Relative contributions of the thalamus and the paraventricular nucleus of the hypothalamus to the cardiac sympathetic afferent reflex

Bo Xu, Hong Zheng, and Kaushik P. Patel
Department of Cellular and Integrative Physiology, University of Nebraska Medical Center, Omaha, Nebraska
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Xu B, Zheng H, Patel KP. Relative contributions of the thalamus and the paraventricular nucleus of the hypothalamus to the cardiac sympathetic afferent reflex. Am J Physiol Regul Integr Comp Physiol 305: R50–R59, 2013. First published April 24, 2013; doi:10.1152/ajpregu.00004.2013.—The cardiac sympathetic afferent reflex (CSAR) is induced by stimulating the cardiac sympathetic afferents, which evokes increases in sympathetic outflow and arterial pressure. In the present study, we attempted to identify the contribution of thalamic and hypothalamic nuclei involved in the CSAR. First, we observed that there was an increase in the number of c-Fos-labeled cells in the paraventricular nucleus (PVN) (190 ± 18 vs. 101 ± 15; P < 0.05), the paraventricular nucleus of the thalamus (PVT) (239 ± 23 vs. 151 ± 15; P < 0.05), and the mediodorsal thalamic nucleus (MD) (92 ± 9 vs. 63 ± 6; P < 0.05) following epicardial application of bradykinin (BK) compared with the control group (P < 0.05). Second, using extracellular single-unit recording, we found 25% of spontaneously active neurons in the thalamus were stimulated by epicardial application of BK or capsaicin in intact rats. However, 24% of spontaneously active neurons in the thalamus were still stimulated by epicardial application of BK or capsaicin despite vagotomy and sinoaortic denervation. None of the neurons in the thalamus responded to baroreflex changes in arterial pressure, induced by intravenous injection of phentolamine or sodium nitroprusside. The CSAR was inhibited by microinjection of muscimol or lidocaine into the PVN. However, it was not inhibited or blocked by microinjection of muscimol or lidocaine into the thalamus. Taken together, these data suggest that the thalamus, while activated, is not critical for autonomic adjustments in response to activation of the CSAR. On the other hand, the PVN is critically involved in the central pathway of the CSAR.

METHODS

General procedures. Male Sprague-Dawley rats weighing between 250 and 300 g were obtained from SASCO Breeding Laboratories (Omaha, NE). This study was approved by the Institutional Animal Care and Use Committee of the University of Nebraska and was carried out under the guidelines of the American Physiological Society and the National Institutes of Health’s Guide for the Care and Use of Laboratory Animals.
Each rat was anesthetized by intraperitoneal injection of urethane (0.75 g/kg ip) and α-chloralose (70 mg/kg ip). Adequate depth of anesthesia was assessed by the absence of corneal reflexes and paw withdrawal response to a noxious pinch. Supplemental doses of anesthesia were administered to maintain an adequate depth of anesthesia during the experiment. The trachea was cannulated for mechanical ventilation using a rodent ventilator (SAR-830; CWE, San Francisco, CA). The right femoral artery and femoral vein were cannulated for the recording of arterial blood pressure and administration of chemicals, respectively. The mean arterial pressure (MAP) and heart rate (HR) were simultaneously recorded on a PowerLab data acquisition system (8SP; ADInstruments, Colorado Springs, CO). A constant infusion of 60 µl/min infusion of saline was instituted to maintain the hydrated status of the animal. With this regimen, there are no changes in renal sympathetic nerve activity (RSNA), MAP, or HR over the usual ~6 h of the experimental protocol (26, 50). Body temperature was maintained at about 37°C with an animal temperature controller (ATC 1000; World Precision Instruments, Sarasota, FL).

Experimental protocols. First, c-fos gene expression was determined following epicardial application of BK (0.3 nmol in 2.0 µl, repeat 6 times at intervals of 20 min) in six rats with vagotomy and sinoaortic denervation. Six applications of epicardial stimulation were chosen on the basis of previous studies (19, 25). It has been reported that c-fos gene expression is induced 30 min after neuronal activation (23). We assume that single application of BK or capsaicin can activate c-Fos via activation of the epicardium was rinsed three times with 10 ml of warm normal saline (38°C). Successive applications of chemicals were separated by at least 15 min for the complete recovery of the neuron discharge and MAP. The activity changes of the BK-sensitive, -insensitive and -inhibitory response neurons were investigated. To exclude the possibility that the increased activity of the BK-sensitive neurons in the thalamus was secondary to the elevation of arterial pressure induced by epicardial application of the chemicals and investigate whether these neurons involve the baroreflex, the response of the thalamic neurons to transient changes in MAP induced by the phenylephrine (PE; 5–25 µg/kg) and sodium nitroprusside (SNP; 4–20 µg/kg) were determined.

Third, the effects of epicardial application of saline, capsaicin, or BK, on the activity of the thalamic neurons were randomly investigated in eight rats with vagotomy and sinoaortic denervation.

Lastly, to determine whether the thalamus is an important component for the cardiogenic sympathoexcitatory response, we determined whether the CSAR could be blocked or inhibited by microinjection of lidocaine (8.5 nmol/side) or the GABA_A agonist muscimol (100 pmol/side) into the MD (bilateral microinjection, n = 6), PVN (bilateral microinjection, n = 6), or PVT (unilateral, n = 6). The PVN was injected since it is known to be involved in regulating sympathetic outflow (52, 56).

CSAR induced by epicardial application of chemicals. A limited left lateral thoracotomy was performed to expose the heart, and the pericardium was removed. The cardiac afferents were activated by application of a piece of filter paper (3×3 mm) saturated with capsaicin (1.0 nmol in 2.0 µl) or BK (0.3 nmol in 2.0 µl) to the epicardial surface of the anterior wall of the left ventricle (27, 56, 57). In the control group, a piece of filter paper saturated with saline (2.0 µl) was used. Each piece of filter paper was removed 1 min later, and the epicardium was rinsed three times with 10 ml of warm normal saline (38°C). Successive applications of chemicals were separated by

Fig. 1. Representative images of c-Fos immunoreactivity in the thalamus and the paraventricular nucleus (PVN) in a control rat following epicardial application of saline (A and B) and a rat following epicardial application of bradykinin (BK) (C and D). Arrows indicate c-Fos-positive cells. PVN, paraventricular nucleus of the thalamus; MD, the mediodorsal thalamic nucleus; 3V, third ventricle; D3V, dorsal 3rd ventricle.
CSAR was induced by epicardial application of BK. Randomly into BK-treated and vehicle-treated (0.9% saline) groups. The MAP or RSNA to epicardial chemical stimulation. at least 15 min. The CSAR was evaluated by the responses of the SE; n with epicardial application of bradykinin. Values are expressed as means ± SE; n = 6. *P < 0.05 compared with control.

c-Fos immunohistochemical labeling. Animals were placed randomly into BK-treated and vehicle-treated (0.9% saline) groups. The CSAR was induced by epicardial application of BK. c-fos gene expression was determined following epicardial application of BK (0.3 mmol in 2.0 μl, repeated 6 times at intervals of 20 min) in 6 rats with vagotomy and sinoaortic denervation. Ninety minutes after the last application of BK or 0.9% saline, animals were deeply anesthetized and perfused transcardially with 100 ml saline, followed by ice-cold 4% paraformaldehyde (Sigma, St. Louis, MO) in 0.1 M phosphate buffer (pH 7.2). Then brains were removed and cryoprotected by storing overnight at 4°C in 0.1 M phosphate buffer (pH 7.2) containing 30% sucrose. The brains were frozen on dry ice and sectioned coronally at 30 μm on a sliding microtome.

The avidin-biotin-peroxidase complex (ABC) method was used for staining of c-Fos protein, as described in our previous studies (55). Briefly, after treatment with 0.5% hydrogen peroxide and 1% normal goat serum (Vector ABC kit; Vector Laboratories, Burlingame, CA), brain sections were incubated in a primary polyclonal rabbit anti-c-Fos antibody (1:5,000 dilution; Santa Cruz Biotechnology, Santa Cruz, CA) at 4°C for 24 h, and in biotinylated anti-rabbit IgG (Vector kit; 1:200) for 60 min. Subsequently, the sections were placed in ABC solution (Vector kit; 1:50) for 30 min and then incubated in 30% hydrogen peroxide and 3,3'–diaminobenzidine (DAB) for 5–8 min. The c-Fos protein was visualized by the DAB reaction products as dark-brown staining.

Quantitative analysis of the c-Fos staining in control and BK-treated rats was performed by counting the number of cells staining for c-Fos in each area of nuclei. Tissue sections from control and BK-treated rats were carefully matched to compare as closely as possible identical fields or nuclei in the brain. These sections were processed simultaneously for immunocytochemistry to minimize differences in staining intensity due to daily variations in the method. The fields were analyzed on a Leica light microscope. Three representative sections, which most closely matched the standard stereotaxic planes of the Paxinos and Watson's atlas (34), were chosen. Bilateral c-Fos-positive cells were counted by a treatment-blinded analyzer using National Institutes of Health Image/ImageJ software. The particle analysis function with equivalent detection thresholds across conditions. The average number of c-Fos-stained cells was taken to represent the number of activated cells within a given nucleus.

Vagotomy and sinoaortic denervation. Bilateral cervical vagal denervation and arterial baroreceptor denervation were carried out. First, the carotid sinus area was exposed bilaterally, each carotid sinus nerve was identified and cut, and all of the other nerve fibers that were visible in this area were also cut. The carotid bifurcation and the common carotid arteries were stripped of adventitial tissues from 4 mm below the bifurcation to 4 mm above. Then, the vessels were painted with 10% phenol solution to destroy any remaining nerve fibers in this area. Each vagus was then identified in the neck, tied, and sectioned. The effectiveness of the denervation was determined by recording the change in HR to intravenous injection of PE (20 μg/kg). This dose of PE evoked an increase in MAP between 25 and 40 mmHg. The arterial baroreceptor denervation was assumed to be completed when the change in the HR was less than 5 beats per minute (bpm) in response to this MAP challenge.

Fig. 2. Number of c-Fos-positive cells in the PVN, median preoptic area (MnPO), subfornical organ (SFO), the PVT, the MD, central medial thalamic nucleus (CM), and paratenial thalamic nucleus (PT) in control rats and rats treated with epicardial application of bradykinin. Values are expressed as means ± SE; n = 6. *P < 0.05 compared with control.

Fig. 3. A–C: approximate locations of neurons in the thalamus, which were sensitive to epicardial application of bradykinin (diamonds) and neurons in the thalamus that were not activated by epicardial application of bradykinin (solid circles) in intact and denervated rats. The distance (in mm) posterior to bregma is shown for each section. PVT, paraventricular nucleus of the thalamus; MD, the mediodorsal thalamic nucleus; D3V, dorsal 3rd ventricle; sm, stria medullaris of the thalamus; PT, paratenial thalamic nucleus; CM, central medial thalamic nucleus; IAM, interanteromedial thalamic nucleus.
**Extracellular single-unit recording in vivo.** Rats were placed in a stereotaxic apparatus (model 620; Stoelting, Chicago, IL). The stereotaxic coordinates for the thalamus were determined according to the atlas of Paxinos and Watson (34). Typically, three tracks were explored for extracellular recording in each rat, from ~1.7 to ~2.2 mm caudal to bregma, 0.2–0.6 mm lateral (right side) to the midline and a depth of 5.4–6.8 mm ventral to the dorsal surface. Extracellular single-unit recording was carried out using a single micropipette (resistance 5–15 MΩ) filled with 0.5 M sodium acetate containing 2% pontamine sky blue. The glass micropipettes were advanced using a microdrive controller (type 860; Hugo Sachs Elektronik, March, Germany) into the thalamus. The spontaneous activity of neurons was recorded using a single micropipette and a depth of 5.4–6.8 mm ventral to the dorsal surface. Extracellular single-unit recording was carried out using a single micropipette and a depth of 5.4–6.8 mm ventral to the dorsal surface. Extracellular single-unit recording was carried out using a single micropipette and a depth of 5.4–6.8 mm ventral to the dorsal surface.

In spontaneously active neurons in the thalamus, the spontaneous neuronal discharge was identified through a retroperitoneal incision, and the nerve-electrode junction was insulated electrically from the surrounding tissues. The effects on RSNA, MAP, and HR were observed before and after treatments. The thalamic neurons were considered to be responsive if their peak discharge frequency after treatment changed >30% from baseline. The baseline pulse pressures, systolic...
arterial pressures, MAP, and HR were averaged over 2 min before treatment. Maximum RSNA, discharge of neurons, and blood pressure responses were measured ~30 s after BK or capsaicin application. Data are presented as means ± SE. Differences between groups were determined by a two-way ANOVA followed by the Newman-Keuls test for post hoc analysis of significance (StatView II; Berkeley, CA). P < 0.05 was considered statistically significant.

RESULTS

c-Fos staining in the thalamus of rats following stimulation of heart with BK. There was an increased number of c-Fos-stained neurons in the thalamus and the PVN of rats with epicardial application of BK, shown in Fig. 1. Figure 2 shows the mean numbers per section of c-Fos-labeled neurons in specific nuclei of the thalamus. Compared with the animals in the control group (n = 6), the numbers of c-Fos-stained neurons were significantly (P < 0.05) increased in several areas in the thalamus following epicardial application of BK compared with the control group, which include the PVT areas in the thalamus following epicardial application of BK, shown in Fig. 1. Figure 2 shows the mean numbers per section of c-Fos-stained neurons in the thalamus and the PVN of rats with epicardial application of BK. There was an increased number of c-Fos-stained neurons in the thalamus following epicardial application of BK compared with the control group.

In contrast to the CSAR being inhibited by microinjection of lidocaine or muscimol into the PVN, the CSAR could not be inhibited or blocked by microinjection of lidocaine or muscimol into the PVT or MD (Fig. 8). This dose and volume of lidocaine and muscimol have been used in the past to block transmission in various areas in the brain (17, 39). Furthermore, doubling the dose of lidocaine (17.0 nmol, 200 nl) injected into the MD and PVT in two rats did not even attenuate the reflex, suggesting that the lack of response in MD and PVT was not due to “incomplete blockade”. The baseline MAP (−15.4 ± 4.6 vs. −1.2 ± 2.9; n = 6; P < 0.05) and
RSNA (−25.9 ± 7.7 vs. −0.9 ± 1.9; n = 6; P < 0.05) were decreased after microinjection of muscimol into the PVN compared with microinjection of aCSF, but not after microinjection of lidocaine. Microinjection of aCSF had no significant effect on the RSNA, MAP, or the CSAR.

DISCUSSION

In the present study, we found that there are increased numbers of c-Fos-labeled cells in the thalamus, which include the PVT and the MD, and in the PVN of the hypothalamus following epicardial application of BK. To confirm this, using extracellular single-unit recording, we found 25% of the neurons in the thalamus were activated by epicardial application of BK or capsaicin in the intact rats. Furthermore, neurons in the thalamus were also activated by epicardial application of BK, despite vagotomy and sinoaortic denervation. However, none of the neurons in the thalamus responded to baroreflex changes in arterial pressure in intact animals. These results indicate that specific regions in the thalamus, including the PVT and MD, are activated by stimulation of cardiac sympathetic afferents, but are independent of vagal and baroreceptor input. Finally, the renal sympathoexcitatory response activated by stimulation of cardiac sympathetic afferents is mediated via the PVN but not the thalamic PVT and MD.

The PVT is a unique midline intralaminar nucleus that has been implicated in the regulation of autonomic and visceral functions (2, 44). PVT neurons also respond to stress and to peripherally administered psychostimulants cocaine and amphetamine (3, 9). In addition, PVT receives inputs from homeostatic control regions of the NTS (33). Previous studies
have shown that the NTS receives sensory inputs from cardiac receptors and is an important area for the central transmission of the CSAR (47, 49, 51). Chemical stimulation of cardiac sympathetic afferents induces \textit{c-fos} expression in the NTS, while dorsal rhizotomy virtually eliminated the \textit{c-fos} expression (19, 21). An electrophysiological study has shown that NTS neurons can be excited by stimulation of cardiac sympathetic afferents (48). These results suggest that cardiac sympathetic afferents terminate in the NTS, which is involved in this sympathoexcitatory cardiovascular reflex. In addition, spinal cord stimulation increases c-Fos and heat shock protein 72 expression in the PVT, PVN, and NTS, both known to be involved in regulation of pain and emotions (8). In this respect, it is possible that the PVT may integrate cardiac sympathetic afferent input that passes through the dorsal horn of the spinal cord to the pons (8). The MD also has connections with the insular cortex and the hypothalamus, which are associated with central autonomic regulation (18, 30, 32).

Our results show that c-Fos-stained neurons were significantly increased in several areas in the thalamus following epicardial application of BK, which include MD and PVT. Furthermore, using extracellular single-unit recording, we found most neurons, which were activated by epicardial application of BK or capsaicin, were localized in the MD and PVT. c-Fos has been widely used as a tool for the study of neural correlates of nociception (4, 6, 20), and its expression in the brain in response to specific afferent input is specific for neurons, but not for glial, ependymal, or endothelial cells (31). The c-Fos immunoreactivity mapping technique allows examination of large numbers of cells in the brain in a single animal after noxious stimulation and/or tissue injury. Extracellular single-unit recording in vivo from selected brain regions is a classic technique for studies of responses of central neurons to sensory stimulation. The interactions between the CSAR and the arterial baroreflex or chemoreflex were investigated with relatively few neurons in the NTS (48). Similarly, relatively few neurons in the RVLM dictate sympathoexcitation as well (1). Thus, it is possible that a seemingly small proportion of activated neurons may play an important role in the CSAR regulation. These results suggest that, specific regions in the thalamus, including the PVT and the MD, are activated by stimulation of cardiac sympathetic afferents.

Both cardiac sympathetic afferents and cardiac vagal afferents can be activated during myocardial ischemia or epicardial application of some chemicals (29). In the present study, 7/29 (24%) neurons in the thalamus were activated by epicardial
application of BK or capsaicin in rats with vagotomy and sinoaortic denervation. Similarly, 25% of the neurons were activated in intact rats, suggesting that the sympathetic afferent nerves contribute to the majority of the afferent input to the thalamus from the heart related to CSAR. Consistent with these observations, none of the neurons in thalamus responded to baroreflex changes in arterial pressure in intact rats, suggesting that this effect was not secondary to the change in blood pressure induced by epicardial applications.

It is well known that the CSAR induced by stimulating the cardiac sympathetic afferents increases sympathetic outflow and arterial pressure. Our results show that bilateral microinjection of an anesthetic, lidocaine, into the thalamus abolished the CSAR. Furthermore, microinjection of muscimol, a GABA<sub>A</sub> receptor agonist, into the PVN significantly attenuated the CSAR. These results are consistent with previous studies (53, 56). Interestingly, in contrast to microinjection of muscimol or lidocaine into the PVN, the CSAR could not be inhibited or abolished by microinjection of muscimol or lidocaine into the MD or PVT. It is known that lidocaine is a local anesthetic, which blocks voltage-gated sodium channels. Microinjections of lidocaine in discrete brain areas have been widely used as a tool for reversible functional inactivation (12, 38). Both the reversible inhibition of the PVN with lidocaine and the irreversible lesion of the PVN with direct current abolished the CSAR, indicating the necessity of the PVN area for the expression of the CSAR (56). On the other hand, microinjection of muscimol into the PVN significantly decreased baseline RSNA and MAP, while microinjection of muscimol into the PVT or MD had no significant effects on baseline RSNA and MAP. There are two possible explanations for the paradox that thalamic neurons are evoked by epicardial BK, while the CSAR cannot be inhibited or abolished by microinjection of muscimol or lidocaine into the thalamus. One is that the present study was done in anesthetized rats and that both the MD and the PVT have connections with the insular cortex, which may have been inhibited by the anesthetic (10, 43). Another possible explanation is that the MD and PVT received sympathetic afferent nerve information but do not play a direct role in modulating sympathetic efferent nerves. Previous studies have indicated that the thalamus may act as a gate to afferent pain signals, with cortical activation being necessary for the sensation of pain (36, 37). Bilateral activation of the thalamus has been shown in both angina and silent ischemia (36, 37). Thus, compared with the PVN, which plays a critical role in CSAR, the thalamus may be considered to be involved in pain pathways, including processing cardiac pain in response to input from the heart, but not as a crucial component of the central neurocircuitry of the CSAR.

The CSAR is an excitatory sympathetic reflex, which can be initiated by myocardial ischemia and in CHF (11). It has been shown that BK activates the kinin B2 receptors on cardiac capsaicin-sensitive afferents to produce a sympathoexcitatory reflex response (15, 29). The CSAR is enhanced in rats and dogs with CHF (11, 29), which, at least, partially contributes to the overexcitation of the sympathetic nervous system (16, 46). Also, the cardiogenic sympathetic reflex involves predominantly brain stem nuclei (15). Although the brain stem nuclei play a critical role in generation and regulation of sympathetic activity, the present study indicated that areas in the thalamus, such as the PVT and MD, are activated by stimulation of cardiac sympathetic afferents. However, the physiological role of the thalamus on the CSAR needs further investigation.

**Perspectives and Significance**

Although some specific regions in the thalamus, including the PVT and the MD, are activated by stimulation of cardiac sympathetic afferents, specific blockade of the activity within these nuclei fails to block the CSAR. These data also confirm and validate the critical role of the PVN in the CSAR. Taken together, these data indicate that the thalamus, although considered to be involved in pain pathways, including processing cardiac pain in response to input from the heart, is not a critical component of the central neurocircuitry, like the PVN, which dictates autonomic outflow in response to activation of the CSAR.

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**DISCLOSURES**

No conflicts of interest, financial or otherwise, are declared by the authors.

**AUTHOR CONTRIBUTIONS**

Author contributions: B.X. and K.P.P. conception and design of research; B.X. performed experiments; B.X. analyzed data; B.X. and K.P.P. interpreted results of experiments; B.X. prepared figures; B.X. drafted manuscript; B.X., H.Z., and K.P.P. edited and revised manuscript; B.X., H.Z., and K.P.P. approved final version of manuscript.
REFERENCES


