system were collected and recrystallized to constant specific activity. [^{3}H]5α-DHP and [^{3}H]3α,5α-THP successfully crystallized, respectively, with authentic unlabeled 5α-DHP and 3α,5α-THP with a product recovery $>$85% and a percentage of error $<$4% (Table 1). In the presence of SP (10^{-6} M), the levels of [^{3}H]5α-DHP and [^{3}H]3α,5α-THP produced from [^{3}H]progesterone were markedly attenuated in the tissue extracts and incubation media (Fig. 2*b*). Exposure of dorsal SC slices to graded concentrations of SP (10^{-12} to 10^{-4} M) induced a dose-dependent decrease in the formation of [^{3}H]5α-DHP (Fig. 3*a*) and [^{3}H]3α,5α-THP (Fig. 3*b*).

To determine whether the inhibitory effect of SP on progesterone metabolism is effectively mediated through rNK1 localized on 5αR-3α-HSOR-containing neurons, pharmacological studies were performed with selective rNK1 agonist {[Sar-9,Met(O_2)^{11}]-SP} and rNK1 antagonist (SR140333). Incubation

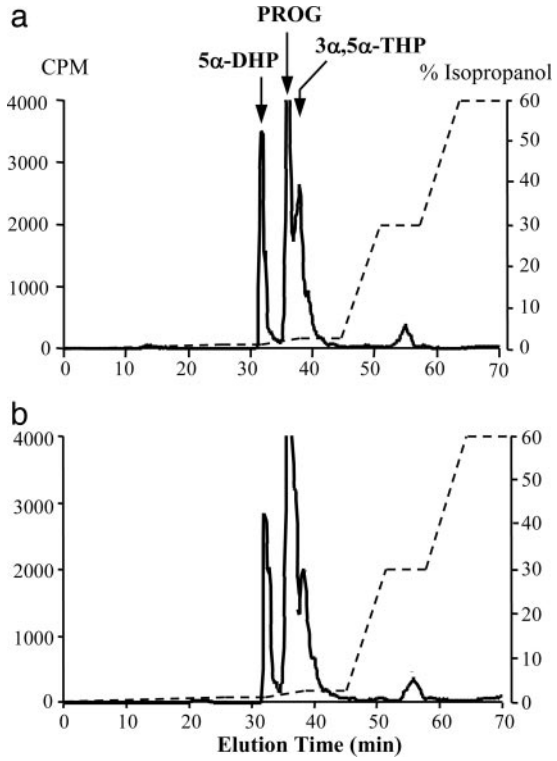

Fig. 2. Effects of SP on progesterone (PROG) metabolism in the spinal DH. Analysis of [^{3}H] steroids extracted from the medium after a 3-h incubation of SC slices with [^{3}H]progesterone in the absence (*a*) or presence (*b*) of 10^{-6} M SP is shown. The ordinate indicates radioactivity measured in the HPLC eluent. The dashed lines represent the gradient of secondary solvent (% isopropanol). The arrows indicate elution positions of standard steroids.

Table 1. Recrystallization assessment of the authenticity of neuroactive metabolites produced from [^{3}H]progesterone in the rat SC

Neuroactive steroids	% recovery, $\frac{SA_{crystals}}{SA_{ML}}$	% error, $\frac{\left\lvert SA_{crystals} - \left(\frac{SA_{crystals} + SA_{ML}}{2}\right)\right\rvert}{\left(\frac{SA_{crystals} + SA_{ML}}{2}\right)}$
5α-DHP	86	2
3α,5α-THP	89	3

ML, mother liquor; SA, specific activity (cpm·mg^{-1}).

of SC slices with [Sar-9,Met(O_2)^{11}]-SP (10^{-6} M) mimicked the inhibitory action of SP on the conversion of [^{3}H]progesterone into [^{3}H]5α-DHP (Fig. 4*a*) and [^{3}H]3α,5α-THP (Fig. 4*b*). In contrast, the inhibitory effect of SP was completely reversed by the specific rNK1 antagonist SR140333 (10^{-5} M); in the absence of SP, SR140333 (10^{-5} M) did not modify on its own the levels of [^{3}H]5α-DHP and [^{3}H]3α,5α-THP newly synthesized from [^{3}H]progesterone (Fig. 4).

Discussion

Thanks to a combination of anatomical, biochemical, and pharmacological approaches, this work has identified a neurochemical mechanism in spinal sensory centers that control important neurobiological functions. The data clearly show that SP, well known as a major neurotransmitter involved in spinally mediated nociceptive processes (4–9), inhibits progesterone conversion into neuroactive metabolites in spinal DH sensory networks. The inhibitory action of SP on progesterone conversion into 5α-DHP and 3α,5α-THP was mimicked by [Sar-9,Met(O_2)^{11}]-SP, a highly selective rNK1 agonist (23, 24). In addition, SR140333, a specific antagonist for rNK1 (25–27), completely blocked the inhibitory effect of SP on 5α-DHP and 3α,5α-THP formation. These results indicate that the action of SP on progesterone conversion into neuroactive derivatives in the spinal sensory circuit is mediated by rNK1, which plays a pivotal role in the generation of inflammatory and neuropathic pain (4–9).

Involvement of sex steroids in the modulation of pain has been reported by previous studies that showed antinociceptive actions of physiological or experimental pregnancy in rats and women (28–30). In particular, elevated plasma levels of estrogen and progesterone have been positively correlated with the stimulation of the spinal opioid system and the increase of pain thresholds (30).

The present report reveals that, having more than a simple modulating role, progesterone is directly involved in basic neurochemical events occurring inside spinal rNK1 neurons that are crucial for pain processing (4–9). In the intracellular domain of rNK1 neurons, enzymatic activities of 5αR and 3α-HSOR that metabolize progesterone were dramatically reduced when SP was applied to spinal DH slices. Within a 3-h incubation period with SP at 10^{-6} M, an important decrease (around −70%) was observed in the amounts of [^{3}H]5α-DHP and [^{3}H]3α,5α-THP released by rNK1–5αR-3α-HSOR-containing neurons in the spinal DH. No difference was observed in the expression of SP inhibitory action on 5α-DHP and 3α,5α-THP formation in spinal rNK1 neurons in males and females, suggesting that neurochemical events occurring in these sensory neurons during nociception may be similar in both sexes. However, it is possible that in pain modulation the involvement of biochemical pathways generating 5α-DHP and 3α,5α-THP may differ in males and females, owing to the availability of the

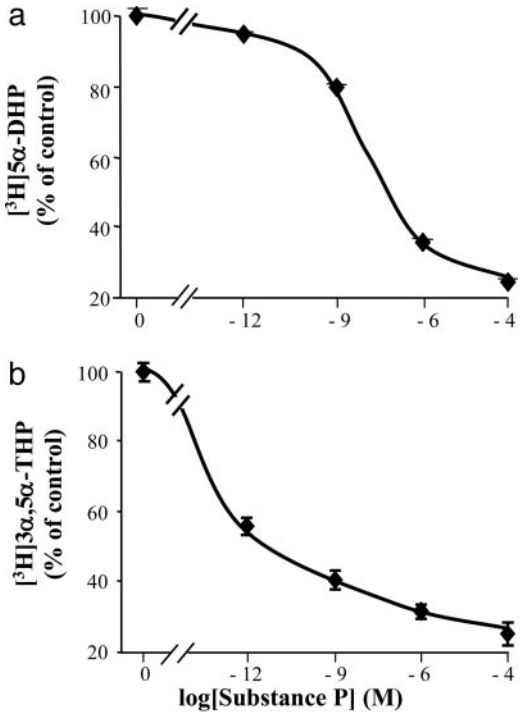

Fig. 3. Action of graded concentrations of SP on the conversion of [^{3}H]progesterone into [^{3}H]5α-DHP (*a*) and [^{3}H]3α,5α-THP (*b*) in the SC. The values were obtained from experiments similar to those presented in Fig. 2. Results are expressed as percentages of the amount of each steroid formed in the absence of SP. Values are the mean ± SEM of four independent experiments.

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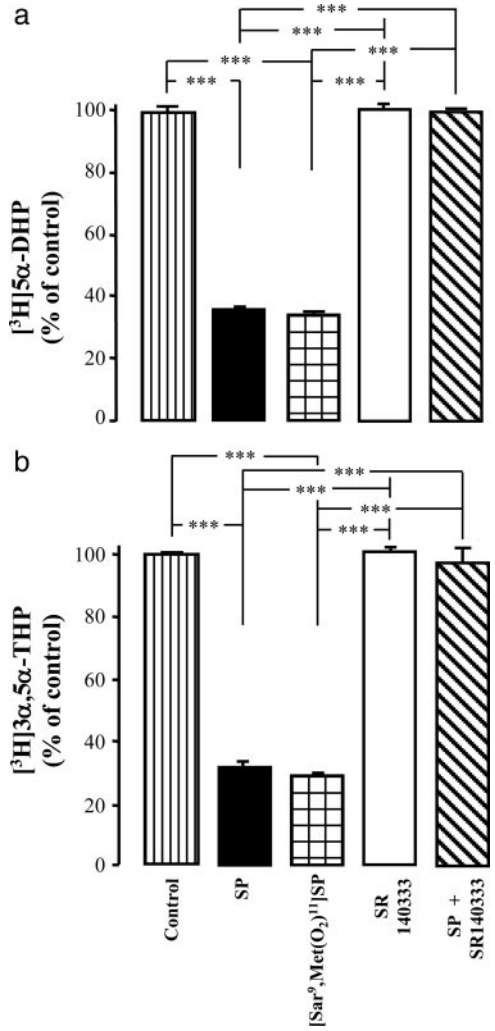

Fig. 4. Effects of rNK1-specific agonist [Sar-9,Met(O_2)^{11}]-SP (10^{-6} M) and antagonist SR140333 (10^{-5} M) on [^{3}H]progesterone conversion into [^{3}H]5α-DHP (*a*) and [^{3}H]3α,5α-THP (*b*) in the SC. The values were obtained from experiments similar to those presented in Fig. 2. Results are expressed as percentages of the amount of each steroid formed in the absence of drugs. Values are the mean ± SEM of four independent experiments. ***, $P < 0.001$.

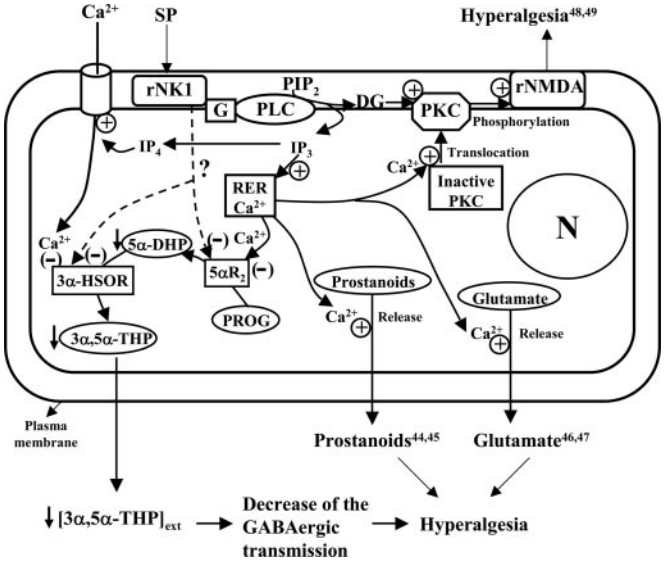

Fig. 5. Neurochemical events occurring in spinal rNK1 neurons under painful state. SP activates via rNK1 the G protein/phospholipase C (PLC)/inositol-triphosphate (IP_3) pathway that increases the intracellular Ca^{2+} concentration. High levels of Ca^{2+}, which stimulate prostanoid/glutamate release or phosphorylation of NMDA receptor (rNMDA), also modify the intracellular Ca^{2+}/H^+ balance. Then, the intracellular pH may become different from the optimal values required for 5α-R and 3α-HSOR activities leading to 5α-DHP and 3α,5α-THP formation (50, 51). Steroid-metabolizing enzymes are sensitive to environmental changes in Ca^{2+} as its chelation with EDTA stimulates their biological activities 2- to 4-fold (52). The question mark indicates that other factors different from Ca^{2+} may also be involved in the inhibitory action of the SP-rNK1 system on 5α-R and in 3α-HSOR activities in spinal DH neurons. N, nucleus. PROG, progesterone.

precursor progesterone, the plasma concentration of which is generally elevated in females. It is also noteworthy that, within the context of neurosteroidogenesis, progesterone may be synthesized *de novo* from cholesterol or pregnenolone in the SC of male and female rats and thus participate in neurochemical events involved in spinally mediated nociceptive processes (10, 16, 31–33).

The neuroactive steroid 5α-DHP acts mainly through genomic progestin receptors (34), and inhibition of its secretion by SP may be correlated with transcriptional events involved in nociceptive mechanisms. For instance, it has been shown that 5α-DHP, acting through progestin nuclear receptors, exerts a potent neuroprotective action in the peripheral and central nervous systems (34–36). Therefore, the down-regulation of 5α-DHP synthesis by SP may possibly facilitate genomic events leading to apoptotic cell death evidenced in the spinal DH in the peripheral neuropathic pain state (37–39).

As has been demonstrated, 3α,5α-THP is a potent modulator of $GABA_A$ receptors that behaves as an allosteric stimulator at nanomolar concentrations or as a pure agonist at micromolar doses (12, 17, 31). Consequently, by decreasing locally 3α,5α-THP concentration in the spinal sensory circuit, the SP-rNK1 system may indirectly interfere with the GABAergic transmission, which is pivotal for the regulation of nociceptive mechanisms (17–19). The fact that 3 h were sufficient for SP at 10^{-6} M to provoke a −70% reduction in the amount of 3α,5α-THP released in the DH suggests the existence of a down-regulatory mechanism of 3α,5α-THP formation during the acute phase of pain processing, which may decrease the GABAergic tone and facilitate the occurrence of spinal hyperexcitability and hyperalgesia. However, the situation may become different during a chronic painful state because in several long-term or chronic pathological states the body itself reacts by triggering various endogenous mechanisms aimed at reducing deleterious consequences for coping with the abnormal situation. Therefore, it is possible that under a chronic painful state, an endogenous process may be activated to counteract the action of SP on 5α-DHP and 3α,5α-THP formation.

In support of this suggestion, we have recently observed that the gene encoding cytochrome P450 side-chain cleavage was overexpressed in spinal sensory networks in chronic-neuropathic rats, leading to a significant increase of local concentrations of pregnenolone and 3α,5α-THP in the SC (16). Consequently, involvement of 5α-DHP and 3α,5α-THP biosynthetic pathways in the regulation of nociception needs to be considered in regard to various neurobiological mechanisms occurring in both acute and chronic phases of pain, even though *in vivo* studies have shown that 3α,5α-THP and its synthetic analogs induce antinociceptive effects in rats and humans (40–43). It is also important to recall that rNK1-mediated hyperalgesia seems to be an extremely complex mechanism involving the release of prostanoids (44, 45) and glutamate (46, 47), and the phosphorylation of NMDA receptor through a protein kinase C transduction process (48, 49).

Therefore, we suggest here an hypothetical model to recapitulate and clarify neurochemical events that may occur in spinal

rNK1–5α-R-3α-HSOR-containing neurons under painful state (see Fig. 5). The model shows the potential importance of the neurochemical mechanism identified herein as this component appears as a possible link between the spinal SP-rNK1 system and activities of NMDA receptor and $GABA_A$ receptors that are crucial for sensory processes (2, 18, 19). We do not contend that the model proposed is an exhaustive one. However, it may be an interesting tool for developing therapeutic strategies because the model suggests a relationship between neuroendocrine factors, neurotransmitters, pharmacological events, and intracellular changes in spinal rNK1 neurons that control nociception.

We thank Sébastien Ducret for his technical assistance during the triple-labeling experiments. This work was supported by grants from the Conseil Régional d'Alsace, Centre National de la Recherche Scientifique, and Université Louis Pasteur de Strasbourg. C.K. was the recipient of a fellowship from the Ministère de l'Education Nationale et de la Recherche.

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