Enhanced NMDA receptor-mediated intracellular calcium signaling in magnocellular neurosecretory neurons in heart failure rats

Javier E. Stern and Evgeniy S. Potapenko
Department of Physiology, Georgia Regents University, Augusta, Georgia

Submitted 1 April 2013; accepted in final form 18 June 2013

Stern JE, Potapenko ES. Enhanced NMDA receptor-mediated intracellular calcium signaling in magnocellular neurosecretory neurons in heart failure rats. Am J Physiol Regul Integr Comp Physiol 305: R414–R422, 2013. First published June 19, 2013; doi:10.1152/ajpregu.00160.2013.—An enhanced glutamate excitatory function within the hypothalamic supraoptic and paraventricular nuclei is known to contribute to increased neurosecretory and presympathetic neuronal activity, and hence, neurohumoral activation, during heart failure (HF). Still, the precise mechanisms underlying enhanced glutamate-driven neuronal activity in HF remain to be elucidated. Here, we performed simultaneous electrophysiology and fast confocal Ca2+ imaging to determine whether altered N-methyl-D-aspartate (NMDA) receptor-mediated changes in intracellular Ca2+ levels (NMDA-ΔCa2+) occurred in hypothalamic magnocellular neurosecretory cells (MNCs) in HF rats. We found that activation of NMDA receptors resulted in a larger ΔCa2+ in MNCs from HF when compared with sham rats. The enhanced NMDA-ΔCa2+ was neither dependent on the magnitude of the NMDA-mediated current (voltage clamp) nor on the degree of membrane depolarization or firing activity evoked by NMDA (current clamp). Differently from NMDA receptor activation, firing activity evoked by direct membrane depolarization resulted in similar changes in intracellular Ca2+ in sham and HF rats. Taken together, our results support a relatively selective alteration of intracellular Ca2+ homeostasis and signaling following activation of NMDA receptors in MNCs during HF. The downstream functional consequences of such altered ΔCa2+ signaling during HF are discussed.

NMDA; vasopressin; supraoptic; glutamate; Ca2+

SYMPATHOHUMORAL ACTIVATION involving augmented sympathethic tone and elevated hormonal plasma levels, including vasopressin (VP) and angiotensin II, among others (37, 43, 73), is a key central nervous system pathophysiological process in congestive heart failure (HF). Chronically elevated plasma VP levels have been reported both in animal models and human patients with HF (15, 19, 52, 62) being an important factor contributing to altered fluid/electrolyte balance, as well as detrimental myocardial effects (17, 18, 40, 44, 53). The importance of VP in HF is also underscored by several clinical trials demonstrating that VP receptor antagonism efficiently improves water balance and hemodynamic parameters in HF patients (2, 9, 41). Thus the VP system is currently emerging as a novel therapeutic target for the treatment of HF (14). Despite its major impact on morbidity and mortality in HF patients (7), the precise mechanisms contributing to neurohumoral activation, including elevated VP release in HF, remain incompletely understood.

The hypothalamic supraoptic (SON) and paraventricular (PVN) nuclei are crucial centers involved in autonomic and neuroendocrine regulation of the circulation (23, 60). Within these nuclei, magnocellular neurosecretory cells (MNCs) directly control VP (and oxytocin) release into the circulation, according to their degree and pattern of electrical activity (6, 51). A growing body of evidence supports an enhanced glutamate excitatory action within the SON and PVN in HF rats (27, 29, 32, 48, 49, 71). This includes elevated endogenous glutamate levels (29, 32), increased expression of glutamate N-methyl-D-aspartate (NMDA) receptors (32), and synaptic remodeling involving an increased predominance of glutamate excitatory over GABA inhibitory inputs in HF rats (24, 48). This exacerbated glutamate excitatory strength was shown to contribute to enhanced presympathetic and neurosecretory neuronal activity (24, 48), as well as the concomitant increase in sympathohumoral activation in HF rats (29, 32, 71).

NMDA receptors are one of the key glutamate receptors influencing neuronal activity and sympathohumoral outflow from the hypothalamus (25, 31, 32, 42, 61, 72). NMDA receptor activation results in an influx of Ca2+, which in addition to evoking a direct membrane depolarization, leads to an increase in intracellular free Ca2+ levels (ΔCa2+) (38). This in turn affects a variety of downstream signaling pathways, including activation of Ca2+-sensitive channels (47, 54) and changes in the intracellular kinase/phosphatase balance (8), all of which can in turn further alter neuronal excitability following NMDA receptor activation. Moreover, other signaling mechanisms known to contribute to altered neuronal function in HF rats, including nitric oxide (NO) and reactive oxygen species (ROS) production (4, 22, 26, 68), are strongly dependent on or influenced by NMDA-mediated increases in intracellular Ca2+. The functional consequences of ΔCa2+ are largely dependent on its magnitude and time course. Still, the precise spatiotemporal dynamics of NMDA-ΔCa2+ in key neurons involved in the regulation of sympathohumoral activation has not been investigated yet. Moreover, whether a change in NMDA-ΔCa2+ dynamics contributes to altered neuronal and thus neurohumoral activation in HF rats, is presently unknown. In this study, we performed simultaneous patch-clamp electrophysiological recordings and fast confocal Ca2+ imaging to characterize NMDA-ΔCa2+ dynamics in MNCs in sham and HF rats. Our results show an enhanced NMDA-ΔCa2+ signal in MNCs of HF rats, which was independent of the magnitude of the NMDA-mediated current, or of the degree of NMDA-evoked firing activity. Moreover, our results suggest that the enhanced NMDA-ΔCa2+ acts as a positive feedback mechanism contributing to increased MNC membrane excitability in HF rats.
MATERIALS AND METHODS

Animals and induction of HF-Male Wistar rats (150–180 g) were purchased from Harlan Laboratories (Indianapolis, IN). Rats were housed at room temperature (24–26°C) in a 12-h light-dark cycle room and given free access to food and water. In a subset of experiments, we also used male heterozygous transgenic VP-eGFP Wistar rats (5–6 wk old), in which VP neurons are endogenously fluorescent (67). All procedures were carried out in agreement with the Georgia Regents University Institutional Animal Care and Use Committee guidelines. All protocols used for these studies were submitted, reviewed, and approved by an independent committee (IACUC). HF was induced by coronary artery ligation as previously described (4). Briefly, animals were anesthetized with isoflurane 4% and intubated for mechanical ventilation. A left thoracotomy was performed and the heart exteriorized. The ligation was placed on the main diagonal branch of the left anterior descending coronary artery.

Buprenorphine (Brupren C3 0.3 mg/kg sc; Butler Schein/NLS, Dublin, OH) was given immediately after surgery to minimize postsurgical pain. Sham animals underwent the same procedure but the coronary artery was not occluded. All animals were used 6 to 7 wk after surgery. Transthoracic echocardiography (Vevo 770 system; Visual Sonics) was performed 4 wk after surgery under light anesthesia. The left ventricle internal diameter, as well as the left ventricle posterior and anterior walls diameter, were obtained throughout the cardiac cycle from the short-axis motion imaging mode. Automatic calculation using the parameters measured was obtained for ejection fraction and fractional shortening. Mean cardiac function values obtained from sham and HF rats are summarized in Table 1.

Hypothalamic slice preparation. Hypothalamic brain slices were prepared according to methods previously described (48, 58). Briefly, rats were deeply anesthetized with pentobarbital sodium (80 mg/kg ip) and perfused through the heart with an ice-cold sucrose solution [containing in mM: 200 sucrose, 2.5 KCl, 3 MgSO4, 26 NaHCO3, 1.25 NaH2PO4, 20 d-glucose, 0.4 ascorbic acid, 1 CaCl2, and 2 pyruvic acid; pH 7.4; 290–310 mosmol/l]. Slices were placed in oxygenated ice-cold artificial cerebrospinal fluid (aCSF) was used to pull patch pipettes (3–4 MΩ) from borosilicate glass (G150TF-3, Warner Instruments, Sarasota, FL) was used to pull patch pipettes (3–4 MΩ) from borosilicate glass (G150TF-3, Warner Instruments, Sarasota, FL). A minimal pressure of 5–8 PSI was used. Cell input resistance and cell capacitance were calculated in voltage clamp using a 5-mV pulse while holding the cells at −70 mV. Spike threshold was calculated based on the third derivative of the action potential wave-form implemented by MiniAnalysis software (5, 56). Repetitive firing activity was evoked by injecting depolarizing current pulses (80 pA) of progressively increasing durations (0.1–0.7 s), and plots of the number of evoked spikes as a function of the current pulse duration were generated. All drugs were purchased from Sigma-Aldrich (St. Louis, MO).

Confocal calcium imaging. SON neurons were loaded through the patch pipette with Fluo-5F pentapotassium salt (100 μM; Molecular Probes, Carlsbad, CA), as previously described (13, 57). Once in the whole cell mode, the dyec was allowed to dialyze into the cell for at least 15 min before the initiation of the recordings to allow complete dialysis of the dye. Imaging was conducted using the Yokogawa real time live cell laser confocal system combined with a highly sensitive EMCCD camera (iXON+885, Andor Technology, South Windsor, CT). Fluorescence images were obtained using diode-pumped solid-state laser (Melles Griot, Carlsbad, CA) at 488 nm and emitted light at >495 nm. Images were acquired at a rate of 2 Hz. The fractional fluorescence (F/F0) was determined by dividing the fluorescence intensity (F) within a region of interest (6 × 6 pixels ~4.8 × 4.8 μm) by a baseline fluorescence value (F0) determined from 30 images before activation of NMDA receptors. Data were analyzed using Andor IQ software (Andor Technology).

Statistical analysis. All values are expressed as means ± SE. Between-group differences (sham vs. HF) were compared using unpaired t-tests or analysis of variance, as indicated by Bonferroni post hoc tests. Differences were considered statistically significant at P < 0.05, and n refers to the number of cells. All statistical analyses were conducted using GraphPad Prism (GraphPad Software, San Diego, CA).

RESULTS

Cardiac function values in sham and HF rats. Mean cardiac function values obtained from sham and HF rats in this study are summarized in Table 1. When compared with sham rats, ligated rats showed a significant increased left ventricle internal dimension throughout the cardiac cycle, a decreased percentage of ejection fraction, and a decreased percentage fractional shortening (P < 0.0001 in all cases).

NMDA receptor activation evokes a similar I(NMDA), but larger ΔCa2+ in MNCs in HF rats. Patch-clamp electrophysiological and/or imaging recordings were obtained from a total of 95 SON MNCs obtained from sham (n = 48 MNCs from 19 rats) and HF rats (n = 47 MNCs from 19 rats). To study NMDA receptor-mediated changes intracellular Ca2+ dynamics (NMDA-ΔCa2+) in MNCs from sham and HF rats, simultaneous patch-clamp recordings (voltage-clamp mode) and fast confocal Ca2+ imaging measurements were obtained from

Table 1. Summary data of echocardiography measurements of left ventricular parameters obtained from sham and heart failure rats

<table>
<thead>
<tr>
<th></th>
<th>EF, %</th>
<th>FS, %</th>
<th>LVIDd, mm</th>
<th>LVIDs, mm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham</td>
<td>80.1 ± 4.0</td>
<td>52.2 ± 4.7</td>
<td>7.6 ± 0.3</td>
<td>3.7 ± 0.5</td>
</tr>
<tr>
<td>HF</td>
<td>34.8 ± 1.6*</td>
<td>17.6 ± 0.9*</td>
<td>10.2 ± 0.2*</td>
<td>8.5 ± 0.2*</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = 19 rats for each group. EF, ejection fraction; FS, fractional shortening; LVIDd and s: left ventricle internal dimension during diastole and systole respectively; HF, heart failure. *P < 0.0001 vs. sham.
neurons loaded with Fluo-5F (100 μM) through the patch pipette. Focal application of NMDA (50 μM, 1 s, 5–8 PSI) onto the recording neurons evoked a transient inward current (I_{NMDA}) and a concomitant ΔCa^{2+} (Fig. 1, A and B). As shown in Fig. 1C, the overall magnitude of I_{NMDA} was not different between MNCs in sham and HF rats (n = 9 and 13, respectively, P = 0.8). Conversely, the magnitude of the NMDA-ΔCa^{2+} was significantly larger in MNCs from HF rats (~85%, P < 0.05). When Ca^{2+} responses were normalized to the underlying I_{NMDA} within each cell, a larger ΔCa^{2+}/unit of I_{NMDA} was still observed in HF compared with sham rats (~125%, P < 0.01, Fig. 1C). Given similar cell capacitance values between MNCs in sham and HF rats (Table 2), similar results were observed when I_{NMDA} and ΔCa^{2+} values were normalized by cell capacitance (not shown). Thus, despite similar I_{NMDA}-evoked currents, a larger increase in intracellular Ca^{2+} was observed in MNCs from HF rats.

A major factor influencing the shape and time course of intracellular Ca^{2+} signal is buffering by the endoplasmic reticulum via the sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA) pump (66). Thus to determine whether the prolonged NMDA-ΔCa^{2+} in HF rats was due to a diminished ER Ca^{2+} buffering capacity, we repeated a set of experiments in slices preincubated with thapsigargin (2 μM, 45 min), an endoplasmic reticulum SERCA pump blocker (35). We found that thapsigargin significantly prolonged the duration of the NMDA-ΔCa^{2+}, in MNCs from both sham and HF rats (ΔCa^{2+} decay time: sham-thapsigargin: 43.4 ± 2.7 ms; HF-thapsigargin: 56.0 ± 3.9 ms; n = 11 and 6, P < 0.05 vs. respective sham and HF groups in control ACSF, see Fig. 1C for comparison). No changes in other ΔCa^{2+} parameters were observed (not shown).

NMDA receptor activation evokes an enhanced firing activity along with larger ΔCa^{2+} per action potential in HF rats. To study NMDA responses under more physiological conditions, experiments were performed also in the current-clamp mode, a condition in which NMDA receptor activation evokes membrane depolarization and firing discharge. As summarized in Table 2, no differences in resting membrane potential, input resistance, or action potential threshold were observed between sham and HF groups. Focal application of NMDA to the recording cell (10 μM, 1 s) evoked a transient membrane depolarization, an increased firing rate, and a concomitant increase in intracellular Ca^{2+} (Fig. 2). The degree of NMDA

Table 2. Summary data of basic intrinsic membrane properties of MNCs in sham and HF rats

<table>
<thead>
<tr>
<th></th>
<th>Resting V_m, mV</th>
<th>Input Resistance, Ω</th>
<th>Cell Capacitance, pF</th>
<th>Spike Threshold, mV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham</td>
<td>−72.9 ± 0.8 (n = 30)</td>
<td>0.51 ± 0.05 (n = 30)</td>
<td>25.7 ± 0.9 (n = 48)</td>
<td>−45.0 ± 1.2 (n = 30)</td>
</tr>
<tr>
<td>HF</td>
<td>−73.4 ± 0.9 (n = 24)</td>
<td>0.52 ± 0.04 (n = 24)</td>
<td>27.3 ± 1.1 (n = 47)</td>
<td>−44.7 ± 1.0 (n = 24)</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = number of rats. V_m, membrane potential.
receptor-mediated membrane depolarization and firing discharge was significantly larger in MNCs from HF rats ($P < 0.05$ in both cases, see Fig. 2B, n = 30 and 24 in sham and HF rats, respectively). In a proportion of these cells, we were able to reliably quantify $\Delta Ca^{2+}$ responses. In these MNCs (n = 16 and 18 in sham and HF rats, respectively), the magnitude and time course of the NMDA receptor-evoked $\Delta Ca^{2+}$ was significantly larger in HF rats. Thus both the $\Delta Ca^{2+}$ peak amplitude and area were significantly larger in HF compared with sham rats ($~145$ and $210\%$, respectively, $P < 0.001$) (Fig. 2C). While a strong tendency for a slower monoexponential decay time course was observed in MNCs from HF rats, differences did not reach statistical significance ($P = 0.08$). Conversely, no differences in the $\Delta Ca^{2+}$ rise time were observed between experimental groups (not shown).

The larger NMDA-$\Delta Ca^{2+}$ observed in HF rats could be the result of the larger depolarization and/or number of action potentials triggered by NMDA receptor activation in these rats. However, when we normalized within each MNC, the overall magnitude of the evoked $\Delta Ca^{2+}$ by either the area of the membrane depolarization or the total number of spikes evoked, a significantly larger $\Delta Ca^{2+}$ response was still observed in MNCs of HF rats ($P < 0.01$ and $P = 0.05$, respectively, n = 16 and 18 in sham and HF rats, respectively, Fig. 3, A and B). In addition, no significant correlation was observed between the magnitude of the NMDA-$\Delta Ca^{2+}$ and the NMDA-ΔmV ($R^2 = 0.19$ and 0.08 for sham and HF rats, respectively). Moreover, the enhanced NMDA-$\Delta Ca^{2+}$ in HF rats persisted when action potentials were blocked in slices pretreated with tetrodotoxin (TTX, 1 μM) ($P < 0.05$, n = 7 and 4 in sham and HF rats, respectively, Fig. 3D). Finally, to verify that these changes occur in vasopressin neurons, we obtained a few recordings from endogenously fluorescent eGFP-VP MNCs. Similar to nonidentified cells, we found a significantly larger NMDA-$\Delta Ca^{2+}$ in identified eGFP-VP neurons in HF rats [$\Delta Ca^{2+}$ area (F/F0 $\times$ s): sham = 20.1 ± 8.9; HF: 63.3 ± 11.2, $P < 0.05$, n = 4 and 11, respectively], as well as larger $\Delta Ca^{2+}$/unit of membrane depolarization (sham = 0.12 ± 0.04; HF: 0.65 ± 0.16, $P < 0.05$, n = 4 and 11, respectively).

Taken together, these results indicate that the enhanced NMDA-$\Delta Ca^{2+}$ in MNCs in HF rats was not due to the larger NMDA-mediated depolarization/firing discharge. Conversely, they support an overall increased $\Delta Ca^{2+}$ entry per unit of NMDA-mediated membrane excitation.

Evoked repetitive action potential firing activity results in similar $\Delta Ca^{2+}$ in MNCs from sham and HF rats. The results above suggest that NMDA receptor activation per se, independent of action potential firing, is sufficient to evoke differences in NMDA-$\Delta Ca^{2+}$ dynamics between MNCs in sham and HF rats. To study in more details the relationship between repetitive action potential firing and Ca$^{2+}$ dynamics in MNCs, repetitive firing was evoked by injecting depolarizing current pulses of incremental durations (80 pA, 0.1, 0.4, and 0.7 s), while monitoring the magnitude of the evoked $\Delta Ca^{2+}$ ($n = 9$ and 10 in sham and HF, respectively). As shown in Fig. 4, the firing discharge in MNCs increased progressively in both groups as a function of the injected current ($P < 0.0001$, two-way ANOVA). However, no differences in the number of
evoked actions potentials were observed between sham and HF rats ($P = 0.5$, two-way ANOVA). Similarly, the mean $\Delta Ca^{2+}$ area increased progressively in both groups as a function of the injected current ($P < 0.0001$, two-way ANOVA), but no differences between groups were observed ($P = 0.8$, two-way ANOVA). Finally, when the magnitude of the evoked $\Delta Ca^{2+}$ was normalized to the number of evoked action potentials for each current step, no differences were observed between experimental groups ($P = 0.2$, two-way ANOVA).

**DISCUSSION**

Using a combination of patch-clamp electrophysiology with simultaneous fast confocal $Ca^{2+}$ imaging, we characterized NMDA-$\Delta Ca^{2+}$ in MNCs from sham and HF rats. We show that: 1) while the magnitude of $I_{NMDA}$ was similar between MNCs in the two experimental groups, a larger $\Delta Ca^{2+}$ was evoked in HF rats; 2) in current-clamp mode, NMDA receptor activation evoked an enhanced membrane depolarization and

---

**Fig. 3.** Enhanced NMDA-mediated $\Delta Ca^{2+}$ per unit of membrane depolarization or evoked action potential in MNCs of HF rats. Summary data showing a significant increase in NMDA-mediated $\Delta Ca^{2+}$ in MNCs from HF rats, when data were normalized either by the total area of the NMDA-mediated depolarization (A) or by the total number of evoked action potentials (B). In C, a plot of the NMDA-evoked $\Delta Ca^{2+}$ area as a function of the NMDA-evoked depolarizing area is shown ($n = 16$ and 18 in sham and HF rats, respectively). D: summary data showing that the larger increase in NMDA-mediated $\Delta Ca^{2+}$ per unit of membrane depolarization in HF rats persisted in the presence of tetrodotoxin (1 $\mu$M, $n = 7$ and 4 in sham and HF rats, respectively). *$P = 0.05$ and **$P < 0.01$ vs. respective sham.

**Fig. 4.** Repetitive action potential firing evoked by direct current injection resulted in similar $\Delta Ca^{2+}$ in MNCs from sham and HF rats. A: representative examples of 3 bursts of action potentials evoked by progressively increasing current pulse duration (0.1, 0.4, and 0.7 s, respectively, top traces), and the respective $\Delta Ca^{2+}$ (bottom traces) obtained from a MNCs in a HF rat. B: summary data showing mean number of evoked action potentials (top), mean changes in $\Delta Ca^{2+}$ area (middle), and mean $\Delta Ca^{2+}$ area/number of action potential (APs, bottom) in MNCs from sham and HF ($n = 9$ and 10 in sham and HF, respectively). Note the lack of differences between sham and HF rats in any of the parameters measured.
firing discharge, along with a larger $\Delta Ca^{2+}$ in HF, compared with sham rats; 3) the NMDA-$\Delta Ca^{2+}$ per unit of membrane depolarization or per individual action potential was larger in HF rats; and 4) the number of action potentials and concomitant $\Delta Ca^{2+}$ evoked by direct delivery of depolarizing steps of increasing durations was similar between sham and HF rats.

While an enhanced glutamate function within the SON/PVN of HF rats was previously reported (27, 29, 32, 48, 71), the precise underlying mechanisms and cellular consequences of the exacerbated glutamate function in HF rats remain to be fully elucidated. In this study, we focused on the functional efficacy of NMDA receptors and their actions on membrane excitability and intracellular $Ca^{2+}$ dynamics. We report here that focal and transient activation of NMDA receptors evoked a similar NMDA-mediated inward current in MNCs from sham and HF rats. Despite a similar $I_{NMDA}$ magnitude between the two experimental groups, a larger NMDA-$\Delta Ca^{2+}$ was observed in HF rats. Multiple factors shape the magnitude and waveform of a $Ca^{2+}$ transient such as those following NMDA receptor activation. These include the total number of NMDA receptors, the single-channel $Ca^{2+}$ permeability of NMDA receptors, release of $Ca^{2+}$ from intracellular stores, as well as intracellular $Ca^{2+}$ buffering and cytosolic clearance mechanisms. The fact that a similar $I_{NMDA}$ current was evoked in sham and HF rats would argue against an increased in NMDA receptor numbers or increased in $Ca^{2+}$ permeability in this conditions, since $Ca^{2+}$ influx is a major component mediating $I_{NMDA}$ (38). This is in agreement with a previous study showing lack of changes in NMDA receptor NR1 subunit mRNA expression in the SON in HF rats (32).

MNCs possess numerous $Ca^{2+}$ buffering/clearance mechanisms, including plasmalemmal (PMCs) and endoplasmic reticulum (ER-SERCa) $Ca^{2+}$-transport ATPases, and the mitochondrial $Ca^{2+}$-selective unipor (10), all of which have been shown to efficiently shape somatic $Ca^{2+}$ transients in these neurons (28, 30). These $Ca^{2+}$ buffering mechanisms slowly decrease the levels of cytosolic free $Ca^{2+}$, resulting in a slow decaying $Ca^{2+}$ time course following the initial transient rise. Thus changes in the efficacy of $Ca^{2+}$ buffering mechanisms typically affect not only the peak, but mostly the decay phase, of the $Ca^{2+}$ transient. In the present study, we found that both the peak and duration of the NMDA-$\Delta Ca^{2+}$ in MNCs from HF rats were significantly enhanced compared with sham rats, suggesting a compromised $Ca^{2+}$ buffering.

To determine whether a blunted ER-SERCa buffering mechanism contributed to the prolonged NMDA-$\Delta Ca^{2+}$ in HF rats, we compared the effects of thapsigargin (TG), a SERCa blocker, between sham and HF rats. Our results showing that TG prolonged the NMDA-$\Delta Ca^{2+}$ signal both in sham and HF rats, indicate that the ER indeed acts as an important intracellular $Ca^{2+}$ buffering mechanism shaping the NMDA-$\Delta Ca^{2+}$ waveform. This is in agreement with previous studies showing that blockade of the ER-SERCa prolonged the decay of a $K^{+}$-induced increase in $Ca^{2+}$ in MNCs (30). However, the fact that TG prolonged the NMDA-$\Delta Ca^{2+}$ waveform in both experimental groups to a similar extent would argue against a blunted ER-SERCa function during HF. Finally, in addition to acting as a $Ca^{2+}$ buffering organelle, the ER can also release stored $Ca^{2+}$, contributing to the overall NMDA-$\Delta Ca^{2+}$ waveform (12). Prolonged blockade of the SERCa with TG, as performed in our study, also leads to the depletion of ER $Ca^{2+}$ store (45). Thus our results showing that TG resulted in an enhancement, rather than a reduction, in the NMDA-$\Delta Ca^{2+}$ signal indicate that $Ca^{2+}$ release from the ER does not contribute to the $\Delta Ca^{2+}$ signals following NMDA receptor activation in MNCs. Clearly, future studies will be needed to elucidate alternative mechanisms contributing to the altered NMDA receptor-mediated $Ca^{2+}$ waveform in HF rats.

We observed a similar enhanced NMDA-$\Delta Ca^{2+}$ in the current clamp mode, in which NMDA receptor activation evoked also a larger membrane depolarization along with a more pronounced firing discharge in MNCs of HF rats. Still, the larger NMDA-$\Delta Ca^{2+}$ response persisted when action potentials were blocked by TTX, indicating that activation of NMDA receptors per se (and not the $Ca^{2+}$ influx associated with action potential firing) was associated to the prolonged $Ca^{2+}$ signal in HF rats. The implications of the more robust NMDA-mediated membrane depolarization and firing discharge in MNCs of HF rats is discussed further below. Notably, no differences in $\Delta Ca^{2+}$ dynamics between sham and HF rats were observed following trains of action potentials evoked by direct membrane depolarization. In this case, most of the $Ca^{2+}$ contributing to the $\Delta Ca^{2+}$ originates from influx via voltage-gated $Ca^{2+}$ channels. Thus these results suggest that $Ca^{2+}$ homeostasis is not globally affected in MNCs from HF rats, but rather, that the source of $Ca^{2+}$ (i.e., NMDA receptors), and their spatial proximity to specific buffering mechanism are key determining factors (46).

What are the possible downstream consequences of the enhanced $\Delta Ca^{2+}$ following NMDA receptor activation in HF? Critical inhibitory signals within the SON and PVN, such as NO and GABA, are both $Ca^{2+}$ dependent and can be influenced directly or indirectly by NMDA receptor activation (3, 50). Moreover, the efficacy of both NO and GABA actions have been shown to be diminished in HF rats, contributing in turn to neurohumoral activation in this disease. For example, a blunted NO production within the PVN is recognized as a key mechanism contributing to sympathohumoral activation in HF rats (4, 68, 73). While a diminished neuronal NO synthase and endothelial NO synthase expression has been reported in the SON/PVN of HF rats (4, 70), the possibility that an enhanced NMDA-$\Delta Ca^{2+}$ response contributes to diminished NOS activity and consequently, NO production, has not been explored. While not directly tested in this study, our results showing an enhanced, rather than a blunted, $Ca^{2+}$ response would argue against this possibility. Similarly, a blunted GABAergic inhibitory function has been reported in the hypothalamus of HF rats (24, 48, 69). We recently demonstrated, both in SON MNCs and presym pathetic PVN neurons, an NMDA-mediated, $Ca^{2+}$ dependent potentiation of GABA$_{A}$ receptor function, which serves as a counterbalancing inhibitory feedback mechanism to restrain overexcitation following NMDA receptor activation (50). Importantly, this NMDA-$Ca^{2+}$-GABA$_{A}$ coupling was blunted in MNCs from HF rats, likely contributing to NMDA-driven neurohumoral activation in HF (50). While the precise mechanisms underlying the blunted NMDA-GABA$_{A}$ receptor coupling are still unknown, the present results argue against a diminished ability of NMDA receptors to evoke a sufficiently large change in intracellular $Ca^{2+}$ as a contributing factor.

Another important finding in this study is that NMDA receptor activation in HF, despite evoking a similar underlying $I_{NMDA}$ current than in sham rats, resulted in a more pronounced membrane depolarization and firing discharge in the former.
One possibility is that the blunted NO and GABA inhibitory mechanisms previously reported to occur during HF (4, 24, 68, 69) enabled a more robust NMDA excitatory response to be elicited in this condition. Alternatively, it is also reasonable to speculate that the enhanced NMDA-\(\Delta\text{Ca}^{2+}\) could itself contribute to altered MNCs neuronal excitability in HF rats, via interactions with \(\text{Ca}^{2+}\)-sensitive ion channels, including \(\text{Ca}^{2+}\)-activated \(\text{K}^+\) channels (e.g., SK and BK channels) or \(\text{Ca}^{2+}\)-activated, nonselective cation channels (CAN) (47, 54). Both types of \(\text{Ca}^{2+}\)-sensitive channels are expressed in MNCs and play important though opposing roles in regulating membrane excitability and firing properties in these neurons. Thus, whereas SK channels mediate an afterhyperpolarization (AHP) that acts to inhibit repetitive firing (21, 59, 64), CAN channels promote firing activity, in part via generation of fast depolarizing after potentials (fDAPs) (16, 63, 65). Importantly, both AHPs and DAPs temporally overlap, being their balance then a critical factor that determines the influence of \(\Delta\text{Ca}^{2+}\) on membrane excitability (1). Thus it is possible that in HF rats, the exacerbated NMDA-\(\Delta\text{Ca}^{2+}\) could tip the balance toward a predominant activation of CAN channels, resulting in an enhanced NMDA-mediated membrane excitability in HF rats. While such interaction between NMDA receptors and CAN channels has been recently shown in substantia nigra neurons (39), future studies addressing the coupling of NMDA-\(\Delta\text{Ca}^{2+}\) to downstream \(\text{Ca}^{2+}\)-sensitive channels in MNCs are needed to more conclusively test their involvement in increased membrane excitability during HF.

**Perspectives and Significance**

Neurohumoral activation is a critical pathological process contributing to morbidity and mortality in patients with heart failure (7). Thus elucidating underlying mechanisms contributing to neurohumoral activation in this disease is of high clinical relevance. While an enhanced glutamate excitatory action has been recognized as a crucial factor in HF, the precise mechanisms contributing to elevated glutamate excitatory function remain to be determined. Activity-dependent changes in neuronal intracellular \(\text{Ca}^{2+}\) levels act as a critical signal capable of affecting multiple neuronal functions including regulation of membrane excitability, neurotransmitter release, neuroplasticity, and gene expression, among others (36). Glutamate NMDA receptors (NMDA receptors) are pivotal molecules that translate activity-dependent signaling between neurons into complex changes intracellular \(\text{Ca}^{2+}\), being thus a major source of \(\text{Ca}^{2+}\) in neurons, including MNCs. Results from the present study provide evidence for an enhanced NMDA-mediated increase in \(\text{Ca}^{2+}\) in MNCs during HF. In addition to contributing to increased membrane excitability, as supported in this study, the exacerbated NMDA-\(\Delta\text{Ca}^{2+}\) is expected to affect other critical functions in MNCs, including dendritic excititation-secretion coupling. It is well recognized that in addition to releasing their peptide content from axonal terminal in the neurohypophysis into the general circulation, MNCs can also release VP and oxytocin from their dendrites in an activity-dependent manner (34). This intraneuronal dendritic peptide release serves as an efficient autocrine mechanism by which MNCs optimize their own firing activity in response to specific physiological challenges (20, 33). Moreover, recent studies from our laboratory indicate that dendritic release of VP can also act in a diffusible manner to stimulate the activity of neighboring presynaptic neurons, leading to an increased renal sympathetic nerve activity (55). Activation of NMDA receptor, and the subsequent increase in \(\text{Ca}^{2+}\), is a powerful mechanism that stimulates dendritic peptide release from MNCs (11). Thus it is reasonable to speculate that the enhanced NMDA-\(\Delta\text{Ca}^{2+}\) reported here during HF may lead to an exacerbated dendritic VP release, resulting in turn not only in further increased VP neuronal activity, but also in a more prominent recruitment of presynaptic neurons, both ultimately contributing to neurohumoral activation in HF. Future studies assessing on one hand the precise mechanisms underlying exacerbated NMDA-\(\Delta\text{Ca}^{2+}\) (e.g., intracellular \(\text{Ca}^{2+}\) buffering capacity), and on the other hand the overall impact of such changes to sympathohumoral activation at the systems level, are warranted.

**GRANTS**

This work was supported by National Heart, Lung, and Blood Institute R01 HL-090948 (to J. E. Stern).

**DISCLOSURES**

No conflicts of interest, financial or otherwise, are declared by the author(s).

**AUTHOR CONTRIBUTIONS**

Author contributions: J.E.S. conception and design of research; J.E.S. and E.S.P. analyzed data; J.E.S. interpreted results of experiments; J.E.S. drafted manuscript; J.E.S. edited and revised manuscript; J.E.S. approved final version of manuscript; E.S.P. performed experiments; E.S.P. prepared figures.

**REFERENCES**


