Facilitation of AMPA receptor-mediated steady-state current by extrasynaptic NMDA receptors in supraoptic magnocellular neurosecretory cells

Yoon Hyoung Pai¹, Chae Seong Lim², Kyung-Ah Park¹, Hyun Sil Cho¹, Gyu-Seung Lee¹, Yong Sup Shin², Hyun-Woo Kim¹, Byeong Hwa Jeon¹, Seok Hwa Yoon²*, and Jin Bong Park¹*

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*Correspondence
Jin Bong Park
E-mail: jinbong@cnu.ac.kr
Seok Hwa Yoon
E-mail: seohwy@cnu.ac.kr

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ABSTRACT
In addition to classical synaptic transmission, information is transmitted between cells via the activation of extrasynaptic receptors that generate persistent tonic current in the brain. While growing evidence supports the presence of tonic NMDA current (I_{NMDA}) generated by extrasynaptic NMDA receptors (eNMDARs), the functional significance of tonic I_{NMDA} in various brain regions remains poorly understood. Here, we demonstrate that activation of eNMDARs that generate INMDA facilitates the α-amino-3-hydroxy-5-methylisoxazole-4-proprionate receptor (AMPAR)-mediated steady-state current in supraoptic nucleus (SON) magnocellular neurosecretory cells (MNCs). In low-Mg²⁺ artificial cerebrospinal fluid (aCSF), glutamate induced an inward shift in I_{holding} (I_{GLU}) at a holding potential (V_{holding}) of ~70 mV which was partly blocked by an AMPAR antagonist, NBQX. NBQX-sensitive I_{GLU} was observed even in normal aCSF at V_{holding} of ~40 mV or ~20 mV. I_{GLU} was completely abolished by pretreatment with an NMDAR blocker, AP5, under all tested conditions. AMPA induced a reproducible inward shift in I_{holding} (I_{AMP}) in SON MNCs. Pretreatment with AP5 attenuated I_{AMP} amplitudes to ~60% of the control levels in low-Mg²⁺ aCSF, but not in normal aCSF at V_{holding} of ~70 mV. I_{AMP} attenuation by AP5 was also prominent in normal aCSF at depolarized holding potentials. Memantine, an eNMDAR blocker, mimicked the AP5-induced I_{AMP} attenuation in SON MNCs. Finally, chronic dehydration did not affect I_{AMP} attenuation by AP5 in the neurons. These results suggest that tonic I_{NMDA}, mediated by eNMDAR, facilitates AMPAR function, changing the postsynaptic response to its agonists in normal and osmotically challenged SON MNCs.

INTRODUCTION
Accumulating evidence over the last few decades suggests that in addition to classical synaptic transmission, information is transmitted between cells via the diffusion of neurotransmitters into the extracellular space and the activation of extrasynaptic receptors in the brain [1,2]. The activation of extrasynaptic ionotropic receptors generates slow, persistent tonic currents that offer unique mechanisms of neuronal control [3,4], while synaptic receptors mediate rapid, phasic excitatory (E) or inhibitory (I) postsynaptic currents (EPSCs or IPSCs, respectively). In this sense, activation of extrasynaptic N-methyl-D-aspartate receptors (eNMDARs) by glutamate in the extracellular space can evoke a persistent tonic NMDA current (tonic I_{NMDA}) [5-8], while synaptic
NMNDA receptors are responsible for classical EPSCs. The idea that a glutamate signaling mechanism can be compartmentalized via synaptic and extrasynaptic NMDARs has been also supported by studies showing that synaptic and extrasynaptic NMDARs are linked to distinct, and even opposing, downstream biological actions [9-11]. However, despite growing evidence that supports the presence of tonic $I_{NMDA}$ generated by eNMDARs, the functional significance of tonic $I_{NMDA}$ in various brain regions remains poorly understood.

In the brain, $\alpha$-amino-3-hydroxy-5-methylisoxazole-4-propionate receptors (AMPARs) are responsible for the bulk of glutamatergic EPSCs and their dynamic regulation ensures dynamic fitting of the receptor function that underlies much of the plasticity of excitatory transmission. For example, calcium influx through open ATP-gated channels leads to reduced surface AMPARs in dendrites and at synapses in the hippocampus [12], while it facilitates AMPAR function in the hypothalamic paraventricular nucleus (PVN) neurons [13,14]. Given that activation of either synaptic or extrasynaptic NMDARs causes changes in intracellular calcium concentration [9], it is plausible that both synaptic and extrasynaptic receptors are involved in the regulation of AMPAR function. Despite the wealth of information regarding the role of synaptic NMDARs, little is known about the role of eNMDARs in regulating AMPAR function in the brain.

Magnocellular neurosecretory cells (MNCs), composed of vasopressin and oxytocin neurons in the hypothalamic PVN and supraoptic nucleus (SON), play a major role in fluid-balance homeostasis and reproductive function [15]. As in other brain regions, glutamate is a critical excitatory neurotransmitter in SON MNCs [16]. Glutamate-generating tonic $I_{NMDA}$ under glial control efficiently influences neuronal activity in the magnocellular neurosecretory system. Enhanced activation of eNMDARs, with blunted glial GLT-1 clearance, contributes to increased MNC activity and hormone release during dehydration and heart failure conditions [4,6]. However, the functional role(s) of eNMDARs that generate tonic $I_{NMDA}$ must be further elucidated in SON MNCs. In the present study, we demonstrated that activation of eNMDARs that generate tonic $I_{NMDA}$ facilitates AMPAR function, resulting in enhanced AMPA-induced steady-state current in SON MNCs.

**METHODS**

**Experimental animals**

All animal experiments adhered to the Chungnam National University policies regarding the care and use of animals. Male Sprague-Dawley rats (60~80 g) were housed under a 12/12-h light/dark schedule. Rats were randomly divided into two groups: euhydrated (EU) and chronic dehydrated (DE) animals. The EU group was allowed free access to normal tap water, whereas DE rats exposed by 2% saline for 7 days. All rats had access to food water ad libitum except for the DE periods throughout the experiments. Plasma osmolality was measured by freezing-point depression (Fiske Associates, Norwood, MA, USA) prior to sacrifice.

**Electrophysiological recordings and data analysis**

Hypothalamic slices (300 µm) were obtained as previously described [17]. Brian slices containing the SON were cut using a vibroslicer (Leica VT 100s, Leica, Bensheim, Germany) and placed in a holding chamber containing standard artificial cerebrospinal fluid (aCSF) until use. Standard aCSF consists of 126 mM NaCl, 26 mM NaHCO$_3$, 5 mM KCl, 2.4 mM NaH$_2$PO$_4$, 2.4 mM CaCl$_2$, 1.2 mM MgCl$_2$, and 10 mM glucose, pH 7.3-7.4. The medium was saturated with 95% O$_2$ and 5% CO$_2$.

Patch-clamp recordings were obtained using an Axopatch200-B amplifier (Axon Instruments, Foster City, CA, USA). For voltage-clamp recordings, a low Mg$^{2+}$ aCSF (20 µM MgCl$_2$) was used to facilitate NMDAR-mediated currents at a holding potential ($V_{holding}$) of ~70 mV. Periods of 180 sec of synaptic activity were analyzed using the Minianalysis 6.0.3 program (Synaptosoft Inc., Decatur, GA). In some cases, recordings were also obtained at a $V_{holding}$ of ~40 mV or ~20 mV in normal standard aCSF. Currents were recorded in the presence of picrotoxin (100 µM) to inhibit ionotropic GABA receptors, if not mentioned. Current output was filtered at 2 kHz and digitized at 10 kHz (Digidata 1322A, Axon Instruments) in conjunction with pClamp 9.2 software. Patch pipettes (3–5 MΩ) were filled with a solution containing 140 mM K-glucanote, 10 mM KCl, 10 mM HEPES, 0.5 mM CaCl$_2$, 5 mM EGTA and 5 mM Mg$^{2+}$ATP, pH 7.3.

Persistent activation of AMPARs and NMDARs was defined as the difference of holding current ($I_{holding}$) before and after application of the receptor agonists: the glutamate AMPA/kainate receptor antagonists, NBQX (2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo[f]quinoxaline-2,3-dione) or DNQX (5,7-dintroquinoxaline-2,3-dione, 10 µM), and the NMDA receptor antagonist AP5 (D,L-2-amino-5-phosphonopentanoic acid, 100 µM), respectively [17-19]. The $I_{holding}$ was measured in 50 ms epochs of traces lacking PSCs using Mini Analysis (Synaptosoft, Decatur, GA, USA).

Series resistance was monitored throughout the experiment. Neurons with series resistance changes >15% during experiments were not included in the analysis.

**Statistics**

Numerical data are presented as the mean±standard error of the mean (SEM). Statistical significance of the data was determined using independent or paired Student’s t-test as needed.
RESULTS

Electrophysiological recordings were obtained from 112 SON MNCs under bright-light microscopy [20]. In a low-Mg$^{2+}$ bath solution, AP5 induces an outward shift in the holding current (I_holding), named tonic I_{NMDA} mediated by eNMDAR in SON MNCs [6,7]. The mean amplitude of tonic I_{NMDA} in SON MNCs was 12.1±2.5 pA (n=7) in our recording conditions (20 μM of MgCl$_2$ and V_holding of −70 mV). In contrast, AP5 caused no significant changes in I_holding in normal aCSF containing 1.2 mM MgCl$_2$.

**Tonic activation of AMPARs by glutamate in SON MNCs**

To determine whether I_{NMDA} generated by eNMDARs alters AMPARs function to change the response to its agonists, glutamate was bath applied with a known concentration of the agonist (10 μM) in low Mg$^{2+}$ aCSF. Glutamate caused a steady-state inward shift in I_holding (I_{GLU}) at V_holding of −70 mV, which was blocked by the sequential application of NBQX and NBQX+AP5 in SON MNCs (Fig. 1A and 1B). Interestingly, glutamate failed to change I_holding in the presence of AP5 in SON MNCs (Fig. 1A and 1B), suggesting that glutamate induced persistent tonic activation of AMPARs in an NMDAR-activation-dependent manner in SON MNCs. In agreement with this, glutamate induced NBQX-sensitive currents in normal aCSF when V_holding was depolarized to ~40 mV or ~20 mV, activating NMDARs. While glutamate caused minimal changes in I_holding (p>0.2, n=5) at V_holding of ~70 mV, it induced NBQX-sensitive I_{GLU} at THE depolarized V_holding (~40 mV, 16.6±2.9 pA, n=7; ~20 mV, 14.6±4.9 pA, n=7) (Fig. 1C and 1D). Furthermore, I_{GLU} was completely inhibited by pretreatment with AP5 at all tested potentials in SON MNCs (data not shown). In a subset of experiments, we further confirmed that I_{GLU} was blocked by the additional application of AP5 (Fig. 1C inset).

**NMDARs blockade reduced AMPA-induced current in SON MNCs**

To determine whether NMDAR activation caused an alteration in the steady state sensitivity of the AMPA receptor-activated channels to its agonists, AMPA was bath-applied in low-Mg$^{2+}$ aCSF and normal aCSF. Application of AMPA (1 μM) induced a reproducible steady-state inward current (I_{AMPA}) in the SON MNCs, which was blocked by an AMPAR antagonist, DNQX or
ESQI (Fig. 2). We recorded and compared I\textsubscript{AMP} in the absence and presence of the NMDAR antagonist.

Although there is a tendency for I\textsubscript{AMP} amplitude to increase with repeated AMPA applications, pretreatment with AP5 consistently attenuated I\textsubscript{AMP} in the first and second AMPA applications (Fig. 2A). The inhibitory effects of AP5 on the first and second I\textsubscript{AMP} were tested in the same number of neurons and combined to compare with the control values (Fig. 2B). In the presence of AP5, I\textsubscript{AMP} amplitude was decreased to 60.3\pm4.06% of that in the absence of the antagonist in low-Mg\textsuperscript{2+} aCSF (control: 134.8\pm17.0 pA; AP5: 77.8\pm8.5 pA; n=12, p<0.001) (Fig. 2A and 2B), while pretreatment with AP5 failed to affect I\textsubscript{AMP} in normal aCSF (control: 91.7\pm10.9 pA; AP5: 86.9\pm11.5 pA; n=10, p>0.15). I\textsubscript{AMP} facilitation by NMDA was also evident in normal aCSF at depolarized V\textsubscript{holding} (Fig. 2C). In agreement with I\textsubscript{AMP} facilitation by NMDA in low-Mg\textsuperscript{2+} aCSF, AP5 reduced I\textsubscript{AMP} amplitudes from 92.0\pm11.2 pA to 65.3\pm11.7 pA (n=12, p<0.001) at V\textsubscript{holding} of –40 mV in normal aCSF. Pretreatment with AP5 also significantly reduced I\textsubscript{AMP} at V\textsubscript{holding} of –20 mV (control: 65.2\pm7.0 pA; AP5: 39.3\pm5.1 pA; n=10, p<0.001) (Fig. 2D). In a subset of experiments, AP5 inhibition of I\textsubscript{AMP} was tested in the absence of picrotoxin (Fig. 2C, inset). Pretreatment with AP5 efficiently reduced I\textsubscript{AMP} in the absence of picrotoxin at V\textsubscript{holding} of –20 mV in normal aCSF (control: 55.8\pm6.7 pA; AP5: 35.3\pm5.3 pA; n=4, p<0.01).

These results suggested that NMDAR activation enhanced the steady-state activation of AMPARs, altering the neuronal response to the receptor agonists in SON MNCs.

Roles of eNMDAR in I\textsubscript{AMP} Potentiation in SON MNCs

In the next experiments, we investigated whether eNMDARs that generate tonic I\textsubscript{NMDA} contribute to I\textsubscript{AMP} facilitation in SON MNCs. To isolate eNMDAR function, we adopted an eNMDAR selective antagonist, memantine [21,22]. To investigate the role of eNMDARs in I\textsubscript{AMP} facilitation, we recorded and compared I\textsubscript{AMP} amplitude in the absence and presence of memantine in low-Mg\textsuperscript{2+} aCSF (Fig. 3). Memantine induced an outward shift in I\textsubscript{holding}.

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\textbf{Fig. 2.} NMDAR activation facilitated AMPA-induced steady-state inward currents (I\textsubscript{AMP}) in SON MNCs. (A) Representative current traces showing I\textsubscript{AMP} in low and normal Mg\textsuperscript{2+} aCSF (V\textsubscript{holding} –70 mV). Note that pretreatment with AP5 consistently reduced I\textsubscript{AMP} in low-Mg\textsuperscript{2+} aCSF, while repeated applications of AMPA increased I\textsubscript{AMP} in normal aCSF. Bold lines at each trace represent the application of an AMPAR antagonist, DNQX or NBQX. (B) The mean I\textsubscript{AMP} amplitudes in low-Mg\textsuperscript{2+} (n=12) and normal (n=10) aCSF are summarized as in A. Effects of AP5 on the first and second I\textsubscript{AMP} were tested and combined from the same number of neurons in each group. (C) Representative current traces showing that the pretreatment of AP5 consistently reduced I\textsubscript{AMP} recorded at V\textsubscript{holding} of –40 mV in normal aCSF. I\textsubscript{AMP} inhibition by AP5 was not affected by picrotoxin in normal aCSF (V\textsubscript{holding} –20 mV, inset). Bold lines at each trace represent the application of AMPAR antagonist as in A. (D) The mean I\textsubscript{AMP} amplitudes at V\textsubscript{holding} of –40 mV (n=12) and –20 mV (n=10) are summarized. Effects of AP5 on the first and second I\textsubscript{AMP} were pooled as in B. ***p<0.001 compared to respective controls.
(13.2±3.8 pA, n=6), which is comparable to tonic I\textsubscript{NMDA} uncovered by AP5 (12.1±2.5 pA, n=7, p>0.7) in low-Mg\textsuperscript{2+} aCSF.

Pretreatment with memantine reversibly and significantly reduced I\textsubscript{AMPA} amplitudes from 152.3±24.4 pA to 114.8±24.0 pA (n=8) in low-Mg\textsuperscript{2+} aCSF (p<0.05) (Fig. 3A and 3B), while it failed to affect I\textsubscript{AMPA} in normal aCSF (control: 108.8±12.7 pA; memantine: 105.0±13.2 pA; n=6). The inhibition rate of I\textsubscript{AMPA} by pretreatment with memantine (65.8±5.4% of control, n=8) was comparable to that of AP5 (60.3±4.06% of control, n=12, p>0.4) in low-Mg\textsuperscript{2+} aCSF. Pretreatment with memantine also significantly reduced I\textsubscript{AMPA} at V\textsubscript{holding} of −40 mV in normal aCSF (control: 59.2±6.1 pA; memantine: 42.3±5.1 pA; n=6, p<0.01) (Fig. 3B). These results suggested that eNMDAR activation facilitates I\textsubscript{AMPA} in SON MNCs.

I\textsubscript{AMPA} potentiation by NMDARs during chronic dehydration

To investigate the functional significance of I\textsubscript{AMPA} facilitation by tonic I\textsubscript{NMDA} in SON MNCs, we examined and compared the I\textsubscript{AMPA} potentiation by I\textsubscript{NMDA} in euhydrated (EU) and chronic dehydrated (DE) rats (Fig. 4). Chronic dehydration with a 7-day salt loading (2% NaCl) protocol increased plasma osmolarity from 306.7±2.3 mOsm (n=4) to 378.5±7.1 mOsm (n=5). Consistent with a previous report [23], DE significantly increased EPSC frequency in SON MNCs (EU: 1.81±0.35 Hz, n=6; DE: 4.09±0.62 Hz, n=7).

Bath application of AMPA induced reproducible I\textsubscript{AMPA} in DE SON MNCs as in EU SON MNCs. Pretreatment with AP5 significantly reduced I\textsubscript{AMPA} in low-Mg\textsuperscript{2+} aCSF at V\textsubscript{holding} of −70 mV (control: 68.6±15.0; AP5: 45.9±12.8 pA; n=8, p<0.05) and in normal aCSF at V\textsubscript{holding} of −40 mV (control: 70.1±10.4 Pa; AP5: 47.9±8.6 pA; n=8, p<0.001) in DE SON MNCs. Although I\textsubscript{AMPA} amplitudes were slightly lower in DE than in EU neurons, the difference did not reach statistical significance (p>0.2 in both cases). Furthermore, pretreatment with AP5 inhibited I\textsubscript{AMPA} at similar rates in EU and DE SON MNCs (Fig 4), suggesting that I\textsubscript{AMPA} potentiation by I\textsubscript{NMDA} was preserved in DE SON MNCs.

DISCUSSION

The main findings of this study may be summarized as follows: 1) glutamate induced the tonic activation of AMPARs in a tonic I\textsubscript{NMDA}-dependent manner; 2) tonic I\textsubscript{NMDA} mediated by eNMDARs facilitated AMPA-induced steady state inward currents (I\textsubscript{AMPA}); and 3) I\textsubscript{AMPA} potentiation by eNMDARs was preserved in SON MNCs during chronic dehydration. To our knowledge, these data are the first to demonstrate that eNMDAR activity modulates the steady-state sensitivity of the AMPA receptor channels to their agonists in neuroendocrine systems.

Tonic I\textsubscript{NMDA} mediated by eNMDARs in SON MNCs

Although receptors are often considered extrasynaptic if they are located more than 100 nm from the postsynaptic density, the precise delineation of the synaptic-extrasynaptic receptors seems to be specific to the parameter under considerations [24]. In terms of electrophysiological activities, synaptic NMDARs...
are defined as receptors recruited in response to spontaneous glutamate release, generating EPSCs, or during low-frequency afferent stimulation (less than 0.05 Hz), while eNMDARs correspond to those not activated during such conditions. In this sense, there is a general consensus that eNMDARs are responsible for the persistent tonic excitatory current in SON MNCs [6,25], while their synaptic counterparts mediate conventional EPSCs. In the present study, Ianing shift by a NMDAR antagonist, AP5, supported the presence of a persistent inward current with basal glutamate release mediated by NMDARs in SON MNCs. Combined with the fact that memantine failed to affect the basic properties of glutamate EPSCs in SON MNCs [6], our results showed that memantine, a selective eNMDAR blocker, mimicked the AP5-induced Ianing shift, which confirmed the notion that eNMDARs generate tonic INMDA in the neurons. The subunit composition of the eNMDAR mediating tonic IGLU, in various brain regions has not been well clarified, while eNMDARs containing the NR2B subunit have been known to be partly responsible for tonic INMDA [6]. Future studies are warranted to delineate the subunit composition of the eNMDARs mediating IAMPA facilitation in SON MNCs.

**Modulation of AMPARs function by tonic INMDA in SON MNCs**

Our results showing that IAMPA was inhibited by AP5 or extracellular Mg2+ in low-Mg2+ aCSF suggested that activated NMDARs facilitated AMPARs function in SON MNCs. Furthermore, similar inhibitory effects of AP5 and memantine on IAMPA facilitation suggested that eNMDARs facilitated AMPAR function in the neurons. Combined with the fact that IGLU amplitudes were dependent on AP5 (Fig. 1), these results suggested that eNMDAR activity modulates the steady-state sensitivity of AMPA receptors to their agonists in SON MNCs. Given that binding glutamate/AMPA to the AMPA receptor results in Na+ influx, which causes depolarization of the membrane, our results showing that AP5 inhibited IAMPA/IGHLU could indicate that AP5 inhibited eNMDAR activated by the agonists. However, our results showing that AMPARs antagonists completely abolished IAMPA in both the absence and presence of NMDAR antagonists (Fig. 2) argued against this possibility. It is not likely that a bath application of AMPA recruited additional NMDARs generating INMDA in our recording conditions. This idea was further supported by the finding that IAMPA was insensitive to the following application of AP5 (data not shown).

In general, there has been a consensus that extrasynaptic glutamate receptors generate slow, persistent tonic currents, while their synaptic counterparts mediate AMPAR- or NMDAR-EPSCs. In the present study, it is not clear whether extrasynaptic AMPARs (eAMPARs) generate IAMPA and, if so, what portion of the currents is mediated by eAMPARs in SON MNCs. However, it is interesting to note that eAMPARs are highly mobile and move rapidly between the plasma membrane and the intracellular compartments by exocytosis and endocytosis, and diffuse laterally to and from synaptic sites [26-29]. Such continuous AMPAR exchanges between synapses and different cellular pools ensures a dynamic fit of synaptic AMPAR numbers in synaptic plasticity, including long-term potentiation (LTP). A prevailing two-step model is that NMDAR-dependent LTP is mediated by surface insertion and synaptic delivery of AMPARs, in which AMPARs deliver to the extrasynaptic sites first, and their synaptic targeting requires synaptic NMDAR activation that likely triggers the signal transduction cascade necessary to anchor AMPARs in the synapse. Given that activated eNMDARs cause Ca2+ influx [9], it is reasonable to assume that eNMDARs regulated AMPARs trafficking in the present study, resulting in IAMPA facilitation. As a gliotransmitter, ATP has been known to activate purinergic receptors in PVN MNCs, promoting the insertion of AMPARs at the surface and, strengthening the excitatory synapses [14]. However, it is noteworthy that NMDAR-induced CaM-KII activation alters AMPARs channel properties in both a Ca2+-dependent and a Ca2+-independent manner [30,31]. It is also possible that INMDA facilitated AMPARmediate currents via increased single channel conductance and/or open channel probability of AMPAR activated channels in SON MNCs. Future studies are warranted to delineate the cellular mechanisms of eNMDAR-mediated IAMPA facilitation in SON MNCs.

**Functional significance of IAMPA potentiation by eNMDARs in SON MNCs**

A peculiar property of the SON is that it undergoes anatomical remodeling under certain physiological conditions, such as lactation and chronic dehydration. This remodeling includes a reduction in astrocytic coverage of neurons [52] and is associated with increased extracellular levels of glutamate in the SON [33]. The increased extracellular level of glutamate in the nucleus is also in agreement with enhanced EPSC frequency [23] and increased numbers of excitatory synaptic contacts in SON MNCs. Combined with our results showing that IAMPA facilitation by INMDA is conserved in DE SON MNCs, these results suggested that IAMPA facilitation by INMDA could contribute to enhanced neuronal activity and hormone release from the neurons during the osmotic challenge. Spontaneous glutamate release from astrocytes synchronizes neuronal activity via eNMDAR activation [34,35], and increases the frequency of AMPAR-mediated EPSCs via the activation of metabotropic glutamate receptors facilitating presynaptic release in CA1 pyramidal neurons [36] and kainite receptors in hippocampal interneurons [37], respectively. Combined with the fact that Ca2+ influx triggers the signal transduction cascade necessary for anchoring AMPARs in synapses [26,38], our results showing that eNMDAR activation potentiated AMPAR-mediated steady-state currents in SON MNCs are in line with astrocytic
glutamate release altering AMPARs, resulting in strengthening of excitatory synapses in SON MNCs.

In summary, our results support the view that tonic $I_{NMDA}$ generated by activated eNMDARs facilitates AMPA receptor function in SON MNCs, which gives the gliotransmitter glutamate the ability to regulate postsynaptic efficacy during normal and physiological challenges, including chronic dehydration.

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CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

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