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Distribution of CRF Activity and Immunoreactive ACTH within the Hypothalamic-Neurophypophyseal Complex in Various Species[1] (40217)

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We previously briefly reported that in the rat the posterior pituitary and the hypophyseal stalk have a higher concentration of CRF activity than does the hypothalamic median eminence or other parts of the brain (1). To see whether this distribution is characteristic only for the rat, we have extended the studies to various other mammalian species (rat, sheep, pig, cattle, and man). We also studied the distribution of immunoassayable ACTH within the hypothalamic–neurohypophyseal complex in these species.

Materials and methods. Sprague–Dawley rats (250 g) were housed 2/cage under controlled lighting (lights on 0600–1800 hr) and temperature (24 ± 1°C). Purina laboratory chow and tap water were allowed *ad lib.* Animal quarters were not entered for 10 hr prior to each experiment to standardize the experimental conditions. Ten to 24 rats per experiment were decapitated under basal unstressed conditions within 15 s after removal from their home cage or after a given experimental procedure. Tissue fragments from various parts of the hypothalamic-pituitary complex or from frontal cerebral cortex were dissected within 3 min after the death of animals and placed in cold 0.1 *N* HCl. After the skull was opened, the brain was gently lifted and turned upside-down. The supradiaphragmatic portion of the pituitary stalk was severed with a fine scissors at its junction with the median eminence. The lower border of the dissected stalk was at the level of the pituitary diaphragm. The median eminence (ME), extending from the posterior border of the optic chiasm to the anterior border of the mammillary bodies (2 × 2 × 0.5 mm, approximately 2 mg), was then excised with a thin blade parallel to the base of the brain. Pars nervosa (PN) of the posterior pituitary was dissected from the pars intermedia tissue with fine forceps and scissors under a dissecting microscope. Cerebral cortex was excised from the frontal pole of the brain. Materials from sheep, pig, and cattle to correspond to the rat tissues mentioned above were obtained at a slaughter house. They were dissected within 40 min or less after death. The technique for dissection was essentially the same as described for the rat. Tissues were pooled from 5 to 8 animals in each species. Human tissues were obtained from two patients who died of causes unrelated to hormonal disorders at routine autopsy at the University of Oregon Health Sciences Center within 20 hr of death. Specimens were also obtained from three dogs after a lethal iv dose of pentobarbital.

Pieces of each tissue were pooled from the animals available at each dissection and homogenized in cold 0.1 *N* HCl. Tubes containing 0.1 *N* HCl were weighed before and after adding tissue fragments so that the average weight of the tissue block from one animal could be calculated for each tissue. Five mg of each tissue from each species was homogenized in 0.1 ml 0.1 *N* HCl except for rat stalk and pars nervosa where it was necessary to use a proportionately larger amount of vehicle (0.3 ml/5 mg) to minimize potential errors during homogenization–extraction procedures. The homogenates were centrifuged at 3000*g* for 30 min at 4° and the supernatants stored at −20° until use. The extracts were thawed and adjusted to pH 7.0 with 1 *N* NaOH immediately before they were tested for CRF activity. CRF bioassay was performed with an *in vitro* system using cultured rat adenohypophyseal cells and ACTH measurement by radioimmunoassay with an antibody against $ACTH^{11-24}$ (West) kindly supplied by the National Pituitary Agency (2, 3). Three-week-adrenalectomized

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female rats maintained on 0.9% saline as drinking water were employed as donors for pituitary cells in all experiments (4). All ACTH values shown in a single graph or table were measured in the same assay. Statistical comparison was made with Student's *t* test and the Newman-Keuls multiple comparison tests (5).

Results. Comparison of CRF activity of neural tissues from various species. CRF activity of extracts from 0.75 mg of hypophyseal stalk (stalk), hypothalamic median eminence (ME), pars nervosa of the posterior pituitary (PN), or cerebral cortex (cortex) were compared in rat, sheep, pig, cattle, and human beings. As shown in Fig. 1 and Table I, stalk had the highest CRF activity in all species studied, although a statistically significant difference was not demonstrated between stalk and PN in rat and pig ($P > 0.05$, Newman-Keuls). In all species studied, the rank order of the CRF potency was stalk > PN > ME > cortex, except for the human where ME was more potent than PN.

Dose-response relationships of CRF activity of ovine and porcine neural tissues. CRF activity of various neural tissues from sheep and pig were compared employing multiple doses of extracts. Again CRF activity was highest in stalk in both species ($P < 0.01$, Newman-Keuls) (Figs. 2, 3). CRF activity of stalk was

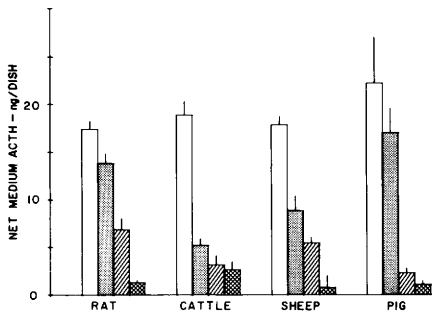

FIG. 1. ACTH secretion from cultured adenohypophyseal cells in response to extracts from 0.75 mg of hypophyseal stalk (open bars), pars nervosa of the posterior pituitary (stippled bars), hypothalamic median eminence (hatched bars), on frontal cerebral cortex (cross-hatched bars) in various species. In this and subsequent Figs., cells were cultured for 4 days and were incubated with each extract for 4 hr. Each bar and vertical line indicate mean and SE of net ACTH secretion induced by each extract in three assay dishes. Statistical comparison by Newman-Keuls test; stalk vs PN: $P < 0.01$ (cattle, sheep), $P > 0.05$ (rat, pig).

TABLE I. ACTH SECRETION FROM CULTURED ADENOHYPOPHYSEAL CELLS IN RESPONSE TO EXTRACTS FROM 1 mg OF EACH HUMAN TISSUE.[a]

	Cortex	ME	Stalk	PN
Net Medium ACTH (ng/dish)	5.4 ± 0.5	9.2 ± 0.7	19.0 ± 2.5	6.2 ± 1.0

[a] Cells were cultured for 4 days and were incubated with each extract for 4 h. Mean and SE of ACTH secretion in three assay dishes are shown. ME = hypothalamic median eminence, Stalk = hypophyseal stalk, PN = pars nervosa of pituitary. Statistical comparison by Newman-Keuls test: stalk *vs* ME: $P < 0.01$, ME *vs* PN: $P > 0.05$, PN *vs* cortex: $P > 0.05$.

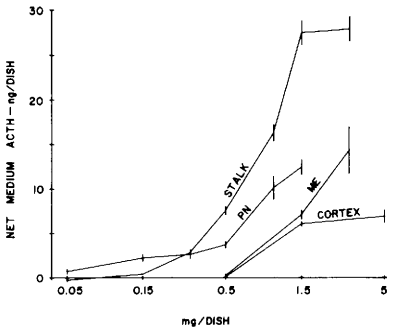

FIG. 2. ACTH secretion from cultured adenohypophyseal cells in response to graded doses of various ovine neural tissues. Stalk = hypophyseal stalk, PN = pars nervosa of the posterior pituitary, ME = hypothalamic median eminence, cortex = frontal cerebral cortex. The weight of tissue extracted for each dose is shown on the abscissa. Mean and SE of ACTH secretion in three assay dishes are shown for each point. Statistical comparison by Newman-Keuls test; stalk *vs* PN: $P < 0.01$, PN *vs* ME: $P < 0.01$.

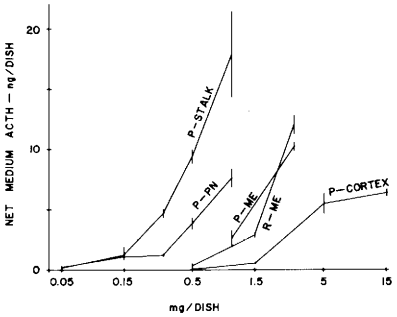

FIG. 3. ACTH secretion in response to graded doses of various porcine neural tissues. The weight of tissue extracted for each dose is shown on the abscissa. Rat median eminence extract was also studied for comparison. P− = Pig, R− = Rat. Statistical comparison by Newman-Keuls test; stalk *vs* PN $P < 0.01$, PN *vs* ME $P < 0.05$.

clearly greater than that of PN ($P < 0.01$, Newman-Keuls) in pig in this experiment.

The dose-response slopes for cortex were significantly flatter ($P < 0.02$) than those for other tissues in both species and we have interpreted this as a "nonspecific" and probably physiologically unimportant CRF effect of cortex (7).

Distribution of CRF activity within the bovine hypophyseal stalk. The supradiaphragmatic portion of the stalk (Fig. 4) was divided horizontally into two approximately equal halves (I, II) and the infradiaphragmatic portion of the stalk (III) was separated from the neural lobe of the pituitary gland. Pools of each segment were made from five animals. CRF activity of the extract from 0.75 mg of stalk tissue from each segment was compared.

The highest CRF activity was in the lower half of the supradiaphragmatic portion (II, $P < 0.01$, Newman-Keuls). The infradiaphragmatic portion (III) had the lowest CRF activity ($P < 0.05$, Newman-Keuls). The immunoreactive ACTH content in each segment of the stalk was 752 ± 104, 768 ± 72, 14954 ± 556 pg/mg for I, II, and III, respectively.

Effect of in vivo perfusion of rat brain with saline on CRF activity of median eminence or stalk. Two groups of 12 male rats were anesthetized with ether. In one group, the brain was perfused with saline before removal. The other group served as nonperfused controls. After the thoracic cage was opened the base of the ascending aorta was clamped and 20 ml of saline was injected through the aorta while the venous drainage was freely expelled from the opened heart. The brain was then removed and the base of each brain was examined with a dissecting microscope before the stalk was dissected. The stalk was extremely pale in the saline-perfused compared to the control brain, indicating a technical success. As shown in Fig. 5, CRF activity of 0.3 mg stalk or 1 mg ME was not altered by saline perfusion ($P > 0.1$). Immunoreactive ACTH content in stalk and ME in the saline-perfused group was 930 and 185 pg/mg, respectively, essentially the same as in the nonperfused control group, 967 and 155 pg/mg, respectively.

Distribution of immunoreactive ACTH activity (Table II). As described previously (2), in our CRF asssay both the ACTH contamination of the test substance and the basal ACTH secretion in the control cells are subtracted from the gross ACTH in the medium to obtain net ACTH secretion induced by the test substance. Therefore, it is important to document the concentration of ACTH in various tissues to allow some estimate of the magnitude of potential error due to contamination of the medium with ACTH added with the extract.

In Group I (rat, cattle, sheep, human), ACTH concentration within the hypothalamic-neurohypophyseal complex was relatively low, with the highest being in PN, the lowest in ME. In Group II (pig, dog) ACTH

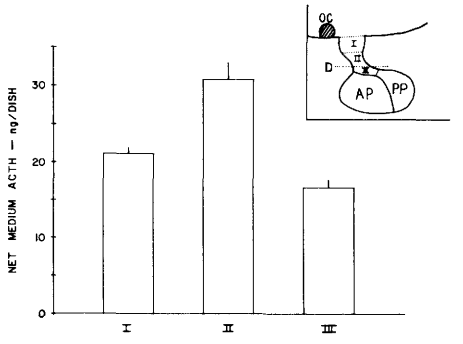

FIG. 4. ACTH secretion from cultured adenohypophyseal cells in response to extracts from 0.75 mg of each segment of bovine hypophyseal stalk as depicted in the right upper corner. Each bar and vertical line indicate mean and SE of ACTH secretion in 6 assay dishes. AP = anterior pituitary, PP = posterior pituitary, OC = optic chiasm, D = diaphragm.

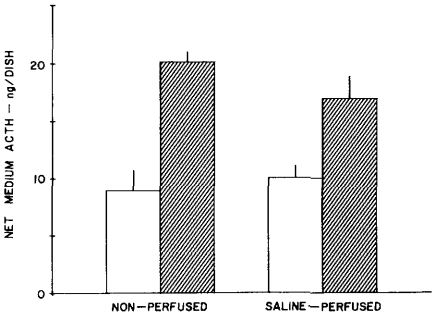

FIG. 5. ACTH secretion from cultured adenohypophyseal cells in response to extracts from 0.3 mg of rat stalk (hatched bars) or 1 mg of rat median eminence (open bars) obtained with or without prior perfusion of the brain with saline. See text for details. Tissues were pooled from 12 male rats in each group. The absolute quantity of ACTH secreted in response to a given dose of extract was greater than usual in this experiment.

TABLE II. CONCENTRATION OF IMMUNOREACTIVE ACTH IN TISSUES IN VARIOUS SPECIES (pg/mg WET WT).

	Group I				Group II	
	Rat	Sheep	Cattle	Human	Pig	Dog
Cerebral cortex	<10*	<10*	<10*		<10*	<10*
Median eminence	130	86	82	354	720	482
Stalk	496	272	380	642	29994	276250
Pars nervosa	10986	9406	4192	10074	13347	1444

* Less than detectable limit of assay.

concentration was higher in the stalk than in PN. ACTH concentration in the stalk was much higher in Group II than in Group I. There was no measureable ACTH immunoreactivity in the cerebral in any species studied.

Discussion. Our present data indicate that CRF activity, as measured by our *in vitro* bioassay, is in highest concentration in the supradiaphragmatic portion of the hypophyseal stalk in a number of mammalian species. Okon and Koch (6), employing a radioimmunoassay for gonadotropin-releasing hormone (GnRH) and thyrotropin-releasing hormone (TRH), found that these hormones were present in the highest concentration in the stalk in human, consistent with our present data for CRF. These authors also found that TRH was detectable only in the upper two-thirds of the stalk, whereas GnRH was more generally distributed throughout the stalk. CRF was found throughout the bovine stalk in our present study, although a higher concentration was found in the upper two-thirds. In more recent studies, Estes *et al.*, employing both radioimmunoassay and bioassay, showed that GnRH exists in highest concentration in the hypophyseal stalk in cattle (7). The data on the CRF concentration of rat stalk compared to that of rat PN in the present investigation are slightly different from our previous data (8), which showed similar concentration in the PN and in the stalk. This slight inconsistency in the rat presumably arises from the methodologic variations due to the tiny size of the stalk in this species. The potent CRF activity in the stalk or posterior pituitary found in the present study cannot be due to vasopressin. In our CRF assay system, neither lysine nor arginine vasopressin stimulates ACTH secretion (9). The greater CRF activity of the posterior pituitary than that of the median eminence shown in this paper agrees with some previous reports (10–13). However, as far as we are aware, there have been no previous studies in which the CRF concentration of the pituitary stalk was specifically examined. Neither the CRF activity nor the concentration of immunoreactive ACTH of the hypophyseal stalk were altered by perfusing rat brain with saline *in vivo*, suggesting that these hormones reside in the extravascular compartment in this tissue. Since the concentration of CRF activity is much higher in the stalk and the posterior pituitary than in the hypothalamic median eminence, these tissues should provide better starting material with higher specific CRF activity for the purification of CRF than the hypothalamic tissue. The average weight of one hypophyseal stalk is approximately 70 mg for cattle and 7–25 mg for human, sheep, pig, and dog.

The distribution of immunoreactive ACTH within the hypothalamic–neurophypophyseal complex was markedly different between Group I (rat, cattle, sheep, human) and Group II (pig, dog). ACTH concentration in the hypophyseal stalk was 100–1000 times greater in Group II than in Group I. The reason for this difference is not clear but it may be related to anatomical differences. In Group I the pituitary diaphragm is well-developed and clearly separates the pituitary fossa from the rest of the cranial cavity, and the stalk is relatively long. In Group II the pituitary diaphragm is poorly developed and only partially covers the pituitary gland, and the stalk is relatively short. This may facilitate migration of ACTH from the pituitary into the stalk by reverse flow in the portal vessels (14).

Summary. Using an *in vitro* CRF assay employing cultured rat adenohypophyseal cells from adrenalectomized female donors and ACTH measurement by radioimmunoassay, the distribution of corticotrophin-releasing factor (CRF) activity and immu-

noreactive ACTH within the hypothalamic–neurohypophyseal complex and cerebral cortex was compared in various mammalian species (rat, sheep, pig, dog, cattle and man). For all species studied the highest concentration of CRF activity was always in the hypophyseal stalk. ACTH concentration was higher in the pars nervosa of the posterior pituitary lobe than in the stalk or hypothalamic median eminence in rat, sheep, cattle, and man, whereas in the pig and dog ACTH concentration was higher in the stalk than in the pars nervosa or median eminence.

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