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ORIGINAL ARTICLE

Functional cardiovascular action of L-cysteine microinjected into pressor sites of the rostral ventrolateral medulla of the rat

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Abstract The endogenous sulfur-containing amino acid L-cysteine injected into the cerebrospinal fluid space of the cisterna magna increases arterial blood pressure (ABP) and heart rate (HR) in the freely moving rat. The present study examined (1) cardiovascular responses to L-cysteine microinjected into the rostral ventrolateral medulla (RVLM), where a group of neurons regulate activities of cardiovascular sympathetic neurons and (2) involvement of ionotropic excitatory amino acid (iEAA) receptors in response. In the RVLM of urethane-anesthetized rats accessed ventrally and identified with pressor responses to L-glutamate (10 mM, 34 nl), microinjections of L-cysteine increased ABP and HR dose dependently (3–100 mM, 34 nl). The cardiovascular responses to L-cysteine (30 mM) were not attenuated by a prior injection of either antagonist alone, MK801 (20 mM, 68 nl) for the NMDA type of iEAA receptors, or CNQX (2 mM) for the non-NMDA type. However, inhibition of both NMDA and non-NMDA receptors with additional prior injection of either antagonist completely blocked those responses to L-cysteine. The results indicate that L-cysteine has functional cardiovascular action in the RVLM of the anesthetized rat, and the responses to L-cysteine involve both NMDA and non-NMDA receptors albeit in a mutually exclusive parallel fashion. The findings may suggest endogenous roles of L-cysteine indirectly via iEAA receptors in the neuronal network of the RVLM for cardiovascular regulation in physiological and pathological situations.

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Introduction

Concentrations of amino acids in the cerebrospinal fluid are lower than those in the blood (Davson et al. 1987), since the blood–brain barrier effectively isolates the brain from varying concentrations of plasma amino acids (Hawkins et al. 2006). A direct injection of high concentration of several amino acid solutions into the cerebrospinal fluid space of the cisterna magna produces changes in arterial blood pressure (ABP) of freely moving rats (Takemoto 1990, 1991, 2012). In addition to the typical amino acid neurotransmitters, L-glutamate and L-aspartate, L-proline, L-arginine, D-arginine, L-asparagine, or L-cysteine injected into the cisterna magna was found to increase ABP, among tested over twenty amino acids (Takemoto 1990, 1995a, b, 2012). Of those pressor amino acids, L-cysteine showed the strongest pressor response, equivalent to the neurotransmitter L-glutamate (Takemoto 1995a). Additional pharmacological investigation suggested a major contribution of autonomic nervous activation with minor vasopressin release from the hypothalamus for the pressor response to L-cysteine, different from L-glutamate (Takemoto 1995a). Double labeling immunohistochemistry combined with c-Fos detection supports minor but significant activation of hypothalamic vasopressinergic neurons with L-cysteine stimulation of the cisterna magna (Takemoto 2013), in a mode different from the major contribution of vasopressinergic activation with the neurotransmitter candidate L-proline (Takemoto 2011). The current study was designed to examine involvement of autonomic nervous system on

ABP and heart rate (HR) responses to L-cysteine. With regard to acting sites on the autonomic nervous activation with L-cysteine injected into the cisterna magna, the outermost medulla along the injected flow route could have possible acting sites (Takemoto 2012). It contains several nuclei of the neuronal network for cardiovascular regulation: the nucleus tractus solitarius in the dorsal medulla as an entrance for visceral information, the caudal ventrolateral medulla (CVLM) as a relay center with inhibitory neurons, and the rostral VLM (RVLM) as an exit to the spinal cord, which are defined with responses to microinjections with the typical multiple excitatory amino acid (EAA) receptors agonist L-glutamate (Dampney 1994; Guyenet 2006; Takemoto 2012). The RVLM is one of the most important nuclei where basic vascular sympathetic tone is produced via direct connections to sympathetic neurons in the spinal cord. Namely, a group of pre-sympathetic vasomotor neurons in the RVLM contributes to maintain ABP at rest (Dampney 1994; Guyenet 2006; Takemoto 2012). The present study, therefore, focused on examination of ABP responses to microinjections of L-cysteine into the RVLM.

In pilot tests, broad type of ionotropic EAA (iEAA) receptors antagonist kynurenic acid completely blocked responses to L-cysteine. Then, depressor responses to L-proline in the CVLM were abolished with non-NMDA receptors inhibitor and attenuated with NMDA receptor inhibitor (Takemoto 2007). For clarification of contribution with iEAA receptor types, effects of NMDA and non-NMDA receptor antagonists on cardiovascular responses to L-cysteine in the RVLM were further examined.

Intracisternal injection of L-cysteine but not D-cysteine increases ABP in the freely moving rat (Takemoto 2013), suggesting stereo-specific substance responsible for producing changes in ABP. Then, a derivative molecule of L-cysteine used as L-cysteine prodrug, *N*-acetyl-L-cysteine, reverses cocaine-induced drug seeking (Baker et al. 2003), to be expected as a useful study tool for addiction. *N*-Acetyl-L-cysteine could have some direct actions in the RVLM to modulate ABP. The current microinjection study into the RVLM also examined responses to stimulations with D-cysteine or *N*-acetyl-L-cysteine.

Methods

All protocols and surgical procedures used in this study were performed in accordance with the Guiding Principles for the Care and Use of Animals approved by the Council of the Physiological Society of Japan and the guidelines of the Committee of Animal Experimentation, Hiroshima University and the Committee of Research Facilities for Laboratory Animal Science, Natural Science Center for Basic Research and Development, Hiroshima University.

Animal preparation

Twenty-three, male Wistar rats (300–350 g) were anesthetized with urethane (i.p., 1.0–1.2 g/kg), and the fur around the ventral neck and inguinal region was shaved with electric clippers. After placing the rat in a supine position on a stereotaxic frame (Narishige, Japan), a tracheal catheter coated with atropine sulfate ointment (Santen, Japan) to prevent secretion was inserted, as described previously (Takemoto 2004, 2007). A microscope was used to visualize the brain stem region. The ventral surface of the medulla oblongata was exposed by opening a window in the basioccipital bone using a dental drill. A ventral approach was employed to the medulla, because there is less variability with a ventral than a dorsal approach and a smaller lesion for brain entities from the ventral approach. Insertions of the right femoral arterial (SP 28, single lumen polyethylene tube of Natsume, Japan, 0.4 and 0.8 mm of the inner and outer diameters) and left femoral venous (SP 10, 0.28 and 0.61 mm of the diameters) tubing were performed. The rats were ventilated by a rodent respirator (Shinano, Japan) to maintain normocapnia ($PaCO_2$ 35–45 mmHg) as described in the previous report (Takemoto 2004), and were then immobilized by an intravenous injection of pancuronium bromide (1 mg/kg, additional doses were given as required). The dura and arachnoid were lifted carefully to avoid tearing of the surface vessels. An adequate depth of anesthesia was assessed based on ABP stability and/or the absence of a withdrawal response to a firm toe pinch during periods of recovery from the neuromuscular blockade. Supplemental doses of urethane were given as needed. The rectal temperature of the rats was maintained at 37.0 ± 0.5 °C with a heating mat. The direct and mean ABP and HR were recorded on a pen-writing oscillograph (RJG4024, Nihon Kohden, Japan) as described previously (Takemoto 2013).

Microinjections

Amino acid solutions, L-cysteine and *N*-acetyl-L-cysteine (Wako pure chemical industries, Japan), and D-cysteine hydrochloride monohydrate (nacalai tesque, Kyoto, Japan), were freshly prepared just before each experiment by dissolving in artificial cerebrospinal fluid (ACSF) (Takemoto 1990) or Artcereb (commercial solution for irrigation and perfusion in cerebrospinal surgery, Otsuka, Japan). D-Cysteine hydrochloride or *N*-acetyl-L-cysteine solution was adjusted around pH 7.4 with NaOH solution to be finally 1 M, and diluted with ACSF or Artcereb. L-Glutamic acid monosodium salt (0.1 M, nacalai tesque) was prepared as stock solution. (+)MK801 (Sigma) and CNQX disodium salt (Santa Cruz) were dissolved in Artcereb. Methylene blue (Merck) was dissolved in

isotonic physiological solution. A glass micropipette (20–50 μm outer diameter at both tips, borosilicate glass capillaries, 1 mm outer diameter and 0.58 mm inner diameter, Clark Electromedical instruments) was made using micropipette processor (PE-2, Narishige, Japan). One end of the micropipette was connected to polyethylene tubing (SP10) with chemical bond and the needle of a Hamilton microsyringe (filled with distilled water) attached to a micromanipulator was tightly inserted into the other end of the tubing. The graduation of the micromanipulator had been calibrated previously using the microsyringe. After the capillary system was filled with the solution to be injected, the micropipette was mounted onto a pipette holder. Injections of drug solutions were repeatedly delivered through the same micropipette. The capillary system was carefully cleaned with distilled water between each drug solution when needed.

Identification of the RVLM

A marker (zero) point was identified at the caudolateral edge of the beginning of the basilar artery as described previously (Takemoto 2004, 2007). When the location of the basilar artery differed markedly from the expected location, the rostral end of the second rootlet of cranial nerve XII was used as an additional reference point, located on average 1.11 mm rostral from the basilar artery zero point (Takemoto 2004). Then, the region sensitive to 10 mM L-glutamate injection (0.34 nm in 34 nl) into the rostral part of the VLM was first detected by observation of an ABP increase over 15 mmHg, avoiding bleeding from vessels on the medulla surface. Injection sites were situated 1.7–2.2 mm lateral to the middle of the basilar artery, 1.5–3 mm anterior to the bifurcation base of the basilar artery, and 0.7 mm in depth from the ventral surface of the medulla.

Concentrations of cysteine solution for microinjections

Doses of L-cysteine were preliminarily examined using lower concentration of 1 mM to higher 100 mM of microinjection into the RVLM identified with pressor responses to 10 mM of L-glutamate (34 nl). Then, doses of D-cysteine and *N*-acetyl-L-cysteine were decided to be 3, 10, and 30 mM in the places where 10 mM of L-glutamate and 30 mM of L-cysteine both produce pressor responses over 15 mmHg before injection of D-cysteine or *N*-acetyl-L-cysteine.

Inhibition tests

For inhibition tests, one capillary was used repeatedly by exchanging solutions. This took about 3–5 min. The RVLM was first identified with a pressor response >15 % of basal ABP to 10 mM L-glutamate (34 nl), then injected with 30 mM L-cysteine (34 nl) as in Fig. 3.

The concentrations of inhibitors MK801 and CNQX were based on previous experiments with similar protocols in the CVLM (Takemoto 2005). A 2 mM concentration of each antagonist completely blocked ABP response to a subsequent injection of NMDA for MK801, and AMPA or Kainate for CNQX, but 2 mM of MK801 was not enough to block depressor responses to L-proline stimulation. The present inhibition test for L-cysteine, therefore, used 10 times that amount, 20 mM, for MK801 but the same 2 mM for CNQX.

After microinjection of either antagonist, the response to repeatedly injected L-cysteine was tested, and then the effect of another antagonist was examined. The response to L-glutamate microinjection was examined before completing the experiments. Metabotropic glutamate receptors in the RVLM could produce the persistent pressor response to L-glutamate even after blockade of ionotropic glutamate receptors (Tsuchihashi et al. 2000).

Marker injection

To locate the responsive site, 5 % methylene blue solution was injected into the site of the RVLM of the rat. Ten minutes later the rat was killed with overdose of pentobarbital sodium (65 mg/kg, i.v.) and the upper body was transcardially perfused with physiological saline containing heparin (20 ml) followed by saturated picric acid-saline solution (50 ml). The brain was removed and a photo of the ventral side with the blue spot was taken. The blocked brainstem was kept in the saturated picric acid-saline solution at 4 °C, then sliced coronally into 50–75 μm thickness with a vibratome. The slices were covered with CC/Mount (Diagnostic BioSystmes) or Mount–Quick "Aqueous" (Daido Sangyo, Japan). The slice with the injection site was recorded with a digital camera system (DP70, Olympus).

The results are analyzed non-parametrically, because parts of data sets do not show equality of variance. Variables are expressed as a median in a box whisker plot (Box plot) for figures, or described as a median (upper quartile, lower quartile) to be concise. The Box plot in figures contains a median, upper quartile, lower quartile, and sample maximum and minimum. The data were analyzed using Jonckheere–Terpstra test for dose–response tendency, Mann–Whitney's *U* test, or Kruskal–Wallis analysis followed by Scheffe's test and Steel's test. A statistical analysis package, Ekuseru-Tokei 2010 developed by Social Survey Research Information Co., Ltd. (Japan) was used. p values <0.05 were considered statistically significant.

Results

Hemodynamic changes with repeated microinjections of L-cysteine

Microinjections of L-cysteine (100 mM, 34 nl), repeated seven times, into the RVLM of a rat, produced a 34 % increase in median (36 of upper quartile, 32 of lower quartile), indicating robust responses to L-cysteine without acute toxic action.

Basal control values of ABP and HR

Table 1 summarizes basal values of ABP and HR before microinjections of amino acid solutions. Those variables showed no difference (Mann–Whitney's *U* test) between values before L-cysteine and L-glutamate microinjections for the dose–response study as shown in A of Table 1. Basal values of HR but not ABP before L-cysteine and L-glutamate microinjections were reduced significantly after inhibition with both blockers of MK801 and CNQX in two inhibition tests with different sequences ($p < 0.01$, Kruskal–Wallis analysis followed by Steel's test), as shown in B of Table 1.

The dose–response relationship of L-cysteine and L-glutamate in the RVLM

ABP and HR responses to microinjections (34 nl) of L-cysteine solution from 3 to 100 mM showed significant concentration-dependent increases ($p < 0.01$ by Jonckheere–Terpstra test) in five rats as in the upper panels of Figs. 1 (ABP) and 2 (HR). For comparison, those to L-glutamate in the same protocol were examined in five rats as in the bottom panels of Figs. 1 (ABP) and 2 (HR). Responses in both variables to L-glutamate also showed significant concentration-dependent increases ($p < 0.01$ by Jonckheere–Terpstra test).

Responses to D-cysteine and *N*-acetyl-L-cysteine

After identifying the pressor response as greater than 15 % in the RVLM with 30 mM L-cysteine and 10 mM L-glutamate, L-cysteine structurally related molecules, D-

Table 1 Basal control values of arterial blood pressure (ABP mmHg) and heart rate (HR beats/min) before microinjections of amino acid solutions

a Basal values before microinjections for the dose–response study

	C	G	By *U* test
ABP	80 (80, 65)	80 (83, 65)	Not significant
HR	485 (492, 458)	462 (495, 411)	Not significant
N	21	23	

b Basal values before microinjections for two type inhibition tests

	G	C	MK-C	MK-CN-C	MK-CN-G
ABP^{ns}	75 (77, 75)	70 (75, 60)	60 (80, 60)	55 (60, 53)	58 (60, 52)
HR$^{\#\#}$	452 (470, 445)	472 (472, 456)	421 (423, 405)	392** (407, 367)	398** (402, 361)
N	5	5	5	5	5
	G	C	CN-C	CN-MK-C	CN-MK-G
ABP^{ns}	72 (73, 70)	70 (75, 66)	70 (73, 70)	67 (75, 60)	70 (70, 65)
HR$^{\#\#}$	425 (479, 411)	413 (464, 400)	385 (427, 380)	347** (400, 347)	352** (366, 346)
N	5	5	5	5	5

Values indicate median (upper quartile, lower quartile)

N the number of microinjections, *C*: L-cysteine, *G* L-glutamate, *MK-C* L-cysteine after inhibition with MK801, *MK-CN-C* L-cysteine after inhibition with MK801 plus additional CNQX, *MK-CN-G* L-glutamate after inhibition with MK801 plus additional CNQX, *CN-C* L-cysteine after inhibition with CNQX, *CN-MK-C* L-cysteine after inhibition with CNQX plus additional MK801, *CN-MK-G* L-glutamate after inhibition with CNQX plus additional MK801

^{ns} and $^{\#\#}$ not significant and $p < 0.01$ by Kruskal–Wallis test among variables, ** $p < 0.01$ by Steel's test against variable in G

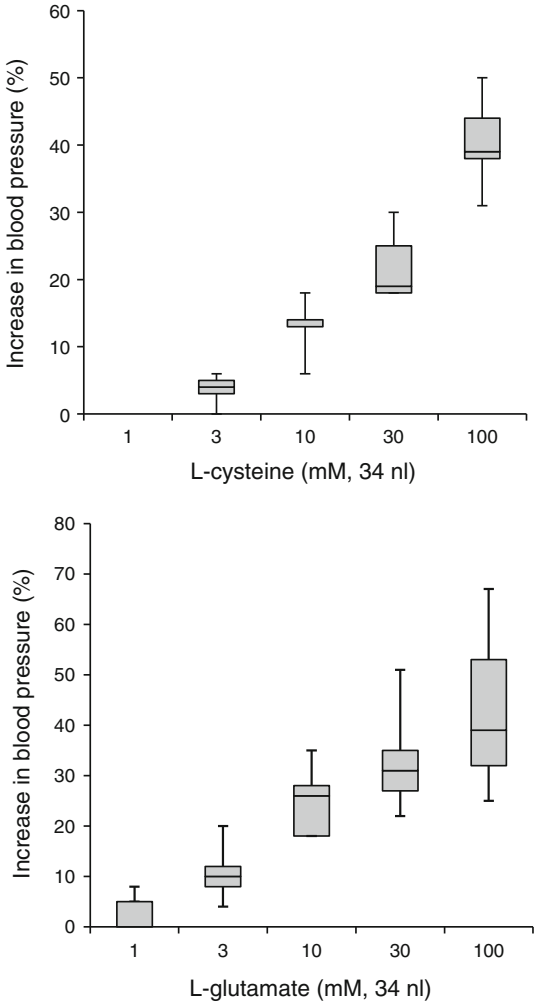

Fig. 1 Dose–blood pressure response relationships for microinjection of L-cysteine and L-glutamate into the RVLM of the anesthetized rat. Data are shown in *box plot*. Responses significantly increased dependent on concentrations in both cases ($p < 0.01$ by Jonckheere–Terpstra test)

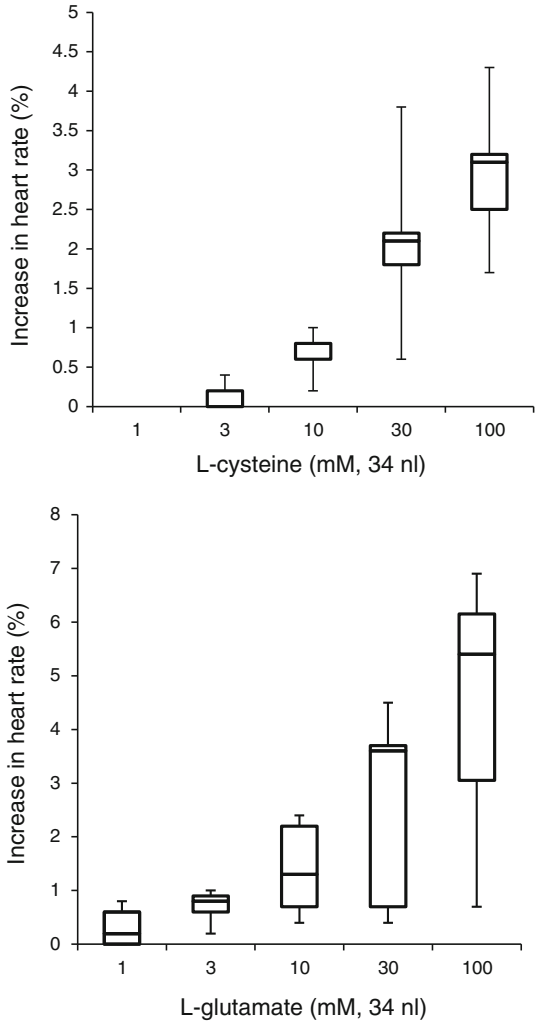

Fig. 2 Dose–heart rate response relationships for microinjection of L-cysteine and L-glutamate into the RVLM of the anesthetized rat. Data are shown in *box plot*. Responses significantly increased dependent on concentrations in both cases ($p < 0.01$ by Jonckheere–Terpstra test)

cysteine and *N*-acetyl-L-cysteine, were microinjected into two rats. Both molecules produced no change in ABP and HR between 3 and 30 mM.

Inhibition tests with MK801 and CNQX

After identification of the RVLM with L-glutamate (10 mM, 34 nl) and L-cysteine (30 mM), antagonist injections of 20 mM MK801 (68 nl) for NMDA receptors or 2 mM CNQX (68 nl) for non-NMDA receptors were performed and the following response to L-cysteine (30 mM) injection was examined in each five rats (Fig. 3). The pressor and tachycardiac responses to L-cysteine following inhibition of either receptor persisted in most trials and in each one case produced augmentation as summarized in Figs. 4 (ABP) and 5 (HR). However, additional blockade with the different type of antagonist, or inhibition of both type receptors, completely abolished the pressor and tachycardiac responses to L-cysteine in all ten trials (five trials for MK801 to CNQX and another five trials for CNQX to MK801) as summarized in Figs. 4 (ABP) and 5 (HR). Statistic analyses with Kruskal–Wallis test followed by Scheffe's test showed significant inhibition with both antagonists but not either antagonist alone for the responses to L-cysteine in both variables as shown in Figs. 4 (ABP) and 5 (HR). There was no significant response to L-glutamate before and after iEAA blockade (Mann–Whitney *U* test). With respect to basal ABP and HR reductions after MK 801 and CNQX inhibitions as seen in Fig. 3, there was significant reduction of HR but not of ABP in all the inhibition tests (Table 1b) as mentioned above.

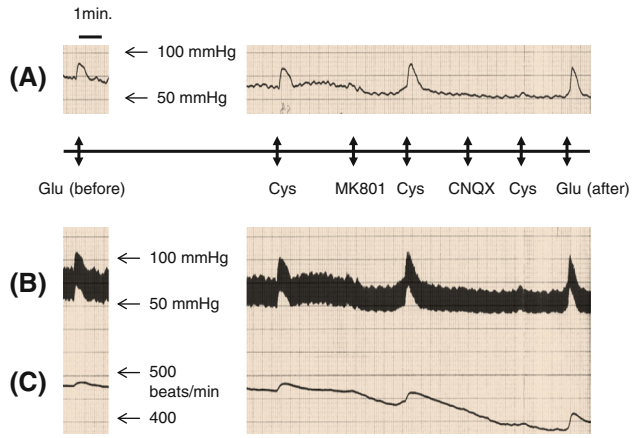

Fig. 3 Recordings of mean **A** and pulsatile **B** blood pressure and heart rate **C** in an inhibition test. Glu; 10 mM L-glutamate (34 nl), *Cys* 30 mM L-cysteine (34 nl), MK801 (20 mM, 68 nl), and CNQX (2 mM, 68 nl) were injected at the time indicated by *arrow heads*

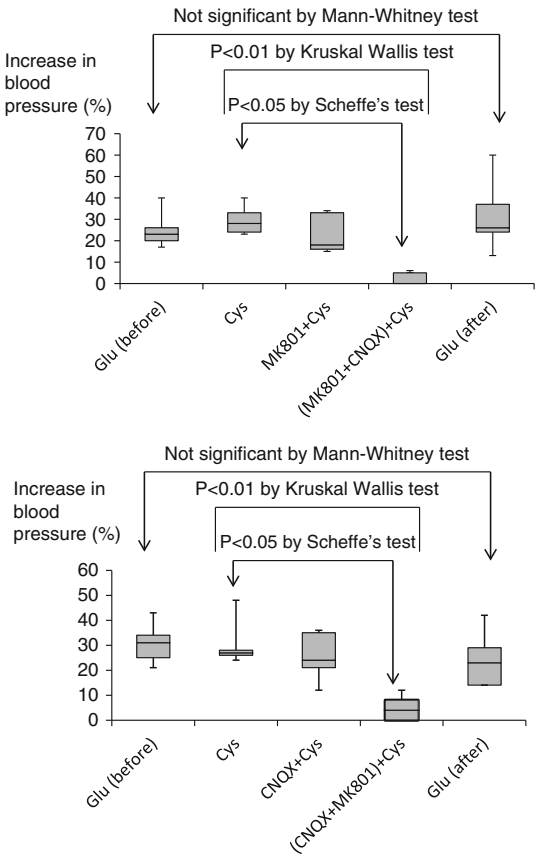

Fig. 4 Inhibition tests with MK801 and CNQX on the pressor response to L-cysteine in the RVLM of the anesthetized rat. Glu; L-glutamate (10 mM, 34 nl), *Cys* L-cysteine (30 mM, 34 nl), *MK801* the NMDA receptor antagonist (20 mM, 68 nl), *CNQX* the non-NMDA receptor antagonist (2 mM, 68 nl). Data are shown in *box plot*

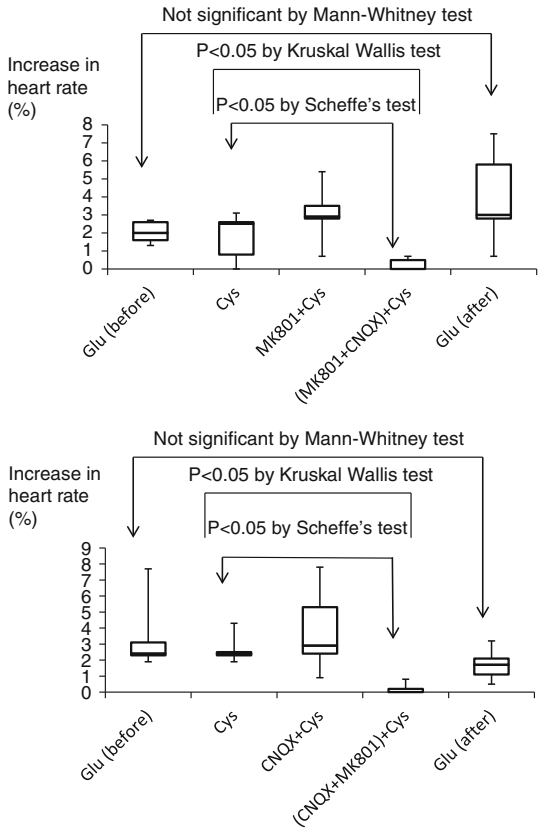

Fig. 5 Inhibition tests with MK801 and CNQX on the tachycardiac response to L-cysteine in the RVLM of the anesthetized rat. Glu; L-glutamate (10 mM, 34 nl), *Cys* L-cysteine (30 mM, 34 nl), *MK801* the NMDA receptor antagonist (20 mM, 68 nl), *CNQX* the non-NMDA receptor antagonist (2 mM, 68 nl). Data are shown in Box plot

Identification of the RVLM with methylene blue marker

Figure 6 shows typical photos of the dye spot in the RVLM. The ventral brain photo also contains the site of the CVLM (a blue spot in the caudal medulla) with a preliminary experiment after the RVLM.

Discussion

The current study demonstrates a functional ABP action of L-cysteine and its action-related receptor types in a specific area of the brain, the RVLM where a group of neurons regulates sympathetic vasomotor neurons. Namely, direct stimulation of L-cysteine produces strong robust pressor and tachycardiac responses in the RVLM of the anesthetized rat, and the cardiovascular responses are uniquely inhibited by both the NMDA and non-NMDA iEAA receptors, but not by either one alone.

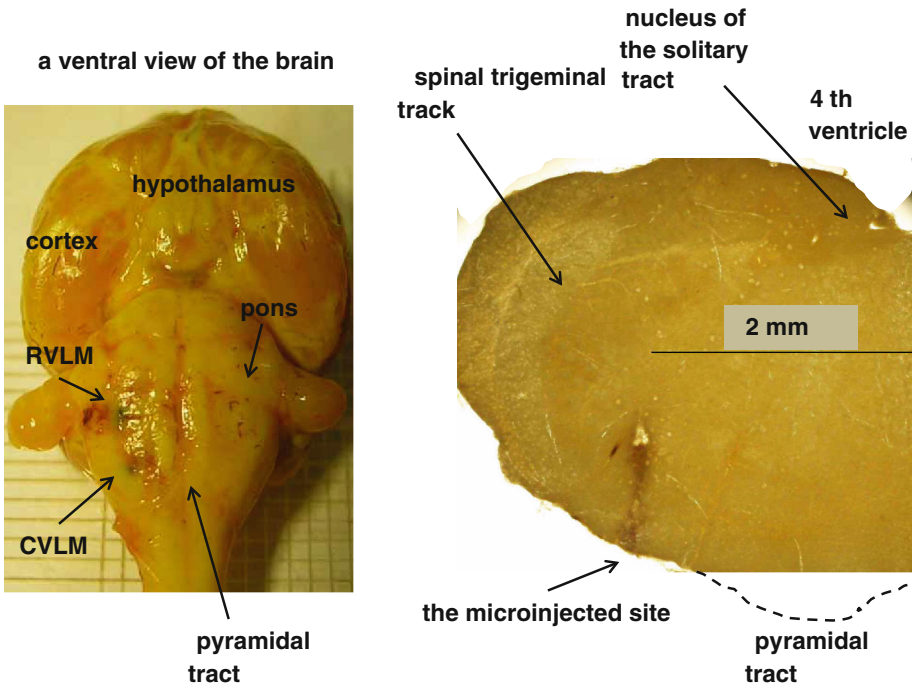

Fig. 6 Typical photos of the RVLM microinjected with methylene blue. In the left photo, two *blue spots* in the ventral medulla are shown; the upper is in the RVLM and the lower in the CVLM. The right photo shows the frontal section of the RVLM (color figure online)

Microinjections of L-cysteine between 3 and 100 mM dose dependently produced an increase in ABP and HR in the RVLM of the urethane-anesthetized rat, identified with 10 mM L-glutamate. As shown in Fig. 1, the dose–ABP response relationship in L-cysteine injection was shifted to the right of that in L-glutamate injection, while it suggests a sufficiently strong central action of L-cysteine on blood pressure regulation. A 20 % increase was obtained with 30 mM of L-cysteine in a concentration three times higher than L-glutamate. Intracisternal injections of L-cysteine in conscious rats had pressor action with doses equivalent to L-glutamate (Takemoto 1995a). The weaker pressor response to L-cysteine than L-glutamate in the RVLM of the current experiment could be produced by different levels of consciousness using the anesthetics urethane and/or suppression of spontaneous breathing with the neuromuscular relaxant pancuronium bromide. Another possibility is the additional acting sites for the L-cysteine injection into the cisterna magna which influences a much larger area.

HR responses to L-cysteine or L-glutamate showed dose-dependent increase (Fig. 2), but responding levels varied in both cases. Those large deviations of HR responses were also obtained in inhibition tests (Fig. 5). Changes in ABP are produced by changes in total peripheral resistance and/or cardiac output. Total peripheral resistance summarizes individual changes of the whole body in resistance vessels where sympathetic nervous activities strongly influence the resistance (Guyenet 2006; Takemoto 2012). Neurons in the RVLM send sympathetic premotor neurons to the spinal cord for the whole body and the sites responding to L-cysteine and L-glutamate stimulation may be varied for different body functions such as muscle, visceral and cardiac sympathetic neurons (Dampney 1994; Campos and McAllen 1997). Changes in ABP (~40 % in Fig. 1) would be produced mainly by changes in total peripheral resistance rather than possible cardiac output augmentation related to HR changes (~3–5 % in Fig. 2) in the RVLM modulation. Then, those sites sensitive to amino acid stimulations could have included the varying numbers of pre-sympathetic neurons sending to the heart, possibly resulting in large variation of HR data.

L-Cysteine but not D-cysteine injected into the cisterna magna of conscious rats produced an increase in ABP and HR in c-Fos experiments (Takemoto 2013). *N*-Acetyl-L-cysteine is a powerful prodrug of L-cysteine to protect addiction via cystine-glutamate exchanger on glia (Baker et al. 2003), suggesting a potential direct action of *N*-acetyl-L-cysteine itself in the brain. However, the present study showed that *N*-acetyl-L-cysteine in addition to D-cysteine (30 mM) did not show significant responses in the RVLM of the anesthetized rat, where L-cysteine (30 mM) produced pressor responses. The results showing a lack in the pressor response to the stereoisomer D-cysteine and the derivative *N*-acetyl-L-cysteine suggest that the acting principle of L-cysteine would need L-type stereoisomer specification and an intact amino group without any acetyl group in the RVLM. The pressor response to L-cysteine does not necessarily depend on a thiol group alone. The responding substance to L-cysteine in the RVLM requires strictly specific molecular structure.

Notorious neurotoxic action of overdosed L-cysteine in the brain of immature animals is attenuated with previous application of the NMDA receptor inhibitor MK801 (Olney et al. 1990), and our preliminary examination using kynurenic acid completely blocked the cardiovascular responses to L-cysteine in the RVLM. Therefore, the abolishment of the cardiovascular responses to L-cysteine was expected after the prior injection of MK801 in the current experiments. However, the results showed the cardiovascular responses to L-cysteine microinjection after the prior MK801 application persisted. Also, unexpectedly, an additional prior injection of CNQX completely blocked the persisting cardiovascular responses to L-cysteine. The same occurrence was observed when the antagonists were applied in the opposite order as CNQX first and MK801s. It appears that one receptor type compensates for another when one cannot operate efficiently, in response to L-cysteine application. Namely, blockade of the cardiovascular responses to L-cysteine requires both of these receptor blocking drugs. The cardiovascular responses to L-cysteine clearly mediate both NMDA and non-NMDA receptors, but it appears there are separate parallel modes that react with L-cysteine. NMDA and non-NMDA receptors themselves may be the responding entities to L-cysteine. However, receptor binding assays using synaptic plasma membrane have reported low affinities of L-cysteine for NMDA, kainate, and AMPA binding sites (Pullan et al. 1987), suggesting a less direct binding action of L-cysteine on NMDA and non-NMDA receptors. It appears there may be more complicated mechanisms.

L-Cysteine is a thiol amino acid with only one acidic group, but several sulfur-containing derivatives of L-glutamate and L-aspartate are suggested as potential excitatory amino acid neurotransmitters (Thompson and Kilpatrick 1996). Those contain sulphinic (–SOO–H) or sulphonic group (–SOO–OH) in the thiol position of L-cysteine. The typical sulfur containing amino acids, L-cysteic acid, L-cysteine sulphinic acid, L-homocysteic acid, and L-homocysteine sulphinic acid have been well examined having activity at EAA receptors (Curtis and Watkins 1963; Thompson and Kilpatrick 1996). However, the other acidic group lacking sulfur-containing L-cysteine has received less attention as an acting substance in the brain until Olney and Ho (1970) presented the first evidence on its neurotoxic activity for the hypothalamic arcuate nucleus after oral administration. Systemic administration (subcutaneously, sc) of high concentration of L-cysteine in the neonatal rat resulted in brain damage especially in the fronto-parietal neocortex, hippocampus, thalamus, and caudate nucleus, due to the immature blood–brain barrier, but previous subcutaneous treatment of the NMDA receptor antagonist MK801 prevented the brain damage (Olney et al. 1990). Further evidence using the in vitro chick embryo retina suggested possible recruitment of two kinds of receptors; one is an NMDA receptor for lower concentrations of L-cysteine and additional non-NMDA receptor for higher concentrations of L-cysteine (Olney et al. 1990). The present functional study monitoring ABP and HR in the RVLM of the anesthetized rat showed stable pressor responses to repeated injections of L-cysteine, indicating no acute toxic action of L-cysteine against neurons in the RVLM responsible for blood pressure regulation. Inhibition tests revealed mediation via two kinds of NMDA and non-NMDA receptors to produce the pressor and tachycardiac responses to L-cysteine microinjections as seen with toxic mechanisms. However, the use of either antagonist alone was ineffective in the current functional study. L-Cysteine appears to influence iEAA receptors on neurons of the RVLM, but through different modes of actions from the toxic one.

The pressor response to L-cysteine was abolished by both of NMDA and non-NMDA receptor antagonists in the RVLM. With respect to iEAA receptors, several sympathetic excitatory reflexes rely on NMDA and/or non-NMDA receptors for activation of pre-sympathetic neurons in the RVLM (Schreihofer and Sved 2011). The chemoreceptor reflex (Koshiya et al. 1993), the somatic pressor reflex (Kiely and Gordon 1994), the somato-sympathetic reflex (Zanzinger et al. 1994), the nasopharyngeal reflex (McCulloch et al. 1999), and the gall bladder-stimulated pressor reflex (Zhou et al. 2006) are considered to use iEAA agonists such as L-glutamate as neurotransmitters in the RVLM, because those reflexes were blocked with kynurenic acid or NMDA/non-NMDA receptor antagonists microinjected in the RVLM. L-Glutamate acts on wide ranges of EAA receptors from ionotropic to metabotropic receptors, suggesting a strictly shielded synapse structure between presynaptic glutamate release and postsynaptic iEAA receptors in question to make only iEAA receptor response. If postsynaptic membrane has metabotropic receptors in addition to iEAA receptors, those receptors would produce compensated cardiovascular responses to endogenously released L-glutamate as seen in exogenous L-glutamate in the current study after both NMDA and non-NMDA receptors blocking in Figs. 3, 4, 5. Recently Llewellyn-Smith and Mueller (2013) have shown widely distributed NMDA receptors over the RVLM pre-sympathetic neurons. However, it remains unknown if densely localized NMDA receptors on the pre-sympathetic neurons could make completely shielded synaptic cleft to glutamate release. Alternative explanation might be the existence of possible endogenous neurotransmitters only for NMDA receptors or non-NMDA receptors. L-Aspartate could be one of endogenous neurotransmitters only for NMDA receptors in the RVLM (Kubo et al. 1997), but it remains

less information on endogenous agonists only for the non-NMDA receptors, kainate and AMPA receptors. Because kynurenic acid abolished the pressor response to intracisternally applied L-proline which has common molecular structure to kainate, L-proline was expected to be an endogenous agonist for one of the non-NMDA receptors (Takemoto 1999) in the RVLM, but giving no ABP response in the RVLM (Takemoto 2004) corresponding to no response with low doses of kainate itself (Takemoto 2007). The results suggest less possibility of L-proline as endogenous agonist only for kainate receptors to respond in the RVLM, although there was some possibility in the hypothalamic supra optic nucleus (Takemoto and Semba 2006; Takemoto 2011; Lopes-Azevedo et al. 2013). With respect to endogenous agonist only for AMPA receptors, it seems to attract less attention than for NMDA receptors possibly being no information thus far. L-Cysteine may indirectly activate iEAA receptors through release of missing endogenous agonists only for non-NMDA receptors together with L-aspartate for NMDA receptors in the RVLM.

Receptor binding assays using synaptic membrane and 15 kinds of sulfur-containing amino acids provided the results that L-cysteine has a strong affinity for L-2-amino-4-phosphonobutanoate (L-AP4) binding receptors with less affinity for three iEAA receptors (Pullan et al. 1987). L-AP4 is recently known as an agonist for group III metabotropic EAA receptors 4, 6, 7, and 8 (Niswender and Conn 2010) and microinjections of L-AP4 increase ABP in the RVLM of the rat (Tsuchihashi et al. 2000). L-Cysteine may bind to metabotropic EAA receptors including group III in the micronetwork or the synapses to result in parallel mobilization of iEAA receptors. The second possibility is unidentified receptors specific to L-cysteine. Acting entity for L-cysteine remains elusive.

We have provided several lines of evidence on the functional action of L-cysteine on blood pressure regulation with intracisternal injections in conscious rats (Takemoto 1990, 1995a, 2012, 2013). Brain slices release L-cysteine with high concentration of potassium stimulation (Keller et al. 1989; Zängerle et al. 1992), and neurons and glial cells rapidly take up this amino acid (Sagara et al. 1993), suggesting a neuromodulatory action of L-cysteine in healthy and/or pathological brains (Janáky et al. 2000). The current study added another line of evidence on a possible functional role of L-cysteine in the RVLM of the anesthetized rat via a parallel mode of iEAA receptors. Responding principles to L-cysteine in addition to physiological roles of it in the brain remain puzzling. Additional multidisciplinary approaches including at molecular levels are required to understand exact roles of L-cysteine in healthy brain and to apply the knowledge as tool for pathological brains.

Conflict of interest The author declares that she has no conflict of interest.

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