Heterogeneity of Vertebrate Luteinizing Hormone–Releasing Hormone

Abstract. *Radioimmunoassay and chromatography analyses of hypothalamic luteinizing hormone-releasing hormone (LHRH) have demonstrated the presence of LHRH-like immunoreactive peptides in a wide range of vertebrates. Contrary to previous reports, the molecule differs in various vertebrates. Avian, reptilian, and teleostean LHRH's are chemically distinct from the mammalian peptide but are in themselves indistinguishable. However, amphibian LHRH appears to be identical to the mammalian peptide. These findings have interesting evolutionary implications.*

Immunoreactive luteinizing hormone-releasing hormone (LHRH) has been demonstrated in the hypothalamus of mammals, birds, amphibians, teleost fish, and cyclostomes (*1–4*). Some studies, in which bird and amphibian LHRH was found to be identical to mammalian LHRH (*2, 3*), suggest that the amino acid sequence of the LHRH decapeptide has been highly conserved during 400 million years of evolution. This contrasts with the considerable variation that has occurred in the related neurohypophysial peptides (oxytocin and vasopressin family) during vertebrate evolution (*5*). We investigated the nature of hypothalamic LHRH in representative species from the five major vertebrate classes (Mammalia, Aves, Reptilia, Amphibia, and Pisces), and demonstrate that, contrary to previous reports (*2, 3*), LHRH in these vertebrates differs in physicochemical properties.

Hypothalami from various vertebrate species were extracted and assayed for LHRH immunoreactivity by means of antiserums to specific regions of LHRH. For effective binding, antiserum 1076 requires the residues Ser^4 to Leu^7, antiserum 743 residues Trp^3 to Pro^9, and antiserum 744 residues Leu^7 to Gly^{10} of the decapeptide (*6–8*). The curves of displacement of ^{125}I-labeled LHRH from antiserum 1076 by synthetic mammalian LHRH and by hypothalamic extracts are illustrated in Fig. 1. Identical results were obtained with antiserums 743 and 744 in assays performed on several occasions and on different extracts. The displacement curves for rat and frog hypothalamic extracts were parallel to the curve for synthetic LHRH, suggesting that in these species the molecule is immunologically indistinguishable from synthetic LHRH. The immunoreactive LHRH content in the rat and frog was 7.5 ± 1.1 (mean ± standard error) and 10.6 ± 3.3 ng per hypothalamus, respectively, in accordance with previous reports (*1, 3*). Displacement curves for pigeon, tortoise, teleost hypothalamic extracts were nonparallel to those of synthetic LHRH, suggesting that LHRH in these species differs from the mammalian and amphibian peptide. Moreover, pigeon, tortoise, and teleost hypothalamic extract displacement curves were parallel to each other, suggesting a possible similarity in the structure of their LHRH. Pigeon hypothalami contained 1.0 ± 0.1 ng of LHRH per hypothalamus, tortoise hypothalami 3.4 ± 1.8 ng, and teleost hypothalami 1.1 ± 0.1 ng. Elasmobranch hypothalamic extracts elicited no decrease in binding of labeled antigen in the assay illustrated in Fig. 1, but immunoreactive LHRH (0.1 ng per hypothalamus) was detected in elasmobranch extracts in two other assays. Insufficient displacement occurred with these extracts to allow assessment of parallelism.

Although nonparallelism of displacement curves suggests structural differences in LHRH, this could have been caused by contaminants in the hypothalamic extracts, such as LHRH degrading peptidases and substances binding LHRH. However, this possibility can be excluded since ^{125}I-labeled LHRH was neither degraded nor bound by pigeon and teleost hypothalamic extracts; a similar result was obtained previously with acid extracts of ovine hypothalamus (*8*). Addition of bacitracin, which inhibits hypothalamic peptidases (*9*), had no effect on the assayed values of LHRH in hypothalamic extracts or on the slopes of the displacement curves. Moreover, pigeon and teleost hypothalamic immunoreactive LHRH purified by affinity chromatography still yielded displacement curves that were nonparallel to the curve for synthetic mammalian LHRH.

To confirm that the structure of immunoreactive LHRH differed in the vertebrate classes, we subjected hypothalamic extracts to chromatographic analysis. The LHRH immunoreactive peptides from the various vertebrates differed in their mobility on a cellulose CM-32 column (Fig. 2). For each hypothalamic extract, run on the same column, there was a single major immunoreactive peak. The elution volumes of rat and frog hypothalamic extracts were identical to the elution volume of synthetic LHRH (115 to 170 ml). Pigeon, tortoise, and teleost

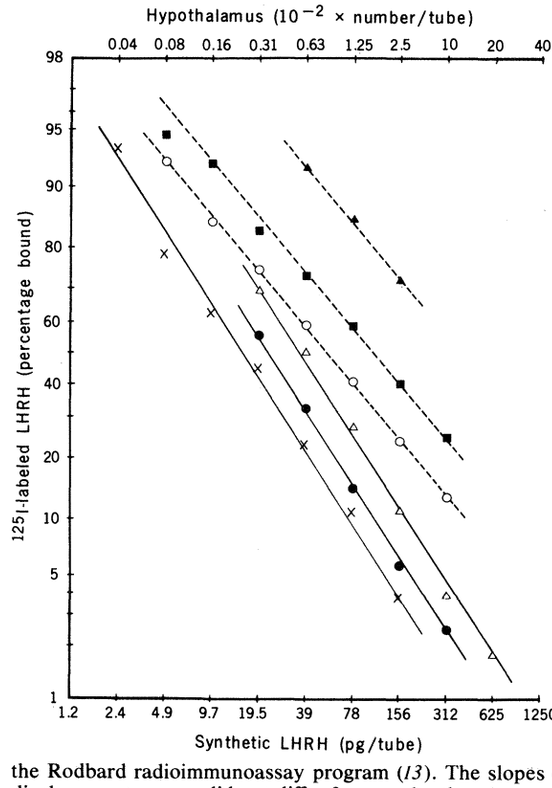

Fig. 1. Comparative displacement of ^{125}I-labeled LHRH from antiserum 1076 by synthetic LHRH and by hypothalamic extracts. Hypothalami were removed from animals immediately after decapitation. Groups of two to three hypothalami were pooled, extracted with 2*N* acetic acid, lyophilized, and reconstituted in radioimmunoassay buffer (*7*). Assays were performed three to four times on different hypothalamic extracts. The linearized (logit transformed) displacement curves are shown. Symbols: ×, synthetic LHRH; ●, rat (Long Evans); ○, pigeon (*Columbia livia*); ▲, tortoise (*Chersine angulata*); △, frog (*Xenopus laevis*); and ■, teleost (*Sarotherodon mossambicus*). Extracts of elasmobranch (*Poroderma africanum*) hypothalami induced no displacement of ^{125}I-labeled LHRH in this assay. The slopes of the displacement curves of synthetic, rat, and frog LHRH were not significantly different as tested by the Rodbard radioimmunoassay program (*13*). The slopes of the pigeon, tortoise, and teleost displacement curves did not differ from each other, but were significantly different from those of the synthetic, rat, and frog curves ($P < .05$). The displacement curves of chicken (*Gallus domesticus*), lizard (*Mabuya capensis*), and toad (*Bufo gariepensis*) hypothalamic extracts were identical to those of pigeon, tortoise, and frog, respectively (separate study).

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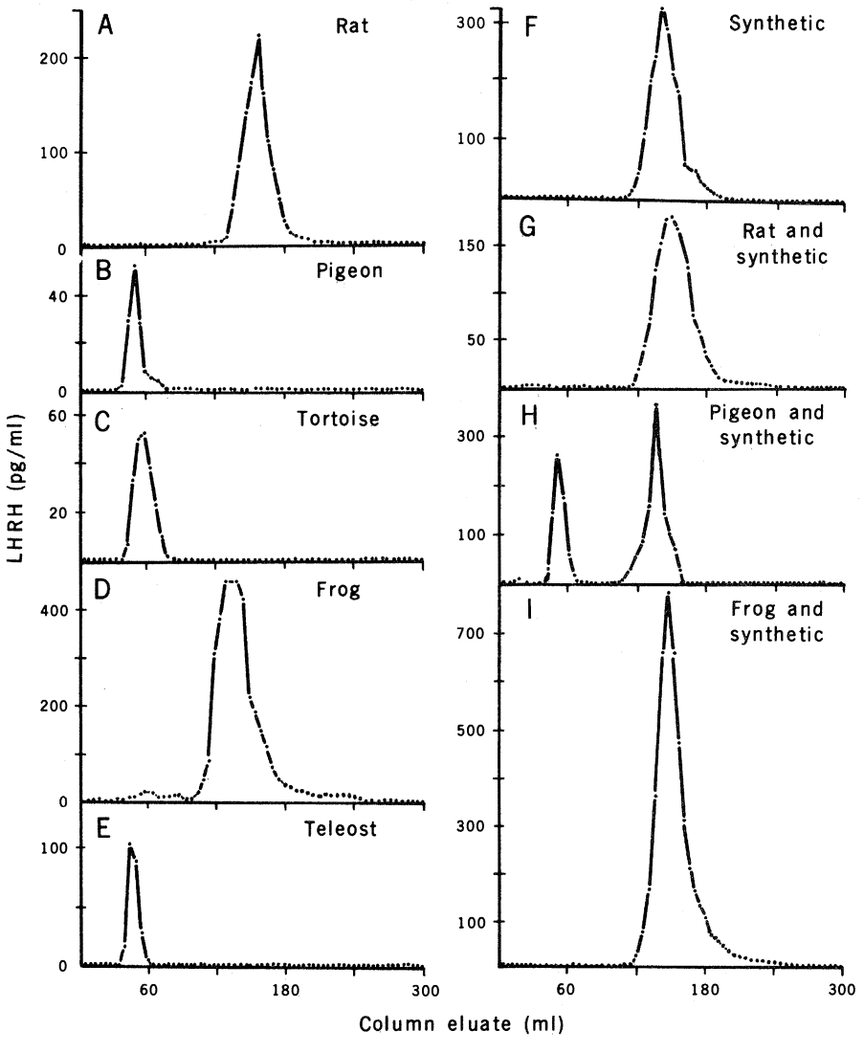
immunoreactive LHRH's, however, consistently eluted much earlier (40 to 60 ml). When synthetic LHRH was added to hypothalamic extracts of some species and rechromatographed, rat and frog LHRH's could not be distinguished from synthetic LHRH, whereas pigeon, tortoise, and teleost LHRH's were again clearly separated from the synthetic material. On high-pressure liquid chromatography, frog immunoreactive LHRH purified by affinity chromatography coeluted with synthetic LHRH (26.4 to 28.8 ml). When tested with the same procedure, chicken and teleost immunoreactive LHRH eluted earlier (22.4 to 24.4 ml), thus confirming the differences shown on cation-exchange chromatography.

Jeffcoate *et al.* (*2*) found that chicken immunoreactive LHRH was indistinguishable from synthetic mammalian LHRH in gel filtration and cation-exchange chromatography and concluded that avian LHRH was the same as, or at least similar to, that of mammals. We suggest that the higher molarity buffer used by Jeffcoate *et al.* (*2*) may account for the earlier elution of synthetic LHRH from a cellulose CM-32 column and for the inability of these workers to separate the two molecular species. Our data are supported by the studies of Jackson (*10*) who, using a lower molarity buffer, demonstrated that LHRH biological activity in chicken hypothalamic extracts eluted earlier from a cellulose CM-32 column than did rat LHRH.

The presence of LHRH-like material in reptiles and in elasmobranch fish has not to our knowledge been described previously, and the present findings establish the universal distribution of LHRH-like peptides in vertebrates. Amphibian hypothalamic immunoreactive LHRH is indistinguishable from the mammalian peptide. However, avian, reptilian, and teleostean hypothalamic immunoreactive LHRH's are chemically distinct from the mammalian and amphibian peptide, both in their interaction with specific antiserums and in chromatographic properties. Moreover, the present study suggests that bird, reptile, and teleost LHRH's are similar compounds.

Our observations have interesting evolutionary implications. The finding that amphibian LHRH is identical to the mammalian peptide and yet different from avian, reptilian, and teleostean LHRH's, which are in themselves indistinguishable by the methods used, supports a contemporary phylogenetic scheme suggesting that mammals and amphibians may be more closely related than are mammals on the one hand and reptiles and birds on the other (*11*). An explanation for our findings is that the



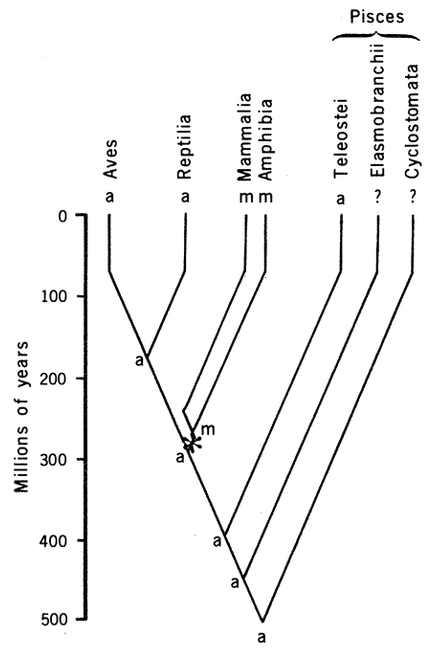


Fig. 2 (left). Elution profiles of hypothalamic immunoreactive LHRH on cation-exchange chromatography. Lyophilized extracts of single hypothalami were reconstituted in 0.002*M* ammonium acetate (NHAc) buffer, *p*H 4.5, applied to a cellulose CM-32 column (1 by 18 cm), and eluted initially with 20 ml of 0.002*M* NHAc buffer, and then 0.06*M* NHAc buffer, *p*H 4.5, at room temperature. Fractions were lyophilized and reconstituted in radioimmunoassay buffer for assay. (A to E) Extracts of a single hypothalamus; (F) 10 ng of synthetic LHRH; (G to I) hypothalamic extracts plus synthetic LHRH. In (H) pigeon extract immunoreactive LHRH was first purified by affinity chromatography to remove contaminating material that might interfere in elution. Recovery of synthetic LHRH was 98 percent. Chicken, lizard, and toad LHRH eluted in identical positions to pigeon, tortoise, and frog LHRH, respectively (separate study). Fig. 3 (right). Evolution of LHRH in vertebrates. The letters *m* (mammalian) and *a* (avian) represent the type of LHRH that is thought to have occurred at various diverging points in evolution, and the type that has been identified in extant species from each group. Question marks indicate that LHRH is present but the type has not been identified. The asterisk indicates the time at which mutation of the ancestral LHRH may have occurred.

LHRH in present-day birds, reptiles, and teleosts might represent the ancestral LHRH molecule that occurred in the ancient osteichthyes and has persisted for about 400 million years. A single mutation in the "parent" molecule might have occurred in a lineage common to amphibians and mammals after its divergence from the common stock from which the extant birds and reptiles arose, and this mutant molecule may have given rise to the mammalian-type analog in extant amphibians and mammals (Fig. 3). In spite of the physicochemical differences in vertebrate immunoreactive LHRH, the biologically active region of the molecule has clearly been conserved during evolution since we have shown (*12*) that when it is purified by affinity chromatography, the hormone from all species stimulates the release of luteinizing hormone from sheep anterior pituitary cells in culture.

J. A. KING

Department of Zoology, University of Cape Town, Rondebosch, 7700, Cape Town, Republic of South Africa

R. P. MILLAR

Department of Chemical Pathology, University of Cape Town Medical School, and *Groote Schuur Hospital, Observatory, 7925, Cape Town*

References and Notes

1. A. V. Schally, A. Arimura, A. J. Kastin, *Science* **179**, 341 (1973).
2. S. L. Jeffcoate, P. J. Sharp, H. M. Fraser, D. T. Holland, A. Gunn, *J. Endocrinol.* **62**, 85 (1974).
3. L. C. Alpert, J. R. Brawer, I. M. D. Jackson, S. Reichlin, *Endocrinology* **98**, 910 (1976); D. J. Deery, *Gen. Comp. Endocrinol.* **24**, 280 (1974).
4. H. J. T. Goos and O. Murathanoglu, *Cell Tissue Res.* **181**, 163 (1977); J. W. Crim, A. Urano, A. Gorbman, *Am. Zool.* **18**, 614 (abstr.) (1978); I. M. D. Jackson, *ibid.*, p. 385.
5. R. Acher, J. Chauvet, M. T. Chauvet, *Eur. J. Biochem.* **29**, 12 (1972).
6. Antiserums 743 and 744 were kindly donated by A. Arimura. The abbreviations Ser, Leu, Trp, Pro, and Gly are for serine, leucine, tryptophan, proline, and glycine, respectively.
7. S. Hendricks, R. Millar, B. Pimstone, *S. Afr. Med. J.* **49**, 1559 (1975).
8. R. P. Millar, P. Denniss, C. Tobler, J. C. King, A. V. Schally, A. Arimura, in *La biologie cellulaire des processus neurosecretoires hypothalamiques*, C. Kordon and J. D. Vincent, Eds. (Publication 280, Centre National de la Recherche Scientifique, Paris, 1978), p. 487.
9. J. F. McKelvy, P. Leblanc, C. Laudes, S. Perrie, Y. Grimm-Jorgensen, C. Kordon, *Biochem. Biophys. Res. Commun.* **73**, 507 (1976).
10. G. L. Jackson, *Endocrinology* **89**, 1460 (1971).
11. A. S. Romer, *Vertebrate Paleontology* (Univ. of Chicago Press, Chicago, ed. 3, 1966), pp. 24–206; P. Licht, H. Papkoff, S. W. Farmer, C. H. Muller, H. W. Tsui, D. Crews, *Recent Prog. Horm. Res.* **33**, 169 (1977).
12. J. A. King and R. P. Millar, in preparation.
13. D. Rodbard, in *Principles of Competitive Protein Binding Assays*, W. D. Odell and W. H. Daughaday, Eds. (Lippincott, Philadelphia, 1971), p. 204.
14. We thank G. N. Louw and M. C. Berman for encouragement. The work was financed by grants from the University of Cape Town, the Medical Research Council, the Council for Scientific and Industrial Research, and the Atomic Energy Board of the Republic of South Africa. L. Purves supplied the Rodbard program and Ayerst Laboratories the synthetic LHRH.

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Cerebral Norepinephrine: Influence on Cortical Oxidative Metabolism in situ

Abstract. *Unilateral lesion of the locus coeruleus and the resultant norepinephrine depletion in the ipsilateral cerebrum alters the relationship between cerebral metabolic demands and local delivery of oxygen and substrates. This effect of norepinephrine depletion is demonstrated by slower recovery of the redox ratio of cytochrome* a,a_3 *during increased metabolic demands induced by local cortical stimulation.*

Monoamines, norepinephrine in particular, are putative central nervous system (CNS) neurotransmitters or modulators. Various physiological functions of the CNS ranging from attentiveness and learning to regulation of muscle tone may be influenced by central noradrenergic mechanisms (*1*). Several states of CNS dysfunction, including mania and depression, appear to be associated with altered metabolism of norepinephrine (*2*). Many of the pharmacologic agents that profoundly affect CNS function, such as antidepressant drugs, amphetamine, and cocaine, are thought to mediate their effects by modifying central norepinephrine metabolism (*3*). Furthermore, cerebral blood flow and cerebrovascular permeability may be regulated by central noradrenergic neurons originating in the locus coeruleus (LC) (*4*). However, it is not known whether this represents a direct effect of noradrenergic neurons on cerebral vasculature or a secondary phenomenon due to an alteration of cerebral metabolic demands.

We have studied the question of whether norepinephrine has an influence on oxidative energy metabolism of the cerebral cortex. We took advantage of the fact that most, if not all, of the noradrenergic supply of the mammalian cerebral cortex emanates from a compact nucleus of noradrenergic cells in the ipsilateral brainstem, the nucleus LC (*1*, *5*). Discrete lesion of the LC offers the opportunity to ascertain the effects of selective cerebral cortical norepinephrine depletion. In view of the evidence that LC neurons are most active during periods of stress and increased alertness and, conversely, are inactive during periods of drowsiness or depressed cortical functioning (*1*, *6*), we elected to study the metabolic effects of LC lesions under conditions of increased cerebral activity induced by direct electrical stimulation of the cerebral cortical surface. To accomplish this, we required a signal of oxidative metabolic activity that allowed kinetic interpretation in intact cerebral cortical tissue. The development of dual wavelength reflection spectrophotometry of changes in the redox ratio of cytochrome a,a_3 offered this ability, because the cytochrome redox ratio has been shown to vary with the tissue respiratory state (*7*).

Experiments were performed on male Wistar rats (250 to 350 g). Unilateral LC lesions were made on either side by local injection of 6-hydroxydopamine as described previously (*8*) with minor modifications. Littermate rats without lesions served as controls. Two weeks later, the rats were anesthetized with sodium pentobarbital (50 mg/kg, intraperitoneally), tracheotomized, paralyzed with curare, and artificially respired with a mixture of 30 percent oxygen and 70 percent nitrogen. Holes, approximately 5 mm in diameter, were drilled in the skull on either side over the frontoparietal cortex leaving the dura intact. Cytochrome a,a_3 redox ratio changes were monitored within fields 3 mm in diameter on either cortex. In half of the rats, recordings were first made on the side ipsilateral to the LC lesion. The cortical surface was stimulated (2-second trains, 20 Hz, 0.5-msec square-wave pulse duration) through forked electrodes 2 mm apart positioned extradurally at the center of the 3-mm optical field. A stainless steel electrode, referenced to neck muscle, was used to monitor shifts in the cortical steady potential.

The spectrophotometric procedure is based on the fact that reduced cytochrome a,a_3 absorbs more light (reflects less) at 605 nm than does its oxidized form. The cerebral cortex was illuminated at 605 nm and also at 590 nm. Reflectance at the latter wavelength provided a reference compensation for changes in light scattering, blood volume and hemoglobin saturation. The voltage record of the reference (590 nm) reflection was subtracted from that of the 605-nm reflection to provide the more accurate cytochrome a,a_3 signal. Changes in cytochrome a,a_3 redox ratios and the compensation signal were displayed on a chart recorder. Because this signal at 590 nm is near the isobestic point for hemoglobin oxygenation-deoxygenation shifts, it has been considered indicative of blood volume changes within the optical field (*7*). Records from the cerebral cortex ipsilateral to the LC lesion were compared to those from the contralateral cortex of the rats with lesions and to

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