Acute neurosteroids inhibit the spinal reflex potentiation via GABAergic neurotransmission

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Recently, we demonstrated a chronic neurosteroid-dependent inhibition of activity-dependent spinal reflex potentiation (SRP), but it remains unclear whether neurosteroids acutely modulate SRP induction. This study shows progesterone as well as two of its 3α,5α-derivatives, allopregnanolone and 3α,5α-tetrahydrodeoxycorticosterone (THDOC), to be capable of producing acute GABA_A receptor (GABA_A R)-dependent inhibition of SRP. When compared with test simulation (1 stimulation/30 s) of pelvic afferent nerves that evoked a baseline reflex activity in an external urethra sphincter electromyogram, repetitive stimulation (RS; 1 stimulation/1 s) induced SRP characterized by an increase in the evoked activity. Intrathecal progesterone (3–30 μM, 10 μl) at 10 min before stimulation onset dose dependently prevented RS induction. Intrathecal allopregnanolone (10 μM, 10 μl) and THDOC (10 μM, 10 μl) also prevented the SRP caused by RS. Pretreatment with the GABA_A R antagonist bicuculline (10 μM, 10 μl) at 1 min before progesterone/neurosteroid injection attenuated the inhibition of SRP caused by progesterone, allopregnanolone, and THDOC. Results suggest that progesterone and its neurosteroid metabolites may be crucial to the development of pelvic visceral neuropathic/postinflammatory pain and imply clinical use of neurosteroids, such as allopregnanolone and THDOC, for visceral pain treatment.

progesterone; urethra; visceral pain; hypergesia; spinal cord

PROGESTERONE. A GONADAL HORMONE that exerts physiological effects on the reproductive system (44), is metabolized by 5α-reductase into 3α,5α-derivatives such as 3α,5α-tetrahydrodeoxycorticosterone (THDOC) and 3α-hydroxy-5α-pregn-20-one (allopregnanolone). Behavioral studies recently revealed that neurosteroids may modulate a broad spectrum of neural activity, such as anxiety, depression (11, 13, 14), and analgesia (12, 15, 47) through their nongenomic effects on membrane ion channels and receptors (15).

Gamma-aminobutyric acid (GABA) is a common inhibitory central nervous system neurotransmitter. Exogenous neurosteroids have been shown to alter type A GABA receptor (GABA_A R) expression in the hippocampus (26, 43). Pharmacological blockade of neurosteroid synthesis prevented changes in GABA_A R mRNA levels in CA1 neurons (8). For the spinal cord, 5α-reduced neurosteroids are presumed to modulate neural activity predominantly via its effects on GABA_A R (24, 39). Studies have been done to develop strategies for pain treatment by facilitating neurosteroid-dependent regulation of spinal GABAergic inhibition (1). Studies investigating nociception demonstrated that peripheral inflammation produces upregulation of spinal 5α-reduced neurosteroid synthesis in the spinal cord (22, 28) and leads to inhibition of synaptic transmission within the substantia gelatinsosa, a pivotal site for pain gating (29). Our laboratory previously reported activity-dependent spinal reflex potentiation (SRP) at the lumbosacral spinal cord level. Although nociceptive behavior was not measured, we demonstrated that acute irritant instillation activates TRPV1/TRPA1 expressing nociceptive afferent fibers to facilitate SRP; this suggests that SRP is a form of pain-related reflex plasticity (31, 34). Moreover, dispersion in the ureter (6) and anus (7) abolishes SRP induction by enhancing spinal GABA_A R-mediated inhibition. A very recent study demonstrated that chronic progesterone administration has an effect on SRP induction via the action of its neurosteroid metabolites on GABA_A R (32). However, the acute effects of progesterone on SRP induction are yet to be established. When considering that neurosteroids acutely modulate neural activity via membrane channels/receptors (13–15), we suspect that progesterone might also acutely affect SRP induction. This study attempts to determine whether acute progesterone administration modulates SRP induction and, if so, whether this involves the actions of its 5α-reduced metabolites on specific spinal GABA_A R subunits. We first tested whether inhibition of SRP induction by progesterone stems from the actions of its 5α-reduced metabolites through administration of progesterone or its metabolites. We then tested whether progesterone’s 5α-reduced metabolites inhibit SRP induction via GABA_A R using a selective GABA_A R antagonist.

MATERIALS AND METHODS

Animal preparations. This study was reviewed and approved by the Institutional Review Board of Chung-Shan Medical University in Taiwan. Eighty-two adult female Sprague-Dawley rats (250–360 g) were used throughout this study. Rats were individually housed in wood chip-lined plastic cages having free access to water and food, and they were maintained on a 12:12-h light-dark cycle with lights on at 0700. The estrous stage was assessed daily at 9 AM by vaginal lavage using the traditional stage nomenclature (2). At least two complete, regular 4-day estrous cycles were checked before the day of the experiment and when the rats were in estrus phase (low-progesterone level) was tested in this study.
Surgical preparations. Animals were anesthetized with urethane intraperitoneally (1.2 g/kg ip). The trachea was intubated to keep the airway patent. A PE-50 catheter (Portex, Hythe, Kent, UK) was placed in the left femoral vein for administration of anesthetics when needed. The atlanto-occipital membrane was incised and a PE-10 catheter was inserted through the slit and passed caudally to the T13 level of the vertebrae (around L6-S2 level of the spinal cord). The left pelvic nerve was carefully dissected from the surrounding tissue and transected, and the central stump of the transected nerve was mounted on a pair of stainless steel wire electrodes for electric stimulation. At the end of the experiments, the animals were killed under deep anesthesia, using an intravenous injection of saturated potassium chloride solution. The position of the tip of the intrathecal catheter was confirmed by a laminectomy at T12-L2 vertebrae. In case that the tip of the catheter deviated more than 2 mm form the target tissue (dorsal aspect of the L6-S2 spinal cord), data were excluded for analysis.

Application of drugs. The drugs used in this study were as follows: progesterone water soluble (3, 10, 30 μM, 10 μl it), a progesterone agonist; THDOC (30 μM, 10 μl it), a 3α,5α-metabolite of progesterone; allopregnanolone (30 μM, 10 μl it), a 3α,5α-metabolite of progesterone; bicuculline (10 μM, 10 μl it), a GABAAR antagonist; and 4,5,6,7-tetra-hydroisoxazolo(5,4-c)pyridine-3-ol (gaboxadol, THIP, 10 μM, 10 μl it), a GABAAR agonist. Drugs were dissolved in artificial cerebrospinal fluid or DMSO and applied in a final DMSO concentration of less than 1%. A solution of identical volume to the tested agents was dispensed to serve as the vehicle. The concentrations of tested agents were adapted from the work of Meyer and colleagues (29), and pilot studies have been performed to test the effective dosages of tested agents.

Reflux activity recording. After a PE-50 catheter was inserted into the urinary bladder to drain it freely, epoxy-coated copper wire (50 μm; M.T. Giken, Tokyo, Japan) electromyogram electrodes were placed intra-abdominally into the external urethra sphincter to make it easier to perform and identify its position. The external urethral sphincter electromyogram activities were amplified 20,000-fold by a preamplifier (Grass P511AC, Cleveland, OH) and then continuously displayed on a recording system with a sampling rate of 20,000 Hz (MP30, Biopac, Santa Barbara, CA). Square wave pulses with an intensity that caused single spike action potentials were used to standardize the baseline reflex activity and were applied to animals throughout each experiment. The protocol for assessing the effects of electrical stimulation and different kinds of reagents on the reflex activity was as follows: protocol 1: pelvic afferent nerve test stimulation (TS): single shock was repeated at 30-s intervals (1 stimulation/30 s) for 30 min. This frequency of stimulation was chosen for sampling data because it did not result in response facilitation. Protocol 2: pelvic afferent nerve repetitve stimulation (RS): after an equilibrium period (usually 30 min), the RS (1 stimulation/1 s, lasting for 30 min) was applied to induce reflex potentiation. Protocol 3: intrathecal reagent injections: after another equilibration (usually 30 min), progesterone, THDOC, or allopregnanolone was injected 10 min before stimulation onset. In some experiments, pretreated bicuculline was injected 1 min before progesterone, THDOC, or allopregnanolone administrations. Furthermore, THIP was injected 1 min before stimulation onset.

Data analysis. Comparisons across different stimulation paradigms as well as all drug- and vehicle-treated groups were determined using two-way ANOVA, followed by a post hoc test (SigmaStat 2.0; Systat Software, San Jose, CA). In all cases, a difference of $P < 0.05$ was considered as a statistically significant difference.

RESULTS

Baseline reflex activity and reflex potentiation. Initial experiments established stable baseline reflex activity and RS-induced reflex potentiation. Single test stimulation pulses (Fig. 1B, TS: 1 stimulation/30 s) evoked baseline reflex activity with single action potentials, while RS (1 stimulation/1 s) induced reflex potentiation. The RS-induced reflex potentiation was characterized by progressive increases in firing that reached a maximum at ~1 min following stimulation onset and remained until stimulation cessation (Fig. 1, B and C). When compared with TS, RS significantly increased the mean spike numbers evoked by each pulse (RS, *$P < 0.05$, **$P < 0.01$ to TS, $n = 7$).

Progesterone-dependent inhibition of reflex potentiation. To elucidate the acute effects of progesterone on RS-induced reflex potentiation, we intrathecally administered progesterone (PRG; 10 μM, 10 μl) to the preparations at 10 min before stimulation. Progesterone, but not vehicle solution (data not shown), inhibited the RS-induced reflex potentiation (RS) by decreasing the spike count evoked by each pulse (Fig. 2A, PRG + RS). When compared with animals that received RS only (RS), progesterone significantly decreased the mean spike count at 30 min after RS onset (Fig. 2C, PRG + RS, ###$P < 0.01$ to RS, $n = 7$). The effects of progesterone on RS-induced reflex potentiation were further investigated by using it at various concentrations. Progesterone dose dependently inhibits RS-induced reflex potentiation at concentrations of 3–30 μM (Fig. 2B; 3 μM PRG + RS, 10 μM PRG + RS, and 30 μM PRG + RS). When compared with the RS group, Fig. 2D shows a dose-dependent decrement in the mean spike count caused by progesterone at 3–30 μM (3, 10, and 30 μM, PRG + RS, #$P < 0.05$, ##$P < 0.01$ to RS, $n = 7$).
Effects of progesterone metabolites. To further determine whether progesterone-mediated inhibition of reflex potentiation is attributable to its neurosteroid metabolites, we intrathecally applied allopregnanolone or THDOC, which are two 3α,5α-derivatives of progesterone, at 10 min before stimulation. Allopregnanolone (ALL; 30 μM, 10 μl i.t) and THDOC (THD; 30 μM, 10 μl i.t; Fig. 3A, ALL + RS and THD + RS), but not the vehicle solution (VEH + RS), inhibited RS-induced reflex potentiation. No statistical differences were seen in mean spike count at 30 min after stimulation onset between RS and RS + VEH (Fig. 3C, RS and RS + VEH, respectively; P > 0.05, n = 7). Progesterone, allopregnanolone, and THDOC all decreased RS-evoked spike count when compared with vehicle (PRG + RS, ALL + RS, THD + RS, and VEH + RS, respectively; ###P < 0.01, RS + VEH, n = 7).

**GABAAR agonist and antagonist.** We next explored GABAAR involvement in progesterone-dependent inhibition of reflex potentiation by intrathecally injecting a GABAAR antagonist before progesterone/neurosteroid administration. While exhibiting no effects on reflex potentiation (data not shown), intrathecal bicuculline (BIC; 10 μM, 10 μl before progesterone, allopregnanolone, or THDOC injection), a GABAAR antagonist, prevented inhibition of the RS-induced reflex potentiation caused by progesterone (Fig. 3A, BIC + PRG + RS), allopregnanolone (BIC + ALL + RS), and THDOC (BIC + THD + RS). When compared with progesterone/neurosteroid-treated animals, mean spike counts at 30 min after stimulation onset were increased significantly by bicuculline with progesterone (Fig. 3C, BIC + PRG + RS, ###P < 0.01, PRG + RS, n = 7), allopregnanolone (BIC + ALL + RS, ###P < 0.01, ALL + RS, n = 7), and THDOC (BIC + THD + RS, ###P < 0.01, THD + RS, n = 7). We further elucidated the role of GABAAR in RS-induced reflex potentiation through intrathecal injection of THIP (10 μM, 10 μl), a GABAAR agonist. THIP pretreatment at 1 min before stimulation onset inhibited RS-induced reflex potentiation (Fig. 3B, THIP + RS) by decreasing mean spike count at 30 min after RS onset (Fig. 3D, THIP + RS, ###P < 0.01 to RS, n = 7).

**DISCUSSION**

A recent in vivo study that investigated activity-dependent neural plasticity demonstrated that through the action of its 5α-reduced metabolites on GABAAR, chronic progesterone administration attenuates SRP induction (36). Our results are in line with this notion that progesterone and its neurosteroid metabolites may impact neural plasticity by affecting GABAergic neurotransmission. Our data further suggest that progesterone and its neurosteroid metabolites acutely modulate GABAAR-dependent inhibition, thus abolishing activity-dependent reflex plasticity induction at the spinal cord level. We found that acute intrathecal progesterone or its neurosteroid metabolites, including allopregnanolone and THDOC, inhibit SRP induction within minutes after administration. We also observed that intrathecal pretreatment with bicuculline, a GABAAR antagonist, prevents said SRP abolition. Acute intrathecal administration of THIP, a GABAAR-selective agonist, also produces acute inhibition of SRP induction in a similar manner to that done by acute progesterone injection. Together, these findings suggested that acute inhibitory modulation of activity-dependent neural plasticity at the spinal cord level can be caused by progesterone through the action of its 5α-reduced metabolites on spinal GABAAR.

Progesterone can bind to intracellular receptors that act as transcription factors and regulate gene expression. On the other hand, progesterone and its neurosteroid metabolites can also act at an array of neurotransmitter receptors, including GABAAR as well as NMDA, AMPA, kainite, glycine, serotonin, sigma-1, nicotinic and muscarinic acetylcholine receptors through rapid nongenomic effects (10, 41, 45). The current study...
demonstrates SRP inhibition caused by progesterone, allopregnanolone, and THDOC at a few minutes after administration. Therefore, we propose that the progesterone-dependent inhibition on SRP is too rapid to be explained by genomic transcription, i.e., nongenomic pathways would account for such a fast onset.

In brain areas such as the cerebellum (21), hippocampus (40), and visual cortex (42), GABAergic inhibitory synapses play substantial roles in the modulation of NMDA-dependent forms of neural plasticity. Our previous studies showed that SRP induction depends on postsynaptic glutamatergic NMDA receptor activation in the spinal dorsal horn (3–5, 7). In the present study, acute inhibition of NMDA-dependent SRP caused by progesterone, allopregnanolone, or THDOC was prevented by bicuculline, a GABAAR antagonist. These results are in line with studies suggesting that GABAAR-mediated inhibition is pivotal in regulating NMDA-dependent neural plasticity, such as hippocampal long-term potentiation (40, 48) and the spinal windup phenomenon (9).

Although there are well-established model systems appropriate for animal studies investigating somatic pain, such as the tail flick and hot plate test. To develop animal models for studying specific mechanisms of visceral pain are worth pursuing because it seems involves mechanisms that have yet to be clarified (16). Our laboratory recently demonstrated activation of nociceptive afferent fibers expressing TRPV/TRPA after instillation of capsaicin or mustard oil into the pelvic organs, including the uterus (36, 38) and descending colon (38), inducing acute reflex sensitization. Despite such an animal model does not fit well to clinical scenarios that visceral pain is usually treated in subacute/chronic patients, in vivo animal studies demonstrated activation of TRPV1/TRPA1 expressing nociceptive afferent fibers innervating the pelvic viscera facilitated SRP (35, 38). We propose that at the spinal cord level,
prolonged sensitization of reflex circuitry might lead to enhanced neural activity that might, at least in part, underlie the development of visceral pain (36). In the current study, we demonstrate attenuated reflex potentiation after acute neurosteroid administration. Since nowadays, a number of synthetic neuroactive steroids that share their endogenous counterparts' characteristics in modulating neuronal activity have been developed (27). We suggest that these synthetic neurosteroids, such as alphaxalone and steroid-3α-hydroxy-5β-pregnan-20-one hemisuccinate, may be possible candidates for clinical visceral pain/hyperalgesia treatment.

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DISCLOSURES
No conflicts of interest are declared by the authors.

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