Proc. Natl. Acad. Sci. USA
Vol. 92, pp. 3774–3778, April 1995
Neurobiology

Neurosteroids, via σ receptors, modulate the [^{3}H]norepinephrine release evoked by *N*-methyl-D-aspartate in the rat hippocampus

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Contributed by Etienne-Emile Baulieu, December 27, 1994

ABSTRACT ***N*-Methyl-D-aspartate (NMDA, 200 μM) evokes the release of [^{3}H]norepinephrine ([^{3}H]NE) from preloaded hippocampal slices. This effect is potentiated by dehydroepiandrosterone sulfate (DHEA S), whereas it is inhibited by pregnenolone sulfate (PREG S) and the high-affinity σ inverse agonist 1,3-di(2-tolyl)guanidine, at concentrations of $\geq$100 nM. Neither 3α-hydroxy-5α-pregnan-20-one nor its sulfate ester modified NMDA-evoked [^{3}H]NE overflow. The σ antagonists haloperidol and 1-[2-(3,4-dichlorophenyl)-ethyl]-4-methylpiperazine, although inactive by themselves, completely prevented the effects of DHEA S, PREG S, and 1,3-di(2-tolyl)guanidine on NMDA-evoked [^{3}H]NE release. Progesterone (100 nM) mimicked the antagonistic effect of haloperidol and 1-[2-(3,4-dichlorophenyl)ethyl]-4-methyl-piperazine. These results indicate that the tested steroid sulfate esters differentially affected the NMDA response *in vitro* and suggest that DHEA S acts as a σ agonist, that PREG S acts as a σ inverse agonist, and that progesterone may act as a σ antagonist. Pertussis toxin, which inactivates the $G_{i/o}$ types of guanine nucleotide-binding protein ($G_{i/o}$ protein) function, suppresses both effects of DHEA S and PREG S. Since σ_1 but not σ_2 receptors are coupled to $G_{i/o}$ proteins, the present results suggest that DHEA S and PREG S control the NMDA response via σ_1 receptors.**

Overlooked for a long time, the physiological relevance of σ receptors has recently attracted attention since selective σ ligands have been documented as neuromodulators in the mammalian central nervous system (CNS; ref. 1). Their capacity to modulate *N*-methyl-D-aspartate (NMDA)-mediated glutamatergic neurotransmission (2–13) has been emphasized and proposed to play a crucial role in major neuroadaptative phenomena, such as long-term potentiation, learning and memory, seizures, acute neuronal death, and neurodegeneration (14). Using the *in vitro* model of NMDA-evoked [^{3}H]norepinephrine ([^{3}H]NE) release from preloaded rat hippocampal slices (15, 16), we have shown that the selective σ ligands (+)-*N*-cyclopropylmethyl-*N*-methyl-1,4-diphenyl-1-ethylbut-3-en-1-ylamine hydrochloride (JO-1784; ref. 17) and 1,3-di(2-tolyl)guanidine (DTG; ref. 18) potentiated and inhibited, respectively, the NMDA response in a concentration-dependent manner (3). Haloperidol, which also displays high affinity for σ binding sites (19, 20) but not spiperone, another butyrophenone devoid of such affinity (21, 22), prevented the effects of JO-1784 and DTG (3).

Pregnenolone (PREG), its sulfate ester PREG S, and progesterone (PROG), testosterone, and estradiol-17β have been reported to displace, under equilibrium binding conditions, both [^{3}H](+)*N*-allylnormetazocine {[^{3}H](+)SKF-10,047} and [^{3}H]haloperidol from the σ sites on rat brain (23) and splenocyte plasma membranes (24). However, these observations have not been further substantiated by functional bioassays. The present study was therefore carried out to evaluate whether neurosteroids modulate NMDA-evoked [^{3}H]NE overflow via action on σ receptors.

MATERIALS AND METHODS

The following drugs were used: NMDA (Sigma), DTG (Aldrich), haloperidol (McNeil Laboratories), spiperone (Research Biochemicals, Natick, MA), and pertussis toxin (PTX; Sigma). 1-[2-(3,4-Dichlorophenyl)ethyl]-4-methylpiperazine (BD-1063) was kindly provided by W. D. Bowen (Laboratory of Medicinal Chemistry, National Institutes of Diabetes and Digestive and Kidney Diseases, Bethesda, MD). The steroids were kindly provided by D. Philibert (Roussel-UCLAF). [^{3}H]NE (3,4-[7-^{3}H]NE; specific activity, 540.2 GBq/mmol/liter) was purchased from Isotopchim (Ganagobie-Peyruis, France).

Eighty-four female Sprague–Dawley rats (180–225 g), purchased from Iffa Credo, were kept at 21°C on a 12 hr:12 hr light/dark cycle with free access to water and Purina chow. At least 4 weeks prior to the release experiments, rats were anesthetized under ether and bilateral ovariectomy was carried out by lateral access. In a subgroup of 16 rats, anesthetized with chloral hydrate (400 mg/kg, i.p.) 3–11 days prior to the experiments, PTX (1 μg/2 μl of physiological saline) was injected (using a 10-μl Hamilton syringe) bilaterally into the dorsal hippocampus at A: 4.5, L: 4, and D: 4, according to the atlas of Paxinos and Watson (25) as described (3). Twelve control rats received an equal volume of the vehicle. When appropriate, the rats were killed by decapitation and their brains were rapidly dissected. Coronal slices (0.4-mm thick) of the hippocampus were prepared with a McIlwain tissue chopper. The slices were incubated in Krebs' solution containing [^{3}H]NE (0.1 μM) and bubbled with a mixture of 95% O_2/5% CO_2 at 37°C for 30 min. The composition of the Krebs' solution (in mM) was NaCl 118, KCl 4.7, $CaCl_2$ 1.3, $MgCl_2$ 1.2, NaH_2PO_4 1, $NaHCO_3$ 25, glucose 11.1, Na_2EDTA 0.04, and ascorbic acid 0.06. At the end of the incubation period, each glass chamber received two slices that were superfused continuously at a rate of 0.5 ml/min with oxygenated Mg^{2+}-free Krebs' solution at 37°C for 68 min. As indicated in *Results*, one steroid and/or one of the σ ligands DTG, haloperidol, BD-1063, or spiperone were added in Mg^{2+}-free Krebs' solution throughout the superfusion period. The prototypic σ ligand DTG was chosen since it acts on both σ_1 and σ_2 receptors (3, 26). The universal σ antagonist haloperidol also binds to dopaminergic, serotoninergic, adrenergic, and cholinergic sites

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Abbreviations: 3α,5α-THP, 3α-hydroxy-5α-pregnan-20-one; CNS, central nervous system; DHEA, dehydroepiandrosterone; DTG, 1,3-di(2-tolyl)guanidine; $G_{i/o}$ protein, guanine nucleotide-binding protein; (+)SKF-10,047, (+)-*N*-allylnormetazocine; JO-1784, (+)-*N*-cyclopropylmethyl-*N*-methyl-1,4-diphenyl-1-ethylbut-3-en-1-ylamine hydrochloride; NMDA, *N*-methyl-D-aspartate; BD-1063, 1-[2-(3,4-dichlorophenyl)ethyl]-4-methylpiperazine; [^{3}H]NE, [^{3}H]norepinephrine; PTX, pertussis toxin; PREG, pregnenolone; PROG, progesterone; S, sulfate ester.

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(21, 22). Therefore, spiperone, another butyrophenone that displays higher affinities for these former sites than haloperidol but has very low affinity for σ binding sites (1, 18, 20–22), and BD-1063, a very potent and selective σ antagonist (27), were used to assess the specificity of the results obtained with haloperidol. Stock solutions of steroids were prepared in ethanol and diluted with Mg^{2+}-free Krebs' solution so as to get the appropriate steroid concentration with a final ethanol concentration of <0.05%. Stock solutions of DTG, haloperidol, BD-1063, and spiperone were prepared in HCl and diluted with Mg^{2+}-free Krebs' solution to the final σ concentration needed, whereas the HCl concentration was kept <0.01%. Forty minutes after the beginning of the experiments, the superfusion medium was switched for 4 min to medium also containing NMDA (200 μM) to evoke [^{3}H]NE efflux. Ten successive 4-min fractions of the superfusate were collected starting 12 min before the NMDA superfusion. At the end of all experiments, the hippocampal slices were dissolved in 0.5 ml of Soluene 350 (Packard Instruments, Rungis, France) and the radioactivity in the slices and superfusate samples was determined by liquid scintillation spectrometry (Packard Tri-Carb 4660). The expression "NMDA-evoked release of [^{3}H]NE" will be used throughout instead of "NMDA (200 μM)-evoked overflow of ^{3}H from hippocampal slices preloaded with [^{3}H]NE." The NMDA-evoked [^{3}H]NE overflow was expressed as the increase in ^{3}H release over spontaneous outflow, determined immediately preceding NMDA superfusion, and expressed as percent of residual tissue ^{3}H content at the end of the superfusion. This method of calculation renders the amount of endogenous NE uninfluential (28). Results are expressed as mean ± SEM. The means were compared using the Student's *t* test and the Dunnett's correction for multiple comparisons with a single control group. Probability values of <0.05 were considered significant.

RESULTS

The fractional release evoked by NMDA (200 μM) from hippocampal slices labeled with [^{3}H]NE varied between 1.29% ± 0.14% (n = 8) and 1.71% ± 0.40% (n = 9) according to experiments (see Figs. 1–3). These values are consistent with results obtained with hippocampal (3, 6, 29) or cortical slices (30, 31). In the present work, none of the steroids, added to the Mg^{2+}-free Krebs' solution from the beginning of the superfusion in the 10 nM to 3 μM concentration range, affected the spontaneous efflux of ^{3}H.

Modulation of the NMDA-Evoked Overflow of [^{3}H]NE by Dehydroepiandrosterone Sulfate (DHEA S) and PREG S. DHEA S potentiated, in a concentration-dependent manner, the release of [^{3}H]NE induced by NMDA (Fig. 1*A*). The lowest effective concentration of DHEA S (30 nM) enhanced the response to NMDA by 37%. Conversely, PREG S inhibited the release of [^{3}H]NE induced by NMDA in a concentration-dependent manner (Fig. 1*B*). The lowest effective concentration of PREG S (100 nM) induced a 60% inhibition of the NMDA response.

Effect of Haloperidol, BD-1063, and Spiperone on the Modulatory Effect of DHEA S and PREG S. Haloperidol (100 nM), inactive by itself on the NMDA response, completely prevented the potentiating effect of DHEA S and the inhibitory effect of PREG S on NMDA-evoked [^{3}H]NE release (Fig. 1). In contrast, spiperone (100 nM) was inactive in suppressing both effects of DHEA S and PREG S (data not shown). Similarly to haloperidol, BD-1063 concentration-dependently prevented the enhancing effect of DHEA S (300 nM) as well as the inhibitory effect of PREG S (300 nM) (Fig. 2 *Inset*).

Effect of PROG on the Modulatory Effect of DHEA S, PREG S, and the σ Ligand DTG. PROG, DHEA, PREG, 3α-hydroxy-5α-pregnan-20-one (3α,5α-THP), and 3α,5α-THP S did not affect NMDA-evoked [^{3}H]NE release in the 10 nM to 1 μM concentration range. Among these five inactive steroids tested, PROG was the only one reported to displace σ ligands from their binding sites (23, 24, 32). This prompted us to investigate whether PROG might counteract the DHEA S-induced potentiation and the PREG S-induced inhibition of NMDA-evoked [^{3}H]NE overflow. Indeed, PROG concentration-dependently inhibited (in the 10 nM to 1 μM range) the potentiation and the inhibition of NMDA-evoked [^{3}H]NE release induced by DHEA S (300 nM) and PREG S (300 nM), respectively (Fig. 2). At 100 nM, PROG decreased the enhancing effect of DHEA S by 69% and abolished the reducing effect of PREG S.

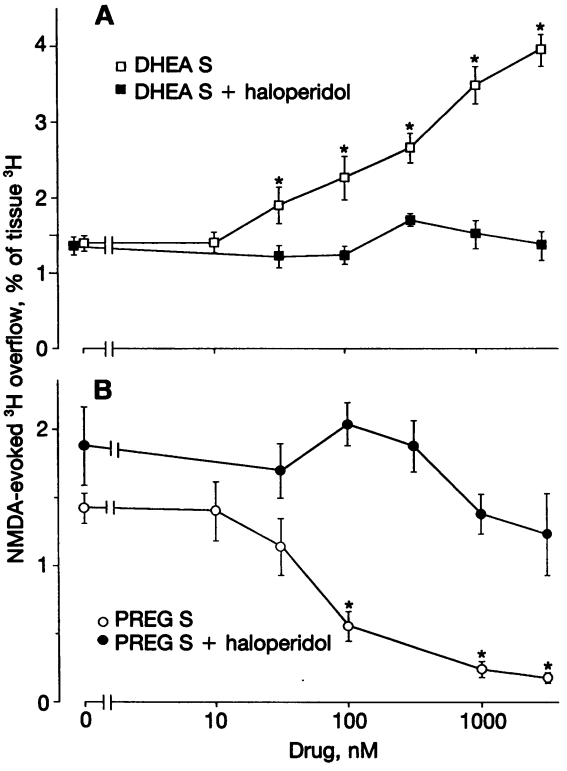

FIG. 1. Effects of DHEA S (*A*) and of PREG S (*B*) without and with haloperidol (100 nM) on NMDA-evoked release of [^{3}H]NE. Hippocampal slices of spayed rats were preincubated with [^{3}H]NE with carboxygenerated Mg^{2+}-free Krebs' medium maintained at 37°C. NMDA (200 μM) was added to the superfusate for 4 min after 40 min. Each point represents the mean ± SEM of six to nine experiments. *, $P < 0.01$; Student's *t* test vs. basal values.

DTG has previously been reported to decrease NMDA-evoked [^{3}H]NE release (3). PROG concentration-dependently reversed (in the 30 nM to 1 μM concentration range) DTG (300 nM)-induced inhibition of NMDA-evoked [^{3}H]NE release (Fig. 2).

Effect of PTX on DHEA S- and PREG S-Induced Modulation of the NMDA-Evoked Release of [^{3}H]NE. The pretreatment with PTX, injected in the dorsal hippocampus 3–11 days prior to sacrifice, according to a previously published protocol (3), affected neither the basal [^{3}H]NE outflow nor the NMDA-evoked release of [^{3}H]NE (Fig. 3). However, PTX totally abolished the effects of DHEA S and PREG S on NMDA-evoked release of [^{3}H]NE (Fig. 3).

DISCUSSION

The present results indicate that none of the steroids tested affected the spontaneous [^{3}H]NE efflux. However, at nanomolar concentrations, DHEA S increased, whereas PREG S reduced, the response to NMDA, while 3α,5α-THP S remained inactive on the NMDA response as well as the other

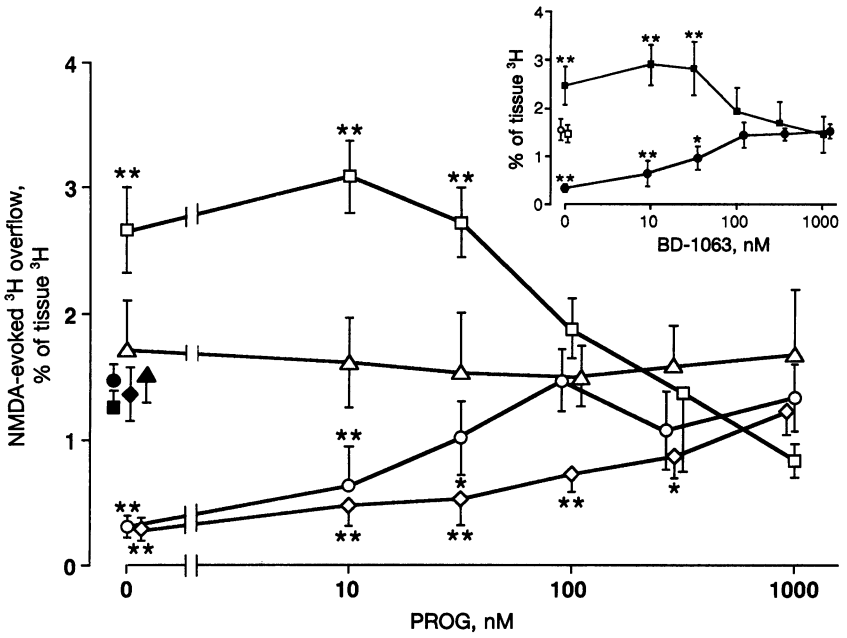

FIG. 2. PROG counteracts PREG S (300 nM)- and DTG (300 nM)-induced inhibition and DHEA S (300 nM)-induced potentiation of NMDA (200 μM)-evoked [^{3}H]NE overflow. Triangles, PROG alone; squares, DHEA S plus competitor; circles, PREG S plus competitor; diamonds, DTG plus PROG. Solid symbols, basal values of NMDA (200 μM)-evoked [^{3}H]NE overflow in the absence of any neurosteroid and DTG; open symbols, values of NMDA-evoked [^{3}H]NE overflow in the presence of various concentrations of PROG alone or in combination with DHEA S, PREG S, or DTG. (*Inset*) BD-1063 counteracts DHEA S (300 nM)- and PREG S (300 nM)-induced potentiation and inhibition of NMDA-evoked release of [^{3}H]NE. Open symbols, basal values of NMDA-evoked [^{3}H]NE overflow in the absence of any neurosteroid and BD-1063; solid symbols, values of NMDA-evoked [^{3}H]NE overflow in the presence of various concentrations of BD-1063 in combination with DHEA S or PREG S. See the legend to Fig. 1 for experimental details. Each point represents the mean ± SEM of seven to nine experiments. *, $P < 0.05$, and **, $P < 0.01$; Student's *t* test vs. basal values.

nonsulfated steroids tested. The modulatory effects of both steroids were suppressed by the high-affinity σ ligands haloperidol and BD-1063 as well as by PROG. Finally, PTX pretreatment, which inactivates the $G_{i/o}$ types of guanine nucleotide-binding proteins ($G_{i/o}$ proteins), prevented both DHEA S- and PREG S-induced effects.

Farb (33), using whole cell recordings from voltage-clamped spinal chord neurons, observed no enhancing effect of DHEA S (at concentrations up to 10 μM) on the basal transmembrane potential and spontaneous firing activity. This is consistent with the present observations. He also observed no modulatory effect of DHEA S on the neuronal response to NMDA. However, Meyer and Gruol (34) showed that DHEA S, in the 10–100 μM concentration range, weakly facilitated the activation of CA_1 neurons in hippocampal slices after stimulation of the Schaffer collaterals. The increase of NMDA-evoked [^{3}H]NE overflow by DHEA S in the present *in vitro* paradigm would seem consistent with the latter findings. In the light of the data of Farb (33) on isolated neurons, the present data obtained with hippocampal slices point to an indirect effect of DHEA S on the NMDA response. Although, to our knowledge, the affinity of DHEA S for σ binding sites has not been assessed, the effect of BD-1063 and haloperidol, which interact with the σ binding sites but not with the NMDA receptor (2, 26, 35, 36) on DHEA S-induced potentiation of NMDA-evoked [^{3}H]NE overflow, indicates that DHEA S most likely acted on σ receptors (Figs. 1 and 2). Spiperone, which shares the widespread binding properties of haloperidol but displays very low affinity for σ binding sites (1, 18, 20–22), failed to suppress the potentiation of the NMDA-evoked [^{3}H]NE overflow by DHEA S, thus supporting the contention that DHEA S modulated the NMDA response via σ receptors.

The prototypic neurosteroids PREG and PREG S (37–39) have no effect on spontaneous firing of hippocampal neurons (40–43), in accordance with our present observations. However, at micromolar concentrations, PREG S allosterically potentiates NMDA-evoked currents in rat hippocampal neurons in culture (40, 41, 43, 44). The latter reports apparently contradict the present results showing that PREG S decreased the response of hippocampal slices to NMDA. Accordingly, Mathis *et al.* (45) have recently shown in a behavioral model that PREG S (0.84–840 pmol, intracerebroventricularly) also concentration-dependently blocked the NMDA response. However, in our experiments, in the presence of haloperidol (100 nM), PREG S tended to enhance (in the 0.1–1 μM concentration range) the NMDA-evoked [^{3}H]NE overflow (Fig. 1*B*) and induced a robust potentiation of the NMDA response following $G_{i/o}$ protein inactivation (Fig. 3*B*). Previous reports have shown that PREG S displaces radiolabeled σ probes (23, 24, 32). Hence, the present inhibitory effect of PREG S most likely corresponds to an indirect σ receptor-mediated modulation of the NMDA response since haloperidol and BD-1063 blocked PREG S-mediated inhibition of the NMDA-evoked [^{3}H]NE overflow. Thus, PREG S would exert two opposite effects on NMDA-induced neuronal activation: the direct potentiation of NMDA–receptor interaction and an indirect σ-mediated inhibition of the NMDA response, which seems to predominate under physiological conditions.

The identification of at least in part opposite effects of the sulfate esters on the NMDA response markedly contrasts with the general opinion that DHEA S and PREG S exhibit similar antagonistic properties on the γ-aminobutyric acid ($GABA_A$) receptor function (42). In fact, Majewska and coworkers (46–49) have previously stated that DHEA S and PREG S behave differently in their capability to displace $GABA_A$ receptor-associated [^{35}S]butylbicyclophosphorothionate binding. Nevertheless, the present *in vitro* release paradigm of NMDA-evoked [^{3}H]NE overflow from preloaded rat hippocampal slices constitutes a relevant approach to discriminate easily the effects of DHEA S, PREG S, and 3α,5α-THP S and allows us to identify precisely their profile of action on the NMDA response.

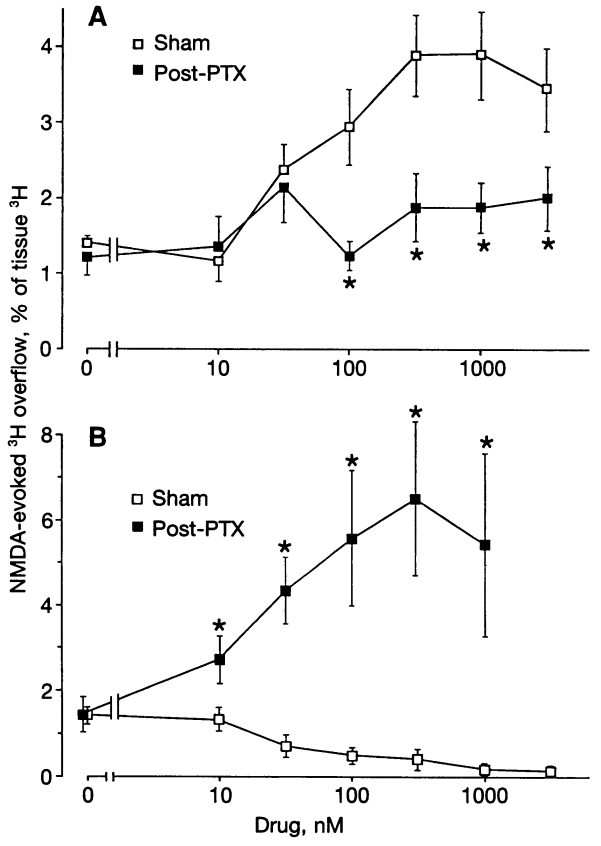

FIG. 3. PTX blocks the effect of DHEA S (*A*) and reverses that of PREG S (*B*) on the release of [^{3}H]NE evoked by NMDA (200 μM). PTX (1 μg) was stereotaxically injected in the hippocampus 3–11 days prior to the experiments. The neurosteroid was added from the beginning of the superfusion. See the legend to Fig. 1 for experimental details. Each point represents the mean ± SEM of six to eight experiments. *, $P < 0.01$; Student's *t* test vs. basal values.

PROG, although inactive by itself, prevented the effects of PREG S, DTG, and DHEA S on the NMDA response (Fig. 2), as did the σ antagonists haloperidol and BD-1063 (Figs. 1 and 2). PROG has been proposed as a potential endogeneous ligand for σ sites (23, 24, 32). Consistent with these and our previous observations, the σ-mediated antagonist-like activity of PROG is supported by *in vivo* experiments showing that stereotaxically administered, PROG, inactive on NMDA-induced neuronal activation, counteracts the DTG-induced modulation of the response of CA_3 hippocampal pyramidal neurons to NMDA (50).

PTX, a blocker of the $G_{i/o}$ types of G proteins (51), has been shown to inhibit the binding of several σ probes (52) as well as the DTG-induced decrease of NMDA-evoked [^{3}H]NE overflow (3). Since PTX pretreatment totally abolished the DHEA S-mediated potentiation of the NMDA-evoked [^{3}H]NE overflow (Fig. 3), it is likely that the modulation of the NMDA response by DHEA S involved σ_1 receptors, which, unlike σ_2 receptors, are coupled to $G_{i/o}$ proteins (26). Accordingly, we also assume that PREG S inhibited the NMDA-induced neuronal activation by acting on σ_1 receptors.

In conclusion, we have shown that DHEA S, PREG S, and PROG affect the neuronal excitability induced by NMDA via three types of interactions: DHEA S acts as a σ agonist, PREG S acts as a σ inverse agonist, and PROG acts as a σ antagonist. This further supports the notion that an important physiological role of neurosteroids might be to modulate the NMDA receptors.

We are grateful to Dr. M. Th. Bluet-Pajot, Prof. R. Fain, and M. Pourprix for their help in stereotaxic procedures, to Dr. W. D. Bowen for BD-1063, and to J. C. Lambert for the illustrations. This work was supported by the Fondation de France and the Ministère de la Recherche–Direction de la Recherche et des Etudes Doctorales (Paris, France; Grant R1/RS2266).

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