EVOLUTION OF THE STRUCTURE AND FUNCTION OF VERTEBRATE BRAIN GONADOTROPIN-RELEASING HORMONE

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SUMMARY

In this study, the structure and function of gonadotropin-releasing hormone (GnRH) in different vertebrate species, in the classes Aves, Reptilia and Pisces was investigated. Acetic acid extracts were subjected to gel filtration chromatography and semipreparative high performance liquid chromatography (HPLC) to partially purify the GnRHs. The GnRH immunoreactivity was then characterized by analytical HPLC, and by assaying HPLC fractions by radioimmunoassay with region-specific antisera generated against mammalian GnRH, Gln^8-GnRH and Trp^7,Leu^8-GnRH and assessing luteinizing hormone (LH)-releasing activity of fractions in a chicken dispersed anterior pituitary cell bioassay.

Five GnRH molecular forms have thusfar been structurally characterized in vertebrate brain. In mammals a GnRH with the structure pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2 has been demonstrated in the hypothalamus (Matsuo *et al.*, 1971; Burgus *et al.*, 1972). Gln^8-GnRH and His^5,Trp^7,Tyr^8-GnRH were present in chicken hypothalamus (King and Millar, 1982a, 1982c; Miyamoto *et al.*, 1983, 1984), Trp^7,Leu^8-GnRH in salmon brain (Sherwood *et al.*, 1983) and $Tyr^3,Leu^5,Glu^6,Trp^7,Lys^8$-GnRH in lamprey brain (Sherwood *et al.*, 1986).

In ostrich (*Struthio camelus*) hypothalamus two GnRHs with identical properties to Gln^8-GnRH and His^5,Trp^7,Tyr^8-GnRH have been demonstrated, as well as four other LH-releasing factors with different chromatographic and immunological properties to any of the known naturally-occurring GnRHs. Since Gln^8-GnRH and His^5,Trp^7,Tyr^8-GnRH were also present in chicken hypothalamus it appears likely that these two GnRHs occur in all birds.

In alligator (Alligator mississippiensis) brain only two GnRHs were detected. These forms co-eluted with Gln^8-GnRH and His^5,Trp^7,Tyr^8-GnRH in two HPLC systems. They cross-reacted similarly to the two synthetic peptides with antisera directed against mammalian GnRH and Gln^8-GnRH and released LH from chicken dispersed anterior pituitary cells in a similar manner to the synthetic peptides. The Archosaurs (alligators and crocodiles) are believed to be closely related to birds and therefore it seems likely that they should have identical GnRHs. In skink (Calcides ocellatus tiligugu) brain one GnRH, which co-eluted with His^5,Trp^7,Tyr^8-GnRH, was demonstrated. Two other lizards (Cordylis nigra and Pordarcis s. sicula) have been studied (Powell et al., 1985; R.C. Powell, G. Ciarcia, V. Lance, R.P. Millar and J.A. King, submitted). In C. nigra four immunoreactive GnRHs were detected, two of which co-eluted with, and released chicken LH similarly to, Trp^7,Leu^8-GnRH and His^5,Trp^7,Tyr^8-GnRH. In P. s. sicula a GnRH molecular form similar to Trp^7,Leu^8-GnRH occurred as well as two novel GnRHs. It thus appears that Gln^8-GnRH does not occur in lower reptiles, but His^5,Trp^7,Tyr^8-GnRH and/or Trp^7,Leu^8-GnRH do. His^5,Trp^7,Tyr^8-GnRH appears to be a widespread GnRH, occurring in vertebrates as diverse as birds and elasmobranch fish.

In dogfish (Poroderma africanum) brain seven factors, which stimulated release of LH from chicken dispersed anterior pituitary cells, were separated on analytical HPLC. Two of these factors were partially characterized as Trp^7,Leu^8-GnRH and His^5,Trp^7,Tyr^8-GnRH. Three of the other forms cross-reacted with GnRH antisera, but appear to be novel GnRHs. In teleost (Coris julis) brain two GnRHs similar to Trp^7,Leu^8-GnRH and His^5,Trp^7,Tyr^8-GnRH were present. These two GnRHs therefore appear to occur in both fish species studied. Trp^7,Leu^8-GnRH

is widespread amongst teleost fish (Jackson and Pan, 1983; Sherwood et al., 1983; Breton et al., 1984; Sherwood et al., 1984; King and Millar, 1985).

From these data it seems evident that the mammalian GnRH molecular form occurs only in mammals and amphibians, Gln^8-GnRH in birds and higher reptiles, and Trp^7,Leu^8-GnRH in gnathostomes. His^5,Trp^7,Tyr^8-GnRH appears to be present in numerous different vertebrates. $Tyr^3,Leu^5,Glu^6,Trp^7,Lys^8$-GnRH has thus far only been detected in lamprey brain. A number of novel GnRHs, whose structures have not been elucidated are present.

CONTENTS

1.0 INTRODUCTION

In mammals, secretion of anterior pituitary hormones is controlled by factors released from the hypothalamus into the portal blood. Gonadotropin-releasing hormone (GnRH) is a decapeptide (Fig. 1) which stimulates release of luteinizing hormone (LH) and follicle-stimulating hormone (FSH) from the anterior pituitary (Schally, 1978). This GnRH was initially isolated from porcine and ovine hypothalamus, and subsequently considerable diversity of GnRH structure has been demonstrated in other vertebrate species (King and Millar, 1982a, 1982b; Miyamoto et al., 1983, 1984; Sherwood et al., 1983, 1986) (Fig. 1).

The portal vascular system linking the hypothalamus and anterior pituitary has also been described in birds (Green, 1951), amphibians and reptiles (Ball, 1981). In fish there appears to be considerable anatomical variation. In teleosts the hypothalamus interdigitates with the anterior pituitary and thus there may be direct neurogenic control of the pituitary by the hypothalamus (Ball, 1981). Elasmobranchs have a well-developed median eminence, however, there is no vascular or nervous relationship with the ventral lobe of the pars distalis, where gonadotropin is released (Holmes and Ball, 1974; Dodd, 1975). Thus control of gonadotropin release may occur via the systemic circulation. In cyclostomes there is either no anatomic relationship of a vascular or neural type, or an inefficient one, for transfer of hypothalamic factors to the anterior pituitary (Ball, 1981). Hypothalamic regulatory factors may reach the anterior pituitary either via the systemic circulation or across connective tissue (Nozaki et al., 1984b; Dodd, 1975). Neurohormones could diffuse through the connective tissue into underlying blood vessels which supply the pars distalis (Belenky et al., 1979).

MAMMALIAN GnRH

	1	2	3	4	5	6	7	8	9	10
porcine and ovine	pGlu.	His.	Trp.	Ser.	Tyr.	Gly.	Leu.	Arg.	Pro.	Gly-NH_2

AVIAN GnRH

	1	2	3	4	5	6	7	8	9	10
chicken I								Gln		
chicken II					His		Trp.	Tyr		

AMPHIBIAN GnRH

	1	2	3	4	5	6	7	8	9	10
frog										

PISCINE GnRH

	1	2	3	4	5	6	7	8	9	10
salmon							Trp.	Leu		
lamprey			Tyr		Leu.	Glu.	Trp.	Lys		

Fig. 1. Known vertebrate GnRHs. Only amino acids which differ from the mammalian decapeptide are indicated.

1.1 PHYLOGENETIC DISTRIBUTION OF GnRH IN VERTEBRATE BRAIN

Mammals

In 1947 Green and Harris postulated that a factor which controls release of LH and FSH from the anterior pituitary existed. This factor was, however, only discovered 25 years later by two groups of workers. Porcine (Matsuo et al., 1971) and ovine (Burgus et al., 1972) hypothalamic GnRH was isolated and sequenced (Fig. 1), and the structure was found to be the same in both mammalian species. The GnRH molecule is a decapeptide with a pyro-glutamic acid on the N-terminus and glycine amide on the C-terminus. This GnRH is thought to occur in all mammals, although in only two species has GnRH been thoroughly investigated. A second, smaller immunoreactive peak was apparent in ovine hypothalamus when chromatographed on CM32 carboxymethyl cellulose cation exchange chromatography (King and Millar, 1981). This GnRH molecular form has not been characterized, but was less positively charged. A number of studies have indicated that GnRH also occurs in extrahypothalamic brain, in particular olfactory bulb (Dluzen et al., 1981; Witkin et al., 1982; Dluzen and Ramirez, 1983; Ibata et al., 1983; Witkin and Silverman, 1983) and ovine pineal gland (King and Millar, 1981; Millar et al., 1981; Piekut and Knigge, 1981). The ovine pineal gland had a GnRH molecular form with identical properties to the hypothalamic GnRH. A second form was structurally different from the hypothalamic peptide (King and Millar, 1981). This GnRH was less positively charged and eluted earlier on cation exchange chromatography. The GnRH in the pineal gland may be involved in regulating the process of protein and/or peptide secretion

(Haldar-Misra and Pevet, 1983). The GnRH in the olfactory bulbs may mediate the response to external chemical cues during social encounters (Dluzen et al., 1981; Dluzen and Ramirez, 1983).

Birds

An early chromatographic and immunological study suggested that mammalian and avian GnRH were the same (Jeffcoate et al., 1974). Studies on chicken (Gallus domesticus), pigeon (Columbia livia) and quail (Coturnix coturnix japonica) hypothalamic GnRHs indicated that avian GnRH differed from the mammalian peptide, being less positively charged (Jackson, 1971; King and Millar, 1979a, 1980; Hattori et al., 1980). The GnRH in chicken and pigeon hypothalamus cross-reacted poorly with middle-directed antisera to mammalian GnRH, but had high quantitation with N- and C-terminus-directed antisera. Additional data showed that the chicken hypothalamic GnRH differs from the mammalian peptide at Arg^8 (King and Millar, 1982b). Amino acid analysis and partial sequence analysis of purified chicken hypothalamic GnRH demonstrated that glutamine replaced arginine in position 8 (King and Millar, 1982a, 1982c). This was confirmed by Miyamoto et al. (1983). A second form of chicken hypothalamic GnRH has been characterized as His^5,Trp^7,Tyr^8-GnRH (Miyamoto et al., 1984). Extrahypothalamic brain GnRH has been demonstrated in the chicken (King and Millar, 1980; Józsa and Mess, 1982; Knight et al., 1983) and pigeon (King and Millar, 1980; Weindl et al., 1982).

Reptiles

Immunoreactive GnRH in hypothalamic extracts of lizard (Mabuya capensis) and tortoise (Chersine angulata) were immunologically different from mammalian GnRH and less positively charged (cation exchange chromatography) (King and Millar, 1979b, 1980), differing from the mammalian GnRH in the vicinity of Leu^7. A recent study investigated lizard (Cordylis nigra) brain GnRH, using high performance liquid chromatography (HPLC) and radioimmunoassay with region-specific antisera (Powell et al., 1985). A GnRH molecular form with identical properties to Trp^7,Leu^8-GnRH was identified. This peptide had LH-releasing activity similar to synthetic Trp^7,Leu^8-GnRH. In addition, three structurally related GnRH molecular forms were detected, one having similar HPLC elution properties and LH-releasing activity to His^5,Trp^7,Tyr^8-GnRH (Powell et al., 1985). A preliminary study on alligator (Alligator mississippiensis) brain GnRH using a N- and C- terminal-directed antiserum and a C- terminal antiserum, demonstrated that the GnRH differs from the mammalian peptide in at least the 8 position (Lance, 1985; Lance et al., 1985). Extrahypothalamic brain GnRH has been described in the tortoise (King and Millar, 1980), but GnRH in different brain regions of reptile brain has thusfar not been studied. GnRH appears to have a role in the reproductive behaviour of certain reptiles. In the lizard, Anolis carolinensis, GnRH increased female sexual receptivity and when female iguanas (Iguana iguana) were treated with a Gln^8-GnRH analogue, male sexual behaviour was stimulated (Alderete et al., 1982; Phillips et al., 1985).

Amphibians

Immunoreactive GnRH is present in the hypothalamus of frogs (Rana pipiens, R. catesbeiana) (Alpert et al., 1976; Eiden et al., 1982), clawed toad (Xenopus laevis) (Deery, 1974; King and Millar, 1979a, 1979b, 1980) and toad (Bufo gariepensis) (King and Millar, 1980). This GnRH had identical chromatographic and immunoreactive properties to mammalian GnRH. Seasonal variation of hypothalamic GnRH has been demonstrated in X. laevis (King and Millar, 1979b), Bufo japonicus (Jokura and Urano, 1985; Urano et al., 1985), and in the newt (Taricha granulosa) (Zoeller and Moore, 1985), corresponding with seasonal reproductive activity.

In frog (R. catesbeiana) brain a single GnRH molecular form with identical amino acid composition to the mammalian hormone has been isolated (Rivier et al., 1981). However, in X. laevis brain two forms of GnRH have been identified, having identical immunological and chromatographic properties to mammalian GnRH and His^5,Trp^7,Tyr^8-GnRH (King and Millar, 1986). A number of studies have demonstrated GnRH in bullfrog (R. catesbeiana) sympathetic ganglion (Jan et al., 1979; Eiden and Eskay, 1980; Branton et al., 1982; Eiden et al., 1982). This GnRH is structurally different from mammalian GnRH (Eiden and Eskay, 1980; Eiden et al., 1982) and seems to be a transmitter for the late slow excitatory postsynaptic potential (Jan et al.,1979, 1980). Branton et al (1982) have shown that ganglionic-like GnRH occurs in brain extracts of metamorphic and mature adults. In the sympathetic ganglia a GnRH molecular form with identical properties to Trp^7,Leu^8-GnRH was present, and is thought to have a neurotransmitter function (Jones et al., 1984).

Fish

Numerous immunological studies have demonstrated GnRH in osteichthyes brain (see Crim and Vigna, 1983). Tilapia (Sarotherodon mossambicus) brain GnRH differed from mammalian GnRH in the vicinity of Leu^7 (King and Millar, 1979a, 1980) and was less positively charged, possibly having no Arg^8. A GnRH in salmon (Oncorhynchus keta) brain was purified and sequenced and had the structure Trp^7,Leu^8-GnRH (Sherwood et al., 1983). Additional GnRH molecular forms were also present in salmon brain (Sherwood et al., 1983). Multiple forms of GnRH also occurred in hake (Merluccius capensis) pituitary gland, and in the brain of tilapia (Tilapia sparrmanii), codfish (Gadus morhua morhua), milkfish (Chanos chanos), trout (Salmo gairdneri) and mullet (Mugil cephalus) (Barnett et al., 1982; Jackson and Pan, 1983; Sherwood et al., 1984; King and Millar, 1985). The nature of the molecular variants of GnRH has not been determined, however, it appears that most teleosts have a GnRH molecular form with similar properties to Trp^7,Leu^8-GnRH (Jackson and Pan, 1983; Breton et al., 1984; Sherwood et al., 1984; King and Millar, 1985).

In chondrichthyes, immunoreactive GnRH has been detected in dogfish (Poroderma africanum and Squalus acanthias) hypothalamus (Jackson, 1980; King and Millar 1980) and ratfish (Hydrolasus colliei) hypothalamus (Jackson, 1980). S. acanthias brain extract has been chromatographed on reverse phase HPLC (Sherwood and Sower, 1985). Two immunoreactive GnRHs (antiserum R-42) occurred, neither co-eluting with mammalian GnRH or Trp^7,Leu^8-GnRH. Other elasmobranch fish (Scyliorhinus canicula and Triakis scyllia) had no detectable immunoreactive GnRH (Deery, 1974;

Nozaki and Kobayashi, 1979). More recently, Nozaki *et al.* (1984a) detected GnRH in *T. scyllia* brain by immunohistochemistry. The structure of elasmobranch GnRH has not been elucidated.

In agnathans, both lamprey and hagfish GnRHs have been studied. The lampreys (*Entosphenus tridentata*, *Lampetra richardsoni*, *E. japonica* and *Petromyzon marinus*) exhibited immunoreactivity in brain preparations (Crim *et al.*, 1979a, 1979b; Nozaki and Kobayashi, 1979; Nozaki *et al.*, 1984a; Sherwood and Sower, 1985). Immunoreactive GnRH occurred in the hagfish *Heptatretus hexatrema* (King and Millar, 1980) and *Eptatretus stouti* (Jackson, 1980), whereas *E. burgeri* brain had no detectable immunoreactive GnRH (Nozaki and Kobayashi, 1979; Nozaki *et al.*, 1984a). Contrary to Jackson's report of immunoreactivity GnRH in *E. stouti*, Sherwood and Sower (1985) did not detect immunoreactive GnRH in *E. stouti* brain. The immunoreactive GnRH in lamprey (*P. marinus*) brain has been chromatographed on reverse phase HPLC and two peaks were detected (Sherwood and Sower, 1985). The major peak eluted in the same position as mammalian GnRH, but sequence analysis of purified material characterized it as Tyr^3,Leu^5,Glu^6,Trp^7,Lys^8-GnRH (Sherwood *et al.*, 1986). Amino acid analysis of the second GnRH form in lamprey has been performed and a number of novel amino acids occurred (Sherwood *et al.*, 1986).

Extrahypothalamic brain GnRH has been detected in tilapia (*S. mossambicus*), dogfish (*P. africanum*) (King and Millar, 1980) and goldfish (*Carassius auratus*) (Stell *et al.*, 1984) brain. In a number of teleosts immunoreactive GnRH seem to occur in the olfactory bulb with processes of the GnRH neurons projecting to the retina (Münz *et al.*, 1982).

1.2 GnRH IN OTHER TISSUES

GnRH has been described in a number of tissues outside the brain and these GnRH-like molecules appear to have specific functions. Human placental GnRH seems to be indistinguishable from the mammalian hypothalamic peptide (Siler-Khodr and Khodr, 1978, 1979; Khodr and Siler-Khodr, 1980; Tan and Rousseau, 1982). The GnRH is released by placental tissue *in vitro* (Siler-Khodr and Khodr, 1979; Khodr and Siler-Khodr, 1980), and the content in the placenta varies with the stages of gestation (Siler-Khodr and Khodr, 1978). A GnRH-like factor with immunological and biological activity also occurred in rabbit fetal placenta (Nowak *et al.*, 1984) and rat placenta (DePalatis *et al.*, 1980). Receptors for GnRH have been described in human placenta (Currie *et al.*, 1981; Belisle *et al.*, 1984). Placental cell cultures incubated with GnRH have stimulated production of hCG, thus suggesting a role for GnRH in human placenta (Belisle *et al.*, 1984).

GnRH in the Leydig cells and seminiferous tubules of rat testes has been detected by immunohistochemistry (Paull *et al.*, 1981; Turkelson *et al.*, 1983). Dutlow and Millar (1981) showed that testicular immunoreactive GnRH differs from the hypothalamic peptide. Two factors with GnRH receptor-binding properties, which were chemically distinct from the native peptide appear to be present in rat testis (Bhasin *et al.*, 1983). The two factors had molecular weights of 68 000 and 6 000. GnRH receptors were present on the Leydig cells, but not on cells in the seminiferous tubules (Bourne *et al.*, 1980; Clayton *et al.*, 1980; Sharpe and Fraser, 1980; Millar *et al.*, 1982; see Sharpe, 1982). It has been speculated that a GnRH-like factor is secreted from Sertoli cells in the

testes and that this factor has an inhibitory effect on Leydig cells (Sharpe, 1982). A small peptide (smaller than 3 500 daltons) found in rat follicular fluid stimulated the pituitary to release LH and FSH *in vitro* and *in vivo*. This GnRH-like peptide eluted differently to mammalian GnRH in HPLC and did not cross-react with two antisera generated against mammalian GnRH (Ying *et al.*, 1981). This peptide is thought to be secreted from the granulosa cells. GnRH receptors have been demonstrated on purified granulosa cells (Jones *et al.*, 1980) and on luteal cells (Clayton *et al.*, 1979). The GnRH in the ovary affected granulosa and luteal cell function (see Sharpe, 1982).

GnRH-like immunoreactivity in pancreatic islets of the rat co-eluted with synthetic and hypothalamic GnRH in cation exchange chromatography and was immunologically identical (Seppälä *et al.*, 1979). Human pancreatic islets also had GnRH immunoreactivity (Wahlström and Seppälä, 1979; Seppälä and Wahlström, 1980). The function of this GnRH-like molecule was not apparent. Immunoreactive GnRH appears to be present in human and bovine milk (Baram *et al.*, 1977; Sarda and Nair, 1981; Hazum, 1983). This GnRH had similar properties to synthetic mammalian GnRH (Sarda *et al.*, 1981). GnRH immunoreactivity occurred in frog (*R. catesbeiana*) and lizard (*C. nigra*) adrenal gland (Eiden *et al.*, 1982; Powell *et al.*, 1985), frog (*R. catesbeiana* and *Bufo marinus*) and fish (carp, goldfish and trout) retina (Eiden *et al.*, 1982; Stell *et al.*, 1984), lizard (*C. nigra*) spinal cord (Powell *et al.*, 1985), human seminal plasma (Chan and Tang, 1983), and eel (*Anguilla anguilla*) and dogfish (*P. africanum*) systemic blood (King and Millar, 1980; Dufour *et al.*, 1982), the functions of these factors being unknown. GnRH receptors appear to be present in rat adrenal gland (Eidne *et al.*, 1985a) and human mammary carcinoma tissue (Eidne *et al.*, 1985b; Miller *et al.*, 1985).

1.3 GnRH-LIKE PEPTIDES IN INVERTEBRATES AND OTHER LOWER ORGANISMS

The protochordate Ciona intestinalis had GnRH-like immunoreactivity in its nervous system (Georges and Dubois, 1980). The hepatopancreas of the grass shrimp (Penaeus monodon) appears to have immunoreactive and biologically active GnRH. No GnRH was detected in other tissues (Wan et al., 1984). No GnRH-like activity was detected in the neural ganglia of the snail Helix sp. (King and Millar, unpublished). Yeast α-mating factor has extensive sequence homology with GnRH and it bound to rat pituitary GnRH receptors and stimulated release of LH from cultured pituitary cells (Loumaye et al., 1982)

1.4 BIOLOGICAL ACTIVITY OF VERTEBRATE GnRHs

Mammals

Synthetic mammalian GnRH has been shown to stimulate release of LH from pituitary cells of numerous mammalian species and this suggests that the structure of GnRH is conserved in mammals. Gln^8-GnRH had 1-5% of the LH-releasing activity of the mammalian peptide in rat pituitary cells in vitro (Hasegawa et al., 1984; Miyamoto et al., 1984) and in vivo (Sandow et al., 1978) and in sheep pituitary cells in vitro (Millar and King, 1983). Trp^7,Leu^8-GnRH had 5% of the LH-releasing activity of mammalian GnRH (Sherwood et al., 1983) and His^5,Trp^7,Tyr^8-GnRH 32% (Miyamoto et al., 1984) in rat pituitary cells. $Tyr^3,Leu^5,Glu^6,Trp^7,Lys^8$-GnRH, which has recently been isolated from lamprey brain, has not been tested for biological activity in the mammalian system. It appears that the mammalian GnRH receptor is highly specific in regard to GnRH structure.

Birds

The five naturally-occurring GnRHs have been used to stimulate release of LH from chicken dispersed anterior pituitary cells. Mammalian GnRH and Gln^8-GnRH were equipotent, with an ED_{50} of 3×10^{-10} M (Millar and King, 1983; Johnson *et al.*, 1984a). Trp^7,Leu^8-GnRH was 2.5 times more potent than mammalian GnRH (Millar and King, 1984) and His^5,Trp^7,Tyr^8-GnRH 5-6 times more potent (Millar and King, 1984; Chou *et al.*, 1985). $Tyr^3,Leu^5,Glu^6,Trp^7,Lys^8$-GnRH had 0.001% of the LH-releasing activity of mammalian GnRH (J.A. King, R.C. deL. Milton and R.P. Millar, unpublished). Mammalian GnRH and Gln^8-GnRH were also equipotent *in vivo* in chickens (Johnson *et al.*, 1984b; Sterling and Sharp, 1984) and in quails (Chan *et al.*, 1983; Hattori *et al.*, 1985). Therefore, it is thought that the GnRH receptor in birds is more promiscuous than the mammalian receptor.

Reptiles

Very few studies have been performed in reptiles with regard to biological activity of the naturally-occurring GnRHs. Mammalian GnRH appears to be inactive in various turtles (Licht *et al.*, 1984), but it was slightly active in stimulating a rise of LH in the turtles *Chrysemys picta* (Callard and Lance, 1977) and *C. mydas* (Licht, 1980). Gln^8-GnRH was inactive in a snake (*Naja naja*) and a turtle (*Sternotherus odoratus*) (Licht *et al.*, 1984).

Amphibians

Numerous studies have shown that synthetic mammalian GnRH stimulates gonadotropin secretion in frogs (Thornton and Geschwind, 1974; Daniels and Licht, 1980; McCreery *et al.*, 1982; Licht *et al.*, 1984; Porter and Licht 1984). Gln^8-GnRH stimulated gonadotropin release similarly to mammalian GnRH in the frog *R. catesbeiana* (Licht *et al.*, 1984).

Fish

Synthetic mammalian GnRH increased plasma gonadotropin concentration *in vivo* in a number of teleosts (Peter and Crim, 1979; reviewed by Peter, 1983). Mammalian GnRH also increased gonadotropin release from rainbow trout (*S. gairdneri*) pituitary gland and goldfish (*C. auratus*) pituitary cells *in vitro* (Crim and Evans, 1980; Crim *et al.*, 1981; MacKenzie *et al.*, 1984). Trp^7,Leu^8-GnRH stimulated gonadotropin in the coho salmon (*Oncorhynchus kisutch*) (van der Kraak *et al.*, 1983) and in the goldfish (*C. auratus*) (MacKenzie *et al.*, 1984). Mammalian GnRH, Trp^7,Leu^8-GnRH and Gln^8-GnRH were equipotent in increasing serum gonadotropin in the goldfish (Peter *et al.*, 1985). The activities of His^5,Trp^7,Tyr^8-GnRH and $Tyr^3,Leu^5,Glu^6,Trp^7,Lys^8$-GnRH in teleosts are not known.

In the dogfish (*S. canicula*), an elasmobranch fish, GnRH stimulated an increase in plasma gonadotropin levels (Dodd, 1975) and in estrogen and androgen (Jenkins and Dodd, 1980). In the lamprey (*P. marinus*), an agnathan fish, an agonist analogue of mammalian GnRH and synthetic $Tyr^3,Leu^5,Glu^6,Trp^7,Lys^8$-GnRH induced ovulation and increased plasma levels of estradiol (Sower *et al.*, 1983, 1985).

Table I summarizes the biological activities of GnRHs in different vertebrates.

TABLE I. BIOLOGICAL ACTIVITY OF VERTEBRATE GnRHs IN DIFFERENT VERTEBRATE CLASSES

GnRH	Mammal	Bird	Reptile	Amphibian	Teleost	Cyclostome
Mammalian	++++	+++	++/0	++++	++++	++++
Chicken I	+	+++	0	++++	++++	
Chicken II	++	++++			++++	
Salmon	+	+++			++++	++++
Lamprey	0	0				++++

Plus signs indicate relative biological activity of GnRH in different vertebrates : ++++ full activity; +++ high activity; ++ intermediate activity; + low activity; 0 no activity.

1.5 BIOSYNTHESIS OF GnRH

A number of studies have indicated that GnRH is processed from a larger precursor molecule to its decapeptide form (Millar et al., 1977; Gautron et al., 1981; Curtis and Fink, 1983; Curtis et al., 1983). The cloned genomic and cDNA sequences for a precursor form of GnRH have been isolated from human placenta (Fig. 2). The DNA sequence coded for a protein with 92 amino acids with a 23 amino acid signal peptide preceding the GnRH decapeptide sequence and a cleavage site Gly-Lys-Arg following the GnRH peptide, with a further 53 amino acids, which formed a peptide of unknown function (Seeburg and Adelman, 1984). This peptide seems to be a potent inhibitor of prolactin secretion and can stimulate release of gonadotropins in rat pituitary cell cultures (Nikolics et al., 1985).

Since only one GnRH sequence was present in the human placental GnRH precursor and a single gene was identified using cDNA probes, it appears that only one GnRH form occurs in humans. However, multiple forms of GnRH occur in a number of submammalian vertebrates including a bird (King and Millar, 1982a, 1982b; Miyamoto et al., 1983, 1984), an amphibian (Branton et al., 1982; King and Millar, 1986), a reptile (Powell et al., 1985) and numerous fish (Jackson and Pan, 1983; Sherwood et al., 1983, 1984; Idler and Crim, 1985; King and Millar, 1985; Sherwood et al., 1986), and therefore it seems likely that mammals also have more than one form of GnRH. The GnRH may, however, differ from the isolated mammalian GnRH to such an extent that the cDNA probes did not detect it.

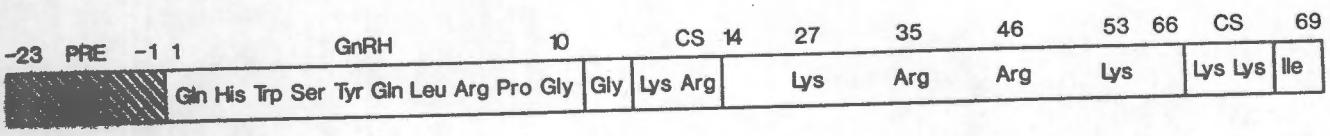


Fig. 2. Schematic diagram of the structure of the human placental GnRH precursor as determined by nucleic acid sequencing of the corresponding cDNA (Seeburg and Adelman, 1984).

2.0 MATERIALS AND METHODS

2.1 ANIMALS

The following animals were used in the study : ostrich (*Struthio camelus*, Aves), alligator (*Alligator mississippiensis*, Reptilia), skink (*Calcides ocellatus tiligugu*, Reptilia), teleost (*Coris julis*, Pisces - Osteichthyes-Teleostei) and dogfish (*Poroderma africanum*, Pisces-Chondrichthyes-Elasmobranchii).

2.2 EXTRACTION PROCEDURES

2.2.1 Ostrich hypothalami

Brains were removed from three hundred and sixty ostriches and the hypothalami were dissected within 20 min of death, frozen on dry ice and stored at -70°C. They were homogenized in ice-cold 2N acetic acid containing 10^{-3} M phenylmethane-sulphonylfluoride (PMSF) and centrifuged for 90 min at 18 000 x g at 4°C. The supernatant was decanted and the pellet re-extracted with 2N acetic acid. The combined supernatants were lyophilized and stored at -20°C.

2.2.2 Alligator brains

Brains were removed from twenty-three freshly-killed animals killed in the legal hunting season in Louisiana, lyophilized, and homogenized in ice-cold 2N acetic acid containing 10^{-4} M bacitracin and 10^{-3} M PMSF. The homogenate was boiled for 10 min, chilled, and centrifuged for 1 hr at 18 000 x g at 4°C. The supernatant was decanted, the pellet re-extracted and the pooled supernatants defatted with petroleum ether (40-60°C) (2 x vol) at 4°C for 1 hr, lyophilized and stored at -20°C.

2.2.3 Skink brains

Brains were removed from four animals, lyophilized, homogenized in ice-cold 2N acetic acid, and centrifuged for 1 hr at 18 000 x g at 4°C. The supernatant was lyophilized (provided by G. Ciarcia, Department of Evolutive and Comparative Biology, University of Naples, Italy).

2.2.4 Dogfish tissues

Twenty pituitaries, hypothalami and extrahypothalamic brains were dissected within 24 hr of capture in March (Batch A), eighty-two whole brains were dissected in April (Batch B), and a further one hundred and nine whole brains were dissected in October (Batch C). The tissues were lyophilized and Batches A and B homogenized with ice-cold 2N acetic acid containing 10^{-4} M bacitracin and 10^{-3} M PMSF. The homogenates were centrifuged for 1 hr at 18 000 x g at 4°C. Supernatants were decanted and pellets re-extracted in ice-cold 2N acetic acid containing 10^{-3} M PMSF. Batch C was homogenized with 2N acetic acid containing 10^{-3} M PMSF, the homogenate boiled for 10 min, chilled, and centrifuged for 1 hr at 18 000 x g at 4°C. The pellet was re-extracted and supernatants were pooled and defatted with petroleum ether (40-60°C) (2 x vol). All the supernatants were lyophilized and stored at -20°C. Dogfish systemic blood was collected in March and October (10 ml and 7 ml, respectively). The cells were spun down and the supernatants extracted with acetone (4 x vol) in 2N acetic acid at 4°C. The extracts were centrifuged at 18 000 x g for 1 hr at 4°C. The supernatants were lyophilized and assayed for immunoreactive GnRH in serial dilutions. The presence of binding substances and peptidases in the serum extract was investigated.

2.2.5 Teleost brain

Eight brains were dissected, lyophilized, and homogenized with ice-cold 2N acetic acid and centrifuged for 1 hr at 18 000 x g at 4°C. The supernatant was lyophilized (provided by G. Ciarcia).

2.3 CHROMATOGRAPHY

2.3.1 Gel filtration

The tissue extract was reconstituted in 2N acetic acid, sonicated, and applied to a Sephadex G-25 fine (5 x 85 cm; Pharmacia) column equilibrated with 2N acetic acid at 4°C. One hundred 20-ml fractions were collected, using 2N acetic acid as solvent. The void volume (Vo) and salt elution volume (Vs) were assessed using bovine serum albumin and sodium chloride, respectively. The elution volume of the immunoreactive GnRH was compared with that of synthetic mammalian GnRH. Aliquots of fractions were lyophilized and assayed by radioimmunoassay with region-specific antisera generated against three of the known naturally-occurring GnRHs.

2.3.2 Semipreparative high performance liquid chromatography

Samples were eluted on a Lichrosorb RP 18 column (0.16 x 25 cm; 10 um particle size; Chrompack) using 0.01 M ammonium acetate, pH 4.0 (A) and

60% acetonitrile in 0.01 M ammonium acetate pH 4.0 (B) as solvents. The conditions used were : 40% B for 20 min, followed by a gradient from 40 to 55% B for 5 min, 25 min at 55% B, a 5-min gradient from 55 to 99% B and 5 min at 99% B. Flow rate was 8 ml/min and sixty 1-min fractions were collected. Aliquots of fractions were lyophilized and assayed with region-specific antisera.

2.3.3 Analytical high performance liquid chromatography

System 1. Samples were eluted on a Vydac C18 reverse phase column (no 1) (0.46 x 25 cm; 5 um particle size; The Separations Group) with 30% B in A + B for 10 min, B being 60% acetonitrile in 0.1% heptafluorobutyric acid and A being 0.1% heptafluorobutyric acid. This was followed by a gradient from 30 to 50% B for 20 min, then isocratically at 50% B for 10 min. This was followed by a 5-min gradient from 50 to 99% B and 5 min at 99% B. Flow rate was 1.5 ml/min. Fifty 1-min fractions were collected. Aliquots of fractions were lyophilized and assayed with various region-specific GnRH antisera.

System 2. The sample was eluted on a second Vydac C18 reverse phase column (no 2) (0.46 x 25 cm; 5 um particle size; The Separations Group) using the same solvent system as in system 1.

System 3. The sample was eluted on a Spherisorb ODS reverse phase column (no 1) (0.4 x 25 cm; 5 um particle size; Phase Separations) with 40% B in A + B for 20 min, A being 0.01 M ammonium acetate, pH 4.0, and B being 60% acetonitrile in 0.01 M ammonium acetate, pH 4.0. This was followed

by a 5-min gradient from 40 to 60% B, 25 min at 60% B, a 5-min gradient from 60 to 99% B and 5 min at 99% B. Flow rate was 1.5 ml/min. Sixty 1-min fractions were collected. Aliquots of fractions were lyophilized and assayed with various region-specific GnRH antisera.

System 4. The sample was eluted on a second Spherisorb ODS reverse phase column (no 2) (0.4 x 25 cm; 5 um particle size; Phase Separations) with 40% B in A + B for 20 min, A being 0.01 M ammonium acetate, pH 4.0, and B being 60% acetonitrile in 0.01 M ammonium acetate, pH 4.0. This was followed by a 10-min gradient from 40 to 60% B, 20 min at 60% B, a 5-min gradient from 60 to 99% B and 5 min at 99% B. Flow rate was 1.5 ml/min. Sixty 1-min fractions were collected. Aliquots of fractions were lyophilized and assayed with various region-specific GnRH antisera.

System 5. The sample was eluted on a Vydac C18 reverse phase column (no 2) (0.46 x 25 cm; 5 um particle size; The Separations Group) with 27% B in A + B for 10 min, A being 0.1% heptafluorobutyric acid and B being 60% acetonitrile in 0.1% heptafluorobutyric acid. This was followed by a 20-min gradient from 27 to 50% B, 10 min at 50% B, a 5-min gradient from 50 to 99% B and 5 min at 99% B. Flow rate was 1.5 ml/min. Fifty 1-min fractions were collected. Aliquots of fractions were lyophilized and assayed with region-specific GnRH antisera.

Mammalian GnRH (R.C. deL. Milton, University of Cape Town, Cape Town, South Africa), Gln^8-GnRH (R.W. Roeske, Indiana University, Indianapolis, IN), Trp^7,Leu^8-GnRH (J.E. Rivier, The Salk Institute, La Jolla, CA) and His^5,Trp^7,Tyr^8-GnRH (R.C. deL. Milton) were chromatographed on these HPLC systems as standards. To ensure that columns were not contaminated with synthetic GnRHs, each run of biological material was preceded by a blank run and was assayed for GnRH immunoreactivity with antiserum 80/1 which cross-reacts with all four of these GnRH forms (King and Millar, 1985).

2.4 RADIOIMMUNOASSAY OF GnRH

Immunoreactive GnRH in the samples was measured as described by Millar *et al.*, 1984.

2.4.1 Region-specific antisera

Antisera 80/1 (S. Blähser, Institut für Anatomie und Zytobiologie, Giessen, Germany) and 1076 (King and Millar, 1980) were generated against mammalian GnRH, antiserum 303 was generated against Gln^8-GnRH (King *et al.*, 1983) and antiserum 802 was generated against Trp^7,Leu^8-GnRH (Powell *et al.*, 1985).

Figure 3 indicates the regions of the decapeptide required for effective binding by these antisera. Antiserum 80/1 requires $pGlu^1$ and $Gly^{10}NH_2$ (King *et al.*, 1983). Substitutions of Gly^6 with bulky amino acids are not tolerated as the conformation of GnRH (Fig. 4) is affected by these substitutions. Antiserum 1076 requires the region Trp^3 to Pro^9 of GnRH (King and Millar, 1982b); antiserum 303 requires Gln^8 as in Gln^8-GnRH (King *et al.*, 1983) and antiserum 802 requires Trp^7 and Leu^8 as in Trp^7,Leu^8-GnRH (J.A. King and R.P. Millar, unpublished). The sensitivities (half-maximal displacement of iodinated peptide) in the radioimmunoassay with antisera 80/1, 1076, 303 and 802 are given in Table II.

2.4.2 Radio-iodination of GnRH with ^{125}I

Synthetic mammalian GnRH, Gln^8-GnRH and Trp^7,Leu^8-GnRH (J.E. Rivier) were labelled with ^{125}I using a modification of the chloramine-T method

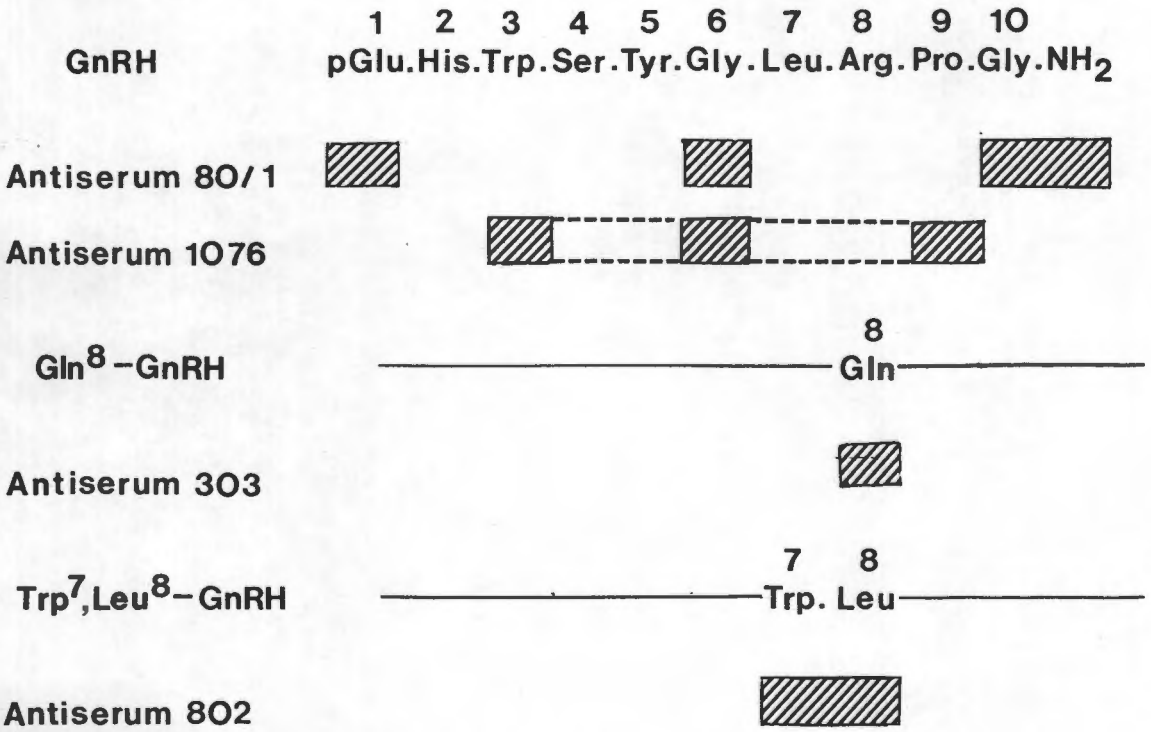


Fig. 3. Regions of the mammalian GnRH, Gln^8-GnRH and Trp^7,Leu^8-GnRH molecules which are known to be essential for full immunoreactivity with antisera 80/1, 1076, 303 and 802.

(Millar et al., 1984) ^{125}I-GnRH was purified on a CM32 carboxymethylcellulose cation exchange column (1 x 20 cm; Whatman) with 0.002 M ammonium acetate, pH 4.6, for 20 ml and then with 0.06 M ammonium acetate, pH 4.6 (Millar et al., 1984). It was used in the radioimmunoassay with the antisera 80/1 and 1076. ^{125}I-Trp^{7},Leu^{8}-GnRH was purified on the same column with a gradient from 0.01 M ammonium acetate, pH 4.6, to 0.1 M ammonium acetate, pH 4.6. It was used with antiserum 802. ^{125}I-Gln^{8}-GnRH was purified on a Sep-Pak C-18 cartridge (Waters Associates), pre-washed with methanol and water. The iodinated peptide was eluted with a gradient of 20 to 50% acetonitrile in 0.002 M ammonium acetate, pH 4.6, over 15 ml (King et al., 1983). It was used with antiserum 303. Fractions containing the iodinated peptides were pooled and stored at -20°C.

2.4.3 Radioimmunoassay protocol

Aliquots of gel filtration and HPLC fractions were reconstituted in 100 ul phosphate-buffered saline (PBS) containing gelatin (0.04 M $NaH_2PO_4.2H_2O$, 0.15 M NaCl, 0.01 M disodium ethylenediaminetetra acetic acid, 0.015 M NaN_3, pH 7.0, with 0.1% gelatin). Standard (mammalian GnRH, Gln^{8}-GnRH or Trp^{7},Leu^{8}-GnRH) was reconstituted in PBS-gelatin buffer and serially diluted. 100 µl of standard or sample, 200 µl PBS-gelatin buffer and 100 µl of antiserum (final dilutions given in Table II) were preincubated in glass test-tubes for 15 min at 20°C. 100 µl of the appropriate iodinated peptide (10 000 cpm) was added and the tubes were incubated overnight at 4°C. Separation of antibody-bound and free iodinated peptide was achieved by adding 750 µl dextran-coated

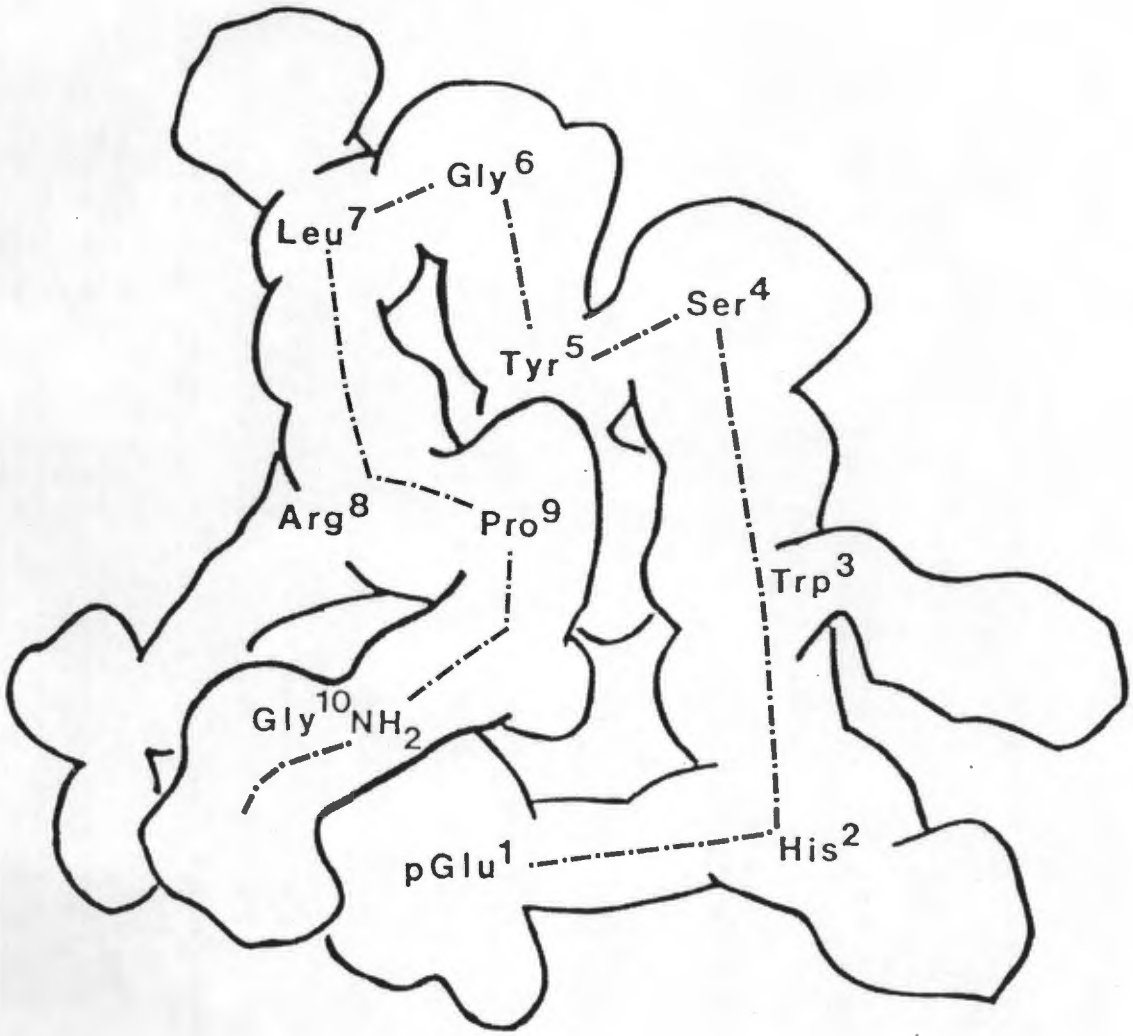


Fig. 4. The proposed conformation of GnRH.

TABLE II. GnRH RADIOIMMUNOASSAY CHARACTERISTICS

Antiserum	Final dilution	Half-maximal displacement (pg)
80/1	1:12 000	32.5
1076	1:100 000	15.7
303	1: 2 000	361
802	1: 1 250	582

charcoal (0.5% charcoal, 0.05% dextran), incubating at 4°C for 15 min, and centrifuging at 3 000 x g at 4°C for 15 min. Supernatants were poured over and counted in a gamma counter. Mammalian GnRH was used as standard with antisera 80/1 and 1076; Gln^8-GnRH with antiserum 303 and Trp^7,Leu^8-GnRH with antiserum 802.

2.5 TESTS FOR PEPTIDASES AND BINDING SUBSTANCES

To test for peptidase activity the tissue extract was incubated with mammalian ^{125}I-GnRH at 20°C. Aliquots were removed at 30-min intervals for 3 hr and binding with excess antiserum 1076 was determined as in a radioimmunoassay. To test for binding substances, the extract was incubated with mammalian ^{125}I-GnRH at 4°C overnight in the absence of antiserum and the radioimmunoassay was performed as usual.

2.6 LH-RELEASING ACTIVITY IN CHICKEN DISPERSED ANTERIOR PITUITARY CELLS

The LH-releasing activity in the HPLC fractions was measured by incubating aliquots of fractions (or pools of fractions) with chicken dispersed anterior pituitary cells (Millar and King, 1983) and measuring the LH content in the cell medium by radioimmunoassay (Follett et al., 1972).

Anterior pituitaries were dissected from chicken heads collected on ice within 2 hr of death, and placed in ice-cold sterile Romanoff's avian Ringer's Albumin buffer (RRA) (147 mM NaCl, 4.15 mM KCl, 1.8 mM $CaCl_2$, 0.49 mM $MgCl_2.6H_2O$, 1.5 mM KH_2PO_4, 2.2 mM $Na_2HPO_4.12H_2O$, 11.1 mM glucose and 0.5% BSA, pH 7.2). The pituitaries were minced with a razor blade

and the fragments incubated with 10 ml Hepes buffer (137 mM NaCl, 5 mM KCl, 0.7 mM $Na_2HPO_4.12H_2O$, 25 mM 4-2-hydroxyethyl piperazine ethanesulphonic acid, 0.36 mM $CaCl_2$, 10 mM glucose, and 0.1% BSA, pH 7.2) containing 0.9% collagenase and 1.8 mg/dl deoxyribonuclease for 1 hr at 37°C. The suspension was then passed up and down a sterile 10 ml pipette to assist dispersion, centrifuged at 500 x g for 5 min, and the pellet was re-suspended in RRA at 20°C. The cells were washed a further two times in RRA and filtered through a Baxter 'Plexitron' nylon mesh. The cells were distributed into polypropylene tubes (12 x 75 mm) at a concentration of approximately 0.7 pituitary equivalents per tube. The tubes were preincubated in 1.5 ml RRA for 20 min at 37°C with gentle shaking under a 90% water-saturated atmosphere of 4% CO_2 - 96% air. The cells were centrifuged at 500 x g for 5 min, and the supernatant poured off. HPLC fractions and synthetic Gln^8-GnRH were diluted in RRA and added to the cells in triplicate in a volume of 1.0 ml. The tubes were incubated for 2 hr at 37°C and centrifuged at 500 x g for 5 min, the supernatant poured over into polypropylene tubes and re-centrifuged at 500 x g for 5 min. An aliquot of the final supernatant was assayed for LH immunoreactivity.

2.7 RADIOIMMUNOASSAY OF CHICKEN LH

Immunoreactive LH was measured as described by Follett *et al.*(1972).

2.7.1 LH antiserum

Antiserum 16/6 (B.K. Follett, University of Bristol, Bristol, U.K.) was raised against chicken pituitary LH in a rabbit.

2.7.2 Preparation of ^{125}I-labelled chicken luteinizing hormone

Purified chicken LH (B.K. Follett; P. Sharp, AFRC Poultry Research Centre, Midlothian, Scotland) was labelled with ^{125}I using chloramine-T (Follett *et al.*, 1972). The labelled LH was purified by adsorption chromatography on a column (0.5 x 2 cm) of CF11 cellulose (Whatman) with 40 ml 0.12 M barbiturate buffer (pH 8.6) and 10 ml human plasma, collecting 1 ml fractions. Fractions containing ^{125}I-LH were pooled and stored at -20°C.

2.7.3 Radioimmunoassay protocol

A double antibody technique of radioimmunoassay was performed in disposable polystyrene tubes (12 x 75 mm). Standard (produced by stimulating chicken pituitary cells with 10^{-6} M Gln^{8}-GnRH) or sample was made up to 300 µl of PBS-gelatin buffer. 100 µl of antiserum 16/6 (1:30 000) was added and tubes were preincubated for 15 min at 20°C. 100 µl ^{125}I-LH (10 000 cpm) was added and the tubes were incubated overnight at 4°C. Separation of antibody-bound and free ^{125}I-LH was achieved by adding 100 µl of Sac-Cel (donkey anti-rabbit, antibody-coated cellulose suspension; Wellcome), incubating at 20°C for 30 min, adding 1 ml of water and centrifuging at 2000 x g for 5 min. The supernatant was aspirated and the pellet counted in a gamma counter.

3.0 IDENTIFICATION OF Gln^8-GnRH, His^5,Trp^7,Tyr^8-GnRH AND NOVEL FORMS OF GnRH IN THE HYPOTHALAMUS OF THE OSTRICH

3.1 ABSTRACT

Gonadotropin-releasing hormone molecular forms were studied in ostrich (*S. camelus*) hypothalamic extract using gel filtration, HPLC, radioimmunoassay with region-specific GnRH antisera and by assessment of LH-releasing activity using chicken dispersed anterior pituitary cells. Two molecular forms of GnRH with chromatographic, immunological and biological properties identical to those of Gln^{8}-GnRH and His^{5},Trp^{7},Tyr^{8}-GnRH were demonstrated in the hypothalamic extract. Three additional novel LH-releasing factors, which did not cross-react with the GnRH antisera used, were identified.

3.2 INTRODUCTORY STATEMENT

Chicken hypothalamic GnRHs have been isolated and characterized as Gln^8-GnRH (King and Millar, 1982a, 1982c; Miyamoto *et al.*, 1983) and His^5,Trp^7,Tyr^8-GnRH (Miyamoto *et al.*, 1984). Hypothalamic immunoreactive GnRH has also been described in a number of other birds (King and Millar, 1979a; Hattori *et al.*, 1980; King and Millar, 1980; Weindl *et al.*, 1982), but the structure of the GnRHs in these birds is not known. It is, therefore, necessary that GnRH in another bird is studied.

3.3 RESULTS

Chromatography and radioimmunoassay of ostrich hypothalamic extract

The concentration of immunoreactive GnRH in the hypothalamus was 18.9 pg/mg dry weight of extract (1.08 ng/hypothalamus) according to N- and C-terminus-directed antiserum 80/1.

On gel filtration chromatography, immunoreactive GnRH (antiserum 80/1) co-eluted with decapeptide mammalian GnRH as a broad peak (Fig. 5). This suggests that the GnRH is a decapeptide with blocked N- and C-termini (antiserum 80/1 requirements for effective binding). The immunoreactive peak was pooled and lyophilized.

Semipreparative HPLC of decapeptide-sized immunoreactive GnRH resolved three immunoreactive peaks (antiserum 80/1) (Fig. 6). The middle-directed antiserum 1076 cross-reacted 73% with peak I, 17% with peak II and 9.2% with peak III (relative to antiserum 80/1). The relative cross-reactivities of synthetic analogues of the five known naturally-occurring GnRHs are given in Table III. It seems possible, therefore, that peak II is Gln^8-GnRH. The three peaks were pooled separately.

On analytical HPLC (system 5), peak I (from semipreparative HPLC) eluted as two peaks of immunoreactive GnRH (antiserum 80/1) (Fig. 7). The first peak did not co-elute with any of the known naturally-occurring GnRHs; the second peak co-eluted with Gln^8-GnRH. As peak II (from semipreparative HPLC) also co-eluted with Gln^8-GnRH, this is likely to be due to overlapping of the peaks (on semipreparative HPLC) when pooling fractions.

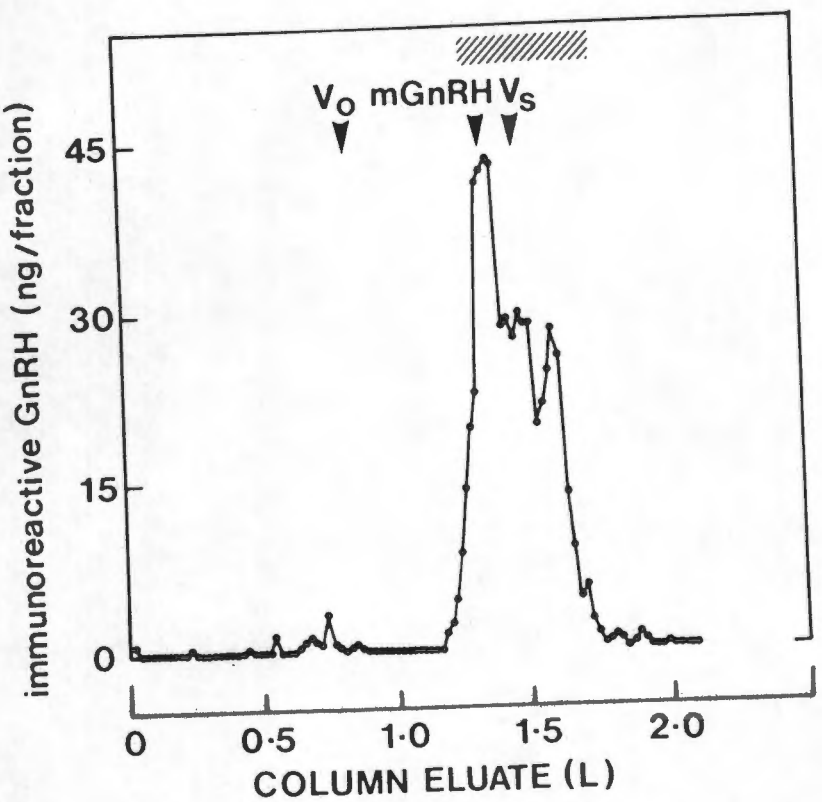


Fig. 5. Gel filtration chromatography of ostrich hypothalamic extract (572 ng GnRH, antiserum 80/1). The void volume (Vo), salt elution volume (Vs) and elution position of mammalian GnRH are indicated. The hatched block indicates the fractions pooled for further analysis.

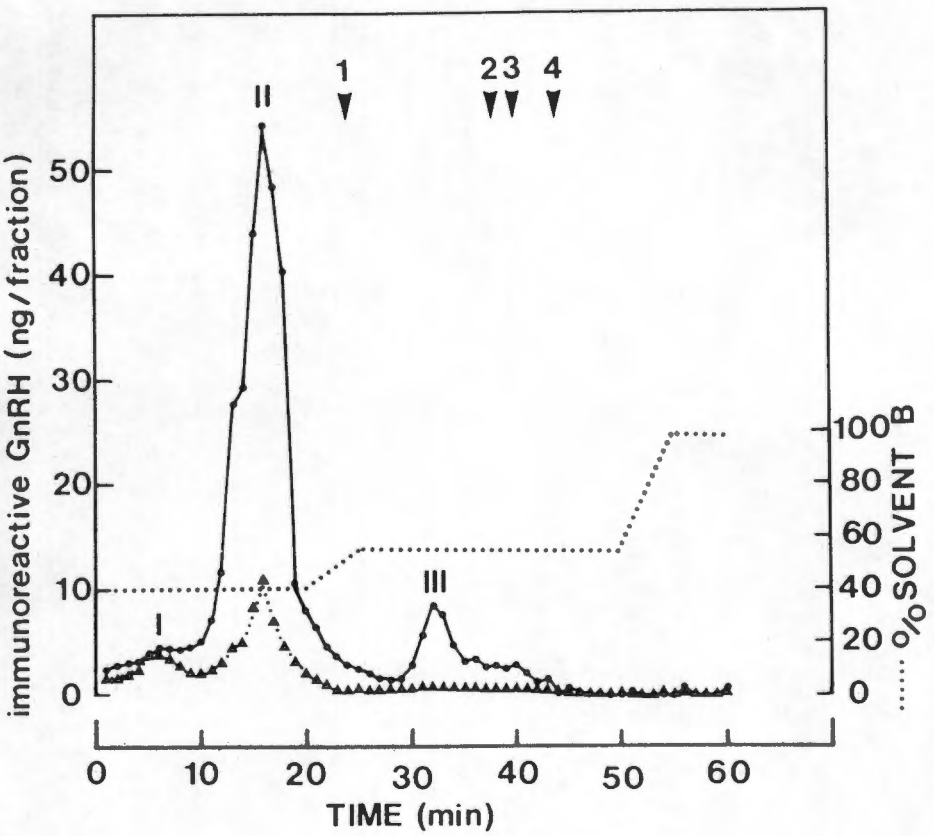


Fig. 6. Semipreparative HPLC of ostrich hypothalamus immunoreactive GnRH (540 ng GnRH, recovery was 73%, antiserum 80/1). Fractions were assayed with antisera 80/1 (●—●) and 1076 (▲····▲).

TABLE III. CROSS-REACTIVITY OF GnRH ANTISERA WITH THE FIVE KNOWN NATURALLY-OCCURRING GnRHs

(*RELATIVE TO CROSS-REACTION WITH ANTISERUM 80/1)

Synthetic peptides	PERCENTAGE RELATIVE CROSS-REACTION	
	antiserum 1076	antiserum 303
GnRH	100	0.02
Gln^8-GnRH	17.7	100
Trp^7,Leu^8-GnRH	0.05	0.2
His^5,Trp^7,Tyr^8-GnRH	2.4	3.4
$Tyr^3,Leu^5,Glu^6,Trp^7,Lys^8$-GnRH	< 1.1	-

* Cross-reaction of each GnRH form with antiserum 80/1 is taken as 100% and relative cross-reaction with the other antisera is a percentage of this.

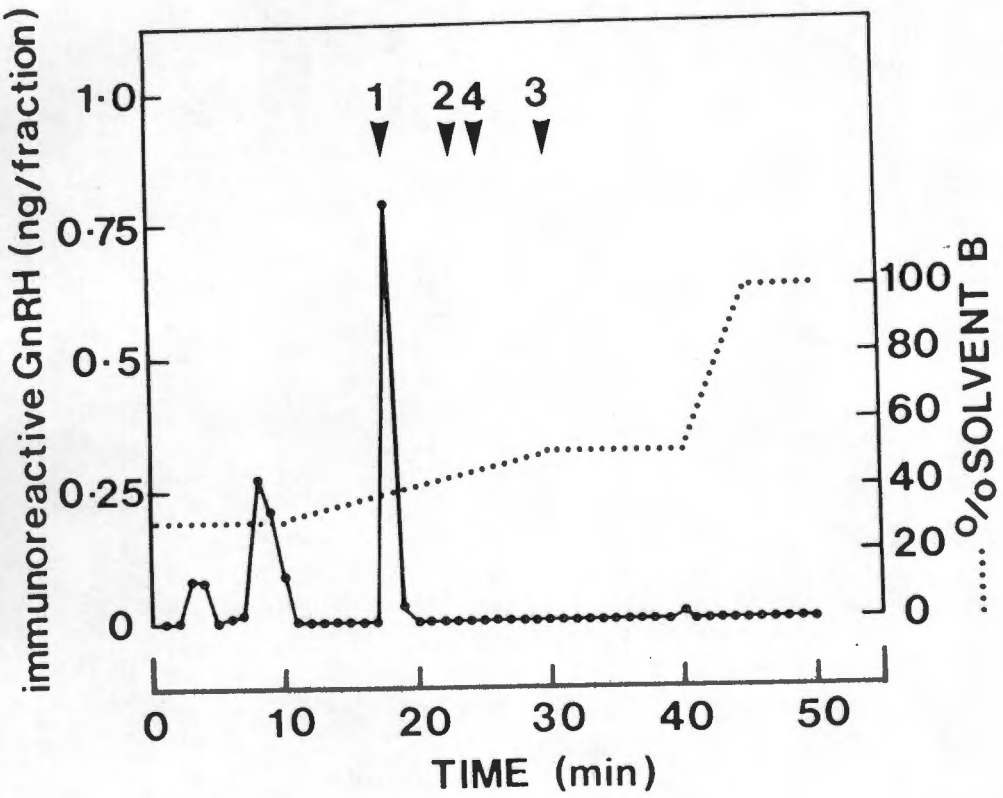


Fig. 7. Analytical HPLC of ostrich hypothalamus immunoreactive peak I (system 5, 854 pg GnRH, recovery was 145%, antiserum 80/1). Fractions were assayed with antiserum 80/1. Elution positions of Gln^8-GnRH (1), mammalian GnRH (2), Trp^7,Leu^8-GnRH (3) and His^5,Trp^7,Tyr^8-GnRH (4) are indicated.

Peak II eluted as a single peak of immunoreactive GnRH (antiserum 80/1) on HPLC system 2, but eluted earlier than any of the four known naturally-occurring GnRHs (Fig. 8A). This was due to overloading of the column and the peak was thus pooled, lyophilized, and reapplied to the same system (Fig. 8B). The peak again eluted early (Fig. 8B). Fraction eight was lyophilized and half of the lyophilizate was reapplied to HPLC system 2. Immunoreactive GnRH eluted as a single peak (antiserum 80/1) (Fig. 8C), which co-eluted with Gln^8-GnRH. The other half of the lyophilizate was applied to HPLC system 4, where the immunoreactive GnRH again co-eluted with Gln^8-GnRH (antiserum 80/1) (Fig. 9). This GnRH molecular form also cross-reacted with antiserum 303 (123% relative to antiserum 80/1) (Fig.9), which requires Gln^8 (Table III) for full immunoreactivity. These data suggest that peak II is Gln^8-GnRH.

Peak III (semipreparative HPLC) eluted as a single peak of immunoreactivity (antiserum 80/1) on HPLC system 2 (Fig. 10A). This peak did not co-elute with any of the four known naturally-occurring GnRHs, due to overloading of the column. The peak was, therefore, pooled and reapplied to the same system. The immunoreactive GnRH eluted as two peaks (antiserum 80/1) (Fig. 10B); the first peak co-eluting with mammalian GnRH and the second peak with His^5,Trp^7,Tyr^8-GnRH. Antiserum 1076 cross-reacted 16% with the first peak (relative to antiserum 80/1, data not shown). This suggests that it is not mammalian GnRH, which cross-reacts equally with antisera 80/1 and 1076 (Table III).

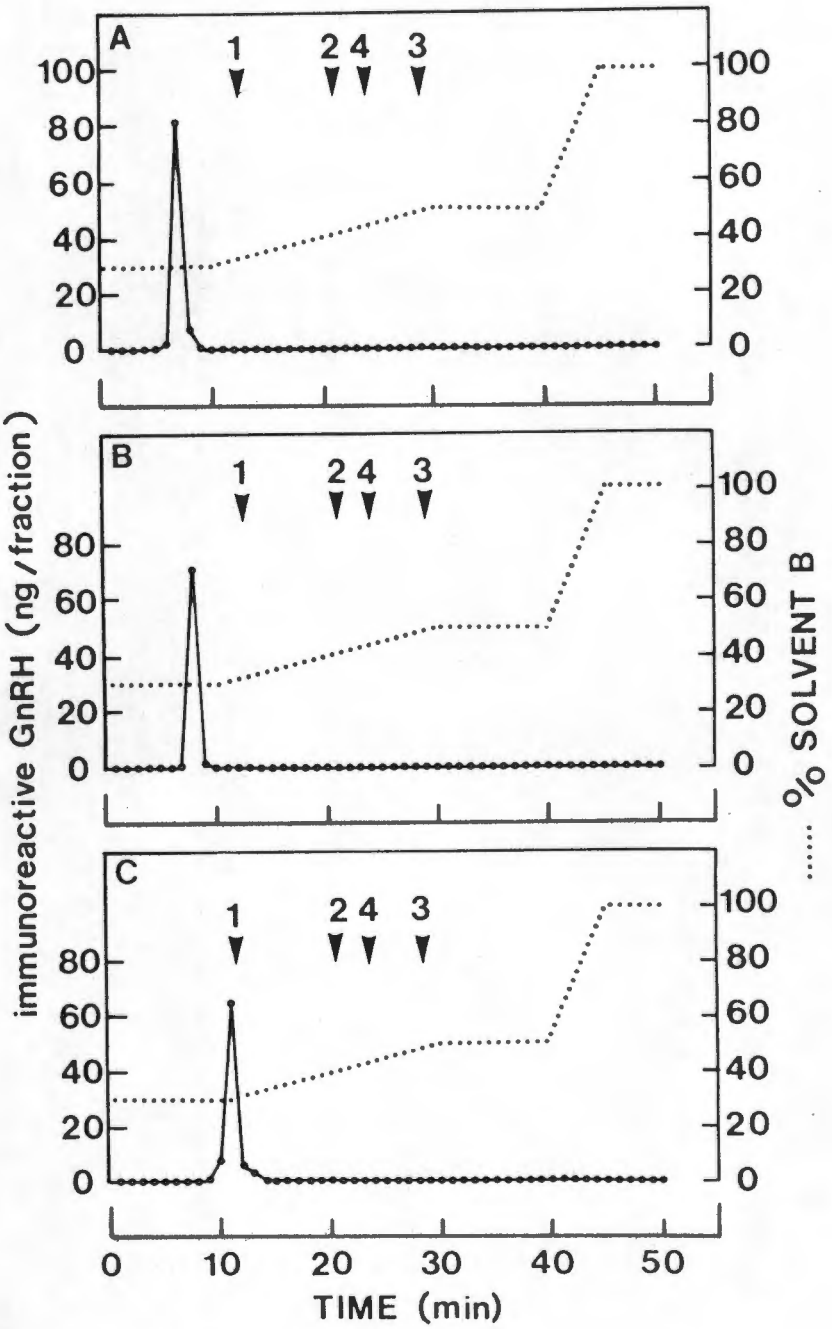


Fig. 8. Analytical HPLC of ostrich hypothalamus immunoreactive peak II (system 2). A. 64 ng GnRH, recovery was 145%, antiserum 80/1. B. 76 ng GnRH, recovery was 95%, antiserum 80/1. C. 69.7 ng GnRH, recovery was 82%, antiserum 80/1. Fractions were assayed with antiserum 80/1. Elution positions of Gln^8-GnRH (1), mammalian GnRH (2), Trp^7,Leu^8-GnRH (3) and His^5,Trp^7,Tyr^8-GnRH (4) are indicated.

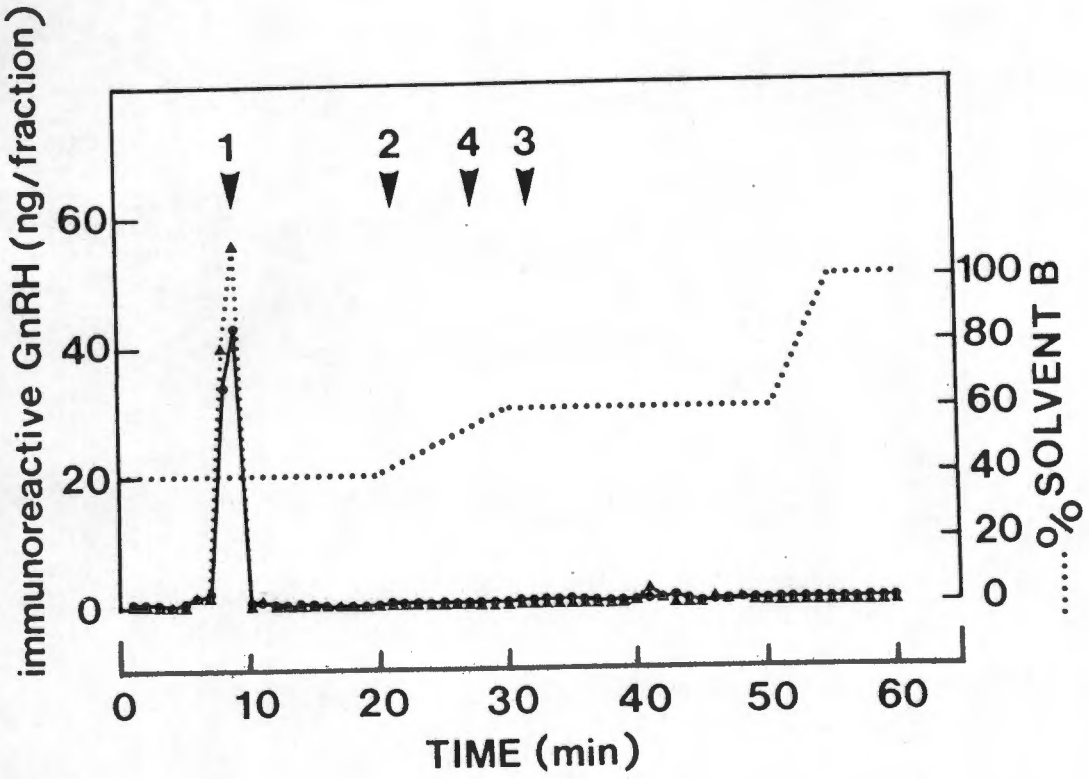


Fig. 9. Analytical HPLC of ostrich hypothalamus immunoreactive GnRH peak II (system 4, 72.6 ng GnRH, recovery was 113%, antiserum 80/1). Fractions were assayed with antisera 80/1 (●—●) and 303 (▲···▲). Elutions positions of Gln^8-GnRH (1), mammalian GnRH (2), Trp^7,Leu^8-GnRH (3) and His^5,Trp^7,Tyr^8-GnRH (4) are indicated.

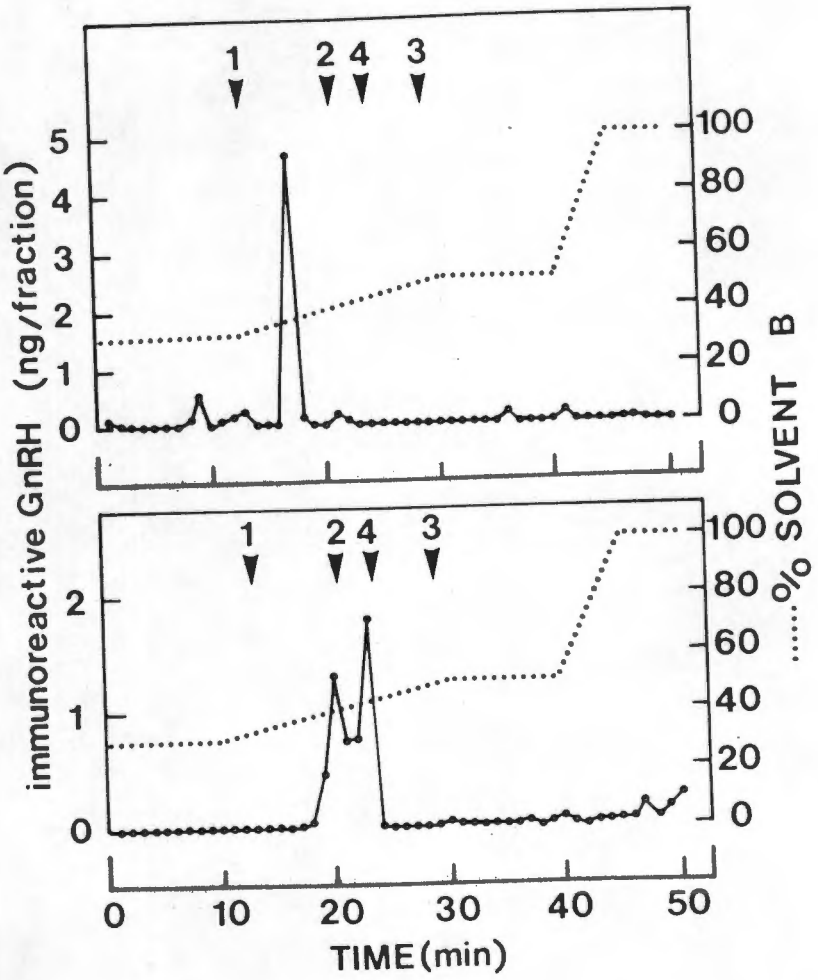


Fig. 10. Analytical HPLC of ostrich hypothalamus immunoreactive peak III (system 2). A. 4.3 ng GnRH, recovery was 109.6%, antiserum 80/1. B. 6.3 ng GnRH, recovery was 89%, antiserum 80/1. Fractions were assayed with antiserum 80/1. Elution positions of Gln^8-GnRH (1), mammalian GnRH (2), Trp^7,Leu^8-GnRH (3) and His^5,Trp^7,Tyr^8-GnRH (4) are indicated.

Chicken LH-releasing activity

The peak which co-eluted with Gln^8-GnRH (peak II from semipreparative HPLC) stimulated the release of LH (Fig. 11). The amount of LH released is consistent with the peak being Gln^8-GnRH. The peak coincident with His^5,Trp^7,Tyr^8-GnRH was twice as potent as Gln^8-GnRH (Fig. 12). Synthetic His^5,Trp^7,Tyr^8-GnRH is 5 times more potent than synthetic Gln^8-GnRH (Millar and King, 1984; Chou et al., 1985). These results thus demonstrate that two GnRHs in ostrich hypothalamus are likely to be Gln^8-GnRH and His^5,Trp^7,Tyr^8-GnRH. The peak coincident with mammalian GnRH (0.44 ng, antiserum 80/1) released a substantial amount of LH (Fig. 12), which is not consistent with the peak being mammalian GnRH as a similar amount of synthetic mammalian GnRH would release far less LH. Therefore, this is a novel GnRH molecular form. Three additional peaks of LH-releasing activity were demonstrated (Fig. 12), which displayed no immunoreactivity with antiserum 80/1. These peaks are likely to be novel forms of GnRH which do not cross-react with the antiserum, or structurally-unrelated peptides which have LH-releasing activity.

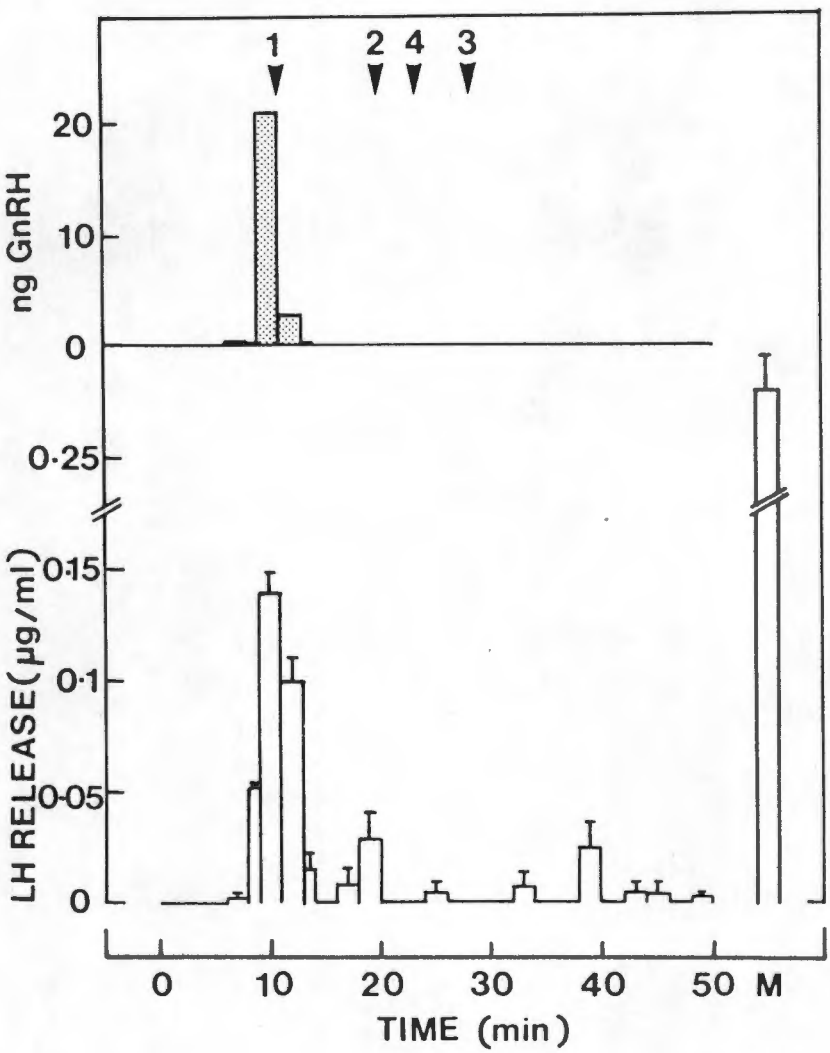


Fig. 11. Chicken pituitary cell bioassay. A. Effect of ostrich hypothalamic peak II GnRH (Fig. 8C) in HPLC pooled fractions (system 2) and 10^{-7} M Gln^8-GnRH (maximal stimualtion, M) on LH release from dispersed chicken pituitary cells. LH release is expressed as ug LH/ml of cell medium with basal release subtracted (open column). Columns represent the mean $\pm$ SEM (n = 3) of a pool of HPLC fractions (in twos). Immunoreactive GnRH (antiserum 80/1) per HPLC fraction pool taken for the bioassay is expressed as ng GnRH/aliquot of HPLC fraction pool (dotted column). Elution positions of Gln^8-GnRH (1), mammalian GnRH (2), Trp^7,Leu^8-GnRH (3) and His^5,Trp^7,Tyr^8-GnRH (4) are indicated.

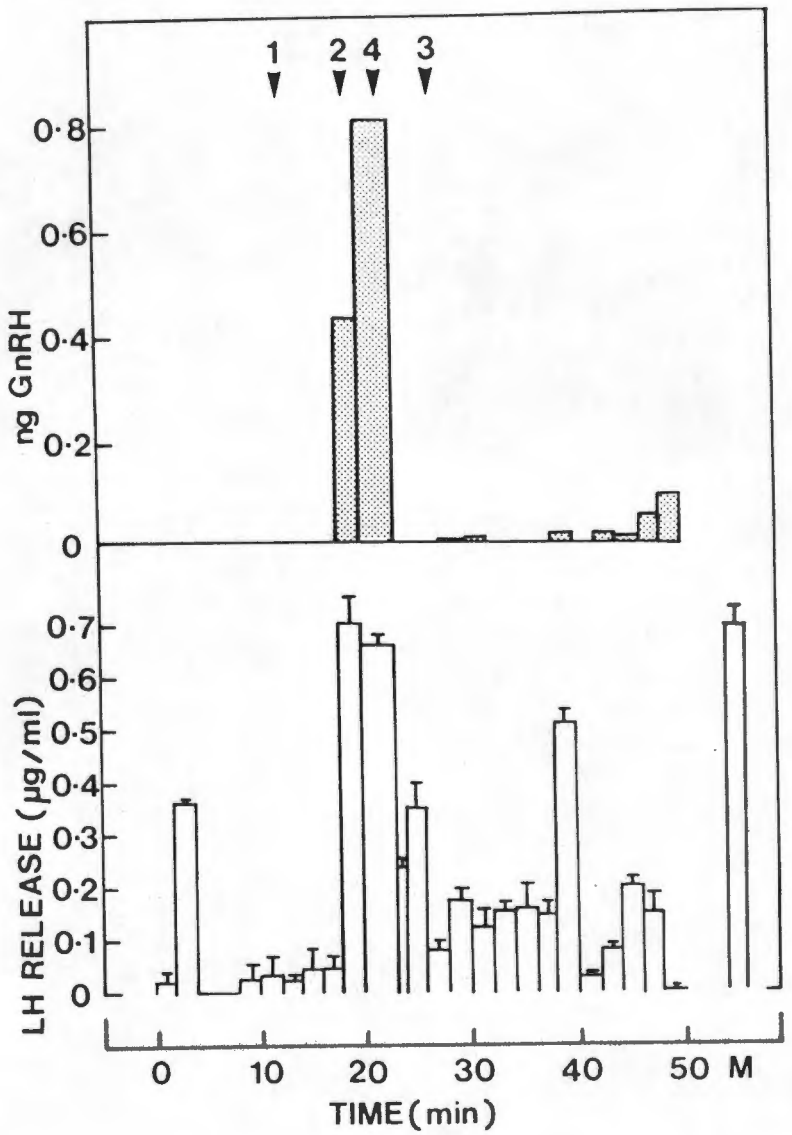


Fig. 12. Chicken pituitary cell bioassay. A. Effect of ostrich hypothalamic peak III GnRH (Fig. 10B) in pooled HPLC fractions (system 2) and 10^{-7} M Gln^8-GnRH (maximal stimulation, M) on LH release from dispersed chicken pituitary cells. LH release is expressed as ug LH/ml of cell medium with basal release subtracted (open column). Columns represent the mean ± SEM (n = 3) of a pool of HPLC fractions (in twos). Immunoreactive GnRH (antiserum 80/1) per HPLC fraction pool taken for the bioassay is expressed as ng GnRH/aliquot of HPLC fraction pool (dotted column). Elution positions of Gln^8-GnRH (1), mammalian GnRH (2), Trp^7,Leu^8-GnRH (3) and His^5,Trp^7,Tyr^8-GnRH (4).

3.4 DISCUSSION

Two immunoreactive GnRH molecular forms were identified in ostrich hypothalamus. One form co-eluted with Gln^8-GnRH in two analytical HPLC systems employing different columns and solvents, which separate the known vertebrate GnRHs and a wide range of GnRH analogues. The interaction of this GnRH with three region-specific antisera was identical to that of Gln^8-GnRH and, moreover, this peak stimulated release of LH similarly to synthetic Gln^8-GnRH. These data strongly suggest that one form of GnRH in ostrich hypothalamus is Gln^8-GnRH. The second immunoreactive (antiserum 80/1) GnRH molecular form co-eluted with His^5,Trp^7,Tyr^8-GnRH on analytical HPLC and did not cross-react with antiserum 1076. It released twice as much LH from chicken anterior pituitary cells than the peak which co-eluted with Gln^8-GnRH, and thus had similar chromatographic, immunological and biological properties to His^5,Trp^7,Tyr^8-GnRH. Four additional LH-releasing substances were present. One of these forms co-eluted with mammalian GnRH in an HPLC system, but had different immunological and biological properties. The other three forms are novel GnRH molecular forms with low immunoreactivity, or unrelated peptides with LH-releasing activity.

Since Gln^8-GnRH and His^5,Trp^7,Tyr^8-GnRH occur in both chicken (King and Millar, 1982a, 1982c; Miyamoto *et al.*, 1983, 1984) and ostrich hypothalamus, it appears that these two GnRHs are conserved in birds. However, since other novel GnRHs are present in the ostrich hypothalamus, it appears that diversity in GnRH structure does occur in birds.

4.0 DIVERSE MOLECULAR FORMS OF GnRH IN REPTILE BRAIN

4.1 ABSTRACT

Gonadotropin-releasing hormone molecular forms in the brains of two reptiles, A. mississippiensis (alligator) and C. ocellatus tiligugu (skink) were characterized by HPLC and radioimmunoassay with region-specific GnRH antisera, and by assessment of LH-releasing activity in chicken dispersed anterior pituitary cells. In alligator brain, two GnRHs had identical properties to the two known forms of chicken hypothalamic GnRH (Gln^8-GnRH and His^5,Trp^7,Tyr^8-GnRH) in their elution on two reverse phase HPLC systems, cross-reaction with region-specific GnRH antisera, and ability to release LH. In skink brain, one immunoreactive and bioactive GnRH form, which co-eluted with His^5,Trp^7,Tyr^8-GnRH on reverse phase HPLC, was identified.

4.2 INTRODUCTORY STATEMENT

Amongst reptiles, in lizard (C. nigra) brain four GnRHs have been partially characterized using HPLC, radioimmunoassay with region-specific antisera and assessment of chicken LH-releasing activity (Powell et al., 1985). Two of these forms had identical properties to Trp^{7},Leu^{8}-GnRH and His^{5},Trp^{7},Tyr^{8}-GnRH, whereas other forms appear to be novel GnRHs. Immunoreactive GnRHs in lizard (M. capensis) and tortoise (C. angulata) hypothalami were immunologically different from mammalian GnRH (King and Millar, 1979a, 1980); however, their structures have not been studied further. Alligator (A. mississippiensis) hypothalamic GnRH was studied using two region-specific GnRH antisera. The GnRH differed from the mammalian peptide in at least the 8 position (Lance, 1985; Lance et al., 1985). Therefore, there is very little data concerning GnRHs in different reptilian species.

4.3 RESULTS

Alligator brain extract

Chromatography and radioimmunoassay of alligator brain extract

Immunoreactive GnRH content and concentration are given in Table IV.

Immunoreactive GnRH (antiserum 80/1) eluted as a broad peak (Fig. 13), coincident with synthetic mammalian GnRH on gel filtration, indicating that they have the same molecular size. The data suggest that the GnRH is a decapeptide with blocked N- and C-termini as in mammalian GnRH (antiserum 80/1 requires the N- and C-termini for binding). The first fifty fractions were also assayed with middle-directed antiserum 1076 to determine the possible presence of high molecular weight GnRH (a precursor or GnRH bound to a protein) (data not shown). No immunoreactivity was detected, indicating that there is no high molecular weight GnRH or that the antiserum does not cross-react with it. The immunoreactive GnRH peak was pooled and lyophilized.

The pooled lyophilized fractions from the gel filtration column were reconstituted in water and sonicated for analytical HPLC. A portion of the sample was applied to HPLC system 1. A single peak of immunoreactive GnRH (antiserum 80/1) co-eluted with synthetic Gln^8-GnRH (Fig. 14). It was well-separated from the other three naturally-occurring GnRHs. This immunoreactive GnRH also cross-reacted with antiserum 303 (114% relative to cross-reaction with antiserum 80/1) (Fig. 14). This antiserum requires Gln^8 for full cross-reaction. It is known that Gln^8-GnRH

TABLE IV. CONTENT AND CONCENTRATION OF IMMUNOREACTIVE GnRH (ANTISERUM (80/1) IN REPTILE BRAIN

Species	Tissue	Immunoreactive GnRH (ng/tissue)	Immunoreactive GnRH (pg/mg dry weight of tissue)
Alligator	whole brain	14.6	19.3
Skink	whole brain	0.1	7.9

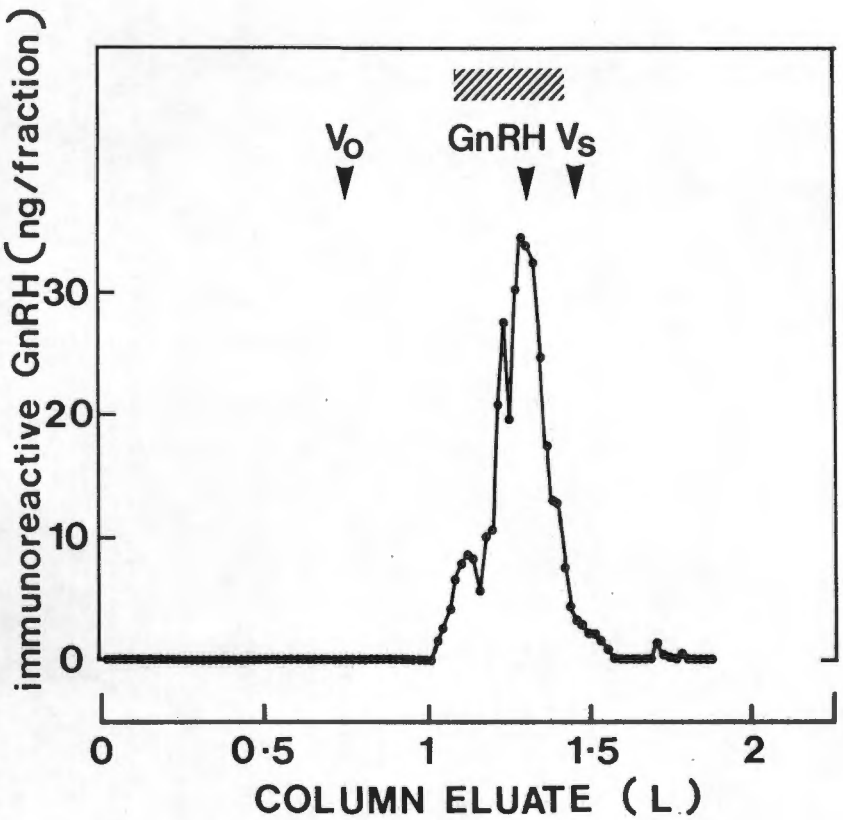


Fig. 13. Gel filtration chromatography of alligator brain extract (336 ng GnRH, antiserum 80/1). The void volume (V_o), salt elution volume (V_s) and elution position of synthetic mammalian GnRH are indicated. The hatched block represents the immunoreactive GnRH region pooled for further analysis.

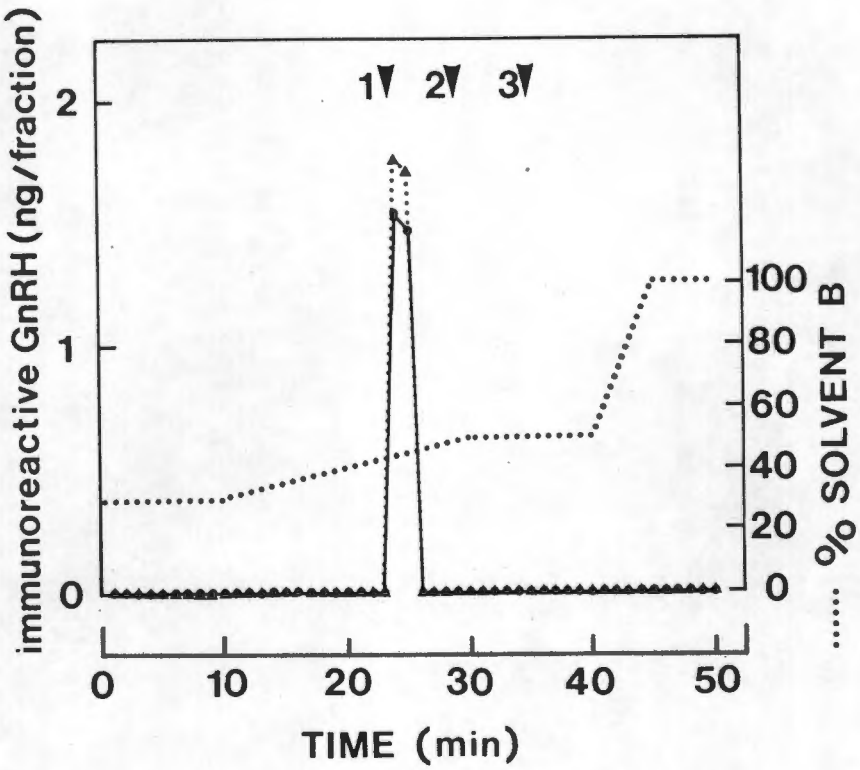


Fig. 14. Analytical HPLC of alligator brain immunoreactive GnRH (system 1, 3.2 ng GnRH, recovery was 94%, antiserum 80/1). Fractions were assayed with antisera 80/1 (●—●) and 303 (▲···▲). Elution positions of Gln^8-GnRH (1), mammalian GnRH (2) and Trp^7,Leu^8-GnRH (3) are indicated.

cross-reacts fully with antisera 80/1 and 303 (King et al., 1983) (Table III). Therefore, the data strongly suggest the presence of Gln^8-GnRH in alligator brain.

The remaining sample was applied to HPLC system 1 in two runs (Fig. 15). Two immunoreactive GnRH peaks (antiserum 80/1) eluted in each of the runs. Peak I (the major immunoreactive peak) and peak II co-eluted with Gln^8-GnRH and His^5,Trp^7,Tyr^8-GnRH, respectively. His^5,Trp^7,Tyr^8-GnRH cross-reacts only 5% with antiserum 80/1 (relative to mass) (King and Millar, 1985). Thus peak II could contain 40.5 ng (Run A) (Fig. 15A) and 10.4 ng (Run B) (Fig. 15B) of His^5,Trp^7,Tyr^8-GnRH. Run B fractions were also assayed with middle-directed antiserum 1076 (Fig. 15B). Peak I cross-reacted 12% with antiserum 1076 (relative to antiserum 80/1) which is similar to the relative cross-reaction of Gln^8-GnRH with these two antisera (King and Millar, 1982b). Peak II did not cross-react with antiserum 1076 (Fig. 15B). This is similar to the relative cross-reaction of His^5,Trp^7,Tyr^8-GnRH with these two antisera. Antiserum 1076 cross-reacts only 0.1% with His^5,Trp^7,Tyr^8-GnRH (relative to mass) (King and Millar, 1985). The two immunoreactive GnRH peaks in Run A were pooled separately and lyophilized for further analytical HPLC. Aliquots of Run B fractions were lyophilized for assessment of LH-releasing activity in the chicken anterior pituitary cell bioassay.

The two peaks from Run A, HPLC system 1, were rechromatographed separately on HPLC system 3 (Fig. 16). Peaks I and II again co-eluted with Gln^8-GnRH (Fig. 16A) and His^5,Trp^7,Tyr^8-GnRH (Fig. 16B) respectively, and were well-separated from the other naturally-occurring GnRHs. These data provide further evidence for the presence of Gln^8-GnRH and His^5,Trp^7,Tyr^8-GnRH in alligator brain.

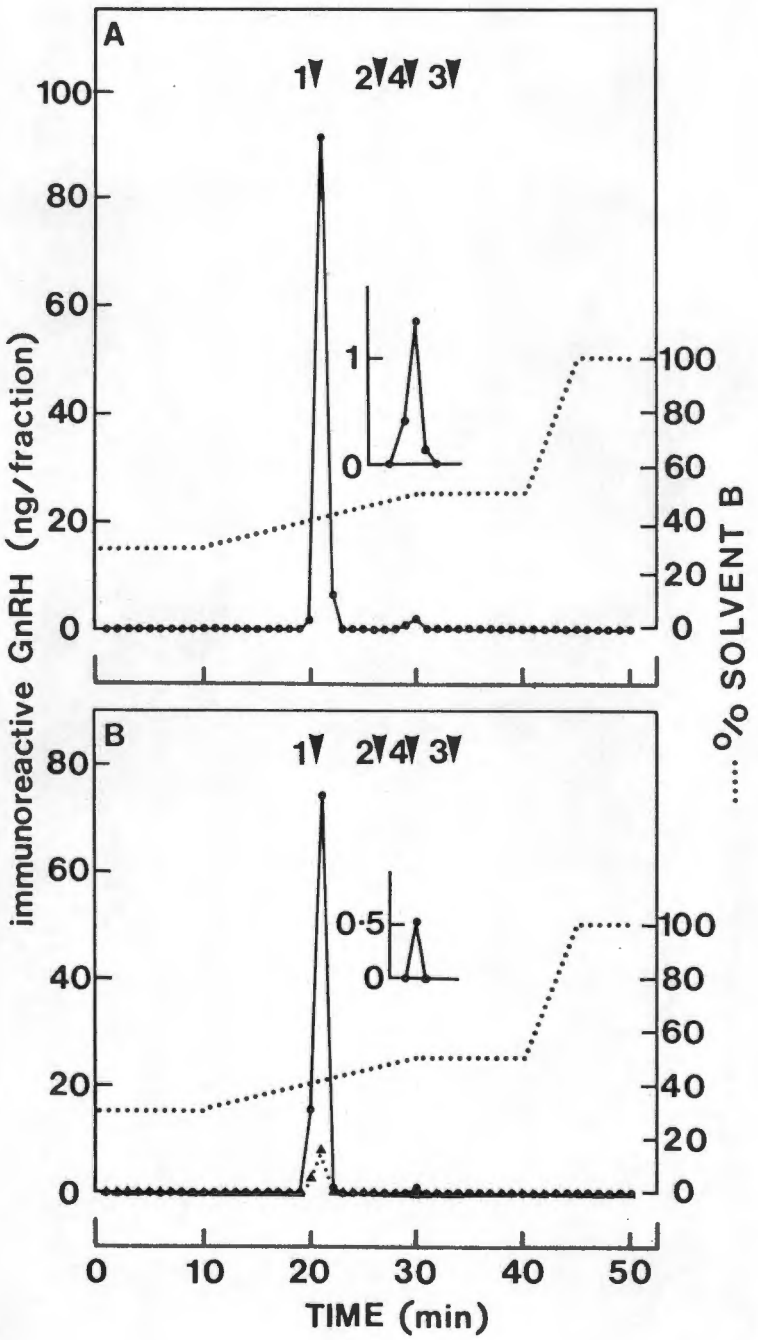


Fig. 15. Analytical HPLC of alligator brain immunoreactive GnRH (system 1). A. 62 ng GnRH, recovery was 150%, antiserum 80/1. Fractions were assayed with antiserum 80/1 (●—●) B. 62 ng GnRH, recovery was 145%, antiserum 80/1. Fractions were assayed with antisera 80/1 (●—●) and 1076 (▲·····▲). Elution positions of Gln^{8}-GnRH (1), mammalian GnRH (2), Trp^{7},Leu^{8}-GnRH (3) and His^{5},Trp^{7},Tyr^{8}-GnRH (4) are indicated. The inserts represent a twenty-fold magnification of peak II.

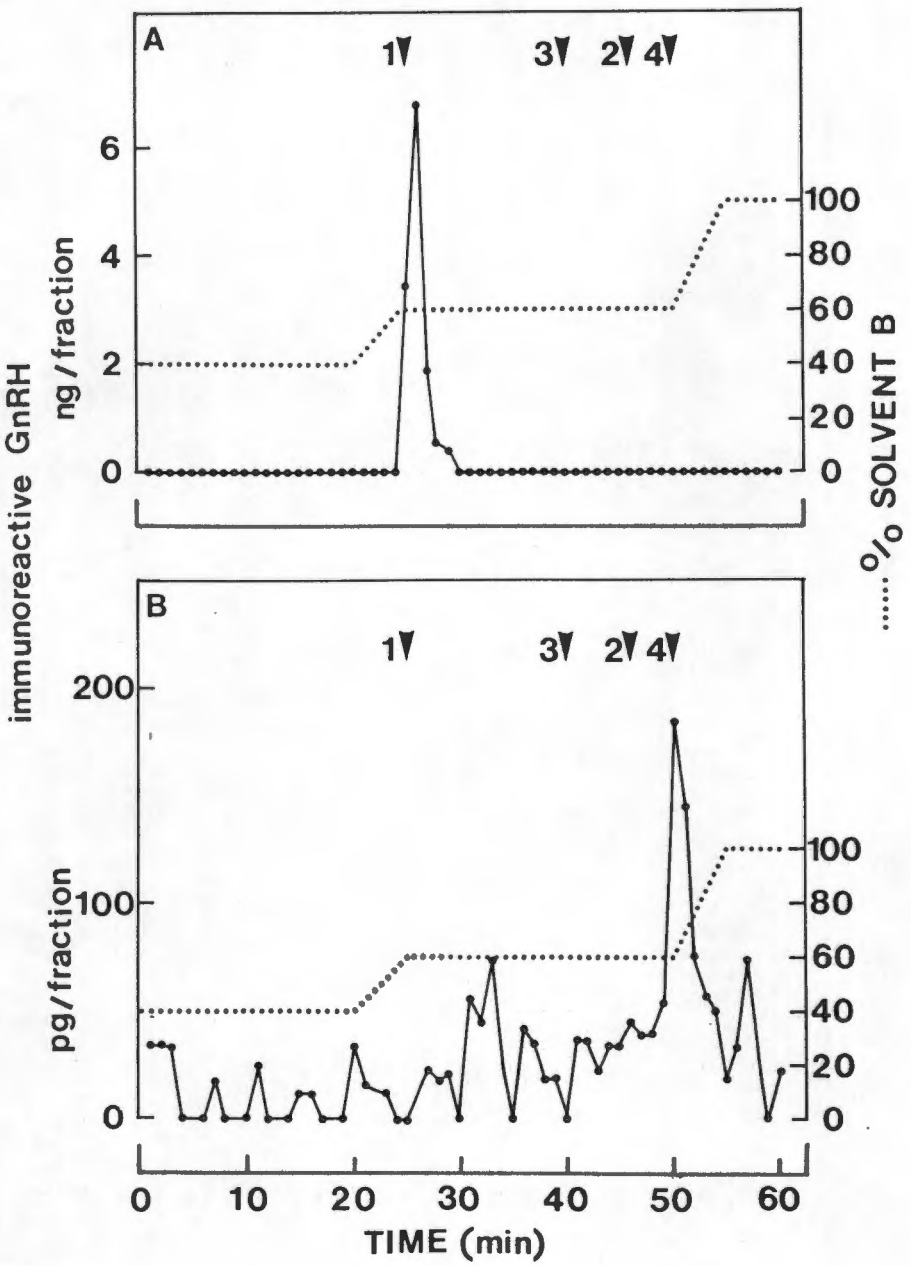


Fig. 16. Analytical HPLC of alligator brain immunoreactive GnRH peaks from system 1 on system 3. A. 11.1 ng peak I GnRH, recovery was 119%, antiserum 80/1. Fractions were assayed with antiserum 80/1. B. 413 pg peak II GnRH, recovery was 80%, antiserum 80/1. Fractions were assayed with antiserum 80/1. Elution positions of Gln^8-GnRH (1), mammalian GnRH (2), Trp^7,Leu^8-GnRH (3) and His^5,Trp^7,Tyr^8-GnRH (4) are indicated.

Chicken LH-releasing activity

Both immunoreactive GnRH peaks (antiserum 80/1) stimulated chicken LH release (Fig. 17). Peak I (5.1 ng immunoreactive GnRH, antiserum 80/1) released 6.36 μg of LH. Peak II (0.075 ng immunoreactive GnRH, antiserum 80/1) released 3.53 μg of LH. His^5,Trp^7,Tyr^8-GnRH has been shown to be 5 times more potent than Gln^8-GnRH in stimulating chicken LH release (Millar and King, 1984; Chou et al., 1985). Thus peak II (if it were His^5,Trp^7,Tyr^8-GnRH) would be twice as potent as peak I. This is consistent with the two GnRHs being Gln^8-GnRH and His^5,Trp^7,Tyr^8-GnRH.

Skink brain extract

Chromatography and radioimmunoassay of skink brain extract

Immunoreactive GnRH content and concentration are given in Table IV. On analytical HPLC system 2, a single immunoreactive GnRH peak eluted (396 pg, antiserum 80/1) (Fig. 18), coincident with His^5,Trp^7,Tyr^8-GnRH. If the peak were His^5,Trp^7,Tyr^8-GnRH, it would contain 7.9 ng GnRH (by mass), as this GnRH analogue cross-reacts only 5% with antiserum 80/1 (King and Millar, 1985). This peak of GnRH was well-separated from the other three naturally-occurring GnRHs chromatographed on this system.

Chicken LH-releasing activity

The LH-releasing activity of the above HPLC fractions was assessed. The immunoreactive GnRH peak stimulated release of LH (Fig. 19) in a chicken dispersed anterior pituitary cell bioassay. His^5,Trp^7,Tyr^8-GnRH has

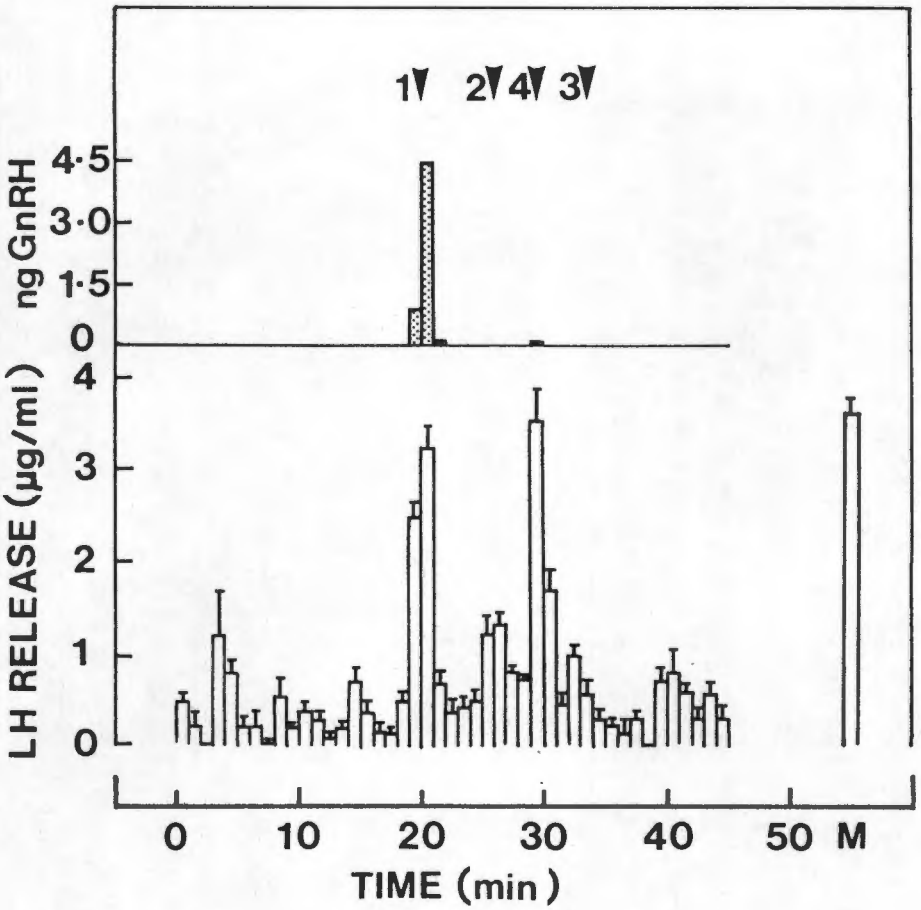


Fig. 17. Chicken pituitary cell bioassay. Effect of alligator brain GnRH in HPLC fractions (system 1) and 10^{-7} M Gln^8-GnRH (maximal stimulation, M) on LH release from chicken pituitary cells. LH release is expressed as µg LH/ml of cell medium with basal release subtracted (open column). Columns represent the mean ± SEM (n = 3) of an assay for each HPLC fraction. Immunoreactive GnRH (antiserum 80/1) per HPLC fraction aliquot taken for the bioassay is expressed as ng GnRH/aliquot of HPLC fraction (dotted column). Elution positions of Gln^8-GnRH (1), mammalian GnRH (2), Trp^7,Leu^8-GnRH (3) and His^5,Trp^7,Tyr^8-GnRH (4) are indicated.

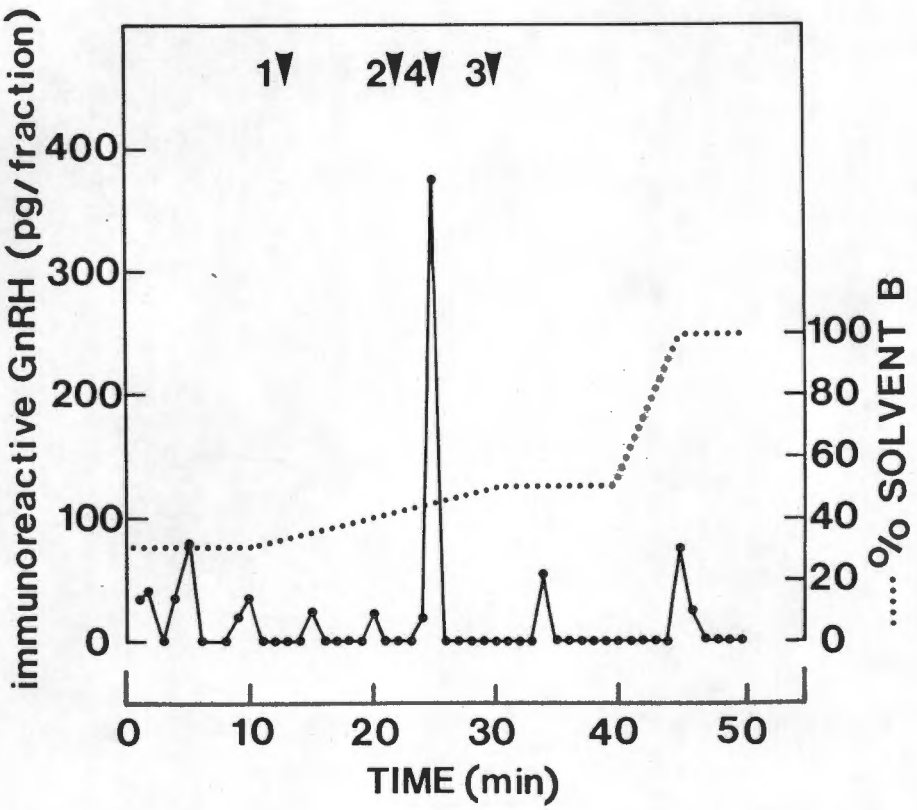


Fig. 18. Analytical HPLC of skink brain GnRH (system 2, 241 pg, GnRH, recovery was 163%, with antiserum 80/1). Elution positions of Gln^8-GnRH (1), mammalian GnRH (2), Trp^7,Leu^8-GnRH (3) and His^5,Trp^7,Tyr^8-GnRH (4) are indicated.

LH-releasing activity in this bioassay (Millar and King, 1984; Chou et al., 1985). The immunoreactive peak had only 396 pg of GnRH (measured by antiserum 80/1) and yet stimulated a substantial release of LH, indicating that this peak could be His^5,Trp^7,Tyr^8-GnRH, which cross-reacts only 5% with antiserum 80/1 (King and Millar, 1985).

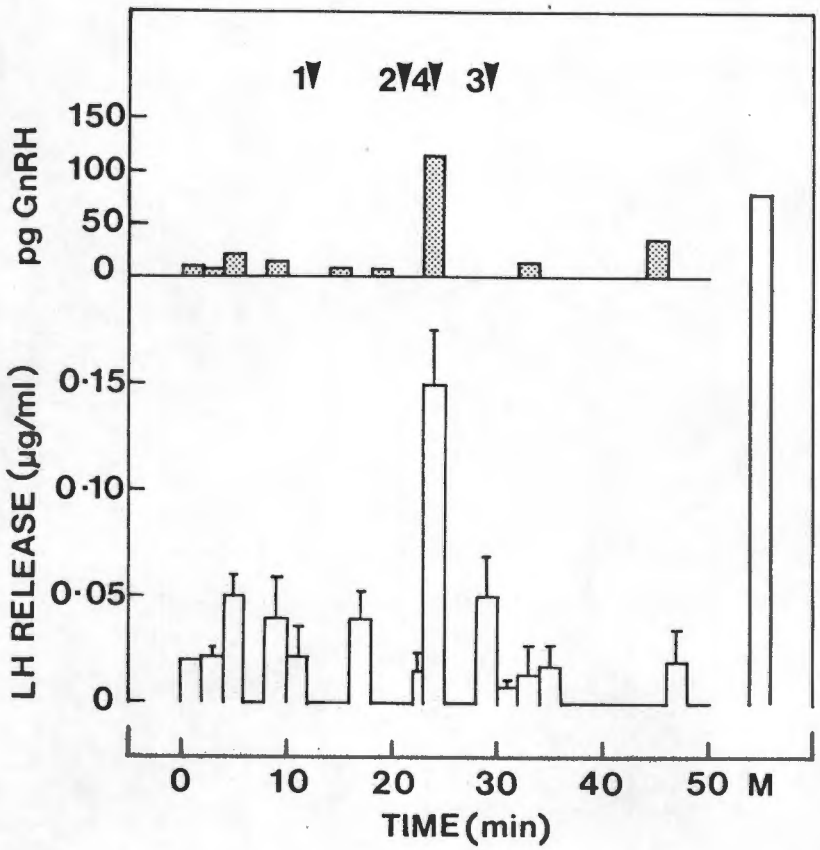


Fig. 19. Chicken pituitary cell bioassay. Effect of skink brain GnRH in pooled HPLC fractions (system 2) and 10^{-6} M Gln^8-GnRH (maximal stimulation, M) on LH release from dispersed chicken pituitary cells. LH release is expressed as µg LH release/ml of cell medium with basal release subtracted (open column). Columns represent the mean ± SEM (n = 3) of an assay for each pool of HPLC fractions (in twos). Immunoreactive GnRH (antiserum 80/1) per HPLC fraction pool taken for the bioassay is expressed as ng GnRH/aliquot of HPLC fraction pool (dotted column). Elution positions of Gln^8-GnRH (1), mammalian GnRH (2), Trp^7,Leu^8-GnRH (3) and His^5,Trp^7,Tyr^8-GnRH (4) are indicated.

4.4 DISCUSSION

These studies demonstrate structural diversity in reptilian GnRH. Two GnRHs, with identical chromatographic, immunological and biological properties to Gln^8-GnRH and His^5,Trp^7,Tyr^8-GnRH, were present in alligator brain. Preliminary studies with GnRH antisera IJ-29 and R-42 indicated that alligator hypothalamic GnRH differs from the mammalian GnRH in at least position 8 (Lance, 1985; Lance *et al.*, 1985). Skink brain had only one detectable form of GnRH, with similar properties to His^5,Trp^7,Tyr^8-GnRH. In the lizard (*C. nigra*) four immunoreactive GnRHs were present, two of which have identical chromatographic, immunological and biological properties to Trp^7,Leu^8-GnRH and His^5,Trp^7,Tyr^8-GnRH (Powell *et al.*, 1985). One of the other two GnRH forms co-eluted with Gln^8-GnRH on reverse phase HPLC, but had different immunological properties (Powell *et al.*, 1985).

The two GnRHs in alligator brain, Gln^8-GnRH and His^5,Trp^7,Tyr^8-GnRH, are also present in chicken hypothalamus (King and Millar, 1982a, 1982c; Miyamoto *et al.*, 1983, 1984). Alligators are believed to be phylogenetically related to birds and possibly they have a common ancestor (Romer, 1970). The fact that alligator and bird GnRHs appear to be the same, is consistent with this theory of evolution.

Skink brain had a GnRH form similar to His^5,Trp^7,Tyr^8-GnRH, which was also present in *C. nigra* and in the alligator. This molecular form of GnRH is widespread among vertebrates and possibly represents an early-evolved and conserved GnRH form (Millar and King, 1986). This form of GnRH seems to occur in most reptiles.

5.0 DIVERSE MOLECULAR FORMS OF GnRH IN BRAIN TISSUE OF AN ELASMOBRANCH AND A TELEOST FISH

5.1 ABSTRACT

Immunoreactive and biologically active gonadotropin-releasing hormones in dogfish (P. africanum) and teleost (C. julis) brain extracts were studied by HPLC, radioimmunoassay with region-specific GnRH antisera and by assessment of LH-releasing activity in a chicken dispersed anterior pituitary cell bioassay. In dogfish brain extract, seven GnRH molecular forms with LH-releasing activity were demonstrated. Three of these forms co-eluted with synthetic mammalian GnRH, His^5,Trp^7,Tyr^8-GnRH and Trp^7,Leu^8-GnRH on HPLC. The peaks coincident with His^5,Trp^7,Tyr^8-GnRH and Trp^7,Leu^8-GnRH had identical immunological and biological properties to the synthetic peptides. However, the molecular form co-eluting with mammalian GnRH had different immunological and biological properties from mammalian GnRH and is thus a novel molecular variant of GnRH. The four remaining forms were also novel GnRHs or structurally unrelated peptides with LH-releasing activity. Immunoreactive GnRH occurred in dogfish systemic blood. In teleost brain extract, three biologically active GnRH forms with LH-releasing activity were present. The major peak of GnRH immunoreactivity co-eluted with Trp^7,Leu^8-GnRH, and a second immunoreactive form co-eluted with His^5,Trp^7,Tyr^8-GnRH. The third biologically active peak is a novel, early-eluting molecular variant of GnRH or a structurally unrelated peptide with LH-releasing activity.

5.2 INTRODUCTORY STATEMENT

Three different classes of fish occur, the agnathans, osteichthyes and chondrichthyes. Numerous studies have been performed on GnRH structure of osteichthyes, and a GnRH in salmon (O. keta) brain has been identified as Trp^7,Leu^8-GnRH (Sherwood et al., 1983). A GnRH with similar properties to Trp^7,Leu^8-GnRH occurs in most teleosts studied (Jackson and Pan, 1983; Sherwood et al., 1983; Breton et al., 1984; King and Millar, 1985; Sherwood et al., 1984). Multiple forms of GnRH have been demonstrated in hake (M. capensis) pituitary gland, and in the brain of tilapia (T. sparrmanii), codfish (G. morhua morhua), milk fish (C. chanos), trout (S. gairdneri) and mullet (M. cephalus) brain (Barnett et al., 1982; Jackson and Pan, 1983; Sherwood et al., 1984; King and Millar 1985). In lamprey (P. marinus) brain, two forms of GnRH are present, one of which has been characterized as Tyr^3,Leu^5,Glu^6,Trp^7,Lys^8-GnRH (Sherwood et al., 1986). However, the structure of GnRH in chondrichthyes has not been thoroughly investigated. Immunoreactive GnRH has been detected in certain chondrichthyes (Jackson, 1980; King and Millar, 1980), whereas no detectable immunoreactive GnRH occurred in others (Deery, 1974; Nozaki and Kobayashi, 1979). On HPLC, S. acanthias brain GnRH eluted as two immunoreactive peaks, neither co-eluting with mammalian GnRH or Trp^7,Leu^8-GnRH (Sherwood and Sower, 1985).

5.3 RESULTS

Dogfish tissue extracts

Quantitation of GnRH

Immunoreactive GnRH content and concentration (antiserum 80/1) are given in Table V. GnRH concentration in the hypothalamus was higher than in extrahypothalamic brain; total content of GnRH was similar. The pituitary gland contained no detectable immunoreactive GnRH. Immunoreactive GnRH concentration in dogfish brain varied seasonally, being higher in dogfish collected in spring (October) than in those collected in autumn (March, April). Immunoreactive GnRH occurred in dogfish systemic blood at a concentration equivalent to that reported in hypophysial portal blood of mammals (Clarke and Cummins, 1982). GnRH binding substances and peptidases, which could yield artificial GnRH immunoreactivity, were not present in serum or hypothalamic extracts.

Chromatography and radioimmunoassay of dogfish brain extract

Immunoreactive GnRH in Batch C (whole brain) eluted as a single broad peak (antiserum 80/1) on gel filtration (Fig. 20), later than mammalian GnRH, possibly due to other constituents of the extract causing adsorption. Batch B (whole brain) immunoreactive GnRH eluted in a similar manner (data not shown).

TABLE V. SEASONAL VARIATIONS IN IMMUNOREACTIVE GnRH (ANTISERUM 80/1) IN FISH BRAIN AND SERUM EXTRACTS

Species	Tissue	Month of collection	Immunoreactive GnRH		
			ng/tissue	pg/mg dry weight	pg/ml
Dogfish	Batch A				
	hypothalamus (20)	March	0.2	8.0	
	extrahypothalamic brain (20)		0.3	0.7	
	pituitary gland (20)		< 0.003	< 0.6	
	Batch B				
	whole brain (82)	April	0.3	0.9	
	Batch C				
	whole brain (109)	October	0.8	1.8	
	serum (10 ml)	March			190
	serum (7 ml)	October			58
Teleost	whole brain		0.4	20.6	

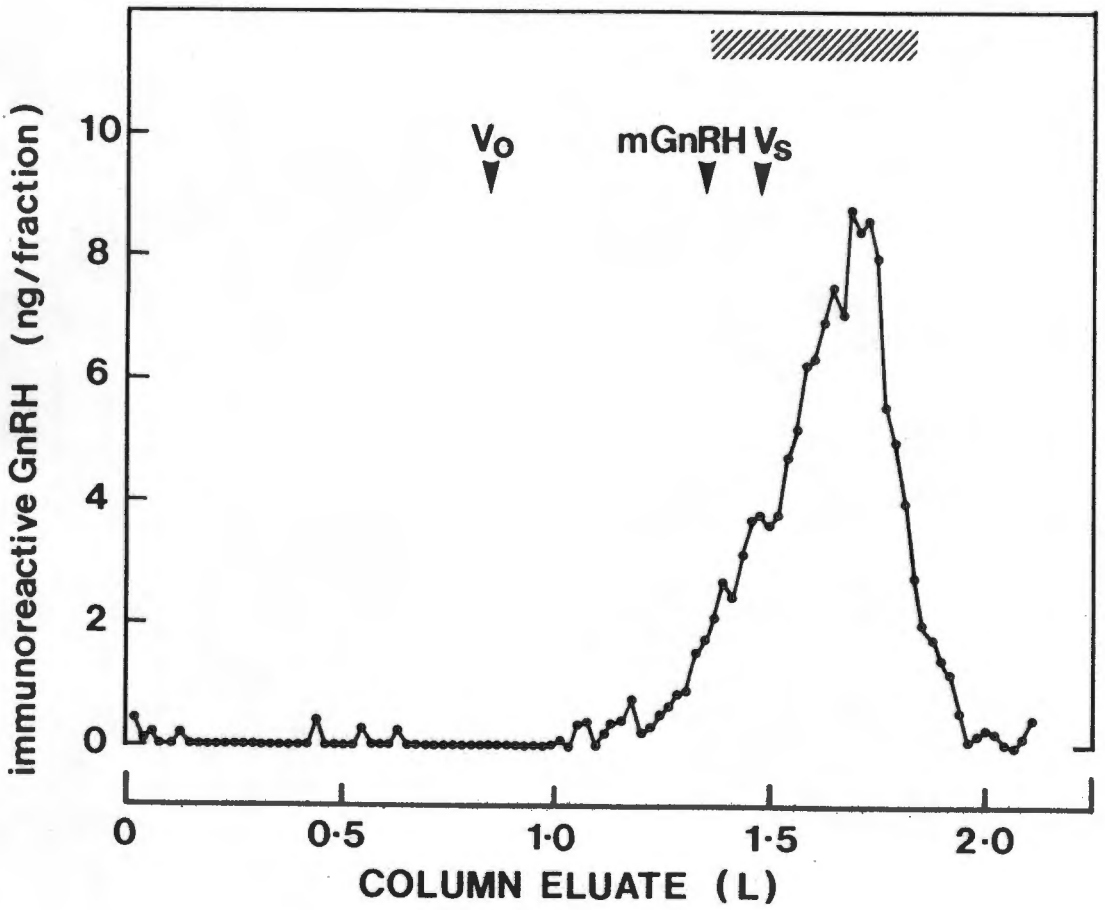


Fig. 20. Gel filtration chromatography of dogfish brain extract (Batch C) (132 ng GnRH, antiserum 80/1). The void volume (Vo), salt elution volume (Vs) and elution position of mammalian GnRH are indicated. The hatched block indicates the fractions pooled for further analysis.

On semipreparative HPLC, the immunoreactive GnRH peak (Batch C) from gel filtration eluted as seven immunoreactive peaks (antiserum 80/1) (Fig. 21).

Immunoreactive peaks from the above run were pooled together and chromatographed on analytical HPLC system 2. Five immunoreactive GnRH peaks (antiserum 80/1) were separated (Fig. 22A). Peaks II and III eluted in similar positions to mammalian GnRH and His^5,Trp^7,Tyr^8-GnRH, respectively. Peak IV eluted in the region of Trp^7,Leu^8-GnRH. Peak II, which eluted in the position of mammalian GnRH, had a relative immunoreactivity of 40% with antiserum 1076 compared with antiserum 80/1 (Fig. 22B) (Table VI). Synthetic mammalian GnRH cross-reacts equally with these two antisera (Table VI); thus peak II is clearly not identifiable as mammalian GnRH. Peak III had a relative immunoreactivity of 0.9% with antiserum 1076 (relative to antiserum 80/1) (Fig. 22B) and 13% with antiserum 802 (relative to Trp^7,Leu^8-GnRH) (Table VI), which is similar to the cross-reaction of synthetic His^5,Trp^7,Tyr^8-GnRH with these antisera (Table VI). Peak IV cross-reacted less than 0.5% with antiserum 1076 relative to antiserum 80/1 and fully with antiserum 802 (Table VI), which is identical to the cross-reaction of synthetic Trp^7,Leu^8-GnRH with these antisera (Table VI). Peaks I and V did not cross-react with antisera 1076 and 802 and therefore have the N-and C-termini in common with mammalian GnRH, but considerable structural differences from the known GnRHs in other regions of the molecule. Batch B immunoreactive GnRH eluted similarly on HPLC system 1 (data not shown).

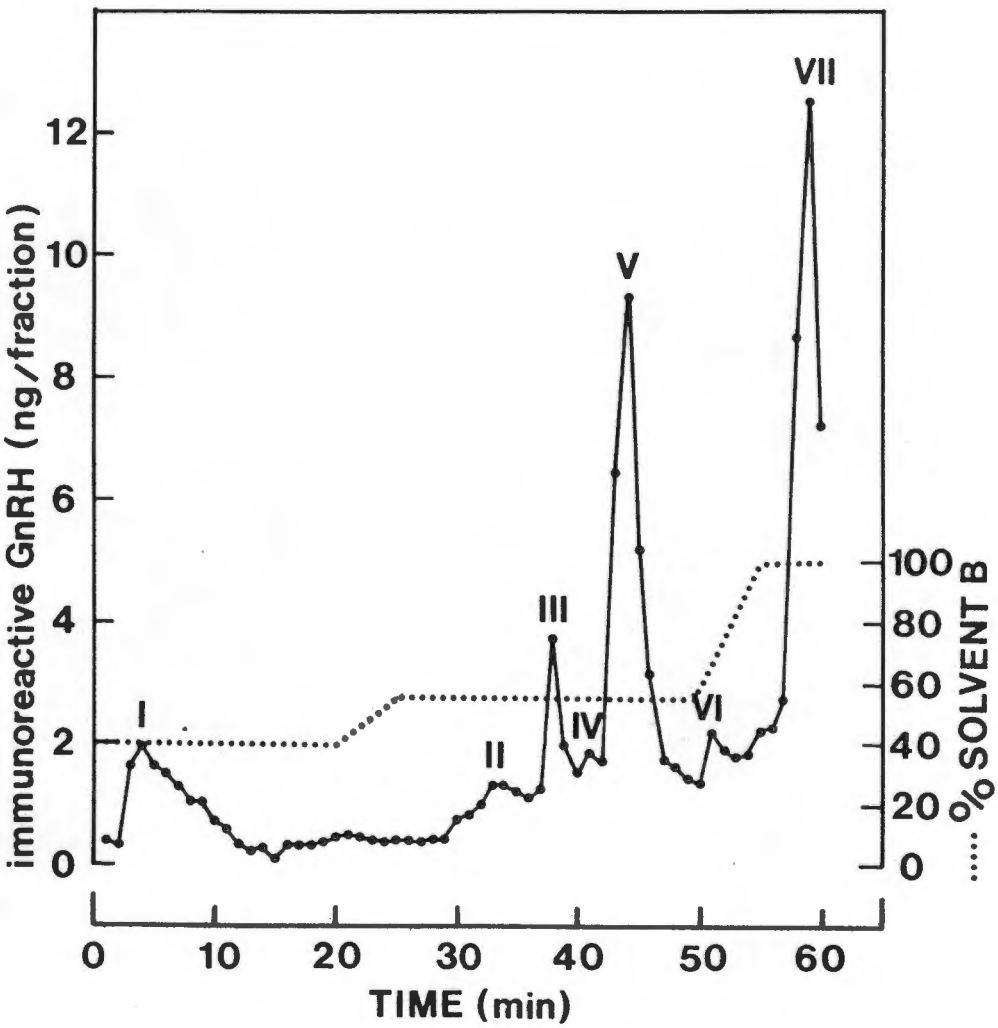


Fig. 21. Semipreparative HPLC of dogfish brain immunoreactive GnRH (Batch C) (90.3 ng GnRH, recovery was 69%, antiserum 80/1). Fractions were assayed with antiserum 80/1. Elution positions of Gln^8-GnRH (1), mammalian GnRH (2), Trp^7,Leu^8-GnRH (3) and His^5,Trp^7,Tyr^8-GnRH (4) are indicated.

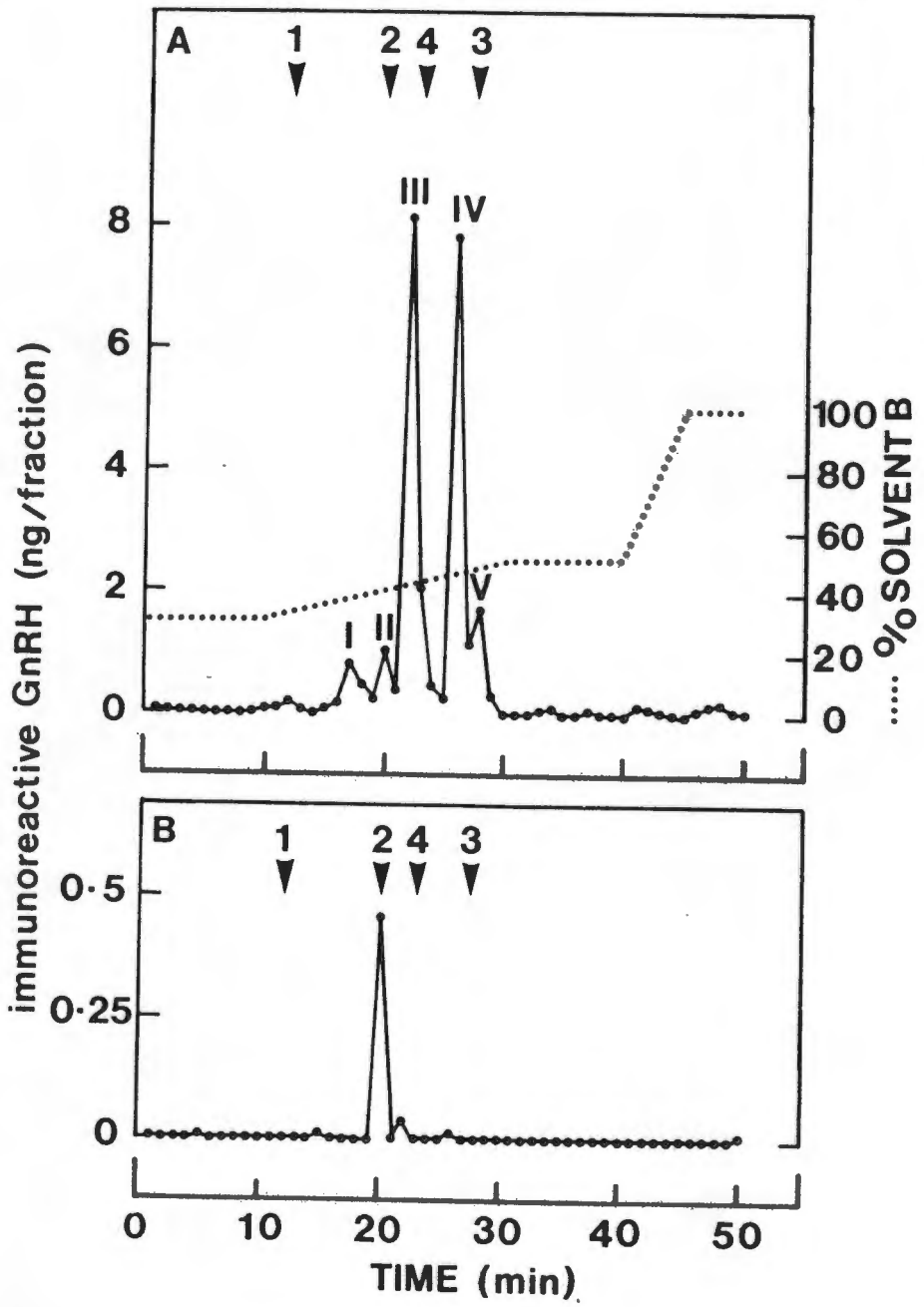


Fig. 22. Analytical HPLC of dogfish brain immunoreactive GnRH (Batch C) (system 2, 31.8 ng GnRH, recovery was 78%, antiserum 80/1). Fractions were assayed with antisera 80/1 (A) and 1076 (B). Elution positions of Gln^{8}-GnRH (1), mammalian GnRH (2), Trp^{7},Leu^{8}-GnRH (3) and His^{5},Trp^{7},Tyr^{8}-GnRH (4) are indicated.

TABLE VI. CROSS-REACTIVITY OF GnRH ANTISERA WITH SYNTHETIC ANALOGUES OF FIVE NATURAL VERTEBRATE GnRHs AND DOGFISH IMMUNOREACTIVE PEAKS (HPLC SYSTEM 2, FIG. 3).

	PERCENTAGE RELATIVE CROSS-REACTIVITY	
	antiserum 1076 (relative to cross-reaction with antiserum 80/1)	antiserum 802 (cross-reaction with Trp^7,Leu^8-GnRH was taken as 100%)
Synthetic peptides		
GnRH	100	< 0.02
Gln^8-GnRH	17.7	< 0.02
Trp^7,Leu^8-GnRH	0.05	100
His^5,Trp^7,Tyr^8-GnRH	2.4	17.6
$Tyr^3,Leu^5,Glu^6,Trp^7,Lys^8$-GnRH	< 1.1	< 0.32
Dogfish immunoreactive GnRH peaks		
I	<4.4	< 0.8
II	40	< 1.2
III	0.9	13.2
IV	0.5	100
V	< 2.2	<1.0

Chicken LH-releasing activity

The five immunoreactive GnRH peaks (Batch C, HPLC system 2) (Fig. 22A) all stimulated chicken LH release (Fig. 23). Several additional bioactive peaks were identified which were not detected by radioimmunoassay. Peak II, which co-eluted with mammalian GnRH (0.37 ng, antiserum 80/1), released 0.2 μg of LH. This confirms that the peak is not mammalian GnRH, as a similar amount of mammalian GnRH (assayed by antiserum 80/1) would not have LH-releasing activity. It appears, therefore, that this peak has substantial LH-releasing activity and is poorly immunoreactive. Peak III, co-eluting with His^5,Trp^7,Tyr^8-GnRH (2.9 ng, antiserum 80/1), stimulated LH release maximally as did 100 ng Gln^8-GnRH. This further supports the identification of this peak as His^5,Trp^7,Tyr^8-GnRH, since a similar amount of synthetic His^5,Trp^7,Tyr^8-GnRH would release LH maximally. His^5,Trp^7,Tyr^8-GnRH cross-reacts only 5% with antiserum 80/1 (King and Millar, 1985) (relative to mass) and is 5 times more potent than Gln^8-GnRH (Millar and King, 1984; Chou *et al.*, 1985). Peak IV, which co-eluted with Trp^7,Leu^8-GnRH (2.1 ng, antiserum 80/1), released a substantial amount of LH. The same amount of synthetic Trp^7,Leu^8-GnRH would release a similar amount of LH. Trp^7,Leu^8-GnRH cross-reacts 30% with antiserum 80/1 (King and Millar, 1985). His^5,Trp^7,Tyr^8-GnRH is twice as potent as Trp^7,Leu^8-GnRH in the chicken pituitary cell bioassay (Millar and King, 1984)

Seven LH-releasing peaks were also present in Batch B dogfish brains (data not shown). These peaks correspond to those demonstrated in Batch C.

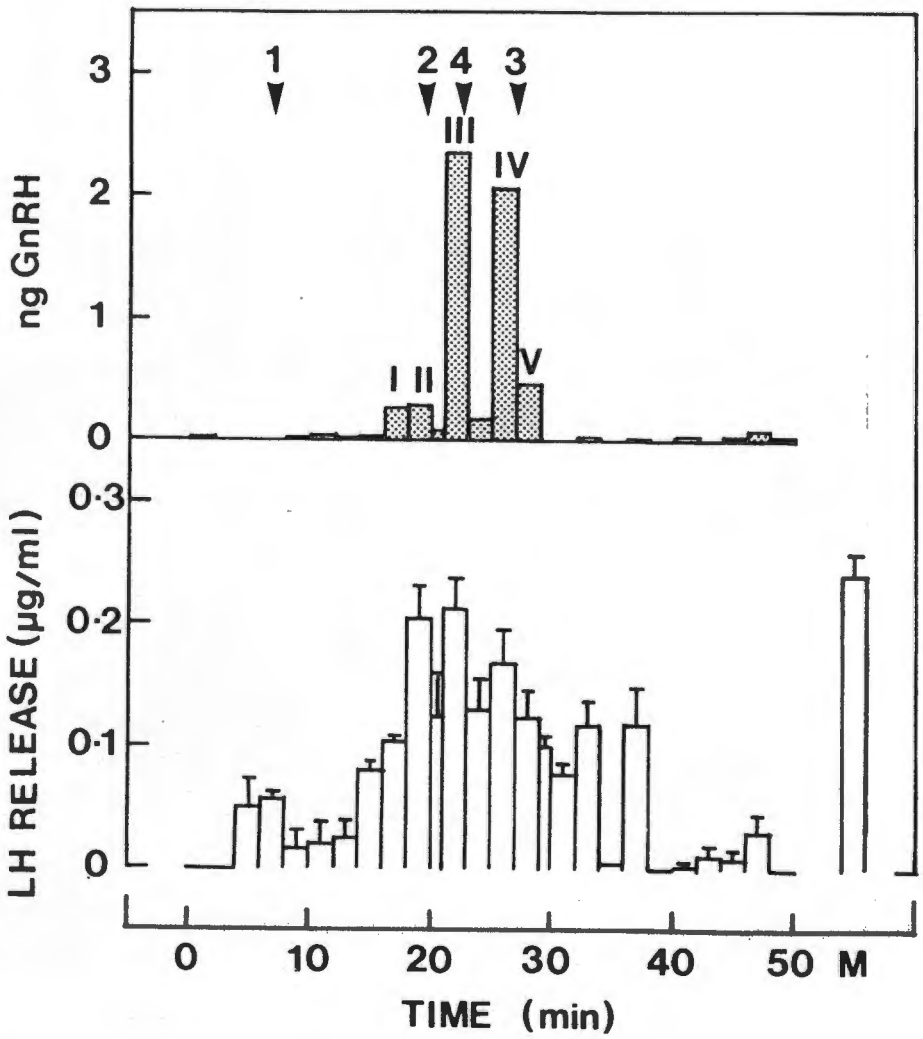


Fig. 23. Chicken pituitary cell bioassay. Effect of dogfish brain GnRH in pooled HPLC fractions (Batch C, system 2) and 10^{-7} M Gln^8-GnRH (maximal stimulation, M) on LH release from chicken anterior pituitary cells. LH release is expressed as µg LH/ml of cell medium with basal release subtracted (open column). Columns represent the mean ± SEM (n = 3) of an assay for each pool of HPLC fractions (in twos). Immunoreactive GnRH (antiserum 80/1) per HPLC fraction pool taken for the bioassay is expressed ng GnRH/aliquot of HPLC fraction pool (dotted column). Elution positions of Gln^8-GnRH (1), mammalian GnRH (2), Trp^7,Leu^8-GnRH (3) and His^5,Trp^7,Tyr^8-GnRH (4) are indicated.

Teleost brain extract

Chromatography and radioimmunoassay of teleost brain extract

The concentration of immunoreactive GnRH in teleost whole brain was 20.6 pg GnRH/mg dry weight (Table V). In HPLC system 2, one major immunoreactive GnRH peak (antiserum 80/1) co-eluted with Trp^{7},Leu^{8}-GnRH (Fig. 24A). A smaller peak (antiserum 80/1) co-eluted with His^{5},Trp^{7},Tyr^{8}-GnRH (Fig. 24A).

Chicken LH-releasing activity

Both immunoreactive peaks (antiserum 80/1) stimulated release of LH. (Fig. 24B). His^{5},Trp^{7},Tyr^{8}-GnRH is twice as potent as Trp^{7},Leu^{8}-GnRH in the chicken anterior pituitary cell bioassay (Millar and King, 1984). However, if the two peaks were His^{5},Trp^{7},Tyr^{8}-GnRH and Trp^{7},Leu^{8}-GnRH respectively, they would be equipotent in this bioassay. A third bioactive GnRH, which had no GnRH immunoreactivity, eluted early on HPLC and appears to be a novel, hydrophilic form of GnRH or an unrelated peptide which releases LH. A large amount of LH was released in the late fractions. As the amount of LH released is above maximal release, it is thought to be due to lysing of the cells, because of residual acetonitrile in the HPLC fractions.

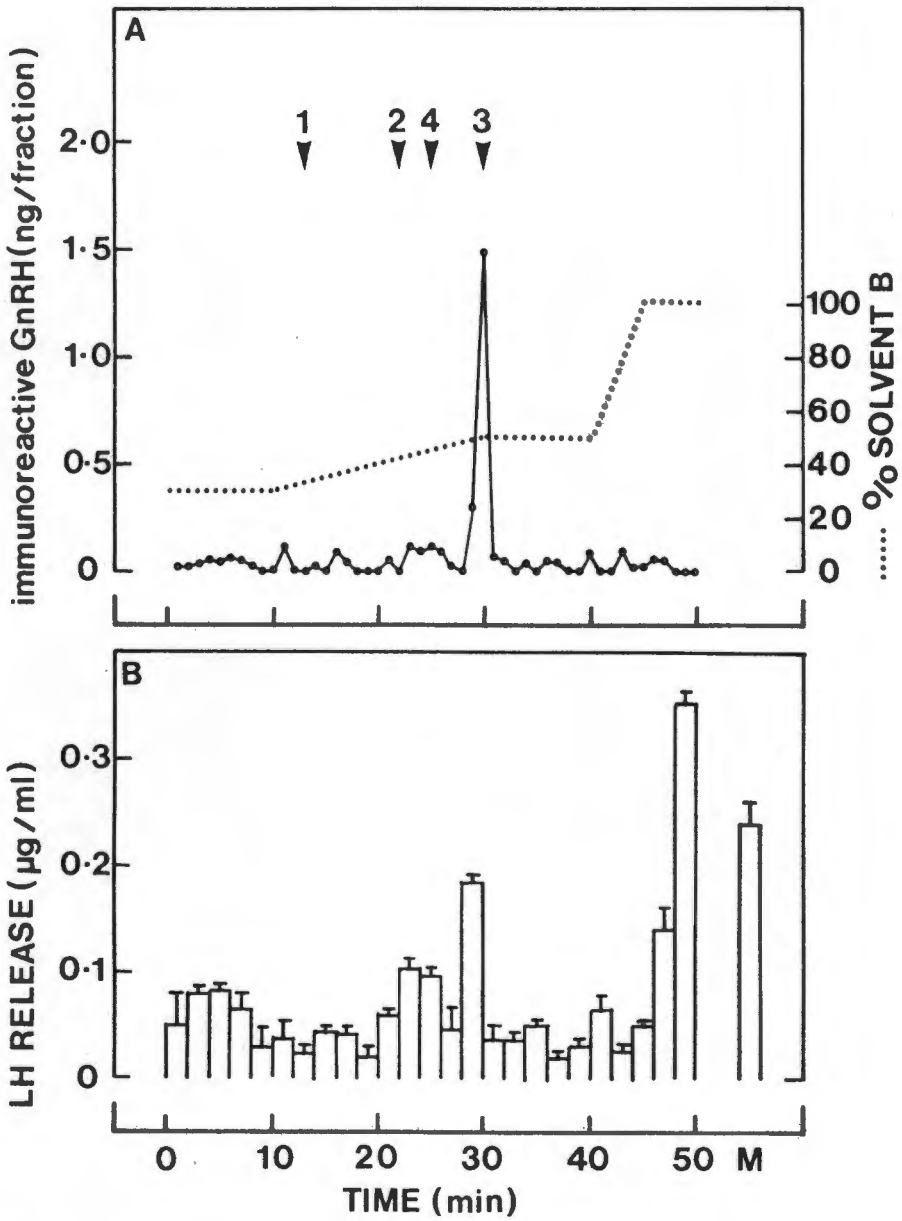


Fig. 24. Analytical HPLC (system 3, 2.98 ng GnRH, recovery was 74.4%, antiserum 80/1) and chicken pituitary cell bioassay of teleost brain GnRH. A. Fractions were assayed with antiserum 80/1. Elution positions of Gln^8-GnRH (1), mammalian GnRH (2), Trp^7,Leu^8-GnRH (3) and His^5,Trp^7,Tyr^8-GnRH (4) are indicated. B. Effect of teleost brain GnRH in pooled HPLC fractions and 10^{-7} M Gln^8-GnRH (maximal stimulation, M) on LH release from dispersed chicken pituitary cells. LH release is expressed as ug LH release/ml of cell medium with basal release subtracted (open column). Columns represent the mean $\pm$ SEM (n = 3) of an assay for each pool of HPLC fraction (in twos).

DISCUSSION

In dogfish brain, GnRH concentration was greater in the hypothalamus than in extrahypothalamic brain. There may be seasonal variations in GnRH concentration as brains of dogfish killed in spring contained more GnRH than those killed in autumn. Dogfish pituitary gland contained no detectable GnRH. In teleost fish, however, a number of immunocytochemical and radioimmunoassay studies have demonstrated the presence of GnRH in the pituitary gland (Goos and Murathanoglu, 1977; Nozaki and Kobayashi, 1979; Schreibman *et al.*, 1979; Margolis-Kazan *et al.*, 1981; Münz *et al.*, 1981; Goos *et al.*, 1985; King and Millar, 1985). In teleosts the median eminence interdigitates with the pituitary gland (Ball, 1981) whereas in elasmobranch fish no vascular or nervous relationship exists between the median eminence and ventral lobe of the anterior pituitary, where gonadotropin is released (Dodd, 1975). GnRH immunoreactive material was not detected in the pituitary gland of the dogfish (*T. scyllia*) (Nozaki and Kobayashi, 1979). Immunoreactive GnRH in dogfish blood has previously been described (King and Millar, 1980), and its presence is confirmed in this study. Whether this indicates systemic activation of the pituitary gland via GnRH released into the general circulation is an intriguing possibility requiring further investigation.

Seven peaks with LH-releasing activity were demonstrated in dogfish brain. Two of these peaks have been partially characterized, by elution position on HPLC, radioimmunoassay with region-specific GnRH antisera and assessment of LH-releasing activity, as His^5,Trp^7,Tyr^8-GnRH and Trp^7,Leu^8-GnRH. Although our findings strongly support this identification, it is possible that there are novel GnRH forms with similar properties.

Trp^7,Leu^8-GnRH appears to be present in all teleost fish studied (Jackson and Pan, 1983; Sherwood *et al.*, 1983; Breton *et al.*, 1984; Sherwood *et al.*, 1984; King and Millar, 1985). His^5,Trp^7,Tyr^8-GnRH is a widespread form and possibly represents an early-evolved and conserved GnRH. A third GnRH form in dogfish brain co-eluted with mammalian GnRH on HPLC, but cross-reacted differently with certain antisera and had bioactivity higher than appropriate for mammalian GnRH. In lamprey brain a GnRH form has been identified, which co-elutes with mammalian GnRH, but differs immunologically (Sherwood and Sower, 1985). The four other LH-releasing peptides in dogfish brain appear to be novel forms of GnRH or unrelated peptides with LH-releasing activity. As none of these forms cross-reacted with antiserum 1076, they are likely to have changes in the middle region of the GnRH peptide.

A GnRH form which co-eluted with Trp^7,Leu^8-GnRH was present in the teleost. This finding adds to other data showing that in all teleost species investigated a GnRH form identical to Trp^7,Leu^8-GnRH occurs (Jackson and Pan, 1983; Sherwood *et al.*, 1983; Breton *et al.*, 1984; Sherwood *et al.*, 1984; King and Millar, 1985). Of the two other LH-releasing factors present in this species, one form co-eluted with His^5,Trp^7,Tyr^8-GnRH.

6.0 CONCLUDING DISCUSSION

The purpose of this study was to gain insight into the possible evolution of the structure of vertebrate brain GnRH and its functions. Over the last few years it has been demonstrated that a number of different molecular forms of GnRH occur. Five different GnRH forms have been isolated from vertebrate brain, and additional novel forms, with different chromatographic, immunological and biological characteristics have been demonstrated. The five structurally characterized and naturally-occurring GnRHs have an effect on the pituitary-gonadal axis in the species/class from which they were isolated, and are thus involved with reproduction.

In birds, GnRHs have been structurally characterized from the hypothalami of chickens only. The data on ostrich hypothalamic GnRHs are thus of considerable interest. In ostrich hypothalamus two immunoreactive GnRHs with identical chromatographic, immunological and biological properties to Gln^{8}-GnRH and His^{5},Trp^{7},Tyr^{8}-GnRH were present. As these two forms are present in chicken hypothalamus, it would appear that GnRH structure in birds has been conserved. Four other factors which released LH from chicken dispersed anterior pituitary cells were also present in ostrich hypothalamic extracts. Only one of these forms cross-reacted with GnRH antisera. These factors might also be present in chicken hypothalamus. King and Millar (1982a, 1982c) used affinity chromatography with antiserum 1076 as an initial step in the purification of Gln^{8}-GnRH, and as these factors have little or low immunoreactivity with antiserum 1076, they would not have been bound by antiserum 1076 coupled to the affinity column. Miyamoto *et al.* (1983, 1984) used a rat anterior pituitary cell bioassay to monitor GnRHs in chicken hypothalamus. However, the factors

found in ostrich hypothalamus are likely to release LH only from chicken pituitary cells, as the mammalian receptor has stringent binding requirements (Milton et al., 1983; Millar and King, 1984).

It is not known which of the GnRH molecular forms described in ostrich hypothalamus has a physiological role in releasing LH and/or FSH. In the chicken, both Gln^8-GnRH and His^5,Trp^7,Tyr^8-GnRH stimulated LH and FSH release from chicken anterior pituitary cells (King and Millar, 1983; Millar and King, 1984; Chou et al., 1985) at physiological concentrations. The two peptides may act synergistically or antagonistically through the same or different receptors. It is possible that the greater activity of His^5,Trp^7,Tyr^8-GnRH in stimulating LH release reflects its role as the primary regulator of gonadotropin secretion. However, in view of the non-stringency of the chicken GnRH receptor, enhanced activity of this form might not imply that this form of GnRH is the (or a) endogenous regulator. Immunocytochemical analysis of the distribution of the two forms of GnRH in the chicken hypothalamus (with antisera specific for the two forms) and the demonstration which form(s) are in nerve endings abutting on the hypophysial portal vessels will help clarify this issue. In addition, the effects of administration of specific antisera in vivo on gonadotropin secretion should reveal whether one or both of the GnRHs serves the role of regulating reproduction. The GnRHs may also regulate pituitary hormones other than the gonadotropins, as GnRH has been shown to be a potent stimulator of growth hormone release in goldfish (T. Marchant and R. Peter, personal communication).

In ostrich extrahypothalamic brain, the presence of both Gln^8-GnRH and His^5,Trp^7,Tyr^8-GnRH has been demonstrated, as in the hypothalamus (R.C. Powell, H. Jach, R.P. Millar and J.A. King, submitted). In extrahypothalamic brain regions, these GnRHs may serve as neurotransmitters or neuromodulators in neuronal pathways subserving reproductive behaviour. Other roles in the central nervous system are possible. Extrahypothalamic brain GnRH has been described in a number of vertebrates (see Introduction). As Gln^8-GnRH and His^5,Trp^7,Tyr^8-GnRH are both present in the hypothalamus and in extrahypothalamic brain, it is possible that they are in a common precursor molecule.

Of the two reptilian species studied, alligator brain contained two GnRHs with identical properties to Gln^8-GnRH and His^5,Trp^7,Tyr^8-GnRH, whereas a single GnRH form with properties similar to His^5,Trp^7,Tyr^8-GnRH was demonstrated in skink brain. In a previous study on lizard (*C. nigra*) brain, four immunoreactive GnRHs were detected, two of which had identical properties to Trp^7,Leu^8-GnRH and His^5,Trp^7,Tyr^8-GnRH (Powell *et al.*, 1985). It appears that His^5,Trp^7,Tyr^8-GnRH is widespread in reptiles. In *Podarcis s. sicula* (a lizard) brain, however, His^5,Trp^7,Tyr^8-GnRH was not detected. A GnRH with identical properties to Trp^7,Leu^8-GnRH was present, as well as two novel forms of GnRH (R.C. Powell, G. Ciarcia, V. Lance, R.P. Millar and J.A. King, submitted). The three species of lizard (*C. ocellatus tiligugu*, *P. s. sicula* and *C. nigra*) therefore seem to be similar with regard to the nature of their GnRH molecular forms, as GnRH with identical properties to His^5,Trp^7,Tyr^8-GnRH and/or Trp^7,Leu^8-GnRH is present in all three, but not Gln^8-GnRH as in the alligator.

The two GnRHs in alligator brain, Gln^8-GnRH and His^5,Trp^7,Tyr^8-GnRH, are also present in chicken (King and Millar, 1982a, 1982c; Miyamoto *et al.*, 1983, 1984) and ostrich hypothalamus. It is believed that alligators,

which belong to the subclass Archosaura (order Crocodilia) are phylogenetically remote from the base of the reptilian family tree and more closely related to birds (Romer, 1970). They show numerous features found in birds and thus the fact that alligator and bird GnRHs are identical is consistent with this theory of evolution. The fact that Gln^8-GnRH was not present in any of the other reptiles studied also supports the theory that alligators are more closely related to the birds than are other reptiles.

In dogfish (*P. africanum*) brain, seven LH-releasing factors were present. Two were partially characterized as Trp^7,Leu^8-GnRH and His^5,Trp^7,Tyr^8-GnRH; the others appear to be novel GnRHs or structurally unrelated peptides with LH-releasing activity. In another dogfish (*S. acanthias*), two immunoreactive GnRHs were present (Sherwood and Sower, 1985), one of which eluted similarly to His^5,Trp^7,Tyr^8-GnRH on HPLC. The other form appears to be a novel GnRH molecular form. In the other fish species studied, the teleost (*C. julis*), a GnRH with identical properties to Trp^7,Leu^8-GnRH was present. A second GnRH form co-eluted with His^5,Trp^7,Tyr^8-GnRH. Trp^7,Leu^8-GnRH appears to be present in all teleost fish investigated (Jackson and Pan, 1983; Sherwood *et al.*, 1983; Breton *et al.*, 1984; Sherwood *et al.*, 1984; King and Millar, 1985). A GnRH in trout (*S. gairdneri*) brain (Sherwood and Sower, 1985) also eluted in the same position as His^5,Trp^7,Tyr^8-GnRH. Thus it seems that the dogfish (*P. africanum*) has similar GnRHs to those in teleost fish, whereas *S. acanthias* differs in lacking Trp^7,Leu^8-GnRH. His^5,Trp^7,Tyr^8-GnRH seems to be a very widespread form of GnRH. A GnRH with identical properties is present in chicken (Miyamoto *et al.*, 1984) and ostrich hypothalamus, in alligator and lizard brain (Powell *et al.*, 1985), in frog (*X. laevis*)

brain (King and Millar, 1986), and possibly also in trout and dogfish brain (Sherwood and Sower, 1985). Therefore, it may be an early-evolved and conserved form of GnRH. It has, however, not as yet been demonstrated in mammalian species. Interestingly, His^5,Trp^7,Tyr^8-GnRH released 32% LH and 41% FSH compared with mammalian GnRH in rat pituitary cells (Miyamoto et al., 1984), which is far greater than the gonadotropin-releasing activity of the other GnRHs found in submammalian vertebrates. This could be an indication that this GnRH molecular form does occur in mammals, but has not been detected.

Multiple forms of GnRH have been described previously in brain tissue of species of bird (King and Millar, 1982a, 1982c; Miyamoto et al., 1983, 1984), reptile (Powell et al., 1985), amphibian (Branton et al., 1982; King and Millar, 1985), numerous species of teleost fish (Barnett et al., 1982; Jackson and Pan, 1983; Sherwood et al., 1983, 1984; King and Millar, 1985), and in an elasmobranch and cyclostome fish (Sherwood and Sower, 1985; Sherwood et al., 1986). In mammals, King and Millar (1981) reported two forms of GnRH in sheep pineal gland and hypothalamus. The structure of the second form has not been established. The function of the various forms of GnRH in these vertebrates has not been determined. It also remains to be discovered which form(s) regulates the release of gonadotropins endogenously. Some of the GnRHs described may occur in extrahypothalamic brain areas and may have neurotransmitter functions (Jones et al., 1984) or be involved with reproductive behaviour (Cheng, 1977; Mauk et al., 1980; Dluzen et al., 1981; Dluzen and Ramirez, 1983). GnRHs in higher vertebrates (birds and mammals) seem to be more conserved

than those in lower vertebrates (reptiles and fish).

In elasmobranch and cyclostome fish, there is no vascular or nervous connection between the median eminence and pars distalis (Dodd, 1975; Nozaki et al., 1984b). However, GnRH-like substances are present in dogfish and lamprey brain. Hagfish seem to have either no or very small amounts of GnRH (Crim et al., 1979a; Nozaki and Kobayashi, 1979; Jackson, 1980; King and Millar, 1980; Sherwood and Sower, 1985). This may be due to the fact that most hagfish are continuous breeders, thus not requiring a GnRH. The hagfish can release eggs for many months after hypophysectomy (Matty et al., 1976; Gorbman, 1980). The GnRH may, however, differ vastly from the known forms, so that it is undetectable by antisera directed against the known GnRHs and inactive in bioassays in the higher vertebrates.

Amongst invertebrates, analysis of earthworm (Lumbricus terrestris) extract by HPLC and radioimmunoassay revealed no immunoreactive GnRH (R. C. Powell, R.P. Millar and J.A. King, unpublished). Yeast α-mating factor (a peptide involved with "gamete" formation in Saccharomyces cerevisiae) has six of thirteen amino acids in common with mammalian GnRH. Table VII indicates the homology of yeast α-mating factor with the other naturally-occurring GnRHs. Lamprey GnRH also has six amino acids homologous, whereas the others have less homology.

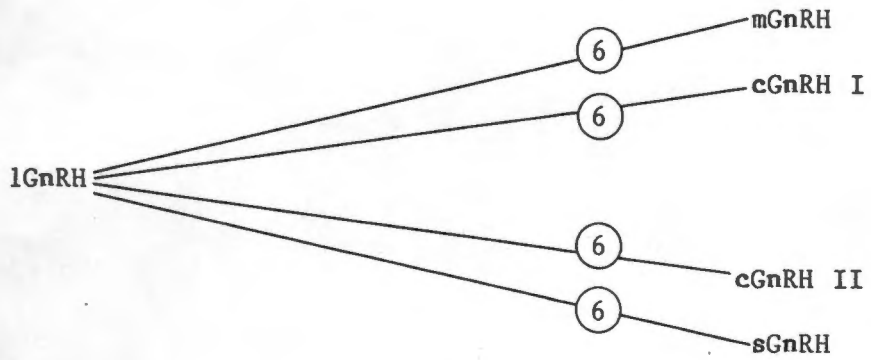
In most of the vertebrate species, two or more GnRH molecular forms appear to be present in the same brain tissue, suggesting that gene duplication occurred during GnRH evolution. Lamprey GnRH is the GnRH form with the most nucleotide base changes with respect to the other GnRHs (Fig. 25), the next being chicken GnRH II. Chicken GnRH II

TABLE VII . HOMOLOGY OF THE KNOWN NATURALLY-OCCURRING GnRHs WITH YEASTα- MATING FACTOR

	1	2	3	4	5	6	7	8	9	10	11	12	13
Yeast α-mating factor	Trp.	His.	Trp.	Leu.	Gln.	Leu.	Lys.	Pro.	Gly.	Gln.	Pro.	Met.	Tyr

	1	2	3	4	5	6	7	8	9	10
Mammalian GnRH	pGlu.	<u>His.</u>	<u>Trp.</u>	Ser.	Tyr.	Gly.	<u>Leu.</u>	<u>Arg.*</u>	<u>Pro.</u>	<u>Gly</u>-NH_2
Chicken GnRH I	pGlu.	<u>His.</u>	<u>Trp.</u>	Ser.	TYr.	Gly.	<u>Leu.</u>	Gln.	<u>Pro.</u>	<u>Gly</u>-NH_2
Chicken GnRH II	pGlu.	<u>His.</u>	<u>Trp.</u>	Ser.	His.	Gly.	Trp.	Tyr.	<u>Pro.</u>	<u>Gly</u>-NH_2
Salmon GnRH	pGlu.	<u>His.</u>	<u>Trp.</u>	Ser.	Tyr.	Gly.	Trp.	<u>Leu.</u>	<u>Pro.</u>	<u>Gly</u>-NH_2
Lamprey GnRH	pGlu.	<u>His.</u>	Tyr.	Ser.	<u>Leu.</u>	<u>Glu.</u>	Trp.	<u>Lys.</u>	<u>Pro.</u>	<u>Gly</u>-NH_2

Sequence homologies to yeastα-mating factor are underlined. * Arg is considered homologous to Lys.



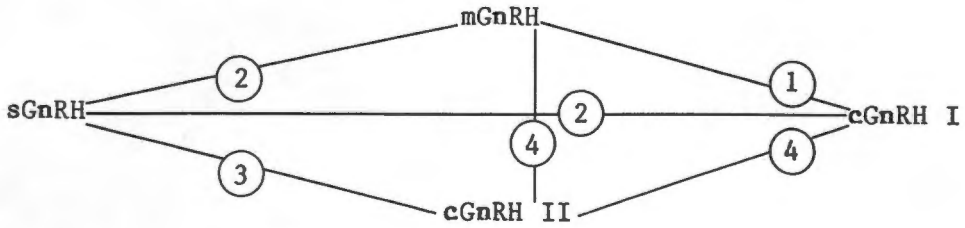


Fig. 25. Minimum number of base changes required to convert Tyr^3,Leu^5,Glu^6,Trp^7,Lys^8-GnRH (l GnRH) into the other four known naturally-occurring GnRHs, and minimum number of base changes required to interconvert mammalian GnRH (m GnRH), Gln^8-GnRH (c GnRH I), Trp^7,Leu^8-GnRH (s GnRH) and His^5,Trp^7,Tyr^8-GnRH (c GnRH II).

(His^5,Trp^7,Tyr^8-GnRH) seems to be the most widespead GnRH form amongst vertebrates and may have arisen early after gene duplication and remained conserved. Trp^7 occurs in three of the known GnRHs, lamprey GnRH (Tyr^3,Leu^5,Glu^6,Trp^7,Lys^8-GnRH), salmon GnRH (Trp^7,Leu^8-GnRH) and chicken GnRH II (His^5,Trp^7,Tyr^8-GnRH), whereas Leu^7 occurs in mammalian GnRH and chicken GnRH I (Gln^8-GnRH). This may indicate how the GnRHs have evolved. It seems possible that lamprey, salmon and chicken GnRH II evolved first as they occur mainly in lower vertebrates.

The GnRH molecule has, however, remained conserved in certain regions of the molecule. The length of the five known naturally-occurring GnRHs is constant. The two C-terminal and two N-terminal amino acids and Ser in position 4 have remained unchanged. These conserved residues may be the most important for activity. The N- and C-termini are thought to bind to the GnRH receptor, as only middle-directed antisera will recognise receptor-bound GnRH (Eidne et al., 1985). Serine is believed to be necessary for maintaining the GnRH conformation (Struthers et al., 1985).

The mammalian-type GnRH seems to occur only in mammals and amphibians, and Gln^8-GnRH in birds and higher reptiles. Trp^7,Leu^8-GnRH is present in gnathostomes (chondrichthyes and osteichthyes fish), but not in cyclostomes. His^5,Trp^7,Tyr^8-GnRH occurs in four of the five vertebrate classes (birds, reptiles, amphibians and gnathostomes); and may have a specific function which has been conserved. Tyr^3,Leu^5,Glu^6,Trp^7,Lys^8-GnRH has only recently been discovered in the lamprey and seems to be a more primitive GnRH form as it differs considerably from the other four forms. It may have changed during 400 million years of separate evolution. A number of novel GnRHs have been demonstrated in different vertebrate species and elucidation of their structures will provide greater clarity in understanding the evolution of GnRH.

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8.0 PUBLICATIONS

1. Powell, R.C., King, J.A. and Millar R.P. (1984). Identification of salmon LH-RH in lizard brain. Program of the 8th South African Biochemical Congress, p. 89, Port Elizabeth, South Africa (Abstract)

2. King, J.A., Meinert, R.C. and Millar R.P. (1984). Molecular heterogeneity of vertebrate luteinizing hormone-releasing hormone: structural analysis of reptile and teleost brain LH-RH. Program of the 7th International Congress of Endocrinology, p. 848, Quebec City, Canada (Abstract).

3. Powell, R.C., King, J.A. and Millar, R.P. (1985). [Trp^7,Leu^8] LH-RH in reptilian brain. Peptides 6: 223-227.

4. Powell, R.C., Millar, R.P. and King, J.A. (1986). Diverse molecular forms of gonadotropin-releasing hormone in an elasmobranch and a teleost fish. Gen. Comp. Endocrinol. (in press).

5. Powell, R.C., Jach, H., Millar, R.P. and King, J.A. Identification of Gln^8-GnRH, His^5,Trp^7,Tyr^8-GnRH and novel forms of GnRH in the hypothalamus and extrahypothalamic brain of the ostrich (Struthio camelus) (submitted).

6. Powell, R.C., Ciarcia, G., Lance, V., Millar, R.P. and King, J.A. Identification of diverse molecular forms of GnRH in reptile brain. (submitted).