Bidirectional Effect of Pregnenolone Sulfate on GluN1/GluN2A *N*-Methyl-D-Aspartate Receptor Gating Depending on Extracellular Calcium and Intracellular Milieu

Divyan A. Chopra, Daniel T. Monaghan, and Shashank M. Dravid

Department of Pharmacology, Creighton University, Omaha, Nebraska (D.A.C., S.M.D.); and Department of Pharmacology and Experimental Neuroscience, University of Nebraska Medical Center, Omaha, Nebraska (D.T.M.)

Received June 16, 2015; accepted July 10, 2015

ABSTRACT

Pregnenolone sulfate (PS), one of the most commonly occurring neurosteroids in the central nervous system, influences the function of several receptors. PS modulates *N*-methyl-D-aspartate receptors (NMDARs) and has been shown to have both positive and negative modulatory effects on NMDAR currents generally in a subtype-selective manner. We assessed the gating mechanism of PS modulation of GluN1/GluN2A receptors transiently expressed in human embryonic kidney 293 cells using whole-cell and single-channel electrophysiology. Only a modest effect on the whole-cell responses was observed by PS in dialyzed (nonperforated) whole-cell recordings. Interestingly, in perforated conditions, PS was found to increase the whole-cell currents in the absence of nominal extracellular Ca^{2+} , whereas PS produced an inhibition of the current responses in the presence of 0.5 mM

extracellular Ca^{2+} . The Ca^{2+} -binding DRPEER motif and GluN1 exon-5 were found to be critical for the Ca^{2+} -dependent bidirectional effect of PS. Single-channel cell-attached analysis demonstrated that PS primarily affected the mean open time to produce its effects: positive modulation mediated by an increase in duration of open time constants, and negative modulation mediated by a reduction in the time spent in a long-lived open state of the receptor. Further kinetic modeling of the single-channel data suggested that the positive and negative modulatory effects are mediated by different gating steps which may represent GluN2 and GluN1 subunit-selective conformational changes, respectively. Our studies provide a unique mechanism of modulation of NMDARs by an endogenous neurosteroid, which has implications for identifying state-dependent molecules.

Introduction

Excitatory neurotransmission mediated by *N*-methyl-D-aspartate (NMDA) receptors (NMDARs) is known to play an important role in synaptic plasticity, learning, and memory (Traynelis et al., 2010). Moreover, NMDAR dysfunction may contribute to a variety of neuropsychiatric and neurological disorders, including schizophrenia, epilepsy, stroke, and trauma (Hedegaard et al., 2012). NMDARs are tetramers composed of two obligatory glycine-binding GluN1 subunits and usually two glutamate-binding GluN2 subunits. There are four types of GluN2 subunits, GluN2A to GluN2D. The function of NMDARs is regulated by endogenous modulators such as magnesium, protons, zinc, and neurosteroids (Traynelis et al., 2010). Pregnenolone sulfate (PS) is one of the most abundant neurosteroids formed by cleavage of the cholesterol side chain in glial cells (Robel and Baulieu, 1994), and it potentiates or inhibits the NMDARs in a subtype-selective manner (Malayev et al., 2002; Horak et al., 2006).

Initial studies in spinal cord neurons suggested that PS potentiation was dependent on the agonist concentration, with the potentiation being reduced at higher agonist concentrations and almost eliminated at 1 mM NMDA (Wu et al., 1991). Based on the agonist-concentration dependent effect, it has been suggested that PS increases the agonist efficacy/potency (Malayev et al., 2002). In oocyte experiments where PS is coapplied with agonists, PS typically potentiates GluN1/GluN2A currents (Yaghoubi et al., 1998; Malayev et al., 2002). However, in fast-jump experiments in a mammalian expression system [human embryonic kidney (HEK293) cells] coapplication of PS with agonists was found to typically slow the desensitization and deactivation kinetics of GluN1/GluN2A receptors but not to increase the steady-state currents (Ceccon et al., 2001; Horak et al., 2006). In contrast, preapplication of PS followed by agonist application leads to significant increase in the peak amplitude of GluN1/GluN2A currents (Bowlby, 1993; Horak et al., 2004). These studies indicate that the effect of PS is partly disuse dependent.

Overall, the effect of PS on GluN1/GluN2A receptors when coapplied with agonists differs in mammalian expression systems compared with oocyte expression systems, and this

This work was supported by the National Institutes of Health National Institute of Mental Health [Grant R01-MH060252].
dx.doi.org/10.1124/mol.115.100396

ABBREVIATIONS: BAPTA, 1,2-bis(*o*-aminophenoxy)ethane-*N,N,N',N'*-tetraacetic acid; DQP-1105, 5-(4-bromophenyl)-3-(1,2-dihydro-6-methyl-2-oxo-4-phenyl-3-quinolinyl)-4,5-dihydro-g-oxo-1*H*-pyrazole-1-butanoic acid; HEK293, human embryonic kidney 293 cells; MIL, maximum interval likelihood; NMDA, *N*-methyl-D-aspartate; NMDAR, *N*-methyl-D-aspartate receptors; PKA, protein kinase A; PS, pregnenolone sulfate; TM, transmembrane.

discrepancy has not yet been resolved, although it may involve the phosphorylation state of the receptor (Petrovic et al., 2009). Studies using site-directed mutagenesis and chimeric receptors suggest that the linker regions connect transmembrane (TM) 3 and 4 to the S2 domain of the ligand-binding domain, and part of TM3 and TM4 is a critical site of action of neurosteroids including PS (Jang et al., 2004; Horak et al., 2006). The action of PS has also been found to be mediated partly by relief of proton inhibition at GluN2A- and GluN2D-containing receptors but not GluN2B- and GluN2C-containing receptors (Kostakis et al., 2011).

The effects of PS on single-channel kinetics and gating of NMDARs remain poorly understood. Using whole-cell and cell-attached electrophysiology, we have identified a novel aspect of PS action. Specifically, our studies indicate extracellular Ca^{2+} - and intracellular milieu-dependent actions of PS, which may provide novel insights into the positive and negative modulatory effects of PS. These novel paradigms also have important implications for our understanding of the physiologic roles of PS.

Materials and Methods

Expression of Recombinant NMDARs. HEK293 cells were maintained as previously described elsewhere (Dravid et al., 2008). The cells were transiently transfected with Viafect reagent (Promega, Madison, WI). Rat GluN1-1a (GenBank U11418, U08261; pCIneo vector; hereafter GluN1, provided by Dr. Stephen Heinemann, Salk Institute, La Jolla, CA), GluN2A (GenBank D13211, pCIneo vector, provided by Dr. Shigetada Nakanishi, Osaka Bioscience Institute, Osaka, Japan), and green fluorescent protein in the ratio of 1:2:0.5 were used as previously described elsewhere (Bhatt et al., 2013). The GluN1-1b splice variant and GluN1-1a-R663A mutant were provided by Dr. Stephen Tryanalis (Emory University, Atlanta, GA) and GluN2B (GenBank U11419, Q00960; pcDNA3.1 vector) was provided by Dr. Peter Seeburg (Max Planck Institute for Medical Research, Heidelberg, Germany). Electrophysiology experiments were performed 16–48 hours after transfection.

Electrophysiology. Electrophysiologic recordings in whole-cell and single-channel mode were obtained from transfected HEK293 cells at room temperature (22–25°C). An external solution containing (in millimolar) 150 NaCl, 3 KCl, 10 HEPES, 0.5 CaCl_2 , and 6 mannitol (to adjust osmolarity) was used for the recordings unless otherwise stated. Recordings were conducted in the absence of nominal extracellular CaCl_2 or in the presence of 0.5 mM CaCl_2 as indicated in the text for each experiment. The external pH was adjusted to 7.4 with NaOH. This solution was supplemented with 0.02 mM EDTA to chelate trace amounts of zinc.

The same external solution was used for whole-cell recordings as well as cell-attached recordings. Recordings were performed under two conditions: 1) 100 μM glutamate, 100 μM glycine (control patches); and 2) 100 μM glutamate, 100 μM glycine, and 100 μM PS (PS patches). Pregnenolone sulfate (β -hydroxy-5-pregnen-20-one 3-sulfate, or 3-hydroxypregn-5-en-20-one sulfate, or 5-pregnen- β -ol-20-one sulfate) sodium salt was obtained from Sigma-Aldrich (St. Louis, MO).

For whole-cell recordings, agonists and PS were added to the extracellular solution, and for cell-attached recordings, these drugs were present only in the pipette solution. Recordings were obtained using an Axopatch 200B amplifier (Axon Instruments/Molecular Devices, Sunnyvale, CA) and digitized with pCLAMP 10 software (Axon Instruments/Molecular Devices). Whole-cell recordings were obtained at -70 mV, filtered at 2 kHz, and digitized at 5 kHz. For cell-attached recordings a potential (V_m) of $+70$ mV was applied, and data were filtered at 5 kHz (-3 dB, 8-pole Bessel) and digitized at 20 kHz. Single-channel amplitude was not corrected for junction potential.

The internal solution used for whole-cell recordings consisted of (in millimolar) 110 cesium gluconate, 30 CsCl_2 , 5 HEPES, 4 NaCl, 0.5 CaCl_2 , 2 MgCl_2 , 5 BAPTA [1,2-bis(*o*-aminophenoxy)ethane-*N,N,N',N'*-tetraacetic acid], 2 Na_2ATP , and 0.3 Na_2GTP (pH 7.3). For perforated whole-cell patch-clamp recordings, 20 $\mu\text{g}/\text{ml}$ of gramicidin was added to the pipette internal solution. Whole-cell configuration after giga-ohm seal was reached typically within 10–15 minutes. Rapid perfusion for whole-cell concentration jumps was achieved with a two-barreled θ glass pipette controlled by a piezoelectric translator (Burleigh Instruments, Fishers, NY). The solution exchange times for 10–90% solution were typically ~ 1 –2 milliseconds. Two concentration profiles were obtained: 1) 100 μM glutamate and 100 μM glycine; and 2) 100 μM glutamate, 100 μM glycine, and 100 μM PS. The cell was moved from the control solution with no drugs to a solution containing agonists $\pm$ PS. Drug application was typically for 2.5 seconds during each 15-second sweep.

Data Processing and Kinetic Modeling. Recordings containing a single active channel were idealized using QUB software (SUNY Buffalo, Buffalo, NY) as previously described elsewhere (Dravid et al., 2008; Bhatt et al., 2013). The idealized data were used for maximum interval likelihood (MIL) fitting (Qin et al., 1996). A 120-microsecond dead time was imposed using QUB. All gating steps were free and not constrained. The C5O2 model, consisting of three closed and two open states in linear configuration and two desensitized states emerging from C1 and C2, respectively (see Fig. 7), provided the best fit to the single-channel data based on the log likelihood values. Other models tested included a C4O2 model with four instead of five closed states and a model where the receptor can transition to either a fast or slow gating step as described previously (Bhatt et al., 2013). The mean open time, mean shut time, and open probability were obtained from the idealized data using ChanneLab (Synaptosoft, Decatur, GA), with an imposed dead time of 120 microseconds. Peak and steady-state responses and deactivation, and desensitization time course for whole-cell recordings were analyzed using Clampfit (pCLAMP 10.2).

Statistical Analysis. All the values are expressed as mean $\pm$ S.E.M. Data were compared using a paired *t* test for macroscopic current profiles and unpaired *t* test for the cell-attached patches. $P \leq 0.05$ was considered statistically significant.

Results

Effect of Pregnenolone Sulfate on Macroscopic Currents Is Dependent on Intracellular Milieu and Extracellular Calcium. We tested the effect of PS on macroscopic GluN1/GluN2A whole-cell currents under dialyzed (nonperforated) conditions. PS (100 μM) was coapplied with glutamate (100 μM) and glycine (100 μM) (Fig. 1) to determine the optimum conditions for carrying out single-channel studies. PS (in the absence of nominal extracellular Ca^{2+}) was found to produce a modest but statistically significant reduction in the peak response ($P = 0.0285$, $N = 6$, paired *t* test, $I_{\text{PS}}/I_{\text{control}} = 0.898 \pm 0.021$) with no statistically significant effect on the steady-state responses ($P = 0.1037$, $I_{\text{PS}}/I_{\text{control}} = 0.855 \pm 0.040$). We further tested whether extracellular Ca^{2+} is a factor for absence of strong responses to PS. In the presence of 0.5 mM Ca^{2+} , PS was found to have no statistically significant effect on the peak response ($P = 0.1481$, $N = 5$, $I_{\text{PS}}/I_{\text{control}} = 1.050 \pm 0.082$) or steady-state responses ($P = 0.0669$, $I_{\text{PS}}/I_{\text{control}} = 1.086 \pm 0.063$).

Previous studies that have evaluated the effect of coapplied PS on whole-cell GluN1/GluN2A currents in HEK293 cells have found modest or no potentiation of steady-state currents when coapplied with agonists (Ceccon et al., 2001; Horak et al., 2006). Thus, our findings in whole-cell conditions are similar to

Fig. 1. Modulation of whole-cell responses by pregnenolone sulfate depends on the intracellular milieu and extracellular calcium. Whole-cell recordings under non-perforated (dialyzed) or perforated modes were obtained from HEK293 cells expressing GluN1/GluN2A receptors in the absence of nominal Ca²⁺ or in the presence of 0.5 mM extracellular Ca²⁺ (holding potential = -70 mV, filtered at 2 kHz, digitized at 5 kHz). Agonists (100 μ M glutamate and 100 μ M glycine) were applied in the absence (black traces) or presence of 100 μ M PS (red traces), and the peak and steady-state responses were evaluated. Responses were compared by paired *t* test. *P < 0.05; **P < 0.01.

these studies. In oocyte recordings, however, an increase in GluN1/GluN2A responses is consistently observed where, unlike in whole-cell recordings, the intracellular milieu is generally undisturbed.

It has previously been shown that NMDAR responses and their modulation by endogenous or synthetic molecules is affected by phosphorylation and dephosphorylation pathways (Petrovic et al., 2009; Acker et al., 2011). In a typical whole-cell recording, dialyzing the intracellular components might affect the phosphorylation/dephosphorylation machinery of the cell. Hence, we performed perforated whole-cell recordings using gramicidin to test whether keeping intracellular milieu intact would affect PS modulatory actions.

Under perforated whole-cell conditions and in the absence of extracellular Ca²⁺, PS statistically significantly increased the peak response (*P* = 0.00104, *N* = 7, $I_{PS}/I_{control}$ = 1.814 ± 0.073) and the steady-state response (*P* = 0.0019, $I_{PS}/I_{control}$ = 1.818 ± 0.097). In the presence of 0.5 mM extracellular Ca²⁺, in perforated patch mode PS significantly reduced the peak response (*P* = 0.0083, *N* = 7, $I_{PS}/I_{control}$ = 0.60 ± 0.036) and

the steady-state response (*P* = 0.0124, $I_{PS}/I_{control}$ = 0.582 ± 0.025). PS did not significantly affect the desensitization or deactivation time constants under any of the conditions tested (data not shown). A transient rise in current was observed in the whole-cell recordings when the solution containing PS was washed out (Fig. 1). This feature is similar to that demonstrated previously when PS and agonists are coapplied (Horak et al., 2004).

Molecular Determinants of Ca²⁺-Dependent Inhibition by Pregnenolone Sulfate. We further assessed the potential molecular determinants of extracellular Ca²⁺-dependent inhibition by PS. We first replicated the observation of inhibition of GluN1/GluN2A currents by PS in the presence of 0.5 mM external Ca²⁺ under perforated whole-cell recording conditions (Fig. 2A). One of the sites where Ca²⁺ binds in the extracellular vestibule is the DRPEER motif (Watanabe et al., 2002; Karakas and Furukawa, 2014). We tested the effect of PS on GluN1R663A/GluN2A receptors in perforated whole-cell patch-clamp recordings. This mutant was chosen based on its most exterior positioning, which may prevent it from having

Fig. 2. Molecular determinants of inhibition by pregnenolone sulfate in the presence of external Ca^{2+} . (A) PS (100 μM) in the presence of extracellular 0.5 mM Ca^{2+} reduced the glutamate (100 μM) + glycine (100 μM) induced responses. PS potentiated current responses at (B) GluN1R663A/GluN2A receptors as well as (C) GluN1-1b/GluN2A receptors. (D) PS did not inhibit current responses at GluN1/GluN2B, but neither did it significantly increase the responses. Traces in black represent control with glutamate and glycine, and traces in red represent responses in the presence of PS. Responses were compared by paired t test. Fold-change in current by PS relative to control is plotted as individual bars with 1 representing the baseline. * $P < 0.05$; ** $P < 0.01$.

basal effects (as indicated in Watanabe et al., 2002) yet may allow for testing the importance of this region in the modulatory effect of PS.

We found that PS potentiated the peak current ($P = 0.0389$, $N = 5$, $I_{\text{PS}}/I_{\text{control}} = 1.654 \pm 0.191$) as well as the steady-state current responses ($P = 0.0264$, $N = 5$, $I_{\text{PS}}/I_{\text{control}} = 1.543 \pm 0.138$) from GluN1R663A/GluN2A receptors in the presence of 0.5 mM Ca^{2+} (Fig. 2B). The degree of potentiation was comparable to the potentiation by PS in the absence of nominal Ca^{2+} . This finding demonstrates a critical role of the DRPEER motif in the bidirectional effect of PS depending on extracellular Ca^{2+} .

We further tested the effect of exon-5 insert (present in GluN1-1b), which is a key molecular determinant for proton and zinc inhibition (Traynelis et al., 2010) as well as proton-dependent differential efficacy of PS at GluN1/GluN2A versus GluN1/GluN2B receptors (Kostakis et al., 2011). The whole-cell responses at GluN1-1b/GluN2A receptors were statistically significantly increased by PS in the presence of 0.5 mM Ca^{2+} (Fig. 2C), showing an increase in the peak current ($P = 0.0357$, $N = 7$, $I_{\text{PS}}/I_{\text{control}} = 1.525 \pm 0.105$) as well as steady-state current responses ($P = 0.0199$, $N = 7$, $I_{\text{PS}}/I_{\text{control}} = 1.360 \pm 0.11$). PS was also found to increase the deactivation kinetics of the GluN1-1b/GluN2A receptors (data not shown).

Together these data demonstrate that conformational changes induced by presence of exon-5 can mask the inhibitory effect of PS produced due to its allosteric interaction with Ca^{2+} binding at the DRPEER motif. However, it should

be noted that we cannot rule out other possibilities, such as a change in proton-sensitivity of the receptor leading to changes in the mechanism of action of PS.

Finally, we tested whether the Ca^{2+} -dependent inhibition is specific for GluN2A-containing receptors. In contrast to GluN1/GluN2A receptors, no statistically significant inhibition by PS was observed in the presence of Ca^{2+} at the GluN1/GluN2B receptors ($N = 5$; Fig. 2D), although no potentiation was observed either. However, PS did statistically significantly increase the decay kinetics of the GluN1/GluN2B receptors (data not shown).

Pregnenolone Sulfate Affects Mean Open Time of GluN1/GluN2A Receptors. After we had identified the conditions where the potentiating and inhibiting effects of PS are robust, we assessed the single-channel effects of PS under these conditions. We obtained cell-attached patches with one active channel for evaluating the effect of PS on GluN1/GluN2A gating (Fig. 3).

In the first set of recordings, CaCl_2 was absent from the pipette internal solution. The mean open time ($\pm$ S.E.M.) in the control patches was found to be 1.52 ± 0.17 milliseconds (114,675 events; $n = 9$). In the presence of PS, the mean open time was statistically significantly higher: 3.11 ± 0.24 milliseconds (93,505 events; $n = 5$, $P = 0.00017$, unpaired t test). The mean shut time was not affected by PS: 17.3 ± 1.8 milliseconds in control patches (115,032 events) and 15.3 ± 2.9 milliseconds in PS patches (93,840 events, $P = 0.528$). The open probability, measured over the entire length of the

Fig. 3. Pregnenolone sulfate increases open probability of GluN1/GluN2A receptors. Representative steady-state, single-channel recording in cell-attached mode from patches containing one active GluN1/GluN2A receptor. The openings are downward for all the traces. The recording was obtained at 100 μ M glutamate and 100 μ M glycine [pipette potential = +70 mV, filtered at 5 kHz (2 kHz for presentation), digitized at 20 kHz] under control conditions or in the presence of PS (100 μ M). PS ($n = 5$) increased the mean open time of the receptor compared with control patches ($n = 9$) ($P = 0.00017$). PS did not have any significant effect on the mean shut time of the receptor ($P = 0.528$). The probability of opening (calculated individually over the length of entire recording) was found to be significantly increased by PS ($P = 0.0022$). Unpaired t test was used for comparison. ** $P < 0.01$; *** $P < 0.001$.

recordings, was found to increase from 0.082 ± 0.007 in control patches to 0.186 ± 0.034 in PS patches ($P = 0.0022$). The amplitude of openings was unaffected by PS: 5.01 ± 0.18 pA for control patches and 5.09 ± 0.16 pA for PS.

Thus, it appears that the major effect of PS is on the mean open time of the GluN1/GluN2A receptors, which leads to higher open probability in the presence of PS. Compared with previous studies the overall open probability of GluN1/GluN2A was found to be lower in our cell-attached patches. This may be due to differences in the recording solutions or mode of recording or a difference in the modal gating of the receptor. However, it should be noted that under our recording conditions the mean open time and open probability for GluN1/GluN2A were higher compared with GluN1/GluN2B (Bhatt et al., 2013), with a similar order of magnitude as previously described elsewhere in outside-out patches (Erreger et al., 2005).

After we included 0.5 mM Ca²⁺ in the extracellular solution, PS was found to reduce the mean open time from 1.53 ± 0.18 milliseconds (57,406 events; $n = 5$; Fig. 4) in control patches to 0.72 ± 0.07 milliseconds (85,892 events; $n = 6$, $P = 0.0012$, unpaired t test). No statistically significant change in the mean shut time was observed in control patches: 19.3 ± 2.0 milliseconds (57,824 events) versus PS patches: 19.4 ± 3.9 milliseconds (85,903 events) ($P = 0.981$). The overall open probability was found to be statistically significantly reduced by PS in the presence of 0.5 mM Ca²⁺. The open probability in control patches was 0.076 ± 0.012 ,

which was statistically significantly reduced in the presence of PS to 0.041 ± 0.006 ($P = 0.023$). The amplitude of openings was unaffected by PS in the presence of extracellular Ca²⁺ (control = 5.24 ± 0.28 pA; PS = 5.27 ± 0.28 pA). Because no change in the mean shut time was seen, the reduction in open probability was primarily due to the shorter mean open time. No statistically significant differences in single-channel properties were observed in control patches obtained under conditions of the absence or presence of extracellular 0.5 mM Ca²⁺. Thus, extracellular Ca²⁺ bidirectionally modulates PS responses similar to the results obtained in perforated whole-cell recordings.

Pregnenolone Sulfate Produces Unique Effects on Open and Shut Time Constants That Underlie Potentiation and Inhibition of GluN1/GluN2A Receptors. We further evaluated the effect of PS on the open and shut time characteristics. We fitted the single-channel data from individual patches using MIL to a model consisting of two open states and five shut states, of which two were the longer desensitized states as previously described elsewhere (Dravid et al., 2008). Both control and PS patches produced reasonably good fits with this scheme, suggesting that this model provides a reasonable description of the receptor gating (assuming that in the presence of 100 μ M PS the receptor binding sites for PS are close to 100% occupied).

We first compared the patches obtained in the absence of extracellular Ca²⁺, which showed ~2-fold potentiation in the open probability. The global fit for all events in the absence of extracellular Ca²⁺ is presented in Fig. 5, and the time

0.5mM Ca²⁺ cell-attached recordings

Fig. 4. Effect of pregnenolone sulfate on the GluN1/GluN2A receptor single-channel properties depends on extracellular Ca²⁺. Cell-attached recordings were obtained with addition of 0.5 mM Ca²⁺ to the pipette internal solution. The representative steady-state, single-channel control recording is from patches containing one active GluN1/GluN2A receptor with the absence or the presence of PS (100 μM). PS ($n = 6$) reduced the mean open time of the receptor compared with control patches ($n = 5$) ($P = 0.0013$). PS did not have any significant effect on the mean shut time of the receptor ($P = 0.981$). The probability of opening was significantly reduced by PS ($P = 0.0229$). Data are compared with unpaired t test. * $P < 0.05$; ** $P < 0.01$.

constants and area of individual patches are presented in Table 1.

A major effect of PS as evident from this analysis is an increase in time constants for the open states [control, $\tau_1 =$

0.31 ± 0.04 , $\tau_2 = 1.76 \pm 0.26$; PS, $\tau_1 = 0.86 \pm 0.27$ ($P = 0.0179$); $\tau_2 = 3.62 \pm 0.37$ ($P = 0.0013$)]. The shift in time constants is consistent with an increase in the mean open time observed in the presence of PS (Fig. 3). No

Fig. 5. Pregnenolone sulfate-mediated potentiation of GluN1/GluN2A receptors involves a shift in open states to longer durations and a reduction in the occupancy of long-lived shut states. The single-channel currents from cell-attached patches in the absence of nominal Ca²⁺ with one active GluN1/GluN2A receptor were idealized for each patch and summed to generate global dwell time histograms. The open time histogram was fitted by a sum of two exponential components: control, 114,675 open events ($n = 9$); PS, 93,505 events ($n = 5$). The time constants and percentage area are shown in the inset. The dwell times from each patch were individually fitted and are presented in Table 1. PS was found to significantly increase τ_1 and τ_2 time constants but not the area (Table 1). The composite shut time histogram was fitted by a sum of five exponential functions: control, 115,032 closed periods ($n = 9$); PS, 93,840 closed periods ($n = 5$). PS was found to significantly reduce the τ_2 and τ_3 time constants and increase τ_5 (Table 1). Only the area of τ_5 was significantly reduced by PS. SQRT, square root.

TABLE 1

Time constants and areas of closed and open components obtained from exponential fits

Comparison of the time constants (τ , in milliseconds) and relative contribution (a, % area of the component) of the open time and shut time components obtained from individual fits to the cell-attached patches. Data are mean $\pm$ S.E.M. The values were compared by unpaired *t* test.

Time Constants and Areas	0 Calcium		0.5 Calcium	
	Control (<i>n</i> = 9)	PS (<i>n</i> = 5)	Control (<i>n</i> = 5)	PS (<i>n</i> = 6)
Open time				
τ_1 (ms)	0.31 $\pm$ 0.04	0.86 $\pm$ 0.27*	0.39 $\pm$ 0.09	0.50 $\pm$ 0.04
τ_2 (ms)	1.76 $\pm$ 0.26	3.62 $\pm$ 0.37**	1.70 $\pm$ 0.21	2.30 $\pm$ 0.61
a1 (%)	22 $\pm$ 4	25 $\pm$ 8	22 $\pm$ 7	87 $\pm$ 5***
a2 (%)	78 $\pm$ 4	75 $\pm$ 8	78 $\pm$ 7	13 $\pm$ 5***
Shut time				
τ_1 (ms)	0.63 $\pm$ 0.04	0.63 $\pm$ 0.16	0.69 $\pm$ 0.12	1.03 $\pm$ 0.09*
τ_2 (ms)	5.80 $\pm$ 0.43	2.93 $\pm$ 0.53**	5.50 $\pm$ 1.08	6.70 $\pm$ 0.79
τ_3 (ms)	19.3 $\pm$ 1.2	12.4 $\pm$ 1.3**	18.7 $\pm$ 3.5	21.3 $\pm$ 2.4
τ_4 (ms)	73.6 $\pm$ 8.8	50.4 $\pm$ 10.1	90.0 $\pm$ 34.6	117.0 $\pm$ 47.9
τ_5 (ms)	1117 $\pm$ 198	3249 $\pm$ 759*	1406 $\pm$ 227	2176 $\pm$ 628
a1 (%)	30 $\pm$ 4	33 $\pm$ 7	37 $\pm$ 4	41 $\pm$ 3
a2 (%)	27 $\pm$ 1	31 $\pm$ 4	24 $\pm$ 3	28 $\pm$ 4
a3 (%)	37 $\pm$ 2	32 $\pm$ 5	32 $\pm$ 5	27 $\pm$ 2
a4 (%)	4 $\pm$ 1	4 $\pm$ 2	7 $\pm$ 2	4 $\pm$ 2
a5 (%)	0.6 $\pm$ 0.1	0.2 $\pm$ 0.04*	0.5 $\pm$ 0.1	0.4 $\pm$ 0.1

*P < 0.05; **P < 0.01; ***P < 0.001.

statistically significant change was observed in the areas of open states.

Analysis of shut time constants revealed that the time constants τ_2 , τ_3 , and τ_5 were statistically significantly different (Table 1). The τ_2 (*P* = 0.0014) and τ_3 (*P* = 0.0038) were reduced in the presence of PS, and τ_5 (*P* = 0.0044) was statistically significantly increased. No change in the area of shut time constants was observed except that of τ_5 , which was reduced in the presence of PS (*P* = 0.0351).

We next evaluated the effect of PS on open and shut times in the presence of 0.5 mM extracellular Ca^{2+} (Fig. 6; Table 1). As clearly evident, a statistically significant reduction in the areas of the longer time constant τ_2 (*P* = 0.00002) and an

increase in areas of τ_1 (*P* = 0.00002) were found in the presence of PS; however, no change of the time constants themselves was observed [control, τ_1 = 0.39 $\pm$ 0.09 (22 $\pm$ 7%), τ_2 = 1.70 $\pm$ 0.21 (78 $\pm$ 7%); PS, τ_1 = 0.50 $\pm$ 0.04 (87 $\pm$ 5%), τ_2 = 2.30 $\pm$ 0.61 (13 $\pm$ 5%)]. These results suggest that the primary inhibitory effect of PS is via reduction in the dwell time in the longer open state. No statistically significant differences were observed in the shut time constants except τ_1 , which was statistically significantly increased in the presence of PS (*P* = 0.0417) (Table 1).

Previous studies suggested that the shut time components consisting of τ_2 and τ_3 likely represent a conformational change in the GluN2 subunit and that in τ_1 may represent

Fig. 6. Inhibitory effect of pregnenolone sulfate on GluN1/GluN2A receptors is primarily due to reduced dwell time in a longer open state. Global dwell-time histograms were generated by summation of idealized data from individual cell-attached patches in the presence of 0.5 mM extracellular Ca^{2+} . The open time histogram was fitted by a sum of two exponential components: control, 57,406 open events (*n* = 5); PS, 85,892 events (*n* = 6). The time constants and percentage area are shown in the inset. The dwell times from each patch were individually fitted and are presented in Table 1. PS was found to significantly increase the area of τ_1 and reduce the area of τ_2 with no change in the time constants themselves (Table 1). The composite shut time histogram was fitted by a sum of five exponential functions: control, 57,824 closed periods (*n* = 5); PS, 85,903 closed periods (*n* = 6). PS was found to significantly increase τ_1 but not other time constants or areas of the components (Table 1). SQRT, square root.

a conformational change in the GluN1 subunit (Banke and Traynelis, 2003; Erreger et al., 2005). Thus, the specific changes in τ_2 and τ_3 by PS in the absence of extracellular Ca^{2+} may indicate GluN2-selective conformational change, and specific change in τ_1 in the presence of 0.5 mM Ca^{2+} may represent conformational change in the GluN1 subunit.

Pregnenolone Sulfate Influences Different Gating Steps to Produce Potentiation and Inhibition of GluN1/GluN2A Receptors. We addressed the specific effect of PS on the gating steps during receptor activation using a model previously described for NMDARs including GluN1/GluN2A receptors (Dravid et al., 2008; Kussius and Popescu, 2009). MIL fitting to the model demonstrated that in the absence of nominal Ca^{2+} , PS increased the rates for forward steps $\text{C}_1 \rightarrow \text{C}_2$ ($P = 0.0025$) and $\text{C}_2 \rightarrow \text{C}_3$ ($P = 0.0264$) and the reverse step $\text{C}_2 \rightarrow \text{C}_1$ ($P = 0.0075$) (Table 2). The reverse rate constant from $\text{O}_1 \rightarrow \text{C}_3$ was reduced in the presence of PS ($P = 0.0004$). These findings are also predicted by the changes in open time constants τ_1 and τ_2 and shut time constants τ_2 and τ_3 (Table 1).

In contrast, PS in the presence of 0.5 mM Ca^{2+} , which reduces open probability and mean open time, leads to an increase in the rate of reverse gating step from $\text{O}_1 \rightarrow \text{C}_3$ ($P = 0.0117$) and to a dramatic reduction in the forward $\text{O}_1 \rightarrow \text{O}_2$ ($P = 0.0029$) rate constant, which is predicted by a reduction in the area of the open time constant τ_2 (Table 1). These results indicate that the positive and negative modulatory effects of PS may not be mediated via effects on the same gating steps.

Discussion

In this study, we found that in dialyzed whole-cell recordings, PS produced only modest effects on the steady-state currents from GluN1/GluN2A receptors; however, the effect of PS was significantly different in perforated whole-cell recordings where the intracellular milieu is likely to be closer to the native system. Under perforated conditions, PS was found to cause potentiation of steady-state currents in the absence of Ca^{2+} and inhibition in the presence of 0.5 mM Ca^{2+} . In single-channel cell-attached recordings, PS was similarly found to

produce bidirectional effects depending on extracellular Ca^{2+} . When PS was coapplied with agonists in the absence of Ca^{2+} , PS led to an increase in the open probability whereas in the presence of 0.5 mM Ca^{2+} PS led to a reduction in the open probability of GluN1/GluN2A receptors.

Mechanism for Positive and Negative Modulatory Effects of Pregnenolone Sulfate. It has been shown that the action of PS on NMDARs is phosphorylation dependent, so intracellular milieu may affect PS-mediated modulation. Previous studies have reported PS modulation to be mediated partly by protein kinase A (PKA) (Petrovic et al., 2009). The potentiating effect of PS in outside-out patches is lost after 2 minutes of obtaining the patch, and this effect can be reversed by addition of PKA (Petrovic et al., 2009). Thus, one possibility is that the potentiating effect on steady-state currents is lost in dialyzed whole-cell mode due to inhibition of intracellular PKA or other changes that affect NMDAR posttranslational modifications.

This phenomenon of intracellular milieu-dependent effects may also be true for other agents. Recent studies have demonstrated that the IC_{50} of a subtype-selective negative allosteric modulator DQP-1105 [5-(4-bromophenyl)-3-(1,2-dihydro-6-methyl-2-oxo-4-phenyl-3-quinolinyl)-4,5-dihydro-g-oxo-1H-pyrazole-1-butanoic acid] is dependent on whole-cell configuration. It was found that the inhibitory action of DQP-1105 was reduced at GluN1/GluN2A receptors and a greater magnitude of subtype-selectivity between GluN1/GluN2D and GluN1/GluN2A receptors was observed in perforated mode of recording using gramicidin compared with under dialyzed condition (Acker et al., 2011). Also of note, the whole-cell dialyzed configuration is known to increase the desensitization of the NMDAR (Sather et al., 1990). Because GluN2A-containing receptors have greater desensitization while GluN2D-containing receptors appear to lack desensitization (Traynelis et al., 2010), it is likely that the difference in open probability in the two-preparations for the two subtypes may also underlie the differential effect of DQP-1105. This may also be relevant to the differential effect of PS in dialyzed and perforated conditions in our present study.

Our data suggest that 0.5 mM Ca^{2+} is sufficient to switch PS modulation from positive to negative effect under perforated whole-cell mode and in cell-attached recordings (Figs. 1–4). Thus, the potential site of Ca^{2+} binding or action may provide understanding of the inhibitory site of PS. Two Ca^{2+} binding sites have been identified in the NMDARs. One is present in the pore of a functional NMDAR (Jahr and Stevens, 1993). The other Ca^{2+} binding site is present in the DRPEER region located in the linker region between the ligand-binding domain and transmembrane domain of the GluN1 subunit (Watanabe et al., 2002). Both sites have been shown to increase the Ca^{2+} -block of the channel and reduce the channel conductance (Jahr and Stevens, 1993; Watanabe et al., 2002). However, we did not find a significant change in single-channel amplitude under control 0.5 mM Ca^{2+} conditions or with PS, which only reduced the mean open time. Thus, the inhibitory mechanism of external Ca^{2+} alone appears to be different from the inhibitory effect of PS.

Using GluN2A and GluN2C chimeras and site-directed mutagenesis, it has been shown that PS does not act by binding to the amino terminal domain, ligand-binding domain, or intracellular carboxyl-terminal domain of GluN2A receptors (Horak et al., 2006; Cameron et al.,

TABLE 2

Hidden Markov maximum interval likelihood fitting of the steady-state currents

Idealized current records were fitted to the gating scheme as described in Fig. 7. All rates have units of s^{-1} . Data are mean $\pm$ S.E.M. from patches containing one active channel fitted individually. The rates were compared by unpaired t test.

Rates	0 Calcium		0.5 Calcium	
	Control ($n = 9$)	PS ($n = 5$)	Control ($n = 5$)	PS ($n = 6$)
	s^{-1}			
$\text{C}_1 \rightarrow \text{C}_2$	95 $\pm$ 5	160 $\pm$ 20**	115 $\pm$ 30	85 $\pm$ 15
$\text{C}_2 \rightarrow \text{C}_1$	45 $\pm$ 5	140 $\pm$ 40**	50 $\pm$ 15	35 $\pm$ 5
$\text{C}_2 \rightarrow \text{C}_3$	260 $\pm$ 10	485 $\pm$ 120*	230 $\pm$ 35	180 $\pm$ 15
$\text{C}_3 \rightarrow \text{C}_2$	865 $\pm$ 90	825 $\pm$ 190	830 $\pm$ 220	410 $\pm$ 40
$\text{C}_3 \rightarrow \text{O}_1$	735 $\pm$ 60	860 $\pm$ 140	825 $\pm$ 115	640 $\pm$ 75
$\text{O}_1 \rightarrow \text{C}_3$	1665 $\pm$ 150	630 $\pm$ 80***	1280 $\pm$ 135	2130 $\pm$ 220**
$\text{O}_1 \rightarrow \text{O}_2$	1230 $\pm$ 340	825 $\pm$ 515	920 $\pm$ 230	70 $\pm$ 30**
$\text{O}_2 \rightarrow \text{O}_1$	1655 $\pm$ 280	885 $\pm$ 230	1745 $\pm$ 480	815 $\pm$ 290
$\text{C}_1 \rightarrow \text{D}_1$	2.1 $\pm$ 0.4	1.4 $\pm$ 0.3	3.0 $\pm$ 1.4	1.7 $\pm$ 0.5
$\text{D}_1 \rightarrow \text{C}_1$	1.3 $\pm$ 0.3	0.4 $\pm$ 0.1	0.8 $\pm$ 0.2	0.7 $\pm$ 0.2
$\text{C}_2 \rightarrow \text{D}_2$	4.6 $\pm$ 1.2	11.3 $\pm$ 4.4	8.4 $\pm$ 1.9	5.5 $\pm$ 2.0
$\text{D}_2 \rightarrow \text{C}_2$	15.8 $\pm$ 1.5	25.7 $\pm$ 6.3	18.0 $\pm$ 4.2	15.6 $\pm$ 4.0

* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

2012). It exerts its effect by acting on the TM3–TM4 loop of the NMDARs (Park-Chung et al., 1997; Horak et al., 2006; Borovska et al., 2012). Additionally, in oocyte studies the potentiating effect of PS on GluN1/GluN2A receptors is influenced by the presence or absence of exon-5 in the GluN1 subunit. In the absence of exon-5 (GluN1-1a, which we have used) the potentiation by PS is lower compared with when exon-5 is present (GluN1-1b) (Kostakis et al., 2011). This finding is relevant to the location of DRPEER Ca^{2+} -binding site on the GluN1 subunit.

Thus, the Ca^{2+} -dependent effects of PS may arise due to an allosteric interaction between Ca^{2+} -binding to the DRPEER site and PS binding to an “inhibitory site” influenced by GluN1 subunit, which together reduce the stability of long-lived open times. In contrast, the absence of Ca^{2+} prevents this allosteric interaction and inhibitory action of PS. Our results with the GluN1R663A mutant in the DRPEER motif and exon-5 insert are in agreement with this hypothesis because PS instead of inhibiting responses in the presence of 0.5 mM Ca^{2+} led to potentiation of the receptor to a similar extent as in the absence of nominal Ca^{2+} .

Bidirectional Actions of Pregnenolone Sulfate Are Produced by Mechanistically Distinct Gating Steps.

Our single-channel data demonstrate a significant effect of PS on mean open time in producing both inhibition and potentiation of the receptor. This is most evident in the free-energy plots where effects on open time are most predominant (Fig. 7). The properties of PS-induced inhibition are quite peculiar in that the longer open state is almost completely abolished. This correlates with a substantial reduction in the forward rate constant from O1 to O2 state in the kinetic scheme (from 920 to 70). Based on our kinetic analysis, the pore dilation may occur in two distinct stable states as represented by O1 and O2. These states can occur in a sequential manner as we and others have represented in our kinetic models (Dravid et al., 2008; Kussius and Popescu, 2009), or these may emerge from two different closed states (Schorge et al., 2005).

Based on our data, PS interaction with Ca^{2+} obstructs the dilation of pore to a more stable conformation representing the longer open time. Removal of nominal Ca^{2+} or presumably preventing Ca^{2+} interaction with DRPEER site or masking the effect of Ca^{2+} with the exon-5 insert (Figs. 1 and 2) unravels the potentiating mechanism of PS. The potentiating mechanism engages molecular determinants close to the TM3 and TM4 regions linker, forming the external vestibule as demonstrated previously elsewhere (Kostakis et al., 2011). Indeed, restricting linkers in TM3–TM4 or using the reducing agent dithiothreitol, which acts on GluN1 cysteines close to the TM3–TM4 linker (Sullivan et al., 1994), affects multiple gating steps not restricted to transitions to open states, similar to our kinetic analysis of effect of PS in the absence of nominal Ca^{2+} (Talukder and Wollmuth, 2011). This difference in PS inhibition mainly affecting fast-gating steps transitioning to open states while PS potentiation affects slow-gating steps in addition to the transition to open states suggests that the inhibitory site of action of PS may be closer to the pore or vestibule of the channel, and that the potentiating effect may involve structural elements involving larger and thus slower conformational changes. Studies using GluN1 and GluN2 subunit partial agonists and Lurcher mutations suggest that a slower gating step may represent GluN2 subunit

Fig. 7. Kinetic mechanism describing the effects of pregnenolone sulfate on GluN1/GluN2A receptor activation. MIL fit of single-channel data to understand the kinetic mechanism of GluN1/GluN2A receptor modulation by PS is shown. All rates are in s^{-1} . Bold numbers with asterisks denote the rates that were significantly different from glutamate/glycine control patches. Rates $\pm$ S.E.M. are presented in Table 2. Data were analyzed using unpaired t test. Free-energy trajectories for the kinetic states in the different models are presented. Control with 0.5 mM Ca^{2+} or without nominal Ca^{2+} produced similar profiles. The free energies of the active open states are most dramatically affected by PS during either inhibition or potentiation of the receptor. Scale bar: 1 k_BT . * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

conformational change while a faster gating step may represent a GluN1 subunit conformational change (Banke and Traynelis, 2003; Erreger et al., 2005; Murthy et al., 2012).

Thus, based on our data, it is possible that the potentiation of the receptor is mediated by modification of the slower putative GluN2-gating step because we observed changes in longer shut time constants τ_2 and τ_3 and slower gating steps in addition to its effect on mean open time. In contrast, the inhibition of the receptor by PS in the presence of 0.5 mM Ca^{2+} may involve modification to the GluN1-gating step because it led to a specific change in τ_1 . This hypothesis is also supported by our results in the DRPEER mutant and GluN1-1b splice variant where the PS inhibition is eliminated.

Conclusion and Remaining Questions. In most of the previous studies in neurons and mammalian expression systems, a potentiating effect is observed with preapplication of PS. In fact at GluN1/GluN2C receptors while coapplication of PS leads to PS-induced inhibition, preapplication of PS followed by agonist-alone application leads to potentiation of currents (Horak et al., 2006). The effect of preapplication is likely

a more relevant phenomenon to the physiology of the normal central nervous system because PS appears to be present under basal conditions (Robel and Baulieu, 1994). Thus future experiments to address the differences in whole-cell responses in dialyzed and perforated conditions upon preapplication of PS may reveal interesting results that may be relevant to neurosteroid physiology in the central nervous system.

Interestingly, PS is also being evaluated for its efficacy for treating cognitive and behavioral impairments in mental disorders (see, for example, Marx et al., 2014; and Wong et al., 2015). Our data suggest that there is a need to better understand the pharmacologic basis of actions of PS and to assess the ability of newly discovered allosteric modulators of NMDAR to serve as intracellular cell state-dependent agents.

Authorship Contributions

Participated in research design: Chopra, Monaghan, Dravid.

Conducted experiments: Chopra.

Performed data analysis: Chopra, Dravid.

Wrote or contributed to the writing of the manuscript: Chopra, Dravid.

References

- Acker TM, Yuan H, Hansen KB, Vance KM, Ogden KK, Jensen HS, Burger PB, Mullasseril P, Snyder JP, and Liotta DC et al. (2011) Mechanism for non-competitive inhibition by novel GluN2C/D N-methyl-D-aspartate receptor subunit-selective modulators. *Mol Pharmacol* **80**:782–795.
- Banke TG and Traynelis SF (2003) Activation of NR1/NR2B NMDA receptors. *Nat Neurosci* **6**:144–152.
- Bhatt JM, Prakash A, Suryavanshi PS, and Dravid SM (2013) Effect of ifenprodil on GluN1/GluN2B N-methyl-D-aspartate receptor gating. *Mol Pharmacol* **83**:9–21.
- Borovska J, Vyklicky V, Stastna E, Kapras V, Slavikova B, Horak M, Chodounska H, and Vyklicky L, Jr (2012) Access of inhibitory neurosteroids to the NMDA receptor. *Br J Pharmacol* **166**:1069–1083.
- Bowlby MR (1993) Pregnenolone sulfate potentiation of N-methyl-D-aspartate receptor channels in hippocampal neurons. *Mol Pharmacol* **43**:813–819.
- Cameron K, Bartle E, Roark R, Fanelli D, Pham M, Pollard B, Borkowski B, Rhoads S, Kim J, and Rocha M et al. (2012) Neurosteroid binding to the amino terminal and glutamate binding domains of ionotropic glutamate receptors. *Steroids* **77**:774–779.
- Ceccon M, Rumbaugh G, and Vicini S (2001) Distinct effect of pregnenolone sulfate on NMDA receptor subtypes. *Neuropharmacology* **40**:491–500.
- Dravid SM, Prakash A, and Traynelis SF (2008) Activation of recombinant NR1/NR2C NMDA receptors. *J Physiol* **586**:4425–4439.
- Erreger K, Dravid SM, Banke TG, Wyllie DJ, and Traynelis SF (2005) Subunit-specific gating controls rat NR1/NR2A and NR1/NR2B NMDA channel kinetics and synaptic signalling profiles. *J Physiol* **563**:345–358.
- Hedegaard M, Hansen KB, Andersen KT, Bräuner-Osborne H, and Traynelis SF (2012) Molecular pharmacology of human NMDA receptors. *Neurochem Int* **61**:601–609.
- Horak M, Vlcek K, Chodounska H, and Vyklicky L (2006) Subtype-dependence of N-methyl-D-aspartate receptor modulation by pregnenolone sulfate. *Neuroscience* **137**:93–102.
- Horak M, Vlcek K, Petrovic M, Chodounska H, and Vyklicky L, Jr (2004) Molecular mechanism of pregnenolone sulfate action at NR1/NR2B receptors. *J Neurosci* **24**:10318–10325.
- Jahr CE and Stevens CF (1993) Calcium permeability of the N-methyl-D-aspartate receptor channel in hippocampal neurons in culture. *Proc Natl Acad Sci USA* **90**:11573–11577.
- Jang MK, Mierke DF, Russek SJ, and Farb DH (2004) A steroid modulatory domain on NR2B controls N-methyl-D-aspartate receptor proton sensitivity. *Proc Natl Acad Sci USA* **101**:8198–8203.
- Karakas E and Furukawa H (2014) Crystal structure of a heterotetrameric NMDA receptor ion channel. *Science* **344**:992–997.
- Kostakis E, Jang MK, Russek SJ, Gibbs TT, and Farb DH (2011) A steroid modulatory domain in NR2A collaborates with NR1 exon-5 to control NMDAR modulation by pregnenolone sulfate and protons. *J Neurochem* **119**:486–496.
- Kussius CL and Popescu GK (2009) Kinetic basis of partial agonism at NMDA receptors. *Nat Neurosci* **12**:1114–1120.
- Malayev A, Gibbs TT, and Farb DH (2002) Inhibition of the NMDA response by pregnenolone sulphate reveals subtype selective modulation of NMDA receptors by sulphated steroids. *Br J Pharmacol* **135**:901–909.
- Marx CE, Lee J, Subramaniam M, Rapisarda A, Bautista DC, Chan E, Kilts JD, Buchanan RW, Wai EP, and Verma S et al. (2014) Proof-of-concept randomized controlled trial of pregnenolone in schizophrenia. *Psychopharmacology (Berl)* **231**:3647–3662.
- Murthy SE, Shogan T, Page JC, Kasperek EM, and Popescu GK (2012) Probing the activation sequence of NMDA receptors with lurcher mutations. *J Gen Physiol* **140**:267–277.
- Park-Chung M, Wu FS, Purdy RH, Malayev AA, Gibbs TT, and Farb DH (1997) Distinct sites for inverse modulation of N-methyl-D-aspartate receptors by sulfated steroids. *Mol Pharmacol* **52**:1113–1123.
- Petrovic M, Sedlacek M, Cais O, Horak M, Chodounska H, and Vyklicky L, Jr (2009) Pregnenolone sulfate modulation of N-methyl-D-aspartate receptors is phosphorylation dependent. *Neuroscience* **160**:616–628.
- Qin F, Auerbach A, and Sachs F (1996) Estimating single-channel kinetic parameters from idealized patch-clamp data containing missed events. *Biophys J* **70**:264–280.
- Robel P and Baulieu E (1994) Neurosteroids biosynthesis and function. *Trends Endocrin Metab* **5**:1–8.
- Sather W, Johnson JW, Henderson G, and Ascher P (1990) Glycine-insensitive desensitization of NMDA responses in cultured mouse embryonic neurons. *Neuron* **4**:725–731.
- Schorge S, Elenes S, and Colquhoun D (2005) Maximum likelihood fitting of single channel NMDA activity with a mechanism composed of independent dimers of subunits. *J Physiol* **569**:395–418.
- Sullivan JM, Traynelis SF, Chen HS, Escobar W, Heinemann SF, and Lipton SA (1994) Identification of two cysteine residues that are required for redox modulation of the NMDA subtype of glutamate receptor. *Neuron* **13**:929–936.
- Talukder I and Wollmuth LP (2011) Local constraints in either the GluN1 or GluN2 subunit equally impair NMDA receptor pore opening. *J Gen Physiol* **138**:179–194.
- Traynelis SF, Wollmuth LP, McBain CJ, Menniti FS, Vance KM, Ogden KK, Hansen KB, Yuan H, Myers SJ, and Dingledine R (2010) Glutamate receptor ion channels: structure, regulation, and function. *Pharmacol Rev* **62**:405–496.
- Watanabe J, Beck C, Kuner T, Premkumar LS, and Wollmuth LP (2002) DRPEER: a motif in the extracellular vestibule conferring high Ca^{2+} flux rates in NMDA receptor channels. *J Neurosci* **22**:10209–10216.
- Wong P, Sze Y, Chang CC, Lee J, and Zhang X (2015) Pregnenolone sulfate normalizes schizophrenia-like behaviors in dopamine transporter knockout mice through the AKT/GSK3 β pathway. *Transl Psychiatry* **5**:e528.
- Wu FS, Gibbs TT, and Farb DH (1991) Pregnenolone sulfate: a positive allosteric modulator at the N-methyl-D-aspartate receptor. *Mol Pharmacol* **40**:333–336.
- Yaghoubi N, Malayev A, Russek SJ, Gibbs TT, and Farb DH (1998) Neurosteroid modulation of recombinant ionotropic glutamate receptors. *Brain Res* **803**:153–160.

Address correspondence to: Dr. Shashank M. Dravid, Department of Pharmacology, Creighton University, School of Medicine, 2500 California Plaza, Omaha, NE 68178. E-mail: shashankdravid@creighton.edu