season are important considerations for a fuller understanding of these two forms of male sexual behavior.

Although the conditions thought to be important for the evolution of divergent male reproductive strategies [for example, a great differential in male reproductive success where females are not a controllable resource (*11*)] are common in anuran breeding systems; previous observations of anuran sexual behavior in the field usually have been anecdotal. Satellite male behavior has been described in fish, iguanid lizards, elephant seals (*6*), and ruffs (*11*); however, the success rates of these satellites in intercepting females are unknown. Our experimental approach not only shows that noncalling male tree frogs try to intercept females but also provides an estimate of their success. The male satellite behavior may be found in other species that utilize well-defined signals as a means of mate location and selection.

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# Adrenocorticotropin in Rat Brain: Immunocytochemical Localization in Cells and Axons

Abstract. *By means of antiserum (purified by affinity chromatography) directed against adrenocorticotropin (ACTH) 11-24, cell bodies and beaded axons were visualized in rat brain. The ACTH-like immunoreactivity (ACTH-LI) was primarily located in the hypothalamus (cells and axons). Fibers were scattered throughout thalamus, amygdala, periaqueductal gray area, and reticular formation. There was no change in the distribution of ACTH-LI in rats that had been subjected to hypophysectomy. This distribution of ACTH-LI parallels that of β-lipotropin and is altered by specific lesions in a similar fashion. The presence of ACTH-LI in cells and beaded axons in brain raises the possibility that it is a neuroregulator functioning as a neurotransmitter, neuromodulator, or neurohormone.*

Adrenocorticotropin (ACTH) is a peptide synthesized in the anterior and intermediate lobes of the pituitary gland (*1*). In addition to its role in regulation of the adrenal cortex, ACTH is thought to have a role in stress, motivation, learning, and memory (*2*), and has been reported to bind to opiate receptors in rat brain,

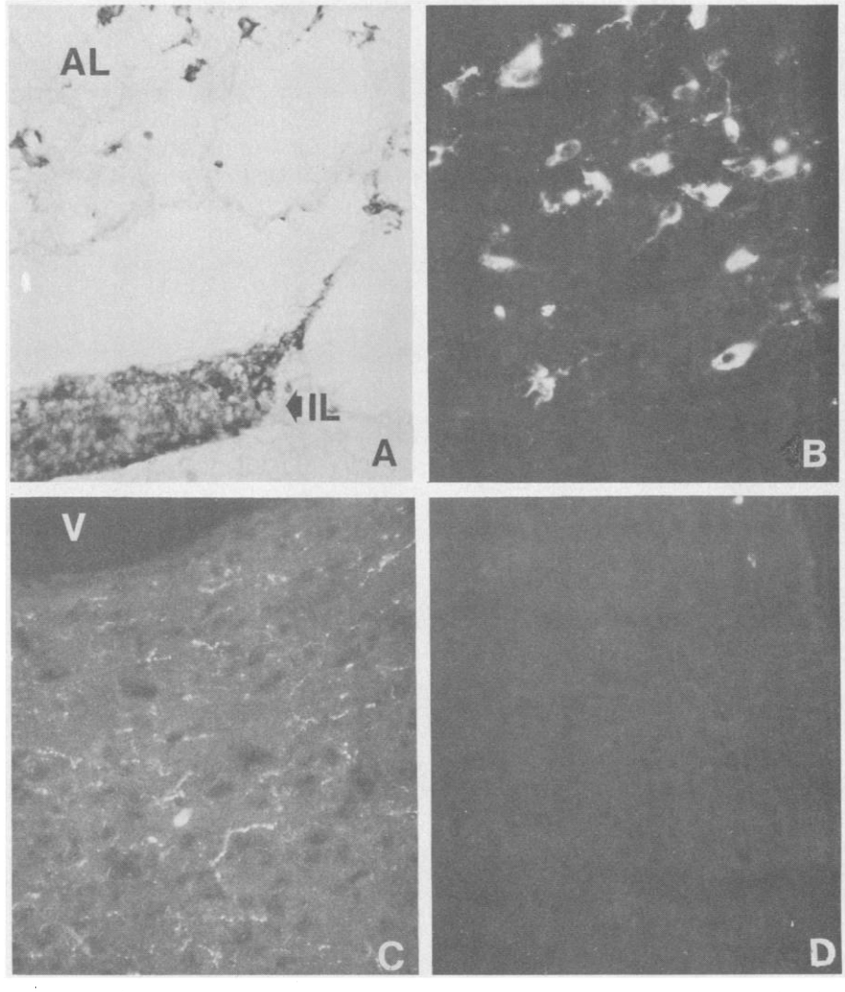


Fig. 1. (A) Adrenocorticotropin-positive cells in the anterior lobe (*AL*) and intermediate lobe (*IL*) of a normal rat pituitary showing unstained nuclei (×270; horseradish peroxidase stain). (B) Adrenocorticotropin-positive cells in the basal hypothalamus (arcuate region) of a colchicine-treated (50 μg, intracerebroventricularly) rat showing unstained nuclei (×270; fluorescein stain). (C) Adrenocorticotropin-positive fibers in the dorsomedial hypothalamic nucleus of a colchicine-treated rat. Note the fine beaded appearance (*V*, third ventricle) (×270; fluorescein stain). (D) Control for (B). Antiserum to ACTH and 100 μ*M* ACTH 1-24 was incubated on a serial section adjacent to that seen in (B). The photograph was taken of the arcuate region similiar to that in (B). There were no ACTH-positive structures in any such slides. (Colchicine-treated rat; section ×270; fluorescein stain.)


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though only at relatively high concentrations (*3*). Recently, immunoreactive ACTH has been detected in several areas of the brain of normal and hypophysectomized rats (*4*). We now report the immunocytochemical localization of this peptide in rat brain.

Six normal and three hypophysectomized male Sprague-Dawley rats were anesthetized with pentobarbital and prepared for immunocytochemistry as described (*5*). Briefly, each animal's chest was opened and a tube inserted through the left ventricle and tied into the aorta. The animal was then perfused for 30 minutes at 4°C in 4 percent formaldehyde and 0.1*M* phosphate buffer. The brain was removed, blocked, and further fixed in cold formaldehyde for 2 hours. It was then placed in a solution of 5 percent sucrose and phosphate-buffered saline for 16 hours at 4°C. The brain was frozen onto a cryostat chuck with liquid nitrogen, and sectioned at 10 $\mu$m at −17°C. The sections were incubated overnight with rabbit antiserum (purified by affinity chromatography) directed specifically against ACTH 11-24 (*6*).

In the present study the antiserum was used at a dilution of 1/150 in phosphate-buffered saline containing 0.3 percent Triton X-100. The slides were washed with phosphate-buffered saline, and incubated with either horseradish peroxidase-conjugated swine antiserum to rabbit immunoglobulin G (IgG) (*7*) or goat antiserum to rabbit IgG conjugated with fluorescein isothiocyanate (FITC). The horseradish peroxidase marker was developed with diaminobenzidine and $H_2O_2$ (*8*). For immunocytochemical controls we used synthetic ACTH 1-24 (*9*) in excess (100 n*M*) to block the antibodies and prevent specific binding to brain ACTH. The ACTH positive staining occurred in pituitary (Fig. 1A) and brain (Fig. 1, B and C). All the ACTH-LI appeared to be specific, in that it was blocked by ACTH 1-24 (Fig. 1D) but remained unaltered by 200 n*M* human $\beta$-lipotropin ($\beta$-LPH), human $\beta$-endorphin, $\beta$-melanocyte stimulating hormone ($\beta$-MSH), methionine-enkephalin and leucine-enkephalin.

Adrenocorticotropin-like immunoreactivity (ACTH-LI) was detected in cells and fine beaded axons throughout the brainstem (Fig. 1, B and C, and Table 1). The area of heaviest concentration was the medial basal hypothalamus. Positively stained cell bodies occurred in the arcuate nucleus and in areas just lateral to that nucleus (Fig. 1, B and C). Dense fiber distributions were seen surrounding the third ventricle with lighter activity in evidence throughout the hypothalamus (Fig. 1C). A dense pattern of fibers was also seen in the periventricular nucleus of the thalamus. Moderate numbers of positive fibers were detected in the medial amygdaloid nucleus, ansa lenticularis, and the periaqueductal central gray area. Positive fibers were scattered throughout the reticular formation. No ACTH-LI was detected in the cerebral cortex, cerebellar cortex, or hippocampus. The same anatomical distribution was observed in animals that had been subjected to hypophysectomy 10 days prior to examination.

In the rat pituitary, ACTH is synthesized and released by cells in the anterior and intermediate lobes. These same cells also produce $\beta$-lipotropin (*10, 11*), a potential biosynthetic precursor for methionine-enkephalin and $\beta$-endorphin (*12*) (endogenous opiate peptides). Pelletier *et al.* (*11*) have shown that ACTH and $\beta$-LPH are located in the same pituitary cells and even the same granules. Recent studies on the structure of the high-molecular-weight precursor to ACTH observed in the mouse pituitary tumor cell line AtT-20/D-16V, have suggested that ACTH, $\beta$-LPH, and $\beta$-endorphin are synthesized as part of one common 31,000-dalton glycoprotein precursor (*13*). Finally, immunoreactive $\beta$-LPH has been detected in bovine and rat brain (*14*). We have shown that brain $\beta$-LPH-like-immunoreactivity is found in cells and beaded axons of the rat brain and have described a system of $\beta$-LPH–containing structures which appears anatomically distinguishable from the enkephalin system (*15*). These various observations suggest that $\beta$-LPH and ACTH share a biosynthetic precursor.

Table 1. Comparison of the localization of ACTH-like immunoreactivity and $\beta$-lipotropin-like immunoreactivity in rat pituitary as indicated by immunohistochemical studies.

| Area | ACTH | $\beta$-LPH* |
|---|---|---|
| Pituitary | | |
| Anterior lobe | Yes | Yes |
| Intermediate lobe | Yes | Yes |
| Posterior lobe | No | No |
| Medulla | | |
| N. tractus solitarius | Yes | Yes |
| N. lateral recticular formation | Yes | Yes |
| N. ambiguus | No | No |
| N. VII | No | No |
| Pons | | |
| N. locus coeruleus | Yes | Yes |
| N. parabrachealis | Yes | Yes |
| N. V | No | No |
| Mesencephalon | | |
| Periaqueductal gray area | Yes | Yes |
| Reticular formation | Yes | Yes |
| N. interpeduncularis | No | No |
| Diencephalon | | |
| Zona incerta | Yes | Yes |
| Medial habenula | No | No |
| Stria terminalis | Yes | Yes |
| Medial hypothalamus | Yes | Yes |
| Lateral hypothalamus | Yes | Yes |
| Arcuate nucleus and periarcuate area | Yes (cells) | Yes (cells) |
| Median eminance | Yes | Yes |
| N. paraventricular thalamus | Yes | Yes |
| N. suprachiasmatic | No | No |
| Telencephalon | | |
| Globus pallidus | No | No |
| Lateral septum (ventral) | Yes | Yes |
| N. accumbens | Yes | Yes |
| Caudate putamen | No | No |
| Amygdala (medial) | Yes | Yes |

*See Watson *et al.* (*15*).

The antiserum directed against ACTH does not cross react with $\beta$-LPH, and that directed against $\beta$-LPH does not cross react with ACTH (*13, 15, 16*); yet the patterns of immunoreactive staining with the two antiserums were very similar (Table 1). Both antiserums demonstrate cells located in the arcuate and periarcuate areas of rat hypothalamus; these cells appear to be distributed in very similar fashion. Further, both antiserums demonstrate beaded axons of similar size and morphology. Using the light microscope and serial sections, we found that ACTH-LI and $\beta$-LPH-like activity was distributed in a very similar fashion throughout the brain (Table 1). When hypothalamic lesions were made to destroy the $\beta$-LPH positive cells on one side of rat brain, the staining for ACTH and $\beta$-LPH fibers was reduced by about the same degree.

The demonstration of ACTH-LI in rat brain depends entirely on the specificity of the antiserums used; this demonstration was blocked by ACTH 1-24 and not by $\beta$-MSH, $\beta$-endorphin, $\beta$-lipotropin, methionine- or leucine-enkephalin. Furthermore this antibody demonstrates specifically the ACTH-containing cells in the anterior and intermediate lobes of the pituitary. However, it is possible other substances might be visualized inadvertently. Our present findings, along with those of Kreiger *et al.* (*4*), raise the possibility that ACTH may have neurotransmitter or neurohormonal roles in brain as well as the pituitary axis.

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## Do Albumin Clocks Run on Time?

About 10 years ago, Sarich and Wilson (*1*) presented evidence from modern primates that serum albumin proteins change at a regular rate, and proposed that observed differences in albumins between species could be used to estimate times of divergence and help reconstruct phylogenies. Despite some criticisms (*2*), the approach has become widely accepted, and current workers cite an impressive body of evidence from a variety of vertebrates [for example, ranid and hylid frogs (*3, 4*), iguanid lizards and crocodylians (*5*), marsupials (*4*), placental carnivores (*6*), and primates (*7, 8*)] that serum albumins change at a regular rate, calculated to be 0.6 million years (m.y.) per immunological distance unit (IDU) (*9*). Demonstration of regularity in albumin evolution and calibration of rate of change ultimately rest on interpretation of paleontological evidence. Contrary to published assertions, the fossil record provides little evidence for the accepted calibration rate, or even for the hypothesis that serum albumins evolve at a regular rate.

The currently accepted albumin clock rate is from Sarich (*7*), based on comparisons between prosimian and anthropoid primates. A mean immunological distance (ID) of about 100 (*10*) and an estimated time of divergence between modern prosimians and anthropoids of about 60 m.y. yielded a rate of 1 IDU per 0.6 m.y. (or 1.67 IDU per m.y.). Two years later, Sarich [table 3 in (*8*)] presented data that increased the prosimian versus anthropoid mean ID to 125, but he retained the original rate of 1 IDU/0.6 m.y., and that is the rate that all later papers cite. I have summarized Sarich's (*7, 8*) primate ID data in Table 1, along with ranges of estimated times of divergence based on current interpretations of the fossil record (*11*), and the resulting estimated rates of albumin change calculated from the mean ID's. The minimum likely rate from the latest prosimian versus anthropoid comparison (1 IDU/0.48 m.y.) is higher than the maximum likely rates suggested by various higher primate comparisons (around 1 IDU/0.60) m.y.). A date older than 60 m.y. for the prosimian-anthropoid split is highly unlikely since at that time (middle Paleocene), the first primate radiation had just gotten under way and modern primates arose from a second, later radiation (*12*). Dates younger than the minimum estimated times of divergence for the various anthropoid comparisons listed in Table 1 are highly unlikely, since by those times (or very shortly thereafter), we have undoubted fossils of the relevant groups involved. Thus the prosimian versus anthropoid data suggest a rate of albumin change that is in contradiction with rates suggested by data from higher primate comparisons. Despite this internal inconsistency, those data remain the cornerstone for the albumin clock calibration.

The placental carnivore data cited as evidence for a rate of serum albumin change of 1 IDU/0.6 m.y. are canoid versus feloid comparisons (*6*) that showed a mean ID of 89. Given the range of possible times of divergence suggested by the fossil record (see Table 1), one can say only that those data are not in contradiction with a rate of 1 IDU/0.6 m.y. However, a full analysis of the data from that study shows within the canoids a *Canis* versus arctoids mean ID of 46. That yields a maximum rate (1 IDU/0.80 m.y.) that is lower than the minimum likely rate of the canoid versus feloid split (1 IDU/0.67 m.y.). It is highly unlikely that canoids and feloids diverged earlier than 60 m.y., since at that time the miacid radiation that later gave rise to modern carnivores was just beginning (*12*), and it is also unlikely that *Canis* and arctoid stocks diverged later than 37 m.y., since about that time we have the earliest members of those groups. Thus,

Table 1. Immunological distances and the fossil record.

| Pair | ID | | Estimates from | |
|---|---|---|---|---|
| | Mean | Range | Fossil record divergence dates (m.y.) | Albumin clock rates (m.y./IDU) |
| Three prosimians versus three anthropoids (*7*) | 103 | 93 to 112 | 45 to 60 | 0.44 to 0.58 |
| Six prosimians versus five anthropoids (*8*) | 125 | 115 to 131 | 45 to 60 | 0.36 to 0.48 |
| Eleven ceboids versus five catarrhines (*7*) | 59 | 43 to 70 | 35 to 55 | 0.59 to 0.93 |
| Six cercopithecoids versus five hominoids (*7*) | 35 | 28 to 42 | 20 to 30 | 0.57 to 0.86 |
| Two gibbons versus four other hominoids (*8*) | 13 | 12 to 15 | No good evidence | |
| *Homo* versus *Pan* + *Gorilla* (*8*) | 8 | 7 to 9 | 5 to 20 | 0.62 to 2.5 |
| Four canoids versus three feloids (*6*) | 89 | 69 to 105 | 37 to 60 | 0.42 to 0.67 |
| *Canis* versus six arctoids (*6*) | 46 | 31 to 56 | 37 to 60 | 0.80 to 1.30 |


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