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ORIGINAL ARTICLE

Cardiovascular actions of L-cysteine and L-cysteine sulfinic acid in the nucleus tractus solitarius of the rat

Yumi Takemoto

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Abstract The sulfur-containing excitatory amino acid (EAA) L-cysteine sulfinic acid (CSA), a neurotransmitter candidate, is endogenously synthesized from L-cysteine (Cys). Exogenous Cys administration into the brain produces cardiovascular effects; these effects likely occur via synaptic stimulation of central nervous system (CNS) neurons that regulate peripheral cardiovascular function. However, the cardiovascular responses produced by CNS Cys administration could result from CSA biosynthesized in synapse. The present study examined the role of CSA in Cys-induced cardiovascular responses within the nucleus tractus solitarius (NTS) of anesthetized rats. The NTS receives input from various visceral afferents that gate autonomic reflexes, including cardiovascular reflexes. Within the NTS, both Cys and CSA microinjections produced decrease responses in arterial blood pressure and heart rate that were similar to those produced by L-glutamate. Co-injection of the ionotropic EAA receptor antagonist kynurenic acid abolished Cys-, but not CSA-, induced cardiovascular responses. This finding suggests that only Cys-induced cardiovascular responses are mediated by kynurenate-sensitive receptors. This study provides the first demonstration that Cys- and CSA-induced cardiovascular responses occur via different mechanisms in the NTS of rats. Further, this study also indicates that Cys-induced cardiovascular responses do not occur via CSA. Thus, within the NTS, endogenous Cys and/or CSA might be involved in cardiovascular regulation.

Y. Takemoto (✉)
Neurophysiology, Basic Life Sciences, Institute of Biomedical and Health Sciences, Hiroshima University, Hiroshima, Japan
e-mail: yumitake@hiroshima-u.ac.jp

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Introduction

Administration of the thiol amino acid L-cysteine (Cys), but not D-cysteine, into the cisterna magna of freely moving rats produces strong increases in arterial blood pressure (ABP) and heart rate (HR) (Takemoto 1990, 2013). The increase in ABP and HR occurs via activation of the autonomic nervous system and vasopressin release (Takemoto 1990, 1995, 2013). L-Cysteine sulfinic acid (CSA) is a well-known sulfur-containing excitatory amino acid (EAA) endogenous to mammals and a neurotransmitter candidate (Griffiths 1990). CSA is synthesized from Cys by cysteine dioxygenase (Baba 1987) as a major metabolite (Griffith 1987). Thus, Cys-induced cardiovascular responses might result from CSA biosynthesis and subsequent CSA-induced receptor stimulation.

Intracisternal Cys injections could activate neurons in the nucleus tractus solitarius (NTS) located in the dorsal medulla (Takemoto 2012). Within the NTS, visceral afferent fibers (including baroreceptor afferent fibers) synapse onto secondary central nervous system (CNS) neurons (Baude et al. 2009; Andresen and Paton 2011). NTS baroreceptor neurons form a reflex loop with neurons in the caudal and rostral medulla. This reflex loop regulates sympathetic nervous activity and stabilizes ABP within limited ranges (Guyenet 2006).

In anesthetized rats, NTS microinjections of either the typical wide-range EAA receptor agonist L-glutamate or the candidate neurotransmitter L-proline produce rapid decrease responses in ABP and HR (Talman et al. 1980;

Takemoto 2001). L-Glutamate and L-proline likely produce these cardiovascular responses via activation of cardiovascular reflex loops (Andresen and Paton 2011). Interestingly, co-injection of the non-selective ionotropic EAA (iEAA) receptor antagonist kynurenate into the NTS differentiates responses to the two amino acids. Kynurenate completely abolishes cardiovascular responses to L-proline, but has no effect on cardiovascular responses to L-glutamate (Talman 1989, 1997; Takemoto 2001). This kynurenate inhibition test could be effective to discriminate cardiovascular responses to Cys and CSA in the NTS.

Thus, the present study used a NTS site identified by L-glutamate-induced depressor and bradycardic responses to determine (1) cardiovascular responses to Cys and CSA microinjections, and (2) whether or not kynurenate co-administration differentiates Cys- and CSA-induced cardiovascular responses.

Methods

All protocols and surgical procedures were performed in accordance with the following two guidelines: (a) the guiding principles for the care and use of animals approved by the Council of the Physiological Society of Japan; (b) the other is Hiroshima University guidelines of the Committee of Animal Experimentation and the Natural Science Center for Basic Research and Development Committee of Research Facilities for Laboratory Animal Science.

Animal preparation

Twenty-two male Wistar rats (weighing between 315 and 340 g, Slc from Shimizu Laboratory Supplies, Kyoto, Japan) were anesthetized with intraperitoneal urethane administration (1.0–1.2 g/kg). Supplemental urethane doses (0.05–0.1 g/kg) were given as needed. An atropine sulfate (Santen, Osaka, Japan) coated tracheal cannula was inserted by tracheotomy. A polyethylene tubing (PE 50) cannula was inserted into the femoral artery for monitoring ABP via a pressure transducer. A pen-writing oscillograph recorded pulsatile ABP waves, mean ABP, and HR (Nihon Kohden, Tokyo, Japan; Takemoto 2001, 2013). Animals were placed in a stereotaxic frame (Narishige, Tokyo, Japan) with the incisor bar set to 11 mm below the interaural line. Throughout the surgery, animals were artificially ventilated with a rodent respirator (Shinano, Tokyo, Japan). Tidal volume was set at 1 ml/100 g body weight at rates of 65–75 cycles/min. A partial occipital craniotomy was performed under microscopic observation to ensure that the dorsal surface of the medulla oblongata was exposed at the level of the calamus scriptorius. Rats were immobilized by an intravenous infusion of pancuronium bromide (0.5 mg/kg/h; ex-Sankyo, Tokyo, Japan). Following neuromuscular blockade, adequate surgical anesthetic depth was determined by ABP stability and toe pinch response. Rats were maintained at a rectal temperature of 37 ± 0.5 °C with a heating pad.

Amino acid microinjections

L-Cysteine and D-cysteine amino acid solutions (Nacalai Tesque, Kyoto, Japan) were dissolved in artificial cerebrospinal fluid (ACSF; pH 7.4) and freshly prepared immediately before each experiment (Takemoto 1990, 2014). Kynurenate was prepared at a final concentration of 100 mM (Takemoto 2001). For NTS microinjections, a glass micropipette (20–30 μm outside tip diameter) was prepared and mounted on a micromanipulator. The outer end of the micropipette was attached to a Hamilton microsyringe via polyethylene tubing (PE20). A cardiovascular responding site was found within the NTS where L-glutamate (10 mM) microinjections (50 nl) produced decrease responses in ABP (~25 %) and HR (~5 %), around 0.5 mm rostral and 0.5 mm lateral to the calamus scriptorius, and 0.5 mm ventral from the dorsal medulla surface. Places on the vessels were avoided during micropipette insertion for bleeding. After establishing a L-glutamate responsive NTS site, experimental solutions were loaded into the micropipette and depressor and bradycardic responses were recorded in at least 5 min intervals.

Mean ABP and HR values prior to microinjections are summarized in Table 1a, b. In the dose–response experiments, pre-injection mean ABP and HR values were not significantly different between Cys and CSA microinjections. Similarly, in the kynurenate inhibition experiments, mean ABP and HR values were not significantly different prior to each amino acid injection alone or prior to co-injection of each amino acid with kynurenate.

Data analysis

Data of mean ABP and HR were analyzed non-parametrically, because parts of the data set did not show equality of variance. Variables of mean ABP and HR were analyzed using the Ekuseru-Tokei 2012 statistical analysis package (Social Survey Research Information Co., Ltd., Tokyo, Japan). Data are expressed in the table as median values and interquartile ranges (IQR). Data are expressed in the figures as median values, IQRs, and additional maximums and minimums. Statistical significance was set at $p < 0.05$.

Table 1 Mean arterial blood pressure (ABP: mmHg) and heart rate (HR: beats/min) values prior to amino acid solution microinjections

	C	CSA	By *U* test
(a) Pre-injection values in the dose–response study			
ABP	89 (100, 84)	91 (93, 86)	Not significant
HR	415 (424, 403)	415 (444, 386)	Not significant
N	22	30	

	C	C + $K^{n.s.}$	CSA	CSA + $K^{n.s.}$	G	G + $K^{n.s.}$
(b) Pre-injection values in the inhibition tests						
ABP	94 (100, 88)	89 (96, 79)	87 (100, 82)	97 (101, 81)	90 (100, 87)	90 (98, 81)
HR	435 (457, 427)	440 (445, 402)	440 (452, 427)	420 (435, 398)	455 (470, 420)	435 (440, 413)
N	7	7	7	7	7	7

Values indicate the median (upper quartile, lower quartile)

Wilcoxon signed-rank test

U test Mann–Whitney's *U* test, *N* the number of microinjections, *C* L-cysteine, *CSA* L-cysteine sulfinic acid, *G* L-glutamate, *+K* co-injection of kynurenate, *n.s.* ABP and HR were not significantly different between amino acid injections alone and amino acid/kynurenate co-injections

Results

Pilot experiments

Four rats previously used in other projects were used in a pilot experiment. These rats received seven repeated Cys and CSA (10 mM) microinjections (50 nl) into NTS sites defined by L-glutamate-induced cardiovascular responses (see "Methods"). Both amino acids produced robust depressor responses: median (IQR) depressor responses for Cys were −31 % (−32 to −30 %) and −35 % (−37 to −33 %); CSA-induced median depressor responses were −26 % (−33 to −24 %) and −32 % (−36 to −28 %). Variables did not display decay tendencies in response to either amino acid, indicating low acute toxicity on NTS neurons. NTS microinjections of D-cysteine (10 mM, 50 nl) did not produce a change in ABP.

Cys and CSA dose–response effects

In NTS sites defined by L-glutamate microinjections, both Cys and CSA produced dose-dependent decreases in ABP and HR (Fig. 1). Lower concentration of CSA (1 mM) than Cys (3 mM) effectively produced both responses.

Effects of kynurenate on Cys- and CSA-induced cardiovascular responses

Similar to L-glutamate, NTS microinjections of Cys and CSA produced rapid depressor and bradycardic responses (Fig. 2). Previously, we demonstrated that NTS co-injections of kynurenate along with L-glutamate and L-proline only blocked L-proline-induced cardiovascular responses in each tested rat (Takemoto 2001). Here we utilized the same

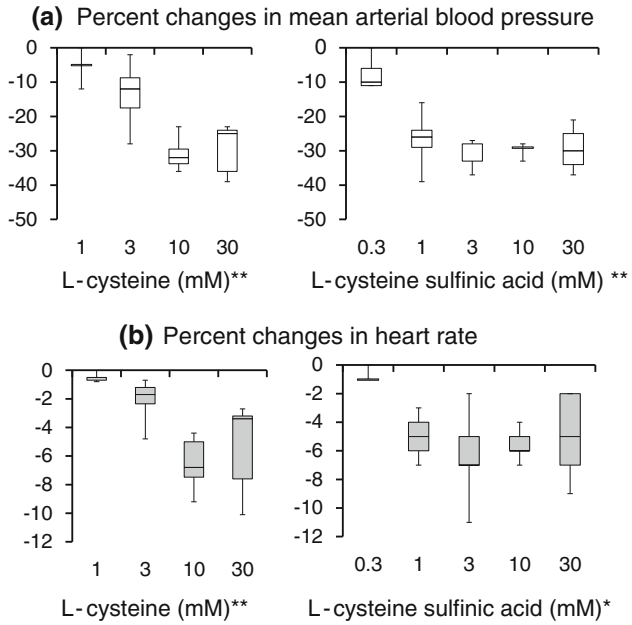

Fig. 1 Dose-dependent cardiovascular responses to L-cysteine and L-cysteine sulfinic acid microinjected (50 nl) into the NTS of rats. When microinjected into defined sites, both amino acids produced significant dose-dependent reductions in arterial blood pressure (**a**) and heart rate (**b**) similar to L-glutamate. Jonckheere–Terpstra test, $^{**}p < 0.01$, $^{*}p < 0.05$. The box plot contains median, *upper* quartile, *lower* quartile, and sample maximum and minimum values. L-cysteine $n = 6$ and L-cysteine sulfinic acid $n = 5$

protocol to determine the effects of kynurenate on Cys- and CSA-induced cardiovascular responses (e.g., as seen in Fig. 2). In our previous experiment (Takemoto 2001), kynurenate did not block depressor responses to L-glutamate. In pilot tests using different rats, kynurenate also did not block depressor response to CSA but blocked the responses to Cys. To avoid possible persistent blocking effects produced

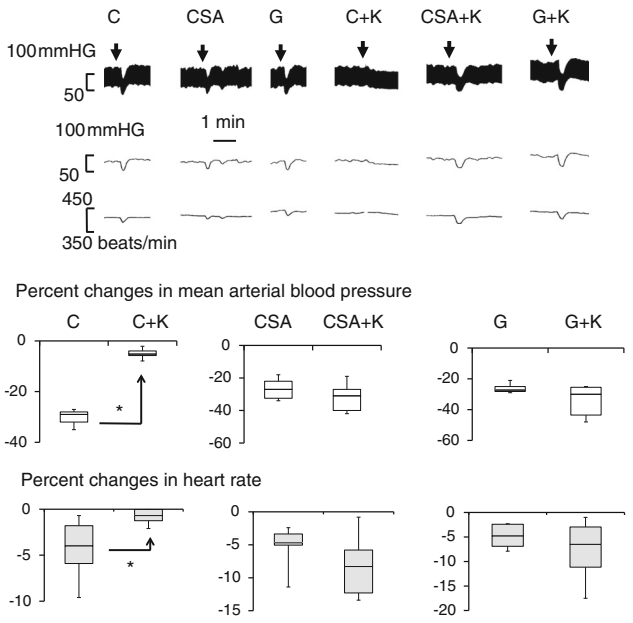

Fig. 2 Inhibition tests of amino acid and kynurenic acid co-administration. The *upper* two recordings show pulsatile and mean arterial blood pressure responses. The *lower* recording shows heart rate. The *arrow* indicates the time of microinjection (50 nl). *C* L-cysteine 10 mM, *CSA* L-cysteine sulfinic acid 10 mM, *G* L-glutamate 10 mM, *K* kynurenic acid (100 mM). The box plot contains median, *upper* quartile, *lower* quartile, and sample maximum and minimum values. Wilcoxon signed-rank test, $*p < 0.05$

by kynurenate, we decided to administer amino acids in sequence (Cys, CSA, and L-glutamate; Fig. 2) to each rat. Kynurenate co-administration completely abolished Cys-induced depressor and bradycardic responses; in contrast, kynurenate co-administration had no effect on CSA-induced depressor or bradycardic responses in seven rats (Fig. 2).

Discussion

The present study demonstrated the cardiovascular effects produced by CSA microinjections into the NTS of anesthetized rats. CSA, an EAA neurotransmitter candidate, satisfies neurotransmitter classification criteria. These criteria include identification of the presence, synthesis, release, inactivation, identity of action, and receptor activation of the neurotransmitter within nerve endings (Griffiths 1990). However, the role of CSA as an EAA receptor agonist and possible CSA effects on central cardiovascular regulation were largely unknown (Takemoto 2012). To our knowledge, there is only one previous report of CSA-induced cardiovascular effects; that study examined the effects of periaqueductal gray CSA administration on blood pressure (Maione et al. 1998). Therefore, we believe this is the first report of the cardiovascular effects of CSA produced by NTS administration.

Cys and CSA are well-known neurotoxic amino acids (Olney et al. 1990; Griffiths 1990). In the present study, repeated Cys and CSA injections produced robust depressor responses without decay, indicating low acute toxicity for NTS neurons responsible for regulating mean ABP. Administration of D-cysteine into the NTS did not produce a change in mean ABP, suggesting the effects of L-cysteine are stereospecific. The effects of NTS Cys administration are consistent with other CNS Cys administration studies; Cys but not D-cysteine produced cardiovascular responses upon administration to the cisterna magna of conscious rats (Takemoto 2013) and to the rostral ventrolateral medulla (RVLM) of anesthetized rats (Takemoto 2014).

NTS microinjections of Cys or CSA both produced dose-dependent decrease responses in ABP and HR and a lower dose of CSA than Cys effectively produced cardiovascular responses (Fig. 1). It was possible that the cardiovascular responses to Cys were mediated by the biosynthesis of CSA in neuronal terminals (Griffiths 1990); however, evidence from the kynurenate experiments makes this explanation unlikely.

Within the RVLM, Cys-induced cardiovascular responses were completely blocked by administration of the NMDA and non-NMDA receptor antagonists MK801 and CNQX, respectively (Takemoto 2014). This blockade suggests that only iEAA receptors are involved in Cys-induced cardiovascular responses. NTS co-administration of the non-selective iEAA receptor antagonist kynurenate blocked L-proline-, but not L-glutamate-, induced cardiovascular responses (Takemoto 2001). This result indicates no inhibition of L-glutamate-induced cardiovascular responses as a positive control. Consequently, the present study focused on the effects of kynurenate on CSA-induced cardiovascular responses.

As expected, kynurenate/Cys co-administration completely abolished Cys-induced cardiovascular responses. In contrast, kynurenate/CSA co-administration had no effect on CSA-induced cardiovascular responses (Fig. 2). This selective blockade suggests that kynurenate-sensitive receptors mediate only Cys-induced cardiovascular effects. In vitro, low concentrations of exogenous kynurenate inhibit α7 nicotinic acetylcholine receptors (Albuquerque and Schwarcz 2013). However, higher concentrations of exogenous kynurenate, as used in this study, block iEAA receptors (Albuquerque and Schwarcz 2013). Similar to in the RVLM, Cys also appears to activate iEAA receptors in the NTS (Takemoto 2014). The lack of kynurenate blockade of CSA- and L-glutamate-induced cardiovascular responses suggests these effects occur via mechanisms separate from Cys-induced cardiovascular effects. If Cys produced cardiovascular responses after conversion to CSA, then kynurenate should have blocked CSA-induced cardiovascular responses as well. Thus, the current findings

suggest that cardiovascular responses produced by Cys administration into the NTS are not mediated by CSA.

Similar to L-glutamate activity in the NTS, it is possible that CSA also activates metabotropic EAA (mEAA) receptors (Porter and Roberts 1993; Maione et al. 1998; Croucher et al. 2001; Foley et al. 1998). Viard and Sapru (2002) examined cardiovascular responses produced by NTS administration of various synthetic mEAA receptor agonists, including all types of group I–group III mEAA receptors. Similar to CSA, all of the synthetic agonists tested produced depressor and bradycardic responses. In addition to iEAA receptors in the NTS, it is possible that CSA also could activate mEAA receptors. CSA might produce cardiovascular-related regulation by working in tandem with L-glutamate in the NTS. However, additional studies with newly developed mEAA receptor antagonists are needed to test this idea.

Visceral afferent terminals in the NTS are thought to release L-glutamate (Talman 1997; Baude et al. 2009). In addition to important metabolic roles in the brain, L-glutamate also is an agonist for both iEAA and mEAA receptors. Vesicular glutamate transporters (VGluTs), which are involved in synaptic vesicle loading, could be a reliable glutamatergic neuronal marker (Baude et al. 2009). Although VGluT2 is more prevalent than VGluT1, VGluTs are widely distributed throughout the NTS (Lin et al. 2004). Within the NTS, glutamatergic projections have been suggested to arise from multiple sources including visceral afferent neurons, local interneurons, and central projections (Baude et al. 2009).

Talman et al. (1980) first suggested L-glutamate as a potential baroreceptor afferent neurotransmitter involved in cardiovascular function. This group demonstrated that L-glutamate-induced depressor and bradycardic responses were the same as responses induced by baroreceptor stimulation. However, the following studies found that kynurenic acid blocked baroreflexive-induced, but not L-glutamate-induced, cardiovascular responses (Talman 1989). This finding led them to suggest two possibilities: (1) baroreceptor afferents contain an endogenous neurotransmitter other than L-glutamate and/or (2) in addition to iEAA receptors, mEAA receptors in the NTS also contribute to baroreflexive-induced cardiovascular responses (Talman 1997). The current study suggests that as alternatives to L-glutamate, NTS neurons could contain the neurotransmitter candidate L-proline (Takemoto 2001) and/or Cys. This is supported by findings that kynurenate administration into the NTS completely blocks the cardiovascular responses produced by L-proline and Cys. In addition to L-proline, however, more detailed studies are needed to find possible neurotransmitter roles of Cys in the NTS.

Intracisternal Cys administration in conscious rats produces opposite cardiovascular responses from NTS Cys administration in urethane-anesthetized rats. These conflicting findings raise a question: are NTS neurons of conscious rats not involved in the cardiovascular responses to intracisternal Cys application? L-Glutamate microinjections into the NTS of anesthetized rats produce depressor responses similar to Cys; however, L-glutamate microinjections into the NTS of conscious rats produce the opposite pressor response (Machado and Bonagamba 1992). Machado et al. (1997) speculate that this ABP response difference results from activation of another chemoreflexive response in conscious states. Within the NTS, activation of the conscious state additional response could override the L-glutamate depressor response observed in anesthetized animals.

In anesthetized animals, anesthetics appear to suppress chemoreflexive pressor neurons to a greater extent than baroreflexive depressor neurons, resulting in L-glutamate-induced depressor responses (Machado et al. 1997). In in vitro oocyte experiments, urethane dose-dependently modulated all ion channel function: neuronal nicotinic acetylcholine, GABA, and glycine receptor-mediated currents were potentiated, whereas NMDA and AMPA receptor-mediated currents were attenuated (Hara and Harris 2002). Hara and Harris (2002) suggest that urethane concentrations used in vivo would affect channel function modestly, because urethane lacks a single predominant target of action. Therefore, urethane could modify various NTS receptors resulting in L-glutamate-induced depressor responses.

Intracisternal Cys or L-glutamate administration to conscious rats augments respiratory rates (Takemoto1995). Without urethane anesthetic interference, Cys might activate respiratory-related cardiovascular neurons located in the NTS, or other respiratory centers, contributing to the pressor response in conscious rats. Further physiological studies without anesthetics are required to determine the modulatory effect of Cys on respiratory function.

Of the several amino acids that produce a pressor response upon intracisternal administration to conscious rats, Cys produces both the strongest and most equivalent response to L-glutamate (Takemoto 1990, 1995). There are at least eight mammalian enzymatic pathways that metabolize Cys. Cys metabolites include CSA, hydrogen sulfide (H_2S), pyruvate, taurine, and glutathione (Griffith 1987). Within the CNS, the metabolites H_2S and taurine have known cardiovascular effects (Ufnal et al. 2008; Dawe et al. 2008; Sgaragli and Pavan 1972; Takemoto 1991). Here we provide new evidence that CSA also modulates cardiovascular function through a mechanism distinct from that of Cys.

Cardiovascular responses to Cys in the RVLM, another important nucleus for sympathetic vasomotor regulation, were mediated by two different ionotropic receptors in parallel alternative fashion (Takemoto 2014). Namely, both NMDA and non-NMDA iEAA receptor antagonists completely blocked cardiovascular responses, but either antagonist alone allows to maintain the responses. When one receptor does not work, another appears to compensate for the function. However, synaptic membrane receptor binding assays have not found that Cys binds to any iEAA receptors (Pullan et al. 1987). Thus, it seemed likely that Cys-induced iEAA receptor-mediated effects were produced by indirect iEAA activation, possibly through a mediator compound. CSA is a probable mediator because of the major metabolite of Cys in the brain (Griffith 1987). The current study, however, demonstrates that Cys-induced NTS responses are independent of CSA. This finding argues against CSA mediating Cys-induced cardiovascular responses. Thus, it remains unknown how Cys produces cardiovascular effects within the NTS.

Cys and CSA concentrations in the cerebrospinal fluid (CSF) are either very low or below the limits of detection. Two groups have reported the CSF concentrations of Cys and CSA from lymphoma patients treated with high doses of the antifolate drug methotrexate (Quinn et al. 1997; Becker et al. 2007). Prior to treatment, neither study detected Cys nor CSA in control CSF. The lower limits of detection in those studies were 50 nM for Cys and ranged from 10 nM to 1.0 μM for CSA (Quinn et al. 1997; Becker et al. 2007). Multiple Cys and CSA reuptake transporters have been identified that are frequently localized on astrocytes (McBean 2007). These astrocytic transporters would rapidly take up any synaptic Cys and CSA release that leaks into the extracellular space. This could result in trace amounts of both amino acids remaining in the CSF. Within the NTS, the current study demonstrates that exogenous Cys (3–30 mM) and CSA (1–30 mM) microinjections produce depressor and bradycardic responses similar to exogenous L-glutamate (10 mM). Even if the concentrations of Cys and CSA are diluted through the course of diffusion, they still could reach the synapse at levels high enough to evoke cardiovascular responses.

The current study revealed that NTS administration of either exogenous Cys or CSA produced similar depressor and bradycardic responses; however, the cardiovascular effects of Cys and CSA occur via different mechanisms. Whereas a kynurenate-sensitive receptor pathway alone is sufficient to mediate Cys-induced cardiovascular responses, CSA-induced responses appear to require a different or additional pathway. Cys-induced cardiovascular responses in the NTS do not seem to occur via CSA biosynthesis. Thus, within the NTS, endogenous Cys and CSA might be involved in EAA receptors-mediated cardiovascular regulation.

Conflict of interest The author declares that she does not have a conflict of interest.

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