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Research report

Yawning responses induced by local hypoxia in the paraventricular nucleus of the rat

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Abstract

Yawing was induced by microinjections of L-glutamate, cyanide and a nitric oxide-releasing compound (NOC12) into the paraventricular nucleus of the hypothalamus (PVN) in anesthetized, spontaneously breathing rats. To evaluate physiological aspects of yawning, we monitored intercostal electromyogram (EMG) as an index of inspiratory activity, digastric EMG, blood pressure and electrocorticogram (ECoG). Microinjection of L-glutamate in the medial parvocellular subdivision (*mp*) elicited a stereotyped yawning response, i.e. an initial depressor response and an arousal shift in ECoG followed by a single large inspiration with mouth opening. The same sequential events were observed during spontaneous yawning, indicating that the *mp* is responsible for triggering yawning. Microinjection of cyanide into the *mp* caused the same yawning responses as the ones elicited by microinjection of L-glutamate, suggesting that the *mp* is sensitive to chemical hypoxia or ischemia within the PVN. Microinjection of NOC12 into the *mp* elicited a single large inspiration with a variable onset delay, suggesting that diffusible nitric oxide (NO) within the *mp* may act as a paracrine agent to cause a yawning response. We hypothesize that the *mp* of the PVN contains an oxygen sensor that causes a yawning response.

Keywords: Yawning; Paraventricular nucleus; Cyanide; Oxygen sensor; Hypoxia; Nitric oxide

1. Introduction

We have recently reported that a stereotyped yawning response can be evoked by electrical and chemical stimulation of the paraventricular nucleus (PVN) in anesthetized, spontaneously breathing rats [12]. A unique aspect of the yawning response is that a depressor response always precedes the yawning behavior, characterized by a single large inspiration with mouth opening. This phenomenon is of particular interest in terms of the relationship between attenuation of cerebral circulation and occurrence of yawning. It is clinically well known that patients suffering from brain ischemia frequently yawn. Another relevant example is the case of orthostatic hypotension, namely a failure in regulating cerebral circulation; yawning frequently occurs in these subjects [14]. These lines of evidence indicate that brain hypoxia/ischemia would be one of the factors causing yawning. Based on these findings, we hypothesize that local hypoxia within the PVN may induce yawning. The present study has been designed to test this hypothesis.

To cause local hypoxia/ischemia, we microinjected cyanide (a widely used model for producing chemical hypoxia [5]) into the PVN. The doses of cyanide were determined according to the study by Sun et al. [15], who used this technique to produce hypoxia/ischemia within the brainstem. Cyanide was injected into the sites of the PVN where a stereotyped yawning response was induced by L-glutamate microinjection in this study.

All microinjections were done with glass micropipettes in the present study, whereas in the previous study yawning was induced by electrical and chemical stimulation with a bipolar concentric microelectrode with a central opening (0.5 mm OD, 0.2 mm ID)

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connected to a fine cannula. The former technique provided a higher resolution, and as a result, we found by chance that the PVN was divided into three functionally distinct neuronal regions related to yawning. We, therefore, focused the first set of experiments on regional differences within the PVN. Thereafter, we examined hypoxia sensitivity within the PVN by microinjecting cyanide into the responsive sites where yawning responses were consistently induced by L-glutamate.

Furthermore, local brain ischemia generates nitric oxide (NO) in several regions such as the hippocampus and cerebral cortex [1,3]. Diffusible NO has been established to act as a paracrine agent [4]. Our earlier histological examination [12] revealed the existence of nitric oxide synthase (NOS)-containing cells in yawning-evoked sites of the PVN. In addition, intravenous administration of a NOS inhibitor prevented the yawning response evoked by chemical stimulation of the PVN [12]. In the present study, we examined a role of diffusible NO in ischemic yawning response, by microinjecting NOC12, a NO-releasing compound, into the PVN sites where cyanide evoked a yawning response.

2. Materials and methods

2.1. Surgical procedure

Experiments were performed on 17 male Wistar rats weighing 300–450 g. The rats were anesthetized with 50 mg/kg pentobarbital sodium intraperitoneally, and additional doses were given as needed. Surgical procedures were essentially the same as in our previous report [12]. In brief, catheters were placed in the femoral artery to monitor arterial blood pressure (BP). Heart rate was measured from the BP pulse with a tachometer (AT-601G, Nihon Kohden, Japan). To monitor inspiratory activity, a pair of twisted wire electrodes, insulated except for 1 mm at the tips, were implanted into the lower intercostal space, by way of a 23-gauge hypodermic needle; the needle was then withdrawn, leaving the wires in the intercostal muscle. Similarly, pairs of wire electrodes were implanted in the digastric muscle to monitor the mouth opening activity. For ECoG recordings, holes were drilled in the skull and two screw electrodes were implanted. ECoG signals were amplified and filtered (0.1–50.0 Hz bandpass). These polygraphic signals were all stored in a DAT data recorder (PC208Ax, Sony, Japan) for further analysis. Rectal temperature was maintained at 37°C with a heating lamp.

The animal was fixed prone in a stereotaxic frame and a parietal craniotomy was performed. For drug microinjections into the paraventricular nucleus of the hypothalamus, we made a four-barrel glass micropipette, which was connected to a fine cannula. The free end of the cannula was attached to a picopump (PV830 Pneumatic, WPI) for injections. The volumes injected were monitored under a dissecting microscope and measured from the movement of the fluid meniscus. The barrels were filled with either 0.1 M L-glutamate, 0.1 M NOC12, 0.02–0.1 M sodium cyanide, or artificial cerebrospinal fluid (ACSF: 147 mM NaCl, 3 mM $NaHCO_3$, 3.5 mM KCl, 1.3 mM $CaCl_2$, 1.1 mM $MgCl_2$, 0.5 mM NaH_2PO_4 and 1.1 mM urea; pH 7.30–7.35). L-glutamate, sodium cyanide and NOC12 were freshly dissolved in ACSF. The chemicals were obtained from Wako, Japan. The dura overlying the cortex was opened for advancement of the micropipette, which was inserted into an area 0.2–0.3 mm lateral to the midline and 1.1–2.4 mm posterior to the bregma, 5.8–7.2 mm vertical from the parietal dura, according to the Paxinos and Watson atlas of the rat brain [10]. We made a systematic search for the sites from which yawning responses were evoked by microinjection of L-glutamate (40 nl). After exploratory tracking with L-glutamate, we identified the sites where maximal yawning responses were obtained. A small volume (8–40 nl) of cyanide or NOC12 solution was then injected into the same responsive site to evaluate whether a yawning response could be induced. On completion of the experiment, successful stimulation sites were marked by 0.5 M acetate–pontamine for histological examination. The rat was then deeply anesthetized with pentobarbital sodium (100 mg/kg) to remove the brain.

2.2. Data analysis

After the experiments, ECoG signals were displayed on an oscilloscope together with intercosteral EMG. For power spectrum analysis the ECoG signal was digitized at a sampling rate of 1024 Hz and subjected to fast Fourier transform. Analysis was performed on data, sampled during pre-injection (control), early yawning and late yawning (recovery) periods of each response. The early yawning period was selected on the basis of visual inspection as the early phase of a yawning response, corresponding to an initial depressor response prior to a final yawning event (a single large inspiration). The late yawning (recovery) period was 15–30 s after the occurrence of a single large inspiration. Each power spectrum was divided into four frequency bands; 0.3–3.0, 3.5–7.0, 7.5–12.0, 13.0–30.0 Hz. These frequency bands were selected as being sensitive to changes induced by microinjection of a drug in an anesthetized rat.

2.3. Statistical analysis

Statistical analyses were carried out by one way analysis of variance (ANOVA), followed by Sheffe's test to correct for multiple comparisons of treatments. A probability value of 0.05 was adopted as a level for significance.

3. Results

3.1. Regional differences in yawning responses within the PVN

In our first experiment, we made a systematic search for responsive sites in and around the PVN by microinjection of L-glutamate. We could find those sites with a stereotyped yawning response as reported in our previous study [12], which was characterized by an initial depressor response followed by a final yawning event (a single large inspiration with mouth opening). A representative example is shown in Fig. 1B–D. In addition to the confirmation of our previous results, we found distinct responses related to yawning in certain regions within the PVN (Fig. 1A and E). The reason for the higher spatial resolution would be that we used a glass micropipette (tip diameter < 5 μm) in the present experiment, whereas in the previous study we stimulated the PVN with a bipolar concentric microelectrode (0.5 mm OD). Since analogous results were obtained from seven rats in this experiment we concluded that the PVN was divided into three functionally distinct regions related to yawning, as illustrated in Fig. 3.

In the experiment searching for the regional differences in yawning responses within the PVN, we moved a glass micropipette 0.1 mm steps along a track from a dorsal region to a ventral one within the PVN and injected 40 nl of L-glutamate at each site. As a result, we found those sites in the dorsal part of the PVN where injection of L-glutamate elicited only a depressor response without yawning behavior (Fig. 1A). Thereafter, we consistently identified a broad PVN region where a stereotyped yawning response was reliably evoked; the response pattern was characterized by an initial depressor response followed by a final yawning event (a single large inspiration). Note that the final yawning event occurred with a long onset latency (18 s) in Fig. 1B. When we advanced the micropipette deeper

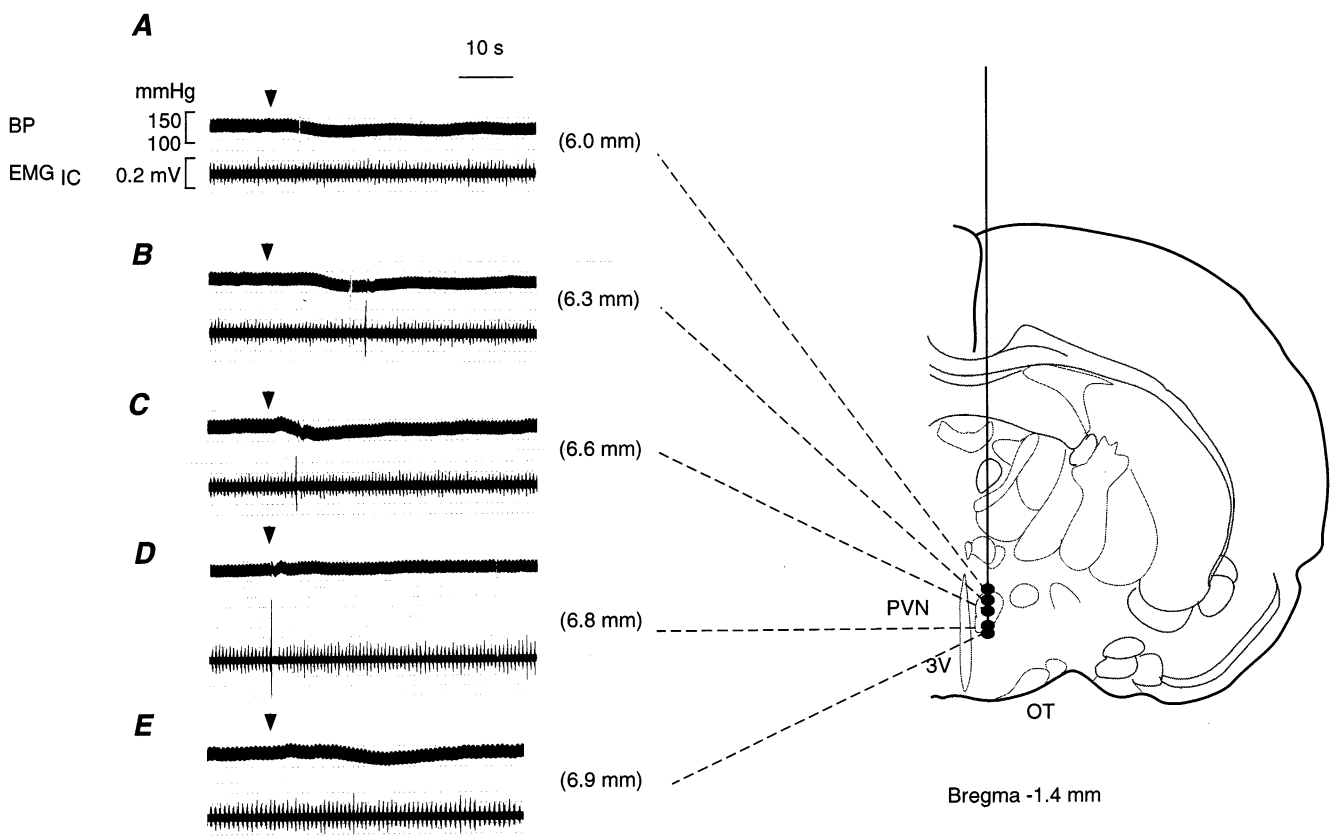

Fig. 1. Representative yawning responses to injection of L-glutamate (4 nmol, inverted triangle in left panel), obtained while moving a glass micropipette along a track from a dorsal region to a ventral one within the PVN (right panel). The latencies for the single large inspiration shortened when advancing the electrode to the ventral PVN. BP, blood pressure; EMCIC, intercostal electromyogram; OT, optic tract; 3V, third ventricle.

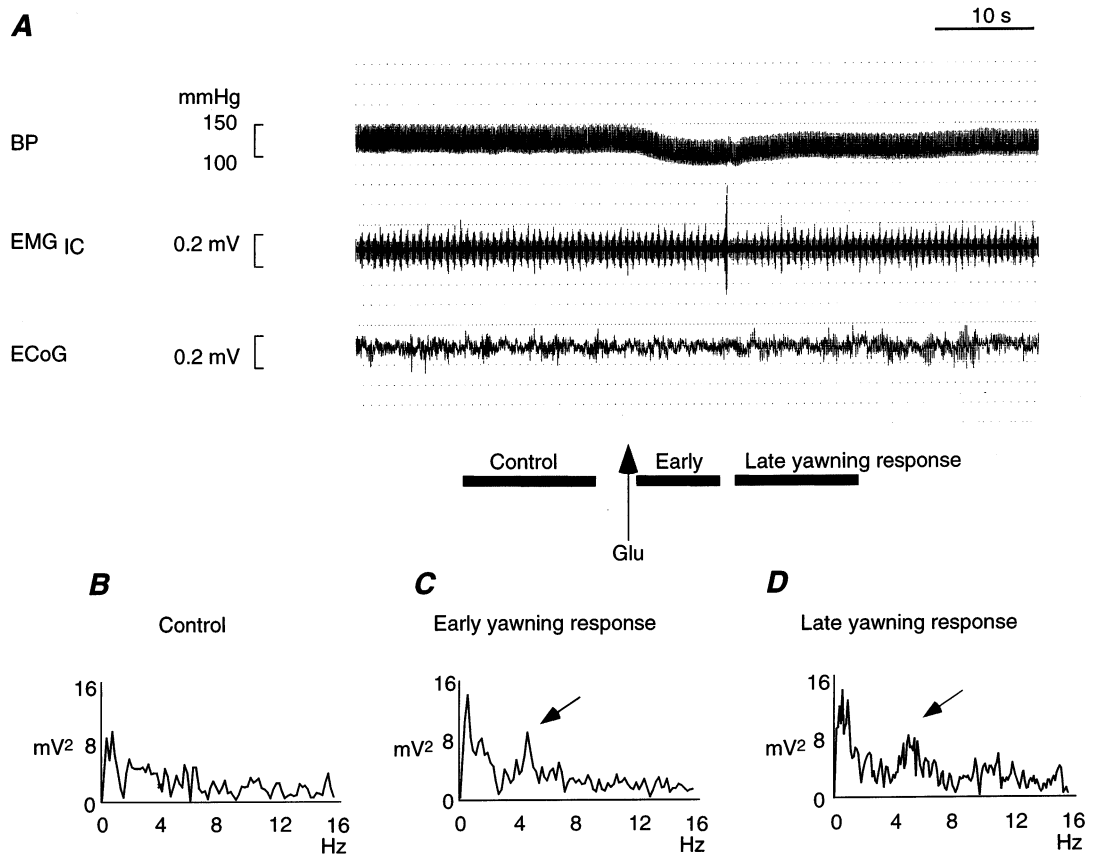

Fig. 2. Stereotyped yawning response evoked by microinjection of L-glutamate (Glu; 4 nmol) into the *mp* of the PVN (A). Changes in the ECoG recording before (B), during (C) and after (D) yawning are shown in the lower panel. Note that the waves shifted to lower faster rhythms (indicated by arrows) after microinjection of L-glutamate into the PVN. BP, blood pressure; EMCIC, intercostal electromyogram; ECoG, cortical electroencephalograph.

along the track, the onset latency of the final yawning event became shorter (Fig. 1C). We then identified a unique region where a yawning event of a single large inspiration occurred shortly after the injection of L-glutamate (Fig. 1D). It is notable that an initial depressor response was not evoked at this region. Such a region was restricted to a small area in the ventral border of the PVN. In fact, we frequently observed that, when the micropipette advanced only one step (0.1 mm) from the responsive site, this unique yawning response disappeared (Fig. 1E).

A typical example of a stereotyped yawning response is shown in Fig. 2. The response pattern was characterized by an initial depressor response followed by a final yawning event (a single large inspiration). Power spectral analysis of ECoG waves revealed that an arousal shift to lower voltage and faster rhythms ($\sim$4.5 Hz) was observed during the initial depressor response, i.e. prior to a sudden development of a final yawning behavior (Fig. 2C). The component of fast rhythms remained after the final yawning behavior (Fig. 2D), and returned gradually to control levels. These results were comparable to those of our previous study [12].

A summary of these three types of yawning-related responses obtained from seven rats is illustrated in Fig. 3. A stereotyped yawning response (open circles) was identified in a broad region of the PVN. A second type of yawning-related response, characterized by a rapid occurrence of a single large inspiration without a depressor response (filled circles) was found in a restricted area near the ventral border of the PVN. A third type of response, i.e. a depressor response without a yawning behavior (filled triangles) was observed predominantly in the dorsal part of the PVN.

3.2. Responses to cyanide

Considering the regional differences within the PVN, we evaluated the effect of cyanide injection in the specific region with the stereotyped yawning. Fig. 4 shows a typical example of the effect of cyanide injection into the site where the stereotyped yawning response was elicited by L-glutamate injection. Cyanide injection caused the same sequential events of a stereotyped yawning response. The initial response represented a fall in BP and an arousal shift in the ECoG to

lower voltage and faster rhythms (~ 5 Hz)(Fig. 4C). This early yawning response was followed by the development of a single large inspiration. After the final yawning behavior, BP returned gradually to control levels. As for ECoG, the component of fast rhythms remained after the final yawning behavior (Fig. 4D), but this also returned gradually to control levels. We examined the effects of cyanide injection in seven sites (one site per one rat) and found that cyanide injection caused the stereotyped yawning response in all the sites examined (Fig. 6, upper panel).

3.3. Responses to NO

We finally evaluated the effect of NOC12 injection in the specific regions where the stereotyped yawning responses were elicited by both L-glutamate and cyanide injections, as shown a typical example in Fig. 5. NOC12 injection caused a single large inspiration with a longer delay of ~ 70 s as compared with the responses by L-glutamate or cyanide. It was also noticed that a fall in BP was weak or negligible. An arousal shift in the ECoG to lower voltage and faster rhythms (~ 4.5 Hz) (Fig. 5C) was observed only during early yawning response and it disappeared shortly after the final yawning behavior (Fig. 5D). Similar yawning responses to NOC12 injection were obtained from all sites examined (five sites from five rats, Fig. 6, lower panel).

4. Discussion

In our previous work [12], we revealed that microinjections of L-glutamate into the PVN elicited yawning responses that were significantly reduced by PVN treatment with a NOS inhibitor. This study extends the earlier observations by providing additional details concerning the location of discrete regions of the PVN that mediate depressor versus respiratory/arousal components of the stereotyped yawning behavior and by evaluating hypoxia sensitivity within the PVN.

4.1. Regional differences in yawning responses within the PVN

The present study demonstrated that two types of responses related to yawning were elicited by microinjections of L-glutamate in the PVN; one type was a stereotyped yawning, consisting of initial depressor response and an arousal shift of ECoG to lower voltage and faster rhythms, followed by a final yawning event (a single large inspiration with mouth opening). The sequential events were the same as those of spontaneous yawn and this type of yawning response was the same as that reported in our previous study [12]. The stereotyped yawning response was identified in the medial parvocellular subdivision (*mp*) of the PVN, based on the terminology of Sawchenko and Swanson [13]. A second type of response was characterized by a rapid

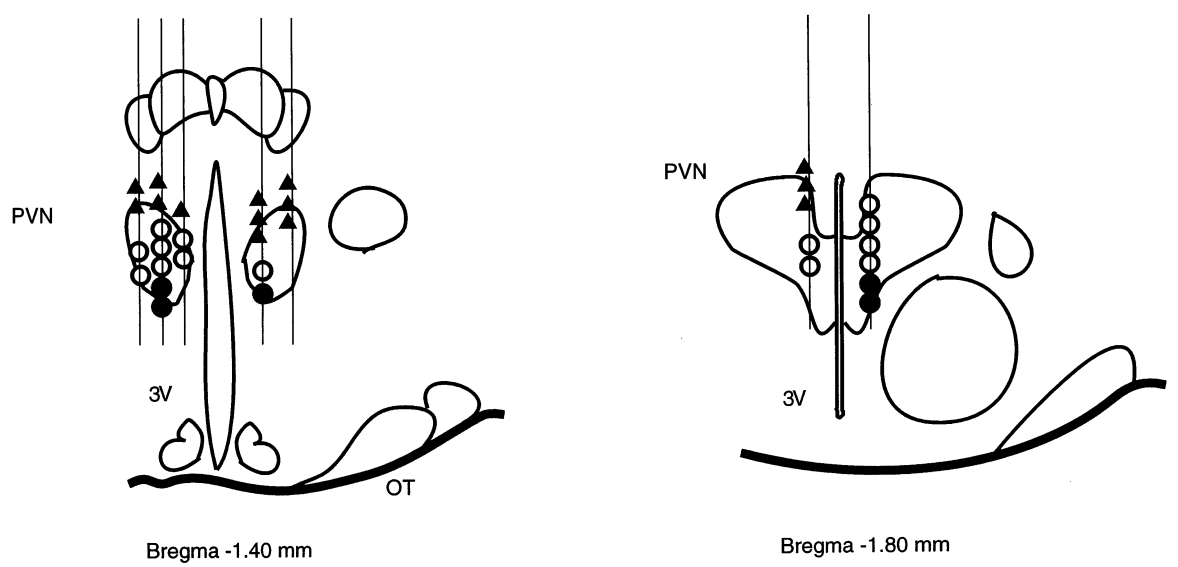

Fig. 3. Schematic drawings of transverse sections of the PVN, showing a summary of three types of responses obtained from seven rats. A stereotyped yawning response defined in Fig. 1 (○) was identified in a broad region within the PVN. A unique yawning response, characterized by a rapid occurrence of a single large inspiration without a depressor response (●) was found near the ventral border of the PVN. A depressor response alone without a yawning response (▲) was observed in the dorsal part of the PVN. OT, optic tract; 3V, third ventricle.

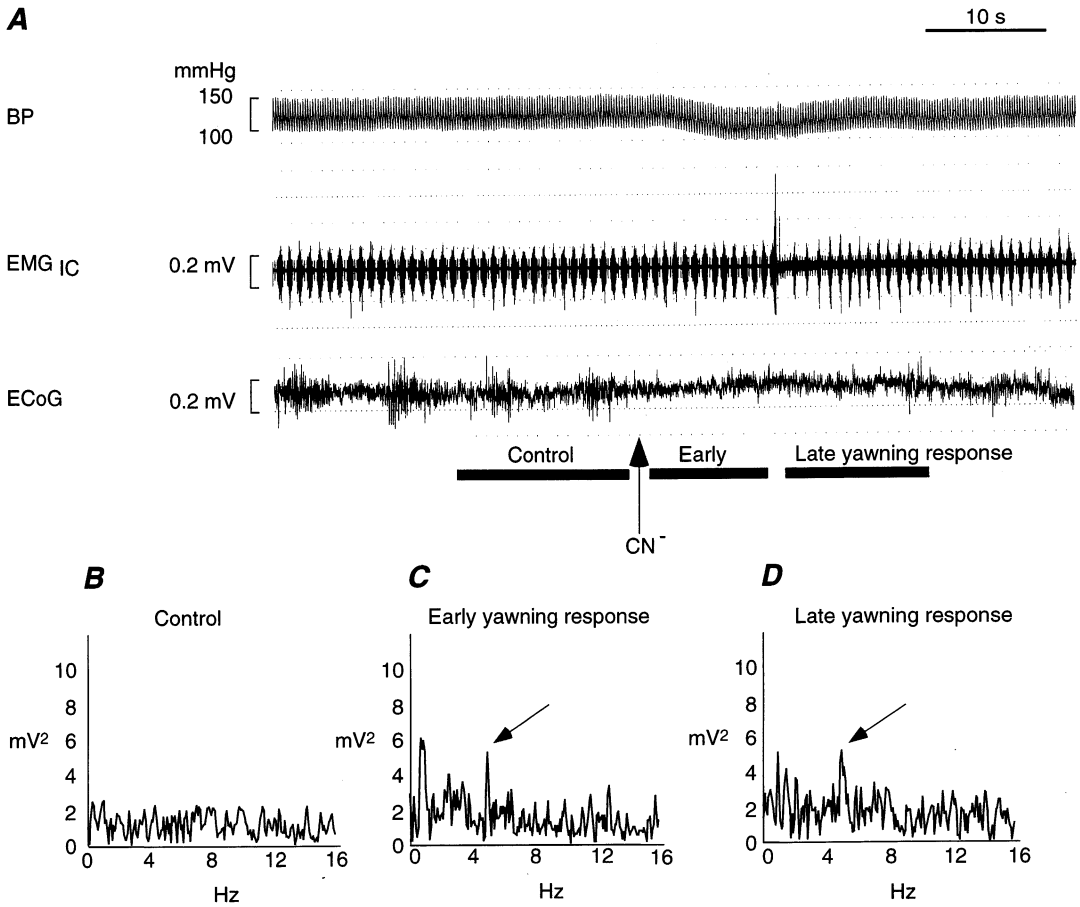

Fig. 4. Stereotyped yawning response evoked by microinjection of cyanide (CN-, 3.2 nmol) into the *mp* of the PVN (A). Changes in the ECoG recording before (B), during (C) and after (D) yawning are shown in the lower panel. Note that the waves shifted to lower faster rhythms (indicated by arrows) after microinjection of cyanide into the PVN. Abbreviations as in Fig. 2.

occurrence of single large inspiration without depressor response. This newly identified response was obtained in a restricted region in the ventral border of the *mp* of the PVN. We failed to find this type of response in our previous study, probably due to the relatively large stimulating electrodes used. By applying the high-resolution technique with multi-barrel glass micropipette, we found the pinpoint area described above.

Our observation that microinjection of L-glutamate in the ventral border of the *mp* produced a rapid occurrence of a single large inspiration without an initial depressor response, led to the hypothesis that the neurons in this region may project directly to the lower brainstem where the respiratory-related neurons are located. In this connection, histological data reported by Sawchenko and Swanson [13] demonstrated that parvocellular neurons in the ventral part of the *mp* send descending axons to the lower brainstem or the spinal cord.

We isolated another functional area where exclusively depressor response without a single large inspiration was evoked. The location of this response was the dorsal parvocellular subdivision (*dp*) of the PVN [13]. Although this area is not primarily related to a yawning behavior, we noticed that the time course of the depressor response was analogous to that observed during the stereotyped yawning response. This suggests that the initial change in BP during the stereotyped yawning response might be mediated through the *dp* of the PVN.

Regarding the depressor response evoked in the PVN, Kannan et al. [7] and Porter and Brody [11] have already demonstrated that depressor (sympathoinhibitory) response are elicited by chemical stimulation in the rostral medial part of the PVN. These earlier investigations seem compatible with our present result. Our result that a depressor response could be evoked without yawning indicates that sympathoinhibitory feedback system via baroreceptor is not responsible for triggering the yawning response.

Among different neuronal structures relating to yawning, the most essential substrate is the *mp* of the PVN where the stereotyped yawning response was elicited by microinjection of L-glutamate and cyanide. Since the same time sequence was observed during spontaneous yawning, the neuronal structure in the *mp* of the PVN might be responsible for triggering yawn. It

is further suggested that the yawning-triggering structure may be linked to the origins of at least three distinct efferent outputs, projecting to the respiratory, cardiovascular and arousal systems.

4.2. Response to cyanide

This study provided a new important finding that the stereotyped yawning response was activated by local application of cyanide in the PVN. Since cyanide has been used as a tool for producing chemical hypoxia [5], the present results can be explained by the notion that the PVN is a specific area that is sensitive to hypoxia. The responsive sites or the sites with oxygen sensitivity were found in the *mp* of the PVN, i.e. the yawning triggering structure described above. We, therefore, hypothesize that the stereotyped yawning response is triggered by local hypoxia in the PVN.

Regarding oxygen sensing cells, peripheral chemoreceptors in the carotid body have long been recognized [16]. Recent studies nominated another oxygen sensing cells in the central nervous system (CNS). These are found in the rostral ventrolateral medulla [2,8,15] or the caudal part of the hypothalamus [6]. The present study further nominated the PVN as a similar hypoxia-sensing site within the CNS.

What is the functional role of the oxygen sensor in the PVN? It has been established that hypoxia in the ventrolateral medulla, caused by brainstem ischemia elicits defense reactions which are characterized by apnea and pressor response. A distinct functional significance may be applied to the oxygen sensor in the PVN. Namely, hypoxia in the PVN, caused by higher brain ischemia may induce a waking behavior, which is characterized by yawning and ECoG arousal.

4.3. Mechanism of hypoxia sensitivity

Accumulating evidence suggests a linkage among hypoxia/ischemia, L-glutamate and NO in the mechanism of hypoxic/ischemic neuronal excitotoxicity [1,9]. Choi [3] summarized in his recent review that hypoxia/ischemia leads to stimulation of L-glutamate (NMDA) receptors, which enhance calcium influx and further activate NOS in the brain. In this case, excess NO formation finally mediates the neurotoxicity. We con-

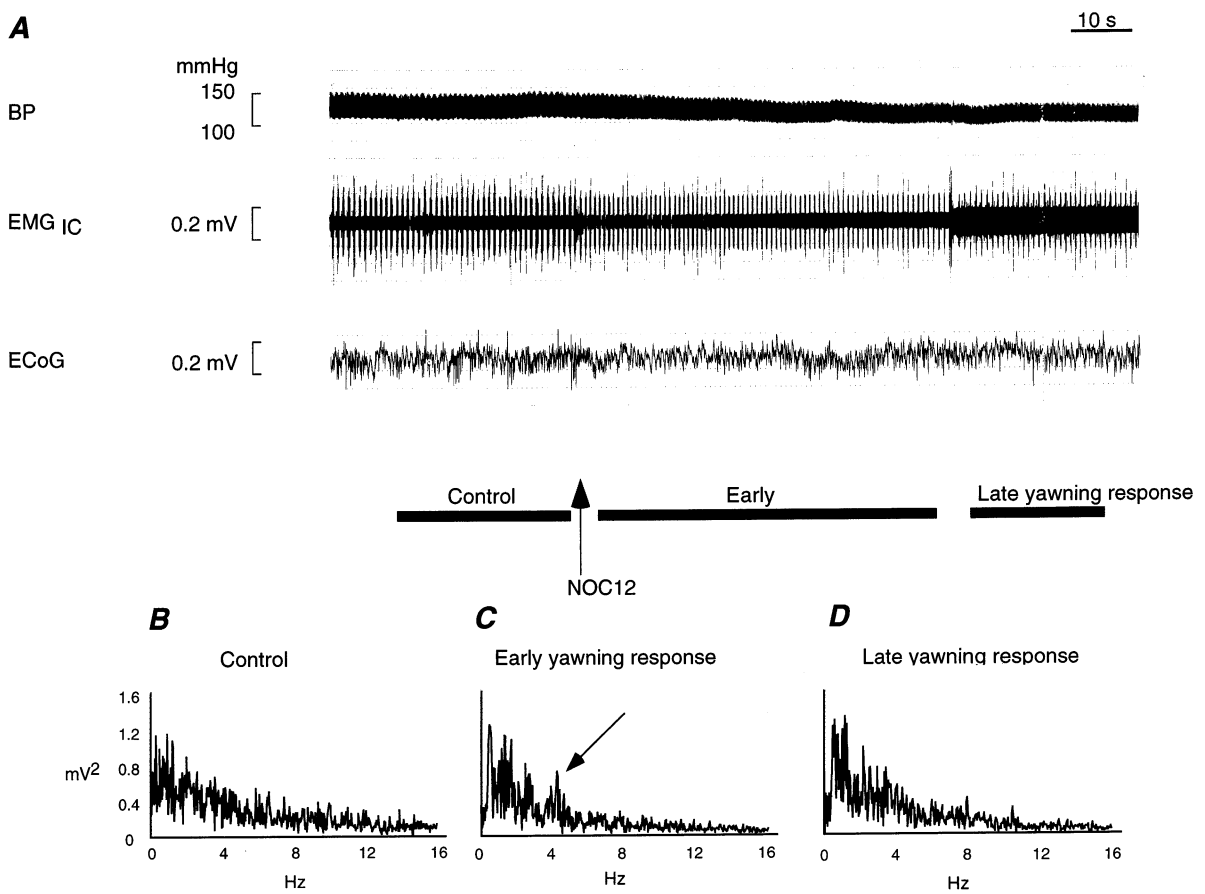

Fig. 5. Stereotyped yawning response evoked by microinjection of NO (NOC12; 4 nmol) into the *mp* of the PVN (A). Changes in the ECoG recording before (B), during (C) and after (D) yawning are shown in the lower panel. Note that the waves shifted to lower faster rhythms (indicated by arrow) after microinjection of NOC12 into the PVN. Abbreviations as in Fig. 2.

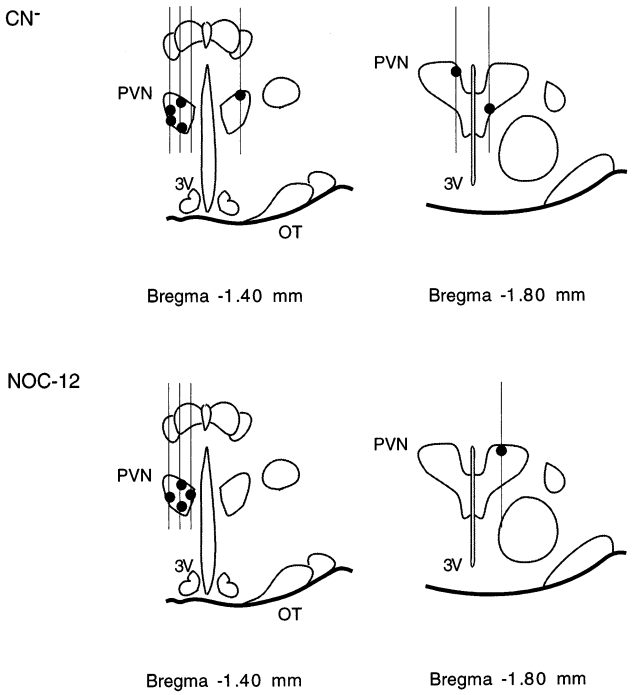

Fig. 6. Schematic drawings of transverse sections of the PVN, showing injection sites (●) where stereotyped yawning responses were obtained by cyanide (upper panel) and NOC12 (lower panel). OT, optic tract; 3V, third ventricle.

sider that analogous neuronal processes might take place within the PVN where yawning was induced by cyanide, NO and L-glutamate, as shown in this study. This idea may be further supported by our previous study [12] that (1) NOS positive cells exist in the PVN and (2) yawning responses were significantly reduced by PVN treatment with an NOS inhibitor. We propose the following hypothesis that hypoxia/ischemia in the higher brain would activate NMDA receptor of NOS-containing cells within the PVN. NO released by activation of NOS would then cause the yawning response.

4.4. Diffusible NO as a paracrine agent

In a stereotyped yawning response, the yawning behaviour (a single large inspiration) was induced with a time lag of ~ 10 s. The delayed respiratory response can not be explained by synaptic transmission but it might be better explained by the time process required for NO diffusion within the PVN, namely NO diffusion from the yawning-triggering structure in the *mp* of the PVN to the neurons projecting to the respiratory portion of the *mp* (the ventral border of the *mp*). As mentioned above, the neurons projecting to the brainstem respiratory-related cells are situated in the ventral border of the PVN. This area is located apart from the *mp* where NOS-containing cells are located. The delay in the respiratory response might be attributable to the time needed for NO diffusion from the NO generating cells to the respiratory efferent cells. It is reasonable to speculate that if the distance to the respiratory efferent cells is shortened then the time required for NO diffusion would be diminished. This notion is supported by the present data showing that the time lag is shortened when advancing the electrode to the ventral PVN where respiratory efferents are located.

In conclusion, yawning was induced by microinjections of L-glutamate and cyanide into the *mp* of the PVN. The results suggest that an oxygen sensor exists within the *mp* of the PVN and that yawning may be an arousal behavior caused by higher brain ischemia.

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