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RESEARCH ARTICLE

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Activation of nociception-sensitive ionotropic glutamate receptor-expressing rostroventrolateral medulla neurons by stimulation of cardiac afferents in rats

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Abstract

Myocardial ischemia causes the release of bradykinin, which activates afferent nerve endings in the ventricular epicardium. This elicits a sympathetically mediated increase in arterial pressure and heart rate, referred to as the cardiogenic sympathetic afferent reflex. The rostroventrolateral medulla (RVLM) is a key sympathetic brain stem site for regulating cardiovascular activity. This study aimed to determine the importance of non-barosensitive nociception sympathetic activity and the role of glutamate receptor activation of RVLM neurons in the cardiogenic sympathetic afferent reflex. We tested the hypothesis that inhibition of barosensitive sympathetic activity attenuates but does not abolish the reflex response to cardiac visceral afferents. Renal sympathetic nerve activity (RSNA), arterial pressure, and heart rate responses to epicardial bradykinin application were recorded in anesthetized rats before and after bilateral RVLM microinjection of either $GABA_A$ agonist muscimol, ionotropic glutamate receptor antagonist kynurenic acid, N-methyl-d-aspartate (NMDA) receptor antagonist 2-amino-5- phosphonopentanoic acid (AP5), or non-NMDA antagonist 6-cyano-7-nitroquinoxaline-2, 3-dione (CNQX). Baroreceptor loading-induced inhibition of barosensitive activity attenuated the bradykinin-induced RSNA response (93 ± 14% increase) and tachycardia (18 ± 3 bpm). While RVLM muscimol microinjection abolished the RSNA response (1.6 ± 4.2% from baseline, 0.49 ± 0.38 μV*s), surprisingly, it did not abolish the tachycardia (27 ± 4 bpm). Kynurenic acid microinjection blocked the arterial pressure and RSNA responses, while AP5 or CNQX only attenuated the responses. These data suggest that nociception-sensitive sympathetic activity that does not appear to be barosensitive is also involved in the cardiogenic sympathetic afferent reflex. Importantly, while muscimol and kynurenic acid abolished the arterial pressure and RSNA response, neither affected the tachycardia, suggesting an alternate cardiac pathway independent of RVLM.

KEYWORDS

bradykinin, cardiogenic reflex, cardiovascular, heart rate, sympathetic

1 | INTRODUCTION

The cardiogenic sympathetic afferent reflex is a cardiovascular reflex characterized by a sympathetically mediated increase in blood pressure and heart rate.[1–8] Myocardial ischemia produces several metabolites, including bradykinin, which activates cardiac sympathetic nerve endings on the anterior left ventricular epicardium of the heart.[9–13] The cardiogenic sympathetic afferent reflex is important for maintaining vascular flow to coronary vessels during myocardial ischemia. However, the increased sympathetic outflow can also be detrimental because it further increases the oxygen demand of the ischemic myocardium.

Vasomotor neurons within the rostral ventrolateral medulla (RVLM) play an important role in the sympathetic regulation of arterial pressure and heart rate and provide a tonic source of sympathetic activity to the heart and blood vessels.[14–21] Increased blood pressure within the carotid sinus and aortic arch stimulates baroreceptor afferent fibers that powerfully inhibit tonic RVLM activity.[16,22] Increasing arterial pressure above 140 mmHg in the rat can practically silence renal sympathetic nerve activity.[8,23–26] However, while baroreceptor activation inhibits tonically active sympathetic activity, not all RVLM activity is tonically active or barosensitive.[2,27]

Li and Pan (2000) demonstrated the role of barosensitive RVLM neurons in the reflex response to epicardial bradykinin.[2] In that study, they recorded tonically active RVLM single-unit neuronal firing and found that only barosensitive neurons responded to epicardial bradykinin application. However, they only studied tonically active neurons in the RVLM, and the significance of non-barosensitive RVLM activity in the cardiogenic sympathetic afferent reflex and the neurotransmitters involved remains unknown.

While most basal sympathetic activity is derived from tonically active barosensitive RVLM neurons, and baroreceptor activation can virtually abolish all tonically active sympathetic activity, we reasoned that any reflex response elicited by activation of cardiac nociceptors during baroreceptor loading is mediated by neurons that are neither barosensitive nor tonically active. We tested the hypothesis that the cardiogenic sympathoexcitatory reflex is mediated by RVLM glutamate receptor activation of both barosensitive and non-barosensitive sympathetic activity. To show that RVLM mediated the reflex response and determine the differential role of RVLM glutamate receptors, we tested the response to epicardial bradykinin after either $GABA_A$ agonist muscimol, ionotropic glutamate receptor antagonist kynurenic acid, NMDA receptor antagonist 2-amino-5-phosphonopentanoic acid (AP5), or non-NMDA antagonist 6-cyano-7-nitroquinoxaline-2,3-di one (CNQX) microinjection into the RVLM bilaterally.

We found that baroreceptor loading-induced inhibition of tonic sympathetic activity only attenuated, but did not eliminate, the reflex response to epicardial bradykinin. This suggests that the cardiogenic sympathetic afferent reflex is also mediated by non-barosensitive, nociception-sensitive RVLM activity. While bilateral RVLM microinjection of either muscimol or kynurenic acid abolished the arterial pressure and RSNA response to epicardial bradykinin, neither affected the tachycardia, suggesting an alternate brain stem cardiac chronotropic pathway.

2 | METHODS

Adult male Sprague–Dawley rats (Envigo, Indianapolis, IN) 10–14 weeks old, weighing 300-400 g, were surgically prepared according to the Guide for the Care and Use of Laboratory Animals using procedures approved by the East Tennessee State University Committee on Animal Care and Use.[28] Data and analyses supporting this study's findings are available from the corresponding author upon reasonable request. Rats were initially anesthetized using 2% isoflurane in O_2 through a nose cone to allow for the cannulation of vessels and tracheostomy. We confirmed the adequate depth of anesthesia before surgical preparations by the absence of a corneal reflex (eye-blink) or withdrawal response to tail pinch. At the end of the experiments, rats were euthanized by an intravenous injection of an overdose of 1% αchloralose and immediately decapitated.

2.1 | Surgical preparation

We maintained each rat's body temperature between 37 and 38°C throughout the surgery with a heating pad and lamp coupled to a TCAT-2 temperature controller and rectal temperature probe (Physitemp Clifton, NJ). The left carotid artery was cannulated with PE-50 tubing (Becton Dickinson, Sparks MD) to measure arterial pressure with a PT300 pressure transducer (Grass Instruments, Quincy, MA) connected to a P122 strain gauge amplifier (Grass Instruments, Quincy, MA). Heart rate was counted by triggering from the arterial pressure pulse. The left jugular vein was cannulated with PE-50 tubing for the I.V. administration of anesthetics and paralytics (see below). We cut the cervical vagus nerve bilaterally to ensure no efferent activity was conducted via the vagus nerve and, therefore, eliminate the possibility of any parasympathetic effect on heart rate after bradykinin application. The left and the right femoral veins were cannulated for the separate administration of vasopressor drugs (for details, see "Epicardial Bradykinin During Baroreceptor Loading").

The trachea was cannulated with a 14G endotracheal tube for mechanical ventilation using a rodent ventilator (CWE, Ardmore, PA). End-tidal O_2 and CO_2 were monitored with a Gemini O_2 & CO_2 gas analyzer (CWE, Ardmore, PA). Minute volume was set at 100 mL/kg, and CO_2 was maintained at 4%–6% by adjusting the respiratory rate, typically between 55 and 70 rpm. We performed a lateral thoracotomy for epicardial bradykinin (10 μg/mL, Sigma, St. Louis, MO) application using an electrosurgical cauterizer (Bantam Pro A952, Bovie, Clearwater, FL.). The pericardium was left intact until we were ready to apply bradykinin to the epicardium.

Rats were placed in a stereotaxic frame (Kopf Instruments, Tujunga, CA) with the incisor bar positioned 5 mm below the interaural level. We removed the skin overlying the dorsal surface of the skull. Then, we drilled a small square window (~1 cm) at the surface of the skull to allow for placement of the micropipette within the right and left RVLM. Stereotaxic coordinates were 12 mm caudal to bregma, 2 mm lateral to the midline, and 8 mm ventral from the surface of the cerebellum.

2.2 | Renal sympathetic nerve recording

A left flank incision was performed to expose the kidney, and the surrounding tissue was retracted to expose the left kidney for renal sympathetic nerve recordings. The left kidney was gently retracted laterally, and the renal sympathetic nerve was carefully dissected. The peritoneum was immersed in mineral oil, and the nerve was mounted on a bipolar hook electrode. The distal end of the renal nerve was cut to avoid recording any afferent activity. We amplified (X10,000) and bandpass filtered (100–3000 Hz) the nerve signal with an alternating current amplifier (model P511, Grass Instruments). The nerve signal was rectified, and the signal was integrated using a 0.5-s time constant.

After the surgical procedures, rats were slowly administered 1% αchloralose (100 mg/kg I.V., Sigma, St. Louis, MO) and weaned from isoflurane over approximately 20 min. We carefully monitored arterial pressure during this infusion to avoid over-anesthetizing the rat. This approach maintained a complete surgical anesthetic state throughout the recording. We verified the depth of anesthesia by the absence of an increase in arterial pressure or RSNA to tail-pinch without any paralytic drug. After determining that rats remained completely surgically anesthetized, they were paralyzed with D-tubocurarine (0.1 mg/kg I.V., Sigma, St. Louis, MO) to block any spontaneous muscle twitching that typically occurs in the retracted external oblique or of the diaphragm. This dose of D-tubocurarine lasted ~30–45 min and was allowed to wear off to assess the adequacy of the anesthetized state of the rat. In all cases, rats' arterial pressure, heart rate, and RSNA remained completely unresponsive to corneal reflex or tail-pinch. At the end of the recordings, background electrical noise was determined by cutting the proximal end of the renal nerve, thereby removing efferent activity. The remaining activity was subtracted from the integrated values of the RSNA.

2.3 | Epicardial bradykinin

As previously demonstrated, we applied bradykinin to the anterior epicardial surface (~1 cm^2) of the left ventricle with a cotton-tipped applicator through the exposed window in the left chest to stimulate cardiac nociceptive afferents.[1,2,6–8] After each bradykinin application, we washed the heart using ~10 mL of room temperature normal saline, and the arterial pressure, heart rate, and RSNA returned to baseline levels over ~10–15 min. To ensure that rats do not respond to the mechanical application of the cotton applicator touching the epicardium, we applied saline to the heart before any reflex trials, as we have in previous studies.[6–8]

2.4 | Epicardial bradykinin during baroreceptor loading

Before microinjection treatment, we tested the heart rate and RSNA reflex response to epicardial bradykinin during baroreceptor loading-induced inhibition of RSNA. To do this, we administered the α-adrenergic agonist phenylephrine (PE; 125 μg/mL) using ramped infusions via the left femoral vein catheter, starting at a rate of 375 μg/h and increasing by 125 μg/h every 5 s until arterial pressure increased to ~160 mmHg as previously described.[25,26,29] We then tested the reflex response to epicardial bradykinin during the PE-induced peak in arterial pressure (~160 mmHg).

2.5 | Epicardial bradykinin after RVLM microinjection

A glass pipette (tip diameter 20–30 μm) was first advanced to the RVLM within the right hemisphere and then the left hemisphere for the bilateral microinjections. Drugs were injected using a calibrated microinjection system (Nanoject III, Drumond Scientific, Broomall, PA), and we observed the movement of the meniscus of the injectant using an operating microscope. After the microinjection, the glass pipette remained in the right RVLM for ~1 min to ensure adequate drug diffusion. After we withdrew the

glass pipette from the right hemisphere, it was placed in the respective stereotaxic coordinates for injection into the RVLM of the left hemisphere and left in place ~1 min after the microinjection. The reflex response to epicardial bradykinin was tested ~5 min after the second microinjection.

2.6 | Histology

We examined the location of the pipette tip within the RVLM and histologically confirmed each injection in all rats. After the experiments, brain stems were removed rapidly, fixed overnight in 4% paraformaldehyde solution, and stored in 30% sucrose at 4°C until cut. Frozen 40 mm coronal sections were cut on a freezing microtome and mounted on slides. All microinjections contained 5% rhodamine-labeled fluorescent microspheres (0.04 μm, Molecular Probes, Eugene, OR). Sections were cut from the florescent microsphere's emergence to the facial nerve's caudal pole, used as the rostrocaudal landmark. Sections were imaged with an Olympus BX43 fluorescence microscope and an Orca-Spark digital camera (Hamamatsu, Bridgewater, NJ). Brightfield and fluorescent images were merged to analyze the location of the glass injection needle tip and the distribution of the injectant. Microinjection locations were plotted on standardized sections from the Paxinos and Watson atlas. Rats with micropipette misplacement outside the RVLM were excluded from the analysis.

2.7 | Data analysis

Arterial pressure, heart rate, and RSNA were recorded with Cambridge Electronic Design Micro1401 hardware and Spike 2® software (Cambridge, UK). After a high-quality nerve recording was obtained, an ~15 min stabilization period was given, followed by a 5 min control period to normalize baseline and reflex RSNA before and after microinjection treatments. RSNA is expressed as both the raw integrated (μV*s) activity as well as activity normalized to the initial control period for each rat and expressed as a percentage change relative to that control period as previously described.[30] To assess the arterial pressure, heart rate, and RSNA responses to epicardial bradykinin, baseline arterial pressure, heart rate, and RSNA were averaged during 30 s of the baseline period before epicardial bradykinin. The peak arterial pressure (not assessed during PE infusion), heart rate, and RSNA responses were measured immediately after the bradykinin application.

Data were analyzed using two-way ANOVA and plotted with GraphPad Prism software. Repeated measures was used to compare treatment and bradykinin response within group, and mixed-effect model was used to compare between groups. We used the Bonferroni post-test to compare the difference between group means when F values were significant. $p < 0.05$ was considered statistically significant.

3 | EXPERIMENTAL DESIGN

3.1 | Protocol 1, epicardial bradykinin during baroreceptor loading

We first recorded control responses to epicardial bradykinin to test the reflex response to epicardial bradykinin application during baroreceptor-mediated inhibition of tonically active sympathetic activity. Control responses to epicardial bradykinin were examined at least twice, separated by ~10–15 min to ensure a reproducible response. After each bradykinin application, the heart was washed using ~10 mL of roomºT normal saline, and the arterial pressure, heart rate, and RSNA returned to baseline levels. The reflex response to epicardial bradykinin during baroreceptor loading was tested during the PE-induced increase in arterial pressure (~160 mmHg). The arterial pressure, heart rate, and RSNA were allowed to recover to baseline over ~10–15 min, and the reflex response to epicardial bradykinin was tested to ensure that it returned to control levels.

3.2 | Protocol 2, epicardial bradykinin after RVLM microinjection

For microinjection studies, arterial pressure, heart rate, and RSNA response to epicardial bradykinin application were tested before (control) and after bilateral microinjection (100 nL) into the RVLM. Microinjections of either vehicle (saline), the $GABA_A$ receptor agonist muscimol (1.0 nmol), broad-spectrum ionotropic glutamate receptor antagonist kynurenic acid (5.0 nmol), non-NMDA receptor antagonist 6-cyano-7-nitroquino xaline-2,3-dione (CNQX, 0.9 nmol), or NMDA receptor antagonist 2-amino-5-phosphonopentanoic acid (AP5, 1.2 nmol), into the RVLM, were performed in separate groups of rats such that each rat only received one treatment. We performed control bradykinin tests at least twice, separated by ~10–15 min before microinjection treatments. After control reflex tests, we inhibited baroreceptor-mediated sympathetic activity with PE infusion by increasing arterial pressure to ~160 mmHg. After bradykinin application, the heart was washed using ~10 mL of room temperature normal saline, and the arterial pressure, heart rate, and RSNA returned to

baseline levels. To ensure that the reflex response to epicardial bradykinin was repeatable, we tested the reflex response after arterial pressure, heart rate, and RSNA had returned to baseline.

4 | RESULTS

We conducted these studies with a total of 51 anesthetized vagotomized rats. We dismissed eight rats from analysis due to inaccurate microinjection outside the RVLM. All microinjections were verified histologically and plotted according to Paxinos and Watson's stereotaxic atlas.[31] Microinjections were located within 12.00–12.24 mm caudal to bregma (Figure 1).

4.1 | Baroreceptor loading attenuates and muscimol inactivation of RVLM silences the arterial pressure and RSNA but not the heart rate response to epicardial bradykinin

Because sympathetic vasomotor activity is powerfully inhibited by baroreceptor loading, we used PE-induced baroreceptor loading to inhibit only barosensitive sympathetic activity followed by bilateral microinjection of muscimol into the RVLM to block all sympathetic activity derived from RVLM. As such, the fraction of sympathetic activity elicited during baroreceptor loading is considered non-barosensitive. Table 1 shows the grouped mean (± SEM) arterial pressure, heart rate, and RSNA values during baseline and the reflex response to epicardial bradykinin during control, the PE-induced inhibition of sympathetic activity, and after RVLM microinjection treatment. During control bradykinin applications, repeated bradykinin application consistently induced a response of similar magnitude as we have previously reported.[6–8] Figure S1A–C shows representative tracings of arterial pressure, heart rate, and RSNA during baseline and the reflex response to epicardial bradykinin during control, baroreceptor loading, and before and ~10 min after bilateral microinjection of vehicle (saline) into the RVLM.

Inactivation of the RVLM by muscimol microinjection abolished the arterial pressure and RSNA but not the heart rate response to epicardial bradykinin. In contrast, baroreceptor loading induced inhibition of sympathetic activity only attenuated the responses. Figure 2 shows representative responses and grouped arterial pressure, heart rate, and RSNA during baseline and the reflex response to epicardial bradykinin during control, baroreceptor loading, and before and ~10 min after bilateral microinjection of muscimol into the RVLM. Prior to PE-induced baroreceptor inhibition of sympathetic activity, or muscimol microinjection, control epicardial bradykinin application significantly increased arterial pressure, heart rate, and RSNA ($p < 0.001$, Figure 2A,D–G). Baroreceptor loading significantly decreased baseline heart rate and RSNA and significantly attenuated the heart rate and RSNA response to epicardial bradykinin (Figure 2B). Muscimol microinjection significantly reduced baseline arterial pressure, heart rate, and RSNA ($p < 0.001$, Figure 2C). While muscimol completely abolished the arterial pressure and RSNA response to epicardial bradykinin, the heart rate response to epicardial bradykinin remained, albeit significantly lower ($p = 0.007$) than that observed during PE-induced sympathoinhibition. Vehicle microinjection did not affect arterial pressure, heart rate, or RSNA during baseline or the reflex response to epicardial bradykinin (See Appendix S1).

4.2 | The arterial pressure and RSNA, but not the heart rate response to epicardial bradykinin, are mediated by ionotropic glutamate receptors within the RVLM

To determine the role of RVLM ionotropic glutamate receptor activation during the cardiogenic sympathetic reflex, we tested reflex response to epicardial bradykinin before and after bilateral microinjection of either kynurenic acid, CNQX, or AP5 into the RVLM. Figure 3 shows representative arterial pressure, heart rate, and RSNA responses during baseline and the reflex response to epicardial bradykinin after either vehicle, kynurenic acid, CNQX, or AP5. Kynurenic acid microinjection did not significantly decrease baseline arterial pressure ($p = 0.203$), heart rate ($p > 0.999$), or RSNA ($p > 0.999$) compared with vehicle. It did, however, block the arterial pressure ($p = 0.136$) and RSNA ($p > 0.999$) reflex response to epicardial bradykinin (Figure 4A,C,D). Kynurenic acid microinjection did not diminish the heart rate response to epicardial bradykinin ($p < 0.001$, Figure 4B).

To determine if the reflex response to epicardial bradykinin is mediated by NMDA versus the non-NMDA ionotropic glutamate receptors within the RVLM, we microinjected either AP5 to block NMDA or CNQX to block non-NMDA receptors in two separate groups of rats, and tested the reflex response to epicardial bradykinin. Neither CNQX nor AP5 microinjection affected baseline arterial pressure (CNQX, $p > 0.999$; AP5, $p = 0.561$), heart rate (CNQX, $p > 0.999$; AP5, $p > 0.999$), RSNA (CNQX, $p > 0.999$; AP5, $p > 0.999$) compared with vehicle. After microinjection of either CNQX or AP5,

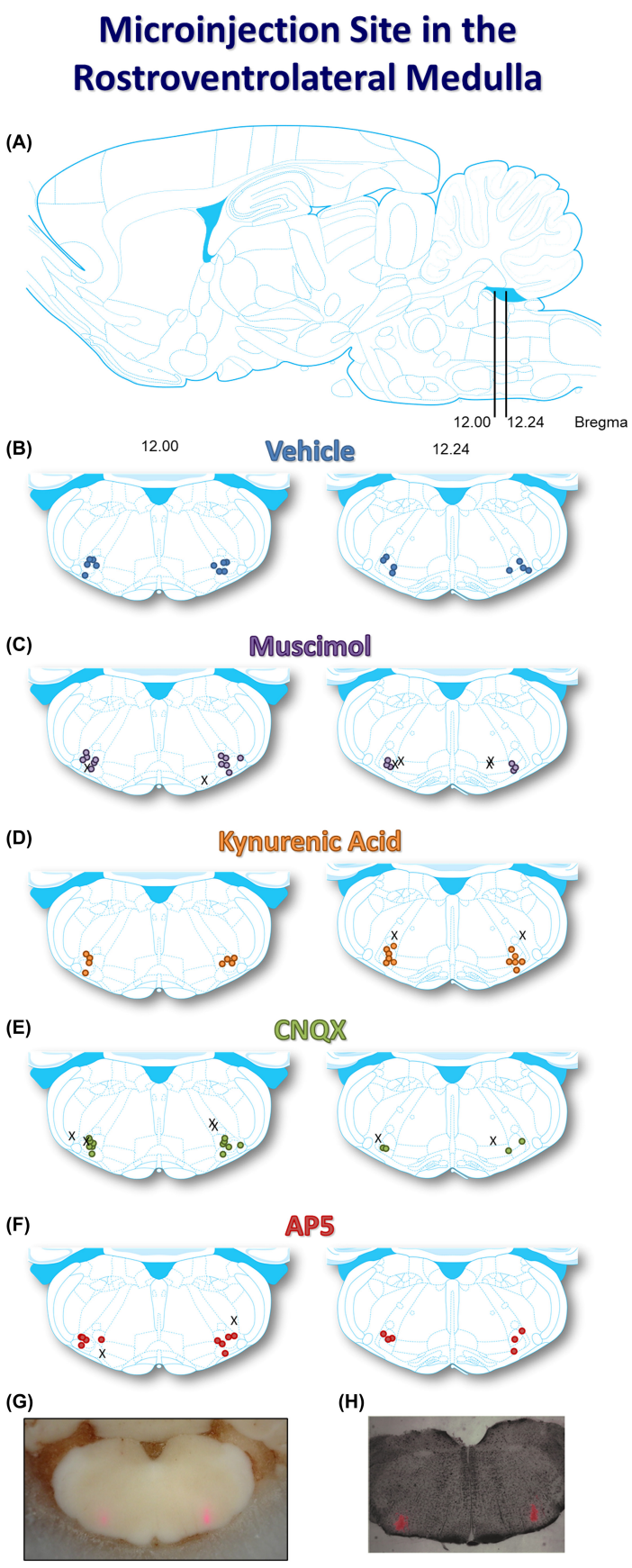

FIGURE 1 A parasagittal image (1.9 mm lateral) from Paxinos and Watson atlas indicating the level of the microinjections between 11.90 and 12.24 caudal to bregma (A). Compiled locations indicating the microinjection sites of vehicle (B, $n=8$), muscimol (C, $n=9$), Kynurenic acid (D, $n=10$), 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX, E, $n=8$), 2 amino-5-phosphonopentanoic acid (AP5, F, $n=8$) 2.90 mm (left panel) to 3.24 mm (right panel) caudal to bregma. Representative coronal section showing 100 nL bilateral microinjections (G). Injectants had 5% Fluorescent microspheres for histological verification. Merged fluorescent and light microscopic (2X) image showing an injection area (H). O indicates bilateral microinjections were within the RVLM, and X indicates microinjections where one or both were outside the RVLM.

bradykinin still elicited a significant blood pressure (CNQX, $p=0.029$; AP5, $p<0.001$), heart rate (CNQX, $p<0.001$; AP5, $p<0.001$), and RSNA (CNQX, $p<0.035$; AP5, $p<0.001$). Whereas the raw integrated RSNA response to epicardial bradykinin after microinjection of either CNQX or AP5 did not significantly diminish

TABLE 1 Cardiogenic sympathetic reflex after RVLM treatment.

	Control	Baroreceptor Loading with phenylephrin			Microinjection treatment	
	Baseline	Reflex	Baseline	Reflex	Baseline	Reflex
Arterial pressure (mmHg)						
Vehicle ($n=8$)	98.8 ± 3.6	132.9 ± 4.3*	166.9 ± 2.4	174.0 ± 3.1	106.5 ± 2.6	137.1 ± 5.6*
Muscimol ($n=9$)	105.4 ± 2.9	145.3 ± 3.6*	162.6 ± 4.0	171.9 ± 3.6	57.9 ± 1.1$^{\#}$	60.7 ± 1.2
Kynurenic acid ($n=10$)	98.7 ± 4.2	137.7 ± 5.7*	161.3 ± 2.7	170.9 ± 3.9	90.0 ± 5.0	97.8 ± 4.5
CNQX ($n=8$)	98.4 ± 3.8	130.9 ± 5.3*	165.0 ± 2.0	174.0 ± 2.2	100.8 ± 5.6	112.4 ± 6.5$^{*,**,\lozenge}$
AP5 ($n=8$)	101.1 ± 4.2	134.4 ± 6.5*	158.5 ± 3.5	172.5 ± 3.8	92.8 ± 6.1	110.0 ± 7.2$^{*,**,\lozenge}$
Heart rate (bpm)						
Vehicle ($n=8$)	344.9 ± 10.0	367.1 ± 12.8*	329.5 ± 10.7$^{\#}$	347.3 ± 9.7*,**	342.1 ± 10.5	359.9 ± 12.4*
Muscimol ($n=9$)	367.7 ± 11.2	389.9 ± 9.9*	350.8 ± 12.0$^{\#}$	369.2 ± 12.8*,**	322.3 ± 10.4$^{\#,\#\#}$	349.1 ± 12.5*,**,***
Kynurenic acid ($n=10$)	352.4 ± 8.3	369.4 ± 8.1*	334.9 ± 6.8$^{\#}$	354.2 ± 7.1*,**	347.7 ± 8.7	363.5 ± 7.0*
CNQX ($n=8$)	343.0 ± 9.8	361.6 ± 11.5*	327.4 ± 8.4$^{\#}$	349.8 ± 7.3*,**	338.9 ± 12.0	359.1 ± 13.8*
AP5 ($n=8$)	344.5 ± 5.0	362.0 ± 3.9*	329.4 ± 6.7$^{\#}$	346.4 ± 7.2*,**	334.4 ± 9.4	350.9 ± 8.9*
Integrated Raw RSNA (μV*s)						
Vehicle ($n=8$)	3.14 ± 0.46	8.20 ± 0.87*	0.90 ± 0.16$^{\#}$	3.22 ± 0.53*,**	3.01 ± 0.31	6.92 ± 0.46*
Muscimol ($n=9$)	5.14 ± 1.05	13.02 ± 2.40*	1.39 ± 0.36$^{\#}$	5.95 ± 1.41*,**	1.56 ± 0.45$^{\#}$	2.05 ± 0.73
Kynurenic acid ($n=10$)	5.12 ± 0.57	11.48 ± 1.33*	1.05 ± 0.15$^{\#}$	5.20 ± 1.02*,**	5.19 ± 0.53	5.58 ± 0.56
CNQX ($n=8$)	4.00 ± 0.76	10.83 ± 1.83*	0.99 ± 0.41$^{\#}$	4.44 ± 1.24*,**	3.98 ± 0.80	5.40 ± 1.00*
AP5 ($n=8$)	4.54 ± 0.31	10.93 ± 0.73*	1.02 ± 0.27$^{\#}$	5.45 ± 1.00*,**	4.52 ± 0.43	7.76 ± 0.59*
Normalized RSNA (% baseline)						
Vehicle ($n=8$)	101.2% ± 1.5%	281.4% ± 23.1%*	31.9% ± 6.2%$^{\#}$	112.6% ± 19.7%*,**	104.5% ± 8.5%	255.2% ± 33.2%*
Muscimol ($n=9$)	99.0% ± 2.3%	274.9% ± 26.0%*	28.3% ± 5.2%$^{\#}$	121.7% ± 14.7%*,**	30.9% ± 4.6%$^{\#}$	36.5% ± 6.4%
Kynurenic acid ($n=10$)	101.1% ± 1.1%	225.5% ± 9.3%*	20.8% ± 1.3%$^{\#}$	100.8% ± 13.8%*,**	106.3% ± 7.9%	113.3% ± 7.1%
CNQX ($n=8$)	98.3% ± 1.2%	276.1% ± 14.3%*	25.9% ± 7.7%$^{\#}$	101.1% ± 15.9%*,**	96.7% ± 4.4%	140.0% ± 11.8%$^{*,**,\lozenge}$
AP5 ($n=8$)	100.2% ± 0.1%	246.4% ± 19.1%*	26.5% ± 7.7%$^{\#}$	139.5% ± 32.2%*,**	107.1% ± 12.1%	183.8% ± 17.4%$^{*,**,\lozenge}$

Note: Arterial pressure (mmHg), heart rate, renal sympathetic nerve activity (RSNA % baseline), and raw RSNA (μV*s) during baseline and the reflex response after epicardial bradykinin (10 μg/mL) application before (control), during phenylephrine-induced baroreceptor loading, and after microinjection of either vehicle, muscimol, kynurenic acid, CNQX, or AP5.

*$p<0.05$ significantly greater than the respective baseline. **$p<0.05$ significantly less than the respective control reflex. ***$p<0.05$ significantly less than reflex during phenylephrine-induced baroreceptor loading. $^{\#}p<0.05$ significantly less than the respective control baseline. $^{\#\#}p<0.05$ significantly less than the respective baseline during phenylephrine-induced baroreceptor loading. $^{\lozenge}p<0.05$ significantly less than the reflex response in the vehicle-treated rat.

the reflex response to epicardial bradykinin (CNQX, $p>0.999$; AP5, $p>0.999$) blood pressure (CNQX, $p=0.020$; AP5, $p=0.008$) and normalized RSNA (CNQX, $p<0.001$; AP5, $p=0.010$) response. The tachycardia elicited by epicardial bradykinin application was unaffected by microinjection of CNQX or AP5 (CNQX, $p>0.999$; AP5, $p>0.999$).

5 | DISCUSSION

During a heart attack, the ischemic myocardium releases several metabolites, one of which is bradykinin.[11] Bradykinin elicits a sympathetic reflex by binding to the bradykinin B2 receptor expressed on thinly myelinated Aδ and unmyelinated C-fibers on cardiac sensory spinal afferents.[9,11,12] Li and Pan (2000) previously demonstrated that barosensitive RVLM neurons mediate the reflex response to epicardial bradykinin.[2] In that study, they recorded the responses of tonically active RVLM single-unit neuronal firing in response to epicardial bradykinin application. They found that only barosensitive neurons responded to epicardial bradykinin application. However, because they could only record tonically active RVLM neurons, it was still unknown if epicardial bradykinin may also activate non-barosensitive neurons.

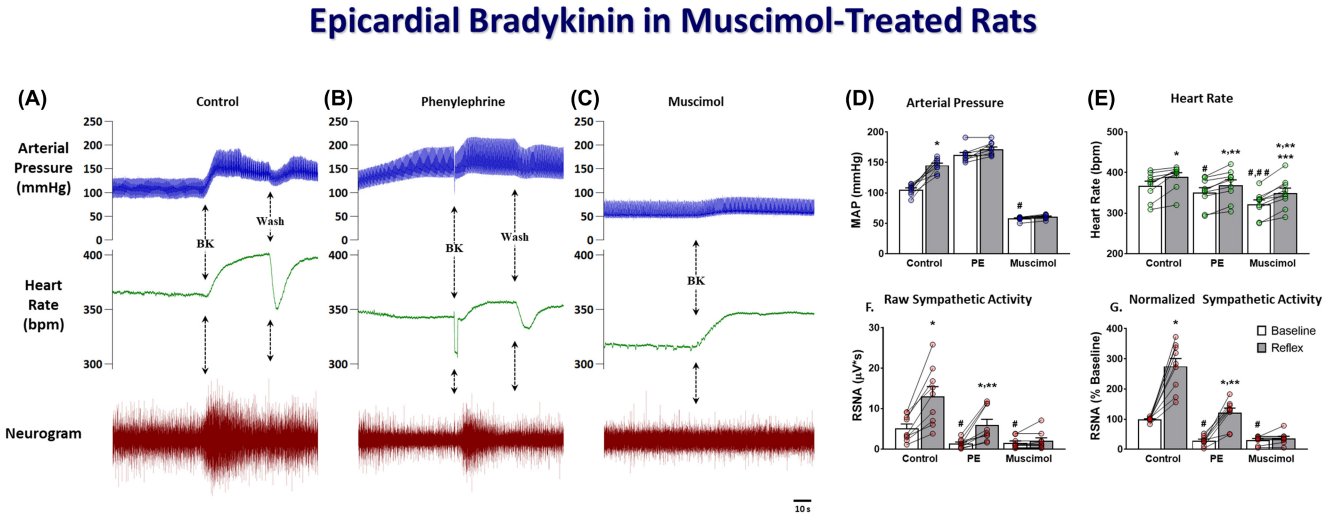

FIGURE 2 Original tracings showing arterial pressure, heart rate, and RSNA responses to epicardial bradykinin (BK, 10 μg/mL) during control (A), PE-induced sympathoinhibition (B), and after bilateral RVLM microinjection (100 nL) of muscimol (1.0 nmol, C). Group data showing the arterial pressure (D), heart rate (E), and the raw (F) and normalized (G) renal sympathetic nerve activity during baseline and the reflex response to epicardial bradykinin. A repeated measures two-way ANOVA was used to identify the main effect of muscimol treatment or bradykinin application and interaction, followed by Bonferroni pos-hoc analysis. * = significant increase from baseline, ** = less than control response, *** = significantly less than reflex during phenylephrine-induced sympathoinhibition, # = less than control baseline, ## = significantly less than baseline during phenylephrine-induced sympathoinhibition. ($n = 9$, $p < 0.05$).

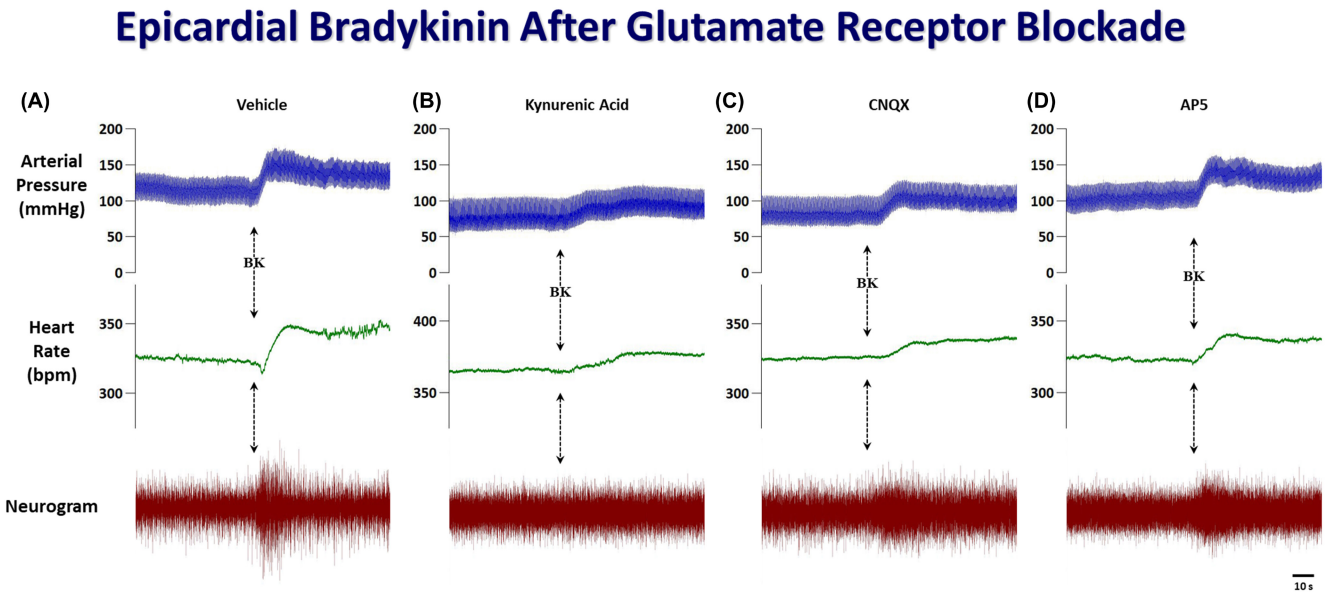

FIGURE 3 Original tracings showing arterial pressure, heart rate, and RSNA responses to epicardial bradykinin (BK, 10 μg/mL) after bilateral microinjection (100 nL) of vehicle (saline, A), kynurenic acid (5.0 nmol, B), CNQX (0.9 nmol, C), or AP5 (1.2 nmol, D).

Tonically active RVLM neurons project to sympathetic preganglionic neurons in the lateral horn of the thoracic and upper lumbar spinal cord to maintain blood pressure via sympathetic activity.[16,32] An increase in arterial pressure increases the discharge rate of baroreceptor afferent fibers, causing the inhibition of bulbospinal sympathoexcitatory RVLM neurons, resulting in decreased sympathetic activity.[14,22] An increase in arterial pressure to ~140 mmHg virtually silences this tonically active RSNA in the rat.[24–26] Based on this, we used the PE-induced increase in arterial pressure to inhibit barosensitive sympathetic activity and test the reflex response to epicardial bradykinin. As such, the remaining fraction of sympathetic reflex activity observed during baroreceptor inhibition is non-barosensitive. Thus, any reflex response to epicardial bradykinin application during the baroreceptor

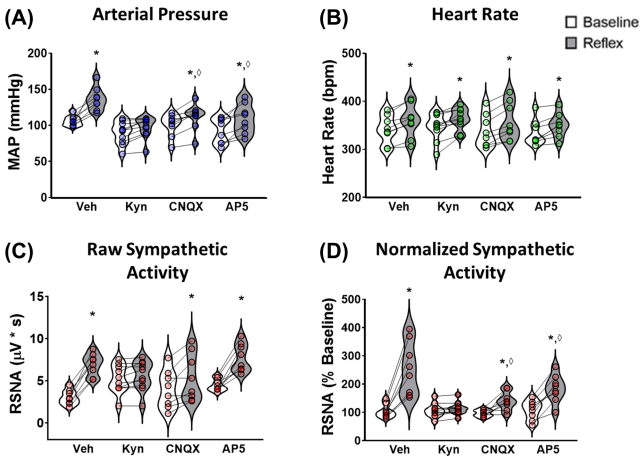

FIGURE 4 Group data showing the arterial pressure (A), heart rate (B), and the raw (C) and normalized (D) renal sympathetic nerve activity during baseline and the reflex response to epicardial bradykinin after bilateral microinjection (100 nL) of vehicle (saline, $n = 8$), kynurenic acid (5.0 nmol, $n = 10$), CNQX (0.9 nmol, $n = 8$) or AP5 (1.2 nmol, n = 8). A mixed-effect (repeated measures for bradykinin application) two-way ANOVA was used to identify a main effect for microinjection treatment or bradykinin application and interaction, followed by Bonferroni pos-hoc analysis. * = significantly greater than its respective baseline, ◊ = significantly less than vehicle reflex. ($p < 0.05$).

loading-induced sympathoinhibition is mediated by non-barosensitive neurons.

To determine the extent to which the reflex may involve nuclei outside the RVLM, we used microinjection of the long-lasting $GABA_A$ agonist muscimol bilaterally into the RVLM. Interestingly, while muscimol treatment completely abolished the arterial pressure and RSNA response to epicardial bradykinin, it only modestly attenuated the heart rate response. This suggests that while the arterial pressure and RSNA response are entirely mediated by neurons located within the RVLM, the heart rate response is at least partially mediated by a population of neurons located outside the RVLM. We show that this is not an intrinsic cardiac response to bradykinin or vagally mediated because these rats were cervically vagotomized bilaterally. Also, in three rats, we performed cervical spinal transection, which completely abolished the heart rate response.

Glutamate is the primary excitatory neurotransmitter in the central nervous system, and the RVLM expresses both NMDA and non-NMDA receptors.[33–36] In this regard, it is unsurprising that the NMDA and non-NMDA receptor antagonists, AP5 and CNQX, partially blocked and that the broad-spectrum ionotropic receptor antagonist kynurenic acid completely blocked the reflex blood pressure and normalized RSNA response to epicardial bradykinin. Zhou W et al. (2006) showed that CNQX and AP5 into the RVLM partially blocked and that kynurenic acid also blocked the sympathetic reflex response to visceral afferent stimulation in the cat by stimulating the gallbladder.[37]

Wang et al. (2006) performed extracellular single-unit recording of NTS neurons and showed that chemosensitive and barosensitive neurons mediate the sympathetic response to stimulation of epicardial afferent fibers.[38] In conditions like heart failure, where there is increased cardiac metabolic byproduct and activation of cardiac afferents, there is also increased firing of chemosensitive neurons that are also not barosensitive.[38] It is clear that barosensitive RVLM neurons are important and partially modulate the reflex response to epicardial bradykinin. To determine the extent of non-barosensitive sympathetic activity, we increased arterial pressure with PE to inhibit sympathetic activity. However, the fraction of sympathetic reflex responsiveness to epicardial bradykinin that remains during baroreceptor inhibition is likely derived from nociception-sensitive neurons that are not tonically active.

Interestingly, while epicardial bradykinin consistently elicited a significant reflex tachycardia, baroreceptor inhibition of sympathetic activity had little effect on the magnitude of the heart rate response to epicardial bradykinin. It is expected that bilateral vagotomy would have diminished the reflex bradycardia observed during baroreceptor loading. However, in this study, it is unknown if intact vagal activity may also affect the reflex tachycardia observed after bradykinin application because we did not

compare the reflex tachycardia before and after bilateral vagotomy. Surprisingly, while muscimol inactivation of the RVLM completely abolished the RSNA response and virtually all the arterial pressure response to epicardial bradykinin, it did not affect the tachycardia. This suggests that tachycardia is mediated by source(s) other than the RVLM. Because the rats were cervically vagotomized, and in the three rats, cervical transection abolished the heart rate response, the source of activity must be supraspinal and sympathetic.

The bulbospinal RVLM neurons are the final output of the sympathetic preganglionic neurons. Additionally, other non-barosensitive brainstem nuclei send projections to preganglionic neurons and can affect vasomotor tone. It is tempting to speculate because there are several sympathetically related supraspinal sites that may influence heart rate independently of the RVLM. For example, the medullary raphe nuclei and ventromedial reticular nuclei are groups of cells along the midline medulla that play a key role in both spinal modulation of pain as well as autonomic function, for review see Mason P (2001) and Hornung JP (2004).[39,40] Stimulation of the medullary raphe pallidus by microinjection of either glutamatergic agonists[41] or disinhibition with $GABA_A$ antagonist bicuculline[42,43] elicits a robust increase in sympathetic nerve activity to intrascapular brown adipose tissue (IBAT) but has minimal effect on splanchnic or renal sympathetic nerve activity. The same stimulation also causes tachycardia, similar to what we observed after stimulation of cardiac nociceptors with bradykinin.[41–43]

There is good evidence to show that the raphe nuclei are not only involved in autonomic cardiac regulation but also in modulating the descending response to cardiac nociception. In the Macaca monkey, activation of a raphe-spinal pathway was shown to modulate the activity of upper thoracic spinothalamic tract neurons after bradykinin stimulation of cardiac nociceptors.[44] In the cat, stimulation of raphe magnus inhibits activation of spinoreticular neurons by painful stimuli.[45] While electrophysiological data show that some raphe nuclei are barosensitive, the same neurons are not also sensitive to nociceptive stimuli.[46] Additionally, PE-induced baroreflex inhibition of sympathetic activity that almost completely inhibits splanchnic sympathetic nerve activity does not affect IBAT sympathetic activity, which is derived mainly from raphe rather than the RVLM.[43]

5.1 | Perspectives

The major novel findings of the present study are that first, baroreceptor-mediated inhibition of tonically active neurons only attenuated the reflex response to epicardial bradykinin, suggesting that non-barosensitive neurons play an important role in the reflex response to epicardial bradykinin. Second, muscimol inactivation of RVLM abolished the arterial pressure and RSNA response. However, it did not abolish the reflex tachycardia, demonstrating a separate supraspinal source. Third, kynurenic acid completely blocked the arterial pressure and raw and normalized RSNA responses, while CNQX or AP5 only attenuated the blood pressure and normalized RSNA responses. Collectively, these data suggest that both barosensitive and nociception-sensitive ionotropic glutamate receptor-expressing neurons within the RVLM mediate the arterial pressure and RSNA component of the cardiogenic sympathetic afferent reflex. In contrast, the reflex tachycardia is mediated in part by an alternate pathway that does not involve neurons in the RVLM.

5.2 | Limitations

In this study, we used a PE-induced increase in arterial pressure to activate baroreceptors and, therefore, inhibit barosensitive sympathetic activity. We refer to the sympathetic reflex activity elicited by epicardial bradykinin application observed during baroreceptor-induced inhibition as non-barosensitive. However, another possible interpretation that must be considered is that epicardial bradykinin may be a more potent stimulus than baroreceptor-mediated inhibition of sympathetic activity. If so, activating those cardiac nociceptors may still stimulate barosensitive sympathetic activity. Because sino-aortic baroreceptor denervation removes baroreceptor-mediated inhibition of sympathetic activity, leading to elevated sympathetic activity, it would be challenging to differentiate bradykinin activation of barosensitive and non-barosensitive activity using this approach. Alternatively, electrical stimulation of the baroreceptor afferent fibers rather than PE-induced sympathetic inhibition may provide more potent inhibition of barosensitive neurons and yield contrasting results.

We expressed RSNA as percent basal activity before any treatments (normalized) to reduce biological and technical variability between rats. In this regard, the quality of the RSNA signal may vary based on the quality of the dissection as well as any biological variable. When we compared the relative magnitudes of the reflex response after microinjection treatment, both CNQX and APS attenuated the reflex response, and kynurenic acid abolished the response. However, when comparing the raw RSNA signal, neither CNQX nor AP5 attenuated the reflex response, while kynurenic acid still abolished the reflex. After normalizing the raw integrated signal to a control period recorded before any treatments, we observed no change in the relative baseline activity, but we did observe

the relative RSNA response to epicardial bradykinin application was attenuated after CNQX or AP5 microinjection.

Another consideration for this study is that it was performed in anesthetized rats. Given the complexity of the experimental approach, these experiments could not be carried out on conscious animals. However, the modality of anesthesia may be considered when interpreting the results. In these experiments, we transitioned the rats from isoflurane after the surgical preparation to α-chloralose for the recordings. Many studies examining the sympathetic regulation of blood pressure have been performed in rats under α-chloralose in a similar fashion.[47–57] Other studies chose to use urethane, a combination of urethane and a-chloralose, or sodium pentobarbital. The use of different anesthetic agents should be considered when comparing studies of this nature.

AUTHOR CONTRIBUTIONS

Matthew R. Zahner conceived and designed research, performed experiments, analyzed data, interpreted results of experiments, prepared figures, drafted the manuscript, edited and revised the manuscript, and approved the final version of the manuscript. Cade C. Oculam performed experiments, analyzed data, interpreted the results of experiments, and approved the final version of the manuscript. Eric Beaumont interpreted the results of experiments, edited and revised the manuscript, and approved the final version of the manuscript.

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CONFLICT OF INTEREST STATEMENT

None declared.

DATA AVAILABILITY STATEMENT

All data and materials are available in the manuscript, the Appendix S1, or are available from the corresponding author upon reasonable request.

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