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Membrane Stretch Gates NMDA Receptors

Sophie Belin, Bruce A. Maki, James Catlin, Benjamin A. Rein, **Gabriela K. Popescu***

Department of Biochemistry, Jacobs School of Medicine and Biomedical Sciences, University at Buffalo, SUNY, Buffalo, NY, 14214, United States

* Corresponding author: popescu@buffalo.edu

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Abstract

N-Methyl-D-aspartic (NMDA) receptors are ionotropic glutamate receptors widely expressed in the central nervous system, where they mediate phenomena as diverse as neurotransmission, information processing, synaptogenesis, and cellular toxicity. They function as glutamate-gated Ca^{2+}-permeable channels, which require glycine as co-agonist, and can be modulated by many diffusible ligands and cellular cues, including mechanical stimuli. Previously, we found that in cultured astrocytes, shear stress initiates NMDA receptor-mediated Ca^{2+} entry in the absence of added agonists, suggesting that more than being mechanosensitive, NMDA receptors may be mechanically activated. Here, we used controlled expression of rat recombinant receptors and non-invasive on-cell single-channel current recordings to show that mild membrane stretch can substitute for the neurotransmitter glutamate in gating NMDA receptor currents. Notably, stretch-activated currents maintained the hallmark features of the glutamate-gated currents, including glycine-requirement, large unitary conductance, high Ca^{2+} permeability, and voltage-dependent Mg^{2+} blockade. Further, we found that the stretch-gated current required the receptor's intracellular domain. Our results are consistent with the hypothesis that mechanical forces can gate endogenous NMDA receptor currents even in the absence of synaptic glutamate release, which has important implications for understanding mechanotransduction and the physiological and pathological effects of mechanical forces on cells of the central nervous system.

Significance Statement

We show that in addition to enhancing currents elicited with low agonist concentrations, membrane stretch can gate NMDA receptors in the absence of the neurotransmitter glutamate. Stretch-gated currents have the principal hallmarks of the glutamate-gated currents including requirement for glycine, large Na^+ conductance, high Ca^{2+} permeability, and voltage-dependent Mg^{2+} block. Therefore, results suggest that mechanical forces can initiate cellular processes presently attributed to glutamatergic neurotransmission, such as synaptic plasticity and cytotoxicity. Given the ubiquitous presence of mechanical forces in the central nervous system, this discovery identifies NMDA receptors as possibly important mechanotransducers during development and across the lifespan, and during pathologic processes such as those associated with traumatic brain injuries, shaken baby syndrome, and chronic traumatic encephalopathy.

Introduction

Cells of the central nervous system (CNS) experience endogenous and environmental mechanical forces *in vivo*, and respond to osmotic and atmospheric pressure *ex vivo* (Tyler, 2012; Koser et al., 2016; Bliznyuk et al., 2020). Mechanical stimuli affect several neurophysiological processes including neuronal firing, vesicle fusion, dendritic spine formation, and synaptic activity (Hill, 1950; Korkotian and Segal, 2001; Star et al., 2002; Kim et al., 2007; Ucar et al., 2021). However, the mechanism of mechanotransduction in the CNS remains poorly understood largely due to experimental, technological, and theoretical challenges unique to examining the effect of mechanical forces in biological tissues. Among these obstacles are the omnipresence of mechanical cues, their diverse three dimensional and dynamic actions, the variety of macromolecules that participate in mechanotransduction, and the multiplicity of mechanisms by which transducers sense and respond to mechanical stimuli (Cox et al., 2019; Le Roux et al., 2019; Kefauver et al., 2020).

On a millisecond time-scale, mechanotransduction is mediated by mechanically-activated and mechanically-sensitive ion-channels (Cox et al., 2019; Kefauver et al., 2020). Mechanically-activated channels are membrane proteins dedicated to scanning the environment for mechanically-encoded information; they represent the molecular basis for a wide array of mechanosensory processes including hearing, touch, and proprioception; and are critical for normal development and adaptation throughout life (Walsh et al., 2015; Murthy et al., 2017). On the other hand, a large swath of ion channels whose primary physiological function is to respond to electrical and chemical signals, while not directly gated by mechanical stimuli, are mechanosensitive. These channels mediate much of the CNS mechanotransduction and are essential to how mechanical forces influence the normal development and functioning of the

54 brain and spinal cord, and also how they initiate or aggravate acute and chronic neuropathologies
55 (Tyler, 2012).

56 N-methyl-D-aspartate (NMDA) receptors are glutamate-gated channels with demonstrated
57 mechanosensitivity (Johnson et al., 2019). NMDA receptors mediate excitatory transmission and
58 plasticity in CNS and are critical for the normal physiology of excitatory synapses; moreover,
59 their overactivation mediates glutamate excitotoxicity, which has been implicated as a causal
60 factor in several neuropathologies. Ambient pressure, membrane stretch, and membrane lipid
61 composition modulate their agonist-gated currents in native preparations, in heterologous
62 systems, and in artificial lipid bilayers (Fagni et al., 1987; Miller et al., 1992; Nishikawa et al.,
63 1994; Paoletti and Ascher, 1994; Casado and Ascher, 1998; Kloda et al., 2007). In addition to
64 mechanosensitivity, we reported recently that shear stress, as applied by shear microfluidic flow
65 onto cultured astrocytes, elicits NMDA receptor-mediated Ca^{2+} influx in the absence of
66 glutamate, suggesting that mechanical stimuli *per se* can gate NMDA receptor currents (Maneshi
67 et al., 2017). This observation has important implications for a potential role of NMDA receptors
68 in mechanotransduction during the normal development and function of the CNS (Tyler, 2012;
69 Goriely et al., 2015; Heuer and Toro, 2019); and also in severe neuropsychiatric pathologies,
70 including those associated with acute traumatic brain and spinal cord injuries, chronic traumatic
71 encephalopathy, shaken baby syndrome, and episodic edema or tumor growth (Bonnier et al.,
72 2004; Shively et al., 2012; Sloley et al., 2021). Therefore, we undertook the work reported here
73 to investigate our novel observation in more depth.

74 Given that shear force can elicit NMDA receptor-dependent Ca^{2+} fluxes in primary cultures of
75 astrocytes in the absence of agonist (Maneshi et al., 2017), here we investigate more specifically
76 the sensitivity of NMDA receptor currents to membrane stretch, using a recombinant system and

77 cultured neurons, with single-channel and whole-cell current recordings. We found that, as with
78 sheer stress in cultured astrocyte, gentle suction applied to a membrane patches elicited currents
79 from recombinant NMDA receptors expressed in HEK cells in the absence of the
80 neurotransmitter glutamate. Importantly, the stretch-gated current maintained the characteristic
81 biophysical properties of the glutamate-gated current, including requirement for glycine, high
82 unitary conductance, Ca^{2+}-permeability, and voltage-dependent Mg^{2+} blockade. In addition, we
83 found that the C-terminus of NMDA receptors is required to initiate stretch-induced currents.

84 **Materials and Methods**

85 *Cells and receptor expression*

86 HEK293 cells (American Type Culture Collection number CRL-1573) were grown and
87 maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum
88 (FBS, Gibco) and 1% penicillin/streptomycin. Cells were grown to 80% confluency, and
89 passages 24 - 31 were used for transfections. Cells were transfected transiently *via* the Ca^{2+}-
90 phosphate method using pcDNA3.1 (+) plasmids encoding rat GluN1-1a (P35439-1), rat
91 GluN2A (Q00959) and GFP (P42212) in a 1:1:1 ratio. When indicated, plasmids encoding
92 GluN1-1a and GluN2A were replaced by plasmids encoding CTD-truncated GluN1-a (GluN1-a
93 838stop) and CTD-truncated GluN2A (GluN2A 844stop), provided by Dr. Westbrook (Krupp et
94 al., 1999, 2002). Alternatively, when indicated, the GluN2A-encoding plasmid was substituted
95 with plasmids expressing rat GluN2B (Q00960), rat GluN2C (Q00961), or rat GluN2D
96 (Q62645). Cells were incubated with the DNA mixture for 2 hours, were washed twice with
97 phosphate buffer saline (PBS), and incubated in growth medium supplemented with 2 mM
98 $MgCl_2$, to prevent excitotoxicity. They were used for electrophysiological recordings within 24
99 hours.

Culture of Dissociated Hippocampal Neurons

Low-density cultures of acutely dissociated hippocampal neurons were prepared from Sprague-Dawley rat embryos (Envigo) of unknown sex, at embryonic day 18 (E18) with minor adjustments from previously described methods (Misonou and Trimmer, 2005; Borschel et al., 2012). Briefly, a pregnant rat was euthanized in a CO_2 chamber and quickly decapitated, and the uterus was surgically removed. Embryos were decapitated, and the hippocampi were removed and placed in ice-cold dissecting solution containing HBSS supplemented with 4 mM sodium bicarbonate (Sigma), 10 mM HEPES (Sigma) and 1% penicillin/streptomycin (Corning). Cells were enzymatically dissociated with 0.25% trypsin (20 min at 37°C), and then gently triturated and filtered through a 40 mm strainer (BD Falcon, Franklin Lakes, NJ). Dissociated cells were counted and plated at a density of 100,000 cell/cm² onto glass cover-slips precoated with poly-D-lysine (Corning) in plating media containing MEM (Gibco) supplemented with 10% FBS, 0.6 % glucose (Sigma), 2 mM GlutaMAX (Gibco), 1 mM sodium pyruvate (Sigma), and 1% penicillin/streptomycin. Within a few hours, after cells have adhered to plates, the medium was gently replaced with Neurobasal A medium (Gibco) supplemented with B27 (Gibco) and 2 mM GlutaMAX. Three days after plating, the proliferation of non-neuronal cells was inhibited by including arabinofuranosylcytosine (5 µM, Sigma). Neurons were used for electrophysiological measurements between 7 and 30 days in vitro.

Electrophysiology

To maintain consistency in seal formation with minimal mechanical disruption to the patch, we used the following procedure. Prior to entering the bath, we applied slight positive pressure (5 mmHg) through the recording pipette with a high speed pressure-clamp system (HSPC-1, ALA Scientific, Farmingdale, NY) (McBride and Hamill, 1993, 1999). Electrical resistance through

the pipette (20 ± 5 MΩ) was monitored by observing the amplitude of the current elicited by a test voltage-pulse. After contacting the cell, the positive pressure was released to 0 mmHg, and slight suction (-5 mmHg) was applied to initiate slow seal formation onto the cellular membrane, which was monitored as an increase in pipette resistance. Finally, after obtaining a high-resistance seal, we released the negative pressure and applied +100 mV to the patch to visualize the activity of NMDA receptors at 0 mmHg, as inward Na^+ currents.

To examine the dependency of channel activity on the level of applied pressure, cells were bathed in PBS; after seal formation, we applied pressure in increments of 10 mmHg, and recorded activity for periods lasting ~5 minutes for each pressure level, over the indicated range. When specified, a 5-minute recovery step was recorded after relaxing the pressure to 0 mmHg. Channel activity was evaluated in cell-attached patches obtained with pipettes filled with (in mM) 150 NaCl, 2.5 KCl, 10 HEPBS, 1 EDTA, pH 8.0 (NaOH) and the indicated agonists glutamate (1 mM), glycine (0.1 mM), or NMDA (0.1 mM), as previously described (Hamill et al., 1981; Maki et al., 2014). Solutions lacking agonists were prepared using double-distilled deionized ultrapure water (Fisher Scientific, Hampton, NH) to prevent contamination (Cummings and Popescu, 2015).

To examine the effect of pressure on the receptor's conductance, Ca^{2+} permeability, and voltage dependency of its Mg^{2+} blockade, cells were bathed in a high K^+ bath solution (in mM): 142 KCl, 5 NaCl, 1.8 CaCl2, 1.7 MgCl2, 10 HEPBS, pH 7.2 (with KOH) to collapse the physiological membrane potential of HEK cells, which is ~10 mV (Borschel et al., 2012). Pipette solution was (in mM): 150 NaCl, 2.5 KCl, 10 HEPBS, 1 EDTA, 10 tricine, pH 8.0, and glycine (0.1) and/or glutamate (1) as indicated. Ca^{2+} and Mg^{2+} were added as chloride salts and were buffered to the indicated free concentration according to MAXCHELATOR software. After seal

146 formation, we applied sustained suction (-40 mmHg) and varied the applied voltage in 20-mV
147 increments, each lasting 1 minute, over the +100 mV to +20 mV range.

148 All current traces were filtered (10 kHz), amplified (Axopatch 200b) and then sampled (40 kHz)
149 and stored as digital files using QuB software (Nicolai and Sachs, 2013).

150 *Data analysis*

151 Current traces were inspected visually off-line and only recordings with low-noise and stable-
152 baseline were selected for analyses. Traces were initially processed to correct for spurious noise
153 events and minor baseline drifts (Maki et al., 2014). Corrected traces were idealized separately
154 for each applied pressure within the QuB suite for kinetic analyses, with the SKM algorithm after
155 applying a digital filter (12 kHz) (Qin, 2004). We estimated the open probability (nPo) in each
156 trace according to the following relationship:

157 $$nP_o = \sum_{n=1}^{N} n \cdot Po(n) / N$$

158 Where P_o is the open probability of each channel, n is the indeterminate number of channels in
159 each patch, and N the minimum number of channels in each patch, estimated
160 as the number of overlapping unitary currents (simultaneous openings) observed in the condition
161 producing maximal activity. Values for nP_o were obtained by averaging activity in each 5-minute
162 segment, and were considered non-zero for a threshold of >1,000 events.

163 Unitary channel conductance (γ) and reversal potential (E_{rev}) were estimated from linear fits to
164 the unitary current-voltage relationship measured over a one-minute period. Ca^{2+} permeability
165 was estimated as a function of the measured Ca^{2+}-induced shifts in E_{rev} using the Lewis Equation
166 below (Lewis, 1979), with the experimental constant α = 25.4 mV.

$$\frac{P_{Ca}}{P_{Na}} = \frac{[Na]\,(e^{\frac{\Delta Erev}{\alpha}} - 1)}{4\,[Ca]}$$

Statistics

Results are given as the mean ± SEM of a minimum of three measurements per condition. Statistical analyses were performed using two-way ANOVA multiple comparisons and the Bonferroni correction, or unpaired Student's *t*-test relative to controls measured at zero pressure, as indicated. Means were considered significantly different for $P < 0.05$.

Results

Membrane stretch substitutes for glutamate in gating NMDA receptors

NMDA receptors are tetrameric transmembrane proteins that assemble from three subfamilies of subunits: glycine-binding GluN1 and GluN3(A, B), and glutamate-binding GluN2(A-D). Functional NMDA receptors assemble as heterotetramers of two obligatory GluN1 subunits, which are widely expressed in cells of the CNS, and two of GluN2 and/or GluN3 subunits whose expression is regulated developmentally and regionally. Of the glutamate-binding GluN2 subunits, GluN2A predominates in adult animals and at mature synapses, whereas GluN2B is expressed mostly in juvenile animals and at immature synapses (Monyer et al., 1992; Goebel and Poosch, 1999; Paoletti et al., 2013).

To examine whether NMDA receptors are simply mechanically sensitive or whether they can be gated by mechanical forces in the absence of neurotransmission, we expressed rat recombinant GluN1/GluN2A receptors in HEK293 cells and recorded inward Na^+ currents from cell-attached patches, while gently varying the pressure applied through the recording pipette in 10-mmHg increments over the -40 mmHg to +40 mmHg range. These pressures are typical for the

187 activation of dedicated mechanotransducers such as Piezo channels (Coste et al., 2012; Kim et
188 al., 2012). Observing NMDA receptor activity over long periods is necessary to reduce patch-to-
189 patch variability due to modal gating, which for NMDA receptors occurs on a minutes time scale
190 (Popescu and Auerbach, 2003; Borschel et al., 2012). Therefore, at each pressure level, we
191 recorded 5 minutes of continuous activity.

192 When the recording pipette included supra-saturating levels of the neurotransmitter glutamate (1
193 mM; Kd, 3 μM) (Popescu et al., 2004) and the obligatory co-agonist glycine (0.1 mM; Kd, 2.5
194 μM) (Cummings and Popescu, 2015), applying +100 mV through the pipette produced large
195 inward unitary currents (8 – 10 pA) indicative of channel activation, at all levels of applied
196 pressure tested (**Figure 1A**, top traces). Often, overlapping openings were apparent, indicating
197 that multiple active channels were trapped in the recorded patch. In these conditions, neither
198 negative nor positive pressure altered channel activity. When glycine was omitted, we observed
199 only minimal and sporadic currents (<1,000 events per 5-min segment), regardless of whether
200 glutamate was present or not, and applying either negative or positive pressure did not alter this
201 low baseline-activity (**Figure 1A**, middle traces). However, when glycine was present, negative
202 but not positive pressure gated substantial current in the absence of glutamate (**Figure 1A**,
203 bottom traces). The suction-gated current increased with increasing pressure in a consistent
204 manner, although the magnitude of the effect varied. On average, -40 mmHg of hydrostatic
205 pressure increased GluN1/GluN2A channel activity (nPo) from 0.10 ± 0.06 to 0.50 ± 0.18 (n = 6,
206 P = 0.007) (**Figure 1A, B**). This result demonstrates that suction alone can gate GluN1/GluN2A
207 receptors, and therefore it is possible to open the NMDA receptor pore mechanically, in the
208 absence of neurotransmission.

To ascertain whether pressure can gate currents from other members of the NMDA receptor family, we co-expressed GluN1 with GluN2B, GluN2C or GluN2D subunits in HEK293 cells and recorded single-channel inward Na^+ currents from cell-attached patches with pipettes containing glycine (0.1 mM) but not glutamate. As with the adult GluN1/GluN2A receptor, we observed a selective increase in channel activity with negative pressure, and no effect with positive pressure of similar magnitude (**Figure 1B**). The application of negative pressure increased the nPo for GluN2B from 0.03 ± 0.02 at 0 mmHg to 0.43 ± 0.15 at -30 mmHg, (n = 4, P = 0.03). We could not detect significant changes for GluN2C and for GluN2D channels, for which measured averages at 0 mmHg and -40mmHg, were: 0.010 ± 0.004 and 0.04 ± 0.01 (n = 5, P > 0.05), and 0.07 ± 0.03 and 0.18 ± 0.04 (n = 4, P > 0.05), respectively. This may reflect in part the well-documented high kinetic variability of GluN2B- and GluN2C-containg receptors (Amico-Ruvio and Popescu, 2010; Khatri et al., 2014), and the low open probability of GluN2D-containing receptors, which makes detection more challenging (Vance et al., 2013), and their much lower maximal open probabilities measured with glutamate: 0.16 ± 0.02 for GluN2B (Borschel et al., 2012); 0.032 ± 0.015 for GluN2C (Khatri et al., 2014), 0.023 ± 0.001, for GluN2D (Vance et al., 2013).

Overall, these results support the hypothesis that mechanical forces in addition to modulating the glutamate-gated current, can by themselves provide the energy necessary to shift the receptor's closed-to-open equilibrium and produce a detectable increase in open probability. We focused next on GluN1/GluN2A receptors, which generally produce more robust and reliable responses (Borschel et al., 2012).

Mindful of the many sources that can contribute to the variability of the observed changes, we aimed to reduce the incidence of confounding effects due to cellular processes over the long

recording period necessary to cover the 80 mmHg-range investigated with the protocol above. For this, we shortened the experiment by limiting observations to negative pressure, which allowed us to add a 5-minute recovery step to test the reversibility of the pressure-dependent effect. As in the first set of experiments, with this shorter protocol we found that negative pressure had no effect on channel activity in the absence of glycine, or in the presence of saturating concentrations of glycine and glutamate (**Figure 2, Table 1**). However, when glutamate was omitted, -40 mmHg of pressure increased the observed current (nPo) from 0.03 ± 0.01 to 0.08 ± 0.05 (n = 4, P = 0.006), which represented 19% of the maximal glutamate-gated current in the same conditions (0.41 ± 0.07, n = 4). The ambient glutamate concentration at extrasynaptic sites in adult rat hippocampal slices is estimated at 25 – 80 nM (Herman and Jahr, 2007; Moldavski et al., 2020), which represents less than 10% of the synaptic concentration (~ 1 mM) (Clements et al., 1992; Wadiche and Jahr, 2001; Budisantoso et al., 2012). Therefore, the level of activity we observed with mild stretch is on par with that reported for extrasynaptic receptors activated by synaptic glutamate spill-over or by glutamate leak from injured neurons (Moldavski et al., 2020), and may be physiologically significant if the stretch-gated currents maintain the biophysical properties of glutamate-gated currents, especially their large unitary conductance, high Ca^{2+} permeability, and voltage-dependent Mg^{2+}-block. Therefore, we next examined these biophysical properties of the stretch-gated current.

Biophysical properties of stretch-gated NMDA receptor currents

Within the larger family of glutamate gated channels, NMDA receptors have characteristically large unitary conductance, high Ca^{2+} permeability, and voltage-dependent Mg^{2+} block (Hansen et al., 2018). These distinctive biophysical properties of the glutamate-gated current are essential for the many roles NMDA receptors play in health and disease (Iacobucci and Popescu, 2017;

Hansen et al., 2018). To estimate the conductance and permeability properties of the stretch-gated receptors from cell-attached recordings, we bathed the cells in a high K^+ solution to collapse the cellular transmembrane potential. After gentle seal formation, we applied -40 mmHg of pressure and recorded activity at several applied pipette potentials for one-minute periods (**Figure 3A).** From these data, we measured unitary current amplitude at each voltage, and estimated the unitary conductance as the slope of the voltage-current relationship (**Figure 3B**). Relative to the glutamate-gated Na^+ currents, which had $\gamma_{Na} = 81 \pm 9$ pS (n = 5), stretch-gated currents had similar unitary Na^+ conductance, $\gamma_{Na} = 87 \pm 6$ pS (n = 3, p > 0.5) (**Figure 3B**). Therefore, stretch-gated currents retain the high unitary conductance characteristic of NMDA receptors.

In physiological conditions, external Ca^{2+} permeates NMDA receptors and concurrently reduces channel conductance (voltage-independent block). To examine how external calcium affects stretch-gated currents, we measured single-channel current amplitudes of glutamate-gated and stretch-gated currents at several applied voltages, in the presence of external calcium. We found that 1.8 mM Ca^{2+} reduced the glutamate-gated conductance to $\gamma = 61 \pm 2$ pS, ($P < 0.05$) indicative of ~25% current blockade (**Figure 3B**), a value consistent with previous reports (Ascher and Nowak, 1988; Maki and Popescu, 2014). Similarly, 1.8 mM Ca^{2+} reduced the amplitude of stretch-gated currents to $\gamma_{1.8} = 51 \pm 6$ pS ($P < 0.01$), and this reduction was not statistically different in magnitude from that observed for glutamate-evoked currents ($P = 0.19$, two-way Anova) (**Figure 3A, B**).

From the same data, we constructed linear fits to the current-voltage relationships obtained in zero and 1.8 mM Ca^{2+}, to estimate reversal potentials for each condition. Relative to 0 Ca^{2+}, in 1.8 mM Ca^{2+}, the reversal potential of glutamate-gated currents shifted by +6 mV, indicative of a

high relative Ca^{2+} permeability, P_{Ca}/P_{Na} = 10.7, as reported previously (Wollmuth and Sakmann, 1998; Maki and Popescu, 2014). For stretch-activated currents the measured shift in reversal potential was +16 mV, corresponding to 2-fold increase in permeability, P_{Ca}/P_{Na} = 21, relative to glutamate-gated currents. Together, these measurements suggest that in physiologic Ca^{2+} concentrations, membrane stretch gates NMDA receptor currents that maintain characteristic high unitary conductance, and voltage-independent Ca^{2+}-block, and may have slightly stronger higher Ca^{2+} permeability relative to the glutamate-gated currents.

Last, we examined the sensitivity of the stretch-gated current to block by external Mg^{2+}. We recorded on-cell single-channel currents from GluN1/GluN2A receptors at several applied voltages, with pipettes containing glycine (0.1 mM), Mg^{2+} (10 μM, Kd = 1 μM) (Premkumar and Auerbach, 1996), and either glutamate (1 mM) or sustained negative pressure (-40 mmHg) (**Figure 3C**). At each voltage, we identified non-overlapping bursts of activity and measured the channel mean open time as a measure of Mg^{2+}-block. We found that glutamate-gated currents were sensitive to block by external Mg^{2+} in a voltage-dependent manner, such that the mean duration of openings decreased from 5.1 ± 2 ms at -20 mV, to 1.0 ± 0.2 ms at -60 mV, as reported previously (Nowak et al., 1984). For stretch-gated currents, we observed a similar shortening of open durations with hyperpolarization, from 4.0 ± 0.6 ms at -20 mV, to 1.0 ± 0.05 ms at -60 mV ($p < 0.05$, paired Student's *t*-test), indicating similar sensitivity to voltage-dependent block (**Figure 3C**) (Premkumar and Auerbach, 1996). At all examined voltages, the difference between the estimated mean open durations for glutamate-gated and stretch-gated currents was not statistically significant ($p > 0.05$, two-way ANOVA). Together, these results indicate that stretch-activated channels maintain the characteristic biophysical properties of

300 glutamate-gated channels, including high conductance, large Ca^{2+} permeability, strong voltage-
301 dependent Mg^{2+} block, and long openings.

302 *Stretch-gated NMDA currents require the receptor's carboxyl terminal*

303 Given the potentially significant physiological implications of a Ca^{2+}-rich currents gated by
304 mechanical forces through NMDA receptors, it will be important to understand the mechanism
305 by which these arise, and more specifically, to identify the allosteric network responsible for
306 mechanotransduction. The existing literature on the mechanosensitivity of NMDA receptors
307 suggests several mechanisms by which mechanical forces may facilitate the glutamate-gated
308 current. These include a reduction of Mg^{2+} block (Zhang et al., 1996; Kloda et al., 2007; Mor and
309 Grossman, 2010), perhaps transmitted through the transmembrane domain (Casado and Ascher,
310 1998), but also allosteric mechanisms that implicate the C-terminal domain (Singh et al., 2012).
311 For the stretch-gated current, our results exclude a mechanism mediated by changes in Mg^{2+}-
312 block. Therefore, we asked whether the C-terminal domain (CTD) influences the receptor's
313 mechanically-elicited current.

314 We recorded single-channel currents from on-cell patches expressing receptors lacking the
315 intracellular C-terminal domain ($GluN1^{\Delta 838}$/$GluN2A^{\Delta 844}$). We reported previously that relative to
316 wild-type receptors (WT, $P_o = 0.54 \pm 0.04$), glutamate-gated currents from these truncated
317 receptors have lower but measurable open probabilities (ΔCTD, 0.08 ± 0.02, $n = 8$, $P < 0.5$)
318 (Maki et al., 2012). Using the pressure protocol described here, with only glycine in the pipette
319 and no external pressure, we observed low spontaneous activity from ΔCTD receptors ($0.05 \pm$
320 0.01, $n = 4$), which was not different from WT GluN1/Glu2A (**Figure 4**, **Table 1)**. However,
321 suction up to -40 mmHg did not increase the basal activity of truncated receptors (0.04 ± 0.01, n
322 = 3) (**Figure 4, Table 1**). This result suggests that the ΔCTD of GluN1/GluN2A receptors is

necessary for their mechanical activation by mild suction. This observation may indicate that the CTD is necessary to transmit force from the cytoskeleton to the gate; alternatively, it may indicate that the energy provided by suction, transmitted by some other unknown mechanism, is enough to gate the channel only when the tethering of the CTD to intracellular structures endow the receptor under observation a certain threshold of rigidity.

Mechanical activation of neuronal NMDA receptors

Regardless of mechanism, our result that the intracellular domain is required for mechanical gating of currents from NMDA receptors suggests that the intracellular milieu in which NMDA receptors operate, and specifically the intracellular interactions mediated by their CTD will influence the effectiveness with which hydrostatic pressure will gate currents from glycine-bound receptors. In addition, lipid composition of membranes varies widely across cell type, development stage, and subcellular location, and can be a critical determinant of mechanotransduction (Perozo et al., 2002; Phillips et al., 2009). We therefore investigated the effectiveness of hydrostatic pressure to gate NMDA receptors in a neuronal environment.

We cultured primary rat hippocampal neurons (P7 - P30), and recorded cell-attached currents with pipette solutions containing low concentrations of NMDA (0.1 mM; EC50, 90 μM) (Erreger et al., 2007) and glycine (0.1 mM) to identify currents mediated by endogenous NMDA receptors. We observed inward Na^+ currents with large unitary amplitudes (8.9 pA ± 0.3) consistent with NMDA receptor activation. Hydrostatic pressure (-40 mmHg) increased substantially the measured nP_o from 0.13 ± 0.02 at rest to 0.40 ± 0.04 ($n = 5$, $P < 0.0001$, one-way ANOVA); this potentiation was fully reversible (**Figure 5B, Table 1**) and mirrored results obtained with low NMDA and glycine from GluN1/GluN2A receptors in HEK293 cells (**Figure 5A, Table 1**). In similar experiments, and with only glycine in the pipette, the average nPo

346 measured in neurons was 0.04 ± 0.01 at rest, and 0.07 ± 0.02 (n = 4) with -40 mmHg, and these
347 values were not statistically different (one-way ANOVA, with Bonferroni correction) (**Figure**
348 **5B, Table 1**).

349 Together, these observations validate the results obtained with recombinant receptors in HEK
350 cells and support our proposal that mild stretch can gate native NMDA receptors in the absence
351 of neurotransmission, and likely potentiate responses elicited by low concentrations of glutamate
352 (< 0.1 mM), as may occur at extrasynaptic locations (Moldavski et al., 2020).

353 ## Discussion

354 Glutamate-gated NMDA receptor currents can be modulated by several types of mechanical
355 perturbations including those generated by changes in environmental pressure (Fagni et al., 1987;
356 Mor and Grossman, 2006), membrane composition (Miller et al., 1992; Nishikawa et al., 1994;
357 Casado and Ascher, 1998), osmotic and hydrostatic pressure (Paoletti and Ascher, 1994; LaPlaca
358 and Thibault, 1998), and microfluidic sheer stress (Maneshi et al., 2017). Although the effects of
359 mechanical stimulation on channel responses vary across stimulation procedure, receptor
360 preparations, and experimental conditions, these results have established that NMDA receptors
361 are mechanosensitive (Paoletti and Ascher, 1994). Here, we report that gentle suction can
362 activate NMDA receptors in the absence of glutamate. This observation establishes that NMDA
363 receptors, in addition to being mechanosensitive, can be activated mechanically, which is
364 consequential to understanding the mechanobiology of the central nervous system. Before
365 addressing this point of impact, we note several caveats.

366 As previously reported for mechano-sensitivity (Paoletti and Ascher, 1994), the mechano-
367 activity we observed here was variable, despite taking a number of experimental precautions.

Among these, we examined recombinant receptors residing in cell-attached membrane patches. This approach minimizes variability due the uncertain molecular composition of endogenous receptors; it maintains cellular integrity and a near-physiologic cellular environment; it allows precise control of the magnitude of the applied pressure with a high-speed pressure clamp; and provides a high-resolution single-molecule readout for receptor activity. Nonetheless, a direct correlation between the applied pressure and the receptor's microscopic properties is complicated by several uncontrollable variables. First, even when using pipettes of specified geometry, the area of the electrically accessible membrane patch (the dome delimited by the seal) varies from patch to patch and can change during a single recording due to membrane creep (Suchyna et al., 2009). Further, the tension experienced by receptors varies with their position within the patch, being highest at the apex and lowest near the perimeter (Bavi et al., 2014). Lastly, the size and mechanical properties of the cytosolic mass pulled within the pipette is not uniform across observations, and can detach from the bilayer upon continuous mechanical stimulation. Such blebbing may produce additional inconsistencies in the magnitude of the force that reaches the receptor and can also modify the receptor's gating properties (Suchyna et al., 2009). Lastly, NMDA receptors display intrinsic gating heterogeneity due to modal gating (Popescu and Auerbach, 2003; Popescu, 2012; Vance et al., 2013), which is responsible for the characteristic biphasic decay in their macroscopic response (Zhang et al., 2008). As such, even in controlled experimental conditions, the measured equilibrium open probability of NMDA receptors varies considerably (Borschel et al., 2012; Vance et al., 2013). Moreover, receptor activity is sensitive to cellular factors that may vary from cell to cell and may change during extended recording periods (Chen and Huang, 1991; Cerne et al., 1993; Tong et al., 1995; Wyszynski et al., 1997). With these considerations in mind, the magnitude of the changes in

activity we observed with gentle suction are consistent with substantial mechano-activation of NMDA receptors.

This observation is important for several reasons. Controlled NMDA receptor-mediated Ca^{2+} is required for the normal physiology of excitatory synapses, and mechanical forces may be important in initiating these processes during development and throughout life (Tyler, 2012). Alternatively, NMDA receptor Ca^{2+} can also initiate synaptic pruning, spine shrinkage, and neuronal death. NMDA receptors are expressed not only at post-synaptic, mechanically stable locations, but also in mechanically active or osmotically sensitive zones, such as growing axons or dendritic boutons, where local deformations in extracellular matrix, membrane tension or curvature, and intracellular cytoskeleton can impinge mechanically on receptors. Therefore, NMDA receptors operate in a mechanically rich landscape and depending on their location may experience differential mechanical forces. Our results show no effect of membrane stretch on currents elicited with maximally effective glutamate concentrations (**Figures 1A** and **2**, and **Table 1**). Therefore, it is unlikely that this mechanism will influence synaptic transmission. However, the levels of mechano-activation we observed with gentle membrane stretch can have a significant impact on signal transduction by neuronal extrasynaptic NMDA receptors, or those expressed in glial cells. Additionally, NMDA receptors, of unknown function, have been identified at non-traditional sites such gastrointestinal, lung, and adrenal tissue during human development (Szabo et al., 2015); and in adult tissues such as kidney (Leung et al., 2002), bone (Itzstein et al., 2001), myocytes (Seeber et al., 2004; Dong et al., 2021), colon (Del Valle-Pinero et al., 2007) and others, such as cancerous tissue (Yan et al., 2021). Therefore, the significance of the mechano-activity described here will vary with the site of NMDA receptor expression and their microenvironment.

For GluN2A-containing receptors, which is the most prevalent NMDA receptor isoform expressed in adult mammals, -40 mM Hg of pressure produced currents that had 19% open probability, 80% unitary conductance, and 200% Ca^{2+} permeability, relative to the current produced by saturating glutamate (1 mM) in similar conditions (1.8 mM Ca^{2+}). With the more sensitive protocol illustrated in **Figure 2**, the response did not appear to plateau with -40 mmHg, therefore it is possible that stronger forces may elicit higher activity. Together with the observation reported here and previously (Paoletti and Ascher, 1994; Casado and Ascher, 1998) that gentle membrane stretch potentiates responses elicited with low concentrations of the GluN2-site agonist (glutamate or NMDA), the mechano-activity of NMDA receptors may represent an important physiologic mechanism, especially in development or at sites of dendritic growth and synaptic formation. Alternatively, inappropriate mechanical activation of extrasynaptic NMDA receptors, due to for example, external mechanical forces experienced by brain or spinal cord, may initiate or aggravate apoptotic or necrotic cell injury through additional Ca^{2+} influx.

In some experimental paradigms, the mechanosensitivity of NMDA receptors reflects mechanically-induced changes in the receptor's sensitivity to voltage-dependent Mg^{2+} block (Zhang et al., 1996; Kloda et al., 2007; Parnas et al., 2009; Cox et al., 2019). Our measurements were done in the absence of external Mg^{2+}, and we were able to demonstrate similar voltage-dependent block for stretch-gated and glutamate-gated currents (**Figure 3C**), therefore we can definitively exclude this mechanism for the stretch-gated activity we examined here. Aside from modulating Mg^{2+}-block, previous reports found mechanosensitivity to depend on the receptor's intracellular CTD (Singh et al., 2012; Bliznyuk et al., 2015). In our hands, the CTD of NMDA receptors was required for mechanical activation by gentle membrane stretch.

Given the modular make-up of NMDA receptors, and their complex interactions with extracellular matrix proteins, membrane proteins and lipids, and with intracellular proteins and cytoskeletal components, it is likely that depending on the type of stimulation, mechanical forces will impinge on separate receptor domains. For example in the experiments reported by the Martinac group (Kloda et al., 2007), when mechanosensitivity was tested on purified recombinant NMDA receptors inserted in liposomal particles, it was reasonable to infer a force-from lipid transduction mechanism, given the absence of interacting proteins or cellular structures. However, when operating in their native environments, receptors are much more mechanically constrained and they can sense membrane deformation not only through direct interactions with lipid but also through their extracellular or intracellular domains. In addition, mechanical constraints imposed by interaction with cellular and/or intracellular structures, even if not serving as mechanical transducers, will limit the receptor's conformational freedom and thus affect their sensitivity to mechanical activation. Therefore, although the CTD appears necessary for mechanical gating of NMDA receptors, it remains to be determined whether this is a case of force-from filament mechanism, or the CTD simply stabilizes the molecule in mechano-sensitive conformations.

Therefore, although unknown at this time, it will be important to determine the mechanism by which mechanical forces gate NMDA receptors currents. Mechano-activation of NMDA receptors may be of importance at extrasynaptic and non-neuronal sites in the CNS, where it may contribute to fundamental processes such as synapse formation, dendrite remodeling, and glial physiology, and outside of the nervous system in gastric and pulmonary development, and cardiac and bone remodeling. Conversely, this knowledge may help better understand, and

459 therefore prevent or address, neuropsychiatric disorders such as shaken baby syndrome, chronic
460 traumatic encephalopathy, and other trauma-associated neuropathologies.

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668

Legends to Figures and Table

Figure 1. Mild suction gates NMDA receptors in the presence of glycine (**A**) Current traces recorded from cell-attached patches expressing GluN1/GluN2A receptors with +100 mV applied through the recording pipette. Downward traces illustrate inward Na^+ currents at the indicated pressure levels, in the presence (+) or absence (-) of glutamate (Glu, 1 mM) and/or glycine (Gly, 0.1 mM). (**B**) Summary of response-dependency on pressure level for each GluN2 subtype in the presence of glycine (0.1 mM) with no glutamate added. *, $P < 0.05$ **, $P < 0.01$ (one-way ANOVA, with Bonferroni correction).

Figure 2. Negative hydrostatic pressure agonizes NMDA receptors (**A**) Effect of suction on GluN1/GluN2A receptors, with the indicated agonists (Glu 1 mM, Gly 0.1 mM) and +100 mV in the cell-attached recording pipette. *, $P < 0.05$ **, $P < 0.01$ (one-way ANOVA with Bonferroni correction). (**B**) Summary of the effects of suction on receptor activity. *, $P < 0.05$ (unpaired Student's *t*-test).

Figure 3. Biophysical properties of stretch-gated currents from recombinant NMDA receptors. (**A**) On-cell patch-clamp current traces recorded from cells expressing GluN1/GluN2A receptors in response to saturating concentrations of Glu (1 mM) (left) and gentle stretch (-40 mmHg). (**B**) Voltage dependency of unitary current amplitude, and summary of Ca^{2+}-dependent reduction in unitary conductance. *, $P < 0.05$; **, $P < 0.01$ (two-way ANOVA, with Bonferroni correction). (**C**) Current traces recorded with external Mg^{2+} (10 μM) and summary of voltage-dependent reduction in mean open durations.

Figure 4. Mechanical activation of NMDA receptors by suction requires their intracellular C-terminal domain. Cell-attached Na^+-current traces recorded from GluN1/GluN2A receptors

lacking the intracellular C-terminal domain (ΔCTD) (left) and summary of results compared with WT receptors. *, $P < 0.05$ (unpaired Student's t-test).

Figure 5. Stretch gates native NMDA receptors. A. Suction potentiates currents elicited from recombinant GluN1/GluN2A receptors with low concentration of NMDA (0.1 mM). **B**. In neurons, suction potentiates NMDA-elicited currents and gates currents of similar unitary amplitude. **, $P < 0.01$; ***; $P < 0.001$; ****, $P < 0.0001$ (one-way ANOVA test with Bonferroni correction).

699 **Table 1** Summary of activity recorded in cell-attached patches

Agonist	Patch activity				P value
	0 mmHg		-40 mmHg		
	nPo / 5 min	n	nPo / 5 min	n	
GluN1/GluN2A					
Glu/Gly	0.41 ± 0.07	4	0.42 ± 0.11	4	nd
-/-	0.00 ± 0.00	3	0.00 ± 0.00	3	nd
Glu/-	0.00 ± 0.00	3	0.00 ± 0.00	3	nd
NMDA/Gly	0.11 ± 0.02	7	0.24 ± 0.04	7	0.005
-/Gly	0.03 ± 0.01	6	0.08 ± 0.02	4	0.006
ΔCTD					
-/Gly	0.05 ± 0.01	4	0.04 ± 0.01	3	>0.05
Neurons					
NMDA/Gly	0.13 ± 0.02	5	0.40 ± 0.04	5	0.003
-/Gly	0.04 ± 0.01	4	0.07 ± 0.03	4	>005

700

701 Summary of observed channel activity in cell attached patches in response to applied force (-40
702 mmHg) for the receptors and agonists indicated. Values are mean ± SEM of activity (nPo/5 min)
703 for the corresponding number of patches (n) used for analyses, and the P value calculated with
704 one-way ANOVA and Bonferroni's pairwise correction; nd, not determined.