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Neurosteroids Enhance Spontaneous Glutamate Release in Hippocampal Neurons

POSSIBLE ROLE OF METABOTROPIC σ_1-LIKE RECEPTORS*

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Pregnenolone sulfate (PREGS), one of the most abundantly produced neurosteroids in the mammalian brain, improves cognitive performance in rodents. The mechanism of this effect has been attributed to its allosteric modulatory actions on glutamate- and γ-aminobutyric acid-gated ion channels. Here we report a novel effect of PREGS that could also mediate some of its actions in the nervous system. We found that PREGS induces a robust potentiation of the frequency but not the amplitude of miniature excitatory postsynaptic currents (mEPSCs) mediated by α-amino-3-hydroxy-5-methylisoxazole-4-propionate receptors in cultured hippocampal neurons. PREGS also decreased paired pulse facilitation of autaptic EPSCs evoked by depolarization, indicating that it modulates glutamate release probability presynaptically. PREGS potentiation of mEPSCs was mimicked by dehydroepiandrosterone sulfate and (+)-pentazocine but not by (−)-pentazocine, the synthetic (−)-enantiomer of PREGS or the inactive steroid isopregnanolone. The σ receptor antagonists, haloperidol and BD-1063, blocked the effect of PREGS on mEPSCs, as did pertussis toxin and the membrane-permeable Ca^{2+} chelator 1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid (acetoxymethyl) ester. These results suggest that PREGS increases spontaneous glutamate release via activation of a presynaptic $G_{i/o}$-coupled σ receptor and an elevation in intracellular Ca^{2+} levels. We postulate that presynaptic actions of neurosteroids have a role in the maturation and/or maintenance of synaptic networks and the processing of information in the central nervous system.

In the 1980s, Baulieu and collaborators (reviewed in Ref. 1) made the important discovery that certain steroids are synthesized in the central and peripheral nervous systems. These compounds, known as neurosteroids, are produced locally in glial and neuronal cells and can exert important modulatory actions in the nervous system. A particularly abundant neurosteroid in the central nervous system is pregnenolone sulfate (PREGS)[1] (1, 2). Although the neurophysiological role of endogenous PREGS has yet to be conclusively established, experiments involving exogenous administration of this compound suggest that it has a promnesic effect (3–7). For example, post-training injection of PREGS into the hippocampus and amygdala of mice improves retention for foot shock active avoidance training (4). In addition, low levels of PREGS in the hippocampus were found to be correlated with a deficiency in cognitive performance in aged rats, which could be ameliorated by intrahippocampal injections of this neurosteroid (5). More recently, it was demonstrated that PREGS attenuates amyloid peptide-induced amnesia in mice (8). Thus, PREGS or its analogs could potentially be used for the treatment of Alzheimer's disease and other neuropsychiatric disorders.

Although the mechanisms by which PREGS produces cognitive effects are not fully understood, numerous studies suggest that this agent modulates several neuronal ion channels (9). For instance, PREGS has been shown to inhibit γ-aminobutyric acid-type A ($GABA_A$) receptors (10), to potentiate *N*-methyl-D-aspartate (NMDA) receptors (11–14), and to inhibit voltage-gated Ca^{2+} channels (15). In addition to ion channels, PREGS has also been shown to target metabotropic receptors, such as σ receptors. These receptors were initially thought to belong to the opioid family of receptors, but they are now categorized separately (16). Two classes of pharmacologically defined σ receptors are widely accepted and are denoted as the σ_1 and σ_2 subtypes (16). σ_1 receptors bind (+)-benzomorphans and haloperidol with high affinity. In contrast, σ_2 receptors bind haloperidol and (+)-benzomorphans with low affinity, and they also bind benzomorphans without enantioselectivity.

σ ligand binding sites can be detected both intracellularly on the endoplasmic reticulum (ER) and extracellularly on the plasma membrane (17). A σ ligand-binding protein has recently been cloned from a number of tissues, including brain (18). This binding protein exhibits the pharmacological profile of σ_1 receptors and is expressed in the ER (19–23). Although its sequence has no known homology to other mammalian proteins, it shows similarity to the yeast sterol C_8-C_7 isomerase involved in ergosterol biosynthesis (24). Plasma membrane σ receptors

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[1] The abbreviations used are: PREGS, pregnenolone sulfate; GABA, γ-aminobutyric acid; NMDA, *N*-methyl-D-aspartate; NMDAR, NMDA receptor; ER, endoplasmic reticulum; mEPSC, miniature excitatory postsynaptic current; PPF, paired pulse facilitation; EPSC, excitatory postsynaptic current; BAPTA-AM, 1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid (acetoxymethyl) ester; ent-PREGS, (−)-enantiomer of PREGS; AMPAR, α-amino-3-hydroxy-5-methylisoxazole-4-propionate receptor; GTPγS, guanosine 5′-3-*O*-(thio)triphosphate; PLC, phospholipase C; IP_3R, inositol 1,4,5-trisphosphate receptor.

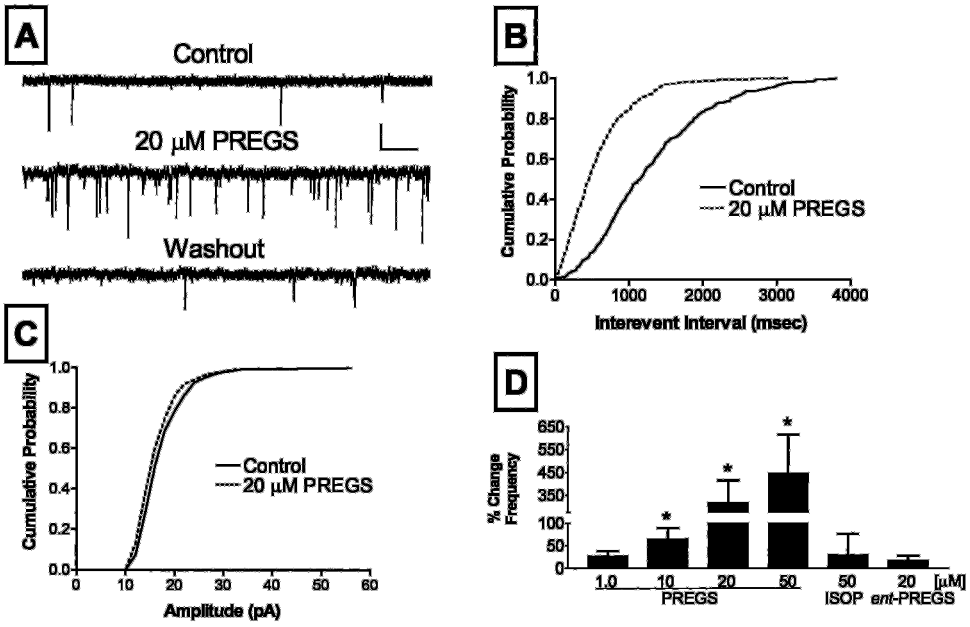

FIG. 1. **Modulation of glutamate release by PREGS.** *A*, sample traces of mEPSC recordings obtained before (*Control*), during administration of PREGS (20 μM), and after washout from a single representative neuron (*scale bars*, 41 pA and 256 ms). *B*, average cumulative probability histograms of control and PREGS (20 μM) interevent intervals from three neurons (error bars have been removed for clarity). Control plots were obtained by averaging base-line and washout data for each of the three neurons. Interevent intervals were significantly reduced by application of PREGS ($p < 0.001$; Kolmogorov-Smirnov two-sample test). *C*, average cumulative probability histogram for the amplitude of the same neurons described for *B*. Amplitude distribution was not significantly affected by PREGS treatment ($p > 0.05$; Kolmogorov-Smirnov two-sample test). Event detection threshold was set at 10 pA, and error bars have also been removed for clarity. *D*, PREGS enhancement of mEPSC frequency is dose-dependent at concentrations between 1 and 50 μM ($n = 5$–10 neurons/group; *, $p < 0.05$ by unpaired *t* test *versus* theoretical mean of zero; one-way analysis of variance yielded a $p < 0.03$; see "Results" for mEPSC amplitude data). Also illustrated in this *panel i*s the lack of an effect of the (−)-enantiomer of PREGS (*ent*-PREGS; $n = 4$) and the inactive steroid isopregnanolone (*ISOP*; $n = 8$) on mEPSC frequency.

can be activated by PREGS and other neurosteroids but, in contrast to σ ligand binding proteins expressed in the ER, appear to be directly coupled to pertussis-sensitive G proteins (25–28). Both the cDNA sequence and physiological role of these G protein-coupled metabotropic σ receptors have yet to be fully characterized.

In this paper, we report a novel effect of PREGS on glutamate release that depends on activation of plasma membrane σ receptors. Specifically, we measured the effects of this neurosteroid on miniature excitatory postsynaptic currents (mEPSCs) mediated by the α-amino-3-hydroxy-5-methylisoxazole-4-propionate subtype of ionotropic glutamate receptors. Miniature synaptic currents are the most elementary forms of synaptic transmission, representing the postsynaptic responses to action potential-independent spontaneous release of single presynaptic vesicles. It is well established that when a modulator affects presynaptic neurotransmitter release, it produces a change in the frequency but not in the amplitude of miniature synaptic events (for instance, see Ref. 29). We found that PREGS selectively induces a robust increase in the frequency of mEPSCs, indicating that it enhances the probability of glutamate release from presynaptic terminals. Moreover, we show that this effect depends on an elevation in intracellular Ca^{2+} levels triggered by activation of presynaptic $G_{i/o}$ protein-coupled σ_1-like receptors.

EXPERIMENTAL PROCEDURES

Cell Culture—Animal procedures were approved by the Institutional Animal Care and Use Committee of the University of New Mexico Health Sciences Center and conform to National Institutes of Health guidelines. Neuronal cultures were prepared in all cases from postnatal day 3–4 Sprague-Dawley rats. These experiments utilized either mixed hippocampal cell cultures (prepared as described previously (30)) or autaptic neuronal cultures grown on glial cells attached to collagen-on-agarose microislands (prepared as described elsewhere (31)). Neurons grown on microislands were used for the studies of paired pulse facilitation (PPF). Neurons were used for electrophysiological experiments 8–14 days after culture.

Electrophysiology—Whole-cell patch clamp experiments were performed using instrumentation and software previously described (30), with the exception that mEPSCs were first recorded on digital audiotape and then digitized by using a Digidata 1200 interface and pClamp 7 software (Axon Laboratories, Foster City, CA). Miniature EPSCs were analyzed using the Mini Analysis Program from Synaptosoft (Decatur, GA). We recorded from pyramidal-like neurons that had large somas and well defined dendritic processes. Neurons were clamped at −70 mV for most experiments and, when indicated, at −90 mV. The external solution contained 130 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM Hepes, 11 mM glucose, and 0.02 mM bicuculine methiodide (pH 7.4, ~320 mosmol). For mEPSC recordings, this solution also contained 600 nM tetrodotoxin. For some recordings of autaptic currents, the concentration of Mg^{2+} was increased to 2 mM to favor PPF (32). For recordings of mEPSCs, the internal solution contained 5 mM CsCl, 140 mM $CsCH_3SO_3$, 10 mM EGTA, 10 mM Hepes, pH 7.4, ~300 mosmol. To record autaptic currents, the composition of the internal solution was 4 mM NaCl, 0.5 mM $CaCl_2$, 5 mM EGTA, 10 mM Hepes, 140 mM potassium gluconate, pH 7.25, ~280 mosmol. Patch pipette electrodes had resistances ranging from 3 to 7 megaohms. Autaptic EPSCs were generated by a 1.5- or 2-ms depolarizing pulse (from −70 mV to +20 mV); for studies of PPF, two pulses separated by 50 or 60 ms were delivered at a frequency of 0.05 Hz. Compounds were dissolved in Me_2SO before dilution into external solution, and equal volumes of Me_2SO were added to control external solutions. Me_2SO concentrations never exceeded 0.05%.

Chemicals—Tetrodotoxin and 6-cyano-7-nitroquinoxaline-2,3-dione were from Alexis Biochemicals (San Diego, CA); 1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid (acetoxymethyl) ester (BAPTA-AM) and pertussis toxin were from Calbiochem; PREGS was from Steraloids (Newport, RI); BD-1063 and DL-2-amino-5-phosphonovalerate were from Tocris (Ellisville, MO). Synthesis of the (−)-enantiomer of PREGS (*ent*-PREGS) has been described elsewhere (33). (+)-Pentazocine succinate was generously provided by Kevin Gormley (National Institute on Drug Abuse). All other chemicals were from Sigma or Fluka (St. Louis, MO).

Statistical Analysis—The effects of all compounds were quantified with respect to the average of control and washout responses. The

Kolmogorov-Smirnov test was used initially to test for significant differences between treatments in individual cells and to determine whether data followed a Gaussian distribution. Statistical comparisons of pooled data were performed by one-way analysis of variance followed by Bonferroni's *post hoc* test or by Student's *t* test. In all cases, a $p < 0.05$ was considered to indicate statistical significance. Statistical analyses were performed with the Mini Analysis program or Prism (GraphPad, San Diego, CA). Data are presented as mean ± S.E. in all cases.

RESULTS

We first measured the effect of PREGS on mEPSCs to determine whether it modulated spontaneous glutamate release. To isolate AMPAR-mediated events, we recorded mEPSCs at −70 mV in 1 mM Mg^{2+}-containing external solution. Under these conditions, 6-cyano-7-nitroquinoxaline-2,3-dione (20 μM) reduced the frequency of events by 96 ± 3% ($n = 7$) with respect to control (data not shown). As illustrated in Fig. 1, PREGS caused a robust, concentration-dependent, increase in mEPSC frequency. The effect of PREGS was significant at concentrations of ≥10 μM (Fig. 1*D*). The onset of this effect occurred within ~1 min after bath application of the neurosteroid and was fully reversible within ~2–4 min after washout. The increase in mEPSC frequency was observed at both −70 and −90 mV. At these membrane potentials, PREGS (50 μM) increased mEPSC frequency by 494 ± 184% ($n = 6$) and 488 ± 192% ($n = 7$), respectively (data not shown). This finding indicates that the effect of PREGS is not due simply to an increase in the detection of subthreshold mEPSCs. The effect of PREGS was also not due to NMDAR activation, because recording in Mg^{2+}-free external solution containing the NMDAR antagonist DL-2-amino-5-phosphonovalerate (50 μM) had no effect on the action of this neurosteroid. PREGS (50 μM) increased mEPSC frequency by 448 ± 151% ($n = 4$) or 485 ± 182% ($n = 9$) with respect to control in the presence of DL-2-amino-5-phosphonovalerate or Mg^{2+}-containing external solution, respectively (data not shown). We did not detect any effect of PREGS on mEPSC amplitude at any of the concentrations examined; mEPSC amplitudes were changed by 1.6 ± 2.8, 0.55 ± 3.6, −1.6 ± 4.4, and −1.1 ± 3.5% with respect to control in the presence of 1, 10, 20, and 50 μM PREGS, respectively (see Fig. 1*C* for an illustration of a lack of an effect of 20 μM PREGS). To eliminate the possibility of a nonspecific action and to determine the enantioselectivity of the PREGS effect, we tested the effect of the inactive neurosteroid, isopregnanolone, and the (−)-enantiomer of PREGS, *ent*-PREGS, respectively. As shown (Fig. 1*D*), these steroids did not induce a change in the frequency of mEPSCs. Since *ent*-PREGS has been shown to exert more potent inverted U-shaped effects than PREGS under some experimental conditions (6), we also tested its effect at a 1 μM concentration and found that it does not affect mEPSC frequency (2.5 ± 0.9% change with respect to control; $n = 3$).

To confirm that PREGS increases the probability of glutamate release, we measured its effect on PPF of autaptic AMPAR-mediated EPSCs evoked by depolarizing pulses (Fig. 2). It is well established that manipulations that enhance the basal probability of release increase the impact of the first action potential (*i.e.* deplete synaptic vesicles), resulting in a reduction in PPF (For an example, see Ref. 34). Accordingly, we found that treatment with PREGS (50 μM) increases the amplitude of the first EPSC by 43 ± 11% ($n = 9$; $p < 0.01$ by one-sample *t* test *versus* a theoretical mean of zero; Fig. 2*A*) and also reduces PPF (Fig. 2*B*), confirming that PREGS increases the probability of presynaptic glutamate release.

PREGS has been shown to inhibit NMDA receptor-dependent [^{3}H]norepinephrine release in hippocampal slices by a mechanism that involves σ_1 receptors (28). We therefore examined the role of these receptors in the effects of PREGS on glutamate release. As shown in Fig. 3, the σ_1 receptor antagonists, haloperidol and BD-1063 (preincubation for 30–45 min at 37 °C), blocked the PREGS-induced increase in mEPSC frequency. Moreover, pretreatment with haloperidol or BD1063 did not affect the average basal frequency of mEPSCs (Fig. 3, *B1* and *C1*). This finding indicates that the decrease in PREGS efficacy in the presence of these two compounds is not due to an overall decline in the spontaneous release probability. We next determined whether the effect of PREGS could be mimicked by other σ receptor agonists. We tested the effect of DHEAS, another neurosteroid that activates σ_1-like receptors in the brain (25), and of (+)-pentazocine, the prototypical σ_1 receptor agonist. As shown in Fig. 4, these compounds produced a similar increase in mEPSC frequency to that produced by PREGS (Fig. 1*D*). Conversely, (−)-pentazocine did not increase mEPSC frequency (Fig. 4*C*). None of these compounds significantly affected mEPSC amplitude (data not shown).

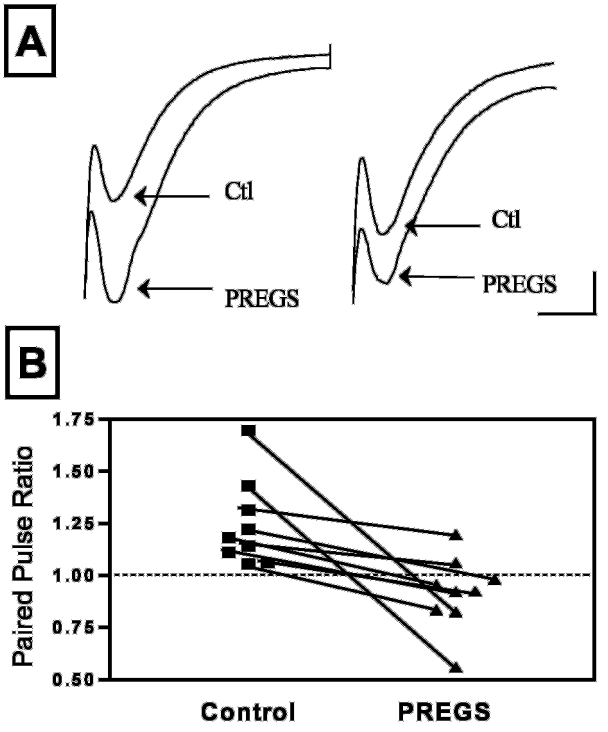

FIG. 2. **PREGS reduces paired pulse facilitation of autaptically induced EPSCs.** *A*, sample traces of paired EPSCs obtained before (*CTL*) and after application of PREGS (50 μM). The stimulus artifacts and sodium spikes are truncated. The interpulse interval was 60 ms (*scale bars*, 100 pA and 12 ms). Note that PREGS induces a large increase in amplitude of the first of the paired EPSCs and reduces the paired pulse ratio. *B*, summary of the effects of PREGS (50 μM) on the paired pulse ratio in nine neurons. The average ± S.E. of the paired pulse ratios were 1.25 ± 0.07 and 0.9 ± 0.06 in the absence and presence of 50 μM PREGS, respectively ($n = 9$; $p < 0.02$ by paired *t* test).

Plasma membrane σ receptors have been shown to be coupled to $G_{i/o}$ proteins (25–28). Therefore, we tested the effect of pertussis toxin treatment on the PREGS-induced increase in mEPSC frequency. As illustrated in Fig. 5, incubation of neurons for 36–48 h at 37 °C with 50 ng/ml pertussis toxin significantly reduced the effect of PREGS on mEPSC frequency. This result indicates that the effect of PREGS requires activation of the $G_{i/o}$ subtype of G proteins. As also shown, pretreatment with pertussis toxin did not affect basal frequency of mEPSCs (Fig. 5*B*). This suggests that the decrease in PREGS efficacy in the presence of pertussis toxin is not due to an overall decline in the spontaneous release probability and is most likely due to a direct effect of pertussis toxin on the PREGS-mediated second messenger cascades that results in increased spontaneous glutamate release.

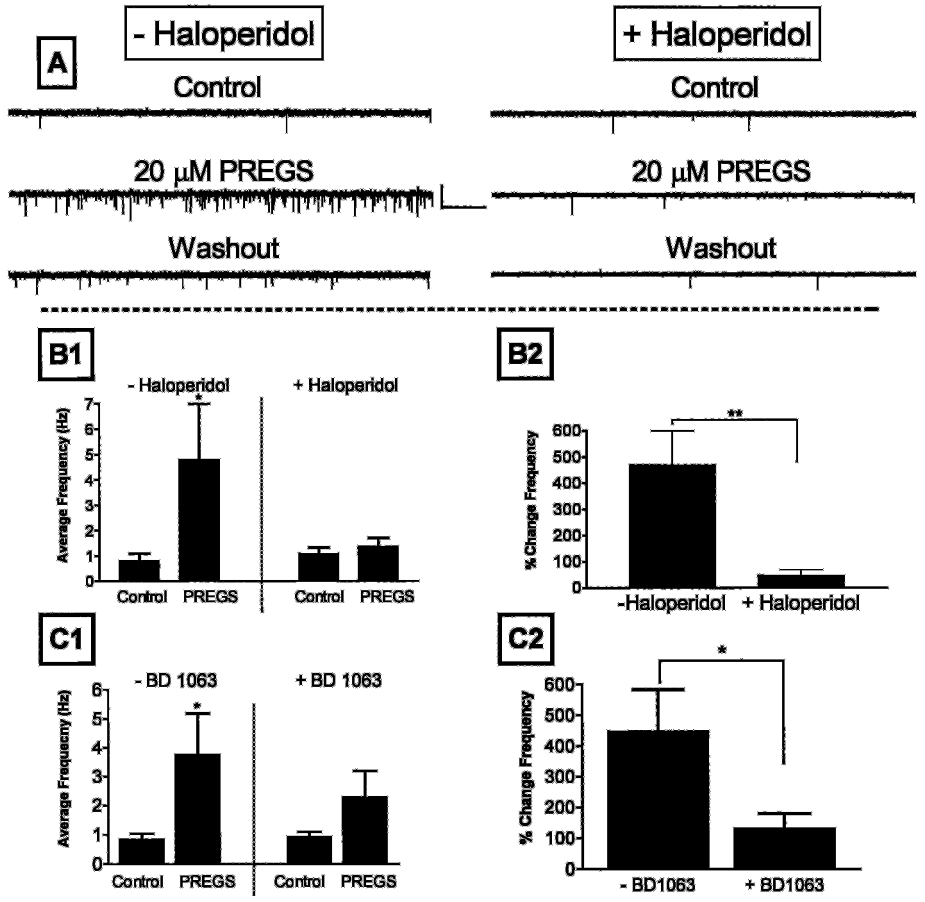

FIG. 3. **PREGS enhancement of mEPSC frequency is blocked by σ_1 receptor antagonists.** *A*, sample traces obtained from untreated (−*haloperidol*) and haloperidol (300 nM)-pretreated (+*haloperidol*) neurons obtained in the absence and presence of 20 μM PREGS and after washout (*scale bars*, 27 pA and 2.6 s). *B* and *C*, summary of the effect of pretreatment with 300 nM haloperidol and 1 μM BD-1063. Neurons were incubated with these inhibitors for 30–45 min at 37 °C. The concentration of PREGS was 20 μM in all cases. *B1*, combined plots of the average frequencies of mEPSCs under control and PREGS treatment conditions for untreated ($n = 8$) and haloperidol-treated ($n = 9$) neurons. Each *bar* represents the mean ± S.E. (*, $p < 0.05$ for control *versus* PREGS in the minus haloperidol group by paired *t* test). *B2*, average percentage change in mEPSC frequency for neurons shown in *B1*. The PREGS-induced percentage change in mEPSC frequency for each individual neuron was calculated with respect to the average of control and washout responses (**, $p < 0.01$ by unpaired *t* test). *C1*, combined plots of the average frequencies of mEPSCs under control and PREGS treatment for untreated (−BD1063; $n = 6$) and BD1063-treated (+BD1063; $n = 8$) neurons. Each *bar* represents the mean ± S.E. (*, $p < 0.05$ for control *versus* PREGS in the minus BD1063 group by paired *t* test). *C2*, average percentage change in mEPSC frequency for the neurons shown in *C1*. The PREGS-induced percentage change in mEPSC frequency for each individual neuron was calculated with respect to the average of control and washout responses (*, $p < 0.05$ by paired *t* test). For all of the experiments shown in this figure, treated neurons were from sister cultures prepared in parallel and used for experiments on the same day.

σ receptors have been shown to regulate intracellular calcium (16, 35); therefore, we examined its role in the mechanism of action of PREGS. The results of these experiments are shown in Fig. 6. As a control, neurons were first exposed to 50 μM PREGS, which produced the expected increase in mEPSC frequency. After washout, the same neurons were incubated for 15–20 min with the membrane-permeable Ca^{2+} chelator, BAPTA-AM (10 or 20 μM). A subsequent exposure to 50 μM PREGS failed to induce an elevation in mEPSC frequency (Fig. 6, *A* and *B*). This result indicates that an elevation in intracellular Ca^{2+} levels is required for the presynaptic actions of PREGS. To eliminate the possibility that the effect of BAPTA-AM was an artifact due to run down of the effect of PREGS, we applied PREGS twice under control conditions (Fig. 6*C*). As shown, the effect of a second application of PREGS closely reproduced that of the first application (same result seen in three additional neurons).

DISCUSSION

PREGS Increases Glutamate Release Probability—In this paper, we report that PREGS induces a robust increase in the frequency but not the amplitude of AMPAR-mediated mEPSCs in hippocampal neurons cultured from neonatal rats. Moreover, we found that PREGS reduces PPF of AMPAR-mediated synaptic responses, indicating that this neurosteroid increases the probability of glutamate release at the presynaptic level. To the best of our knowledge, this is the first report of a modulatory effect of PREGS on the basal probability of glutamate release in central nervous system neurons. It is noteworthy, however, that we previously found that PREGS exerts presynaptic modulatory actions on glutamatergic terminals in the rat hippocampus. Specifically, we demonstrated that PREGS enhances PPF of NMDAR- and AMPAR-mediated EPSPs in CA1 pyramidal neurons in hippocampal slices from adult rats (36). Importantly, we did not find evidence indicating that PREGS affects the basal probability of glutamate release in these slices (36). Thus, PREGS appears to exert distinct effects on glutamate release depending on either the neuronal developmental stage or the type of neuronal preparation being used (*i.e.* it increases the probability of spontaneous glutamate release in hippocampal neurons cultured from neonatal rats and increases facilitation of evoked glutamate release in CA1 pyramidal neurons in hippocampal slices from adult rats).

Our finding that PREGS affects glutamate release contributes to the growing evidence that this neurosteroid has impor-

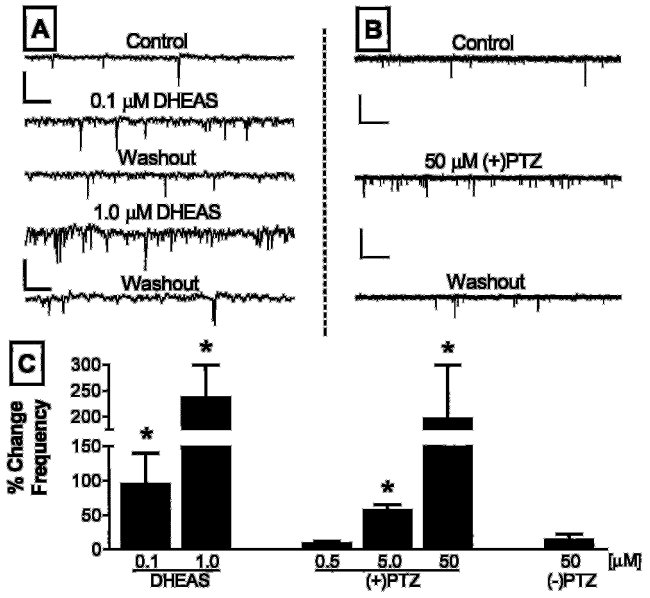

FIG. 4. **σ_1 receptor agonists DHEAS and (+)-pentazocine (*PTZ*) mimic the PREGS-dependent enhancement of mEPSC frequency.** *A*, sample traces of mEPSC recordings obtained before (*Control*) and during administration of DHEAS (0.1 and 1.0 μM) and after washout of both concentrations from a single representative neuron (*scale bars*, 41 pA and 256 ms). *B*, sample traces of mEPSC recordings obtained before (*Control*) and during administration of (+)-pentazocine (50 μM) and after washout from a single representative neuron (*scale bars*, 41 pA and 1.3 s). *C*, summary graph of average percentage change in mEPSC frequency obtained with 0.1 ($n = 6$) and 1.0 μM ($n = 6$) DHEAS. Also shown are the effects of 0.5 ($n = 2$), 5 ($n = 6$), and 50 μM (+)-pentazocine ($n = 5$) and 50 μM (−)-pentazocine ($n = 5$). Each *bar* represents the average ± S.E. (*, $p < 0.05$ by one-sample *t* test *versus* a theoretical mean of zero)

tant regulatory actions on the release of a number of neurotransmitters. In agreement with the results of our study, *in vivo* microdialysis experiments have demonstrated that PREGS increases basal acetylcholine release in the hippocampus and cortex of rats (37–39) and that it also increases basal dopamine release in the rat nucleus accumbens (40). Not all studies, however, have shown a potentiating effect of PREGS on basal neurotransmitter release. Teschemacher *et al.* (41) found that PREGS (1–50 μM) reduces the probability of GABA release in cultured hippocampal neurons. Taken together with our finding that PREGS increases glutamate release in the same type of neurons, the results of Teschemacher *et al.* (41) indicate that this neurosteroid differentially regulates basal neurotransmitter release from GABAergic *versus* glutamatergic axonal terminals. It should also be emphasized that another study found that PREGS (10 nM to 3 μM) does not affect basal [^{3}H]norepinephrine release in hippocampal slices from adult rats (28). Thus, the effects of PREGS appear to depend on the neurotransmitter specificity of a particular presynaptic terminal.

Role of σ_1-like Receptors in the Mechanism of Action of PREGS—Monnet *et al.* (28) determined that PREGS decreases NMDA-evoked overflow of [^{3}H]norepinephrine in hippocampal slices of adult rats and that σ receptor antagonists block this effect. This finding prompted us to evaluate the role of σ receptors in the mechanism of the PREGS-induced increase in spontaneous quantal glutamate release. We found that the σ receptor antagonists, haloperidol and BD-1063, block the effect of PREGS on the probability of glutamate release. In addition, the effect of PREGS was mimicked by DHEAS, another neurosteroid that binds to σ receptors. Importantly, (+)-pentazocine, the prototypical σ_1 receptor agonist, also mimicked the effects of PREGS, albeit at higher concentrations (5–50 μM) than expected; lower concentrations of (+)-pentazocine (0.1–10 μM) have been shown to activate metabotropic σ_1-like receptors in brain membranes (25). However, it is possible that a relatively higher level of σ receptor occupancy may be required to induce an increase in spontaneous glutamate release in presynaptic terminals of developing neurons. Despite this uncertainty, our finding that (−)-pentazocine did not affect mEPSC frequency clearly argues for an involvement of a σ_1-like receptor in this process. Thus, our results and those reported by Monnet *et al.* (28) indicate that σ receptors are key players in the mechanism of the presynaptic actions of PREGS in the central nervous system. Studies by Ueda and collaborators (25, 27) have recently shown that PREGS and other σ receptor agonists stimulate binding of [^{35}S]GTPγS to synaptic membranes from the mouse brain in a pertussis toxin-sensitive manner. Reconstitution experiments performed by the same group of investigators showed that G_i couples to brain metabotropic σ receptors (25, 27). Consequently, our finding that the effect of PREGS on quantal glutamate release is blocked by pertussis toxin suggests that these G_i-coupled metabotropic σ receptors mediate the actions of PREGS on glutamate release in cultured hippocampal neurons.

Our finding that PREGS increases mEPSC frequency at concentrations of ≥10 μM is in agreement with the estimated binding affinity (~3 μM) of this neurosteroid for brain σ receptors (42), although there is an apparent contradiction between our findings and the results of Ueda *et al.* (25). These investigators reported that PREGS increases [^{35}S]GTPγS binding in brain homogenates in the 10 nM to 10 μM range, which indicates that this neurosteroid has a more potent interaction with metabotropic σ receptors. However, the prefrontal cortex and amygdala of adult mice were used to prepare the homogenates for those studies, and it is possible that higher concentrations of PREGS are required to activate metabotropic σ receptors in axonal terminals of developing hippocampal neurons. In these neurons, presynaptic metabotropic σ receptors could be associated with different proteins or could be regulated by posttranslational mechanisms that are only active during development.

It seems unlikely that the recently cloned σ binding protein is directly involved, at least initially, in the mechanism of the presynaptic actions of PREGS for several reasons (19–23). First, the σ binding protein sequence contains an ER retention signal (18), and, therefore, PREGS would not be expected to be able to reach this intracellular protein in intact neurons because of its limited lipid solubility. Second, cloning of the σ binding protein revealed that it only has a single transmembrane domain and also that it lacks a G protein binding domain (18). Therefore, our finding that the effect of PREGS is blocked by pertussis toxin treatment suggests that the σ binding protein is not involved in this process. Finally, a direct interaction of neurosteroids with cloned σ binding proteins has not been conclusively demonstrated. For instance, DHEAS, which mimicked the effects of PREGS on glutamate release probability, did not significantly displace [^{3}H](+)-pentazocine binding from recombinant σ binding proteins expressed in Sf21 cell membranes (25). However, Maurice *et al.* (43) found that intracerebroventricular infusion of a 16-mer oligodeoxynucleotide antisense to the ER σ binding protein blocked the antiamnesic effect of DHEAS. Thus, this neurosteroid may interact with the σ binding protein *in vivo* but not *in vitro*. Interestingly, the antiamnesic effect of PREGS was not affected by antisense treatment, which argues against an interaction, at least *in vivo*, of PREGS with the ER σ binding protein.

Our studies do not exclude the participation of σ binding proteins at later stages of the signal transduction cascade mediating the PREGS-induced increase in glutamate release

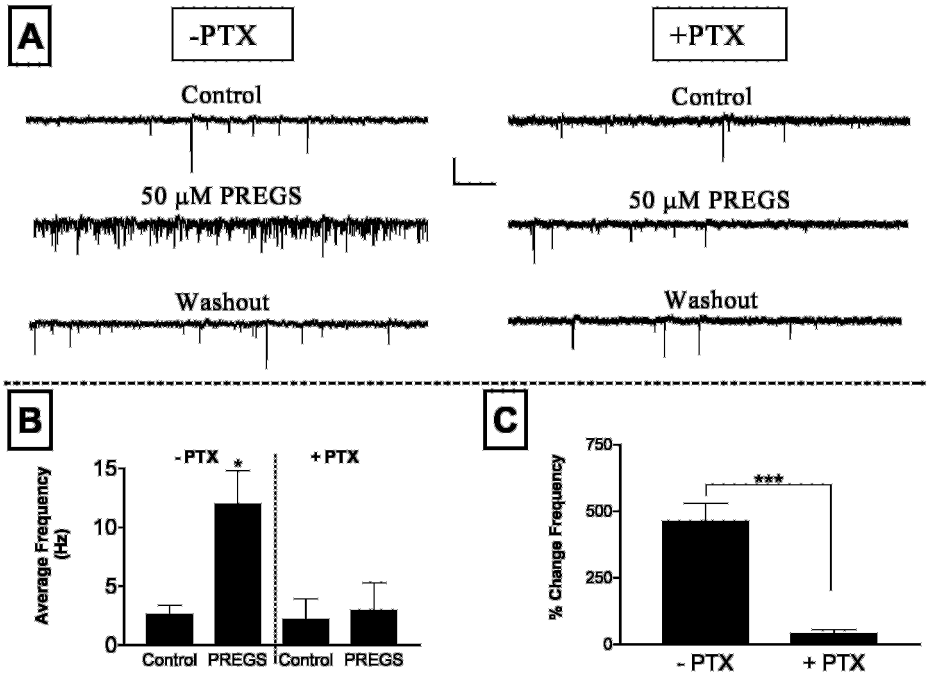

FIG. 5. **PREGS enhancement of mEPSC frequency is blocked by pertussis toxin.** *A*, sample traces obtained from untreated (−*PTX*) and pertussis toxin-pretreated (+*PTX*; 50 ng/ml for 36–48 h at 37 °C) neurons (*scale bars*, 16 pA and 1.3 s). *B* and *C*, summary of the effect of pretreatment with pertussis toxin. *B*, average frequencies of mEPSCs under control and 50 μM PREGS treatment conditions for untreated ($n = 5$) and PTX-treated ($n = 7$) neurons. Each *bar* represents the mean ± S.E. (*, $p < 0.05$ for control (−*PTX*) *versus* PREGS (−*PTX*) by paired *t* test). *C*, average percentage change in frequency for the neurons shown in *B*. The PREGS-induced percentage change for each individual neuron was calculated with respect to the average of control and washout responses (***, $p < 0.001$ by unpaired Student's *t* test).

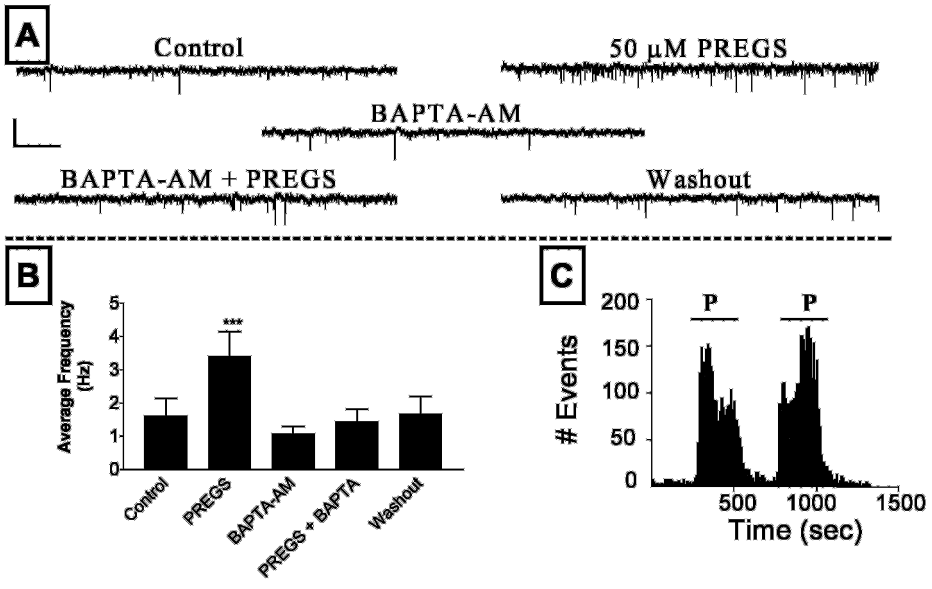

FIG. 6. **Treatment with the membrane-permeable calcium chelator BAPTA-AM blocks the effect of PREGS on mEPSC frequency.** *A*, sample traces obtained from a neuron under control conditions followed by the sequential addition of PREGS (50 μM), BAPTA-AM (20 μM for 15–20 min), PREGS plus BAPTA-AM, and then control external solution (*scale bars*, 16 pA and 1.3 s). *B*, summary graph of the result of these experiments. Each *bar* represents the mean ± S.E. of five neurons (***, $p < 0.001$; PREGS *versus* all other treatments by repeated measures analysis of variance followed by Bonferroni's *post hoc* test). *C*, time frequency histogram illustrating a control experiment in which the sequential application of PREGS (*P*; 35 μM) causes a reproducible increase in mEPSC frequency (see "Results" for more details).

probability. Morin-Surun *et al.* (44) found that treatment of an adult guinea pig brain stem preparation with σ receptor ligands resulted in translocation of σ binding proteins from the cytoplasm to the plasma membrane. This translocation was correlated with inhibition of hypoglossal spontaneous motor rhythmic activity, which was prevented by incubation with a phospholipase C (PLC) inhibitor. The authors of this study postulate that the translocation of the single transmembrane domain σ binding protein regulates the activity of pertussis toxin-sensitive G proteins by an unconventional mechanism, which, in turn, activates PLC (44). Interestingly, it has been recently demonstrated that σ binding proteins form a trimeric complex with ankyrin and inositol 1,4,5-trisphosphate receptors (IP_3Rs) in the ER and that σ ligands cause translocation of ankyrin-IP_3R complexes to other organelles, including the plasma membrane (35, 45, 46). Importantly, dissociation of ankyrin from IP_3Rs enhances efflux of Ca^{2+} from the ER via these receptors, which would be consistent with our finding that a Ca^{2+} chelator blocks the actions of PREGS (see below for a more detailed discussion). Therefore, it is possible that PREGS interacts with a membrane-bound receptor that initiates a signal transduction cascade, leading to translocation of σ binding proteins and activation of pertussis-sensitive G proteins.

Role of Ca^{2+} in the Mechanism of Action of PREGS—We found that the membrane-permeable Ca^{2+} chelator BAPTA-AM blocks the PREGS-induced increase in glutamate release. This finding demonstrates that the mechanism of action of this neurosteroid involves an elevation in $[Ca^{2+}]_i$. This finding is consistent with several reports demonstrating that σ receptors regulate $[Ca^{2+}]_i$ (35, 45, 47–49). It is also in agreement with recent reports that a significant percentage of miniature synaptic currents in hippocampal neurons are regulated by changes in $[Ca^{2+}]_i$ in axonal terminals (50, 51). Enhancement of mEPSC frequency in cultured hippocampal neurons by brain-derived neurotrophic factor was shown to require an elevation in $[Ca^{2+}]_i$ mediated by activation of a signal transduction cascade involving PLC-γ and IP_3Rs (29). Miniature postsynaptic currents in retinal ganglion cells were also shown to

depend on Ca^{2+} released from internal stores via the PLC-β/IP_3R pathway (52). Although G_q proteins have been linked to activation of this pathway, recent evidence suggests that $G_{i/o}$ can also activate PLC$-\beta$ in neurons (53). Thus, it is possible that the mechanism by which activation of a $G_{i/o}$-coupled σ receptor triggers an elevation in $[Ca^{2+}]_i$ involves activation of PLC-β, and we are currently investigating this possibility as well as the contribution of different Ca^{2+} sources to the mechanism of action of PREGS.

It was recently demonstrated that σ_2, rather than σ_1, receptors modulate $[Ca^{2+}]_i$ in a neuroblastoma cell line that expresses both types of receptor (49). However, we do not think that these receptors play a significant role in the mechanism of action of PREGS for several reasons. First, the effect of PREGS was blocked by BD-1063, which has been demonstrated to have preferential affinity for σ_1 sites (54). Second, the prototypical σ_1 ligand, (+)-pentazocine, mimicked the actions of PREGS. Third, haloperidol has been shown to act as an agonist of σ_2 receptors (55). For instance, it has been shown to elevate $[Ca^{2+}]_i$, which would have been expected to increase basal mEPSC frequency and/or to potentiate the effect of PREGS in cultured hippocampal neurons (49). Since haloperidol did not have any of these effects under our experimental conditions, it is unlikely that σ_2 receptors mediate the effects of PREGS on glutamate release. Finally, binding of PREGS, DHEAS, and other neurosteroids to σ_2 receptors has not been demonstrated (55). Thus, we conclude that a metabotropic receptor with the pharmacological profile of the σ_1 subtype mediates the actions of PREGS on the probability of glutamate release.

An important finding of our study is that *ent*-PREGS, the synthetic (−)-enantiomer of PREGS, did not affect mEPSC frequency. This result indicates that the interaction between presynaptic σ_1-like receptors and PREGS is enantioselective. This finding is not surprising given that the action of pentazocine and other benzomorphans on σ_1 receptors is also enantioselective. Interestingly, the interaction of PREGS with NMDA and $GABA_A$ receptors is not enantioselective in cultured neurons (7, 33). Since *ent*-PREGS has been shown to exert either more potent (6) or less potent (7) effects on memory in rodents than PREGS, it would be important to determine whether the lack of interaction of *ent*-PREGS with presynaptic metabotropic σ_1-like receptors contributes to its differential effects *in vivo*.

Significance—PREGS modulated glutamate release probability at concentrations of $\geq$10 μM, which appears to be a higher concentration than expected for a physiologically relevant action. Recent papers reported concentrations of ~8 ng/g of rat brain tissue (2) and ~13 ng/g of rat hippocampal tissue (5). However, these concentrations of PREGS were determined in homogenates of brain tissue from adult rats, and the actual synaptic concentrations of this neurosteroid are unknown. It is entirely possible that PREGS reaches micromolar levels in synaptic regions under certain conditions. Moreover, PREGS levels could be higher in the immature nervous system where the NMDAR activity is higher than in the mature nervous system. Indeed, Ca^{2+} influx through NMDARs has been shown to stimulate PREGS synthesis in cultured hippocampal neurons (56) and in isolated rat retinas (57). Thus, we believe that our findings have significant physiological implications. It should also be emphasized that we detected a clear effect of DHEAS at concentrations as low as 0.1 and 1 μM, which also lends support to the physiological relevance of our findings.

In conclusion, we postulate that the effects of PREGS and DHEAS on quantal glutamatergic synaptic transmission may have an impact on synaptic development in the central nervous system. Recent studies with knockout mice lacking proteins involved in regulation of neurotransmitter vesicle exocytosis have demonstrated that quantal transmitter release is not required for neuronal differentiation and axonal pathfinding but is essential for the persistence of synaptic neuronal networks (58–60). Thus, it is possible that the effects of neurosteroids on spontaneous glutamate release in developing synapses could contribute to the maturation and/or maintenance of these specialized structures.

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