Endogenous 24S-hydroxycholesterol modulates NMDAR-mediated function in hippocampal slices

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Abstract

N-methyl-D-aspartate receptors (NMDARs), a major subtype of glutamate receptors mediating excitatory transmission throughout the CNS, play critical roles in governing brain function and cognition. Because NMDAR dysfunction contributes to the etiology of neurological and psychiatric disorders including stroke and schizophrenia, NMDAR modulators are potential drug candidates. Our group recently demonstrated that the major brain cholesterol metabolite, 24S-hydroxycholesterol (24S-HC), positively modulates NMDARs when exogenously administered. Here, we studied whether endogenous 24S-HC regulates NMDAR activity in hippocampal slices. In CYP46A1−/− (knockout; KO) slices where endogenous 24S-HC is greatly reduced, NMDAR tone, measured as NMDAR to α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPAR) excitatory post-synaptic current (EPSC) ratio, was reduced. This difference translated into more NMDAR-driven spiking in wild-type (WT) slices compared with KO slices. Application of SGE-301, a 24S-HC analogue, had comparable potentiating effects on NMDAR EPSCs in both WT and KO slices, suggesting that endogenous 24S-HC does not saturate its NMDAR modulatory site in ex vivo slices. KO slices did not differ from WT slices in either spontaneous neurotransmission or in neuronal intrinsic excitability, and exhibited LTP indistinguishable from WT slices. However, KO slices exhibited higher resistance to persistent NMDAR-dependent depression of synaptic transmission induced by oxygen-glucose deprivation (OGD), an effect restored by SGE-301. Together, our results suggest that loss of positive NMDAR tone does not elicit compensatory changes in excitability or transmission, but it protects transmission against NMDAR-mediated dysfunction. We expect that manipulating this endogenous NMDAR modulator may offer new treatment strategies for neuropsychiatric dysfunction.
**Introduction**

N-methyl-D-aspartate receptors (NMDARs) are non-selective cation channels that are gated by the neurotransmitter glutamate and that exhibit voltage dependence and high calcium permeability. Normal NMDAR function is required for proper synapse formation, maturation, elimination, and plasticity (Cohen and Greenberg 2008; Hunt and Castillo 2012; Sanz-Clemente et al. 2013). Dysfunction of NMDARs has significant impact on brain function and cognition and has been implicated as a major contributor to several neurological and psychiatric diseases, including epilepsy, Alzheimer’s disease, depression, stroke and schizophrenia (Lau and Zukin 2007; Hardingham and Bading 2010; Ghasemi and Schachter 2011; Paoletti et al. 2013). Interestingly, NMDAR hypofunction is linked to psychotic symptoms associated with schizophrenia (Tamminga 1998; Coyle 2006; Coyle et al. 2010). Therefore, elevating NMDAR activity may be an effective treatment for key symptoms of schizophrenia (reviewed in Coyle et al., 2010). On the other hand, there is longstanding interest in reducing NMDAR function as a therapeutic strategy for excitotoxic injury from stroke and from neurodegenerative diseases (Danysz and Parsons 2012; Lai et al. 2014). Thus, both down-regulation and up-regulation of NMDAR function may have a place in the therapeutic repertoire.

NMDARs exhibit multiple binding sites for endogenous ligands and regulators, through which channel activity can be positively and negatively regulated. Besides its major agonist and co-agonist—glutamate and glycine, NMDAR activity is allosterically modulated by a variety of modulators, including polyamines, redox agents, arachidonic acid, and neurosteroids (Yamakura and Shimoji 1999; Vyklicky et al. 2014). Neurosteroids, produced endogenously in the brain, have rapid modulatory effects on γ-aminobutyric acid receptors (GABARs) and
NMDARs to influence neuronal excitability and plasticity (Paul and Purdy 1992; Benarroch 2007), with resulting effects on learning and memory (Vallee et al. 2001a; Vallee et al. 2001b). Pregnenolone sulfate (PREGS) is an endogenous neurosteroid that enhances NMDAR-dependent synaptic plasticity and behaviors (Flood et al. 1992; Mathis et al. 1994; Sliwinski et al. 2004; Smith et al. 2014). However, the importance of its neuromodulatory role under physiological conditions is still questionable, given the extremely low level of endogenous PREGS in the rodent brain (Wu et al. 1991; Liere et al. 2009).

We recently identified a novel class of positive NMDAR modulators, 24S-hydroxycholesterol (24S-HC) and synthetic analogues (Paul et al. 2013). Application of 24S-HC or the synthetic analogues, SGE-201 and SGE-301, directly enhances NMDAR currents in hippocampal neurons and HEK cells in culture. Moreover, synaptic and cognitive deficits induced by NMDAR channel blockers were reversed following SGE-201 or SGE-301 treatment in hippocampal slices and in vivo (Paul et al. 2013). 24S-HC is the major brain cholesterol metabolite and is produced by cholesterol 24-hydroxylase (CYP46A1), a neuron-specific enzyme localized to dendrites (Russell et al. 2009). The abundant level of endogenous 24S-HC, up to several micromolar (30-60 ng/mg tissue) in adult brain, suggests that it may be critical for maintaining normal NMDAR tone under physiological conditions (Smith et al. 1972; Meljon et al. 2012). Based on this, we hypothesize that endogenous 24S-HC contributes to normal NMDAR function, and manipulating 24S-HC level may regulate NMDAR tone, thus offering a potential strategy for treating physiological abnormalities resulting from NMDAR hypo-function and hyper-function.
The endogenous 24S-HC concentration can be up or down-regulated by genetic alteration of CYP46A1 expression. Mice lacking CYP46A1 enzyme have normal development and basal brain function but exhibit deficits of synaptic plasticity, learning and memory (Kotti et al. 2006; Kotti et al. 2008; Russell et al. 2009). On the other hand, mice overexpressing CYP46A1 enzyme showed increased levels of synaptic proteins in hippocampus, as well as enhanced memory retention in aged female mice (Maioli et al. 2013). Despite circumstantial evidence from the above studies, the direct effect of endogenous 24S-HC on NMDAR function has not previously been explored. In the present study, we tested whether reduction of endogenous 24S-HC leads to NMDAR hypofunction in mouse hippocampal slices. Results from our studies suggest that endogenous 24S-HC does indeed regulate NMDAR tone without detectable secondary/compensatory effects on neurotransmission. The actions of endogenous 24S-HC are apparently not saturated and contribute to a form of NMDAR-dependent synaptic depression induced by pathophysiological conditions in hippocampal slices.

Materials and Methods

Slice preparation

Hippocampal slices were prepared from postnatal day 18 (P18) to P32 wild type (WT) or CYP46A1−/− (KO) mice. WT C57Bl/6J or KO mice on a similar genetic background (Jackson Laboratories) were used in all studies. Slices for all studies were cohort and age matched to the extent possible. In accordance with protocols approved by the Washington University Animal Studies Committee, mice were anesthetized with isoflurane and decapitated. The brain was removed and glued onto a Leica VT1200 specimen holder. Sagittal (300 μm) slices were cut in ice-cold, modified artificial cerebrospinal fluid (ACSF) (in mM: 87 NaCl, 75 sucrose, 25
glucose, 25 NaHCO₃, 2.5 KCl, 1.25 NaH₂PO₄, equilibrated with 95% oxygen-5% CO₂ plus 0.5 CaCl₂, 3 MgCl₂; 320 mosmol). Slices were then incubated at 32–34°C for 30 min in choline-based ACSF (in mM: 92 choline chloride, 25 glucose, 30 NaHCO₃, 2.5 KCl, 1.2 NaH₂PO₄, 20 HEPES, 2 thiourea, 5 Na ascorbate, and 3 Na pyruvate, 2 CaCl₂ and 1 MgCl₂, equilibrated with 95% oxygen-5% CO₂; 300 mosmol), and subsequently stored at room temperature in regular ACSF (in mM: 125 NaCl, 25 glucose, 25 NaHCO₃, 2.5 KCl, 1.25 NaH₂PO₄, equilibrated with 95% oxygen-5% CO₂ plus 2.6 CaCl₂, 1.2 MgCl₂; 310 mosmol), allowing for at least one hour recovery prior to experiments. Except for noted exceptions, drugs were obtained from Sigma (St. Louis, MO).

**Analysis of endogenous 24S-HC level in mouse hippocampal tissues and slices**

Three mouse brains from each group of WT or CYP46A1 KO animals were harvested. Each brain was bisected into two hemispheres. Hippocampi from one of the hemispheres were frozen at -70 °C immediately. The other hemisphere from the same brain was cut into 300 μm hippocampal slices and stored in ACSF at room temperature for 5-6 h before being frozen at -70 °C. For measurement of 24S-HC level, the frozen tissue and slice samples were homogenized with 300 μL of PBS buffer. 24S-HC in 25 μL (50 μL of the sliced hippocampus tissue homogenate) of the whole hippocampus tissue homogenate was extracted with 200 μL of methanol. Deuterated 24S-HC (24S-HC d₇: 100 ng) was used as an internal standard and was added to the samples before extraction. The four point calibration standards of 24S-HC, containing 24S-HC d₇ were also prepared for the absolute quantification. 24S-HC and 24S-HC d₇ were derivatized to N,N-dimethylglycinate (DMG) to increase the mass spectrometry (MS) sensitivity. Sample analysis was performed with a Shimadzu 20AD HPLC system, a LeapPAL.
autosampler coupled to a triple quadrupole mass spectrometer (API 4000) operated in multiple reaction monitoring (MRM) mode. The positive ion electrospray ionization (ESI) mode was used for detection of 24S-HC and deuterated 24S-HC d7. The study samples were injected in duplicate for data averaging. Data processing was conducted with Analyst 1.5.1 (Applied Biosystems). Bicinchoninic acid (BCA) assay was performed on all the tissue samples for protein determination. The values reported for the hippocampal samples were normalized to protein content.

**Whole-cell patch-clamp recording**

Slices were transferred to a recording chamber and continuously perfused with oxygenated, ACSF at 2 ml min⁻¹. Experiments examining tonic NMDAR activation upon glutamate transporter inhibition and LTP/OGD studies were performed at 30-32° C. Other recordings were performed at 23-25°C. For measuring dual-component EPSCs, picrotoxin (PTX, 100 μM) and D-serine (10 μM) were included in extracellular solutions to inhibit GABA-mediated neurotransmission and to enhance NMDAR-mediated currents. In some experiments, 2,3-dioxo-6-nitro-1,2,3,4-tetrahydrobenzo[f]quinoxaline-7-sulfonamide (NBQX; Tocris, Bristol, United Kingdom) at 10 μM was included in extracellular solutions to isolate NMDAR EPSCs. For isolating AMPAR EPSCs or GABAR inhibitory post-synaptic currents (IPSCs), 100 μM PTX and 50 μM D-(-)-amino-5-phosphonopentanoic acid (D-APV; Tocris, Bristol, United Kingdom) or 10 μM NBQX and 50 μM D-APV were included in extracellular solutions, respectively. For measuring tonic NMDAR current, 100 μM PTX, 10 μM NBQX and 10 μM D-serine were included in extracellular solutions. 50 μM DL-threo-β-Benzylxaspartic acid (TBOA) Tocris, Bristol, United Kingdom) was bath-applied to evoke tonic NMDAR current. 40 μM (+)-5-methyl-
10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine (MK-801; Tocris, Bristol, United Kingdom) was bath-applied to examine the time constant of NMDAR channel block. For measuring neuronal intrinsic excitability, experiments were performed in the presence of 100 μM PTX, 10 μM NBQX and 50 μM D-APV to block synaptic transmission.

Somatic, whole-cell patch-clamp recordings were performed using standard differential contrast interference microscopy under infrared illumination. In the experiments where PSCs or PSPs were evoked, a small cut was made to separate CA1 from CA3 area to prevent recurrent synaptic activity. CA1 pyramidal cells were identified on an upright Nikon Eclipse E600FN microscope and a QImaging camera controlled with QCapture (QImaging, Surrey, Canada). Somatic whole cell recordings were made with borosilicate patch pipettes (World Precision Instruments, Sarasota, FL; Sutter Instruments, Novato, CA), having open tip resistance of 3–7 MΩ. After a whole-cell configuration was established, cells were allowed to fill with the intracellular solution for ~5 min. Intracellular EPSCs or EPSPs were evoked by a stimulating electrode placed in stratum radiatum, ~50 μm away from CA1 cell body layer and 50-100 μm away from the recorded cell. Stimulation was applied at 0.033-0.05 Hz, with 0.1 ms pulse width. Recordings were obtained using a MultiClamp 700B amplifier (Molecular Devices; Sunnyvale, CA) and pClamp 10.4 software (Molecular Devices; Sunnyvale, CA).

For voltage-clamp experiments, the intracellular pipette solution contained in mM: 120 cesium methanesulfonate, 20 HEPES, 10 EGTA, 2 MgATP, 0.3 Na₂GTP, and 5 QX-314; pH was adjusted with CsOH to pH 7.25; 290 mosomol. Cells were voltage-clamped at -30 mV, -70 mV and 0 mV for evoked NMDAR EPSCs (including dual-component EPSCs), isolated AMPAR EPSCs and GABA IPSCs, respectively. For measuring sEPSCs and sIPSCs, cells were
voltage-clamped at -70 mV and 0 mV, respectively, and data were acquired in gap-free mode. For measuring tonic NMDAR current, cells were voltage-clamped at -30 mV. For current-clamp experiments, the intracellular pipette solution contained in mM: 140 K-glucuronate, 4 MgCl₂, 10 HEPES, 0.4 EGTA, 4 MgATP, 0.3 Na₂GTP, and 10 phosphocreatine; pH was adjusted with KOH to pH 7.25; 290 mosmol. For measuring intrinsic excitability, CA1 neurons were initially maintained at -74 mV with constant current injection when necessary, and 500 ms current steps with 100 pA increment intervals were injected somatically, with a range of 0-800 pA. Current threshold was defined as the minimal injected current needed for firing a single spike. For measuring dual-component EPSPs and the resultant firing, a repetitive stimulation protocol containing 10 pulses at 50 Hz was applied to promote dual-component EPSPs. Neurons were initially maintained at -65 mV with constant current injection before application of stimulation. Stimulation intensity was adjusted to evoke 5-6 spikes (half of the pulse number) for each cell. Somatic bridge balance and pipette capacitance were adjusted using MultiClamp 700B Commander software.

Data were acquired at 100 kHz, filtered at 4 kHz using an 8-pole Bessel filter, and digitized using a DigiData1550 16-bit A/D converter (Molecular Devices; Sunnyvale, CA). Somatic access resistance was monitored continuously, and cells with unstable access resistance (> 20% change) were excluded from analysis. SGE-301 obtained from SAGE Therapeutics (Cambridge, MA) was dissolved at 1.25 mM in 100% DMSO to make concentrated stock solutions.
Field EPSPs (fEPSPs), Long-term potentiation (LTP), and oxygen-glucose deprivation (OGD) recordings

The hippocampus was dissected from postnatal day 28–32 WT or KO mice under isoflurane anesthesia. Slices (400 µm) were cut from the septal hippocampus with a rotary slicer in ACSF containing (in mM): 124 NaCl, 5 KCl, 2 MgSO₄, 2 CaCl₂, 1.25 NaH₂PO₄, 22 NaHCO₃, 10 glucose, bubbled with 95% oxygen- 5% CO₂ at 4–6 °C; 296 mosmol. After recovering for at least one hour at 30° C, hippocampal slices were transferred to a submerged recording chamber with continuous bath perfusion of ACSF at 2 ml min⁻¹ at 30 °C. Extracellular recordings were obtained from the apical dendritic layer of the CA1 region elicited with 0.1 ms constant current pulses through a bipolar stimulating electrode placed in stratum radiatum. During an experiment, excitatory postsynaptic potentials (EPSPs) were monitored using a half-maximal stimulus based on a baseline input-output curve. For LTP studies, tetanic stimulation (100 Hz for 1 s) was delivered to a bipolar extracellular stimulating electrode placed in the Schaffer collateral pathway. fEPSPs were monitored for 60 min subsequent to the tetanus. As a measure of LTP, the slope of the fEPSP 50-60 min following tetanic stimulation was compared with the fEPSP slope for the 10 min immediately preceding tetanic stimulation. For OGD experiments, ASCF was replaced with identical fluid without glucose and gassed with 95% N₂/5% CO₂ for 20 min. An input-output curve was repeated 90 min following OGD for statistical comparisons of changes in EPSP slopes at half-maximal intensity. In some experiments, KO slices were treated with 1 μM bath SGE-301 for 20 min before OGD and during the 20 min OGD challenge. SGE-301 was then washed out after OGD challenge.
Data analysis

Data analysis was performed in Clampfit 10.4 (Molecular Devices; Sunnyvale, CA). For measurement of NMDAR/AMPAR, NMDAR current averaged from 10 traces was estimated as mean value at 50-55 ms following peak of dual-component EPSC (Hestrin et al. 1990), while AMPAR current averaged from 10 traces was calculated as EPSC peak amplitude following 5 min D-APV (50 µM) treatment. For measuring TBOA-induced tonic NMDAR current, the mean value of holding current 12 sec immediately preceding TBOA application was calculated as the baseline, and the mean current at the end of a 6 min TBOA application was subtracted from the baseline. For estimating MK-801 induced decay of tonic NMDAR current, the decay tau of current was calculated by fitting the current trace to a single exponential decay function. For calculating SGE-301 effects on synaptic currents, baseline EPSC or IPSC peak amplitude was determined from averaging 20 traces before SGE-301 bath-application. After 6 min SGE-301 wash-in, EPSC or IPSC peak amplitude was averaged from the following 20 traces, and level of potentiation was calculated by normalizing this value to baseline. For comparing intrinsic excitability, spike firing threshold was determined as the minimal intensity of injected current needed for eliciting the first spike. Frequency of sEPSCs and sIPSCs was calculated using Mini analysis software (Synaptosoft; Fort Lee, NJ). For determining levels of OGD-induced synaptic depression, slope of fEPSP calculated at 90 min following OGD was normalized to fEPSP slope at the 10 min baseline.

Student’s independent, two-tailed t-test was performed to determine statistical significance between the means of two groups. For data normalized to baseline (percentage changes) and for data sets that failed to pass the Shapiro-Wilk normality test, two-tailed non-parametric
Wilcoxon tests were performed. Mann-Whitney tests were used for non-paired comparison of drug effects between two groups, and a Kruskal-Wallace test with Dunn’s post hoc analysis were used to compare non-parametric data with >2 groups (see text and figure captions). For detection of drug and genotype effects within an experiment, a two-way ANOVA and Bonferroni post hoc analysis were performed. Significance is described at the level of *p≤0.05, **0.01, and ***0.001. Values were presented as mean ± standard error of the mean (SEM).

Results

*Ex vivo* slices retain similar 24S-HC levels as fresh tissue.

The level of endogenous 24S-HC is dramatically reduced in mice genetically deficient in *CYP46A1* (Russell et al. 2009). To confirm the reduction of endogenous 24S-HC in the CYP46A1 KO mice used in our study, we analyzed and compared 24S-HC level in WT and CYP46A1 KO mouse hippocampal slices. Data from LC-MS analysis showed that average 24S-HC level in freshly isolated hippocampus is significantly lower in KO (1.8 ± 0.2 ng/mg protein; N = 3) than in WT (385.0 ± 11.6 ng/mg protein; N =3; Fig. 1A, ***p < 0.001), suggesting that, as expected (Lund et al. 2003; Russell et al. 2009; Meljon et al. 2014), endogenous 24S-HC level is greatly reduced in the KO mice. The estimated concentration of endogenous 24S-HC level in WT hippocampus was in the tens of micromolar, sufficient to modulate NMDAR function, assuming homogenous distribution of the oxysterol (Paul et al. 2013). The results showed a slightly lower level of 24S-HC in WT slices than in intact WT tissue; however, 24S-HC was still much higher in WT slices (326.0 ± 16.2 ng/mg protein; N = 3) than in KO slices (2.0 ± 0.5 ng/mg protein; N = 3; Fig. 1A, ***p < 0.001), suggesting retention of high 24S-HC levels despite continuous perfusion of slices (Fig.1A). This is consistent with
strong retention of 24S-HC in cells (Paul et al. 2013) but could also imply ongoing synthesis. The observation that slices have nearly the same 24S-HC concentration as fresh tissue implies that slices are an appropriate model for exploring the potential role of endogenous 24S-HC in regulating NMDAR function.

**NMDAR EPSCs, tonic current and NMDAR induced spiking are reduced in CYP46A1 KO slices**

24S-HC and its analogues potentiate NMDAR activity when applied exogenously (Paul et al. 2013). However, the role of endogenous 24S-HC in modulating NMDAR activity has not been established. To address this, we first examined effects of endogenous 24S-HC on NMDAR tone, measured in evoked, dual-component EPSCs. CYP46A1 KO slices exhibited a significantly smaller NMDAR to AMPAR EPSC ratio than WT slices (Fig. 1B-C), suggesting that reducing endogenous 24S-HC in KO slices dampens NMDAR function. The results further suggest that basal NMDAR contribution to EPSCs reflects not only glutamate and glycine co-agonist presence, but also the presence of the positive allosteric modulator, 24S-HC.

Although the effect of CYP46A1 KO on NMDAR EPSCs was significant, it was not a strong effect. To confirm that endogenous 24S-HC modulates the basal NMDAR current tone, we examined whether tonic NMDAR current induced by ambient glutamate was also reduced in CYP46A1 KO slices. We reasoned that 24S-HC’s modulatory effects may be more evident at the lower, ambient glutamate concentrations than that at saturating, synaptic concentrations (Linsenbardt et al. 2014). Consistent with this idea, addition of the glutamate transporter blocker TBOA for 6 min, in the presence of blockers of GABARs and AMPARs, induced larger
currents in WT neurons than in KO neurons (Fig. 2A-C). The contribution of NMDARs to these currents was verified with MK-801, an activation dependent blocker of NMDARs.

MK-801 blocking rate is proportional to channel open probability (Huettner and Bean 1988; Rosenmund et al. 1995). Consistent with our interpretation that larger TBOA-induced currents in WT slices represent channel openings with higher open probability, we found that MK-801 induced blocking rate was faster in WT slices than in KO slices (Fig. 2D).

Although NMDARs play a limited role in basal synaptic transmission at voltages near the resting membrane potential (~-60 to -70 mV), the NMDAR contribution is amplified when neurons receive repetitive excitatory synaptic drive, resulting in persistent depolarization, relief from physiological Mg$^{2+}$ block of the NMDAR channel, and increased participation of NMDARs in spike generation (reviewed in Hunt and Castillo 2012). To address whether loss of CYP46A1 and the associated reduced NMDAR contribution to EPSCs (Fig. 1) affect the role of NMDARs in driving spike firing, we current clamped WT or KO neurons and adopted a repetitive synaptic stimulation protocol to elicit NMDAR dependent spiking. Trains of 10 pulses at 50 Hz were delivered to promote dual-component EPSPs with stimulation intensity adjusted to evoke 5-6 spikes (approximately half of the pulse number) for each cell at baseline (Fig. 3, A-C). Input/output curves verified that WT and KO cells produced similar maximum firing rates with saturated levels of synaptic stimulation (8.7± 0.3 for WT, 8.7 ± 0.4 for KO, Fig. 3D). Therefore, the criterion of 5-6 spikes at baseline represented ~60-70% of maximum for both groups. After baseline recording of 10 sweeps, 50 μM D-APV was washed-in to block NMDAR-mediated EPSPs (Fig. 3A, B, E), and the same stimulus protocol and intensity used at
baseline was applied again to evoke spiking. D-APV more strongly reduced spiking in WT cells than in KO cells (Fig. 3A, B, E), suggesting more NMDAR contribution to EPSP-spike generation in WT animals. These results are consistent with the reduced NMDAR synaptic tone in KO animals (Fig. 1,2).

An exogenous analogue demonstrates lack of saturation and specificity

24S-HC appears to potentiate NMDAR activity at a unique binding site specific for oxysterols (Paul et al. 2013). To address whether the endogenous level of 24S-HC saturates this NMDAR binding site, the synthetic 24S-HC analogue SGE-301 (10 μM) was bath-applied to slices after a stable 10 min baseline recording, and its effect on NMDAR function was examined. When overall drug effect across genotypes was evaluated, SGE-301 significantly potentiated NMDAR EPSCs, but there was no interaction between genotype and drug condition (Fig. 4A, B). This suggests that SGE-301 potentiation was statistically indistinguishable in KO versus WT slices and that the NMDAR allosteric site is therefore not saturated by endogenous 24S-HC despite estimated high levels of the oxysterol (Fig. 1A).

In hippocampal cultures, potentiation by 24S-HC and its analogues is selective for NMDARs, given that no potentiation of evoked AMPAR EPSCs or GABAR IPSCs was observed when the compounds were exogenously applied to WT neurons (Paul et al. 2013). However, selectivity has not been examined \textit{in situ} or in the CYP46A1 null background, where saturation of putative sites by endogenous 24S-HC is not a concern. To address this, we studied effects of SGE-301 on AMPAR EPSCs and GABAR IPSCs in CYP46A1 KO slices, where potential effects of endogenous 24S-HC can be excluded. We found that, while significantly potentiating
NMDAR EPSCs relative to baseline (Fig. 4A, E), SGE-301 failed to potentiate AMPAR EPSCs (Fig. 4C, E; Wilcoxon test; P > 0.2).

SGE-301 effects on GABAR IPSCs were more complex (Fig. 4D-E). Overall, there was no significant change in IPSCs (Wilcoxon test, P > 0.05, N = 11). When one outlying cell exhibiting potentiation during SGE-301 presentation was excluded from analysis, we found a small but significant decrease (29.7± 4.4%, N = 10, Wilcoxon test, **P< 0.01) in IPSC amplitude following 6 min application of SGE-301. However, analysis of the time course of IPSC depression in these cells revealed that IPSC depression was not clearly linked to SGE-301 application. Instead, IPSCs typically showed a gradual, time-dependent decrease, and this decrease did not differ from that observed in 7 KO cells treated with vehicle (DMSO) only. These IPSCs exhibited a 17.4± 8.7% depression over 6 min, not statistically different from the depression exhibited by SGE-301 (Mann-Whitney test, P > 0.2; data not shown). Thus, we conclude that IPSCs exhibit time-dependent rundown but, like AMPAR EPSCs, show no evidence for SGE-301 related potentiation or inhibition.

Reducing endogenous 24S-HC does not affect neuronal intrinsic excitability or spontaneous transmission

Increased neuronal intrinsic excitability has been reported in several GluN1 KO mouse models (Pagadala et al. 2013; Tatard-Leitman et al. 2015). This effect is apparently a homeostatic response to loss of the obligatory GluN1 subunit, without which NMDAR currents are eliminated. Other studies using sensory deprivation or pharmacological NMDAR block have also observed compensatory changes in intrinsic excitability and/or synaptic transmission.
(Turrigiano et al. 1994; Zhang and Linden 2003; Turrigiano and Nelson 2004; van Welie et al. 2004; Fan et al. 2005). It remains unclear whether the more subtle manipulation of altering NMDAR positive allosteric modulation also elicits homeostatic compensation from intrinsic conductances. To test this, we compared WT and KO responses to 500 ms current steps, ranging from 0 – 800 pA (Fig. 5A, B). Neurons were current clamped to -74 mV prior to steps using a small bias current when needed. We found no significant difference in any of the parameters related to intrinsic excitability, including firing rates vs injected current intensity, threshold of action potential firing, resting membrane potential with no injected bias current, or membrane resistance (Fig. 5C-F). We conclude that loss of oxysterol positive allosteric modulation is not sufficiently severe to invoke homeostatic changes in excitability.

Increased spontaneous basal excitatory synaptic transmission has been reported in GluN1 KO mouse models (Pagadala et al. 2013; Tatard-Leitman et al. 2015), possibly arising as a result of altered intrinsic excitability or representing a separate homeostatic effect on transmission. To elucidate whether changing 24S-HC levels alters basal spontaneous synaptic transmission, we measured frequency of spontaneous EPSCs (sEPSCs) and IPSCs (sIPSCs) in individual neurons, and we compared the frequency ratio of sEPSCs to sIPSCs (E/I ratio) in WT versus KO slices. sEPSCs and sIPSCs were recorded at holding potentials of -70 mV and 0 mV, respectively (Fig. 6A,B). We observed no difference of sEPSC and sIPSC frequency and E/I ratio between WT and CYP46A1 KO neurons (Fig. 6C). Furthermore, no significant change of sEPSC amplitude was observed between WT and KO neurons (data not shown). Taken together, we find that loss of positive allosteric modulation is not sufficiently severe to invoke compensatory changes in intrinsic conductances or in spontaneous synaptic transmission.
Reducing endogenous 24S-HC does not affect LTP induction

Impaired NMDAR dependent LTP in CYP46A1 KO mice has been demonstrated previously in 5-8 week old mice (Kotti et al. 2006; Kotti et al. 2008; Russell et al. 2009). We compared LTP in slices prepared from 30-day-old WT and KO animals. Field EPSPs (fEPSPs) were evoked at 50% maximal level to obtain a stable 10 min baseline recording. Slices were then stimulated at 100 Hz for 1 s to induce LTP, after which the fEPSP was monitored for an additional 60 min.

Surprisingly, KO slices exhibited LTP indistinguishable from LTP in WT slices (Fig. 7A), suggesting that endogenous 24S-HC is not necessary for LTP that is induced by tetanic stimulation. Thus, it appears that basal NMDAR function in 30-day-old KO slices is sufficient for normal LTP induction.

CYP46A1 KO slices exhibit higher resistance to OGD-induced synaptic depression

Despite our results showing intact LTP in CYP46A1 KO slices, we found that tonic NMDAR currents, perhaps generated largely by extrasynaptic receptors, are strongly affected by 24S-HC deletion (Fig. 2). Such conditions may be met by pathophysiological overstimulation of NMDARs during ischemia in situ or by oxygen-glucose deprivation in vitro (Soria et al. 2014).

Given that NMDAR overactivation participates in ischemia-mediated excitotoxicity and synaptic depression (Weilinger et al. 2013), and a form of NMDAR dependent synaptic depression can be induced in response to oxygen deprivation (Izumi et al. 1998), we hypothesized that 24S-HC reduction may alter OGD-induced synaptic depression or its recovery. To test this hypothesis, we examined synaptic depression caused by 20 min OGD in hippocampal slices. Field EPSPs (fEPSPs) were evoked at 50% maximal level, and fEPSP slopes were compared before and after OGD in WT vs KO slices. OGD resulted in > 80% depression of fEPSPs from both WT and KO slices (30’). 90 min following OGD wash-out (120’), recovery of fEPSPs was
significantly greater in KO slices compared with WT slices (Fig. 7B), suggesting that reduction of 24S-HC protects neurotransmission from OGD-induced synaptic depression. To test if 24S-HC is likely responsible for the persistent synaptic depression in WT slices, we treated KO slices with 1 μM SGE-301 before and during OGD. Similar to WT slices, KO slices treated with SGE-301 exhibited fEPSP depression that failed to recover to the level observed from untreated KO slices after OGD wash-out (Fig. 7B). Thus, acute application of SGE-301 on KO slices restores persistent OGD-induced synaptic depression observed in WT slices.

Discussion

Our results revealed reduced NMDAR activity and function in mice lacking endogenous 24S-HC. We found evidence for decreased NMDAR contributions to evoked EPSCs, to TBOA-induced tonic currents, and to NMDAR-dependent action potential generation, and we found resistance to NMDAR-mediated synaptic depression during OGD when endogenous 24S-HC level is greatly reduced. These changes occur in a context in which overall synaptic transmission and intrinsic excitability are not detectably altered. This pattern of results suggests that endogenous 24S-HC contributes to NMDAR dependent transmission in both physiological and pathophysiological conditions. The findings suggest some optimism for the strategy of therapeutic manipulation of 24S-HC levels, which minimizes collateral side effects.

One possible interpretation of our main finding could be that altered NMDAR function is a secondary consequence of CYP46A1 deletion, resulting perhaps from altered cholesterol levels or from transcriptional or post-transcriptional effects of the oxysterol (Wang et al. 2002; Maioli et al. 2013; Hong and Tontonoz 2014) and a consequent reduction in NMDAR
expression or fewer functional synaptic NMDARs. Previous study of CYP46A1 knockout animals revealed no overall changes in cholesterol levels, attributed to feedback regulation of cholesterol synthesis (Lund et al. 2003; Russell et al. 2009; Meljon et al. 2014). CYP46A1 KO mice are outwardly normal without significant changes in physiological measures compared to WT mice (Lund et al. 2003). CYP46A1 KO mice exhibit normal cellular architecture of the hippocampus, indistinguishable synapse morphology compared to WT mice, and unchanged endogenous GluN1 levels (Kotti et al. 2006). Our own results suggest no impairment of AMPAR EPSCs and GABAR IPSCs or alteration in excitability (see below). Furthermore, MK-801 blocking rates of tonic NMDAR currents are slower in KO slices than in WT (Fig. 2D), supporting our hypothesis that channel open probability (Huettner and Bean 1988; Rosenmund et al. 1995; Emnett et al. 2015), not receptor number, is the major difference between WT and KO cells. Although we cannot fully exclude more complicated explanations of reduced NMDAR EPSCs in KO mice, the most parsimonious explanation of reduced NMDAR function consistent with known acute effects of 24S-HC (Paul et al. 2013; Linsenbardt et al. 2014) is that NMDARs in KO slices show reduced positive allosteric tone.

NMDAR-dependent spiking was reduced in 24S-HC deficient slices, consistent with the reduced NMDAR EPSCs. NMDARs play essential roles in information processing in the brain, and their contribution is frequency-dependent, as a result of physiological Mg$^{2+}$ block of the channel (Herron et al. 1986; Daw et al. 1993; Hunt and Castillo 2012). The slow decay and voltage-dependent activation of NMDAR EPSCs contribute to temporal summation of EPSPs and resultant spike generation following repetitive synaptic activity (Dingledine et al. 1986; Augustinaite and Heggelund 2007). Therefore, NMDARs integrate synaptic inputs (Larkum and...
Nevian 2008), promote recurrent excitation of cortical networks (Wang et al. 2008), and generate persistent activity of neural assemblies (Major and Tank 2004; Hunt and Castillo 2012). In the present study, KO cell spiking showed reduced sensitivity to D-APV (Fig. 3A-E), suggesting that the relatively small changes in NMDAR tone translate into functionally relevant effects on activity.

NMDARs have important long-term effects on synaptic development, pruning, and plasticity of established synapses (Cohen and Greenberg 2008; Hunt and Castillo 2012; Sanz-Clemente et al. 2013). Therefore, it may be surprising that we failed to observe evidence for compensatory or Hebbian effects of 24S-HC loss on spontaneous transmission and/or intrinsic excitability (Fig. 5,6). Also surprising is the lack of effect of 24S-HC reduction on LTP induction in slices (Fig. 7A). A recent study showed that forebrain-selective GluN1 deletion altered basal synaptic transmission and intrinsic excitability of CA1 neurons (Tatard-Leitman et al. 2015). Furthermore, NMDAR activation has been associated with changes in intrinsic excitability (Zhang and Linden 2003), as well as triggering AMPAR insertion underlying LTP (Malinow and Malenka 2002). On the other hand, blocking NMDARs is not a particularly effective trigger for homeostatic plasticity (Turrigiano and Nelson 2004), and our results in slices suggested no effect of 24S-HC reduction on evoked LTP (Fig. 7A). Overall, our inability to detect secondary changes in transmission, excitability, or LTP may arise from the weaker NMDAR manipulation in our study than in other studies. For instance, forebrain GluN1 deletion will cause complete loss of NMDAR function in affected regions. A role of 24S-HC in LTP induction may only be evident in older animals or under certain stimulation protocols (Kotti et al. 2006; Kotti et al. 2008). Thus, we propose that mild manipulation of NMDARs by allosteric modulation in our
study prevented strong compensatory changes and is consistent with the relatively mild phenotype for KO animals (Kotti et al. 2006; Russell et al. 2009).

Enhancing NMDAR activity is a potential treatment strategy for schizophrenia (Hashimoto 2014) and for age-related dementia (Burgdorf et al. 2011). Interestingly, elevating endogenous levels of 24S-HC has been reported effective for improving learning and memory deficits associated with aging (Maioli et al. 2013), suggesting a possible link between 24S-HC level and NMDAR function/activity. However, it is unclear whether increasing endogenous 24S-HC levels would be an effective treatment if 24S-HC modulatory sites on NMDARs are fully occupied. Our results suggest that in the ex vivo slice, sites are not fully occupied, as indicated by SGE-301 potentiation of NMDAR function in WT tissue (Fig. 4). This suggests that endogenous 24S-HC does not saturate NMDARs in situ. A possible explanation for this finding is that certain amount of 24S-HC may be lost during slice preparation. However, the endogenous 24S-HC level, when measured from WT hippocampal slices (326.0 ± 16.2 ng/mg protein; Fig.1A), was similar to fresh tissue (385.0 ± 11.6 ng/mg protein; Fig.1A). Our measured levels were also similar to previously reported measurements from freshly prepared brain tissues (30-60 ng/mg total tissue in adult mice brain) (Karu et al. 2007; Griffiths and Wang 2011; Meljon et al. 2012), assuming protein is ~10% of tissue weight. Overall, our slice measurements suggest that net loss of 24S-HC with slice preparation and perfusion is not a concern in our experimental conditions and that 24S-HC is retained and/or synthesized locally for up to 5-6 h ex vivo. The lack of functional saturation in the context of high tissue 24S-HC levels suggests that a large fraction of 24S-HC may be sequestered and not active at NMDARs. An implication of the lack of saturation is that compounds like SGE-301, with
selective potentiating effects at NMDARs (Fig. 4), may be effective drug candidates (Paul et al. 2013).

Down-regulation of oxysterol function also may have therapeutic potential, for instance in stroke. NMDAR-mediated excitotoxicity is a major outcome of the bio-energetic failure associated with stroke (reviewed in Hardingham and Bading 2010). Unfortunately, clinical trials aimed at directly inhibiting/blocking NMDARs have not experienced much success due to multiple undesired side effects (Muir 2006). Here, we found that KO slices exhibited improved recovery of synaptic transmission from NMDAR-dependent, OGD-induced synaptic depression (Fig. 7B), suggesting potential therapeutic benefit of 24S-HC reduction. This beneficial effect was prevented by slice pretreatment with a 24S-HC analogue, suggesting that levels of endogenous 24S-HC directly modulate NMDAR-mediated synaptic depression in WT tissue.

Approaches for reducing endogenous 24S-HC effects on NMDARs might include targeting CYP46A1 function genetically or pharmacologically (Mast et al. 2010; Shafaati et al. 2010), although both shRNA and pharmacological inhibition of CYP46A1 have yielded secondary consequences that would have to be addressed in a therapeutic setting (Fourgeux et al. 2014; Chali et al. 2015). An alternative approach is to directly antagonize endogenous 24S-HC’s positive allosteric effects on NMDARs (Linsenbardt et al. 2014). Taken together, our findings suggest that reducing 24S-HC’s action on NMDARs may offer a novel therapeutic approach for conditions involving NMDAR over-activity.

In summary, results from this study support the hypothesis that endogenous 24S-HC selectively modulates NMDAR function. We find little evidence for secondary consequences of
either genetic CYP46A1 deletion or of the reduced positive NMDAR tone. Our evidence suggests that the endogenous oxysterol site is not saturated by endogenous 24S-HC levels, so that up-regulation and down-regulation of 24S-HC levels may be used endogenously to govern normal NMDAR-dependent plasticity, and both upregulation and downregulation might represent viable therapeutic strategies.
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Conflicts. CFZ is a member of the Scientific Advisory Board for Sage Therapeutics, Inc. and holds equity in Sage Therapeutics. Washington University in St. Louis has licensed technology to Sage Therapeutics. The research herein was not supported by or licensed to Sage Therapeutics.
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Figure Captions

Figure 1. Decreased NMDAR positive tone in CYP46A1 KO slices. A: Endogenous 24S-HC was significantly reduced in KO tissue and slices (N = 3 WT and 3 KO animals; ***P < 0.001). B: Dual-component NMDAR and AMPAR EPSCs (red) were evoked in WT and KO slices at -30 mV. NMDAR EPSCs, measured 50 ms following peak EPSC, were completely blocked following 50 μM D-APV application (black). AMPAR EPSC peak amplitude was calculated after D-APV application. C: NMDAR/AMPAR ratio was compared between WT and KO slices (N = 15 cells from WT and 9 cells from KO slices; *P < 0.05).

Figure 2. Induced tonic NMDAR currents are smaller and represent decreased channel open probability in KO slices. A and B: WT and KO responses to TBOA (50 μM) application in the presence of AMPAR and GABAR blockers (10 μM NBQX and 100 μM PTX), followed by co-application of 40 μM MK-801. The solid red line is a fit to a single exponential decay. C. Summary of TBOA induced current (N = 13 WT and 13 KO cells, *P < 0.05). D. Time constant of current decay in the presence of MK-801 (n = 9 WT and 10 KO cells; **P < 0.01). The time constant was not obtainable in 4 WT and 3 KO cells due to a large, prolonged EPSC at the onset of MK-801 application in this subset of cells. Although the source of the contaminating current is not completely clear, it may represent network disinhibition upon initial MK-801 application.

Figure 3. NMDAR induced spiking is reduced in CYP46A1 KO slices. A and B: EPSPs and associated action potentials were evoked in WT (A) and KO (B) slices at -65 mV during a 10-pulse, 50 Hz presynaptic stimulus train. Neither NBQX nor D-APV was present at baseline.
EPSPs were then re-evoked with the same stimulus parameters after 50 µM D-APV wash-in. 

C: Spike number at baseline in WT or KO neurons. D: Input-output curve for spikes evoked by repetitive synaptic stimulation in WT and KO neurons at baseline. WT and KO neurons share similar maximum firing capacity at a saturated level of synaptic stimulation (N = 18 WT and 17 KO cells). E: Normalized (after/before D-APV) spike number following D-APV wash in showed a weaker D-APV effect on KO slices (N = 18 cells from WT and 17 cells from KO slices; *P < 0.05).

Figure 4. Endogenous 24S-HC does not saturate oxysterol-mediated potentiation, and is selective for NMDAR EPSCs. A: NMDAR EPSCs were evoked at -30 mV in WT and KO slices in the presence of 100 µM PTX and 10 µM NBQX. After a 10 min stable baseline was established (black traces), SGE-301 was bath-applied for 15-30 min. After 6 min of SGE-301 perfusion, potentiation of EPSCs had stabilized and was averaged from traces in the next 10 min (red traces). B: Summary of SGE-301 (10 µM) effect in WT and KO slices. Data from 10-15 min following drug onset were averaged for each cell and expressed relative to the average baseline responses (10 min) before drug delivery. A two-way ANOVA and Bonferroni post hoc analysis revealed a significant effect of SGE-301 (df = 1; F = 7.8; **P < 0.01) but no effect of genotype and no significant interaction between genotype and drug. (N = 10 cells from WT and 10 cells from KO in DMSO group; N = 13 cells from WT and 14 cells from KO slices in SGE-301 group; df = 1; F = 0.8; P > 0.2). C: AMPAR EPSCs were evoked at -70 mV in KO slices in the presence of 100 µM PTX and 50 µM D-APV. After a 10 min stable baseline was established (black trace), SGE-301 was bath-applied for 15-30 min. Potentiation was
measured after 6 min of SGE-301 perfusion (red trace). D: GABAR IPSCs were evoked at 0 mV in KO slices in the presence of 10 μM NBQX and 50 μM D-APV. After a 10 min stable baseline was established (black trace), SGE-301 was bath-applied for 15-30 min. Potentiation was measured after 6 min of SGE-301 perfusion (red trace). E: Normalized (after/before) peak amplitude was compared for NMDAR, AMPAR and GABAR PSCs after 6 min SGE-301 application (N = 14 cells for NMDAR, 7 cells for AMPAR, and 11 cells for GABAR; Wilcoxon test ***P< 0.001).

**Figure 5. Intrinsic excitability is unaffected by CYP46A1 deletion. A-B**: A 500 ms current pulse, ranging from 0 – 800 pA to evoke trains of action potentials, was injected into WT or KO neurons held at -74 mV. C: The number of spikes evoked by injected current at certain intensity was compared between WT and KO neurons. D-F: Parameters related to intrinsic excitability, including threshold of action potential firing, resting membrane potential, and membrane resistance were compared between WT and KO neurons (N = 21 WT cells and 18 KO cells, P > 0.1).

**Figure 6. Spontaneous transmission is not detectably altered in CYP46A1 KO slices. A-B**: Representative traces of sEPSCs and sIPSCs recorded from WT and KO neurons. C: Frequency of sEPSC, sIPSC, and E/I ratio were compared between WT and KO neurons (N = 10 WT cells and 9 KO cells; P > 0.1).

**Figure 7. NMDAR dependent LTP is unaltered, while synaptic depression induced by OGD is abrogated in CYP46A1 KO slices. A**: fEPSPs were evoked at 50% maximal level.
fEPSP slopes were compared before and after 100 Hz, 1s tetanic stimulation in WT (open squares) and KO slices (open circles). At 60 min following tetanic stimulation, potentiation of fEPSPs was indistinguishable between WT and KO slices (N = 5 WT slices and 5 KO slices, P > 0.2). B. fEPSPs were evoked at 50% maximal level. fEPSP slopes were compared before/after 20 min OGD in WT (open squares) and KO slices (open circles), and KO slices treated with 1 μM SGE-301 before and during OGD for 40 min total (closed circles). OGD produced > 80% depression of fEPSPs (30’) from all the three groups. After 90 min OGD wash-out (120’), recovery of fEPSPs was significantly greater in KO slices, but not in WT slices or KO slices treated with 1 μM SGE-301 (N = 5 WT slices, 5 KO slices, and 5 KO slices treated with SGE-301. *P < 0.05; Kruskal-Wallis test and Dunn’s post hoc analysis). Insets show representative traces from the various conditions at the indicated time points. Scale bar: 1 mV, 5 ms.
A  WT    sEPSC

B  KO    sIPSC

C  sEPSC  sIPSC  E/I ratio

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