The mapped pattern of kainate on the blood pressure responses is similar to that of L-proline in the ventrolateral medulla of the rat

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<Abstract>

Kainate is an excitatory amino acid receptor agonist with a structure similar to the amino acid L-proline. Our previous studies demonstrated that microinjections of L-proline into the ventrolateral medulla (VLM) of the rat induce a mapped pattern of blood pressure responses distinct from L-glutamate, and the depressor response to L-proline in the caudal VLM (CVLM) is abolished by the kainate/AMPA receptor antagonist CNQX. The present study investigated whether kainate produces the L-proline-mapped pattern of responses in the VLM, compared with the pattern by AMPA. Kainate is known to activate AMPA receptors at higher concentrations. Therefore, responses to kainate were investigated at a low concentration. Microinjections of AMPA or NMDA showed the pattern of the L-glutamate-type; a pressor response in the rostral VLM and caudal pressor area (CPA) and a depressor response in the CVLM. Microinjections of kainate showed depressor responses in the CVLM but minor pressor responses in the rostral VLM, suggesting the same responses to L-proline. However, the response sites in the CPA did not enable us to clearly determine the L-proline-type. Further trials at sites defined by a pressor response to L-glutamate in the CPA, successive injections of L-proline and kainate produced no response, indicating that L-glutamate responding neurons in the CPA are not sensitive to L-proline and kainate. These results suggest that kainate stimulation in the VLM produces a mapped pattern of ABP responses similar to the mapped pattern with L-proline. Kainate receptors could therefore be involved in the depressor response to L-proline in the medulla.

Key words; kainate, L-proline, amino acid, ventrolateral medulla, arterial blood pressure, rat

Kainate is a typical exogenous agonist for kainate receptors. Kainate receptors are defined as one of the ionotropic excitatory amino acid (EAA) receptors which include the NMDA (N-methyl-D-aspartate) and AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid) receptors [2, 5, 8-11, 15]. Interestingly, the molecular structure of kainic acid is similar to that of the non-essential amino acid L-proline. Previous studies have demonstrated that injection of L-proline into the rat brain induces various cardiovascular responses similar to those by induced by L-glutamate [20-23]. However, a study of mapped pattern of arterial blood pressure (ABP) responses to microinjections of L-proline into the ventrolateral medulla (VLM) in the rat showed a distinct result from that by L-glutamate [25]. The VLM contains three loci that regulate cardiovascular responses, including the rostral (R) VLM, the caudal (C) VLM and the caudal pressor area (CPA, [6]). The RVLM sends pre-sympathetic efferent projections to the intermediolateral cell column. In addition, the cellular activity in the CVLM and CPA can influence the basal ABP through RVLM efferent projections [3, 4, 7, 16, 19]. Microinjections of L-glutamate in the RVLM and CPA increase ABP, whereas ABP is decreased following L-glutamate injections into the CVLM; however the mapped pattern for L-proline has depressor responses in the caudal areas of VLM with minor pressor responses in the RVLM [25]. Because L-glutamate stimulates all known EAA receptors [2, 5, 8-11, 15], local injections of L-glutamate may result in a complicated response pattern in a region containing all subtypes of the ionotropic EAA receptors. Many physiological experiments have used ionotropic EAA receptor agonists to stimulate neurons. However, there are no systematic data on the mapped patterns of the responses in the VLM including the CPA with ionotropic EAA receptor agonists especially at low concentrations. L-Proline may influence the responses of a subgroup of EAA receptors, such as the structurally related kainate receptors. Indeed, if medullary kainate receptors are largely responsible for the ABP response to L-proline, selective microinjections of kainate into the VLM would be expected to result in the same pattern of

ABP responses as those seen following L-proline administration. Kainate at high concentrations (0.1–4 nmol/ microinjection), in the medulla, has commonly been used to destroy neurons that regulate cardiovascular output [12, 13, 28]. Kainate is also known to be a stimulant of AMPA receptors at higher concentrations [11]. To avoid damaging cells and to evaluate the physiological role of kainate at kainate receptors in cardiovascular regulation, lower picomolar concentrations of kainate were employed. Therefore, the mapped pattern of ABP responses to microinjections of kainate in the VLM of the anesthetized rat were initially investigated and compared to those following AMPA or NMDA receptor stimulation. The results from kainate injections indicated a marked response pattern in the RVLM and CVLM as L-proline produced, but unclear in the CPA. Therefore, the responses to L-proline or kainate following precise injections into a locus of the CPA were subsequently evaluated, after confirmation of the pressor response to AMPA or L-glutamate injection. Brief reports of this work have appeared previously in abstract form [24, 27].

<Materials and 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 Guideline for 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 (N-BARD), Hiroshima University.

Animal procedures. Male Wistar rats, weighing 325-370 g, were anesthetized with urethane (1.1-1.3 g/kg, I.P., Sigma), 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), insertions of carotid arterial and femoral venous tubing (PE50) and a tracheal catheter were performed as described previously [25]. The tracheal catheter was coated with atropine sulfate ointment (Santen) to prevent secretion. 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. The dura and arachnoid were lifted carefully to avoid tearing the surface vessels. The rats were ventilated by a rodent respirator (Shinano) to maintain normocapnia ($PaCO_2$: 35-45 mmHg) as described in a previous report [25], and were then immobilized by an I.V. infusion of pancuronium bromide (Sankyo) at a flow rate of 0.4 mg/kg/hr. 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 a stoppage of the infusion of the neuromuscular blockade. Supplemental doses of urethane (75 mg/kg/injection, I.V.) were given as needed. The rectal temperature of the rats was maintained at 37.0 ± 0.5 °C by a cooling bag or a heating mat. The direct and mean ABP was recorded on a pen-writing oscillograph (RJG4024, Nihon Kohden).

Preparation for microinjections. A glass micropipette (20-30 μm o.d. at both tips, borosilicate glass capillaries, 1 mm o.d. and 0.58 mm i.d,. Clark Electromedical instruments) was made using a micropipette processor (PE-2, Narishige). One end of the micropipette was tightly connected to polyethylene tubing with a chemical bond and the needle of a Hamilton microsyringe (filled with distilled water) attached to a micromanipulator was 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. Artificial cerebrospinal fluid (ACSF, see [23]) was used as the vehicle for all drug solutions: L-proline and L-glutamate were obtained from nacalai tesque (Japan), and NMDA and AMPA were obtained from Sigma (St. Louis, Mo). Kainic acid (nacalai tesque, Japan) was first dissolved in a small volume of distilled water and then diluted with ACSF. Injections of several doses of kainic acid and drug solutions were repeatedly delivered through the same micropipette. The capillary system was carefully cleaned with distilled water between each drug concentration or solution. A marker (zero) point was identified at the caudolateral edge of the beginning of the

basilar artery as described previously [25]. 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 [25].

Experiments. The concentration of kainic acid that effectively evoked changes in ABP was determined in the CVLM and compared with the depressor response to L-glutamate (0.34 nmol, 34 nl) in 3 rats. The concentrations of AMPA (0.4 pmol/ 34nl) and NMDA (2 pmol/ 34 nl) were determined in a previous study [26]. The ABP responses to microinjections of kainic acid (59 loci in 10 rats), AMPA (52 in 5 rats) and NMDA (42 in 7 rats) in the RVLM, CVLM, and CPA were subsequently mapped. Micropipettes were lowered 0.7-1.0 mm in the CVLM and CPA, and 0.5-0.7 mm in the RVLM to the ventral surface of the medulla [25]. The microinjections were repeated when the ABP was stable and returned to the pre-injection level. In the case of no change in the ABP, the intervals between injections were at least 3 min. The patterns of the responses to AMPA and NMDA were clearly determined to be L-glutamate-type based on the response geometry, but the responses to kainate were unclear geometrically in the CPA but not in the RVLM and CVLM. In the second experiment, the loci in the functionally defined CPA that produce the pressor response to L-glutamate and/or AMPA were identified and successive injections of L-proline and kainic acid were performed to determine the effects of these compounds on ABP in 6 rats. The functionally identified CPA showed consistently no response to L-proline and kainic acid in all rats, indicating that the geometrically deduced CPA, as L-proline induced depressor acting area, in the previous study [25] could be involved in the CVLM depressor region or include neurons not sensitive to L-glutamate/AMPA. To locate sites after the injections, methylene blue (0.1%, 68 nl) was injected into the functionally defined CPA in two rats. Ten minutes later the rat was euthanized with an overdose of sodium pentobarbital (100 mg/kg, I.V., Dainippon). The brain stem was removed and

stored in a saturated picric acid-saline solution for 24 hr, then sectioned at 50 or 100 μm thickness with a vibratome. The sections were covered with Crystal/Mount (Biomeda Corp.) and images were captured with a digital camera system (DP70, Olympus).

Data analysis. Data are presented as the mean values ± SEM. A one-way repeated measure analysis of variance combined with multiple comparison of Tukey's method or the Student t-test was employed to detect significant differences. In all cases, P values of less than 0.05 were considered to be statistically significant.

<Results>

Determination of kainate concentration. In the CVLM sites where microinjections of L-glutamate (0.34 nmol, 34 nl) evoked a depressor response (-20 ± 9 mmHg, n=3), the depressor responses were -5 ± 0 mmHg to kainate solutions at 0.04 pmol (34 nl), -11 ± 2 mmHg at 0.4 pmol, and –22 ± 3 mmHg at 4 pmol. The ABP following injections of 4 pmol of kainate remained at the low level for 10 minutes. The ABP response to microinjections of L-proline (3.4 nmol, 34 nl) following kainate injections (4 pmol) were -13 ± 2 mmHg, a smaller response ($p<0.05$ by t-test) than that (-26 ± 4 mmHg, n=5) in the previous paper [25], possibly suggesting a toxic effect of kainate at 4 pmol. Taking the possible toxity or action on AMPA receptors of kainate at high concentrations into account, 0.4 pmol/ 34 nl was determined to be a suitable concentration of kainate for subsequent experiments.

ABP response mapped patterns to ionotropic EAA receptor agonists. Examples of ABP recordings for responses to kainate and AMPA are shown in **Figure 1**. **Figure 2** illustrates the mapped results of the ABP responses to microinjections of kainate, AMPA, and NMDA, including the loci where ABP recordings were determined for **Figure 1**. The depressor response was -16 ± 2 mmHg (22 trials) with kainate, -16 ± 2 mmHg (18) with AMPA, and -21 ± 2 mmHg (13) with NMDA. The pressor response was obtained at 10 sites (8 ± 1 mmHg) for kainate, at 27 sites (26 ±

2 mmHg) for AMPA, and at 23 sites (20 ± 4mmHg) for NMDA. The basal ABP was 90 ± 1 mmHg in these trials (113). The number of the injected points giving no change was 27 for kainate, 7 for AMPA, and 6 for NMDA. The mapped patterns of the responses to AMPA and NMDA revealed a stimulated pressor response in the RVLM and CPA and a depressor response in the CVLM, clearly equivalent to the response pattern following L-glutamate microinjection [25]. The mapped pattern of the responses to kainate was different from those patterns induced by AMPA and NMDA. Kainate evoked a strong depressor response in the CVLM with minor pressor response in the RVLM (**Fig. 2**), which was equivalent to the responses following L-proline [25]. However, the border between the CVLM and CPA includes both the pressor and depressor responses to kainate, by reference to the CPA as the pressor response sites to AMPA and NMDA in **Figure 2**, thus resulting in the unclear response type to kainate in the CPA.

Responses to L-proline and kainate in the CPA defined with L-glutamate and/or AMPA.

To compare the ABP responses to L-proline and kainate in the CPA at the same location which produces a pressor response to L-glutamate and/or AMPA, successive microinjections were performed with the same pipette after exchanging the solution. A pressor responding site to AMPA or L-glutamate was first determined. After the effect of L-proline injection (3.4 nmol, 34 nl) was observed, kainate was tested and again AMPA or L-glutamate was injected. In 6 rats, microinjections of L-proline and kainate into the CPA defined by the AMPA and L-glutamate produced no pressor response (**Fig. 3**). The injection site in the CPA of one rat is also shown in **Figure 3.** The CPA site was located caudally, approximately -14.2 to -14.5 mm from Bregma, corresponding to coordinates used by Sun and Panneton [17, 18].

<**Discussion**>

The present study describes, for the first time the mapped pattern of the ABP responses in the rat VLM, including the CPA, to microinjections of low concentration of kainate. These responses

include a major depressor response in the CVLM with a minor pressor response both in the RVLM and CPA, to the same low concentration of kainate. Mapping patterns induced by AMPA and NMDA showed both a depressor response in the CVLM and a pressor response in the RVLM and CPA, equivalent to the mapped pattern of responses obtained following microinjections of glutamate [25]. In the CVLM, the three agonists produced depressor responses of almost equivalent strength. In the RVLM and CPA, almost all the injection sites for AMPA and NMDA produced the pressor response, but kainate failed to evoke the pressor response in many sites (**Fig. 2**). Higher concentrations of kainate stimulate both AMPA and kainate receptors [11]. If kainate stimulates the AMPA receptors in the present study, it should have resulted in a strong pressor response to kainate stimulation in a manner similar to that for AMPA stimulation, both in the RVLM and CPA. However, kainate at a low concentration (0.4 pmol/ 34 nl) in the present study produced only minor ABP responses in the RVLM and CPA, although it was equivalently effective in the depressor response in the CVLM, probably suggesting that a proper and effective low dose of kainate was used which was sufficient to distinguish the responses from kainate and AMPA receptors. Therefore, the present results underscore the unique ABP response pattern to microinjections of kainate, a pattern that is distinct from the other ionotropic EAA receptor agonists. The heterogeneous distribution of the ionotropic EAA receptors has been observed in the brain with *in situ* hybridization and immunohistochemistry [15], but there is no detailed information available regarding EAA receptor distribution throughout the entire medulla other than for NMDA and AMPA receptors in the RVLM [1]. Kainate receptors involved in vasomotor control might be distributed throughout the rat VLM in a manner distinct from AMPA and NMDA receptor distribution, in part as suggested functionally in the CVLM by Miyawaki et al. [14].

The primary goal of the present study was to compare the mapped pattern of the ABP responses to kainate with those to L-proline. The mapped pattern of ABP responses to L-proline is

characterized by a depressor response in the caudal area of VLM with a minor pressor response in the RVLM, distinct from the pattern seen following L-glutamate injection, as in the previous study [25]. The pattern induced by AMPA was identical to that of L-glutamate, thus indicating that it is unlikely that AMPA receptors are responsible for the depressor response to L-proline microinjections into the VLM. Importantly, similar mapped patterns of ABP responses were obtained following kainate and L-proline in the RVLM and CVLM, but the responses were unclear in the CPA. The CPA as L-proline induced depressor acting region in the previous study [25] is judged based on the mapped pattern of responses and geometry but not the functional identification by L-glutamate. The border between the CVLM and CPA is not clearly defined on the mapped pattern as seen in **Figure 2** (AMPA). The mapped responses to AMPA stimulation showed intermingled distribution of pressor and depressor responding sites at the border area between the CPA and CVLM, as seen in the pattern with L-glutamate [25]. Considering these deviations of responding sites, the geometrical CPA region marked as the responding sites to L-proline from the reference of responding site map to L-glutamate in the previous study [25] may be distinct from the functional sites in the present study. Therefore, a further investigation of neurons in the CPA, defined with the pressor response to L-glutamate and/or AMPA, was performed and revealed that neither L-proline nor kainate microinjection evoked a response. Although it remains possible that neurons in the far caudal CVLM not sensitive to L-glutamate produced the depressor response to L-proline but not kainate, L-proline and kainate induce the same ABP responses within the investigated VLM in the present study.

The molecular structure of kainate is similar to that for L-proline, and the depressor responses to L-proline are completely blocked by the AMPA/kainate receptor antagonist CNQX [26], thus suggesting that responses to L-proline are mediated by either AMPA or kainate receptors. The present study indicated that the mapped pattern of responses to kainate and L-proline were similar,

however, they were distinct from those to AMPA. Taken together, the present results support the hypothesis that the responses to L-proline are mediated via kainate receptors in the rat medulla. However, stimulations in the in vivo experiment could affect a wide range of the neuronal circuit elements including kainate receptors relating to the ABP control. There are possibilities that some structures activated by L-proline may therefore intervene in the response to kainate receptors. Alternatively, it remains possible that some unknown proline specific receptors may exist that are sensitive to CNQX. To locate the responding site(s) to L-proline at either the molecular or cellular level and identify details about the receptor type and specificity, further investigations are thus required.

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Figure legends

Figure 1 Examples of traces of direct (upper panel) and mean (lower panel) arterial blood pressure. Solutions (0.4 pmol, 34 nl) were injected at the times marked with arrows in panel, (a) - (d) as shown in **Figure 2**. AMPA traces in (B) were taken in one rat. RVLM: rostral ventrolateral medulla; CVLM: caudal ventrolateral medulla; CPA: caudal pressor area. The solid bars above ABP traces are time marker lines with ticks indicating every 1 min.

Figure 2 The mapped pattern of arterial blood pressure (ABP) responses to the microinjection of kainate (0.4 pmol in 34 nl), AMPA (0.4 pmol in 34 nl), NMDA (2 pmol in 34 nl) into the ventrolateral medulla. Open circles indicate the sites which induced an increase in ABP. Closed circles indicate sites which induced a decrease in ABP. The circle sizes are proportional to the degrees of response. The arrow indicates examples of the circle size equivalent to changes in ABP and sites (a)-(d) in **Figure 1**. The dots indicate the sites which induced no change in ABP. RVLM: rostral ventrolateral medulla; CVLM: caudal ventrolateral medulla; CPA: caudal pressor area.

Figure 3 (upper panel) Arterial blood pressure responses (mean ± SEM, n=6) to microinjections of AMPA, L-proline, and kainate into the L-glutamate defined caudal pressor area within the rat ventrolateral medulla. There were statistically significant differences between the changes induced by proline/kainate and glutamate/AMPA injections, but not between proline and kainate or between AMPA and glutamate injections.

(lower panel) The outlined frontal section of the medulla with a part of an original photograph showing a spot of methylene blue that had been injected into the caudal pressor area (CPA). The outline was drawn from three photographs.

Figure 1

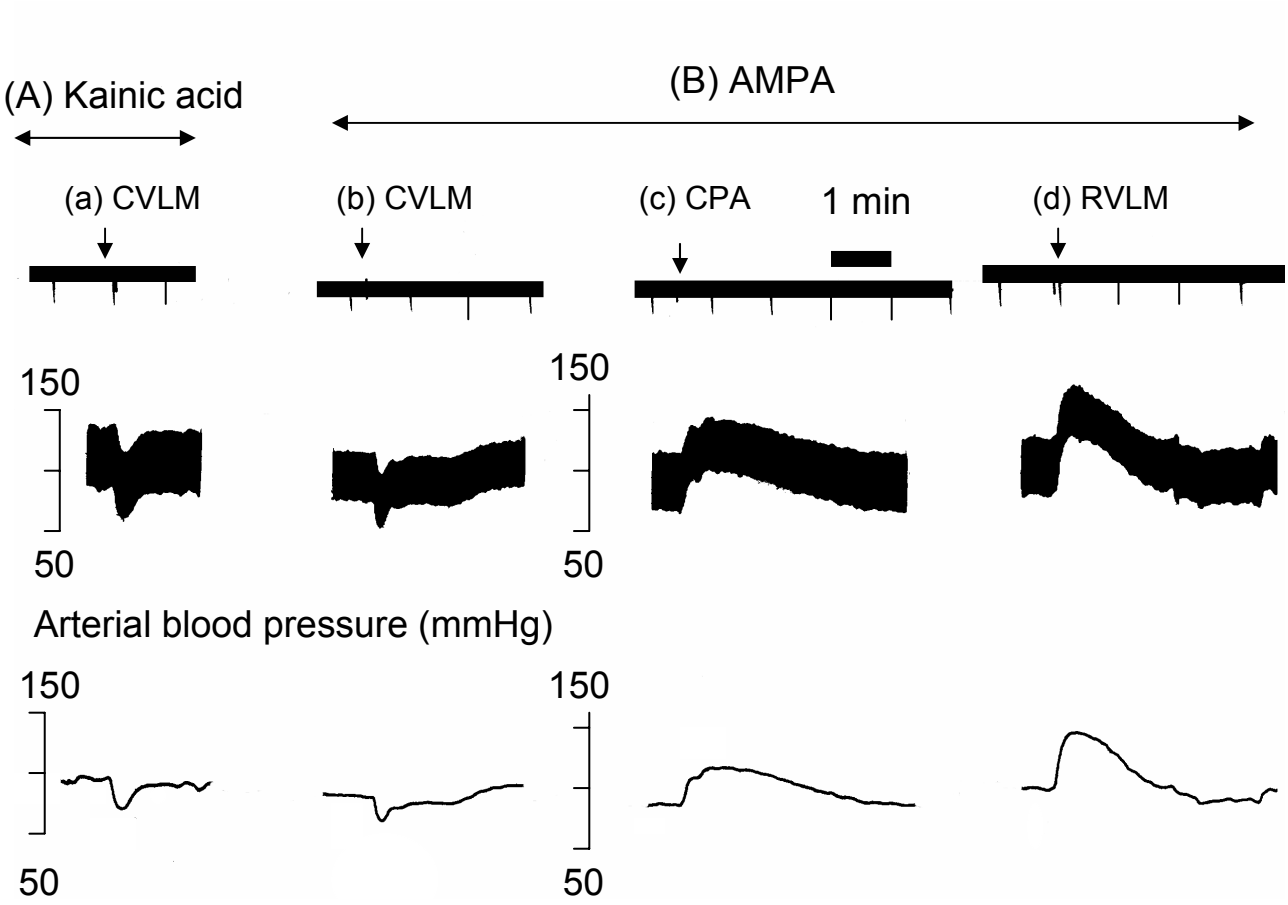

(A) Kainic acid
(B) AMPA
(a) CVLM
(b) CVLM
(c) CPA
1 min
(d) RVLM
150
50
150
50
Arterial blood pressure (mmHg)
150
50
150
50

Figure 2

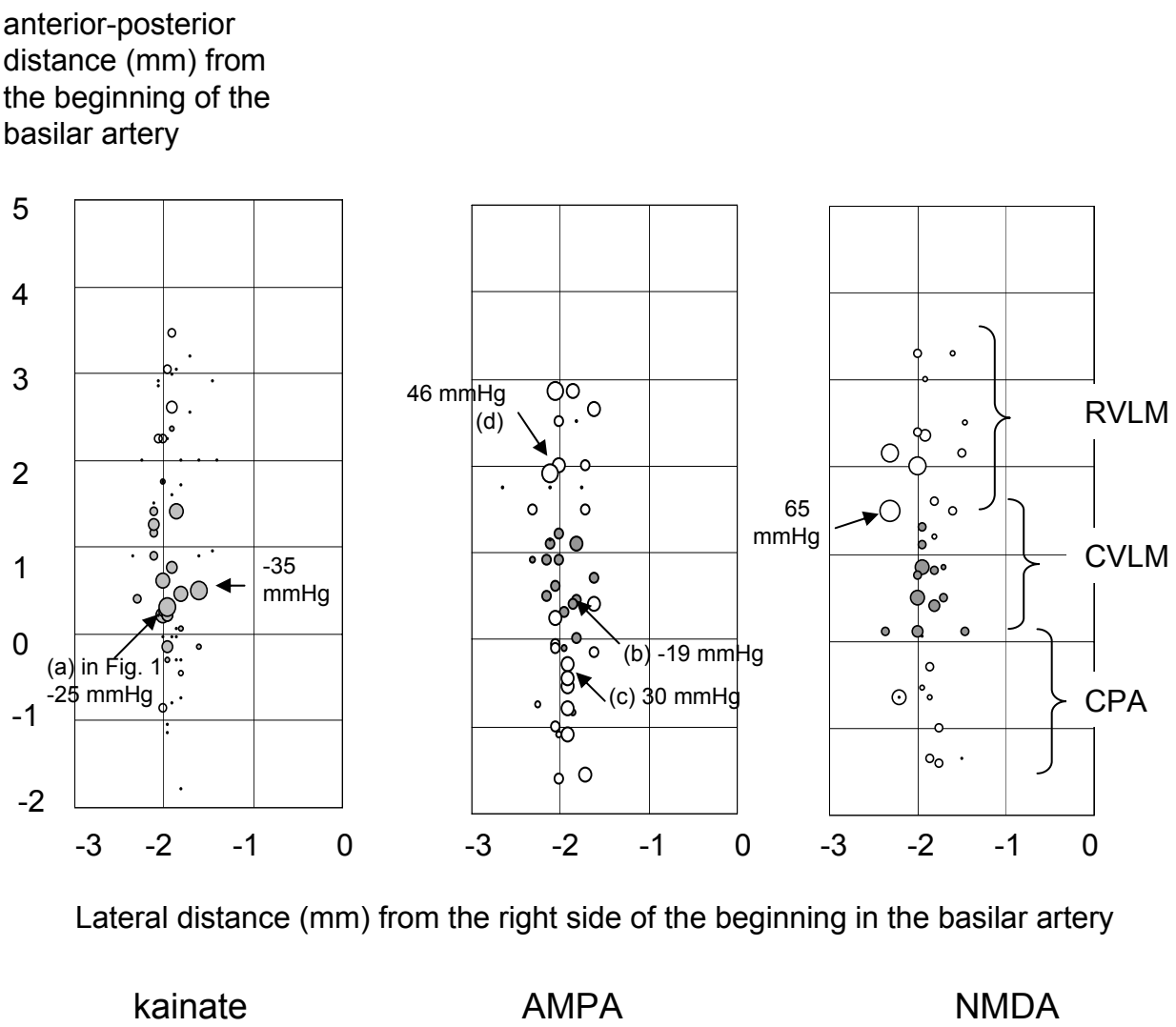
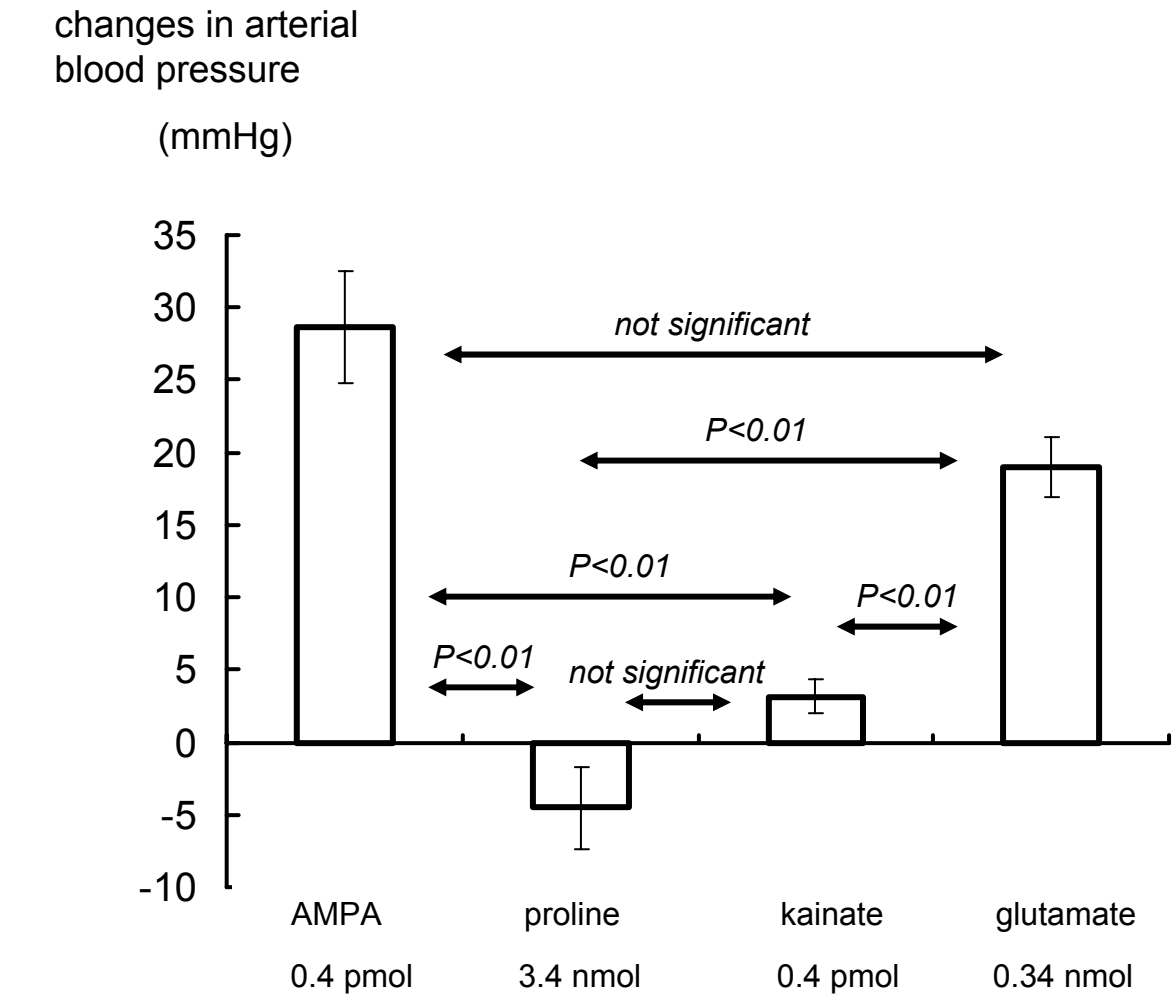
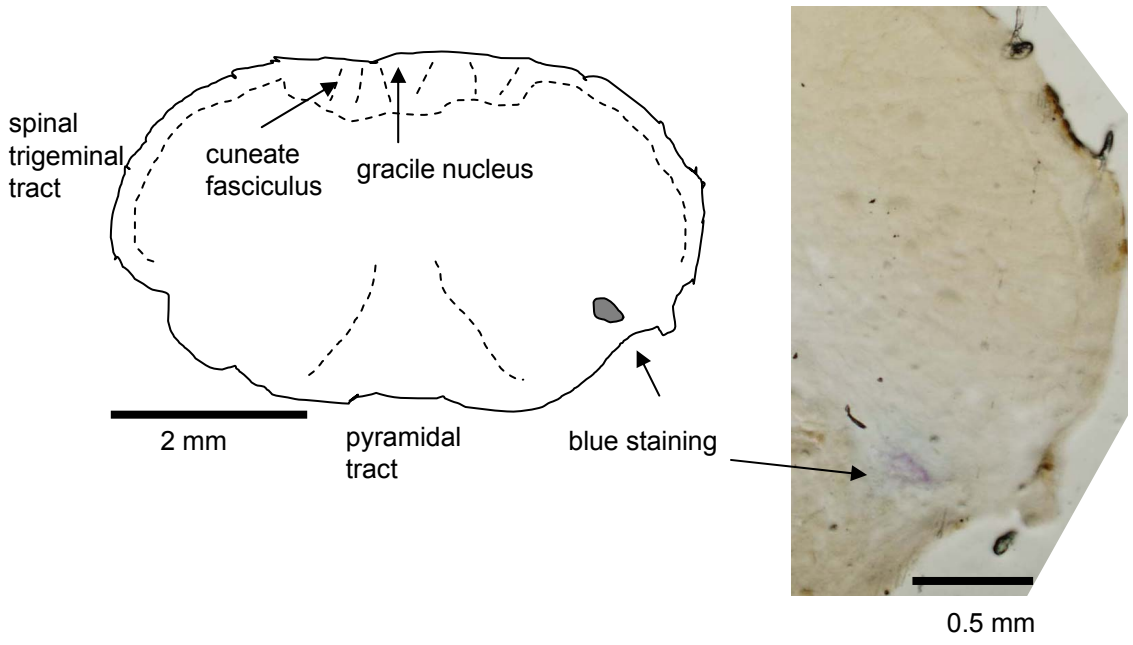

anterior-posterior distance (mm) from the beginning of the basilar artery
5
4
3
2
1
0
-1
-2
-35 mmHg
(a) in Fig. 1
-25 mmHg
46 mmHg
(d)
(b) -19 mmHg
(c) 30 mmHg
65 mmHg
RVLM
CVLM
CPA
-3
-2
-1
0
Lateral distance (mm) from the right side of the beginning in the basilar artery
kainate
AMPA
NMDA

Figure 3


changes in arterial blood pressure
(mmHg)
35
30
25
20
15
10
5
0
-5
-10
not significant
P<0.01
P<0.01
P<0.01
P<0.01
not significant
AMPA
0.4 pmol
proline
3.4 nmol
kainate
0.4 pmol
glutamate
0.34 nmol


spinal trigeminal tract
cuneate fasciculus
gracile nucleus
2 mm
pyramidal tract
blue staining
0.5 mm