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Sulfated steroids as endogenous neuromodulators

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Abstract

Central nervous system function is critically dependent upon an exquisitely tuned balance between excitatory synaptic transmission, mediated primarily by glutamate, and inhibitory synaptic transmission, mediated primarily by GABA. Modulation of either excitation or inhibition would be expected to result in altered functionality of finely tuned synaptic pathways and global neural systems, leading to altered nervous system function. Administration of positive or negative modulators of ligand-gated ion channels has been used extensively and successfully in CNS therapeutics, particularly for the induction of sedation and treatment of anxiety, seizures, insomnia, and pain. Excessive activation of excitatory glutamate receptors, such as in cerebral ischemia, can result in neuronal damage via excitotoxic mechanisms. The discovery that neuroactive steroids exert rapid, direct effects upon the function of both excitatory and inhibitory neurotransmitter receptors has raised the possibility that endogenous neurosteroids may play a regulatory role in synaptic transmission by modulating the balance between excitatory and inhibitory neurotransmission. The sites to which neuroactive steroids bind may also serve as targets for the discovery of therapeutic neuromodulators.

Keywords: Pregnenolone sulfate; Neurosteroid; Glutamate receptors; NMDA receptors; GABA receptors; Pregnanolone hemisuccinate; Dopamine; Stroke; Pain

1. Neurotransmitter receptor modulation by sulfated steroids

Modulation of $GABA_A$ receptors by pregnenolone sulfate (PS). A physiological role for PS as an endogenous neuromodulator was initially suggested by Majewska and Schwartz (1987), based upon their discovery that PS inhibited GABA mediated ^{36}Cl-uptake by rat brain synaptosomes, as well as previous reports indicating that PS is one of the more abundant steroids in brain (Corpéchot et al., 1983). Electrophysiological studies demonstrated that PS inhibited GABA responses of rat cortical neurons (Majewska et al., 1988), confirming that PS is an inhibitor of the $GABA_A$ receptor.

Modulation of $GABA_A$ receptors by dehydroepiandrosterone sulfate (DHEAS). Along with PS, DHEAS was reported to be abundant in rat brain, and to persist following adrenalectomy and gonadectomy (Corpéchot et al., 1985). DHEAS was found to function as an allosteric negative modulation of $GABA_A$ receptors (Majewska et al., 1990).

Inverse modulation of $GABA_A$ receptors by sulfated and unsulfated steroids occurs at distinct sites. $GABA_A$ receptors are also subject to positive modulation by nonsulfated neurosteroids, of which the most extensively studied are allopregnanolone and pregnanolone, which potently potentiate $GABA_A$ receptor function (Harrison et al., 1987). Although structural similarities might lead one to expect that these steroids would share a common binding site with PS, this is not the case. Structure activity relationships for $GABA_A$ receptor modulation are different for sulfated inhibitory steroids vs. nonsulfated potentiating steroids (Park-Chung et al., 1999). Potentiation by nonsulfated steroids requires 3α stereochemistry. Pregnenolone is inactive at $GABA_A$ receptors, as are 3β isomers of pregnanolone. In contrast, PS is inhibitory, as are both the 3α and 3β isomers of pregnanolone sulfate. Although the addition of a negatively charged sulfate or hemisuccinate group at the C-3 position converts a number of neuroactive steroids from potentiating to inhibitory (Park-Chung et al., 1999), a negatively charged group at C-3 is not absolutely essential for inhibition, as the nonsulfated neurosteroid dehydroepiandrosterone (DHEA) is also inhibitory, although

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less potent than its sulfated derivative DHEAS (Park-Chung et al., 1999; Imamura and Prasad, 1998).

The inhibitory potency of DHEAS is unaltered in the presence of a high concentration of the nonsulfated potentiating neurosteroid allopregnanolone, demonstrating that these two neuroactive steroids modulate $GABA_A$ receptors via distinct sites (Park-Chung et al., 1999). A recent study of the effects of PS and allopregnanolone on GABA-mediated spontaneous IPSCs of neurons from rat medial preoptic nucleus similarly concluded that PS and allopregnanolone do not interact competitively. However, whereas 10 μM PS alone had only a small effect on IPSC amplitude or time course, PS antagonized prolongation of IPSC decay by allopregnanolone, suggesting that PS and allopregnanolone interact indirectly (Haage et al., 2005).

Mechanism of $GABA_A$ receptor modulation by sulfated steroids. Inhibition of $GABA_A$ receptor function by PS and DHEAS is not voltage-dependent (Majewska et al., 1988; Spivak, 1994), indicating that these sulfated steroids do not need to penetrate significantly into the membrane field to reach their sites of action, and suggesting that they act allosterically rather than by physically occluding the ion pore of the $GABA_A$ receptor. An early single-channel study of rat cortical neurons in culture concluded that PS had no effect on channel conductance or open time, but reduced the frequency of channel opening (Mienville and Vicini, 1989). A subsequent single-channel patch clamp study of α1β2γ2L $GABA_A$ receptors expressed in HEK293 cells found that PS had no effect on the rate constants for channel closing and opening, and concluded PS produces a block that is independent of GABA binding, channel activation state, or membrane potential, with an IC_{50} of 100 nM, suggesting that PS acts essentially as a slow noncompetitive antagonist (Akk et al., 2001). In contrast, studies of macroscopic $GABA_A$ receptor currents have reported that PS accelerates the development of desensitization, that the potency of PS as an inhibitor of macroscopic $GABA_A$ receptor current is enhanced in the presence of GABA, and that PS block develops more rapidly in the presence of GABA, leading to the suggestion that PS enhances the rate of entry into a desensitized state (Eisenman et al., 2003; Shen et al., 2000). The disagreement between microscopic and macroscopic studies of PS inhibition of $GABA_A$ receptor function remains to be resolved.

The site of action of sulfated neurosteroids on $GABA_A$ receptors remains unclear. Based upon the observation that PS reduced the apparent affinity of ^{35}S-TBPS, Sousa and Ticku (1997) suggested that DHEAS might bind at the picrotoxin/cage convulsant site; however, the inhibitory effects of PS and DHEAS persist in the presence of a mutation to the transmembrane M2 channel domain that eliminates picrotoxin sensitivity (Shen et al., 1999). The absence of voltage sensitivity or alteration of single-channel open time argues against a binding site within the pore. Akk et al. (2001) identified a valine residue in the channel domain of the α1 subunit that slowed the development of PS inhibition when mutated to serine, but concluded that this residue is unlikely to be part of the binding site and likely influences PS action indirectly. A recent study in *C. elegans* identified multiple residues in transmembrane domain 1 (M1), as well as a residue near the extracellular end of the M2 helix, that are critical for low-

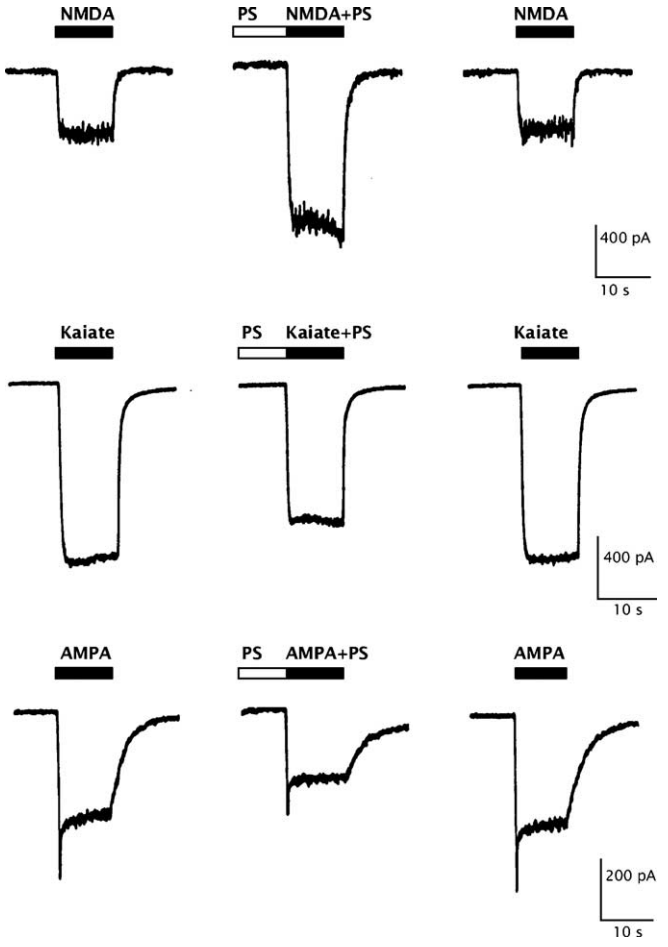

Fig. 1. PS potentiates the NMDA response and inhibits kainate and AMPA responses of chick spinal cord neurons in culture. A, 100 μM PS dramatically potentiates the current induced by 30 μM NMDA. B, PS (100 μM) inhibits the current induced by 50 μM kainate. C, PS (100 μM) inhibits the current induced by 25 μM AMPA. Reprinted with permission from (Wu et al., 1991).

μM inhibition of *C. elegans* $GABA_A$ receptors by PS. This latter residue is of particular interest, as it is a positively charged arginine that could potentially coordinate with the negatively charged sulfate of PS. The *C. elegans* receptor exhibits some pharmacological differences as compared to mammalian $GABA_A$ receptors (for example, pregnanolone is inhibitory), so these results may or may not be relevant to mammalian receptors; however, it is notable that an arginine residue is also found in this region of mammalian $GABA_A$ receptor subunits (Wardell et al., 2006).

Inhibition of Glycine Receptors by PS. Wu et al. reported that PS inhibits glycine receptors of spinal cord neurons with an IC_{50} of 3.7 μM (Wu et al., 1990, 1997). Based upon the lack of voltage- or use-dependence and the observation that PS shifted the glycine concentration-response curve to the right, PS was proposed to act as a competitive inhibitor for the glycine site, although an allosteric mechanism of action is also possible. Maksay et al. (2001) reported that the potency of PS inhibition was dependent upon glycine receptor subunit composition, with an IC_{50} ranging from 2 μM for homomeric α1 receptors to 10 μM for α2β receptors.

Bidirectional Modulation of Glutamate Receptors by PS. The effect of PS on glutamate receptors was examined by Wu et al.,

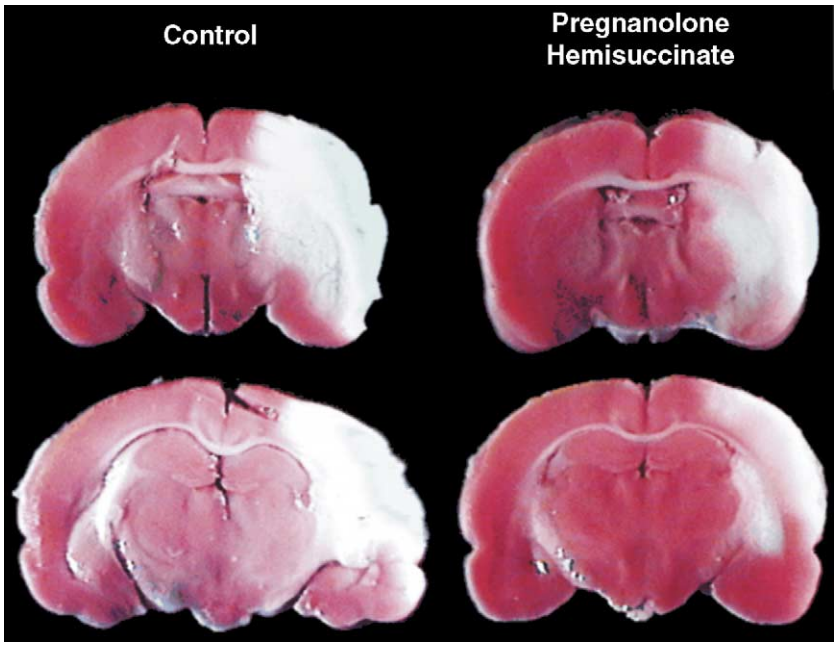

Fig. 2. Pregnanolone hemisuccinate is neuroprotective in an *in vivo* model of stroke. Rats were infused with vehicle (control) or pregnanolone hemisuccinate (6.9 mg/kg/h; 6.9 mg/kg loading dose), beginning immediately or 30 min after initiation of ischemia. Infusion of pregnanolone hemisuccinate was continued for an additional 22 h, at which time the rats were killed and their brains were stained with the vital dye 2,3,5-triphenyl tetrazolium Cl. Representative sections are from animals receiving pregnanolone hemisuccinate immediately after initiation of ischemia. Infarct area appears pale. The volume of cortical and subcortical infarct was reduced by 47±10% and 26±6%, respectively in animals receiving pregnanolone hemisuccinate ($n=10$). When infusion was delayed until 30 min after the onset of ischemia the volume of cortical infarct was reduced by 39±7%, with no significant reduction in the subcortical region ($n=13$). Reprinted with permission from Weaver et al. (1997).

who found that PS rapidly enhanced the NMDA induced current of chick spinal cord neurons by as much as 200%, with an EC_{50} of 57 μM, while inhibiting responses to AMPA and kainate (Wu et al., 1991; Wu and Chen, 1997; Fig. 1). Based upon the observation that initiation of PS potentiation takes place on a time scale of less than a second, PS was proposed to act as a direct positive allosteric modulator of NMDA receptors. In a subsequent electrophysiological study of hippocampal neurons, Bowlby demonstrated that PS increases the efficacy of the NMDA receptor coagonists NMDA and glycine. Single-channel analysis using excised patches indicated that PS increased the fractional open time (nP_o) by 200%, primarily by increasing the rate of channel opening, although there was also a small increase in channel open time (Bowlby, 1993). A study by Wong and Moss (1994), also in hippocampal neurons, reached similar conclusions. The observation that PS potentiation could be observed in excised patches provides further support for the hypothesis that PS exerts its potentiating effect by a direct allosteric interaction with the NMDA receptor. Interestingly, potentiation of the NMDA response was observed by Bowlby (1993) not only in the excised outside-out patch configuration, when PS and NMDA were both applied to the external membrane surface, but also in the cell-attached configuration when NMDA was present in the recording pipette and PS was applied to the outside of the cell, suggesting that in this latter configuration PS was able to reach its site of action on the NMDA receptor either by crossing the cell membrane or by lateral diffusion within the membrane. However, Park-Chung et al. (1997) reported that inclusion of a saturating concentration of PS within the whole cell recording pipette did not alter the response of spinal cord neurons to PS applied extracellularly at the same concentration. This indicates that the recognition site for rapid potentiation of the NMDA response by PS is directed extracellularly.

In contrast to the rapid potentiating effect of PS, the analogs pregnanolone sulfate and epipregnanolone sulfate, which differ from PS primarily by the lack of a C-5–C-6 double bond, inhibit the NMDA response of chick spinal cord neurons. Surprisingly, interaction studies showed that PS and epipregnanolone sulfate do not compete for a common site, and that inclusion of epipregnanolone sulfate in the patch pipette did not block the effect of extracellular application of steroid, demonstrated that there are at least two distinct extracellularly oriented steroid modulatory sites on or associated with the NMDA receptor (Park-Chung et al., 1997). Pregnenolone itself is without modulatory activity at NMDA receptors, as is pregnanolone, arguing that the presence of the negatively charged sulfate group at C-3 is important for activity. Additional structure-activity studies showed that other negatively charged groups at C-3 can substitute for the sulfate group. In particular, both pregnenolone hemisuccinate and pregnanolone hemisuccinate are active (Weaver et al., 2000). Pregnanolone hemisuccinate is of particular interest in this regard, as it is neuroprotective against NMDA induced excitotoxicity in tissue culture, and exhibits sedative and analgesic properties in rodents *in vivo*, as well as being neuroprotective against middle cerebral artery occlusion stroke damage (Weaver et al., 1997; Fig. 2).

Malayev et al. demonstrated that modulation of NMDA receptors expressed in *Xenopus laevis* oocytes by PS is subunit-

dependent, with NR1/NR2A and NR1/NR2B receptors exhibiting potentiation by PS, whereas NR1/NR2C and NR1/NR2D receptors exhibit inhibition (Malayev et al., 2002; Fig. 3). Both positive and negative modulation by PS is voltage-independent, indicating that neither effect requires penetration of PS into the field of the channel. Based upon concentration-response analysis, PS was proposed to act by stabilizing or destabilizing the active state of the receptor via a two-state allosteric model for NMDA receptor modulation (Fig. 4). In contrast to PS, 3α5βS was found to be inhibitory at all four subunit combinations tested, although it was somewhat more potent at receptors containing NR2C or NR2D than at receptors containing the NR2A or NR2B subunits (Malayev et al., 2002). A subsequent study by Horak et al. (2004) examined NR1/NR2B NMDA receptors expressed in HEK 293 cells, and similarly concluded that PS enhances open probability. Utilizing fast perfusion, Horak et al. found that potentiation of the NMDA response by PS is reduced when PS is applied during a glutamate application, as compared to when PS application precedes exposure to glutamate. Based on this result, the investigators proposed a model in which binding of glutamate reduces the affinity of the receptor for PS. Although this model was able to fit the kinetic data, it is not in thermodynamic balance, and it is difficult to understand how such a nonreciprocal scheme, in which PS stabilizes the open channel state (which necessarily has high affinity for glutamate), but glutamate destabilizes the binding of PS, could be made to work thermodynamically. Additional studies are clearly required to elucidate the detailed kinetics of NMDA receptor modulation by PS.

Exploiting the opposite polarity of modulation of NR1/NR2B and NR1/NR2D receptors by PS, Jang et al. (2004) constructed a series of chimeric NR2 subunits in which various corresponding regions of the NR2B and NR2D subunits were exchanged. Based upon analysis of PS modulation of receptors containing these chimeric subunits, a region including the M4 transmembrane domain and the adjacent region of the M3–M4 extracellular loop which they termed Steroid Modulatory Domain 1 (SMD1) was identified as critical for PS potentiation (Fig. 5). Interestingly, the same region apparently influences pH sensitivity, but this is an

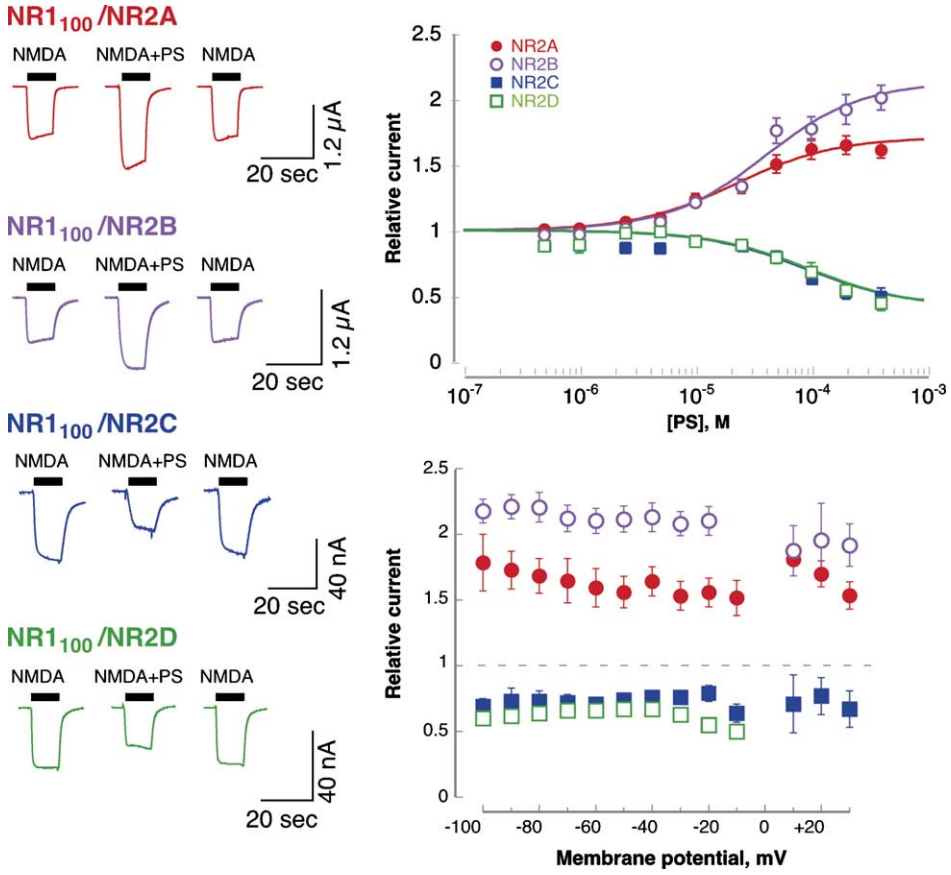

Fig. 3. Inverse modulation of NMDA receptor subtypes by PS. (A–D), examples of traces obtained from *Xenopus laevis* oocytes previously injected with (A) NR1-4b/NR2A, (B) NR1-4b/NR2B, (C) NR1-4b/NR2C, or (D) NR1-4b/NR2D mRNAs. The *bar* indicates the period of drug application. Interval between consecutive current traces was 45 s. Receptors were activated by co-application of 10 μM glycine plus 80 μM NMDA (NR1/NR2A), 25 μM NMDA (NR1/NR2B and NR1/NR2C), or 10 μM NMDA (NR1/NR2D). Co-application of 100 μM PS to NR1/NR2A or NR1/NR2B receptors resulted in an increase in the agonist response, whereas co-application of 100 μM PS to NR1/NR2C or NR1/NR2D resulted in a decrease in the agonist response. (E) Concentration-response curves for PS effect on NR1/NR2 receptors. Data points are averaged values of normalized peak current responses from oocytes injected with NR1/NR2A (n=8), NR1/NR2B (n=8), NR1/NR2C (n=4) or NR1/NR2D (n=4) RNAs. Responses were normalized to the control response obtained by application of 10 μM glycine plus 80 μM NMDA (NR2A), 25 μM NMDA (NR2B, NR2C) or 10 μM NMDA (NR2D). (F) Effect of holding potential on modulation of the NMDA/glycine response by PS. Points are averaged relative currents obtained in the presence of 100 μM PS, standardized relative to the response induced from the same oocyte by 10 μM glycine plus 80 μM (NR1/NR2A, n=4), 25 μM (NR1/NR2B, n=7; NR1/NR2C, n=3), or 10 μM NMDA (NR1/NR2D, n=3). Symbols are defined as in E. Error bars indicate s.e.m. Reprinted with permission from Malayev et al. (2002).

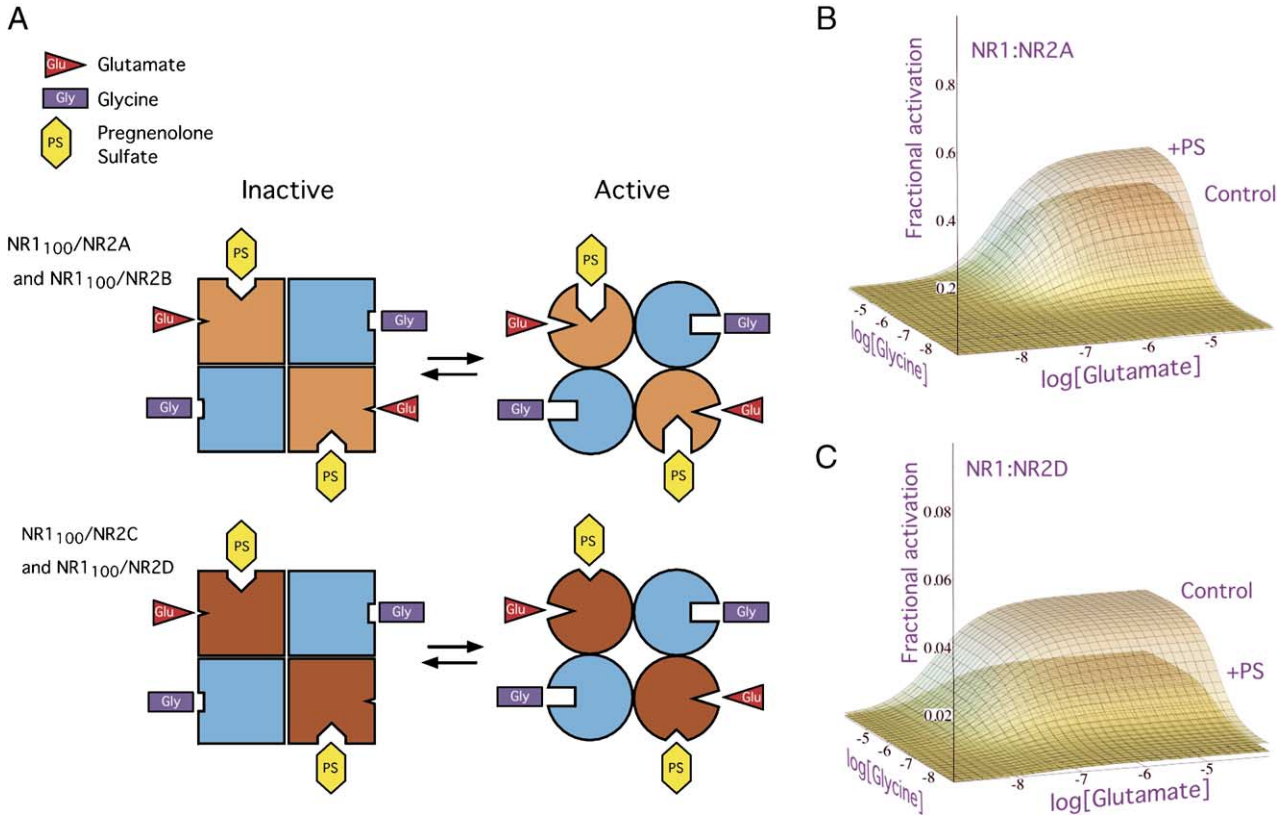

Fig. 4. Allosteric model of NMDA receptor modulation by PS. A, Activation of the receptor (gating) is assumed to be concerted and described by a two-state model. The model includes 6 binding sites, two each for glutamate/NMDA, glycine, and PS. High affinity is indicated by a deep "slot" for the corresponding ligand, while low affinity is indicated by a shallow slot. Affinity of the active state for PS is greater than that of the resting state for NR1/NR2A and NR1/NR2B, but less than that of the resting state for NR1/NR2C and NR1/NR2D. Reprinted with permission from Malayev et al. (2002). B and C, Theoretical glutamate–glycine dose-effect surfaces calculated based on the allosteric model, illustrating how PS can enhance efficacy of glycine and glutamate at NR1/NR2A receptors, while reducing efficacy at NR1/NR2D receptors.

independent effect; in contrast to ifenprodil, relief of proton inhibition does not play a role in the potentiating effect of PS. Molecular modeling based upon a crystal structure of the AMPA receptor extracellular domain (Sun et al., 2002) indicated that SMD1 contributes to a hydrophobic pocket at the NR1/NR2B interface, which may constitute a PS binding site (Jang et al., 2004). A subsequent study by Horak et al. (2006) examined NR2A/NR2C chimeras and confirmed that the M3–M4 region is critical for PS potentiation. Using rapid perfusion, these investigators were able to detect a rebound of the glutamate-induced current of NR1/NR2A receptors when PS was washed out. This suggests that PS exerts an inhibitory effect on NR1/NR2A receptors that is largely masked by its potentiating effect (Horak et al., 2006). Given that inhibition by PS is also observed for AMPA and kainite receptors (Fig. 6), and that all glutamate receptor types tested to date exhibit inhibition by pregnanolone sulfate (Wu et al., 1991; Yaghoubi et al., 1998; Wu and Chen, 1997), it may be that an inhibitory steroid site is a universal feature of ionotropic glutamate receptors, whereas the potentiating site may be restricted to NMDA receptors containing the NR2A or NR2B subunits. Based upon studies of chimeric NR2A/NR2C subunits, Petrovic et al. (2005) report that the M3–M4 loop also controls the inhibitory potency of pregnanolone sulfate, indicating that this region plays an important role in both positive and negative modulation of the NMDA receptor by sulfated steroids.

2. Modulation of neurotransmitter release by sulfated steroids

Numerous reports indicate that sulfated steroids are capable of acting presynaptically to modulate the release of a variety of neurotransmitters. In some, but not all cases, evidence suggests the involvement of σ receptors. Despite having been first described in 1976 (Gilbert and Martin, 1976), the nature and physiological function of σ receptors remains obscure (Gibbs and Farb, 2000; Monnet and Maurice, 2006). Initially confused with opiate and NMDA receptors, σ receptors were eventually shown to be a distinct entity (Quirion et al., 1987). A remarkably wide variety of pharmacologically significant compounds have been reported to bind to σ receptors, including PS, DHEAS, progesterone, phencyclidine, ifenprodil, haloperidol, dextromethorphan, and pentazocine. The cloned σ1 receptor does not resemble any other mammalian protein, although it does exhibit 33% sequence identity to a fungal sterol isomerase (Hanner et al., 1996). However, no enzymatic activity has been identified for the mammalian σ1 receptor. σ receptors have been shown to directly associate with Kv1.4 K^+ channels, conferring sensitivity to σ1 ligands, leading to the suggestion that the σ1 receptor may function as a regulatory subunit for ion channels (Aydar et al., 2002). Other evidence suggests that σ receptors couple through $G_{i/o}$, despite the lack of structural or sequence similarity to known G-protein coupled receptors (Itzhak, 1989).

PS at nM concentrations has been reported to reduce the frequency of GABA-mediated mIPSCs in cultured hippocampal neurons, and this effect is mimicked by the σ receptor agonist SKF 10047 and is blocked by the σ receptor antagonist BD-1063 and the $G_{i/o}$ antagonist pertussis toxin, suggesting involvement of σ and G-protein coupled receptors (Mtchedlishvili and Kapur, 2003). PS and DHEAS are reported to increase the frequency of glutamate-mediated EPSCs in hippocampal cultures (Meyer et al., 2002). As with the effect of PS on GABA-mediated transmission, enhancement of glutamate-mediated transmission by PS is sensitive to σ receptor antagonists and pertussis toxin, but this effect is only evident at PS concentrations >10 μM (although DHEAS was active down to 100 nM). The similar sensitivity to inhibitors suggests a common mechanism of action for these two effects, but this is difficult to reconcile with the large differences in potencies for PS modulation.

In superfused rat hippocampal synaptosomes, PS at nM concentrations inhibits NMDA-stimulated release of ^{3}H-norepinephrine, but does not affect basal release, while DHEAS is inactive (Cannizzaro et al., 2003). Similarly, PS at nM concentrations inhibits, while DHEAS enhances, NMDA induced ^{3}H-norepinephrine release from preloaded hippocampal slices, and the effects of both steroids are sensitive to pertussis toxin and to σ receptor antagonists (Monnet et al., 1995).

When infused into rat striatum in vivo by reverse microdialysis, PS at concentrations as low as 10 nM increases dopamine overflow, whereas pregnenolone is without effect. The increase in dopamine overflow is blocked by D-AP5, but is resistant to BD-1063, indicating the involvement of NMDA receptors, but not σ receptors (Sadri-Vakili, 2003).

PS at nM concentrations has been reported to enhance LTP in hippocampal slices and also to enhance the effect of exogenously applied NMDA (Sliwinski et al., 2004; Fig. 7). Partridge et al. have shown that paired pulse facilitation (PPF) of AMPA-mediated responses of CA1 neurons in response to Schaffer collateral stimulation is enhanced at high nM concentrations of PS, arguing for a specific modulatory effect of PS on presynaptic facilitation of glutamate release. PPF enhancement by PS was sensitive to pertussis toxin and to σ1 receptor antagonists (Partridge and Valenzuela, 2001; Schiess and Partridge, 2005; Fig. 8). Similar potentiation of PPF by PS was observed in dentate granule cells in response to stimulation of the perforant pathway (Thomas et al., 2005). In the absence of exogenous PS, PPF enhancement could be reproduced by perfusion with the steroid sulfatase inhibitors DU-14 or PKL-32, arguing for the presence of an endogenous PS-like sulfated steroid capable of modulating glutamate release (Thomas et al., 2005).

Mameli et al. (2005) found that PS at concentrations of 17 μM or above increased AMPA receptor-mediated mEPSC frequency in hippocampal slice recordings from postnatal day 3–4 rats via a presynaptic action. This effect of PS was blocked by NMDA receptor antagonists but not by σ antagonists. Notably, a similar increase in mEPSC frequency was induced by postsynaptic depolarization, and this latter

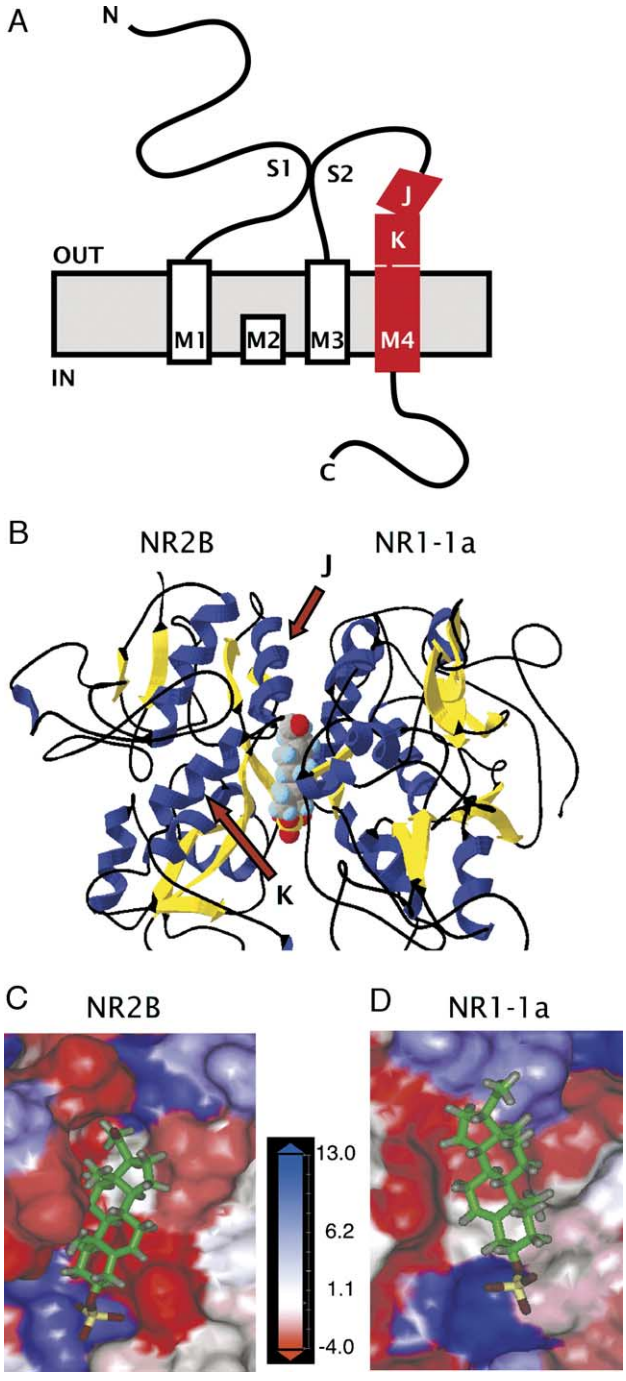

Fig. 5. Identification of a Steroid Modulatory Domain, SMD1, required for PS potentiation. A, The topological representation of the NR2B subunit and the location of SMD1 (colored in red) are depicted. Membrane domains are denoted as M1–M4. The amino terminus (N) is located on the extracellular side and the carboxyl terminus (C) on the intracellular side of the plasma membrane. B, Molecular modeling of a dimer comprising the S1–S2 domains of NR2B and NR1-1a reveals a potential binding pocket for PS. The 3D model is depicted in a ribbon configuration with helices colored in blue and sheets colored in yellow. PS is docked at the interface between twos subunits. Detailed views of the potential binding pocket for PS on NR2B (C) and NR1-1a (D) are illustrated. NR1-1a or NR2B is removed from the model to show the hydrophobic pocket on NR2B (C) or NR1-1a (D), respectively. The receptor surface is colored according to a hydrophobicity scale, with hydrophobic residues colored in red and charged residues in blue. PS is depicted in a stick configuration and colored by the atom type, with hydrogen in white, carbon in green, oxygen in red, and sulfur in yellow. Reprinted from Jang et al. (2004).

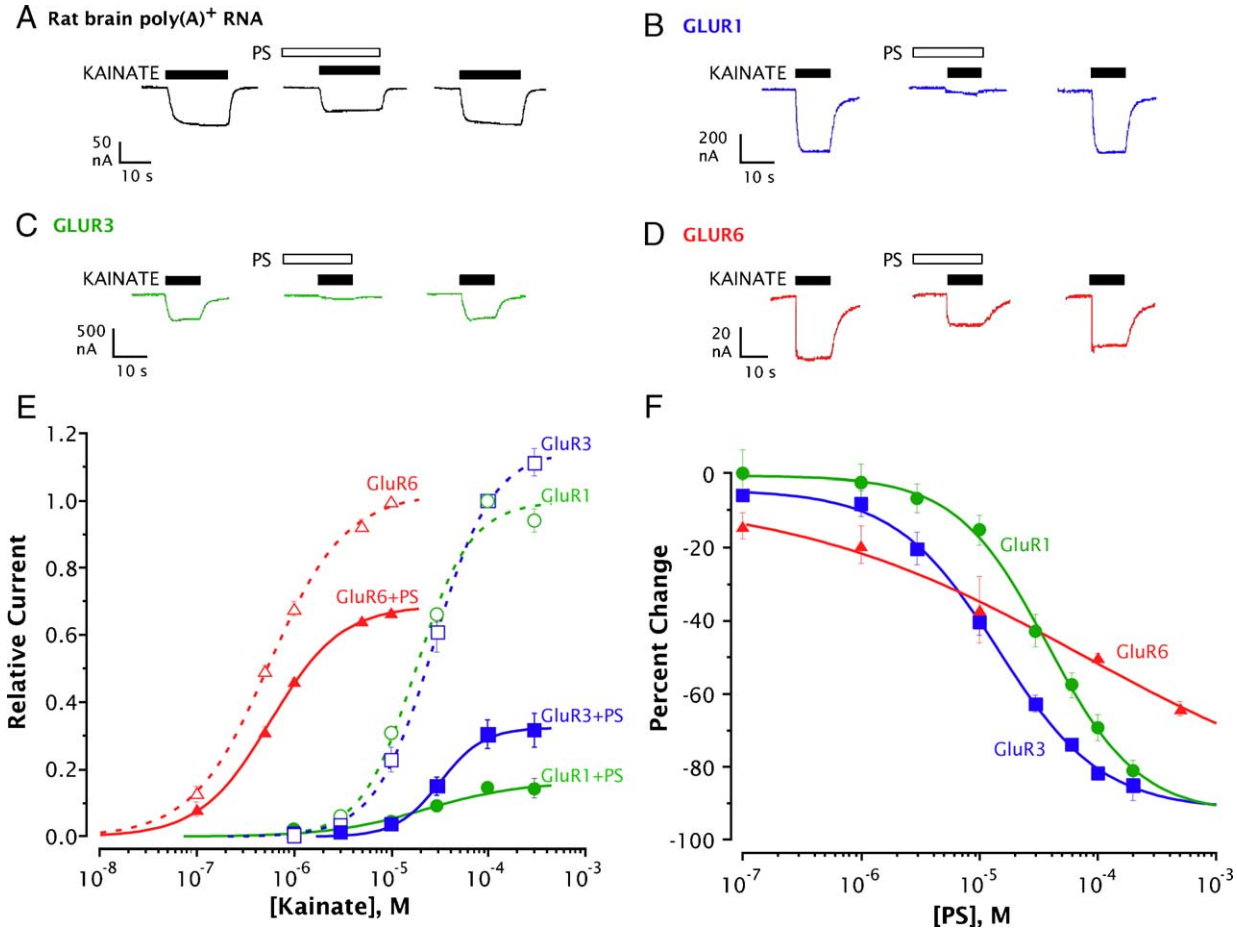

Fig. 6. PS inhibits AMPA and kainate receptor function. A–D Representative traces showing inhibitory effect of 100 μM PS on kainate-induced currents of *X. laevis* oocytes injected with (A) rat brain poly A+RNA, (B) GluR1 cRNA, (C) GluR3 cRNA, D GluR6 cRNA. Kainate concentration was 100 μM in traces A – C, 10 μM for trace D. *Solid bar* shows period of kainate application; *open bar* shows period of PS exposure. E, PS (closed symbols) decreases maximum kainate responses of GluR1, GluR3, and GluR6, receptors. F, Concentration-response curve for inhibition by PS. Each *data point* represents the mean of three experiments. *Error bars* represent s.e.m. Results are expressed as percentage change in the peak 100 μM (GluR1 and GluR3) or 10 μM (GluR6) kainate-induced current in the presence of PS. Each *data point* is the mean of three experiments; *error bars* indicate s.e.m. Reprinted from Yaghoubi et al. (1998), Copyright 1998 by Elsevier, with permission from Elsevier.

effect was blocked by perfusion with an antibody that reacts with pregnenolone and PS, suggesting that postsynaptic depolarization induces the release of a PS-like retrograde messenger. Facilitation of neurotransmission by PS was not observed in slices from rats older than postnatal day 5, suggesting that PS may play a specific role in plasticity of immature synapses (Fig. 9).

In cultures of immortalized GTI-7 hypothalamic neurons, PS at concentrations >10 μM stimulated the release of gonadotrophic releasing hormone (GnRH), while DHEAS was inactive. No glutamatergic agonists were added, but PS-induced GnRH was antagonized by NMDA receptor selective inhibitors AP5 and MK-801, suggesting that release was likely due to PS-induced potentiation of the action of endogenous glutamate (El-Etr et al., 2006).

Taken together, these results indicate that sulfated steroids are capable of modulating neurotransmission in a variety of receptor systems and by multiple mechanisms. Moreover, there is strong suggestive evidence in some cases for modulation by endogenous sulfated steroids.

3. What are levels of PS and DHEAS in brain?

Much of the early interest in PS and DHEAS as possible endogenous neuromodulators derives from early reports that they are among the most abundant steroids in brain, but this has recently become a topic of controversy. Corpéchot et al. found that PS was present in anterior rat brain at a level of 16 ng/g of brain tissue (Corpéchot et al., 1983), corresponding to an average concentration of 35 nM, while DHEAS was present at somewhat lower levels (Corpéchot et al., 1981). Levels of PS and DHEAS in rat brain remained high 15 days after adrenalectomy and castration, arguing that PS and DHEAS are made within the brain and are therefore neurosteroids. Lanthier and Patwardhan (1986) found similarly high levels of PS and DHEAS in post-mortem human brain, with the highest levels in pituitary (PS, 5–38 ng/g tissue; DHEAS, 29–71 ng/g), and even higher levels of PS in some cranial nerves. Neither of these studies assayed PS or DHEAS directly; both used liquid–liquid extraction to separate unconjugated and conjugated steroids into organic and aqueous phases, followed by acid

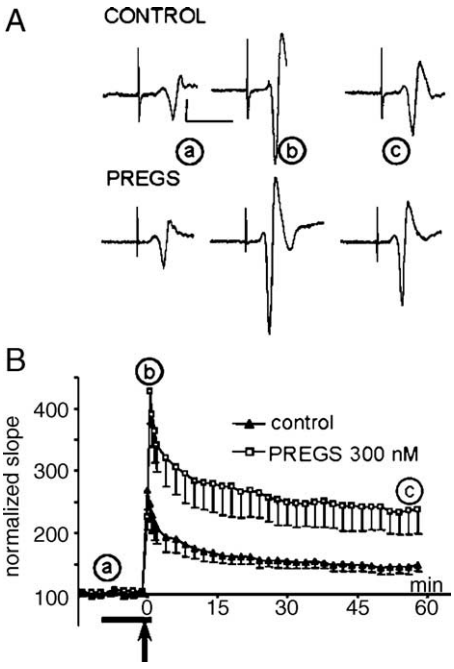

Fig. 7. PS enhances LTP in CA1 of rat hippocampus. A, PS effect on representative recordings of the somatic fEPSPs during LTP paradigm under baseline conditions (A) and during potentiation (B) and maintenance (C; 60 min) phases (vertical scale: 1 mV, horizontal scale: 10 msec). B, Normalized fEPSPs slopes following tetanic stimulation (3 × 300 Hz/1 sec; arrow) in control and PS-exposed slices (10 min, horizontal bar). Note the significant enhancement of LTP in slices exposed to PS (300 nM; ANOVA, $P \leq 0.025$, vs. control LTP, $n = 12$ in each group). Reprinted with permission from Sliwinski et al. (2004).

solvolysis of the aqueous phase to release pregnenolone and DHEA, which was then measured by radioimmunoassay using antisera directed against pregnenolone or DHEA.

A subsequent study by Mathur et al. (1993) utilized gas-chromatography-mass spectrometry (GC-MS) to measure pregnenolone and DHEA following a similar liquid–liquid extraction and solvolysis, and reported similarly high levels of PS and DHEAS in rat, rabbit, and dog brain. Liere et al. (2000) utilized solid-phase extraction (SPE) on a C18 column, followed by elution in either 40%/60% or 85%/15% methanol/water to obtain presumptive sulfated steroid and unconjugated steroid fractions. The sulfated steroid fraction was then subjected to solvolysis and derivatization with heptafluorobutyric anhydride (HFBA) and was assayed by GC-MS, yielding an estimate of 8.3 ng PS and 2.5 ng DHEAS/g rat brain.

In contrast, a study in which PS was derivatized directly and assayed without solvolysis by liquid chromatography/electrospray ionization-mass spectrometry (LC/ESI-MS) found only very low levels (ca. 0.5 ng/g tissue) in rat brain, close to the limit of sensitivity of the assay, even though the assay was able to detect PS in samples of brain homogenate that were "spiked" with exogenous PS at levels comparable to those reported in earlier studies of brain tissue (Mitamura et al., 1999). A subsequent study utilizing nanoscale LC/ESI-MS to measure levels of PS without derivatization concluded that the level of PS in rat brain was below the detection limit of 0.3 ng/g tissue (Liu et al., 2003). Higashi et al. (2003a) developed an ELISA based upon a polyclonal antibody directed against PS. This method does not require solvolysis, although an initial solid phase extraction step was used to remove unconjugated pregnenolone because the assay exhibited 4% cross-reactivity toward unconjugated pregnenolone. Based upon the assay of samples spiked with 0.5 ng PS/g tissue, recovery of PS was 60%, while endogenous PS in rat brain was estimated as 0.05–0.4 ng/g brain tissue. Similarly, using a DHEAS ELISA, Higashi et al. reported low DHEAS levels in rat brain, with a maximum level of 0.41 ng/g tissue in diencephalon (Higashi et al., 2001). Ebner et al. (2006) used anion exchange chromatography to separate free and sulfated steroids prior to solvolysis and GC-MS, and failed to detect PS in rat brain at a detection limit of 0.3 ng/g tissue, although DHEAS was detected.

The discrepancy between the high levels of PS and DHEAS in rat brain estimated by measuring pregnenolone and DHEA following solvolysis versus the lower tissue levels estimated using direct methods of PS and DHEAS measurement led to the suggestion that much of the pregnenolone and DHEA obtained after solvolysis may be derived from steroid conjugates that co-purify with sulfated steroids, but which are distinct from PS and DHEAS (Liu et al., 2003; Higashi et al., 2003b). To address this question, Liere et al. (2004) revisited their SPE method of purifying steroid sulfates. They found that introducing a water wash of the C18 column prior to methanol/water elution improved recovery of ^{3}H-PS in the 40%/60% methanol/water fraction, but with this revised procedure they detected little or no PS in rat brain. Analysis of the SPE water wash revealed large amounts of phospholipids, while solvolysis and derivatization of this fraction followed by GC-MS revealed substantial levels of pregnenolone. Liere et al. concluded that the water wash contained a lipoidal pregnenolone derivative, possibly in the form of micelles. Furthermore, these lipoidal derivatives

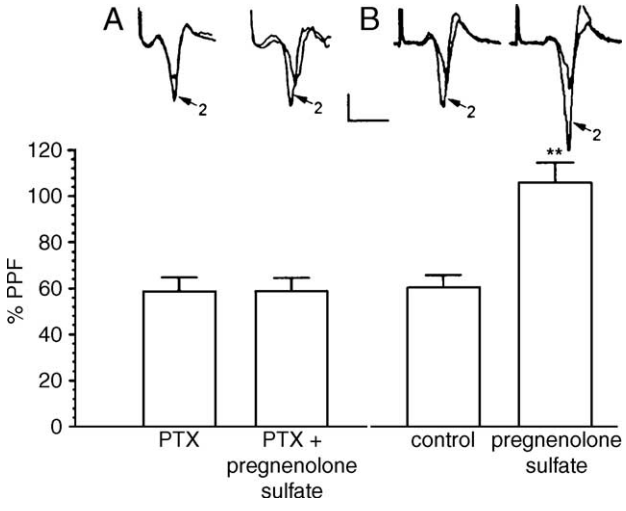

Fig. 8. Blocking Gi/o activation prevents PS enhancement of PPF. Effect of pregnenolone sulfate on PPF in slices maintained for a minimum of 12 h compared to PPF in sister slices maintained under the same conditions. Recordings were made between 30 and 48 min after switching to PS + pertussis toxin or to PS. A, Response to 1 μM pregnenolone sulfate in sister slices maintained in 50 ng/ml pertussis toxin (paired t-test $P > 0.5$ $n = 5$). B. Response to 1 μM PS in sister slices maintained in aCSF (paired t-test $P < 0.005$, $n = 5$). Scale bars for inset: 1 mV, 5 ms. Reprinted from Schiess and Partridge (2005), Copyright 2005 by Elsevier, with permission from Elsevier.

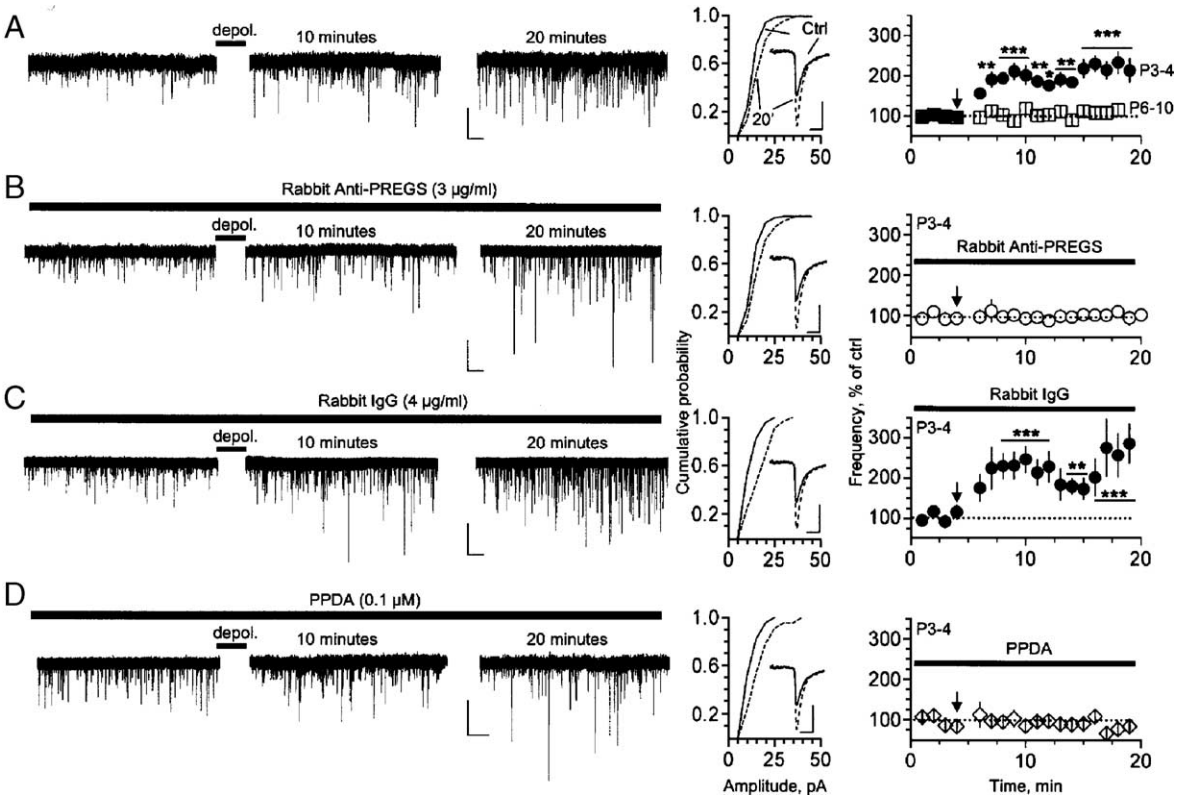

Fig. 9. A PS-like neurosteroid, which retrogradely modulates NMDA receptors, mediates the depolarization-induced increase of mEPSC frequency. A, Depolarization (depol.)-induced increase of mEPSC frequency can be observed in CA1 pyramidal neurons from P3–P4 ($n=19$) but not P6–P10 ($n=7$; traces not shown) rats. The cumulative probability plots and average traces (inset) illustrate that mEPSC amplitude also increased 20 min after depolarization in P3–P4 neurons (compare solid vs dashed traces). A similar result was obtained with P6–P10 neurons ($n=7$; data not shown). B, Preincubation (14 min) with rabbit anti-PS IgG blocks the depolarization-induced increase of mEPSC frequency but not the increase in amplitude in P3–P4 neurons ($n=14$). C, Incubation with rabbit IgG neither affects the depolarization-induced increase in frequency nor the increase in amplitude in P3–P4 neurons ($n=6$). D, Incubation with 1-(phenanthrene-2-carbonyl)piperazine-2,3-dicarboxylic acid (PPDA), a compound that preferentially antagonizes NR2C/D subunit-containing receptors, blocks the depolarization-induced increase of mEPSC frequency but not the increase in amplitude in P3–P4 neurons ($n=5$). Calibration: A–D, 20 pA, 5 s (left); 8 pA, 12 ms (right). *$p<0.05$; **$p<0.01$; ***$p<0.001$. Ctrl, Control. Error bars represent SEM. Reprinted with permission from Mameli et al. (2005), Copyright 2005 by the Society for Neuroscience.

also appear in the aqueous phase after a liquid–liquid extraction similar to that used in earlier studies. Similar results were obtained for DHEAS. It thus appears likely that the estimates of brain PS and DHEAS from earlier studies employing solvolysis were inflated due to contamination by these lipoidal pregnenolone derivatives.

Unfortunately, Liere et al. were unable to identify the chemical nature of the interfering lipoidal derivatives of pregnanolone. In particular, because HFBA derivitization results in hydrolysis of the sulfate group, if present, it is not clear whether these lipoidal derivatives contain pregnenolone in its sulfated or nonsulfated form. The presence of lipophilic steroid sulfoconjugates in plasma and brain has been suggested by a number of investigators (Kurtenbach et al., 1973; Oertel et al., 1970; Mathur et al., 1993). Such conjugates could conceivably represent a storage or "depot" form of sulfated neurosteroids. Liere et al. (2004) did not favor this hypothesis, based on the indirect evidence that HFBA hydrolysis/derivitization of PS, DHEAS, and pregnenolone tosylate was inhibited by triethylamine, which enhanced derivitization of pregnenolone from the lipoidal fraction. Although Liere et al. did not detect appreciable amounts of PS or DHEAS in rat brain, they did find significant amounts of both steroids at in two samples of aged human brain (PS, 1.11 and 1.85 ng/g tissue; DHEAS, 13.4 and 7.9 ng/g) (Liere et al., 2004), suggesting that the rat may not be the best mammalian model for studying the potential role of sulfated neurosteroids in human.

Until the nature of the lipoidal steroid fraction is elucidated, the question of sulfated neurosteroid levels in brain remains unresolved. It seems clear that bulk or average regional levels of free PS and DHEAS may be lower than previously reported, although a more conclusive investigation will necessarily await the development of better inhibitors of sulfatase activity and the development of selective antibodies for the identification of PS in situ. Studies of PS and DHEAS abundance in brain have not in general employed inhibitors of steroid sulfatases, which could possibly degrade these steroids. Recovery of PS standards added to brain tissue or homogenate has generally been good, arguing against degradation by brain sulfatases as an explanation for low levels, but the possibility that rapid degradation of PS could occur as cells are disrupted cannot be excluded. The supposed abundance of PS in rat brain has been interpreted as supporting a functional role for PS in the nervous system. The finding that PS levels are lower than previously believed raises doubts about this interpretation; however, an important caveat is that measurements of bulk tissue levels shed little light upon the

concentration of PS at the synapse, which could be much higher if PS is compartmentalized, made and released locally, or synthesized in response to stimulation.

4. Synthesis and degradation of sulfated steroids by sulfotransferases and sulfatases

A related question concerns the presence in neural tissue of steroid sulfotransferase enzymes capable of the synthesis of sulfated neurosteroids. Using RT-PCR, Shimada et al. detected mRNA in rat brain for ST2A1, a rat steroid sulfotransferase. The presence of the enzyme in brain was confirmed by Western blot using an anti-ST2A1 antibody. Immunoprecipitated enzyme was able to catalyze transfer of radioactivity from ^{35}S-PAPS to pregnenolone or DHEA, supporting the identification of the enzyme as a neurosteroid sulfotransferase. ST2A1 expressed in *E. coli* exhibited the ability to catalyze sulfation of pregnenolone, as well as DHEA, allopregnanolone, and corticosterone (Shimada et al., 2001). In rat and mouse, the mRNA for the steroid sulfotransferase SULT2B1a, which has high activity toward pregnenolone, is expressed almost exclusively in brain (Kohjitani et al., 2006; Shimizu et al., 2003), although in human it seems to be expressed only in fetal brain (Geese and Raftogianis, 2001). Kimoto et al reported immunochemical staining for hydroxysteroid sulfotransferase, as well as other neurosteroidogenic enzymes in rat hippocampal pyramidal and granule neurons (Fig. 10).

Moreover, tissue content of both pregnenolone and PS was found to increase following stimulation of hippocampal cubes by NMDA (Kimoto et al., 2001), although PS content was measured using the earlier method of Liere et al. (2000), which is

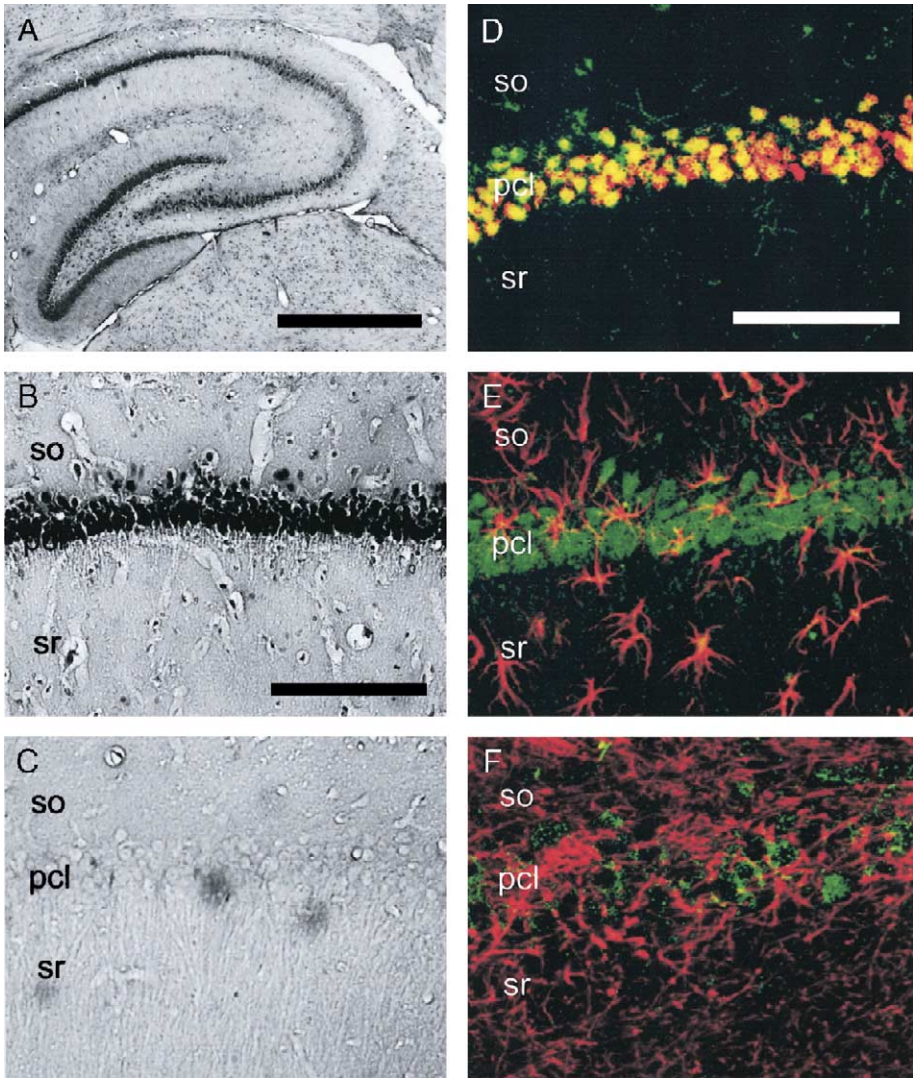

Fig. 10. Immunohistochemical staining of the hydroxysteroid sulfotransferase in hippocampal slices of an adult male rat. A, Low magnification image of the whole hippocampus, stained with antibodies against rat hydroxysteroid sulfotransferase. B, The hippocampal CA1 region, stained with antibodies against rat sulfotransferase. C, Staining with anti-sulfotransferase IgG, preincubated with a saturating concentration of purified hydroxysteroid sulfotransferase in the CA1 region. D, Fluorescence dual staining of the sulfotransferase (*green*) and NeuN (*red*). E, Fluorescence dual staining of the sulfotransferase (*green*) and GFAP (*red*). F, Fluorescence dual staining of the sulfotransferase (*green*) and MBP (*red*). A superimposed region of green and red fluorescence is represented in *yellow*. B and C, and D–F are at the same magnification. so, Stratum oriens; pcl, pyramidal cell layer; sr, stratum radiatum. A–C, Immunoreactive cells were visualized by diaminobenzidine-nickel staining. *Scale bar*, 800 μm (A), 120 μm (B and C), and 100 μm (D–F). Reprinted with permission from Kimoto et al. (2001).

potentially subject to interference by lipoidal pregnenolone derivatives (Liere et al., 2000, 2004). Using an antiserum against a conserved region of rat hydroxysteroid sulfotransferase, Beaujean et al. found immunoreactivity in the brain of frog *Rana ridibunda*. Immunoreactivity was present in neuronal cell bodies in hypothalamus and anterior preoptic area, as well as in nerve fibers of diencephalon and telencephalon. Incubation of hypothalamus or telencephalon homogenates with ^{3}H-pregnenolone and ^{35}S-PAPS resulted in incorporation of both labels into a HPLC peak with the mobility of PS. Similar experiments indicated the formation of DHEAS from DHEA and PAPS (Beaujean et al., 1999). Moreover, formation of these two neurosteroids by hypothalamic explants was inhibited by neuropeptide Y, which is also present in hypothalamus, and this inhibition was blocked by specific neuropeptide Y antagonists, suggesting that synthesis of sulfated steroids is dynamically regulated (Beaujean et al., 2002).

If PS does play a role as an endogenous regulator of synaptic transmission, a mechanism for removal is required. Because pregnenolone appears to be inactive, both as a receptor modulator and as a presynaptic modulator of neurotransmitter release, a plausible pathway for inactivation of PS is by removal of the sulfate by brain steroid sulfatase. Steroid sulfatase activity has been reported in rodent (Mortaud et al., 1996; Compagnone et al., 1997), bovine (Park et al., 1997), monkey (Kríz et al., 2005) and human (Steckelbroeck et al., 2004) brain. DHEAS levels in rat brain have been reported to increase following chronic treatment with the 3β-hydroxysteroid sulfatase inhibitor DU-14 (Johnson et al., 1997). More recently, a study by Caldeira et al. (2004) examined the effect of DU-14 on levels of PS in coronal brain slices from neonatal rats, using liquid/liquid extraction followed by radioimmunoassay with an antibody that recognizes both PS and pregnenolone. They found levels of PS ranging from ca. 0.5–6 ng/g tissue, which increased 276% in tissue treated with DU-14. Even given the uncertainties regarding the measurement of sulfated steroids, it is difficult to understand how nervous tissue could contain the biosynthetic machinery for synthesizing and degrading PS, and how such increases in PS levels following inhibition of steroid sulfatase activity could occur unless nervous tissue contained the ability to express substantial amounts of sulfated steroids.

5. Summary

The question of whether sulfated neurosteroids play a physiological role as regulators of ongoing synaptic transmission remains a provocative and appealing area for further exploration. With presynaptic effects upon release of a variety of transmitters, combined with postsynaptic effects upon multiple neurotransmitter receptors, PS and DHEAS remain attractive candidates for a role as endogenous neuromodulators regulating the balance between excitatory and inhibitory neurotransmission. Important questions remain, however. It remains unclear whether sulfated neurosteroids ever attain the nanomolar to micromolar concentrations required for some modulatory effects upon receptors. This would be more likely if PS or DHEAS is produced locally in response to stimulation. This has not been demonstrated directly, but the presence of steroid sulfotransferases in neurons, combined with indirect evidence for a PS-like retrograde messenger that is released in response to depolarization (Mameli et al., 2005), suggests that this may occur. The question of the physiological role of sulfated neurosteroids would be easier to answer if specific antagonists were available, but so far none have been identified. Given the complexity of PS action on multiple receptor types, and perhaps multiple sites on NMDA receptors, a clean antagonist is perhaps too much to hope for. Another approach that may have promise is mutagenesis of specific receptor subtypes *in vivo* to disable steroid modulation. A chimeric NR2B/NR2D subunit constructed by Jang et al. confers insensitivity to PS modulation on NMDA receptors, but it also alters pH sensitivity of the receptor, which could be a confounding factor. Molecular modeling suggests specific contact residues for PS docking that should be effective targets for future exploratory mutagenesis studies (Jang et al., 2004).

Independently of whether sulfated neurosteroids play a physiological role, the investigation of the modulatory effects of these neuroactive steroids has identified novel potential targets for pharmacological intervention. In particular, PS and DHEAS enhance memory retention in mice (Flood and Roberts, 1988; Flood et al., 1998, 1992, 1995; Markowski et al., 2001) and block learning deficits induced by NMDA antagonists or scopolamine (Urani et al., 1998; Mathis et al., 1996; Meziane et al., 1996; Maurice et al., 1997), suggesting that sulfated neurosteroids or analogs may be useful in treatment of cognitive impairment. Weaver et al. have illustrated the potential for utilizing pregnanolone hemisuccinate and related neuroactive steroids for the treatment of ischemic brain injury, chronic pain, and seizures (Weaver et al., 1997, 2000) while Sadri-Vakili et al. (2003) illustrated its potential for modulating cocaine-induced behavioral activation and possibly cocaine addiction.

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