Contents lists available at ScienceDirect





Neuropharmacology

journal homepage: www.elsevier.com/locate/neuropharm

Visualizing pregnenolone sulfate-like modulators of NMDA receptor function reveals intracellular and plasma-membrane localization



Mariangela Chisari^{a,1}, Timothy J. Wilding^b, Samuel Brunwasser^c, Kathiresan Krishnan^d, Mingxing Qian^d, Ann Benz^a, James E. Huettner^b, Charles F. Zorumski^{a,e}, Douglas F. Covey^{d,e,*}, Steven Mennerick^{a,e,**}

^a Department of Psychiatry, Washington University School of Medicine, St. Louis, MO, USA

^b Department of Cell Biology and Physiology, Washington University School of Medicine, St. Louis, MO, USA

^c Medical Scientist Training Program, Washington University School of Medicine, St. Louis, MO, USA

^d Department of Developmental Biology, Washington University School of Medicine, St. Louis, MO, USA

e Taylor Family Institute for Innovative Psychiatric Research, Washington University School of Medicine, St. Louis, MO, USA

HIGHLIGHTS

• KK-169 is a potent and efficacious positive allosteric modulator of NMDA receptors.

- KK-169 is a clickable analogue of pregnenolone sulfate.
- KK-169 reveals intracellular accumulation of sulfated steroids.

1. Introduction

Positive modulators of NMDA receptors (NMDARs) are important candidates for therapeutic development to treat psychiatric disorders (Hansen et al., 2017; Reddy, 2010; Smith et al., 2014; Strong et al., 2014; Sun et al., 2015). Sulfated neurosteroids are of particular interest because they modulate both inhibition and excitation through effects on GABA_A receptors (GABARs) and NMDARs respectively. Effects on

https://doi.org/10.1016/j.neuropharm.2018.10.015

Available online 15 October 2018

ARTICLE INFO ABSTRACT Positive modulators of NMDA receptors are important candidates for therapeutic development to treat psy-Keywords: Pregnenolone sulfate chiatric disorders including autism and schizophrenia. Sulfated neurosteroids have been studied as positive Glutamate allosteric modulators of NMDA receptors for years, but we understand little about the cellular fate of these NMDA compounds, an important consideration for drug development. Here we focus on a visualizable sulfated neu-GABA rosteroid analogue, KK-169. As expected of a pregnenolone sulfate analogue, the compound strongly potentiates Click chemistry NMDA receptor function, is an antagonist of GABA_A receptors, exhibits occlusion with pregnenolone sulfate Photolabeling potentiation, and requires receptor domains important for pregnenolone sulfate potentiation. KK-169 exhibits somewhat higher potency than the natural parent, pregnenolone sulfate. The analogue contains a side-chain alkyne group, which we exploited for retrospective click labeling of neurons. Although the anionic sulfate group is expected to hinder cell entry, we detected significant accumulation of KK-169 in neurons with even brief incubations. Adding a photolabile diazirine group revealed that the expected plasma membrane localization of KK-169 is likely lost during fixation. Overall, our studies reveal new facets of the structure-activity relationship of neurosteroids at NMDA receptors, and their intracellular distribution suggests that sulfated neurosteroids could have unappreciated targets in addition to plasma membrane receptors.

Abbreviations: D-APV, D-(–)-2-Amino-5-phosphonopentanoic acid; GABAR, γ-amino-butyric acid receptor; NBQX, 2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo[*f*] quinoxaline-2,3-dione; NMDAR, N-methyl-*p*-aspartate receptor; PREGS, pregnenolone sulfate

^{*} Corresponding author. Department of Developmental Biology, Washington University School of Medicine, St. Louis, MO, USA.

^{**} Corresponding author. Department of Psychiatry, Washington University School of Medicine, St. Louis, MO, USA.

E-mail addresses: dcovey@wustl.edu (D.F. Covey), menneris@wustl.edu (S. Mennerick).

¹ Present address: Section of Pharmacology, Department of Biomedical and Biotechnological Sciences, University of Catania, Catania, Italy.

Received 26 April 2018; Received in revised form 7 September 2018; Accepted 12 October 2018

^{0028-3908/ © 2018} Elsevier Ltd. All rights reserved.

NMDARs depend on both subunit composition and on steroid structure. Pregnane sulfates produce mainly NMDAR inhibition, and pregnenolone sulfate (PREGS) induces potentiation (Korinek et al., 2011; Malayev et al., 2002; Park-Chung et al., 1994, 1997). Although modulators of NMDARs may have clinical utility (Abdallah et al., 2015; Collingridge et al., 2013; Lai et al., 2011; Sun et al., 2015; Vyklicky et al., 2016), our understanding of the effects of PREGS at these receptors remains limited, including basic structure-activity information (Burnell et al., 2018). How PREGS-like compounds are trafficked or compartmentalized in neurons is also unclear, even though compartmentalization may affect receptor access (Jiang et al., 2016) and may hint at additional targets. For instance, in some cases neuroactive compounds may sequester in intracellular organelles, followed by release into the extracellular space (Tischbirek et al., 2012). An important barrier to understanding is the lack of biologically active, visualizable analogues to address cellular distribution. Such compounds may also yield new insights into structure-activity relationships. We have begun to address these fundamental issues with novel, biologically active probes.

Previous work has been equivocal regarding the ability of NMDARactive sulfated neurosteroids to enter cells and sequester therein. The electronegativity of the sulfate group would seem to prohibit cell entry (Eisenman et al., 2007), compared with un-sulfated GABA_A receptor potentiators, which sequester in specific intracellular organelles (Jiang et al., 2016). However, unknown carrier molecules, active transport mechanisms, or other routes of cell entry may exist for sulfated neurosteroids.

Previous efforts to evaluate subcellular distribution have utilized fluorescently labeled neurosteroid analogues (Borovska et al., 2012; Eisenman et al., 2007). Because fluorescent moieties themselves may alter the physical properties, cell permeability and trafficking of steroid analogues, here we took the approach of a more modest chemical alteration that permitted retrospective click chemistry for visualization. This alteration retained modulatory potency and efficacy at NMDARs compared with PREGS and the oxysterol class of NMDAR modulators. One lead compound, KK-169, exhibited readily reversible NMDAR potentiation. Other analogues with longer carbon side-chain lengths exhibited slowly reversible potentiation. KK-169 showed expected features of PREGS neuroactivity with no discernible presynaptic activity at concentrations employed. Retrospective click chemistry revealed that KK-169 accumulates inside neurons, a feature associated with its hydrophobicity based on comparison with other analogues. Our results begin to reveal advantages and limits of chemical biology tools for probing the pharmacology of NMDAR positive allosteric modulators.

2. Material and methods

2.1. Xenopus oocytes

Stage V–VI oocytes were obtained from sexually mature female *Xenopus laevis* (Xenopus One) anesthetized with 0.1% tricaine (3-aminobenzoic acid ethyl ester). To achieve defolliculation, oocytes were incubated for 20 min at 37 °C in a calcium-free collagenase (2 mg/ml) solution containing (in mM): NaCl (96), KCl (2), MgCl₂ (1), and HEPES (5) at pH 7.4. cRNA for receptor subunits was prepared using the mMESSAGE mMachine in vitro transcription kit (Ambion, Austin, TX, USA). Capped mRNA, encoding rat NMDAR GluN1a and GluN2A subunits or rat GABA_A receptor α 1, β 2, γ 2L subunits were injected in equal parts (3–13 ng RNA for each receptor subunit). Oocytes were incubated at 18 °C in ND96 medium containing (in mM): NaCl (96), KCl (1), MgCl₂ (1), CaCl₂ (2) and HEPES (10) at pH 7.4, supplemented with pyruvate (5 mM), penicillin (100 U/ml), streptomycin (100 µg ml⁻¹) and gentamycin (50 µg ml⁻¹) for up to 5 days.

2.2. Cell cultures

All animal care and experimental procedures were consistent with National Institutes of Health guidelines and were approved by the Washington University Animal Studies Committee. Studies involving animals are reported in accordance with the ARRIVE guidelines (Kilkenny et al., 2010; McGrath et al., 2010). Sprague-Dawley rat pups (postnatal day 1–3), were anesthetized with isoflurane. Hippocampal slices ($500 \mu m$ thickness) were digested with 1 mg/ml papain in



Fig. 1. Biological activity at NMDARs is retained with varied side chain lengths in PREGS analogues. **A.** The structures of first-generation alkyne-conjugated PREGS analogues. MQ-154 differed from others most notably in the presence of an amide linkage to a side-chain diazirine group. **B.** Effect of varied concentrations of respective compounds on NMDA-induced current in *Xenopus* oocytes expressing recombinant GluN1 and GluN2A subunits. NMDA (5 μ M) was applied for 50 s in the absence of compound to establish baseline responsiveness, followed by 50 s co-application. Each application/co-application was followed by a 25 s wash period before the ensuing application. Responses are displayed normalized to the original NMDA current (dotted line). N = 4–7 oocytes per compound.

oxygenated Leibovitz L-15 medium (Life Technologies, Gaithersburg, MD), followed by mechanical trituration in modified Eagle's medium (MEM; Life Technologies) containing 5% horse serum, 5% FCS, 17 mM $_{\rm D}$ -glucose, 400 μ M glutamine, 50 U/ml penicillin, and 50 μ g ml $^{-1}$ streptomycin. Combined astrocytes and neurons were seeded at a density of ~600 cells/mm² onto 35-mm dishes or 25-mm cover glasses coated with 0.1 mg/ml poly-D-lysine and 1 mg/ml laminin. Cultures subsequently developed at 37 °C in a chamber with 5% CO₂ and controlled humidity. Glial proliferation was inhibited by adding cytosine arabinoside (6.7 μ M) 3–4 days after plating. The following day, culture medium was switched to Neurobasal medium (Life Technologies) plus B27 supplement (Life Technologies).



2.3. Click labeling

Cell cultures were incubated with $10 \,\mu$ M KK-169, KK-181 or MQ-189 for 15 min in saline containing (in mM): NaCl (138), KCl (4), CaCl₂ (2), MgCl₂ (1), glucose (10), HEPES (10), pH 7.25 with NaOH, 1 μ M 2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo[*f*]quinoxaline-2,3-dione (NBQX) and 50 μ M D-(-)-2-Amino-5-phosphonopentanoic acid (D-APV). For some experiments cells were exposed to 365 nm light using a 0.15 A Blak-Ray lamp for 15 min at a distance of 2 cm. Afterward, cells were immediately fixed for click-chemistry labeling with 4% paraformaldehyde plus 0–0.05% glutaraldehyde in phosphate buffered saline at room temperature for 10 min. After a phosphate-buffer saline (PBS) wash, fixed cells were incubated in 1 μ M AlexaFluor 488-azide, a click reagent for fluorescence visualization (ThermoFisher Scientific), in 100 μ M Tris[(1-benzyl-1*H*-1,2,3-triazol-4-yl)methyl]amine, 2 mM ascorbic acid,1 mM CuSO₄ for 1 h in the dark, followed by three washes in PBS. Cells were coverslipped for subsequent imaging. Epifluorescence



Fig. 2. Potency and efficacy of KK-169. A. Responses of a GluN1 and GluN2A-expressing oocyte to 5 μ M NMDA and increasing concentrations of KK-169, co-applied with NMDA. The traces in panel A are color coded according to concentrations in panel C. B. 30 μ M KK-169 alone (blue trace) did not gate a current in oocytes with a confirmed response to 5 μ M NMDA (black trace). C. A fit of the Hill equation to the responses to increasing KK-169 concentrations yielded an EC₅₀ value of 6.2 μ M \pm 0.8 and a Hill coefficient of 0.6 \pm 0.1. N = 4 cells at 100 μ M and 8–11 cells for other concentrations.

Fig. 3. Effect of KK-169 on glutamate (Glu) concentration-response parameters. A. Baseline NMDA responses in the absence of KK-169 from an oocyte expressing GluN1 and GluN2A subunits. B. Response to the lowest concentration of glutamate co-applied with 30 μ M KK-169. C. Full glutamate concentration response for peak current recorded in the same cells in the absence and presence of 30 μ M KK-169. Responses were normalized to peak current evoked by 100 μ M glutamate in the absence of KK-169. Fits to the Hill equation yielded EC₅₀ values of 4.8 vs. 0.22 μ M for glutamate alone and plus KK-169 respectively, and Hill coefficients of 1.2 and 0.85 respectively. N = 4 oocytes for each condition.

photomicrographs were acquired using a Nikon TE2000 inverted microscope with a PlanFluor 40x microscope objective (0.6 N.A.) and a Coolsnap ES2 camera (Photometrics). Images were analyzed using the Fiji distribution of ImageJ imaging software (http://fiji.sc/Fiji).

2.4. Electrophysiology recordings in Xenopus oocytes

Evaluation of receptor modulation in *Xenopus* oocytes was performed with an OC725 amplifier (Warner Instruments) 2–5 d after RNA injection, in a two-electrode voltage-clamp configuration at -70 mV. Oocytes were transferred to a chamber containing unsupplemented ND96 solution. For NMDA recordings, Ba²⁺ was substituted for Ca²⁺ to prevent contributions of endogenous Ca²⁺ activated current, MgCl₂ was omitted, and 10 μ M p-serine was added to the bath. Recording pipettes were filled with 3 M KCl and had open tip resistances of ~ 1 M Ω . Drug application was achieved using a gravity-driven multibarrel delivery system with a common tip. Each drug was applied for 30 s and current elicited by each compound was evaluated at the peak current or the current at the end the application (as indicated).

2.5. Electrophysiology recordings in neuronal cultures and N2a cells

Current recordings were made with an Axopatch 200B amplifier (Molecular Devices) in whole-cell configuration, from neurons 6-13 days in vitro. 6-8 day-old hippocampal cells were used for recordings of exogenous agonist responses because of improved spatial voltage clamp in younger neurons with limited processes; 10-13 day-old cultures were used for EPSC recordings because of synaptic maturity. N2a cells were used 24 h following transfection. For recordings, cells were transferred to an extracellular solution containing (in mM): NaCl (138), KCl (4), CaCl2 (2), glucose (10), HEPES (10), D-serine (0.02), NBQX (0.001), and D-APV (0.01), bicuculline (0.01) at pH 7.25. NMDAR autaptic current was recorded in a D-APV-free solution, whereas AMPA current was recorded in NBQX free solution and GABAA autaptic current in bicuculline free solution. No antagonists were used for N2a cell recordings. The open-tip resistance of patch-pipette for recordings was $2.5-3.5 \text{ M}\Omega$ when filled with an internal solution containing (in mM): KCl (140), NaCl (4), EGTA (0.1), and HEPES (10) at pH 7.25, adjusted with KOH. Access resistance $(8-10 \text{ M}\Omega)$ was compensated 80-100% for synaptic recordings. For autaptic responses, cells were stimulated with 1.5-ms pulses to 0 mV from -70 mV to evoke transmitter release. Drugs were applied using a multibarrel, gravity-driven local perfusion system with a common tip placed 0.5 mm from the center of the microscope field. For recordings using exogenous drug application, patch-pipettes were



Fig. 5. Effect of KK-169 on GABA_A receptor function. **A.** Effect of co-application of KK-169 at the indicated concentrations with GABA (10 μ M) in a hippocampal neuron. The peak GABA current showed little inhibition, consistent with a PREGS-like mechanism (Eisenman et al., 2003; Shen et al., 2000). **B.** Summary of effects on ending GABA current during the co-application protocol shown in A. N = 9 neurons. Fit to the data with the Hill equation (solid line) predicts an IC₅₀ of 2.2 μ M.

filled with a solution containing in (mM): cesium methanesulfonate (120), HEPES (20), EGTA (10) at pH 7.25 adjusted with CsOH. For these experiments, cells were clamped at -70 mV. All electrophysiology recordings were performed at room temperature.

2.6. Chimeric receptors and cell transfection

Recombinant chimeric receptors were as described (Wilding et al., 2014, 2016). HEK cells were propagated in 25 cm^2 flasks with MEM plus 10% fetal bovine serum and passaged once each week with protease XXIII (Sigma-Aldrich). Cells used for transfection were seeded onto 12-well plates and transfected the following day. cDNAs were expressed by transient transfection in HEK 293 cells using Lipofectamine 2000 (Invitrogen). Coexpression of GFP from a second vector was used to identify transfected cells. The day after transfection, cells were plated at low density on 35 mm plates coated with nitrocellulose; recordings were obtained on the following 2 d.

Neuro2A (N2A) cells were propagated in 25 cm² flasks with DMEM plus 10% fetal bovine serum and passaged with 0.05% trypsin-EDTA (Life Technologies). Cells were seeded onto 35 mm dishes and, the following day cDNAs for NMDAR subunits (GluNR1/GluNR2A-D) were expressed by transient transfection along with GFP plasmid using Lipofectamine 2000 (Invitrogen).



Fig. 4. Effect of KK-169 on saturating agonist responses. **A.** A cultured hippocampal neuron was challenged with indicated concentrations and exposure times of glutamate and KK-169. **B.** To explicitly test the effect of KK-169 on steady-state responses to saturating glutamate, KK-169 was applied during the NMDA application in another cell. **C.** Summary of results from the protocols in A and B on peak and steady state NMDA responses respectively. Both peak and steady state were significantly potentiated by KK-169. N = 7 cells. Asterisks indicate paired t-tests, p < 0.05.

2.7. Chemistry

Novel steroid analogue synthesis was through a multi-step process described in the supplemental material. LogP values were calculated using the freely available ALOGPS 2.1 software available from the Virtual Computational Chemistry Lab: http://www.vcclab.org/. Detailed chemical synthesis is supplied as Supplemental material.

2.8. Data analysis

Data were acquired and initially analyzed with pCLAMP 10 software (Molecular Devices) for electrophysiology and ImageJ for images. Data were subsequently processed with Microsoft Excel and are presented as means \pm S.E. Statistical significance was determined using a Student's two-tailed *t*-test, ANOVA, or Mann-Whitney test as indicated in figure legends. Statistical analysis and fitting were executed with GraphPad



Fig. 6. Effect of KK-169 and PREGS on NMDAR EPSCs. **A-C.** Influence of 30 μ M KK-169 on the indicated parameters of pharmacologically isolated, evoked NMDAR EPSCs. Decay time was measured from 10 to 90% of the peak response. (N = 6 autaptic neurons). Asterisks denote results of paired t tests. **D.** Summary of concentration-response effects on normalized peak NMDAR EPSCs (N = 7–9 cells per condition, except 2 cells at the 5 μ M concentration, EC₅₀ = 3.7 μ M with a Hill slope of 3.7. **E-G.** Effect of equimolar concentrations (30 μ M) of KK-169 and PREGS on evoked NMDAR EPSCs (N = 6 comparisons by paired *t*-test).

Prism (GraphPad Software, La Jolla, CA) or Sigma Plot (San Jose, CA). For fitting of memantine onset and offset kinetics a weighted time constant was calculated from bi-exponential fits using the equation as $\Sigma Ai^* \tau i$, where Ai is the fractional amplitude and τi is the time constant of the component. p values are indicated in Figures as * p < 0.05, **p < 0.01, ***p < 0.005.

2.9. Drugs

All commercial drugs and salts were obtained from Sigma (St. Louis, MO), except for D-APV and NBQX, which were obtained from Tocris.

3. Results

3.1. Bioactivity of KK-169 and related compounds at NMDARs

Based on the ability of sulfated steroids to increase NMDAR function allosterically during agonist activation, we studied the effect of newly synthetized compounds with different side chain lengths at carbon 17. These modifications supported an alkyne group for retrospective click chemistry (Fig. 1A). We tested the NMDAR activity of these new compounds in *Xenopus* oocytes co-expressing GluN1a and GluN2A subunits. Oocytes were challenged with NMDA (5 μ M) and with increasing concentrations of test compounds (0.3 μ M up to 30 μ M) (Fig. 1B). Each compound increased NMDA current in a concentration-dependent manner. Among the active compounds, KK-169 exhibited reversible potentiation of NMDA current. Other compounds such as KK-171 and KK-181 increased NMDA current more effectively than KK-169 (1–3 fold), but such potentiation was not efficiently removed upon wash (Fig. 1B), perhaps because longer side chains increased lipophilicity and promoted retention. We prepared MQ-154 as a potential photolabeling compound (Fig. 1A). However, the changes made to permit attachment of the photolabeling group on the side chain abolished NMDAR activity (Fig. 1B). Because of its strong biological activity and favorable reversibility, we selected KK-169 as a click-accessible lead compound to study NMDA potentiation by sulfated steroids.

Representative traces for KK-169 potentiation of glutamate-gated current are shown in Fig. 2A. In the absence of glutamate, KK-169 did not detectably gate a current (Fig. 2B). Analysis of potentiation at varied KK-169 concentrations suggested an EC₅₀ concentration of 6.2 µM (Fig. 2C). We next tested KK-169 while varying the concentration of the endogenous agonist glutamate (Fig. 3). Responses to low glutamate concentration (e.g., 1 µM) were strongly potentiated, producing a desensitizing response at a concentration of glutamate that did not desensitize in the absence of KK-169 (Fig. 3A and B). The primary effect of the modulator was to shift the concentration-response for peak glutamate responses to lower agonist concentrations (from $\sim 10 \,\mu\text{M}$ to \sim 3 μ M), and also shifted the Hill coefficient (limiting slope) to a lower value (Fig. 3C). We acknowledge that strong desensitization at the highest concentrations of glutamate and KK-169 may affect accuracy of measurements, given that drug delivery to oocytes may be slow relative to desensitization rate.

To evaluate whether, in addition to increasing the potency of glutamate, KK-169 also increases agonist efficacy, we challenged native



Fig. 7. Effect of KK-169 on evoked AMPAR and GABA_AR PSCs. **A-C.** Effect of 30 μ M KK-169 on parameters of pharmacologically isolated, evoked autaptic AMPAR EPSCs (N = 5 cells tested in the absence and presence of KK-169). **D-F.** Effect of 30 μ M KK-169 on parameters of pharmacologically isolated autaptic evoked GABA_AR IPSCs. Pipette solution included KCl (N = 6 cells tested in the absence and presence of KK-169).

receptors in young cultured hippocampal neurons, where more rapid applications of drug could be achieved, thereby allowing more accurate measurement of peak current. Fig. 4 shows that 30 μ M KK-169 had modest but statistically significant effects on both peak and steady-state responses to 100 μ M glutamate. We conclude that KK-169 increases agonist efficacy, although this effect alone is unlikely to account for the shift in agonist EC₅₀.

Previous work has shown that prolonged incubation in PREGS at low nM concentrations potentiates NMDA currents in *Xenopus* oocytes, suggesting high-affinity interaction (Kostakis et al., 2013). Consistent with these observations, we found that hippocampal neurons incubated for 3 min in 10 nM (n = 9 cells) or 100 nM (n = 11 cells) KK-169 exhibited modest potentiation over baseline NMDA responses (1.2 \pm 0.09; p = 0.04 and 1.6 \pm 0.16, p = 0.007 respectively, paired one-sample t tests).

Based on the presence of the sulfate group at carbon 3, we expected that KK-169 may inhibit GABA_A receptor activity since the structural demands for GABA_A receptor inhibition by sulfated steroids are low (Mennerick et al., 2001; Nilsson et al., 1998) and are evolutionarily conserved (Twede et al., 2007). In hippocampal neurons, GABA (10 μ M) co-applied with KK-169 elicited a rapidly decaying current with hallmarks expected of sulfated steroid inhibition (Eisenman et al., 2003; Shen et al., 2000) (Fig. 5A). Testing a concentration series for KK-169 produced an IC₅₀ at 10 μ M GABA of 2.2 μ M for KK-169 (Fig. 5B). These data suggest that KK-169 retains features of other sulfated neuroactive steroids, including actions at both NMDA and inhibition of GABA_A receptors.

3.2. Effects on synaptic transmission

To explore the impact of KK-169 on neuronal transmission, including potential off-target effects, we evaluated the effects of KK-169 on EPSCs in hippocampal neurons (Figs. 6 and 7). KK-169 significantly increased the amplitude and decay of recurrent (autaptic) evoked NMDA EPSCs (Fig. 6). These effects were readily reversible (Fig. 6A and B). The concentration response relationship for KK-169 on evoked NMDAR EPSCs was similar to that expected from effects observed on agonist-evoked currents in oocytes (Fig. 6D).

To compare KK-169 with the effect of a canonical positive allosteric modulator, we compared effects of equimolar PREGS (30μ M) on individual cells' evoked NMDAR EPSCs. Effects on peak current EPSCs observed in the presence of KK-169 were compared to PREGS (Fig. 6E–G). KK-169 increased peak current ~1.5 fold more than PREGS (Fig. 6F). EPSC decay time in the presence of both drugs was approximately doubled compared to control conditions, and no statistical difference between effects of the two drugs on this parameter was found (Fig. 6G).

To test selectivity, we tested KK-169 on pharmacologically isolated AMPAR EPSCs (in saline lacking NBQX but with D-APV added). Neither peak current nor decay time was altered by $30 \,\mu$ M KK-169 (Fig. 7A–C). These results effectively exclude an important effect of KK-169 on presynaptic glutamate release or on AMPAR function. A presynaptic effect would affect NMDAR and AMPAR EPSCs similarly. The result also suggests a difference from PREGS, which inhibits AMPARs and kainate receptors (Wu and Chen, 1997; Yaghoubi et al., 1998). As expected, KK-169 ($30 \,\mu$ M) also inhibited and speeded pharmacologically isolated inhibitory postsynaptic current (IPSCs) mediated by GABA_A receptors (Fig. 7D).

3.3. Mechanistic features of KK-169

A characteristic of many NMDAR positive allosteric modulators is that they alter the kinetics of open-channel trapping blockers such as ketamine and memantine (Emnett et al., 2015), suggesting a mechanism by which the positive modulator increases channel open probability and thus increases access of the channel to blocker.



Fig. 8. KK-169 speeds kinetics of open-channel trapping block. **A, B.** Sample traces showing the effect of the trapping blocker memantine (10 μ M) applied during the steady-state response to 10 μ M NMDA of a hippocampal neuron. Panel A shows current in the absence of KK-169, and panel B shows current in the presence of 10 μ M KK-169. Artifacts of electronic valve activation have been removed for clarity **C.** Summary of the effect of KK-169 on the amplitude of steady-state current in the absence of memantine and the alteration of block onset and offset kinetics (N = 10 neurons). The first bar shows the effect of KK-169 normalized to baseline NMDA current amplitude (dotted line), and the other bars show the memantine-induced onset and offset weighted time constants in the presence of KK-169 normalized to values in the absence (dotted line).

Consistent with this idea, we found that the presence of KK-169 decreased the time constant for memantine onset and offset during receptor activation by exogenous NMDA (Fig. 8). These results support a mechanism by which KK-169 increases NMDAR channel open probability, although we cannot exclude a role for an increase in surface NMDAR number, as observed for recombinant NMDARs expressed in oocytes exposed to prolonged incubation of PREGS (Kostakis et al., 2013).

To test the assumption that KK-169 is a PREGS-like allosteric modulator, we performed a pharmacological occlusion test (Fig. 9). In the presence of a near-saturating concentration of KK-169, PREGS lost its ability to potentiate NMDAR currents (Fig. 9A, B, E). As a negative comparator, we employed another class of NMDAR positive modulator, SGE-201, an analogue of the oxysterol cholesterol metabolite 24(*S*)-hydroxycholesterol (Paul et al., 2013). In the presence of KK-169, SGE-201 retained potentiation. (Fig. 9C, D, F). These results are consistent with a shared mechanism between PREGS and KK-169.

Because PREGS differently potentiates NMDAR based on subunit combination (Kostakis et al., 2013), we expressed NMDAR subunits in murine neuronal N2A cells in order to assess KK-169 effect (Fig. 9G–K). Cells were challenged with 10 μ M NMDA and 30 μ M KK-169 and a significant potentiation was observed when GluN2A and GluN2B subunits were expressed, but not for GluN2C and GluN2D subunits. This pattern of results is consistent with PREGS-like potentiation but not with oxysterol-like potentiation (Paul et al., 2013).

As an independent test of KK-169's mechanism, we turned to chimeric receptors (Wilding et al., 2016) that distinguish oxysterol from sulfated steroid potentiation. We used constructs that contained GluN1 and GluN2B domains, representing receptors sensitive to oxysterol and PREGS potentiation, and kainate receptor (GluK2) subunit domains, representing receptors insensitive to oxysterol and PREGS potentiation. Previous work has shown that the transmembrane domain alone from GluN subunits, with the balance of domains from the kainate receptor, can sustain oxysterol potentiation, but GluN transmembrane and ligand-binding domains are required for PREGS potentiation (Wilding et al., 2016). Other receptors tested included WT homomeric kainate-gated GluK2 unedited Q and edited R subunits, as well as chimeric subunits with GluN extracellular amino terminal and ligand binding



domains linked to the transmembrane and cytoplasmic domains from GluK2 Q (Wilding et al., 2016). As expected, we observed that potentiation by KK-169 (Fig. 10A) closely matched the pattern previously elucidated for PREGS-like potentiation (Wilding et al., 2014). NMDAR inhibition, which apparently requires the GluN ligand-binding domain, was evident at high concentrations of KK-169 (Fig. 10B).

> Fig. 9. KK-169 occlusion of PREGS potentiation and KK-169 subunit selectivity suggest a shared mechanism with PREGS. A-D. Traces show the effect of modulators at the indicated concentrations in the presence or absence of a high KK-169 concentration (30 µM). E-F. Summary of effects of modulators during baseline (E, N = 9 cells) and KK-169 incubations (F, N = 9 cells). G-J. Representative responses to 10 µM NMDA alone (black traces) or NMDA + 30 µM KK-169. Responses were acquired from the indicated GluN2 subunits expressed with GluN1a in N2a cells. K. Summary of potentiation for each combination. A two-way ANOVA followed by Dunnett's multiple comparison test revealed that GluN2A and GluN2B containing NMDARs were increased above baseline (n = 12)and 8 respectively; p < 0.0001 for both conditions) but GluN2C and GluN2D receptors were not (n = 7 and 8 respectively; p = 0.54 and 0.26).



Fig. 10. Potentiation of NMDA current in HEK cells expressing chimeric receptors **A**, **B**. Summaries of the effect of 10 μ M KK-169 (A) or of varied KK-169 concentrations (B) on agonist-induced responses. Similar to PREGS, potentiation by KK-169 requires both the LBD and TMD domains from GluN subunits (teal). In contrast, the GluN TMD domain alone, which is sufficient for oxysterol potentiation, failed to support potentiation by KK-169 (violet). In addition, KK-169 caused no significant potentiation of homomeric WT GluK2 Q (orange) or R (red), or of chimeric subunits that combined the NMDAR amino terminal and ligand-binding domians with the kainate receptor transmembrane and cytoplasmic domains (green) (paired *t*-test, number of cells shown in each bar in A). KK-169 concentrations higher than 30 μ M yielded less potentiation or suppressed agonist-evoked currents in constructs that lacked potentiation. The agonist(*s*) employed depended on the LBD of the construct, either 10 μ M NMDA/10 μ M glycine or 10 μ M kainate. Data for black, teal, and green symbols in B were fit to the following equation for mixed activation/inhibition, where x is agonist concentration, M is maximum response, EC₅₀ is concentration to half of maximum, IC₅₀ is the concentration exhibiting half inhibition, and n and b are Hill coefficients for the activation and inhibition functions respectively:

$$y = \left| \frac{100 + \left(\frac{M}{1 + \left(\frac{EC_{50}}{x}\right)^n}\right)}{1 + \left(\frac{x}{IC_{50}}\right)^b} \right|$$

For WT (black): EC_{50} = 16 \pm 4 $\mu M,$ n = 1.3 \pm 0.3. IC_{50} = 110 \pm 52 $\mu M,$ b = 0.75 \pm 0.08, M = 510 \pm 100. For LBD

TMD (teal): EC_{50} = 22 $\pm~7\,\mu\text{M},\,n$ = 1.6 $\pm~0.2.~\text{IC}_{50}$ = 92 $\pm~26\,\mu\text{M},\,b$ = 1.4 $\pm~0.2,\,M$ = 364 $\pm~84.$ For ATD

LBD (green): $EC_{50} = 3 \pm 1 \mu M$, $n = 1.5 \pm 0.3$. $IC_{50} = 11 \pm 9 \mu M$, $b = 0.87 \pm 0.07 M = 100 \pm 62$. Data for TMD (violet), K2Q (orange), and K2R (red) symbols were fit with an inhibition equation of the form:

$$y = \left\lfloor \frac{100}{1 + \left(\frac{x}{IC_{50}}\right)} \right\rfloor$$

TMD (violet): $IC_{50} = 410 \pm 120 \,\mu\text{M}$; K2Q (orange): $IC_{50} = 890 \pm 260 \,\mu\text{M}$; K2R (red): $IC_{50} = 340 \pm 48 \,\mu\text{M}$.

3.4. KK-169 accumulation and retention

Extracellularly applied sulfated steroids might be expected to lack intracellular access, as a result of the charged sulfate group (Eisenman et al., 2007). To test this, we incubated cells for different time periods in the presence of KK-169. We then fixed cells and performed retrospective in situ click chemistry using an azide conjugated fluorophore (Emnett et al., 2016; Jiang et al., 2016). The method employs coppercatalyzed cycloaddition of an azide-containing fluorophore to the alkyne group contained on KK-169. To our surprise, all incubation times yielded intracellular staining above background levels (Fig. 11A-C). These results suggest that KK-169 in fact enters cells at detectable levels despite the anionic sulfate group. To avoid loss of steroid, we did not wash cells prior to incubation in fixative. We cannot exclude the possibility that dilute KK-169 enters cells due to fixative-induced permeabilization of the membrane. However, the increase in fluorescence with increased incubation time of live cells in KK-169 suggests that a good fraction of KK-169 fluorescence is due to entry into live neurons.

It is unclear how KK-169 is retained upon fixation. One possibility is that aldehyde cross-linking prevents KK-169 efflux. We found that cold methanol fixation also fostered retention of KK-169, visualized by retrospective click labeling. Intracellular fluorescence in methanol-fixed neurons (n = 24) was 0.93 \pm 0.08 that of cells fixed in parallel with the standard protocol (n = 22 cells). To address whether a permeabilized membrane fosters efflux of KK-169 following labeling, we incubated cells in 0.1% Triton-X 100 detergent for 10 min following fixation. Permeabilized cells (n = 26) showed 1.23 \pm 0.06 of the fluorescence of cells treated in parallel with the standard aldehyde fixation protocol. We conclude that fixation permanently retains the steroid analogue and renders it mostly non-diffusible, but not necessarily through aldehyde-mediated protein cross-linking.

To examine whether lipophilicity is a factor in the intracellular accumulation, we compared KK-169 accumulation and retention with one of the more lipophilic sulfated compounds, KK-181 (Fig. 1), using retrospective click labeling (Fig. 11D–H). Cells were either immediately fixed following compound incubation, to assay initial accumulation



Fig. 11. Intracellular accumulation of KK-169 as revealed by retrospective click chemistry. **A**, **B**. Photomicrographs of cells that were incubated in the absence or presence of unlabeled KK-169 for 15 min, then fixed with paraformaldehyde/glutaraldehyde for click labeling with an azide-conjugated fluorophore (see Methods). Bottom panels show phase-contrast images of the fields. Scale bar = $25 \,\mu$ m. **C**. Summary of normalized cytoplasmic fluorescence intensity as a function of different incubation times in the unlabeled compound. Each symbol color represents an independent experiment. A repeated-measures one-way ANOVA revealed a significant effect of incubation time on labeling intensity (P < 0.01). **D-H**. Strong accumulation and retention of lipophilic analogue KK-181. **D-G**. Each panel represents a different field of cells incubated for 15 min in the indicated unlabeled compound, fixed, then click labeled. Top rows were immediately fixed. Bottom rows were washed for 2 min with saline in the absence of compound before fixation. Underlying fluorescence in G represents astrocyte accumulation. Each color represents an independent experiment, normalized to the t = 0, KK-169 condition. Each color represents an independent experiment of 5 fields of cells per condition. Open and gray bars represent immediate fixation following a 2 min saline rinse respectively. ANOVA analysis revealed a significant main effect of compound (p < 0.05), and a significant effect of wash (p < 0.05). A post-hoc Bonferroni corrected *t*-test revealed a significant difference between the control and wash conditions of only KK-169 (p < 0.01).

(Fig. 11D, E, H), or were washed with saline for 2 min prior to fixation to evaluate retention (Fig. 11F, G, H). We found that saline wash significantly reduced KK-169 retention, suggesting significant efflux of KK-169 in the absence of continued incubation. However, washing following KK-181 incubation had almost no effect on retention of this compound (Fig. 11).

It is surprising that KK-169 and KK-181 both exhibited intracellular accumulation. There appeared to be little evidence for preferential plasma membrane accumulation/retention (Fig. 11), the location of target GABA_A receptors and NMDARs. We hypothesized that the plasma-membrane pool of KK-169 is not fixed in place and that we therefore failed to observe plasma-membrane associated steroid. To test this hypothesis, we prepared another analogue, MQ-189, which has a similar structure to KK-169 except for a diazirine photolabeling group at carbon 6 to allow covalent linkage to proteins and/or lipids prior to fixation (Fig. 12A and B). We have previously used this strategy to explore localization of other classes of neuroactive steroids (Jiang et al., 2016). We avoided diazirine placement in the side chain, given the loss of activity in MQ-154 (Fig. 1). Unlike MQ-154, MQ-189 (10 µM) potentiated NMDA (10 µM) currents in hippocampal neurons by $38 \pm 10\%$ (N = 8, p = 0.007, paired *t*-test), demonstrating that biological activity was retained. Cells incubated in MQ-189 or in KK-169 were irradiated with 365 nm light for 15 min during compound incubation. Subsequently, cells were fixed and click labeled as in previous experiments. We found that photolinkage increased plasma-membrane associated fluorescence compared with KK-169 labeling, which remained localized primarily to intracellular compartments (Fig. 12).

Although the mean intracellular fluorescence intensity for KK-169 trended toward higher values than for MQ-189, the difference did not reach statistical significance (Fig. 12F). Thus, the major difference between compounds was the presence of a plasma membrane-associated component for MQ-189. We conclude that although considerable intracellular KK-169 is retained intracellularly following fixation, the plasma-membrane pool is only readily observable with photo-attachment.

4. Discussion

Positive NMDAR modulation is of interest to basic and clinical neuroscience for several reasons. Modest increases in NMDAR activation enhance memory and learning and may benefit cognitive symptoms in schizophrenia (Collingridge et al., 2013; Sun et al., 2015). Exogenous PREGS or its precursor pregnenolone enhances learning and memory (Akwa et al., 2001; Petit et al., 2011; Vallee et al., 1997) and may reduce symptoms in schizophrenia patients (Marx et al., 2009). For development of therapeutics that capitalize on these effects, the field requires more information about cellular actions of the compounds. Novel chemical biology approaches, such as ours, represent one way forward. Our work represents an initial exploration of the pharmacological activity and cellular level distribution of a new class of tools for exploring sulfated neurosteroids. Our results provide new information about structure-activity relationships while revealing some of the structural properties of compounds important for cellular retention and accumulation. As with other recent results (Krausova et al., 2018), our



Fig. 12. Enhanced membrane labeling by a photoaffinity click analogue. A, B. Structures of a photoaffinity analogue (MQ-189) and KK-169. C. D. Representative results from incubation in unlabeled analogue in the presence of ultraviolet irradiation (see Methods), followed by fixation and retrospective click fluorescence. D1 and D2 represent fluorescence intensity of the images through the straight lines drawn in C1 and C2. E. Summary of the peak fluorescence in membrane regions expressed as a ratio to cytoplasmic fluorescence from 11 to 12 fields in 4 experiments. MQ-189 showed a significantly higher ratio of membrane to cytoplasmic labeling (N = 11-12 fields in 4 Mann-Whitney experiments. test. p = 0.001). F. In these same fields the absolute level of intracellular fluorescence trended higher for KK-169 compared to MQ-189 but this difference did not reach statistical significance (Mann-Whitney test, p = 0.13

data show that NMDAR potentiation can be retained with alterations to the PREGS structure. Specifically, our results demonstrate that bioactivity tolerates side chain alterations to add a click label (KK-169, KK-181) or click label plus photolabel (MQ-189). Core features of PREGS activity are retained without detectable off target effects.

PREGS has complex actions at NMDARs (Chopra et al., 2015; Korinek et al., 2011), and the binding site remains elusive. The site has been assumed to be extracellular (Park-Chung et al., 1997), and the M3-M4 extracellular loop of GluN2 subunits was initially implicated in sulfated steroid action (Horak et al., 2006). More recent work has demonstrated that both the ligand-binding and transmembrane domains of GluN subunits are required (Wilding et al., 2016). The concentration of intracellular Ca²⁺ may determine the direction of PREGS modulation of GluN1/2A receptors (Chopra et al., 2015), but this does not imply an intracellular PREGS site. In general, it is unclear whether any previously identified domain is essential for binding or rather for translating the binding of modulator elsewhere into a change in channel open probability. The balance of evidence suggests an external site of interaction in the plasma membrane, so although we found evidence for intracellular accumulation/retention, the basis for currently known neuroactive actions is likely through external, membrane-associated sites.

The primary goal of this study was to develop chemical biology tools for the exploration of NMDAR modulators of the PREGS class. An unexpected outcome of the work was the identification of potent and efficacious analogues (Fig. 1), which suggest new aspects of the structure-activity relationship between steroids and NMDARs. Previous work has explored mostly steroid inhibitors of NMDAR function (Adla et al., 2017). Based on the strong activity of multiple analogues with a hydrophobic side chain, we conclude that side chain hydrophobicity is likely to increase potentiation. The hydrophobic chain may foster a steroid orientation that promotes interaction with a hydrophobic (e.g., transmembrane) PREGS site on the receptor. It remains unclear whether introducing lipophilicity in other parts of the steroid structure would exhibit similar benefit. It is possible that strong activity requires hydrophobicity at the opposite end of the molecule from the sulfate charge.

Off-target effects at GABA_A receptors, presynaptic sites, and at AMPA/kainate receptors represent a potential disadvantage of sulfated steroids as therapeutics (Lee et al., 2010; Seljeset et al., 2015; Yaghoubi et al., 1998; Zamudio-Bulcock and Valenzuela, 2011). At concentrations that have maximum NMDAR effect, we found little evidence that KK-169 has presynaptic or AMPAR effects. Unfortunately, GABAergic actions are quite ubiquitous among anionic lipophilic compounds (Chisari et al., 2010; Mennerick et al., 2001), so it seems unlikely that this off target effect will be eliminated by additional chemical modifications.

Our visualization approach included *in situ* variants of click chemistry and photo-labeling, chemical biology techniques that have gained increasing traction in recent years (Baskin et al., 2007; Hofmann et al., 2014; Hou et al., 2012). Our results include the new observation that the negative charge of sulfated steroids does not prevent their rapid intracellular accumulation. Possible explanations include active transport mechanisms or a small amount of passive influx across the membrane through lipophilic interactions. Based on calculated logP values KK-169 is marginally more lipophilic than PREGS, in either its neutral or anionic form (clogP ~ 2.6–2.9). Thus, KK-169 should give a reasonable reflection of the ability of the natural compound PREGS to penetrate membranes through passive mechanisms. Intracellular targets may be involved in some of the reported actions of PREGS, including presynaptic effects. On the other hand, KK-181 is predicted to be considerably more lipophilic (clogP ~ 4), explaining its slow reversibility.

As with our work with KK-169, other cellular studies have localized intracellular compounds by virtue of cellular retention in the absence of photolabeling (Jao et al., 2015; Peyrot et al., 2014; Viertler et al., 2012). In those cases, high-affinity protein interactions may drive retention. Our results do not completely exclude a role for protein targets in accumulation and retention. Presumed membrane localization of compound was rapidly lost before and/or after fixation, and plasma-membrane labeling was revealed only with photo-labeling (Fig. 12). This suggests that the plasma membrane pool of KK-169 is more labile than the intracellular pool. Thus, although the precise targets and mechanisms remain elusive, our series of structural analogues provide initial clues of relevant properties responsible for KK-169 accumulation and retention.

5. Conclusions

In summary our work characterizes optically and biochemically accessible pharmacological tools to study the behavior of sulfated steroids as modulators of NMDAR function. Given the importance of NMDARs to neuronal function, and given the interest in NMDAR ligands to therapeutic applications, these tools may help advance understanding of pharmacological actions and aid the rational design of therapeutics.

Funding

NIH grants MH101874 (SM, CFZ), MH110550 (DFC), and NS30888 (JEH).

Acknowledgements

The authors thank Amanda Taylor for technical help and George Elias for early oocyte experiments. The authors thank members of the Taylor Family Institute for Innovative Psychiatric Research for discussion. CFZ is a member of the scientific advisory board for Sage Therapeutics, and DFC and CFZ hold stock in Sage Therapeutics. Sage Therapeutics was not involved in the design or analysis of experiments herein.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.neuropharm.2018.10.015.

References

- Abdallah, C.G., Sanacora, G., Duman, R.S., Krystal, J.H., 2015. Ketamine and rapid-acting antidepressants: a window into a new neurobiology for mood disorder therapeutics. Annu. Rev. Med. 66, 509–523.
- Adla, S.K., Slavikova, B., Smidkova, M., Tloustova, E., Svoboda, M., Vyklicky, V., Krausova, B., Hubalkova, P., Nekardova, M., Holubova, K., Vales, K., Budesinsky, M., Vyklicky, L., Chodounska, H., Kudova, E., 2017. Physicochemical and biological properties of novel amide-based steroidal inhibitors of NMDA receptors. Steroids 117, 52–61.
- Akwa, Y., Ladurelle, N., Covey, D.F., Baulieu, E.E., 2001. The synthetic enantiomer of pregnenolone sulfate is very active on memory in rats and mice, even more so than its physiological neurosteroid counterpart: distinct mechanisms? Proc. Natl. Acad. Sci. U. S. A. 98, 14033–14037.

- Baskin, J.M., Prescher, J.A., Laughlin, S.T., Agard, N.J., Chang, P.V., Miller, I.A., Lo, A., Codelli, J.A., Bertozzi, C.R., 2007. Copper-free click chemistry for dynamic in vivo imaging. Proc. Natl. Acad. Sci. U. S. A. 104, 16793–16797.
- Borovska, J., Vyklicky, V., Stastna, E., Kapras, V., Slavikova, B., Horak, M., Chodounska, H., Vyklicky Jr., L., 2012. Access of inhibitory neurosteroids to the NMDA receptor. Br. J. Pharmacol. 166, 1069–1083.
- Burnell, E.S., Irvine, M., Fang, G., Sapkota, K., Jane, D.E., Monaghan, D.T., 2018. Positive and negative allosteric modulators of N-Methyl-d-aspartate (NMDA) receptors: structure-activity relationships and mechanisms of action. J. Med. Chem. https://doi. org/10.1021/acs.jmedchem.7b01640. (PMID: 29446949).
- Chisari, M., Shu, H.J., Taylor, A., Steinbach, J.H., Zorumski, C.F., Mennerick, S., 2010. Structurally diverse amphiphiles exhibit biphasic modulation of GABA_A receptors: similarities and differences with neurosteroid actions. Br. J. Pharmacol. 160, 130–141.
- Chopra, D.A., Monaghan, D.T., Dravid, S.M., 2015. Bidirectional effect of pregnenolone sulfate on GluN1/GluN2A N-Methyl-D-Aspartate receptor gating depending on extracellular calcium and intracellular milieu. Mol. Pharmacol. 88, 650–659.
- Collingridge, G.L., Volianskis, A., Bannister, N., France, G., Hanna, L., Mercier, M., Tidball, P., Fang, G., Irvine, M.W., Costa, B.M., Monaghan, D.T., Bortolotto, Z.A., Molnar, E., Lodge, D., Jane, D.E., 2013. The NMDA receptor as a target for cognitive enhancement. Neuropharmacology 64, 13–26.
- Eisenman, L.N., He, Y., Fields, C., Zorumski, C.F., Mennerick, S., 2003. Activation-dependent properties of pregnenolone sulfate inhibition of GABA_A receptor-mediated current. J. Physiol. 550, 679–691.
- Eisenman, L.N., Shu, H.J., Akk, G., Wang, C., Manion, B.D., Kress, G.J., Evers, A.S., Steinbach, J.H., Covey, D.F., Zorumski, C.F., Mennerick, S., 2007. Anticonvulsant and anesthetic effects of a fluorescent neurosteroid analog activated by visible light. Nat. Neurosci. 10, 523–530.
- Emnett, C., Li, H., Jiang, X., Benz, A., Boggiano, J., Conyers, S., Wozniak, D.F., Zorumski, C.F., Reichert, D.E., Mennerick, S., 2016. A clickable analogue of ketamine retains NMDA receptor activity, psychoactivity, and accumulates in neurons. Sci. Rep. 6, 38808.
- Emnett, C.M., Eisenman, L.N., Mohan, J., Taylor, A.A., Doherty, J.J., Paul, S.M., Zorumski, C.F., Mennerick, S., 2015. Interaction between positive allosteric modulators and trapping blockers of the NMDA receptor channel. Br. J. Pharmacol. 172, 1333–1347.
- Hansen, K.B., Yi, F., Perszyk, R.E., Menniti, F.S., Traynelis, S.F., 2017. NMDA receptors in the central nervous system. Methods Mol. Biol. 1677, 1–80.
- Hofmann, K., Thiele, C., Schött, H.-F., Gaebler, A., Schoene, M., Kiver, Y., Friedrichs, S., Lütjohann, D., Kuerschner, L., 2014. A novel alkyne cholesterol to trace cellular cholesterol metabolism and localization. J. Lipid Res. 55, 583–591.
- Horak, M., Vlcek, K., Chodounska, H., Vyklicky Jr., L., 2006. Subtype-dependence of Nmethyl-D-aspartate receptor modulation by pregnenolone sulfate. Neuroscience 137, 93–102.
- Hou, J., Liu, X., Shen, J., Zhao, G., Wang, P.G., 2012. The impact of click chemistry in medicinal chemistry. Expet Opin. Drug Discov. 7, 489–501.
- Jao, C.Y., Nedelcu, D., Lopez, L.V., Samarakoon, T.N., Welti, R., Salic, A., 2015. Bioorthogonal probes for imaging sterols in cells. Chembiochem 16, 611–617.
- Jiang, X., Shu, H.J., Krishnan, K., Qian, M., Taylor, A.A., Covey, D.F., Zorumski, C.F., Mennerick, S., 2016. A clickable neurosteroid photolabel reveals selective Golgi compartmentalization with preferential impact on proximal inhibition. Neuropharmacology 108, 193–206.
- Kilkenny, C., Browne, W.J., Cuthill, I.C., Emerson, M., Altman, D.G., 2010. Improving bioscience research reporting: the ARRIVE guidelines for reporting animal research. PLoS Biol. 8, e1000412.
- Korinek, M., Kapras, V., Vyklicky, V., Adamusova, E., Borovska, J., Vales, K., Stuchlik, A., Horak, M., Chodounska, H., Vyklicky Jr., L., 2011. Neurosteroid modulation of Nmethyl-D-aspartate receptors: molecular mechanism and behavioral effects. Steroids 76, 1409–1418.
- Kostakis, E., Smith, C., Jang, M.K., Martin, S.C., Richards, K.G., Russek, S.J., Gibbs, T.T., Farb, D.H., 2013. The neuroactive steroid pregnenolone sulfate stimulates trafficking of functional NMDA receptors to the cell surface via a non-canonical G-protein and Ca⁺⁺ dependent mechanism. Mol. Pharmacol. 84, 261–274.
- Krausova, B., Slavikova, B., Nekardova, M., Hubalkova, P., Vyklicky, V., Chodounska, H., Vyklicky, L., Kudova, E., 2018. Positive modulators of the N-Methyl-d-aspartate receptor: structure-activity relationship study of steroidal 3-hemiesters. J. Med. Chem. 61, 4505–4516.
- Lai, T.W., Shyu, W.C., Wang, Y.T., 2011. Stroke intervention pathways: NMDA receptors and beyond. Trends Mol. Med. 17, 266–275.
- Lee, K.H., Cho, J.H., Choi, I.S., Park, H.M., Lee, M.G., Choi, B.J., Jang, I.S., 2010. Pregnenolone sulfate enhances spontaneous glutamate release by inducing presynaptic Ca2+-induced Ca2+ release. Neuroscience 171, 106–116.
- Malayev, A., Gibbs, T.T., Farb, D.H., 2002. Inhibition of the NMDA response by pregnenolone sulphate reveals subtype selective modulation of NMDA receptors by sulphated steroids. Br. J. Pharmacol. 135, 901–909.
- Marx, C.E., Keefe, R.S., Buchanan, R.W., Hamer, R.M., Kilts, J.D., Bradford, D.W., Strauss, J.L., Naylor, J.C., Payne, V.M., Lieberman, J.A., Savitz, A.J., Leimone, L.A., Dunn, L., Porcu, P., Morrow, A.L., Shampine, L.J., 2009. Proof-of-concept trial with the neurosteroid pregnenolone targeting cognitive and negative symptoms in schizophrenia. Neuropsychopharmacology 34, 1885–1903.
- McGrath, J.C., Drummond, G.B., McLachlan, E.M., Kilkenny, C., Wainwright, C.L., 2010. Guidelines for reporting experiments involving animals: the ARRIVE guidelines. Br. J. Pharmacol. 160, 1573–1576.
- Mennerick, S., Zeng, C.M., Benz, A., Shen, W., Izumi, Y., Evers, A.S., Covey, D.F., Zorumski, C.F., 2001. Effects on γ -aminobutyric acid (GABA)_A receptors of a neuroactive steroid that negatively modulates glutamate neurotransmission and

M. Chisari et al.

augments GABA neurotransmission. Mol. Pharmacol. 60, 732-741.

- Nilsson, K.R., Zorumski, C.F., Covey, D.F., 1998. Neurosteroid analogues. 6. The synthesis and GABA_A receptor pharmacology of enantiomers of dehydroepiandrosterone sulfate, pregnenolone sulfate, and $(3\alpha,5\beta)$ -3-hydroxypregnan-20-one sulfate. J. Med. Chem. 41, 2604–2613.
- Park-Chung, M., Wu, F.S., Farb, D.H., 1994. 3α-Hydroxy-5β-pregnan-20-one sulfate: a negative modulator of the NMDA-induced current in cultured neurons. Mol. Pharmacol. 46, 146–150.
- Park-Chung, M., Wu, F.S., Purdy, R.H., Malayev, A.A., Gibbs, T.T., Farb, D.H., 1997. Distinct sites for inverse modulation of N-methyl-D-aspartate receptors by sulfated steroids. Mol. Pharmacol. 52, 1113–1123.
- Paul, S.M., Doherty, J.J., Robichaud, A.J., Belfort, G.M., Chow, B.Y., Hammond, R.S., Crawford, D.C., Linsenbardt, A.J., Shu, H.J., Izumi, Y., Mennerick, S.J., Zorumski, C.F., 2013. The major brain cholesterol metabolite 24(S)-hydroxycholesterol is a potent allosteric modulator of N-methyl-D-aspartate receptors. J. Neurosci. 33, 17290–17300.
- Petit, G.H., Tobin, C., Krishnan, K., Moricard, Y., Covey, D.F., Rondi-Reig, L., Akwa, Y., 2011. Pregnenolone sulfate and its enantiomer: differential modulation of memory in a spatial discrimination task using forebrain NMDA receptor deficient mice. Eur. Neuropsychopharmacol 21, 211–215.
- Peyrot, S.M., Nachtergaele, S., Luchetti, G., Mydock-McGrane, L.K., Fujiwara, H., Scherrer, D., Jallouk, A., Schlesinger, P.H., Ory, D.S., Covey, D.F., Rohatgi, R., 2014. Tracking the subcellular fate of 20(s)-hydroxycholesterol with click chemistry reveals a transport pathway to the Golgi. J. Biol. Chem. 289, 11095–11110.
- Reddy, D.S., 2010. Neurosteroids: endogenous role in the human brain and therapeutic potentials. Prog. Brain Res. 186, 113–137.
- Seljeset, S., Laverty, D., Smart, T.G., 2015. Inhibitory neurosteroids and the GABAA receptor. Adv. Pharmacol. 72, 165–187.
- Shen, W., Mennerick, S., Covey, D.F., Zorumski, C.F., 2000. Pregnenolone sulfate modulates inhibitory synaptic transmission by enhancing GABA_A receptor desensitization. J. Neurosci. 20, 3571–3579.
- Smith, C.C., Gibbs, T.T., Farb, D.H., 2014. Pregnenolone sulfate as a modulator of synaptic plasticity. Psychopharmacology 231, 3537–3556.
- Strong, K.L., Jing, Y., Prosser, A.R., Traynelis, S.F., Liotta, D.C., 2014. NMDA receptor modulators: an updated patent review (2013-2014). Expert Opin. Ther. Pat. 24, 1349–1366.

- Sun, M.Y., Linsenbardt, A.J., Emnett, C.M., Eisenman, L.N., Izumi, Y., Zorumski, C.F., Mennerick, S., 2015. 24(S)-Hydroxycholesterol as a modulator of neuronal signaling and survival. Neuroscientist 22 (2), 132–144.
- Tischbirek, C.H., Wenzel, E.M., Zheng, F., Huth, T., Amato, D., Trapp, S., Denker, A., Welzel, O., Lueke, K., Svetlitchny, A., Rauh, M., Deusser, J., Schwab, A., Rizzoli, S.O., Henkel, A.W., Muller, C.P., Alzheimer, C., Kornhuber, J., Groemer, T.W., 2012. Usedependent inhibition of synaptic transmission by the secretion of intravesicularly accumulated antipsychotic drugs. Neuron 74, 830–844.
- Twede, V.D., Covey, D.F., Tartaglia, A.L., Bamber, B.A., 2007. The neurosteroids dehydroepiandrosterone sulfate and pregnenolone sulfate inhibit the UNC-49 GABA receptor through a common set of residues. Mol. Pharmacol. 72, 1322–1329.
- Vallee, M., Mayo, W., Darnaudery, M., Corpechot, C., Young, J., Koehl, M., Le Moal, M., Baulieu, E.E., Robel, P., Simon, H., 1997. Neurosteroids: deficient cognitive performance in aged rats depends on low pregnenolone sulfate levels in the hippocampus. Proc. Natl. Acad. Sci. U. S. A. 94, 14865–14870.
- Viertler, M., Schittmayer, M., Birner-Gruenberger, R., 2012. Activity based subcellular resolution imaging of lipases. Bioorg. Med. Chem. 20, 628–632.
- Vyklicky, V., Smejkalova, T., Krausova, B., Balik, A., Korinek, M., Borovska, J., Horak, M., Chvojkova, M., Kleteckova, L., Vales, K., Cerny, J., Nekardova, M., Chodounska, H., Kudova, E., Vyklicky, L., 2016. Preferential inhibition of tonically over physically activated NMDA receptors by pregnane derivatives. J. Neurosci. 36, 2161–2175.
- Wilding, T.J., Lopez, M.N., Huettner, J.E., 2014. Radial symmetry in a chimeric glutamate receptor pore. Nat. Commun. 5, 3349.
- Wilding, T.J., Lopez, M.N., Huettner, J.E., 2016. Chimeric glutamate receptor subunits reveal the transmembrane domain is sufficient for NMDA receptor pore properties but some positive allosteric modulators require additional domains. J. Neurosci. 36, 8815–8825.
- Wu, F.S., Chen, S.C., 1997. Mechanism underlying the effect of pregnenolone sulfate on the kainate-induced current in cultured chick spinal cord neurons. Neurosci. Lett. 222, 79–82.
- Yaghoubi, N., Malayev, A., Russek, S.J., Gibbs, T.T., Farb, D.H., 1998. Neurosteroid modulation of recombinant ionotropic glutamate receptors. Brain Res. 803, 153–160.
- Zamudio-Bulcock, P.A., Valenzuela, C.F., 2011. Pregnenolone sulfate increases glutamate release at neonatal climbing fiber-to-Purkinje cell synapses. Neuroscience 175, 24–36.