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Preferential enhancement of GluN2B-containing native NMDA receptors by the endogenous modulator 24S-hydroxycholesterol in hippocampal neurons

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Abstract

24S-hydroxycholesterol (24HC) is the major metabolic breakdown product of cholesterol in the brain. Among its other effects on neurons, 24HC modulates N-methyl-D-aspartate (NMDA or GluN) receptors, but our understanding of this mechanism is poor. We used whole-cell patch clamp recordings and various pharmacological approaches in mouse brain slices to record isolated NMDAR-mediated (I_{NMDA}) tonic and evoked synaptic currents. 24HC (1 μM) significantly enhanced tonic, but not evoked, I_{NMDA} of dentate gyrus granule cells. The I_{NMDA} had both GluN2A and GluN2B-mediated components. Preincubation of the slices with PEAQX (a GluN2A antagonist) or Ro25–6981 (a GluN2B antagonist) dramatically changed the I_{NMDA} modulatory potential of 24HC. Ro25–6981 blocked the enhancing effect of 24HC on tonic I_{NMDA} , while preincubation with PEAQX had no effect. In cholesterol 24-hydroxylase (CYP46A1) knockout mice, in sharp contrast to WT, 24HC slightly decreased the tonic I_{NMDA} of granule cells. Furthermore, 24HC had no effect on tonic I_{NMDA} of dentate gyrus parvalbumin interneurons (PVINs), known to express different GluN subunits than granule cells. Taken together, our results revealed a specific enhancement of GluN2B-containing NMDARs by 24HC, indicating a novel endogenous pathway to influence a subclass of NMDARs critically involved in cortical plasticity and in numerous neurological and psychiatric disorders.

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Keywords

24S-hydroxycholesterol; NMDAR; CYP46A1; GluN2B; parvalbumin; dentate gyrus

1. Introduction

The N-methyl-D-aspartate receptors (NMDARs or GluNRs) are hetero-tetrameric ligand-gated ion channels of the ionotropic glutamate (Glu) receptor family (Regan et al., 2015). So far, seven genetically encoded subunits of NMDARs have been found: GluN1, four GluN2 (GluN2A-D) and two GluN3 (GluN3A-B). Activation of NMDARs requires the presence of the agonist Glu, as well as one other co-agonist: glycine or D-serine. A functional NMDAR is thought to be assembled of two GluN1 glycine binding and two GluN2 Glu-binding subunits (Tajima et al., 2016). GluN1 is expressed throughout the brain, in almost all neurons and in some glia. GluN2A and GluN2B have an overlapping expression in the cortex and hippocampus, with expression primarily, though not exclusively, in principal neurons (Monyer et al., 1994). It is noted that expression of GluN2A and GluN2D containing NMDARs is largely confined to cortical and hippocampal GABAergic neurons, mainly parvalbumin-containing (von Engelhardt et al., 2015). Synaptic GluN2A containing receptors appose presynaptic release sites, whereas GluN2B containing receptors are considered to be located predominantly outside the postsynaptic density (Tovar and Westbrook, 2017). Previous work has shown that a persistent activation of NMDARs is usually mediated by GluN2B *in vivo* (Monaco et al., 2015). Because of the complex composition and function of NMDARs and their numerous subunit assembly possibilities, identifying the modulation of specific receptor subunit combinations is highly desirable.

Excessive activation or inactivation of NMDARs in the brain is involved in multiple neuropsychiatric disorders including schizophrenia, epilepsy, depression (Machado-Vieira et al., 2017), Alzheimer's diseases (AD) (Olivares et al., 2012), and ischemic brain injury (Amantea and Bagetta, 2017). NMDARs can be regulated by various endogenous and exogenous factors, and have been regarded as coincidence detectors because of their ligand-gated and voltage-dependent properties. The activation of NMDAR requires both binding of glutamate and a coagonist (glycine or D-serine) and postsynaptic depolarization. In addition, NMDARs also contain several regulatory sites sensitive to endogenous elements such as polyamines, Zn^{2+} , protons, glutathione, 24-hydroxycholesterol (24HC) and others (Wyllie et al., 2013).

In addition to the liver, the brain is the second organ in the body that can synthesize cholesterol. Cholesterol-derived neurosteroids are positive allosteric modulators (PAM) of NMDAR in the brain. In neurons, cholesterol hydroxylase enzymes metabolize cholesterol into the oxysterols 24-hydroxycholesterol (24HC), 25-hydroxycholesterol, and 27-hydroxycholesterol (Zhang and Liu, 2015). Only these metabolic products of cholesterol can cross the blood-brain barrier, and thus are responsible for the removal of excess cholesterol from the brain (Bjorkhem, 2006).

24HC is considered the most abundant cholesterol metabolite found in the brain. Previous reports show that 24HC, as well as its synthetic derivatives are both modulators of NMDAR

(Korinek et al., 2015; Sun et al., 2017). In cultured hippocampal neurons, 24HC shows a highly selective NMDAR modulation and interacts with a site distinct from other neuroactive steroids (Linsenhardt et al., 2014), and may be critical for maintaining the NMDARs tone under normal physiological conditions (Sun et al., 2016a). Here, by using whole-cell patch clamp recordings in mouse brain slices, and pharmacological methods, we recorded isolated NMDAR-mediated (I_{NMDA}) tonic and evoked postsynaptic currents from dentate gyrus granule cells of both wild type and cholesterol 24-hydroxylase (CYP46A1) deficient mice, and from parvalbumin-containing interneurons (PV-INs). Our goal was to understand the effects of 24HC on I_{NMDA} mediated by native NMDAR assemblies present in the mature brain, and to uncover potential subunit-specific interactions of 24HC with NMDARs.

2. Materials and Methods

2.1 Slice Preparation.

Male C57BL/6J wild type (WT), CYP46A1 knockout (KO) mice, and PV cre X Ai14 mice (ages: 10 – 16 weeks) were used according to protocols approved by the UCLA Chancellor's Animal Research Committee. WT C57BL/6J or CYP46A1 KO (From Dr. David Russell, UT Southwestern) mice on a similar genetic background (JAX) were used in most studies. PV cre X Ai14 transgenic mice were generated by crossing PV-Cre (JAX Stock No: 008069) and Ai14 (JAX Stock No: 007914), both back-crossed for >10 generations on C57BL/6J background. Horizontal 350 μm hippocampal brain slices were prepared exactly as our previously reported (Ferando et al., 2016). For recording, brain slices were transferred to a submerged recording chamber at 34°C and perfused at 5 ml/min with a modified artificial cerebrospinal fluid (ACSF_{NMDA}) containing (in mM): 126 NaCl, 10 D-glucose, 26 NaHCO₃, 0.05 MgCl₂, 2.5 KCl, 1.25 NaH₂PO₄, 2 CaCl₂, 1.5 C₃H₃NaO₃, 1 L-Glutamine; and 0.005 D-Serine, 0.005 Glycine, 0.01 6,7-dinitroquinoxaline-2,3-dione (DNQX, Tocris Biosciences, Minneapolis, MN, USA), 0.03 picrotoxin (PTX, Sigma-Aldrich, St. Louis, MO, USA) were added to block ionotropic glutamatergic and GABA-ergic transmission. All salts were purchased from Sigma-Aldrich.

2.2 Patch Clamp.

Slices were visualized under IR-DIC upright microscope (Olympus BX-51WI, 20x XLUMPlan FL N objective) and whole-cell recordings were obtained from granule cells or PV-INs of the dentate gyrus with borosilicate patch pipettes (4 – 6 M Ω , King Precision Glass) containing of internal solutions (ICS) (in mM): 135 CsCl, 10 HEPES, 0.2 EGTA, 2 Na₂-ATP, 0.5 Na₂-GTP, 5 QX-314, 5 tetraethyl ammonium chloride (TEA). The pH of the ICS was adjusted to 7.2 with CsOH and its osmolarity was 285 – 290 mOsm. ICS were stored at –80°C in 1 ml aliquots. Before each experiment, ICS aliquots were thawed to room temperature and kept on ice during recording.

2.3 Drugs.

24 (S)-Hydroxycholesterol (24HC) (Focus Biomolecules, Plymouth Meeting, PA, USA, stock dissolved in DMSO to 10 mM). PEAQX tetrasodium salt, Ro25–6981 maleate and D-APV (Tocris Biosciences, Minneapolis, MN, USA).

2.4 Recording and Analysis.

Recordings were obtained using an Axon-patch 200B amplifier (Molecular Devices, San Jose, CA, USA), low-pass filtered at 5 kHz (Bessel, 8-pole) and digitized at 10 kHz with a National Instruments data acquisition board (BNC 2110, National Instruments, Austin, TX, USA). All data were acquired with EVAN (custom-designed LabView-based software).

Tonic current measurement. A custom written procedure (Wavemetrics, IGOR Pro 6.22A, Lake Oswego, OR, USA) was used to perform the analysis. An all-points histogram of a randomly selected recording segment of 10 s during the period of interest was plotted. A Gaussian was fitted to the part of the distribution from the minimum value at the left to the rightmost (largest) value of the histogram distribution. The mean of the fitted Gaussian was considered to be the tonic current (I_{tonic}). This process was repeated for all segments of interest.

Evoked current measurement. Evoked currents were extracted from continuous current recording and baseline corrected. The measurement of amplitude and decay time is described in legend of Fig. S2.

2.5 Statistical Analyses.

Data are presented as mean $\pm$ SEM, unless otherwise noted, two-tailed Wilcoxon paired rank test or two-tailed Mann-Whitney test, or one-way non-parametric ANOVA (Kruskal-Wallis) test was used for all data comparisons. Significance levels was set to $p < 0.05$. All statistics were calculated and using GraphPad Prism 6 (GraphPad software, La Jolla, CA, USA) and Excel 2016. The p values for significance levels are indicated in the text and/or figures.

3. Results

3.1 24HC increases APV-sensitive tonic I_{NMDA} and its variance

Isolated I_{NMDA} were recorded in a modified ACSF ($\text{ACSF}_{\text{NMDA}}$). The GABA_A, AMPA, and kainate receptors were pharmacologically blocked by PTX (30 μM) and DNQX (10 μM) respectively. In the recorded cells, Na⁺ and K⁺ channels were blocked by QX314, Cs⁺ and TEA in the intracellular solution. The I_{NMDA} could be blocked by applying of 50 μM APV (Fig. 1a). The right panel of Fig. 1a shows the all-points histograms of a representative 10 s long recordings in control, 1 μM 24HC and 50 μM APV. The Gaussian fit to the histograms demonstrated that 24HC right-shifted (increased) the tonic current (I_{tonic}) when compared to control. While holding the cells at -70 mV, an isolated I_{NMDA} (APV-sensitive I_{NMDA}) was obtained by subtracting the I_{tonic} in the presence of 50 μM APV at the end of each experiment. The openings of NMDA channels mostly contributed to the baseline APV-sensitive current (tonic I_{NMDA}) and its variance. 1 μM 24HC significantly increased the I_{tonic} of APV-sensitive I_{NMDA} to 220 % of control level (Fig. 1b). The variance (σ^2) of the APV-sensitive tonic I_{NMDA} was also significantly increased by 1 μM 24HC to 296 % of control level (Fig. 1c).

3.2 24HC predominantly affects NMDARs

We first performed a non-stationary noise analysis (NSNA) to estimate the single channel conductance of tonically active NMDARs. The transitional period during APV application was selected, covering the I_{NMDA} (either in control or during 24HC application) until APV was fully effective. The mean I_{tonic} and its variance (σ^2) were calculated every 1 s, and were scaled by subtracting the mean and the σ^2 respectively of the last 3 s segments recorded in the presence of APV. After plotting the σ^2 against the mean I_{tonic} , we wanted to fit the data with the relationship $\sigma^2 = iI_m - I_m^2/N$; where $I_m = iNp$, $\sigma^2 = i^2Np(1-p)$, and σ^2 is the variance, I_m is the mean I_{tonic} , i is the single channel current, N is the total number of channels. However, it was evident, that only the initial part of the parabola was present in our recordings, most likely due to a low p and/or N . Therefore, we resorted to fitting only the early linear segment of the parabola using linear regression to $\sigma^2 = iI_m$. The linear slope, giving a reasonable approximation of i , was $i = 1.72 \pm 0.51$ pA, $n = 5$ from transitional recording periods of control to APV applications; and $i = 3.25 \pm 0.56$ pA, $n = 9$ from transitional recording periods of 24HC to APV applications. The slight increase in single channel current by 24-HC was not statistically significant ($p = 0.11$, Mann-Whitney test). The pooled value of i from all $n = 14$ experiments was 2.71 ± 0.44 pA, which, assuming a driving force of 70 mV in our recordings ($V_h - V_{\text{rev}}$), yields an estimate of the single NMDARs conductance (γ) of 39 pS. This is marginally less than previously reported (Maki and Popescu, 2014), but our measurements were done in the presence of residual Mg^{2+} .

We next wanted to determine if 24HC had any action on the currents in the absence of NMDARs. Therefore, we applied $1\mu\text{M}$ 24HC after blocking NMDARs by $50\mu\text{M}$ APV. In the presence of APV, $1\mu\text{M}$ 24HC failed to change the I_{tonic} or its variance (Fig. S1a and b).

We also tested if 24HC affected spontaneous excitatory activity (sEPSCs), predominantly mediated by non-NMDA type glutamate (AMPA) receptors. EPSCs were recorded at $V_h = -70$ mV during perfusion of regular ACSF and of ACSF + $50\mu\text{M}$ APV (to block NMDARs). The amplitudes and frequencies of sEPSCs were evaluated. Results shown in Fig. S1c, d indicate that, under regular ACSF perfusion, $1\mu\text{M}$ 24HC had no effect on sEPSCs amplitudes, but it decreased their frequency. Under ACSF + $50\mu\text{M}$ APV perfusion, $1\mu\text{M}$ 24HC also did not change the amplitudes of non-NMDAR mediated sEPSCs, but still decreased the frequency. The amplitudes and frequencies of sEPSCs prior to 24HC applications in both regular ACSF and ACSF + $50\mu\text{M}$ APV were similar. In addition to sEPSCs, we also recorded spontaneous inhibitory activity (sIPSCs) from the same cells before and after 24HC application at $V_h = 0$ mV. Under regular ACSF perfusion, $1\mu\text{M}$ 24HC had no effect on sIPSCs amplitudes, but slightly decreased sIPSCs frequencies. In ACSF + $50\mu\text{M}$ APV perfusion, $1\mu\text{M}$ 24HC changed neither the amplitudes of sIPSCs nor their frequencies. The amplitudes and frequencies of sIPSCs prior to 24HC applications in regular ACSF and in ACSF + $50\mu\text{M}$ APV were similar (Mann-Whitney test, data not shown). For the recordings of the spontaneous synaptic events, CsCl in the recording pipette was replaced by an equimolar concentration of Cs-methanesulfonate (Cs-met).

3.3 Effect of 24HC on evoked synaptic I_{NMDA}

To record evoked synaptic I_{NMDA} , a paired pulse stimulation was delivered near the hippocampal fissure. The approximate locations of stimulation and recording are shown in Fig. S2a. During control, 1 μM 24HC, and 50 μM APV perfusion periods, 3 or more sets of paired stimuli were delivered. Traces in Fig. S2b show the averaged evoked I_{NMDA} recorded in a cell during each period. The amplitude of the evoked I_{NMDA} induced by the first pulse (P1), the second pulse (P2), paired-pulse ratio (PPR = P2/P1) and the decay time of P1 (τ_1) were measured and analyzed. The evoked I_{NMDA} were blocked by applying 50 μM APV, indicating that they were also predominantly mediated by NMDARs. The APV-sensitive evoked I_{NMDA} then was obtained by subtracting the responses remaining in the presence of 50 μM APV. Application of 1 μM 24HC did not change any of the measured parameters of the evoked I_{NMDA} (Fig. S2).

3.4 Voltage-dependence of the tonic I_{NMDA}

To analyze if the voltage-dependence of I_{NMDA} was altered by 24HC, we recorded tonic I_{NMDA} in granule cells while delivering a voltage ramp from +40 mV to -100 mV. Raw tonic I_{NMDA} traces with ramp stimulation with and without APV are shown in Fig. S3a. All data were fitted to the Woodhull model (Woodhull, 1973) using the equation $g/g_{\text{max}} = 1 - [\text{Mg}^{2+}]_o / (\text{Kd}_{\text{Mg}} \exp(\delta z F V / RT)) + [\text{Mg}^{2+}]_o$, to quantify the voltage dependence of the Mg^{2+} block. There was no significant change in the values derived from the fits after applying 24HC (Fig. S3b). Therefore, it appeared that 24HC does not change the Mg^{2+} affinity and/or voltage-dependence of NMDAR activation in our experiments.

3.5 Effects of GluN2A and GluN2B antagonist on tonic and synaptically evoked I_{NMDA}

Because of the residual Mg^{2+} -sensitivity of the NMDARs in our recording condition at a $V_h = -70$ mV, we wanted to remove all blocking effects of Mg^{2+} to avoid any confounding factors in the following pharmacological experiments. Therefore, we recorded the I_{NMDA} at a $V_h = +40$ mV. The tonic I_{NMDA} was more variable at this V_h when compared to recordings at $V_h = -70$ mV, but the effects of 24HC were similar: at $V_h = +40$ mV, 1 μM 24HC significantly increased the APV-sensitive tonic I_{NMDA} to 193 % of control (Fig. 3a and d) and its variance to 176 % of control (Fig. 3a and e), respectively. This result was in good agreement with our data obtained at $V_h = -70$ mV.

To explore the NMDAR subunit contributions to the recorded tonic and evoked synaptic responses, we used PEAQX (300 nM) to block NMDARs containing GluN2A subunits, and Ro25-6981 (1 μM) to block NMDARs with GluN2B subunits (Aroniadou-Anderjaska et al., 2018).

To verify the blocking effects of the two NMDAR subunit blockers, we first perfused either 300 nM PEAQX or 1 μM Ro25-6981 directly onto the slices during I_{NMDA} recording of the granule cells at a $V_h = +40$ mV. At the end of the recording, 50 μM APV was perfused to fully block I_{NMDA} . During each recording period, paired pulse stimulations were delivered to obtain evoked synaptic I_{NMDA} , as performed at the $V_h = -70$ mV. The GluN2A blocker PEAQX failed to affect significantly the APV-sensitive tonic I_{NMDA} (Fig. 2a and c) and its

variance (Fig. 2a and d). In contrast, the GluN2B antagonist Ro25–6981 significantly reduced both the APV-sensitive tonic I_{NMDA} (Fig. 2b and c) and its variance (Fig. 2b and d).

In contrast to its lack of effect on tonic I_{NMDA} , PEAQX decreased the amplitudes of APV-sensitive I_{NMDA} P1 and P2, but did not change the PPR or the τ_1 of P1. The GluN2B-specific antagonist Ro25–6981, also decreased the amplitude of P1 and of P2, but enhanced the PPR, and failed to affect the τ_1 of P1 (Fig. S4). Our results were consistent with the idea that tonic I_{NMDA} were predominantly mediated by Ro25–6981-sensitive GluN2B containing NMDARs, while both GluN2A and GluN2B subunits were involved in generating synaptically evoked I_{NMDA} .

3.6 24HC selectively potentiates tonic I_{NMDA} mediated by GluN2B-containing receptors

Since in both of our experiments at $V_h = -70$ mV and at $+40$ mV, 24HC acted predominantly on the tonic I_{NMDA} , pharmacologically identified to be mediated almost exclusively by GluN2B containing NMDARs, it follows that 24HC might preferentially potentiate the activity of such native receptors. Next, to ensure that GluN2A or GluN2B were completely blocked during the recordings, we preincubated all slices in the respective specific antagonists for at least 30 min before recordings, and continuously exposed the slices at $V_h = +40$ mV to the same concentration of specific antagonist during the recordings. PEAQX treatment did not alter the effect of $1 \mu\text{M}$ 24HC on tonic I_{NMDA} , since applying 24HC in the presence of PEAQX, the 24HC still significantly increased the APV-sensitive tonic I_{NMDA} (Fig. 3b and d) and its variance (Fig. 3b and e). In sharp contrast, Ro25–6981 treatment blocked the enhancing effect of 24HC on the APV-sensitive tonic I_{NMDA} (Fig. 3c and d), and reduced the 24HC effect on its variance (Fig. 3c and e). After preincubation and perfusion of the specific antagonists, the residual I_{NMDA} were sensitive to $50 \mu\text{M}$ APV. We compared the values of the I_{NMDA} recorded prior to the perfusion of 24HC among the three conditions (no drug, and two NMDAR blockers), by normalizing the tonic I_{NMDA} by the membrane capacitance (C_m). There were no significant differences among the C_m -normalized I_{NMDA} values in GluN2A/B blockers and those during control (no drug) recordings (I_{NMDA} normalized by C_m (A/F): 8.62 ± 1.41 in no drug, 6.10 ± 1.47 in PEAQX, 6.24 ± 0.86 in Ro25–6981, $p = 0.3771$; I_{NMDA} variance normalized by C_m (pA^2/pF): 17.11 ± 3.5 in no drug, 11.71 ± 3.34 in PEAQX, 8.69 ± 2.74 in Ro25–6981, $p = 0.1681$; $n = 9$ respectively, one-way Kruskal-Wallis test). The apparent discrepancy between this result and the effects of acute perfusions of the antagonists may be explained by the more robust statistical power of the paired comparisons (see Fig. 2). Also, the C_m of the recorded granule cells were indistinguishable among groups (C_m (pF): 22.56 ± 3.70 in no drug, 22.56 ± 1.60 in PEAQX and 23.0 ± 2.06 in Ro25–6981, $n = 9$ respectively, $p = 0.9279$, one-way Kruskal-Wallis test).

We also analyzed the effects of 24HC on evoked I_{NMDA} in the three pharmacological conditions. In slices without drugs, 24HC had no effect on the amplitude of P1, P2, PPR, or the τ_1 of P1. Those data are highly consistent with our results on evoked I_{NMDA} recorded at $V_h = -70$ mV. In PEAQX preincubated slices, 24HC decreased the amplitude of P1, and the amplitude of P2, but had no effect on PPR or the τ_1 of P1. In Ro-6981 preincubated slices, 24HC only decreased the amplitude of P2; but failed to affect either the amplitude of P1, or

PPR, the τ_1 of P1 was slightly elevated (Fig. S5). The precise contributions of GluN2A or GluN2B to the synaptically evoked I_{NMDA} by acute or chronic GluN2A/B antagonist application were impossible to determine. Thus the effects of 24HC on synaptically evoked I_{NMDA} are ambiguous, but future experiments using null mutants may be useful in addressing this issue.

3.7 No effects of 24HC on tonic I_{NMDA} in CYP46A1 KO mice

CYP46A1 is the main enzyme responsible for the production of 24HC from cholesterol in the brain. We used CYP46A1 KO mice to examine the role of endogenous 24HC production on I_{NMDA} . We recorded isolated I_{NMDA} from granule cells as in WT mice. After a control recording period, 1 μM 24HC was applied to the slices, and 50 μM APV was applied at the end of the recordings to fully block I_{NMDA} , $V_h = -70$ mV. Fig. 4a shows a typical recording from a control period up to the end of APV application. The all-point histograms represent distributions during 10 s recording segments from control, 1 μM 24HC and 50 μM APV conditions, respectively. In CYP46A1 KO mice slices, 1 μM 24HC slightly reduced the APV-sensitive tonic I_{NMDA} (Fig. 4b), but had no effect on its variance (Fig. 4c). We next compared the C_m -normalized tonic I_{NMDA} between WT and CYP46A1 KO mice. Surprisingly, there was a significant difference between the C_m itself of the granule cells recorded in the two preparations (Fig. 4d). This may be due to an increased membrane cholesterol concentration (Ohki, 1969), as CYP46A1 KO mice have heightened brain levels of cholesterol (Mast et al., 2017b). However, when normalized by the C_m values, there were no differences in the C_m -normalized APV-sensitive tonic I_{NMDA} (Fig. 4e) or its variance (Fig. 4f).

We also recorded and measured the evoked I_{NMDA} of CYP46A1 KO mice. Fig. S6a shows an example of the averaged evoked traces (averaged 3 raw traces each) from control, 24HC and APV applications recording period. 1 μM 24HC had no effect on amplitude of P1, amplitude of P2 and PPR; but slightly increased the τ_1 of P1 (Fig. S6).

3.8 24HC has no effect on tonic I_{NMDA} of PV-INs

We used td-Tomato fluorescence to identify PV-INs in slices prepared from PV-cre X Ai14 mice. Even without fluorescence, most PV-INs could be recognized based on their characteristic pyramidal shape that distinguishes them from the granule cells in the cell layer. Perfusion and recording solutions were identical as for the granule cells, V_h was +40 mV. As shown in Fig. 5a, a representative example of the raw data (left) and current histogram distributions (right), the tonic I_{NMDA} and its variance of PV-INs was unaffected by 1 μM 24HC (Fig. 5b and c). Prior to comparing the C_m -normalized APV-sensitive I_{NMDA} between granule cells (GCs) and PV-INs, we assessed the C_m in the two cell types and found no significant difference (Fig. 5d). In contrast, the C_m -normalized APV-sensitive tonic I_{NMDA} was considerably smaller in PV-IN than in GC (Fig. 5e). The variance of the C_m -normalized tonic I_{NMDA} was similar between GC and PV-IN (Fig. 5f).

For evoked I_{NMDA} of PV-INs (example averaged traces of three recording periods showed in Fig. S7a), 1 μM 24HC decreased the amplitudes of P1 and P2, but did not change the PPR or the τ_1 of P1 (Fig. S7).

4. Discussion

We explored the modulatory mechanisms of 24HC on native NMDARs in mouse brain slices. The data support the conclusion that in the dentate gyrus, 24HC has a cell type- and GluN2B subunit-specific enhancement of tonic I_{NMDA} .

Some of the neuromodulatory effects of 24HC have been previously described, demonstrating a strong PAM at NMDARs (Sun et al., 2016b), but an NMDAR subunit-specific modulation has not been reported. In cultured hippocampal neurons, sub-micromolar concentrations of 24HC potentially enhanced NMDAR mediated currents, an effect that did not overlap with that of other allosteric modulators (Paul et al., 2013). Although the exact mechanism of the potentiation could not be elucidated, it was clear that 24HC increased agonist efficacy at NMDARs by partitioning itself in the plasma membrane at its extracellular side and interacting with the receptor at a unique site. From chimeric receptor studies, it appears that 24HC potentiation requires only the transmembrane domain (TMD) of GluN2 subunits in combination with GluN1 (Wilding et al., 2016). Many studies using brain slices have showed that, exogenous 24HC enhanced LTP (Paul et al., 2013) and its synthetic analog SGE-201 increased neuronal activity (Emnett et al., 2015), which points to an enhancing effect of 24HC on synaptic activity, but lacks insights into its mechanism of action. But, when focusing on NMDA receptors, which are highly involved in synaptic plasticity and activity, most studies of the effects of 24HC or its related synthetic products relied on cultured neurons. In contrast to the cultured cells, our *ex vivo* studies in fully mature dentate gyrus granule cells showed that NMDAR-mediated evoked synaptic EPSCs are not much affected by 1 μM 24HC. This could be due to differences in the subunit composition of the synaptic receptors in the two preparations, as early on in development GluN2B receptors predominate (Monyer et al., 1994). The main effect of 24HC was to potentiate the tonic I_{NMDA} , an effect we showed to be predominantly mediated through GluN2B receptors, which are likely situated at extrasynaptic sites. The subunit composition of synaptic and extra-synaptic NMDARs is different and highly specific, and also varies with cell type, circuit and brain region (Tovar and Westbrook, 1999). Interestingly, in NMDARs expressed in HEK cells, the 24HC analog SGE-201 significantly potentiated all four types of GluN2 expressed with GluN1. Although no significant differences were noted between the various GluN2 subunit-containing receptors, the potentiation of GluN2B and GluN2C containing receptors appeared to be larger than that of the other two GluN2s. It could also be the case that the NMDARs responsible for generating the tonic I_{NMDA} in dentate gyrus granule cells are triheteromeric of the GluN1/GluN2A/GluN2B combination, that would also be effectively blocked by Ro25-6981 (Ayciriex et al., 2017).

Among GluN2A, GluN2B or GluN2D subunits, GluN2B is the most important target in modulating the function of NMDARs despite its predominant extrasynaptic distribution (Paoletti et al., 2013; Shipton and Paulsen, 2014). In clinical settings, a single, low dose of ketamine could produce rapid antidepressant actions in treatment-resistant depressed patients, which was related to reducing the activity of GluN2B subunit containing NMDARs in the cortex (Miller et al., 2014). Memantine, one of the few compounds approved to treat AD, also affects extra-synaptic GluN2B subunit-containing NMDARs (Xia et al., 2010). In contrast, the predominantly synaptically localized GluN2A mostly plays a role in mediating

synaptic plasticity, Ca^{2+} concentration changes in the postsynaptic cell, and neuron-glia interactions through a variety of intracellular signaling pathways (Kaufman et al., 2012). Segregation of GluN2A and GluN2B to synaptic and extra-synaptic sites may be incomplete (Petrálie et al., 2010; Thomas et al., 2006), but our results are consistent with GluN2B subunits of granule cells vastly contributing to the tonic I_{NMDA} which also constitutes the predominant modulatory target of 24HC.

The synthesis of 24HC by the cytochrome P450 enzyme CYP46A1 is strictly neuronal. Genetic deletion of CYP46A1 reduced brain 24HC levels by ~95% or more, indicating that there is normally little non-enzymatic 24HC production (Russell et al., 2009). The lack of CYP46A1 influences many intracellular signaling pathways, protein expression and other processes. Lack of 24HC synthesis also decreased NMDAR activity (Sun et al., 2016a), which is consistent with endogenous 24HC contributing to NMDAR modulation. In our experiments, the isolated tonic I_{NMDA} recorded from granule cells of CYP46A1 KO mice showed similar amplitude to those recorded in WT, after normalization to C_m , however its sensitivity to 24HC was lost. Our study, limited by the paucity of KO animals, used only a single concentration (1 μM) of 24HC, and we did not measure actual levels of endogenous 24HC in the slices. In WT slices, eluting 24HC from the membrane with γ -cyclodextrin is not realistic, and therefore we could not recreate the KO condition with low levels of endogenous 24HC. However, it is clear that endogenous 24HC does not fully saturate NMDARs *in vivo* (Griffiths and Wang, 2011), thus the added 24HC concentration in our experiments may have made the difference in WT but not in KO slices. Certainly, a proper dose-response study of the effects of 24HC on native NMDARs in both WT and KO slices is high on the list of future experiments.

It needs to be emphasized that the isolated I_{NMDA} recorded from brain slices *ex vivo* under our experimental conditions required no added exogenous NMDA or Glu to activate NMDARs, and no blocking of Glu uptake was necessary. Most of I_{NMDA} appeared as a tonic current with very small phasic components, just as we have reported before (Dalby and Mody, 2003). We obtained smaller single channel NMDAR conductance than the previously reported 50–60 pS, with either a single channel recordings from layer 2/3 pyramidal neuron of cortex or cell-attached recording from CA3 pyramidal cell (Scheppach, 2016). This apparent mismatch may be caused by a residual Mg^{2+} block in our recording conditions that was also evident in the voltage-dependence of the tonic currents.

Altering NMDAR activity, especially in a subtype specific manner, is a potential treatment strategy for schizophrenia (Hashimoto, 2014), age-related dementia (Burgdorf et al., 2011), synapse formation and plasticity, and other physiological and pathological neuronal events. The metabolism of 24HC is the most important pathway to balance the level of cholesterol in the brain (Bielska et al., 2012), and pathological alterations in cholesterol metabolism can lead to severe conditions, such as Niemann-Pick C (NPC) disease, which is characterized by accumulation of cholesterol and other lipids in the CNS (Tangemo et al., 2011). Altered cholesterol metabolism is also involved in the generation of plaques and neurofibrillary tangles in AD, and the progressive extensive atrophy in AD is associated to a progressive reduction of plasma 24HC (Solomon et al., 2009). Moreover, plasma 24HC reduction is negatively correlated with the severity of dementia in AD patients (Papassotiropoulos et al.,

2000). Elevated endogenous levels of 24HC have been reported to improve learning and memory deficits associated with aging. When lowering CYP46A1 levels using AAV-shCYP46A1 injections, sphingolipids and specific enzymes involved in phosphatidylcholine and sphingolipid metabolism are rapidly increased in the hippocampus, which can be associated with many neurodegenerative disorders (Ayciriex et al., 2017). A recent report also shows that elevating CYP46A1 expression by efavirenz, can significantly improve long term spatial memory and decrease A β expression in 5XFAD mice (Mast et al., 2017c). According to a very recent study, patients with Autism Spectrum Disorders (ASD) had significantly higher plasma levels of 24HC than controls, making plasma 24HC a potential diagnostic tool for ASD (Grayaa et al., 2018). All of the above suggest a possible link between 24HC levels and the initiation of various neurological and psychiatric disorders, including learning and memory deficits, which tightly associate with the function and activation of NMDARs in CNS. Recently, treatments of major depressive disorders are also focusing on subtype-selective NMDAR modulators (Henter et al., 2018).

Our results prompt that 24HC should be further explored as a potential GluN2B subunit-specific NMDARs PAM, not just in dentate gyrus granule cells. Much of basic and clinical research focuses on drugs that can either diminish or facilitate the function of specific subtypes of NMDARs, but have numerous side effects. Perhaps one of the most beneficial aspects of the 24HC induced modulation of NMDARs is that 24HC and its synthetic enzyme CYP46A1 are both endogenous to the brain. Presently, in addition to using genetic technology, drugs and endocrine mechanisms exist that can directly modulate the expression of CYP46A1 (Mast et al., 2017a). Taken together, our findings demonstrate that 24HC and CYP46A1 could be considered as novel therapeutical targets to modulate the function of GluN2B-containing NMDARs in the brain.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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I.M. and X.W. designed research. T.N., S.K., H.K., contributed research ideas. X.W. performed research, X.W. and I.M. analyzed data, X.W. and I.M. wrote the paper. We are grateful to the members of the ModyLab for valuable discussions.

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Highlights:

- 24HC acts as an endogenous modulator of NMDA receptors in dentate gyrus.
- 24HC enhances tonic, but not evoked, NMDA receptor-mediated currents (I_{NMDA}) recorded in dentate gyrus granule cells.
- The effect of 24HC on tonic I_{NMDA} of granule cell is specific to GluN2B containing NMDA receptors.
- 24HC has no effect on the tonic I_{NMDA} of granule cells in CYP46A1 KO mice.
- 24HC has no effect on the tonic or phasic I_{NMDA} of dentate gyrus PV interneurons.

Fig 1.

Effects of 1 μ M 24S-hydroxycholesterol (24HC) on NMDAR-mediated currents (I_{NMDA}) as measured by whole-cell patch clamp recordings (at $V_h = -70$ mV) from brain slices of WT mice. a) Left: raw trace of the recording, showing control recording period, 1 μ M 24HC application, and during 50 μ M APV application. Segments labeled with 1, 2 and 3 indicate the 10 s segments recorded from control, 24HC and APV application periods, respectively. Right: All-point histograms and Gaussian fits of the histograms during the three different recording periods. 1: control, 2: 24HC, 3: APV. Changes in b) tonic APV-sensitive I_{NMDA} (29.31 ± 11.06 pA in control, 64.56 ± 14.18 pA in 24HC, $n = 15$); and c) variance (σ^2) of the APV-sensitive I_{NMDA} (31.06 ± 6.09 pA 2 in control, 92.16 ± 23.26 pA 2 in 24HC, $n = 15$). p-values of two-tailed Wilcoxon paired rank tests between control and application of 24HC are indicated. Paired symbols connected with dashed lines indicate values from the cell in a.

Fig 2.

Action of GluN2A antagonist (PEAQX, 300 nM) and GluN2B antagonist (Ro25–6981, 1 μ M) on tonic I_{NMDA} ($V_h = +40$ mV). **a**) Left: recordings of raw tonic I_{NMDA} in a cell during control, PEAQX and APV (50 μ M) period; 1, 2 and 3 indicate the randomly selected 10 s segments used from each period for the analysis. Right: histograms and Gaussian fits of each of the 10 s selections during the three different recording periods. **b**) Same as **a**, but for the application of Ro25–6981. **c**) Tonic APV-sensitive I_{NMDA} (from 124.10 ± 27.36 to 109.90 ± 31.38 pA by PEAQX application, $n = 10$; from 130.40 ± 38.61 to 60.59 ± 16.48 pA by Ro25–6981 application, $n = 10$), and **d**) variance of APV-sensitive I_{NMDA} (from 231.0 ± 54.48 to 207.60 ± 61.30 pA² by PEAQX application, $n = 10$; from 265.40 ± 82.75 to 126.20 ± 31.26 pA² by Ro25–6981 application, $n = 10$). p-values of two-tailed Wilcoxon paired rank test between control and PEAQX or Ro25–6981 applications are indicated. Paired symbols connected with dashed lines indicate values from the cells in **a**, and **b**.

Fig 3.

Effects of 24HC on tonic I_{NMDA} in untreated slices, and in GluN2A antagonist (PEAQX) or GluN2B antagonist (Ro25-6981) preincubated and perfused slices, recorded at $V_h = +40$ mV. a, b, c): Raw data traces as in Fig. 1a, but at $V_h = +40$ mV. The three conditions of the slices are: untreated (No drug), preincubated and perfused with PEAQX, or with Ro25-6981. d) Tonic APV-sensitive I_{NMDA} (No drug: control: 183.32 ± 33.42 pA, 24HC: 353.85 ± 61.34 pA, $n = 9$; PEAQX: control: 135.50 ± 37.11 , 24HC: 244.0 ± 50.0 pA, $n = 9$; Ro25-6981: control: 132.96 ± 13.43 pA, 24HC: 168.22 ± 27.32 pA, $n = 9$), and e) variance of APV-sensitive I_{NMDA} (No drug: control: 353.80 ± 82.94 pA², 24HC: 621.90 ± 137.50 pA², $n = 9$; PEAQX: control: 266.80 ± 89.63 pA², 24HC: 493.80 ± 129.00 pA², $n = 9$; Ro25-6981: control: 173.20 ± 44.07 pA², 24HC: 270.10 ± 40.80 pA², $n = 9$). p-values of two-tailed Wilcoxon paired rank test between control (C) and 24HC (24) are indicated. Paired symbols connected with dashed lines indicate values from the cells in a, b, and c.

Fig 4.

1 μM 24HC has no significant effect on tonic I_{NMDA} recorded in slices obtained from CYP46A1 KO mice. All tonic I_{NMDA} are recorded from granule cells of CYP46A1 KO mice ($V_h = -70$ mV). a, b, and c) As in Fig. 1, showing the changes of APV-sensitive I_{NMDA} in CYP46A1 KO mice. b) Tonic APV-sensitive I_{NMDA} (control: 34.46 ± 12.72 pA, 24HC: 27.49 ± 11.53 pA, 80 % of control, $n = 6$) and c) its variance (control: 80.05 ± 21.90 pA², 24HC: 69.17 ± 17.59 pA², 86 % of control, $n = 6$). p-values of two-tailed Wilcoxon paired rank test between control and 24HC application are indicated. Paired symbols connected with dashed lines indicate values from the cell depicted in a. d, e, f) Summary of the data obtained from WT ($V_h = -70$ mV, $n = 15$) and CYP46A1 KO mice ($V_h = -70$ mV, $n = 6$). Two-tailed Mann-Whitney test are performed on d) the membrane capacitance (C_m) of recorded granule cells (WT: 19.80 ± 1.23 pF, CYP46A1 KO: 27.33 ± 1.36 pF), e) the C_m -normalized APV-sensitive I_{NMDA} (WT: 1.61 ± 0.62 A/F, CYP46A1 KO: 1.22 ± 0.46 A/F), and f) the C_m -normalized variance of APV-sensitive I_{NMDA} (WT: 1.84 ± 0.45 pA²/pF, CYP46A1 KO: 2.89 ± 0.74 pA²/pF), and p values comparing WT and KO are indicated.

Fig 5.

24HC (1 μ M) has no effect on tonic I_{NMDA} recorded from dentate gyrus PV-INs at $V_h = +40$ mV. a) Left: raw trace of tonic I_{NMDA} recorded from a PV-IN, showing control recording period, 1 μ M 24HC application, and during application of 50 μ M APV. The labels 1, 2 and 3 indicate the 10 s segments during control, 24HC and APV periods, respectively. Right: all-points histograms and Gaussian fits of the 10 s segments of the three different recording periods. b) Tonic APV-sensitive I_{NMDA} with recording of PV-INs (control: 88.38 ± 26.71 pA, 24HC: 103.67 ± 33.26 pA, $n = 7$), and c) its variance (control: 184.30 ± 40.23 pA^2 , 24HC: 173.0 ± 56.33 pA^2 , $n = 7$). p values of two-tailed Wilcoxon paired rank test between control and 24HC application are indicated. Dash line paired dots denote the values from cell depicted in a. d, e, f) Summary graphs comparing 24HC effects in granule cells (GCs) ($V_h = +40$ mV, $n = 9$) and PV-INs ($V_h = +40$ mV, $n = 7$). Two-tailed Mann-Whitney test is performed on d) the membrane capacitance (C_m) of recorded cells (GC: 22.56 ± 3.69 pF, PV-IN: 20.86 ± 1.82 pF), e) the C_m -normalized APV-sensitive I_{NMDA} (GC: 8.62 ± 1.41 A/F, PV-IN: 4.88 ± 2.09 A/F), and f) the C_m -normalized variance of APV-sensitive I_{NMDA} (GC: 17.11 ± 3.50 pA^2/pF , PV-IN: 9.85 ± 3.01 pA^2/pF), and p values are indicated.