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## Endogenous 5-HT outflow from chicken aorta by 5-HT uptake inhibitors and amphetamine derivatives

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**ABSTRACT.** Chemoreceptor cells aggregating in clusters in the chicken thoracic aorta contain 5-hydroxytryptamine (5-HT) and have voltage-dependent ion channels and nicotinic acetylcholine receptors, which are characteristics typically associated with neurons. The aim of the present study was to investigate the effects of 5-HT uptake inhibitors, fluvoxamine, fluoxetine and clomipramine (CLM), and amphetamine derivatives, *p*-chloroamphetamine (PCA) and methamphetamine (MET), on endogenous 5-HT outflow from the isolated chick thoracic aorta *in vitro*. 5-HT was measured by using a HPLC system with electrochemical detection. The amphetamine derivatives and 5-HT uptake inhibitors caused concentration-dependent increases in endogenous 5-HT outflow. PCA was about ten times more effective in eliciting 5-HT outflow than MET. The 5-HT uptake inhibitors examined had similar potency for 5-HT outflow. PCA and CLM increased 5-HT outflow in a temperature-dependent manner. The outflow of 5-HT induced by PCA or 5-HT uptake inhibitors was independent of extracellular Ca<sup>2+</sup> concentration. The 5-HT outflow induced by CLM, but not that by PCA, was dependent on the extracellular NaCl concentration. These results suggest that the 5-HT uptake system of 5-HT-containing chemoreceptor cells in the chicken thoracic aorta has characteristics similar to those of 5-HT-containing neurons in the mammalian central nervous system (CNS).

**KEY WORDS:** 5-HT outflow, amphetamine derivative, chicken, thoracic aorta, uptake inhibitor

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A large amount of 5-hydroxytryptamine (5-HT) is found in the enterochromaffin cells of the mucosal epithelium in the mammalian gastrointestinal tract, whereas a small amount is found in the raphe nuclei neurons of the central nervous system (CNS), the axons of which innervate various regions of the CNS including the spinal cord. Dysfunction of these neurons is considered to be one of the causes of depression and neurological disorders. There is still a lack of complete understanding of the pathology of depression; however, tricyclic antidepressant therapies were shown to increase 5-HT and/or noradrenaline amounts in the synaptic regions of monoaminergic neurons of the brain by inhibiting the re-uptake of 5-HT released from the neurons. It is also well-known that amphetamine derivatives increase monoamine levels in synaptic regions via the inhibition of the uptake of released monoamines through plasma membrane transporters, depletion of monoamines from secretory vesicles and then elicitation of their outflow from them, and inhibition of monoamine oxidase [8, 11, 18, 24, 29]. The operation of 5-HT transporters on the cell membrane in the reverse mode is thought to result in the outflow of large amounts of 5-HT from neurons into the synapse [11].

In the chicken aorta, 5-HT-containing epithelioid cells aggregate in clusters on the inner wall of the thoracic aorta [19], and are excitable chemoreceptor cells because they contain voltage-dependent Na<sup>+</sup> and K<sup>+</sup> channels, L-type and N-type Ca<sup>2+</sup> channels [13], and nicotinic ACh receptors [14], which cause the release of endogenous 5-HT in response to hypoxia and depolarization [12]. From these facts, similar to 5-HT containing neurons in the mammalian CNS, it is likely that these cells in the chicken aorta have uptake mechanisms for 5-HT. If 5-HT transporters for 5-HT uptake are present in these cells, spontaneous outflow of 5-HT is expected to be facilitated by amphetamine derivatives and 5-HT uptake inhibitors.

However, the pharmacological and functional characterization of 5-HT uptake system in chicken aorta has not been demonstrated. The aim of the present study was to examine the effects of 5-HT uptake inhibitors, clomipramine (CLM), fluoxetine and fluvoxamine, and amphetamine derivatives, methamphetamine (MET) and *p*-chloroamphetamine (PCA), on endogenous 5-HT release to investigate the 5-HT uptake systems in 5-HT-containing cells of the chicken thoracic aorta.

### MATERIALS AND METHODS

**Preparation of tissue samples:** Male chickens (14–28 days after hatching) were deeply anesthetized by placing them in a small chamber in which ether or isoflurane was vaporized, and then were decapitated. The chick thoracic aorta with 5-HT-containing chemoreceptor cells was isolated and freed from surrounding tissues. Aortic strips containing chemoreceptor cells (about 5 mm in length) were cut longitudinally

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to open them and were kept in oxygenated Hepes-buffered saline solution on ice until use. All experiments were performed under the regulation of the Institutional Animal Care and Use Committee of the Graduate School of Veterinary Medicine, Hokkaido University, Japan. The animal facilities and animal care programs are accredited by AAALAC international in the U.S.A.

**5-HT outflow experiments:** Hepes-buffered saline contained (mM): NaCl 140, KCl 6, CaCl<sub>2</sub> 2.5, MgCl<sub>2</sub> 1.2, Hepes 10 and glucose 10. The pH was adjusted to 7.3 with NaOH. In Ca<sup>2+</sup>-free solution, CaCl<sub>2</sub> was removed, and 0.5 mM EGTA was added. In low Na<sup>+</sup> solution, NaCl was iso-osmotically replaced with sucrose. The measurement of 5-HT outflow from an aortic strip was described previously [13]. Briefly, the aortic strip was put in a sample tube on ice containing Hepes-buffered saline solution (0.1 ml) with and without secretagogues and then incubated at 37°C for 10 min to stimulate the 5-HT-containing cells of aortic tissues. In some experiments, temperature or incubation time was altered. The secretory response was terminated by placing the tubes on ice. MET (Sumitomo Dainihon Pharma, Osaka, Japan), PCA (Sigma, St. Louis, MO, U.S.A.), CLM (Sigma), fluvoxamine, fluoxetine (Tooris, Bristol, U.K.),  $\omega$ -conotoxin GVIA (Peptide Institute, Osaka, Japan) and nifedipine (Wako, Osaka, Japan) were prepared from 0.1 M stock solutions and dissolved in Hepes-buffered saline solution.

**Measurement of 5-HT:** After termination of the secretory response, the aortic tissue was transferred to another sample tube containing 0.4 N perchloric acid (0.2 ml) to extract 5-HT and 5-hydroxyindole acetic acid (5-HIAA), a 5-HT metabolite, remaining in the tissue. To measure the amounts of 5-HT and 5-HIAA in the incubation medium, 4.4 N perchloric acid (10  $\mu$ l) was added to the medium to obtain a final concentration of 0.4 N. The sum of the amounts of 5-HT and 5-HIAA in the tissue and incubation medium was regarded as the total amount of 5-HT. After centrifugation of the sample tube containing tissue extract or incubation medium, K<sub>2</sub>HPO<sub>4</sub> was added to the supernatant to obtain a final concentration of 580 mM (pH 5–6). After removal of potassium perchlorate by centrifugation, the clear supernatant was applied to a high-performance liquid chromatography apparatus. The mobile phase was composed of the following: KH<sub>2</sub>PO<sub>4</sub>-H<sub>3</sub>PO<sub>4</sub> buffer, 100 mM (pH 3.5), EDTA, 40  $\mu$ M, sodium octasulfonic acid, 1.16 mM and methanol, 15–17%. The mobile phase was degassed by using DG-350 (EICOM, Kyoto, Japan), and the flow rate was adjusted to 0.5 ml/min. The samples were applied using autosampler model 33 (System Instruments, Tokyo, Japan) to an ODS-column (EICOMPAK SC-50DS, 3.0  $\times$  150 mm, EICOM), and 5-HT and 5-HIAA were separated and detected by an electrochemical detector, ECD-300 (EICOM). 5-HT release (% of content) was expressed as a percentage of total 5-HT content in the aortic strip.

**Data analysis:** All data were expressed as means  $\pm$  S.E.M. Statistical comparisons between two groups were performed by the unpaired Student's *t*-test. For multiple comparisons, ANOVA, followed by Dunnett's test, was used. A *P* value of less than 0.05 was considered significant.

## RESULTS

**Time- and temperature-dependent outflow of 5-HT:** The chicken aortic tissue incubated at 37°C gradually released 5-HT in the absence of drugs (control), and about 5% of 5-HT content in the tissue was released in 20 min (Fig. 1A). In the presence of PCA (1 mM), the outflow of 5-HT increased to 20% in 20 min. Under this condition, the 10 min incubation time was enough to measure 5-HT outflow in the medium in response to PCA. In the following experiments, therefore, aortic tissues were incubated for 10 min in the presence of various drugs.

If 5-HT in incubation medium resulted from its leakage from damaged chemoreceptor cells, spontaneous or evoked outflow of 5-HT would be independent of incubation temperature. Therefore, we examined the effect of PCA and CLM on 5-HT outflow by changing the temperature. The outflow of 5-HT increased with the increase in temperature in the presence and absence of these drugs (Fig. 1B). A slight increase in 5-HT was observed at 27°C, indicating that the secretory responses to PCA and CLM from 5-HT-containing chemoreceptor cells were not due to the leakage from damaged cells.

**Concentration-dependent outflow of 5-HT:** The aortic tissues were incubated for 10 min with MET, PCA, CLM, fluoxetine or fluvoxamine at various concentrations to examine their effects on 5-HT release. The resting outflow of 5-HT was  $3.6 \pm 0.6\%$  (*n*=4) for the experiments of MET and PCA, but this varied slightly from preparation to preparation. Both amphetamine derivatives (1  $\mu$ M–1 mM) caused concentration-dependent increases in 5-HT outflow (Fig. 2A). PCA was about ten times more effective in eliciting 5-HT outflow than MET.

The resting outflow of 5-HT was  $4.0 \pm 0.4\%$  (*n*=10) for the experiments of 5-HT uptake inhibitors. These drugs (1  $\mu$ M–1 mM) also increased 5-HT outflow in concentration-dependent manners (Fig. 2B), but maximal responses to them could not be obtained. The secretory responses to all drugs began to appear at concentrations above 0.1 mM, and fluvoxamine at 1 mM was more effective than at the same concentration of fluoxetine or CLM. These results suggest that 5-HT-containing chemoreceptor cells in the chick aorta respond to amphetamine derivatives and antidepressants similar to the neurons in the CNS.

**Extracellular Ca<sup>2+</sup>-dependent outflow of 5-hydroxytryptamine:** We examined the effect of extracellular Ca<sup>2+</sup> removal on 5-HT outflow in response to PCA (1 mM) or 5-HT uptake inhibitors (0.1 mM) (Fig. 3). The resting outflow of 5-HT was not affected by Ca<sup>2+</sup> removal. The secretory responses to PCA and 5-HT uptake inhibitors were not significantly attenuated by the removal of extracellular Ca<sup>2+</sup>. The response to PCA (1 mM) was not affected by 1  $\mu$ M  $\omega$ -conotoxin VIA, a N-type Ca<sup>2+</sup> channel blocker, or 1  $\mu$ M nifedipine, a L-type Ca<sup>2+</sup> channel blocker (not shown), the concentration of which effectively inhibited 5-HT outflow induced by excess KCl [13]. These results indicate that extracellular Ca<sup>2+</sup> independent mechanisms are involved in the 5-HT outflow induced by these agents from aortic chemoreceptor cells.

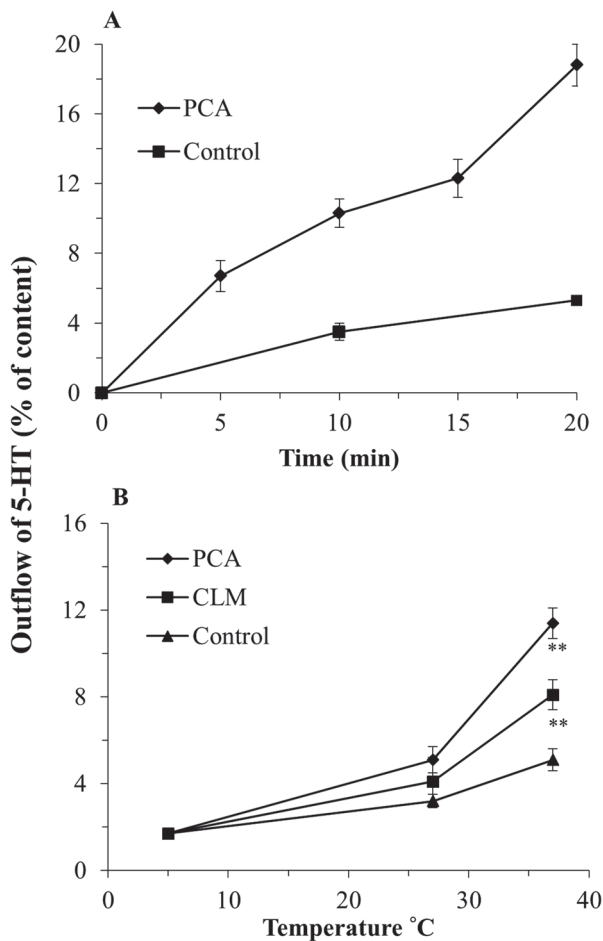


Fig. 1. Time- and temperature-dependent outflow of 5-HT from the chemoreceptor cells of chicken aorta induced by PCA and CLM. The time-course of the resting outflow (control,  $n=4-5$ ) and *p*-chloroamphetamine (PCA) (1 mM,  $n=4-5$ )-induced outflow of 5-HT (A). Temperature-dependence of resting 5-HT outflow (control,  $n=4-8$ ) and PCA- (1 mM,  $n=4-7$ ) and clomipramine (CLM) (0.1 mM,  $n=4$ )-induced 5-HT outflow (B). Error bars are S.E.M. \*\*:  $P<0.01$ , different from the control (Dunnett's test).

**Extracellular NaCl-dependent outflow of 5-hydroxytryptamine:** It is reported that 5-HT is taken up into the cells through 5-HT transporters in an extracellular NaCl-dependent manner [25–27]. Therefore, the effects of NaCl on 5-HT outflow in response to PCA (1 mM) and CLM (0.1 mM) were examined (Fig. 4). When NaCl was substituted for sucrose, the spontaneous 5-HT outflow in the presence of NaCl at 10 mM or absence of NaCl appeared to be greater, but not significantly, than that in the presence of NaCl at 20 mM or 140 mM. CLM failed to produce outflow of 5-HT at NaCl concentrations under 10 mM. However, CLM induced a significant increase in 5-HT outflow in the presence of 20 mM NaCl to the same extent as that in the presence of 140 mM NaCl. PCA elicited 5-HT outflow regardless of the presence or absence of extracellular NaCl.

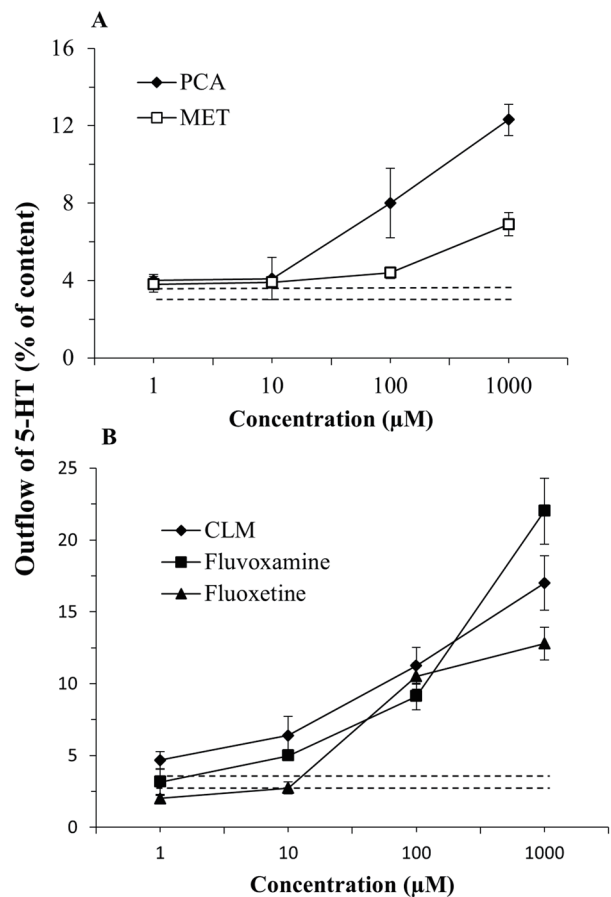


Fig. 2. Effects of amphetamine derivatives and 5-HT uptake inhibitors on 5-HT outflow. The concentration-dependent increases in 5-HT outflow in response to *p*-chloroamphetamine (PCA,  $n=4-6$ ) and methamphetamine (MET,  $n=4$ ) (A), and clomipramine (CLM,  $n=8$ ), fluvoxamine ( $n=8$ ) and fluoxetine ( $n=5$ ), for 10 min at 37°C (B). Error bars are S.E.M. Dashed lines indicate upper and lower limits of S.E.M. of the resting outflow ( $n=10$ ).

## DISCUSSION

The present results clearly indicate that amphetamine derivatives, MET and PCA, and 5-HT uptake inhibitors, fluoxetine, fluvoxamine and CLM, caused concentration-dependent increases in endogenous 5-HT outflow from chemoreceptor cells of chicken thoracic aorta. The outflows of 5-HT induced by PCA and 5-HT uptake inhibitors were independent of extracellular  $\text{Ca}^{2+}$ . CLM but not PCA caused an increase in 5-HT outflow which is dependent on extracellular NaCl.

In this study, amphetamine derivatives and 5-HT uptake inhibitors increased 5-HT outflow from chicken thoracic aorta. It has been shown that 5-HT is localized in the epithelioid cells in the wall of the chicken aorta, forming a band ~1mm in width [19], indicating that 5-HT outflow from the chicken aorta arises from these epithelioid cells. Our group has reported that these cells are chemoreceptor

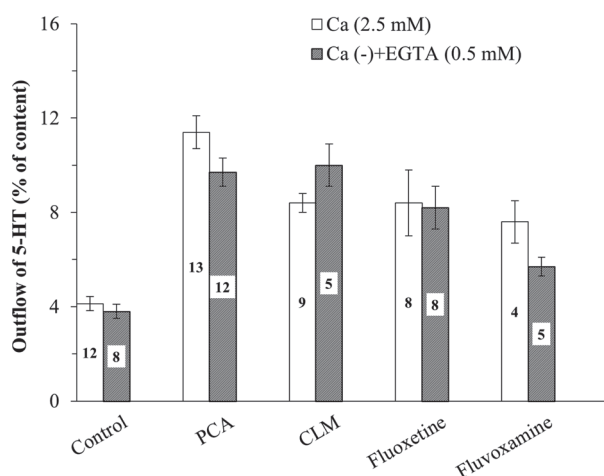


Fig. 3. Effects of extracellular  $\text{Ca}^{2+}$  removal on 5-HT outflow induced by *p*-chloroamphetamine and 5-HT uptake inhibitors. The resting 5-HT outflow (control,  $n=4-5$ ) and the outflow of 5-HT in response to *p*-chloroamphetamine (PCA, 1 mM), clomipramine (CLM, 0.1 mM), fluvoxamine (0.1 mM) and fluoxetine (0.1 mM) for 10 min at 37°C in the presence (open columns) and absence (hatched columns) of extracellular  $\text{Ca}^{2+}$ . Error bars are S.E.M., and numbers in the column indicate numbers of experiments.

cells and that chicken aorta containing these cells releases 5-HT in response to nicotinic agonists, depolarization and hypoxia [12–14]. It has been reported that MET and PCA are capable of releasing  $^3\text{H}$ -dopamine and  $^3\text{H}$ -5-HT from rat brain synaptosomes [5, 17], and thus, amphetamine derivatives are well-established as compounds that decrease tissue concentrations of these neurotransmitters in several brain regions [20]. In this experiment, PCA was more effective in increasing 5-HT outflow than MET. It is also reported that PCA is about ten times more potent for 5-HT release than MET in synaptosomes [5] and that PCA exerts greater neurotoxic effects on the serotonergic system than on the dopaminergic one. Conversely, MET exerts greater neurotoxic effects on the dopaminergic system than on the serotonergic one. [4]. Taken together, it is reasonable to suggest that PCA is a potent 5-HT releaser in chemoreceptor cells of chicken thoracic aorta, as demonstrated previously in 5-HT-containing neurons.

5-HT uptake inhibitors are reported to elicit 5-HT outflow from various regions of the brain [3, 6, 9] and cultured brain slice preparations [21]. These drugs are well-known to inhibit plasma membrane 5-HT transporters involved in the uptake of extracellular 5-HT released from neurons [16, 28]. This was also the case in the present study of chemoreceptor cells where these inhibitors were also effective in increasing extracellular 5-HT. These results suggest that, like in the case of 5-HT-containing neurons, 5-HT-containing chemoreceptor cells express 5-HT transporters on their plasma membrane. However, there were no differences in the potency of 5-HT release between CLM, fluoxetine and fluvoxamine, although they had different  $K_i$  values for binding to human 5-HT transporters [2]. This discrepancy may be explained

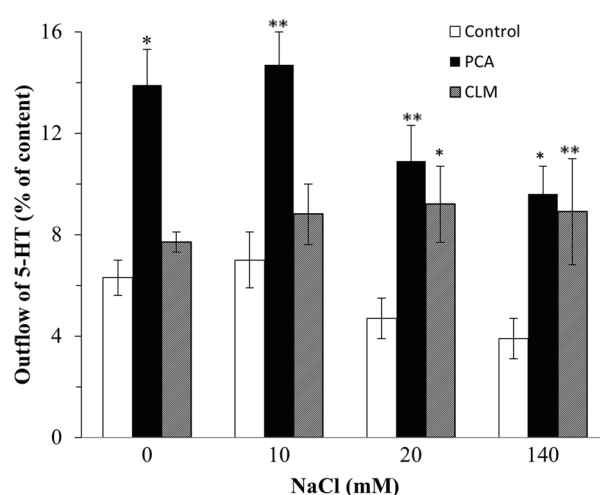


Fig. 4. Effects of extracellular NaCl on 5-HT outflow induced by *p*-chloroamphetamine and clomipramine. The resting 5-HT outflow (control,  $n=4-8$ , open columns) and the 5-HT outflow in response to *p*-chloroamphetamine (PCA, 1 mM,  $n=4-7$ , filled columns) and clomipramine (CLM, 0.1 mM,  $n=4$ , hatched columns) at 0, 10, 20 and 140 mM NaCl. Error bars are S.E.M. \*:  $P<0.05$  and \*\*:  $P<0.01$ , different from the control (Dunnett's test).

by (1) the short drug incubation period of 10 min and (2) the reduction of inward currents through basal  $\text{K}^+$  channels by high concentrations of antidepressants [15]. Alternatively, (3) chicken 5-HT transporters have different sensitivity to these drugs from human ones. Further investigation is needed to address this issue.

In 5-HT-containing chemoreceptor cells, spontaneous 5-HT outflow was not affected by extracellular  $\text{Ca}^{2+}$  removal. The released 5-HT is likely to be taken up into the cells through 5-HT transporters, because 5-HT uptake inhibitors caused increases in 5-HT in the incubation medium even in the absence of extracellular  $\text{Ca}^{2+}$ . Amphetamine derivatives are reported to promote 5-HT overflow in an extracellular  $\text{Ca}^{2+}$  independent manner [31] and in both  $\text{Ca}^{2+}$ -dependent and independent manners in synaptosomes, suggesting that the former is associated with  $\text{Ca}^{2+}$  influx through voltage-dependent  $\text{Ca}^{2+}$  channels and the latter with carrier-mediated release [7]. In this study, however, we did not observe  $\text{Ca}^{2+}$ -dependent release in response to PCA. This difference may be explained by different preparations, synaptosomes and chemoreceptor cells, which may have resulted in differences in the compartmentalization of 5-HT [10].

In 5-HT-containing chemoreceptor cells, the lowering of extracellular NaCl to 10 mM or below caused an increase in spontaneous 5-HT outflow, which was similar to that of catecholamine release from the adrenal medulla [30]. The activity of 5-HT transporters on the cell membrane is reported to be coupled with extracellular NaCl [22, 25, 27], a half maximal concentration of which was about 15 mM [26] or less [27]. It is likely that the increase in spontaneous 5-HT outflow at low NaCl external concentrations results from the inhibition of 5-HT transporters, because a 5-HT transporter

inhibitor, CLM, failed to induce 5-HT outflow at low NaCl concentrations, under which 5-HT transporter activity has already been attenuated. This hypothesis may be supported by the fact that CLM produces 5-HT outflow at 20 mM NaCl to the same extent as that at 140 mM. Unlike CLM, however, 5-HT outflow induced by PCA was not affected by the presence or absence of extracellular NaCl, indicating that the mechanisms producing 5-HT outflow by PCA and CLM may be different. It is reported that amphetamine derivatives are capable of enhancing transporter reversal, resulting in monoamine efflux from neurons [1, 11, 23], as a consequence of the effect of amphetamines on vesicular monoamine transporters and monoamine oxidase in 5-HT-containing neurons [8, 24, 29]. Taken together, it is suggested that PCA promotes 5-HT outflow by reversing the transport of 5-HT in chicken chemoreceptor cells.

In conclusion, the present results indicate that 5-HT-containing chemoreceptor cells in the chicken thoracic aorta have 5-HT uptake activity similar to those of 5-HT-containing neurons in the CNS. The chicken thoracic aorta maybe becomes a useful model for examining the effects of drugs on 5-HT outflow from 5-HT-containing neurons *in vitro*, because endogenous 5-HT release from aortic strips was easily detected with HPLC equipped with an electrochemical detector.

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