JNeurosci
THE JOURNAL OF NEUROSCIENCE

JNeurosci Accepted Manuscript

Research Articles: Neurobiology of Disease

Positive allosteric modulation as a potential therapeutic strategy in anti-NMDA receptor encephalitis

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3
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18 **Abbreviated title:** Anti-NMDAR therapeutic strategy
19
20 **Pages: 33**
21 **Figures: 8**
22 **Abstract words: 229**
23 **Introduction words: 371**
24 **Discussion words: 1219**
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44 **ABSTRACT**

45 N-methyl-D-aspartate receptors (NMDARs) are ionotropic glutamate receptors important for

46 synaptic plasticity, memory, and neuropsychiatric health. NMDAR hypofunction contributes to

47 multiple disorders, including anti-NMDAR encephalitis (NMDARE), an autoimmune disease of

48 the central nervous system associated with GluN1 antibody-mediated NMDAR internalization.

49 Here we characterize the functional/pharmacological consequences of exposure to cerebrospinal

50 fluid (CSF) from female human NMDARE patients on NMDAR function, and we characterize

51 the effects of intervention with recently described positive allosteric modulators (PAMs) of

52 NMDARs. Incubation (48 h) of rat hippocampal neurons of both sexes in confirmed NMDARE

53 patient CSF, but not control CSF, attenuated NMDA-induced current. Residual NMDAR

54 function was characterized by lack of change in channel open probability, indiscriminate loss of

55 synaptic and extrasynaptic NMDARs, and indiscriminate loss of GluN2B-containing and

56 GluN2B-lacking NMDARs. NMDARs tagged with N-terminal pHluorin fluorescence

57 demonstrated loss of surface receptors. Thus, function of residual NMDARs following CSF

58 exposure was indistinguishable from baseline, and deficits appear wholly accounted for by

59 receptor loss. Co-application of CSF and PAMs of NMDARs (SGE-301 or SGE-550, oxysterol-

60 mimetic) for 24 h restored NMDAR function following 24 h incubation in patient CSF.

61 Curiously, restoration of NMDAR function was observed despite wash-out of PAMs prior to

62 electrophysiological recordings. Subsequent experiments suggested that residual allosteric

63 potentiation of NMDAR function explained the persistent rescue. Further studies of the

64 pathogenesis of NMDARE and intervention with PAMs may inform new treatments for

65 NMDARE and other disorders associated with NMDAR hypofunction.

66

67 **Significance statement:** Anti-N-methyl-D-aspartate receptor encephalitis (NMDARE) is

68 increasingly recognized as an important cause of sudden-onset psychosis and other

69 neuropsychiatric symptoms. Current treatment leaves unmet medical need. Here we demonstrate

70 cellular evidence that newly identified positive allosteric modulators of NMDAR function may

71 be a viable therapeutic strategy.

72

73 **INTRODUCTION**

74 Anti-N-methyl-D-aspartate receptor encephalitis (NMDARE) is increasingly recognized as a

75 cause of sudden-onset psychosis and other neuropsychiatric symptoms (Fischer et al., 2016;

76 Dalmau et al., 2017). In fact, NMDARE is now reported as the most common cause of

77 autoimmune encephalitis, with comparable incidence and prevalence to infectious encephalitis

78 (Gable et al., 2012; Dubey et al., 2018). Indicated treatment for NMDARE includes

79 immunosuppressive therapies. With current treatment, approximately 80% of patients improve,

80 while relapses are reported in 14-25% of cases (Gabilondo et al., 2011; Titulaer et al., 2013).

81 Although reassuring, documented improvement may take place over protracted periods (Titulaer

82 et al., 2013), with persistent psychiatric and cognitive sequelae of disease increasingly

83 recognized (Finke et al., 2016). These findings emphasize the need for complementary

84 approaches to treatment (Panzer and Lynch, 2013), including interventions capable of mitigating

85 symptoms during the acute phase of disease, and strategies that promote receptor recovery and

86 improve longer-term outcomes.

87

88 At the cellular level, binding of NMDAR antibodies leads to capping and cross-linking of

89 NMDARs, resulting in receptor dimerization and internalization (Hughes et al., 2010). Other

90 results suggest that human NMDAR antibodies may alter surface NMDAR retention at synapses

91 (Mikasova et al., 2012). The combined effects are thought to be responsible for the unique

92 manifestations of signs and symptoms that characterize patients with NMDARE, a hypothesis

93 reinforced by the observation that several clinical features are reproduced with pharmacological

94 blockade of NMDARs (Krystal et al., 2002; Anticevic et al., 2012; Zorumski et al., 2016).

4

95 Although loss of surface receptors is key to antibody effects, whether remaining surface

96 receptors exhibit altered function because of antibody binding is unclear.

97

98 We examined additional effects of NMDAR antibody exposure by challenging primary cultures

99 of rodent hippocampal neurons through incubation with cerebrospinal fluid (CSF) from

100 NMDARE patients. We found no evidence of altered function of remaining surface NMDARs

101 following 24-48 h of CSF exposure, nor preferential loss of synaptic versus extrasynaptic

102 NMDARS or GluN2A versus GluN2B-containing NMDARs. We provide proof-of-concept, *in*

103 *vitro* evidence that a class of oxysterol mimetics, selective positive allosteric modulators (PAMs)

104 of NMDAR function (Paul et al., 2013), rescue the effect of patient CSF when administered mid-

105 course of a 48 h incubation. The results implicate a novel drug class for rapid intervention in

106 NMDARE.

107

108 **MATERIALS AND METHODS**

109 **Hippocampal Cell Culture:** Rat hippocampal neuron-astrocyte co-cultures were created and

110 maintained as previously described (Mennerick et al., 1995; Moulder et al., 2007; Emnett et al.,

111 2013) Briefly, hippocampi and cortical (astrocyte) tissue were dissected from PND 0-3 rats of

112 either sex using protocols approved by the Institutional Animal Care and Use Committee

113 (IACUC). Cells were seeded in modified Eagle's medium (Life Technologies) containing 5%

114 horse serum, 5% fetal calf serum, 17 mM D-glucose, 400 mM glutamine, 50 U/ml penicillin, and

115 50 mg/ml streptomycin. Mass cultures were plated at 650 cells/mm onto 25-mm cover glasses

116 coated with 5 mg/ml collagen or 0.1 mg/ml poly-D-lysine with 1 mg/ml laminin. Microisland

117 cultures were seeded on microdots of collagen on 0.15% agarose background as described

5

118   (Mennerick et al., 1995; Moulder et al., 2007; Emnett et al., 2013). Experiments were performed

119   at 7-11 DIV.

120

121   **Patient material:** CSF was collected from patients admitted to Barnes-Jewish Hospital

122   (Washington University in St. Louis; Saint Louis, Missouri) with definite autoimmune

123   encephalitis, including symptoms and signs consistent with NMDARE (Titulaer et al., 2013), and

124   antibodies against the GluN1 subunit of the NMDAR (clinical testing performed by the Mayo

125   Clinic; Rochester, Minnesota). The clinical assay for NMDARE is a cell-based assay using

126   specific anti-IgG immunofluorescence detection methods on transfected cells (EUROIMMUN,

127   Lübeck, Germany). We confirmed with anti-human IgG staining of tissue, as described below.

128   Patient A also presented with ovarian teratoma, a classic co-morbidity for NMDARE. CSF

129   samples were obtained prior to induction of appropriate immunotherapy. Antibody titers were

130   1:4 for patient A, 1:10 for patient B, and 1:32 for patient C. Additional CSF samples were

131   obtained from female patients between 29 and 33 years of age with multiple sclerosis (MS), a

132   chronic neuroinflammatory/autoimmune disease (Polman et al., 2011), as well as from

133   neurologically normal volunteers. Patients were enrolled in ongoing prospective observational

134   research studies at Washington University in St. Louis. Neurologically normal volunteers were

135   recruited from the local community, and CSF was obtained following a brief interview (to

136   establish status as controls) and neurological examination. Study protocols were approved by the

137   Washington University School of Medicine Human Research Protections Office. Written

138   informed consent was obtained from patients (or their delegates) and neurologically normal

139   volunteers, permitting collection, storage, and use of CSF in appropriate clinical research studies.

140   CSF was stored at -70^0 C until use.

6

141

142   In electrophysiological recording and immunohistochemistry experiments, cells were incubated

143   in 1:12 or 1:24 dilutions of patient CSF, neurologically normal CSF, or artificial CSF (aCSF)

144   containing in mM: 138 NaCl, 4 KCl, 2 $CaCl_2$, 10 glucose, 10 HEPES 2 $CaCl_2$, 1 $MgCl_2$, and 10

145   glucose. Glutamate receptor antagonists D-APV (10 μM) and NBQX (1 μM) were included to

146   prevent excitotoxicity. Due to limited CSF sample, aliquots were harvested following

147   experiments and reused up to four times; controls and experimental samples were matched for

148   number of uses.

149   **N2a Cell Transfection:** Murine neuro-2a (N2a; ATCC #CCL-131) cells were cultured in

150   Dulbecco's Modified Eagle's medium (DMEM) supplemented with 10% (v/v) FBS, 2 mM

151   glutamine plus 100 U/ml penicillin and 0.1 mg/ml streptomycin in an atmosphere of 5% CO2

152   and 95% air. Cells were maintained at sub-confluent densities and transiently transfected with

153   GluN1 (0.34 μg) and GluN2B-SEP (2 μg) subunit DNA. GluN1a in pcDNA3 plasmid was a gift

154   kindly provided by Dr. Elias Aizenman (Department of Neurobiology, University of Pittsburgh

155   School of Medicine, Pittsburgh, PA 15261) The pCI-SEP_NR2B subunit was a gift from

156   Roberto Malinow (Addgene plasmid # 23998)(Kopec et al., 2006). The transfection protocol

157   consisted of 4 h incubation at 37° C in serum-free medium containing plasmids and

158   Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA). Following the incubation, the medium

159   was exchanged for serum-containing medium including 100 uM ketamine to prevent receptor-

160   mediated cell death (Boeckman and Aizenman, 1996).

161   **Slice Staining:** Rodent brain tissue was fixed, permeabilized and incubated in a 1:20 dilution of

162   patient CSF, followed by application of anti-human IgG secondary antibody conjugated to Alexa

7

163 Fluor 555. Images were taken of hippocampus using a 4x objective under epifluorescence

164 illumination.

165 **Electrophysiology:** Whole-cell recording pipettes were pulled from borosilicate glass capillary

166 tubes (World Precision Instruments) and exhibited 2-6 MΩ final open-tip resistances. Unless

167 otherwise stated, neurons were voltage clamped to -70 mV. Electrophysiological extracellular

168 recording solution typically contained: 138 mM NaCl, 4 mM KCl, 2 mM CaCl$_2$, 10 mM glucose,

169 10 HEPES, 20 μM D-serine, 15 μM gabazine, 1 μM NBQX, and 0.2 μM tetrodotoxin, pH 7.25.

170 Extracellular Mg^{2+} was excluded during electrophysiological evaluation of NMDAR function.

171 The whole-cell pipette internal solution contained (in mM): 140 cesium methanesulfonate, 5

172 NaCl, 0.5 CaCl$_2$, 5 EGTA, and 10 HEPES, pH 7.25. NMDA receptor function was probed by

173 applying 10 μM NMDA for 5 s.

174 Evoked EPSCs were obtained from solitary neurons in microcultures with a 1.5 ms

175 depolarization to 0 mV to elicit a breakaway action potential in the cell's axon. For these

176 experiments potassium gluconate replaced cesium methanesulfonate in the pipette solution.

177 Tetrodotoxin was excluded from bath solution, and NBQX was added or excluded as indicated.

178 Access resistance was compensated to 90-95% for evoked autaptic PSC recordings. Some

179 synaptic recordings were obtained in presence of 1 μM NBQX as indicated.

180 **Fluorescence imaging and anti-GFP immunostaining:** To assess surface receptor presence,

181 we performed acid quenching of superecliptic pHluorin (SEP)-tagged receptor subunits in live

182 cells during time-lapse imaging. Imaging was performed 24-48 h post-transfection. Cells were

183 perfused with electrophysiological extracellular saline containing 2 mM CaCl$_2$, 1 mM MgCl$_2$, 10

184 mM glucose and 10 μM D-APV. 2-[N-morpholino] ethane sulphonic acid (MES) was then

8

185 applied by replacing HEPES in the standard saline and setting the pH to 5.5 with HCl.

186 Fluorescence images were acquired with conventional epifluorescence on a Nikon TE2000

187 inverted microscope and a CCD camera (Roper Scientific) and analyzed using Fiji/ImageJ. For

188 MES-application experiments, fluorescence intensity was measured in a fixed region of interest

189 (ROI) near the membrane over the entirety of perfusion (22.5 seconds). Baseline fluorescence

190 was calculated as the average of pre-MES and post-MES fluorescence to account for time-

191 dependent decreases. This absolute baseline was compared with that at the final image during

192 MES challenge to measure the MES-induced fluorescence change.

193 For anti-GFP labeling of SEP-tagged surface receptors, transfected N2a cells were incubated in

194 control or patient CSF 24 h post-transfection. After 24 or 48 h of treatment, cultures were fixed

195 for 5 minutes in 4% paraformaldehyde in phosphate buffered saline. Cells were blocked with

196 10% normal goat serum (NGS) and incubated with rabbit anti-GFP antibody (Life Technologies,

197 catalogue number A6455) for 3 hours at a 1:1000 dilution. Cells were incubated in secondary

198 antibody conjugated to Alexa Fluor 568 for 30 minutes, and mounted onto coverslips for

199 visualization. Cells were imaged on a Nikon TE2000 microscope equipped with a C1 laser

200 scanning confocal module. Single confocal sections through the middle of the cell were analyzed

201 with Fiji/ImageJ. To ensure objectivity, cells were selected for analysis based on SEP

202 fluorescence, without reference to anti-GFP labeling. To assess anti-GFP surface labeling, ROI

203 lines at 30-pixel thickness were drawn through the cell excluding the nucleus. Fluorescence

204 intensity was assessed along the line, using cell exterior as background. Peak fluorescence value

205 within the first 50 pixels was taken as membrane fluorescence, while mean center values (middle

206 50 points) corresponded to intracellular fluorescence.

9

207   **Experimental Design and Statistical Analysis:** For all experiments we employed a 'yoked'

208   design to guard against ancillary causes of NMDAR alteration. Each set of experimental

209   conditions was performed in parallel on sibling cultures. Each replicate experiment contributed a

210   similar number of cells per experimental condition to overall pooled results.

211

212   Electrophysiology data acquisition and analyses were performed using pCLAMP 10 software

213   (Molecular Devices). Image processing and measurement of fluorescence intensity was acquired

214   in Fiji/ImageJ. Data were processed and plotted using Excel 2011 (Microsoft), and Prism 7

215   (GraphPad). Statistical significance was determined using Student's t tests, one-way ANOVA, or

216   two-way ANOVA as indicated in text and figure legends and as dictated by experimental design.

217   Unless otherwise noted, *post hoc* two-tailed Student's paired or unpaired *t* tests with Bonferroni

218   correction were used for multiple comparisons of only those comparisons important for testing *a*

219   *priori* hypotheses. Significance was defined as a corrected *p* value equivalent to <0.05.

220   NMDARE-A CSF-induced depression of NMDA current relative to control was demonstrated in

221   the first figure in 2 independent replications. In subsequent figures, this statistical comparison

222   was not repeated to reduce the possibility of type II errors in testing of additional hypotheses.

223

224   Least-squares minimization algorithms performed in GraphPad Prism or Clampex were used to

225   estimate rate constants using single exponential functions (Emnett et al., 2013).

226

227   **RESULTS**

228   **Characterization of receptor changes with patient CSF exposure**

10

229 CSF from NMDARE patient A (32 year-old female, NMDARE-A) yielded staining of rat brain

230 reminiscent of NMDAR expression (Figure 1A), in contrast with staining using CSF from age-

231 and sex-matched MS patient A (Figure 1B, MS-A). These results parallel those of previous

232 studies (Moscato et al., 2014; Dalmau et al., 2017). We next confirmed that 48 h incubation of

233 hippocampal cultures in NMDARE-A CSF (1:12 dilution in conditioned culture medium)

234 depressed NMDAR responsiveness compared with a CSF control (Figure 1C-D). Strong

235 depression of NMDAR current was observed with dilutions as low as 1:24, and with incubations

236 as brief as 24 h (Figure 1E). By contrast 1:12 dilution of CSF from neurologically normal

237 volunteers (NN-A, NN-B) or from MS patients (MS-A, MS-B) produced no decrease in

238 NMDAR function relative to aCSF (48 h incubation, Figure 1F-G). Subsequently, aCSF was

239 used as a comparator for most experiments.

240

241 Although surface NMDAR internalization is associated with depression of NMDAR current

242 induced by purified patient antibodies or by NMDARE patient CSF (Hughes et al., 2010;

243 Gleichman et al., 2012; Castillo-Gomez et al., 2017), altered function of remaining surface

244 NMDARs could influence clinical expression of disease or response to therapy. To investigate

245 the function of residual receptors, we examined the effect of patient CSF incubation on NMDAR

246 EPSCs, including decay kinetics recorded from solitary cultured neurons. Because of the brief

247 presence of glutamate in the synaptic cleft, EPSC decay renders a good estimate for the

248 elementary lifetime of NMDAR channels when bound by transmitter (Lester et al., 1990). We

249 found that NMDARE-A CSF incubation selectively decreased the NMDAR component of

250 evoked EPSCs (Figure 2A-C). However, decay kinetics of residual EPSCs were

11

251 indistinguishable from control (Figure 2D). Thus, it appears that channel lifetime in response to

252 brief glutamate exposure is not altered by incubation with patient CSF.

253

254 We also evaluated channel open probability of the total NMDAR population by examining onset

255 and offset kinetics of memantine, an uncompetitive open-channel blocker of NMDARs, during

256 application of exogenous NMDA after 24 h incubation in CSF (Figure 2E-F). In this protocol,

257 decreased P_{open} of channels results in slower onset/offset kinetics for memantine (Emnett et al.,

258 2015). However, antibody-depressed NMDAR function did not mediate a change in kinetics of

259 memantine block/unblock (Figure 2G-H). This finding further suggests that residual surface

260 NMDARs exhibit no substantial change in functionality.

261

262 Synaptic NMDAR activation is generally considered trophic, while extrasynaptic NMDAR

263 activation is thought to mediate excitotoxic death (Hardingham and Bading, 2010; but see Wroge

264 et al., 2012). Patient-derived NMDAR autoantibodies may differentially affect trafficking of

265 synaptic versus extrasynaptic receptor populations (Mikasova et al., 2012). To determine

266 whether altered balance between synaptic and extrasynaptic receptors might contribute to

267 dysfunction associated with NMDARE, we estimated the contribution of synaptic NMDARs to

268 the total population of NMDARs following incubation with patient CSF. To do so, we used the

269 very slowly dissociating NMDAR open-channel blocker MK-801 to enrich for extrasynaptic

270 receptors (Rosenmund et al., 1995). MK-801 requires channel opening for binding and once

271 bound is nearly irreversible (Huettner and Bean, 1988). Thus, when applied during ongoing

272 synaptic activity, MK-801 preferentially blocks synaptic NMDARs. Upon MK-801 removal,

273 NMDA-elicited current thus arises mainly from the extrasynaptic NMDAR population. Synaptic

12

274   activity was elevated during MK-801 application (10 μM, 15 min) with co-incubation in the

275   GABA$_A$ receptor antagonist bicuculline (15 μM), ensuring that subsequent agonist-elicited

276   responses would come nearly exclusively from extrasynaptic receptors (Rosenmund et al., 1995;

277   Hardingham et al., 2002; Wroge et al., 2012). MK-801 treatment was preceded by 24 h

278   incubation with control aCSF or NMDARE patient A CSF. Following MK-801 removal, our

279   evaluation of NMDA current revealed no difference in the fraction of pharmacologically isolated

280   extrasynaptic receptors in NMDARE-A CSF versus control-incubated cells (Figure 3A-D). Thus,

281   patient CSF exposure does not induce preferential loss of synaptic receptors or extrasynaptic

282   receptors.

283

284   Two major GluN2 subunits, GluN2A and GluN2B, contribute to hippocampal NMDAR

285   responses. The two classes localize and function differently (Chen et al., 1999; Tovar and

286   Westbrook, 1999; Izumi et al., 2005). To determine whether one class is preferentially lost with

287   patient CSF incubation, we used pharmacological isolation with the GluN2B-selective antagonist

288   ifenprodil (10 uM). NMDARE-A CSF incubation caused no change in the ifenprodil sensitivity

289   (Figure 3E-H), suggesting that the fractional contribution of GluN2A/GluN2B NMDARs does

290   not change as a result of patient CSF incubation. In summary, our evidence supports no

291   preferential loss of NMDARs associated with localization or with particular GluN2 subunit

292   presence.

293

294   Our data suggest that residual NMDAR behavior remains unperturbed, and therefore functionally

295   intact following exposure to patient CSF. We would expect from previous work that NMDAR

296   surface expression is diminished to account for the downregulation of NMDAR responses

13

297   observed (Hughes et al., 2010; Moscato et al., 2014). To test surface expression in live cells, we

298   utilized a biological assay with N2a cells transfected with heteromeric NMDA receptors

299   composed of GluN1 and super ecliptic pHluorin (SEP)-tagged GluN2B subunits. The SEP-tag is

300   a pH-sensitive GFP variant located at the N-terminus. To test functionality of the SEP-tagged

301   subunit, we tested N2a cells in whole-cell, patch-clamp studies. GFP-transfected NMDARs

302   yielded no response to NMDA application while cells transfected with wild type GluN1 and

303   GluN2B-SEP exhibited robust current in response to NMDA application (219 ± 23 pA, n=6

304   control and 6 GluN-transfected cells; $t(10)=8.9$, $p = <1E-4$, Student's unpaired t test).

305

306   In live cells fluorescence is quenched by the acidity of intracellular organelles but is brighter in

307   surface receptors where the tag is exposed to the neutral pH of the extracellular space (Kopec et

308   al., 2006). Live transfected cells showed robust fluorescence that was quenched by MES, a

309   membrane impermeant acid (Kopec et al., 2006)(Figure 4A). The baseline fluorescence of SEP-

310   transfected cells was weaker in transfected cells incubated in NMDARE-A CSF, and MES

311   quenching was reduced accordingly (Figure 4B). These results implicate a robust loss of surface

312   receptors with patient CSF treatment.

313

314   As an additional check on surface NMDAR presence in GluN1/GluN2B-SEP transfected N2a

315   cells, we used anti-GFP antibody labeling in fixed, non-permeabilized cells (Figure 4C-J). Cells

316   transfected with GluN1/GluN2B-SEP exhibited significantly higher membrane-associated anti-

317   GFP labeling than control cells transfected with cytosolic GFP (Figure 4C-G). In

318   GluN1/GluN2B-SEP-transfected cells incubated in NMDARE-A and MS-A CSF, anti-GFP

319   surface labeling was strongly reduced (Figure 4H-J). Thus, both live-cell imaging and

320   immunocytochemistry results support loss of surface receptors following incubation with

321   NMDARE patient CSF.

322

323   **Effects of oxysterol-like PAMs on receptor function following patient CSF exposure.**

324   The above results demonstrate that exposure to NMDARE patient CSF mediates broad loss of

325   surface NMDARs, regardless of synaptic localization or subunit composition. It follows,

326   therefore, that treatment with a broad spectrum NMDAR PAM may be a rational approach to

327   enhancing function of remaining surface NMDARs. The major brain cholesterol metabolite 24S-

328   hydroxycholesterol and several synthetic analogues represent a new class of broad-spectrum

329   PAMs (Sun et al., 2015). Subsequently, we evaluated the ability of SGE-301, a previously

330   characterized synthetic oxysterol analogue (Paul et al., 2013), and SGE-550, a similar but

331   previously unreported synthetic PAM, to rescue NMDAR function following exposure to patient

332   CSF.

333

334   As expected, the acute (30 second) application of either SGE-301 or SGE-550 (2 μM)

335   potentiated responses to 10 μM NMDA (Figure 5A-C). We explored effects of PAM incubation

336   as an intervention during patient CSF exposure. Following 24 h incubation of cultured

337   hippocampal cells in NMDARE-A CSF, we intervened with a co-application of one of the PAMs

338   for an additional 24 h. Following the full 48 h challenge, we removed the culture medium

339   containing CSF and PAM and placed cells in recording saline free of PAM. Despite the absence

340   of PAM in recording solutions, NMDA responses in both aCSF control conditions and

341   NMDARE-A CSF conditions were significantly augmented (Figure 5D, E). Statistically, there

342   was no interaction between CSF incubation status and PAM treatment status (Figure 5D, E),

15

343   indicating that PAMs may not interfere directly with the mechanisms of depressed NMDA

344   current amplitude, including receptor internalization. We followed-up this observation with

345   experimental tests of the mechanism of persistently potentiated NMDA currents following

346   prolonged PAM incubation.

347

348   We have previously shown that oxysterols are resistant to washout, potentially explaining the

349   persistent effects of PAMs (Paul et al., 2013; Linsenbardt et al., 2014). However, we found no

350   correlation between time of recording following PAM removal and the size of NMDA current in

351   our dataset (not shown). Therefore, we entertained the possibility that oxysterol analogues induce

352   a permanent change in NMDAR function (e.g., by fostering receptor insertion or inhibiting

353   internalization in control and antibody-treated conditions). We used the open-channel blocker

354   memantine to test if persistently increased NMDA current following PAM incubation is

355   associated with increased channel open probability, as expected of PAM action (Emnett et al.,

356   2015). Indeed, cells incubated 24 h with SGE-301 or with SGE-550, but recorded in the absence

357   of PAM, exhibited faster onset and offset memantine kinetics (Figure 6A-D). We further

358   evaluated whether the potentiation of NMDA current results from acute drug presence by briefly

359   incubating cells with the steroid scavenger γ-cyclodextrin (γ-CDX; 500 uM, 2 min)(Akk et al.,

360   2005; Shu et al., 2007; Paul et al., 2013). Although γ-CDX incubation alone appeared to have a

361   mild potentiating effect, the scavenger effectively reversed the persisting PAM potentiation

362   (Figure 6E-G). The rapid reduction produced by the scavenger causes us to conclude that

363   persisting potentiation is most readily explained by lingering compound, likely retained within

364   the membrane and/or sequestered intracellularly.

365

366   As a final test of whether PAMs interfere directly with the mechanisms of NMDAR removal, we

367   returned to N2a cells transfected with SEP tagged GluN subunits to visualize surface receptors.

368   We imposed the same CSF challenge and PAM intervention as in Figure 4 on cells to assess

369   surface receptor presence. Cells were incubated for 48 h in NMDARE-A or MS-A CSF with

370   SGE-301 intervention initiated at 24 h. Following the treatment, cells were fixed and labeled

371   with anti-GFP antibody (Figure 7A-D). We again observed significant depression of surface

372   receptor presence in cells treated with NMDARE patient A CSF. SGE-301 did not affect

373   membrane or intracellular labeling, nor was there a statistically significant interaction between

374   CSF condition and SGE-301 treatment (Figure 7E-F). This suggests that PAMs do not alter

375   surface receptor presence, and the observed PAM-associated persisting NMDAR potentiation is

376   most likely a result of SGE-301's direct interaction with NMDARs.

377

378   To extend the proof-of-principle for the PAM therapeutic approach, we examined the effect of

379   SGE-301 on CSF incubation from two additional NMDARE patients (NMDARE-B, C; Figure

380   8). Incubation in NMDARE-B and NMDARE-C CSF (23 year and 50 year old females,

381   respectively; 1:12 dilution for 48 h) decreased responses to NMDA, and the depression was

382   circumvented by incubation in SGE-301, similar to results with NMDARE-A CSF (Figure 8E).

383   These results suggest that the PAM approach may be broadly applicable in patients with

384   NMDARE.

385

386   **DISCUSSION**

387   Our proof of concept studies support the use of NMDAR PAMs as adjunct or complementary

388   treatments for NMDARE and possibly for other disorders associated with NMDAR

17

389   hypofunction, including schizophrenia (Olney and Farber, 1995; Krystal et al., 2002; Lin et al.,

390   2012; Cioffi, 2013). Using CSF obtained from a patient with a confirmed clinical diagnosis of

391   NMDARE, we functionally characterized NMDARs in cultured rat hippocampal cells following

392   incubation with patient CSF, and we quantified characteristics of rescue with two allosteric

393   modulators of NMDAR function. Persistent effects of compounds restore NMDAR function

394   through allosteric potentiation of residual NMDARs and suggest that signs and symptoms of

395   NMDARE may be ameliorated by this therapeutic approach. These cellular studies set a course

396   for future *in vivo* tests of the therapeutic approach.

397

398   Our study relied primarily on CSF from a single NMDARE patient for which we had sufficient

399   CSF to permit multiple studies. This patient presented with classic features of NMDARE (see

400   Methods). Nevertheless, we designed several experiments to test that CSF from NMDARE

401   patient A possesses features characteristic of NMDARE. Features confirmed in our studies

402   included CSF labeling (using IgG-specific secondary antibody) of rodent brain tissue in a pattern

403   expected of NMDAR distribution, functional selective depression of NMDAR current, and loss

404   of surface NMDARs. We replicated key results in two additional patients with IgG against

405   NMDARs, confirmed by testing in commercial clinical labs and by our own staining (Figure 8).

406

407   In addition, our experiments characterized several new features of NMDAR suppression studied

408   using NMDARE patient A sample. In contrast with some recent studies (Mikasova et al., 2012),

409   our results suggest that both synaptic and extrasynaptic receptors are equally targeted for loss.

410   Although the dimensions and molecular complexity of the synaptic cleft might reduce antibody

411   access, direct access of various antibodies to the synapse has been demonstrated in numerous

412 previous studies (e.g., Ehlers, 2000; Martens et al., 2008; Mikasova et al., 2012). Thus, it seems

413 likely that both populations of NMDARs are directly targeted by human GluN antibody.

414 Alternatively, interchange between synaptic and extrasynaptic populations (Tovar and

415 Westbrook, 2002) may allow indirect antibody access to synaptic receptors by targeting the

416 extrasynaptic pool. Future time course studies may distinguish these possibilities.

417

418 Other previous studies have suggested that antibody may alter the function of remaining surface

419 NMDARs. For instance, within minutes following patient CSF exposure, increased channel open

420 duration was detected (Gleichman et al., 2012). Our studies revealed no evidence for increased

421 or decreased open probability of NMDAR channels following prolonged patient CSF exposure,

422 using an open-channel blocker as a probe of channel open probability in whole-cell recordings

423 (Huettner and Bean, 1988). Discrepancies with previous work may reflect differences in the time

424 at which measurements were made or heterogeneity in the clinical population. These hypotheses

425 can be tested as additional patient material becomes available.

426

427 Taken together, our findings suggest that treatment with PAMs may represent a potential strategy

428 to rescue NMDAR hypofunction, with no detectable direct effect on the mechanisms of NMDAR

429 internalization that characterize the cellular pathophysiology. A more direct approach to maintain

430 or restore NMDAR function would be to target the cell biological mechanisms responsible for

431 receptor endocytosis and insertion; however, at this time, pharmacological interventions

432 targeting these mechanisms are not practical. Therefore, we made use of emerging understanding

433 of a class of positive regulators of NMDAR function that has resulted from study of the

434 endogenous cholesterol metabolite, 24S-hydroxycholesterol (Sun et al., 2015).

435

436   SGE-301 and SGE-550 represent synthetic analogues of 24S-hydroxycholesterol with selective

437   NMDAR effects in the sub-to-low micromolar concentration range (Paul et al., 2013;

438   Linsenbardt et al., 2014). These compounds appear to bind to a site independent of other known

439   allosteric modulators of NMDAR function (Paul et al., 2013; Wilding et al., 2016) and increase

440   channel open probability. Results with chimeric GluN –GluK subunits suggest that GluN

441   transmembrane domains are critical for oxysterol modulation (Wilding et al., 2016). A

442   transmembrane interaction site would be consistent with the lipophilic nature of these modulators

443   and with their sensitivity to cyclodextrin extraction (Shu et al., 2007). Interaction of oxysterols

444   with NMDARs results in a slight reduction in the EC50 of agonists and increased agonist

445   efficacy (Linsenbardt et al., 2014). Here we show that several dilutions of NMDARE patient

446   CSF result in similar presumed steady-state NMDAR reductions (Figure 1). The mechanisms

447   responsible for a fixed steady-state level of functional receptors despite varied antibody

448   concentration are unclear. We found that low micromolar concentrations of allosteric modulators

449   restored the activity of NMDARs from this steady-state to near or above baseline levels (Figures

450   5,6,8).

451

452   A puzzling observation initially was that potentiated NMDAR function persisted beyond the

453   period of direct exposure to compounds in all cells incubated in SGE-301 or SGE-550. We found

454   that the enhanced function was associated with increased channel open probability, was sensitive

455   to a scavenger of lipophilic compounds, and was not associated with increased surface

456   NMDARs. Thus, we attribute the persistence to the strong lipophilicity of the modulators. We

457   have previously shown evidence of modulator persistence for at least tens of seconds (Paul et al.,

458 2013). The present studies confirm that some oxysterol-like compounds can persist for many

459 minutes beyond removal, presumably by adhering to cells or accumulating intracellularly,

460 potentiating NMDAR responses despite constant local perfusion with compound-free saline.

461 This persistence of drug binding becomes relevant when assessing therapeutic viability,

462 recognizing that strong lipophilicity may be advantageous to dosing schedules. However, drug

463 exposure in a clinical setting would presumably last beyond the 24 h incubation period used

464 herein and could result in other changes not appreciated here. Additional *in vivo* studies are

465 required to clarify the time-dependent effects, and effect of dosing regimen on NMDAR

466 function.

467

468 Our work focused on the restorative actions of NMDAR modulators following exposure to CSF

469 from NMDARE patients. The idea that NMDAR hypofunction accounts for the broad spectrum

470 of clinical symptomatology described in NMDARE is supported by two primary lines of

471 evidence. First, pharmacologically-induced NMDAR hypofunction produces psychotomimetic

472 symptoms, not unlike those observed in NMDARE. Second, intrathecal injection of recombinant

473 antibodies can recapitulate key aspects of the disorder in rodents (Malviya et al., 2017). We

474 acknowledge, however, that additional pathogenic processes downstream of NMDAR

475 internalization, or other CSF constituents (e.g., other disease-associated antibodies or

476 inflammatory mediators; Kreye et al., 2016) may affect neuronal function and contribute to

477 symptomatology. Some of these may account for apparent differences in the degree of

478 suppression of NMDAR function (e.g., the stronger suppression observed in NMDARE patient

479 A vs. patients B and C). Others may be unrelated to NMDAR function ant thus not modulated

480 by NMDAR PAMs. Furthermore, sample quantities limited culture studies to relatively brief

21

481 periods of exposure, unlike the clinical situation where antibody is constantly renewed, resulting

482 in prolonged exposure. For these reasons, it will be important to adopt robust animal models and

483 follow-up the cellular level studies herein with circuit-level and *in vivo* tests of the therapeutic

484 potential of NMDAR PAMs in NMDARE (Planaguma et al., 2015; Malviya et al., 2017).

485

486 Our work shows that indiscriminate loss of NMDARs occurs following exposure to NMDARE

487 patient CSF, and suggests that PAMs may be developed as a potential therapeutic strategy.

488 Restoration of NMDAR function was achieved with application of oxysterol analogues, which

489 are potent, selective, but broad spectrum PAMs of NMDAR function. The restorative effects

490 were fully accounted for by the acute pharmacological effects of the compounds.

491

492

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619

620

621 **FIGURES AND LEGENDS**

622 **Figure 1.** Depression of NMDA current in hippocampal neurons following patient CSF

623 incubation. **A.** NMDARE-A CSF (1:20 dilution) labels mouse hippocampus in a pattern typical

624 of NMDAR distribution. Human antibody binding to the tissue section was visualized with anti-

625 human secondary antibody conjugated to Alexa Fluor 555. **B.** CSF from an age- and sex-

626 matched MS patient (1:20 dilution), failed to exhibit an NMDAR-like pattern of labeling. **C.**

627 NMDA (10 µM)-elicited current from a hippocampal neuron following 48 h incubation with

628 either aCSF (1:12, black trace) or NMDARE-A CSF (1:12, red trace). **D.** Summary of NMDA

629 current density from cells treated as in C; (t(46)=6.951, *p=1.08E-8) (n=21 for control and 27

630 for NMDARE-A). **E.** Comparison of effects of two dilutions of NMDARE-A CSF: 24 h in either

631 1:12 dilution (n=10) or 1:24 dilution (n=10) in sibling cultures. A one-way ANOVA showed a

632 significant NMDARE-A CSF-mediated current depression (F(2,27)=14.1, *p= 6.6E-5). Asterisks

633 denote $p<0.05$ versus aCSF, determined with Student's unpaired t test, Bonferroni corrected for

634 multiple comparisons. **F.** Representative traces from an experiment examining NMDA (10 µM)-

635 elicited current from a hippocampal neuron following 48 h incubation of hippocampal cultures in

636 aCSF (1:12 black trace, n=15) or CSF from MS-A (1:12, gray trace, n=10). **G.** Control CSF

637 from age- and sex-matched MS patients (MS-A, MS-B) and neurologically normal controls (NN-

638 A, NN-B). There was no significant difference between any treated group and control; p>0.05

639 uncorrected t tests.

640

641 **Figure 2.** Lack of change in key functional properties of residual NMDARs. **A** and **B**. Evoked

642 autaptic EPSCs from a hippocampal neuron incubated with aCSF (A) or NMDARE-A CSF (B).

643 Dual-component AMPAR and NMDAR EPSCs (light traces) and pharmacologically isolated

29

644 NMDAR EPSCs (dark traces) are shown. **C.** Summary of ratio of NMDAR to AMPAR peak

645 EPSC; (t(14)=4.2, *p=1E-9, Student's unpaired *t* test. **D.** Summary of weighted time constant

646 values (τ_w) obtained from a bi-exponential fit to the decay phase of the NMDAR EPSCs;

647 (t(14)=1.15, p=0.27, Student's unpaired *t* test). **E** and **F**. The rapid onset/offset open-channel

648 blocker memantine was used to probe changes in NMDAR channel open probability. **G**. and **H**.

649 Summary of the memantine onset and offset kinetics (obtained from exponential fits) revealed no

650 change following NMDARE-A CSF-mediated depression; (G: t(7)=1.15, p=0.29;

651 H:t(13)=1.33,p=0.21 respectively, Student's unpaired *t* tests).

652

653 **Figure 3.** No evidence of selective effect of NMDARE-A CSF on proportion of extrasynaptic

654 NMDARs or on the proportion of GluN2B-containing receptors. **A** and **B**. NMDA-elicited

655 current from neurons without (black) and with (blue) preceding MK-801 challenge to block

656 synaptic receptors. **C.** Summary of raw NMDA current density of respective ±MK-801

657 conditions. Analysis of raw current density revealed no interaction between condition

658 (aCSF/NMDARE-A CSF) and treatment (± MK-801; F (1, 52) = 0.36, two-way ANOVA,

659 *p*>0.55). **D.** Summary of MK-801 effect, normalized to the mean current density of the

660 respective -MK-801 condition (right). Analysis of normalized data also revealed no change in the

661 proportion of MK-801 insensitive current following NMDARE-A CSF incubation; (t(42)=0.93,

662 *p*>0.36, Student's unpaired *t* test). **E** and **F**. Representative examples of ifenprodil (10 μM)

663 sensitivity of NMDA-elicited current. **G.** Summary of raw NMDA current density in baseline

664 and ifenprodil conditions. There was no statistical interaction between condition

665 (aCSF/NMDARE-A CSF) and treatment (± ifenprodil; F (1, 48) = 0.7209, p=0.4, two-way

666 ANOVA, *p*>0.05). **H.** Summary of ifenprodil effect, normalized to the respective baseline
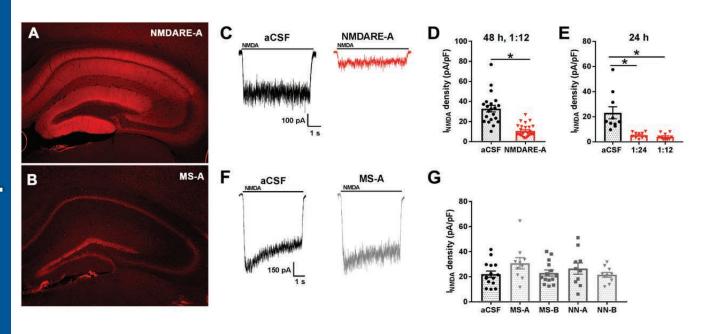
667   current density. Analysis of normalized data also revealed no change in the proportion of

668   ifenprodil-sensitive current following NMDARE-A CSF incubation; (t(24)=0.74, *p=0.47*,

669   Student's unpaired *t* test).

670

671   **Figure 4:** NMDARE-A CSF depresses surface NMDAR presence. **A.** Fluorescence quenching

672   associated with surface NMDARs. N2a cells transfected with GluN1/GluN2B-SEP (super-

673   ecliptic pHluorin) were used to visualize NMDARs on the plasma membrane. Transient wash

674   with cell-impermeant MES quenched fluorescence in control cells. Scale bar 10 µm. **B.**

675   Comparison of MES quenching in control cells and cells incubated in NMDARE-A CSF for 24

676   hours. The absolute MES-induced change in fluorescence between cells treated with MS-A CSF

677   (*n* = 21 cells) vs. NMDARE-A CSF (*n* = 21 cells from 4 independent replicates) was

678   significantly different (t(40)=2.4, *p=0.02, Student's unpaired *t* test). **C-G.** Surface receptors

679   detected using anti-GFP labeling of fixed, non-permeabilized transfected N2a cells. Cells were

680   transfected with GluN1/GluN2B-SEP subunits or with cytosolic GFP as a control. Analysis lines

681   (yellow) through labeled cells in various conditions yielded evidence for surface receptors (red

682   anti-GFP antibody labeling) in GluN2B-SEP-transfected cells labeled with primary antibody but

683   not in other control conditions (Pri = primary antibody incubated). Significant membrane

684   labeling was detected in SEP-transfected cells labeled with primary antibody, compared with

685   other indicated conditions (Bonferroni corrected Student's unpaired *t* test, *p<0.05). For **G**

686   numbers on bars indicate pooled cell number from 4 experiments. Scale bar 10 µm. **H-J.** Anti-

687   GFP used to label surface NMDARs in transfected N2a cells incubated for 24 h in MS-A CSF or

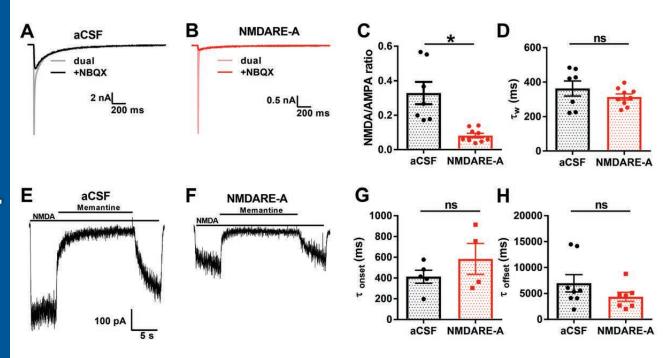688   in NMDARE-A CSF, prior to fixation. NMDARE-A CSF reduced anti-GFP membrane labeling

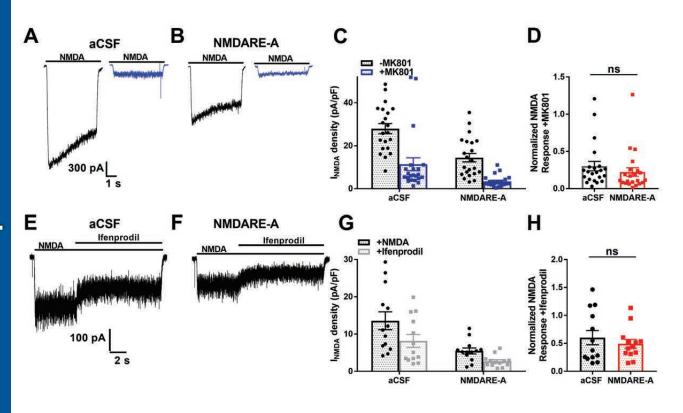689   (Bonferroni corrected Student's unpaired *t* test, *p<0.05).

31

690   **Figure 5.** Intervention by oxysterol-mimetic PAMs. **A** and **B**. Acute potentiation of NMDA-

691   elicited current by SGE-301 (2 µM, A) and by SGE-550 (2 µM, B). PAM was pre-applied for 30

692   s before co-application with NMDA (colored traces). **C.** Normalized current showed significant

693   potentiation by both SGE-301($t_{(27)}$=4.3, *p=2E-4) and SGE-550 ($t_{(63)}$=4.3, *p <1E-4, paired

694   Student's *t* tests). **D** and **E**. Summary of NMDA-elicited current density recorded following 48 h

695   of incubation with either aCSF or NMDARE-A CSF, with or without SGE-301 (D) or SGE-550

696   (E) treatment at 24 h. Two-way ANOVA with Bonferroni corrected post hoc *t* tests indicated

697   significant potentiation in aCSF and CSF conditions for both SGE-301 and SGE-550 (**p*<0.05).

698   There was no statistical interaction between CSF condition and either SGE-301 (D: $F_{(1, 56)}$ =

699   0.5132, p=0.48) or SGE-550 (E: $F_{(1, 125)}$ = 2.121, p=0.15) treatment. Currents were obtained in

700   recording saline, following removal of CSF and PAM in the culture medium.

701

702   **Figure 6.** Tests of residual PAM activity to explain persisting potentiation. **A** and **B**. Memantine

703   test of NMDAR channel open probability following 24 h aCSF incubation (A) or incubation with

704   SGE-301 (B). **C** and **D**. Summary of memantine onset and offset time constant values, obtained

705   from exponential fits; C onset: $t_{(13)}$=3.1, *p=1.6E-3; C offset $t_{(25)}$=3.9, *p=6E-4; D

706   onset:$t_{(12)}$=2.8, *p=1.6E-2; D offset: $t_{(12)}$=3.9, *p=2E-3, Student's unpaired *t* tests. **E.** Sample

707   traces from cultures incubated under control or in SGE-301 (2 µM) for 24 h then rinsed with

708   saline or γ-CDX (500 µM) for 2 min before recording. **F** and **G**. Summary of effect of brief CDX

709   incubation on residual SGE-301 (F) and SGE-550 (G). A two-way ANOVA showed a significant

710   main effect of SGE-301 ($F_{(13,39)}$ = 11.35, *p < 1E-4). Post hoc testing revealed significant

711   potentiation by SGE-301 and SGE-550 and significant reversal of potentiation by CDX

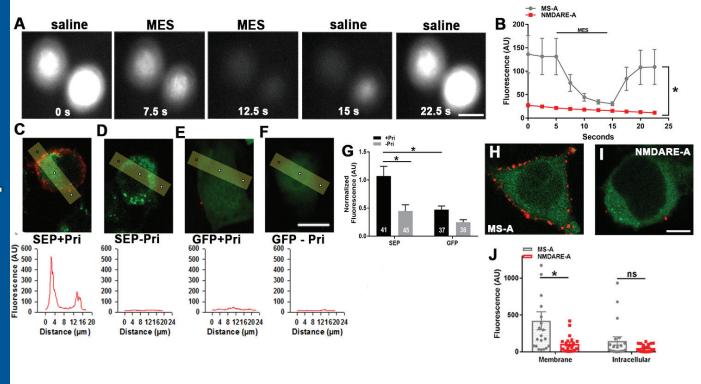712   (*p<0.05, Bonferroni corrected Student's unpaired *t* tests).

713   **Figure 7:** No effect of SGE-301 intervention on surface NMDAR presence. **A-D.** Examples of

714   GluN1/GluN2B-SEP-transfected N2a cells incubated under the indicated conditions. Cells were

715   incubated in CSF for 48 h and incubation with SGE-301 commenced 24 h following CSF

716   initiation. Anti-GFP labeling (red) in non-permeabilized cells was used to quantify surface

717   NMDARs. Green labeling represents SEP fluorescence following fixation. **E** and **F.** Two-way

718   ANOVA with Bonferroni corrected post hoc *t* tests indicated significant depression of surface

719   labeling after incubation in NMDARE-A CSF (E: t(38)=3.0, *p=4.8E-3). There was no statistical

720   interaction between SGE-301 and CSF condition (E: F(1,76)=0.75, p=0.39). SGE-301 did not

721   affect membrane (E) or intracellular anti-GFP labeling (F) in post hoc testing (p > 0.05,

722   Bonferroni corrected *t* tests).

723

724   **Figure 8.** Effect of SGE-301 intervention on depression induced by CSF from a second

725   NMDARE patient. **A-C.** Staining performed as in Figure 1A,B for 2 additional patient samples

726   and a control sample. **D.** Sample traces representative of the indicated conditions. NMDARE-B

727   CSF was incubated for 48 h at 1:12 dilution. Intervention with 2 μM SGE-301 commenced at 24

728   h. **E.** Summary of effect of incubation with NMDARE-B CSF and intervention with SGE-301. A

729   two-way ANOVA showed a significant main effect of SGE-301 (F(1,59)=11.9, *p=1.0E-3)

730   (asterisk associated with legend) but no interaction with CSF incubation condition

731   (F(1,59)=1.02, p=0.32). Post hoc testing revealed significant depression of NMDA-mediated

732   current induced by NMDARE-B CSF compared with the aCSF condition (t(29)=2.38, *p=.02,

733   Student's unpaired *t* test). **F.** Same protocol and statistics as depicted in D and E applied to

734   patient NMDAR-C. A two-way ANOVA showed a significant main effect of SGE-

735   301(F(1,48)=9.71, *p=3.1E-3) but no interaction with CSF incubation condition (F(1,48)=0.26,
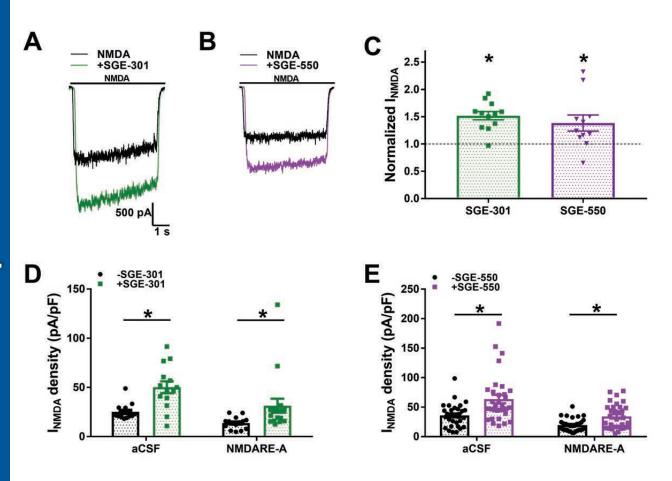
736   p=0.61). Post hoc testing revealed significant depression of NMDA-mediated current induced by

737   NMDARE-B CSF compared with the aCSF condition (t(24)=2.99, *p=6.4E-3, Student's

738   unpaired *t* test). One data point in the aCSF+SGE-301 condition >120 pA/pF is not shown on the

739   graph for clarity but was used in statistics.

740

741

A aCSF
dual
+NBQX
2 nA
200 ms

B NMDARE-A
dual
+NBQX
0.5 nA
200 ms

C
NMDA/AMPA ratio
0.6
0.4
0.2
0.0
*
aCSF   NMDARE-A

D
τ_w (ms)
600
400
200
0
ns
aCSF   NMDARE-A

E aCSF
NMDA   Memantine
100 pA
5 s

F NMDARE-A
NMDA   Memantine

G
τ onset (ms)
1000
800
600
400
200
0
ns
aCSF   NMDARE-A

H
τ offset (ms)
20000
15000
10000
5000
0
ns
aCSF   NMDARE-A

A MS-A

B MS-A+SGE-301

C NMDARE-A

D NMDARE-A+SGE-301

E

-SGE-301
+SGE-301

Membrane

*

ns

ns

Fluorescence (AU)

1000
800
600
400
200
0

MS-A   NMDARE-A

F

-SGE-301
+SGE-301

Intracellular

ns

ns

Fluorescence (AU)

1000
800
600
400
200
0

MS-A   NMDARE-A

A NMDARE-B **B** NMDARE-C **C** Control

D

aCSF
NMDA

aCSF
+SGE-301
NMDA

NMDARE-B
NMDA

NMDARE-B
+SGE-301
NMDA

200 pA | ___
2s

E

Current density (pA/pF)

□ -SGE-301
□ +SGE-301 ✳

80
60
40
20
0

aCSF   NMDARE-B

F

Current density (pA/pF)

□ -SGE-301
□ +SGE-301 ✳

120
80
40
0

aCSF   NMDARE-C