

## Immunohistochemical Study of the Distribution of Corticotropin Releasing Factor and Serotonin in the Cerebellar Nuclei of Monkeys<sup>+</sup>

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**=Abstract=**Immunohistochemical methods, employing a specific antiserum against human corticotropin releasing factor (CRF) and serotonin, were applied to determine the distribution of corticotropin releasing factor-immunoreactivity (CRF-IR) and serotonin-IR in the deep cerebellar nuclei of the Squirrel monkey. CRF-IR labeled fibers were demonstrated in three all deep cerebellar nuclei, including the dentate, interposed and fastigial nucleus. All these fibers were varicose and greater density was observed in the dentate and the interposed nuclei. The serotonin-IR fibers were observed in all deep cerebellar nuclei. But the distribution of these fibers was denser than that of CRF-IR fibers. Labeled fibers were varicose, and there was no evidence of greater density within deep cerebellar nuclei.

**Key Words:** *Corticotropin releasing factor (CRF), Serotonin, Cerebellar nuclei, Immunohistochemistry*

### INTRODUCTION

Corticotropin-releasing factor (CRF) is a 41-amino acid peptide which is known to act as a hypothalamic releasing factor, stimulating the secretion of adrenocorticotrophic hormone and beta-endorphin from the anterior pituitary (Vale et al., 1983). In addition, several lines of evidence (biochemical, histochemical and electrophysiological) indicate that CRF may function as a neurotransmitter in extrahypophysial neuronal projections. For example, there have been several immunohistochemical studies characterizing the anatomic distribution of CRF-like immunoreactivity in rodent

brains (Bloom et al. 1982; Merchaenthaler et al. 1982; Olschowka 1982; Cummings et al. 1983; Joseph & Knigge 1983; Swanson et al. 1983; Fellman et al. 1984; Skofitsch & Jacobwitz 1985; Sakanaka et al. 1987). These reports have described extensive, widely distributed systems of CRF in extrahypophyseal neuronal perykarya and fibers.

However, limited information is available concerning the distribution of CRF in primate brains. For example, immunohistochemical studies have been limited in monkeys to examinations of the hypothalamus (Kawata et al. 1982; Paull et al. 1984) and circumventricular organs (Kawata et al. 1983) and in humans to the hypothalamus (Bresson et al. 1987), inferior olive, and cerebellum (Powers et al. 1987).

Recently Cha and Foote (1988) observed the presence of CRF immunoreactivity in the olivocerebellar climbing-fiber systems of monkey by

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immunohistochemistry. Thus, their results reinforced many previous reports indicating that CRF is localized in extrahypophysial circuits, where it may serve as a neurotransmitter (Vale *et al.* 1983; Emeric-Sauval 1986).

Axons projecting from the inferior olive constitute the olivocerebellar pathway which provides climbing-fiber input throughout the cerebellar cortex as well as collateral innervation of deep cerebellar nuclei. Deep cerebellar nuclei consists of the dentate, emboliform, globose and fastigial nuclei. The axons of the Purkinje cells communicate with few exceptions with the deep cerebellar nuclei. The lateral (hemispheric) zone connects with the dentate nucleus, the medial (vermal) zone connects with the fastigial nucleus, the paravermal zone connects with the emboliform and globose nucleus. Throughout these three longitudinal zones, most Purkinje cell axons pass out of the cerebellum and end on the brainstem and cerebral cortex.

It is important to investigate the distribution of CRF in cerebellar nuclei and compare this with the distribution of serotonin because the cerebellar output is influenced not only by deep cerebellar nuclei but also by climbing and mossy fibers.

## MATERIALS AND METHODS

Immunohistochemical material was obtained from five adult New World squirrel monkeys. The animals were deeply anesthetized with ketamine (25 mg/kg, IM) and sodium pentobarbital (15 mg/kg, IP). They were then perfused transcardially with ice-cold 1% paraformaldehyde in phosphate buffer (0.15 M) for 1.0 minute followed by perfusion with ice-cold 4% paraformaldehyde in phosphate buffer for 9 minutes at a flow rate of 250-500 ml/minute (depending on body size). The brain was removed immediately and cut into blocks 3-5 mm thick. These blocks were immersed in cold fixative for 6-12 hours and then washed in a series of cold sucrose solutions of increasing concentration. They were then stored in 18% sucrose in phosphate buffer for 1-7 days. Forty-micron frozen sections were cut in the coronal plane and incubated, freely floating, for 48-72 hours at 4°C in primary antiserum. The primary antiserum was visualized with the avidin-biotin-com-

plex (ABC) method by using an ABC kit available from Vector Labs (Burlingame, CA, USA). These kits utilize as a secondary antiserum biotinylated antirabbit IgG that is subsequently bound to biotinylated horseradish peroxidase by use of an avidin bridge. The sections were developed for peroxidase reactivity with 3,3-diaminobenzidine (LaVail *et al.* 1973).

The distribution of perykarya and fibers exhibiting CRF-LI was evaluated by careful comparison of the immunohistochemical sections with adjacent Nissl-stained sections.

The primary antiserum used in these studies was raised in rabbits and was directed against the human form of CRF which is identical to the rat form (Vale *et al.* 1981; Rivier *et al.* 1983; Shibihara *et al.* 1983). The antiserum was generously furnished by W. Vale and J. Rivier of the Salk Institute. For the antiserum utilized in the present studies, a dilution series of 1 : 1,000, 1 : 2,000 and 1 : 4,000 was evaluated. The staining from the 1 : 2,000 dilution was found to be optimal. The optimal dilution of the antiserum against serotonin was the same. As controls for nonspecific immunoreactivity, a sample of sections was incubated without primary antiserum and a different sample was exposed to a 3% hydrogen peroxide solution prior to the HRP reaction. Sections processed without primary antiserum did not exhibit any immunoreactivity, while those exposed to hydrogen peroxide, to destroy endogenous peroxidase activity, exhibited the same immunoreactivity as normally treated sections. Also, 25 sections from different levels throughout the cerebellum were exposed to 1 : 2,000 primary antiserum which had been preabsorbed for 24 hours with human CRF (Penninsula Laboratories, Belmont, CA, USA) at a concentration of 0.1 mg/ml ( $2.1 \times 10^{-5}$  M). Sections from this sample did not exhibit any immunoreactivity.

## RESULTS

### Distribution of CRF-IR fibers in deep cerebellar nuclei:

CRF-IR labeled fibers were observed in all deep cerebellar nuclei of the Squirrel monkey. All these fibers were varicose. The fibers of the dentate

and interposed nucleus were denser than those of the fastigial nucleus (Figs. 1, 2 & 3). There was no difference in the density of fibers between the interposed and the dentate nucleus, but finer fibers were observed in the dentate nucleus (Fig. 4).

Immunoreactive fibers in the molecular layer of the cerebellar cortex exhibited a higher density than those of the cerebellar nuclei (Fig. 5).

#### **Distribution of serotonin-IR fibers in deep cerebellar nuclei:**

The distribution of serotonin-IR fibers was similar to the point that these fibers were observed in all deep cerebellar nuclei. But the distribution of the serotonin-IR fibers was denser than that of CRF-IR fibers. These labeled fibers were varicose and also, some exhibited a long running appearance. There was no evidence of greater density in any given area of the three deep cerebellar nuclei. The caliber of fibers in the fastigial and the interposed nuclei was generally constant (Figs. 6 & 7). But fine and thick fibers were mixed together in the dentate nucleus (Figs. 8 & 9).

Immunoreactive fibers were observed, with varying densities, throughout all of the major regions of the cerebellar cortex. These were especially prominent in the granule cell layer (Fig. 10). A lower density of immunoreactive fibers was observed in the molecular layer and occasional long fibers were seen in the white matter (Fig. 11). A network of immunoreactive fibers was also observed in the cerebellar commissure which connects the right and left cerebellar nuclei (Fig. 12).

### **DISCUSSION**

In the present study, details of the distribution of CRF- and serotonin-immunoreactive nerve fibers in the cerebellum of the monkey were demonstrated without any pharmacological pretreatment of the animals. The pattern of the serotonin innervation in the monkey cerebellum as revealed in this study differs from the results from rats. It has previously been reported that in the rat, the pool of serotonin nerve fibers mainly consisted of tangential elements, which were predominantly in the molecular layer, while in the cat only a few serotonin fibers

were found in the molecular layer of the cerebellar cortex but dense networks of 5-HT nerve fibers were present in the granular layer (Takeuchi *et al.* 1982). In our studies in monkeys the serotonin fibers were predominantly in the granular layer, so the distribution pattern of serotonin in the monkey and cat may be similar.

In the monkey cerebellar nuclei in our studies the density of serotonin innervation was similar to that of the cortex but, the density of CRF innervation in the cerebellar nuclei was relatively low compared with the density in the cerebellar cortex. CRF and serotonin fibers in the cerebellar nuclei are collateral fibers of the climbing and mossy fibers (Eccles *et al.* 1974; Andersson and Oscarsson 1978; Wiklund *et al.* 1984), so these findings indicate that the collateral fibers of serotonin-immunoreactive mossy fibers are more profuse than the collateral fibers of CRF-immunoreactive climbing fibers. In the rat, cat and opossum, cells double-labeled with HRP retrogradely transported from the cerebellar cortex and immunostained for serotonin have been mapped within the paramedian, lateral and gigantocellular reticular nucleus (Bishop and Ho 1985; Walker *et al.* 1988). In contrast to suggestions from other investigators (Shinnar *et al.* 1973; Chan-Palay 1975), comparatively few serotonin-containing cerebellar afferents appear to arise from the raphe nucleus in those species.

The function of serotonin fibers in the cerebellum is unclear but the paucity of synaptic contacts between diffuse fibers and cerebellar neurons in the rat (Chan-Palay 1977; Beaudet and Sotelo 1981) has led to the suggestion that the actions of serotonin are mediated through a nonsynaptic neurohormonal interaction (Strahlendorf *et al.* 1979).

Cummings *et al.* (1989) has previously reported that in the cat, density of CRF fibers was somewhat greater in interposed nucleus than within medial nucleus although CRF fibers were less dense in posterior interposed nucleus than anterior interposed nucleus. These results are similar to those with revealed in our study.

It is suggested that CRF is contained within the olivocerebellar pathway of rats, cats, sheep, monkeys and humans. Both immunohistochemical and *in situ* hybridization techniques have yielded



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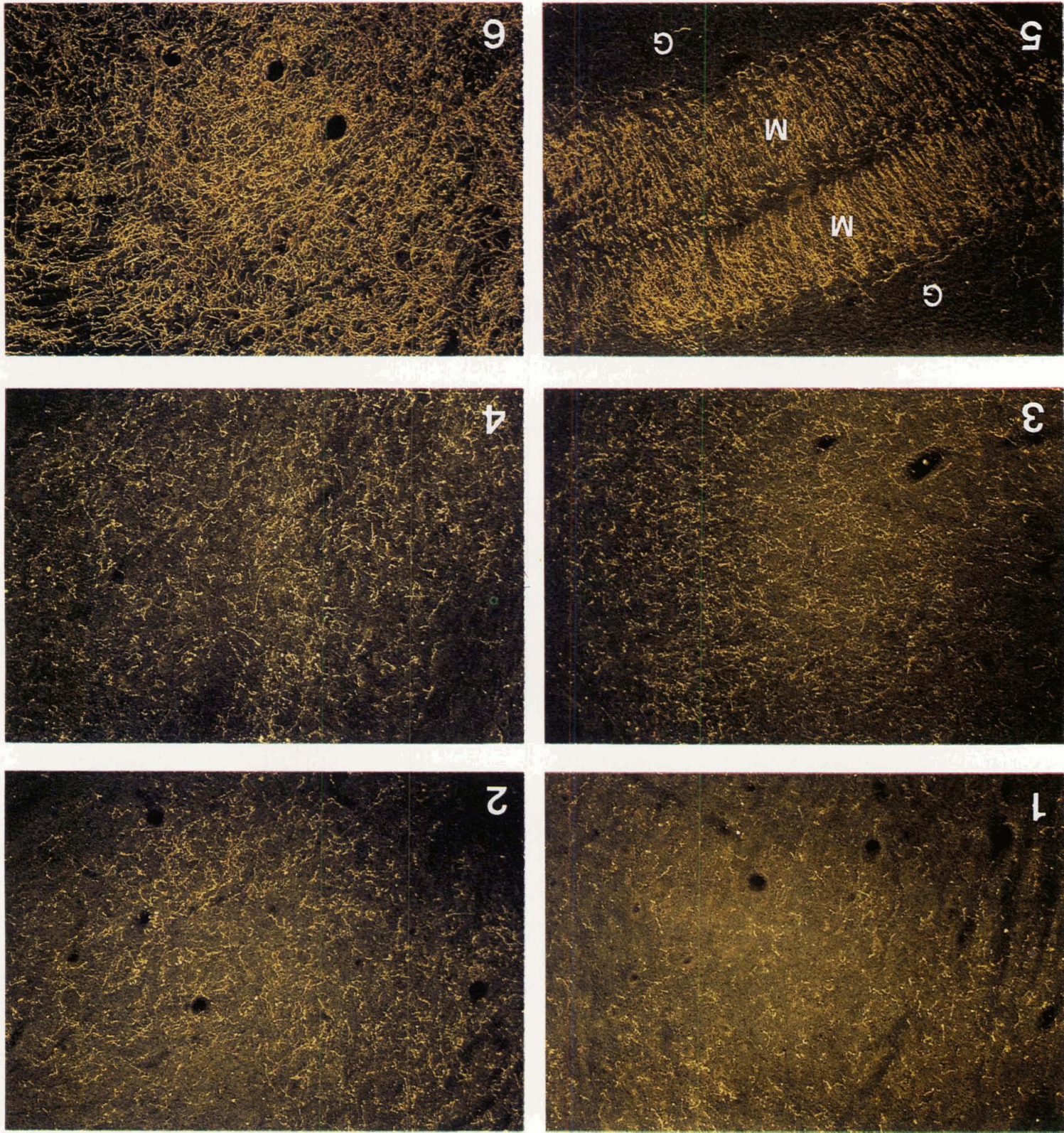
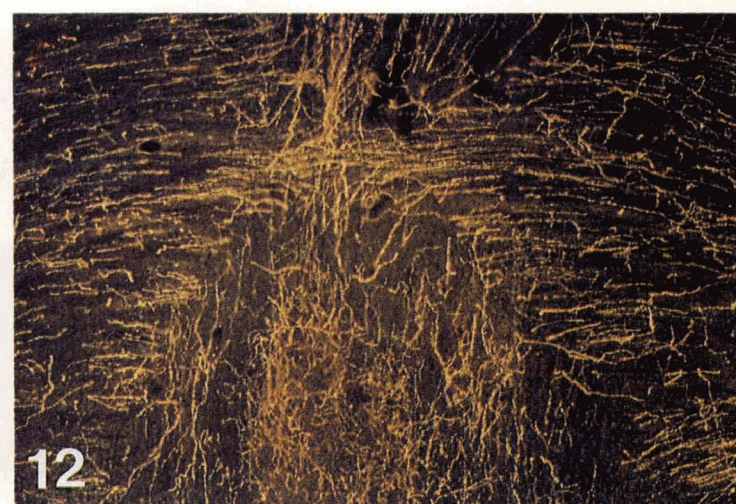
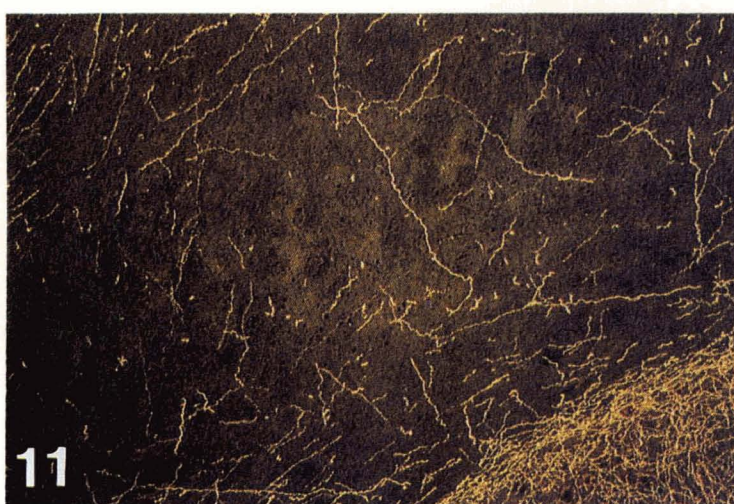
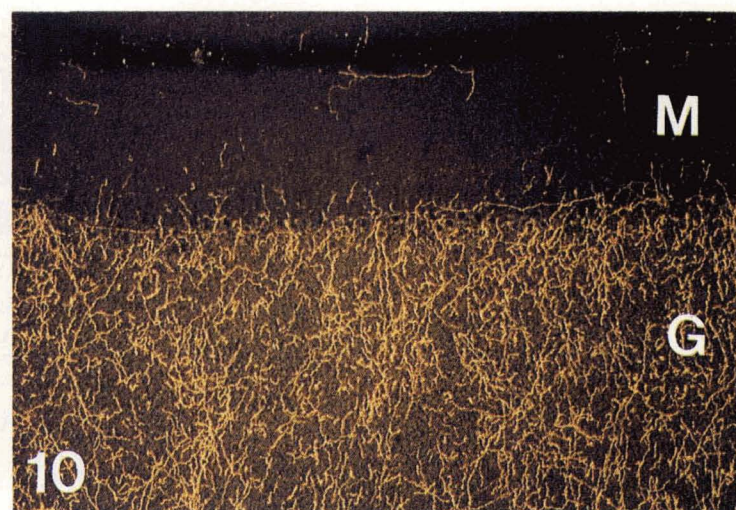
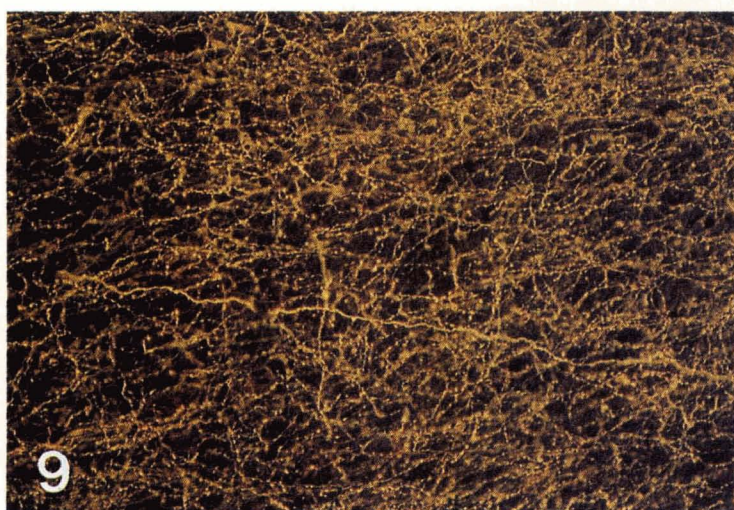
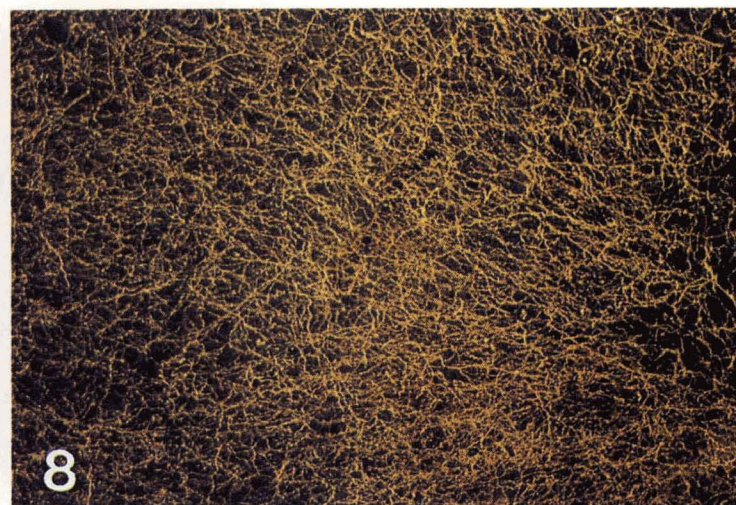
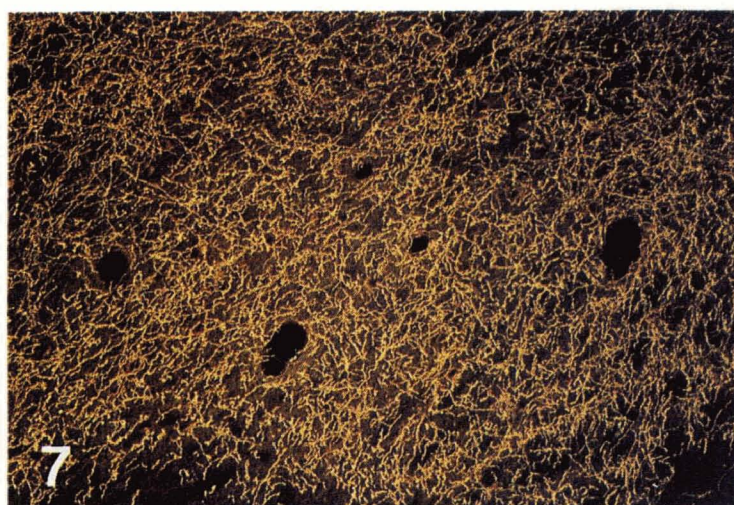


Fig. 1. CRF fibers in the fastigial nucleus. These fibers are varicose and their density is lower than that of other nuclei (x 100).  
Fig. 2. CRF fibers in the interposed nucleus. These fibers also showed varicosity (x 100).  
Fig. 3. CRF fibers in the dentate nucleus. Note the varicosity of the fibers (x 100).  
Fig. 4. CRF fibers in the dentate nucleus. Note the presence of fine fibers (x 100).  
Fig. 5. CRF fibers in the molecular layer. These fibers exhibited higher density than those of the cerebellar nuclei. M : molecular layer, G : granular layer (x 100).  
Fig. 6. Serotonin fibers in the fastigial nucleus. The caliber of fibers in this area is generally constant and labeled fibers are varicose (x 100).





**Fig. 7.** Serotonin fibers in the interposed nucleus. Labeled fibers are similar in appearance to those of Fig. 6 (x 100).

**Fig. 8.** Serotonin fibers in the dentate nucleus. The fine and thick fibers are mixed together (x 100).

**Fig. 9.** Serotonin fibers in the dentate nucleus. The fine fibers are easily detected at higher magnification (x 200).

**Fig. 10.** Serotonin fibers in the granular layer. Serotonin fibers are especially prominent in this layer. M : molecular layer, G : granular layer (x 100).

**Fig. 11.** Serotonin fibers in white matter. Occasional long fibers are seen in the white matter (x 100).

**Fig. 12.** Serotonin fibers in the cerebellar commissure. These fibers connect the right and left cerebellar nuclei (x 100).



= 국 문 초 록 =

원숭이 소뇌핵의 Corticotropin Releasing Factor와  
Serotonin 분포에 대한 면역조직화학적 연구

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이 은 영

원숭이 소뇌핵에서의 CRF와 Serotonin의 분포를 분석하고자 관류고정시킨 후 소뇌의 냉동절편을 만들어 CRF와 Serotonin에 대한 각각의 1차 항체를 적용한 후 ABC 방법으로 면역조직화학 염색을 시행하였다. CRF 신경섬유는 소뇌핵 전반에 걸쳐 분포하였다. CRF 신경섬유는 거의 모두 염주알 모양으로 염색되었으며, 꼭지핵(fastigial nuc.)에서의 분포 밀도는 다른 부위보다 낮았으며, 치아핵(dentate nuc.)에서는 보다 가는 신경섬유가 많이 분포하였다. Serotonin 신경섬유도 소뇌핵 전반에 걸쳐 분포하였으며, 전반적인 분포밀도가 CRF 신경섬유보다 높았다. Serotonin 신경섬유 역시 염주알 모양이 많았으며, 소뇌핵 별로의 분포밀도에는 차이가 없었다. 치아핵에서는 다른 부위와는 달리 가는 섬유가 많이 관찰되었다.