MOLECULAR EVOLUTION OF NEUROPEPTIDES ISOLATED FROM THE BRAIN OF THE PACIFIC SALMON (ONCORHYNCHUS SPP.).

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ABSTRACT

The molecular evolution of three neuropeptides genes isolated from Pacific salmon (*Oncorhynchus* spp.) brain was investigated using both immunocytochemistry and molecular biology. The distribution of gonadotropin-releasing hormone (GnRH), a key reproductive neurohormone, was studied in protochordates, fishes and rat. The presence of GnRH-like immunoreactivity in the protochordates suggests that this hormone is phylogenetically ancient. The similar distribution of the peptide in the brains of fishes and mammals suggests that its location, and possibly function, has been conserved during evolution. The molecular structure of GnRH in salmon was investigated by isolation and characterization of one form, salmon (s) GnRH, from a sockeye salmon genomic library using a complementary (c) DNA for sGnRH originally isolated from cichlid. The entire protein coding region was sequenced and found to be distributed on three exons interrupted by two introns. The gene shows a high degree of sequence identity to an Atlantic salmon sGnRH gene and structural similarity to rat and human mammalian (m) GnRH genes. This suggests that both genes may be derived from a common ancestor. Two cDNAs encoding vasotocin, an important osmoregulatory neurohormone, were

isolated from a chum salmon brain cDNA library using short degenerate probes against fish vasotocin. These clones have 65% sequence identity to each other at the protein level. The presence of two vasotocin precursors is probably due to the ancestral salmonid line becoming tetraploid at some point. Both GnRH and vasotocin exhibit typical features of members of evolutionarily ancient and diverse neuropeptide families. The hormone coding portion of the precursor is highly conserved in terms of protein sequence but the associated proteins, the signal and cryptic peptides, are not. This implies that the hormone portion of the precursor is under stricter constraint that the other regions. Three different cDNAs encoding α-tubulin were also isolated from the chum salmon brain cDNA library. These clones showed very high sequence identity with each other and with previously isolated α-tubulins from a variety of organisms. It appears that structural proteins, such as tubulin, are under strict constraint and limited in the extent to which their sequences can evolve before they become non-functional. Tubulin genes appear to have been duplicated repeatedly producing a multigene family. The tubulin clones show variations in their carboxy terminal regions and possess typical microtubule associated binding protein sites. These studies demonstrate that proteins, and portions of protein precursors, can evolve at different rates, which may be influenced by events such as gene duplication or tetraploidy.

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ATP: Adenosine Triphosphate.

Denhardts solution (50 X): 1% (w/v) Ficoll, 1% (w/v) polyvinylpyrrolidone, 1% (w/v) BSA (Pentax Fraction V).

dNTP: Deoxyribonucleotide triphosphate.

DTT: Dithiotreitol.

EDTA: Disodium ethylene diamine tetracetate.

Klenow: Klenow fragment of *E.coli* DNA polymerase.

PEG: Polyethylene glycol.

PNK: Polynucleotide kinase.

pfu: Plaque forming units.

SDS: Sodium dodecyl sulfate.

SET: Saline EDTA Tris.

SSC: Saline Sodium citrate.

TAE: Tris Acetate EDTA.

TBE: Tris Borate EDTA.

TE: Tris EDTA.

TMAC: Tetramethylammonium chloride.

Tris: Tris(Hydroxymethyl)aminomethane

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Chapter 1

General Introduction

Since Linnaeus (1758) proposed the first systematic organization of the natural world, scientists have been attempting to improve and refine the ways in which organisms are grouped and phylogenetic relationships determined. Traditionally, such groupings and relationships have been based on phenotypic data (such as morphological or behavioral characteristics). Some of these data have the advantage of allowing comparisons between extant groups and the fossilized remains of extinct organisms. In the last 50 years, improved biochemical techniques for isolating and sequencing proteins have allowed comparisons of protein sequence data. Differences in sequence identity between proteins isolated from phylogenetically distant (based on traditional methods of determining relationships) organisms gave rise to the concept of the molecular clock (Zuckerkandl and Pauling 1962). The premise of this concept is that proteins evolve in direct proportion to absolute time (rather than generation time), so that differences between two orthologous proteins in two species can be used to estimate the time since the two species last shared a common ancestor. This concept naturally led to the idea that there was now a quantitative foundation for evolutionary biology which could be expressed in terms of the unit evolutionary period or UEP (Wilson *et al.* 1977).

More recently, the development of molecular biological techniques and recombinant DNA technology has enabled comparisons to be made between DNA and RNA sequences isolated from very different organisms. As sequence data have accumulated for various genes in different species, it has become clear that the original concept of the molecular clock is an oversimplification and that rates of molecular change can vary considerably, within and among genes and proteins. Alternative types of clocks, such as local clocks (Wilson *et al.* 1987) or episodic clocks (Gillespie 1984) have been proposed. These timepieces are based on statistical analyses which suggest that molecular evolution is episodic, with short bursts of rapid evolution followed by long periods of slow evolution. It has been suggested that the rate of evolution for a protein depends both on the probability that a substitution will be compatible with the biochemical function of the protein and on the dispensibility of the protein to the organism (*i.e.* the probability that the organism can survive and reproduce without it) (Wilson *et al.* 1977). The existence and usefulness of the molecular clock is still a controversial area (Bulmer *et al.* 1991, Runnegar 1991). In addition, the proposal of the neutral theory (Kimura 1968), has incited extensive debate on the exact nature of molecular evolution. This theory suggests that the presence of the great majority of evolutionary substitutions at the molecular level are not due to Darwinian selection acting on advantageous mutations

but due to random fixation of selectively neutral or selectively equivalent mutations (for recent review see Kimura 1991). It can also be argued that the survival of an individual species may be depend on environmental events and be completely unlinked to the genes under study. Thus, any particular species may accumulate a considerable amount of (neutral) change in a particular segment of DNA but the variation in sequence is unrelated to the survival and has not been selected for (*i.e.* there is correlation but not causation) (Runnegar 1991). Another thorny issue is whether selection acts directly on proteins and genes, since they are effectively screened from the environment, or on the whole animal and thereby only indirectly on genes. It is also important to note that mutations causing variations in regulatory regions of genes may be an extremely significant area of molecular evolution (Wilson 1985). Any change in a regulatory region which affects expression, particularly those that turn genes on or off, may lead to the expression of a protein in a new tissue and may have more effect on the adaptive evolution of a species than changes in the gene itself. Mutations causing alterations in the concentration of a protein such as an enzyme can have enormous implications for the overall metabolic processes of a cell. For this reason, it is extremely important to analyze and understand the promoter and controlling regions of genes.

Statistical modelling has been widely applied to the analysis of the evolution of entire genomes, but comparison

of compositional patterns can also be used (Bernardi and Bernardi 1986, Bernardi *et al.* 1988, Ticher and Graur 1989). These methods involve the analyses of compositional distributions (guanosine/cytosine usage in codons) of large DNA fragments (30-100 kilobases), coding sequences, codon usage and rates of synonymous substitutions. Other workers have investigated the structure of vertebrate genes in terms of the size distribution and number of introns and exons, the placement of introns, and other aspects of gene organization (Smith 1988). Most of these studies have found that some of the patterns in question are not random in nature (such as the existence of a bias toward guanosine or cytosine in the third position of codons of protein-coding genes) which implies there are some selective constraints. However, the functional basis (if any) underlying such observations is unclear.

One way to avoid problems associated with the study of the evolution of genomes is to compare smaller sections of coding material such as mitochondrial (mt) DNA or ribosomal RNA. Studies on MtDNA have been used to determine phylogenetic relationships in many groups including the teleosts (Normark *et al.* 1991, Kocher *et al.* 1989, Meyer and Wilson 1990, Irwin *et al.* 1991). However, as with most methods of determining phylogenetic relationships, there are limits to the amount of information that can be deduced from mtDNA sequences. MtDNA seems to have a high rate of synonymous (silent) substitution (Brown *et al.* 1982) and may

also have a relatively high average rate of amino acid replacement, suggested by the presence in the mtDNA genome of some, apparently, rapidly evolving genes (Jacobs *et al.* 1988). Ribosomal RNA was recently used to do an `extensive' phylogenetic analysis of prokaryotic and eukaryotic lineages using data from many bacteria but few (mainly mammalian) vertebrates and no teleosts (Cedergren *et al.* 1988). Another study, which determined the phylogenetic relationships of tetrapods, was done using ribosomal RNA data, with the coelacanth as an outgroup against which the others were compared (Hedges *et al.* 1990). In general, these studies tend to support the conventional eukaryotic lineages based on fossil data, although dates of divergence and rates of evolution may vary.

Perhaps, a more accessible way of looking at molecular evolution for many biologists is to compare sequences (genomic and cDNA) of proteins with known functions, in a variety of organisms and thereby deduce the evolutionary history of protein or peptide families rather than whole genomes or lineages (Acher 1980). In combination with biochemical and physiological data, sequence comparisons may provide clues as to which regions of a particular gene or mRNA are under functional constraints and whether alterations in sequence have biological significance. There are several groups of proteins which are good candidates for such studies and some, such as the globins, cytochrome c, and the histones have been widely used for determining

extensive phylogenetic relationships based on amino acid sequence comparisons. Such data can now be supplemented with comparisons of the underlying coding sequences of these proteins. Other proteins which allow comparisons to be made between widely different groups are the so-called housekeeping proteins. These are proteins, such as the tubulins and actins, which maintain structural integrity and are involved in the general up-keep of the cell. Metabolic enzymes can also be considered housekeeping proteins. Such proteins are often ubiquitously expressed and found in all eukaryotes making them easy to isolate and identify from a wide variety of animals. As housekeeping proteins, it is assumed that they are indispensible for the survival of cells and therefore are under strict functional constraints, which would imply less sequence variation than in some other kinds of proteins. In contrast, some endocrine proteins can vary in sequence and function considerably. Endocrine proteins, particularly those involved with reproduction (since success in this area presumably ensures genes are passed to the next generation) are particularly good candidates for investigations into molecular evolution.

When studying molecular evolution, it is important to consider the context within which the peptides or proteins exist and possible effects of molecular variation at phenotypic and population levels. Phylogenetic relationships based on combinations of biochemical, physiological and morphological data are likely to be the

strongest. To this end, it is useful to study not only the molecular nature of a given peptide, but also its temporal and spatial distribution within both individuals and species, and across phyla. As proteins evolve their functions and patterns of expression may change. New roles may have arisen, as appears to be the case for a number of endocrine proteins. In particular, the neurohormones, many of which exist as families of proteins with similar structures, are, as their name implies, found in an environment where they can act as both neurotransmitters and releasing factors. The number and type of roles these peptides or proteins play in various tissues remains unclear for many neurohormone families in many species. The neurohormone, gonadotropin-releasing hormone (GnRH), is found in the brain but also in such divergent tissues as the placenta in mammals, the retina in fish and birds, and the sympathetic ganglia in frogs, suggesting a variety of roles in addition to the one for which it is named (see chapter 2). Similarly, vasopressin and oxytocin, although very similar in structure, have very different roles in mammals. Vasopressin is involved in the control of water balance and oxytocin is responsible for the contraction of smooth muscle in the uterus and mammary glands. Clearly, in lower vertebrates, the oxytocin-like molecules, which show remarkable similarity in sequence to oxytocin in mammals, cannot be acting in these capacities. For some neurohormone families, members have been identified in the nervous

systems of invertebrates. Whether these proteins originally acted as neurotransmitters or neurohormones is unclear. Some of these proteins seem to act in non-neural capacities in the invertebrates since the putative vasopressin/oxytocin family members identified in invertebrates seem to act as venoms or toxins (see chapter four). Until a more complete phylogenetic study is done, this remains a murky area.

Most endocrine hormones are translated as larger precursor proteins which consist of a leading signal peptide (if the protein is secreted), the hormone (or hormones) and a variable number of other proteins or peptides. These associated proteins are often cryptic in function but some, such as the C-peptide in insulin, appear to be involved in providing correct conformational structures for the subsequent cleavage and release of the hormone (Eipper *et al.* 1986). It is also possible that functions change as different portions of the precursor molecule become biologically active so it is important to determine which portions of precursor proteins have physiological or biological significance and whether this is the same in all species. The natural corollary of these observations is that the associated receptors must be evolving in some sort of complementary fashion. In terms of determining phylogenetic relationships, overall gene structure (position of introns) or architecture (location of signal and cryptic peptides) may be more informative than sequence similarity, which may be fairly low for widely divergent groups (Brenner

1988). This has proved to be the case for endocrine proteins such as the GnRH family and the insulin/insulin-like growth factor family.

Most vertebrate sequence data collected to date comes from mammalian species and, although phylogenetic relationships within this class are beginning to be studied using molecular data (*eg.* Bulmer *et al.* 1991), there are still comparatively few data for other vertebrate groups. The lack of investigation into the molecular evolution of other groups reflects both the recent introduction of these techniques and the emphasis of research on mammals. Although the teleosts are the most numerous group of vertebrates (>20,000 species, Nelson 1984), they have not been as extensively studied at the molecular level as mammals, other vertebrates such as *Xenopus*, or invertebrates such as *Drosophila*.

In order to understand the molecular evolution of many vertebrate proteins it is necessary to compare sequences found in fish as well as other vertebrates. In fish some neuropeptides are relatively well characterized at the protein level but not the nucleic acid level. I therefore isolated and characterized the cDNAs or genes for several proteins from Pacific salmon. The GnRH and vasopressin/oxytocin families of neuropeptides are relatively well described at the protein level, in teleosts as well as in a variety of other vertebrates. They are clearly both ancient and physiologically important families

and may be useful in helping to elucidate the patterns of evolution of other ancient and widely distributed neurohormone families. It therefore seemed appropriate to investigate the molecular evolution of these two families, specifically GnRH and vasotocin (which is related to vasopressin). Clearly the distribution of these molecules within the animal may have changed during evolution so to provide a morphological context for the molecular studies on GnRH, I also investigated the distribution of GnRH immunoreactivity in a variety of vertebrates and a protochordate. As a comparison to the endocrine proteins, I isolated and characterized a tissue-specific tubulin, an important structural `housekeeping' protein, from the brain of salmon. In contrast to the endocrine proteins, these proteins seem to evolve at different, relatively slow rates, and exist as multiple similar forms in most animals studied. They also have a different molecular architecture. Since they are not secreted, they lack signal peptides and also do not seem to possess associated cryptic proteins. Almost no information was available on the existence, structure or sequence of tubulins in teleosts. Although it is not necessarily possible to deduce evolutionary relationships of the different phyla from such molecular data, it would provide information as to how these proteins have evolved over time.

Literature Cited

Acher, R. 1980. Molecular evolution of biologically active polypeptides. Proceedings of the Royal Society, London, Series B. **210**:21-43.

Bernardi, G., Mouchiroud, D., Gautier, C., and Bernardi, G. 1988. Compositional patterns in vertebrate genomes: Conservation and change in evolution. Journal of Molecular Evolution **28**:7-18.

Bernardi, G., and Bernardi, G. 1986. Compositional constraints and genome evolution. Journal of Molecular Evolution **22**:1-11.

Brenner, S. 1988. The molecular evolution of genes and proteins: a tale of two serines. Nature (London) **334**:528-530.

Brown, W.M., Prager, E.M., Wang, A., and Wilson, A.C. 1982. Mitochondrial DNA sequences of primates: Tempo and mode of evolution. Journal of Molecular Evolution **18**:225-239.

Bulmer, M., Wolfe, K.H. and Sharp, P.M. 1991. Synonymous nucleotide substitution rates in mammalian genes: Implication for the molecular clock and relationship of mammalian orders. Proceedings of the National Academy of Sciences (USA) **88**:5974-5978.

Cedergren, R., Gray, M.W., Abel, Y., and Sankoff, D. 1988. The evolutionary relationships among known life forms. Journal of Molecular Evolution **28**:98-112.

Eipper, B.A., Mains, R.E., and Herbert, E. 1986. Peptides in the nervous system. Trends in Neuroscience **19**:463-468.

Gillespie, J.H. 1984. The molecular clock may be an episodic clock. Proceedings of the National Academy of Sciences (USA) **81**:8009-8013.

Hedges, S.B., Moberg, K.D., and Maxson, L.R. 1990. Tetrapod phylogeny inferred from 18S and 28S ribosomal RNA sequences and a review of the evidence for amniote relationships. Molecular Biology and Evolution **7**:607-633.

Irwin, D.M., Kocher, T.D., and Wilson, A.C. 1991. Evolution of the cytochrome b gene of mammals. Journal of Molecular Evolution **32**:128-144.

Jacobs, H.T., Elliot, D.J., Math, B., and Farquharson, A. 1988. Nucleotide sequence and gene organization of sea urchin mitochondrial DNA. Journal of Molecular Biology. **202**:185-217.

Kimura, M. 1968. Evolutionary rate at the molecular level. Nature (London) **217**:624-626.

Kimura, M. 1991. Recent development of the neutral theory viewed from the Wrightian tradition of theoretical population genetics. Proceedings of the National Academy of Sciences (USA) **88**:5969-5973.

Kocher, T.D., Thomas, W.K., Meyer, A., Edwards, S.V., Pääbo, S., Villablanca, X., and Wilson, A.C. 1989. Dynamics of mitochondrial DNA evolution in animals: Amplification and sequencing with conserved primers. Proceedings of the National Academy of Sciences (USA) **86**:6196-6200.

Linnaeus, C. 1758. Systema Naturae, 10th Ed. Stockholm

Meyer, A., and Wilson, A.C. 1990. Origin of tetrapods inferred from their mitochondrial DNA affiliation to lungfish. Journal of Molecular Evolution **31**:359-364.

Nelson, J.S. 1984. Fishes of the World, 2nd Edition, John Wiley and Sons, New York, 523 pp.

Normark, B.B., McCune, A.R., and Harrison, R.G. 1991. Phylogenetic relationships of neopterygian fishes, inferred from mitochondrial DNA sequences. Molecular Biology and Evolution. **8**:819-834.

Runnegar, B. 1991. Nucleic acid and protein clocks. Philosophical Transactions of the Royal Society of London. Series B. **333**:391-397.

Smith, M.W. 1988. Structure of vertebrate genes: A statistical analysis implicating selection. Journal of Molecular Evolution **27**:45-55.

Ticher, A., and Graur, D. 1989. Nucleic acid composition, codon usage, and the rate of synonymous substitution in protein-coding genes. Journal of Molecular Evolution **28**:286-298.

Wilson, A.C. 1985. The molecular basis of evolution. Scientific American **253**:164-173.

Wilson, A.C., Carlson S.S., and White, T.J. 1977. Biochemical evolution. Annual Review of Biochemistry **46**:573-639.

Wilson, A.C., Ochman, H, and Prager, E.M. 1987. Molecular time scale for evolution. Trends in Genetics 3:241-247.

Zuckerkandl, E., and Pauling, L. 1962. Molecular disease, evolution and genic heterogeneity. *In* Horizons in Biochemistry (Kasha, M., and Pullman, B., eds.), pp. 189-225. Academic Press, New York.

Chapter 2

Comparative study of the distribution of GnRH-like immunoreactivity in a protochordate, fishes and a rat.

Parts of this chapter have been already been published in two papers:

Kelsall, R., Coe, I.R., and Sherwood, N.M. 1990. Phylogeny and ontogeny of gonadotropin-releasing hormone: Comparison of guinea pig, rat and a protochordate. General and Comparative Endocrinology **78**:479-494.

Coe I.R., Grier, H.J., and Sherwood, N.M. (in press) Gonadotropin-releasing hormone in the molly *Poecilia latipinna*: Molecular form, quantity and location. Journal of Experimental Zoology.

The techniques outlined in this chapter have also been used in another study published in:

Lovejoy, D.A., Ashmead, B.J., Coe, I.R., and Sherwood, N.M. (in press) Presence of gonadotropin-releasing hormone immunoreactivity in dogfish and skate brains. Journal of Experimental Zoology.

Introduction

Gonadotropin-releasing hormone (GnRH) was originally named for its ability to cause the release of gonadotropins from the pituitary but a survey of the distribution of the hormone within and across a wide range of organisms, in addition to other studies, suggests that GnRH has a variety of functions. As our understanding of neuroendocrine systems increases, it appears that multiplicity of function is a common feature to many neurohormones. The origin of gonadotropin-releasing hormone in either vertebrates or their predecessors is not known. However, GnRH has been identified by immunological methods in representatives of every class of vertebrates. As with many neuropeptides, the GnRH family appears to be phylogenetically ancient. A peptide with a GnRH-like structure has been identified in yeast and acts as a mating factor (see Kurjan and Herskowitz 1982) although this peptide has relatively low sequence identity at the protein level and cannot be conclusively identified as a member of the GnRH family. The presence of GnRH has not yet been demonstrated in non-chordate invertebrates but it may be that a GnRH-like peptide is not recognized by antisera currently used for immunocytochemistry and radioimmunoassay (RIA). The presence of peptides related to vertebrate peptides within the invertebrates has already been demonstrated for such families as the enkephalins (see Haynes 1980, O'Shea and

Schaeffer 1985). Recently the cDNA sequence of the precursor molecule for an insulin-related peptide was described in the mollusc *Lymnaea* sp. (Smit *et al.* 1989). It is possible that similar recombinant DNA techniques will lead to the discovery of a GnRH-like peptide in the invertebrates. The sea squirt is important as a living representative of ancestral animals common to the deuterostome line of evolution that led from invertebrates to vertebrates. It has been suggested that their neural gland is similar to and possibly homologous with the vertebrate anterior pituitary (see Thorndyke and Georges 1988). There is evidence for the presence of GnRH-like peptides in the sub-phylum Urochordata which contains the tunicates (Georges and Dubois 1980). In these studies GnRH-like immunoreactivity was found within the neural or cerebral ganglion but since our understanding of the functioning of this organ is poor the actual role of the peptide remains obscure. The cephalochordate, amphioxus, which may resemble an ancestral vertebrate, also possesses an immunoreactive GnRH-like molecule (Schreibman *et al.* 1986). Additionally it has been shown that GnRH agonists increase the levels of the sex steroids (Chang *et al.* 1985) leading to the suggestion that GnRH is involved in reproduction in this sub-phylum. The importance of the cephalochordates in helping to establish evolutionary relationships of hormone families was recently demonstrated by the isolation and characterization from amphioxus of a

protein that appears to be ancestral to both insulin and insulin-like growth factors (Chan *et al.* 1990).

Very low levels of a GnRH-like molecule have been found in the brains of the craniotes, the hagfish (Jackson 1980, King and Millar 1980, Sower 1990, Lovejoy 1991). Neither the distribution nor the structure of this molecule are known.

There is a considerable body of literature on the distribution of GnRH within the chordates. Immunocytochemistry, high performance liquid chromatography (HPLC) and RIA using a variety of GnRH antisera, have demonstrated the presence of GnRH-like molecules in the brains of representatives of every vertebrate group (Crim *et al.* 1979a, Nozaki and Kobayashi 1979, 1980, Sherwood and Sower 1985). Within the vertebrates, GnRH-like immunoreactivity exhibits a similar overall pattern of distribution in the brain (Barry 1979, Nozaki and Kobayashi 1979, Nozaki *et al.* 1984a, Demski 1984, Demski 1987). In general, GnRH cell bodies are concentrated in the preoptic area (POA) and send fibers to the median eminence or pituitary (depending on the vertebrate group). Some vertebrates possess a midbrain nucleus of GnRH cells. Fibers are also often found in olfactory bulbs and in the ventral telencephalon. In many groups GnRH fibers and cells have been found in the terminal nerve (although this structure has not been conclusively identified in all animals, see Demski 1987). The exact function of this nerve

has not been established and it was originally suggested that, in fishes, it may be involved in the transmission (and possible modulation) of signals derived from external stimuli (probably pheromones received at the olfactory epithelium) to a specific region of the brain or retina (Stell *et al.* 1984, Kyle *et al.* 1985). However, this has not been supported by more recent physiological data which suggests that, in fishes, the medial olfactory tract (which runs alongside the terminal nerve) mediates responses to sex pheromones (Sorensen *et al.* 1991).

The primitive agnathans, the lampreys, possess two forms of GnRH, lGnRH I and lGnRH II (Sherwood and Sower 1985, Sherwood *et al.* 1986a). The amino acid sequence is known for one form, generally known as lGnRH, whereas only the amino acid composition is known for the other. The distribution of GnRH immunoreactivity in the brain has been examined in this group and is similar to the general vertebrate plan (Crim *et al.* 1979b, King *et al.* 1988). In the cartilaginous fish, GnRH-like immunoreactivity has been found in cell bodies and fibers in the brain of the leopard shark, *Triakis* sp. (Nozaki *et al.* 1984b, Wright and Demski 1991), the round stingray, *Urolophalus halleri*, (Demski *et al.* 1987, Wright and Demski 1991), the thornback guitarfish, *Platyrhinoidis triseriata*, (Wright and Demski 1991), the spiny dogfish, *Squalus acanthias*, (Stell 1984, Lovejoy *et al.*, in press) and the black skate, *Bathyraja kincaidii* (Lovejoy *et al.*, in press). GnRH-immunoreactivity seems to

be concentrated in the terminal nerve system in elasmobranchs (Stell 1984, Lovejoy *et al.*, in press) with relatively few cell bodies in the POA, and some scattered fibers in the telencephalon and the median eminence in some species. However, more recent studies with a variety of antisera have shown the existence of a large and strongly GnRH immunoreactive nucleus in the midbrain of some sharks and rays (see Wright and Demski 1991). The cartilaginous fishes possess at least two forms of GnRH, chicken (c) GnRH-II, which is found in many other vertebrates, and a novel form, isolated from dogfish (Lovejoy 1991).

Within the teleosts, GnRH-like immunoreactivity exhibits a similar overall distribution in the brain of all species studied so far (Blahser *et al.* 1985, Munz and Claas 1987, Garcia Ayala *et al.* 1989, Nozaki *et al.* 1985, Schafer *et al.* 1989) suggesting that GnRH may have similar multiple roles in different species. GnRH positive cells and fibers are found in the ventral parts of the olfactory bulb and in the olfactory nerve. Fibers and cells are also found in the ventral telencephalon and the POA. Some fibers extend to the optic tectum and cerebellum. GnRH-like immunoreactivity in the brain of teleosts has been studied in a variey of species including the goldfish, *Carassius auratus*, (Kah *et al.* 1984, Stell *et al.* 1984, Kyle *et al.* 1985, Kah *et al.* 1986), the platyfish, *Xiphophorus*, sp. (Schreibman *et al.* 1979, Munz *et al.* 1981, 1982, Halpern-Sebold and Schreibman 1983), the blue-gill sunfish, *Leponis macrochirus*, (Munz *et*

al. 1982), the cichlids, *Haplochromis burtonii* (Davis and Fernald 1990) and *Cichasoma biocellatum*, and Japanese eel, *Anguilla japonica*, (Nozaki and Kobayashi 1979, 1980, Nozaki *et al.* 1985), the carp, *Cyprinus carpio*, (Pan *et al.* 1979), the three-spined stickleback, *Gasterosteus aculeatus*, (Borg *et al.* 1982), the trout, *Salmo* sp. (Goos and Murathanoglu 1977, Dubois *et al.* 1979, Breton *et al.* 1986), the sole (Nunez-Rodiguez *et al.* 1985), the catfish (Blahser *et al.* 1985) and the molly, *Poecilia latipinna*, (Batten 1986). In the goldfish, GnRH fibers have been found running between the retina (via the optic nerve) and the terminal nerve (Stell *et al.* 1984, 1987). Within the retina a plexus of fibers is present. This final feature seems to be unique to the teleosts (although a similar system may have evolved independently in birds) and suggests that GnRH may have a role in the transmission and modulation of visual stimuli (Stell *et al.* 1984). The importance and influence of visual input to the GnRH system in teleosts is demonstrated by the cichlids (Davis and Fernald 1990). In these fish, dominant males possess large and intensely staining GnRH cells in the POA. However, these individuals can be visually intimidated into becoming subordinate males and the GnRH cells decrease in size and staining intensity. In other teleosts, GnRH cell bodies are found in the midbrain and GnRH containing fibers extend to the brainstem and other spinal areas.

Of the seven known forms of GnRH, at least four, mammalian (m) GnRH, salmon (s) GnRH, cGnRH-II and catfish

GnRH (in addition to some novel unidentified forms), have been identified in teleosts (Sherwood and Lovejoy 1989, Lovejoy 1991). Most species studied to date possess more than one form. The more advanced bony fish tend to have sGnRH, cGnRH-II and, in some species, a third form, which can be novel or similar to one of the other known forms. The functional basis for the presence of multiple GnRH forms within one species is not clear, although it has been suggested that they could be acting in different capacities such as hormone or neuromodulator. The differential distributions of multiple GnRH forms within the brain of the goldfish and the rainbow trout have been studied using HPLC and RIA (Yu *et al.* 1988, Okuzawa *et al.* 1990). Differential immunocytochemical studies have not been done in fish and rely on antisera which are reliably exclusive in their targets.

In the molly, *Poecilia* sp., GnRH cell bodies are found in the midbrain in a region where many cells are known to send fibers along the spinal cord (Miller and Kriebel 1986). GnRH positive fibers, extending to the urophysis, are found in the spinal cord in mollies although they could not be conclusively linked to the GnRH positive cell bodies in the midbrain. The poeciliids (*e.g.* platyfish and guppies) have been used in many studies of the distribution and ontogeny of GnRH within the brain (Munz *et al.* 1981, Munz *et al.* 1982, Halpern-Sebold and Schreibman 1983, Schreibman *et al.* 1983, Zentel *et al.* 1987). Despite their popularity as

models for the study of GnRH systems, GnRH forms within the brain have not been investigated. Additionally, the location and distribution of GnRH fibers and cells are similar but not identical between poeciliid species. In another poeciliid, the black molly (*Poecilia latipinna*) a considerable amount of information is available regarding the cytophysiology, ultrastructure and innervation of the gonadotropic cells of the pituitary and urophysis (Goos *et al.* 1985, Batten 1986, Peute *et al.* 1986, Miller and Kliebel 1986). However, neither the nature of the GnRH present in the molly brain nor the complete distribution of the peptide in the brain have been investigated.

Most amphibians possess several forms of GnRH including sGnRH, cGnRH-II and mGnRH (Sherwood *et al.* 1986b) and have a pattern of GnRH-like immunoreactivity similar to other tetrapods, with immunoreactive cells found in the terminal nerve (Alpert *et al.* 1976, Wirsig and Getchell 1986, Muske and Moore 1988) and positive fibers extending to the olfactory epithelia and POA (Demski 1984, Demski 1987). In *Xenopus*, GnRH positive perikarya are found in the olfactory bulbs and optic tectum (Doerr-Schott and Dubois 1976, Nozaki *et al.* 1984b, Muske and Moore 1987). In contrast to other vertebrates, GnRH has been established as a neuromodulator in frogs, specifically in the sympathetic ganglia (Jan *et al.* 1979).

Reptiles generally possess several forms of GnRH including cGnRH-I and cGnRH-II (Lovejoy *et al.* 1991). The

distribution of GnRH-like immunoreactivity in the brain is similar to other vertebrates although a terminal nerve has not been conclusively identified in this group (Nozaki *et al.* 1979, Nozaki *et al.* 1980, Nozaki *et al.* 1984b). In addition, like the elasmobranchs, a lizard (the chameleon) has been shown to possess a large, intensely GnRH-immunoreactive midbrain nucleus (Bennis *et al.* 1989)

Birds possess at least two forms of GnRH, cGnRH-I and cGnRH-II, (King and Millar 1982a, 1982b, 1984, Miyamoto *et al.* 1984) and show approximately the same brain GnRH distribution as other vertebrates although there is some evidence of differential distribution (Katz *et al.* 1990). However, they are unusual in possessing GnRH positive fibers in the retina (see Demski 1987). This suggests that GnRH may have a similar role as in the teleost retina but that this system has evolved a second time and may be an independent amacrine system.

Metatherian and prototherian mammals (the marsupials and monotremes) appear to possess both cGnRH-II and mGnRH (King *et al.* 1989) while eutherian (placental) mammals have only mGnRH (Matsuo *et al.* 1971, Burgus *et al.* 1972). The distribution of GnRH-like immunoreactivity in mammals has been well studied (see Silverman 1988 for review) and is concentrated in the forebrain-preoptic-hypothalamic pathways and the terminal nerve which is an elaborate plexiform system extending from the olfactory epithelium to the ventral forebrain. GnRH cells have been shown to originate

in the medial olfactory placode of the developing nose. They then migrate (developmentally) through the forebrain with the terminal nerve, eventually settling in the septal-preoptic area and hypothalamus (Schwanzel-Fukada and Pfaff 1989). In contrast, the origin of the GnRH cell bodies found in the midbrain is unknown. GnRH fibers also extend to other areas and it has been suggested that there are multiple sites of hormone secretion (Anthony *et al* 1984). Since GnRH receptors are found in various areas of the brain, it is postulated that the peptide may be acting as a neurotransmitter as well as a hormone (Millan *et al*. 1986).

The similarity in distribution of GnRH-like immunoreactivity across such a broad phylogenetic range emphasizes the importance of the GnRH system. It also suggests that the main role of GnRH has been conserved within these phyla. However, the presence of GnRH-like immunoreactivity in areas such as the retina in fish and birds, in the sympathetic ganglia in amphibians, in large midbrain nuclei in reptiles and some elasmobranchs, and in the placenta in mammals suggests that additional or novel functions may have arisen for the peptide in select groups.

In order to determine whether GnRH-like immunoreactivity was present in protochordates, I used two antisera made against two forms of GnRH to examine the distribution of GnRH in a local tunicate, *Chelyosoma productum*. This is a representative of the group that may have some resemblance to members of the line that gave rise to the vertebrates.

Since different forms as well as distributions may imply functional evolution, I undertook a survey of GnRH-like immunoreactivity in salmon (*Oncorhynchus* sp.) and mollies (*Poecilia* sp.), neither of which had previously been studied this way. As positive controls for the immunocytochemistry, I used goldfish and rat, which are both well characterized in terms of the GnRH distribution in the brain. If patterns of distribution within these two species matched well with previous findings then I could be confident that patterns found in the other groups were specific for GnRH. It is useful to understand the distribution of GnRH across the vertebrates when trying to derive an evolutionary history for the GnRH family. If the distribution of the molecule within the brain has changed as the structure of the molecule has evolved, this may imply a change in function. If very different species, which possess distinct forms of GnRH, have similar neural systems which always seem to contain GnRH, then perhaps function is maintained despite changes in peptide sequence.

Materials and Methods.

ANIMALS

Tunicates

Adult specimens of a tunicate or sea squirt (*C. productum*/class Ascidea/subphylum Urochordata/phylum

Chordata) were collected in 1987 by a diver at Ten Mile Point, Victoria, British Columbia. The tunicates were transferred and kept in marine aquaria in the laboratory. For each individual the neural ganglion, neural gland, and part of the basket were removed and pinned out in Sylgard lined dishes. Tissue was fixed in 4% paraformaldehyde in phosphate buffered saline (PBS, recipe according to Vectastain ABC Kit, Vector Labs., Burlingame, CA.), pH 7.2, for 2 hr. It was then rinsed in PBS, 3 x 10 min, and cryoprotected in 30% sucrose overnight at 4°C.

Fish

Salmon

Chum salmon (*Oncorhynchus keta*) brains were collected at the Qualicum Hatcheries, Qualicum, B.C. in the fall of 1987 as they returned to spawn. Male fish only were taken. Fish were anesthetized with carbon dioxide and brains quickly removed. Brains were too big to be fixed whole so were cut into pieces and fixed in 4% paraformaldehyde or Zamboni's fixative for several hours to several days at 4°C. Tissue was paraffin embedded and sectioned using standard protocols. These sections were subsequently used for fluorescence or peroxidase anti-peroxidase (PAP) immunostaining. In addition, steelhead (*O. mykiss*) brains were also collected in the summer of 1988 from San Mateo Bay, B.C. These animals were anesthetized with MS-222 and perfused with heparinized teleost saline then Zamboni's

fixative. Brains were removed and left in fresh fixative for an additional 24-48 hours, then transferred to 30% (w/v) sucrose for cryoprotection overnight at 4°C.

Sockeye salmon (*Oncorhynchus nerka*) brains were collected in August 1988 near Nanaimo. Animals were perfused via the conus arteriosus with heparinized teleost saline then Zamboni's fixative. Brains were removed and allowed to continue fixing for an additional 24 hr. Tissue was rinsed in PBS and cryoprotected in the same way as the steelhead brains.

Mollies

Mollies (*Poecilia* sp.) were acquired from local pet stores. They were anesthetized with MS-222 and then killed by decapitation. Animals were too small to perfuse so the brain was dissected out (leaving behind the pituitary) and immersed in Zamboni's fixative for 24-36 hours.

Goldfish

Goldfish (*Carassius auratus*) were brought from local pet stores or from commercial suppliers. They were examined for reproductive condition and those considered to be approaching the spawning stage (white dots on gills, presence of eggs) were used for immmunocytochemistry. Individuals were anesthetised with MS-222 then perfused through the heart with heparinized saline for 5-10 minutes followed by Zamboni's fixative until the gills had turned yellow. The brain was then removed and left immersed in Zamboni's fixative for several more hours. Tissue was then

either processed for paraffin embedding using standard techniques or for cryosectioning by rinsing in PBS for 1.5 hours, then in fresh buffer for another 1.5 hours followed by cryoprotection as outlined previously. Sections were immunostained using several techniques including immunofluorescence with fluorescein isothiocyanate (FITC) labelled secondary antibody, and light microscopy with peroxidase anti-peroxidase (PAP) and avidin biotin conjugate (ABC) staining.

Rats

Rats (*Rattus norvegicus*) were otained from the University of Victoria Animal Care Facility. They were generally about 5 months old and weighed approximately 300g. Animals were anesthetized with chloral hydrate (400mg/ml, 1 - 1.5 ml per rat). The chest wall was opened and the heart exposed. The heart was perfused via the left ventricle with heparinized saline (NaCl 9g/l) until the lungs turned white. The animal was perfused with Zamboni's fixative until perfusion was considered complete as indicated by the yellow colour of the toes and nose. The animals were left undisturbed for 1 - 2 hours to allow fixation to take place. The head was then cut off and top of the skull removed. The head was placed in a stereotaxic frame with the ear bars and tooth bar in the same horizontal plane. Frontal cuts were made just anterior to the pineal and posterior to the olfactory bulb. Sagittal cuts were also made to remove the

lateral regions of the cerebrum. The trimmed brains were left in fixative for 24 hours then transferred to 30% sucrose for cryoprotection overnight at 4°C and embedded in OCT (Ames).

Cryosectioning

For tunicates, steelhead, sockeye, goldfish, mollies and rats, brains were embedded in OCT mounting medium and frozen in liquid nitrogen. For tunicates, cryostat sections were cut at 10μm, thaw mounted onto gelatin-coated slides, and stored at -80°C. For the fish brains, sagittal and frontal cryostat sections were cut at 10 - 20 μm, thaw-mounted onto poly-L-lysine coated slides and stored at -80°C. For the rats, cryostat sections were cut at approximately 30 μm for sagittal sections and 10 -20 μm for frontal sections. These were thaw-mounted onto poly-L-lysine coated slides and processed in the same manner for ABC immunostaining as for the fish sections. Steelhead and sockeye sections were stored at -80°C for several months before processing. Tunicate, goldfish, molly and rat sections were processed within a month of fixation

IMMUNOSTAINING

Antisera

All the antisera used for immunostaining were raised in our lab and have been characterized by RIA (see Kelsall *et al.* 1990 for more details) although this does not ensure

specificity in immunocytochemical studies (Swanson, *pers. comm.*). Antiserum GF was raised in rabbits against sGnRH and the numerical suffix (GF-4, GF-5) represents the bleed from which the antiserum was isolated. Antiserum Bla-4 was also raised in rabbits against lamprey GnRH. This antiserum was used for immunostaining of the tunicates since it was postulated that the GnRH-like molecule in this group may be closer in structure to the form found in a primitive vertebrate such as the lamprey than to other forms.

Tunicates

A Vectastain ABC kit was used for avidin-biotin immunostaining. Slides were allowed to come to room temperature before being rehydrated in PBS (2 x 15 min). They were incubated in blocking serum for 1 hr and then in primary antiserum solution for 48 hr at 4°C. The primary antiserum solution consisted of Bla-4 diluted 1:50 in PBS containing 0.3% Triton X-100. Slides were then rinsed in PBS as before and incubated in biotinylated goat anti-rabbit secondary antibody for 1 hr, rinsed again, and incubated in ABC reagent for 2 hr. Slides were rinsed in PBS and incubated in 0.05% diaminobenzidine solution containing 0.04% nickel ammonium sulfate for 10 min. Reaction product was visualized by adding 60μl of 30% hydrogen peroxide per slide. Sufficient color usually developed within 10 min. Slides were then rinsed in tap water for 5 min, counterstained in 1% methyl green for 2 min, rinsed,

dehydrated and mounted in Histoclad. To ensure staining was specific, controls were included in which the primary antiserum was omitted.

Fish

Peroxidase anti-peroxidase (PAP) immunostaining

Paraffin-embedded sections and cryosections of chum and goldfish were used for PAP immunostaining. Cryosections were allowed to come to room temperature and ringed with rubber cement to keep solutions in place throughout the immunostaining. Endogenous peroxidase was inactivated by incubating the sections in 0.05M sodium phosphate buffer (PB) containing 0.3% H_2O_2 for 10 min, rinsed in PB twice and incubated in PB containing 2% goat serum. Primary antiserum, either GF-4 or GF-5, was diluted 1:250 in PB with goat serum. Sections were covered with primary antibody solution overnight at 4°C. Slides were rinsed in PB for 10 min then covered with goat anti-rabbit gamma globulin (GARGG) diluted 1:100 in PB with the addition of 0.05% sodium azide (PBZ) for 2-3 hours at 4°C. Peroxidase anti-peroxidase solution diluted 1:500 in PBZ was added to the sections which were left at room temperature for 2 hours. To visualize reaction product, PBZ containing 0.05% diaminobenzidine and 0.03% $NiNH_4SO_4$ was added to the sections for 5 min followed by 0.03% H_2O_2 in PBZ for 10 min. Cryosections and paraffin-embedded sections were then counterstained in 1% methyl green, rinsed, dehydrated and mounted in Histoclad.

Fluorescein Isothiocyanate (FITC) immunostaining

Cryosections and paraffin-embedded sections of chum salmon and goldfish were immunostained using the FITC technique. Slides with cryosections were warmed and ringed as for PAP immunostaining. Sections were incubated in PB containing 2% goat serum then in the same primary antibody solution under identical conditions as for PAP. Slides were then rinsed in PB, incubated in FITC-GARGG diluted 1:50 in PB at room temperature for several hours. They were then rinsed in PB and mounted in a glycerol/water (3:2) solution.

Avidin-Biotin Conjugate Immunostaining

A Vectastain ABC kit was used for avidin-biotin immunostaining of sockeye, steelhead, molly, goldfish and rat sections. Slides were warmed and ringed as for PAP and FITC staining then rehydrated in PBS (Vectastain recipe). Slides were rinsed in PBS (2 x 15 min), immersed in 0.3% H_2O_2 in absolute methanol (2-5 min) and rinsed again in PBS (2 x 5 min). They were then immersed in blocking serum for 1 hour at room temperature and then in primary antiserum solution for 48 hours at 4°C. The primary antiserum solution consisted of GF-4 diluted 1:500 in PBS containing 0.3% Triton X-100. Slides were rinsed as before, incubated in biotinylated secondary antibody for 1 hour at room temperature, rinsed again and incubated in ABC reagent for 2 hours at room temperature. Slides were rinsed in PBS again and immersed for 10 min in 0.05% diaminobenzidine solution

containing 0.04% nickel ammonium sulfate. Reaction product was visualized by adding approximately 60 µl of 30% H_2O_2 to each slide. Sufficient color usually developed within 7-10 min. Slides were then rinsed in water, counterstained in 1% methyl green (3 min), rinsed, dehydrated and mounted in Histoclad. To ensure that staining was specific, controls were included in which the primary antiserum was omitted.

Results

Tunicates

GnRH-like immunoreactivity was found in nerve fibers in the neural ganglion and in at least one anterior and posterior root (fig. 1). Immunoreactive cell bodies could not be definitively identified. The neural gland did not appear to contain any specific GnRH-like immunoreactivity.

Salmon

Chum

Paraformaldehyde fixation and paraffin embedding appeared to destroy or mask the antigenicity of any endogenous GnRH. Additionally, the presence of widespread non-specific staining could not be abolished when using PAP immunostaining without, apparently, also destroying the antigenicity of any endogenous GnRH. The quality of the tissue which was fixed using Zamboni's fixative was poor.

There were many tangled fibers and a general deterioration in the integrity of the tissue. This was may be partly due to poor fixation but may also be linked to the physiological state of the animals at the time of collection. In addition, these animals may have been post-ovulatory and levels of GnRH within the brain may have peaked several weeks before collection. I was therefore unable to detect any GnRH immunoreactivity in chum salmon brains.

Steelhead and Sockeye

I was also unable to detect any GnRH immunoreactivity in these sections despite the different processing methods from those used for the chum. This may be due to the reproductive state of the animals (possibly post-ovulatory) at the time of collection or could be related to the size of tissue and efficiency of fixation. GnRH may not be present in sufficient quantities in the brain to be detected by immunocytochemistry. In addition these sections were stored for an extended period of time before processing which may have destroyed, or depleted beyond detection, any endogenous stores of GnRH.

Mollies

Immunocytochemical studies showed the presence of GnRH-immunoreactive cells in the posterior ventral telencephalon and preoptic area. One male fish possessed immunoreactive cells in the nucleus glomerulosus (in the thalamus), an area

which has not previously been reported to contain GnRH containing cells. GnRH fibers were found extensively throughout the brain, located within the telencephalon, th optic tectum, the hypothalamus and the midbrain.

Considerable differences in presence and distribution of ir-fibers and ir-cell bodies were noted between individuals. One male animal possessed stronger overall ir-staining than other animals and had a more extensive ir-fiber network. This may be related to the reproductive state of the individual at the time of sacrifice although all animals were considered to be in reproducitve condition.

Immunoreactive fibers were distributed extensively throughout the brain. Many short ir-fibers were found in the telencephalon and olfactory bulbs. They extended in all directions forming a loose network with no strong orientation. A number of ir-fibers extended from the ventral midline area of the telencephalon both dorsally and laterally. In coronal sections it was possible to follow ir-fibers across the anterior commissure from one side of the brain to the other. In some animals, varicose ir-fibers followed a nerve bundle which ran between the telencephalon and the preoptic area (fig. 2 a). Within the optic tectum, a loose plexus of ir-fibers was found primarily between the stratum album centrale and the stratum album griseum (fig. 2 b). A network of ir-fibers, with no particular directionality, was present throughout the ventral hypothalamus or inferior lobe (fig. 2 c). An extensive

network of ir-fibers was also found running between the preoptic area, the inferior lobe, the optic tectum and the midbrain (fig. 2 d). The highest density of ir-fibers was found within the anterior diencephalon. Some ir-fibers appeared to lie within the optic tract although others extended both rostrally and caudally. Fibers also extended through the medulla oblongata and along the spinal cord. Neither ir-fibers nor ir-cell bodies were found in the cerebellum.

Immunoreactive cell bodies were generally located fairly close to the midline (although not exclusively). A few isolated cell bodies were found in the posterior ventral telencephalon and in the preoptic area (fig. 4). None were found in the anterior telencephalon. Several cell bodies were found within or on the margin of the nucleus glomerulosus in one individual (fig. 3 a,b). Isolated cell bodies were also found in the midbrain (fig.3 d). A generalized pattern of overall immunoreactivity is shown in figure 4.

Goldfish

Differences in extent and intensity of GnRH immunoreactive staining were noted between different individuals. Fibers were found throughout the brain and retina using both immunofluorescence and ABC immunostaining. Numerous fibers were present throughout the telencephalon. Most were not organized into well defined tracts. However,

a number of fibers did extend along the olfactory tract between the ventral telencephalon and preoptic region (fig. 5 a,b, and fig. 6 b) and a plexus was found along the base of the brain within the inferior lobe. In the olfactory bulb, many short fibers, extending in all orientations, were present (fig. 6 c). A few small cell bodies were identified in the ventral telencephalon (fig. 6 a). A network of fibers was also present in the optic tectum, apparently extending across many cell layers (fig. 7).

A faint and diffuse immunoreactive plexus was found in the retina (not shown).

Rats

Terminology is according to König and Klippel (1963). GnRH-immunoreactive cell bodies and fibers were found in the olfactory tract and olfactory tubercle (figs. 8 and 9) of the rat brain. Immunoreactive fibers were present in the tractus olfactorius intermedius (TOI), tuberculum olfactorium, pars corticalis, lamina plexiformis (TULP), tuberculum olfactorium, pars corticalis, lamina pyramidalis (TULC), and tractus olfactorius lateralis, pars ventralis (TOLV). These fibers were typically varicose and oriented in line with the tract (fig. 8). A single section usually only contained one or a few immunoreactive fibers although adjacent sections would also contain fibers in this area, suggesting a loose, scattered network of GnRH immunoreactive fibers existed throughout the olfactory tract. In some

cases, immunoreactive fibers were found running just under the surface of the brain inside the meninges (fig. 8 d)). In the anterior olfactory nucleus (medial (oam), lateral (oal) and posterior (oap)) small immunoreactive cell bodies were found either individually or in groups of two cells (fig. 9 and fig. 10 a,b). Generally these cells were strongly stained throughout the cytoplasm leaving the nucleus clearly visible (fig. 9 b,e). Most cells did not have visible immunoreactive extensions although in one case a very long single immunoreactive process was visible (fig. 10 a,b). A few intensely immunoreactive unipolar cells with long processes were found in the olfactory tract (fig. 10 c,d).

Immunoreactive bipolar cells and fibers were also found in the the medial preoptic area (figs. 11 and 12). Coronal sections clearly showed GnRH positive cells in the nucleus preopticus lateralis and nucleus lateralis hypothalami and a plexus of immunoreactive fibers around the third ventricle. GnRH-positive bipolar cells were found in the preoptic area (fig. 13 a,b) and sagittal sections showed that an immunoreactive plexus extended between the anterior preoptic area and median eminence and within the ventral midbrain (fig. 13 c). Bipolar cells with small cell bodies (fig. 14 a,b) and single isolated fibers (fig. 14 c,d) were found in the diagonal band of Broca. A GnRH-immunoreactive plexus extended along the ventral surface of the brain within the hypothalamus and was particularly dense and intensely

stained in the median eminence (fig. 15). The strongest staining appeared to be along the tissue bordering the third ventricle (fig. 15 b,c). Small blood capillaries were visible in this region and immunoreactive fibers were seen densely packed around them (fig. 15c).

Figure 1. Immunoreactive GnRH-like fibers in the cerebral ganglion (cg) and neural roots of the sea squirt, *C. productum*.

(a) The arrow indicates the posterior root of the ganglion: the arrowheads show oblique sections through the anterior roots. The neural gland (ng) lies below the cerebral ganglion. The antiserum, Bla-4, was made against lamprey GnRH. Scale bar = 200μm.

(b) An enlargement of the cerebral ganglion and posterior root from (a) is shown. Arrow heads are putative cell bodies. Scale bar = 100μm.


a

Figure 2. GnRH immunoreactive fibers in the brain of the molly, *Poecila* sp.

(a) An immunoreactive fiber lying within a tract extending between the telencephalon and the preoptic area.

(b) Immunoreactive plexus within the optic tectum, lying within the stratum album centrale (sac) near the stratum periventriculare (sp).

(c) Short immunoreactive fibers with no particular directionality within the hypothalamus (or inferior lobe).

(d) Fibers extending between the midbrain and the spinal cord.

Scale bar = 15 μm.

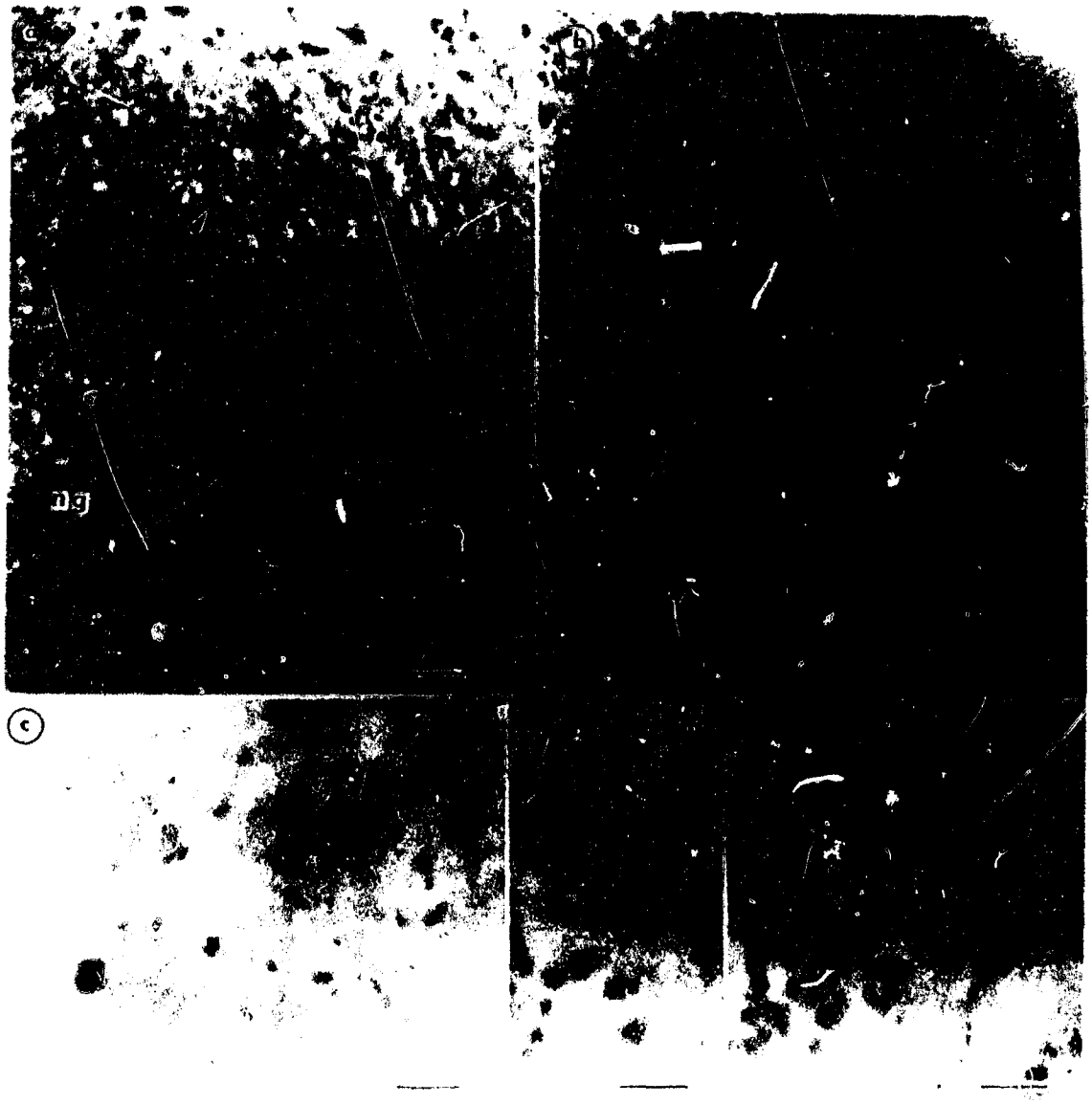

a
b
c

Figure 3. GnRH immunoreactive cell bodies in the brain of the molly, *Poecilia* sp.

(a)-(b) GnRH immunoreactive cell bodies (arrows) within the nucleus glomerulus (ng). Note fibers lying to the lower right of cell body in (b). Enlargements of the above cell bodies in (a) and (b) are shown in (c) and (e) respectively. (d) GnRH immunoreactive cell body found in the midbrain.

(a)-(b) Scale bar = 25 μm.

(c)-(e) Scale bar = 10 μm.

Figure 4. Schematic representation of the GnRH immunoreactive system found within the brain of the molly, *Poecilia* sp.
Black circles represent cell bodies and dotted lines represent fibers.

Abbreviations

C cerebellum

IL inferior lobe (hypothalamus)

MO medulla oblongata

MT midbrain tegmentum

NG nucleus glomerulosus.

OT optic tectum

POA preoptic area

T telencephalon

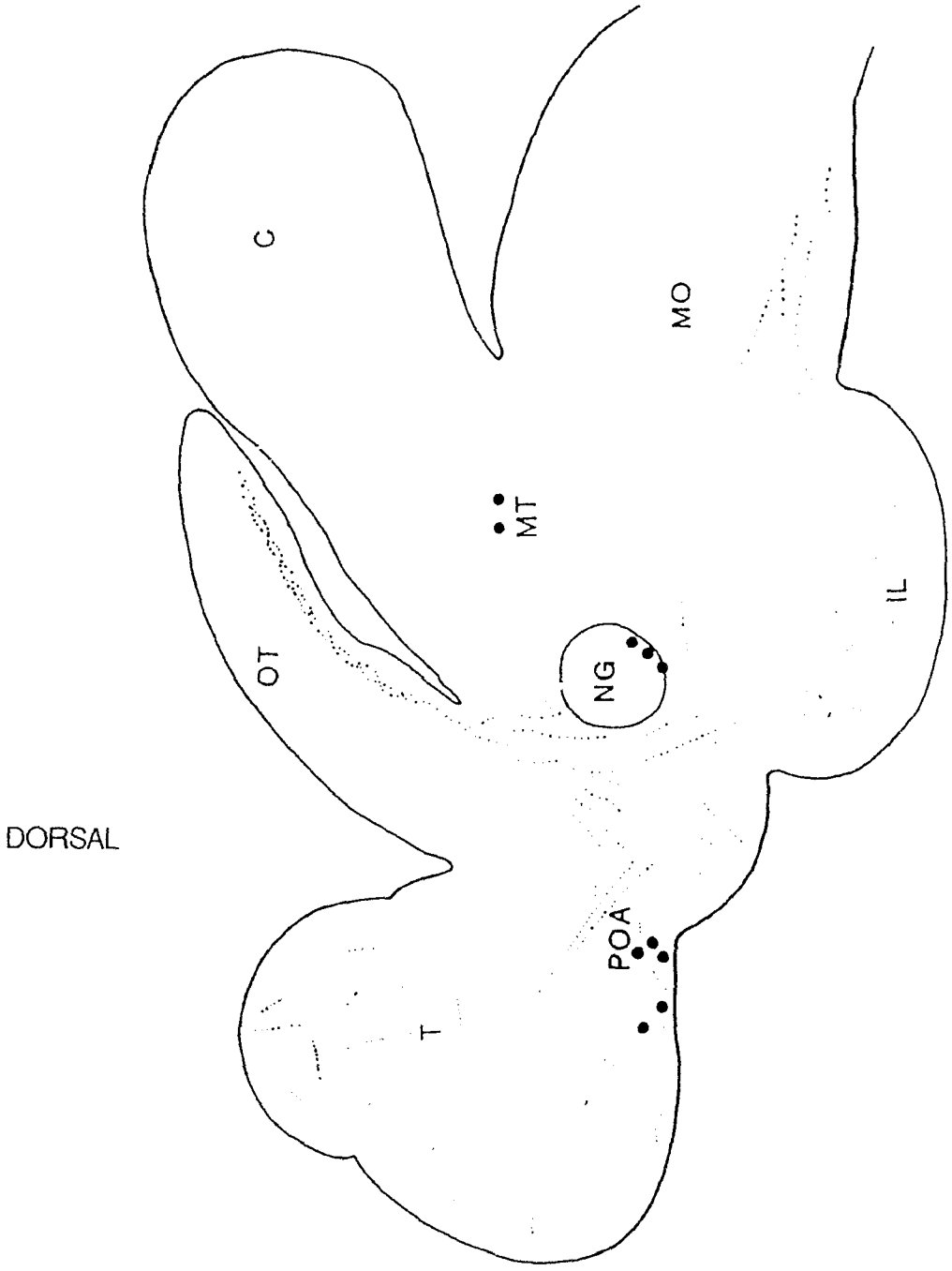

C
MO
MT
IL
OT
NG
POA
T
DORSAL
ANTERIOR

Figure 5. GnRH immunoreactivity in the olfactory tract of the goldfish, *Carassius auratus*.

(a) Fluorescein isothiocyanate (FITC) staining of immunoreactive fibers.

(b) Avidin biotin conjugate (ABC) staining of immunoreactive fibers. Both fine varicose fibers and thick, intensely staining fibers can be seen.

Scale bar = 10μm

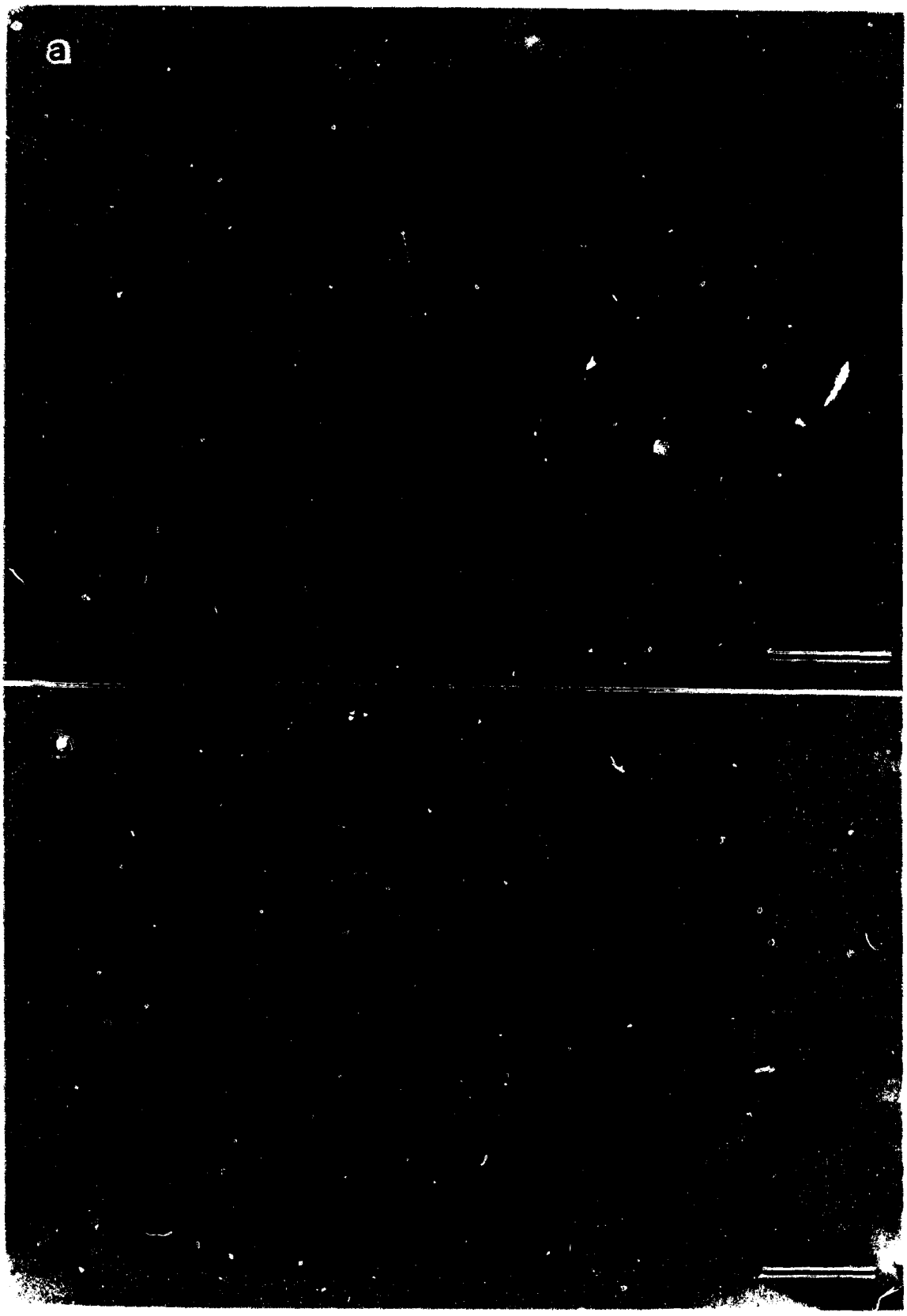

a

Figure 6. GnRH immunoreactivity in the telencephalon of the goldfish, *Carassius auratus*.

(a) GnRH immunoreactive cell body in the ventral telencephalon. Scale bar = 2.5 μm.

(b) GnRH (fluorescein isothiocyanate (FITC) stained) immunoreactive fiber in the ventral telencephalon. Scale bar = 10 μm.

(c) GnRH immunoreactive fibers throughout the telencephalon. Scale bar = 10 μm.


a
b
c

Figure 7. GnRH immunoreactive fibers in the optic tectum of the goldfish, *Carassius auratus*.

(a) GnRH immunoreactive fibers. Scale bar = 10μm.

(b) Diffuse network of GnRH immunoreactive fibers. Scale bar = 10μm.

(c) Higher magnification of (b). Scale bar = 10 μm.

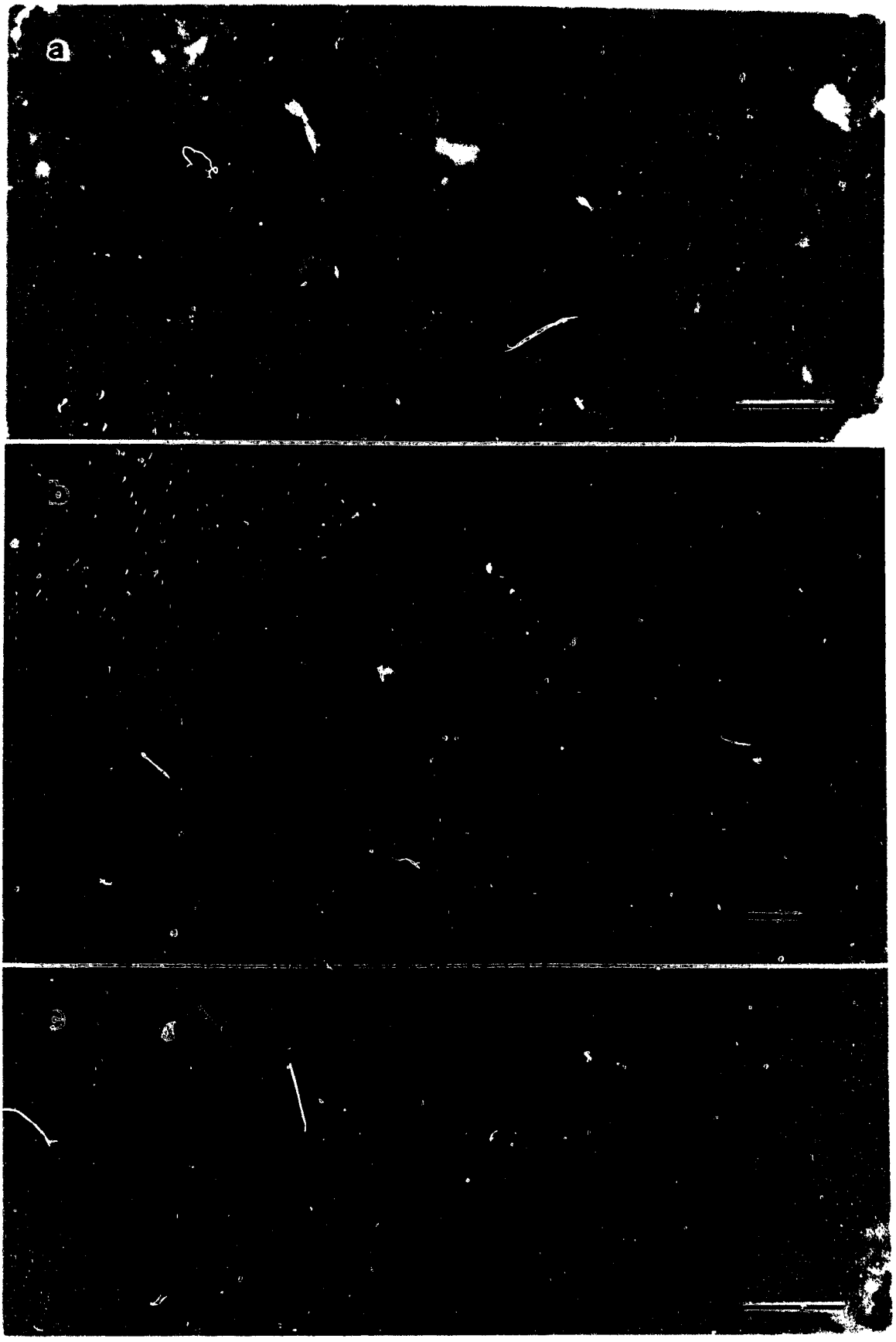

a
b
c

Figure 8. Mid-sagittal section of the rat (*Rattus norvegicus*) brain showing GnRH immunoreactivity in the olfactory tract and olfactory tubercle. Anterior is towards the left.

(a) Single fiber (arrow heads) present within the olfactory tract. Scale bar = 25 μm.

(b) Same fiber at higher magnification. Scale bar = 5 μm.

(c) Immunoreactive fiber in an adjacent section. Scale bar = 5 μm.

d). Immunoreactive fiber present just under the surface of the brain. Scale bar = 2 μm.

Figure 9. GnRH immunoreactive cell bodies in the olfactory nucleus of the rat, *Rattus norvegicus*.

(a) Immunoreactive fibers (arrow heads) and cell bodies. Scale bar = 5 μm.

(b) Immunoreactive cell body showing unstained nucleus. Scale bar = 2 μm.

(c) Magnification of (a). Scale bar = 2 μm.

(d) Single immunoreactive cell body. Scale bar = 2 μm.

(e) - (f) Single immunoreactive cell bodies. Scale bar = 5 μm.

a
b
d

Figure 10. GnRH immunoreactive cell bodies in the olfactory tract and olfactory nucleus of the rat, *Rattus norvegicus*.
(a) Single immunoreactive cell body with long process in olfactory nucleus. Scale bar = 10 μm.
(b) Magnification of (a). Scale bar = 5 μm.
(c) Single immunoreactive cell body and process in olfactory tract. Scale bar = 5 μm.
(d) Magnification of (c). Scale bar = 5 μm.

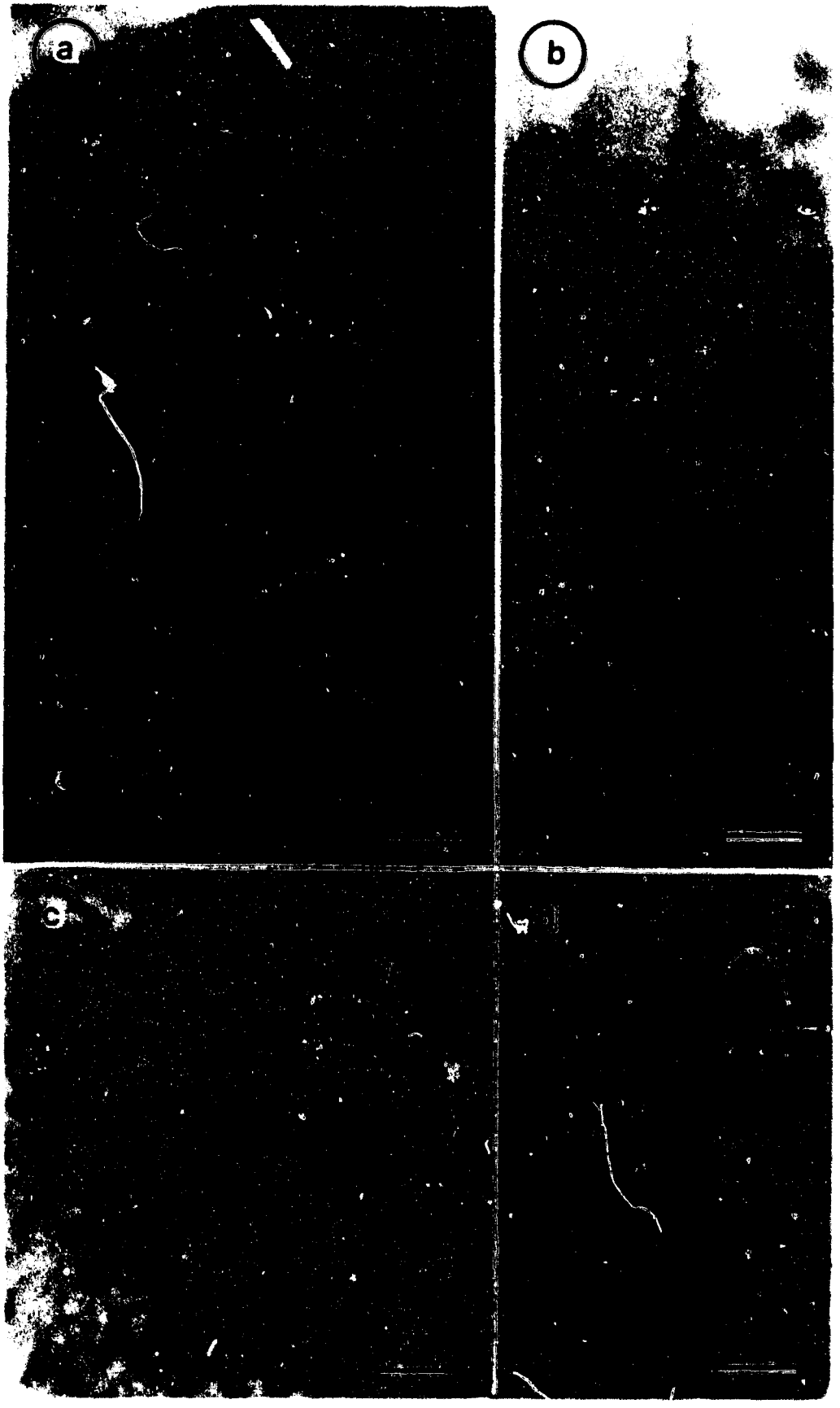

a
b
c

Figure 11. Coronal sections showing GnRH immunoreactivity in the preoptic area of the rat, *Rattus norvegicus*.
(a) GnRH immunoreactive plexus (open arrow heads) and cell bodies (closed arrow heads) in the medial preoptic area (mpoa). Scale bar = 25 μm.
OC = optic chiasm
III = third ventricle
(b) Adjacent section showing plexus (open arrow heads) and cell bodies (closed arrow heads). Scale bar = 25 μm.

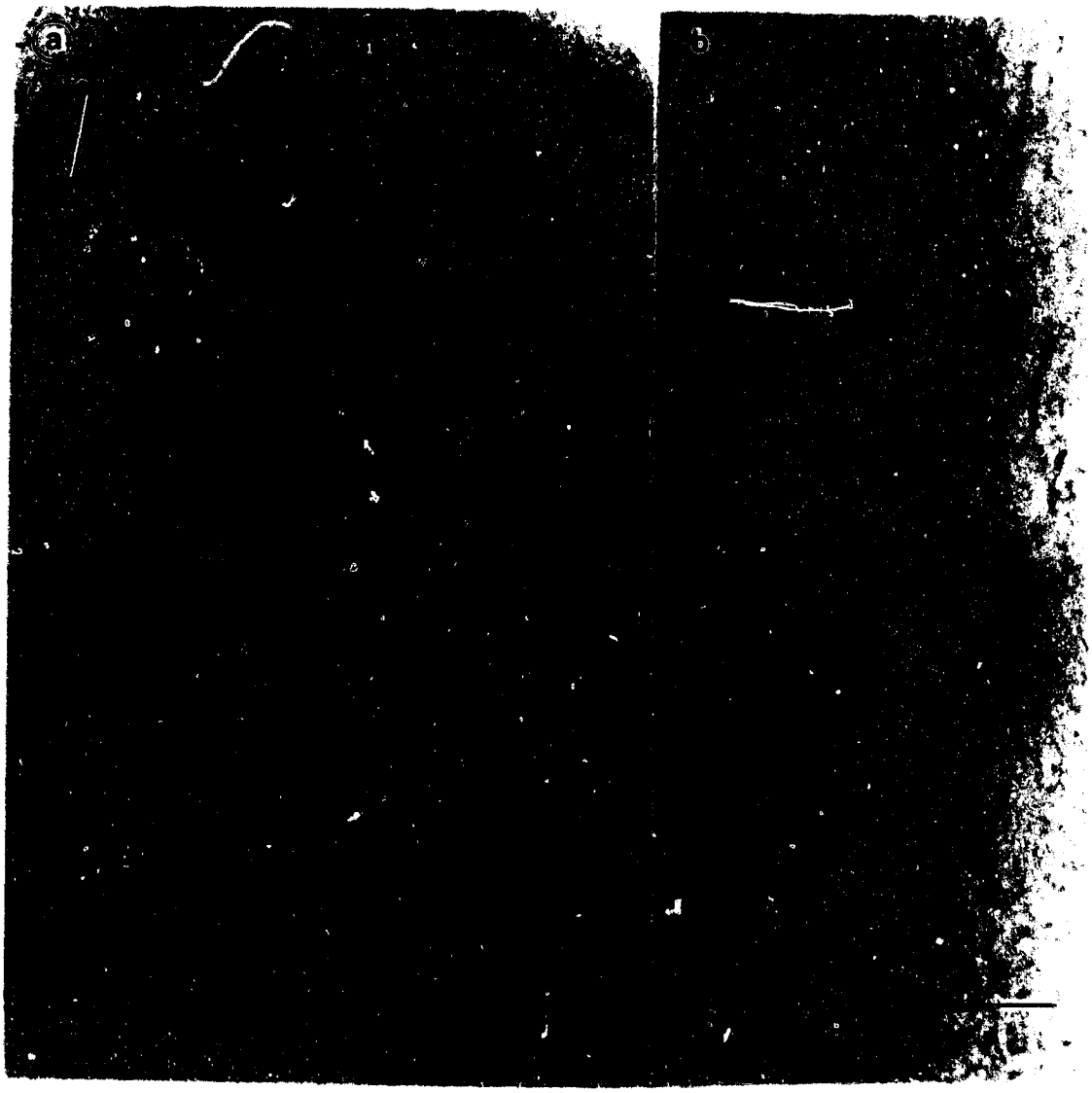

a
b

Figure 12. Magnification of GnRH immunoreactivity in preoptic area of the rat, *Rattus norvegicus*.
(a) Immunoreactive cell bodies (arrow heads) and plexus. Scale bar 12 = μm.
(b) Adjacent section showing immunoreactive cell bodies (arrow heads) and plexus. Scale bar = 12 μm.


a

Figure 13. Sagittal sections showing GnRH immunoreactive cells and plexus in the preoptic area of the rat, *Rattus norveigus*.

(a) Bipolar GnRH immunoreactive cell. Scale bar = 10 μm.

(b) Two GnRH immunoreactive cells. Scale bar = 10 μm.

(c) Dense GnRH immunoreactive plexus in the anterior ventral preoptic area which extends between the ventral telencephalon and the median eminence. Scale bar = 10 μm.


a

Figure 14. GnRH immunoreactive fibers in the diagonal band of Broca of the rat, *Rattus norvegicus*.

(a) Two bipolar GnRH cells. Scale bar = 10 μm.

(b) Higher magnification of (a). Scale bar = 5 μm.

(c) Single GnRH immunoreactive fiber. Scale = 10 μm.

(d) Higher magnification of (c). Scale bar = 5 μm.

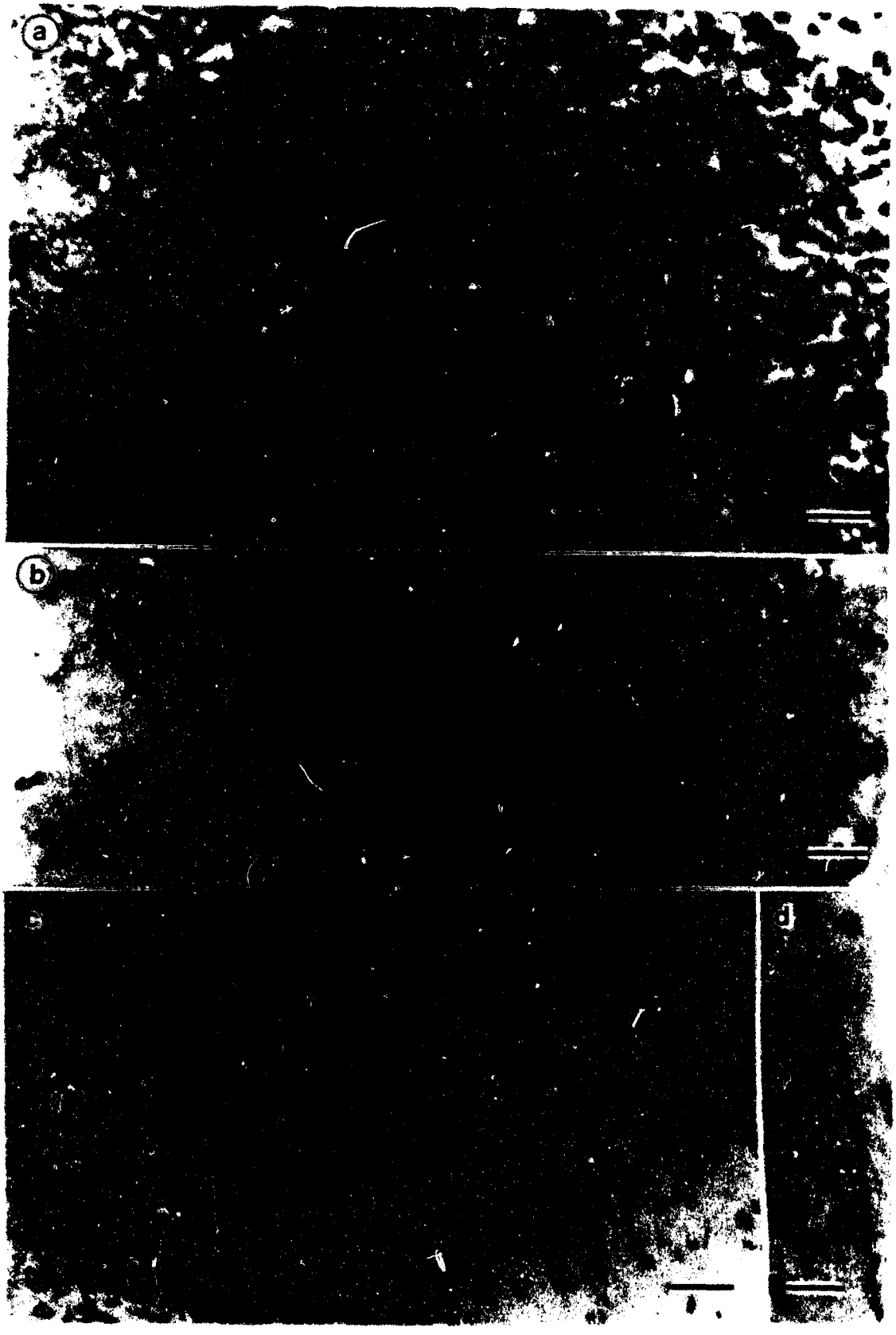

a
b
c
d

Figure 15. Mid-sagittal section showing GnRH immunoreactivity in the hypothalamus of the rat, *Rattus norvegicus*.

(a) The GnRH immunoreactive plexus extends along the ventral surface of the brain between the preoptic area, anterior to the optic chiasm (oc), and the median eminence (me). Densely packed immunoreactive fibers are found throughout the median eminence and particularly along the periphery (arrows) of the third ventricle (III). Scale bar = 70 μm.

(b) Magnification of anterior portion of median eminence showing plexus and intense staining near third ventricle. Scale bar = 25 μm.

(c) Magnification of posterior portion of median eminence showing plexus and intense staining around third ventricle and small blood vessel (arrow head). Scale bar = 25 μm.

(d) Magnification of anterior portion of median eminence showing GnRH immunoreactive plexus consisting of fine, extensive network of varicose fibers. Scale bar = 12.5 μm.


a
oc
III
me
b
c
d

Discussion

It is clear that a GnRH-like molecule is present in a wide range of vertebrates and is distributed throughout the brain. This supports the concept that GnRH is common to most vertebrates and suggests that the antisera used in the study detect several forms of GnRH. The patterns of GnRH-like immunoreactivity within the brain appear to be strongly conserved throughout the vertebrates suggesting that perhaps the function or functions of GnRH within the vertebrate brain have also been conserved.

Vertebrates may have evolved from larval, fish-like protochordates perhaps 600 million years ago. The presence of GnRH-like material in the tunicate nerve fibers suggests that the peptide may be an ancient neurotransmitter or neurohormone. The existence of GnRH-like immunoreactivity in the tunicate confirms earlier studies by a number of workers in different species (Georges and Dubois 1980, 1985, Fritsch *et al*. 1982, Schreibman *et al*. 1986, Thorndyke and Georges 1988).

The failure to detect GnRH-like immunoreactivity in the salmon may be, in large part, due to technical problems associated with the nature of the tissue and method of collection. It is not easy to perfuse effectively these animals, especially in the field. If the brain cannot be fixed *in situ*, then extensive dissection followed by fixation must take place. This makes subsequent

interpretation and reconstruction of the immunoreactive systems more difficult. It is also possible that levels of GnRH were very low (due to the fish being post-ovulatory) at the time of collection although this was approximately the same reproductive stage as the original protein analysis (Sherwood *et al.* 1983). The integrity and quality of the brain tissue for histology was poor which may have been due to the poor condition of the fish at this stage in their life cycle or to inadequate fixation.

Within the goldfish and mollies, the distribution of GnRH-like immunoreactivity throughout the telencephalon, POA, optic tectum, retina and hypothalamus is similar to the findings of previous studies (Kah *et al.* 1984, 1986). Fewer cell bodies and a slightly less extensive immunoreactive network were found in the goldfish in this study compared to previous reports. This may be due to individual variation in reproductive state. Studies on the tropical fish, the cichlid, indicate quite clearly that there can be considerable variation in the amount and distribution of GnRH within the brain of an individual fish with social status and during its reproductive cycle (Davis and Fernald 1990, Fernald, pers. comm.). This has rarely been taken into account in studies on the distribution of GnRH within the teleost brain but may, in part, be responsible for variations in reported GnRH distributions of similar species by different groups.

The distribution of GnRH within the molly brain

corresponds, to a limited extent, with that found for other poeciliids, namely the platyfish, *Xiphophorus* sp. and the guppy, *Poecilia reticulata* (Schreibman *et al.* 1983, Zentel *et al.* 1987). In platyfish, GnRH perikarya are found in the nucleus olfactoretinalis (NOR), the nucleus preopticus periventricularis (NPP) and the nucleus lateralis tuberis (NLT). In contrast, in guppies no ir-perikarya were found in the NLT but an extensive network of ir-fibers is present in this part of the brain both in mollies and in guppies. It is known that the concentration of GnRH found in the brain varies considerably with the maturity and reproductive phase of the individual (Breton *et al.* 1986, Schreibman *et al.* 1983, Zentel *et al.* 1987) so it is possible that GnRH containing cells are present in this part of the brain but the levels of the peptide were too low to be detected in the mollies using our antiserum. In the platyfish, staining in the NPP was consistently stronger than in the other two regions and this is also the only region in the molly that consistently showed the presence of ir-cells. The presence of GnRH containing cells in the nucleus glomerulosus (in the thalamic area) is the first report of ir-GnRH being found in this area in fish and needs to be substantiated by further work on more individuals. The functional significance of ir-cells in this area is not known and, since they were only observed in one male fish, they may indicate the existence of a very precise and short-lived reproductive or maturational phase during which cells in this region produce

GnRH. A correlative study of both the reproductive state of the molly and the distribution of GnRH cells and fibers in the brain would be highly informative. GnRH cell bodies were found in the midbrain which is an area that has previously been reported to contain immunoreactive cells (Miller and Kriebel 1986). The same study reported GnRH fibers extending down the spinal cord to the urophysis which may be the final destination of the fibers observed here in the medulla oblongata. More recently, an extensive study of neuropeptide-immunoreactive systems was done in the molly brain (Batten *et al.* 1990). Similar findings were reported to those described here although, at the junction of the olfactory bulb and ventral telencephalon, additional cell bodies were found which sent fibers both to the telencephalon and olfactory bulb. No cell bodies were reported in the nucleus glomerulosus perhaps supporting the suggestion that the presence of these indicates a very short-lived reproductive or maturational stage. Differences in the studies could be due to differences in antisera specificity.

Although the antisera used in this study recognize more than one form of GnRH (Kelsall *et al.* 1989) and thus cannot be used to determine differential distributions of GnRHs at a histological level, it is already well established that multiple forms of GnRH exist within the teleost brain (Sherwood 1987, Sherwood *et al.* 1984, Sherwood and Coe 1991). Obviously this raises the question of their

respective roles. To date, there is no known difference in the function of the sGnRH and cGnRH II within the teleost brain although there does appear to be some spatial difference in distribution in goldfish and rainbow trout (Yu *et al.* 1988, Okuzawa *et al.* 1990) and some differential efficacy in releasing gonadotropins (Peter *et al.* 1990).

Although the precise distributions of the different forms of GnRH are not known, the presence of GnRH-like immunoreactivity in a number of distinct areas within the teleost brain suggests that the different forms present may be acting in different capacities within the brain (*i.e.* one as a releasing factor acting on the pituitary and one as a neurotransmitter elsewhere in the brain) or that each form has multiple roles. In the rainbow trout, cGnRH-II levels were higher in the cerebellum and medulla while sGnRH was the only form in the pituitary and the predominant form in the hypothalamus (Okuzawa *et al.* 1990). The concept that a peptide can act as a systemic hormone, central neurotransmitter and modulator of pituitary function has already been suggested for another neuropeptide, melanin-concentrating hormone (MCH) in *Poecilia latipinna* (Batten and Baker 1988). MCH cells are located within the nucleus lateralis tuberis and fibers extend both to the neurohypophysis and to many other parts of the brain.

In birds, the two GnRH forms (cGnRH-I and cGnRH-II) are differentially distributed suggesting that cGnRH-II may not act as a gonadotropin-releasing factor (Millam *et al.* 1989).

In teleosts, it is also possible that the two forms present are restricted to different systems which are functionally, spatially and temporally separate. There is some evidence in platyfish to suggest that the various GnRH immunoreactive centers may have different roles in regulating the brain-pituitary-gonad axis since hypophysectomy has a very different effect on the nucleus olfactoretinalis (increasing ir-staining) compared to the nucleus lateralis tuberis and nucleus preopticus periventricularis (reducing the ir-staining) (Schreibman *et al.* 1983). Since the ontogeny of the GnRH systems is well described in poeciliids it would be interesting to see if the NOR, which shows ir-GnRH earliest in development (in platyfish), possesses a different form of GnRH than other systems in the brain. This would, however, depend on the availability of antisera which recognize each form exclusively.

The distribution of GnRH immunoreactivity in the rat is consistent with previous findings (Silverman 1988) and supports the use of these animals as positive controls. GnRH immunoreactivity was particularly evident around the median eminence. This reflects the difference in route taken by GnRH to the pituitary between teleosts and mammals (and other tetrapods). In teleosts, GnRH fibers extend from the POA down into the pituitary so that GnRH is released at the site of the gonadotropic cells. In mammals, GnRH is released into the portal vessels of the median eminence to

be carried to the anterior pituitary. In this study, GnRH immunoreactivity was found in the ventral telencephalon and hypothalamus of both mammals and fish (mollies and goldfish). These are strongly conserved systems involved in the control and release of neurohormones in vertebrates suggesting that the role of GnRH as releasing hormone is also strongly conserved. In mammals, the ventral telencephalon also contains the terminal nerve, which is another highly conserved GnRH immunoreactive system in vertebrates (Demski 1984). This structure was not specifically examined in this study but has been extensively surveyed in many vertebrates (Schwanzel-Fukuda and Silverman 1980, Stell 1984, Stell *et al.* 1984, 1987, Demski 1984, 1987, Demski *et al.* 1987, Munz and Claas 1987, Schwanzel-Fukuda *et al.* 1985, 1987, Muske and Moore 1988). The precise functions or roles of this nerve have not been identified for any group but the presence of GnRH immunoreactivity within it suggests that it may be involved in reproduction and the strong conservation of this GnRH system across vertebrates suggests that it is an essential and necessary component of the vertebrate brain.

Literature Cited

Alpert, L.C., Brawer, J.R., Jackson, I.M.D., and Reichlin, S. 1976. Localization of LHRH in neurons in frog brain (*Rana pipiens* and *Rana catesbeiana*). Endocrinology **98**:910-921.

Anthony, E.L.P., King, J.C., and Stopa, E.G. 1984. Immunocytochemical localization of LHRH in the median eminence, infundibular stalk and neurohypophysis: Evidence for multiple sites of releasing hormone secretion in humans and other mammals. Cell and Tissue Research **236**:5-14.

Barry, J. 1979. Immunohistochemistry of luteinizing hormone-releasing hormone-producing neurons of vertebrates. International Review of Cytology **60**:179-221.

Batten, T.F.C. 1986. Ultrastructural characterization of neurosecretory fibres immunoreactive for vasotocin, isotocin, somatostatin, LHRH and CRF in the pituitary of a teleost fish, *Poecilia latipinna*. Cell and Tissue Research **244**:661-672.

Batten, T.F.C., and Baker, B.I. 1988. Melanin-concentrating hormone (MCH) immunoreactive hypophysial neurosecretory system in the teleost *Poecilia latipinna*: Light and electron microsopic study. General and Comparative Endocrinology **70**:193-205.

Batten, T.F.C., Cambre, M.L., Moons, L., and Vandesande, F. 1990. Comparative distribution of neuropeptide-immunoreactive systems in the brain of the green molly, *Poecilia latipinna*. The Journal of Comparative Neurology **302**:893-919.

Bennis, M., Dubourg, P., Gamrani, H., Calas, A., and Kah, O. 1989. Existence of a GnRH immunoreactive nucleus in the dorsal midbrain tegmentum of the chameleon. General and Comparative Endocrinology **75**:195-203.

Blahser, S., Goos, H.J.Th., de Leeuw, R., Zoeten-Kamp C., and Peute, J. 1985. Gonadotropin-releasing hormone-immunoreactive neuronal structures in the brain and pituitary of the African catfish, *Clarias gariepinus* (Burchell). Cell and Tissue Research **241**:595-596.

Borg, B., Goos, H.J.Th., and Terlou, M. 1982. LHRH-immunoreactive cells in the brain of the three-spined stickleback, *Gasterosteus aculeatus* L. (Gasterosteidae). Cell and Tissue Research **226**:696-699.

Breton, B., Motin, A., Billard, R., Kah O., and Geoffre, S. 1986. Immunoreactive gonadotropin-releasing hormone-like material in the brain and pituitary gland during the periovulatory period in the brown trout (*Salmo gairdneri*): Relationships with the plasma and pituitary gonadotropin. General and Comparative Endocrinology **61**:109-119.

Burgus, B., Butcher, M., Amoss, M., Ling, N., Monahan, M., Rivier, J., Fellows, R., Blackwell, R., Vale, W., and Guillemin, R. 1972. Primary structure of ovine hypothalamic luteinizing hormone-releasing hormone (LRF). Proceeding of the National Academy of Sciences (USA) **69**:278-282.

Chan, S.J., Cao, Q-P., and Steiner, D.F. 1990. Evolution of the insulin/insulin-like growth factor cDNA from amphioxus. Proceedings of the National Academy of Sciences (USA) **87**:9319-9323.

Chang, C.Y., Liu, Y., and Zhu, H. 1985. Steroid sex hormones and their functional regulation in amphioxus. *In* Current Trends in Comparative Endocrinology (Lofts, B., and Holmes, W.N., eds.) Hong Kong University Press, Hong Kong. pp. 205-207.

Crim, J.W., Urano, A., and Gorbman, A. 1979a. Immunocytochemical studies of luteinizing hormone-releasing hormone in brains of agnathan fishes. I. Comparisons of adult Pacific Lamprey (*Entosphenus tridentata*) and the Pacific hagfish (*Eptatretus stouti*). General and Comparative Endocrinology **37**:294-305.

Crim, J.W., Urano, A., and Gorbman, A. 1979b. Immunocytochemical studies of luteinizing hormone-releasing hormone in brains of agnathan fishes. II. Patterns of immunoreactivity in larval and maturing western brook lamprey (*Lampetra richardsonii*) General and Comparative Endocrinology **38**:290-299.

Davis, M.R., and Fernald, R.D. 1990. Social control of neuronal soma size. Journal of Neurobiology **21**:1180-1188.

Demski, L.S. 1984. The evolution of neuroanatomical substrates of reproductive behavior: sex steroid and LHRH-specific pathways including the terminal nerve. American Zoologist **24**:809-830.

Demski, L.S. 1987. Phylogeny of luteinizing hormone-releasing hormone systems in protochordates and vertebrates. *In* The Terminal Nerve (Nervus Terminalis): Structure, Function and Evolution. (Demski, L.S., and Schwanzel-Fukuda, M., eds.) Annals of the New York Acadamy of Science **519**:1-14.

Demski, L.S., Fields, R.D., Bullock, T.H., Schreibman M.P., and Margolis-Nunno, H. 1987. The terminal nerve of sharks and rays: EM, immunocytochemical and electrophysiological studies. *In* The Terminal Nerve (Nervus Terminalis): Structure, Function and Evolution. (Demski, L.S., and Schwanzel-Fukuda, M., eds.) Annals of the New York Academy of Science **519**:15-33.

Doerr-Schott, J., and Dubois, M.P. 1976. LHRH-like systems in the brain of *Xenopus laevis* Daud. Cell and Tissue Research **172**:477-486.

Dubois, M.P., Billard, R., Breton, B., and Peter, R.E. 1979. Comparative distribution of somatostatin, LH-RH, neurophysin and alpha-endorphin in the rainbow trout: an immunocytological study. General and Comparative Endocrinology **37**:220-232.

Fritsch, H.A.R., Van Noorden, S., and Pearse, A.G.E. 1982. Gastro-intestinal and neurohormonal peptides in the alimentary tract and cerebral complex of *Ciona intestinalis* (Ascidiaceae). Cell and Tissue Research **223**:369-402.

Garcia Ayala, A., Zandbergen, M.A., and Peute, J. 1989. Immunocytochemical localization of gonadotropin and gonadotropin releasing hormone in pituitary and brain of the gilthead seabream, *Sparus aurata* L., (Teleost). Netherlands Journal of Zoology **39**:41-55.

Georges, D., and Dubois, M.P. 1985. Presence of vertebrate-like hormones in the nervous system of a tunicate, *Ciona intestinalis*. *In* Current Trends in Comparative Endocrinology (Lofts, B., and Holmes, W.N., eds.) Hong Kong University Press, Hong Kong pp. 55-57.

Georges, D., and Dubois, M.P. 1980. Mise en evidence par les techniques d'immunofluoresence d'un antigene de type LH-RH dans le systeme nerveux de *Ciona intestinalis* (Tunicier ascidiace). Comptes Rendus, Academie Sciences (Paris) Serie D **290**:29-31.

Goos, H.J.Th., and Murathanoglu, O. 1977. Localizaion of gonadotropin releasing hormone (GRH) in the forebrain and neurohypophysis of the trout (*Salmo gairdneri*). Cell and Tissue Research **181**:163-168.

Goos, H.J.Th., De Leeuw, R., De Zoeten-Kamp, C., Peute, J., and Blahser, S. 1985. Gonadotropin-releasing hormone-immunoreactive neuronal structures in the brains and pituitary of the African catfish, *Clarias gariepinus* (Burchell). Cell and Tissue Research **241**:593-596.

Halpern-Sebold, L.R., and Schreibman, M.P. 1983. Ontogeny of centers containing luteinizing hormone-releasing hormone in the brain of platyfish (*Xiphophorus maculatus*) as determined by immunocytochemistry. Cell and Tissue Research **229**:75-84.

Haynes, L.W. 1980. Peptide neuroregulators in invertebrates. Progress in Neurobiology **15**:205-245.

Jackson, I.M.D. 1980. Distribution and evolutionary significance of the hypophysiotropic hormones of the hypothalamus. Frontiers in Hormone Research 6:35-69.

Jan, Y.N., Jan, L.Y., and Kuffler, S.W. 1979. A peptide as a possible transmitter in sympathetic ganglia of the frog. Proceedings of the National Academy of Sciences (USA) **76**:1501-1505.

Kah, O., Breton, B., Dulka, J.G., Nunez-Rodriguez J., and Peter, R.E. 1986. A reinvestigation of the Gn-RH (gonadotropin-releasing hormone) systems in the goldfish brain using antibodies to salmon Gn-RH. Cell and Tissue Research **244**:327-337.

Kah, O., Chambolle, P., Dubourg, P., and Dubois, M.P. 1984. Immunocytochemical localization of luteinizing hormone-releasing hormone in the brain of the goldfish, *Carassius auratus*. General and Comparative Endocrinology **53**:107-115.

Katz, I.A., Millar, R.P., and King, J.A. 1990. Differential regional distribution and release of two forms of gonadotropin-releasing hormone in the chicken brain. Peptides **11**:443-450.

Kelsall, R., Coe, I.R., and Sherwood, N.M. 1990. Phylogeny and ontogeny of gonadotropin-releasing hormone: Comparison of guinea pig, rat, and a protochordate. General and Comparative Endocrinology **78**:479-494.

King, J.A., and Millar, R.P. 1984. Isolation and structural characterization of chicken hypothalamic luteinizing hormone-releasing hormone. Journal of Experimental Zoology **232**:419-424.

King, J.A., and Millar, R.P. 1982a. Structure of chicken hypothalamic luteinizing hormone releasing hormone. I. Structural determination on partially purified material. Journal of Biological Chemistry **257**:10722-10728.

King, J.A., and Millar, R.P. 1982b. Structure of chicken hypothalamic luteinizing hormone releasing hormone. II. Isolation and characterization. Journal of Biological Chemistry **257**:10729-10732.

King, J.A., and Millar, R.P. 1980. Comparative aspects of luteinizing hormone-releasing hormone structure and function in vertebrate phylogeny. Endocrinology **106**:707-717.

King, J.C., Sower, S., and Anthony, L.P. 1988. Neuronal systems immunoreactive with antiserum to lamprey gonadotropin-releasing hormone in the brain of *P. marinus*. Cell and Tissue Research **253**:1-8.

King, J.A., Mehl, A.E.I., Tyndale-Biscoe, C.H., Hinds, L., and Millar, R.P. 1989. A second form of gonadotropin-releasing hormone (GnRH), with chicken GnRH II-like properties, occurs together with mammalian GnRH in marsupial brains. Endocrinology **125**:2244-2252.

König, J.F.R., and Klippel, R.A. 1963. The Rat Brain. Waverly Press, Inc. Baltimore, USA. 162 pp.

Kurjan, J., and Herskowitz, I. 1982. Structure of a yeast pheromone gene (*MFα*): A putative *α*-factor precursor contains four tandem copies of mature *α*-factor. Cell **30**:933-943.

Kyle, A.L., Stell, W.K., and Kah, O. 1985. Luteinizing hormone-releasing hormone (LH-RH)-immunoreactive (ir) cells of the terminal nerve, but not other LHRH-ir neurons in the goldfish brain, are also FMRFamide- and tachykinin-ir. Abstract: Society for Neuroscience Vol. 11, p. 350, Abst. 107-8

Lovejoy, D.A. 1991. Gonadotropin-releasing hormone: Structure and function in cartilaginous fish and radiation in the Vertebrata. Unpublished Ph.D. Dissertation. University of Victoria, Victoria, B.C.

Lovejoy, D.A., Fischer, W.H., Parker, D.B., McRory, J.E., Park, M., Lance, V., Swanson, P., Rivier, J.E., and Sherwood, N.M. 1991. Primary structure of two forms of gonadotropin-releasing hormone from brains of the American alligator (*Alligator mississippiensus*). Regulatory Peptides **33**:105-116.

Lovejoy, D.A., Ashmead, B.J., Coe, I.R., and Sherwood, N.M. *in press*. Presence of gonadotropin-releasing hormone immunoreactivity in dogfish and skate brains. Journal of Experimental Zoology.

Matsuo, H., Baba, Y., Nair, R.M.G., Arimura, A., and Schally, A.V. 1971. Structure of porcine LH- and FSH-releasing hormone. I. The proposed sequence. Biochemical and Biophysical Research Communications **43**:1334-1339.

Millam, J.R., Craig-Veit, C.B., Adams, T.E., and Adams, B.M. 1989. Avian gonadotropin-releasing hormones I and II in brain and other tissues in turkey hens. Comparative Biochemistry and Physiology A **94**:771-776.

Millan, M., Aguilera, G., Wynn, P.C., Mendelsohn, F.A.O., and Catt, K.J. 1986. Autoradiographic localization of brain receptors for peptide hormones: Angiotensin II, corticotropin-releasing factor, and gonadotropin-releasing hormone. Methods in Enzymology **124**:590-606.

Miller, K.E., and Kriebel, R.M. 1986. Peptidergic innervation of caudal neurosecretory neurons. General and Comparative Endocrinology **64**:396-400.

Miyamoto, K., Hasegawa, Y., Nomura, M., Igarashi, M., Kangawa, K., and Matsuo, H. 1984. Identification of the second gonadotropin-releasing hormone in chicken hypothalamus: Evidence that gonadotropin secretion is probably controlled by two distinct gonadotropin releasing hormones in avian species. Proceedings of the National Academy of Sciences (USA) **81**:3874-3878.

Munz, H., and Claas, B. 1987. The terminal nerve and its development in the teleost fishes. *In* The Terminal Nerve (Nervus Terminalis): Structure, Function and Evolution. (Demski, L.S., and Schwanzel-Fukuda, M., eds.) Annals of the New York Academy of Science **519**:50-59.

Munz, H., Claas, B., Stumpf, W.E., and Jennes, L. 1982. Centrifugal innervation of the retina by luteinizing hormone-releasing hormone (LHRH)-immunoreactive telencephalic neurons in teleostean fishes. Cell and Tissue Research **222**:313-323.

Munz, H., Stumpf, W.E., and Jennes, L. 1981. LHRH systems in the brain of platyfish. Brain Research **221**:1-13.

Muske, L.E., and Moore, F.L. 1988. The nervus terminalis in amphibians: Anatomy, chemistry and relationship with the hypothalamic gonadotropin-releasing hormone system. Brain Behavior and Evolution **32**:141-150.

Muske, L.E., and Moore, F.L. 1987. Luteinizing hormone-releasing hormone-immunoreactive neurons in the amphibian brain are distributed along the course of the nervus terminalis. *In* The Terminal Nerve: Structure, Function and Evolution. (Demski, L.S. and Schwanzel-Fukuda, M., eds.) Annals of the New York Academy of Sciences **519**:433-446.

Nozaki, M., and Kobayashi, H. 1979. Distribution of LHRH-like substance in the vertebrate brain as revealed by immunocytochemistry. Archivum Histologicum Japonicum **42**:201-219.

Nozaki, M., and Kobayashi, H. 1980. LH-RH-like substances in the brain of lower vertebrates. *In* Neurosecretion, Molecules, Cells, Systems. (Farner, D.S., and Lederis,K., eds.) Plenum Press, New York, pp. 452-453.

Nozaki, M., Fujita, I., Saito, N., Tsukahara T., and Kobayashi, H. 1985. Distribution of LHRH-like immunoreactivity in the brain of the Japanese eel (*Anguilla japonica*) with special reference to the nervus terminalis. Zoological Science **2**:537-547.

Nozaki, M., Tsukahara, T., and Kobayashi, H. 1984a. Neuronal systems producing LHRH in vertebrates. *In* Endocrine Correlates of Reproduction. (Ochiai K., Arai, Y., Shioda T., and Takahashi, M., eds.) Japan Scientific Society Press, Tokyo, Springer-Verlag, Berlin. pp. 3-27.

Nozaki, M., Tsukahara, T., and Kobayashi, H. 1984b. An immunocytochemical study on the distribution of neuropeptides in the brain of certain species of fish. Biomedical Research **4**:135-145.

Nunez-Rodriguez, J., Kah, O., Breton, B., and Le Menn, F. 1985. Immunocytochemical localization of GnRH (Gonadotropin releasing hormone) systems in the brain of a marine teleost fish, the sole. Experimentia **41**:1574-1576.

Okuzawa, K., Amano, M., Kobayashi, M., Aida, K., Kanyu, I., Hasegawa, Y., and Miyamoto, K. 1990. Differences in salmon GnRH and chicken GnRH-II contents in discrete brain areas of male and female rainbow trout according to age and state of maturity. General and Comparative Endocrinology **80**:116-126.

O'Shea, M., and Schaeffer, M. 1985. Neuropeptide function: The invertebrate contribution. Annual Review of Neuroscience **8**:171-198.

Pan, C.H., Feng, M.Q., Ling, N.C., Pao S., and Xu, W.Q. 1979. Immunocytochemical studies on gonadotropin releasing hormone (GnRH) secretory nucleus of the carp (*Cyprinus carpio*). Acta Biologicae Experimentalis Sinica **12**:305-310.

Peter, R.E., Habibi, H.R., Chang, J.P., Nahorniak, C.S., Yu, K.L., Huang, Y.P., and Marchant, T.A. 1990. Actions of gonadotropin-releasing hormone (GnRH) in the goldfish. Progress in Comparative Endocrinology. **342**:393-398.

Peute, J., de Bruyn, M.G.A., Seldenrijk, R., and van Oordt, P.G.W.J. 1986. Cytophysiology and innervation of gonadotropic cells in the pituitary of the black molly (*Poecilia latipinna*). Cell and Tissue Research **174**:35-54.

Schafer, H., Schulz, R., and Blum, V. 1989. Immunoreactivity to gonadotropin-releasing hormone and gonadotropic hormone in the brain and pituitary of the rainbow trout *Salmo gairdneri*. Cell and Tissue Research **257**:227-235.

Schreibman, M.P., Demski, L.S., and Margolis-Nunno, H. 1986. Immunoreactive (ir) LHRH in the "brain" of amphioxus. Abstract: American Zoologist **26**:abstr.30A.

Schreibman, M.P., Halpern-Sebold L., Ferin,M., Margolis-Kazan, M., and Goos, H.J.Th. 1983. The effect of hypophysectomy and gonadotropin administration on the distribution and quantity of LH-RH in the brains of platyfish: A combined immunocytochemistry and radioimmunoassay study. Brain Research **267**:293-300.

Schreibman, M.P., Halpern-Sebold, L.R., and Goos, H.J.Th. 1979. Identification of luteinizing hormone releasing-hormone (LH-RH) in the brain and pituitary gland of a fish by immunocytochemistry. Journal of Experimental Zoology **210**:153-160.

Schwanzel-Fukada, M., Soledad Garcia, M., Morrell, J.I., and Pfaff, D.W. 1987. Distribution of luteinizing hormone-releasing hormone in the nervus terminalis and brain of the mouse detected by immunocytochemistry. The Journal of Comparative Neurology **255**:231-244.

Schwanzel-Fukuda, M., Morrell, J.I., and Pfaff, D.W. 1985. Ontogenesis of neurons producing luteinizing hormone-releasing hormone (LHRH) in the nervus terminalis of the rat. The Journal of Comparative Neurology **238**:348-364.

Schwanzel-Fukuda, M. and Silverman, A.J. 1980. The nervus terminalis of the guinea-pig: A new luteinizing hormone-releasing hormone (LHRH) neuronal system. The Journal of Comparative Neurology **191**:213-225.

Schwanzel-Fukuda, M., and Pfaff, D.W. 1989. Origin of luteinizing hormone-releasing hormone neurons. Nature (London) **338**:161-164.

Sherwood, N.M. 1987. The GnRH family of peptides. Trends in Neuroscience **10**:129-132.

Sherwood, N.M., and Coe, I.R. 1991. Neuropeptides and their genes in fish. *In* Proceedings of the Fourth International Symposium on the Reproductive Physiology of Fish, University of East Anglia, Norwich, U.K., 7-12 July (eds. Scott, A.P., Sumpter, J.P., Kime, D.E., and Rolfe, M.S.) FishSymp 91, Sheffield, U.K. pp. 38-40.

Sherwood, N.M., and Lovejoy, D.A. 1989. The origin of the mammalian form of GnRH in primitive fishes. Fish Physiology and Biochemistry **7**:85-93.

Sherwood, N.M., Sower, S.A., Marshak, D.R., Fraser, B.A., and Brownstein, M.J. 1986a. Primary structure of gonadotropin-releasing hormone from lamprey brain. Journal of Biological Chemistry **261**:4812-4819.

Sherwood, N.M., Zoeller, R.T., and Moore, F.L. 1986b. Multiple forms of gonadotropin-releasing hormone in amphibian brains. General and Comparative Endocrinology **61**:313-322.

Sherwood, N.M., and Sower, S.A. 1985. A new family member for gonadotropin-releasing hormone. Neuropeptides **6**:205-214.

Sherwood, N.M., Harvey, B., Brownstein, M.J., and Eiden, L.E. 1984. Gonadotropin-releasing hormone (Gn-RH) in striped mullet (*Mugil cephalus*), milkfish (*Chanos chanos*) and rainbow trout (*Salmo gairdneri*): Comparison with salmon Gn-RH. General and Comparative Endocrinology **55**:174-181.

Sherwood, N., Eiden, L., Brownstein, M., Spiess, J., and Vale, W. 1983. Characterization of a teleost gonadotropin-releasing hormone. Proceedings of the National Academy of Sciences (USA) **80**:2794-2798.

Silverman, A.J. 1988. The gonadotropin-releasing hormone (GnRH) neuronal systems: Immunocytochemistry. *In* The Physiology of Reproduction (Knobil, E. and Neill, J., eds.) Raven Press, Ltd. New York. pp. 1283-1304.

Smit, A.B., Vreugdenhil, E., Ebberink, R.H.M., Geraerts W.P.M., Klootwijk, J., and Joose, J. 1989. Growth-controlling molluscan neurons produce the precursor of an insulin-related peptide. Nature (London) **331**:535-538.

Sorensen, P.W., Goetz, F.W., Scott, A.P., and Stacey, N.E. 1991. Recent studies indicate that goldfish use mixtures of unmodified hormones and hormonal metabolites as sex pheromones. *In* Proceedings of the Fourth International Symposium on the Reproductive Physiology of Fish, University of East Anglia, Norwich, U.K., 7-12 July (Scott, A.P., Sumpter, J.P., Kime, D.E., and Rolfe, M.S., eds.) FishSymp 91, Sheffield, U.K. pp. 191-193.

Sower, S.A. 1990. Gonadotropin-releasing hormone in primitive fishes. *In* Progress in Comparative Endocrinology (Epple, A., Scanes, C.G., and Stetson, M.H., eds.) Wiley-Liss, New York. pp. 79-81.

Stell, W.K. 1984. Luteinizing-hormone releasing hormone (LHRH)- and pancreatic polypeptide (PP)-immunoreactive neurons in the terminal nerve of spiny dogfish, *Squalus acanthias*. Anatomical Record **208**:173A-174A.

Stell, W.K., Walker, S.E., and Ball, A.K. 1987. Functional-anatomical studies on the terminal nerve projection to the retina of bony fishes. *In* The Terminal Nerve: Structure, Function and Evolution. (Demski, L.S., and Schwanzel-Fukuda, M. eds.) Annals of the New York Academy of Sciences **519**:80-96.

Stell, W.K., Walker, S.E., Chohan, K.S., and Ball, A.K. 1984. The goldfish nervus terminalis: A luteinizing hormone-releasing hormone and molluscan cardioexcitatory peptide immunoreactive olfactoretinal pathway. Proceedings of the National Academy of Sciences (USA) **81**:940-944.

Thorndyke, M.C., and Georges, D. 1988. Functional aspects of peptide neurohormones in protochordates. *In* Neurohormones in Invertebrates (Thorndyke, M.C., and Goldsworthy, G.J.), Society for Experimental Biology Seminar Series No.33, Cambridge University Press, London.

Wirsig, C.R., and Getchell, T.V. 1986. Amphibian terminal nerve: Distribution revealed by LHRH and AChE markers. Brain Research **385**:10-21.

Wright, D.E., and Demski, L.S. 1991. Gonadotropin hormone-releasing hormone (GnRH) immunoreactivity in the mesencephalon of sharks and rays. The Journal of Comparative Neurology **307**:49-56.

Yu, K.L., Sherwood, N.M., and Peter, R.E. 1988. Differential distribution of two molecular forms of gonadotropin-releasing hormone in discrete brain areas of goldfish (*Carassius auratus*). Peptides **9**:625-630.

Zentel, H.J., Jennes, L., Reinboth, R., and Stumpf, W.E. 1987. Ontogeny of gonadotropin releasing hormone and gonadotropin immunoreactivity in brain and pituitary of normal and estrogen-treated guppies, *Poecilia reticulata* Peters. Cell and Tissue Research **249**: 227-234.

Chapter 3

Isolation and characterization of the DNA and mRNA sequences encoding gonadotropin-releasing hormone (GnRH) from Pacific salmon, *Oncorhynchus nerka*.

Introduction

Gonadotropin-releasing hormone (GnRH) is a key hormone in the reproduction of most vertebrates. There are seven forms of GnRH which have been identified at the protein level. These are the mammalian form, mGnRH, (Matsuo *et al.* 1971, Burgus *et al.* 1972, Tan and Rousseau 1982, Seeburg and Adelman 1984, Adelman *et al.* 1986), two forms found in chicken, cGnRH-I and cGnRH-II, (King and Millar 1982a, 1982b, Miyamoto *et al.* 1982, 1983, 1984), one form found in salmon, sGnRH, (Sherwood *et al.* 1983), one form found in lamprey, lGnRH (Sherwood and Sower 1985, Sherwood *et al.* 1986), one form found in Thai catfish (Ngamvongchon *et al.*, in press) and one form found in dogfish shark (Lovejoy *et al.*, submitted). All seven GnRHs are decapeptides and have in common a pyroglutamyl residue at the amino-terminus and an amidated glycine at the carboxyl terminus. These end-group modifications, which are a common feature of many bioactive peptides, probably protect against enzymatic degradation. In all characterized forms, residues 1, 2, 4, 9 and 10 are the same, residue 8 is the most variable. It is thought that GnRH exists as a folded peptide with a beta

turn at positions 5 and 6. This brings the ends of the molecule together and allows residues 2 and 9, histidine and proline, to bind to each other and stabilize the conformation. In all vertebrates studied, except for ratfish and eutherian mammals, multiple forms of GnRH seem to be present (Sherwood 1987). The functional basis for the presence of multiple forms in a single species is not well understood. However, it does appear that different forms of GnRH are differentially distributed within the brain in fish (Yu *et al.* 1988a, Okuzawa *et al.* 1990) and birds (Katz *et al.* 1990) and that their effectiveness in releasing pituitary hormones varies in birds (Katz *et al.* 1990) and, to some degree, in fish (Peter *et al.* 1990).

The GnRH precursor and gene were initially studied in mammals. The mRNA and gene encoding mGnRH in human hypothalamus and placenta, rat brain and mouse brain have been characterized (Seeburg and Adelman 1984, Adelman *et al.* 1986, Mason *et al.* 1986, Seeburg *et al.* 1987, Radovick *et al.* 1990). As with many other bioactive peptides (Eipper *et al.* 1986), GnRH exists as part of a larger precursor protein. The cDNA consists of mGnRH extended at the N-terminus by a signal peptide, a feature characteristic of many secreted proteins, and at the C-terminus by a Gly-Lys-Arg sequence, characteristic of an enzymatic amidation and precursor processing site, followed by a gene-associated peptide (GAP) which is 53 amino acids long. The GnRH gene in mammals consists of four exons. The first encodes the

5'untranslated region. The second, the signal peptide, mGnRH, the enzymatic amidation and precursor processing site and the first 11 amino acids of GAP. The third exon encodes GAP amino acids 12-43, and the fourth exon encodes the remaining amino acids and the 3' untranslated region (Seeburg and Adelman 1984). A second gene, SH, is transcribed from the opposite strand of DNA to produce an RNA of undefined function (Bond *et al.* 1989). GAP has been shown to co-exist with GnRH in hypothalamic neurons (Phillips *et al.* 1985, Sar *et al.* 1987, Silverman *et al.* 1987, Rubin *et al.* 1987, Merchentaler *et al.* 1989) and was initially thought to inhibit the secretion of prolactin (Nikolics *et al.* 1985, Yu *et al.* 1988b) but this has been disputed (Fink 1985, Thomas *et al.* 1988) and it is certainly not clear what the relationships between GnRH, GAP and prolactin are *in vivo*. Similarly a peptide composed of the first 13 amino acids of GAP has been shown to stimulate GnRH release from rat anterior pituitary cells in culture (Millar *et al.* 1986), but this has been disputed by some of the same group (Thomas *et al* 1988). It is possible that GAP, in common with other cryptic peptides associated with neurohormones, has no specific physiological role but is involved in the correct processing and packaging of the hormone. Many years of attempting to identify a function for C-peptide of insulin have revealed none, beyond its role in correct folding and disulfide bond formation in proinsulin (Eipper *et al.* 1986). The sequences of

associated peptides are considerably less conserved across the phylogenetic spectrum compared to their concomitant endocrine peptides (Sherwood and Parker 1990). Peptides involved in mechanical roles within the cell may be under less constraint and less likely to be strongly conserved than endocrine peptides which bind with specific receptors.

The first example of differential tissue-specific expression of GnRH was made in humans (Seeburg and Adelman 1984) and has subsequently been shown to occur in other mammals (Adelman *et al.* 1986, Mason *et al* 1986a). For reasons that are not clear mGnRH (or mGnRH-like immunoreactivity) is present in human non-neural tissue such as the pancreas (Seppälä and Wahlström 1980), breast tissue (Harris *et al.* 1991) and ovary (Oikawa *et al.* 1990). It is also found in the placenta, where it may act as a local hormone that stimulates synthesis of chorionic gonadotropin (Tan and Rousseau 1982, Khodr and Siler-Khodr 1980, Radovick *et al.* 1990, Kelly *et al.* 1991). Comparison of the mRNAs encoding mGnRH in the brain and those in the placenta showed that the first intron is not removed from the primary transcript in the case of the latter (Seeburg and Adelman 1984, Radovick *et al.* 1990). This results in an unusually long 5'untranslated region of mGnRH mRNA in such tissues. Additionally, a recent study (Kelly *et al.* 1991) found that the placenta expressed two to three times more antisense mGnRH RNA than sense mGnRH. Whether this antisense mGnRH is translated and what the function of the resultant protein is

remain to be determined. It appears that different promotors are activated for the expression of the mGnRH gene in neural and non-neural tissues (Seeburg *et al.* 1987, Radovick *et al.* 1990). The functional basis underlying differential tissue expression remains unclear. In the case of species exhibiting multiple forms of GnRH differential tissue expression may be of considerable importance in determining where and when different forms of GnRH are expressed.

For a number of years the nucleotide coding sequences of only mGnRH was known. However, recently the cDNA structure for sGnRH in the cichlid, *Haplochromis burtonii*, (Bond *et al.* 1991) and the cDNA and gene for sGnRH in the Atlantic salmon, *Salmo salar*, (Klungland *et al.* in press) have been reported. The GnRH precursors in both these species show similar structure to their mammalian counterpart with a signal peptide, GnRH, amide-donating glycine, processing site and GAP. The GnRH portion of the molecule is strongly conserved whereas the signal and GAP portions are not when compared to the mammalian precursor. There is an overall sequence similarity of 63.9% between the protein coding regions (signal, GnRH and GAP) of the cichlid and Atlantic salmon (Klungland *et al.*, in press). There is some sequence similarity (64.2%) between the GAP of the cichlid (53 amino acids) and the Atlantic salmon (46 amino acids) but very little of either to the mammalian GAP which is longer (56 amino acids) than both fish GAPs. However, if the reading

frame is shifted by one base in the cichlid GAP, a small block of sequence identity (10.7%) to a conserved region of the mammalian GAP appears, with a total of six identical amino acids (including one conservative substitution) (Bond *et al.* 1991). This suggests that the mammalian GAP may be derived, via a frameshift event, from an ancestral fish-like GAP molecule. The two fish signal peptides are quite similar to each other (71%), especially towards the carboxy half of the molecule. The signal peptides also show some similarity, particularly in the hydrophobic core of the molecule, to the mammalian signal sequence (Bond *et al.* 1991, Klungland *et al.* in press). The position of the introns in the sGnRH gene of Atlantic salmon is similar to the mammalian gene although intron 1 is longer and introns 2 and 3 are strikingly shorter in the fish. A promotor sequence, which contains the putative TATA and CAT boxes, is found upstream of exon II. Two binding sites for transcription factor SP1 were also identified and one of these is located in a similar position to a promotor sequence in the mammalian gene (Klungland *et al.* in press).

Although cichlids (Sherwood, unpubl.) and probably Atlantic salmon, both possess additional forms of GnRH , these do not appear to be encoded with the same gene as the sGnRH. Multiple forms of a peptide encoded within one gene have been observed for other peptides such as the yeast pheromone gene MFα (Kurjan and Herskowitz 1982). The peptide which is produced from this precursor bears some

sequence identity to the GnRH family but has not been conclusively identified as being a member. Multiple copies within one gene are also found in the FMRFamide gene in *Aplysia* (Schaefer *et al.* 1985). In this gene multiple, non-identical copies of the tetrapeptide are present, most coding for FMRFamide but some coding for FLRFamide.

In order to establish the actual structure of unidentified forms and therefore understand more fully the evolution of the GnRH family I isolated the coding sequences for GnRH in salmon. In doing this, several alternative approaches were used which are outlined below.

Strategies

Several different strategies were used in attempting to clone the GnRH cDNA or gene from salmon.

1) cDNA library construction.

(i) screening with pools of degenerate, short (21 bases) oligonucleotides.

(ii) screening with pools of semi-degenerate long (38 bases) oligonucleotides.

(iii) screening with a cichlid (cross-species) probe

2) Polymerase chain reaction (PCR) using a variety of primers.

3) Genomic library construction and screening with the cichlid probe.

Rationale

When this study was started in 1987, it was clear from the work by Adelman and Seeburg (1984, 1986) that GnRH in mammals is expressed at very low levels and is present as a single copy gene making it a difficult clone to isolate. Additionally, at this time, the full-length mammalian clone was not available and would have been of limited use for screening given the evolutionary distance between fish and mammals and the difference in peptide sequence between mammalian GnRH and salmon GnRH. Since the salmon GnRH peptide sequence was already known (Sherwood *et al.* 1983) the problem was approached by synthesizing pools of degenerate oligonucleotides based on the salmon GnRH sequence and using these to screen a cDNA library made from brain tissue collected from salmon as they returned to spawn. This was the same stage at which tissue was collected for the protein analysis (Sherwood *et al.* 1986). A cDNA library was used because we assumed that the message encoding GnRH, although rare (perhaps 1 in 10^6), would be present at a higher frequency in the mRNA than the gene was in the genome. In addition to the short oligonucleotide pools, long semi-degenerate pools of oligonucleotides were synthesized and used for screening (see Methods and Materials).

In November 1990, the cDNA for the salmon form of GnRH

was isolated from *Haplochromis burtonii*, a cichlid (Bond *et al.* 1991) and was obtained from Dr. John Adelman for screening the salmon libraries.

In addition, during the time this study was conducted, the polymerase chain reaction (PCR) was introduced as a powerful tool in molecular biology. This technology is well suited for isolating rare messages and allows for fast analysis of a considerable number of clones (see Eidne 1991 for review). It also aids in rapid analysis of putative positive clones isolated from libraries. Primers based on the known salmon GnRH protein sequence and published mammalian cDNA sequence for GnRH were synthesized and used to prime salmon brain cDNA. Other primers used included an oligo-dT (strings of thymidine residues) primer, which hybridizes with the poly $(A)^+$ tail found on most mRNAs, and later, primers made against the signal and 3' untranslated regions of the cichlid cDNA.

Finally, as a third option, I decided to screen a genomic library with the cichlid probe since this eliminated the problems associated with low GnRH copy number in the mRNA and the false positives which were isolated with short oligonucleotide probes. The gene or genes for GnRH should, presumably, be present in genomic DNA regardless of the tissue from which it was collected or the time of collection.

Materials and Methods

All glassware was baked (220°C overnight) to destroy residual RNAses, disposable plasticware was used and all solutions for RNA work were either treated with diethylpyrocarbonate (DEPC) or made up in DEPC treated water to ensure an RNAse free environment. Ultrapure guanidium isothiocyanate and cesium chloride were purchased from Bethesda Research Laboratories (BRL, Gaithersburg, MD). The highest quality available (usually molecular biology grade) was used for other reagents. Radioactive nucleotides were purchased from New England Nuclear (NEN)/Dupont (Boston, MA). Lambda Zap II vector, Gigapack Gold packaging mixes and XL-1 cells were obtained from Stratagene (La Jolla, CA). Oligo-dT cellulose, EcoRI adaptors, spin columns and NAP-5 columns were purchased from Pharmacia (Montreal, Quebec). Nylon filters for screening of the cDNA library with oligonucleotides were purchased from Schleicher and Schuell (Keene, NH). For screening with the cichlid probe NEN/Dupont filters were used. Northern and Southern blots were done using Zetaprobe membrane produced by BioRad (Mississauga, Ontario). Restriction enzymes were purchased from either Stratagene or Pharmacia.

1) cDNA library preparation.

Whole brains and pituitaries were collected from 20 reproductively mature male chum salmon that had returned to fresh water to spawn. Fish were obtained from the Qualicum Hatchery on Vancouver Island, BC. RNA was extracted using

standard techniques (Sambrook *et al.* 1989). In brief, tissue was pounded to a fine powder while being kept frozen in liquid nitrogen. The powder was then dissolved in guanidium isothiocyanate homogenisation solution (4 M guanidium isothiocyanate, 1 M β-mercaptoethanol, 1% sarcosyl, 25 mM sodium acetate, pH 6.0, 1 mM EDTA,. This solution was then layered onto a cesium chloride (5.7M CsCl, 100mM EDTA, pH6.5) gradient in ultracentrifuge tubes (Beckman SW-27 or SW-28) and spun at 26,000 rpm for 30 hours at 20°C. RNA moves through the gradient and is sedimented at the bottom of the tube. Tubes were cut open and the gradient solution carefully removed. The RNA pellet was dissolved in DEPC-treated water and any residual protein removed by phenol/chloroform extraction. RNA was then precipitated at -20°C for several hours with 0.1 volume of sodium acetate, pH 6.0 and at least 2 volumes of 100% ethanol. The RNA was then sedimented, washed, dried and redissolved in DEPC-treated H_2O. Aliquots of this solution were used to measure the concentration of the RNA in the solution spectrophotometrically.

Poly $(A)^+$ mRNA was isolated by oligo-dT cellulose chromatography. The poly $(A)^+$ mRNA was used to make cDNA according to standard techniques (Gubler and Hoffman 1983). To make the library, 3 μg salmon poly $(A)^+$ mRNA was reverse-transcribed into cDNA which was then ligated to EcoRI adaptors synthesized on an Applied Biosystems (Foster, CA) Nucleic Acid synthesizer at the Institut für Zellbiochemie

und klinische Neurobiologie, at the University of Hamburg. Excess adaptors were removed by passing the cDNA solution over Pharmacia spin columns according to the manufacturer's instructions. cDNA was then ligated into the EcoRI site of lambda ZAP II cloning vector and packaged using Gigapack Gold packaging mixes according to manufacturer's instructions. Dilutions of the library were used to infect XL-1 cells and plated out onto Luria broth (LB) agar plates in LB top agar. LB medium consists of 10g tryptone, 5g yeast extract, 5g NaCl, and 1 ml of 1N NaOH per litre. Agar for plates is LB medium with the addition of 15g agar per liter. Top agar is LB medium with the addition of 7g agar per liter. The resulting number of plaques indicated that the library consisted of 1.6×10^6 independent recombinants. To determine the proportion of phage with inserts, 48 individual plaques were picked at random from the plates used for determining titre. Plaque cores were immersed in Murray buffer (50 mM Tris, pH 7.5, 100 mM NaCl, 10 mM $MgSO_4$, 0.01% gelatin) with the addition of a few drops of chloroform. Cores were stored overnight at 4°C then plated out at a density to give confluent lysis on 15 cm LB agar plates. Following overnight incubation at 37°C, the plates were removed to a cold room and flooded with about 12 ml of Murray buffer. Plates were allowed to stand for about 8 hours then the Murray buffer from each plate collected into individual Falcon tubes. Plates were rinsed with 3 ml of Murray buffer for an additional hour and the buffer was

removed and combined with the original lysate. These plate lysates were used to isolate lambda DNA using DEAE cellulose and cetyltrimethylammoniumbromide solution (Manfioletti and Schneider 1988). The lambda DNA was then cut using EcoRI to release any insert. Digests were analysed by standard agarose electrophoresis. Results indicated that the library was about 60% recombinant with an average size of insert of about 3 kb. The library was then amplified (Maniatis *et al.* 1982) and the final titer measured as 3.5×10^{10} plaque forming units (pfu) per μl.

Screening

i) Initial screening involved using pools of degenerate oligonucleotides (named GRH) against the first seven amino acids of salmon GnRH: Gln His Trp Ser Tyr Gly Trp.
Since it was speculated that the second form of GnRH in the salmon was likely to be the chicken II form, pools of degenerate probes (named CHK) were also made against that sequence: Gln His Trp Ser His Gly Trp.
Each probe pool, was itself synthesized in two sub-pools because of the presence of the internal serine. These probes were synthesized in 0.2 μM quantities on the synthesizer at the Institut.

GRH-1, (against sGnRH 1-7, antisense),

CCA	TCC	GTA	TGA	CCA	GTG	CTG
	G	A	G		A	T
	C		C			
	A		A			

GRH-2, (against sGnRH 1-7, antisense),

CCA	TCC	GTA	GCT	CCA	GTG	CTG
	G	A	A		A	T
	C					
	A					

CHK-1, (against chicken GnRH-II 1-7, antisense),

CCA	TCC	GTG	TGA	CCA	GTG	CTG
	G	A	G		A	T
	C		C			
	A		A			

CHK-II, (against chicken GnRH-II 1-7, antisense),

CCA	TCC	GTG	GCT	CCA	GTG	CTG
	G	A	A		A	T
	C					
	A					

The amplified chum cDNA library was plated out onto L-agar plates in LB top agarose (LB medium with the addition of 7g agarose per liter) at a density of 25,000 pfu per plate. Duplicate filter lifts were made. Primary filters were left on the plates for 1 min and secondary filters for 1.5 min. Filters were allowed to air dry and then the DNA fixed by baking the filters at 80°C, under vacuum, for 2 hours. Filters were pre-wet in 6 x SET (20 x SET is 3M NaCl, 30 mM EDTA, 300 mM Tris, pH 7.5) then washed for 2 x 1

hour in pre-warmed wash solution consisting of 50 mM Tris, pH 8.0, 1 M NaCl, 1 mM EDTA, 0.1% (w/v) SDS. Filters were prehybridized at 37°C for 16-24 hours in prehybidization solution (6 x SSC, 50 mM sodium phosphate, pH 6.8, 5 x Denhardts), 0.1 mg/ml boiled denatured calf thymus DNA. Approximately 250 ml of prehybridization solution was used for 25 filters. Prehybridization solution was then replaced with fresh warm prehybridization solution (100 ml per 25 filters) with the addition of 100 mg/ml dextran sulfate and the labelled oligonucleotides. Before the labelling reaction, the GRH-1 and CHK-1 pools were mixed together in equal ratios to produce one pool labelled GRH/CHK-1. The GRH-2 pool was added to the CHK-2 pool to give GRH/CHK-2. There were now two pools of mixed (salmon and chicken) probes. Each mixed pool (GRH/CHK-1 and GRH/CHK-2) was then end labelled. To maximize efficiency of labelling, equimolar ratios of label to probe were used in each reaction. Both reactions were done in volumes of 50 μl. For GRH-1/CHK-1 the reaction was as follows: 5 μl 10 x kinase buffer (600 mM Tris, pH 7.6, 10 mM spermidine HCl, 100 mM $MgCl_2$, 100 mM DTT, 2 mg/ml BSA), 6.8 μl oligonucleotides (5 pmoles/μl), 20 μl γ ATP (6000Ci/mmol, 1.7 pmoles/μl), 16.2 μl H_2O, 2 μl T4 PNK (20 units). For the other pool, reaction conditions were identical except that 10 μl of γ ATP was used to label 3.4 μl of oligonucleotides with H_2O making the final volume to 50 μl. Reactions were incubated for 30 min at 37°C then another 10

units of enzyme was added and incubation allowed to continue for another hour. Excess free nucleotide was removed by passing the probe solution over a NAP-5 column according to the manufacturer's instructions. The pools were then combined and added to the hybridization solution. Hybridization took place for 36 hours at 37°C with gentle rotation. Following hybridization, the probe solution was poured off and stored at -20°C for future use. Filters were rinsed briefly (2 x 5 min) with 6 x SSC at 4°C then with pre-warmed (37°C) tetramethylammonium chloride (TMAC, Aldrich, Milwaukee, WI) solution to remove all remaining sodium chloride solution and finally with TMAC solution at the appropriate temperature for the probe length for 2 x 20 min. TMAC solution consists of 3M $(CH_3)_4NH_4Cl$, 50 mM TrisHCl, pH 8.0, 2 mM EDTA, 0.1% SDS. The pools of oligonucleotides used for this screening were all 20 bases in length so the filters were rinsed at 56-58°C based on the data provided by Wood *et al.* (1985) and Jacobs *et al.* (1988).

ii) A second set of semi-degenerate pools of oligonucleotides was also synthesized in Hamburg against the salmon and chicken II GnRH forms and their putative processing sites. These were again synthesized in two sub-pools for each form because of the internal serine.

GnRH salmon 1 and 2

1) Gln His Trp Ser Tyr Gly Trp Leu Pro Gly Gly Lys Arg
CAG CAC TGG AGC TAT GGC TGG CTG CCA GGA GGC AAG AG
C

2) CAG CAC TGG TCC TAT GGC TGG CTG CCA GGA GGC AAG AG
C

GnRH chicken II 1 and 2

1) Gln His Trp Ser His Gly Trp Tyr Pro Gly Gly Lys Arg
CAG CAC TGG AGC CAT GGC TGG TAC CCA GGA GGC AAG AG
C

2) CAG CAC TGG TCC CAT GGC TGG TAC CCA GGA GGC AAT AG
C

These probes were end labelled as outlined above and used to screen the amplified chum cDNA library according to conditions described by Ulrich and co-workers (1984). Prehybridization and hybridization solutions consisted of 5 x SSC, 5 x Denhardts, 50mM sodium phosphate (pH 6.8), 1 mM sodium pyrophosphate, 100μM ATP, 20% formamide, 50μg/μl blocking DNA. Filters were washed in 0.2 x SSC/0.1% SDS at 30°C four times.

iii) For screening with the cichlid probe the amplified chum cDNA library was plated out onto FRM plates at a density of 50,000 pfu per plate. FRM media consists of 5g NaCl, 2g $MgCl_2.6H_2O$, 10g NZ amine, 5g yeast extract, 2g casamino acids and 2g maltose per liter, and is used for both agar plates (15g agar per liter) and top agarose (7g

agarose per liter). Plates were incubated at 37°C overnight. Duplicate filter lifts were made using NEN/Dupont Plaque/Colony Screen Nylon Filters. Primary filters were left on the plates for 1.5-2 minutes and secondary filters were left on for 5 minutes. Filters were then immersed in denaturing solution for 10 min., followed by neutralizing solution for 2 x 10 min. and finally into 2 x SSC for 2 x 5 min. Filters were then air dried and the DNA permanently fixed to the filters by UV cross-linking using a Stratagene Stratalinker. Filters were prehybridized in a solution consisting of 1M NaCl, 1% SDS, 50% formamide, 1x yeast tRNA (100x yeast tRNA is 10 mg/ml yeast tRNA, 10mM Tris, pH 7.5, 5mM EDTA, 1% SDS). About 200 ml of prehybridization solution were used for 24 filters. Prehybridization took place at 37°C with gentle shaking for one hour. The probe used for hybridization consisted of the cichlid GnRH cDNA or a PCR fragment (signal peptide, primer 345, to 3' untranslated region, primer 338). These were random primed (using standard methods, Ausubel *et al.* 1989) and generally contained 1-1.2 x 10^7 cpm. Hybridization took place in the same solution, overnight at 37°C, with gentle shaking. Following hybridization the probe solution was removed and stored at -20°C. Filters were rinsed briefly in 2 x SSC, then in a solution consisting of 0.5 x SSC, 0.1% SDS, 1-2mM EDTA at 42°C for 2 x 30 min. Filters were packed damp and exposed to X-ray film (Kodak X-OMAT, Rochester, N.Y.) at -70°C with intensifying screens for about 3 days.

2) GnRH PCR primers. An assortment of oligonucleotide primers were synthesized for use in PCR. Non-degenerate primers based on the salmon GnRH sequence and the mammalian gene-associated peptide (GAP) were synthesized at the Regional DNA Synthesis Laboratory at the University of Calgary.

GnRH 5

(encoding sGnRH 1-7, sense),

Gln His Trp Ser Tyr Gly Trp
CAG CAC TGG TCC TAC GGC TGG

GnRH 3

(against mGAP 48-55, antisense),
(Glu Glu Glu Thr/Ala Gly Gln Lys Lys)

CTT CTT CTG GCC GGT/C CTC CTC CTC

Nested primers against sGnRH and cGnRH-II 2-11 and 5-11 were also synthesised in Calgary.

cGnRH-II and sGnRH 5-11, (antisense),

His Gly Trp Tyr Pro Gly Gly
Tyr Leu

ACC	ACC	AGG	AAG	CCA	ACC	GT
C	C	C	CTA		C	A
G	G	G	G		G	
T	T	T	T		T	

cGnRH-II and sGnRH 2-11, (antisense)

His	Trp	Ser	His	Gly	Trp	Tyr	Pro	Gly	Gly
			Tyr			Leu			

ACC	ACC	AGG	AAG	CCA	ACC	GTG	IGA	CCA	ATG
C	C	C	CTA		C	A A	CT		G
G	G	G	G		G				
T	T	T	T		T				

In addition, nested primers for PCR, encoding chicken GnRH-II, with an adaptor with a Sal restriction site, were synthesized at Institut für Zellbiochemie und klinische Neurobiologie, at the University of Hamburg.

GnRH 27 (sense)

Sal	Gln	His	Trp	Ser	His	Gly	Trp
GTC GAG	CAG	CAC	TGG	AGC	CAC	GGA	TGG
	A	T		TCI	T	C	
						G	
						T	

GnRH 29 (sense)

Sal	His	Trp	Ser	His	Gly	Trp	Leu	Pro
GTC CAG	CAC	TGG	AGC	CAC	GGC	TGG	CTA	CC
	T		TC	T	I		T	C
								G
								T

An oligo dT primer, GAATTC$(T)_{36}$, with an EcoRI adaptor was also provided for use as a primer against the poly $(A)^+$ tail of cDNA, synthesised directly from mRNA or isolated from cDNA library lysates.

Following disclosure of the putative Atlantic salmon sequence for salmon GnRH, additional primers were synthesized at the Regional DNA Synthesis Laboratory in Calgary.

GnRH S1 (sense)

(encoding the Atlantic salmon GnRH 1-10)

Gln His Trp Ser Tyr Gly Trp Leu Pro Gly

CAG CAC TGG TCG TAT GGC TGG CTA CCT GG

GnRH AS10

(against the Atlantic salmon GnRH 1-10, antisense)

CC AGG TAG CCA GCC ATA CGA CCA GTG CTG

GnRH SIG1

(encoding the beginning of the Atlantic salmon signal peptide)

Met Asp Leu Ser Asn Arg Thr Val

ATG GAT CTT AGC AAC AGA ACG GT

GnRH ASGAP1

(against the end of the Atlantic salmon GAP)

TTA TTT ATG GGG CAT CCA

In addition, Dr. John Adelman at Oregon Health Sciences University, provided primers against the cichlid GnRH cDNA sequence.

GnRH 345 (encoding amino acids 16-21 of the cichlid signal peptide of the sGnRH cDNA, sense)

Leu Val Val Gln Val Thr

TTG GTG GTT CAG GTC ACC

GnRH 338 (against bases 19-38 of the 3' untranslated region of the cichlid sGnRH cDNA, antisense)

CG ATC TTT CCC ATG TGC TGC

A wide variety of combinations of primers and reaction conditions were used on cDNA synthesized from salmon mRNA (chum, sockeye and chinook) and on cDNA isolated from the chum cDNA library. Typically, 5 pmoles of each primer were used in reaction volumes of 25-100 µl with annealing temperatures of 45-55°C. Following PCR, the total reaction volume was extracted once with chloroform:isoamylalcohol (24:1 v/v) to remove the mineral oil then precipitated at -20°C for at least 2 hours with sodium acetate and 100% ethanol. Samples were then spun at 12,000 rpm for about 20 mins to pellet the DNA, rinsed with 80% ethanol and dried. Pellets were redissolved in approximately 10 µl of sterile H_2O and analysed on a 1.5-2.0% Seakem agarose (FMC Bioproducts, Rockland, ME) gel. Distinct PCR bands were cut out as gel slices and the DNA electroeluted from the agarose into 1 x TAE for about 45 min in dialysis tubing bags. This DNA was then precipitated as before. To clone PCR products the most effective method was found to be the following procedure. DNA pellets were redissolved in 4 µl H_2O. To this the following reagents were added: 2 µl each of 5 x ligase buffer (BRL recipe minus the ATP or DTT) and 1.25 mM dNTPs, 1 µl 10mM ATP. 0.5 µl each of T4 PNK and Klenow (both

Pharmacia). The reagents were mixed gently then incubated at 12°C for 45 min followed by 37°C for 30 min. The enzymes were denatured by heating at 70°C for 10 min. The blunt-ended and phosphorylated PCR products were then cloned by adding the following: an appropriate amount of EcoRV cut, de-phosphorylated, blunt-ended Bluescript KS (Stratagene), usually 0.3-0.5 μl, 0.5 μl 10 mM ATP, 2 μl 100mM DTT, 2 μl 5 x ligase buffer, 1 μl T4 DNA ligase (BRL) and H_2O to a final volume of 20 μl. This reaction was mixed gently and left to incubate overnight at room temperature. The ligations were made to 100 μl volume with H_2O, then phenol:chloroform extracted and chloroform extracted once. If quantities of DNA were very low, the phenol:chloroform phase was back extracted and the combined aqueous phases were chloroform extracted. Ligations were precipitated as usual then spun down, washed, dried and redissolved in 10-20 μl H_2O. Competent Xl-1 cells (Stratagene), made according to BioRad instructions for the Gene Pulser, were used for electrotransformation of 1 μl or 1 μl of a 1:10 dilution of the ligation. Electrotransformations were carried out using the BioRad Gene Pulser according to manufacturer's instructions. Transformants were plated onto L-amp plates in the presence of X-Gal (20μl of 10% per small plate) and IPTG (50μl of 0.5M per small plate) to allow blue/white colour selection of putative recombinants. Plates were incubated overnight at 37°C, then white colonies (putative recombinants) were picked and grown up, overnight, in 3 ml

of FRM media with ampicillin at 37°C with vigorous shaking. Saturated cultures were "mini-prepped" using standard alkaline lysis methods (Ausubel *et al.* 1989) to isolate plasmid DNA. Clones were then subjected to PvuII digestion and analysed on agarose gels to determine whether they contained inserts or not. Recombinant clones were then alkali denatured (according to USB Sequenase directions) and sequenced using standard dideoxy sequencing techniques (Sanger *et al.* 1977) and a United States Biochemicals (USB, Cleveland, OH) Sequenase sequencing kit. Sequencing primers (SK, KS, T3, T7, M13 -20, M13 Reverse) were purchased from Stratagene.

3) Genomic Screening

In August 1991, an amplified genomic sockeye salmon library was obtained from Dr. Robert Devlin at West Vancouver Laboratories, Department of Fisheries and Oceans. This library was constructed from about 10 μg of male sockeye DNA in lambda Fix II cloning vector (Stratagene). The library had been amplified and successfully screened for a number of other genes. Southern blotting, using reaction conditions outlined in Church and Gilbert (1984), of the ciclid probe against sockeye genomic DNA digests determined the level of stringency at which the library was screened with the cichlid probe. The entire cichlid clone and a PCR fragment of the cichlid clone (primer 345 to primer 338) were both labelled using a modified Vogelstein reaction

(Hodgson and Fisk 1987), boiled and added to hybridization solution. Hybridization took place at 37-42°C overnight. Sonicated, boiled sea urchin DNA (final concentration 35-50 μg/ml) was added to both prehybridization and hybridization solutions to block non-specific binding. The probe solution was removed and stored at -20°C. Probe solutions were routinely reused successfully. Solutions were allowed to thaw then denatured by heating to at least 90°C for 20 min. or longer. Old solutions were occasionally `spiked' by the addition of freshly labelled probe. Southern blots were washed in 2 x SSC/0.1% SDS briefly to remove excess radioactivity, then washed for several hours with several changes of 2 x SSC/0.1% SDS at 37C. The level of signal on the filter was routinely checked to assess whether further washes at higher stringency were required. Filters were usually packed damp in Saran Wrap once the level of signal had dropped to just above background. For screening of the genomic library, filters were prehybridized in 6 x SSC, 0.1% SDS, 5 x Denhardts and hybridized in 6 x SSC, 0.1% SDS (Maniatis *et al.* 1982). As for the Southern blots, sea urchin DNA was added as a blocking agent. Hybridization took place at 37-42°C for 24-36 hours. Hybridization solution was then removed, stored and reused as outlined above. Filters were rinsed briefly in 2 x SSC/0.1% SDS, then for several hours at 37-42°C in 2 x SSC/0.1% SDS with several changes of solution, then in one or more hour-long rinses of 1 x SSC/0.1% SDS at 37-42°C. If the signal on the

filters was still high then additional rinses were done at a higher temperature until the signal was reduced to just above background. Filters were allowed to dry, wrapped in Saran Wrap and exposed to X-ray film with intensifying screens for 5 days at -80°C. Positive plaques were cored, placed in lambda diluent (10mM Tris, pH 7.5, 10mM $MgSO_4$) and plated at lower density until well-isolated individuals could be identified. The DNA of positive clones (lambda FIX II plus insert) was isolated by making plate lysates of positive clones and subjecting the plate lysate to RNase and DNase treatment followed by PEG precipitation (Maniatis *et al.* 1982). The phage pellet was then redissolved and phage coats removed by proteinase K digestion. The DNA solution was phenol/chloroform extracted until no interface was present then precipated with 3M sodium acetate and 100% ethanol. This DNA was then used as a substrate for PCR using primers 345, S1, AS10, SIG1, and ASGAP1. PCR bands were electroeluted, cloned into pBluescript and sequenced as outlined above. Various digests using different combinations of Xba, SacI, and EcoRI (according to manufacturer's instructions) produced fragments which were blotted and probed with the cichlid probe to determine which clone contained the putative GnRH sequence. Positive fragments were electroeluted and cloned into pBluescript (KS) using the cloning and transformation strategy outlined above.

Results

cDNA library screening

Short probes

A total of 975,000 independent clones were screened from the amplified cDNA library. Weak positives isolated in the first rounds failed to produce any positives in subsequent rounds. Long probes were then used in the hope that they would be more specific.

Long probes

A total of 500,000 clones were screened using the long probe producing several positive clones which were screened until individual plaques could be isolated. These were then rescued (using helper phage) as inserts in pBluescript and sequenced. However, sequence data failed to indicate the presence of a GnRH-like molecule in any of the positive clones. All sequences were compared to sequences in the GenBank database, version 68.0, mammals and other vertebrates, to see if they had sequence identity to anything in the data base but none appeared to have any `significant' similarity. Significant similarity was generally assessed to be 60% or greater sequence identity, (although it should be noted that members of gene superfamilies from widely divergent groups can have less than 60% similarity). Since none of these clones appeared to be anything of interest they were not pursued. In addition, the long probes hybridized extremely effectively

to unidentified, highly repetitive sequences which seem to be common in fish genomes. Based on these results, I did not pursue screening using this strategy any further.

Cichlid probe

A total of 2.4×10^6 clones were screened producing two positives which failed to produce positives in the following round of screening.

PCR.

Extensive and exhaustive attempts to isolate a GnRH-like molecule from freshly prepared cDNA, and cDNA isolated from the chum cDNA library, using PCR failed. All the primers successfully amplified bands at relatively high annealing temperatures (which should promote specificity) but when these were cloned and sequenced no GnRH-like molecules were found. Virtually identical, unidentified sequences were amplified from sockeye, chinook and chum cDNA suggesting that the primers were hybridizing to the same region in all species. Again, all these sequences were processed through GenBank but did not appear to be strongly similar to anything in the database.

Since it was now apparent that there was little or no GnRH present in the mRNA used to make the library or cDNA for PCR, I tried isolating the gene for GnRH from a genomic library.

Genomic Screening.

Southern blots of sockeye genomic DNA, hybridized with the cichlid cDNA for sGnRH and washed at low stringency (2 x SSC/0.1% SDS, 37°C, 5 x 60 min), showed the presence of positive bands (fig. 16). This provided the first indication that the cichlid probe could be used to isolate a GnRH-like molecule. The genomic library was screened using similar hybridization conditions and slightly more stringent washing conditions, 2 x SSC/0.1% SDS at 37°C for several washes, followed by a few washes at 1 x SSC/0.1% SDS at either room temperature or 37°C. Approximately 700,000 clones were screened and 4 positives identified. These were numbered as clones 3_{131}A, 11_{315}A, 26_{5}A, and 27_{23}A. Each positive clone was subjected to 5 rounds of screening to ensure that they were individual plaques. Plate lysates were used to isolate lambda DNA containing the insert. Lambda DNA was also digested with Xba and SacI together (which are unique sites in lambda Fix II on either side of the insert) and also Xba, SacI and EcoRI. These digests produced fragments of varying sizes in each clone. These were separated by agarose gel electrophoresis and blotted across to nylon membrane which was then probed with the cichlid probe. This blot was washed in 2 x SSC/0.1% SDS at 37°C for one hour, followed by two washes in 1 x SSC/0.1% SDS at 37°C for one hour each and a final one hour wash in 1 x SSC/0.1% SDS at 45°C. Overnight exposure was sufficient

to see which Xba/SacI and Xba/SacI/EcoRI fragments were hybridizing with the cichlid cDNA (fig. 17). The difference in restriction patterns between all four clones suggests that they were different. Clone 11_{315}A (panel A, lane b) appears to lose part of the hybridizing portion of the insert when digested with EcoRI (panel B, lane b). Clone 26_{5}A (panel A, lane c) also appears to be reduced to a smaller size following EcoRI digestion (panel B, lane c) but the strength of the hybridization signal does not seem to be affected, suggesting that most of the hybridizing portion of the insert remains intact. The difference in intensity of signal of clone 3_{131}A in panel A, lane a and panel B, lane a may be due to the amount of DNA on the gel. Digests were repeated, separated under identical conditions and the positively hybridizing DNA fragments isolated and subcloned into pBluescript. Plasmid DNA from the subclones and lambda DNA from each clone was used as a substrate for PCR. Asymmetric PCR using the S1 primer on lambda DNA of all clones produced bands which were isolated, made double stranded by random priming, and cloned. Sequence analysis of one band from 26_{5}A showed a nucleotide sequence, following the primer, which suggested that it was indeed a GnRH-like molecule (fig. 18). Based on the downstream sequence, an additional primer was synthesized, ASGAP2, which encoded part of the GAP-like molecule and the first three bases of the intron. This primer was used in combination with SIG1 to amplify the putative GnRH coding

region from 26_5A lambda DNA. SIG1 was also used with ASGAP1 to amplify the entire protein coding regions of the putative GnRH. These PCR bands were cloned and sequence analysis confirmed that 26_5A was indeed sGnRH. From the start of the signal to the end of the GAP (as determined by the PCR primers) is 810 nucleotide bases. This region is split by two introns of 253 nucleotides and 305 nucleotides respectively (figs. 19 and 20). The protein coding region of 252 nucleotides (including the termination codon) predicts a protein precursor of 82 amino acids (fig. 21). This precursor consists of a signal peptide of 23 amino acids, sGnRH which is 10 amino acids in length, a C-terminal glycine which donates an amide group followed by a processing site and a GnRH-associated peptide (GAP) of 46 amino acids. The approximate molecular weight of the precursor is 9,130 Daltons. A comparison of the GAPs from all precursors characterized to date is shown in figure 22.

Figure 16. Southern blot of sockeye (*Oncorhynchus nerka*) genomic DNA probed with the cichlid cDNA for sGnRH. Lanes a-d are BamHI, EcoRI, HindIII and KpnI digests respectively. Panel A shows a filter washed at a slightly lower stringency than the filter in panel B. Positively hybridizing genomic fragments are indicated by black dots. Size markers are indicated in kilobases on the left.

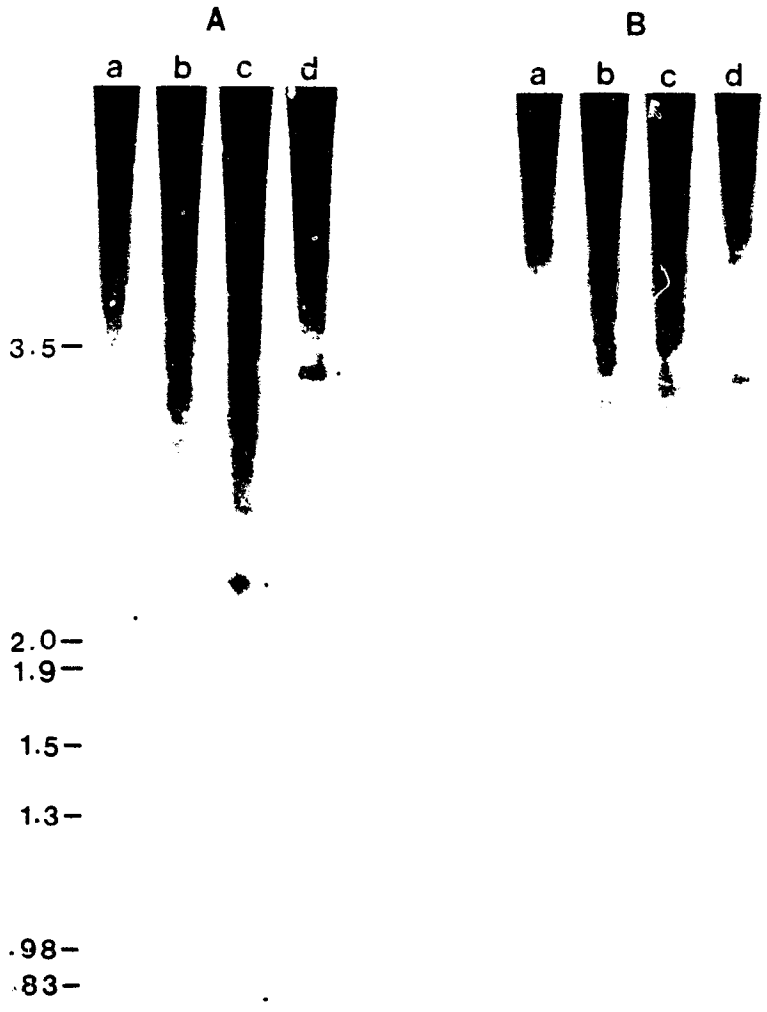

A
B
a
b
c
d
a
b
c
d
3.5
2.0
1.9
1.5
1.3
.98
.83

Figure 17. Southern blot of restriction digests of positive clones isolated from the genomic library. Panel A shows Xba/SacI digests and panel B shows Xba/SacI/EcoRI digests. Lanes a,b,c and d are clones $3_{131}A$, $11_{315}A$, $26_{5}A$ and $27_{23}A$ respectively.

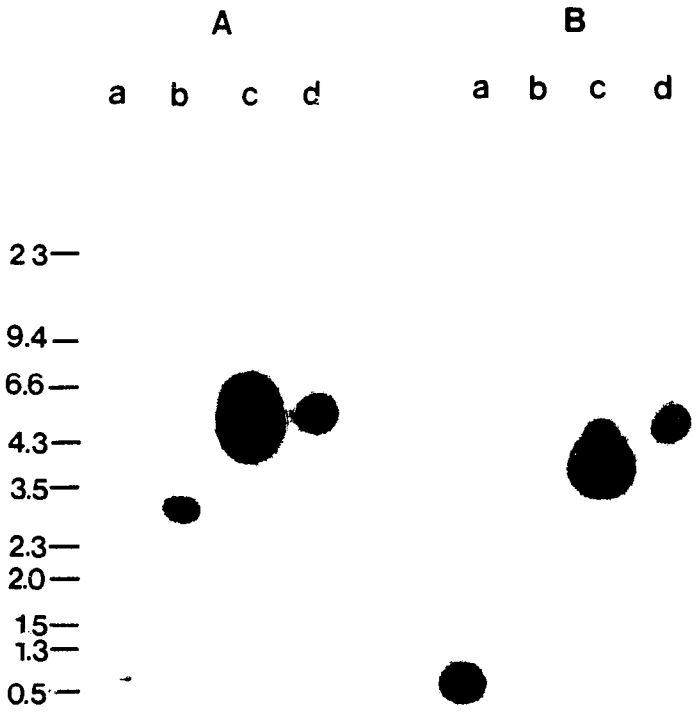

A
B
a b c d
a b c d
23
9.4
6.6
4.3
3.5
2.3
2.0
1.5
1.3
0.5

Figure 18. Autoradiograph of sequencing gel showing sequence of clone generated by asymmetric PCR on 26_5A using the S1 primer. Primer (S1) sequence is indicated, as are first 13 bases following the end of the primer. The 5' end of the clone is at the bottom of the figure. Putative amino acid sequence (single letter code) is shown on the right of the figure. Arrow indicates guanosine residue which is presumed to be the first nucleotide of the intron.

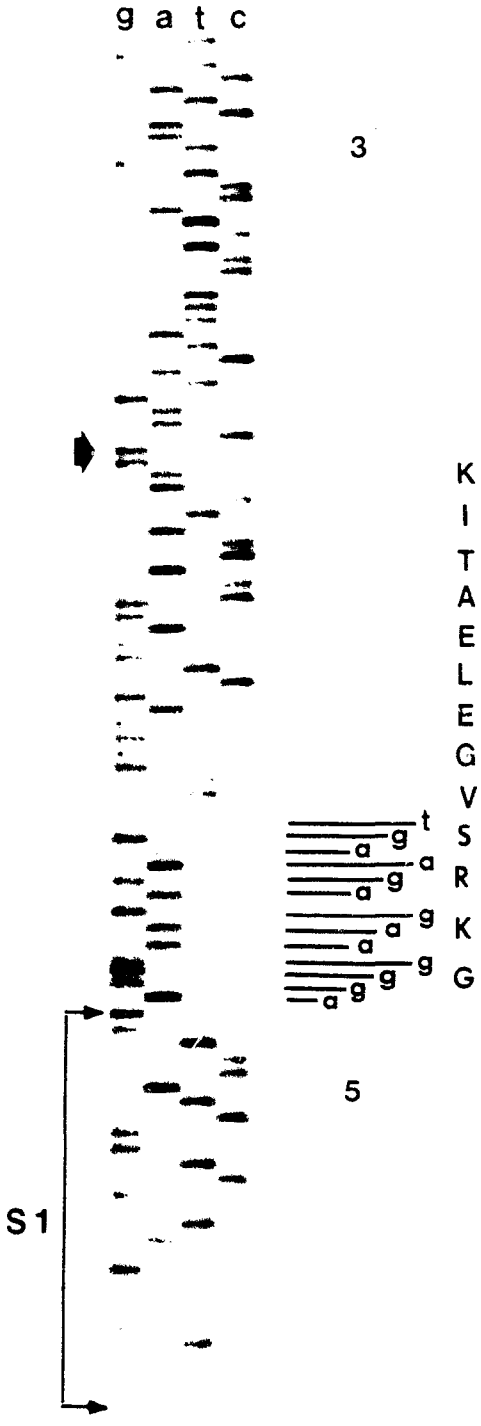
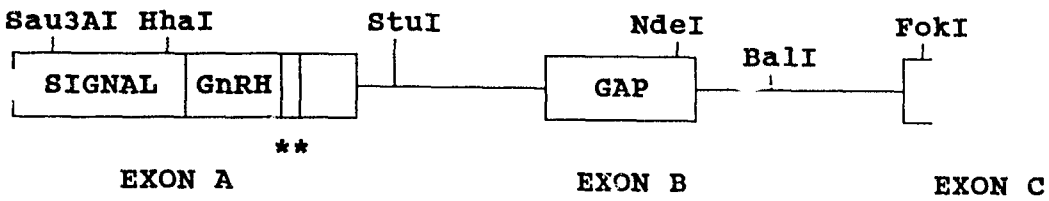

g a t c
3
K
I
T
A
E
L
E
G
V
S
R
K
G
t
g
a
a
g
a
g
a
a
g
g
g
a
5
S1

Figure 19. Schematic representation of the sGnRH in the Pacific salmon, *Oncorhynchus nerka*, and comparison to other GnRH genes.

A. Diagram of the sGnRH gene. Exons are boxed and introns are shown as lines. The processing signal following the GnRH is indicated by asterisks. The 5' end of exon A and the 3' end of exon C have not been definitively identified.

B. Comparison of the GnRH genes of Atlantic salmon, Pacific salmon, human and rat. Exons I, II, III and IV are labelled. Untranslated regions and the processing site following GnRH are shown as clear boxes.

A.


Sau3AI
HhaI
StuI
NdeI
FokI
BalI
SIGNAL
GnRH
GAP
**
EXON A
EXON B
EXON C

B.

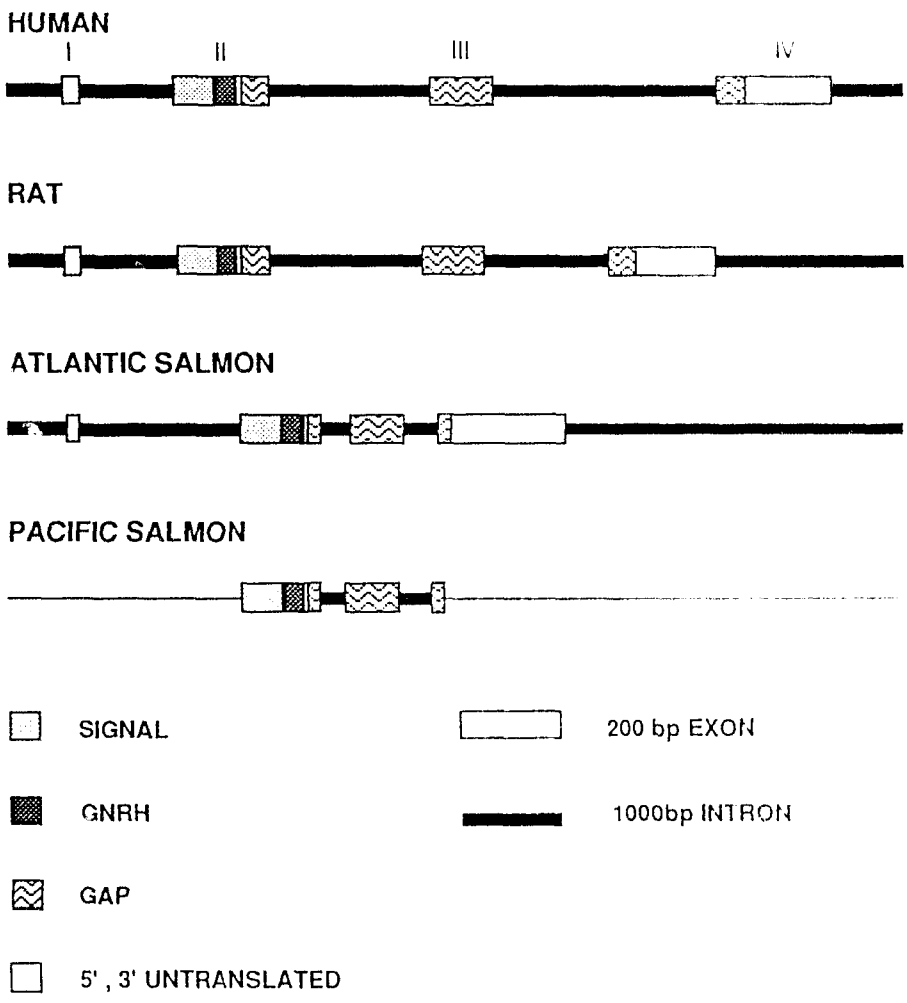

HUMAN
I
II
III
IV
RAT
ATLANTIC SALMON
PACIFIC SALMON
SIGNAL
GNRH
GAP
5', 3' UNTRANSLATED
200 bp EXON
1000bp INTRON

Figure 20. DNA sequence of sockeye (*Oncorhynchus nerka*) sGnRH gene determined by PCR on genomic clone 26_5A. Protein coding sequences are indicated in bold. sGnRH is boxed. The glycine residue which donates an amide group to the C-terminal of the GnRH is underlined. The asterisks indicates putative processing sites. Number of nucleotides is shown above the sequence.

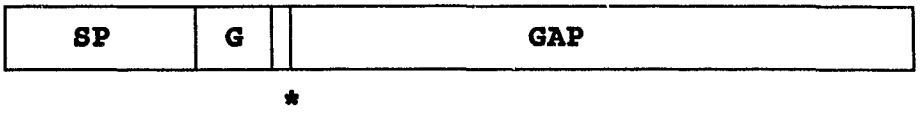
```
          10         20         30         40         50
ATGGATCTTGGCTACAGAATGGTCGTGCAGGTTGTGGTGTTGGCGTTGGTAGCGCAGGTC

          70         80         90         100        110
ACGCTCTCT CAGCACTTGGTCGTATGGCTGGCTACCTGGA GGGAAGAGAAGTGTTGGG
                                          ******

 120        130        140        150        160        170
GAGCTGGAGGCCACCATCAAGGCAAGTACTATTTACCTCTACCTGTAACTACTGTTACAG

 180        190        200        210        220        230
CTATGTCTACATGTGCATACTGTACATGTAATTGTCCTTTAAGGAAATTACTGTGTCTTG

 240        250        260        270        280        290
AAAACCTGTGAACATTTAAATGTGGCATTTATAGGGATAGTTTGTGTGTGAATATTAGTT

 300        310        320        330        340        350
CAAGAGGCCTTGTATATTAATTCAACTCTGTAAGGGTGTTGCATAAAGGGTATATGGGTA

 360        370        380        390        400        410
ATAGCATTACAAATTTCCTACATATTGCTATCCAGATGATGGACACAGGAGGTGTAGTGG

 420        430        440        450        460        470
CTCTTCCTGAGGAGACAAGTGCACATGTCTCAGAGAGACTGAGACCATATGATGTAGTAA

 480        490        500        510        520        530
GTAGTCATATTAATTTATTAGGTTAGATAATGTATTCATTGTATTCCTAATGGCCAATTA

 540        550        560        570        580        590
TTTATGTTTCATCTGAAGATGCTACATTTTCTCCTGGTAAAATATGTTGCAAACAATATT

 600        610        620        630        640        650
CCAAAGTATCTAAATAAATGTTTAGATAAGAGTAATGGCTGACAAATGGAAATCTTCCCT

 660        670        680        690        700        710
GAATTTAGCGAAGTGATACCTATAAATGTAGGGAGACAATGTTGCTCATGTCTGACACAT

 720        730        740        750        760        770
CAGTATAATATAGATATACAACATAAAGTAGACTTCTGAAGTTAACGCTTTGATTTCCCT

 780        790        800        810
AGATATTGAAGAAATGGATGCCCCATAAATAA
```

Figure 21. The sockeye salmon (*Oncorhynchus nerka*) putative cDNA and protein precursor for gonadotropin-releasing hormone (GnRH) determined from the gene.

A. Schematic representation of the sockeye salmon prepro-GnRH precursor protein. The signal peptide (SP) is followed by the GnRH (G), processing signal (*), and the GnRH-associated protein (GAP).

B. The cDNA and amino acid sequence determined from the gene. Arrows indicate the position of introns. Amino acid numbers appear above the sequences, with negative numbers corresponding to the presumed signal sequence.

A.



B.

-23
SIGNAL PEPTIDE
ATG GAT CTT GGC TAC AGA ATG GTC
Met Asp Leu Gly Tyr Arg Met Val

GTG CAG GTT GTG GTG TTG GCG TTG GTA GCG CAG GTC ACG CTC TCT
Val Gln Val Val Val Leu Ala Leu Val Ala Gln Val Thr Leu Ser

+1 GnRH 10 GAP

CAG CAC TGG TCG TAT GGC TGG CTA CCT GGA GGG AAG AGA AGT GTT
Gln His Trp Ser Tyr Gly Trp Leu Pro Gly Gly Lys Arg Ser Val
*** ***

20 30

GGG GAG CTG GAG GCC ACC ATC AAG ATG ATG GAC ACA GGA GGT GTA
Gly Glu Leu Glu Ala Thr Ile Lys↑Met Met Asp Thr Gly Gly Val

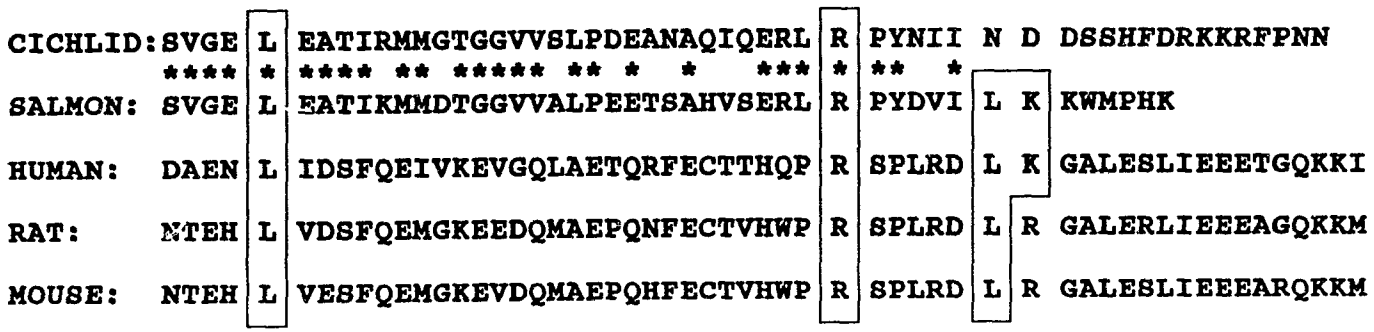
40

GTG GCT CTT CCT GAG GAG ACA AGT GCA CAT GTC TCA GAG AGA CTG
Val Ala Leu Pro Glu Glu Thr Ser Ala His Val Ser Glu Arg Leu

50 60

AGA CCA TAT GAT GTA ATA TTG AAG AAA TGG ATG CCC CAT AAA TAA
Arg Pro Tyr Asp Val↑Ile Leu Lys Lys Trp Met Pro His Lys Ter

Figure 22. Comparison of the GnRH-associated proteins (GAPs) from cichlid, Pacific and Atlantic salmon, human, rat, and mouse GnRH precursors. Single letter codes are used for amino acids. Identical residues in the fishes are indicated by asterisks. Identical residues in all or most GAPs are boxed.


CICHLID: SVGE L EATIRMMGTGGVVSLPDEANAQIQERL R PYNII N D DSSHFDRKKRFPNN
SALMON: SVGE L EATIKMMDTGGVVALPEETSAHVSERL R PYDVI L K KWMPHK
HUMAN: DAEN L IDSFQEIVKEVGQLAETQRFECTTHQP R SPLRD L K GALESLIEEETGQKKI
RAT: NTEH L VDSFQEMGKEEDQMAEPQNFECTVHWP R SPLRD L R GALERLIEEEAGQKKM
MOUSE: NTEH L VESFQEMGKEVDQMAEPQHFECTVHWP R SPLRD L R GALESLIEEEARQKKM

Discussion

The GnRH gene for the salmon form of GnRH isolated from the Pacific salmon, *Oncorhynchus nerka*, suggests the typical structure of GnRH precursors demonstrated to date. This consists of signal peptide followed by hormone, amide-donating glycine residue, processing site and relatively long GnRH-associated peptide (GAP).

The isolation of rare messages such as GnRH from cDNA libraries and through PCR is technically demanding and usually requires several approaches. Synthetic oligonucleotides have been successfully used to isolate many peptides and there is a considerable body of literature on the theoretical aspects of oligonucleotide screening (see Lathe 1985). They do not provide the best probes for screening either Northerns or Southerns and can be extremely problematic in terms of hybridizing with false positives. However, the failure to isolate GnRH from either the cDNA library or through PCR after numerous efforts is probably due to the absence of the message in the RNA. Although the tissue used to isolate mRNA for library construction and PCR was collected as the fish returned to spawn, this does not appear to be the point of optimum expression of GnRH. Evidence is now accumulating which suggests that mRNA, for neuroendocrine peptides, can be axonally transported and possibly stored (Jirikowski *et al.* 1990). This also implies that translation can take place at axon terminals and, possibly, that protein can be stored in the nerve terminals

long after the mRNA has been degraded. This would explain the observation that spawning salmon possess enough GnRH peptide to isolate and purify (Sherwood *et al.* 1983), but do not appear to have detectable (by the methods used in this study) levels of mRNA for GnRH. In addition, studies on GnRH mRNA expression in the cichlid, using the cichlid sGnRH cDNA, have shown that mRNA levels are highest just before ovulation and subsequently drop sharply (Fernald, pers. comm.).

The advantage of using genomic libraries is the guaranteed presence of the gene of interest and, clearly, this proved to be the case for GnRH. In addition, the use of a long probe is preferential for screening of both genomic and cDNA libraries. Compared to the oligonuclectide screening of the cDNA library and PCR, isolation of the sGnRH from the genomic library using the cichlid probe was relatively straightforward.

The sGnRH isolated from the sockeye salmon shows extremely high sequence identity and exon/intron similarity with the Atlantic salmon sGnRH gene sequence. The signal peptide shows the characteristic hydrophobic core, and the GnRH portion of the precursor is identical in amino acid sequence to the salmon GnRH that was originally isolated from Pacific salmon. The protein coding regions of the Atlantic and Pacific salmon are virtually identical, with 96.3% identity at the amino acid level. There are eight base changes, five in the third base position of codons

which do not affect amino acid use. One change at postion 20, the second base of a codon, results in a methionine residue whereas a threonine is present in the Atlantic salmon. Two bases are different at positions 10 and 13, both being the first base of a codon, resulting in a glycine and tyrosine as amino acids four and five of the signal peptide. In the Atlantic salmon these positions have serine and asparagine (Klungland *et al.*, in press), although the cichlid signal peptide has a glycine in position four (Bond *et al.* 1991). Overall, this results in three amino acid differences (87% identity at the amino acid level) in the signal peptide compared to the Atlantic salmon. There is 60.8% identity at the amino acid level compared to the cichlid signal peptide. The amino acids which differ compared to the Atlantic salmon are located at the beginning of the signal peptide which is also the region of hybridization of the SIG1 PCR primer. Since the PCR primer effectively masks the actual sequence of the DNA, it is not possible to be certain that this is the true sequence of the gene although, based on the cichlid sequence, the glycine may be the amino acid of choice at that position. Work continues in the direction of identifying more of the gene sequence.

Comparing the introns of the two different salmon shows the presence of many individual base differences, deletions and insertions. Although some of these differences could be due to PCR error, the extremely high fidelity thoroughout

the protein coding region suggests that most if not all of these differences reflect true variation between the two salmon genes. The Atlantic salmon introns are 277 and 307 base pairs (bp) in length compared to the Pacific salmon introns of 255 and 305 bp. The combined introns of the Pacific salmon have 91% identity with the Atlantic salmon at the nucleic acid level. As would be expected for introns, these parts of the gene are not as highly conserved as the protein coding portions. Both introns show the typical donor and acceptor splice sites with a GT donor and AG acceptor except at the end of exon A, position 475, where the donor site is GC. The consensus splice site sequence of 5' AGGCAAGT 3' is present allowing for the substitution described above. This is also the case for the Atlantic salmon and rainbow trout sGnRH gene (Klungland *et al.*, in press). The GAP portion of the precursor is 46 amino acids long, ten amino acids shorter than the mammalian GAPs (Adelman *et al.* 1986, Mason *et al.* 1986) and eight amino acids shorter than the cichlid GAP (Bond *et al.* 1991). The Pacific salmon GAP is identical to the Atlantic salmon, but varies from the cichlid and mammalian GAPs. Comparing the cichlid and salmon GAPs, serine to final lysine, there are 27 identical amino acids resulting in 58.7% identity at the protein level. There is 8.6% identity compared to the mammalian GAPs. Interestingly, all the GAPs identified so far have a leucine residue in position 5, and all except the cichlid, have a leucine in postion 39. In human GAP and

both salmon GAPs, this residue is followed by a lysine whereas rodents have an arginine which is a conservative substitution. The cichlid appears to be different, although a single base insertion into the GAP would produce a reading frame with a leucine in postion 39 in addition to five other residues similar to mammalian GAP (Bond *et al.* 1991). The conservation of the leucine and arginine residues may be an indication that some sort of secondary structure is being maintained, similar to the retention of cysteine residues in the cryptic (neurophysin) portion of the vasotocin precursor (see chapter four). However, clearly the majority of the molecule has altered in sequence over evolutionary time.

The extremely high degree of similarity at the nucleic acid level between the Pacific and Atlantic salmon sGnRH genes is interesting given their geographical and phylogenetic separation. These two groups have traditionally been considered different genera, the Atlantic salmon belonging to the trout genus, *Salmo*, and the Pacific salmon and related Pacific trout, to the salmon genus, *Oncorhynchus*. A recent mitochondrial DNA study of Pacific salmon noted how similar in sequence the different Pacific salmon and trout species were suggesting they are all derived from a fairly recent common ancestor (Thomas *et al.* 1986). Sockeye salmon, *Oncorhynchus nerka*, from which the sGnRH was isolated, was placed closer to the evolutionary ancestor in this study and is therefore, perhaps, closer to the Atlantic salmon line than other Pacific species of

salmon. However, geographically separate populations of the same species may exhibit sequence polymorphisms as has been recently been demonstrated for vasotocin precursors isolated from chum salmon in Canada and Japan (see chapter four). Further work to characterize the 5' and 3' flanking regions may uncover greater sequence variability between the sGnRH genes in Atlantic and Pacific salmon.

The overall similarity in exon/intron structure suggests that mGnRH and sGnRH are derived from a common ancestor. Within the GnRH coding region, only two base changes are required to convert sGnRH to mGnRH. The presence of peptide similar to mGnRH has been reported in primitive bony fish such as sturgeon, alligator gar and reedfish (Sherwood *et al.* 1991). Thus the mGnRH form is found in bony fish that are close to the line of tetrapod descent but is not found in any other fish studied to date. Most modern bony fish such as salmon and goldfish possess sGnRH, in addition to other forms. Gene duplication of an ancestral GnRH, perhaps similar in form to cGnRH-II, may have given rise to mGnRH. After the modern bony fish separated from the line leading to tetrapods, the mGnRH may have undergone nucleotide substitution to produce sGnRH (Sherwood and Coe 1991). Characterization of more GnRH precursors in fish and phylogenetically significant species such as sturgeon may help to elucidate further the evolutionary history of the GnRH family.

Literature Cited

Adelman, J.P., Mason, A.J., Hayflick, J.S., and Seeburg, P.H. 1986. Isolation of the gene and hypothalamic cDNA for the common precursor of gonadotropin-releasing hormone and prolactin release-inhibiting factor in human and rat. Proceedings of the National Academy of Sciences (USA) **83**:179-183.

Ausubel, F., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A., Struhl, K. 1989. Current Protocols in Molecular Biology. Greene Publishing Associates and Wiley-Interscience, New York.

Bond, C.T., Francis, R.C., Fernald, R.D. and Adelman, J.P. 1991. Characterization of complementary DNA encoding the precursor for gonadotropin-releasing hormone and its associated peptide from a teleost fish. Molecular Endocrinology **5**:931-937.

Bond, C.T., Hayflick, J.S., Seeburg, P.H., and Adelman, J.P. 1989. The rat gonadotropin-releasing hormone: SH locus: Structure and hypothalmic expression. Molecular Endocrinology **3**:257-262.

Burgus, B., Butcher, M., Amoss, M., Ling, N., Monahan, M., Rivier, J., Fellows, R., Blackwell, R., Vale, W., and Guillemin, R. 1972. Primary structure of ovine hypothalamic luteinizing hormone-releasing hormone (LRF). Proceeding of the National Academy of Sciences (USA) **69**:278-282.

Church, G.M., and Gilbert, W. 1984. Genomic sequencing. Proceedings of the National Academy of Sciences (USA). **81**:1991-1995.

Eidne, K.A. 1991. The polymerase chain reaction and its uses in endocrinology. Trends in Endocrinology and Metabolism **2**:169-175.

Eipper, B.A., Mains, R.E., and Herbert, E. 1986. Peptides in the nervous system. Trends in Neuroscience. **19**:463-468.

Fink, G. 1985. Has the prolactin inhibiting peptide at last been found? Nature (London) **316**:487-488.

Gubler, U., and Hoffman, B.J. 1983. A simple and very effective way for generating cDNA libraries. Gene **25**:263-269.

Harris, N., Dutlow,C., Eidne, K., Dong, K.W., Roberts, J., and Millar, R. 1991. Gonadotropin-releasing hormone gene expression in MDA-MB-231 and ZR-75-1 breast carcinoma cell lines. Cancer Research **51**:2577-2581.

Hodgson, C.P and Fisk, R.Z. 1987. Hybridization probe size control: optimized `oligolabelling'. Nucleic Acids Research **15**:6295.

Jacobs, K.A., Rudersdorf, R., Neill, S.D., Dougherty, J.P., Brown, E.L., and Fritsch, E.F. 1988. The thermal stability of oligonucleotide duplexes is sequence independent in tetraalkylammonium salt solutions: application to identifying recombinant DNA clones. Nucleic Acids Research **16**:4637-4650.

Jirikowski, G.F., Sanna, P.P., and Bloom, F.E. 1990. mRNA coding for oxytocin is present in axons of the hypothalmo-neurohypophysial tract. Proceedings of the National Academy of Sciences (USA) **87**:7499-7404.

Katz, I.A., Millar, R.P., and King, J.A. 1990. Differential regional distribution and release of two forms of gonadotropin-releasing hormone in the chicken brain. Peptides **11**:443-450.

Kelly, A.C., Rodgers, A., Dong, K.W., Barrezueta, N.X., Blum, M., and Roberts, J.L. 1991. Gonadotropin-releasing hormone and chorionic gonadotropin gene expression in human placental development. DNA and Cell Biology **10**:411-422.

Khodr, G.S., and Siler-Khodr, T.M. 1980. Placental luteinizing hormone-releasing factor and its synthesis. Science **207**:315-317.

King, J.A., and Millar, R.P. 1982a. Structure of chicken hypothalamic luteinizing hormone releasing hormone. I. Structural determination on partially purified material. Journal of Biological Chemistry **257**:10722-10728.

King, J.A., and Millar, R.P. 1982b. Structure of chicken hypothalamic luteinizing hormone releasing hormone. II. Isolation and charaterization. Journal of Biological Chemistry **257**:10729-10732.

Klungland, H., Lorens, J.B., Andersen, O., Kisen, G.O., and Alestrom, P. (in press) The Atlantic salmon prepro-gonadotropin releasing hormone gene and mRNA. Molecular and Cellular Endocrinology.

Kurjan, J., and Herskowitz, I. 1982. Structure of a yeast pheromone gene (MFα): A putative α-factor precursor contains four tandem copies of mature α-factor. Cell **30**:933-943.

Lathe, R. 1985. Synthetic oligonucleotide probes deduced from amino acid sequence data: Theoretical and practical considerations. Journal of Molecular Biology **183**:1-12.

Lovejoy, D.A., Fischer, W.H., Ngamvongchon, S., Craig, A., Rivier, J.E., and Sherwood, N.M. (submitted). A novel form of gonadotropin-releasing hormone (GnRH) provides insight into GnRH evolution. Proceedings of the National Academy of Sciences (USA).

Maniatis, T., Fritsch, E.F., and Sambrook, J. 1982. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor.

Mason, A.J., Hayflick, J.S., Zoeller, R.T., Young W.S. III, Phillips, H.S., Nikolics, K., and Seeburg, P.H. 1986. A deletion truncating the gonadotropin-releasing hormone gene is responsible for hypogonadism in the hpg mouse. Science **234**:1366-1371.

Manfioletti, G., and Schneider, C. 1988. A new and fast method for preparing high quality lambda DNA suitable for sequencing. Nucleic Acids Research **16**:2873-2884.

Matsuo, H., Baba, Y., Nair, R.M.G., Arimura, A., and Schally, A.V. 1971. Structure of porcine LH- and FSH-releasing hormone. I. The proposed sequence. Biochemical and Biophysical Research Communications **43**:1334-1339.

Merchentaler, I., Culler, M.D., Petrusz, P., Flerko B., and Negro-Vilar, A. 1989. Immunocytochemical localization of the gonadotropin releasing hormone-associated peptide portion of the LHRH precursor in the hypothalamus and extra-hypothalamic regions of the rat central nervous system. Cell and Tissue Research **255**:5-14.

Millar, R.P., Wormald, P.J., Milton, R.C. de L. 1986. Stimulation of gonadotropin release by a non-GnRH peptide sequence of the GnRH precursor. Science **232**:68-70.

Miyamoto, K., Hasegawa, Y., Nomura, M., Igarashi, M., Kangawa, K., and Matsuo, H. 1984. Identification of the second gonadotropin-releasing hormone in chicken hypothalamus: Evidence that gonadotropin secretion is probably controlled by two distinct gonadotropin releasing hormones in avian species. Proceedings of the National Academy of Sciences (USA) **81**:3874-3878.

Miyamoto, K., Hasegawa, Y., Igarashi, M., Chino, N., Sakakibara, S., Kangawa, K., and Matsuo, H. 1983. Evidence that hypothalamic luteinizing hormone-releasing hormone is [Gln^8]-LH-RH. Life Sciences **32**:1341-1347.

Miyamoto, K., Hasegawa, Y., Minegishi, T, Nomura, M., Takahashi, Y., and Igarashi, M. 1982. Isolation and characterization of chicken hypothalamic luteinizing hormone-releasing hormone. Biochemical and Biophysical Research Communications **107**:820-827.

Ngamvongchon, S., Lovejoy, D.A., Fischer, W.H., Craig, A.G., Rivier, J.E., and Sherwood, N.M. (in press). Primary structure of two forms of gonadotropin-releasing hormone, one distinct and one conserved, from catfish brain. Molecular and Cellular Neurosciences.

Nikolics, K., Mason, A.J., Szonyi, E., Ramachandran, J, and Seeburg, P.H. 1985. A prolactin-inhibiting factor within the precursor for human gonadotropin-releasing hormone. Nature (London) **316**:511-517.

Oikawa, M., Darga, C., Ny, T., and Hsueh, A.J.W. 1990. Expression of gonadotropin-releasing hormone and prothymosin-alpha messenger RNA in the ovary. Endocrinology **127**:2350-2356

Okuzawa, K., Amano, M., Kobayashi, M., Aida, K., Hanyu, I., Hasegawa, Y., and Miyamoto, K. 1990. Differences in salmon GnRH and chicken GnRH-II contents in discrete brain areas of male and female rainbow trout according to age and state of maturity. General and Comparative Endocrinology **80**:116-126.

Peter, R.E., Habibi, H.R., Chang, J.P., Nahorniak, C.S., Yu, K.L., Huang, Y.P., and Marchant, T.A. 1990. Actions of gonadotropin-releasing hormone (GnRH) in the goldfish. Progress in Comparative Endocrinology. **342**:393-398.

Phillips, H.S., Nikolics, K., Branton, D., and Seeburg, P.H. 1985. Immunocytochemical localization in rat brain of a prolactin release-inhibiting sequence of gonadotropin-releasing prohormone. Nature (London) **316**:542-546.

Radovick, S., Wondisford, F.E., Nakayama, Y., Yamada, M., Cutler, G.B., and Wientraub, B.D. 1990. Isolation and characterization of the human gonadotropin-releasing hormone gene in the hypothalamus and placenta. Molecular Endocrinology **4**:476-480.

Rubin, B.S., King, J.C., Millar, R.P., Seeburg P.H., and Arimura, A. 1987. Processing of luteinizing hormone-releasing hormone precusor in rat neurons. Endocrinology **121**:305-309.

Sambrook, J, Fritsch, E.F., and Maniatis, T. 1989. Molecular Cloning: A Laboratory Manual. 2nd Edition. Cold Spring Harbor Laboratory Press, Cold Spring Harbor.

Sanger, F., Nicklen, S., and Coulson, A.R. 1977. DNA sequencing with chain-terminating inhibitors. Proceedings of the National Academy of Sciences (USA) **74**:5463-5467.

Sar, M., Culler, M.D., McGimsey, W.C., and Negro-Vilar, A. 1987. Immunocytochemical localisation of the gonadotropin-releasing hormone-associated peptide of the LHRH-precursor. Neuroendocrinology **45**:172-175.

Schaefer, M., Picciotto, M.R., Kreiner, T., Kaldany R., Taussig, R., and Scheller, R.H. 1985. Aplysia neurons express a gene encoding multiple FMRFamide neuropeptides. Cell **41**:457-467.

Seeburg, P.H., and Adelman, J.P. 1984. Characterization of cDNA for precursor of human luteinizing hormone-releasing hormone. Nature (London) **311**:666-668.

Seeburg, P.H., Mason, A.J., Stewart, T.A., and Nikolics, K. 1987. The mammalian GnRH gene and its pivotal role in reproduction. Recent Progress in Hormone Research **43**:69-98

Sepälä, M., and Wahlström, T. 1980. Identification of luteinizing hormone-releasing factor and alpha-subunit of glycoprotein hormones in human pancreatic islets. Life Sciences **27**:395-397.

Sherwood, N.M. 1987. The GnRH family of peptides. Trends in Neuroscience 10:129-132.

Sherwood, N.M., and Coe, I.R. 1991. Neuropeptides and their genes in fish. *In* Proceedings of the Fourth International Symposium on the Reproductive Physiology of Fish, University of East Anglia, Norwich, U.K., 7-12 July (eds. Scott, A.P., Sumpter, J.P., Kime, D.E., and Rolfe, M.S.) FishSymp 91, Sheffield, U.K. pp. 38-40.

Sherwood, N.M., and Parker, D.B. 1990. Neuropeptide families: An evolutionary perspective. Journal of Experimental Zoology Supplement **4**:63-71.

Sherwood, N.M., and Sower, S.A. 1985. A new family member for gonadotropin-releasing hormone. Neuropeptides **6**:205-214.

Sherwood, N.M., Doroshov, S., and Lance, V. 1991. Gonadotropin-releasing hormone (GnRH) in bony fish that are phylogenetically ancient: Reedfish (*Calamoichthys calabaricus*), sturgeon (*Acipenser transmontanus*), and alligator gar (*Lepisosteus spatula*). General and Comparative Endocrinology **84**:44-57.

Sherwood, N.M., Sower, S.A., Marshak, D.R., Fraser, B.A., and Brownstein, M.J. 1986. Primary structure of gonadotropin-releasing hormone from lamprey brain. Journal of Biological Chemistry **261**:4812-4819.

Sherwood, N., Eiden, L., Brownstein, M., Spiess, J., and Rivier, J., and Vale, W. 1983. Characterization of a teleost gonadotropin-releasing hormone. Proceedings of the National Academy of Sciences (USA) **80**:2794-2798.

Silverman, A.J., Silverman, R., Lehman, M.N., Witkin, J.W., and Millar, R.P. 1987. Localization of a peptide sequence contained in the precursor to gonadotropin releasing hormone (GnRH). Brain Research **402**:346-350.

Tan, I., and Rousseau, P. 1982. The chemical identity of the immunoreactive LHRH-like peptide biosynthesized in the human placenta. Biochemical and Biophysical Research Communications **109**:1061-1071.

Thomas, W.K., Withler, R.E., and Beckenbach, A.T. 1986. Mitochondrial DNA analysis of Pacific salmonid evolution. Canadian Journal of Zoology **64**:1058-1064.

Thomas, G.B., Cummins, J.T., Doughton, B.W., Griffin, N., Millar, R.P., Milton, R.C.L., and Clarke, I.J. 1988. Gonadotropin-releasing hormone associated peptide (GAP) and putative processed GAP peptides do not release luteinizing hormone or follicle stimulating hormone or inhibit prolactin secretion in the sheep. Neuroendocrinology **48**:342-350.

Ulrich, A., Berman, C., Dull, T.J., Gray, A., and Lee, J.M. 1984. Isolation of the human insulin-like growth factor gene using a single synthetic DNA probe. European Molecular Biology Organisation Journal **3**:361-364.

Wood, W.I., Gitshier, J.M., Lasky, L.A., and Lawn, R.M. 1985. Base composition-independent hybridization in tetramethylammonium chloride: A method for oligonucleotide screening of highly complex gene libraries. Proceedings of the National Academy of Sciences (USA) **82**:1585-1588.

Yu, K.L., Sherwood, N.M., and Peter, R.E. 1988a. Differential distribution of two molecular forms of gonadotropin-releasing hormone in discrete brain areas of goldfish (*Carassius auratus*). Peptides **9**:625-630.

Yu, W.H., Seeburg, P.H., Nikolics, K., and McCann, S.M. 1988b. Gonadotropin-releasing hormone associated peptide exerts a prolactin-inhibiting and weak gonadotropin releasing activity in vivo. Endocrinology **123**:390-395.

Chapter 4

Isolation and characterization of two vasotocin precursors from chum salmon (*Oncorhynchus keta*) brain.

Parts of this chapter have already been published as:
Heierhorst, J., Mahlmann, S., Morley, S.D., Coe, I.R., Sherwood, N.M., and Richter, D. 1990. Molecular cloning of two distinct vasotocin precursor cDNAs from chum salmon (*Oncorhynchus keta*) suggests an ancient gene duplication. Federation of European Biochemical Societies Letters **260**:301-304.

Introduction

The first neuropeptide in the mammalian brain to be identified, isolated and sequenced was vasopressin (see Acher and Chauvet 1988, Richter 1988, Acher 1990). Vasopressin and oxytocin, which are very similar in structure, were also among the first neurohormones to be characterized at the molecular level in mammals (Ivell and Richter 1984). These peptides have subsequently become a model for the study of the structure, expression, regulation and evolution of neurohormones. Vasopressin- and oxytocin-like molecules have been identified in a wide variety of phyla including invertebrates (see Sherwood and Parker 1990). Within the invertebrates, family members have

diverse functions as a diuretic hormone in the locust (Proux *et al.* 1987), as conpressins in the venom of fish-hunting marine *Conus* snails (Cruz *et al.* 1987), and as a toxin in the sea anenome, *Anemonia sulcata*, (Rathmayer 1979). In humans, vasopressin and oxytocin differ by only two (out of nine) amino acids and the striking similarity of both sequence and precursor structure suggests that both peptides arose from a single ancestral peptide that underwent gene duplication to produce the vasopressin lineage and the oxytocin lineage. These lines have been evolving independently resulting in peptides with variable sequences and different functions in the vertebrates. Since the lampreys only possess one vasopressin/oxytocin-like peptide (Lane *et al.* 1988), this duplication may have taken place after the appearance of the agnathans. In mammals, the genes encoding vasopressin and oxytocin have been characterized and shown to reside on complementary strands of chromosome 20, separated by 8 to 11 kilobases in humans and rats (Ivell and Richter 1984, Richter 1988). These neurohormones provided some of the first evidence that peptides are secreted as larger presursor proteins which are subsequently cleaved to release the peptide hormone. Both vasopressin and oxytocin precursors consist of a signal peptide, the nine amino acid hormone and a larger cryptic peptide known as neurophysin. In addition, the vasopressin precursor possesses another cryptic peptide following the neurophysin which has a glycosylation site and is known as

copeptin. Studies on the Brattleboro rat, which has a genetic defect in the vasopressin gene leading to diabetes insipidus, have shown that this condition is due to a single nucleotide base deletion in the neurophysin region. This leads to a new reading frame predicting a hormone precursor extended at its mutated C-terminus by a poly-lysine sequence (Schmale and Richter 1984). Since vasopressin is barely detectable in these animals, it has been suggested that the altered precursor protein cannot be correctly processed, lending weight to the contention that the associated proteins act to maintain correct conformation for processing and transport (Schmale *et al.* 1989). Another feature of this family, which is proving to be typical of other neurohormones, is the presence of the protein or messenger RNA in tissues outside of the central nervous system. In rats, arginine vasopressin has been found in the adrenal, ovaries, thymus, uterus, pancreas and testis (see Lefebvre and Zingg 1991). The role it plays in these tissues is unclear.

In non-mammalian tetrapods such as the amphibians, the vasopressin-like peptide, vasotocin, has a similar precursor structure to the mammalian form and is thought to act as an antidiuretic hormone while mesotocin is the equivalent peptide to oxytocin (Nojiri *et al.* 1987). Amphibians also seem to produce additional extended forms of vasotocin by differential processing of the vasotocin presursor. These are known as hydrins and have osmoregulatory roles (Rouillé

et al. 1989). The coding sequences for vasotocin and an oxytocin-like peptide, known as isotocin, have been isolated more recently from the teleost, *Catostomus commersoni*, the white sucker, (Heierhorst *et al.* 1989, Morley *et al.* 1990) and show similar organization to their mammalian counterparts. However, both neurophysins are extended at their C-termini by about 30 amino acids. These extensions show some sequence similarity to the copeptin portion of the vasopressin precursor in mammals, suggesting that the copeptin in mammals may have arisen from the C-terminal of an ancestral neurophysin. In addition, two distinct precursors for vasotocin were isolated (Morley *et al.* 1990) suggesting that each gene has undergone a gene duplication. This may not be surprising, since the catostomid fish is thought to have evolved from a diploid cyprinid-like ancestor which became tetraploid (Uyeno and Smith 1972) perhaps 50-100 million years ago. It has been suggested that related gene pairs diverge at a rate of 1% per 10 million years at the amino acid level (Wilson *et al.* 1977). However, the degree of difference between the two sucker precursors, 45% at the amino acid level, does not seem to be consistent with this time scale. Therefore, either the rates of divergence are greater or the genes duplicated prior to the tetraploid event. The line giving rise to the salmon (which are also tetraploid) separated from that of the suckers approximately 70 million years ago (Carroll 1988) prior to either group attaining a tetraploid

karyotype. We were interested to see if the vasotocin precursors in salmon mirrored the divergent precursors in the sucker. In addition, the role of vasotocin, in fish, is poorly understood, although it may be involved with regulating water balance and ion concentration (Hontela *et al.* 1990, Perrott *et al.* 1991, Haruta *et al.* 1991), or controlling blood pressure (Le Mevel *et al.* 1991). In fish, such as salmon, which migrate between salt and fresh water, such an osmoregulatory hormone would be of critical importance, and isolation of clones for this hormone would allow analyses of differential temporal and spatial expression to begin.

Since an oligonucleotide pool of probes against sucker vasotocin had already been successfully used to isolate the precursor cDNAs for vasotocin from a sucker library (Heierhorst *et al.* 1989) the same approach was used to isolate vasotocin precursors from salmon.

Methods and Materials

cDNA library preparation and screening

The salmon brain cDNA library was constructed as outlined in chapter three. The library was screened using a fully degenerate 20-mer oligonucleotide pool corresponding to the first 7 amino acids of the mature vasotocin. This was used instead of the catostomid clone since the degree of

similarity between the two species was unknown and therefore the level of stringency for screening would have to be established empirically. In contrast, all the parameters for oligonucleotide screening of a cDNA library with tetramethylammonium chloride (TMAC) were already known. The library was plated out at a density of approximately 25,000 plaque forming units (pfu) per plate on LB-agar plates in LB-top agarose. The oligonucleotide pool was end-labelled using T4 polynucleotide kinase (Pharmacia) to a specific activity of 10^8 cpm/μg. Duplicate filter lifts were treated exactly as for the TMAC screening described in chapter three. In brief, they were hybridized under non-stringent conditions at 37°C for about 36 hours then washed stringently at 58°C in TMAC solution. In the presence of tetramethylammonium ions, at this temperature, only exact matches to the target sequence should remain hybridized on the filters. Filters were allowed to dry then packed in Saran Wrap and exposed to X-ray film (Kodak X-OMAT) in cassettes at -70°C with intensifying screens for 5 days. Films were then developed, positively hybridizing phage identified and cores taken. These cores were stored in Murray buffer (recipe in chapter three) with chloroform, titered and then plated out at a lower density (5,000 pfu/plate) for rescreening. Screening continued until well isolated single positive plaques could be identified. These plaques were then subjected to helper phage rescue according to Stratagene's instructions for lambda Zap II. This

procedure recovered the insert as a subclone in the plasmid Bluescript SK(-) which could then be analysed. cDNA inserts or restriction fragments of inserts were sequenced by standard dideoxy sequencing either directly or after subcloning into M13 mp18.

Northern blot analysis

Poly $(A)^+$ RNA (4μg) was glyoxylated (Thomas 1983), separated in a 1.2% (w/v) agarose gel and transferred to Hybond-N membranes (Amersham, Oakville, Ontario), according to the supplier's instructions. Blots were hybridized with ^{32}P-labelled cDNA inserts and finally washed at 65°C with 0.5 x SSPE (20 x SSPE is 3M NaCl, 5M $NaH_2PO_4.H_2O$, 0.025M EDTA, pH 7.4).

Results

A total of 1.8 x 10^5 recombinants were screened producing three positive clones. Sequence analysis separated these into two classes, designated VT-1 and VT-2, which both predict a distinct chum salmon vasotocin precursor (fig. 23). The longest cDNA encoding the VT-1 precursor is 821 bases long, excluding the poly A tail. It has in-frame start codons (A'G) at positions 10-12 and 67-69. The latter is more likely to be used for translational initiation, since an exceptionally long signal peptide would otherwise be generated. Neither of these start codons is

preceded by the consensus sequence, GCCGCC$^{A}/_{G}$CC START, for the initiation of translation found in many vertebrate mRNAs (Kozak 1987). However, a purine residue is found three bases upstream of the start codon, in both clones, which does appear to be a strongly conserved feature of translational start sites in vertebrates (Kozak 1984, 1987). The first stop codon is found at positions 526-528, giving rise to a predicted VT-1 precursor of 153 amino acids and a molecular weight of 15,997 Da. A typical polyadenylation signal is found at positions 799-804. The VT-2 precursor is encoded by a cDNA clone of 1199 nucleotides excluding the poly A tail. The first start codon is found at nucleotide residue 45. This is followed by an open reading frame of 474 nucleotide residues predicting a precursor protein of 158 amino acids with a molecular weight of 16,672 Da. A typical polyadenylation signal (AATAAA) is present at nucleotide residues 1005-1010, but an alternative signal (ATTAAA) is found at residues 1174-1179. A diagram showing the overall layout of both precursor proteins is shown in figure 24A. Northern blot analysis of the chum salmon poly (A^{+}) RNA used to construct the library, revealed RNA bands of 1110 bases for VT-1 mRNA and 1450 bases for VT-2 mRNA (see paper for data). The difference in sizes of hybridization signals on the Northern and the lengths of the two clones determined by sequencing may be due to extensive polyadenylation of the messages *in vivo*.

A comparison of amino acid similarity of the salmon

vasotocin precursors to each other and to the sucker precursors in shown in figure 24B. A possible evolutionary history for vasotocin and isotocin is outlined in figure 25.

Figure 23. Comparison of the chum salmon (*Oncorhynchus keta*) VT-1 (top line) and VT-2 cDNA sequences and their predicted precursor structures. The signal peptides are followed by the regions encoding vasotocin, which is shown in bold. The processing sequence (Gly-Lys-Arg) is indicated by asterisks. This is followed by the neurophysin moeity. The polyadenylation signals are underlined. The numbers on the right refer to amino acids (upper and lower) and nucleotide bases (center).

```
VT-1GCCCTTTGAATGAGTTCAGTTGTAGCCGACAGTATCAATTGGACGAAGCACTTC54
VT-2                          GAAGAGCAAGAGGAATATAGGTGTGCTTTTTC32

                 SIGNAL
                 M  P  Y  S  T  F  P  L  L  W  V  L  G  L  L15
AGACTGAACAAGATGCCATATTCTACGTTTCCACTGCTGTGGGTCCTGGGGCTCCTC111
TGCACAATTGCAATGCCACATTCCAC---CTTCTACTGTGCGTCATCGGACTCCTA86
                 M  P  H  S  T  -  L  L  L  C  V  I  G  L  L14

                          VASOTOCIN
A  L  S  S  A  C  Y  I  Q  N  C  P  R  G  G* K* R* S 33
GCGCTCTCCTCCGCGTGCTACATCCAGAACTGTCCGCGAGGGGGGAAGCGCTCT165
GCGTTCTCCTCTGCGTGCTACATCCAGAACTGTCCCAGAGGGGGGAAGCGCGCG140
A  F  S  S  A  C  Y  I  Q  N  C  P  R  G  G* K* R* A 32

NEUROPHYSIN
F  P  D  L  P  -  R  Q  C  M  S  C  G  P  G  D  R  G  R51
TTTCCTGATCTTCCA---CGACAGTGCATGTCATGTGGCCCCGGGGACAGGGGCCGC219
TTACAGGACACCGGCATCAGACAGTGCATGACATGTGGACCAGGGGACCAGGGCCAC197
L  Q  D  T  G  I  R  Q  C  M  T  C  G  P  G  D  Q  G  H51

C  F  G  P  N  I  C  C  G  E  G  M  G  C  Y  M  G  S  P70
TGCTTTGGCCCCAATATCTGCTGTGGGGAGGGAATGGGCTGTTACATGGGCTCCCCA276
TGCTTTGGCCCCAGTATCTGCTGTGGGGAGGGTCTGGGCTGCTGGATGGGCTCCCCA254
C  F  G  P  S  I  C  C  G  E  G  L  G  C  W  M  G  S  P70

E  A  A  G  C  V  E  E  N  Y  L  P  S  P  C  E  A  G  G89
GAGGCAACTGGTTGTGTGGAGGAGAACTACCTGCCCTCCCCCTGCGAGGCTGGAGGA333
GAGACAGCCCGCTGCTTTGAGGAGAATTACCTGCCCACCCCATGCCAGACAGGAGGG311
E  T  A  R  C  F  E  E  N  Y  L  P  T  P  C  Q  T  G  G89

R  V  C  G  S  E  -  G  S  C  A  A  S  G  V  C  C  D  S107
AGAGTGTGTGGCTCTTCT---GGAAGCTGTGCTGCATCCGGAGTCTGCTGTGACTCA387
AGACCTTGTGGATCTGATGCAGGACGCTGCGCTGCACCAGGAGTTTGCTGTGACTCA368
R  P  C  G  S  D  A  G  R  C  A  A  P  G  V  C  C  D  S108

E  S  C  V  L  D  P  D  C  L  E  D  S  K  R  Q  S  P  S126
GAGAGTTGTGTGCTAGACCCAGACTGCCTAGAGGACAGTAAACGTCAGTCACCCAGC444
GAGAGTTGTGTTCTAGATCCAGACTGTTTGTCGGAGGAGTCGTACCACTCTCCTGCG425
E  S  C  V  L  D  P  D  C  L  S  E  S  R  Y  H  S  P  A127

E  Q  N  A  A  L  M  G  G  L  A  G  D  -  L  L  R  I  L144
GAACAGAACGCTGCCTTAATGGGTGGTGGTGCAGGAGAC---CTGCTGCGGATCCTA498
GATCACAGCGCTGGCGCCACAAGTGACTCACCGGGGGAACTGTTGCTGCGCCTGCTA482
D  H  S  A  G  A  T  S  D  S  P  G  E  L  L  R  L  L146

H  A  T  S  R  G  R  P  Q  Ter                          153
CATGCCACCAGCAGAGGGAGACCTCAGTAACCAACCACTGCCCATCCCTCACCTGAA555
CACTTTGCCACCAGGGGGCAAATGGAATACAAACAGTAACAATCTCACTCCAGAGAT539
H  F  A  T  R  G  Q  S  E  Y  K  Q  Ter                 158
```

CACACCCAGAATAGAGCTTAAATTCACCATTTCACATGCACTACTACAAAAACAAAC 612
TCGCTTACATGTTGCCTAATTCAAAATCAACCCTTACTAGCCTTTGACACTTCTCGT 596

CTCACATAGATTCAGACACACAGCAGAAGTAGAGAGCAGGCTTGCTACATAAGGGTG 669
AGATCTGAAAAGATTGAAATTAATTACTTATTTTTTTCAGAAGGAACTTCAAGTAAT 653

AAATTGATCAGCTCTACATGAATGTTTACTGTGTGCTCAGTAGCCTTCAGTAAGTTG 726
TGGATATGTTTTAGCAATATGGTAATTGTTCCACCTTGCCTAGTGATGGCGATTTCG 710

TCATTTATAATGCTTCAACTCAAATATGCATTGTACAATCTACTGGGGTGGAAGGGA 783
CCATATTGTTGACCACTACTATCAAAACCTTTTACACCTATACAGTACCTAGGCTAG 767

TGTAAATATGTAGTTAATAAATATTTTCTTGCACTATT(A)n 821
GGGCAGCGGGCGATTTGGAATCAGGCAAAATGGTTAAACTGACAGAAATAACAGGAT 824

AAAATGGATACAGTTATAAACTGTCAACAAGAGTGTTTTTTTCCACCTAAGAATGTA 881

TATGCACACACATACATTACATATATACGTTTTATGTTCAACAAGACATTGTTTTTC 938

CTAATCGCCTCAATATAAATAGGTCATGTATACACCACAGAAATACAGACATTTAAA 995

TATGTAGGAAATAAATAGTTTTCTTGCAGTGTTGTAGAATGATCAGATTAATGTTC 1051

AACAATCATTGTTGTTAGTATGTGAAGTGCTTGTAGATCACTACATTAATGCAGTG 1107

CATTACAGATATTACTATTAATTATGAATCACCATTTACTGAGACTCACTGCTGTT 1163

GTATAATTGAATTAAAGATATCAAGAAATCACTACC(A)n 1199

Figure 24. Schematic diagram showing the overall structure of both vasotocin precursors and percent amino acid similarity compared to the sucker precursors.

A. Structure of both vasotocin precursors (boxed region). The 5' untranslated region (utr) is followed by the signal peptide (SP), vasotocin (VT), processing site (*), neurophysin, and copeptin-like region (CP). The partial vertical line between the neurophysin and CP indicates that it is not currently known whether this region is cleaved away from the precursor.

B. Comparison of the salmon and sucker vasotocin precursors showing percent amino acid identity between each preprohormone.

A.


5' utr
SP
VT
NEUROPHYSIN
CP
3' utr
*

B.

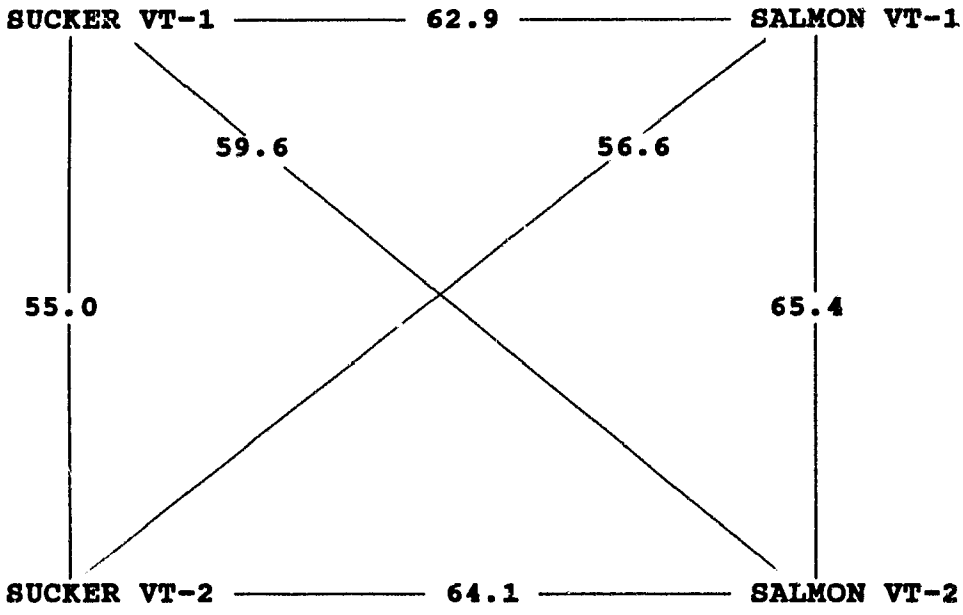
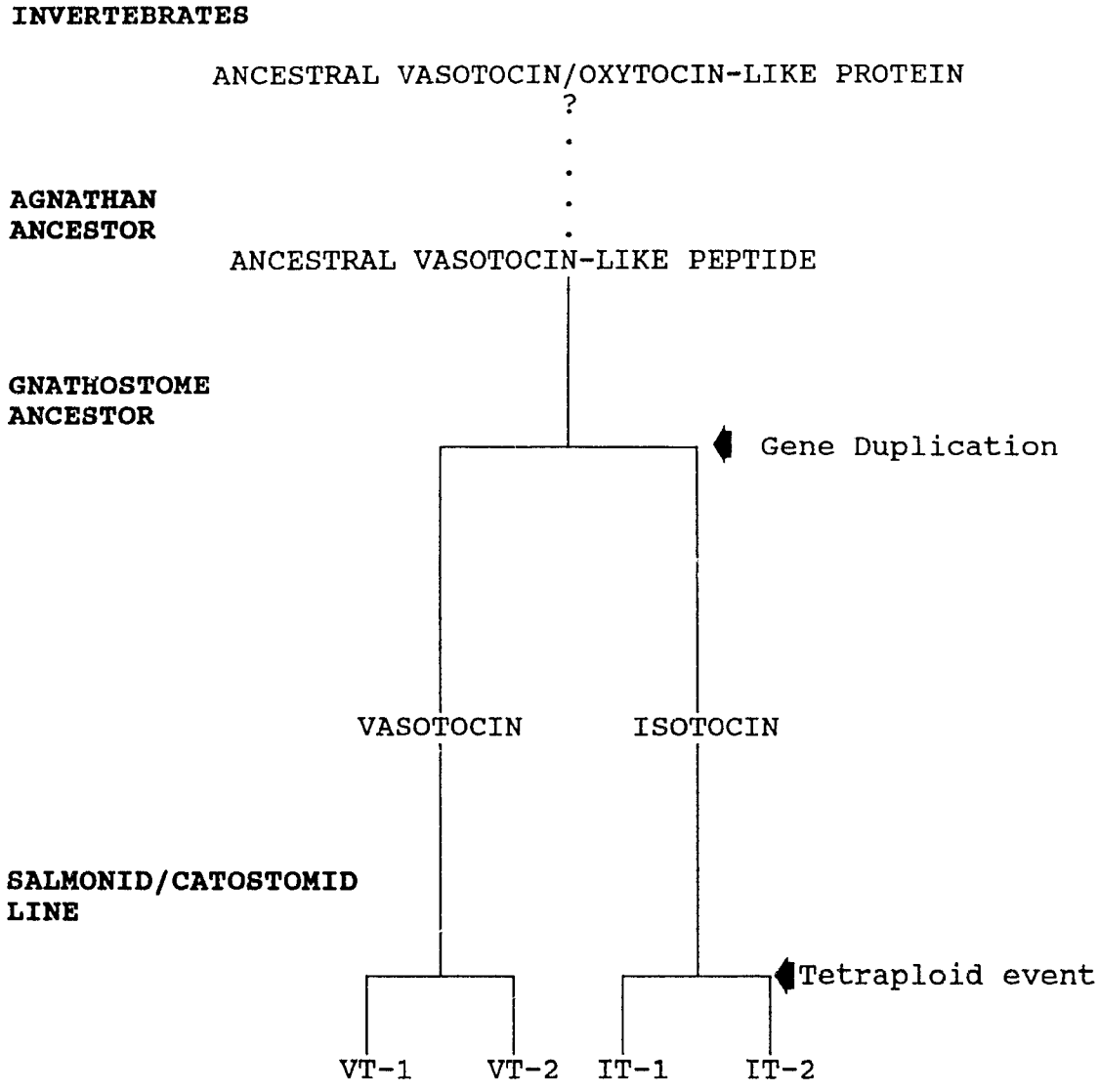

SUCKER VT-1
62.9
SALMON VT-1
59.6
56.6
55.0
65.4
SUCKER VT-2
64.1
SALMON VT-2

Figure 25. Schematic diagram showing possible evolutionary history for isotocin and vasotocin.


INVERTEBRATES
ANCESTRAL VASOTOCIN/OXYTOCIN-LIKE PROTEIN
?
AGNATHAN ANCESTOR
ANCESTRAL VASOTOCIN-LIKE PEPTIDE
GNATHOSTOME ANCESTOR
Gene Duplication
VASOTOCIN
ISOTOCIN
SALMONID/CATOSTOMID LINE
Tetraploid event
VT-1
VT-2
IT-1
IT-2

Discussion

The two classes (two clones in one and one in the other) of cDNAs isolated from the chum library both predict identical vasotocin peptides. However, they are clearly different clones since there is considerable sequence variation in regions flanking the hormone. Both salmon vasotocin precursors show similar structural organization to their mammalian counterpart (Richter 1988). The precursors consist of a signal peptide of either 19 (VT-2) or 20 (VT-1) amino acids, followed by the nine amino acid hormone with a C-terminal donor glycine, which is separated by a typical processing signal from a cysteine rich protein resembling the mammalian neurophysin. The neurophysin-like protein is followed by a sequence of about 30 amino acids which shows similarity to the glycopeptide or copeptin in the mammalian vasopressin precursor. The vasotocin portion of the precursor is conserved compared to the mammalian vasopressin with sequence identity of 89% (at the amino acid level) suggesting that there are functional constraints on variability within this peptide. Other portions of the molecule are not so highly conserved. Unlike the mammalian and amphibian precursors, the salmon precursors, like the sucker precursors (Heierhorst *et al.* 1989), lack the N-linked glycosylation sites in the copeptin-like region. In contrast with the sucker precursors, the salmon precursors possess putative processing signals (VT-1, amino acid 121-122: VT-2, amino acids 122) which could result in post-

translational cleavage of the copeptin-like moiety from the C-terminal region of the precursor. To date there has been no investigation as to whether this cleavage occurs in the salmon. However, it has been shown that conformational constraints preclude this second cleavage in an amphibian vasotocin precursor. It is suggested that use of this site may only occur after the transition of vasotocin in non-mammalian vertebrates to vasopressin in mammals (Chauvet *et al.* 1988). The salmon neurophysin moieties show the strong conservation of the 14 cysteine residues found in all other neurophysins analysed to date. These cysteine residues form a series of disulfide bridges between different parts of the polypeptide (Burman *et al.* 1989). The salmon vasotocin precursors show considerable sequence similarity to the sucker precursors (Morley *et al.* 1990). Both salmon and sucker VT-2 precursors have additional amino acids in identical postions within the first variable region (amino acid residue 38) and the middle conserved region (amino acid residue 96) of their neurophysins. The insertion of an extra amino acid between the ninth and tenth cysteine residues of the salmon and sucker VT-2-associated neurophysins is unique among members of the vasopressin precursor family analysed to date although the toad mesotocin precursor shows the same addition (Nojiri *et al.* 1987). This addition probably disrupts the otherwise conserved spacing of the cysteine residues and may interfere with the folding of the second neurophysin domain (Burman *et*

al. 1989). The two salmon precursors show 35% amino acid variation, half of which are isofunctional substitutions. This is similar to the sucker precursors which show 45% amino acid variation (Morley *et al*. 1990). Interestingly the sucker precursors, VT-1 and VT-2, show greater sequence similarity to their salmon counterparts than to the other precursor in the sucker. The salmon VT-1 and VT-2, however, are more similar to each other than to the sucker precursors. Recently, two vasotocin and isotocin precursors were isolated from chum salmon collected in Japan (Hyodo *et al*. 1991). The vasotocin precursors show considerable sequence identity with the vasotocin precursors described in this study although the Japanese sVT-2 possesses a stop codon in the middle of the neurophysin moeity. This is probably a reflection of the heterogeneity in nucleotide sequence found between members of the same species that are separated by considerable distance. The two isotocin precursors possess neurophysin-like regions which are cysteine rich and have sequence identity to the mammalian and amphibian neurophysins. The C-terminal regions are like the sucker precursors and show similarity to the copeptins of arginine vasopressin and amphibian vasotocin except that they lack the glycosylation site (Hyodo *et al*. 1991). Based on a statistical analysis of the vasotocin and isotocin sequences, the Japanese group estimated the vasotocin precursors to be evolutionarily closer to each other than to either isotocin precursor, and the isotocin precursors to be

approximately as close to each other as the vasotocins were (Miyata *et al.* 1986). Based on these values, this group has suggested a similar evolutionary history for these neuropeptides. They proposed that the salmon vasotocins and isotocins are derived from an ancestral vasotocin-like peptide which underwent gene duplication (approximately 250 million years ago) to produce the isotocin form. Another duplication event, possibly tetraploidy (approximately 100 million years ago), led to the existence of two precursors for vasotocin and isotocin. The different methods of evaluating rates of evolution of neuropeptides make it difficult to determine the accurate evolutionary history. The identification and characterization of more members of the vasotocin-isotocin family in divergent teleosts would be helpful in discerning the actual evolutionary history of this group.

Literature Cited

Acher, R. 1990. Structure, evolution and processing adaptation of neurohypophysial hormone-neurophysin precursors. Progress in Comparative Endocrinology **342**:1-9.

Acher, R., and Chauvet, J. 1988. Structure, processing and evolution of the neurohypophysial hormone-neurophysin precursors. Biochimie **70**:1197-1207.

Burman, S., Wellner, D., Chait, B., Chaudhary, T., and Breslow, E. 1989. Complete assignment of neurophysin disulfides indicates pairing in two separate domains. Proceedings of the National Academy of Sciences (USA) **86**:429-433.

Carroll, R.L. 1988. Vertebrate Paleontology and Evolution, Freeman, New York. 698pp.

Chauvet, J., Michel, G., Chauvet, M.-T., and Acher, R. 1988. An amphibian two-domain `big' neurophysin: Conformational homology with the mammalian MSEL-neurophysin/copeptin intermediate precursor shown by trypsin-sepharose proteolysis. Federation of European Biochemical Societies Letters **230**:77-80.

Cruz, L.J., de Santos, V., Zafafalla, G.C., Ramilo, C.A., Zeikus, R., Gray, W.R., and Olivera, B.M. 1987. Invertebrate vasopressin/oxytocin homologs. Journal of Biological Chemistry **262**:15821-15824.

Haruta, K., Yamashita, T., and Kawashima, S. 1991. Changes in arginine vasotocin content in the pituitary of the medaka (*Oryzias latipes*) during osmotic stress. General and Comparative Endocrinology **83**:327-336.

Heierhorst, J., Morley, S.D., Figueroa, J., Krentler, C., Lederis, K., and Richter, D. 1989. Vasotocin and isotocin precursors from the white sucker, *Catostomus commersoni*: Cloning and sequence analysis of the cDNAs. Proceedings of the National Academy of Sciences (USA) **86**:5242-5246.

Hontela, A., Rasmussen, J.B., Ko, D., Lederis, K., and Chevalier, G. 1990. Arginine vasotocin, an osmoregulatory hormone, as a potential indicator of acid stress in fish. Canadian Journal of Fisheries and Aquatic Science **48**:238-242.

Hyodo, S., Kato, Y., Ono, M., and Urano, A. 1991. Cloning and sequence analyses of cDNAs encoding vasotocin and isotocin precursors of chum salmon, *Oncorhynchus keta*: Evolutionary relationships of neurohypophysial hormone precursors. Journal of Comparative Physiology B **160**:601-608.

Ivell, R., and Richter, D. 1984. Structure and comparison of the oxytocin and vasopressin genes from rat. Proceeding of the National Academy of Sciences (USA) **81**:2006-2010.

Kozak, M. 1984. Compilation and analysis of sequences upstream from the translational start site in eukaryotic mRNAs. Nucleic Acids Research **12**:857-872.

Kozak, M. 1987. An analysis of 5'-noncoding sequences from 699 vertebrate messenger RNAs. Nucleic Acids Research **15**:8125-8148.

Lane, T.F., Sower, S.A., and Kawauchi, H. 1988. Arginine vasotocin from the pituitary gland of the lamprey (*Petromyzon marinus*): Isolation and amino acid sequence. General and Comparative Endocrinology **70**:152-157.

Lefebvre, D.L., and Zingg, H.H. 1991. Novel vasopressin gene-related transcripts in rat testis. Molecular Endocrinology **5**:645-652.

Le Mevel, J.C., Mabin, D., and Vaudry, H. 1991. Intracerebroventricular injection of arginine vasotocin induces elevation of blood pressure in anesthetized trout. Peptides **12**:477-482.

Miyata, T., Hayashida, H., Kikuno, R., and Yasunaga, T. 1986. Computer analysis of homology between genes. *In* Methods in Genetics (Takanami, M., Nishimura, S., and Muramatsu, M. eds) Tokyo Kagaku Dojin, Tokyo. pp 381-425.

Morley, S.D., Schönrock, C., Heierhorst, J., Figueroa, J., Lederis, K., and Richter, D. 1990. Vasotocin genes of the teleost fish *Catostomus commersoni*: Gene structure, exon-intron boundary, and hormone precursor organization. Biochemistry **29**:2506-2511.

Nojiri, H., Ishida, I., Miyashita, E., Sato, M., Urano, A., and Deguchi, T. 1987. Cloning and sequence analysis of cDNAs for neurohypophysial hormones vasotocin and mesotocin from the hypothalamus of toad, *Bufo japonicus*. Proceedings of the National Academy of Sciences (USA) **84**:3043-3046.

Perrott, M.N., Carrick, S., and Balment, R.J. 1991. Pituitary and plasma arginine vasotocin levels in teleost fish. General and Comparative Endocrinology **83**:68-74.

Proux, J.P., Miller, C.A., Li, J.P., Carney, R.L., Girardie, A., Dellate, M., and Schooley, D.A. 1987. Identification of an arginine-vasopressin-like diuretic hormone from *Locusta migratoria*. Biochemical and Biophysical Research Communications **149**:180-186.

Rathmayer, W. 1979. Sea anenome toxins: Tools in the study of excitable membranes. *In* Advances in Cytopharmacology, Volume 3. (Ceccarellic B., and Clementi, F., eds.) Raven Press, New York, pp. 335-344.

Richter, D. 1988. Molecular events in expression of vasopressin and oxytocin and their cognate receptors. American Journal of Physiology **255**:F207-219.

Rouillé, Y., Michel, G., Chauvet, M.T., Chauvet, J., and Acher,R. 1989. Hydrins, hydroosmotic neurohypophysial peptides: Osmoregulatory adaptation in amphibians through vasotocin precursor processing. Proceedings of the National Academy of Sciences (USA) **86**:5272-5275.

Schmale, H., and Richter, D. 1984. Single base deletion in the vasopressin gene is the cause of diabetes insipidus in Brattleboro rats. Nature **308**:705-709.

Schmale, H., Borowiak, B., Holtgreve-Grez, H., and Richter, D. 1989. Impact of altered protein structures on the intracellular traffic of a mutated vasopressin precursor from Brattleboro rats. European Journal of Biochemistry **182**:621-627.

Sherwood, N.M., and Parker, D.B. 1990. Neuropeptide families: An evolutionary perspective. The Journal of Experimental Zoology Supplement **4**:63-71.

Thomas, P.S. 1983. Hybridization of denatured RNA transferred or dotted to nitrocellulose paper. Methods in Enzymology **100**:255-266.

Uyeno, T., and Smith, G.R. 1972. Tetraploid origin of the karyotype of the catostomid fishes. Science **125**:644-646.

Wilson, A.C., Carlson, S.S., and White, T.J. 1977. Biochemical evolution. Annual Review of Biochemistry **46**:573-579.

Chapter 5

Isolation and characterization of different brain-specific α-tubulins isolated from chum salmon (*Oncorhynchus keta*) brain.

Introduction

As a component of the cellular matrix which forms and maintains subcellular architecture, tubulin plays an important role in intracellular communication. Microtubules may be an important element in effecting receptor mediated responses within the cell and it was recently suggested that tubulin may play a role in GnRH receptor-G protein coupling in the rat anterior pituitary lobe (Ravindra and Aronstam 1990). Thus, isolation of a brain (or pituitary) specific tubulin may provide a tool for further study of the peptide/receptor interactions of GnRH in fish. Microtubules also seem to be responsible for the integrity and intracellular distribution of the Golgi apparatus, which is an important element in the secretion and processing of neurohormones (Skoufias *et al.* 1990). In addition, the study of differential tubulin expression may prove to be an important model system for understanding eukaryotic gene regulation (Cleveland and Sullivan 1985).

Microtubules are involved in a number of cellular processes, such as chromosomal movement, cell migration, cell division, cell motility, intracellular transport,

maintenance of cell shape and secretion. The major protein constituent of microtubules is tubulin which is a heterodimer composed of α- and β-subunits, each having a molecular mass of 50 kDa. These important structural proteins are encoded by multigene families, each containing approximately equal numbers of α- and β- genes. Tubulin genes have been identified in a diverse array of organisms including various fungi, trypanosomes, invertebrates and higher vertebrates (Raff 1984, Cleveland and Sullivan 1985, Sullivan 1988). In mammals, both α- and β- tubulins appear to be encoded by about 6 or 7 functional genes (Wang *et al.* 1986, Villasante *et al.* 1986). The presence of pseudogenes has also been noted (Lemischke and Sharp 1982, Lee *et al.* 1983). Sequence data from many species have revealed that a complex array of different isotypes are expressed in an individual. The multitubulin hypothesis (Cleveland *et al.* 1980) suggests that a variety of tubulin isotypes is needed to provide distinct structures and functions. The α- and β-tubulins are probably derived from a single ancestral sequence since the amino acid sequence identity between them is generally about 40-50% (Cleveland and Sullivan 1985). This low level of similarity and the presence of distinct α- and β-tubulins in yeast and lower invertebrates suggests that the divergence is probably ancient (Raff 1984). However, α-tubulin and β-tubulin appear to have been separately, strongly conserved throughout evolution (Sullivan 1988).

Different isotypes may have different patterns of expression during development and differentiation. In mammals, one particular isotype, Tα1 tubulin, is associated with development of neural growth cones (Miller *et al.* 1990) and studies on the control and regulation of its expresion have been especially important in trying to understand axonal regeneration (Tetzlaff *et al.* 1991). However, in general, the relationship between microtubule heterogeneity and function remains obscure, as do the mechanisms by which cells regulate the molecular properties of their microtubules. In addition, many tubulin isotypes have not yet been isolated and completely characterized. It is also becoming clear that considerable tubulin heterogeneity is generated by post-translational modifications such as addition of glutamic acid residues (Edde *et al.* 1990), tyrosination and detyrosination (Schulze *et al.* 1987), and acetylation (L'Hernault and Rosenbaum 1985).

There is a considerable body of work using antibodies to various isotypes and regions of isotypes that suggests that cellular microtubules are assembled as copolymers from all available isotypes and that there is no preferential utilization of any particular isotype in any subset of microtubles (Lewis *et al.* 1987, Lopata and Cleveland 1987). In *Drosophila*, it has been shown that a single testis-specific β-tubulin gene product may fulfill a number of different microtubule functions (Raff 1984). Thus, although there is a high degree of evolutionary conservation of

primary sequence and patterns of expression, multiple tubulin isotypes may not contribute to specifying essential functional distinctions. Such interpretations may result from the ambiguous nature of these antibody techniques, but if this is the case, it is not clear exactly what the evolutionary pressures are that maintain these families. It has been suggested that the presence of different isotypes in a single species does not have any underlying functional basis but sequence variations simply reflect the slow ticking of the evolutionary clock since the time of gene duplication (Lopata and Cleveland 1987). However, this does not explain the observation that isotypes from the same tissue of different species have more sequence identity with one another than do isotypes from different tissues in the same species (Cleveland and Sullivan 1985). In addition, post-translational modifications have been shown to be important in the diversification of function of microtubules in certain tissues such as the seminiferous epithelium (Hermo *et al.* 1990). Perhaps what is more likely is that the variety of post-translational modifications and variations in sequence at the carboxy terminal of the tubulins provide the basis and mechanisms for subtle functional differences which are difficult to detect.

Despite the importance of this protein, especially in the brain (where tubulin heterogeneity is particularly high perhaps reflecting its numerous roles) and its putative role in mediating responses to receptor activation, no tubulin

isotypes have been isolated from this tissue in fish. While there is a growing body of work on tubulins in other vertebrates and many invertebrates, the fish have been largely ignored. For this reason we decided to isolate and characterize an α-tubulin from salmon brain.

In addition, tubulin is a highly expressed and ubiquitous molecule. It would, therefore, be relatively easy to isolate various isotypes. This allowed us to develop and refine techniques and procedures in advance of attempting the isolation of mRNAs of extremely low abundance.

Materials and Methods

Materials

Nylon filters, Bluescript plasmid and restriction enzymes were purchased from Stratagene. Klenow enzyme was purchased from Boehringer Mannheim (Laval, Quebec). Random primer (dp(N)6), dNTPs, and T4 polynucleotide kinase were purchased from Pharmacia. T4 DNA ligase was purchased Bethesda Research Laboratories. A Sequenase sequencing kit was purchased from United States Biochemicals. Radioisotopes were purchased from New England Nuclear/Dupont.

Library Construction and Screening

A trout testis α-tubulin clone, kindly provided by Dr. Tony Garber, University of Calgary Medical School, was used

to screen an amplified chum salmon brain cDNA library constructed in lambda ZAPII (see chapter three). Approximately 25,000 pfu were plated out onto a single LB plate in LB top agarose with 10mM Mg^{2+}. The plate was incubated at 37°C overnight. Plaques were transferred to nylon filters for either 1 min (first filter) or 1.5 min (second filter). Filters were then placed in denaturing solution (1.5M NaCl, 0.5M NaOH) DNA side up for 1 min., followed by neutralising solution (1.5M NaCl, 0.5M Tris, pH 8.0) for 5 min. and finally into 2 x SET (20 x SET is 3M NaCl, 30mM EDTA, 300mM Tris, pH7.5) for at least 5 min. After being allowed to dry, filters were then baked at 80°C for two hours. Prehybridization was carried out at 65°C for approximately 24 hrs. Prehybridization solution consisted of 5 x Denhardts solution, 6 x SET and 0.1% (w/v) SDS. Hybridization took place at 65°C for 12-24 hrs. Hybridization solution consisted of fresh prehybridization solution with the addition of labelled probe to a final concentration of approximately 1 x 10^6 cpm/ml hybridization solution.

Probe Labelling

The probe was supplied as a clone in pTZ19R. In order to avoid hybridization with the Bluescript portion of lambda ZapII, the probe was excised from the plasmid using EcoRI. The EcoRI fragment was then labelled using a modified Vogelstein reaction (Hodgson and [illegible] 1987). Briefly, 60 ng

of random primer was combined with approximately 50-200 ng of probe in 2.5 μl of TE and placed in a boiling water bath for two minutes then on ice. To this mixture was added: 2 μl of 10X oligolabelling buffer (0.5 M Tris, pH 6.9,), 0.1 M $MgSO_4$, 1mM DTT and 0.6 mM each of dCTP, dGTP, and dTTP), 50 μCi of ^{32}P-dATP and 2-10 units of Klenow. The reaction was incubated at room temperature for one hour and unincorporated nucleotides removed by passing the reaction over a NAP-5 column (Pharmacia) equilibrated in TE (according to manufacturer's instructions). The labelled probe was boiled before addition to the hybridization solution.

Sequencing

Positive lambda clones were subjected to helper phage rescue to isolate the Bluescript plasmid containing the putative tubulin insert. Plasmid DNA was prepared using standard alkaline lysis miniprep techniques. Plasmid DNA was alkaline denatured and sequenced using a Sequenase (USB) sequencing kit according to manufacturer's instructions. The insert was sequenced from both ends using Reverse, -20, SK and KS primers (Stratagene). Positive inserts were also excised with EcoRI, subjected to restriction digests using HincII and PvuII and resulting fragments subcloned and sequenced. A nested deletion kit (Stratagene) was also used in an attempt to sequence the entire clone and finally, 17 base primers were synthesized to internal regions of the

clone and used to PCR and sequence the middle part of the whole insert in both directions.

Southern Blots

Genomic DNA was extracted from chum salmon livers using standard techniques (Ausubel *et al*. 1989). Briefly, approximately 13 g of tissue was ground up and added to 100 ml digestion buffer (100 mM NaCl, 10mM Tris, pH 8.0, 25mM EDTA, pH8.0, 0.5% SDS, 0.1 mg/ml proteinase K). This mixture was incubated overnight at 50°C with gentle shaking. It was then phenol/chloroform extracted once and precipitated with ammonium acetate and 100% ethanol. The resulting DNA was spun down, washed with 80% ethanol and dried. The DNA was redissolved in TE and dialyzed against several volumes of TE overnight. The OD of the DNA solution was measured to determine the concentration and quality. Genomic digests were set up using 13 μg of DNA in a total volume of 20 μl according to the manufacturers' recommendations for each restriction enzyme. Digests were run on a 1% agarose gel in 0.5 x TBE for 12-20 hours.

Northern Blots

Total RNA was isolated from a variety of tissues by homogenization in guanidium isothiocyanate buffer followed by repeated extraction with phenol and chloroform (Chomczynski and Sacchi 1987). The RNAs (5 μg) were fractionated on a 1.2% agarose-formaldehyde gel (Ausubel *et*

al. 1989) and transferred to Zeta-Probe membrane (BioRad) using standard methods except that the transfer took place in 10 X SSC (instead of 20 X SSC). Filters were prehybridized and then probed with a random primed EcoRI fragment of pTUB 5.2. Hybridization took place overnight and filters were then washed in 1 X SSC/0.1% SDS (2 X 15 min.) followed by 0.2 X SSC/0.1% SDS (2 X 15 min.) then exposed for 48 hr with intensifying screens. Prehybridization solution consisted of 25mM KH_2PO4, pH7.4; 5 X SSC; 5 X Denhardts solution; 50% formamide. Hybridization solution consisted of prehybridization solution with the addition of 10% (final concentration) dextran sulfate.

Results

From an initial screen of 25,000 pfu's, 14 positive clones were identified of which four were then purified to sequence. These four all proved to be α-tubulin based on comparison with the trout testis sequence. One clone, pTUB 5.2, contained the entire protein coding sequence for α-tubulin whereas the others, pTUB 6.1, 6.2 and 9, were each missing part of the 5' region of the protein coding sequence. The overall structures of all clones are shown in figure 26 along with a restriction map of pTUB 5.2. The cDNA sequence of the full length clone, pTUB 5.2, along with its predicted amino acid sequence is shown in figure 27. This clone contained the entire coding sequence, 444 amino acids, molecular weight of approximately 48,840 Daltons, of

a fish α-tubulin sequence and has 15 bp of 5'-untranslated sequence and 149 bp of 3'-untranslated sequence excluding the poly(A) tail. The start codon is not preceded by the consensus sequence GCCGCC$^{A}/_{G}$CC for the initiation of translation found in many vertebrate mRNAs (Kozak 1984, 1987). This consensus sequence is not present in the trout tubulin either (Garber *et al.* 1991). The pTUB 5.2 cDNA sequence was compared against other tubulins in both the GenBank database, version 70 and the EMBL database, version 29, using Intelligenetics software. The top scoring match was with Chinese hamster α-tubulin III (locus name HAMTUBAC) which showed 81% sequence identity at the nucleic acid level. The trout testis α-tubulin (locus name SMOTUBATS), which was used to screen the library, has 80% sequence identity with the salmon brain α-tubulin at the nucleic acid level. The trout testis protein possesses six more amino acids at the N-terminal compared to the salmon tubulin. When the trout and salmon tubulins were aligned it became apparent that the trout tubulin possessed an isoleucine in the position at which the methionine start codon in the salmon tubulin was found. It therefore appears that the salmon tubulin has an alternative, later start site and is slightly shorter as a result. However, given the short 5' untranslated region, it is possible that an additional methionine, which acts as the start codon, is present in the same position as the the trout clone.

Initial sequencing indicated that clones 6.1 and 6.2

were identical so only 6.1 was analysed further. These truncated clones and the relatively short 5'-untranslated region of pTUB 5.2 are probably due to incomplete cDNA synthesis (*i*.e. the reverse transcriptase had not synthesized a full length transcript from the oligo dT primer). Analysis and comparison of the carboxy terminal region of these proteins (fig. 28) as well as their 3'-untranslated regions (fig. 29) indicates that they are different isotypes which have a high degree of sequence identity at the protein level, but which vary considerably in their 3'-untranslated regions (fig. 29).

All the isotypes have a characteristic microtubule associated protein (MAP) and dynein interaction sites, encoded by amino acid sequence EEGEEY, at the carboxy terminus of the protein (fig. 28).

Northern blot analysis indicated that the brain isotypes are highly tissue specific with apparently much greater expression occurring in the brain than in either muscle, heart or liver (fig. 30). It should be noted that, given the sequence similarity of the three isotypes isolated from salmon brain, the signal seen on the Northern blot in the brain sample is probably due to hybridization of pTUB 5.2 to all three isotypes (and possibly others of almost identical sequence). Weak hybridization signals of approximately similar sizes were observed in the other tissues indicating either low level expression of the brain isotypes or weak cross-hybridization to other isotypes with low sequence

similarity.

Southern blot analysis (fig. 31) indicates that there may be up to four genes encoding α-tubulin in the salmon genome. There is probably considerable cross-hybridization between pTUB 5.2 and other α-tubulin genes therefore it is not possible to determine how many genes encode pTUB 5.2 only. Restriction enzymes NotI, EcoRV, EcoRI and BglI do not cut pTUB 5.2. Restriction enzymes PstI, HindIII and BamHI cut pTUB 5.2 at one site each (fig. 26). HaeIII cuts at 10 sites.

Figure 26. Schematic diagram showing structures of chum salmon (*Oncoryhnchus keta*) brain α-tubulin clones, pTUB 5.2, pTUB 6.1, and pTUB 9. The 5' untranslated region (utr) is followed by the protein coding region, the 3' untranslated region and polyadenosine tail (A). Restriction sites for three enzymes are shown for clone pTUB 5.2. The asterisks indicate the variable region which is involved in interaction with microtubule associated proteins and dynein. Not to scale.

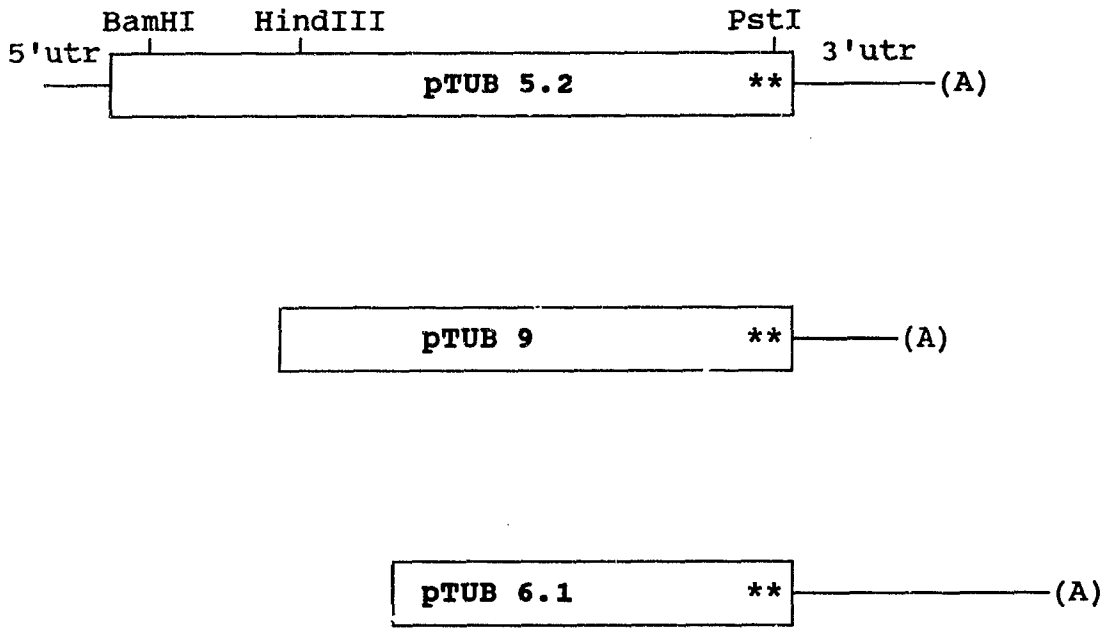

BamHI
HindIII
PstI
5'utr
3'utr
pTUB 5.2
**
(A)
pTUB 9
**
(A)
pTUB 6.1
**
(A)

Figure 27. The complete cDNA coding sequence of the pTUB 5.2 insert aligned with its predicted amino acid sequence.

```
          10         20         30         40         50
CGCGAGGTTATTTCCATGCATGTGGGCCAAGCCGGAGTCCAGATGGGTAACGCCTGTTGG
                M  H  V  G  Q  A  G  V  Q  M  G  N  A  C  W

          70         80         90         100        110
GAGCTGTACTGCCTGGAGCATGGGATCCAGCCGGACGGACAGATGCCCAGTGACAAGACC
E  L  Y  C  L  E  H  G  I  Q  P  D  G  Q  M  P  S  A  K  T

          130        140        150        160        170
TGTGGAGGTGGAGACGACTCCTTCAACACCTTCTTCAGTGAGACCGGAGCTGGAAAGCAT
C  G  G  G  D  D  S  F  N  T  F  F  S  E  T  G  A  G  K  H

          190        200        210        220        230
GTCCCCCGTGCCATCTTCGTTGATCTGGAGCCCACTGTCATCGATGAGGTGAGGACAGGT
V  P  R  A  I  F  V  D  L  E  P  T  V  I  D  E  V  R  T  G

          250        260        270        280        290
ATTTATCGTCAGTTGTTCCACCCTGAGCAGCTGATCACTGGTAAGGAAGATGCTGCCAAC
I  Y  R  Q  L  F  H  P  E  Q  L  I  T  G  K  E  D  A  A  N

          310        320        330        340        350
AACTACGCCCGCGGTCACTACACCATCGGCAAGGAGATCATTGACATTGTGCTGGACAGG
N  Y  A  R  G  H  Y  T  I  G  K  E  I  I  D  I  V  L  D  R

          370        380        390        400        410
ACACGCAAACTGGCTGACCAGTGTACAGGTCTCCAGGGGTTCCTCATCTTCCACAGCTTC
T  R  K  L  A  D  Q  C  T  G  L  Q  G  F  L  I  F  H  S  F

          430        440        450        460        470
GGAGGAGGCACCGGTTCTGGTTTTACCTCCCTCCTGATGGAACGTCTGTCTGTCGACTAC
G  G  G  T  G  S  G  F  T  S  L  L  M  E  R  L  S  V  D  Y

          490        500        510        520        530
GGAAAGAAGTCTAAGCTTGAGTTTGCCGTTTACCCAGCTCCCCAGGTGTCCACGGCTGTG
G  K  K  S  K  L  E  F  A  V  Y  P  A  P  Q  V  S  T  A  V

          550        560        570        580        590
GTGGAGCCCTACAACTCCATCCTGACCACTCACACCACCCTGGAGCACTCCGACTGTGCC
V  E  P  Y  N  S  I  L  T  T  H  T  T  L  E  H  S  D  C  A

          610        620        630        640        650
TTCATGGTGGACAATGAGGCCATCTATGTCATCTGCCGTAGAAACCTTGACATTGAGCGT
F  M  V  D  N  E  A  I  Y  V  I  C  R  R  N  L  D  I  E  R

          670        680        690        700        710
CCCTCGTACACCAACCTCAACAGGCTCATTGGTCAGATCGTCTCCTCCATCACTGCTTCC
P  S  Y  T  N  L  N  R  L  I  G  Q  I  V  S  S  I  T  A  S

          730        740        750        760        770
CTGCGTTTCGATGGAGCCCTGAATGTGGATCTGACAGAGTTCCAGACCAACTTGGTGCCC
L  R  F  D  G  A  L  N  V  D  L  T  E  F  Q  T  N  L  V  P
```

```
         790       800       810       820       830
TACCCCCGTATCCACTTCCCTCTCGCCACATATGCCCCGGTCATCTCGGCTGAGAAGGCC
Y  P  R  I  H  F  P  L  A  T  Y  A  P  V  I  S  A  E  K  A

         850       860       870       880       890
TATCACGAGCAGCTGTCAGTCGCTGACATCACCAACGCCTGCTTCGAGCCGGCCAATCAG
Y  H  E  Q  L  S  V  A  D  I  T  N  A  C  F  E  P  A  N  Q

         910       920       930       940       950
ATGGTGAAGTGTGACCCTCGTCACGGCAAATACATGGCCTGCTGTCTCCTGTACCGTGGT
M  V  K  C  D  P  R  H  G  K  Y  M  A  C  C  L  L  Y  R  G

         970       980       990       1000      1010
GACGTTGTTCCCAAAGATGTCAACTCTGCCATCGCTGCCATCAAAACCAAGAGGAGCATC
D  V  V  P  K  D  V  N  S  A  I  A  A  I  K  T  K  R  S  I

         1030      1040      1050      1060      1070
CAGTTTGTGGACTGGTGTCCCACTGGCTTCAAGGTGGGTATCAACTACCAGCCCCCTACG
Q  F  V  D  W  C  P  T  G  F  K  V  G  I  N  Y  Q  P  P  T

         1090      1100      1110      1120      1130
GTGGTTCCTGGAGGAGACCTGGCCAAGGTGCAGAGGGCTGTGTGCATGCTGAGTAACACC
V  V  P  G  G  D  L  A  K  V  Q  R  A  V  C  M  L  S  N  T

         1150      1160      1170      1180      1190
ACAGCCATCGCTGAGGCCTGGGCCCGTCTGGACCACAAGTTTGACCTGATGTATGCCAAG
T  A  I  A  E  A  W  A  R  L  D  H  K  F  D  L  M  Y  A  K

         1210      1220      1230      1240      1250
AGAGCCTTTGTACACTGGTACGTTGGTGAGGGCATGGAGGAGGGAGAGTTCTCTGAGGCC
R  A  F  V  H  W  Y  V  G  E  G  M  E  E  G  E  F  S  E  A

         1270      1280      1290      1300      1310
AGAGAAGACATGGCAGCCCTGGAGAAGGATTATGAAGAGGTGGGCACTGACAGCGTGGGA
R  E  D  M  A  A  L  E  K  D  Y  E  E  V  G  T  D  S  V  G

         1330      1340      1350      1360      1370
GAAGAGGATGAGGAGGGAGAGGAATATTAAAAGGCTAAAAACATGCTTTCCACCCTACAT
E  E  D  E  E  G  E  E  Y  Ter

         1390      1400      1410      1420      1430
TCCAACACTGTTTAGTCCTACACTTAACATGACAGCAACACATTCCCCACTGCAGTGTTA

         1450      1460      1470      1480      1490
ACAGTCTCTGTTGCATCTCGTCATGTTGTGTGTATTCCCAATAAACGCATCGTAAC(A)n
                                        ------
```

Figure 28. Comparison of the 3' coding regions of α-tubulin clones isolated from chum salmon (*Oncorynchus keta*) brain. MAP2 and dynein interaction sites shown in bold. Variations in amino acids between clones are indicated with an asterisk.

```
..............F  S  E  A  R  E  D  M  A  A  L  E  K  D
pTUB5.2:.......TTCTCTGAGGCCAGAGAAGACATGGCAGCCCTGGAGAAGGAT

..............F  S  E  A  R  E  D  M  A  A  L  E  K  D
pTUB9:.........TTCTCAGAGGCCAGAGAAGACATGGCAGCCCTGGAGAAGGAT

..............F  S  E  A  R  E  D  M  A  A  L  E  K  D
pTUB6.1:.......TTCTCTGAAGCCAGAGAGGACATGGCAGCCCTGGAGAAGGAT
```

```
.............Y  E  E  V  G  T  D  S  V  G  E  E  D  E
pTUB5.2:.....TATGAAGAGGTGGGCACTGACAGCGTGGGAGAAGAGGATGAG
                                       *           *
.............Y  E  E  V  G  T  D  S  I  G  E  E  A  E
pTUB9:.......TATGAAGAGGTGGGTACTGACAGCATCGGAGAAGAGGATGAA
                              *           *  *     *
.............Y  E  E  V  G  V  D  S  I  E  G  E  G  E
pTUB6.1:.....TACGAGGAGGTAGGGGTTGACTCCATTGAGGGGGAGGGAGAG
```

```
.............E  G  E  E  Y  Ter
pTUB5.2:.....GAGGGAGAGGAATATTAA

.............E  G  E  E  Y  Ter
pTUB9:.......GAAGGAGAGGAGTACTAA
             *  *     *  *
.............E  E  G  E  E  Y  Ter
pTUB6.1:.....GAGGAGGGAGAGGAGTATTGA
```

Figure 29. Comparison of 3' untranslated regions of chum salmon (*Oncorhynchus keta*) brain and trout (*Oncorhynchus mykiss*) testis α-tubulins. Putative polyadenylation signals are underlined. The polyadenylation signal of pTUB9 and pTUB6.1 could not be defined.

```
                                    10        20        30        40
pTUB 5(testis) :TerTGAATCCTGACCTGATTGAAAAATGTTTTACTGTTGATTGG

pTUB 5.2(brain):TerAAGGCTAAAAACATGCTTTCCACCCTACATTCCAACACTGT

pTUB 6.1(brain):TerAATGTCCCTGCAGGCAAGACATTGTC(A)n

pTUB 9(brain)  :TerAGGGTGTAATTCCTGATCTGCTGATGTTCCATTAATATAGA


                  50        60        70        80        90
pTUB 5..:TAATTTTAATAAAAGTTCTGTTTTC(A)n

pTUB 5.2:TTAGGCCTACACTTAACATGACAGCAACACATTCCCCACTGCAGTGTTAAC

pTUB 9..:CTACCAGTCCCGGATAGTGCAAGTCGTAAGAAAACAACAAAAAGGTTTTAA


                 100       110       120       130       140
pTUB 5.2:AGTCTCTGTTGCATCTCGTCATGTTGTGTGTATTCCCAATAAACGCATCGT

pTUB 9..:CGGACTAATATTGGAAATCATTCCATAGGAAGCACATTCCACCCAGCTACA


               150       160       170       180       190
pTUB 5.2:AGC(A)n

pTUB 9..:AGAAGAACCTTCCTGCCCTCACATCAGCCATTTTGATAAATTATG(A)14
```

Figure 30. Northern blot analysis of chum salmon (*Oncorhynchus keta*) tissue hybridized to the complete pTUB 5.2 insert. Tissue was extracted from brain (B), heart (H), liver (L) and muscle (M). The top arrow on the left indicates the position of the pTUB5.2 transcript in the brain RNA and similar signals in the other tissues. The arrow on the right indicates the presence of an additional band in muscle. The lower arrows on the left indicate the presence of additional bands hybridizing to the pTUB5.2 clone in brain RNA.

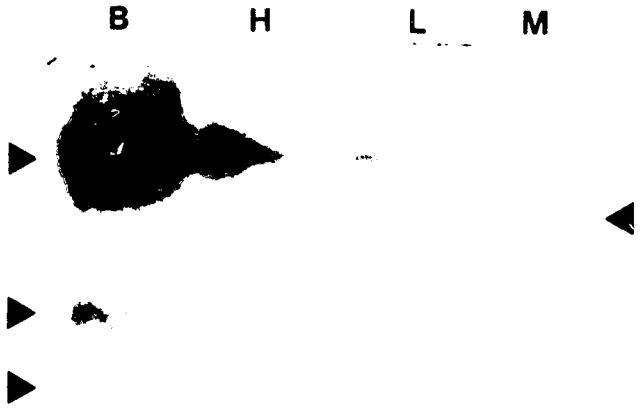
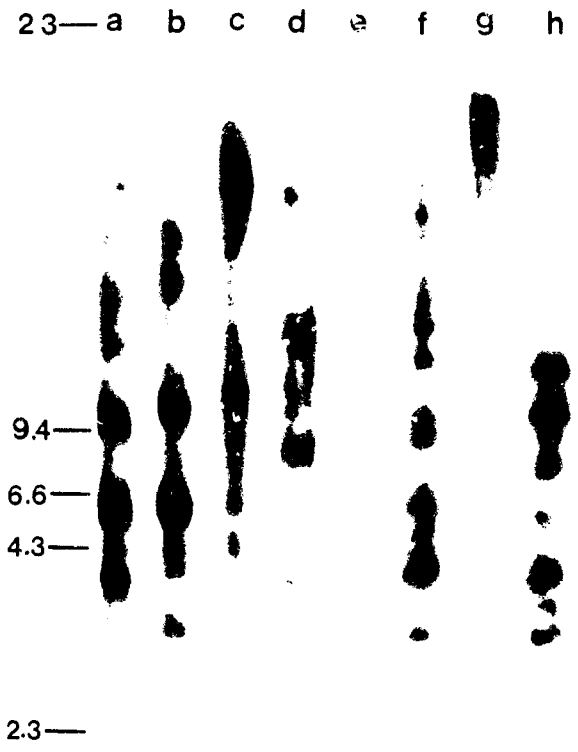

B
H
L
M

Figure 31. Southern blot analysis of chum salmon (*Oncorhynchus keta*) genomic DNA hybridized with the entire pTUB 5.2 insert. Lanes a-h represent digests with the following enzymes repectively, BamHI, BglI. EcoRI, EcoRV, HaeIII, HindIII, NotI and PstI.
Sizes are indicated in kilobases on the left.


23
a
b
c
d
e
f
g
h
9.4
6.6
4.3
2.3

Discussion

The α-tubulins isolated from the chum salmon brain show considerable sequence identity with previously identified α-tubulin and especially with other brain specific α-tubulins (Valenzuela *et al.* 1981). The presence of a number of α-tubulin genes in the salmon is consistent with findings for other vertebrates (Raff 1984, Wang *et al.* 1986, Villasante *et al.* 1986, Sullivan 1988). The high degree of sequence identity with the trout testis α-tubulin is not surprising given the close phylogenetic relationship of the two groups and conservation of tubulins in general.

It is interesting to note that the salmon brain tubulin may have acquired an alternative start codon compared to the testis clone. It is impossible to say whether this is the same for the other clones given the truncated 5' regions. The substitution between an isoleucine in the testis tubulin and a methionine in the brain tubulin is a conservative one requiring only a single base change in the third position.

It has been shown for mouse neuronal α-tubulin that a small proportion of transcripts (<5%) have a post-translational modification which is the acetylation (by tubulin acetyltransferase) of a lysine at position 40 (Eddé *et al.* 1990). There is no lysine at this numerical position in the salmon clone although there is a lysine at position 34. However, if the salmon tubulin possesses a start codon in the same position as the trout clone then this lysine would be residue 40 suggesting that it may be available for

post-translational modification. The function of this modification is not clear although it may enhance stability.

It is already apparent that most of the variation in the protein sequence of α-tubulins occurs between residues 35 and 55 and at the carboxy end of the protei (Sullivan 1988). Only clones pTUB 5.2 and pTUB 9 extend into this region and are virtually identical (only 3 out of 20 amino acids are different). Carboxy terminal variation is much more apparent for the three isotypes isolated from the salmon brain. Clone pTUB 6.1 appears to be a highly conserved isotype (the major neural development isotype in a number of other vertebrates) based on its carboxy terminal sequence (Sullivan 1988). Clones pTUB 9 and pTUB 5.2 do not seem to be highly conserved isotypes based on their C-terminal sequences although pTUB 5.2 may be similar to cα8, a minor constitutive protein identified in many chicken tissues (Pratt and Cleveland 1988). It is possible that pTUB 6.1, as a major constituent of the neural α-tubulin available in the brain, is the source of the strong signal on the Northern blot, although pTUB 5.2 is probably also being expressed at detectable levels. In contrast, the trout testis α-tubulin clone has enough sequence similarity to other isotypes to cross-hybridize with tubulins in many tissues and only the distinct 3'untranslated region could be used to demonstrate tissue-specific expression (Garber *et al.* 1991).

While there may be sequence similarities between

isotypes from different species, they may not imply functional similarities. Indeed, even tissue specificity of expression may not necessarily imply any restriction of functional specificity (Raff 1984).

The C-terminal region of tubulin is exposed on the outer surface of the microtubule lattice and has been shown to be important in interactions with dynein and calcium (Littauer *et al.* 1986, Serrano *et al.* 1986, Maccioni *et al.* 1988, Edde *et al.* 1990) and microtubule-associated proteins (MAPs) (Paschal *et al.* 1989). The sequence, Glu-Gly-Glu-Glu (EGEE), has been implicated as site of the interaction with both MAP2 and dynein (Paschal *et al.* 1989). This sequence is highly conserved amongst tubulins from many different organisms and is also found in the salmon isotypes. These interactions may affect and regulate microtubule function and dynamics.

Post-translational modifications provide a broader spectrum on which the regulatory mechanisms can act and a number of these modifications take place at the C-terminus. Progressive addition of glutamyl units onto a glutamate residue at this end represents a major modification of neuronal α-tubulin in other vertebrates and all the salmon brain tubulins have this modification to varying degrees. Addition and removal of a tyrosine (Y) residue at the carboxy terminus (whether or not it is encoded) is another modification which takes place in a cyclic fashion in the cell as microtubules are depolymerized and formed (Webster

et al. 1987, Sullivan 1988, Edde *et al.* 1990). All the salmon isotypes possess the final tyrosine residue indicating that they are probably part of the soluble cellular pool of available tubulins. In the vertebrate central nervous system, differentiation of certain neuronal populations appears to be associated with extensive detyrosination of axonal microtubules (Sullivan 1988). Areas around Golgi apparatus and nerve growth cones in particular appear to be enriched with tyrosinated α-tubulin (Skoufias *et al.* 1990, Matten *et al.* 1990). Whether these modifications are permanent and the functional basis underlying such changes is unclear since tyrosination does not seem to be associated with microtubule stability (Idriss *et al.* 1991).

These small and subtle changes in base sequence may be an indication of how the molecular evolution of a particular DNA sequence may produce different proteins allowing for alternative regulatory or functional options. It is difficult to show, however, that such minor changes have any significant biochemical or physiological effects. Many structural proteins such as actin, collagen, keratin and globin exist as small families of distinct but related genes. The selective pressures that maintain these families are not clear although two mechanisms have been proposed (Raff 1984). The first is that the slightly different gene products have slightly different biochemical characteristics allowing subtly different functional variations.

Alternatively, the different genes may have arisen so that cells could exert alternate regulation of expression of basic structural proteins. What actually happens within the organism is probably a combination of both. In addition, there may be a degree of redundancy within the genome which allows proteins with slightly different sequences to function within the same capacity while gradually drifting genetically. Various tubulin mutants which possess altered or reduced functions have been identified in *Drosophila*, *Caenorhabditis elegans* and *Chlamydomonas* which indicates that such drifting occurs (Raff 1984). The presence of multiple tubulin genes may allow eukaryotes to co-ordinate tubulin synthesis with multiple programs of expression (Raff 1984). Redundancy may also exist because of the need for large amounts of gene products (Ohta 1991). This may be particularly true for important structural proteins such as tubulin.

Literature Cited

Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A., and Struhl, K. Current Protocols in Molecular Biology. Greene Publishing Associates and Wiley-Interscience, New York.

Chomczynski, P., and Sacchi, N. 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Analytical Biochemistry **162:** 156-159.

Cleveland, D.W., Lopata, M.A., MacDonald, R.J., Cowan, N.J., and Rutter, W.J., and Kirschner , M.W. 1980. Number and evolutionary conservation of alpha- and beta-tubulin and cytoplasmic beta- and gamma-actin genes using specific cloned cDNAs probes. Cell **20**:95-105.

Cleveland, D.W. and Sullivan, K.F. 1985. Molecular biology and genetics of tubulin. Annual Review of Biochemistry **54**:331-365.

Eddé, B., Rossier, J., Le Caer, J.-P., Desbruyeres, E., Gros, F., and Denoulet, P. 1990. Posttranslational glutamylation of α-tubulin. Science **247**:83-85.

Garber, A.T., Hawkins, N., and Dixon, G.H. 1991. The predicted protein sequence of a fish testis-specific α-tubulin cDNA shows conservation of isotype-specific amino acid substitutions. DNA Sequence **2**:47-51.

Hermo, L., Oko, R., and Hecht, N.B. 1990. Differential post-translational modification of microtubules in cells of the seminiferous epithelium of the rat: A light and electron microscope immunocytochemical study. Anatomical Record **229**:31-50.

Hodgson, C.P and Fisk, R.Z. 1987. Hybridization probe size control: optimized `oligolabelling'. Nucleic Acids Research **15**: 6295.

Idriss, H., Stammers, D.K., Ross, C.K., and Burns, R.G. 1991. The dynamic instability of microtubules is not modulated by alpha-tubulin tyrosinylation. Cell Motility and the Cytoskeleton **20**:30-37.

Kozak, M. 1984. Compilation and analysis of sequences upstream from the translational start site in eukaryotic mRNAs. Nucleic Acids Research **12**:857-872.

Kozak, M. 1987. An analysis of 5'-noncoding sequences from 699 vertebrate messenger RNAs. Nucleic Acids Research **15**:8125-8148.

Lee, M G.-S., Lewis, S.A., Wilde, C.D. and Cowan, N.J. 1983. Evolutionary history of a multigene family: expressed human β-tubulin gene and three processed pseudogenes. Cell **33**:477-487.

Lemischke, I.R., and Sharp, P.A. 1982. The sequences of an expressed rat α-tubulin gene and a pseudogene with an inserted repetitive element. Nature (London) **300**:330-335.

Lewis, S.A., Gu, W., and Cowan, N.J. 1987. Free intermingling of mammalian β-tubulin isotypes among functionally distinct microtubules. Cell **49**:539-548.

Littauer, U.Z., Giveon, D., Thierauf, M., Ginzburg, I. and Postingl, H. 1986. Common and distinct tubulin binding sites for microtubule-associated proteins. Proceedings of the National Academy of Sciences (USA) **83**:7162-7166.

Lopata, M.A., and Cleveland, D.W. 1987. In vivo microtubules are copolymers of available β-tubulin isotypes: Localization of each of six vertebrate β-tubulin isotypes using polyclonal antibodies elicited by synthetic antigens. The Journal of Cell Biology **105**:1707-1720.

Matten, W.T., Aubrey, M., West, J., and Maness, P.F. 1990. Tubulin is phosphorylated at tyrosine by pp60-c-src in nerve growth cone membranes. The Journal of Cell Biology **111**:1959-1970.

Miller, F.D., Mathew, T.C., and Toma, J.G. 1991. Regulation of nerve growth factor receptor gene expression by nerve growth factor in the developing peripheral nervous system. The Journal of Cell Biology **112**:303-312.

Ohta, T. 1991. Multigene families and the evolution of complexity. Journal of Molecular Evolution. **33**:34-41.

Paschal, B.M., Obar, R.A., and Vallee, R.B. 1989. Interaction of brain cytoplasmic dynein and MAP2 with a common sequence at the C terminus of tubulin. Nature (London) **342**:569-572.

Raff, E.C. 1984. Genetics of microtubule systems. The Journal of Cell Biology **99**:1-10.

Ravindra, R., and Aronstam, R.S. 1990. Possible role of tubulin in GnRH receptor-G protein coupling in the rat anterior pituitary lobe. Society of Neuroscience Abstracts, **16**, 167.5.

Schulze, E., Asai, D.J., Bulinski, J.C., and Kirschner, M. 1987. Posttranslational modification and microtubule stability. The Journal of Cell Biology **105**:2167-2177.

Skoufias, D.A., Burgess, T.L., and Wilson, L. 1990. Spatial and temporal colocalization of the Golgi apparatus and microtubules rich in detyrosinated tubulin. Journal of Cell Biology **111**:1929-1938.

Sullivan, K.F. 1988. Structure and utilization of tubulin isotypes. Annual Review of Cell Biology **4**:687-716.

Tetzlaff, W., Alexander, S.W., Miller, F.D., and Bisby, M.A. 1991. Response of facial and rubospinal neurons to axotomy: Changes in messenger RNA expresssion for cytoskeletal proteins and GAP-43. Journal of Neuroscience **11**:2528-2544.

Valenzuela, P., Quiroga, M., Zaldivar, J., Rutter, W.J., Kirscher, M.W., and Cleveland, D.W. 1981. Nucleotide and corresponding amino acid sequences encoded by α and β tubulin mRNAs. Nature (London) **289**:650-655.

Villasante, A., Wang, D., Dobner, P., Dolph, P., Lewis, S.A., and Cowan, N.J. 1986. Six mouse α-tubulin mRNAs encode five distinct tubulin isotypes: Testis specific expression of two sister genes. Molecular and Cellular Biology **6**:2409-2419.

Wang, D., Villsante, A., Lewis, S.A., and Cowan, N.J. 1986. The mammalian β-tubulin repertoire: Hematopoietic expression of a novel, heterologous β-tubulin subtype. The Journal of Cell Biology **103**:1903-1910.

Webster, D.R., Gundersen, G.G., Bulinski, J.C., and Borisey, G.G. 1987. Assembly and turnover of detyrosinated tubulin in vivo. The Journal of Cell Biology **105**:265-276.

Chapter 6

General Discussion

The neuropeptides isolated from the salmon brain in this study provide examples of the different levels at which molecular evolution can take place. Changes can occur in entire coding regions, in gene organization, in protein coding regions and in individual nucleotides during evolution. Such changes at any of these levels, in combination or alone, can affect protein function and organismal survival.

Gene Duplication.

The simplest way for an organism to acquire proteins with new functions is probably through the duplication of a whole gene with subsequent differentiation by nucleotide substitution. Many multigene families such as the immunoglobulins, cytochrome P450, hemoglobins, growth hormone, interferon α, tubulin and others, are examples of this (Ohta 1991). Many of the homeobox genes appear to have been duplicated in higher vertebrates (Marx 1992). Duplicated coding sequences are free to differentiate and provide new functions that are useful to the organism, as in the immunoglobulins and, apparently, the tubulins. For others, differentiation of regulatory regions such as enhancers or promoters has occurred as in lactate

dehydrogenase and esterase (Ohta 1991).

Gonadotropin releasing hormone (GnRH) is an example of a peptide whose gene has apparently undergone duplication to give rise to an additional protein coding region which has evolved to produce a second form of the peptide. Figure 32 shows a simple evolutionary scheme for the formation of the GnRH family based on this scenario. The evidence for this is based on the observation that virtually all non-mammalian vertebrates possess multiple forms of GnRH. It is difficult to determine exactly when this duplication occurred but it probably predates the appearence of the cartilaginous fish since most of these species possess two forms (Lovejoy 1991). This extremely ancient duplication has presumably allowed considerable divergence to occur between the two genes accounting for the lack of hybridization between the cichlid sGnRH clone and non-sGnRH genes in the Pacific salmon. Within the teleosts there are a number of additional GnRH forms which have not been sequenced at the protein level (fig. 33). Whether these are due to additional gene duplications or nucleotide substitutions remains to be determined. Gene duplications usually result in the duplicated sequences being situated next to each other, in tandem. This can still be seen for some of the α-tubulins in the parasites, *Leishmania* and *Trypanosomes*. In these organisms the genes are tandemly duplicated either

Figure 32. Schematic diagram showing possible evolutionary history for the gonadotropin-releasing hormone (GnRH) family. The first time each form of GnRH appears it is underlined. Horizontal arrows indicate possible points of gene duplication. Vertical arrows indicate possible points of nucleotide substitution. The ancestral form of GnRH is not known. It is not clear where dogfish GnRH came from although it could possibly be due to a gene duplication. Within the teleosts, novel forms of GnRH are known to exist but there are not enough data to deduce the origin of the novel forms.

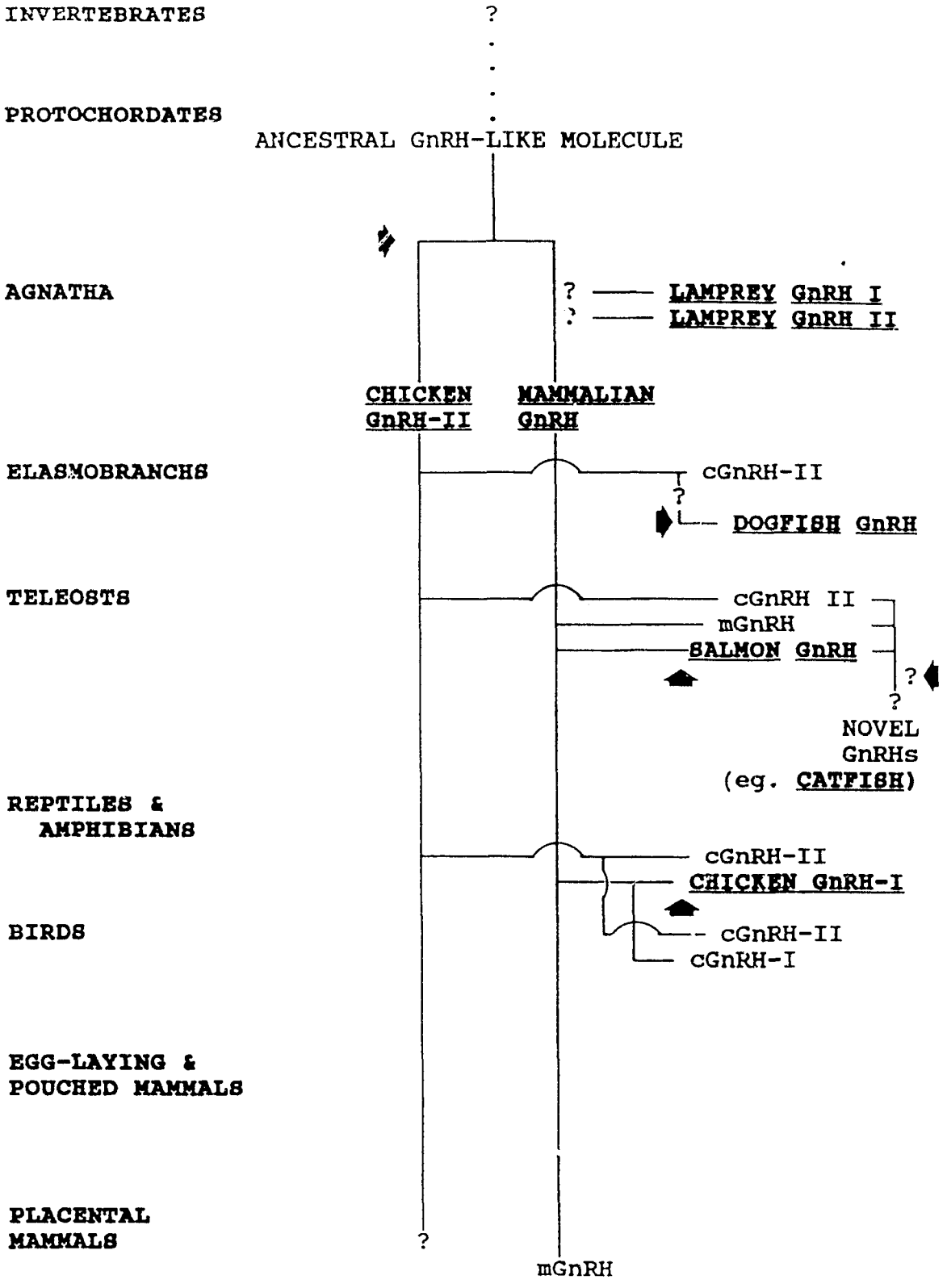
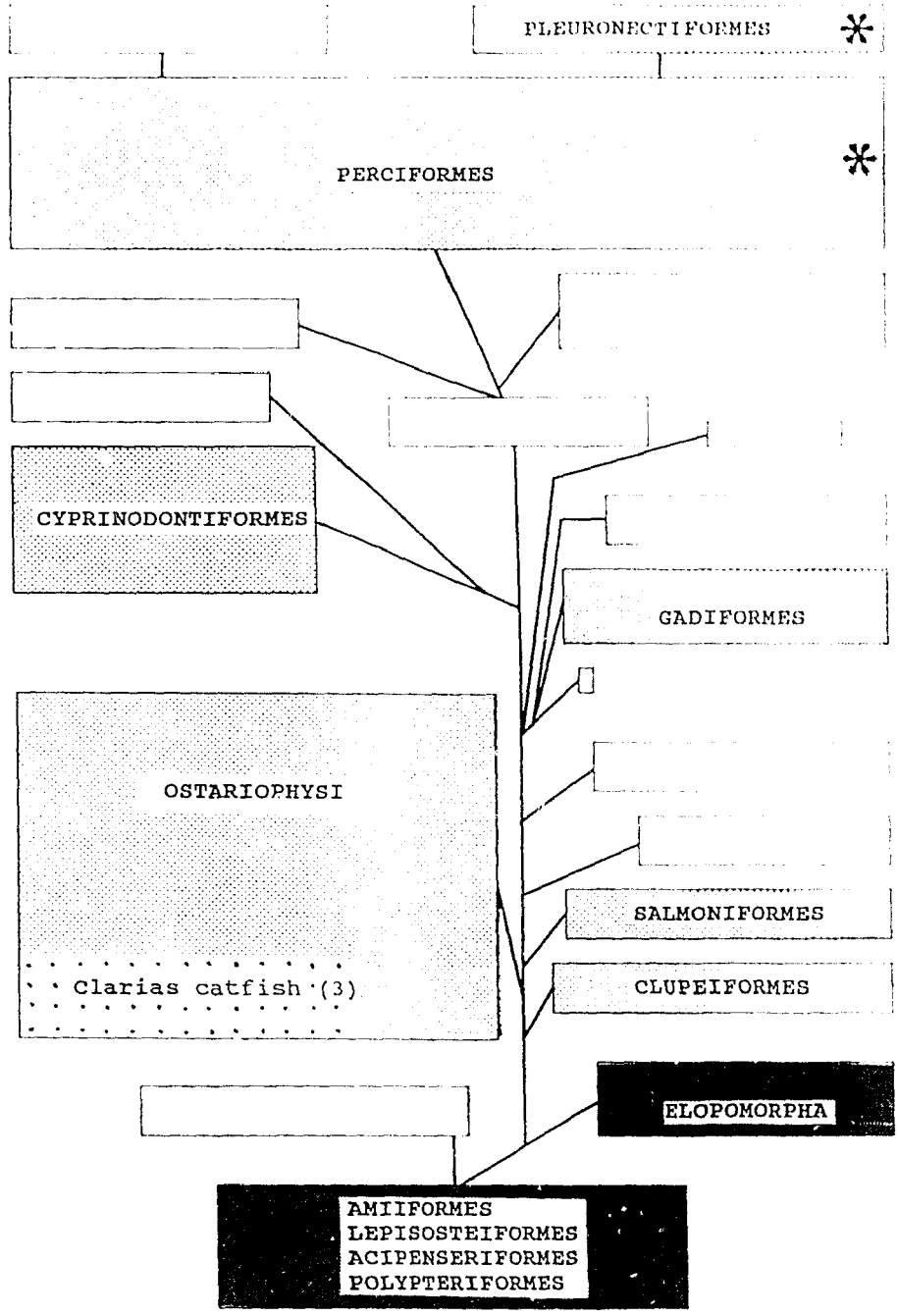

INVERTEBRATES
?
PROTOCHORDATES
ANCESTRAL GnRH-LIKE MOLECULE
AGNATHA
? LAMPREY GnRH I
? LAMPREY GnRH II
CHICKEN GnRH-II
MAMMALIAN GnRH
ELASMOBRANCHS
cGnRH-II
?
DOGFISH GnRH
TELEOSTS
cGnRH II
mGnRH
SALMON GnRH
?
?
NOVEL GnRHs
(eg. CATFISH)
REPTILES & AMPHIBIANS
cGnRH-II
CHICKEN GnRH-I
BIRDS
cGnRH-II
cGnRH-I
EGG-LAYING & POUCHED MAMMALS
PLACENTAL MAMMALS
?
mGnRH

Figure 33. Distribution of gonadotropin-releasing hormone (GnRH) in Osteichthyes. The presence of the mammalian form of GnRH is shown as black; salmon GnRH as shaded; catfish GnRH as dotted, and novel unidentified GnRH as a star. Open boxes indicate orders in which fish have not been analyzed for the forms of GnRH. The chart is an adaptation from Nelson, 1984 and is taken from Sherwood and Coe 1991.


PLEURONECTIFORMES
PERCIFORMES
CYPRINODONTIFORMES
GADIFORMES
OSTARIOPHYSI
Clarias catfish (3)
SALMONIFORMES
CLUPEIFORMES
ELOPOMORPHA
AMIIFORMES
LEPISOSTEIFORMES
ACIPENSERIFORMES
POLYPTERIFORMES

individually or as α/β pairs (Cleveland and Sullivan 1985). The function (if any) of this arrangement is unclear. Over time, however, duplicated genes may relocate to another portion of the genome. This appears to have been the case for the genes encoding GnRH in the Atlantic salmon since the cGnRH-II form was not found situated close by the sGnRH (Klungland *et al.*, in press). Some teleosts possess three forms of GnRH suggesting that additional duplications may have occurred. Gene duplication also occurs through a complete doubling of the chromosomal content without subsequent cell division, an event known as tetraploidy. The salmonids seem to have undergone a tetraploid event since their chromosome number is relatively high compared to other fish (Chevassus 1979, Schmidtke amd Kandt 1981). Since this effectively duplicates the entire genome, second copies of all genes are produced which can subsequently evolve freely. This seems to be the mechanism which has produced two vasotocin precursors in the salmon with identical hormone coding regions. Tetraploidy could also account for the multiple forms of GnRH in the salmon. However, species which are not considered tetraploid, such as the cichlid, the molly and the catfish also possess multiple forms of GnRH (Sherwood and Coe 1991). However, this raises the possibility of additional precursors with identical hormone coding regions as is the case for the vasotocin. The search for additional GnRHs continues in fish but they appear to be located away from the first gene

and of low sequence identity to the sGnRH except for the GnRH coding region since they have not been readily isolated using the sGnRH clones.

The teleosts and other vertebrates, including the non-eutherian mammals (the marsupials and monotremes), all appear to possess at least two genes for GnRH, raising the possibility that this second gene is also present in placental mammals. A mutation in a regulatory region could lead to a failure to express the gene, or if it is expressed, it has diverged to an extent that renders it unrecognizable as a GnRH and undetectable by a probe based on the mammalian form of GnRH (mGnRH). Similarly, extensive divergence within the gene would make it difficult to detect in a genomic library using the other cDNA as a probe. This may be an example of gene `loss' (either through mutation or extensive divergence). Whereas two forms of GnRH in other vertebrates may serve two purposes, perhaps mGnRH is able to act in both capacities resulting in the second form becoming redundant and evolving into oblivion. Recently, two cDNAs were isolated from rat preoptic area and testis and show limited sequence similarity to the mGnRH precursor (Nowak 1990). If these are indeed derived from a second form of GnRH in mammals, they have diverged to an extent which makes them barely recognizable as GnRH family members. A second gene, if present, is probably the relic of a cGnRH-II-like peptide since cGnRH-II is the most widely spread form of GnRH in the vertebrates and a cGnRH-II-like peptide is found

in the prototherian (monotreme) and metatherian (marsupials) mammals.

It is clear that variations in the rate of evolution of genes can occur following gene duplication, but it has also been observed that a protein which has two functions may lose one of them and thereby be less constrained and able to evolve slightly faster than before (Wilson *et al.* 1987). Examples of this phenomenon include α-crystallin, which is a structural protein in the mammalian lens and has unknown functions in other tissues. In the blind mole rat, α-crystallin has evolved faster than in similar lineages which have retained highly developed eyes (de Jong and Hendriks 1986).

Gene Organization

GnRH provides an example of how gene organization, in terms of exon/intron location, is maintained through evolution, despite changes in the size and sequence of introns. Although the whole gene has not been characterized for Pacific salmon GnRH, introns II and III are in the identical position to the Atlantic salmon sGnRH gene and the rat, mouse and human GnRH genes. Intron size has increased in the mammals for introns II and III but intron I is longer in the salmon. The overall similarity in gene organization suggests that mGnRH and sGnRH are derived from the same ancestral gene. A minimum of only two base changes is required to convert mGnRH to sGnRH. It appears that

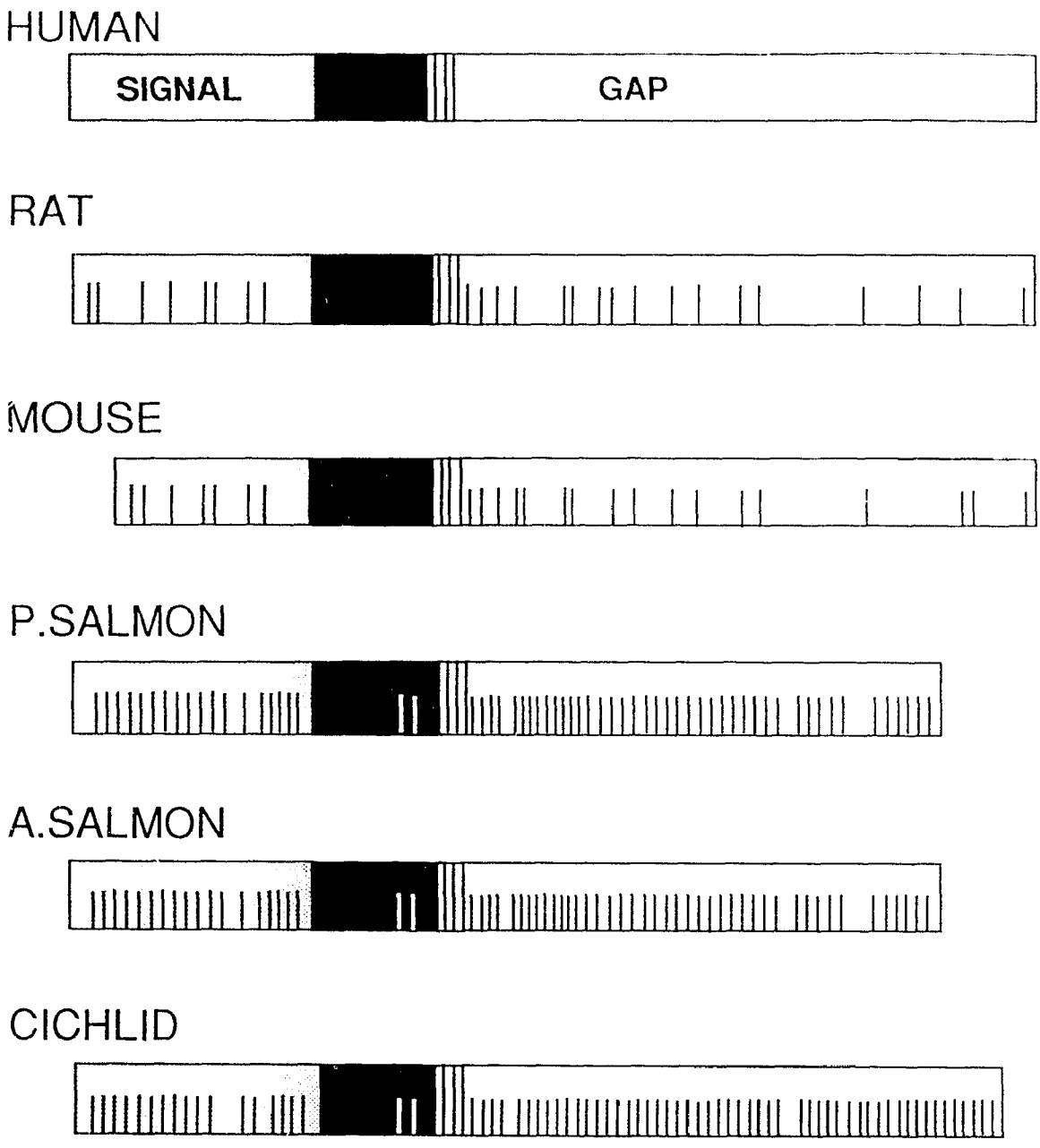
intron size has increased during evolution for certain gene families. Examples of this phenomenon include the hypothalamic peptides such as GnRH, growth hormone-releasing hormone and the α-tubulin family. Fungi have been shown to have shorter introns than either plants or animals (Hawkins 1988). Within the tubulin family it has been shown that the budding yeast, *Sacchromyces cervisiae*, possesses one intronless β-tubulin and the fission yeast, *Schizosaccharomyces pombe*, possesses two genes, one intronless and the other containing an intron. The size and distribution of exons and introns are not known for any fish tubulin but the mammalian tubulins tend to be split on several exons separated by introns. Exon shuffling and reassortment were originally considered to be extremely important in protein evolution. If exons encode specific protein domains or functions, shuffling and reassortment produces novel proteins. This concept was based, in part, on the observation that each domain of the constant region of the heavy chain of immunoglobulins is encoded by an individual exon. Similarly, other, mainly globular, proteins such as albumin, hemoglobin, lysozyme and ovomucoid have functional domains that reside on exons which, in some cases, appear to have been duplicated (Blake 1985). The low-density lipoprotein receptor gene resides on 18 exons some of which have sequence similarity to regions of epidermal growth factor, the blood-clotting factors or the blood protein, complement nine (Sudhof *et al.* 1985).

However, while the exon/domain hypothesis is well supported by data from some of the large, multidomain, globular proteins, it does not seem to explain the exon/intron organization of single domain proteins such as the peptide hormones. Analysis of the size distribution of exons and introns suggests that there are limits to their maximum and minimum sizes implying some constraint perhaps in terms of maintaining efficient splicing mechanisms (Hawkins 1988, Traut 1988). Although their size may vary, the position of introns appears to be fairly stable over evolutionary time, as demonstrated by the GnRH genes, and other genes such as calmodulin (Traut 1988). Maintaining split genes allows cells to exert tissue-specific, temporal and spatial control over the expression of particular genes via alternative splicing which results in different proteins products. Some proteins such as calcitonin exhibit differential splicing which is tissue specific (Breitbart *et al*. 1987). Another way to produce new proteins in through point mutations at the intron/exon splice sites which can result in the deletion or addition of whole blocks of amino acids (Blake 1985).

Evolution of protein coding regions.

Rates of change can vary considerably within different regions of the protein coding portion of genes as demonstrated by both GnRH and vasotocin. A comparison of the preprohormones for GnRH in fish and mammals (fig. 34)

Figure 34. Gonadotropin-releasing hormone (GnRH) precursors for six species. The short vertical lines indicate an amino acid substitution compared to the human precursor. The three vertical lines following the GnRH portion of the precursor indicate the Gly-Lys-Arg processing site which is identical in all six precursors.


HUMAN
SIGNAL
GAP
RAT
MOUSE
P.SALMON
A.SALMON
CICHLID

clearly shows how the different portions of the protein have evolved at different rates. A corresponding analysis of vasotocin and vasopressin (fig. 35) produces similar results. The role of the signal peptides is well characterized and it would appear that a fairly substantial change in amino acid sequence can take place without affecting the secretion of the neuropeptide. In both GnRH and vasotocin, the constraint on the signal peptide is to maintain a hydrophobic core (figs. 36 and 38). Therefore, this region can change as long as the substitutions in the core are conservative. Clearly, the neurohormones are under strict constraint. However, the associated peptides appear to be evolving at a high rate based on the number of substitutions found in fish precursors compared to other vertebrates. This obviously raises questions as to the function of the associated peptides. The retention of the cysteine residues in the vasotocin precursor suggests that they are important and, since their role appears to be in maintaining correct conformation, the role of the associated peptides is probably conformational rather than physiological. This has already been suggested for the C-peptide in insulin (Eipper *et al.* 1986). Hydrophobicity plots of the different GnRH GAPs do not indicate any conservation of a specific conformational profile (fig.37) although, as has already been described in chapter three, a few residues do appear to be conserved in all species examined to date. Whether these confer a similar

Figure 35. Vasopressin (VP) or vasotocin (VT) precursors for four species. Salmon and sucker each have two precursors in one species. The short lines indicate an amino acid substitution compared with the human precursor. The black circle shows a possible glycosylation site. The number of amino acids of the total that are identical with the human form is shown for the neurophysins and glycopeptides (taken from Sherwood and Coe 1991).

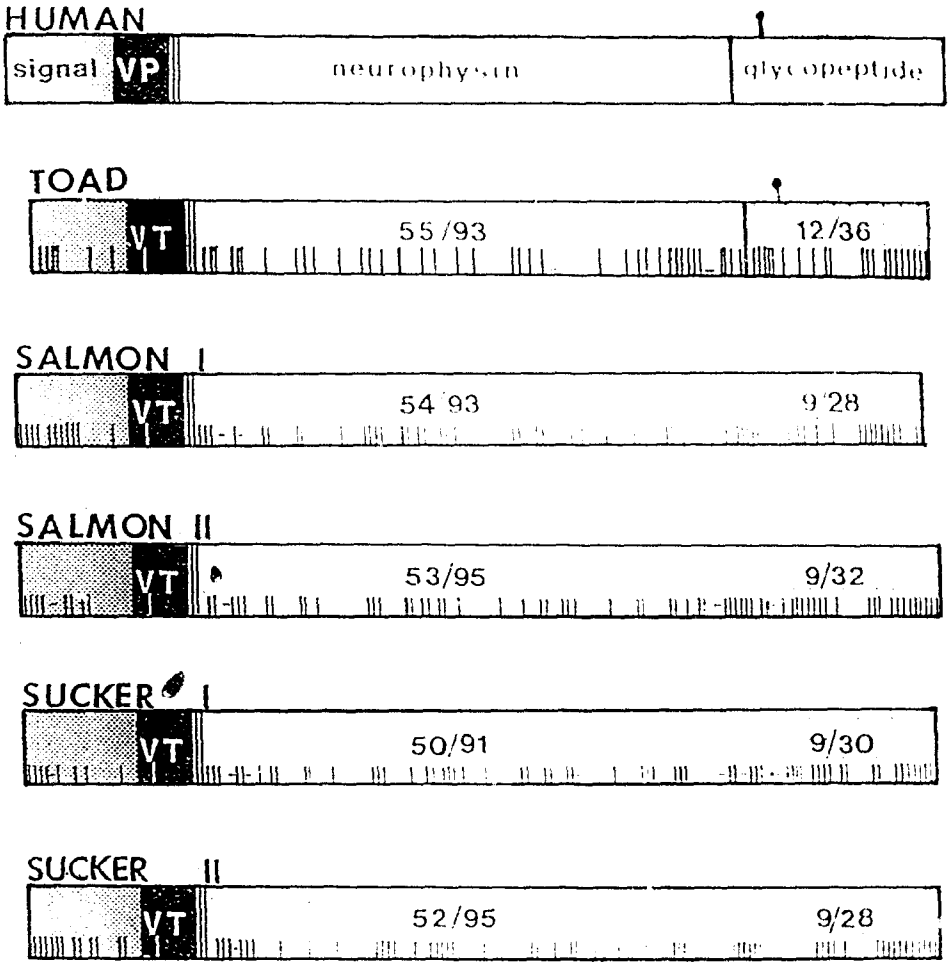

HUMAN
signal
VP
neurophysin
glycopeptide
TOAD
VT
55/93
12/36
SALMON I
VT
54/93
9/28
SALMON II
VT
53/95
9/32
SUCKER I
VT
50/91
9/30
SUCKER II
VT
52/95
9/28

Figure 36. Composite plots showing hydrophobicity and hydrophilicity of the signal peptides of the mammalian and fish gonadotropin-releasing hormone (GnRH) precursors. The horizontal line within each box represents neutrality, with increasing hydrophilicity (to a maximum of 100%) above the line and increasing hydrophobicity (to a maximum of 100%) below the line. Relative values are determined using a combination of methods. These plots were done using the SEQPRO system in the Dept. of Biochemistry and Microbiology at the University of Victoria and also take into account accessibility and flexibility of amino acids. All peptides are plotted along the same horizontal axis regardless of their length. All species have signal peptides which are 23 amino acids in length except the mouse signal peptide which is 21 amino acids long.

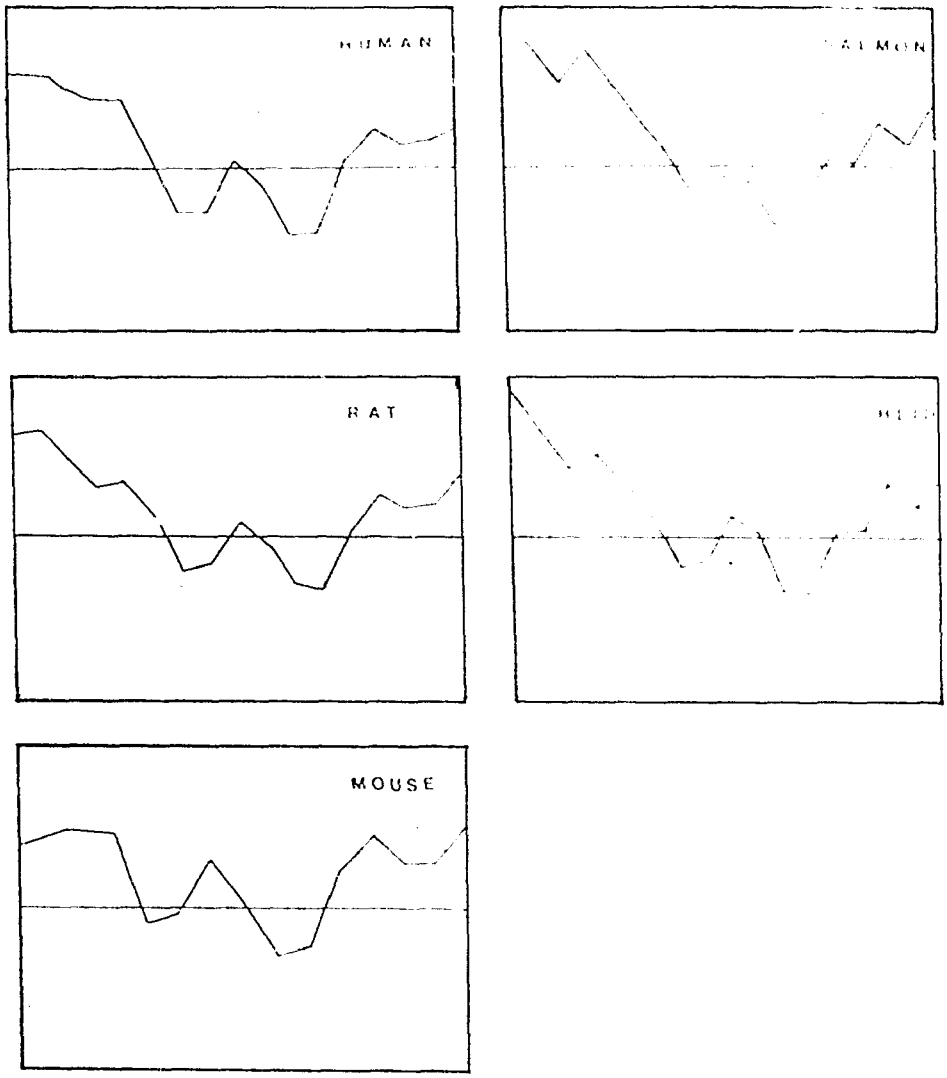

HUMAN
RAT
MOUSE

Figure 37. Composite plots showing hydrophobicity and hydrophilicity of the GnRH-associated peptides of the mammalian and fish gonadotropin-releasing hormone precursors. The horizontal line within each box represents neutrality, with increasing hydrophilicity (to a maximum of 100%) above the line and increasing hydrophobicity (to a maximum of 100%) below the line. Relative values are determined using a combination of methods. These plots were done using the SEQPRO system in the Dept. of Biochemistry and Microbiology at the University of Victoria and also take into account accessibility and flexibility of amino acids. All peptides are plotted along the same horizontal axis regardless of their length. The mammalian species each possess GAPs of 56 amino acids in length, the cichlid GAP is 54 amino acids in length and the salmon GAP is 46 amino acids long.

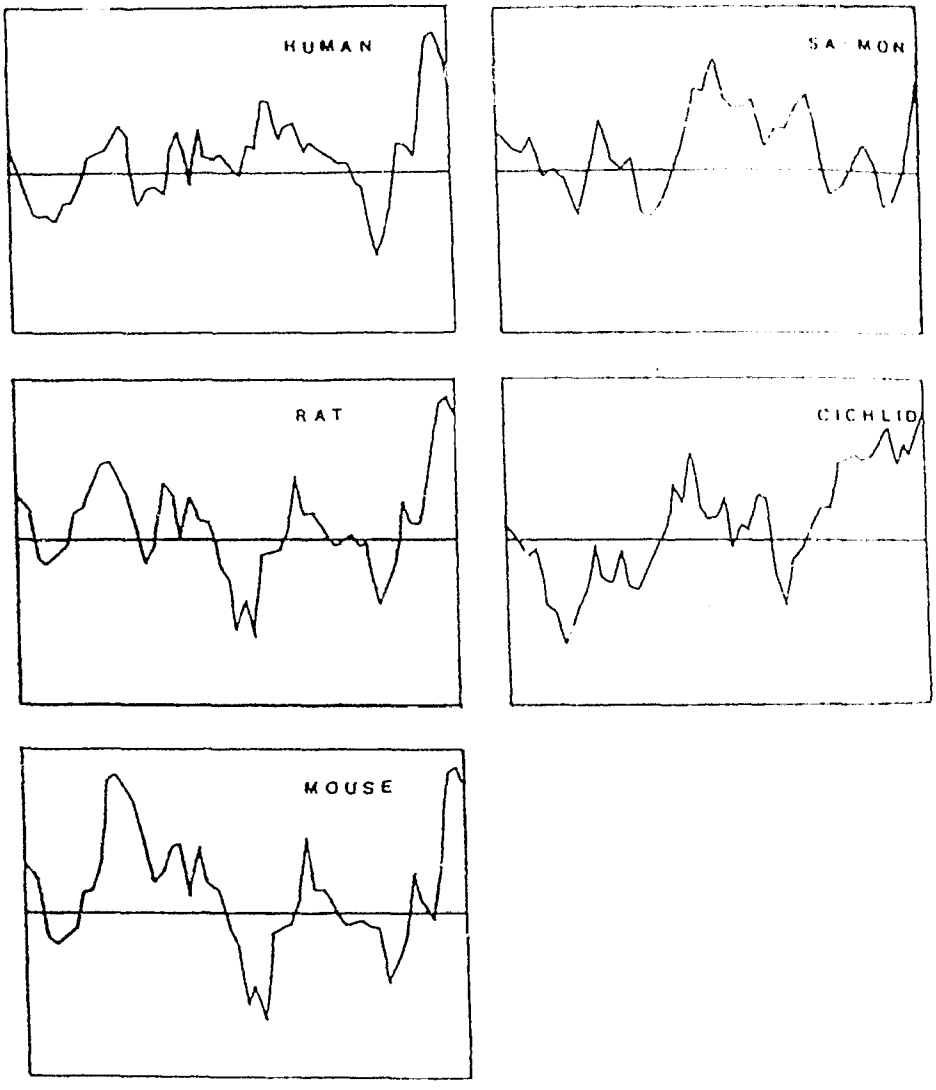

HUMAN
SALMON
RAT
CICHLID
MOUSE

Figure 38. Composite plots showing hydrophobicity and hydrophilicity of the signal peptides of mammalian, toad and fish vasotocin precursors. The horizontal line within each box represents neutrality, with increasing hydrophilicity (to a maximum of 100%) above the line and increasing hydrophobicity (to a maximum of 100%) below the line. Relative values are determined using a combination of methods. These plots were done using the SEQPRO system in the Dept. of Biochemistry and Microbiology at the University of Victoria and also take into account accessibility and flexibility of amino acids. All peptides are plotted along the same horizontal axis regardless of their length. The human, salmon II, and sucker I signal peptides are 19 amino acids long, the salmon I signal peptide is 20 amino acids long and the toad signal peptide is 17 amino acids long.

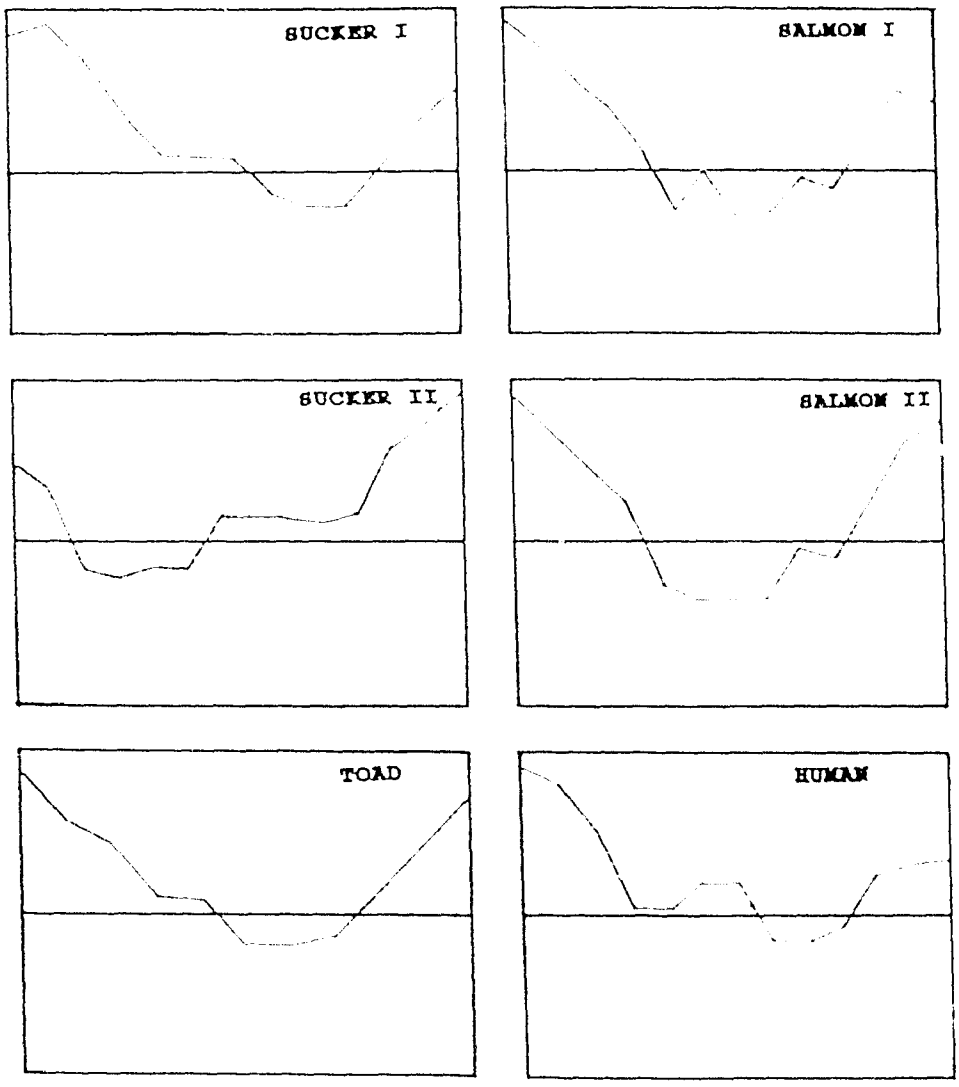
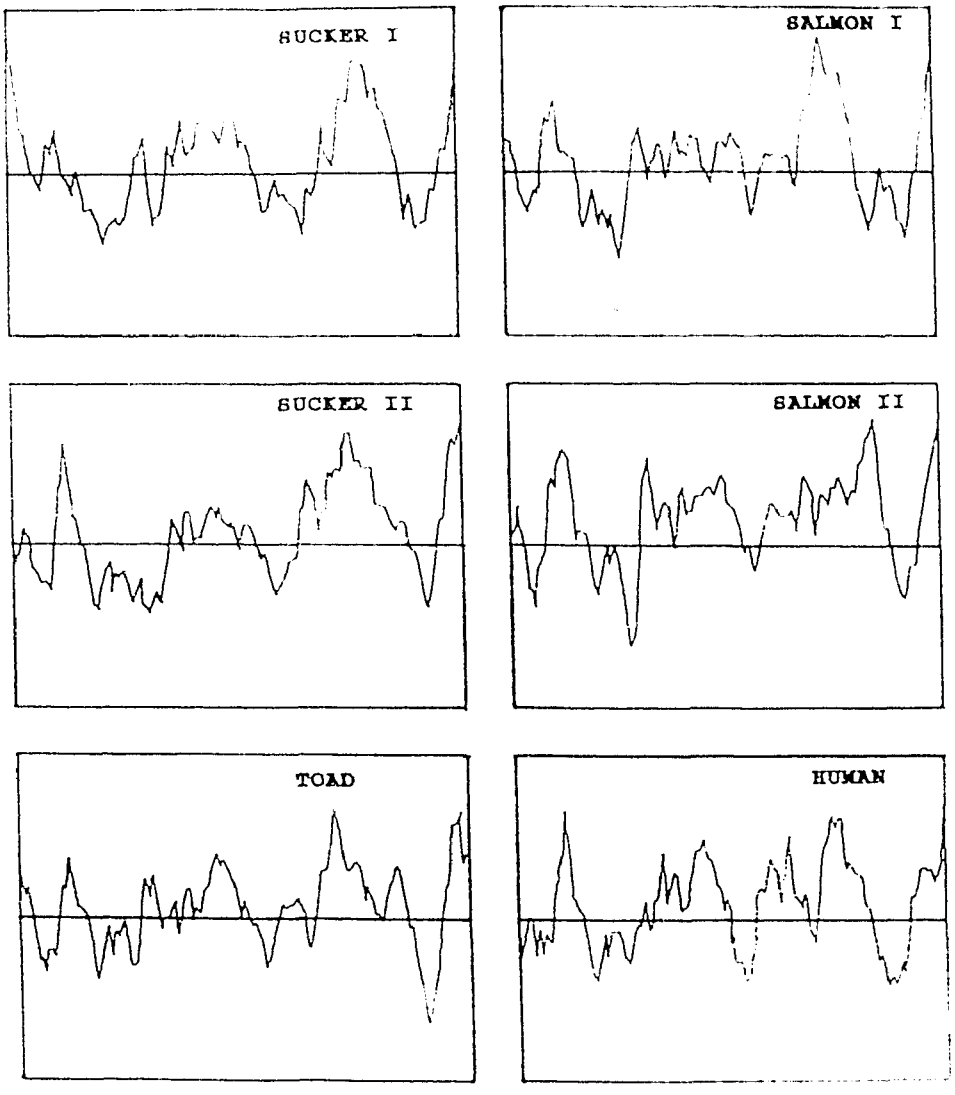

SUCKER I
SALMON I
SUCKER II
SALMON II
TOAD
HUMAN

Figure 39. Composite plots showing hydrophobicity and hydrophilicity of the neurophysin peptides of mammalian, toad and fish vasotocin precursors. The horizontal line within each box represents neutrality, with increasing hydrophilicity (to a maximum of 100%) above the line and increasing hydrophobicity (to a maximum of 100%) below the line. Relative values are determined using a combination of methods. These plots were done using the SEQPRO system in the Dept. of Biochemistry and Microbiology at the University of Victoria and also take into account accessibility and flexibility of amino acids. All peptides are plotted along the same horizontal axis regardless of their length. The human neurophysin/copeptin is 133 amino acids long, the toad complete neurophysin is 129 amino acids long, the salmon I and II neurophysins are 121 and 127 amino acid long respectively and the sucker I and II neurophysins are 121 and 123 amino acids long respectively.


SUCKER I
SALMON I
SUCKER II
SALMON II
TOAD
HUMAN

conformation to all the GAPs is unknown. Similar plots for the vasotocin cryptic peptides (fig. 39) show more conservation of pattern due to the presence of the cysteine residues in all precursors. One way to establish whether the associated peptides have a physiological role is to look for receptors but this has not been reported either for GAP or neurophysin. An endocrine disorder resulting from the loss of part of the GnRH gene in mammals may provide some evidence that the GAP is indeed acting as a conformational assistant. In `hypogonadal' mice, the third and fourth exons have been deleted from the GnRH gene. Although exon two which encodes GnRH remains intact, these mice do not appear to release GnRH, circulating gonadotropins are undetectable and the animals are sexually immature (Seeburg *et al.* 1987). This strongly suggests that the GAP portion of the precursor is required for effective processing of the GnRH peptide.

Different rates of evolution for various portions of neurohormone precursors have already been demonstrated in a number of other neuropeptide families such as corticotropin-releasing factor, somatostatin and growth-hormone releasing hormone (see Sherwood and Parker 1990). The conservation of sequence of the neuropeptide and the lack of conservation of the associated peptides found in GnRH and vasotocin appear to be typical of neuropeptide families.

Evolution of Specific Nucleotide Sites.

Single amino acid changes in protein sequences may not appear to be significant enough to alter function at least for larger proteins. The extensive sequence identity between the α-tubulin from the salmon brain compared to other tubulins may not seem sufficient to explain the existence of numerous tubulins in terms of differential regulation and expression. However, analysis of expression of two rat α-tubulins which only differ by a single amino acid, indicates that one (Tα1) is constitutively expressed at low levels throughout development and is not affected by neuronal development and that the other (T26) is highly expressed during neurite outgrowth, during ontogeny and after injury (Sullivan 1988). This evidence suggests that very small changes in primary sequence information are enough to lead to significant changes in function, either through regulators which presumably recognize the subtle sequence changes or through altered secondary and tertiary structures. The variations in the carboxy terminal region of the different α-tubulins isolated from the salmon brain may be enough to enable regulators and microtubule associated peptides (MAPs) to distinguish between them and to employ them in different capacities. Alternatively, the flanking regions for the various tubulins are different and hence regulated by different transcription factors.

A single nucleotide base change has been shown to be responsible for the endocrine disorder, diabetes insipidus,

as outlined in chapter four. A single base deletion in the neurophysin portion of the vasopressin precursor causes a failure to effectively release the vasopressin resulting in the affliction (Schmale and Richter 1984).

Future Directions

Clearly, we have only begun to unravel the evolutionary history of many neuropeptide families. Critical questions which remain to be answered cover areas such as the origin of the neuropeptide genes, existence of related members and superfamilies, and role of cryptic peptides. It is already apparent that vasopressin family members are present in invertebrates but this has not been investigated for GnRH. Does the origin of GnRH lie within the invertebrates and is it still present in a recognizable form? The additional forms of GnRH in fishes remain elusive, despite the number of groups working in this area. It is still not clear what the roles of multiple forms of neuropeptides are within an individual and when or where in evolution gene duplication and losses occurred. The chromosomal locations of the non-mammalian GnRHs are not known. The differential spatial and temporal locations of multiple forms within the brain are also not known, nor is it clear whether such locations have changed as peptide function has been altered. Is more than one form of GnRH expressed per neuron or is expression mutually exclusive as has been demonstrated for vasopressin and oxytocin? If expression is exclusive, then how is this

controlled? The existence of many neuropeptides in non-neural tissue has raised questions as to tissue-specific expression and control but many more regulatory regions, promotors and enhancers need to be identified. Once regulation is better understood, it will be possible to begin to investigate the evolution of regulatory regions and see how this compares with the evolution of the protein coding regions of neuropeptide genes. The roles of the cryptic peptides that accompany most neuropeptides is still enigmatic and controversial. Identifying more neuropeptide precursors from phylogenetically diverse organisms may help in clarifying any sequence or structural similarities in cryptic peptides which have, until now, not been seen. Identification of receptors for cryptic peptides would also be a breakthrough. Alternatively, sequence variation in the associated peptides, between diverse organisms, would lend weight to the hypothesis that these peptides have no physiological role. Site-directed mutagenesis studies in which the cryptic peptide is altered may help determine whether the peptide hormone is still processed efficiently. Comparison of neuropeptide genes from different organisms may shed more light on the evolution of exon/intron structure and the reasons (if any) for the increase in intron size from fishes to mammals. Studies on non-mammalian vertebrates are critical if evolutionary questions of this nature are to be answered.

Literature Cited

Blake, C.C.F. 1985. Exons and the evolution of proteins. International Review of Cytology **93**:149-185.

Breitbart, R.E., Andreadis, A., and Nadal-Ginard, B. 1987. Alternative splicing: A ubiquitous mechanism for the generation of multiple protein isoforms from single genes. Annual Review of Biochemistry **56**:467-495.

Chevassus, B. 1979. Hybridization in salmonids: Results and perspectives. Aquaculture **17**:113-128.

Cleveland, D.W. and Sullivan, K.F. 1985. Molecular biology and genetics of tubulin. Annual Review of Biochemistry **54**:331-365.

de Jong, W.W., and Hendriks, W. 1986. The eye lens crystallins: Ambiguity as evolutionary strategy. Journal of Molecular Evolution **24**:121-129.

Hawkins, J.D. 1988. A survey on intron and exon lengths. Nucleic Acids Research **16**:9893-9908.

Klungland, H., Lorens, J.B., Andersen, O., Kisen, G.O., and Alestrom, P. (in press) The Atlantic salmon prepro-gonadotropin releasing hormone gene and mRNA. Molecular and Cellular Endocrinology.

Lovejoy, D.A. 1991. Gonadotropin-releasing hormone: Structure and function in cartilaginous fish and radiation in the Vertebrata. Unpublished Ph.D. Dissertation. University of Victoria, Victoria, B.C.

Marx, J. 1992. Homeobox genes go evolutionary. Science **255**:399-401.

Nelson, J.S. 1984. Fishes of the World, 2nd Edition, John Wiley and Sons, New York, 523 pp.

Nowak, F.V. 1990. Cloning of two hypothalamic cDNAs encoding tissue-specific transcripts in the preoptic area and testis. Molecular Endocrinology **4**:1205-1210.

Ohta, T. 1991. Multigene families and the evolution of complexity. Journal of Molecular Evolution **33**:34-41.

Seeburg, P.H., Mason, A.J., Stewart, T.A., and Nikolics, K. 1987. The mammalian GnRH gene and its pivotal role in reproduction. Recent Progress in Hormone Research **43**:69-98.

Schmale, H., and Richter, D. 1984. Single base deletion in the vasopressin gene is the cause of diabetes insipidus in Brattleboro rats. Nature **308**:705-709.

Schmidtke, J., and Kandt, I. 1981. Single-copy DNA relationships between diploid and tetraploid teleostean fish species. Chromosoma **83**:191-197.

Sherwood, N.M., and Coe, I.R. 1991. Neuropeptides and their genes in fish. *In* Proceedings of the Fourth International Symposium on the Reproductive Physiology of Fish, University of East Anglia, Norwich, U.K., 7-12 July (eds. Scott, A.P., Sumpter, J.P., Kime, D.E., and Rolfe, M.S.) FishSymp 91, Sheffield, U.K. pp. 38-40.

Sudhof, T.C., Russell, D.W., Goldstein, J.L., Brown, M., Sanchez-Pescador, R., and Bell, G.I. 1985. Cassette of eight exons shared by genes for LDL receptor and EGF precursor. Science **228**:893-895.

Sullivan, K.F. 1988. Structure and utilization of tubulin isotypes. Annual Review of Cell Biology **4**:687-716.

Traut, T.W. 1988. Do exons code for structural or functional units in proteins. Proceedings of the National Academy of Sciences (USA) **85**:2944-2948.

Wilson, A.C., Ochman, H., and Prager, E.M. 1987. Molecular time scale for evolution. Trends in Genetics 3:241-247.