Hypothalamic Corticotropin-Releasing Hormone (CRH) Secretion into Hypophysial Portal Blood Is Regulated by Cutaneous Sensory Stimulation in Anesthetized Rats

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Abstract  The effects of noxious and non-noxious mechanical stimulation of various segmental skin areas (face, forelimb and forepaw, abdomen, hindlimb and hindpaw) on the secretion of immunoreactive corticotropin-releasing hormone (iCRH) from the hypothalamus into hypophysial portal blood was examined in artificially ventilated rats under halothane anesthesia. Secretion of iCRH was calculated from the iCRH concentration in hypophysial portal plasma and the plasma flow rate. Noxious mechanical stimulation of the skin was delivered by pinching using surgical clamps, while non-noxious mechanical stimulation was provided by brushing with tooth brushes. Pinching of the bilateral forepaws or hindpaws and brushing of the bilateral hindlimbs for 20 min increased hypothalamic iCRH secretion. In contrast, pinching of the face or abdomen and brushing of the face, forelimbs, or abdomen for 20 min did not significantly influence it. These results indicate that cutaneous mechanical sensory stimulation contributes to the reflex regulation of CRH secretion from the hypothalamus into hypophysial portal blood, and also that this effect is highly dependent on the site of stimulation.

Key words: reflex, corticotropin-releasing hormone, cutaneous stimulation, cutaneous segment, rat.

Corticotropin-releasing hormone (CRH) was initially purified from ovine hypothalamic extracts and is a polypeptide composed of 41 amino acids [1]. CRH is a major hypothalamic releasing hormone that regulates the secretion of adrenocorticotropic hormone (ACTH) from the anterior hypophysis and it has the high potency to augment ACTH secretion [2–5]. Immunoreactive CRH (iCRH) is present at a high concentration in the hypophysial portal blood [6, 7]. iCRH

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secretion into the hypophysial portal system increases in response to hemorrhage [7, 8] and hypoglycemia [9, 10] in parallel with an increase in the plasma ACTH level.

It has been reported that various types of somatic sensory stimulation can excite the pituitary-adrenocortical system. For example, the electrical stimulation of somatic afferent nerves increases plasma ACTH [11–13] and plasma corticosterone [14, 15] levels and noxious mechanical stimulation of the skin increases plasma corticosterone levels [16] in anesthetized rats. Suckling of the breast increases plasma corticosterone levels in conscious lactating rats [17] or sows [18], and exercise (which stimulates various somatic afferents) increases plasma ACTH and cortisol levels in dogs [19] or in human beings [20, 21]. On the basis of these previous findings, the hypothalamic neurosecretion of CRH into hypophysial portal blood would be expected to be affected by somatic afferent stimulation. However, this has not yet been proven.

In our laboratory, we have demonstrated the reflex responses of various autonomic and endocrine systems to somatic afferent stimulation in anesthetized animals disconnected from conscious and emotional involvement. These responses depend strongly on the modality of sensory stimulation used (e.g., noxious or non-noxious) as well as on spinal segments of the sensory nerves involved [22]. For example, secretion of catecholamines from the adrenal medulla is reflexly regulated by somatic sensory stimulation via adrenal sympathetic efferents in anesthetized animals. Noxious mechanical stimulation of the skin increases catecholamine secretion, while non-noxious mechanical stimulation reduces catecholamine secretion [23, 24].

The present experiment aimed to examine the reflex effect of cutaneous mechanical stimulation on the secretion of CRH from the hypothalamus into hypophysial portal blood in anesthetized rats. We focused on the reflex effects of two different types of mechanical stimulation (i.e., noxious and non-noxious) provided to four different cutaneous segments (i.e., face, forelimb and forepaw, abdomen, hindlimb and hindpaw).

MATERIALS AND METHODS

The experiments were performed on 27 male Wistar rats weighing 300–440 g. The animals were housed at a constant ambient temperature of 22±2°C under artificial light (0800 h–2000 h), and were fed laboratory chow and water ad libitum.

Rats were anesthetized with halothane (Takeda Chemical Industries, Ltd., Osaka). The halothane concentration was 1.5% during surgery, and this was reduced to 1.0% after surgery during the collection of experimental data. Halothane was added to a gas mixture containing 22–25% O₂ and 75–78% N₂ using a halothane vaporizer (Halothan Vapor 19.1, Dräger, Germany). The trachea was cannulated for ventilation using a respirator (Harvard Pump 681, U.S.A.), and the animals were immobilized with gallamine triethiodide (10–20 mg/kg, i.v.). The

Japanese Journal of Physiology
end-tidal CO₂ concentration was maintained at 3.0–4.0% by monitoring with a gas analyzer (model 1H26, NEC San-ei, Tokyo). The femoral vein and artery of one hindleg were cannulated for infusing chemicals and measuring systemic arterial blood pressure, respectively. Systolic arterial blood pressure was maintained above 90 mmHg by infusion of 4% Ficoll 70 (Pharmacia Fine Chemicals, Sweden) in saline, and the rectal temperature was maintained at 37–38°C using a direct current heating pad and an infrared lamp.

Collection of hypophysial portal blood. Hypophysial portal blood was collected by the parapharyngeal method originally developed by Porter and Smith [25]. After exposing the pituitary gland, animals were kept under resting conditions without any external stimulation for approximately 1 h. Then, heparin solution (400–500 IU/kg, Shimizu Pharm. Co. Ltd., Shizuoka) was injected intravenously, and the pituitary stalk was transected and the anterior pituitary was removed from the fossa. A mixture of 4% Ficoll 70 and heparin (20 IU/ml) was next infused intravenously at a speed of 0.8–2.0 ml/h, and the collection of the hypophysial portal blood was started 15 min after the cutting of the pituitary stalk. The blood samplings were done between 1300 h and 1730 h over sequential 20-min periods for 100–140 min. After centrifuging the collected blood samples for 10 min at 3,000 rpm and 4°C, the plasma was stored at −80°C.

Measurement of CRH. We extracted and measured plasma CRH according to the method of Suda et al. [26] with slight modifications.

Extraction of CRH: A plasma sample was applied to the immunoadfinity column (0.15 ml), which consisted of resin (Activated CH-Sepharose 4B, Pharmacia Fine Chemicals, Sweden) covalently linked to CRH antibodies. Then the CRH retained in the column was eluted with 5 ml of 0.5 M acetic acid containing 0.25% human serum albumin (HSA), and the eluate was lyophilized and reconstituted in 0.3 ml of assay buffer. The assay buffer consisted of 0.1 M phosphate buffer (pH 7.4) containing 0.1% HSA and 0.1% Triton X-100. The recovery of CRH with this system was about 90%.

Radioimmunoassay: We used a synthetic rat CRH (Peninsula Laboratories, U.S.A.) as the standard, and anti-CRH serum (RC-12; provided by Dr. Toshihiro Suda, Tokyo Women’s Medical College, Tokyo) at a final dilution of 1:1,200,000. As the tracer, synthetic rat CRH was iodinated with ¹²⁵I by the chloramine T method. The standard solution of CRH (prepared by serial dilution of CRH with the assay buffer) or the sample, the antiserum, and the tracer were all incubated together for 48 h at 4°C. Thereafter, a second antibody (a sheep antiserum to rabbit γ-globulin) was added to separate the free and bound tracer. After incubation for another 24 h at 4°C, the pellet and supernatant were separated by centrifugation, and then both were counted for radioactivity. The intra-assay and inter-assay coefficients of variation were 6 and 13%, respectively. Serial dilutions of the plasma extract inhibited binding in parallel with synthetic rat CRH, as shown in Fig. 1.

Cutaneous stimulation. Cutaneous stimulation, either noxious or non-
noxious, was delivered for 20 min. Non-noxious stimulation was delivered by brushing one of the four skin areas bilaterally (i.e., the lateral parts of the face, the forelimbs, the lateral parts of the abdomen, or the hindlimbs) with tooth brushes at a frequency around 1 Hz. Noxious stimulation was delivered by pinching one of the four skin areas bilaterally (i.e., the lateral parts of the face, the forepaws, the lateral parts of the abdomen, or the hindpaws) with surgical clamps using a force of approximately 3 kg. The skin areas subject to pinching were changed slightly every 5 min.

Statistical analysis. Results are given as the mean±SE, and data were evaluated statistically using the paired t-test.

RESULTS

1. Secretion of iCRH from the hypothalamus into hypophysial portal blood under resting conditions

The basal iCRH concentration in hypophysial portal plasma collected during the first 20 min in the 27 rats was 2,020±330 pg/ml. This value was much higher than the value in systemic blood plasma that has been reported previously (5.6±0.9 pg/ml, mean±SD) [27]. The large difference between the iCRH concentration in hypophysial portal plasma and that in systemic plasma permitted us to neglect the systemic plasma level when calculating iCRH secretion from the hypothalamus.
The basal hypophysial portal blood plasma flow rate during the first 20 min in the 27 rats was $136 \pm 11 \mu l/20$ min. The secretion rate of iCRH was calculated from the concentration in hypophysial portal plasma and the portal plasma flow rate, and was found to be $185 \pm 11$ pg/20 min.

Consecutive iCRH secretion rates measured for sequential 20-min periods totalling 100 min under resting conditions were stable (the mean values determined in 3 rats were 189, 211, 213, 189, and 189 pg/20 min, respectively).

2. **Response of iCRH secretion to cutaneous stimulation**

   A total of 48 trials of cutaneous stimulation, either pinching or brushing, were employed in 24 rats for examining the stimulus effect on iCRH secretion. Each animal received stimulation one to three times, although often twice.

   1) **Effect of non-noxious mechanical stimulation.** Figure 2A and Table 1A summarize the effect of bilateral brushing of the four skin areas for 20 min on iCRH secretion. Brushing of the hindlimbs significantly increased iCRH secretion during stimulation ($p < 0.05$), while brushing of the face or abdomen produced no significant response. Brushing of the forelimbs did not produce a statistically

Fig. 2. Summary of the responses of the iCRH secretion rate from 24 rats to bilateral brushing (A) or pinching (B) of various cutaneous regions for 20 min expressed as a percentage of the prestimulus value. Numbers in parentheses indicate the number of trials. Each animal received stimulation one to three times, although often twice. Circles and vertical bars show the mean ± SE. Significant differences were determined by comparison with the prestimulation values using the paired $t$-test ($* p < 0.05$, $** p < 0.01$).

Vol. 42, No. 3, 1992
Table 1. Hypothalamic iCRH secretion rate (pg/20 min) before, during and after cutaneous brushing (A) or pinching (B).

<table>
<thead>
<tr>
<th>Type of stim.</th>
<th>Stimulated area</th>
<th>Before</th>
<th>During</th>
<th>After</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. Brushing</td>
<td>Face</td>
<td>205±37</td>
<td>202±42</td>
<td>167±37</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>Forelimb</td>
<td>167±22</td>
<td>231±46</td>
<td>176±26</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>Abdomen</td>
<td>207±15</td>
<td>216±20</td>
<td>220±29</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td>Hindlimb</td>
<td>147±18</td>
<td>220±31*</td>
<td>154±18</td>
<td>6</td>
</tr>
<tr>
<td>B. Pinching</td>
<td>Face</td>
<td>191±24</td>
<td>165±22</td>
<td>194±37</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>Forepaw</td>
<td>205±20</td>
<td>397±70*</td>
<td>246±68</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>Abdomen</td>
<td>196±79</td>
<td>224±29</td>
<td>227±37</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>Hindpaw</td>
<td>202±15</td>
<td>301±18**</td>
<td>174±20</td>
<td>6</td>
</tr>
</tbody>
</table>

Values are mean ± SE; n, number of trials. *p<0.05, ** p < 0.01; significantly different from prestimulus control values using paired t-test.

significant response over 6 trials, but iCRH secretion was increased in 2 of the 6 trials. During stimulation of the hindlimbs, iCRH secretion increased to 149±10% of the prestimulation control value. It returned to the prestimulation level after the cessation of stimulation. Simultaneous measurement of systemic arterial blood pressure showed no significant response to brushing stimulation of all the skin areas tested.

2) Effect of noxious mechanical stimulation. Figure 2B and Table 1B summarize the effect of bilateral pinching of one of the four skin areas for 20 min on iCRH secretion. Pinching of either the forepaws or the hindpaws significantly increased iCRH secretion during stimulation by 189±17 (p < 0.05) and 151±10% (p < 0.01), respectively. A return to the prestimulation control level was noted after the cessation of stimulation. Pinching of the face or abdomen was ineffective in producing a change in iCRH secretion. The systemic arterial blood pressure increased significantly during pinching of the face, forepaws, or hindpaws, and the maximum systolic pressure reached 109±3, 136±3, and 121±2% of the prestimulus control value, respectively. Blood pressure did not alter significantly in response to pinching of the abdomen.

When the same stimulation, either brushing or pinching, was twice employed in the same animal and there was a response, the response was reproducible.

DISCUSSION

This is the first demonstration that iCRH secretion from the hypothalamus into hypophysial portal blood is reflexly controlled by cutaneous stimulation in anesthetized rats. Plasma ACTH and corticosterone levels have been shown to increase following somatosensory stimulation under anesthesia [11–16], as well as in the conscious state [17–21]. Since CRH is the predominant ACTH releasing factor, CRH secretion has also been suggested to be increased by somatic stimu-
lation and the present findings supported this hypothesis. Somatosensory stimulation can regulate the plasma levels of several pituitary hormones such as luteinizing hormone (Tsuchiya et al., in press in Jpn. J. Physiol.) and prolactin [28, 29] as well as ACTH [11–13] in anesthetized animals. However, somatic modulation of the hypothalamic hormones regulating these pituitary hormones has not yet been investigated in detail. The present study demonstrated that a hypothalamic releasing hormone, CRH, could be regulated by somatosensory stimulation.

Tsuchiya et al. [16] reported that plasma corticosterone levels increased following pinching of the hindpaws in anesthetized rats. Our present finding of an increase in iCRH secretion following pinching of the hindpaws is considered to be responsible for the previously-noted increase in adrenocortical function. Tsuchiya et al. [16] could not find any response of plasma corticosterone to brushing of the hindlimbs, while the present study demonstrated an increase in iCRH secretion. The discrepancy between the two experiments may be due to differences in the anesthetic used (halothane vs. pentobarbital) or the stimulus duration (Tsuchiya et al. used a stimulation of 10-min duration, whereas we used 20 min). There is also the possibility that other ACTH-regulating factors, such as vasopressin, vasoactive intestinal peptide, catecholamines, and somatostatin [30], might modulate the somatically-induced ACTH-adrenocortical response. Further investigation is needed to clarify which of these possibilities is the correct one.

Secretion of iCRH was significantly increased following noxious and non-noxious stimulation of selected skin areas. Noxious stimulation of the hindlimbs elicited a reflex increase in iCRH secretion which was equivalent to that produced by non-noxious stimulation of the same area. Thus, we may conclude that the central connections between nociceptive afferents and CRH neurons are as strong as those between non-nociceptive afferents and CRH neurons, at least in the hindlimb skin.

The stimulatory effect on iCRH secretion was highly dependent on the skin region stimulated, since noxious stimulation of the forepaws or hindpaws but not the face or abdomen significantly increased iCRH secretion. These differences appear to depend either on variation in the density of afferent innervation of the skin or on differences in the connections to CRH neurons in the hypothalamus from various skin regions. It is noteworthy that cerebral cortical blood flow [31] and the neuronal activity of the nucleus basalis of Meynert (NBM), the cholinergic fibers of which contribute to the neural regulation of cerebral cortical blood flow [32], have been reported to increase significantly following pinching of a forepaw or hindpaw, and to a lesser degree after pinching of the face or back. On the other hand, adrenal sympathetic activity increases less markedly following pinching of a forepaw or hindpaw than following pinching of the chest or abdominal skin [33]. In addition, sympathetic activity in the interscapular brown adipose tissue is increased in response to pinching of the face, ear, neck and forelimb, but not by pinching of the hindlimb [34]. Considering these facts together, the somatically-induced changes of iCRH secretion were similar to those affecting cerebral cortical
blood flow and NBM neurons but were not similar to those of the sympathetic nerves supplying the adrenal medullary and brown adipose tissue with regard to the efficacy of limb stimulation.

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