ERK1/2 MAPK signaling in hypothalamic paraventricular nucleus contributes to sympathetic excitation in rats with heart failure after myocardial infarction

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Submitted 4 September 2015; accepted in final form 11 January 2016

Yu Y, Wei SG, Zhang ZH, Weiss RM, Felder RB. ERK1/2 MAPK signaling in hypothalamic paraventricular nucleus contributes to sympathetic excitation in rats with heart failure after myocardial infarction. Am J Physiol Heart Circ Physiol 310: H732–H739, 2016. First published January 22, 2016; doi:10.1152/ajpheart.00703.2015.—Brain MAPK signaling pathways are activated in heart failure (HF) induced by myocardial infarction and contribute to augmented sympathetic nerve activity. We tested whether decreasing ERK1/2 (also known as p44/42 MAPK) signaling in the hypothalamic paraventricular nucleus (PVN), a forebrain source of presympathetic neurons, would reduce the upregulation of sympathoexcitatory mediators in the PVN and augmented sympathetic nerve activity in rats with HF. Sprague-Dawley rats underwent left anterior descending coronary artery ligation to induce HF, with left ventricular dysfunction confirmed by echocardiography. One week after coronary artery ligation or sham operation, small interfering (si)RNAs targeting ERK1/2 or a nontargeting control siRNA were microinjected bilaterally into the PVN. Confocal images revealed reduced phosphorylated ERK1/2 immunofluorescence in the PVN of HF rats treated with ERK1/2 siRNAs compared with HF rats treated with control siRNA. Western blot analysis confirmed significant reductions in both total and phosphorylated ERK1/2 in the PVN of HF rats treated with ERK1/2 siRNAs along with reduced expression of renin-angiotensin system components and inflammatory mediators. HF rats treated with ERK1/2 siRNAs also had reduced PVN neuronal excitation (fewer Fos-related antigen-like-immunoreactive neurons), lower plasma norepinephrine levels, and improved peripheral manifestations of HF compared with HF rats treated with control siRNAs. These results demonstrate that ERK1/2 signaling in the PVN plays a pivotal role in mediating sympathetic drive in HF induced by myocardial infarction and may be a novel target for therapeutic intervention.

NEW & NOTEWORTHY

In rats with heart failure induced by myocardial infarction, phosphorylation of ERK1/2, also known as p44/42 MAPK, contributes to the upregulation of renin-angiotensin system activity and inflammation in the hypothalamic paraventricular nucleus and to augmented sympathetic nerve activity.

Heart failure (HF) induced by myocardial infarction is characterized by an increase in renin-angiotensin system (RAS) activity and proinflammatory cytokines (PICs) in the paraventricular nucleus (PVN) of the hypothalamus (5, 15, 36), a forebrain center that integrates neural and humoral signals driving the sympathetic nervous system (4) and plays a major role in the dysfunctional autonomic regulation in HF (23, 29, 35, 44, 45, 61). Central interventions that reduce RAS and PIC activity in the PVN reduce sympathetic nerve activity and improve the peripheral manifestations of HF (11, 14, 15, 53, 55).

Ang II and the prototypical PIC TNF-α both activate the ubiquitous intracellular MAPK signaling cascades that regulate numerous cellular functions via cytoplasmic, nuclear and ion channel effects (19, 24, 30, 32, 37). Thus, as might be expected, HF rats have increased activated phosphorylated (p-)ERK (also known as p44/42 MAPK), p38 MAPK, and JNK in the PVN (45). A recent study (44) revealed that inhibition of brain MAPK signaling, like inhibition of brain RAS (6, 57) or PIC (15, 59), and the chemokine stromal cell-derived factor (SDF)-1 (46), factors that contribute to sympathetic activation in HF (10, 13, 47, 61).

p-ERK1/2 has numerous cytoplasmic and nuclear effects (39, 49). Among its nuclear effects, p-ERK1/2 can activate multiple nuclear transcription factors (3, 18, 39, 49) whose products may include RAS components and inflammatory mediators (3, 12, 25). By that mechanism, ERK1/2 signaling may perpetuate the central excitatory neurochemical milieu driving sympathetic activity in HF.

The present study focused specifically on the role of ERK1/2 signaling in the PVN as a mechanism driving sympathetic nerve activity in HF. We sought to determine whether knockdown of ERK1/2 in the PVN of HF rats would reduce the local expression of sympathoexcitatory mediators, attenuate sympathetic drive, and ameliorate the peripheral manifestations of HF.

METHODS

Animals. Adult male Sprague-Dawley rats weighing 250–300 g were purchased from Harlan Sprague Dawley (Indianapolis, IN). Animals were housed in temperature- (23 ± 2°C) and light-controlled rooms, and standard rat chow and water were provided ad libitum. Experiments were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. The experimental procedures were approved by the Institutional Animal Care and Use Committee of the University of Iowa.

Induction of HF. Rats were anesthetized [ketamine (100 mg/kg) + xylazine (10 mg/kg) ip] and underwent left anterior descending coronary artery ligation to induce HF or sham operation (sham), as previously described (53, 55). Echocardiography was performed under ketamine sedation (60 mg/kg ip) within 24 h of coronary ligation to assess the extent of ischemic injury in HF rats. The percent ischemic zone, left ventricular (LV) ejection fraction, and LV end-diastolic volume were measured as previously described (53, 55). Animals with small infarctions (ischemic zone ≤ 30%) were excluded from the study.

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Experimental protocol. One week after coronary artery ligation or sham operation, rats were anesthetized [ketamine (100 mg/kg) + xylazine (10 mg/kg) ip] and received bilateral PVN microinjections of either pooled ERK1/2 small interfering (si)RNAs [28 ng in 0.5 μl 10 mM JetSTTM (Polyplus-transfection), n = 11], a dose previously determined to be effective in reducing total ERK1/2 and p-ERK1/2 in ANG II-infused rats (52), or a scrambled siRNA (control, n = 11). Sham rats received bilateral PVN microinjections of the scrambled siRNA only (n = 4). PVN microinjections were performed as previously described (52). Five to seven days after the PVN microinjections, a second echocardiogram was performed in some HF rats (n = 4 for each group). Rats from each group were then euthanized for biochemical, molecular, immunohistochemical, and anatomic experiments.

Western blot analysis. Brains were quickly removed, frozen in liquid nitrogen, and stored at −80°C before study. Brains were cut into 500-μm coronal sections. The PVN region including a small amount of the surrounding hypotalamic tissue, and the cerebral cortex at the same rostral-caudal level were punched with a 15-gauge needle stub (inner diameter: 1.5 mm). PVN and cortical tissues were homogenized in cell lysis buffer (Cell Signaling Technology, Beverly, MA), and protein concentration was measured using the BCA protein assay kit (Pierce, Rockford, IL).

Total ERK1/2 and p-ERK1/2 were determined by Western blot analysis in normal rats (n = 4), sham rats treated with control siRNA (n = 4), HF rats treated with control siRNA (n = 7), and HF rats treated with ERK1/2 siRNAs (n = 7). Angiotensin-converting enzyme (ACE), ANG II type 1 receptors (AT1Rs), TNF-α, IL-1β, and cyclooxygenase (COX)-2 were determined only in HF rats treated with control siRNA (n = 7) or ERK1/2 siRNAs (n = 7).

Protein levels for p-ERK1/2, total ERK1/2, RAS components (ACE and AT1Rs), inflammatory mediators IL-1β, TNF-α, and COX-2, and β-actin were analyzed by Western blot analysis using primary antibodies to p-ERK1/2, total ERK1/2 (Cell Signaling Technology, Danvers, MA), ACE, AT1R, IL-1β, TNF-α (Santa Cruz Biotechnology, Santa Cruz, CA), COX-2 (Cayman Chemical, Ann Arbor, MI), and β-actin (Cell Signaling Technology). The density of the bands was quantified using Image Lab analysis software (Bio-Rad, Hercules, CA).

Immunohistochemistry. In HF rats (n = 4 rats/group), p-ERK1/2 immunoreactivity in the PVN was determined by immunofluorescent staining using antibodies for p-ERK1/2/2 MAPK (Cell Signaling Technology). PVN neurons were identified using antibodies to NeuN (Millipore, Billerica, MA).

In HF rats (n = 4 rats/group), Fos-related antigen-like immunoreactivity (Fra-LI), a marker of neuronal activation (40), was detected by diaminobenzidine staining using a rabbit polyclonal antibody (c-fos K-25, Santa Cruz Biotechnology). Fra-LI-positive neurons within a window (4 × 104 μm2) superimposed over the posterior magnocellular, ventrolateral parvocellular, medial parvocellular, and dorsal parvocellular subregions of the PVN were counted manually in two representative 16-μm transverse sections approximately −1.8 mm from the bregma, and an average value for each subregion was used for data analysis.

Analysis of plasma norepinephrine. Plasma norepinephrine (NE) levels in HF rats (n = 7 rats/group) were measured by an ELISA kit (Rocky Mountain Diagnostics, Colorado Springs, CO) according to the manufacturer’s instructions.

Anatomic indicators of HF. In HF rats treated with control siRNA or ERK1/2 siRNAs and euthanized for molecular and biochemical experiments, the ratios of LV weight to body weight, right ventricular (RV) weight to body weight, and wet lung weight to body weight were determined to assess the extent of cardiac remodeling and pulmonary venous congestion.

Statistical analysis. All data are expressed as means ± SE. The significance of differences in mean values was analyzed by one-way ANOVA and Tukey multiple-comparison test or Student’s t-test. P values of <0.05 were considered statistically significant.

RESULTS

ERK1/2 expression in the PVN and cerebral cortex. Our previous studies have demonstrated significant increases in p-ERK1/2 expression in the PVN of HF rats studied 4 wk after coronary artery ligation (44, 45). In the present study, the level of p-ERK1/2 was significantly increased in the PVN of HF rats that received control siRNA 2 wk after myocardial infarction, compared with normal rats or sham rats that received control siRNA. The level of total ERK1/2 was not different across these three groups (Fig. 1). Total ERK 1/2 and p-ERK1/2 were both significantly lower in the PVN of HF rats treated with ERK1/2 siRNAs, compared with HF rats treated with control siRNA. The level of p-ERK1/2 in the PVN of HF rats treated with ERK1/2 siRNAs did not differ significantly from the low levels of p-ERK1/2 that were detectable in the PVN of normal rats and sham rats treated with control siRNA. There were no differences in the levels of total ERK1/2 and p-ERK1/2 in the brain cortex across the four experimental groups.

Confocal imaging revealed abundant immunofluorescent staining for p-ERK1/2 in PVN neurons of HF rats that received control siRNA. Some p-ERK1/2 staining was also observed in non-neuronal (likely glial) elements of the PVN (Fig. 2), consistent with our previous findings in HF rats studied 4 wk after coronary artery ligation (44, 45). The fluorescent intensity for p-ERK1/2 in the PVN was substantially lower in HF rats treated with ERK1/2 siRNAs.

Effect of ERK1/2 knockdown on PVN neuronal activity and sympathetic excitation in HF rats. In HF rats, we examined the effects of ERK1/2 knockdown on PVN expression of ACE, AT1R, TNF-α, IL-1β, and COX-2, factors that are upregulated in the PVN and contribute to sympathetic excitation in this model of HF (6, 16, 17, 36, 54, 57). HF rats treated with ERK1/2 siRNAs had substantially lower PVN levels of the RAS components ACE and AT1R and of the inflammatory mediators IL-1β, TNF-α, and COX-2 than HF rats treated with control siRNA (Fig. 3).

Effect of ERK1/2 knockdown on PVN neuronal activity and sympathetic excitation in HF rats. Our previous studies have consistently shown increased Fra-LI in the PVN of HF rats, correlating with an increase in indexes of sympathetic nerve activity. In the present study, HF rats treated with ERK1/2 siRNAs had significantly fewer Fra-LI-positive neurons in the posterior magnocellular, medial parvocellular, and ventrolateral parvocellular regions of the PVN than HF rats treated with control siRNA (Fig. 4, A and B). No difference between groups was observed for Fra-LI in the dorsal parvocellular region of the PVN.

Plasma NE levels were also significantly lower in HF rats treated with ERK1/2 siRNAs, compared with HF rats treated with control siRNA (Fig. 4C).

Effect of reducing ERK1/2 expression in the PVN on peripheral indexes of HF. Echocardiography revealed similar infarct sizes and degrees of LV systolic dysfunction in HF rats assigned to treatment with ERK1/2 siRNAs and control siRNA (Table 1). In HF rats (n = 4 rats/group) that underwent a second echocardiogram, bilateral PVN microinjections of control siRNA or ERK1/2 siRNAs had no apparent effect on the percent ischemic zone, LV end-diastolic volume, or LV ejection fraction. There were also no significant differences in body weight or LV weight-
to-body weight ratio between the two experimental groups (Fig. 5). However, HF rats treated with ERK1/2 siRNAs had a lower wet lung weight-to-body weight ratio, indicating less pulmonary congestion, and a lower RV weight-to-body weight ratio, indicating less RV hypertrophy, than HF rats treated with control siRNA.

**DISCUSSION**

We have previously reported that ERK1/2 activity is increased in the PVN of rats with ischemia-induced HF (44, 45), where it is associated with an increase in the expression of
AT1Rs (45), upregulation of inflammatory mediators (42), and increased sympathetic nerve activity. Acute central administration of agents that inhibit the phosphorylation of ERK1/2 causes a dramatic reduction in sympathetic nerve activity in rats with established HF but has no effect on sham-operated control rats (44). Chronic central administration of an ERK1/2 inhibitor normalizes plasma NE in HF rats (42).

The present study sought to determine whether ERK1/2 activity within the PVN, an important forebrain locus of presympathetic neurons, contributes to sympathetic activation in HF rats. Rats were treated with bilateral PVN microinjections of siRNAs for ERK1/2 MAPK or control siRNA 1 wk after coronary artery ligation and studied 5–7 days later, within the window of effective siRNA knockdown (31). At this time

*Fig. 3. Bilateral PVN microinjection of ERK1/2 MAPK siRNAs reduced expression of renin-angiotensin system and inflammatory mediators in the PVN in HF rats. A: representative Western blots. B–F: quantitative comparison of protein levels for angiotensin-converting enzyme (ACE; B), ANG II type 1 receptors (AT1R; C), IL-1β (D), TNF-α (E), and cyclooxygenase (COX)-2 (F) in the PVN. Values were corrected by β-actin and are expressed as means ± SE; n = 7 rats/group. *P < 0.05 vs. HF + CON siRNA.

*Fig. 4. Effect of bilateral PVN microinjection of ERK1/2 MAPK siRNAs on PVN neuronal excitation and plasma noradrenaline (NE). A: representative sections from each group showing Fos-related antigen-like immunoreactivity (Fra-LI) among neurons in the PVN. Dark dots indicate single activated neurons. B: quantification of Fra-LI-positive neurons in subnuclear regions of the PVN in each treatment group (n = 4 rats/group). pm, posterior magnocellular PVN; mp, medial parvocellular PVN; vlp, ventrolateral parvocellular PVN; dp, dorsal parvocellular PVN. C: plasma levels of NE, a marker of sympathetic nerve activity, in each treatment group (n = 7 rats/group). Values are expressed as means ± SE. *P < 0.05 vs. HF + CON siRNA.
We previously reported that ANG II upregulates the expression and COX-2 (38), the inducible enzyme that produces PGE2. ANG II is the precursor of ANG II, AT1R (12, 25), TNF-α, IL-1β, and COX-2, elements commonly associated with increased sympathetic drive in HF, reduced Fra-LI in the PVN, reduced plasma NE levels, and lower wet lung weight-to-body weight and RV weight-to-body weight ratios than HF rats treated with control siRNAs. The reduction in ERK1/2 expression in the PVN induced by ERK1/2 siRNAs had no effect on echocardiographic indexes of LV systolic function in HF rats. However, there was a significant reduction in the wet lung weight-to-body weight ratio, indicating an improvement in pulmonary congestion, and in the RV weight-to-body weight ratio, indicating less RV hypertrophy. These anatomic findings reflect the influence of a reduced sympathetic drive on LV end-diastolic pressure via reduced sympathetically mediated renal Na\(^+\) retention (preload reduction) and arterial vasoconstriction (afterload reduction), resulting in lower pulmonary venous, pulmonary arterial, and RV pressures. In previous studies using this model of HF, we have consistently observed an association of RV weight-to-body weight and wet lung weight-to-body weight ratios with the level of LV end-diastolic pressure (8, 16, 17, 42, 45, 50, 53). In the presence of scarred myocardium, a moderate reduction in LV end-diastolic pressure may not result in a change in LV end-diastolic volume by echocardiography (16, 17, 42).

Notably, a low level of p-ERK1/2 was detectable in both the PVN and cerebral cortex in normal rats, suggesting that ERK1/2 signaling is necessary for normal brain function. However, HF induces a marked upregulation of p-ERK1/2 in the subfornical organ and PVN (44, 45), which are key cardiovascular regulatory regions of the brain, with no apparent effect on the immediately surrounding structures or on cerebral cortex. Pending further studies, if the upregulated ERK1/2 signaling in HF is found to be confined to cardiovascular regions of the brain, it may emerge as a potential novel therapeutic target in HF.

It is now well established that increased brain RAS activity and inflammation both contribute to sympathetic excitation in HF. How MAPK signaling fits into this excitatory scenario is less well understood. We have observed that many of the excitatory agonists that are upregulated in the PVN in HF, including ANG II (43), aldosterone (58), TNF-α (46), and the chemokine SDF-1 (46), can upregulate ERK1/2 signaling in the PVN in normal rats. Others have reported that PGE2, corticotropin-releasing hormone, and the (pro)renin receptor are also capable of activating the ERK1/2 signaling pathway (26–28). These observations suggest that ERK1/2 signaling can be evoked by a wide range of excitatory ligands. The present study suggests that ERK1/2 signaling contributes significantly to the excitatory milieu in the PVN that drives sympathetic nerve activity in HF.

There are at least two mechanisms by which ERK1/2 signaling might contribute to sympathetic excitation. The present study focused on ERK1/2-mediated upregulation of excitatory mediators in the PVN. Once activated, p-ERK1/2 can stimulate several transcription factors, e.g., activator-protein 1, Elk-1, nuclear factor-κB, and cAMP response element-binding protein (3, 18, 39, 49), whose downstream products may include such key excitatory elements as angiotensinogen (2), the precursor of ANG II, AT1R (12, 25), TNF-α and IL-1β (3, 22, 33), and COX-2 (38), the inducible enzyme that produces PGE2. We previously reported that ANG II upregulates the expression in lower pulmonary venous, pulmonary arterial, and RV pressures. In previous studies using this model of HF, we have consistently observed an association of RV weight-to-body weight and wet lung weight-to-body weight ratios with the level of LV end-diastolic pressure (8, 16, 17, 42, 45, 50, 53). In the presence of scarred myocardium, a moderate reduction in LV end-diastolic pressure may not result in a change in LV end-diastolic volume by echocardiography (16, 17, 42).

Table 1. Echocardiographic measurements

<table>
<thead>
<tr>
<th>Variables 24 h after MI</th>
<th>HF + Control siRNA</th>
<th>HF + ERK1/2 siRNA</th>
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</thead>
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<tr>
<td></td>
<td>n</td>
<td></td>
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<tr>
<td>LVEDV, ml</td>
<td>7</td>
<td>7</td>
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<tr>
<td>LVEF</td>
<td>0.71 ± 0.03</td>
<td>0.70 ± 0.04</td>
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<tr>
<td>IZ, %</td>
<td>44 ± 2</td>
<td>43 ± 1</td>
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Values are expressed as means ± SE; n, number of rats/group. HF, heart failure; siRNA, small interfering RNA; LVEDV, left ventricular (LV) end-diastolic volume; LVEF, LV ejection fraction; IZ, size of the ischemic zone as a percent of LV circumference.

Fig. 5. Effect of bilateral PVN microinjection of ERK1/2 MAPK siRNAs on peripheral manifestations of HF 2 wk after coronary artery ligation. A: body weight (BW). B: left ventricular (LV) weight-to-BW ratio. C: right ventricular (RV) weight-to-BW ratio. D: wet lung-to-BW ratio. Values are expressed as means ± SE; n = 7 rats/group. *P < 0.05 vs. HF + CON siRNA.
of AT₁Rs in the PVN in an ERK1/2-dependent manner (43, 45). In addition, we demonstrated that siRNA knockdown of ERK1/2 in the PVN significantly attenuated the increased AT₁R expression in the PVN and the hypertension induced by a slow pressor dose of ANG II (56). The present study extends our understanding of the generative role of ERK1/2 signaling by demonstrating that phosphorylation of ERK1/2 mediates upregulation of the expression of another RAS component, ACE, and of the inflammatory mediators TNF-α, IL-1β, and COX-2, in the PVN of HF rats. Thus, it appears that ERK1/2 signaling augments the very systems that stimulate its own activity, a feedforward mechanism that may perpetuate the augmented sympathetic nerve activity in HF. The present study suggests that this excitatory circuit can be effectively interrupted by inhibiting ERK1/2 activity at the PVN level.

A second mechanism by which ERK1/2 signaling may contribute to increased sympathetic activation in HF, not addressed in this study, is by disinhibiting presympathetic PVN neurons. We have observed that prior treatment with acute intracerebroventricular injections of selective inhibitors of ERK1/2 signaling can block the immediate sympathoexcitatory effects of ANG II, aldosterone, TNF-α, or SDF-1 (44, 46, 58, 59) in normal rats. These acute effects are not easily reversed, or SDF-1 (44, 46, 58, 59) by inhibiting ERK1/2 activity at the PVN level. The excitability of PVN neurons is regulated by the inhibitory A-current (20, 34), with the K⁺ channel subunit Kv4.2 playing a prominent role (21). p-ERK1/2 has been shown to phosphorylate and inactivate Kv4.2 in hippocampal neurons (1, 32), where it may contribute to long-term potentiation (37). Phosphorylation of existing proteins (ERK1/2 and Kv4.2) can occur rapidly and might account for the ability excitatory agonists to mediate acute sympathoexcitatory effects and the ability of ERK1/2 inhibitors to reduce sympathetic activity in HF rats over a relatively short time course (44). While this hypothesis remains to be tested, we speculate that a combination of a channel-mediated increase in presympathetic PVN neuronal excitability and a genomic-mediated increase in the the mediators that excite those neurons might account for the influence of ERK1/2 signaling on sympathetic activity in HF.

The cells mediating the effects of ERK1/2 signaling in the PVN and other cardiovascular regulator regions remain to be determined. Confocal imaging in this study revealed a prominent localization of p-ERK1/2 in PVN neurons, where both genomic and channel effects influencing neuronal activity and sympathetic drive may occur. However, ERK1/2 signaling also contributes to glial responses to stressful circumstances (41, 48, 60), and a close examination of our data reveals p-ERK1/2 labeling in non-neuronal elements of the PVN. Thus, ERK1/2 signaling in astrocytes and microglia may well contribute to the generation of excitatory and inflammatory mediators in the PVN in HF. Further studies are needed to determine how this signaling mechanism contributes to the neurochemical milieu of the PVN and other cardiovascular-related nuclei in HF and hypertension.

ERK1/2 is not the only MAPK signaling pathway that is activated in cardiovascular regions of the brain in HF. Our previous studies have demonstrated upregulation of p-p38 MAPK and p-JNK as well as p-ERK1/2 in the PVN of HF rats (44, 45). However, central inhibition of p-p38 or p-JNK seems less effective than central inhibition of p-ERK1/2 in reducing sympathetic nerve activity in HF (42, 44). Whether these two MAPK signaling pathways are truly less important to sympathetic activation or whether these observations simply reflect inadequate doses of inhibitors remains to be determined. Silencing p38 MAPK or JNK, as done here for ERK1/2, might be the most effective way of addressing that issue.

**Perspectives**

Activation of the ERK1/2 MAPK signaling pathway appears to be essential for the full expression of sympathetic excitation in HF. The available literature suggests that phosphorylation of ERK1/2 is both an immediate response mechanism, driving excitation of presynaptic sympathetic neurons, and a stimulus to the production of more agonists to perpetuate sympathetic excitation. As such, brain ERK1/2 MAPK signaling may be an ideal target for therapeutic intervention. The present study suggests that interventions that interfere with ERK1/2 signaling in the PVN may be effective in reducing the adverse effects of augmented sympathetic nerve activity in HF.

**GRANTS**

This material is based on work supported in part by the Department of Veterans Affairs, Veterans Health Administration, Office of Research and Development, Biomedical Laboratory Research and Development, by the University of Iowa, and by National Institutes of Health Grants R01-HL-096671 (to R. B. Felder), R01-HL-073986 (to R. B. Felder), and S10-OD-019941 (to R. M. Weiss).

**DISCLAIMERS**

The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Heart, Lung, and Blood Institute or National Institutes of Health.

**DISCLOSURES**

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

**AUTHOR CONTRIBUTIONS**

Author contributions: Y.Y. and R.B.F. conception and design of research; Y.Y., S.-G.W., R.M.W., and R.M.W. performed experiments; Y.Y. and R.M.W. analyzed data; Y.Y. and R.B.F. interpreted results of experiments; Y.Y. prepared figures; Y.Y. drafted manuscript; S.-G.W. and R.B.F. edited and revised manuscript; R.M.W. and R.B.F. approved final version of manuscript.

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