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5β-Reduced Neuroactive Steroids Are Novel Voltage-Dependent Blockers of T-Type Ca^{2+} Channels in Rat Sensory Neurons in Vitro and Potent Peripheral Analgesics in Vivo

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ABSTRACT

T-type Ca^{2+} channels are believed to play an important role in pain perception, and anesthetic steroids such as alphaxalone and allopregnanolone, which have a 5α-configuration at the steroid A, B ring fusion, are known to inhibit T-type Ca^{2+} channels and cause analgesia in a thermal nociceptive model (Soc Neurosci Abstr **29:**657.9, 2003). To define further the structure-activity relationships for steroid analgesia, we synthesized and examined a series of 5β-reduced steroids for their ability to induce thermal antinociception in rats when injected locally into the peripheral receptive fields of the nociceptors and studied their effects on T-type Ca^{2+} channel function in vitro. We found that most of the steroids completely blocked T-type Ca^{2+} currents in vitro with IC_{50} values at a holding potential of −90 mV ranging from 2.8 to 40 μM. T current blockade exhibited mild voltage-dependence, suggesting that 5β-reduced neuroactive steroids stabilize inactive states of the channel. For the most potent steroids, we found that other voltage-gated currents were not significantly affected at concentrations that produce nearly maximal blockade of T currents. All tested compounds induced dose-dependent analgesia in thermal nociceptive testing; the most potent effect (ED_{50}, 30 ng/100 μl) obtained with a compound [(3β,5β,17β)-3-hydroxyandrostane-17-carbonitrile] that was also the most effective blocker of T currents. Compared with previously studied 5α-reduced steroids, these 5β-reduced steroids are more efficacious blockers of neuronal T-type Ca^{2+} channels and are potentially useful as new experimental reagents for understanding the role of neuronal T-type Ca^{2+} channels in peripheral pain pathways.

It is well established that the neuroactive steroids can modulate neuronal activity in the peripheral and central nervous system, causing a variety of behavioral and neuroendocrine changes in humans and animals (e.g., general anesthesia, analgesia, cognitive and mood disturbances) (Zorumski et al., 2000). It is believed that effects on neurosensory processing and neuronal excitability are primarily mediated by actions at various ligand-gated ion channels, with much attention focused on the modulation of γ-aminobutyric acid ($GABA_A$) receptors (Lambert et al., 1995; Zorumski et al., 2000).

It is also becoming evident that certain neuroactive steroids can modulate voltage-gated ion channels (ffrench-Mullen et al., 1994; Nakashima et al., 1998). In particular, we previously reported that 5α-reduced neuroactive steroids [e.g., alphaxalone and (3α,5α,17β)-17-hydroxyestrane-3-carbonitrile, (+)-ECN] are potent blockers of voltage-gated Ca^{2+} channels in vitro (Todorovic et al., 1998). Voltage-gated Ca^{2+} channels are classified as low-voltage-activated (LVA or T-

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ABBREVIATIONS: LVA, low-voltage-activated; HVA, high-voltage activated; DRG, dorsal root ganglion; DMSO, dimethyl sulfoxide; PWL, paw withdrawal latency; 3αCN, (3α,5β,17β)-17-hydroxyandrostane-3-carbonitrile; 19-Nor3αCN, (3α,5β,17β)-17-hydroxyestrane-3-carbonitrile; 3βCN, (3β,5β,17β)-17-hydroxyandrostane-3-carbonitrile; 19-Nor3βCN, (3β,5β,7β)-17-hydroxyestrane-3-carbonitrile; 3αOH, (3α,5β,17β)-3-Hydroxyandrostane-17-carbonitrile; 19-Nor3αOH, (3α,5β,17β)-3-hydroxyestrane-17-carbonitrile; 3βOH, (3β,5β,17β)-3-hydroxyandrostane-17-carbonitrile; 19-Nor3βOH, (3β,5β,17β)-3-hydroxyestrane-17-carbonitrile.

type) and high-voltage-activated (HVA) based on the membrane potentials at which the channels open (Bean, 1989; Hess, 1990; De Waard et al., 1996). T-type channels, which are of particular interest for this study, were first described in sensory neurons of the dorsal root ganglion (DRG) (Carbone and Lux, 1984) and activate with small membrane depolarizations, raising the possibility that they play crucial roles in the control of sensory neuron excitability (White et al., 1989). Although their unique biophysical properties (e.g., low activation threshold, slow deactivation, complete inactivation) make in vitro studies of T-currents relatively easy, the paucity of selective pharmacological tools for modulating their function impedes study of their function in vivo. Hence, despite their presence in peripheral sensory neurons (Schroeder et al., 1990; Scroggs and Fox, 1992; Cardens et al., 1995), the function of T-type Ca^{2+} channels in sensory processing in general, and in nociceptive processing in particular, remains poorly understood.

Recent evidence suggests that modulation of peripheral T channels influences somatic (e.g., thermal and mechanical) and visceral nociceptive inputs and that inhibition of T currents results in significant antinociception in a variety of animal pain models (Todorovic et al., 2001, 2002, 2003a, 2004; Kim et al., 2003). Therefore, T channels in peripheral nociceptors may be important, although previously unrecognized, targets for antinociceptive therapeutic agents.

The development of novel neuroactive steroids that are selective and potent blockers of neuronal T-type Ca^{2+} channels may greatly aid in revealing roles for these channels in sensory pathways (nociception in particular) and in the development of novel analgesics that might be safer and more effective for pain therapy. In the present study, we extend our earlier structure-activity studies of 5α-reduced steroids to 5β-reduced steroids (Fig. 1). We examined the in vitro effects of the compounds on T-type Ca^{2+} channels in freshly dissociated rat primary sensory neurons. In addition, we evaluated antinociceptive potential in vivo by measuring thermal nociception after injection of the steroids into peripheral receptive fields of sensory neurons. We found that 5β-reduced steroids had activity in both experimental paradigms and that the potency of 5β-reduced steroids to induce antinociception in vivo correlates well with their potency to block T currents in vitro.

Materials and Methods

Electrophysiological Methods, Solutions, and Current Isolation Procedures. Freshly dissociated DRG neurons from adult Sprague-Dawley rats of either sex (100–300 g) were obtained using enzymatic treatment and standard whole-cell, patch-clamp techniques as described elsewhere (Todorovic and Lingle, 1998; Todorovic et al., 1998). Glass coverslips with adherent DRG cells were transferred to a 35-mm culture dish with a total volume <1 ml. The solution application system consisted of multiple independently controlled glass capillary tubes; solution was removed from the opposite end of the chamber by constant suction. Manually controlled valves accomplished switching between solutions. Test solutions were maintained in all-glass syringes and allowed to flow by gravity. Use of glass syringes and capillary tubes minimized loss of lipophilic steroid compounds during perfusion. Changes in Ca^{2+} current amplitude in response to rapidly acting drugs or ionic changes were typically complete in 10 to 20 s. Switching between separate perfusion syringes, each containing control saline, resulted in no changes in Ca^{2+} current.

Most data from DRG cells were obtained from smaller diameter neurons (< 30 μm) with no visible processes. Voltage commands and digitization of membrane currents were done with Clampex 8.2 of the pClamp software package (Axon Instruments, Foster City, CA) running on an IBM-compatible computer. Membrane currents were recorded with an Axopatch 200B patch-clamp amplifier (Axon Instruments). Reported series resistance and capacitance values were taken from the amplifier settings. The average uncompensated series resistance was 7.6 ± 2.5 (mean ± S.D.) MΩ, and the average membrane capacitance was 15 ± 4 pF in the 171 small DRG neurons studied. These were typically compensated 40 to 80% without oscillation in current traces. In 5 medium-sized DRG neurons (cell diameter 31–35 μm), the average membrane capacitance was 36 ± 2 pF, and series resistance was 1.4 ± 0.2 MΩ. Although some medium-sized DRG neurons express "gigantic" T currents (e.g., Fig. 6A in Scroggs and Fox, 1992), we did not observe differences in the response to 5β-reduced neuroactive steroids based on differences in the size of baseline T currents. To record T-currents, the membrane potential was held at −90 mV and stepped to −30 mV to evoke inward currents that inactivate almost completely during a 200-ms test pulse. The intracellular saline for recording T-currents consisted of 135 to 140 mM tetramethyl ammonium hydroxide, 10 mM EGTA, 40 mM HEPES, and 2 mM $MgCl_2$, titrated to pH 7.15 to 7.20 with hydrofluoric acid. In the presence of intracellular F^-, L-type HVA currents were blocked (Todorovic and Lingle, 1998), whereas N-type HVA currents were blocked by preincubation in 1 μM ω-conotoxin-GVIA. Because of the possibility of some residual HVA current contamination, all measurements of T-current amplitude in DRG cells were made from the peak of the inward current to the current remaining at the end of a 200-ms test step. For recording HVA Ca^{2+} currents, we used an intracellular solution containing 110 mM cesium methane sulfonate, 14 mM phosphocreatine, 10 mM HEPES, 9 mM EGTA, 5 mM Mg-ATP, and 0.3 mM Tris-GTP, pH adjusted to 7.15 to 7.20 with CsOH (standard osmolarity, 300 mOsM). When this

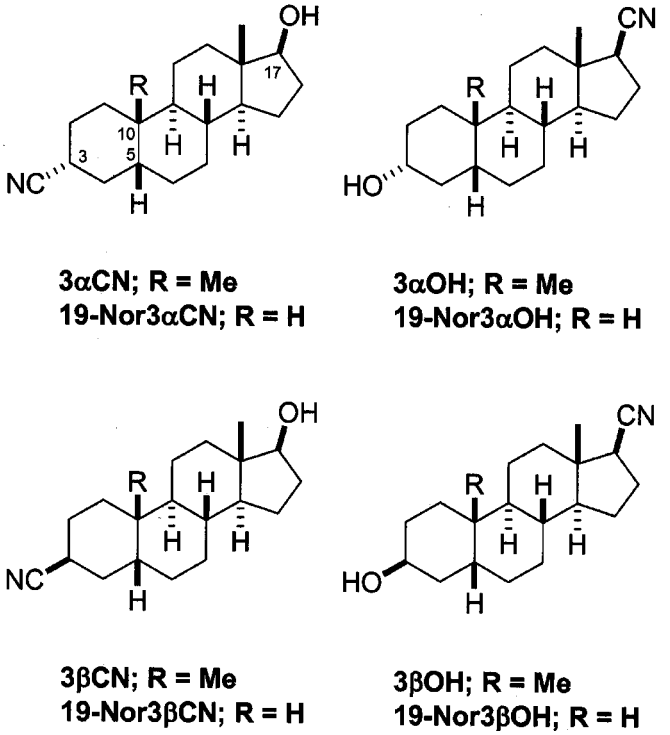

Fig. 1. The structures of 5β-reduced steroids evaluated in this study. Compounds with the 3-cyano group and 17β-hydroxyl groups (3αCN, 19-Nor3αCN, 3βCN, 19-Nor3βCN) are shown on the left. Compounds with the 3-hydroxyl and 17β-cyano groups (3αOH, 19-Nor3αOH, 3βOH, 19-Nor3βOH) are shown on the right.

internal saline was used for recording T-currents, most of the HVA current in these cells was blocked by preincubating cells with 1 μM ω-conotoxin-GVIA, 2 μM ω-conotoxin-MVIIC and including 5 μM nifedipine in the external solution to block N-, P/Q-, and L-type HVA, respectively. The amount of block of T current by steroids was not affected by using either internal saline (n = 2–4 cells for each steroid; data not shown). The standard extracellular saline for recording T- and HVA Ca^{2+} currents contained 152 mM tetraethylammonium chloride, 10 mM HEPES, and 10 mM $BaCl_2$, adjusted to pH 7.4 with tetraethylammonium hydroxide; osmolarity, 316 mOsM.

Voltage-gated Na^+ currents were recorded using electrodes that were pulled from borosilicate glass and had resistances of 0.6 to 1.0 MΩ when filled with pipette solution containing 140 mM CsF, 2 mM $MgCl_2$, 1 mM EGTA, 10 mM Na-HEPES (pH adjusted to 7.3 mM with CsOH, osmolarity adjusted to 310 mM mOsM using sucrose). Cells were superfused with solution containing 30 mM NaCl, 110 mM $CoCl_2$, 3 mM KCl, 1 mM $CaCl_2$, 0.1 mM $CdCl_2$, 2 mM $MgCl_2$, and 10 mM HEPES (pH adjusted to 7.4 with NaOH). On establishing whole-cell configuration, a series resistance compensation of 75% was applied, and cells were held at −90 mV. Cells that showed evidence of poor voltage control, as reflected by the shape of the current-voltage curve, were excluded from the study.

The standard external solution for recording voltage-gated K^+ currents contained 140 mM NaCl, 5 mM KCl, 2 mM $MgCl_2$, 2.0 mM $CaCl_2$, 10 mM glucose, and 10 mM HEPES, pH 7.4, with NaOH. The standard pipette solution used to record voltage-gated K^+ currents contained 110 mM KCl, 14 mM phosphocreatine, 10 mM HEPES, 9 mM EGTA, 5 mM Mg-ATP, and 0.3 mM Tris-GTP, 2 mM QX 314, pH adjusted to 7.2 with CsOH. To record voltage-gated K^+ currents cells were held at −60 mV and depolarized to +60 mV by a 150-ms depolarizing step.

Analysis of Current Blockade. The percentage reduction in peak Ca^{2+} inward current carried by Ba^{2+} ions at a given steroid concentration was used to generate concentration-response curves. For each of these curves, all points are averages of multiple determinations obtained from at least five different cells. On all plots, vertical bars indicate standard errors. Mean values on all concentration-response curves were fit to the following function: PB ([Neurosteroid]) = $PB_{max}/(1 + (IC_{50}/[Neurosteroid])^{n_H})$, where PB_{max} is the maximal percentage block of peak T current, IC_{50} is the concentration that produces 50% maximal inhibition, and n_H is the apparent Hill coefficient for blockade. Fitted values are typically reported with 95% linear confidence limits.

The voltage-dependence of steady-state activation and inactivation was described by the Boltzmann distribution, $I(V) = I_{max}/(1 + \exp[-(V - V_{50})/k])$, where I_{max} represents maximal activatable current, V_{50} represents the voltage where half of the current is activated or inactivated, and k (units of millivolts) represents the voltage dependence of distribution.

Steroids and Reagents. The synthesis, spectroscopic and physical properties of 3αOH, 19-Nor3αOH, 3βOH, and 19-Nor3βOH have been reported previously (Han et al., 1996). The starting material for the preparation of 3αCN and 3βCN was (5β,17β)-17-hydroxyandrostan-3-one. The 17-hydroxy group of this starting material was esterified using pyridine/acetic anhydride, and the 3-cyano group was introduced using a method described previously (Han et al., 1996). The resultant epimeric 3-cyanosteroids were separated by column chromatography on silica gel, and the 17-acetate group was hydrolyzed to regenerate the 17-hydroxy group of the final products, 3αCN and 3βCN. Likewise, 19-Nor3αCN and 19-Nor3βCN were prepared from (5β,17β)-17-hydroxyestran-3-one. The spectroscopic and physical properties of the previously unknown 3-cyanosteroids are given below.

3αCN: colorless crystals, m.p., 176 to 178°C; IR, 3485, 2945, 2931, 2868, 2248, 1459, 1450, 1071, 1055 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$) δ 3.64 (1 H, t, J = 8.7 Hz, C*H*OH), 2.42 (1 H, tt, J = 12.3 Hz, 3.9 Hz, C*H*CN), 0.94 (3H, s), 0.71 (3 H, s); ^{13}C NMR (75 MHz, $CDCl_3$) δ 122.92, 81.77, 50.90, 42.98, 42.52, 40.58, 36.69, 35.95, 35.71, 34.60, 30.43, 30.14, 28.76, 26.53, 25.71, 24.67, 23.51, 23.23, 20.26, 10.94. Anal. calculated for $C_{20}H_{31}NO$: C, 79.68; H, 10.36; N, 4.64. Found: C, 79.75; H, 10.51; N, 4.74.

3βCN: m.p., 150 to 151°C; IR, 3496, 2931, 2867, 2238, 1451, 1377, 1330, 1249, 1055, 955 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$) δ 3.62 (1 H, t, J = 8.4 Hz, C*H*OH), 3.00 (1 H, s, $w_{1/2}$ = 11.5 Hz, C*H*CN), 1.01 (3H, s), 0.72 (3H, s); ^{13}C NMR (75 MHz, $CDCl_3$) δ 122.54, 81.82, 51.02, 42.96, 40.50, 39.24, 36.77, 35.66, 35.11, 32.63, 30.44, 28.57, 27.82, 26.31, 25.81, 23.57, 23.22, 23.05, 20.33, 10.95. Anal. calculated for $C_{20}H_{31}NO$: C, 79.68; H, 10.36; N, 4.64. Found: C, 79.80; H, 10.51; N, 4.31.

19-Nor3αCN: m.p., 150 to 151.5°C; IR, 3427, 2917, 2868, 2238, 1452, 1377, 1263, 1073, 1053 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$) δ 3.67 (1 H, t, J = 8.5 Hz, C*H*OH), 2.47 (1 H, tt, J = 12.3 Hz, 3.9 Hz, C*H*CN), 0.74 (3 H, s); ^{13}C NMR (75 MHz, $CDCl_3$) δ 122.90, 81.92, 49.98, 43.15, 41.77, 39.96, 38.48, 36.66, 36.11, 31.35, 30.53, 30.09, 28.78, 27.07, 25.61, 25.23, 23.92, 23.21, 11.04. Anal. calculated for

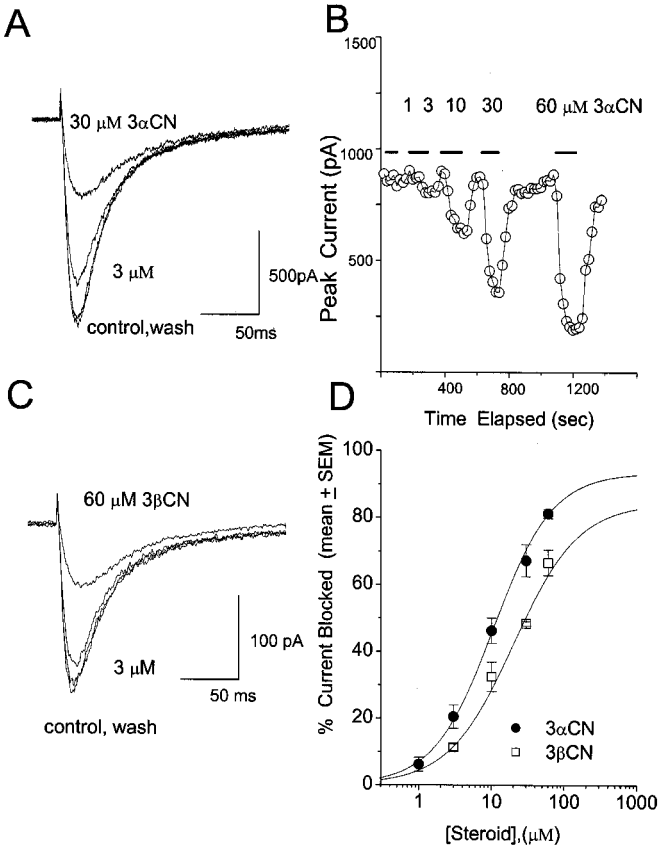

Fig. 2. Inhibitory effects of 3αCN and 3βCN on T current in small-size DRG cells. A, traces of T-currents before (control), during, and after (wash) the application of two different concentrations of 3αCN (3 and 30 μM). The application of 3αCN depressed the current amplitude in a dose-dependent fashion but did not have an effect on the apparent kinetics of the current. B, the time course of T-current blockade in the same DRG cell induced by escalating concentrations of 3αCN (from 1 to 60 μM), demonstrates a rapid and dose-dependent current blockade that was almost complete at 60 μM. The return to baseline was fast after each washout. The horizontal bars indicate the time of drug application. C, traces of T-current blockade in a DRG cell induced by two concentrations (3 and 60 μM) of 3βCN demonstrates reversible and dose-dependent current blockade. D, concentration-response curves for T current blockade by 3αCN (●) or 3βCN (□). The symbols indicate the average of multiple determinations ± S.E.M., with the solid line representing the best fit of the Hill equation. The fitted values for the curves are: 3αCN, IC_{50} = 11 ± 2 μM, n = 1.0 ± 0.1, and max block = 94 ± 7% (n = 10 cells); 3βCN, IC_{50} = 20 ± 10 μM, n = 1.0 ± 0.1, and max block = 84 ± 17% (n = 7 cells).

$C_{19}H_{29}NO$: C, 79.39; H, 10.17; N, 4.87. Found: C, 79.60; H, 9.97; N, 4.94.

19-Nor3βCN: m.p., 148–149°C; IR, 3469, 2915, 2870, 2236, 1453, 1380, 1264, 1073, 1055 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$) δ 3.64 (1 H, t, J = 8.5 Hz, C*H*OH), 3.02 (1 H, s, $w_{1/2}$ = 10 Hz), 0.75 (3 H, s); ^{13}C NMR (75 MHz) δ 122.55, 81.99, 50.11, 43.15, 41.73, 40.60, 38.40, 36.73, 32.83, 31.19, 30.56, 28.54, 27.87, 25.72, 25.35, 24.05, 23.20, 22.42, 11.02. Anal. calculated for $C_{19}H_{29}NO$: C, 79.39; H, 10.17; N, 4.87. Found: C, 79.50; H, 10.05; N, 4.95.

All steroids were dissolved in DMSO to make 10 to 30 mM stock solutions. Aliquots of the stock solutions were added to the standard external solution to achieve the final concentrations stated in the text. The final concentration of DMSO was less than 0.6% in these experiments; this concentration of DMSO did not affect I_{Ba} (data not shown, n = 5).

Behavioral Experiments. All experimental protocols were approved by the University of Virginia Animal Care and Use Committee, Charlottesville, VA, and in accordance with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the US National Institutes of Health. Every effort was made to minimize animal suffering and the number of animals used.

Chemicals and Animals. Adult female Sprague-Dawley rats (weight, 300–330 g) were used for all in vivo experiments because female rats are less aggressive and easier to handle during pain testing. For behavioral experiments, most steroids were dissolved as a stock solution (in 100% DMSO) and diluted in saline so that the highest final DMSO concentration was 3%. Injections of 3% DMSO in saline did not affect latency for paw withdrawal in rats (n = 6 rats, data not shown). For experiments with 3βOH and 3αOH, 20% cyclodextrin in saline was used to dissolve steroids because higher doses of these steroids (e.g., 3 μg/100 μl) crystallized in DMSO and saline after several minutes. Intraplantar injections of 20% cyclodextrin and saline in control experiments did not change thermal paw withdrawal latencies (PWLs) in six animals (data not shown). Likewise, in three cells, 20% cyclodextrin did not affect T currents or the effects of 3 μM 3βOH on T currents (data not shown).

Assessment of Thermal Nociception. The nociceptive response to thermal stimulation was measured using a paw thermal stimulation system consisting of a clear plastic chamber (10 × 20 × 24 cm) sitting on a clear, elevated glass floor and temperature regulated at 30°C (Hargreaves et al., 1988; Jevtovic-Todorovic et al., 1998, 2001; Jevtovic-Todorovic et al., 2003). Each animal was placed in the plastic chamber to accommodate for 15 min. A radiant heat source mounted on a movable holder beneath the glass floor was positioned to deliver a thermal stimulus to the plantar side of the hind paw. When the animal withdraws the paw, a photocell detects interruption of a light beam reflection and the automatic timer shuts off. This method has a precision of ± 0.05 s for the measurement of PWL. To prevent thermal injury, the light beam is automatically discontinued at 20 s if the rat fails to withdraw its paw.

To test the effects of neurosteroids in peripheral receptive fields, we injected 100 μl of test compounds intradermally in the ventral side of the right hind paw of animals. The noninjected side (left hind paw) was used as a control in each animal. All solutions were pH-balanced to 7.4 to avoid skin irritation. No signs of skin inflammation, discoloration, or irritation were noted at the sites of injection with test compounds. All doses are expressed in micrograms per 100 μl. At 10, 20, and 60 min after drug administration, the thermal stimulus was applied and PWLs were measured. The investigator assessing behavior measures was kept unaware of pharmacological interventions.

Statistical Analysis. Baseline values (B) were compared with thermal PWLs of noninjected and injected paws at various times during the testing as indicated in the figures (post-treatment values). In the data displayed, every point is an average of at least nine animals and values represent mean ± S.E.M. Statistical analysis

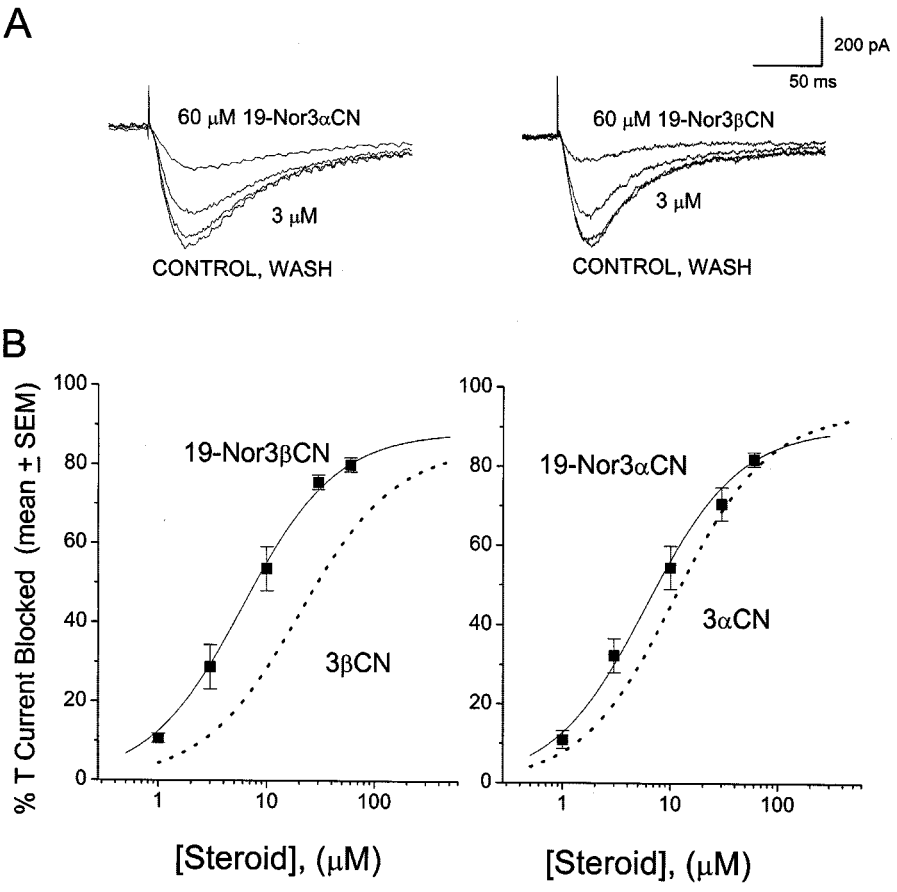

Fig. 3. The replacement of a methyl group with a hydrogen atom at position 10 of the 3-cyano,17-hydroxy steroid nucleus increases the potency of T-current blockade. A, raw traces on the left depict the effects of 19-Nor3αCN at two concentrations, 3 and 60 μM, on T currents in a rat DRG neuron. Raw traces on the right depict the effects of 19-Nor3βCN (an analog of 3βCN with a hydrogen atom instead of a methyl group at position 10) on T currents at the same concentrations, 3 and 60 μM. Note that 19-Nor3αCN and 19-Nor3βCN induced T-current blockade of similar magnitude but 19-Nor3βCN caused more profound blockade of T currents than 3βCN (Fig. 2C) at each concentration (at 3 μM, 25 and 10% blockade, respectively, and at 60 μM, 80% and 65% blockade, respectively). B, left, concentration-response curve for 19-Nor3βCN (fitted solid line was derived from the Hill equation). The calculated IC_{50} was 6.3 ± 0.7 μM, and the Hill coefficient was n = 1.0 ± 0.1, with a maximal block of T-current of 88 ± 3% (n = 7 cells). The dotted line, which represents the concentration-response curve for 3βCN (the actual data points are presented in Fig. 2 D), demonstrates the lower potency of 3βCN (3-fold rightward shift). Right, concentration response curve for 19-Nor3αCN (an analog of 3αCN with a hydrogen atom instead of a methyl group at position 10). Again, the solid line was derived from the Hill equation, and the calculated IC_{50} = 6.0 ± 0.9 μM with n = 1.0 ± 0.1 and the maximal block of T current = 89.9 ± 9.0% (n = 8 cells). The dotted line, which represents the concentration-response curve for 3αCN (the actual data points are presented in Fig. 2D), indicates the lower potency of 3αCN (about 2-fold rightward shift).

was performed using an analysis of variance comparing within-subject variables: paw condition (injected versus noninjected) and test session (before drug administration or 10, 20, and 60 min after treatment). Pair-wise comparisons were also conducted, and α levels were adjusted using the Bonferroni procedure when appropriate.

Dose-response data were fit to the function: $PI([Neurosteroid]) = PI_{max}/ (1+(ED_{50}/[Neurosteroid])^{nH})$, where PI_{max} is the maximal increase in PWLs caused by a drug in the injected versus noninjected paw 10 min after injection; the ED_{50} is the dose that produces half-maximal increase in PWLs indicating an analgesic effect; and n_H is the apparent Hill coefficient indicating the slope of the curve. Fitted values are reported with 95% linear confidence limits. Fitting was done with Origin 7.0 software (OriginLab Corp., Northampton, MA).

Results

In Vitro Effects of 5β-Reduced Neuroactive Steroids on T-Currents in Acutely Dissociated Rat DRG Neurons

Structure-Activity Studies. The DRG contains cell bodies of primary afferent (sensory) fibers with long processes that originate as sensory endings in the periphery and terminate in the dorsal horn of the spinal cord. Whole-cell recordings from dissociated DRG neurons of adult rats are used to study peripheral nociceptive mechanisms in vitro, because the small size of the peripheral nerve endings precludes direct measurement of the currents from the sensory endings. We limited our experiments to small-diameter (20–30 μm) and smaller medium-diameter (31–35 μm) acutely dissociated neurons because the majority of these cells are involved in peripheral nociceptive processing (Coderre et al., 1993; Levine et al., 1993; Snider and McMahon, 1998).

T-type Ca^{2+} currents were isolated as described under *Materials and Methods* and typically monitored with voltage steps to −30 mV from a holding potential of −90 mV. In our previous study with 5α-reduced steroids, we found that neuroactive steroids with a cyano group at position 3 of the steroid were most potent in blocking isolated T currents [e.g., (+)-ECN] (Todorovic et al., 1998). Therefore, in our studies of 5β-reduced neuroactive steroids, we initially focused on 5β-reduced analogs with a cyano group at position 3 of the steroid (i.e., 3αCN and 3βCN).

3αCN is an effective and concentration-dependent blocker of T currents in sensory neurons. Figure 2A shows representative tracings of the inhibitory effects of two 3αCN concentrations—3 and 30 μM—on T-currents in DRG cells. The time course of the blocking effects of 3αCN on T-currents demonstrates the stability of responses during the recordings and the fast kinetics (e.g., fast onset and offset) of T current blockade by 3αCN (Fig. 2B). Similar fast kinetics were observed with other 5β-reduced neuroactive steroids. The calculated IC_{50} for T-current blockade is 11 ± 2 μM; the maximal blocking effect (81 ± 5%) is achieved with 60 μM, and the fitted concentration-response curve approaches 100% blockade (Fig. 2D).

3βCN, the 3β-epimer of 3αCN, was an effective although less potent (~2-fold) blocker of T currents (Fig. 2C). The calculated IC_{50} for 3βCN was 20 ± 10 μM, with similar maximal blockade as estimated with 3αCN (Fig. 2D). This result indicates that the stereochemistry of the cyano group at position 3 affects the potency of T current blockade.

Because the presence or absence of a methyl group (numbered carbon 19 according to the rules of steroid nomenclature) at steroid position 10 has been shown to affect the potency of steroid modulation of 5β-steroids at $GABA_A$ receptors (Han et al., 1996), we investigated the effect of this structural modification on the potency of T current blockade by 5β-steroids. We found that 19-Nor3αCN and 19-Nor3βCN are potent blockers of T currents and, as indicated in Fig. 3, are more potent blockers of T currents than their structural analogs containing the methyl group at position 10, 3αCN and 3βCN, respectively. For example, 19-Nor3βCN at 3 and 60 μM induced 24 and 80% blockade of T current (right tracing), respectively, whereas its analog, 3βCN, induced less blockade at the same concentrations (10 and 65%, respectively) (Fig. 2C). Indeed, both 19-Nor3βCN and 19-Nor3αCN produced more potent blockade of T-currents than their structural analogs, as demonstrated by 3- and 2-fold leftward shifts from the concentration-response curves for 3βCN and 3αCN, respectively (the calculated IC_{50} values for 19-Nor3βCN and 19-Nor3αCN was 6.3 ± 0.3 and 6.0 ± 1.0 μM, respectively) (Fig. 3B). These results indicate that a methyl group at position 10 has a negative effect on potency and that the stereochemistry of the cyano group at position 3 has no

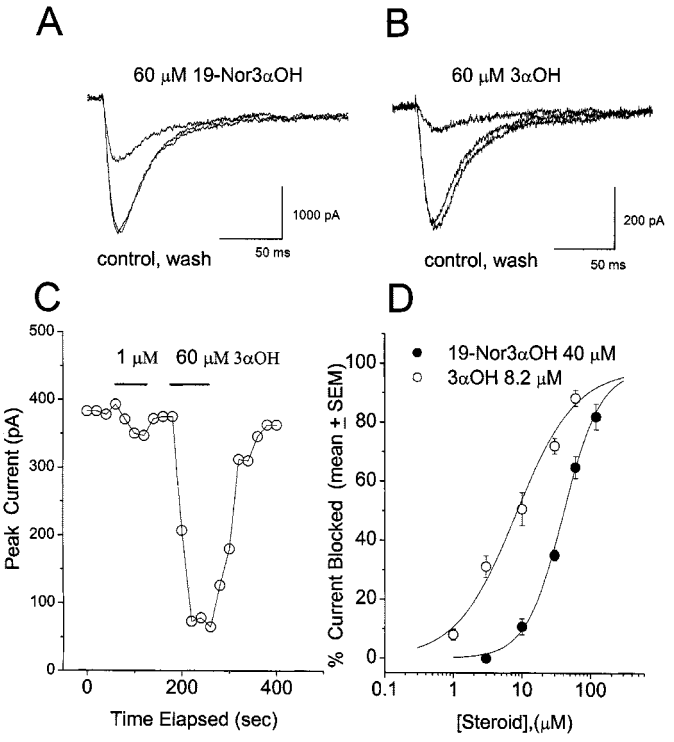

Fig. 4. The replacement of a hydrogen atom with a methyl group at the 10 position of 3β-hydroxy, 17α-cyano steroid nucleus increases the potency of T-current blockade. Raw traces of T currents before (control), during, and after the application (wash) of 60 μM 19-Nor3αOH (A) or 60 μM 3αOH (B) (an analog of 19-Nor3αOH with a methyl group instead of a hydrogen atom in position 10), demonstrate about 60 and 85% blockade of the peak current, respectively. C, the time course of T current blockade induced by two concentrations of 3αOH, 1 and 60 μM, demonstrates a rapid and dose-dependent current blockade that was almost complete at 60 μM. The return to baseline was fast after drug washout. D, concentration-response curves for T current blockade by 3αOH (○) or 19-Nor3αOH (●). The symbols indicate the average of multiple determinations ± S.E.M., with the solid line representing the best fit of the Hill equation. The fitted values for the curves are: 3αOH, IC_{50} = 8.2 ± 0.9 μM, n = 1.0 ± 0.1, and max block = 100 ± 4% (n = 8 cells); 19-Nor3αOH, IC_{50} = 40 ± 10 μM, n = 1.8 ± 0.3, and max block = 98 ± 11% (n = 8 cells).

significant effect on the potency of these 19-norsteroids for block of T-channel current.

5β-Reduced steroids that have a cyano group in the β-configuration at position 17 and a hydroxyl group at position 3 are known modulators of GABA-A receptors. The steroid 3αOH is a potent enhancer and the steroid 3βOH is a potent activation-dependent blocker of GABA-mediated currents (Han et al., 1996; Wang et al., 2002). To determine whether steroids with this functional group substitution pattern were also modulators of T-channels, we evaluated 3αOH, 3βOH, and the corresponding 19-norsteroids (19-Nor3αOH, 19-Nor3βOH).

We found that the two structural analogs 3αOH and 19-Nor3αOH, which differ only by the presence or absence of a methyl group in position 10 (Fig. 1), have substantially different T-current blocking potential. 3αOH was more potent in blocking T-currents than 19-Nor3αOH (Fig. 4). For example, 3αOH, at 60 μM, blocked 80% of the baseline T current (Fig. 4B), whereas 19-Nor3αOH blocked only about 58% of the T current at the same concentration (Fig. 4A). The blockade of T current was concentration-dependent (Fig. 4C), and the calculated IC_{50} was 8.2 ± 0.9 and 40 ± 10 μM for 3αOH and 19-Nor3αOH, respectively, with the fitted value for the maximal block of 98 ± 3% for both analogs (Fig. 4D).

Finally, we examined 19-Nor3βOH and 3βOH. 19-Nor3βOH blocks about 25% of T currents at a concentration of 3 μM (Fig. 5A, left tracing), whereas 3βOH blocks about 55% of T current in sensory neurons at the same concentration (Fig. 5A, right tracing). Figure 5B depicts the time course of T-current blockade caused by the three escalating concentrations of 3βOH—1, 3, and 30 μM. At 30 μM, 3βOH completely blocks T currents. At higher concentrations, the effects of 3βOH were only partially reversible (Fig. 5B), presumably caused by slow washout of this compound. The blockade of T current was concentration-dependent, and the calculated IC_{50} was 2.8 ± 0.6 and 6.0 ± 0.6 μM for 3βOH and 19-Nor3βOH, respectively (Fig. 5C). Overall, 3βOH is the most potent blocker of T current in rat sensory neurons (i.e., 3βOH is approximately 2- to 13-fold more potent than the other 5β-reduced neuroactive steroids examined in this study).

Mechanisms of Blockade of T-type Ca^{2+} Currents in Rat Sensory Neurons. We next determined whether 5β-reduced steroids affect kinetic properties of T currents. Figure 6A depicts a family of inward currents evoked from a holding potential of −90 mV in the absence (left) and presence (right) of 3 μM 3βOH. 3βOH did not significantly alter the time course of T current activation, measured as 10-to-90% rise time (Fig. 6B, n = 5 cells), or inactivation (Fig. 6C; n = 5 cells per data point), measured by the fit of a single exponential function to the decaying phase of the current at potentials from −50 to −10 mV.

Because many blockers of ion channels exhibit voltage-dependent features, we examined whether 3βOH alters voltage-dependent inactivation of T channels at different potentials. For these studies, we selected 3βOH, 3αOH, and 3αCN. T currents were evoked by a voltage step to −30 mV after a 5-s conditioning step at potentials from −110 to −50 mV in the presence and absence of a neuroactive steroid. This protocol defines the voltage-dependence of T current fractional availability in rat sensory neurons (Todorovic and Lingle, 1998). The normalized maximal current elicited from each conditioning potential is plotted as a function of the conditioning potential (Fig. 6D) (n = 6 cells). Based on the best fits using the Boltzmann equation, we found that, under control conditions, half availability (V_{50}) occurred at −69 mV with a slope factor of 8 mV. However, in the presence of 3 μM 3βOH, V_{50} occurred at −80 mV with a slope factor of 10 mV, thus

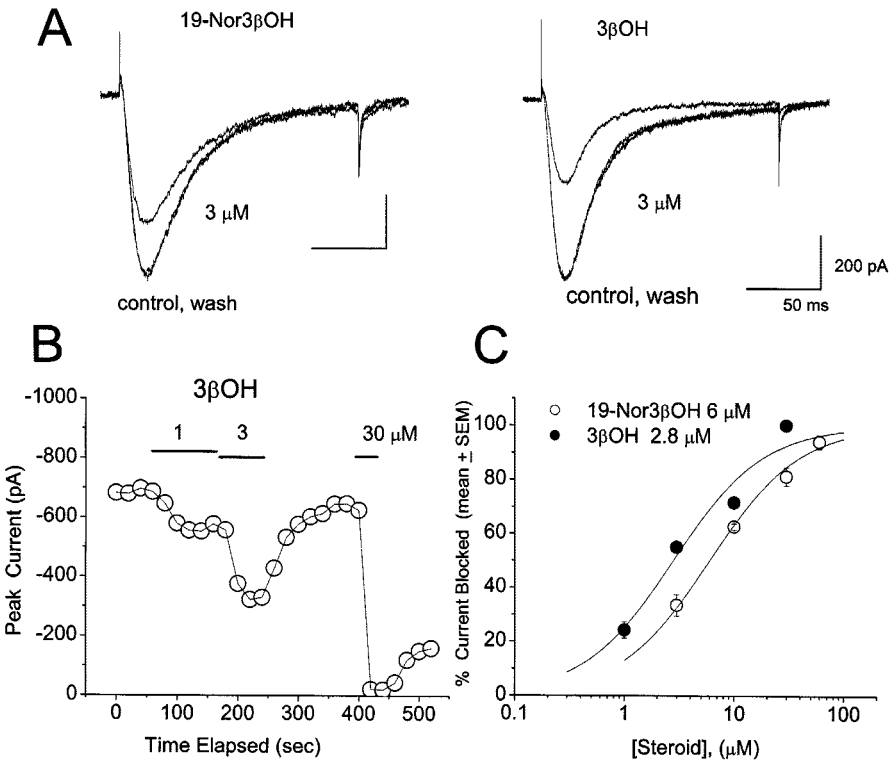

Fig. 5. The replacement of a hydrogen atom with a methyl group at position 10 of the 3α-hydroxy,17β-cyano steroid nucleus increases the potency of T-current blockade. A, raw traces of T currents before (control), during, and after the application (wash) of 3 μM 19-Nor3βOH (left) or 3 μM 3βOH (right) (an analog of 19-Nor3βOH with a methyl group instead of a hydrogen atom in position 10), demonstrate approximately 25% and more than 50% blockade of the peak current, respectively. B, the time course of T-current blockade in the same DRG cell induced by the three concentrations (1, 3, and 30 μM) of 3βOH demonstrates potent and dose-dependent current blockade (about 55% current blockade with 3 μM, and a complete block with 30 μM). A full recovery of the current is observed only after 3 μM 3βOH. Higher concentrations (e.g., 30 μM) are only partially reversible, presumably because of slow washout of this compound. C, concentration-response curves for T current blockade by 19-Nor3βOH (○) or 3βOH (●). The symbols indicate the average of multiple determinations ± S.E.M., with the solid line representing the best fit of the Hill equation. The fitted values for the curves are: 19-Nor3βOH, IC_{50} = 6.0 ± 0.6 μM, n = 1.0 ± 0.1, and max block = 99 ± 11% (n = 9 cells); 3βOH, IC_{50} = 2.8 ± 0.6 μM, n = 1.0 ± 0.2, and max block = 100 ± 4% (n = 8 cells).

shifting the steady state inactivation to more negative potentials. Likewise, 3αOH (n = 5 cells) and 3αCN (n = 4 cells) also exhibited mild voltage-dependent block, shifting the steady-state inactivation to more negative potentials by about 10 mV (data not shown). These experiments indicate that 5β-reduced neuroactive steroids exert more prominent blocking effects on T currents at more positive conditioning potentials. This may be functionally important because it suggests that these agents could be more effective under in vivo conditions when neuronal membrane potentials are in a more depolarized state (e.g., tissue injury, where the nociceptive fibers are more excitable and actively firing). On the other hand, there was very little effect of 3βOH on the voltage dependence of activation (n = 4 cells, Fig. 6E).

Selectivity of Blockade of T-Type Ca^{2+} Currents in Rat Sensory Neurons. Various voltage-gated ion channels (e.g., HVA Ca^{2+} channels and voltage-gated Na^+ and K^+ channels) have been shown to play roles in nociceptive processing (Kirchhoff et al., 1992; Caterina and Julius, 1999; Silbert et al., 2003; Bell et al., 2004). In addition, some neuroactive steroids (e.g., 5α-reduced steroids) are known to modulate HVA Ca^{2+} currents (ffrench-Mullen et al., 1994; Nakashima et al., 1998). To investigate whether 5β-reduced steroids also block other voltage-gated ion channels, we examined the effects of 3βOH, one of the most potent T-type Ca^{2+} currents blockers (Table 1), against HVA Ca^{2+} currents and voltage-gated Na^+ and K^+ channels. We studied the effects of this steroid on inward currents evoked by voltage steps from −70 mV to a test potential of −10 mV in cells that did not express T-type Ca^{2+} currents. Traces from a typical experiment with HVA currents are shown in Fig. 7A, and the time course of effect is depicted in Fig. 7B. At the lowest concentration (3 μM), 3βOH did not significantly alter HVA currents (8 ± 4% block, $p > 0.05$, n = 10 cells); at the highest concentration (30 μM), 3βOH blocked about 72 ± 8% of HVA currents (n = 4 cells). Note that the blocking effects of 3βOH were substantially more pronounced upon T currents in DRG cells recorded under similar conditions. When the membrane potential was held at −70 mV (near the resting membrane potential) followed by membrane depolarization to −30 mV (test potential), most of the evoked T current was blocked by 3 μM 3βOH (73 ± 6%, n = 7 cells) (Fig. 7C). The inhibitory effect of 3βOH was concentration-dependent, as shown in Fig. 5D, and the calculated IC_{50} for T-current blockade under these voltage conditions was 0.76 ± 0.16 μM, with maximal block approaching 100%. The calculated IC_{50} for HVA current

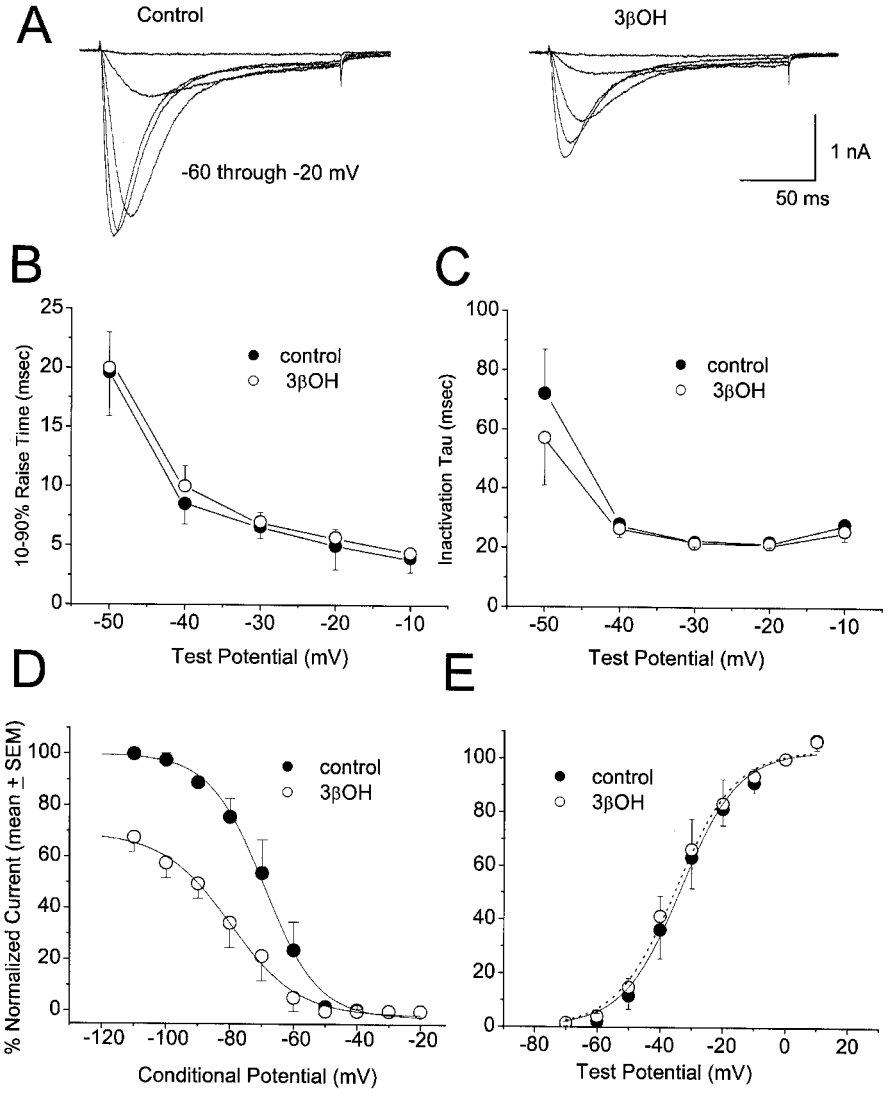

Fig. 6. The most potent 5β-reduced neuroactive steroid, 3βOH, causes voltage-dependent block of T currents. A, a family of T currents was evoked by voltage steps from a holding potential of −90 to −60 through −20 mV in a smaller medium-sized DRG cell before (left) and during (right) the application of 3βOH. B, activation times, measured as the 10-to-90% rise time of peak currents at potentials from −50 to −10 mV in experiments similar to those presented in A (n = 5 cells), were not significantly different before (filled symbols) and during the application of 3 μM 3βOH (○. Vertical lines indicate SE. C, inactivation time constants (τ) were determined from a single exponential fit of the decaying portions of T currents in the same cells as in B of this figure and plotted against test potential for control (●) and 3βOH (○). 3βOH had very little effect on the time course of the inactivation. D, the steady state inactivation curves obtained in the absence (●) and presence of 3 μM 3βOH (○) show a shift of 11 mV in the hyperpolarizing direction by 3βOH (n = 5 cells). The solid lines are the best fit of the Boltzmann distribution with a calculated V_{50} of −69.0 ± 1.0 mV and a slope factor of 8.4 ± 0.6 mV under control conditions. The V_{50} in the presence of 3 μM 3βOH was −80.0 ± 1.0 mV, with a slope factor of 10.0 ± 1.0 mV. E, 3βOH has very little effect on the voltage-dependence of T current activation in DRG cells. The best fit of the Boltzmann equation before (solid line) and during (dotted line) the application of 3 μM 3βOH (n = 4 cells per data point) was obtained by measuring tail currents at the end of a 20-ms depolarizing pulse to activate T channels at different potentials as indicated on the x-axis (from V_h of − 90 mV). Under these conditions, the tail current amplitude is directly proportional to the number of open channels at the end of the activating pulse. In the absence of 3βOH (●), half-maximal activation was −33.3 ± 1.2 mV with a slope factor of 9.2 ± 0.9 mV. In the presence of 3βOH (○, half-maximal activation was −34.9 ± 1.0 mV, and slope factor was 9.4 ± 0.8 mV.

blockade was 10 ± 1 μM, with a fitted maximal block of 79 ± 1%. We also examined the effects of 10 μM 19-Nor3αCN on HVA currents in 3 DRG cells and found no significant effect (3 ± 2% block, $p > 0.05$, data not shown). Thus, our data indicate that 5β-reduced steroids are more than 10-fold less effective in blocking DRG HVA than T-type Ca^{2+} currents.

At 3 μM, a concentration that blocks most of the T current, 3βOH did not significantly affect either voltage-gated Na^+ currents (4 ± 2% block, $p > 0.05$, $n = 7$ cells; Fig. 7E) or voltage-gated K^+ currents (2 ± 1% increase, $p > 0.05$, $n = 5$ cells; Fig. 7F).

To examine the selectivity of another representative 5β-reduced steroid that was also a very potent T-current blocker (Table 1), we studied 19-Nor3αCN and found that this steroid, when applied at a concentration that caused near maximal blockade of T current (10 μM) (Fig. 3B, right), had no

TABLE 1

Structure-activity relationships for the blockade of T-currents in rat DRG cells and peripheral antinociception by 5α -reduced neuroactive steroids

A summary of effects of steroids used in this study on isolated T currents and peripheral thermal nociception.

Compound Name	T Current Inhibition in DRG Cells (IC_{50})	n_H for T Current Blockade	Peripheral Analgesia (ED_{50})	n_H for Analgesia	Max Increase in PWL
	μM		*μg/100 μL*		*s*
3βOH	2.8 ± 0.6	1.0 ± 0.2	0.03±0.02	0.5 ± 0.1	5.9
19-Nor3αCN	6.0 ± 1.0	1.0 ± 0.1	0.30±0.20	0.8 ± 0.3	5.4
19-Nor3βOH	6.0 ± 0.6	1.0 ± 0.1	0.36±0.06	0.5 ± 0.1	4.55
19-Nor3βCN	6.3 ± 0.3	1.0 ± 0.1	0.16±0.10	0.7 ± 0.3	3.99
3αOH	8.2 ± 0.9	1.0 ± 0.1	0.24±0.09	0.6 ± 0.2	5.6
3αCN	11 ± 2	1.0 ± 0.1	0.78±0.50	0.8 ± 0.4	5.0
3βCN	20 ± 10	1.0 ± 0.1	0.26±0.01	0.9 ± 0.1	3.5
19-Nor3αOH	40 ± 10	1.8 ± 0.4	1.05±0.40	0.6 ± 0.3	3.66

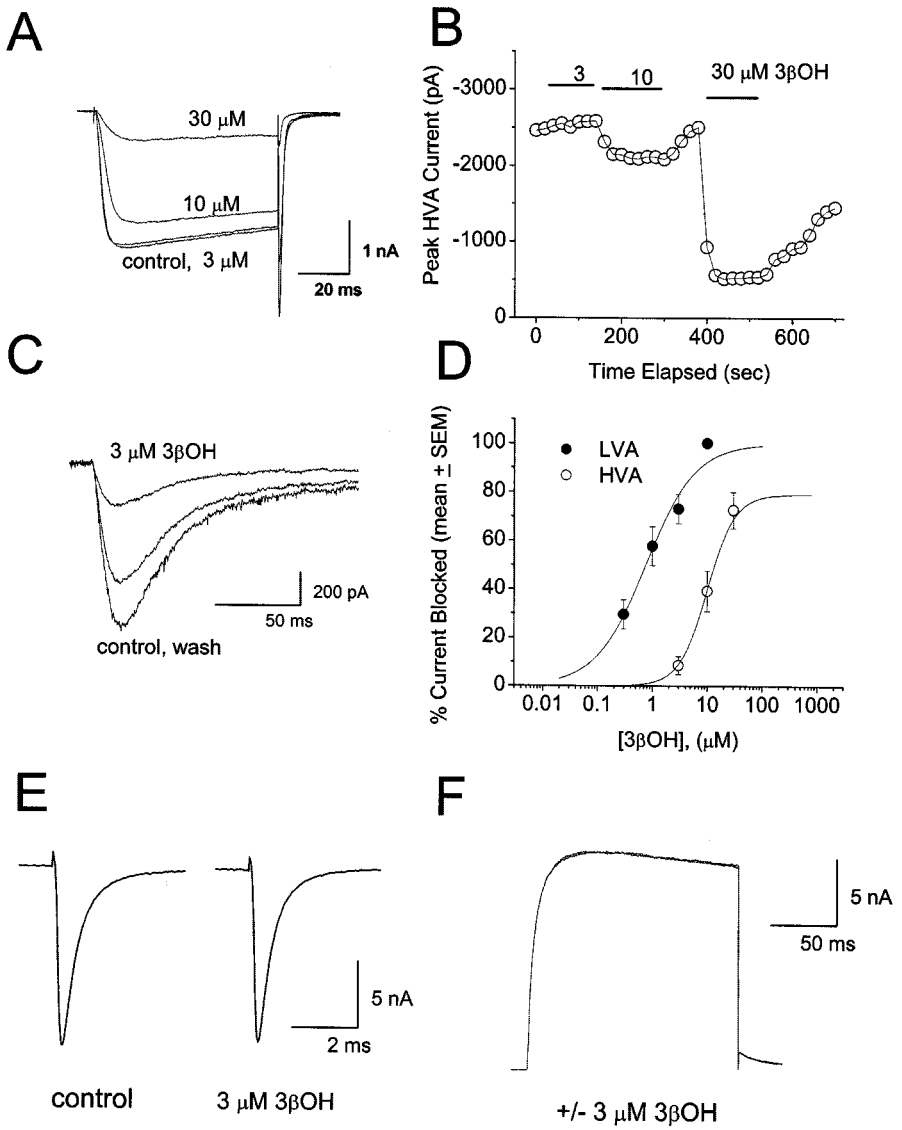

Fig. 7. Effects of 3βOH on LVA and HVA Ca^{2+} and voltage-dependent Na^+ and K^+ currents in rat DRG neurons. A, traces of HVA currents in a small DRG cell evoked from a holding potential of −70 to a test potential of 0 mV before and during the application of 3, 10, and 30 μM 3βOH. Note that 3βOH, at 3 μM, had very little effect on peak HVA currents. At this concentration, 3βOH almost completely blocked T currents evoked from the same holding potential (C). B, time course of the effects of escalating concentrations of 3βOH upon HVA currents from the same cell depicted in A. Horizontal bars indicate times of drug application. Note the partial recovery after the application of 30 μM 3βOH, which blocked about 80% of HVA current. C, at 3 μM, 3βOH almost completely blocked T currents evoked by the membrane depolarization from −70 to −30 mV. D, the average concentration-response curves for the effects of 3βOH on HVA (open symbols) and LVA currents (closed symbols) in experiments where holding potential was −70 mV (see traces in A and C). Symbols indicate the average of multiple determinations, vertical lines are S.E., and solid line is best fit of the Hill equation. The fitted values for the curves shown are: LVA (T) current: $IC_{50} = 0.76 \pm 0.20$ μM, $n = 1.0 \pm 0.2$, and max block = 100 ± 4% ($n = 9$ cells); HVA current: $IC_{50} = 10 \pm 1$ μM, $n = 1.6 \pm 0.5$, and max block = 79 ± 1% ($n = 12$ cells); E, traces from an experiment where voltage-gated Na^+ currents were recorded in a DRG cell from a holding potential of −90 mV to a test potential of −10 mV. The left trace depicts the control, and the right trace depicts a very small effect of 3 μM 3βOH upon peak of the inward current (about 4% block). F, traces of outward voltage-gated K^+ currents in a different DRG cell evoked from V_h of −60 mV to V_t of +60 mV. At 3 μM, 3βOH had no effect on the current.

effect upon either HVA currents (3 ± 2% block, $p > 0.05$, $n = 3$ cells; data not shown), Na^+ currents (3 ± 3% increase, $p > 0.05$, $n = 7$ cells; data not shown), or K^+ currents (no change, $n = 6$ cells, data not shown).

In Vivo Effects of 5β-Reduced Neuroactive Steroids on Peripheral Thermal Nociception in Adult Rats

Structure-Activity Studies. To determine whether 5β-reduced neuroactive steroids modify nociceptive responses in vivo, we designed a series of in vivo studies whereby 100 μl of test compounds were injected directly into the peripheral receptive fields of skin nociceptors located in the hind paw of adult rats, and the latency to paw withdrawal in the presence of a radiant heat stimulus was measured (Hargreaves et al., 1988; Jevtovic-Todorovic et al., 1998, 2003; Todorovic et al., 2001, 2002, 2004).

Intraplantar injections of 5β-reduced steroids into the right paw produced potent and dose-dependent antinociceptive responses in vivo at doses ranging from 0.003 to 3 μg/100 μl (Fig. 8). The maximum increase in PWLs (up to 60% from the pretreatment) was achieved 10 min after injection. This local analgesic effect lasted up to 60 min. Thermal PWLs on the noninjected (left) paw (■) remained unchanged during 60-min testing in all experiments, indicating a lack of systemic antinociceptive effect. The injection of the same volume of vehicle (3% DMSO dissolved in saline) had no effect on PWLs ($n = 9$ animals, data not shown).

Based on our in vivo study, the antinociceptive potency of the 3-cyano steroids is little influenced by the stereochemistry of the cyano group but is increased by the absence of the methyl group at position 10. For example, the maximal increase in PWLs at 10 min induced with 3 μg/100 μl 19-Nor3αCN and 19-Nor3βCN is about 6 s, whereas the maximal increase in PWLs induced with the same doses of 3αCN and 3βCN is about 3.5 to 4.0 s. On the other hand, although the antinociceptive potency of the 3-hydroxy steroids is once again little affected by the stereochemistry of the hydroxyl group, the presence of the methyl group at position 10 increases potency. For example, the maximal increase in PWLs at 10 min induced with 3 μg/100 μl of 3αOH and 3βOH is about 6 s, whereas the maximal increase in PWLs induced with 19-Nor3αOH and 19-Nor3βOH is about 4.5 s.

To further analyze the functional consequences of the presence or absence of the methyl group at position 10, we constructed dose-response curves (solid lines) that were plotted against the corresponding increases in PWLs (as measured by the right-left paw difference in PWLs at 10 min) (Fig. 7).

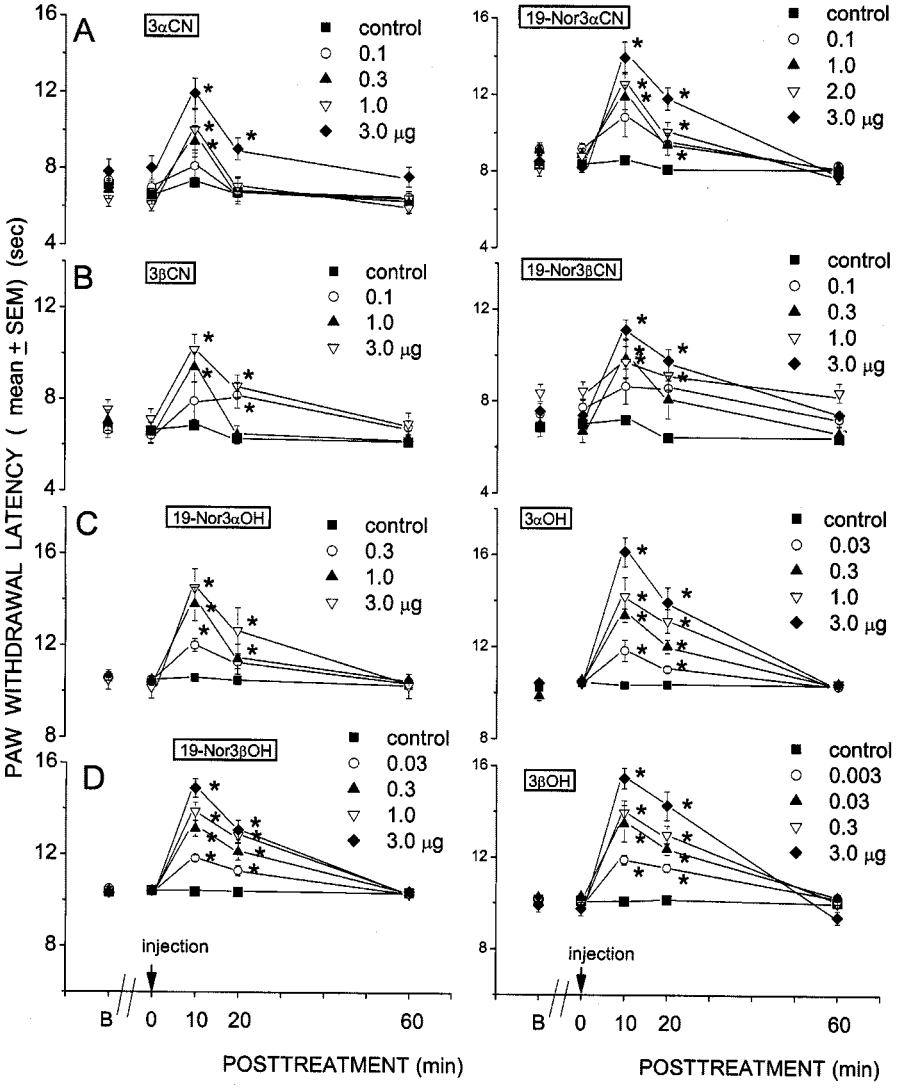

Fig. 8. 5β-reduced steroids induce time- and dose-dependent peripheral antinociception in thermal PWL testing. The plantar side of the right paw was injected with 100 μl of a test solution containing a given 5β-reduced steroid at doses ranging from 0.003 to 3 μg, as indicated on the graphs. The peak antinociceptive effect was recorded at 10 min with a complete return to baseline by 60 min. The left (noninjected) paw showed stable withdrawal latencies throughout the testing (denoted control in the graphs). Baseline testing (B) was performed 2 days before actual drug testing and compared with 0 time PWLs to confirm the stability of PWLs recordings. Significant differences in PWLs between injected (right) and noninjected (left) paws are indicated by asterisks (*) with $p < 0.05$. The significant overall analysis of variance values and p values from subsequent pair-wise comparisons are listed in Table 2. The arrows indicate the time of injection ($n = 9$–12 animals per group).

As shown in Fig. 9, the antinociceptive dose-response curves of 19-Nor3αCN and 19-Nor3βCN were shifted approximately 2- to 2.5-fold to the left compared with 3αCN and 3βCN (Fig. 9, A and B). Even more impressively, the antinociceptive dose response curves of 3αOH and 3βOH were shifted approximately 4- to 10-fold to the left compared with 19-Nor3αOH and 19-Nor3βOH (Fig. 9, C and D). Note that the most potent blocker of T currents in rat sensory neurons in vitro, 3βOH (IC_{50} of 2.8 μM) (Fig. 5C) is also the most potent antinociceptive steroid in vivo (ED_{50} of 0.03 μg/100 μl) (Fig. 9D); conversely, the least potent blocker of T currents in vitro, 19-Nor3αOH (IC_{50} of 40 μM) (Fig. 4D), is also the least potent antinociceptive steroid in vivo (ED_{50} of 1.05 μg/100 μl; Fig. 9C).

Our structure-activity studies in vitro and in vivo suggest a strong correlation between the potency of 5β-reduced neuroactive steroids for blocking T currents in vitro and their potency for antinociception in vivo. To further examine this correlation, we conducted linear regression analyses by plotting the ED_{50} values for analgesia versus IC_{50} values for T current blockade for each 5β-reduced neuroactive steroid (Fig. 10A). We found that there is an excellent correlation ($r = 0.78$) (solid line on the graph), demonstrating a statistically significant relationship between antinociceptive effects in vivo and T current blockade in vitro ($p = 0.02$). Behavior pain testing is often evaluated using an analgesia index that allows comparison of multiple parameters in determining the potency of tested agents (e.g., Buerkle and Yaksh, 1996). When the analgesic effect of 5β-reduced neuroactive steroids was expressed using the analgesia index (calculated as the ED_{50}/maximal increase in PWLs $\times$ 100), a measurement that includes both the potency and magnitude of the maximal antinociceptive response, we found that there is an even better correlation ($r = 0.88$) (solid line on the graph) between antinociceptive effects in vivo and T current blockade in vitro ($p = 0.004$) (Fig. 10B). Assuming that the nerve terminals and soma of sensory neurons express a similar repertoire of ion channels, our data would suggest that the ability of 5β-reduced neuroactive steroids to block T currents in vitro may underlie, at least in part, their analgesic effects in vivo when they are injected into peripheral receptive fields of these neurons.

Discussion

We demonstrate that the newly synthesized 5β-reduced neuroactive steroids examined in this study are potent blockers of the T-type Ca^{2+} channels in rat peripheral sensory neurons in vitro and very potent antinociceptive agents in vivo. Furthermore, we found an excellent correlation between the potency of T-current blockade in vitro and antinociceptive potency in vivo, strongly suggesting that T-type Ca^{2+} channels play an important role in peripheral somatic nociception. We also note that $GABA_A$ receptor potentiation does not correlate with the antinociceptive activity of these 5β-reduced neuroactive steroids because 3βOH, a compound known to be a potent activation-dependent blocker of $GABA_A$ receptors (Wang et al., 2002), was the most potent antinociceptive steroid identified.

Even though the existence of T channels was initially described in sensory neurons (Carbone and Lux, 1984) and subsequently confirmed in many in vitro studies using small size sensory neurons, most of which are nociceptors (Schroeder et al., 1990; Scroggs and Fox, 1992; Cardens et al., 1995; Todorovic et al., 2001), their role in nociception was not previously recognized. It has been shown that T-type Ca^{2+} channels in peripheral nociceptors can enhance both polymodal somatic nociceptive signals (Todorovic et al., 2001, 2002) and peripheral visceral nociceptive signals (Kim et al., 2003). Here, we not only show that the blockade of T currents

TABLE 2

Thermal nociceptive results for the statistically significant differences in injected (R) versus noninjected (L) paw

A two-way analysis of variance was performed for all dose-response experiments presented in Fig. 8 and, if found significant, was followed by subsequent pair-wise comparison analysis. The calculated F and p values are listed only for the times after injection at which they were significantly different from the non-injected side.

5β-Reduced Neuroactive Steroids	Dose	Overall ANOVA	Time after Injection	p Values for Pair-wise Comparisons (R vs. L)
	μg/100 μl		*min*	
19-Nor3αCN	1	$F(1,4) = 26.88$, $p < 0.001$	10 or 20	$p < 0.001$ each
	2	$F(1,4) = 31.837$, $p < 0.001$	10 or 20	$p < 0.001$ each
	3	$F(1,4) = 12.57$, $p < 0.001$	10 or 20	$p < 0.001$ or $p = 0.015$
3βCN	1	$F(1,4) = 15.62$, $p < 0.001$	10 or 20	$p < 0.001$ each
	3	$F(1,4) = 85.70$, $p < 0.001$	10 or 20	$p < 0.001$ each
19-Nor3βCN	0.3	$F(1,4) = 5.705$, $p < 0.001$	10	$p < 0.001$
	1	$F(1,4) = 4.392$, $p < 0.001$	10 or 20	$p < 0.001$ each
	3	$F(1,4) = 17.78$, $p < 0.001$	10 or 20	$p < 0.001$ each
19-Nor3αOH	0.3	$F(1,4) = 12.52$, $p < 0.001$	10	$p < 0.001$
	1	$F(1,4) = 12.31$, $p < 0.001$	10 or 20	$p < 0.001$ each
	3	$F(1,4) = 13.78$, $p < 0.001$	10 or 20	$p < 0.001$ each
3αOH	0.03	$F(1,4) = 11.20$, $p < 0.001$	10 or 20	$p < 0.001$ or $p = 0.003$
	0.3	$F(1,4) = 46.33$, $p < 0.001$	10 or 20	$p < 0.001$ each
	0.1	$F(1,4) = 15.83$, $p < 0.001$	10 or 20	$p < 0.001$ each
	3	$F(1,4) = 47.83$, $p < 0.001$	10 or 20	$p < 0.001$ each
19-Nor3βOH	0.03	$F(1,4) = 28.94$, $p < 0.001$	10 or 20	$p < 0.001$ each
	0.3	$F(1,4) = 32.89$, $p < 0.001$	10 or 20	$p < 0.001$ each
	1	$F(1,4) = 58.22$, $p < 0.001$	10 or 20	$p < 0.001$ each
	3	$F(1,4) = 38.28$, $p < 0.001$	10 or 20	$p < 0.001$ each
3βOH	0.03	$F(1,4) = 32.125$, $p < 0.001$	10 or 20	$p < 0.001$ each
	0.03	$F(1,4) = 18.66$, $p < 0.001$	10 or 20	$p < 0.001$ each
	0.3	$F(1,4) = 35.78$, $p < 0.001$	10 or 20	$p < 0.001$ each
	3	$F(1,4) = 86.65$, $p < 0.001$	10 or 20	$p < 0.001$ each

caused by 5β-reduced neuroactive steroids in vitro correlates very well with their in vivo antinociceptive potency, we also provide the first structure-activity data for the steroid effects. Compounds having either the 3-cyano and 17β-hydroxyl groups (3αCN, 3βCN, 19-Nor3αCN, and 19-Nor3βCN) or the 3-hydroxyl and 17β-cyano group (3αOH, 3βCN, 19-Nor3αOH, and 19-Nor3βCN) are effective T-channel blockers and antinociceptive agents. In addition, the stereochemistry of the group at position 3 on the steroid A-ring does not seem to be of major importance. Finally, the effect of the steroid 19-methyl group at position 10 of the steroid depends on the properties of the functional group at position 3. For example, the 19-norsteroids with the 3-cyano, 17β-hydroxy groups are more potent than those that have the 19-methyl group, whereas the 19-norsteroids with the 3-hydroxy, 17β-cyano groups are less potent than those having the 19-methyl groups.

As in our previous reports with 5α-reduced steroids (Todorovic et al., 1998), we find that 5β-reduced neuroactive steroids exhibit mild voltage-dependent blockade of T type Ca^{2+} channels. This voltage dependence may indicate their preference for inactive states of the channel, resulting in a higher fractional block of the channel at depolarized membrane potentials. For example, we found that the IC_{50} for 3βOH, the most potent T- current blocker, was decreased by about 4-fold at a holding potential of −70 mV compared with a holding potential of −90 mV (0.76 versus 3 μM, respectively). Although direct measurement of resting membrane potentials in skin nociceptive endings in vivo is not technically possible, measurement of the resting membrane potentials from the intact nociceptors in the nerve-DRG preparation in vitro indicates that their resting membrane potential is approximately at −74 mV (Todorovic and Anderson, 1992; Scroggs et al., 1994). Therefore, it is reasonable to propose that T channels in peripheral nociceptors are operational and highly susceptible to blockade by neuroactive steroids. It is noteworthy that most 5β-reduced steroids examined in this study cause almost complete block of neuronal DRG T currents, whereas 5α-reduced neuroactive steroids block T currents only partially (up to 40%; Todorovic et al., 1998).

There are reports in the literature that the modulation of HVA Ca^{2+} currents by some 5α-reduced neuroactive steroids is G-protein dependent (ffrench-Mullen et al., 1994). However, it is unlikely that the modulation of T currents observed in our experiments is through G-protein-mediated pathways because the exclusion of ATP and GTP (two constituents necessary for maintenance of G-protein signaling pathways) in the intracellular solution did not influence the magnitude of T current blockade by 5β-reduced neuroactive steroids. This observation suggests that related but structurally different steroids exhibit distinct mechanisms of blocking neuronal voltage-gated Ca^{2+} currents.

Previous studies suggest that other families of voltage-gated ion channels (e.g., HVA Ca^{2+}, K^+, and Na^+ channels) play important roles in nociceptor excitability. Our findings indicate that 3βOH and 19-Nor3αCN, the most potent representative T-current blockers in vitro, as well as the most potent representative peripheral analgesics in vivo, when applied at concentrations that effectively suppress T currents (e.g., 3 and 10 μM, respectively), do not significantly affect either HVA Ca^{2+}- or voltage-gated Na^+ and K^+ currents in rat sensory neurons. However, further structure-activity studies will be necessary to establish the relative importance of T channels and other voltage- and ion-gated channels in the peripheral antinociceptive effects of 5β-reduced neuroactive steroids.

Although the role of $GABA_A$ receptors in mediating central analgesic effects of the neuroactive steroids was recently suggested (Nadeson and Goodchild, 2000), the role of $GABA_A$ receptors in peripheral nociception remains poorly understood. It has been reported that local intraplantar injection of the GABAergic agent, muscimol, failed to increase analgesic threshold in vivo (Carlton et al., 1999). Based on these findings it seems that, despite the presence of $GABA_A$ receptors on peripheral nociceptors (Carlton et al., 1999), $GABA_A$-mediated effects per se play a less significant role in peripheral nociception under physiological conditions. However, it seems that $GABA_A$-mediated responses play a more promi-

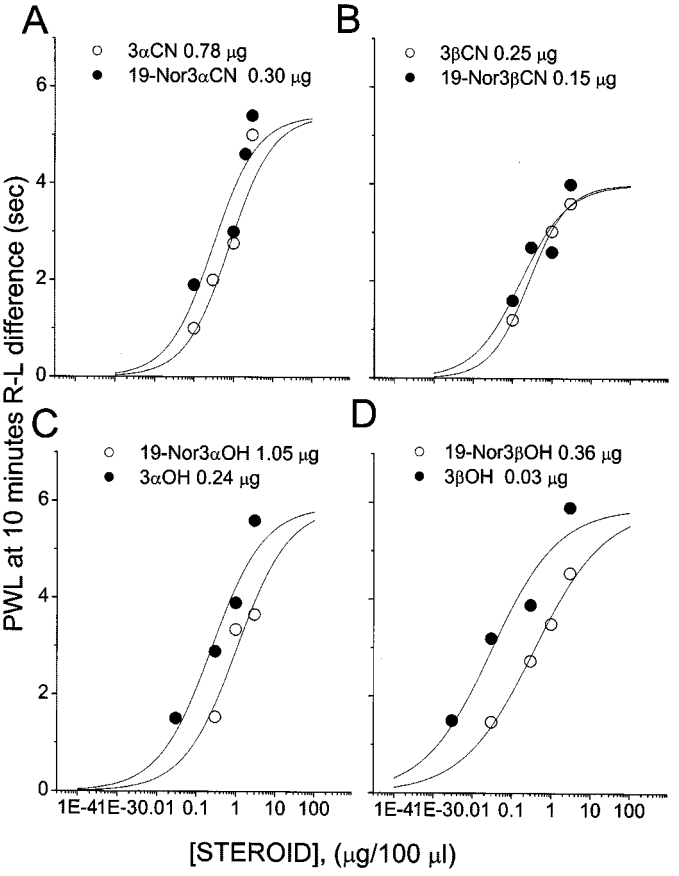

Fig. 9. Dose-response curves for thermal antinociception after local intraplantar injections of 5β-reduced neuroactive steroids. The average difference in PWLs (seconds) between injected and noninjected paws at 10 min (the maximal effect) was plotted against the corresponding doses of a given 5β-reduced neuroactive steroid (in micrograms per 100 μl) and the best fits were obtained using the Hill equation (solid lines). A, 19-Nor3αCN is a more potent antinociceptive agent than its analog, 3αCN, as demonstrated by the leftward shift in the dose-response curve and a 2.5-fold decrease in the ED_{50} (0.3 ± 0.05 μg and 0.78 ± 0.15 μg). B, 19-Nor3βCN is a slightly more potent antinociceptive agent than its analog, 3βCN, as demonstrated by the small leftward shift in the dose-response curve and a 1.5-fold decrease in the ED_{50} (0.15 ± 0.04 and 0.25 ± 0.08 μg). C, 3αOH is more potent than its analog, 19-Nor3αOH, as demonstrated by the leftward shift in the dose-response curve and an over 4-fold decrease in the ED_{50} (0.24 ± 0.04 and 1.05 ± 0.26 μg). D, 3βOH is substantially more potent than its analog, 19-Nor3βOH, as demonstrated by a 12-fold decrease in the ED_{50} (0.03 ± 0.005 and 0.36 ± 0.08 μg). All values for ED_{50}, Hill coefficient and the maximal increase in thermal PWLs by the steroid analogs are in Table 1.

nent role in the nociception caused by the peripheral tissue inflammation (caused by local intraplantar injections of formalin) (Carlton et al., 1999).

Local injections of 5β-reduced neuroactive steroids cause prolongation of thermal PWLs in the injected paws but not in the contralateral (control) paws of rats, indicating that the observed antinociceptive effect results from a direct action on nociceptive nerve endings, rather than a systemic effect. In considering these novel 5β-reduced steroids as local analgesics, it is important to note that T channels are preferentially located on the smaller size sensory neurons that play an important role in nociceptive transmission but not in other modalities of sensory transmission (e.g., touch, vibration) or motor transmission, making selective and potent blockade of T currents a desirable therapeutic objective. By blocking pain sensation preferentially, without causing undesirable motor weakness, locally injected 5β-reduced neuroactive steroids may offer a breakthrough in safe and effective pain therapy. It is noteworthy that the potency of the 5β-reduced steroids that were examined in vivo ranges from 0.03 (for 3βOH) to 1 μg/100 μl (e.g., 19-Nor3αOH). To our knowledge, this is one of the most potent groups of local analgesics yet described. For instance, using the same in vivo model of peripheral thermal nociception, we found that the clinically used voltage-gated Na^+ channel blockers, phenytoin and carbamazepine, were 10-fold less potent than 3βOH in inducing antinociception (Todorovic et al., 2003b). Furthermore, lidocaine, another local anesthetic that is also a Na^+ channel blocker, is used clinically at concentrations 1000-fold higher. Thus, 5β-reduced neuroactive steroids may be an important addition to the class of local analgesics commonly used for regional anesthesia. The steroids could also be amenable to delivery by direct applications in the form of skin patches or local infiltration (at the site of an acute tissue injury, e.g., thermal coagulation, sunburns) because these agents are highly lipid soluble and should be able to easily access peripheral nerve endings. In addition to the potential usefulness of T channel blockers for the treatment of short-term pain, recent studies suggest that local administration of T channels blockers can be beneficial in the treatment of chronic pain (Dogrul et al., 2003; Todorovic et al., 2004).

In summary, we report that the 5β-reduced neuroactive steroids examined in this study are potent blockers of T-type Ca^{2+} channels in rat sensory neurons in vitro and potent peripheral antinociceptive agents in vivo. The correlation between T-channel block and antinociceptive activity strongly suggests that blockade of T-type Ca^{2+} currents in sensory neurons may underlie, at least in part, the potent analgesic properties of 5β-reduced steroids. Thus these steroids are promising new tools for studying the role of T-type Ca^{2+} channels in peripheral nociception and are potentially useful targets for development as novel pain therapies.

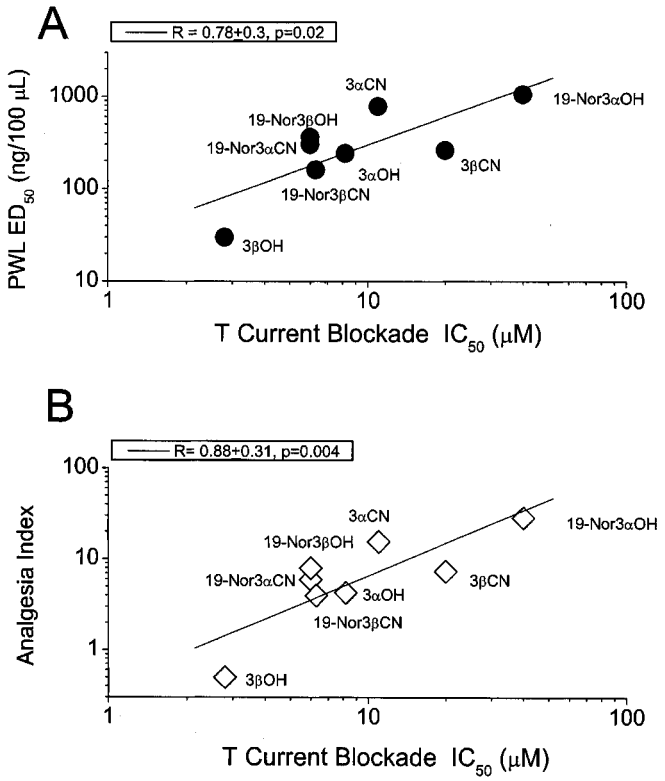

Fig. 10. Correlation between the antinociceptive effects of 5β-reduced neuroactive steroids and T-current blockade. A, there is a statistically significant correlation between the antinociceptive potency of the steroids in vivo (expressed as ED_{50} values calculated at the peak effect − 10 min) and their ability to block T currents in small sensory neurons in vitro (expressed as IC_{50} values). The solid line represents the best fit of the linear regression plot ($r = 0.78 \pm 0.30$ and $p = 0.02$). B, there is a statistically significant correlation between the antinociceptive potency of the steroids in vivo, expressed as the analgesia index [ED_{50}/maximal increase in PWLs (seconds) × 100] and their ability to block T currents in sensory neurons (expressed as the IC_{50} values). The solid line represents the best fit of the linear regression plot ($r = 0.88 \pm 0.31$ and $p = 0.004$).

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