The Negative Allosteric Modulator EU1794-4 Reduces Single-Channel Conductance and Ca\textsuperscript{2+} Permeability of GluN1/GluN2A N-Methyl-D-Aspartate Receptors

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ABSTRACT

NMDA receptors are ligand-gated ion channels that mediate a slow, Ca\textsuperscript{2+}-permeable component of excitatory synaptic currents. These receptors are involved in several important brain functions, including learning and memory, and have also been implicated in neuropathological conditions and acute central nervous system injury, which has driven therapeutic interest in their modulation. The EU1794 series of positive and negative allosteric modulators of NMDA receptors has structural determinants of action near the preM1 helix that is involved in channel gating. Here, we describe the effects of the negative allosteric modulator EU1794-4 on GluN1/GluN2A channels studied in excised outside-out patches. Coapplication of EU1794-4 with a maximally effective concentration of glutamate and glycine increases the fraction of time the channel is open by nearly 1.5-fold, yet reduces single-channel conductance by increasing access of the channel to several subconductance levels, which has the net overall effect of reducing the macroscopic current. The lack of voltage-dependence of negative modulation suggests this is unrelated to a channel block mechanism. As seen with other NMDA receptor modulators that reduce channel conductance, EU1794-4 also reduces the Ca\textsuperscript{2+} permeability relative to monovalent cations of GluN1/GluN2A receptors. We conclude that EU1794-4 is a prototype for a new class of NMDA receptor negative allosteric modulators that reduce both the overall current that flows after receptor activation and the flux of Ca\textsuperscript{2+} ion relative to monovalent cations.

SIGNIFICANCE STATEMENT

NMDA receptors are implicated in many neurological conditions but are challenging to target given their ubiquitous expression. Several newly identified properties of the negative allosteric modulator EU1794-4, including reducing Ca\textsuperscript{2+} flux through NMDA receptors and attenuating channel conductance, explain why this modulator reduces but does not eliminate NMDA receptor function. A modulator with these properties could have therapeutic advantages for indications in which attenuation of NMDA receptor function is beneficial, such as neurodegenerative disease and acute injury.

Introduction

Neuronal death that is associated with cerebral ischemia and traumatic brain injury can produce profound disabilities with long term consequences for families and caregivers. Despite decades of work in both industry and academia, there are no approved therapies for treatment of ischemic stroke other than the dissolution or removal of the vessel clot in a fraction of patients (Awad et al., 2020; Turner et al., 2020). Likewise, there are no approved therapies that can reduce neuronal death after traumatic brain injury (Crupi et al., 2020).

N-methyl-d-aspartate receptors (NMDARs) belong to the ionotropic glutamate receptor family and are heteromeric assemblies of GluN1 and GluN2 subunits, of which there are four subtypes, GluN2A-D (Traynelis et al., 2010; Paoletti et al., 2013). NMDARs are activated by synaptically released

ABBREVIATIONS: ACRIT, critical amplitude; EU1794-4, ethyl 2-[2-(2-imino-4-oxothiazolidin-5-yl)acetamido]-4,5,6,7-tetrahydrobenzo[b]thiophene-3-carboxylate; AMPA, \(\alpha\)-amino-3-hydroxy-5-methyl-4-isoxazole-propionate; GluN, NMDAR subunits; NAM, negative allosteric modulator; NMDA, N-methyl-d-aspartate; NMDAR, NMDA receptor; TBI, Traumatic Brain Injury.
glutamate and mediate a slow component of excitatory synaptic transmission throughout the brain. Under resting conditions, NMDARs are strongly blocked by extracellular Mg$^{2+}$ ions, and this block can be relieved when a neuron is moderately depolarized, typically by $\alpha$-amino-3-hydroxy-5-methyl-4-isoxazole-propionate (AMPA) receptor activation, allowing the influx of Na$^+$ and Ca$^{2+}$ ions through the NMDAR. During acute injury, extracellular glutamate levels are increased by multiple mechanisms in animal models (Benveniste et al., 1984; Nilsson et al., 1990) and humans (Bondoli et al., 1981; Persson and Hillered, 1992; Baker et al., 1993; Yamamoto et al., 1984; Nilsson et al., 1990) and humans (Bondoli et al., 1981; Beal, 1992; Bullock et al., 1998). This leads to persistent activation of NMDARs, excessive entry of cations, including Ca$^{2+}$, into neurons, and subsequent neurotoxicity (Lai et al., 2014). Preclinical studies have implicated over-activation of NMDARs as a contributing factor to neuronal death associated with acute central nervous system injury (Olney, 1969; Choi et al., 1988; Wroge et al., 2012) and demonstrated that inhibition of NMDAR function is neuroprotective in dozens of animal models (summarized in Table S2 of (Yuan et al., 2015)).

Despite strong rationale and preclinical data, multiple clinical trials evaluating treatment with NMDAR antagonists of TBI (Yurkewicz et al., 2005) and ischemic stroke (Grotto et al., 1995; Davis et al., 1997; Lees, 1997; Morris et al., 1999; Albers et al., 2001; Farin and Marshall, 2004; Muir, 2006; Warach et al., 2006) have been unsuccessful. Some of the potential reasons underlying these failures include patient heterogeneity, delays in administering treatment, and dose lowering to eliminate on-target side effects associated with strong, nonselective block of NMDARs (Morris et al., 1999; Albers et al., 2001; Sacco et al., 2001; Gladstone et al., 2002; Farin and Marshall, 2004; Muir, 2006).

Although advances in clinical trial design and new endo-vascular procedures have improved treatment prospects for ischemic stroke (Saver, 2013; Patel et al., 2020), neuroprotective strategies still offer the potential to spare neurons and improve patient outcome if they can be administered with minimal side effects. The complications associated with complete block of all NMDARs arise from ubiquitous expression of NMDARs throughout the brain together with the important roles they serve (Paoletti et al., 2013; Lohmann and Kessels, 2014). The NMDAR antagonists and channel blockers tested in the clinic produce both strong block of NMDA receptors in all brain regions and, not surprisingly, significant adverse effects (Lees et al., 2000; Sacco et al., 2001; Diener et al., 2002; Rowland, 2005; Wood, 2005; Muir, 2006; Blagrove et al., 2009). One strategy to circumvent strong, nonselective block is to target NMDARs that contain specific GluN2 subunits, which are differentially expressed throughout the central nervous system (Monyer et al., 1994; Wyllie et al., 2013; Shipton and Paulsen, 2013). This strategy will reduce the risk of producing local block of NMDA receptors throughout the brain, which should reduce side effects, but may still produce strong block in some cell types if only one NMDAR subtype is present. This could also reduce therapeutic actions in some brain regions that lack the targeted GluN2 subunit. An alternative approach is to develop a compound that acts at all NMDARs to reduce but not eliminate NMDAR-mediated currents throughout the brain. Such a submaximal allosteric modulator might be neuroprotective by limiting Ca$^{2+}$ influx during overactivation of NMDARs without producing intolerable side effects that result from complete NMDAR block.

Here, we investigate ethyl 2-(2-(2-imino-4-oxothiazolidin-5-yl)acetamido)-4,5,6,7-tetrahydrobenzo[b]thiophene-3-carboxylate (EU1794-4), a negative allosteric modulator (NAM) of the NMDAR that contains an iminothiazolidinone ring connected to a thio-phone moiety through an acetamide linkage. EU1794-4 acts in a voltage-independent manner to produce submaximal inhibition at saturating concentrations at all NMDAR subtypes (Katzman et al., 2015; Perszyk et al., 2018). We show at the single-channel level that the mechanism of EU1794-4 modulation involves a reduction in channel conductance coupled with a reduction in Ca$^{2+}$ permeability (Perszyk et al., 2020). These two features should render a NAM neuroprotectant with potentially fewer undesirable on-target actions because of its incomplete inhibition of NMDARs. Thus, our results suggest that EU1794-4 represents a new class of NMDAR NAM with a unique mechanism and intriguing therapeutic possibilities.

### Materials and Methods

#### Molecular Biology and Reagents

The coding DNA (cDNAs) segments for rat wild-type NMDAR subunits GluN1-1a (hereafter GluN1, GenBank U11418, U08261), GluN2A (D12311), GluN2B (U11419), GluN2C (M91563), and GluN2D (L52611) were provided by Drs. S. Heinemann (Salk Institute), S. Nakanishi (Kyoto University), and P. Seeburg (University of Heidelberg), subcloned into the pcI-neo plasmid vector (GluN1, GluN2A, GluN2B) or the pRK plasmid vector (GluN2C, GluN2D). In some experiments, we used a pcI-neo plasmid harboring GFP between the promoter and open reading frame of GluN1 with three start codons (ATGs) inserted between GFP and GluN1 (Yi et al., 2018).

EU1794-4 was synthesized at Emory University as previously described (>95% purity, Katzman et al. (2015)) and was purchased from Millipore Sigma (SML2412; Burlington, MA) or Life Chemicals (F1065-0436; Niagara-on-the-Lake, ON, Canada). All other drugs were purchased from Tocris (Bristol, UK) or Millipore Sigma (Burlington, MA).

#### Two-Electrode Voltage-Clamp Recordings in Xenopus Oocytes

Coding RNA was transcribed in vitro from plasmids containing NMDAR cDNAs according to manufacturer’s instructions (Message mMachine, Ambion; Thermofisher Scientific, Waltham, MA). Xenopus laevis stage VI oocytes (Ecocyte Biosciences) were isolated as previously described (Hansen et al., 2013), injected with 5–10 ng coding RNA (GluN1:GluN2 ratio 1:2), and stored at 15°C in media containing (in millimolar) 88 NaCl, 2.4 NaHCO$_3$, 1 KCl, 0.33 Ca(NO$_3$_)$_2$, 0.41 CaCl$_2$, 0.82 MgSO$_4$, 5 HEPES, 1U/ml penicillin, 0.1 mg/ml gentamicin sulfate, and 1 µg/ml streptomycin (pH 7.4, adjusted with NaOH). Between 2 and 7 days after injection, two-electrode voltage-clamp recordings were performed at room temperature (23°C) in extracellular solution containing (in mM) 90 NaCl, 1 KCl, 10 HEPES, 0.5 BaCl$_2$, and 0.01 EDTA (pH 7.4, adjusted with NaOH). NMDAR current responses from oocytes were recorded at a holding potential of −40 mV; only oocyte recordings with a maximal response amplitude greater than 50 nA were analyzed. Concentration-response curves for EU1794 compounds were generated by applying glutamate and glycine at the concentrations stated in figure legends or text, followed by variable concentrations of test compound, prepared as 20 mM stock solutions in DMSO and diluted to the final concentration in recording solution. DMSO content was 0.05%−0.5% (v/v).

Concentration-response data were analyzed using OriginPro 9.0. For inhibition concentration-response curves, the response evoked by maximally effective concentration of glutamate and glycine in the

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presence of test compounds was given as a percentage of the initial response to glutamate and glycine alone. Data for individual cells were fitted with the Hill equation:

$$\text{Response}(\%) = \left( \frac{100 - \text{minimum}}{1 + (\text{[inhibitor]}/[IC_{50}])^N} \right) + \text{minimum},$$

where minimum is the residual response in saturating concentrations of the negative allosteric modulator, $IC_{50}$ is the concentration that produces a half-maximal inhibition, $[\text{inhibitor}]$ is the concentration of negative allosteric modulator, and $N$ is the Hill slope. For the graphical representation of the results, the data were normalized to the current response to agonist alone, averaged across all cells, and fitted with the Hill equation. $IC_{50}$ values are given as the mean ± S.E.M.

**Tissue Culture.** HEK-293 cells (American Type Culture Collection, ATCC 1573, hereafter HEK cells) were prepared as previously described (Perszik et al., 2018) and maintained in Dulbecco’s modified Eagle’s medium (DMEM, 10566016; ThermoFisher Scientific) supplemented with 10% FBS, 10 U/ml penicillin, and 10 μg/ml streptomycin at 5% CO₂ in a 37°C incubator. At 48 hours prior to recording, the HEK cells were plated onto 50 μg/ml poly(lysine)-coated 5-mm glass coverslips (Warner Instruments) placed in a 24-well plate. The calcium phosphate transfection method (Hansen et al., 2013) was used to transiently transfect these cells 24 hours after plating with GluN1 and GluN2A cDNA at a 1:1 ratio for a total of 200 ng of DNA per well. To decrease the cytotoxic effect of NMDAR expression in cultured HEK cells, the competitive antagonists DL-APV (20 μM; Tocris) and 7-chlorokynurenic acid (20 μM, catalog number 023710; Tocris) were added to the culture medium.

**Single-Channel Recordings.** Single-channel recordings were made from outside-out patches excised from HEK-293 cells 18–24 hours after transfection. All recordings were performed at room temperature (23°C). Thick-walled borosilicate micropipettes (outer diameter 1.5 mm, inner diameter 0.86 mm; Warner Instruments) were pulled and fire-polished to a resistance of 9–14 MΩ, coated with heat-cured SYLGARD 184 (World Precision Instruments), and used to obtain outside-out patches from cells with low NMDAR expression, estimated by the level of GFP intensity. Pipettes were filled with the same internal solution described below for whole-cell recordings. The extracellular recording solution consisted of (in millimolar) 150 NaCl, 10 HEPES, 3 KCl, 0.5 CaCl₂, 0.01 EDTA, and 30 mM-mannitol (pH 7.4). 1 mM glutamate and 0.1 mM glycine were used to activate the NMDARs. Excised outside-out patches were voltage-clamped at a holding potential of ~80 mV, filtered at 8 kHz (8-pole Bessel filter, 3 dB), digitized at 40 kHz, and analyzed offline.

**Single-Channel Analysis.** Recordings were digitally filtered at 2–4 kHz (~3 dB) and idealized by time course fitting, which involved fitting a filtered step response function to each transition [SCAN, David Colquhoun, University College London; Colquhoun and Sigworth (1983)]. To determine the amplitude of the channel conductance levels, only the amplitudes of events with a duration longer than 2.5 filter rise times (Colquhoun and Sigworth, 1983) were analyzed to ensure that the unitary current reflects the fully open channel. Pairs of control and drug-treated recordings from the same patch were processed identically. All-points histograms were fitted by the sum of three or four Gaussian components using least-squares fitting; the S.D. for all components were free to vary. Opening amplitude determined by time course fitting were fitted by the sum of two or three Gaussian components using an expectation-maximization algorithm; the S.D. for all components were constrained to be equal. All resolvable openings were used to estimate the parameters of the Gaussian mixture distribution. From the estimated parameters of the Gaussian mixture distribution, each opening greater than 2.5 filter rise times was classified to one of the components of the Gaussian mixture distribution. To minimize the total number of misclassified openings while keeping the number of misclassified openings equal, Newton’s method regression (root finding) was used to find the critical amplitude ($A_{\text{crit}}$) for a pair of Gaussians. This method for calculating $A_{\text{crit}}$ yielded similar results to minimization of the total number of events. Direct transitions between openings were defined as an opening longer than 2.5 filter rise times (Colquhoun and Sigworth, 1983) that transitioned directly to another open state with a nonzero amplitude that was also longer than 2.5 filter rise times. We only evaluated the first two transitions within every sequence of direct transitions and determined the percent of events for every possible direct transition between conductance levels. Open and closed time distributions were fitted by the sum of multiple exponential components using the maximum likelihood method (ChannelLab), with an imposed resolution of 50 μs. The amplitude of events briefer than the imposed resolution was replaced by the preceding amplitude (Colquhoun and Sigworth, 1983). Open times were defined as contiguous open periods to any conductance level. We also determined the dwell times for individual conductance levels classified by a critical amplitude calculated to minimize the equal number of misclassified events in each Gaussian component. The time a channel remained at an individual conductance was determined regardless of whether the preceding state or subsequent state were open or closed. These dwell times allow comparison of stability of the different conductance levels.

**Whole-Cell Current Recording.** For variance analysis, whole-cell voltage-clamp recordings were performed with thin-walled borosilicate glass electrodes (3 to 4 MΩ, TW150F-4; World Precision Instruments) filled with a solution containing (in millimolar) 110 Cs-glucuronate, 30 CsCl, 5 HEPES, 4 NaCl, 0.5 CaCl₂, 2 MgCl₂, 5 BAFTA (1,2-bis(o-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid), 2 NaATP, and 0.3 NaGTP (pH 7.35). The extracellular recording solution contained (in millimolar) 150 NaCl, 10 HEPES, 3 KCl, 1.0 CaCl₂, and 0.01 EDTA (pH 7.4). Cells were held under voltage-clamp at ~60 mV (not corrected for the calculated junction potential of +12 mV) at 23°C. Currents were recorded using an Axopatch 200B (Molecular Devices), filtered at 2 kHz (~3 dB), and digitized at 5 kHz using pClamp 10.

Solution exchange was performed by lifting an individual HEK cell from the coverslip. The solution flowing into the bath provided a slow exposure and washout of agonist. Variance analysis was performed on current responses to slow application of 10 μM glutamate plus 30 μM glycine supplemented with vehicle or positive modulator at a holding potential of ~60 mV for GluN1/GluN2A and GluN1/GluN2B. For GluN1/GluN2C and GluN1/GluN2D, we applied 1 μM glutamate plus 10 μM glycine. Agonist exchange proceeded slowly over 20–60 seconds and, for some cells, only the rising and falling phases of the current response were analyzed, depending on whether artifacts were present. The current was divided into 100 equal time increments, the mean current as well as the variance of the current were calculated for each time period, and the variance of the pregastion baseline was subtracted. The plot of variance versus current was fitted by Eq 1

$$\text{Variance} = \mu - I^2 / \left( N \mu \right),$$

where $\mu$ is the weighted mean unitary current, $I$ is the macroscopic current amplitude for each of the 100 segments, and $N$ is the number of channels. The weighted mean unitary current (Traynelis and Jaramillo, 1998) is related to the unitary currents $i_j$ for multiple $j$ subconductance levels each with a relative probability of $p_j$, by Eq 2:

$$\text{Weighted mean unitary current } \mu = \sum_i \frac{\xi_i}{p_i} \xi_i / \sum_i \xi_i p_i.$$
a range of positive and negative holding potentials in a set of solutions to determine the ratio of permeability of the channel for Ca\(^{2+}\) to the permeability for monovalent ions [see Jatzke et al. (2002)]. The monovalent ion concentration was constant, and the external Ca\(^{2+}\) concentration varied (0.1 or 10 mM), the base solution being (in millimolar) 150 NaCl, 3 KCl, and 10 HEPES, adjusted to pH 7.2 with NaOH and a final osmolality of 290–300 mOsm. The pipette was filled with a KCl-based solution, which consists of (in millimolar) 140 KCl, 10 HEPES, and 10 BAPTA, adjusted to pH 7.2 with KOH and a final osmolality of 280–290 mOsm. We assume that the relative permeabilities of monovalent ions (K\(^{+}\), Na\(^{+}\), Ca\(^{2+}\)) are the same (Burnashev et al., 1995; Schneggenburger and Ascher, 1997; Jatzke et al., 2002). The current-voltage curve was established using the same protocol as before, and the reversal potential was determined from a fourth-order polynomial function fitted to the I-V curve. The average GluN1/GluN2A current response amplitude used was 1200 pA with an average leak of 180 pA; leak current was typically increased in test solutions that lacked divalent ions. The reversal potentials were used to determine the ratio of the permeability of the channel to Ca\(^{2+}\) and to monovalent ions (eqs. 3 and 4) from modified versions of the Lewis equation (Lewis, 1979):

\[
V_{\text{rev,Ca}} - V_{\text{rev,Na}} = \Delta V_{\text{rev}} = \frac{RT}{F} \ln \left( 1 + \frac{4 \left( \frac{\Delta V_{\text{rev}}}{RT} \right) \left( \frac{\Delta V_{\text{rev}}}{RT} \right)}{\left( \frac{\Delta V_{\text{rev}}}{RT} \right) + 1 + \exp \left( \frac{\Delta V_{\text{rev}}}{RT} \right) \frac{\left( \frac{\Delta V_{\text{rev}}}{RT} \right)}{4 \left( \frac{\Delta V_{\text{rev}}}{RT} \right)} \right) \tag{3}
\]

\[
P_{\text{Ca}} = \frac{\exp \left( \Delta V_{\text{rev,Na}} - 1 \right) \left( \frac{\Delta V_{\text{rev,Na}}}{RT} \right) + 1 + \exp \left( \frac{\Delta V_{\text{rev,Na}}}{RT} \right) \frac{\left( \frac{\Delta V_{\text{rev,Na}}}{RT} \right)}{4 \left( \frac{\Delta V_{\text{rev,Na}}}{RT} \right)}}{P_{\text{Na}} \exp \left( \Delta V_{\text{rev,Na}} - 1 \right) \left( \frac{\Delta V_{\text{rev,Na}}}{RT} \right) + 1 + \exp \left( \frac{\Delta V_{\text{rev,Na}}}{RT} \right) \frac{\left( \frac{\Delta V_{\text{rev,Na}}}{RT} \right)}{4 \left( \frac{\Delta V_{\text{rev,Na}}}{RT} \right)}} \tag{4}
\]

Results

EU1794-4, (compound 5 in Katzman et al., 2015), is a negative allosteric modulator of NMDA receptors. It inhibits all GluN1/GluN2 combinations with IC\(_{50}\) values ranging from 0.28 to 3.1 μM (Fig. 1). EU1794-4 has a number of intriguing properties, including incomplete inhibition at saturating concentrations (with residual responses ranging from 27% to 55% of control, Fig. 1) and the ability to enhance responses to low agonist concentration through an allosteric interaction that increases agonist potency (Perszyk et al., 2018). NMDA receptor inhibition by this series, which is uncharged at physiologic pH (Fig. 1), is voltage-independent and can have neuroprotective effects (Katzman et al., 2015). Moreover, structural determinants of action involve the preM1 helix,
arguing against a channel blocking mechanism (Katzman et al., 2015; Perszyk et al., 2018).

To gain further insight into the mechanism of action, we recorded single unitary currents from GluN1/GluN2A channels present in outside-out patches excised from transiently transfected HEK cells (Fig. 2). Channel activity was recorded in response to maximally effective concentrations of glutamate and glycine (1, 0.1 mM) supplemented with vehicle (0.15% DMSO) or 30 μM EU1794-4 in vehicle. All recordings were performed at a holding potential of −280 mV. As expected, in 0.5 mM extracellular calcium (Premkumar et al., 1997) that was Zn2+- and Mg 2+-free (10 μM EDTA), single-channel

Fig. 2. Single-channel recording from a HEK-293 cell expressing GluN1/GluN2A. (A) Representative unitary currents (filtered at 1 kHz for display) recorded from an outside-out patch expressing GluN1/GluN2A (A1) in control (top) or in the presence of EU1794-4 (bottom). A portion, illustrated by the box in A1, of the same recording on an expanded time scale are shown in (A2). (B) All-point response amplitude histograms of the patch recording in (A) (of the single-channel range) reveals lower conductance levels in the presence of EU1794-4, including a brief state that overlaps with the baseline patch noise. The control histogram was fitted by three Gaussian distributions and the EU1794-4 histogram was fitted by four Gaussian distributions.

TABLE 1
EU1794-4 decrease GluN1/GluN2A current amplitudes in all-point histograms.

<table>
<thead>
<tr>
<th>Open State 1</th>
<th>Control</th>
<th>EU1794-4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Closed State</td>
<td>Control</td>
<td>EU1794-4</td>
</tr>
<tr>
<td>Closed Amplitude (pA)</td>
<td>Closed SD (pA)</td>
<td>Closed Area (%)</td>
</tr>
<tr>
<td>Closed State</td>
<td>EU1794-4</td>
<td>EU1794-4</td>
</tr>
<tr>
<td>Open Amplitude (pA)</td>
<td>Open SD (pA)</td>
<td>Open Area (%)</td>
</tr>
</tbody>
</table>

All point histograms were constructed from the digitized data of all portions of the recording that were analyzed. These distributions were fitted by the sum of 3 or 4 Gaussian components, one of which was 0 (the closed state), and the fitted parameters averaged for 5 patches. In some cases there were multiple active channels, the fitting parameters were constrained to the range of a single channel. The relative fold-reduction of the all-points sum of the entire data record (normalized to record length) is a reflection of charge transfer in the patch, and was reduced in the presence of EU1794-4 to 43 ± 9%, consistent with the reduction in macroscopic current amplitude.
analysis revealed that GluN1/GluN2A NMDARs primarily opened to one large conductance with occasional sojourns to a lower conductance level (Fig. 2). Coapplication of 30 μM EU1794-4 with saturating coagonist visibly shifted the relative conductance levels to which GluN1/GluN2A receptors open, with lower conductances becoming much more prominent than observed in glutamate and glycine alone (Fig. 2B). An all-point histogram for a representative patch shows that the relative time spent in two open states with distinct channel amplitudes in control (5.16 pA, 64%; 3.76 pA, 36%) shifts to three different open channel amplitudes in the presence of EU1794-4 (5.24 pA, 10%; 3.36 pA, 27%; 0.84 pA, 62%; Fig. 2, A2 and B; Table 1). Summation of the all-points data (normalized to record length) shows a reduction in total charge transfer in the presence of EU1794-4 (43% ± 9%), despite the channel spending more time open (i.e., less time closed) in the presence of EU1794-4. Despite the overall reduction in charge transfer, the percentage of time in the closed state was reduced in the presence of EU1794-4 (control 78% ± 8%, EU1794-4 49% ± 7%). This reduction in current passed closely matches the 64% reduction produced by 30 μM EU1794-4 (to 36% of control) in the macroscopic current response of GluN1/GluN2A (Fig. 1). Openings to the smallest conductance level in the presence of EU1794-4 (0.84 pA) were brief and flickery, and often difficult to distinguish from the baseline noise.

We idealized these channel records using the time course fitting method to determine the amplitude and duration of each transition to an open state. We found that, under control conditions in 0.5 mM Ca2+, the receptor opened to a large conductance level (85 ± 9%). This analysis suggested that the largest conductance level is destabilized (Table 4; mean dwell time; control 0.62; EU1794-4, 0.45; P = 0.0036 paired t test), whereas the middle conductance level is more stable (mean dwell time; control 0.32; EU1794-4, 0.51; P = 0.039 paired t test). The lowest conductance level had the briefest mean dwell time of the three conductance levels observed in EU1794-4 (Table 4).

By contrast, no clear change was observed in the briefest channel closed times, which are intraburst closed times that likely reflect duration required to retroactively pregating states after channel closure [Fig. 3, Table 3; Gibb et al. (2018)]. The proportion of closures for the briefest components did not change (τ1, ∼0.04 millisecond, control 33%, EU1794-4 33%), as well as the next briefest closed time (τ2, ∼0.5 millisecond, control 31%, EU1794-4 33%). In contrast, the longest fitted closed durations were altered in EU1794-4 (τ4, control 24.1 millisecond, EU1794-4 85.2 millisecond; τ5, control 1970 millisecond, EU1794-4 973 millisecond). Given the presence of multiple channels in the patches, time constants describing these longer components cannot be interpreted mechanistically and reflect overall channel opening frequency.

Direct transitions between different conductance levels are often taken as evidence that the different conductance levels arise from the same receptor. We used the ACRIT values determined from the fitted amplitude histogram (Table 1) to determine when two contiguous open periods represented a direct transition (Fig. 4). Direct transitions were observed in the presence of EU1794-4 between the large 60 pS conductance state and the 47 pS state (Fig. 4, B and C) and between the 47 pS state and the 23 pS state (Fig. 4, B and C). Direct transitions were observed in the presence of EU1794-4 between the large 60 pS conductance state and the 47 pS state (Fig. 4, B and C) and between the 47 pS state and the 23 pS state (Fig. 4, B and C).
transition between the largest (60 pS) and smallest (23 pS) state were detected, but were rare, perhaps because of the difficulty in idealizing openings to the lowest amplitude because of their brief lifetime. Under control conditions, at least 4.2% ± 1.5% of the transitions after initial channel opening were a direct transition to another conductance level. In the presence of EU1794-4, the direct transitions between two conductance levels increased to at least 8.0% ± 0.67% of all transitions to the open state. We evaluated these direct transitions between the different levels to better understand the relationship between the conductance levels observed for the EU1794-4–bound channel. There was no detectable asymmetry in transitions, as we found equal proportions of transitions from high to medium and medium to high conductance levels in both control and EU1794-4 (Fig. 4C, P = 0.051). Similarly, we found equal proportions of transitions from medium to low and low to medium levels.

### Table 3

<table>
<thead>
<tr>
<th>State</th>
<th>Condition</th>
<th>ms (area, %)</th>
<th>ms (area, %)</th>
<th>ms (area, %)</th>
<th>ms (area, %)</th>
<th>ms (area, %)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Open</td>
<td>Control</td>
<td>0.080 ± 0.013 (8.3 ± 3.1)</td>
<td>3.03 ± 0.61 (92 ± 3)</td>
<td>1.27 ± 0.22 (66 ± 4)</td>
<td>3.73 ± 0.69 (24 ± 2)</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>EU1794-4</td>
<td>0.042 ± 0.013 (10 ± 3)</td>
<td>1.27 ± 0.22 (66 ± 4)</td>
<td>3.73 ± 0.69 (24 ± 2)</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Closed</td>
<td>Control</td>
<td>0.047 ± 0.010 (39 ± 11)</td>
<td>0.51 ± 0.07 (31 ± 6)</td>
<td>4.56 ± 1.37 (18 ± 3)</td>
<td>24.1 ± 7.3 (10 ± 3)</td>
<td>1970 ± 1090 (1.6 ± 0.4)</td>
</tr>
<tr>
<td></td>
<td>EU1794-4</td>
<td>0.055 ± 0.012 (33 ± 9)</td>
<td>0.56 ± 0.10 (33 ± 6)</td>
<td>6.33 ± 1.56 (25 ± 4)</td>
<td>85.2 ± 22.9 (7.3 ± 0.9)</td>
<td>973 ± 236 (1.8 ± 0.3)</td>
</tr>
</tbody>
</table>

**Fig. 3.** EU1794-4 alters several parameters of GluN1/GluN2A receptor channel gating. (A) Open state duration histograms for control (top) and EU1794-4 (bottom) fitted by the sum of two to three exponential components (individual components are shown with dotted lines). Open durations were determined as the duration of each open period regardless of opening conductance level. (B) Closed state duration histograms for control (top) and EU1794-4 (bottom) fitted by the sum of two to three exponential components (individual components are shown with dotted lines). The histogram are plotted using the square root (SQRT) of the counts.

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in EU1794-4. There was an apparent 2-fold imbalance favoring low to high over high to low transitions, however, the difficulty in idealizing the low conductance state due to its low amplitude and brief open time led to more variability between patches and uncertainty in this parameter. More data from low noise patches is needed to draw conclusions about any potential asymmetry involving the lowest conductance level.

To determine whether EU1794-4 could alter the single-channel conductance for other NMDAR subunit combinations, we used variance analysis of macroscopic current responses to estimate weighted mean unitary current in the absence and presence of drug for GluN1/GluN2A, GluN1/GluN2B, GluN1/GluN2C, and GluN1/GluN2D (Fig. 5; Table 5). We observed a reduction of weighted mean conductance in GluN1/GluN2A and GluN1/GluN2B with the application of EU1794-4. However, we could not detect a change in conductance for GluN1/GluN2C and GluN1/GluN2D receptors (Fig. 5; Table 5). We subsequently screened for changes to weighted mean conductance produced by several EU1794-4 analogs, all of which are negative allosteric modulators that included alterations to the thiophene and/or alkyl ester chain (Table 5). We found that adding a heteroatom to the fused ring system (O or S, EU1794-21 and EU1794-15, respectively) retained the ability to reduce channel conductance. EU1794-21 reduced conductance of GluN1/GluN2A, however, EU1794-15 was less efficacious in reducing conductance of GluN1/GluN2A than EU1794-4. Reducing the ester alkyl chain to a methyl, EU2100 retained the ability to reduce channel conductance in GluN1/GluN2A. In addition, analogs that contained a methyl substitution on the fused ring system (EU1794-2 and EU1794-19) or a difluoro substitution (EU1794-23) also retained the ability to reduce channel conductance of GluN1/GluN2A. However, not all analogs could reduce channel conductance. For example, compounds EU1969 and EU1794-1, which did not contain the fused ring structure, did not reduce channel conductance of GluN1/GluN2A. These results suggest a structure-activity relationship for the ability of EU1794 analogs to alter GluN1/GluN2A conductance that is distinct from their ability to reduce the current response. We also evaluated the actions of these analogs on GluN1/GluN2D NMDARs and could only detect reduced conductance for a subset of modulators, including EU2100, EU1794-2, EU1794-19, and EU1794-21. Although these data suggest that the EU1794 series of modulators are less efficacious at reducing conductance at GluN1/GluN2D, the smaller and briefer unitary currents will also decrease the signal-to-noise ratio for variance analysis, perhaps diminishing our ability to detect modest changes in conductance for some analogs.

Given that EU1794-4 can reduce single-channel conductance, we tested whether relative ionic permeability of the ion...
conducting pore might be altered by the modulator, similar to what has been observed for the Ca\textsuperscript{2+}/Na\textsuperscript{+} permeability ratio in response to EU1622-14, a positive allosteric modulator that also reduced channel conductance and had similar molecular determinants of action in the preM1 region (Perszyk et al., 2018, 2020). We assessed the relationship between current and voltage from whole-cell patch-clamp recordings in solutions containing different concentrations of Ca\textsuperscript{2+} to calculate the Ca\textsuperscript{2+} permeability relative to monovalent cations (Fig. 6, see Materials and Methods). EU1794-4 reduced the current response similarly at all voltages, consistent with its voltage-independent mechanism. We determined the reversal potential for different ionic solutions and from this calculated the relative permeability ratio for Ca\textsuperscript{2+} and monovalent cations of GluN1/GluN2A receptors, which was reduced from 3.4 to 2.0 in the presence of EU1794-4 (\(P = 0.021\), unpaired \(t\) test). Thus, in addition to reducing whole-cell current and single-channel conductance, the Ca\textsuperscript{2+} permeability relative to monovalent cations for the residual current is also reduced.

### Discussion

The most important finding of this study is that the negative allosteric modulator EU1794-4 reduces both single-channel conductance and the permeability of Ca\textsuperscript{2+} relative to monovalent ions. EU1794-4 promotes GluN1/GluN2A channel openings to lower sublevels, the lowest of which is exceptionally brief (Fig. 1). This effect is, in part, similar to that described for the positive allosteric modulator EU1622-14, which robustly enhances channel open probability but also reduces single-channel conductance. Interestingly, EU1794-4 appears to destabilize the open states of the channel, yet still paradoxically spends more time in an open state. This dichotomy in which a negative allosteric modulator can reduce charge transfer yet increase time in the open state is unique and has not been described before for ligand-gated ion channels. The two series of modulators that alter conductance (EU1794, EU1622) share structural determinants of action that include the preM1 linker (Strong et al., 2020), suggesting that the properties that promote sublevels may be specific to this binding pocket within the receptor complex. However, not all modulators with structural determinants near preM1 linker helices of both GluN1 and GluN2 subunits, which are outside of the pore (Perszyk et al., 2018, 2020), suggesting that the properties that promote sublevels may be specific to this binding pocket within the receptor complex.

### Table 4

EU1794-4 stabilizes the intermediate conductance level of GluN1/GluN2A channels

Dwell times were calculated using amplitude histogram ACRIT values to subdivide openings for each conductance level for each channel recording. Data are mean \(\pm\) S.E.M. (\(N = 7 \text{ and } 8\) patches). Dwell times were determined for a specific sublevel, regardless of whether the channel was open or closed in the preceding or following time period. For comparison, the mean open time is given, which was determined from the sum of time spent open for all contiguous open periods.

<table>
<thead>
<tr>
<th>Conductance Level</th>
<th>Mean Dwell Time (ms)</th>
<th>Open Time (ms)</th>
</tr>
</thead>
<tbody>
<tr>
<td>~60 pS level</td>
<td>Control: 0.62 ± 0.12</td>
<td>EU1794-4: 0.45 ± 0.09</td>
</tr>
<tr>
<td>~45 pS level</td>
<td>Control: 0.32 ± 0.04</td>
<td>EU1794-4: 0.51 ± 0.08</td>
</tr>
<tr>
<td>~23 pS level</td>
<td>Control: 0.43 ± 0.09</td>
<td>EU1794-4: 0.32 ± 0.08</td>
</tr>
<tr>
<td>All levels</td>
<td>Control: 2.44 ± 0.25</td>
<td>EU1794-4: 1.92 ± 0.30</td>
</tr>
</tbody>
</table>

**Fig. 4.** Direct transitions between EU1794-4 conductance states are primarily between adjacent levels. (A) The amplitude histograms from time course fitting from the same patch as shown in Fig. 1 are given for control (left) and EU1794-4 (right), fitted by the sum of two to three Gaussian components (individual components are shown with dotted lines). (B) Examples of direct transitions between the 60 and 47 pS states (top) and between the 47 and 23 pS states (bottom) are shown. (C) A scatterplot of the contiguous openings with direct transitions between the channel sublevels (only durations greater than 2.5 filter rise times were included) for the patch shown in Fig. 2 (control, left; EU1794-4, right). For each transition, the initial amplitude is on the x-axis and the final amplitude on the y-axis. The ACRIT values (broken lines) were calculated from the amplitude histogram fitting in (A). The relative proportion of all direct transitions (determined by ACRIT) is given for all possible transitions between sublevels in all patches. When multiple direct transitions between subconductance levels occurred, only the first two were analyzed.
We also explored the degree to which this effect on conductance was shared at NMDA receptors with other subunit combinations. Whereas most EU1794 analogs reduced weighted mean unitary conductance at GluN2A, they usually did not produce a detectable effect on GluN2D unitary conductance. It is not clear whether there was a modest effect that was beneath the threshold for detection with variance analysis, or whether this reflected a lack of effect at GluN2D receptors. By contrast, the EU1622 series reduced conductance at all diheteromeric NMDARs. Thus, there appears to be a unique set of structural requirements for modulators that can alter the channel conductance and calcium permeation at all NMDARs that has yet to be fully delineated.

The mechanism of this conductance modulation by EU1794-4 remains unknown, although a few possibilities seem plausible. All potential mechanisms postulate that the conducting states produced by the modulator possess altered pore geometries or alterations in their conducting paths due to modulator binding. Subtle changes in the pore can have profound effects on Ca$^{2+}$ permeability, as observed with human mutations in the pore-forming region and other structure-activity studies (Amin et al., 2018; Li et al., 2019). One possibility that might cause these effects is that the residues that coordinate Ca$^{2+}$ ions [DRPEER, Watanabe et al. (2002)] could be positionally altered, and thus have a different affinity for the divalent ion. We know from previous reports that the presence of Ca$^{2+}$ promotes different conductance levels (Premkumar and Auerbach, 1996; Wyllie et al., 1996; Premkumar et al., 1997). A related possibility is that ions pass through the access ports near the linker regions at the modulator binding site en route to the pore, and the presence of modulator alters the nature of the access ports to limit Ca$^{2+}$ entry to the channel (Wollmuth, 2018). This phenomenon might also reflect conversion of a previously nonconducting state to a conducting state when EU1794-4 binds, such as a nonconducting desensitized state is modified to possess incomplete pore closure while drug is bound. The subconductance states of a mutant NMDA receptor have been shown to have different cation permeabilities (Schneggenburger and Ascher, 1997), as do wild-type and mutant K$^+$-channels (Zheng and Sigworth, 1997, 1998), raising the possibility that the mechanism for EU1794-4 reduction of calcium permeability could be the result of changing the relative proportion of the two subconductance states (47 and 23 pS) with inherently different Ca$^{2+}$ permeability. That is, the change in both Ca$^{2+}$ permeability and single-channel conductance could reflect an increase in the proportion of subconductance states that have a lower Ca$^{2+}$ permeability. Lastly, the altered state of the modulated receptor could have an altered size or nature of the pore leading to both altered conductance and Ca$^{2+}$ permeability. This could be through rearrangement of the transmembrane domain, perhaps preventing the pore from fully dilating (Amin et al., 2021). Alternatively, EU1794 could directly interact with the pore cavity, as has been observed with an AMPA receptor modulator (Yelshanskaya et al., 2020).

![Fig. 5. EU1794-4 reduces weighted mean conductance in NMDARs.](https://example.com)

(A) Example GluN1/GluN2A current responses to slow perfusion of glutamate with constant exposure to glycine supplemented with vehicle or EU1794-4. The increase in variance of the NMDAR response (black trace, below) is apparent in the 1 Hz high-pass filtered current (gray trace, above). (B) Current-variance relationship determined from the onset and washout of the current response to low concentration of agonist from (A). The smooth line is the fit of the current-variance equation to the data (see Materials and Methods). (C) Weighted mean conductance is given for all diheteromeric NMDARs with and without EU1794-4 (30 μM). Conductance was 55 ± 2.8 pS (control) and 39 ± 2.2 pS (EU1794-4) for GluN1/ GluN2A, 50 ± 1.2 pS (control) and 31 ± 0.86 pS (EU1794-4) for GluN1/ GluN2B, 19 ± 2.8 pS (control) and 15 ± 2.5 pS (EU1794-4) for GluN1/ GluN2C, and 28 ± 1.4 pS (control) and 28 ± 2.5 pS (EU1794-4) for GluN1/ GluN2D. *P < 0.05 by paired t test.
The degree of maximal inhibition varies among EU1794 analogs, and it is unclear how the ability to change channel conductance relates to this parameter. EU1794-4 displays the least amount of inhibition at saturating concentrations compared with all other EU1794 analogs that we have tested. By contrast, EU1794-1 and EU2100 reduce activity the most, producing essentially complete inhibition (Katzman et al., 2015). Moreover, EU1794-1 and EU2100 are the only analogs tested that show complete inhibition at saturating concentrations and apparently no decrease in channel conductance. This raises the possibility that perhaps full inhibition could occlude effective variance analysis measurement in that it reduces either channel opening duration or channel conductance to a degree such that the channel activity no longer contributes to the variance, and individual channel openings do not arise above noise in outside-out patches. That is, the inhibited channel is essentially inactive.

EU1794-4 and EU1794-21 reduce the weighted mean conductance of GluN1/GluN2A the most, whereas other analogs produce similar levels of submaximal inhibition, or produce more inhibition with less change in single-channel conductance. This net effect of modulation must result from a complex interplay of channel conductance modulation and channel open/closed kinetics modulation. As shown previously, the EU1794 series has use dependence associated with their modulation, which may impact the ability to precisely determine the full effect of reduced conductance with this assay. Single-channel data will be required for more analogs to determine the relationship between submaximal inhibition to both pore properties and

<table>
<thead>
<tr>
<th>Structure</th>
<th>Compound Name</th>
<th>GluN2A IC50</th>
<th>GluN2D IC50</th>
<th>Conc</th>
<th>GluN2A % control</th>
<th>GluN2D % control</th>
</tr>
</thead>
<tbody>
<tr>
<td>EU2100c</td>
<td>3.4</td>
<td>1.1</td>
<td>30</td>
<td>55 ± 0.94 [12]</td>
<td>49 ± 1.8 [89 ± 4]³</td>
<td>32 ± 1.1 [15]</td>
</tr>
<tr>
<td>EU1794-19d</td>
<td>1.1</td>
<td>0.4</td>
<td>10</td>
<td>58 ± 2.7 [6]</td>
<td>43 ± 3.3 [74 ± 6]³</td>
<td>32 ± 1.6 [5]</td>
</tr>
<tr>
<td>EU1794-23c</td>
<td>5.2</td>
<td>1.5</td>
<td>30</td>
<td>54 ± 2.5 [8]</td>
<td>42 ± 2.2 [78 ± 6]³</td>
<td>26 ± 5.5 [5]</td>
</tr>
<tr>
<td>EU1969c</td>
<td>6.2</td>
<td>2.6</td>
<td>30</td>
<td>55 ± 0.93 [10]</td>
<td>53 ± 1.7 [95 ± 2]</td>
<td>31 ± 1.8 [8]</td>
</tr>
<tr>
<td>EU1794-1c</td>
<td>4.8</td>
<td>2.6</td>
<td>20</td>
<td>56 ± 2.7 [10]</td>
<td>57 ± 2.3 [100 ± 4]</td>
<td>30 ± 2.7 [6]</td>
</tr>
</tbody>
</table>

³IC50 value from Fig. 1. ⁴The calculated weighted mean conductance from the time course fitting of the single-channel data for control is 58.6 ± 2.5 pS and for EU1794-4 is 47.5 ± 2.2 pS, which reflects a 0.81-fold reduction (n = 8, P = 0.0002 paired t test). Calculated weighted mean conductance from the all-points histograms of the single-channel records for control is 58.7 ± 1.2 pS and for EU1794-4 is 24.6 ± 3.0 pS, which reflects a 0.42-fold reduction (n = 5, P = 0.0003 paired t test).

⁵IC50 values from Katzman et al. (2015). ⁶IC50 values from Perszyk et al. (2018). ⁷P < 0.05 by paired t test.
channel-gating kinetics. Moreover, single-channel analysis may be a necessary means for advancing the structure-activity relationship since the multiple actions of these agents cannot be distinguished from macroscopic analysis.

In terms of physiologic relevance, it remains to be determined whether the attenuation of net current flow coupled with the reduction in Ca\textsuperscript{2+} permeability by EU1794-4 could protect against neurotoxic levels of glutamate encountered during acute brain injury. The ability of EU1794-4 to increase glutamate potency could drive increased current through extrasynaptic receptors that encounter low concentrations of glutamate. Although this might mitigate neuroprotective actions at low (i.e., 100’s of nanomolar) concentrations of glutamate, rising levels of glutamate encountered during acute injury at some point will begin to reduce any potential increase in current observed due to increased potency (Benveniste et al., 1984; Nilsson et al., 1990).

In summary, these molecules create an interesting precedent, as they are nonselective NAMs of NMDARs that reduce the conductance of the channel pore and limit permeability of Ca\textsuperscript{2+}, which is the trigger for excitotoxic damage (Choi et al., 1988; Wroge et al., 2012; Parsons and Raymond, 2014). These are desirable therapeutic properties for treatment of acute injury, including TBI and stroke (Yurkewicz et al., 2005). Moreover, four features of EU1794-4 and related analogs are relevant to potential therapeutic implications. First, the submaximal activity of EU1794 analogs could reduce on-target side effects by preventing strong, global NMDA receptor block. Second, the reduction in channel conductance and prolongation of the channel activation, as suggested by enhanced agonist potency, might alter the role of NMDARs in neuron physiology and pathology due to changes in temporal and spatial impact of receptor activation. Third, the agonist-dependence of this modulator might result in this compound being more active in areas of high neuronal activity that results from elevated extracellular glutamate during TBI or stroke. Fourth, the reduction of calcium permeation may reduce neuronal stress in neurodegenerative disease states such as Parkinson and Alzheimer disease given the role of calcium in the pathways that lead to neuronal cell death in these conditions. The actions of this compound in a physiologic setting still need to be established, and more information is needed to understand how modulator properties impact NMDAR activity in the context of circuit function. However, EU1794-4 is an intriguing modulator that has a variety of actions on NMDARs that could be exploited for therapeutic gain using rational structure-based design.

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Authorship Contributions

**Participated in research design:** Perszyk, Zheng, Xie, McDaniel, Yuan, Traynelis.

**Conducted experiments:** Perszyk, Zheng, Zhang, Xie, McDaniel, Pelly.

**Contributed new reagents or analytic tools:** Katzman, Liotta.

**Performed data analysis:** Perszyk, Zheng, Banke, Zhang, Xie, McDaniel, Pelly, Yuan, Traynelis.

**Wrote or contributed to the writing of the manuscript:** Perszyk, Zheng, Banke, Zhang, Xie, McDaniel, Katzman, Pelly, Yuan, Liotta, Traynelis.

References


**Fig. 6.** EU1794-4 reduces the relative Ca\textsuperscript{2+} permeability of GluN1/GluN2A receptors. (A) A representative current-voltage relationship for GluN1/GluN2A current responses activated by maximally effective agonist coapplied with vehicle (left) or EU1794-4 (right) when extracellular solutions contained low or high concentration of Ca\textsuperscript{2+}. The reversal potentials (determined by least-squares fitting using a fourth-order polynomial) were used with the Lewis equation (see Materials and Methods) to determine the relative Ca\textsuperscript{2+}/monovalent permeability ratio. (B) The average reversal potentials are shown for vehicle and EU1794-4. (C) The mean ΔReversal potentials (high Ca\textsuperscript{2+} minus low Ca\textsuperscript{2+}) are given. (D) The average Ca\textsuperscript{2+} permeability ratio to monovalent ions was calculated from the Lewis equation. *P < 0.05 (unpaired t test).


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