

Tonic NMDAR Currents of NR2A-Containing NMDARs Represent Altered Ambient Glutamate Concentration in the Supraoptic Nucleus

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Abstract

NMDA receptors (NMDARs) modulate glutamatergic excitatory tone in the brain via two complementary modalities: a phasic excitatory postsynaptic current and a tonic extrasynaptic modality. Here, we demonstrated that the tonic NMDAR-current (I_{NMDA}) mediated by NR2A-containing NMDARs is an efficient biosensor detecting the altered ambient glutamate level in the supraoptic nucleus (SON). I_{NMDA} of magnocellular neurosecretory cells (MNCs) measured by nonselective NMDARs antagonist, AP5, at holding potential (V_{holding}) -70 mV in low concentration of ECF Mg^{2+} ($[\text{Mg}^{2+}]_o$) was transiently but significantly increased 1-week post induction of a DOCA salt hypertensive model rat which was compatible with that induced by a NR2A-selective antagonist, PEAQX (I_{PEAQX}) in both DOCA- H_2O and DOCA-salt groups. In agreement, NR2B antagonist, ifenprodil, or NR2C/D antagonist, PPDA, did not affect the holding current (I_{holding}) at V_{holding} -70 mV. Increased ambient glutamate by exogenous glutamate (10 mM) or excitatory amino acid transporters (EAATs) antagonist (TBOA, 50 mM) abolished the I_{PEAQX} difference between two groups, suggesting that attenuated EAATs activity increased ambient glutamate concentration, leading to the larger I_{PEAQX} in DOCA-salt rats. In contrast, only ifenprodil but not PEAQX and PPDA uncovered I_{NMDA} at V_{holding} $+40$ mV under 1.2 mM $[\text{Mg}^{2+}]_o$ condition. $I_{\text{ifenprodil}}$ was not different in DOCA- H_2O and DOCA-salt groups. Finally, NR2A, NR2B, and NR2D protein expression were not different in the SON of the two groups. Taken together, NR2A-containing NMDARs efficiently detected the increased ambient glutamate concentration in the SON of DOCA-salt hypertensive rats due to attenuated EAATs activity.

Key words: NMDARs; NR2A; SON MNCs

Significance Statement

The NMDAR-mediated excitatory tone between cells is transmitted via phasic activation of synaptic NMDARs (EPSCs) and tonic activation of extrasynaptic NMDARs (I_{NMDA}) in the brain. The activation of NMDARs depends on the glutamate concentration, NMDAR subunit composition, and their subcellular localization, as well as the membrane potential. Therefore, the mechanism of NMDAR-mediated excitatory tone varies in different pathophysiological conditions. Our results show that the I_{NMDA} in nondepolarized and depolarized neurons is dominantly mediated by NR2A- and NR2B-containing NMDARs, respectively, and the former efficiently detects the ambient glutamate concentration in the supraoptic magnocellular neuroendocrine cells of normal and hypertensive rats. This study shows that NR2A-containing NMDARs could be a biosensor detecting ambient glutamate concentration in the brain.

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Introduction

Glutamate is a major excitatory neurotransmitter in the supraoptic nucleus (SON; van den Pol et al., 1990), which is composed of vasopressin and oxytocin magnocellular neurosecretory cells (MNCs). These neurons are known to play critical roles in fluid balance and cardiovascular and reproductive homeostasis (Silverman and Zimmerman, 1983). Neurohumoral activation has a direct impact on morbidity/mortality in cardiovascular diseases (Cohn et al., 1984; Yemane et al., 2010). In addition to the classical transient excitatory postsynaptic currents (EPSCs) mediated by synaptic receptors, glutamate generates a tonic, sustained excitatory current (I_{NMDA}) when it binds to extrasynaptic NMDARs (eNMDARs) that strongly stimulate firing activity in SON MNCs (Fleming et al., 2011). NMDARs are heterotetramers composed of two NR1 subunits and two NR2 subunits. NMDARs containing NR2A, B, C, and D subunits encoded by four different genes (*GluN2A-D*) exhibit distinct electrophysiological and pharmacological properties as well as different subsynaptic distributions and expression profiles. For example, eNMDARs containing NR2B or NR2D subunit could mediate I_{NMDA} with their tonic activation (Fleming et al., 2011; Neupane et al., 2021), while NR2A-containing NMDARs, predominantly found in synaptic space, mediate EPSCs. NR2D-containing eNMDARs even generate a “Mg²⁺-resistant” I_{NMDA} activated in nondepolarized SON MNCs under the physiological concentration of $[\text{Mg}^{2+}]_o$ (Neupane et al., 2021). Given that elevated glutamatergic excitatory tone supports exacerbated activity of MNCs (Biancardi et al., 2010; Li et al., 2014; Glass et al., 2015; Zhang et al., 2017), which in turn contributes to neurohumoral activation during cardiovascular diseases, elucidating the precise roles of different NMDARs and their subunit plasticity altering MNCs activity in diseases such as hypertension and heart failure is of critical importance.

Under conditions requiring strong secretion of neurohypophysial hormones, there is a pronounced reduction in the astrocytic coverage of SON MNCs (Theodosis and Poulain, 1993; Bobak and Salm, 1996). Neuroglia remodeling in response to physiological challenges resulted in blunted glutamate transporter (GLT) activity leading to increased ambient glutamate in the SON (Fleming et al., 2011), which may depolarize the neurons despite the Mg²⁺ block of NMDARs (Mayer et al., 1984; Nowak et al., 1984). Given that robust NR2D protein expression in the SON MNCs is uncommon in the adult brain (Doherty and Sladek, 2011) and that NR2D generates an “Mg²⁺-resistant” I_{NMDA} of MNCs in a state-dependent manner (Neupane et al., 2021), we investigated whether NR2D-containing eNMDARs contribute to exacerbated I_{NMDA} in SON neurons during hypertension and whether this finding could be a mechanism contributing in turn to neurohumoral activation in this cardiovascular disease. Unexpectedly, our results demonstrate that in DOCA-salt hypertensive rats, a decreased activity of excitatory amino acid transporters (EAATs) resulted in increased ambient glutamate levels to potentiate a Mg²⁺-sensitive I_{NMDA} mediated by NMDAR-containing NR2A rather than NR2B and NR2D.

Materials and Methods

DOCA-salt hypertension model. All animal experimentation was approved by the Institutional Animal Care and Use Committee and was conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Male 5-week-old Sprague Dawley (SD) rats weighing 120–180 g were purchased from Animals. All animals were housed on a 12-hour light/dark cycle and had access to food ad libitum throughout the experiments. After 1 week of acclimatization, rats were anesthetized with avertin (250 mg/kg, i.p.; Millipore Sigma) and underwent unilateral nephrectomy (left kidney) as previously described (Prahalthan et al., 2012). Briefly, a lateral abdominal incision was made to access the left kidney for its resection and sutured after the removal of the kidney. After 1 week, DOCA (D7000-5G, Sigma-Aldrich) was implanted subcutaneously in all nephrectomized rats, and animals were randomly assigned to either the H₂O group (DOCA-H₂O) or salt group (DOCA-salt). The DOCA-salt group had their water replaced with a mixture of 0.8% NaCl and 0.2% KCl in tap water to drink until killed (after 1 or 4 weeks).

Blood pressure measurement. Systolic blood pressure was measured once a week for a month by tail cuff method as previously described (Kubota et al., 2006). Rats were restrained in a cylindrical restrainer at 37°C for 30 min to acclimatize them to the apparatus

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(noninvasive blood pressure system, CODA; Kent Scientific Corporation) before blood pressure recordings were made. Systolic blood pressure was measured in awake rats using a noninvasive tail cuff blood pressure measuring system (PowerLab/8SP data acquisition system, ADInstruments) before DOCA treatment and on the 1, 2, 3, and 4 weeks of DOCA treatment. The physiological data were analyzed using the LabChart 6.1 Pro software (ADInstruments). Averaged blood pressure from at least five consecutive readings obtained from each rat was recorded as final blood pressure.

Water intake, urine output, serum osmolality, and urine osmolality measurements. After 6th day of DOCA treatment, both DOCA-H₂O and DOCA-salt groups were individually housed in metabolic cages provided with H₂O or salt for 24 hours before sample collection. Urine output and water intake were measured within the following 24 hours. In addition, on the 7th day, a fresh urine sample was collected in a tube, and animals were anesthetized with avertin (250 mg/kg, i.p.; Millipore Sigma) for blood collection by cardiac puncture. Both urine and blood were centrifuged. After centrifugation, urine and serum osmolality were measured using the freezing point depression method and a micro-osmometer (model 210, Fiske Associates).

Electrophysiology and data analysis. Patch-clamp recordings from SON MNCs were obtained from acutely prepared hypothalamic slices (300 μ m) as previously described (Neupane et al., 2021). Briefly, rats were decapitated under avertin anesthesia (Avertin, 200 mg/kg, i.p.), and the brains were quickly extracted. Sectioned slices were incubated in artificial CSF (aCSF) containing (in mM) 126 NaCl, 5 KCl, 1.2 MgCl₂, 26 NaHCO₃, 1.2 NaH₂PO₄, 10 glucose, and 2.4 CaCl₂, pH 7.3–7.4, and saturated with 95% O₂ and 5% CO₂ within the slice holder for 1 h at 34°C in the presence of 3 μ M glutamic acid. Single hemisectioned slices were transferred to a recording chamber perfused with aCSF saturated with 95% O₂ and 5% CO₂ and maintained at 34°C. All electrophysiological measurements were recorded using a MultiClamp 700B (Molecular Devices). The current output was filtered at 1 kHz and digitized at 10 kHz (Digidata 1440 and pClamp 10.2 software, Molecular Devices). Data were excluded if the series resistance was not stable throughout the entire recording (20% change) or if neuronal input resistance (IR) was <550 M Ω at the beginning of the recording. The NMDA receptor-mediated tonic current (tonic I_{NMDA}) was defined as changes in the holding current (I_{holding}) in the presence of ionotropic GABA receptor antagonists and was calculated by the difference in I_{holding} measured as the average of a 2-minute steady-state baseline segment obtained before and after the application of NMDAR antagonists. I_{NMDA} was recorded and calculated at -70 mV with low $[\text{Mg}^{2+}]_o$ (20 μ M) or $+40$ mV with normal aCSF containing 1.2 mM $[\text{Mg}^{2+}]_o$ unless otherwise stated. Event detection and analysis of spontaneous EPSCs were carried out using MiniAnalysis software (Synaptosoft) at $V_{\text{holding}} -70$ mV, as previously described (Park et al., 2007). The detection threshold was set at -20 pA and 75 pA/ms for EPSC amplitude and area, respectively. From extracted EPSCs, frequency, amplitude, and decay time constant were calculated. EPSC decay time constants were calculated from single exponential fits.

Drugs were added to the aCSF perfusing solution at differing concentrations. The final concentration of dimethylsulfoxide (DMSO) was <0.05%, when used as a vehicle. DL-2-amino-5-phospho-nopentanoic acid (AP5), (2S*, 3R*) -1-(phenanthren-2-carbonyl) piperazine-2, 3-dicarboxylic acid (PPDA), [[[(1S)-1-(4-bromophenyl)ethyl]amino](1,2,3,4-tetrahydro-2,3-dioxo-5-quinoxaliny)methyl] phosphonic acid tetrasodium salt (PEAQX) and DL-*threo*- β -benzyloxyaspartic acid (TBOA) were all purchased from Tocris Bioscience. All other drugs were purchased from Sigma-Aldrich.

Western blotting. Brain tissue punches containing the SON were collected from 300 μ m coronal hypothalamic slices (three sequential slices/rats) from each brain as described previously (Potapenko et al., 2012). Protein was extracted from the SON punches using a mixture of a protease inhibitor and radioimmunoprecipitation assay (RIPA) lysis buffer and quantified using a bicinchoninic acid (BCA) assay kit (Thermo Fisher Scientific). Approximately 50 μ g of protein was separated on a 10% sodium dodecyl sulfate (SDS)-polyacrylamide gel and transferred to a nitrocellulose membrane. The membranes were then blocked with TBST (0.1% Tween 20 in 1 \times Tris-buffered saline) containing 5% skimmed milk for 1 h at room temperature. The blot was then probed with primary antibodies against NMDAR NR2A (1:1,000; catalog #AGC-002, Alomone Labs; RRID: AB_2756596), NR2B (1:1,000; catalog #06-600, Millipore; RRID: AB_310193), and NR2D subunits (1:1,000; catalog #AGC-020, Alomone Labs; RRID: AB_10658334) overnight at 4°C. Next, the membranes were exposed to horseradish peroxidase-conjugated goat anti-rabbit (catalog #7074, Cell Signaling Technology; RRID: AB_2099233) and anti-mouse (catalog #31430, Thermo Fisher Scientific; RRID: AB_228307) secondary antibody (1:1,000) at room temperature for 1 h. Proteins were visualized using a pierce enhanced chemiluminescence detection kit (Thermo Fisher Scientific), and the intensity of the bands was measured using ImageJ software 1.42q (National Institutes of Health).

Statistical analysis. Numerical data are presented as the mean \pm SEM. To assess the differences in tonic I_{NMDA} under DOCA-salt conditions, we performed a hierarchical testing procedure. In the first step, a Shapiro–Wilk test was used to test the null hypothesis that the data distribution was normal with a significance level of 5%. For data with a normal distribution, the statistical significance of comparisons was assessed using either a two-sample *t* test or a one-way ANOVA followed by a post hoc test (e.g., Bonferroni's test). If the null hypothesis was rejected, nonparametric tests were used with Microcal Origin software (RRID: SCR_002815). For all experiments, male rats were used to avoid effects of hormonal changes on the results. Tonic I_{NMDA} was categorized into two modalities: either the Mg²⁺-sensitive tonic I_{NMDA} , measured by I_{holding} shift induced by increasing the extracellular Mg²⁺ concentration from 20 μ M to 1.2 mM at V_{h} , -70 mV, or the Mg²⁺-resistant tonic I_{NMDA} , measured by I_{holding} shift induced by NMDAR antagonists at V_{h} , -70 mV in normal aCSF. Electrophysiological

recordings were taken from three or more animals per group, and three to four slices were collected per animal. The Mann-Whitney test was used to compare two groups if the data were not normally distributed. The pharmacological sensitivity of tonic I_{NMDA} to various NMDAR antagonists was investigated. To compare I_{NMDA} amplitude and protein expression (NR2A, NR2B, and NR2D) in the H₂O group and DOCA-salt group, we used one-way ANOVA followed by a post hoc test.

Results

Transient increase of I_{NMDA} in SON MNCs in DOCA-salt rats

DOCA-salt treatment successfully induced hypertension as shown by a time-dependent increase in systolic arterial pressure (SAP) in rats. The nephrectomy/DOCA-salt treatment (DOCA-salt) elicited a significant increase in the SAP ($F_{(2,72)} = 48.03$; $p = 0.01$; two-way ANOVA). SAP tended to increase at 1 week, increased significantly at 2 weeks, reached the maximal hypertension at 3 weeks, and was maintained up to 4 weeks post-DOCA implantation (PDI) in DOCA-salt group, while the nephrectomy/DOCA alone with no NaCl and KCl (DOCA-H₂O) did not affect SAP during the period compared with normal control animals ($p = 0.62$; Bonferroni's post hoc test following two-way ANOVA; Fig. 1A).

Increased plasma vasopressin (VP), a neurohypophysial hormone, level may affect the fluid homeostasis (Boone and Deen, 2008; Parekh et al., 2021). Although SAP increase did not reach statistical significance at 1 week PDI (DOCA-H₂O 119.34 ± 0.975 mmHg, $n = 5$ vs DOCA-salt 123.99 ± 2.69 mmHg, $n = 18$ rats), the volume of water intake and urine output were significantly increased in DOCA-salt than DOCA-H₂O groups at 1 week PDI (Table 1). In addition, the urine osmolality was significantly lower in DOCA-salt than that in DOCA-H₂O rats, meanwhile the serum osmotic pressure was not different in the two groups (Table 1).

In next experiment, we investigated I_{NMDA} in SON MNCs in DOCA-H₂O and DOCA-salt groups at 1, 2, and 4 weeks PDI. Increasing $[\text{Mg}^{2+}]_o$ from 20 μM to 1.2 mM induced a significantly larger I_{holding} in DOCA-salt (15.17 ± 2.32 pA, $n = 19$ neurons from 7 rats) compared with DOCA-H₂O group (6.25 ± 0.98 pA, $n = 9$ neurons from 3 rats) at 1 week PDI (Fig. 1B,C), while the difference was not observed at 2 and 4 weeks PDI. An additional NMDAR antagonist, AP5, failed to cause further I_{holding} shift in all tested groups (Fig. 1B,C). These results suggested that more NMDARs could be activated to generate Mg^{2+} -sensitive I_{NMDA} in SON MNCs in DOCA-salt rats at 1 week PDI.

Pharmacology of the enhanced I_{NMDA} in DOCA-salt rats

To assess the composition of NMDARs that potentiate Mg^{2+} -sensitive I_{NMDA} at 1 week PDI, we investigated I_{NMDA} at $V_{\text{holding}} -70$ mV in low Mg^{2+} aCSF and compared its sensitivity with NR2A, NR2B, and NR2C/D subunit selective antagonists in DOCA-H₂O and DOCA-salt group (Fig. 2).

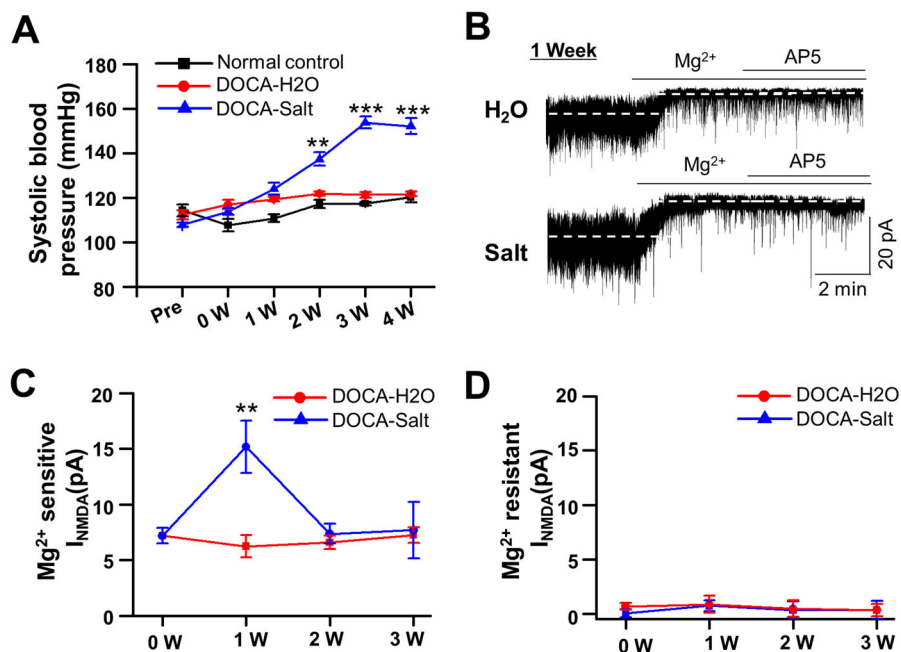


Figure 1. Increased I_{NMDA} of SON MNCs in DOCA-salt rats. **A**, Systolic blood pressure at different times in naive control (normal control, $n = 6$), DOCA-H₂O ($n = 6$), and DOCA-salt model rats (DOCA-salt, $n = 18$) ($*p < 0.05$, $***p < 0.001$, compared with normal control). **B**, Representative current traces showing effects of Mg^{2+} , followed by the sequential application of AP5 (200 μM), a NMDA receptor antagonist, in holding current in DOCA-H₂O and DOCA-salt model rats at 1 week. Representative current traces show the effect of sequential application of Mg^{2+} (1.2 mM) and additional AP5 (100 mM) on the holding current of SON MNCs at 1, 2, and 4 weeks. **C, D**, Summarized bar graph showing average tonic current amplitude block by Mg^{2+} (**C**) and additional AP5 (**D**) in each week, respectively. $**p < 0.01$, compared with DOCA-H₂O.

Table 1. Changes in metabolic parameters after 1 week in DOCA-salt induced hypertensive model

Parameters	DOCA-H ₂ O	DOCA-Salt	<i>p</i> value
Serum osmolality (mOsm/L)	313 ± 3.26	310 ± 1.45	0.40
Urine osmolality (mOsm/L)	1,098 ± 60.24	657 ± 31.49	<0.001
Water intake (ml/d)	15 ± 4.97	145 ± 13.67	<0.001
Urine output (ml/d)	13.66 ± 2.58	127 ± 14.56	<0.001

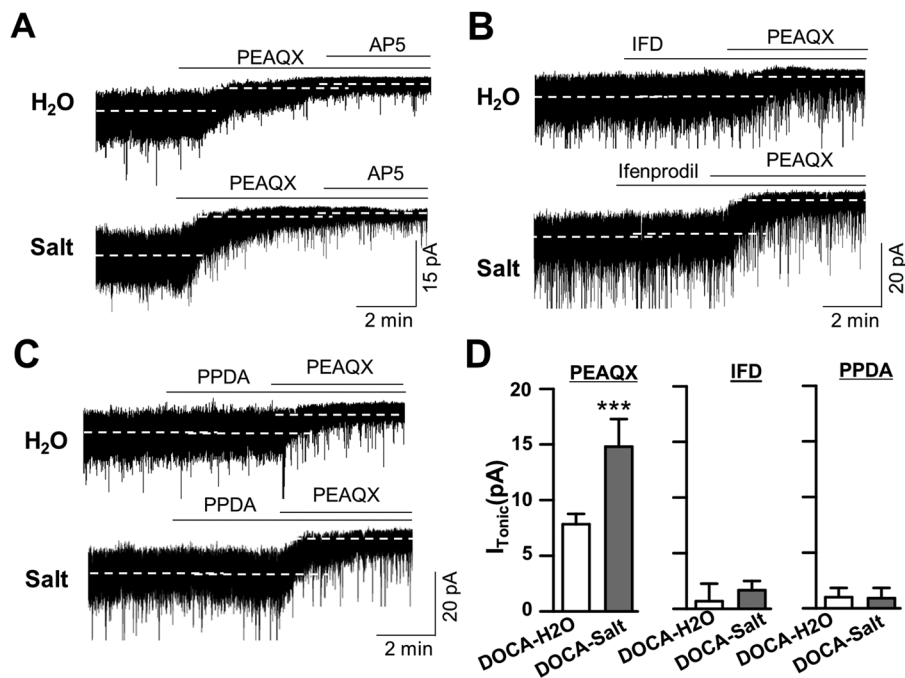


Figure 2. Pharmacology of I_{NMDA} in DOCA-H₂O and DOCA-salt group. **A–C**, Representative current traces showing the effect of PEAQX (1 μM), a NR2A receptor antagonist (**A**), ifenprodil (30 μM), an NR2B receptor antagonist (**B**), and PPDA (1 μM), an NR2C/D antagonist (**C**) on holding current. Note that ifenprodil and PPDA caused minimal effects on $I_{holding}$, while additional PEAQX caused larger $I_{holding}$ changes in DOCA-salt groups. **D**, Summarized bar graph showing mean of holding current change by each antagonist. * $p < 0.05$, compared with DOCA-H₂O; $n = 6$ and 8 in DOCA-H₂O and DOCA-salt, respectively.

PEAQX, an NR2A subunit antagonist, uncovered I_{NMDA} (I_{PEAQX}) of nondepolarized SON MNCs under low Mg²⁺ condition in both DOCA-H₂O group (7.84 ± 0.92 pA; $n = 6$ neurons from 3 rats; $F_{(2,15)} = 16.23$; $p = 0.003$; Bonferroni's post hoc test following one-way RM-ANOVA) and DOCA-salt (14.80 ± 2.49 pA; $n = 8$ neurons from 3 rats; $F_{(2,15)} = 33.14$; $p < 0.001$; Bonferroni's post hoc test following one-way RM-ANOVA). I_{PEAQX} was significantly larger in DOCA-salt than that in DOCA-H₂O group ($p < 0.001$, two-sample t test). However, additional AP5 (PEAQX + AP5) caused minimal $I_{holding}$ shift in both groups (DOCA-H₂O, 0.80 ± 1.42 pA, vs DOCA-salt, 0.77 ± 0.94 pA; Fig. 2A,D). In contrast, ifenprodil (IFD, a selective NR2B antagonist) failed to cause $I_{holding}$ shift in both DOCA-H₂O ($F_{(2,15)} = 9$; $n = 6$ neurons from 3 rats; $p = 0.93$; Bonferroni's post hoc test following one-way RM-ANOVA) and DOCA-salt ($F_{(2,18)} = 33.22$; $n = 8$ neurons from 4 rats; $p = 0.99$; Bonferroni's post hoc test following one-way RM-ANOVA), while additional PEAQX uncovered I_{NMDA} in both groups (DOCA-H₂O, $p = 0.01$, and DOCA-salt, $p < 0.001$; Bonferroni's post hoc test following one-way RM-ANOVA in both cases). Notably, additional PEAQX (IFD + PEAQX) uncovered larger I_{NMDA} in DOCA-salt compared with DOCA-H₂O group (DOCA-H₂O, 6.80 ± 1.73 pA vs DOCA-salt, 14.27 ± 2.17 pA; $p < 0.001$; two-sample t test).

Similarly, PPDA (a selective NR2C/2D antagonist) failed to cause $I_{holding}$ shift in the both DOCA-H₂O ($F_{(2,21)} = 10.99$; $n = 8$ neurons from 3 rats; $p = 0.65$; Bonferroni's post hoc test following one-way RM-ANOVA) and DOCA-salt ($F_{(2,27)} = 42.56$; $n = 10$ neurons from 4 rats; $p = 0.84$; Bonferroni's post hoc test following one-way RM-ANOVA), while additional PEAQX uncovered I_{NMDA} in both groups (DOCA-H₂O, $p = 0.009$, and DOCA-salt, $p < 0.001$; Bonferroni's post hoc test following one-way RM-ANOVA in both cases). Consistently, additional PEAQX (PPDA + PEAQX) uncovered larger I_{NMDA} in DOCA-salt compared with DOCA-H₂O group (DOCA-H₂O, 5.45 ± 1.73 pA vs DOCA-salt, 12.58 ± 1.62 pA; $p < 0.001$; two-sample t test). These results suggested that Mg²⁺-sensitive I_{NMDA} predominantly represented the activation of NR2A-containing NMDARs in nondepolarized SON MNCs in both DOCA-H₂O and DOCA-salt groups.

Decreased EAAT activity in the SON of DOCA-salt rats

EAATs that uptake glutamate, particularly the astrocytic glutamate transporter-1 (GLT1) and glutamate/aspartate transporter (GLAST) isoforms, potentially regulate glutamate clearance to maintain ambient glutamate levels in the central

nervous system (Fleming et al., 2011; Sun et al., 2014). Increased neurohumoral drive such as heart failure condition-induced glial remodeling caused decreased GLT1 and increased GLAST to elevate ambient glutamate level; thus, increased I_{NMDA} of SON MNCs (Potapenko et al., 2012) and similar changes in GLT1 was also observed in SON MNCs during dehydration (Fleming et al., 2011). To know if this is the case in DOCA-salt rats, we compared endogenous EAAT activity by quantifying the magnitude of the I_{NMDA} evoked by a EAAT blocker in DOCA-H₂O and DOCA-salt rats (Fig. 3).

Bath application of the nonselective EAAT blocker TBOA (100 μM) induced a large inward shift in I_{holding} (I_{TBOA}) in SON MNCs in both DOCA-H₂O ($F_{(2,15)} = 54.09$; $n = 6$ neurons from 3 rats; $p < 0.001$; Bonferroni's post hoc test following one-way RM-ANOVA) and DOCA-salt ($F_{(2,18)} = 34.57$; $n = 7$ neurons from 3 rats; $p < 0.001$; Bonferroni's post hoc test following one-way RM-ANOVA). Interestingly, TBOA induced inward shift in I_{holding} (I_{TBOA}) was significantly smaller in DOCA-salt (DOCA-salt, -92.74 ± 14.48 pA) than that in DOCA-H₂O group (-164.47 ± 20.92 pA; $p = 0.01$; two-sample t test). I_{TBOA} was mostly blocked by AP5; thus, the additional application of kynurenic acid, KynA (AP5 + KynA), induced only a minimal I_{holding} shift in both groups (Fig. 3A). These results suggested that attenuated glutamate transporter activity increased extracellular glutamate concentration, resulting in turn in the activation of a Mg²⁺-sensitive I_{NMDA} in DOCA-salt group.

Exogenous glutamate equalizes I_{NMDA} and I_{PEAQX} in DOCA-H₂O and DOCA-salt rats

Next, we further tested whether increasing extracellular glutamate levels potentiated the Mg²⁺-sensitive I_{NMDA} in SON MNCs in DOCA-salt group compared with those in DOCA-H₂O rats (Fig. 4).

In the presence of glutamate (10 μM), both PEAQX and additional AP5 (PPDA + AP5) caused significant changes in both DOCA-H₂O ($F_{(2,15)} = 6.09$; $p = 0.05$; one-way RM-ANOVA, $n = 6$ neurons from 3 rats) and DOCA-salt ($F_{(2,21)} = 12.82$; $p < 0.001$; one-way RM-ANOVA; $n = 8$ neurons from 3 rats). I_{PEAQX} was not different in DOCA-H₂O (31.87 ± 10.89 pA) from DOCA-salt group (33.25 ± 9.3 pA; $p = 0.867$; two-sample t test). Additional AP5 (PEAQX + AP5) induced similar outward shift in I_{holding} in both groups (DOCA-H₂O, 21.16 ± 10.38 pA vs DOCA-salt, 14.47 ± 4.47 pA; two-sample t test; $p = 0.351$). As a result, total I_{NMDA} was not different in DOCA-H₂O (53.03 ± 21.14 pA) and DOCA-salt groups (47.73 ± 12.43 pA; two-sample t test; $p = 0.820$).

These results suggested that the increased I_{NMDA} in DOCA-salt rats was due to blunted GLUT activity, leading to increased levels of endogenous glutamate, but not due to increase or changes in extrasynaptic NMDARs. Moreover, these results indicate that I_{NMDA} is mediated by NR2A receptors in both groups.

To determine whether changes in the expression of NMDAR subunits contributed to altered I_{NMDA} in DOCA-salt rats, we compared the expression of NR2A-D in DOCA-H₂O and the DOCA-salt groups at 1 week PDI. Western blot results showed no significant difference in NR2A-D protein expression between the groups (Fig. 5A,B).

$I_{\text{ifenprodil}}$ in depolarized SON MNCs did not differ between DOCA-H₂O and DOCA-salt rats

We next investigated the sensitivity of I_{NMDA} to NR2A, NR2B, and NR2C/D subunit selective antagonists in depolarized neurons ($V_{\text{holding}} +40$ mV; Fig. 6).

In agreement with the previous reports (Fleming et al., 2011; Neupane et al., 2021), IFD caused an inward I_{holding} shift ($I_{\text{ifenprodil}}$) in depolarized SON MNCs in both DOCA-H₂O ($F_{(2,15)} = 19.25$; $n = 6$ neurons from 3 rats; $p < 0.001$; Bonferroni's post hoc test following one-way RM-ANOVA) and DOCA-salt ($F_{(2,18)} = 32.16$; $n = 7$ neurons from 3 rats; $p < 0.001$; Bonferroni's post hoc test following one-way RM-ANOVA). $I_{\text{ifenprodil}}$ was not different in DOCA-H₂O (26.93 ± 4.46 pA) and DOCA-salt group (26.25 ± 12.74 pA; two-sample t test; $p = 0.960$). In contrast, PEAQX and PPDA failed to cause significant I_{holding} changes in both DOCA-H₂O (PEAQX, $p = 0.96$ and PPDA, $p = 0.91$; Bonferroni's post hoc test

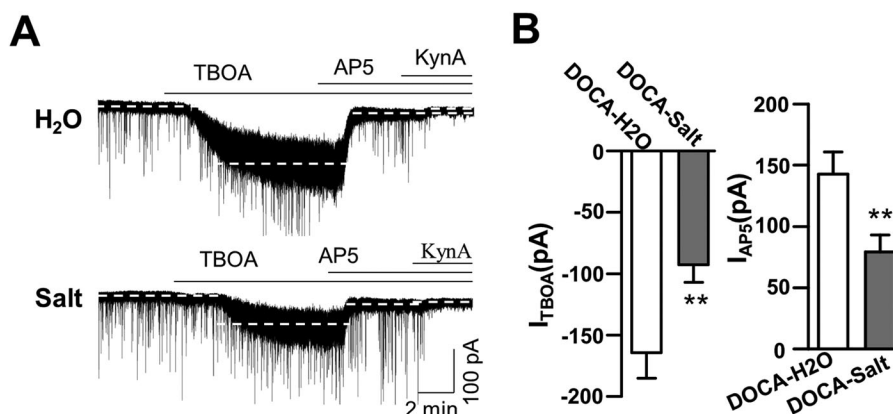


Figure 3. Comparison of I_{NMDA} in the presence of EAAT antagonist, TBOA, in DOCA-H₂O and DOCA-salt models. **A**, Representative current traces showing the effects of TBOA, a glutamate transporter blocker (100 μM), followed by the sequential application of AP5 (200 μM), an NMDA receptor antagonist and kynurenic acid (5 mM), a glutamate receptor antagonist, in holding current. Note that AP5 completely reversed I_{holding} change induced by TBOA in both DOCA-H₂O and DOCA-salt. **B**, Summarized bar graph showing the mean of holding current change by TBOA (left) and additional AP5 (right). * $p < 0.05$, compared with DOCA-H₂O; $n = 6$ and 7 in DOCA-H₂O and DOCA-salt, respectively.

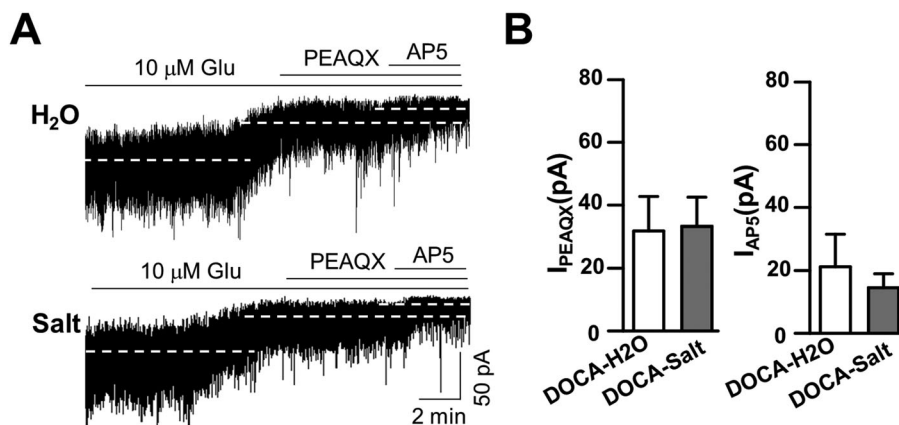


Figure 4. Comparison of I_{NMDA} in the presence of exogenous glutamate in DOCA-H₂O and DOCA-salt groups. **A**, Representative current traces show the effect of PEAQX and additional AP5 (100 μ M) on the holding current of SON MNCs. Note that PEAQX and AP5 caused similar changes in $I_{holding}$. **B**, Summarized bar graph showing mean of holding current change by PEAQX (left) and additional AP5 (right); $n=7$ and 9 in DOCA-H₂O and DOCA-salt, respectively.

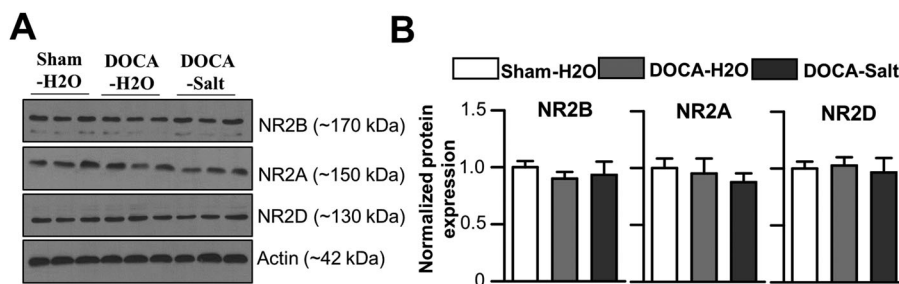


Figure 5. The expression of NMDAR subunit in the DOCA-H₂O and DOCA-salt groups. **A**, Representative image of a Western blot showing NR2A, NR2B, and NR2D subunit expression in DOCA-H₂O and DOCA-salt groups. **B**, Summarized bar graph showing the relative expression of NMDARs in the SON of DOCA-H₂O and DOCA-salt groups. The protein expression was normalized to the level detected in the DOCA-H₂O group and compared with the expression in DOCA-salt animals. Summarized data shown are the mean \pm SE ($n=3$ rats).

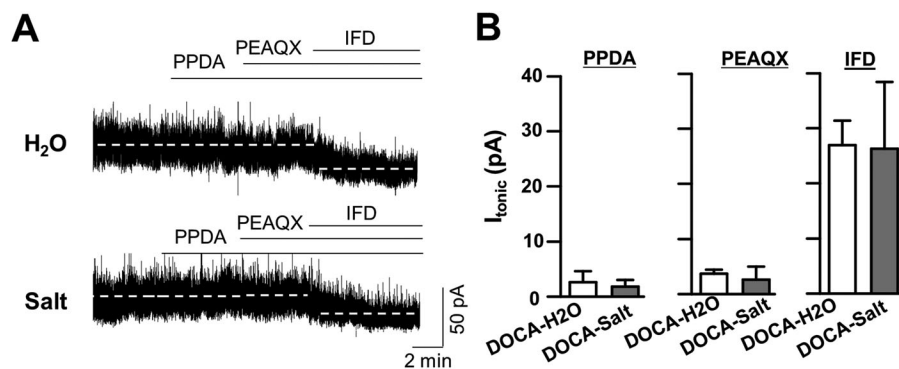


Figure 6. Pharmacology of I_{NMDA} in depolarized SON MNCs. **A**, Representative traces showing the $I_{holding}$ changes induced by the sequential addition of 1 μ M PPDA, 1 μ M PEAQX, and 10 μ M IFD in DOCA-H₂O and DOCA-salt groups with a depolarized membrane potential (V_h , +40 mV). The dotted lines indicate the mean $I_{holding}$ under each condition. **B**, Summarized $I_{holding}$ changes induced by PPDA, PEAQX, and IFD in SON MNCs from both DOCA-H₂O and DOCA-salt groups; $n=6$ in each group.

following one-way RM-ANOVA) and DOCA-salt rats (PEAQX, $p=0.95$ and PPDA, $p=0.89$; Bonferroni's post hoc test following one-way RM-ANOVA in both cases; Fig. 5C,D). These results suggested that $I_{ifenprodil}$ in depolarized SON MNCs did not contribute to the I_{NMDA} mediated by increased ambient glutamate at 1 week PDI.

I_{PEAQX} but not $I_{ifenprodil}$ sensed an increased ambient glutamate concentration in DOCA-salt rats

In the next experiments, we directly investigated the hypothesis that altered ambient glutamate concentration could be sensed by I_{PEAQX} in nondepolarized SON MNCs. For this, we compared the effects of PEAQX on I_{NMDA} in the absence and presence of exogenous glutamate (10 μ M; Fig. 7).

Low Mg²⁺ aCSF, -70 mV

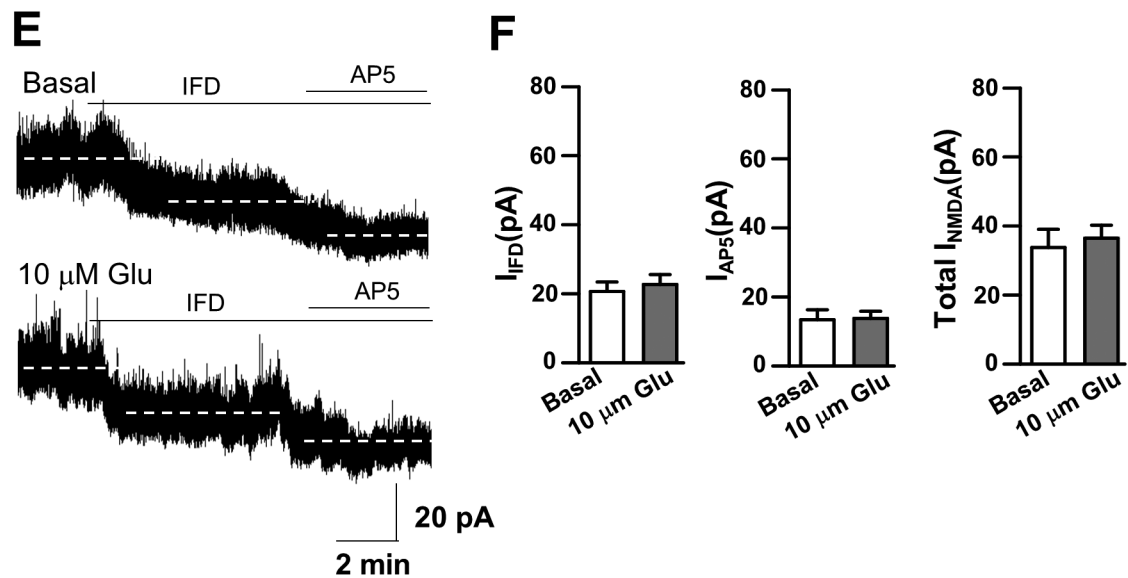
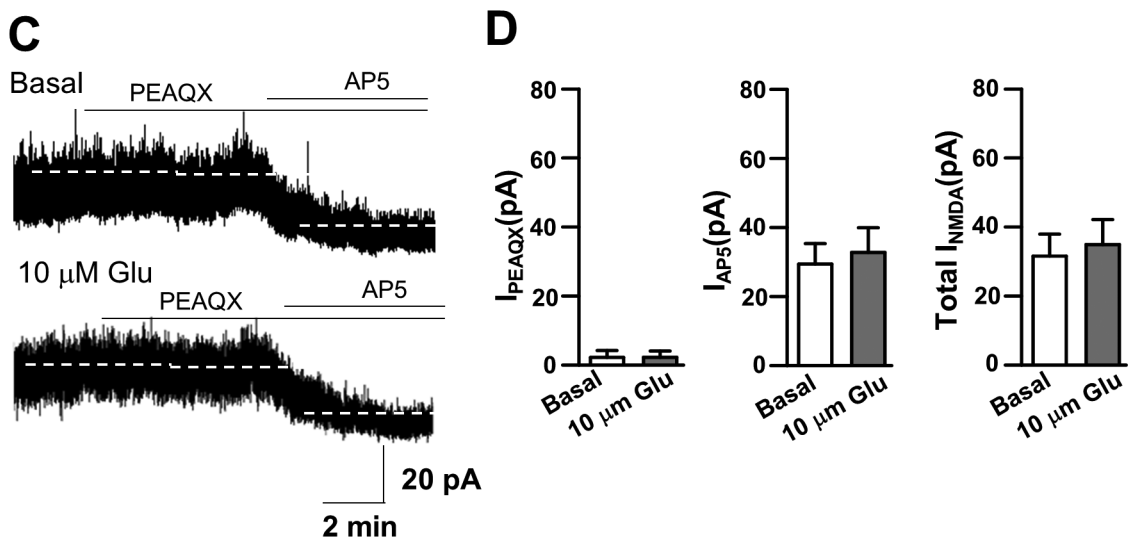
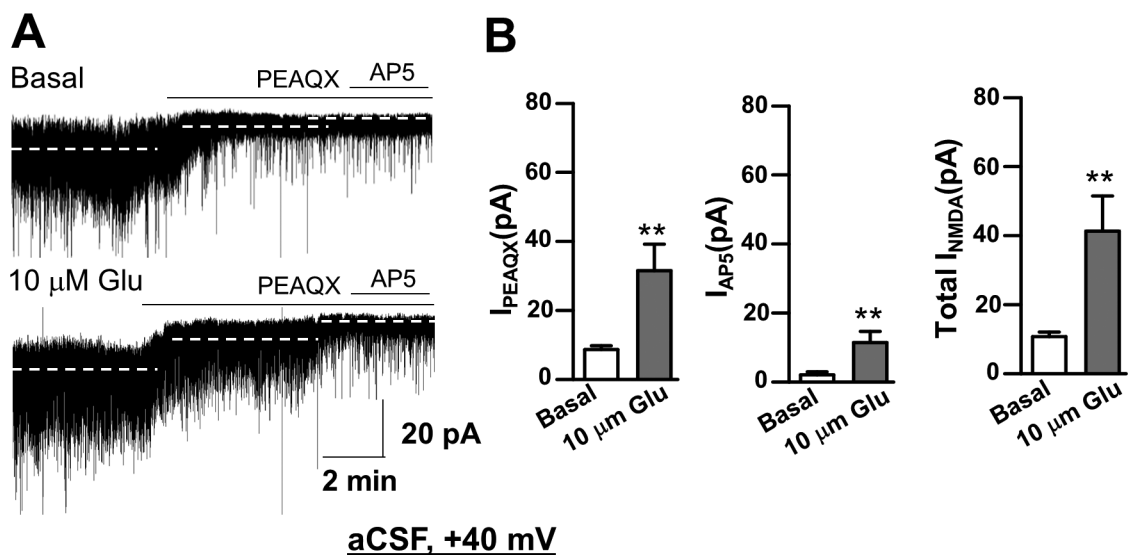


Figure 7. Continued.

As expected, exogenous glutamate significantly increased I_{PEAQX} in nondepolarized SON MNCs from 8.67 ± 1.10 pA ($n = 7$ neurons from 3 rats) to 31.49 ± 7.71 pA ($n = 6$ neurons from 3 rats). Additional AP5 (PEAQX + AP5) caused a minimal but significant I_{holding} shift in the presence of exogenous glutamate (control, 2.06 ± 0.85 pA, $n = 7$ neurons from 3 rats, vs $10 \mu\text{M}$ glutamate, 11.37 ± 3.24 pA, $n = 6$ neurons from 3 rats; $p = 0.01$ in both cases; two sample t test). As a result, total I_{NMDA} in the nondepolarized condition was significantly larger in the presence of $10 \mu\text{M}$ glutamate (10.73 ± 1.35 pA; $n = 6$ neurons from 3 rats) compared with the control condition (41.24 ± 10.25 pA, $n = 7$ neurons from 3 rats; Fig. 7B; $p = 0.01$; two-sample t test), while the portion of I_{PEAQX} to the total I_{NMDA} was not different in the absence and presence of glutamate ($82 \pm 6.67\%$, $n = 6$ vs $76 \pm 5.49\%$, $n = 7$; $p = 0.50$; two-sample t test). Noted that IFD did not affect I_{holding} in nondepolarized SON MNCs even in the presence of exogenous glutamate (control, 1.67 ± 0.95 vs $10 \mu\text{M}$ glutamate, 1.25 ± 1.21 pA). These results supported the idea that I_{NMDA} predominantly represented the activation of NR2A-containing NMDARs in nondepolarized SON MNCs.

We further investigated whether the increased ambient glutamate concentration could be sensed by I_{PEAQX} and $I_{\text{ifenprodil}}$ in depolarized SON MNCs. In contrast to I_{PEAQX} in nondepolarized neurons, exogenous glutamate failed to increase I_{PEAQX} of depolarized SON MNCs (control, 2.18 ± 1.98 pA, $n = 6$ neurons from 3 rats; $F_{(2,15)} = 24.59$; $p = 0.90$; Bonferroni's post hoc test following one-way RM-ANOVA and $10 \mu\text{M}$ glutamate, 2.20 ± 1.84 pA, $n = 6$ neurons from 3 rats; $F_{(2,15)} = 20.97$; $p = 0.92$; Bonferroni's post hoc test following one-way RM-ANOVA). Additional AP5 (PEAQX + AP5) uncovered a similar I_{NMDA} in both conditions (control, 29.39 ± 5.92 pA and $10 \mu\text{M}$ glutamate, 6.67 ± 7.25 pA; Fig. 7C,D). As a result, the total I_{NMDA} of depolarized SON MNCs was not different in the absence and presence of exogenous glutamate (Fig. 7C,D).

In addition, although IFD caused an inward I_{holding} shift in depolarized SON MNCs (Figs. 6, 7C,D), exogenous glutamate failed to affect $I_{\text{ifenprodil}}$ in depolarized SON MNCs (control, 20.38 ± 2.68 pA, $n = 7$ neurons from 3 rats vs glutamate, 22 ± 2.90 pA, $n = 6$ neurons from 3 rats). Additional AP5 (IFD + AP5) uncovered a similar magnitude of I_{NMDA} in both conditions (Fig. 7E,F). As a result, total I_{NMDA} in depolarized SON MNCs was not different in the absence and presence of exogenous glutamate (Fig. 7E,F) suggesting that membrane depolarization to V_{holding} of $+40$ mV maximized I_{NMDA} in our recording conditions, thus making it insensible to increased ambient glutamate at 1 week PDI.

Discussion

The main findings of this study are that (1) Mg^{2+} -sensitive I_{NMDA} was increased significantly but transiently at post 1 week in DOCA-salt rats; (2) the enhanced I_{PEAQX} is an agreement with attenuated EAAT activity with no changes in NMDAR subunit expression at 1 week PDI; and (3) $I_{\text{ifenprodil}}$ in depolarized SON MNCs did not respond to increased ambient glutamate in normal and DOCA-salt groups. These findings indicate that the I_{NMDA} in nondepolarized and depolarized SON MNCs is dominantly mediated by NR2A- and NR2B-containing NMDARs, respectively, and the former efficiently sensed the increased ambient glutamate concentration in the SON of normal and hypertensive rats. One limitation of this study is that only male rats were used to avoid the potential hormonal changes in female rats, which directly influence the hypertension pathophysiology. To the best of our knowledge, this is the first evidence that NR2A-containing NMDARs could contribute to tonic excitation mediated by extrasynaptic NMDARs.

I_{NMDA} generated by NR2A-containing NMDARs

Glutamate can generate a tonic I_{NMDA} when it binds to eNMDARs, while it evokes classical EPSCs via the activation of their synaptic counterparts. Tonic NMDAR current, I_{NMDA} , is a hallmark of eNMDAR activity, and NR2A subunit expression is more localized at synaptic sites and exclusively observed in postsynaptic sites (Groc et al., 2006; Paoletti et al., 2013; Cercato et al., 2016; Franchini et al., 2020). Although the presence of NR2A in both sites has been reported (Thomas et al., 2006; Gladding and Raymond, 2011), it was surprising to observe that PEAQX, an NR2A subunit antagonist, blocked I_{NMDA} in the present study (Fig. 2). These results suggest that synaptic NR2A-containing NMDARs rather than eNMDARs containing NR2B subunit could generate the Mg^{2+} -sensitive I_{NMDA} in nondepolarized SON MNCs. The idea is supported by our results that eNMDAR antagonists including ifenprodil and PPDA failed to affect I_{holding} of nondepolarized SON MNCs in low Mg^{2+} condition (Fig. 2). It is noteworthy that the tonic activation of GABA_A receptors generating tonic GABA_A inhibition were identified superimposed to high-frequency synaptic events (Otis et al., 1991; Salin and Prince, 1996; Hausser and Clark, 1997). Thus, I_{PEAQX} could represent the superimposition of high-frequency synaptic NMDAR currents especially in nondepolarized neurons under low $[\text{Mg}^{2+}]_o$ conditions. The idea is also in line with the fact that Mg^{2+} enhances the

Figure 7. Mechanism of generating I_{NMDA} in the presence of exogenous glutamate in nondepolarized and depolarized SON MNCs. **A**, Representative current traces show the effect of PEAQX and additional AP5 (100 μM) on the holding current of nondepolarized SON MNCs in basal condition and in the presence of $10 \mu\text{M}$ glutamate. **B**, Summarized bar graph showing mean of holding current change by PEAQX (left), additional AP5 (middle), and total I_{NMDA} (right); $n = 7$ and 9 in basal and in the presence of $10 \mu\text{M}$, respectively. **C**, Representative current traces show the effect of PEAQX and additional AP5 (100 μM) on the holding current of depolarized SON MNCs in basal condition and in the presence of $10 \mu\text{M}$ glutamate. **D**, Summarized bar graph showing mean of holding current change by PEAQX (left), additional AP5 (middle), and total I_{NMDA} (right); $n = 6$ in both condition. **E**, Representative current traces show the effect of ifenprodil and additional AP5 (100 μM) on the holding current of depolarized SON MNCs in basal condition and in the presence of $10 \mu\text{M}$ glutamate. **F**, Summarized bar graph showing mean of holding current change by IFD (left), additional AP5 (middle), and total I_{NMDA} (right); $n = 7$ and 6 in basal and in the presence of $10 \mu\text{M}$, respectively.

desensitization of NMDARs (Kampa et al., 2004), while the kinetics of NMDARs are faster at more negative holding potentials (Konnerth et al., 1990; Keller et al., 1991).

Combined with their extrasynaptic location (Lozovaya et al., 2004), NR2B-containing eNMDARs have been known to generate I_{NMDA} when exposed to low concentration of ambient glutamate. However, ifenprodil failed to uncover I_{NMDA} in nondepolarized SON MNCs even in the presence of exogenous glutamate (Figs. 2, 7). The apparent discrepancy may be reconciled with the idea that Mg^{2+} unblock, coupled with membrane potential depolarization, is essential to activate NR2B-containing receptors in our recording condition. This idea is in agreement with the fact that ifenprodil uncovered I_{NMDA} in depolarized SON MNCs (Figs. 6, 7). Given that NR2A and NR2B confer similar glutamate sensitivity to NMDARs (Erreger et al., 2007; Hansen et al., 2008) and low $[\text{Mg}^{2+}]_o$ (20 μM) efficiently blocked NMDARs in nondepolarized SON MNCs, these results are also supportive of the idea that EPSCs causing a transient increase of glutamate concentration in the synaptic cleft activates synaptic NR2A receptors to generate I_{PEAQX} in nondepolarized SON MNCs.

However, combined with the fact that EPSC frequency was not different in the DOCA-H₂O and DOCA-salt groups (Table 2), the significantly larger I_{PEAQX} in DOCA-salt rats argues against a contribution of superimposed EPSCs mediating I_{PEAQX} . This is further supported by our results showing that increased ambient glutamate equalized I_{PEAQX} in the DOCA-salt and DOCA-H₂O groups (Fig. 4). Thus, it is noteworthy that peri- as well as extrasynaptic receptors contribute tonic NMDAR currents (Papouin and Oliet, 2014) as GABA_A tonic inhibition (Farrant and Nusser, 2005; Glykys and Mody, 2007; Belelli et al., 2009). Future studies are warranted to investigate whether perisynaptic NMDARs containing NR2A subunit could generate PEAQX-sensitive I_{NMDA} in low $[\text{Mg}^{2+}]_o$ condition and, thus, also respond to an increased ambient glutamate concentration such as in DOCA-salt 1 week.

I_{PEAQX} but not by $I_{\text{ifenprodil}}$ contribute to sensing altered ambient glutamate concentrations in DOCA-salt rats

In the present study, NR2A-containing NMDARs generated I_{PEAQX} in nondepolarized SON MNCs in low $[\text{Mg}^{2+}]_o$ condition, which sensed an increased ambient glutamate, thus generating in turn a larger I_{NMDA} in DOCA-salt rats. In contrast, $I_{\text{ifenprodil}}$ in depolarized SON MNCs was not different in DOCA-H₂O and DOCA-salt groups (Fig. 6). One possible explanation for this difference is that $I_{\text{ifenprodil}}$ in depolarized SON MNCs was saturated in our recording condition. This possibility is actually supported by our results showing that exogenous glutamate failed to increase $I_{\text{ifenprodil}}$ in depolarized SON MNCs (Fig. 7). This result is also in line with the fact that $I_{\text{ifenprodil}}$ was not potentiated following an increase in ambient glutamate concentration in DOCA-salt rats (Fig. 2).

NMDAR phosphorylation reduces the voltage-dependent Mg^{2+} block of the channels (Chen and Huang, 1992), leading to, as recently shown (Pham et al., 2022), an increase in $I_{\text{ifenprodil}}$. The lack of increase in $I_{\text{ifenprodil}}$ in this study suggests that NR2B phosphorylation is not a likely mechanism contributing to changes in NMDAR function reported in DOCA-salt rats. Overall, our results showed that $I_{\text{ifenprodil}}$ represents the saturated activity of NR2B-containing NMDARs in depolarized SON MNCs.

Although it is purely speculative, it is interesting to note that “the extrasynaptic space” comprising separate domains was proposed instead of one large homogeneous volume (Papouin and Oliet, 2014). Given that NR2A and NR2B are preferentially located in synaptic and extrasynaptic regions (Kew et al., 1998; Tovar and Westbrook, 1999; Barria and Malinow, 2002), I_{PEAQX} and $I_{\text{ifenprodil}}$ may preferentially sense glutamate concentrations in the synaptic cleft over the ambient glutamate concentration in extrasynaptic space, respectively. However, our results showing that exogenous glutamate increased I_{PEAQX} argue against the notion of a functional barrier discriminating “PEAQX-sensitive” synaptic NMDARs and “ifenprodil-sensitive” extrasynaptic regions. Future studies are warranted to identify the spatial component differentiating “PEAQX-sensitive regions” over “ifenprodil-sensitive regions” in the SON.

An increase in ambient glutamate was insufficient to generate a Mg^{2+} -resistant I_{NMDA}

In a previous report (Neupane et al., 2021), it was shown that eNMDARs containing homodimeric NR2D subunit were the best candidate generating the Mg^{2+} -resistant I_{NMDA} in both nondepolarized and depolarized SON MNCs. However, an “NR2D recall” is essential to generate the Mg^{2+} -resistant I_{NMDA} in the matured brain, because NR2D expression is gradually decreased with brain maturation (Monyer et al., 1994; Dunah et al., 1996; Wenzel et al., 1996; Liu and Wong-Riley, 2010). In the present study, DOCA-salt failed to generate an Mg^{2+} -resistant I_{NMDA} in SON MNCs, despite the fact that an increase in ambient glutamate concentration was observed at 1 week PDI. Combined with the results that DOCA-salt did not affect NR2D subunit expression (Fig. 5), these results strengthened the idea that “NR2D recall” is essential to generate Mg^{2+} -resistant I_{NMDA} in the matured brain (Neupane et al., 2021).

Table 2. Phasic current properties of SON MNCs

EPSC properties	DOCA-H ₂ O	DOCA-salt	<i>p</i> value
Frequency (Hz)	0.99 ± 0.33	0.88 ± 0.20	0.76
Amplitude (pA)	43.02 ± 3.74	40.46 ± 1.95	0.51
Weighted τ (ms)	3.49 ± 0.65	3.28 ± 0.34	0.75

Physiological significance of increased glutamate in SON MNCs in hypertension

Elevated ambient glutamate due to diminished glutamate clearance modifies the excitatory tone that plays a critical role in regulating hypothalamic neurohumoral activation (Potapenko et al., 2012). In hypertension, increased NMDAR-Ca²⁺ responses upregulate neuronal firing in SON MNCs (Zhang et al., 2017; Zhang and Stern, 2017). In the present study, elevated glutamate in the prodromal stage of hypertension was detected by NR2A subunit-containing NMDARs. Given that I_{NMDA} mediated by NR2A-containing NMDARs detected only in the physiological atypical concentration of Mg²⁺, it may not directly affect neuronal firing in the SON MNCs. However, normal resting potential appears to be poised in the region of maximal sensitivity to small changes in ambient glutamate and that NR2B subunit-containing NMDARs-mediated tonic I_{NMDA} is limited and saturated at depolarized SON MNCs (Neupane et al., 2021; Fig. 7), our results showed that I_{NMDA} mediated by NR2A-containing NMDARs is an efficient biosensor for detecting altered ambient glutamate level in the brain.

Taken together, our results suggest that synaptic NMDARs containing NR2A subunit generate I_{NMDA} in nondepolarized SON MNCs under low Mg²⁺ condition, while NR2B-containing receptors mediate I_{NMDA} in depolarized SON MNCs under normal Mg²⁺ condition. Thus, I_{PEAQX} represents the tonic activation of NR2A-containing receptors in SON MNCs, standing thus as a useful biomarker for the detection of ambient glutamate concentration in the SON during normal and pathological neurohumoral overdrive.

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