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Original Article

Amino acid release from the rat oral pontine reticular nucleus across the sleep-wakefulness cycle

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To elucidate a functional involvement of amino acids in the regulation of vigilance states, we investigated time-course changes of glycine (Gly), glutamate (Glu), and glutamine (Gln) in the extracellular fluid of the oral pontine reticular nucleus (PnO) in freely behaving rats by an in vivo microdialysis technique. The average concentration of Gly in dialysates collected during rapid eye movement sleep (REMS) was significantly higher than that collected during wakefulness (W) and non rapid eye movement sleep (NREMS) under perfusion with normal artificial cerebrospinal fluid (aCSF). These state-dependent changes in Gly, however, failed to occur when the PnO was perfused with Ca^{2+}-free aCSF. In contrast, the average concentration of neither Glu nor Gln exhibited state dependency under the perfusion with normal or Ca^{2+}-free aCSF; however, the concentrations of all three amino acids showed no consistent time-sequential changes specifically in the first three minutes from the beginning of each vigilance state. Although it is suggested that in the PnO the average concentration of Gly was higher during REMS than during NREMS and W by a Ca^{2+}-dependent mechanism, however, time-course changes in the amino acids are complicated and other mechanisms may also be influential.

Key words: Glycine, Reticular formation, Rapid eye movement sleep, Microdialysis

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Introduction

Across the sleep-wakefulness cycle, neurons in the central nervous system (CNS) as a whole change their mode of activity. It is possible that the activities of the neurons are simultaneously controlled by some integrated systems such as glia or groups of neurons that project to wide areas in the CNS. Thus, to understand the generation of the sleep-wakefulness cycle, it is beneficial to monitor the extracellular changes of substances that influence neuronal activity, such as neurotransmitters or hormones. For example, extracellular levels of acetylcholine and serotonin, which are essential for setting the vigilance states[1], change in the brainstem according to each vigilance state[2,3]. Some amino acids in the extracellular fluid can also exert an influence on neuronal activities as neurotransmitters, and they are reported to be involved in controlling the sleep-wakefulness cycle[4-6].

The brainstem is one of the most crucial parts in the brain for controlling the sleep-wakefulness cycle. Among the vigilance states [wakefulness (W), non rapid eye movement sleep (NREMS), and rapid eye movement sleep (REMS)], the firing patterns of most neurons are different in the brainstem. These are characterized by more frequent and variable activities during REMS and active waking than during

NREMS[7]. Especially, the pontine reticular formation in the brainstem is considered to be important for generating REMS, because the administration of cholinergic agents into it has been shown to induce REMS in cats and rats[8-11]. In the rat, the oral pontine reticular nucleus (PnO) is supposed to be the most critical region for REMS induction[10] or theta rhythm generation[12]. There are no reports about the dynamics of the amino acids in the rat brainstem, however, even though there are some in cats[6,13].

In the present study, we analyzed the dynamics of changes in the amino acids not only across the vigilance states but also within each vigilance state in the rat PnO to understand the relationship between the extracellular amino acids and the vigilance states. We used an in vivo microdialysis method and high performance liquid chromatography (HPLC) to measure extracellular levels of three amino acids [glycine (Gly), glutamate (Glu), and glutamine (Gln)]. Previous investigators reported that their minimum sampling time was 5 min. In the rat, however, the duration of most vigilance episodes is much shorter than 5 min, and the REMS episodes rarely last for longer than 4 min. Here, we made the sampling time as short as 1 min at the beginning of each vigilance state.

Materials and methods

I. Subjects and surgery

Eight male Sprague-Dawley rats at the age of 10-12 weeks weighing 350-450 g were used in the present study. They were raised in our closed colony and kept on a 12-h light and 12-h dark schedule (lights on at 0800 h) in a constant air-conditioned environment of 23 ˚C and 55% relative humidity with free access to rat chow and water.

Under pentobarbital sodium anesthesia (50 mg/kg intraperitoneally), rats were fixed stereotaxically and implanted with three stainless-steel screw electrodes for electrocorticogram through the skull on the frontal cortex, a pair of stainless-steel electrodes for electromyogram in the nuchal muscles, and one or two pairs of silver electrodes for electrooculogram near the eye ball(s). A guide cannula for a microdialysis probe was inserted so that the probe was placed in the left PnO. The stereotaxic coordinates of the guide tip were 8.0 mm posterior, 1.4 mm left, and 8.0 mm ventral to the Bregma, according to the brain atlas of Paxinos and Watson[14].

II. Vigilance states

Vigilance states were classified as W, NREMS, and REMS at 6-second intervals according to the criteria published elsewhere by polygraphic recording charts simultaneously with the recording[15]. We defined the termination of an episode as an occurrence of interruption by a different state that continued for more than 30 seconds.

III. Sample collection

After allowing 7-10 days for recovery from surgery, each rat was transferred into an individual recording cage and connected with a revolving slip ring so that it could move without twisting lead wires and perfusion tubes. Thus, rats could move around in the cage freely and could take water and food *ad libitum*. The microdialysis membranes were 1.0 mm long, with a diameter of 0.24 mm and a 6000 molecular weight cut-off (CMA11, BAS, Tokyo, Japan). Perfusion started approximately 12 hours before sample collection at a rate of 3 µl/min with normal artificial cerebrospinal fluid (aCSF), modified Ringer bicarbonate solution of the following composition: NaCl 122.0 mM, KCl 3.0 mM, $MgCl_2$ 1.2 mM, NaH_2PO_4 0.4 mM, $NaHCO_3$ 25.0 mM, and $CaCl_2$ 1.2 mM. The perfusion buffer was adjusted to pH 7.40 with 1N HCl and further sterilized by 0.2 µm syringe filters (Whatman 13 mm Syringe Filters, Whatman, Clifton, NJ, U.S.A.).

The first series of sampling was performed from 1000 h to 1200 h into twelve vials (W1-4, NREM1-4, REM1-4). Dialysates collected at every vigilance episode were fractionated manually into the vials for 0-1 min (W1, NREMS1, or REMS1), 1-2 min (W2, NREMS2, or REMS2), 2-3 min (W3, NREMS3, or REMS3), and 3 min or more (W4, NREMS4, or REMS4) from the initiation of every episode of each vigilance state, repeatedly. Then, the perfusion buffer was switched at 1200 h from normal aCSF to Ca^{2+}-free aCSF, which differed from the normal one in the compositions of $MgCl_2$ 20 mM and $CaCl_2$ 0 mM. The second series of sampling was performed from 1230 h to 1430 h, and the collection was made into another set of 12 vials.

A delay for the perfusate to travel from the probe to the outlet of the collecting tube was timed precisely by making an air gap in the pathway. The average delays were about 8 min, and errors of the delay for each experiment were as little as about 10 sec. The flow rate of perfusion was constant.

The collected samples were kept at 4 ˚C until the end of each session of sample collection and were stored at – 20 ˚C.

IV. Measurement of the amino acids

The amino acid concentrations were analyzed by HPLC with an electro-chemical detector (300 series, EICOM, Kyoto, Japan)[4]. A small portion (6 µl or 3 µl) of collected perfusate from each sample was diluted into 30 µl with distilled water for an HPLC analysis and reacted with 10 µl of a reagent consisting of 4 mM *o*-phthaldialdehyde and 2-mercaptoethanol at 10 ˚C for 10 min by a sampling injector (Model 231 XL, Gilson, Middleton, WI, U.S.A.). The samples less than 3 µl were excluded from measurement. The derivatized sample mixture (30 µl) was then injected into the HPLC system. The amino acid derivatives were separated in a liquid chromatography column (EICOMPAK MA-5 ODS, EICOM; 2.1 mm in diameter, 150 mm in length) at 30 ˚C with 30% methanol in 0.1 M phosphate buffer (pH 6.0) which was degassed by an on-line degasser (DG-300, EICOM). Electrical current was detected under the condition that the graphite electrode was held at + 550 mV against the Ag/AgCl electrode. External amino acid standards were combined every 5-15 samples. Glu, Gly, and Gln were quantified on a computer system from the chromatogram in reference to the external standards (Power Chrom, AD Instruments, Castle Hill, Australia).

The average concentration (C_m) of each amino acid during each state was calculated according to the following formula.

$$C_m = \Sigma(C_{fk} \times V_{fk})/V_t,$$

where C_{fk}, V_{fk}, and V_t stand for the concentration of the amino acid in each fraction (1-4), volume of dialysate of the fraction, and total volume of all fractions, respectively.

V. Histology

After the experiment, each animal was deeply anesthetized with pentobarbital sodium and perfused transcardially with 30 ml of saline followed by 10% formalin in 0.1 M phosphate buffer solution[16]. The brain was removed, postfixed with the formalin-phosphate solution, and then sliced into serial coronal sections 60 µm in thickness. They were stained with cresyl violet to confirm that the probe was placed in the correct position (Fig. 1). All eight probes in the eight rats were placed within the PnO.

Results

I. Sleep architecture under normal and Ca2+-free aCSF perfusion

There were no significant differences in amounts of

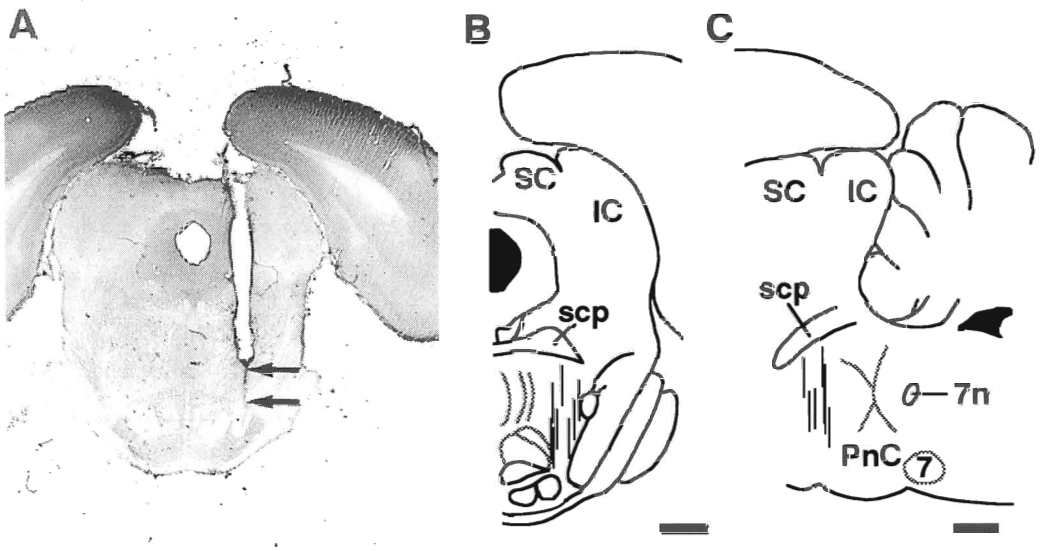

Fig 1. A) Photomicrograph of a coronal section localizing the placement of a microdialysis probe. The arrows indicate the position of the probe membrane.
B and C) Schematic drawings of a coronal (B: 8.0 mm posterior to the Bregma) and a parasagittal (C: 1.4 mm lateral to the Bregma) section summarizing the localization of the probes. Each vertical bar indicates the position of the membrane. The magnification bars represent 1 mm. IC, inferior colliculus; 7, facial nucleus; 7n, facial nerve; PnC, caudal pontine reticular nucleus; SC, superior colliculus; scp, superior cerebellar peduncle.

NREMS, REMS, or W between the two sessions (Table 1).

II. Mean volume of samples in the 12 vials

The results in Table 2 indicate that 41.9% and 42.8% of the total volume of dialysates were collected in the first 3 min of the vigilance states under normal and Ca^{2+}-free aCSF perfusion, respectively. Thus, about 40% of all the dialysates was collected into vials for samples collected at 1 min intervals.

III. Time-sequential changes in the amino acids

In Fig. 2, the amino acid concentrations in the first 3 min during each vigilance state are illustrated. No difference in the concentrations of any of the three amino acids was statistically significant among the samples for 0-1, 1-2, and 2-3 min for all 3 vigilance states under both normal and Ca^{2+}-free aCSF perfusion (Friedman signed rank test or Student *t* test). Thus, there were no consistent time-sequential changes in any of the three amino acids in the first 3 min of any vigilance state. Under normal aCSF perfusion, however, there were several remarkably high values of Gly or Glu restricted to REMS and W in 4 out the 8 rats. These high values were not seen under Ca^{2+}-free aCSF perfusion. No such high values were recognized in Gln during any vigilance state.

IV. Average concentration of the amino acids during each vigilance state

Under normal aCSF perfusion, the average concentration of Gly during REMS was significantly higher than that during NREMS and W ($p = 0.02$, Friedman signed rank test; $p < 0.05$ and $p = 0.02$, respectively,

Table 1. The mean ± standard error of time spent in each vigilance state (min)

	Normal aCSF (n = 8)	Ca^{2+}-free aCSF (n = 7)	Control*(n = 5)
W	40.2 ± 6.9	31.9 ± 7.5	42.6
NREMS	64.4 ± 5.4	73.2 ± 5.2	65.3
REMS	14.6 ± 1.3	15.4 ± 2.2	12.1

*Calculated from the baseline values of time spent for each vigilance state during the light period, which was cited from Honda *et al* [17].

Table 2. The mean ± standard error of sample volume (μl) in each fraction

	Fraction	W	NREMS	REMS	Total
Normal aCSF	1	16.3 ± 2.4	29.2 ± 2.4	15.6 ± 2.2	61.1 ± 5.1
	2	9.1 ± 1.4	25.4 ± 2.5	12.6 ± 1.4	47.1 ± 3.8
	3	6.2 ± 0.9	22.2 ± 2.2	8.6 ± 1.1	36.9 ± 2.9
	4	88.7 ± 19.1	105.7 ± 12.0	6.7 ± 2.1	202.0 ± 11.7
Ca^{2+}-free aCSF	1	16.0 ± 1.1	31.9 ± 2.8	18.7 ± 2.8	64.7 ± 4.5
	2	6.3 ± 0.8	28.6 ± 2.1	15.0 ± 2.2	49.2 ± 3.7
	3	4.3 ± 0.6	25.6 ± 1.7	8.5 ± 1.7	37.8 ± 2.5
	4	78.9 ± 19.6	125.1 ± 8.9	2.7 ± 1.0	206.7 ± 14.4

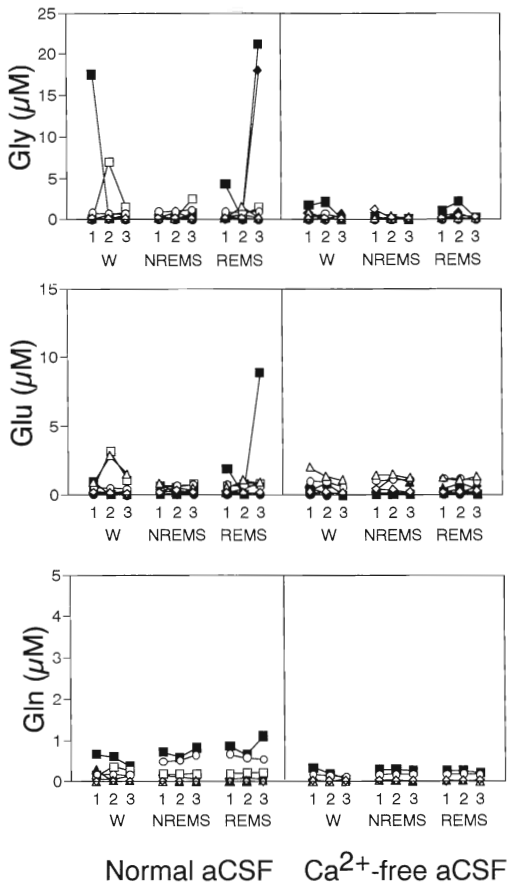

Fig 2. Time-course changes in each amino acid concentrations in the first 3 min of W, NREMS, and REMS episodes under normal (the left side) and Ca^{2+}-free (the right side) aCSF perfusion. Values for a certain rat were represented by the same symbol throughout.

Wilcoxon signed rank test) (Fig. 3). Under Ca^{2+}-free aCSF perfusion, the level of Gly during REMS was not higher than that during NREMS or W. Instead, the level of Gly during W was higher than NREMS ($p < 0.05$, Wilcoxon signed rank test). The average concentrations of Glu or Gln, however, had no state dependency under both normal and Ca^{2+}-free aCSF perfusion.

As shown in Fig.2, the concentrations of Gly and Glu in 2 rats (■ and ◆) were extremely high. However, even if the data of these rats were excluded, the average concentration of Gly during REMS was still significantly higher than W under normal aCSF perfusion ($p < 0.05$, Wilcoxon signed rank test).

Discussion

In the present study, we demonstrated for the first time that the average extracellular concentration of Gly in the PnO was higher during REMS than that during NREMS and W. Further, under Ca^{2+}-free aCSF perfusion, there was no evidence of a REMS-dependent elevation of the average level of Gly. As for Glu and Gln, there were no such changes in the average concentrations under either normal or Ca^{2+}-free aCSF perfusion. The present study, however, failed to show any specific intrastate changes in the amino acid concentrations at least in the first 3 min of each vigilance state.

I. Reliability of the sampling technique

The delay for perfusate to travel from the probe to the outlet of the collecting tube was considered. When the sampling delay was estimated by inserting an air gap, errors in the delay were as little as about 10 sec, and the flow rate of the perfusion was constant. Since substances with low molecular weights pass through the probe membrane and reach an equilibrium within a few seconds, the sampling interval of 1 min was appropriate.

In the present study, dialysis samples from every epoch of a certain vigilance state were collected in the same sequence of vials. This was done in order to because clarify any significant changes in the amino acid concentrations specific to the time sequence of a certain vigilance state. Thus, this method was expected to detect minute-to-minute changes in the amino acid levels. Eventually, however, we failed to detect any time-sequential differences in the amino acid changes.

II. Mechanisms of the amino acid release and their state dependency

We perfused Ca^{2+}-free aCSF in order to inhibit Ca^{2+}-dependent mechanisms of amino acid release. The extracellular amino acid levels are not necessarily influenced by the Ca^{2+} concentration in the environment. Extracellular fluid with this composition does, however, inhibit the release of amino acids in brain slices[18,19]. In the rat striatum, however, exclusion of Ca^{2+} in the extracellular fluid exerts no consistent influence on the basal levels of the amino acids[20,21]. This is partly because the transporter systems of amino acids might be involved in the regulation of the extracellular amino acid level in rats. In the present study, REMS-dependent elevation of the average Gly level was abolished by the Ca^{2+}-free solution. It was hard to say, however, that the level of Gly appeared to be constantly higher during REMS than that during NREMS or W. Furthermore, under normal aCSF perfusion, there were several considerably high levels of Gly or Glu

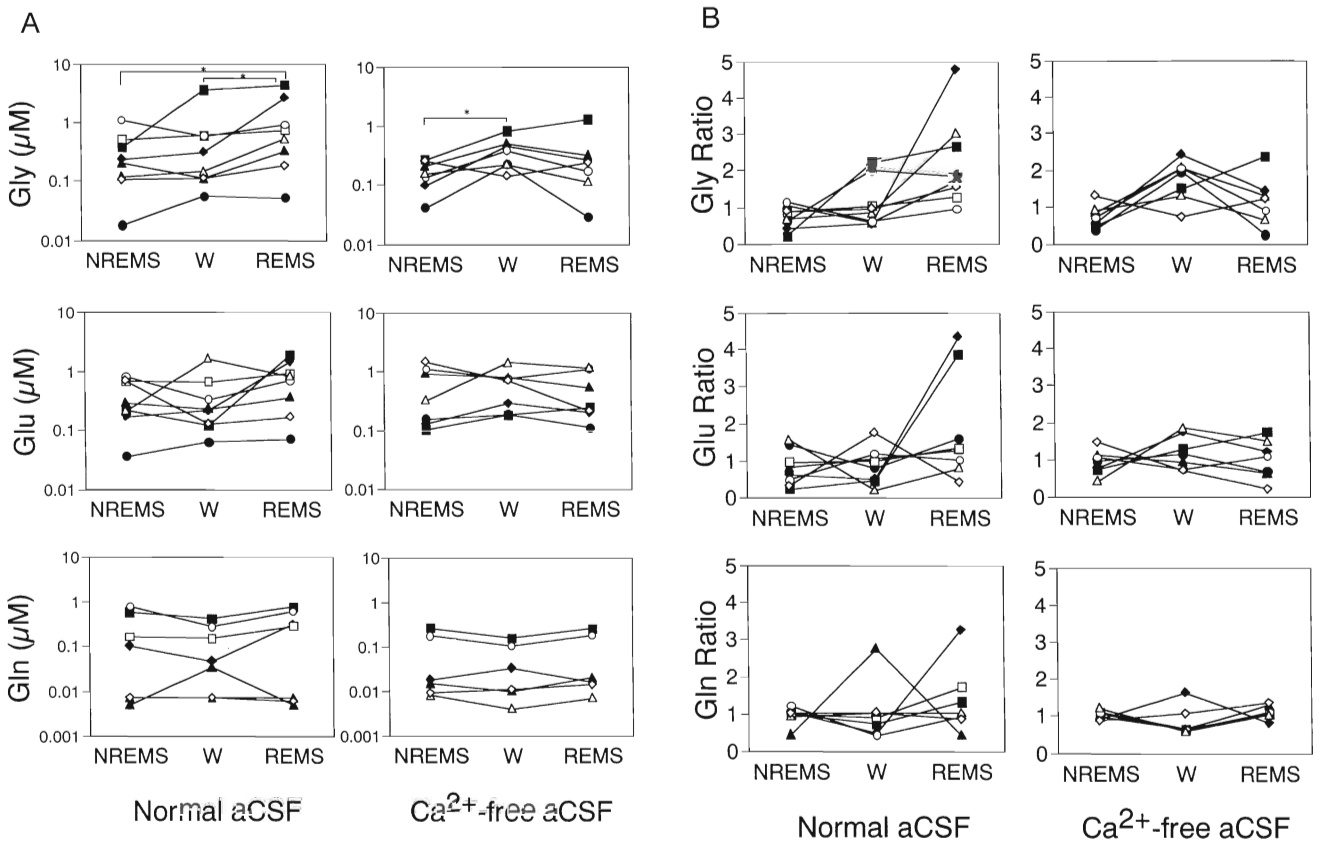

Fig 3. Changes in the amino acids during each vigilance state. A) Each point represents the average concentration of each amino acid during a certain vigilance state in each rat. Values for a certain rat were represented by the same symbol. The left and right panels show the data collected under normal (n = 7-8) and Ca^{2+}-free aCSF (n = 6-7) perfusion, respectively. *: $p<0.05$, Wilcoxon signed rank test. B) Each point represents the ratio of the values in A against the average concentration through all the vigilance states in each rat, which shows the changes in the amino acids among the vigilance states clearer.

restricted to REMS and W in 4 rats out of the 8. Thus, mechanisms that control extracellular levels of Gly or Glu may be quite complicated, and the Ca^{2+} dependent mechanism, which was activated during a certain state, may be only a small part of them. This may be one of the reasons why the time-sequential level of Gly concentration did not differ among the vigilance states, although its average level during REMS was highest.

III. Gly in the PnO and the vigilance states

Gly is thought by some investigators to play an important role in REMS generation[22,23]. A Gly-ergic projection to the pontine reticular formation has been identified[22,23,24], and Gly microinjected into the pontine reticular formation was reported to alter the firing pattern of neurons in the area[25].

In the present study, the average and maximum concentrations of Gly in the dialysates were 0.38 μM and 21 μM, respectively. Considering the probe recovery for amino acids, the extracellular concentration of Gly could be estimated to be at least about 10 times as high as those concentrations in the dialysates. Thus, Gly in the PnO reached a sufficient level to influence NMDA receptors[26].

Because the average extracellular level of Gly was found to be highest during REMS in the rat PnO, Gly may have some roles in REMS generation or maintenance.

IV. Amino acid dynamics within a vigilance state

Although the average level of Gly was found to be elevated during REMS, the current sampling method failed to demonstrate a consistent time-sequential pattern in the concentrations of Gly within any vigilance state at least in the first 3 min from its initiation. We did not focus on specific changes in the amino acid release by the end of each epoch, but our sampling technique must have detected the primary changes specific to the entrance of a new vigilance episode. In

order to understand more precisely the dynamics of the amino acid changes dependent on the vigilance state, it is necessary to develop another method of sampling and then integrate the results from both methods.

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