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Different oxysterols have opposing actions at N-methyl-D-aspartate receptors

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Abstract

Oxysterols have emerged as important biomarkers in disease and as signaling molecules. We recently showed that the oxysterol 24(S)-hydroxycholesterol, the major brain cholesterol metabolite, potently and selectively enhances NMDA receptor function at a site distinct from other modulators. Here we further characterize the pharmacological mechanisms of 24(S)-hydroxycholesterol and its synthetic analogue SGE201. We describe an oxysterol antagonist of this positive allosteric modulation, 25-hydroxycholesterol. We found that 24(S)-hydroxycholesterol and SGE201 primarily increased the efficacy of NMDAR agonists but did not directly gate the channel or increase functional receptor number. Rather than binding to a direct aqueous-accessible site, oxysterols may partition into the plasma membrane to access the NMDAR, likely explaining slow onset and offset kinetics of modulation. Interestingly, oxysterols were ineffective when applied to the cytosolic face of inside-out membrane patches or through a whole-cell pipette solution, suggesting a non-intracellular site. We also found that another natural oxysterol, 25-hydroxycholesterol, although exhibiting slight potentiation on its own, non-competitively and enantioselectively antagonized the effects of 24(S)-hydroxycholesterol analogues. In summary, we suggest two novel allosteric sites on NMDARs that separately modulate channel gating, but together oppose each other.

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Keywords

NMDA Receptor; Modulation; Allosteric; Gating; Neurodegeneration

1. Introduction

The N-methyl-D-aspartate receptor (NMDAR) is a heterotetrameric ligand-gated ion channel. Its role in synaptic plasticity, specifically in long-term potentiation (LTP) and long-term depression (LTD), makes it a target of intense focus in the study of learning and memory. However, activation of NMDARs can also be detrimental, as NMDARs are implicated in multiple neuropsychiatric disorders including schizophrenia, epilepsy, depression, Alzheimer's disease, and ischemic brain injury. As such, NMDARs remain potentially important targets for drug development (Collingridge et al., 2013; Coyle, 2006; Olivares et al., 2012).

Neurosteroids represent one of several known classes of endogenous positive and negative allosteric modulators of NMDAR function. These natural or synthetic compounds directly and rapidly modulate several types of ion channels, including NMDARs and GABA_A receptors (GABA_ARs). Pregnenolone sulfate (PREGS) is one such neuroactive steroid, acting to positively modulate NMDARs and negatively modulate GABA_ARs (Majewska and Schwartz, 1987; Wu et al., 1991). Though PREGS is an endogenous neuroactive steroid, it requires supraphysiological concentrations to affect NMDARs, and thus its relevance to brain function is controversial (Liere et al., 2009; Reddy, 2010; Wu et al., 1991).

The brain harbors other cholesterol derivatives, including oxysterols. Oxysterols are direct metabolic bi-products of cholesterol (Mast et al., 2010). The enzyme responsible for production of the major brain oxysterol is cholesterol-24-hydroxylase, localized primarily to postsynaptic compartments of neurons (Lund et al., 1999; Ramirez et al., 2008). Cholesterol-24-hydroxylase produces 24(*S*)-hydroxycholesterol (24(*S*)-HC), the most abundant cholesterol metabolite found in the brain (Russell et al., 2009). Other oxysterols, including 25-hydroxycholesterol (25-HC), are also present in brain, though at lower concentrations (Griffiths et al., 2006). The role of oxysterols in nervous system functioning has only begun to be investigated (Freemantle et al., 2013; Wong et al., 2007).

We have recently shown that 24(*S*)-HC, as well as its synthetic derivatives, SGE201 and SGE301, are both potent positive allosteric modulators (PAMs) of NMDARs (Paul et al., 2013). 24(*S*)-HC appears to be a highly selective NMDAR modulator and interacts with a site that is distinct from that of other neuroactive steroids and known NMDAR modulators. Its relatively high brain concentration suggests that 24(*S*)-HC may endogenously modulate NMDARs, although its physiological relevance remains unknown. Synthetic oxysterol analogues also represent important lead compounds for treating NMDAR-related neuropsychiatric disorders. Here, we describe details of the pharmacological mechanisms of oxysterol modulation of NMDARs and provide evidence that 25-HC, another natural oxysterol, non-competitively antagonizes the actions of both natural and synthetic oxysterol PAMs. Taken together, we provide evidence for a rather intricate bidirectional modulation of NMDARS by oxysterols.

2. Materials and Methods

2.1 Neuron Cultures

All animal care and experimental procedures were consistent with National Institutes of Health guidelines, approved by the Washington University Animal Studies Committee, and were similar to previously published methods (Mennerick et al., 1995). Hippocampal neurons were obtained from 1 to 3 day postnatal male and female Sprague-Dawley rats anesthetized with isoflurane. After rats were decapitated, hippocampi were removed, cut into 500- μ m-thick slices, and digested with 1 mg/ml papain in oxygenated Leibovitz L-15 medium (Life Technologies, Gaithersburg, MD). Tissue was mechanically dissociated in modified Eagle's medium (Life Technologies) containing 5% horse serum, 5% fetal calf serum, 17 mM D-glucose, 400 μ M glutamine, 50 U/ml penicillin, and 50 μ g/ml streptomycin. Cells were plated in modified Eagle's medium at a density of $\sim$ 650 cells/mm² as mass cultures onto 25-mm coverslips coated with 5 μ g/cm² poly-D-lysine and 1.7 μ g/cm² laminin, or were plated as "microisland" cultures for autaptic recordings at a density of $\sim$ 100 cells/mm² onto 35 mm plastic culture dishes stamped with collagen microdroplets on a layer of 0.15% agarose. Cytosine arabinoside (6.7 μ M) was added 3–4 days after plating to arrest glial proliferation, and one-half the culture medium was replaced with Neurobasal medium (Life Technologies) containing 500 μ M glutamine, 50 U/ml penicillin, 50 μ g/ml streptomycin and B27 supplement (Life Technologies) 4–5 days after plating.

2.2 *Xenopus* oocyte expression

cRNAs encoding rat NMDAR subunits was injected into stage V–VI oocytes harvested from sexually mature female *Xenopus laevis* frogs (*Xenopus* 1, Northland, MI). Frogs were anesthetized with 0.1% tricane (3-aminobenzoic acid ethyl ester) during a partial ovariectomy. Oocytes were defolliculated by shaking for 20 minutes at 37 °C in 2 mg/ml collagenase dissolved in a calcium-free solution containing (in mM): 96 NaCl, 2 KCl, 1 MgCl₂, and 5 HEPES, pH 7.4. Capped RNA for GluN1a and GluN2A subunits was prepared *in vitro* (mMESSAGE mMachine kit, Ambion, Austin, TX) from linearized pBluescript vectors. Subunit RNA was injected in equal parts (50 ng of total RNA). After injection, oocytes were incubated at 18 °C in ND96 solution containing (in mM): 96 NaCl, 2 KCl, 2 CaCl₂, 1 MgCl₂, and 5 HEPES, pH 7.4. ND96 was supplemented with pyruvate (5 mM), penicillin (100 U/ml), streptomycin (100 μ g/ml), and gentamycin (50 μ g/ml).

2.3 Electrophysiology

Whole-cell recordings from neuronal cultures were performed 3–12 days following plating using an Axopatch 200B amplifier (Molecular Devices, Sunnyvale, CA). For recordings, cells were bathed in extracellular solution containing (in mM): 138 NaCl, 4 KCl, 2 CaCl₂, 10 glucose, 10 *N*-(2-hydroxyethyl)piperazine-*N'*-ethanesulfonic acid (HEPES) at pH 7.25. Patch pipettes were filled with solution containing (in mM): 130 cesium methanesulfonate, 4 NaCl, 10 HEPES, 0.5 CaCl₂, and 5 EGTA at pH 7.25, unless otherwise indicated. When filled with this solution, pipette tip resistance was 3–6 M Ω . Cells were usually clamped at –70 mV or as indicated in figure legends. Drugs were applied with a multibarrel, gravity-driven local perfusion system that provides a laminar local stream for solution exchanges. Most NMDA-containing solutions were applied in low Ca²⁺ saline (0.25–0.5 mM) to

diminish Ca^{2+} -dependent desensitization (Clark et al., 1990; Tong et al., 1995) and were nominally devoid of Mg^{2+} and D-APV. In experiments analyzing synaptic transmission, solutions contained 2 mM Ca^{2+} . The common tip was placed 0.5 mm from the center of the microscope field. Solution exchange times were 120 ± 14 ms (10–90% rise) estimated from junction current rises at the tip of an open patch pipette. Experiments were performed at room temperature.

In general, recordings were obtained from young neurons to limit spatial clamp artifacts associated with complex neuronal morphology of older cells. However, synaptic studies were necessarily performed at >DIV10 to allow synaptic maturation. Hippocampal neurons are known to shift GluN2 subunit distributions as they develop from predominantly GluN2B to GluN2A (Traynelis et al., 2010). We evaluated our neuronal cultures for age-dependent differences in response to oxysterols, but observed equivalent potentiation in both young (DIV4) and old (DIV14) cultures (Supplemental Figure 1).

For recordings in inside-out patch configuration, bath solution consisted of (in mM): 120 gluconic acid, 4 NaCl, 0.5 CaCl_2 , 5 EGTA, 10 HEPES, pH 7.2 with CsOH. For recordings in cell-attached configuration, pipette solution included (in mM): 138 NaCl, 4 KCl, 10 HEPES, 1 EDTA, 0.3 NMDA, 0.02 glycine and 0.001 NBQX at pH 8.0. The elevated pH was used to reduce proton inhibition of NMDARs (Borschel et al., 2012).

Oocyte responses to NMDA were recorded 2–5 days following RNA injection using a two-electrode voltage clamp (OC725C amplifier; Warner Instruments, Hamden, CT) at a membrane potential of -70 mV. The bath solution was ND96 solution with the following changes: Mg^{2+} was omitted, Ba^{2+} replaced Ca^{2+} to avoid activation of endogenous Ca^{2+} -dependent currents, and 10 μM glycine was added. Glass recording pipettes (~ 1 M Ω resistance) were filled with 3 M KCl. Compounds were applied to the oocytes using a multibarrel pipette with a common output tip. Data acquisition and analysis were performed with pCLAMP software (Molecular Devices).

2.4 Data Analysis

Data acquisition and analysis were performed primarily using pCLAMP 10 software (Molecular Devices). Inhibition by antagonists was measured by quantifying the peak response to NMDA or, in the case of sustained antagonist application, by quantifying the average current during the final 50–100 ms of drug application. Extended analysis was done using Microsoft Excel 2007 (Microsoft), GraphPad Prism 5 (GraphPad Software), or QuB 2.0.0.20 (QuB Suite). Graphical figures and curve fitting were conducted with Clampfit 10 (Molecular Devices) or SigmaPlot 10 (Systat Software). Data are expressed as mean $\pm$ S.E.M. Statistical significance was determined using a Student's two-tailed *t*-test or ANOVA as indicated. Concentration-response data were fit with the Hill equation [$I = I_{\text{max}} C^n / (\text{EC}_{50}^n + C^n)$], where *C* is the agonist concentration, I_{max} is the maximum current amplitude, EC_{50} is the agonist concentration that yields a 50% response relative to I_{max} , and *n* is the Hill coefficient.

2.5 Materials

24(*S*)-hydroxycholesterol and SGE201 were obtained from Sage Therapeutics (Cambridge, MA). Enantiomeric 25-hydroxycholesterol (*ent*-25-HC) was prepared from *ent*-testosterone as described previously, except that the 26-methyl group was trideuterated (Westover and Covey, 2006). The *ent*-testosterone was made by total synthesis as described previously and is enantiomerically pure (Hu et al., 1997). There is no possibility of racimization of *ent*-25-HC to *nat*-25-HC during the conversion of *ent*-testosterone to *ent*-25-HC and no known biochemical pathway to interconvert natural and enantiomeric steroids. *ent*-Steroids do not occur naturally in nature (Biellmann, 2003; Covey, 2009). All other compounds were obtained from Sigma-Aldrich (St. Louis, MO).

3. Results

3.1 Allosteric Characteristics of Oxysterols

Because of its higher potency, faster reversibility, and sensitivity to cyclodextrin extraction, we employed the synthetic 24(*S*)-HC analogue SGE201 for most of our studies (Paul et al., 2013). To evaluate the pharmacological effects of oxysterols on NMDARs, we first expressed recombinant GluN1a/GluN2A NMDARs in oocytes, where complete glutamate concentration-response curves could be readily obtained in the presence and absence of pre-applied modulator (Figure 1). To address whether the mechanism of modulation might involve a change in potency of co-agonist, we first examined the glycine concentration-response relationship in oocytes in the presence of a fixed, sub-maximum glutamate concentration of 3 μ M (Figure 1A). The EC₅₀ for glycine shifted only slightly in the presence of SGE201 (1 μ M), with a strong increase in the maximum current (Figure 1B). We conclude that SGE201 is unlikely to interact directly with the co-agonist site.

To address the effect of SGE201 on the agonist concentration-response relationship, we evaluated the effect of SGE201 on varied glutamate concentrations in the presence of saturating glycine. SGE201 shifted the EC₅₀ for glutamate and significantly increased the estimated maximum glutamate response (Figure 1C, D). This combination of effects suggests that the mechanism of oxysterol potentiation may involve an increase in agonist efficacy (Colquhoun, 1998), although multiple actions, such as separate effects on agonist potency and channel number, could account for the effects on EC₅₀ and on maximum response. We never observed a current in direct response to 1 μ M SGE201 application, indicating that the oxysterol analogue does not directly gate the NMDAR channel (not shown).

In oocytes, concentration-response characteristics may be affected by technical issues such as slow agonist application, which could influence estimates of EC₅₀ and saturating agonist concentration. To validate our conclusion that oxysterols enhance maximum agonist responses, we turned to neonatal hippocampal neurons, where timing of solution applications can be more precisely controlled. Peak current responses to 300 μ M NMDA were indistinguishable from responses to 1 mM NMDA ($6.73 \pm 7.63\%$ increase; $N = 4$, not significantly different than no change), verifying the previous demonstration that 300 μ M NMDA gates a maximum response at native receptors (Patneau and Mayer, 1990).

Furthermore, responses to 1 mM NMDA were potentiated by both 2 μ M SGE201 (Figure 2A; $78.7 \pm 8.83\%$; $N = 4$) and by the natural oxysterol 24(*S*)-HC (10 μ M; Figure 2B; $66.6 \pm 11.7\%$; $N = 4$).

Potentiation of responses to saturating agonist concentration (Figures 1D, 2A–B) could be achieved by increasing the open probability of active channels or by recruitment of dormant receptors that are otherwise silent. Receptor insertion is excluded by our previous demonstration of potentiation in outside-out patches (Paul et al., 2013). However, oxysterol activation of functionally silent membrane channels was not excluded. To test this possibility, we challenged hippocampal neurons with the uncompetitive NMDAR open-channel blocker MK-801 in the presence of saturating NMDA (300 μ M). The probability of channel opening is proportional to the rate constant of MK-801 block onset, since the dissociation rate of MK-801 is negligible (Huettner and Bean, 1988; Rosenmund et al., 1995). Thus, we compared SGE201 potentiation of NMDA receptor currents at a saturating NMDA concentration with the change in kinetics of channel block by MK-801 (Figure 2C – E). In one series of experiments NMDA receptors were activated in the absence or presence of SGE201 with a pre-application of NMDA, followed by co-administration with MK-801 (Figure 2C). In sister experiments, NMDA was applied in the absence and presence of SGE201 to measure potentiation of steady-state NMDA current (Figure 2D). The increase in MK-801 onset time constant in SGE201 was statistically indistinguishable from the degree of potentiation by SGE201 (Figure 2E). This suggests that the increase in NMDAR current at saturating NMDA concentration can be solely accounted for by an increase in channel open probability, and there is no need to invoke receptor unsilencing to explain the increased maximum NMDA response. As an extension of this conclusion, we suggest that the shift in glutamate EC₅₀ (Figure 1D) is likely a by-product of a change in efficacy rather than a direct effect on agonist binding (Colquhoun, 1998). Taken together with results from Figure 1, the results of Figure 2 show that SGE201 increases agonist efficacy as a primary mechanism of action.

Some negative modulators of NMDA receptors exhibit voltage dependence (Linsenbardt et al., 2013; MacDonald et al., 1991; Parsons et al., 1993). Some positive modulators are voltage independent (Wu et al., 1991), but other lipid modulators are voltage dependent and interact with Mg²⁺ block of the channel (Parnas et al., 2009). To explore the voltage dependence of oxysterol modulation of NMDA receptors, we recorded current responses to 300 μ M NMDA in the absence and presence of SGE201 during a voltage-pulse protocol from potentials of –90 mV to +90 mV (Figure 3). To limit spatial clamp artifacts, small cells from young cultures (DIV 3–5) were used. We digitally subtracted a family of baseline current responses in the absence of NMDA from responses to the same stimuli in the presence of NMDA or NMDA plus SGE201 (2 μ M; Figure 3A). EDTA (0.1 mM) was used to chelate any residual Mg²⁺ or Zn²⁺ present in solutions. SGE201 potentiated NMDA-induced current at all voltages tested, suggesting that potentiation is voltage independent (Figure 3B, C).

In separate experiments, the same protocol was applied in the presence of 0.1 mM Mg²⁺ to test an interaction between oxysterols and Mg²⁺ block of the channel (Figure 3D). In the presence of Mg²⁺, SGE201 still potentiated currents (Figure 3E, F), but potentiation did not

interact with Mg^{2+} block. SGE201 thus potentiates NMDAR current at all voltages, and there was no interaction between voltage and the potentiation induced by SGE201.

3.2 NMDA Receptor Access by Oxysterols

Oxysterols share common structural features with neuroactive steroids and are derived from cholesterol (Akk et al., 2005; Russell et al., 2009). Because neurosteroids access their sites on NMDA and $GABA_A$ receptors through membrane partitioning rather than by direct aqueous diffusion (Akk et al., 2005; Borovska et al., 2012), we investigated routes by which oxysterols associate with NMDA receptors. One possibility is that oxysterols access an aqueous domain exposed to the extracellular solution, similar to the agonist binding site. Another possibility is that oxysterols associate with their site indirectly, through membrane partitioning. A final possibility is that oxysterols interact with a cytosolic or intracellular compartment and need to enter the cell to reach their modulatory site.

As 24(S)-HC is thought to be synthesized primarily within neurons (Ramirez et al., 2008), we tested whether oxysterols potentiate NMDA receptor currents when applied to the cytosolic side of the membrane. First, we excised inside-out membrane patches from hippocampal neurons and administered SGE201 directly by bath application. SGE201 did not discernibly affect channel behavior (Figure 4A, B). In contrast, SGE201 robustly potentiates NMDAR channel activity in outside-out patches (Paul et al., 2013). The result contrasts with the effect of anesthetic neurosteroids, which effectively potentiate $GABA_A$ receptors when applied to the cytosolic face (Akk et al., 2005).

To reduce the possibility that alterations related to patch excision affected our results, in separate whole-cell recordings, we filled neurons directly with SGE201 (50 μ M) added to the patch pipette. If SGE201 is effective from the intracellular compartment, then extrinsic application of γ -cyclodextrin (CDX), which scavenges hydrophobic compounds such as SGE201, should decrease NMDAR current potentiation, effectively reducing the total current observed (Akk et al., 2005; Paul et al., 2013). When CDX was applied, however, we observed no change in NMDAR current, further suggesting that intracellular application of SGE201 does not affect NMDAR potentiation (Figure 4C). To confirm that the cells filled with SGE201 were indeed capable of oxysterol potentiation, a lower concentration of SGE201 was applied extracellularly and CDX-sensitive potentiation was observed (Figure 4C, D). In order to show that this method of intracellular filling viably delivers lipophilic molecules, separate neurons were filled with 50 μ M (3 α ,5 α ,17 β)-3-hydroxyandrostane-17-carbonitrile (ACN), a $GABA_A$ neuroactive steroid positive modulator (Akk et al., 2005). ACN potentiated current responses to GABA, as demonstrated by a CDX-extractable component of GABA currents (Figure 4E, F). In summary, two independent protocols suggest that SGE201 is ineffective when applied intracellularly.

Despite the lack of NMDAR potentiation induced by oxysterol presented to the cytosolic membrane face, slow onset of oxysterol effects suggests that membrane partitioning may be an important step in reaching the NMDAR. To test this, channels were isolated in cell-attached mode, whereby the receptor(s) being monitored were within the patch pipette and exposed directly to 300 μ M NMDA (Figure 5A inset) but were isolated from the bath solution surrounding the cell. This recording configuration requires a bath-applied

modulator to partition into the membrane in order to reach the channel. Figure 5A shows that bath-applied SGE201 increased channel activity in cell-attached mode. Furthermore, the increase in activity affected the open probability of the NMDAR but not the unitary current amplitude of single-channel currents (Figure 5B, C). The degree of potentiation was similar to that observed in outside-out membrane patches and in whole-cell recording (Paul et al., 2013).

In total, these data suggest that oxysterols effectively potentiate NMDA receptor currents when applied extracellularly or when applied to the external leaflet of the plasma membrane. Thus, it is unlikely that the intracellular domain of the NMDA receptor contains a binding site for these modulators.

3.3 Receptor Antagonism by 25-hydroxycholesterol

Although 24(S)-HC is a positive allosteric modulator of NMDA receptors, and is the cholesterol metabolite of highest concentration in the brain, it is not the only oxysterol produced endogenously in brain (Russell et al., 2009). Although our previous screen failed to identify another cholesterol metabolite with significant activity at NMDARs (Paul et al., 2013), 25-hydroxycholesterol (25-HC) marginally but non-significantly increased NMDAR function. The latter prompted testing whether there was an interaction between 25-HC and the positive allosteric modulators, 24(S)-HC and SGE201. 25-HC is synthesized in the brain at rates approximately one-fourth that of 24(S)-HC (Lund et al., 1999). Therefore, we sought to explore the actions of 25-HC in more detail. In further experiments, we found that co-application of 10 μ M 25-HC for up to 40 s caused a small but statistically significant increase in average response to 10 μ M NMDA ($20.4 \pm 5.3\%$; $N = 6$; $p < 0.01$; Figure 6A, B). Lower concentrations of 25-HC potentiated by $9.9 \pm 3.9\%$ (0.1 μ M; $N = 6$) and $17.8 \pm 4.7\%$ (1.0 μ M; $N = 6$; $p < 0.01$), suggesting that 25-HC is a high-potency, low efficacy modulator. Furthermore, in 37 cells incubated for over 5 min in 10 μ M 25-HC, we found only a subtle effect on NMDAR current compared with 42 cells from sibling cultures not exposed to 25-HC ($42.0 \pm 19.2\%$; $p = 0.0537$; Figure 6C). Thus, prolonged incubation in 25-HC leads to little or no potentiation beyond that observed with acute application. Overall, we conclude that 25-HC has a mild potentiating action on NMDARs.

We next evaluated whether 25-HC affects presynaptic or postsynaptic signaling by examining evoked AMPAR EPSCs, NMDAR EPSCs and GABA_AR IPSCs in the absence and presence of 25-HC (Figure 6D – F). In all three cases, 25-HC had no effect on peak current, but it did slightly slow decay time course of NMDAR EPSCs, suggesting that 25-HC is nearly but not completely silent at NMDARs when administered alone (Figure 6G, H). The lack of 25-HC effect on evoked transmission at AMPARs and GABA_ARs also suggests that 25-HC does not modulate the voltage-gated channels and other potential targets responsible for action potential initiation, propagation, or neurotransmitter exocytosis.

We subsequently tested whether 25-HC interacts with potentiation by SGE201. Despite its modest effects alone on NMDAR currents, 25-HC inhibited NMDAR potentiation by 0.2 μ M SGE201 in a concentration-dependent manner, and at 10 μ M 25-HC, almost completely eliminated potentiation. The IC₅₀ of this antagonistic effect was approximately 100 nM

(Figure 7A). In a separate experiment, where neurons were challenged with potentiator in either the absence or the presence of saturating 25-HC (10 μ M; Figure 7B), we found that 25-HC effectively occluded potentiation by both SGE201 (0.2 μ M) and 24(*S*)-HC (1 μ M; Figure 7C, D). However, 25-HC was ineffective at blocking NMDAR potentiation by pregnenolone sulfate (PREGS; 50 μ M) and arachidonic acid (AA; 5 μ M), suggesting it probably interacts with a site unrelated to these other known NMDAR modulators (Figure 7E, F).

The selectivity of 25-HC antagonism for oxysterol potentiation suggests that the interaction could be competitive. However, when we analyzed 25-HC (10 μ M) against the concentration-response profile of SGE201, we observed a suppressed maximal response to SGE201, reflecting non-competitive inhibition (Figure 8A).

To confirm this result, we tested the offset kinetics of high and low oxysterol concentrations in the presence of the antagonist. This experiment assumes that the slow offset kinetics of SGE201 potentiation results from trapping of modulator in the plasma membrane rather than a slow dissociation from a receptor site. The assumption is supported by the sensitivity of SGE201 offset to CDX scavenging (Figure 8B, E, F) (Chisari et al., 2010; Paul et al., 2013). If we trap a higher modulator concentration in the membrane, a competitive antagonist would be at a larger disadvantage and would be slower to displace potentiator.

To probe competition, we established NMDAR potentiation by either 0.2 or 2 μ M SGE201 (Figure 8B). SGE201 was then removed from the bath, and cells were challenged with 10 μ M 25-HC. Compared with saline wash (Figure 8B top), 25-HC accelerated loss of potentiation, but the degree and rate of loss were similar regardless of potentiator concentration (Figure 8C–F). In comparison, CDX extraction led to a faster removal of potentiation, revealing the upper limit for dissociation of potentiator (Figure 8E, F). These results are most consistent with a non-competitive interaction between 25-HC and SGE201.

Oxysterols are known to perturb the lipid bilayer and “mobilize” membrane cholesterol (Olsen et al., 2009). One way to distinguish actions of drugs at a specific receptor site from effects on the membrane is through the use of enantiomers. Enantiomers share identical physiochemical properties and behave identically in membrane environments, while diastereomers may behave differently (Covey, 2009). Thus if membrane perturbation explains the antagonist effects of 25-HC on potentiation, we might expect the unnatural enantiomer of 25-HC (*ent*-25-HC) to mimic the effects of natural 25-HC. Conversely, if the effect involves a chiral protein site, the effect of 25-HC should exhibit enantioselectivity. (Akwa et al., 2001; Gale et al., 2009; Westover and Covey, 2006). To that end, we repeated our occlusion experiments using *ent*-25-HC in place of *nat*-25-HC. Interestingly, *ent*-25-HC retained the ability to inhibit the potentiation of SGE201, though not as effectively as natural 25-HC across three orders of magnitude of concentration (Figure 9). The demonstration of enantioselectivity suggests that 25-HC may interact with a specific receptor site to prevent the NMDAR conformational changes underlying potentiation, rather than perturbing lipids in the plasma membrane.

4. Discussion

The most abundant cholesterol metabolite in the brain is 24(*S*)-HC, produced by cholesterol-24-hydroxylase (Russell et al., 2009). Until recently, it was largely assumed that this metabolite's primary function was in maintaining cholesterol homeostasis in brain (Bielska et al., 2012). However, we recently showed that 24(*S*)-HC acts as a NMDAR PAM, suggesting that its formation in brain could participate in the physiological function of receptors associated with learning and memory (Paul et al., 2013) and possibly with various neuropsychiatric disorders. Synthetic analogues of 24(*S*)-HC, like SGE201, have potential as treatments for schizophrenia and autism (Collingridge et al., 2013; Coyle, 2006), among other neuropsychiatric disorders. In addition, 24(*S*)-HC levels have been reported to be altered in a number of neurodegenerative disorders (Leoni and Caccia, 2013; Leoni et al., 2013; Porter et al., 2010). Our results suggest that these altered levels could represent more than just biomarkers and could be quite relevant to disease pathophysiology. Here, we have described further aspects of oxysterol pharmacology in neurons showing that oxysterols have more than one action at NMDARs, including a form of non-competitive antagonism between two naturally occurring oxysterols.

NMDAR PAMs include pregnenolone sulfate, arachidonic acid, and oxysterols (Miller et al., 1992; Paul et al., 2013; Wu et al., 1991). Our results show that SGE201 potentiates NMDAR current over a range of glutamate and glycine concentrations (Figure 1). Potentiation of responses to saturating agonist concentration is a common feature of other NMDAR PAMs (Horak et al., 2004) and may reflect the weak baseline efficacy of NMDAR agonists, including glutamate (Chen et al., 1999; Rosenmund et al., 1995). The fully liganded receptor does not operate near a P_o of 1.0, so there is ample dynamic range for potentiation.

The route of access of oxysterols to the NMDAR has implications for a role of 24(*S*)-HC in neuronal signaling and for the design of effective analogues. The metabolic enzyme responsible for producing 24(*S*)-HC, cholesterol-24-hydroxylase, is localized primarily to the endoplasmic reticulum of neurons, so we were interested in testing whether intracellular oxysterols potentiate NMDAR current, similarly to neurosteroids (Akk et al., 2005). Neither excised-patch experiments nor whole-cell experiments revealed potentiation of NMDAR function by cytosol-applied SGE201. This result suggests that the binding site is not intracellular. Furthermore, SGE201 is either unable to partition into the membrane when delivered via the cytoplasm or unable to orient correctly to gain access to the NMDAR site. One speculative explanation for this behavior is that membrane asymmetries, such as differences in lipid composition between the inner and outer leaflet, affect oxysterol access to the NMDAR site. The physiological implication of the observation is potentially important, as oxysterols such as 24(*S*)-HC must be released from the cell of origin in order to modulate excitation.

One intriguing possibility is the potential for antagonistic or cooperative actions of oxysterols acting at NMDARs. To that end, we tested another oxysterol found endogenously, 25-HC, for effects on NMDA receptor function. Although 25-HC had little effect alone on NMDARs, it inhibited the potentiating effects of both SGE201 and 24(*S*)-HC

(Figure 7). 25-HC did not inhibit potentiation by PREGS or AA, suggesting selectivity for oxysterol potentiation (Paul et al., 2013).

Given the selectivity of 25-HC for oxysterol potentiation, but not pregnenolone sulfate or arachidonic acid modulation, it was perhaps surprising that our data also suggest a non-competitive interaction between 25-HC and SGE201 and thus likely separate binding sites. Oxysterols are known to have strong effects on cholesterol accessibility and perhaps other aspects of lipid behavior (Olsen et al., 2012). We hypothesized that such a non-receptor effect could give rise to a non-competitive 25-HC profile. If this is the case, these effects on perturbing lipids should be mimicked by the unnatural enantiomer of 25-HC (Gale et al., 2009; Westover and Covey, 2006). However, the enantioselectivity of 25-HC antagonist actions (Figure 9) suggests two NMDAR sites, one for positive allosteric modulation and an interacting site for its reversal by the nearly silent, non-competitive antagonist 25-HC.

Our results raise the prospect, though still speculative, that the interplay between endogenous oxysterols, including 24(S)-HC and 25-HC, may be important for the *in vivo* function of NMDARs. 24(S)-HC is found in brain tissues of various species at remarkably high concentrations (10 – 20 µg/g tissue or ~25 µM) (Meljon et al., 2012). Though 25-HC is reported at far lower concentrations (0.7 µg/g tissue or ~1 µM), it has been shown to increase in inflammatory conditions (Diczfalusy, 2013). Indeed, disorders such as schizophrenia may involve NMDAR hypofunction, since dissociative anesthetics including ketamine mimic positive symptoms of the disease (Lin et al., 2012; Olney et al., 1999). It is interesting to consider that endogenous oxysterol modulators of NMDAR function in brain could participate in the pathophysiology of a variety of neuropsychiatric disorders where NMDAR hypofunction or hyperfunction has been implicated.

In conclusion, 24(S)-HC and related synthetic oxysterol PAMs increase NMDAR agonist efficacy by partitioning from the extracellular side of the plasma membrane and interacting with the receptor at a unique site distinct from co-agonist and other previously described modulatory sites. 25-HC antagonizes oxysterol PAM activity non-competitively. Our results demonstrate that oxysterols represent novel pharmacological and potentially physiological modulators of neuronal excitation, acting to fine-tune NMDAR function.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

NMDA	N-methyl-D-aspartate
24(S)-HC	24(S)-hydroxycholesterol
PREGS	pregnenolone sulfate
PAM	positive allosteric modulator

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Highlights

- Oxysterols represent new modulators of NMDARs that increase agonist efficacy
- Oxysterols do not need direct access to the NMDAR; are ineffective from the cytosol
- 24(S)-HC and 25-HC may compete in a physiological “tug-of-war” for NMDAR modulation

Figure 1. SGE201 increases agonist and co-agonist efficacy of NMDA receptors

A. Current response in *Xenopus* oocytes injected with RNA encoding GluN1a/GluN2A subunits to glutamate (3 μM) plus varied glycine concentrations (0.1, 3 and 10 μM) in the absence (left) or presence (right) of SGE201 (1 μM ; right). **B.** Glycine concentration-response curve derived from oocytes exposed to glycine and glutamate (closed circles; $N = 8$), then with the addition of SGE201 (open circles; $N = 8$). Normalized peak current is plotted. Solid line represents fit to the Hill equation of the form $I = I_{\text{max}} * C^n / (EC_{50}^n + C^n)$. Glycine EC_{50} was $2.3 \pm 0.5 \mu\text{M}$ in the absence of SGE201 and $1.5 \pm 0.1 \mu\text{M}$ in the presence of SGE201 (not significant). Responses to maximum glycine increased significantly (1.3 ± 0.1 vs. 2.0 ± 0.2 , $p < 0.05$). **C.** Current response of an oocyte expressing GluN1a/GluN2A subunits to glutamate (1, 10 and 100 μM ; left), or glutamate in the presence of SGE201 (1 μM ; right). Bath glycine concentration was 10 μM . **D.** Glutamate concentration-response curve derived from oocytes exposed to glutamate alone (closed circles; $N = 8$), then to glutamate and SGE201 (open circles; $N = 8$). Solid lines represent fits to the Hill equation. From fits to individual oocytes, the glutamate EC_{50} was significantly shifted by SGE201 ($24.3 \pm 1.2 \mu\text{M}$ vs. $9.9 \pm 1.0 \mu\text{M}$, $p < 0.05$). The predicted maximum response was also significantly augmented (1.1 ± 0.01 vs. 1.3 ± 0.1 , $p < 0.05$).

Figure 2. SGE201 accelerates rate of MK-801 block and potentiates whole cell NMDA current equivalently

A. Responses from a hippocampal neuron to saturating NMDA application (1 mM) in the absence and presence of SGE201 (2 μ M). **B.** Currents to NMDA (1 mM) in the absence and presence of 24(S)-HC (10 μ M). **C.** Response to 300 μ M NMDA and MK-801 (5 μ M onset) in a control cell (black trace; N = 10) and a sibling culture pre-incubated with 2 μ M SGE201 (red trace; N = 10). **D.** Representative traces indicating responses from a neuron to a saturating NMDA (300 μ M) application (Black: saline; Red: SGE201, 2 μ M; N = 10). **E.** Normalized change in whole-cell current and the change in weighted rate constant (Arrows in C) of MK-801 block derived from C and D. There was no significant difference between the two measurements ($p = 0.46$, unpaired t-test). Whiskers represent 10th and 90th percentiles. The box boundaries represent 25th and 75th percentiles. The lines within the boxes mark the median value.

Figure 3. Oxysterol potentiation lacks voltage dependence

NMDA receptor current was measured at different potentials from hippocampal neurons in the presence of NMDA (300 μ M) before and after administration of SGE201 (2 μ M) for 2 min. **A.** In the absence of Mg^{2+} (chelated with 0.1 mM EDTA) cells were held at -30 mV, then subjected to 50 ms voltage pulses from -90 mV to $+90$ mV in 10 mV increments. Odd-numbered responses are displayed. Currents in the absence of NMDA were subtracted. **B.** NMDA currents in the absence of Mg^{2+} were plotted vs. pulse potential (V_m) and normalized to the response pre-SGE201 at $+90$ mV. In the absence of Mg^{2+} ,

indistinguishable potentiation of NMDAR current was observed at -90 mV and at $+90$ mV ($N = 5$). Potentiation values at -90 mV did not differ significantly from those at $+90$ mV ($p > 0.05$). **C.** The current-voltage relationship was re-plotted normalized to $+90$ mV within each condition to better visualize voltage dependence. **D.** In the presence of Mg^{2+} (0.1 mM) cells were held at -70 mV and subjected to voltage jumps as in **A**, yielding characteristic rectification. **E–F.** Data from experiments in Mg^{2+} were normalized and plotted as in panels **B** and **C** ($N = 5$).

Figure 4. Intracellular application of SGE201 does not potentiate NMDAR current

A. Representative records from hippocampal membrane patches in inside-out configuration. Tip command potential was +100 mV. The extracellular solution, inside the pipette, included NMDA (300 μ M) and glycine (20 μ M). During and after excision of the patch, low- Ca^{2+} cesium gluconate solution (see Materials & Methods for full composition) was applied to the intracellular face of the membrane (top), followed by application of SGE201 (2 μ M) to the intracellular face (bottom). The recording was digitally sampled at 40 kHz with a 5 kHz low pass filter. Traces are displayed with a 2 kHz low pass filter and with minor baseline adjustment to account for drift. **B.** Channel open probability remained unchanged after application of SGE201 to the intracellular face of the membrane ($N = 5$ inside-out patches). **C.** Tests of intracellular effectiveness in whole-cell recordings. Hippocampal neurons were clamped in whole-cell configuration and filled with vehicle (black trace) or intracellular (Int) SGE201 (50 μ M; red trace) and tested for NMDA response (300 μ M). To test for oxysterol potentiation, γ -cyclodextrin (CDX; 500 μ M) was applied acutely to the neuron to extract SGE201 from the membrane and reduce any NMDAR

potentiation. Extracellular CDX did not alter the agonist-induced current (top black trace). As a positive control, each neuron from **C** was also treated with extracellular (Ext) SGE201 (0.2 μ M) for 60 s, then tested as before for extractability (**C**; cyan traces). **D**. NMDAR potentiation by extracellular SGE201 was alleviated by CDX despite the presence of intracellular SGE201 (cyan bars). **E**. In additional positive control experiments, GABA (1 μ M) current was recorded in hippocampal neurons filled with either vehicle (black trace) or ACN, a neurosteroid analogue that is a positive allosteric modulator of GABA_A receptors (50 μ M; red trace). We have previously shown that ACN is effective when administered through a whole-cell pipette (Akk et al., 2005). **F**. CDX alleviated potentiation of GABAR current resulting from intracellular ACN administration. N = 4 for each experiment, ** $p < 0.01$; *** $p < 0.001$.

Figure 5. Indirect access to NMDARs is effective for potentiation

A. Cell-attached recordings were performed with 300 μ M NMDA in the recording pipette and SGE201 (2 μ M) applied to the bath surrounding the patch. Inset schematic shows the configuration of recording and the compartments to which agonist and SGE201 were applied. Representative examples of NMDAR channel activity before (left) and after (right) bath application of SGE201 are shown. **B.** All points histograms derived from the above saline (black) and SGE201 (red) traces depicting the increase in channel openings after treatment with SGE201. **C.** NPo analysis of channel activity in 12 cell-attached patches. *** $p < 0.001$.

Figure 6. 25-HC is a nearly silent modulator of NMDARs

A. NMDA (10 μ M) was applied to neurons (top) to establish baseline response, followed by a 20 s pre-treatment with 25-HC (10 μ M) and subsequent co-administration with NMDA (bottom). **B.** Peak NMDAR current was potentiated in the presence of 0.1 μ M 25-HC ($9.9 \pm 3.9\%$; N = 6); 1 μ M 25-HC ($17.8 \pm 4.7\%$; N = 6); and 10 μ M 25-HC ($20.4 \pm 5.3\%$; N = 6). **C.** In separate experiments, neurons were incubated in the absence or presence of 25-HC (10 μ M) for at least 5 min and responses to NMDA (10 μ M) were then recorded (N = 37). Compared with control sibling cultures not exposed to 25-HC (N = 42), a minor increase in

NMDAR response was observed ($42.0 \pm 19.2\%$; $p = 0.05$). **D – F.** Isolated hippocampal neurons were used to elicit recurrent EPSC/IPSCs. Representative traces in the absence (left) or presence (right) of 25-HC (10 μ M) depict pharmacologically isolated AMPAR EPSCs (**D**), NMDAR EPSCs (**E**), or GABA_AR IPSCs (**F**). **G.** Normalized peak PSCs are unchanged by 25-HC. **H.** The decay time constant for AMPARs and the weighted decay time constants for GABA_ARs and NMDARs were derived from mono-exponential fits to AMPAR EPSCs and to bi-exponential fits to GABA_AR IPSCs and NMDAR EPSCs. Only NMDAR PSCs were significantly but slightly slowed by 25-HC. $N = 5 - 10$.

Figure 7. 25-HC selectively blocks oxysterol potentiation

A. Normalized response to NMDA (10 μ M) plus SGE201 (0.2 μ M) with progressive concentrations of 25-HC. **B.** Examples of the effect of the highest concentration (10 μ M) on SGE201 (0.2 μ M) potentiation in an occlusion protocol. Cells were pre-incubated in vehicle (-25-HC) or in 25-HC, then challenged with the PAM (red traces). **C–F.** Summary of the effect of 25-HC pre-incubation on potentiation by SGE201 (**C**; 0.2 μ M; N = 10), 24(S)-HC (**D**; 1 μ M; N = 5), PREGS (**E**; 50 μ M; N = 47), and AA (**F**; 5 μ M; N = 10). ** $p < 0.01$; *** $p < 0.001$.

Figure 8. Lack of dependence on positive allosteric modulator concentration suggests a non-competitive interaction

A. SGE201 concentration-response curve in the absence and presence of 10 μ M 25-HC. Neurons were challenged with NMDA (10 μ M) and SGE201 (0.2 μ M; closed circles), then to NMDA, SGE201 and 25-HC (open circles). Solid line represents fit to the Hill equation of the form $I = I_{\max} * C^n / (EC_{50}^n + C^n)$. For the fits to the closed circles, $I_{\max} = 1.22$, $EC_{50} = 62.8$ nM, and $n = 0.54$. For the fits to the open circles, $I_{\max} = 0.34$, $EC_{50} = 97.3$ nM, and $n = 1.28$. **B.** Representative traces from hippocampal neurons pre-treated with SGE201 (0.2 μ M) for 60 sec, then administered NMDA (5 μ M) alone to establish a baseline level of SGE201-induced potentiation and to document slow reversibility in the absence of antagonist (top). After re-treatment with SGE201 to restore potentiation, 25-HC (10 μ M; middle) was applied as indicated. Finally, the same neuron was again re-treated with SGE201, the level of NMDAR potentiation was restored, and then CDX (500 μ M) was

administered to extract SGE201 (bottom). The dotted line in each trace represents the baseline NMDAR current recorded prior to SGE201 introduction. **C.** NMDAR current was recorded as in **B** and expressed as the ratio of current at the end of the trace to the current just prior to 25-HC or CDX application ($N = 6$). **D.** The protocol, **B**, was repeated with a higher concentration of SGE201 ($2 \mu\text{M}$; $N = 6$). **E.** Time constants derived from single exponential fits of the offset of potentiation by SGE201 ($0.2 \mu\text{M}$) in the presence of either 25-HC or CDX. **F.** Time constants were derived from fits to the offset of potentiation by SGE201 ($2 \mu\text{M}$) in the presence of 25-HC or CDX. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

Figure 9. Antagonism lacks enantioselectivity

Representative traces from hippocampal neurons in saline control (A), ent-25-HC (0.1 μ M, B) or 25-HC (0.1 μ M, C) at a near IC_{50} concentration. Baseline NMDA response (10 μ M) was obtained (top), then neurons were treated for 60 s with SGE201 (0.2 μ M) and NMDA re-applied (bottom). D. Normalized response to NMDA (10 μ M) plus SGE201 (0.2 μ M) with progressive concentrations (0.1 – 10 μ M) of either 25-HC (red) or ent-25-HC (blue). There was a significant main effect of both drug ($p = 0.002$) and concentration ($p = 0.027$), but no significant interaction (two-way ANOVA; $N = 3 - 10$).