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Estradiol Regulation of the Neurohypophysial Hormones in the Goldfish, *Carassius Auratus*

by

© Fadi Ayoub

A thesis submitted to the School of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Master of Science

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Summary

The neurohypophysial hormones (NHs) consist of a superfamily of nonapeptide hormones that are structurally conserved throughout vertebrates. These hormones are synthesized in neurons of the hypothalamus that project to the posterior lobe of the pituitary where they are stored and released. Most species possess two members of the superfamily that are homologous to the mammalian, arginine vasopressin (AVP) and oxytocin (OXT). In teleosts, arginine vasotocin (AVT) and isotocin (IST) replace AVP and OXT, respectively. In mammals, the NHs have distinct functions as AVP is involved with fluid homeostasis and blood pressure regulation whereas OXT is involved with reproductive physiology. In teleosts, AVT plays a role in blood pressure regulation and both AVT and IST possess some overlapping actions in the induction of spawning or parturition in various fishes. Because of these functions in teleostean reproduction, the regulation of NH gene expression by estradiol was examined in the goldfish, *Carassius auratus*.

Using *in situ* hybridization followed by quantitation of autoradiographic silver grains, the effects of estradiol (E2) administration on AVT and IST mRNA expression in the nucleus preopticus (NPO) was determined. Expression of AVT mRNA was elevated in fish treated with E2 for 12 hours, compared to saline treated fish, for NPO neurons of all sizes grouped together. Quantitating gene expression in three sizes of NPO neurons, separately, produced different results. No significant effects were seen in parvocellular neurons. AVT mRNA levels were increased in magnocellular and intermediate neurons 12 hours after E2 treatment. The combined data for both the magnocellular and intermediate neurons showed an elevation of AVT mRNA levels at 12 hours.

It was concluded that the elevation in AVT mRNA levels offset a decrease in AVT mRNA levels due to saline injection and a possible diurnal rhythm of AVT mRNA concentrations. No significant effects of E2 administration on IST mRNA were found for neurons of all sizes examined separately or grouped together. The results of this study support existing evidence that AVT plays a role in teleostean reproduction.

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List of Abbreviations

ACTH: adrenocorticotropin hormone

Arg: arginine

AVP: arginine vasopressin

AVPv: anteroventral periventricular nucleus

AVT: arginine vasotocin

A (nucleotide): deoxyadenylate

C (nucleotide): deoxycytidylate

Ca^{2+}: calcium

CCK: cholecystokinin

cDNA: complementary deoxyribonucleic acid

$CoCl_2$: cobalt choride

CRF: corticotropin-releasing factor

DEPC: diethyl pyrocarbonate

DNA: deoxyribonucleic acid

E2: estradiol

EDTA: ethylenediaminetetraacetic acid

ERE: estrogen response element

G (amino acid): glycine

G (nucleotide): deoxyguanylate

GH: growth hormone

GSI: gonadosomatic index

HCl: hydrochloric acid

hnRNA: heteronuclear ribonucleic acid

Ile: isoleucine

IST: isotocin

K (amino acid): lysine

Lys: lysine

$MgCl_2$: magnesium chloride

MnPO: median preoptic nucleus

MPN: medial preoptic nucleus

mRNA: messenger ribonucleic acid

M: molar

mM: millimolar

NaCl: sodium chloride

$NaH_2PO_4.H_2O$: sodium phosphate, monobasic

NAPv: nucleus anterior periventricularis

NAT: nucleus anterior tuberis

NH: neurohypophysial hormone

NH (Fig.9): nucleus habenularis

NLTa: nucleus lateralis tuberis pars magnocellularis anterior

NLTd: nucleus lateralis tuberis pars dorsalis

NLTl: nucleus lateralis tuberis pars lateralis

NLTp: nucleus lateralis tuberis pars parvocellularis

NPO: nucleus preopticus

NPOmc: nucleus preopticus pars magnocellularis

NPOpc: nucleus preopticus pars parvocellularis

NP: neurophysin

NPP: nucleus preopticus periventricularis

NRL: nucleus recessus lateralis

NRP: nucleus recessus posterioris

OVLT: organum vasculosum laminae terminalis

OXT: oxytocin

PI: phosphatidylinositol

PMg: nucleus preopticus magnocellularis pars gigantocellularis

PMm: nucleus preopticus magnocellularis pars magnocellularis

PMp: nucleus preopticus magnocellularis pars parvocellularis

Phe: phenylalanine

PVN: paraventricular nucleus

R (amino acid): arginine

RNase: ribonuclease

SFO: subfornical organ

SON: supraoptic nucleus

SSPE: buffer - sodium chloride, sodium phosphate, EDTA

T (nucleotide): deoxythymidylate

TRH: thyrotropin-releasing hormone

V1a: arginine vasopressin receptor, type 1a

V1b: arginine vasopressin receptor, type 1b

V2: arginine vasopressin receptor, type 2

Introduction

The neurohypophysial hormones (NHs), arginine vasopressin (AVP) and oxytocin (OXT), are members of a superfamily of nonapeptides that are structurally conserved among vertebrates. These hormones have well defined roles in water retention, blood pressure regulation (AVP), lactation, and parturition (OXT; reviewed in Cunningham and Sawchenko, 1991) in mammals. AVP also works at the level of the anterior pituitary as a secretagogue, stimulating ACTH release (Gillies *et al.*, 1982) and acts on the liver to promote glycogenolysis (Keppens and DeWulf, 1975). In addition to these functions in the periphery, the NHs also act centrally, influencing processes such as learning and memory. Furthermore, AVP is involved in temperature regulation and cardiovascular function and both AVP and OXT facilitate reproductive and social behaviours (reviewed in De Wied *et al.*, 1993).

Structure of the neurohypophysial hormones

Homologues of these peptides are found throughout the vertebrate classes and within invertebrates. In teleosts, arginine vasotocin (AVT) and isotocin (IST) are the structural homologues of AVP and OXT, respectively (Fig. 1). Structurally AVT and IST possess characteristic cysteines in positions 1 and 6 required to produce a disulphide bond in forming a ring structure. Positions 5, 7, and 9 are also conserved and contain asparagine, proline and an amidated glycine, respectively. Positions 2, 3, 4, or 8 vary between different members of the peptide families. Differences in position 3 and 8 distinguish between members of the OXT and AVP families, respectively. If isoleucine or a related hydrophobic amino acid is found in position 3 the hormone will have an OXT-type action whereas an arginine or lysine in position 8 yields it an AVP-type action (Acher *et al.*, 1995).

AVT is considered to be the ancestral prototype of NHs in vertebrates as it is the only representative present in the most primitive vertebrate, the hagfish (Heierhorst *et al.*, 1992). It is

believed that a gene duplication occurred to produce OXT-type peptides, the second line of hormones that can be detected from bony fishes to mammals (Acher, 1980). Peptides with homologous structures to vertebrate NHs have been characterized in several phyla of invertebrates as well (Acher, 1993). In the mollusc, *Lymnaea stagnalis*, an AVP-like peptide, lysine conopressin (Phe2, Ile3, Arg4, Lys8 - AVP) (Van Kesteren *et al.*, 1995a), and its receptor (Van Kesteren *et al.*, 1995b) have been characterized. This peptide possesses both OXT-like reproductive functions as well as AVP-like metabolic functions (Van Kesteren *et al.*, 1995b).

AVT is present in all non-mammalian vertebrates and in fetal sheep (Vizsolyi and Perks, 1976) whereas IST is replaced by mesotocin (Ile8 - OXT) in non-mammalian tetrapods. Mesotocin is found in lungfishes, which are considered the predecessor of land vertebrates (Acher *et al.*, 1995).

Structures of Vertebrate Neurohypophysial Hormones[a]

	1	2	3	4	5	6	7	8	9	
Vasotocin/vasopressin family										
Vasotocin	Cys	Tyr	Ile	Gln	Asn	Cys	Pro	Arg	Gly (NH_2)	All non-mammalian vertebrates, fetal sheep
Vasopressin	—	—	Phe	—	—	—	—	—	—	Mammals
Lysipressin	—	—	Phe	—	—	—	—	—	—	Pig, Macropodids, Didelphids, Peramelids
Phenypressin	—	Phe	Phe	—	—	—	—	—	—	Macropodids
Oxytocin-like hormone family										
Oxytocin	—	—	—	—	—	—	—	Leu	—	Ratfish, placental mammals
Mesotocin	—	—	—	—	—	—	—	Ile	—	Lungfishes, nonmammalian tetrapods
Isotocin	—	—	—	Ser	—	—	—	Ile	—	Bony fishes
Glumitocin	—	—	—	Ser	—	—	—	Gln	—	Rays
Aspargtocin	—	—	—	Asn	—	—	—	Leu	—	Spiny dogfish
Valitocin	—	—	—	—	—	—	—	Val	—	Spiny dogfish
Asvatocin	—	—	—	Asn	—	—	—	Val	—	Spotted dogfish
Phasvatocin	—	—	Phe	Asn	—	—	—	Val	—	Spotted dogfish

Figure 1. [a]Residues identical with those of vasotocin are indicated by dashes. Modified from Acher *et al.* (1996).

Neurohypophysial hormone precursors

The precursor protein which gives rise to the NHs also produces other molecules, including a cysteine rich protein called neurophysin (NP) and a glycopeptide found only in the AVP precursor in mammals, termed copeptin (Fig. 2; reviewed in Urano *et al.*, 1994). In teleosts, the precursors for AVT and IST have a carboxyl-terminus extension of the NP containing a leucine core that resembles the copeptin but lacks a processing signal and the consensus sequence for N-glycosylation (Heierhorst *et al.*, 1989; Heierhorst *et al.*, 1990; Hyodo *et al.*, 1990; Suzuki *et al.*, 1992; Hiraoka *et al.*, 1993). The glycopeptide extension is lost in both the prooxytocin and promestocin precursor proteins.

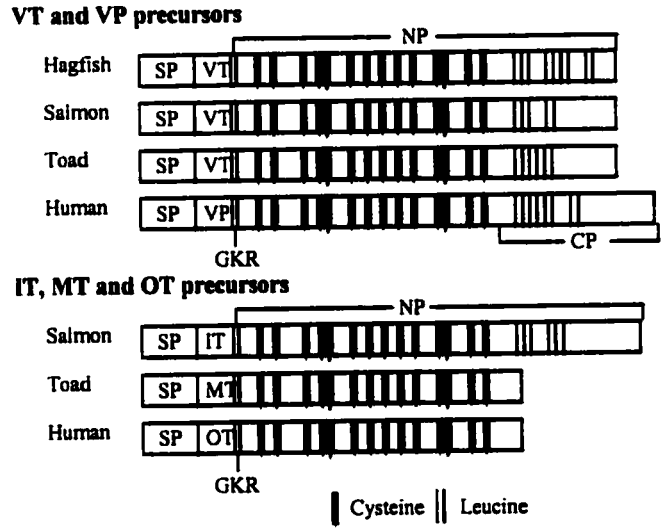


Figure 2. Structural organization of neurohypophysial hormone precursors in vertebrates. Abbreviations indicate moieties of precursors: SP, signal peptide; VT, vasotocin; VP, vasopressin; IT, isotocin; MT, mesotocin; OT, oxytocin; NP, neurophysin; CP, copeptin. GKR, implies Gly-Lys-Arg residues connecting hormone moiety to neurophysin. Modified from Urano *et al.* (1994).

Enzymes involved in processing NH precursors into the hormone and NP have been identified in neurohypophysial secretory granules in ox and rat (Fig. 3; reviewed in Acher, 1993). Four such enzymes generally take part in the biosynthesis of any alpha-amidated peptide messenger. The hormone moiety follows the hydrophobic signal peptide and is linked to the NP by the residues glycine (G)-lysine (K)-arginine (R). These residues are known to serve as processing signals for hormone cleavage. A dibasic endopeptidase cleaves the processing signal after the arginine, then a carboxypeptidase B-like exopeptidase removes the lysine and arginine. A peptidylglycine monooxygenase adds an oxygen atom to the glycine residue and a lyase splits the hydroxyglycine into the amide group and glyoxylic acid.

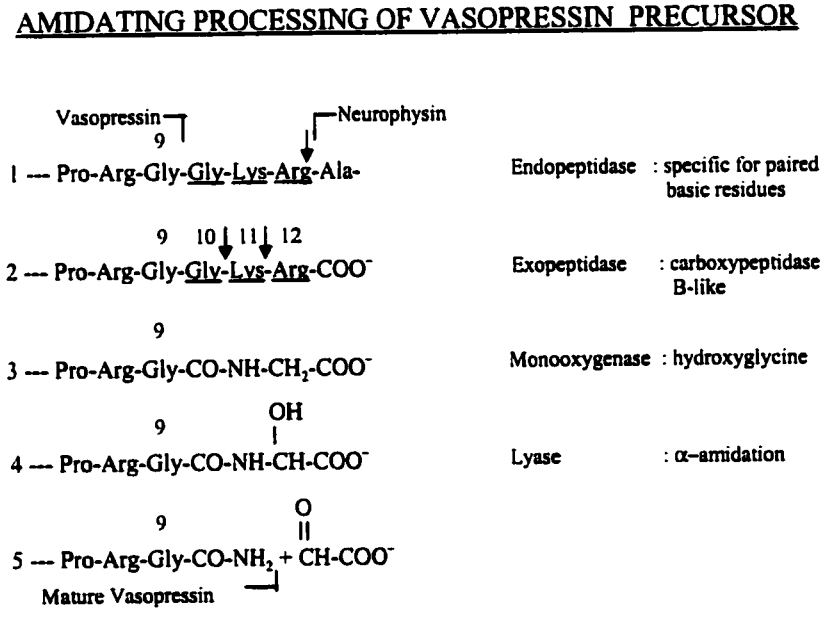


Figure 3. The cascade of the four processing enzymes leading from the vasopressin-neurophysin. Modified from Acher *et al.* (1993).

Intermediates in the NH processing cascade have been identified in several species. In the fetal rat, oxytocinyl-GKR, oxytocinyl-GK, and oxytocinyl-G have all been identified (Alstein *et al.*, 1988). In anuran amphibians, vasotocin related peptides have been detected. Vasotocinyl-GKR (hydrin 1) and vasotocin are both found in similar amounts in the neurohypophysis of the permanently aquatic African clawed toad, *Xenopus laevis*. In semi-aquatic toads and frogs, both vasotocin and vasotocinyl-G (hydrin 2) are present in the neurohypophysis (Rouille *et al.*, 1989). Both hydrin 1 and 2 appear to function in water absorption and reabsorption on the frog urinary bladder and skin, however, they do not act on the frog kidney in promoting water retention (Rouille *et al.*, 1995).

Originally oxytocic (hastening birth), vasopressor (causing vasoconstriction and an increase in blood pressure), and anti-diuretic (reducing urine volume by retaining water) activities were attributed to a single molecule purified from ox pituitary glands which was determined to possess a high sulphur content (Van Dyke *et al.*, 1942). With improved isolation techniques molecules were separated into two biologically active principles as well as non-active binding proteins, which contained several cysteine residues (reviewed in Acher and Chauvet, 1995). The binding proteins were given the name neurophysins (NPs) and they generally occurred in two forms, one bound to OXT (Neurophysin I) and the other to AVP (Neurophysin II). Functions for the NPs have been proposed but not firmly established. An extracellular growth-factor-like function has been considered but not yet thoroughly proven (Worley and Pickering, 1984). Because the NPs can bind the NHs with high affinity it was proposed that they act as carrier proteins for the hormones in the secretory granules, presumably to reduce the osmotic pressure of the relatively small peptides (Sawyer, 1961). However, as the NPs bind the hormone in a 1:1 ratio there would be no reduction in the number of osmotically active particles (reviewed in Hope and Pickup, 1974). NPs may protect the hormones from proteolytic enzymes during transport through the secretory pathway (Acher and Chauvet, 1995) while ensuring folding for accessibility by processing enzymes. In addition, NP-hormone association may be necessary for sorting in the trans-Golgi network during hormone processing (Burbach *et al.*, 1995a).

Neurohypophysial hormone genes

Genes encoding NHs have been cloned in more than 20 different vertebrate species (reviewed in Urano *et al.*, 1994). In all cases, except for the white sucker IST gene, there exists a conserved three exon and two intron organization. The first exon encodes a signal peptide, the hormone moiety, and the N-terminal portion of the NP molecule. The second exon encodes the central portion of the NP molecule while the third exon encodes the C-terminal portion of the neurophysin including the leucine-rich segment, which corresponds to mammalian copeptin. The IST gene of the white sucker differs from the typical NH gene organization in that there is a single exon that encodes the entire precursor protein (Figueroa *et al.*, 1989). In the Pacific hagfish, there is a slight variation, as there exists a third intron in the 5' untranslated region of the AVT gene (Heierhorst *et al.*, 1992), however, the 3 exon, 2 intron organization is present.

In salmonids and cyprinids, two genes are usually present for each NH. In the white sucker and salmonids, where cDNAs have been sequenced, there are two IST and AVT genes termed IST-I, IST-II, AVT-I, and AVT-II . This gene duplication is thought to have taken place due to the tetraploidization of these families (reviewed in Urano *et al.*, 1994). The two white sucker IST genes (Figueroa *et al.*, 1989) predict a 90% similarity between the precursors at the amino acid level whereas the two AVT genes (Morley *et al.*, 1990) possess a 55% similarity. Two AVT and IST genes are also present in salmonid species such as rainbow trout, masu salmon, sockeye salmon, and chum salmon (reviewed in Urano *et al.*, 1994; Venkatesh and Brenner, 1995).

The AVT-I and AVT-II gene products from the chum salmon differ by 35% at the amino acid level (Heierhorst *et al.*, 1990). The nucleotide sequences of the sockeye salmon IST-I cDNA shows greater that 96% homology to those of chum salmon and masu salmon (Hiraoka *et al.*, 1993). Using Northern Blot Analysis, it was determined that both the IST-I and IST-II genes are highly expressed in the chum salmon, rainbow trout, masu salmon, and sockeye salmon. The hybridizing signal for AVT-I mRNA was more intense in masu salmon and rainbow trout whereas

the AVT-II gene is expressed more abundantly in chum salmon and sockeye salmon (Hiraoka *et al.*, 1993).

Promoter sequences of the neurohypophysial hormone genes

Cloning and sequencing of the genes encoding the NHs have yielded information on the precursor protein and the 5' flanking region (reviewed in Mohr *et al.*, 1995). The 5' flanking region is involved with regulating transcription by binding transcription factors that can either stimulate or inhibit mRNA synthesis. Regulatory elements in the 5' flanking region of the NH genes have been sequenced and analyzed in mammals, but not in teleosts.

Both the AVP and OXT genes possess nucleotide sequences within the 5' flanking region which interact with several types of transcription factors including those of the nuclear steroid receptor superfamily. When a lipophilic steroid crosses the cell membrane it can bind and activate its intracellular receptor. The activated hormone-receptor complex translocates to the nucleus to interact with appropriate DNA sequences in the 5' flanking region (reviewed in Parker, 1995). The rat AVP gene has been shown to possess a glucocorticoid response element that can bind both the glucocorticoid and progesterone receptors (Mohr and Richter, 1990). The OXT promoter region displays sequences that can bind receptors for thyroid hormones, retinoids, and estrogens (reviewed in Mohr *et al.*, 1995). The consensus estrogen response element (ERE) consists of the palindrome GGTCAnnnTGACC where nnn represents any three nucleotides (Beato, 1989). The activated estrogen receptor binds to this sequence as a homodimer to stimulate OXT transcription (see below). The rat (Ivell and Richter, 1984) and human (Richard and Zingg, 1990) OXT gene promoters possess a sequence that resembles the consensus ERE except for 1 mismatch in position 4 where a G replaces a C. The bovine (Ruppert *et al.*, 1984) and mouse (Hara *et al.*, 1990) OXT 5' flanking regions contain three and two mismatches, respectively. Therefore, steroids have the potential to regulate NH gene expression in a direct genomic fashion.

Localization of the neurohypophysial hormones

The NHs are synthesized in the cell bodies of neurons located in the hypothalamus as described by *in situ* hybridization (reviewed in Meister, 1993). Reports have suggested that during lactation (Mezey and Kiss, 1991) and salt-loading (Kiyama and Emson, 1990) there is synthesis of both AVP and OXT mRNAs in the same neuron. This is in contrast to the study of Mohr *et al.*, (1988) which demonstrated that AVP and OXT mRNAs were not colocalized within the same neuron during salt-loading. Immunocytochemical studies in mammals have shown that separate neurons contain AVP and OXT and that the bovine supraoptic nucleus (SON) contains predominantly AVP neurons whereas the paraventricular nucleus (PVN) possesses more OXT neurons than AVP neurons (Vandesande *et al.*, 1975).

After synthesis, the peptides are transported along axons to nerve endings localized within the neurohypophysis. In teleosts, AVT and IST are produced and stored in separate neurons in the nucleus preopticus (NPO) as determined by immunocytochemistry (Goossens *et al.*, 1977) and *in situ* hybridization (Hyodo and Urano, 1991; Lederis *et al.*, 1994). This nucleus consists of a parvocellular division made up of relatively small cells and a magnocellular division made up of larger cells (Peter and Gill, 1975; see Results section). In mammals, reptiles, and birds, the NHs are manufactured in the PVN and SON nuclei, which are homologous to the NPO of teleosts. The magnocellular neurons project to the neurohypophysis whereas the parvocellular neurons send their axons mainly to the median eminence to secrete their products into the hypophysial portal system (De Mey *et al.*, 1975). The secreted hormones make their way to the anterior lobe of the pituitary via the portal system to exert their effects upon this gland. Parvocellular neurons also send their axons to the brainstem and spinal cord (Sawchenko and Swanson, 1982) possibly to influence autonomic functions. In addition, magnocellular neurons receive synaptic inputs that modulate their activity in response to viscerosensory information. Inputs from the brainstem relay information concerning blood pressure and body fluids which can stimulate AVP release (reviewed in Renaud *et al.*, 1992).

AVP is colocalized with CRF in neurons of the PVN (Tramu *et al.*, 1983) and potentiates the ACTH releasing ability of CRF (Gillies *et al.*, 1982) from corticotropes in the anterior pituitary. AVP, but not OXT, is found in the suprachiasmatic nucleus. This small nucleus of the hypothalamus maintains circadian rhythms. In goldfish, AVT mRNA and peptide have been colocalized with CRF in both the parvocellular and magnocellular divisions of the NPO. AVT stimulates ACTH secretion from the adenohypophysis in the goldfish (Fryer and Lederis, 1986) but does not potentiate the effects of CRF on release of ACTH as in mammals. Regulation of adenohypophysial function in teleosts is different from mammals as teleosts lack a true hypophysial portal system. Instead of secreting hormones into a portal system, teleost hypothalamic neurons make direct synaptic connections with secreting cells in the anterior pituitary (reviewed in Peter and Fryer, 1983).

Anterior pituitary function can also be influenced by OXT as it can act as a prolactin releasing factor (Samson *et al.*, 1986). Prolactin secretion is usually under inhibitory control by dopamine. Dopamine inhibition is withdrawn during secretion of prolactin. OXT can act on prolactin secreting cells during the brief periods when dopamine inhibition is not present. OXT can find its way to the anterior pituitary either by the portal system or from the short portal vessels connecting the posterior and anterior lobes of the pituitary (Samson and Schell, 1995).
Aside from the localization of NHs in the hypothalamus, AVP and OXT peptides have been detected in brain areas where they may act as neurotransmitters or neuromodulators (reviewed in De Wied *et al.*, 1993). In mammals, CCK, TRH, galanin, and dynorphin have been detected in the same neurons as OXT or AVP (reviewed in Meister, 1993) where they can regulate the secretion of OXT or AVP (reviewed in Meister, 1993). In the periphery, both AVP and OXT are synthesized in the adrenal glands and testes (Ivell, 1986). In addition, OXT mRNA has been detected in the uterus (Lefebvre *et al.*, 1992a), placenta, and amnion (Lefebvre *et al.*, 1992b). It has been suggested that OXT synthesized in the uterus is responsible for uterine smooth muscle contractions during parturition (Higuchi *et al.*, 1995).

Neurohypophysial hormone receptors

The NHs mediate their effects via receptors localized on the cell membrane of target organs. In mammals, four members of the AVP/OXT receptor family have been cloned (reviewed in Burbach *et al.*, 1995b). Two receptors have been cloned in the white sucker (Hausmann *et al.*, 1995; Mahlmann *et al.*, 1994). All are members of the G protein linked receptor superfamily that possess seven trans-membrane domains. AVP can bind to three different receptors designated V1a, V1b and V2. The V1a receptor is located on vascular epithelium and in the liver and is responsible for the vasoconstrictive and glycogenolytic effects of AVP. The V1b receptor is found on the cell membrane of pituitary corticotropes and mediates the ACTH releasing effects of AVP. Both V1a and V1b receptors are linked to the phosphatidylinositol (PI)/Ca++ second messenger pathway but are distinguishable by their pharmacological properties. Receptor occupation of the V2 receptor activates the enzyme adenylate cyclase to initiate the protein kinase A cascade. It is localized to the kidney collecting ducts and acts to increase H_2O permeability (Elalouf *et al.*, 1986) and at the nephron ascending limb to promote ion reabsorption (Wittner *et al.*, 1988). Studies using the techniques of immunocytochemistry and *in situ* hybridization have localized the V1 type receptors in the brain, which mediate AVP's central effects (reviewed in De Wied *et al.*, 1993).

Only one OXT receptor subtype has been cloned and it is localized in the mammary gland and uterus where it mediates smooth muscle contraction. The OXT receptor is also found in the brain and the anterior pituitary and like the V1 receptors is linked to the PI/Ca++ second messenger system (reviewed in Burbach *et al.*, 1995b).

In the white sucker, both the IST (Hausmann *et al.*, 1995) and AVT (Mahlmann *et al.*, 1994) receptors are linked to the PI/Ca++ pathway. Using *in situ* hybridization, the AVT receptor mRNA (Mahlmann *et al.*, 1994) was localized in the liver, bladder, gills, and lateral line and the IST receptor mRNA (Hausmann *et al.*, 1995) was localized in reproductive organs, brains, gills, and lateral line in the white sucker. Although the AVT and IST receptors were determined to be linked to the inositol phosphate pathway, direct action of these hormones on rainbow trout gill

epithelium was shown to be inhibitory on adenylate cyclase activity (Guibbolini and Lahlou, 1992). In addition, receptors related to the two types of AVP receptors have been recognized in the trout (Balment *et al.*, 1993), therefore, subtypes of these receptors may exist in teleosts.

Functions of neurohypophysial hormones in teleosts

In teleosts, characterization of the biological activities of NHs has been limited. AVT, but not IST, is able to increase blood pressure in conscious trout when administered centrally (Le Mevel *et al.*, 1993). The NHs may also be involved in osmoregulation in teleosts as NH mRNA expression in the NPO of rainbow trout was affected by a change in environmental salinity (Hyodo and Urano 1991). AVT mRNA levels were increased for up to two weeks and IST mRNA levels increased for 1 day when rainbow trout were transferred from 80% seawater to freshwater. In flounder and rainbow trout, freshwater-acclimated fish had about twice the plasma levels of AVT than seawater-acclimated fish (Perrot *et al.*, 1991). Such a difference was not seen in carp (Perrot *et al.*, 1991). AVT injected intraperitoneally produced a diuresis in goldfish by increasing the glomerular filtration rate (reviewed in Maetz and Lahlou, 1974). Injected IST also produced the same diuretic effect but at a much higher dose. The above evidence suggests NHs may have a diuretic function in certain teleosts as opposed to an anti-diuretic function seen in mammals. This would mean that the function of the NHs evolved to allow mammals to osmoregulate in their different environment compared to fishes.

NH involvement in teleostean reproduction has received some attention. In guppies (Venkatesh *et al.*, 1992) and other viviparious (fertilization and development of egg occurs within the maternal body) fish (reviewed in Maetz and Lahlou, 1974), administration of AVT and/or IST led to premature parturition. Spawning in oviparious (fertilization and development of egg occurs outside the maternal body) fish was induced by AVT and/or IST. In addition, contraction of genital tracts in both oviparious and viviparious fish has been observed with a physiological dose

of AVT and/or IST (reviewed in Maetz and Lahlou, 1974). Rainbow trout testis in culture has been shown to produce testosterone after application of AVT (Rodriguez and Specker, 1991).

Steroid regulation of neurohypophysial hormone gene expression

In mammals, there is an increase of total brain OXT mRNA in female rat brains concomitant with puberty, a time when levels of ovarian steroids, such as E2, are increasing (Miller *et al.*, 1989). In male and female rats, a pubertal up-regulation of OXT mRNA in the brain is significantly reduced by gonadectomy prior to the onset of puberty (Chibbar *et al.*, 1990). Replacement with either E2 or testosterone reversed the effect of gonadectomy in female and male rats, respectively. Estrogen increases plasma levels of OXT immunoreactivity and OXT-NP (Amico *et al.*, 1981) and stimulates the release of OXT from the pituitary (Yamaguchi, *et al.*, 1979).

In a unique paradigm that mimics certain mammalian physiological events, ovariectomized rats were given E2 implants followed by progesterone 48 hours later (Amico *et al.*, 1995). Both E2 and progesterone were sustained for the next 12 days and the progesterone implant was removed 48 hours prior to sacrifice. This type of hormonal environment is seen during pregnancy and during lactation. With this hormonal regimen, hypothalamic OXT mRNA levels increased compared to sham treated cohorts. If progesterone was not removed or if a blank implant was administered instead of E2 there was no increase.

Estrogen stimulation for 2 days induced OXT-immunoreactivity in hypothalamic sites other than the SON and PVN (Jirikowski *et al.*, 1988) and increased OXT mRNA levels in the SON and the anterior commisural nucleus (Chung *et al.*, 1991). Therefore, estrogen may influence OXT's control of neuroendocrine and behavioural responses.

These *in vivo* data are supported by *in vitro* results in which rat (Adan et *al.*, 1991) and human (Richard and Zingg, 1990) OXT gene promoters, linked to various reporters, showed

increased activity when cell lines transfected with estrogen receptors and the gene promoter constructs were administered E2. However, the bovine potential ERE was shown to be non-functional in these experiments (Adan *et al.*, 1991). These results suggest that the ERE (see Introduction: *promoter sequences of NH genes*) found in the mammalian OXT gene can be directly stimulated by E2 in some species.

Studies on the effects of gonadal hormones on AVP mRNA expression have been limited. Salt-loaded rats with intact gonads increased their levels of AVP and OXT mRNAs in the PVN and SON compared to the control, non-salt-loaded animals (Crowley and Amico, 1993). Salt-loaded gonadectomized rats had levels comparable to controls, indicating that gonads are required to respond to salt loading. Administration of testosterone brought the levels of AVP mRNA back to what was seen with the osmotically stimulated, intact rats. In the male rat, gonadectomy reduced AVP mRNA concentrations in brain areas other than the PVN and SON (reviewed in De Vries *et al.*, 1992). Testosterone replacement reversed the decrease.

Objectives of the study

Since no information is yet available regarding the effects of sex steroids on the neurohypophysial hormones in non-mammalian vertebrates, the objective of the present study was to assess the effects of estradiol administration on AVT and IST gene expression in the goldfish, *Carassius auratus*. From the evidence presented above it appears that both AVT and IST play a role in teleostean reproduction. Elucidating the regulation of AVT and IST gene expression by physiological stimuli may lead to a better understanding of their endocrine role, specifically in reproduction, and possibly other functions in teleosts.

Materials and Methods

Experimental Animals

Goldfish, *Carassius auratus*, of both sexes were obtained from Tropical Fish Supplies (Fort Erie, Ontario) and held in 200 litre tanks supplied with flowing, dechlorinated City of Ottawa tap water (15^0 C). The average weight of the goldfish was 33.4 ± 1.3 g. All fish were maintained in the tanks for a minimum of four weeks prior to the experiments and were fed fish chow to satiation once daily. The fish were kept on a 10-hour light: 14-hour dark photoperiod.

Treatment

All treatments were given in July 1995 - a time when goldfish gonads are regressed and blood levels of sex steroids are low. Fish were divided into six groups according to treatment received: control (non-injected, 0 hours), estradiol treated for 12, 24 and 48 hours, and saline treated for 12 and 24 hours.

Because it is difficult to determine the gender of goldfish when the gonads are sexually regressed, treatments were administered in the following manner. Approximately 12 fish were placed into different sections of the tanks until the time of injections. Each group of 12 goldfish was given its injection and euthanized, by spinal transection, at the appropriate times. The abdominal cavity was then opened to view the gonads and to determine the sex of the fish. For each group of 12 goldfish, 4-5 were used for the study. The males, females with sexually mature gonads, or fish that did not appear normal were not included in this study.

17 Beta-estradiol (E2; Sigma) was dissolved in vehicle, 1:1 propylene glycol (BDH): absolute ethanol (BDH), to make a stock solution of 10 µg/20 µl and subsequently diluted in 0.6 % saline to give a final concentration of 20 µg/100 µl, according to Huggard (personal

communication; as described in Huggard *et al.*, 1996). Each E2 treated fish was given a 100 µl intraperitoneal injection of the steroid solution (therefore 20 µg of steroid). Saline treated fish were given a 100 µl injection of 0.6% saline including vehicle.

Radioimmunoassay

At the end of each experimental period, blood samples were collected, in 0.1 ml syringes, from the dorsal aorta following section of the spinal cord immediately posterior to the cranium. Blood was allowed to clot at room temperature for 1 hour. Following centrifugation, at 11 500 x g for 5 minutes, serum was removed and stored at -20^{o}C until time of assay. Serum was assayed for E2 levels using antibody-coated tubes from the Coat-A-Count kit (Diagnostic Products Corporation).

Tissue Preparation

After sacrifice, brains were removed and immediately immersion-fixed in 4% paraformaldehyde for *in situ* hybridization. Brains were kept in fixative for 48 hours at 4^{0}C and then dehydrated in a graded series of ethanol (48 hours in 70% ethanol at 4^{0}C, 1 hour in 95% ethanol at room temperature and overnight in 100% ethanol at room temperature) then in 1:1 xylene: absolute ethanol for 30 minutes at room temperature, 2 x 45 minutes in xylene at room temperature, and finally 2 x 45 minutes in melted paraffin at 60^{0}C. Brains were then embedded in melted paraffin and left to stand at room temperature allowing the wax to solidify.

Wax blocks were trimmed and then brain sections were cut on a microtome at 7 µm thickness. Sections containing the NPO of the goldfish hypothalamus were mounted onto Superfrost (Fisher Scientific) slides and dried on a slide warmer overnight.

Determination of gonadosomatic index

At the time of sacrifice, the gonads were dissected from the abdominal cavity, blotted on tissue paper, and weighed. The gonadosomatic index (GSI) was determined as follows: weight of gonad x 100/ total body weight. The GSI was measured to ensure that all fish possessed gonads that were regressed.

In situ Hybridization

An 18 mer IST oligonucleotide probe, 5'-ACC GAT GGG GCA GTT GGA-3', corresponding to nucleotides 10-27 of the peptide encoding region of the sucker IST precursor (Heierhorst *et al.*, 1989) and a 20 mer AVT probe, 5'-GGA CAG TTC TGG ATG TAG CA-3', corresponding to nucleotides 1-20 of the sucker AVT precursor (Heierhorst *et al.*, 1989) were synthesized by the University of Ottawa Biotechnology Research Institute.

Slides were immersed in xylene to dissolve the paraffin and then rehydrated through a graded series of ethanol: 100%, 2 x 2 minutes, 95%, 2 minutes, and 70% for 2 minutes and then in 0.1% diethyl pyrocarbonate (DEPC; Sigma) treated water for 2 minutes. Slides were then treated with Protease K (Sigma; 1 µg/µl in 0.1 M Tris buffer, pH 7.9) at 37^{0}C for 30 minutes to digest proteins surrounding mRNA targets. Slides were subsequently treated with 0.25% acetic anhydride in 0.1 M triethanolamine-HCl in phosphate buffer (pH 8.0), rinsed in 0.1 M Tris-HCl (pH 7.0), water and then air dried.

Radioactive procedures

The oligonucleotide probes were 3'-end labelled with ^{35}S-dATP (New England Nuclear). The reaction mixture contained 3.5 µl DEPC treated water, 5 µl 5 x terminal transferase buffer

[Boehringer Mannheim (BM)], 2.5 µl 25 mM $CoCl_2$ (BM), 2 µl probe (100 ng), 10 µl ^{35}S-dATP (125 µCi) and 2 µl terminal transferase (50 Units, BM). The mixture was placed in a 37^{0}C water bath for 1.5 hours. The probe was purified using a Nensorb 20 purification column. The column was washed first with 3 ml methanol (BDH) and then with 3 ml of 0.3 M Tris-HCl (pH 8.3; Sigma). The probe mixture was diluted in 0.5 ml of 0.3 M Tris-HCl and put through the column. The column was then washed with 1.5 ml of the Tris buffer. Finally, the probe was eluted from the column with 0.5 ml of 25% ethanol (BDH) and after the first couple of drops were discarded, approximately 0.3 ml was collected as labelled probe. The radioactivity was determined by mixing 3 µl of labelled probe with 3 ml of Ecolite scintillation liquid and counting in a Beckman scintillation counter. The specific activities of the labelled probes were 7 x 10^9 cpm/µg (IST) and 5 x 10^8 cpm/µg (AVT).

Labelled probe was added to each slide at a concentration of 2500 cpm/µl in hybridization buffer consisting of: 50% formamide (BDH), 6 x SSPE (1 x SSPE= 0.15 M NaCl, 0.01 M $NaH_2PO_4 \cdot H_2O$, 0.001 M EDTA), 50 mM Tris-HCl (pH 7.0), 2 x Denhardt's Solution (1 x= 0.02% of Bovine Serum Albumin, Ficoll and Polyvinylpyrolodine; Sigma), 0.2% Sodium Dodecyl Sulfate (Sigma) and DEPC treated water. Fifty µl of probe in hybridization buffer was added to each slide and the slides were left overnight at room temperature in a humidified box containing 50% formamide in DEPC treated water.

After hybridization, slides were washed in 2 x SSPE (30 minutes at room temperature), 2 x SSPE (30 minutes at 37^{0}C), 0.5 x SSPE (2 x 30 minutes at 37^{0}C), rinsed briefly in water and then in 70% ethanol with ammonium acetate (BDH; pH 8.3) for 2 minutes and then air dried. Slides were dipped in NTB2 nuclear track emulsion (Kodak), at 42^{0}C, diluted 1:1 with water. The emulsion was allowed to dry before slides were placed in a light tight box. Slides were exposed at 4^{0}C for 3 days (IST) or 34 days (AVT) before being developed with D-19 developer (Kodak) and fixed with fixer (Kodak). After fixation, slides were counterstained lightly with cresyl violet (Fisher Scientific).

Images from regions of the slides were digitized on a Macintosh computer using a Zeiss microscope and a Sony CCD video module camera. Quantitation of hybridization signal was carried out by counting autoradiographic silver grains using the public domain NIH image program (developed at the U.S. National Institutes of Health and available at http://rsb.info.nih.gov/nih-image/). Silver grains were counted over 10-20 cells where a nucleus was present in the perikaryon (cell soma), throughout the NPO.

For determination of non-specific or background counts, the number of grains in nuclear areas adjacent to the NPO (nucleus preopticus periventricularis, nucleus anterioris periventricularis; Peter and Gill, 1975) were counted, the mean determined and subtracted from the grain counts in the NPO neurons.

Non-radioactive procedures

Validation of the *in situ* hybridization protocol and the specificity of the probe-mRNA hybrids were assessed using a non-radioactive *in situ* hybridization method. For this method, the probes were 3' end-labelled with digoxigenin-dUTP (BM). The reaction mixture contained 4 µl 5 x terminal transferase buffer (BM), 4 µl 25 mM $CoCl_2$ (BM), 1 µl of 100 pmol probe, 1 µl of 1 nmol/µl digoxigenin-dUTP, 1 µl of 9 nmol/µl dATP, 2 µl of terminal transferase (50 U; BM) and filled to 20 µl with DEPC treated water according to Schmitz *et al.*, 1991. The mixture was placed in a 37^{0}C waterbath for 1.5 hours and then stored at -20^{0}C until use.

Labelled probe was added to each slide at a concentration of 100 pmol/µl in the same hybridization buffer and conditions as for the radioactive procedures. Post hybridization washes consisted of 2 x SSPE (10 minutes at room temperature), 2 x SSPE (10 minutes at 37^{0}C), 0.5 x SSPE (2 x 15 minutes at 37^{0}C). Slides were then placed in Buffer #1 (100 mM Tris, 150 mM NaCl, pH 7.5) and then incubated for 2 hours at room temperature with an anti-digoxigenin antibody coupled to Alkaline Phosphatase (BM) diluted 1:500 in Buffer #2 (Buffer #1 containing 0.3% Triton X-100). After incubation, slides were washed in Buffer #1 (2 x 10 minutes at room

temperature) and then placed in Buffer #3 (100 mM Tris, 100 mM NaCl, 50 mM $MgCl_2$, pH 9.5) for 2 minutes. A colour reaction was produced when the slides were placed in Buffer #3 containing the Alkaline Phosphatase substrates, 4-nitro-blue-tetrazolium (0.33 mg/ml in 70% dimethylformamide; BM) and 5-bromo-4chloro-3-indolylphosphate-4-tolusin salt (0.166 mg/ml in 100% dimethylformamide; BM). Slides were left in the colour reaction solution for 4 hours (IST) and 6 hours (AVT) and then cleared, dehydrated and coverslipped.

Controls used to check the specificity of the probes include: 1) hybridizing with a 100-fold excess unlabelled probe, 2) hybridizing with a 100-fold excess mismatched, unlabelled probe (unlabelled vasotocin with labelled isotocin and vice versa), and 3) RNase treatment (100 µg/ml) for one hour prior to hybridizing.

Morphometry of NPO perikarya

The diameters of the perikarya of neurons from the rostral to caudal extent of the NPO were determined to see if there were any differences, with regard to cell size, in the regulation of NH neurons by estradiol. The diameters were measured on the computer screen and then divided by the magnification of the microscope and camera to obtain the actual cell sizes. Using 15 fish from the study, 40-60 cell somata/fish were measured. Based on cell size the NPO was separated into 3 groups: a group of small cells called parvocellular neurons, a group of large cells called magnocellular neurons and an intermediate group of cells (see Results). The proportion of the NPO cells made up by the different cell groups was determined. In addition, the mean diameter of perikarya from each cell group was determined.

Statistics

Results are expressed as mean ± SEM. Differences in the silver grain count between treatment groups were determined by a one-way analysis of variance (ANOVA). If the ANOVA produced significant differences ($P < 0.05$), a *post-hoc* Tukey's test was performed on pairs of means.

Results

Cell Morphometry

The NPO of the goldfish is located in the preoptic area of the hypothalamus, dorsal to the optic chiasm and consists of relatively small cells rostroventrally and larger cells dorsocaudally. Diameters of cell somata in the NPO ranged from 8 to 48 μm. A histogram of cell number versus cell diameter (grouped within 2 μm bins) showed 3 major peaks (Fig. 4). From the peaks, 3 different cell groups were distinguished: 8-20 μm, 20-28 μm, and 28-48 μm. The smallest group of neurons is the parvocellular neurons, which make up the nucleus preopticus pars parvocellularis (NPOpc) according to Peter and Gill (1975). The NPOpc constitutes the nucleus preopticus magnocellularis pars parvocellularis (PMp), according to Braford and Northcutt (1983) where cell diameters were described as ranging from 9-12 μm. The largest sized neurons are the magnocellular neurons of the NPO pars magnocellularis, according to Peter and Gill (1975) and of the PM pars gigantocellularis (cell diameter of 50-55 μm), according to Braford and Northcutt (1983). The intermediate neurons populate both the NPOpc and NPOmc and constitute the PM pars magnocellularis (cell diameter of 20-25 μm) of Braford and Northcutt's map.

The parvocellular neurons accounted for 52.5% of the neurons in the NPO whereas; the intermediate and magnocellular neurons made up 23.44 and 24.44% of the neurons, respectively. The mean diameter of a parvocellular neuron was 13.6 ± 0.5 μm, an intermediate neuron, 24.6 ± 0.5 μm and a magnocellular neuron, 33.6 ± 0.5 μm.

The parvocellular neurons, located just caudal and ventral to the nucleus preopticus periventricularis (Peter and Gill, 1975), dorsal to the optic chiasm and along the third ventricle, are found at the most rostroventral aspect of the NPO (Fig. 5). As the nucleus extends dorsocaudally the cells become larger and there is a mixture of parvocellular and intermediate neurons and then intermediate and magnocellular cells extending along each side of the third ventricle (Figs. 6 and 7). Finally, there are only a few magnocellular cells with diameters of up to 48 μm located at the

most caudal tip of the NPO (Fig. 8 and 9), which is dorsal to the nucleus anterioris periventricularis of Peter and Gill [1975; (nucleus preopticus parvocellularis posterioris of Braford and Northcutt, 1983)].

Estradiol blood levels and gonadosomatic index

In non-injected control fish, the circulating E2 level was 0.151 ± 0.002 ng/ml and the GSI was 1.67 ± 0.170%, which is indicative of sexually regressed animals (Fig. 10A and B; Kagawa *et al.*, 1983). Injection of E2 produced a rise in blood levels of E2, which was highest at 12 hours (5.75 ± 2.94 ng/ml) and decreased to 3.8 ± 1.23 ng/ml at 24 hours and then to 0.613 ± 0.32 ng/ml at 48 hours (Fig. 10A). Fish treated with E2 for 12 and 24 hours possessed levels of E2 above saline treated and control fish and were within physiological range of sexually mature fish, according to Kagawa *et al.*, 1983. E2 treatment had no effect on the GSI of fish, which was similar to both saline treated and control groups (Fig. 10B).

In situ Hybridization

Validation of the *in situ* hybridization method and specificity of the probes were assessed using a non-radioactive *in situ* hybridization procedure. Addition of a 100-fold excess unlabelled probe to the hybridization buffer produced a signal with much reduced intensity (Fig. 11). Addition of a 100-fold excess of an unlabelled mismatched probe (i.e. labelled AVT probe with unlabelled IST probe and vice versa) produced no change in the intensity of the labelling as compared to a hybridization buffer with only labelled probe (Fig. 12). Prehybridization treatment of sections with 100 µg/ml of RNase for 1 hour abolished the labelling (Fig. 13).

Autoradiographic silver grains which represent detection of NH mRNAs were localized throughout the perikarya of the NPO. The localization patterns of IST and AVT mRNA were similar to the non-radioactive procedure. Silver grains were more numerous in neurons hybridized

with the IST probe than those exposed to the AVT probe despite the shorter exposure time for the IST probe (see discussion).

Neurons containing the IST mRNA were tightly packed close to the ependymal cells lining the third ventricle with an occasional neuron positioned away from the cluster. On the other hand, neurons containing AVT mRNA were scattered and positioned further laterally from the third ventricle compared to IST neurons. Examination of adjacent sections showed that localization of IST mRNA containing neurons differed from that of AVT mRNA containing neurons, suggesting that IST and AVT mRNA are synthesized in separate neurons (Fig. 14). These results agree with studies performed in goldfish (Lederis *et al.*, 1994) and in rainbow trout (Hyodo and Urano, 1991).

Quantitation of AVT mRNA

To determine if there were any differences between the different sized cell groups with regard to regulation of NH mRNA levels by E2, the silver grains were counted for each of the three different sized cell groups separately and for the whole NPO.

Looking at the NPO as a whole, the number of AVT silver grains per perikaryon displayed significant differences between the E2 and saline groups at 12 hours (Fig. 15A; ANOVA: P=0.043; Tukey: P=0.025). The number of silver grains in the saline group at 12 hours (17.7 $\pm$ 1.98 grains) tended to be lower than the control group (23.7 $\pm$ 1.14) but was not statistically different.

Looking at the NPO with respect to the three different sized cell groups showed different results. The number of silver grains in the parvocellular neurons was not statistically different between E2, saline, and control groups using the AVT probe (Fig. 15B). The intermediate cell group showed a pattern similar to the whole NPO (Fig. 15C). There was an increase in silver grains for the E2 group at 12 hours (34.8 $\pm$ 5.75) compared to the saline group at 12 hours (15.11 $\pm$ 0.863; ANOVA: P=0.007; Tukey: P=0.003).

There were statistical differences between several treatment groups when considering the magnocellular neurons (Fig. 15D and 16). There were fewer silver grains in the saline group at 12

hours (26.56 ± 2.999) as compared to the control group (43.35 ± 3.444; ANOVA: P=0.016; Tukey: P=0.031), and the E2 group at 12 hours (45.02 ± 3.279; Tukey: P=0.015). In addition, combining the counts for the intermediate and magnocellular neurons there is a significant decrease in the saline group at 12 hours (Fig. 15E; ANOVA: P=0.007; Tukey: P=0.003). Taken together, there is stimulation in relative levels of AVT mRNA in the intermediate-sized and magnocellular neurons 12 hours after E2 treatment compared to saline treated fish.

Quantitation of IST mRNA

The NPO as a whole showed no significant differences in the number of IST silver grains between treatment groups (Fig. 17A). Considering the different sizes of neurons, there were no significant differences between treatment groups for the parvocellular (Fig. 17B) and intermediate (Fig. 17C) neurons.

Looking at the number of IST silver grains, the ANOVA for the magnocellular neurons did not reach statistical significance (P=0.088) despite a tendency for the counts to be higher in all E2 treated groups compared to the control group (Fig. 17D and 18). At 24 hours, the silver grain count tended to be higher compared to the control group but did not reach statistical significance (Tukey: P=0.051) due to variation in data and a small sample size. The combined silver grain counts of the intermediate and magnocellular neurons showed no significant differences between treatment groups.

Figure 4. Number of cells versus diameter of goldfish nucleus preopticus cell bodies. Measurements were compiled within 2 μm bins. Three major peaks (a, b, c) distinguished 3 different cell sizes: parvocellular neurons (ranging from 8-20 μm), intermediate neurons (ranging from 20-28 μm), and magnocellular neurons (ranging from 28-48 μm).

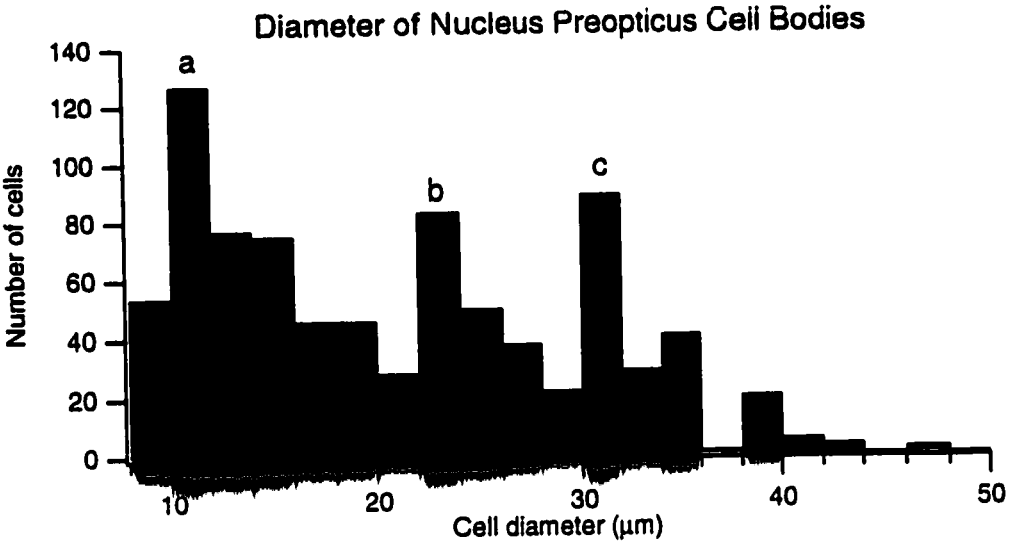

Diameter of Nucleus Preopticus Cell Bodies
Number of cells
140
120
100
80
60
40
20
0
a
b
c
10
20
30
40
50
Cell diameter (µm)

Figure 5. Coronal section of the goldfish brain at a rostral region of the NPO showing *in situ* hybridization for IST mRNA in parvocellular neurons (arrows) with a few intermediate neurons (arrowheads). The IST mRNA was detected using a digoxigenin labelled oligonucleotide probe detected with a digoxigenin antibody conjugated with alkaline phosphatase as a reporter. V represents the third ventricle. Magnification: 125 x

Figure 6. Coronal section of the goldfish brain at a rostral to middle region of the NPO showing *in situ* hybridization for IST mRNA in parvocellular (arrows) and intermediate (arrowheads) neurons. V represents the third ventricle. Magnification: 125 x

Figure 7. Coronal section of the goldfish brain at a middle to caudal region of the NPO showing *in situ* hybridization for IST mRNA in intermediate (arrowheads) and magnocellular neurons (arrows). V represents the third ventricle. Magnification: 125 x

Figure 8. Coronal section of the goldfish brain at a caudal region of the NPO showing *in situ* hybridization for IST mRNA in magnocellular neurons (arrows). V represents the third ventricle. Magnification: 125 x

Figure 9. Distribution of neuronal somata within the goldfish NPO, shown in schematic drawings of a parasagittal and 3 coronal sections. The coronal sections correspond roughly to Figs. 5-7. Large dots represent magnocellular perikarya; small dots parvocellular perikarya. Intermediate neurons (not shown on drawings) are mixed in with both the parvocellular and magnocellular neurons. Abbreviations can be found in List of Abbreviations. Modified from Fryer and Maler (1981).

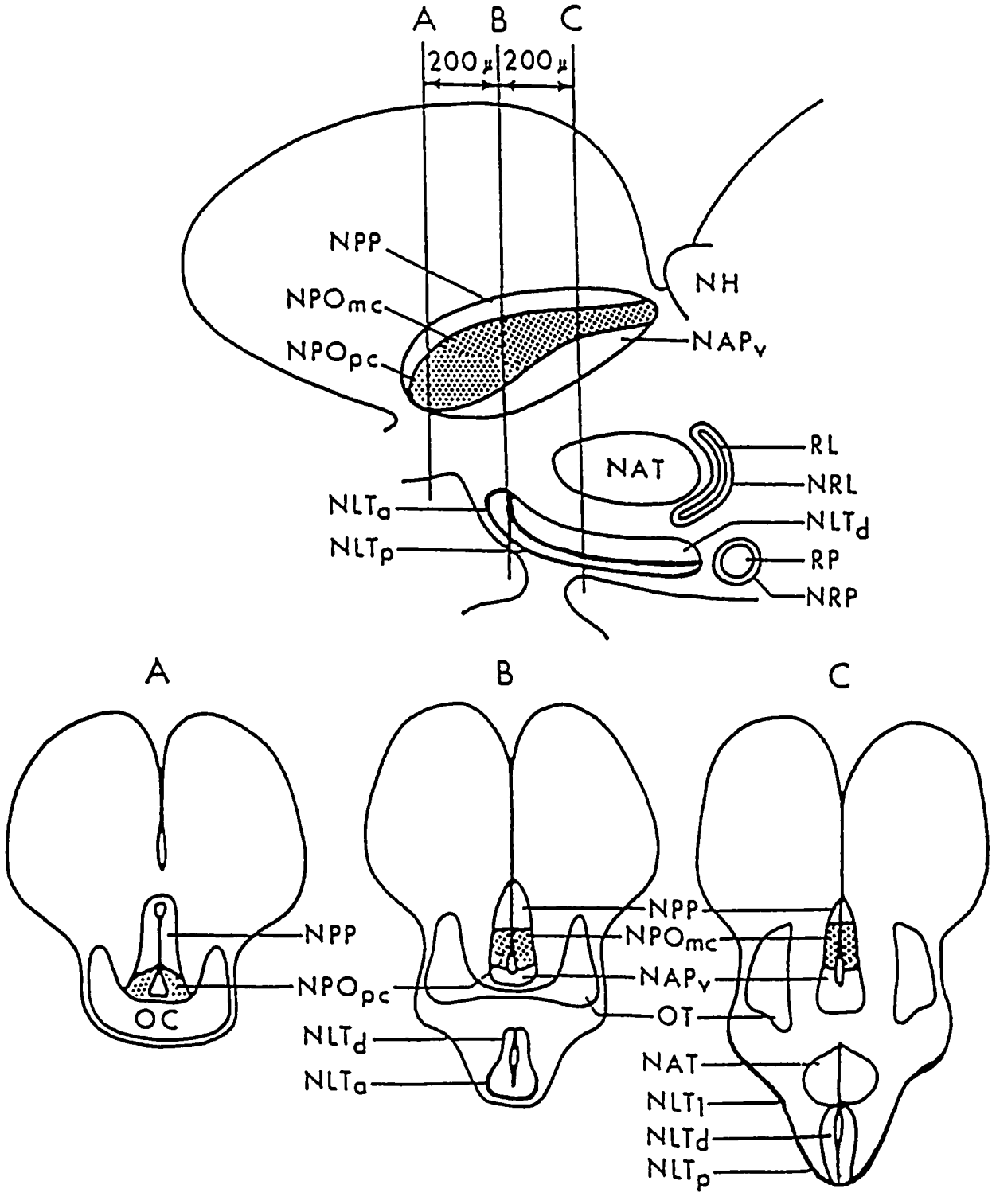
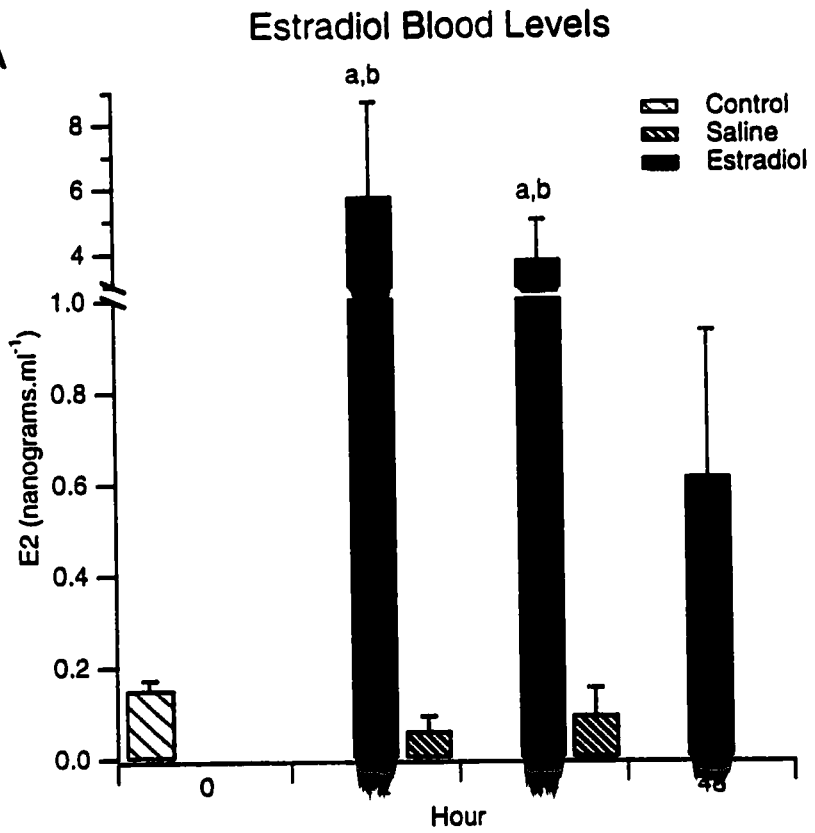

A
B
C
200 μ
200 μ
NPP
NPOmc
NPOpc
NH
NAPv
NAT
RL
NRL
NLTd
RP
NRP
NLTa
NLTp
A
B
C
NPP
NPOpc
OC
NLTd
NLTa
NPP
NPOmc
NAPv
OT
NAT
NLTl
NLTd
NLTp

Figure 10. A) Blood levels of E2 with time in goldfish given: no treatment (control), E2 treatment, or saline treatment. B) Gonadosomatic index (GSI= % of total body weight represented by the gonads; see Materials and Methods for formula) for the different treatment groups. Values represent mean $\pm$ SEM for each treatment group (n=4-5). *a*, $P < 0.05$ compared to saline group at same time period; *b*, $P < 0.05$ compared to control group.

A


Estradiol Blood Levels
a,b
a,b
Control
Saline
Estradiol
8
6
4
1.0
0.8
0.6
0.4
0.2
0.0
E2 (nanograms.ml-1)
0
48
Hour

B

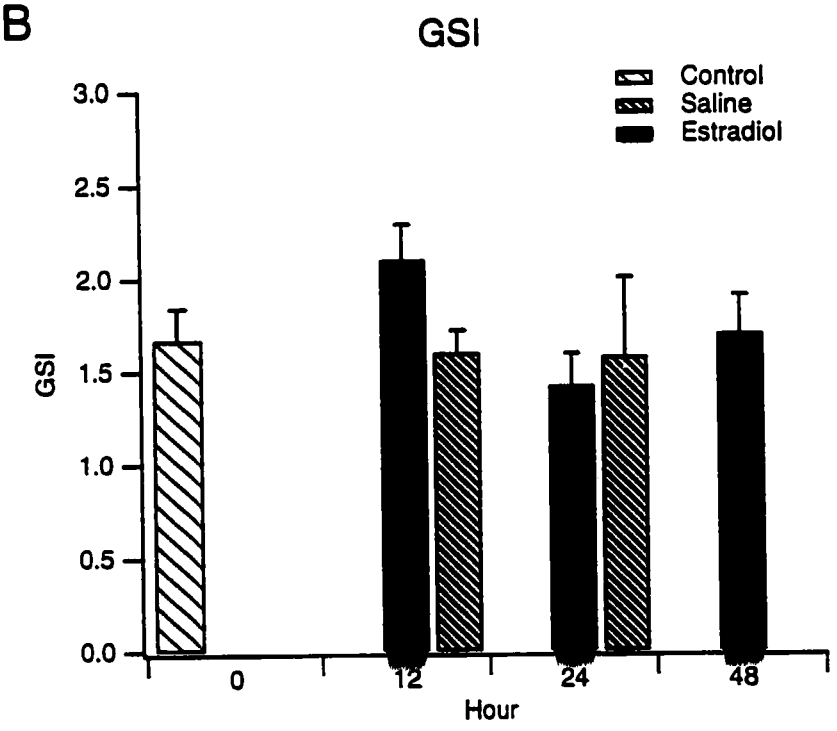

GSI
Control
Saline
Estradiol
3.0
2.5
2.0
1.5
1.0
0.5
0.0
GSI
0
12
24
48
Hour

Figure 11. Validation of the *in situ* hybridization protocol. *In situ* hybridization on adjacent sections of the goldfish NPO using a digoxigenin-labelled probe for IST (A, B) and AVT (C, D) mRNAs with (B, D) or without (A, C) the addition of a 100-fold excess unlabelled probe to the hybridization medium. Signal intensity is reduced substantially with the addition of the unlabelled probe. Magnification: 55 x (A, B), 110 x (C, D).

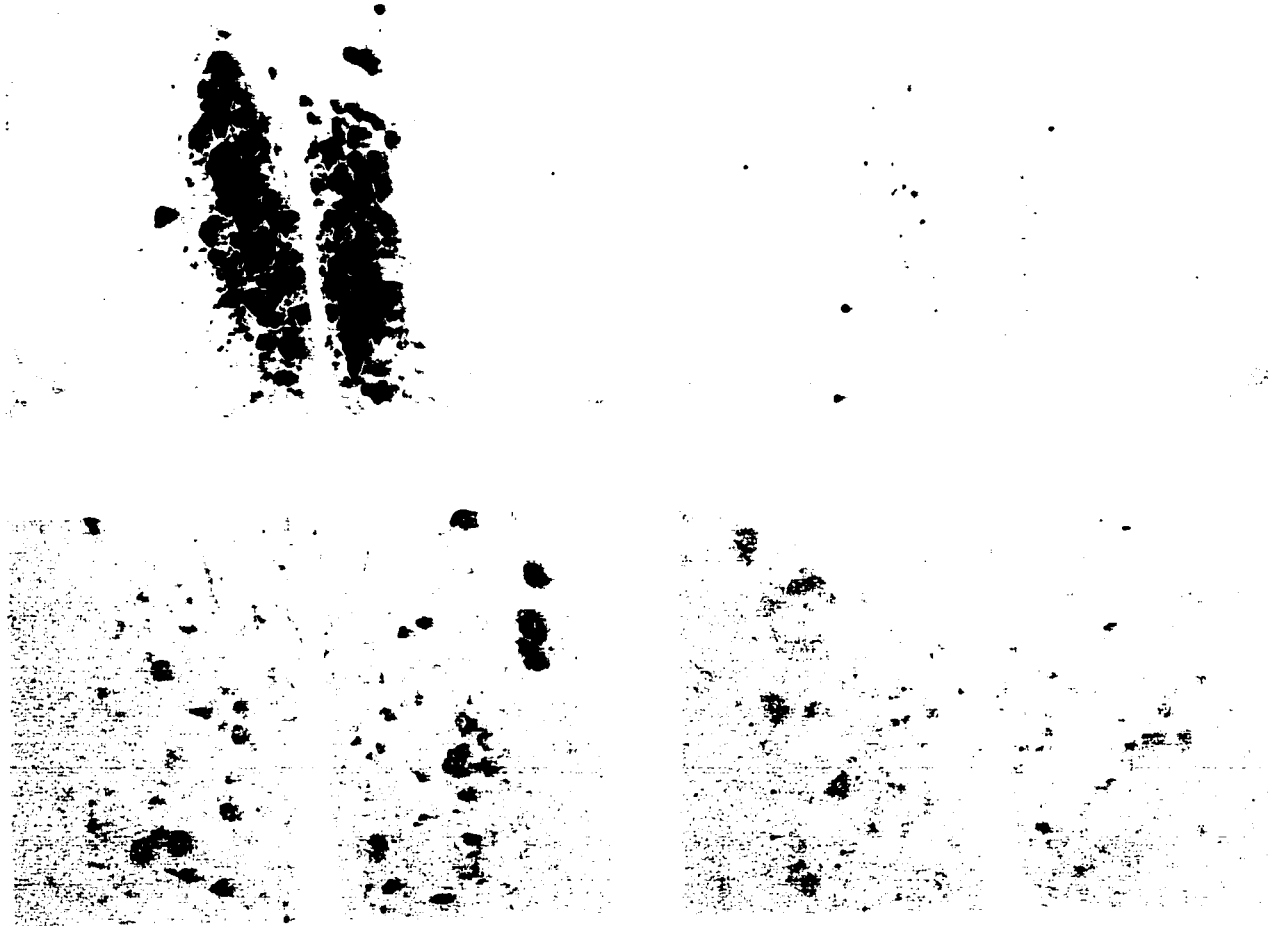


Figure 12. Validation of the *in situ* hybridization protocol. *In situ* hybridization on adjacent sections of the goldfish NPO using a digoxigenin-labelled probe for IST (A, B) and AVT (C, D) mRNAs with the addition of a 100 fold excess of unlabelled probe for AVT (B) or IST (D) into the hybridization medium. Signal intensity is unaltered with the addition of unlabelled heterologous probe. Magnification: 55 x (A, B), 110 x (C, D).

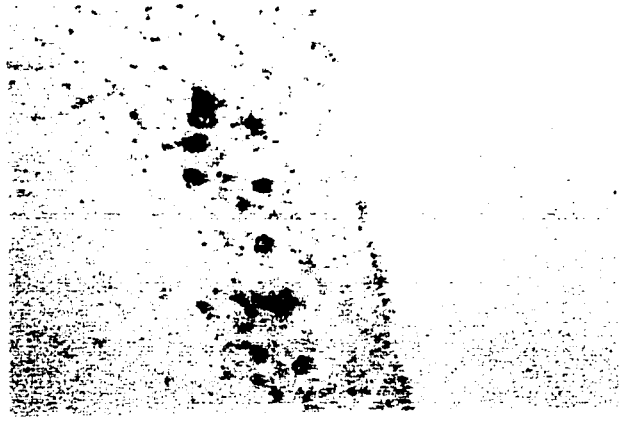
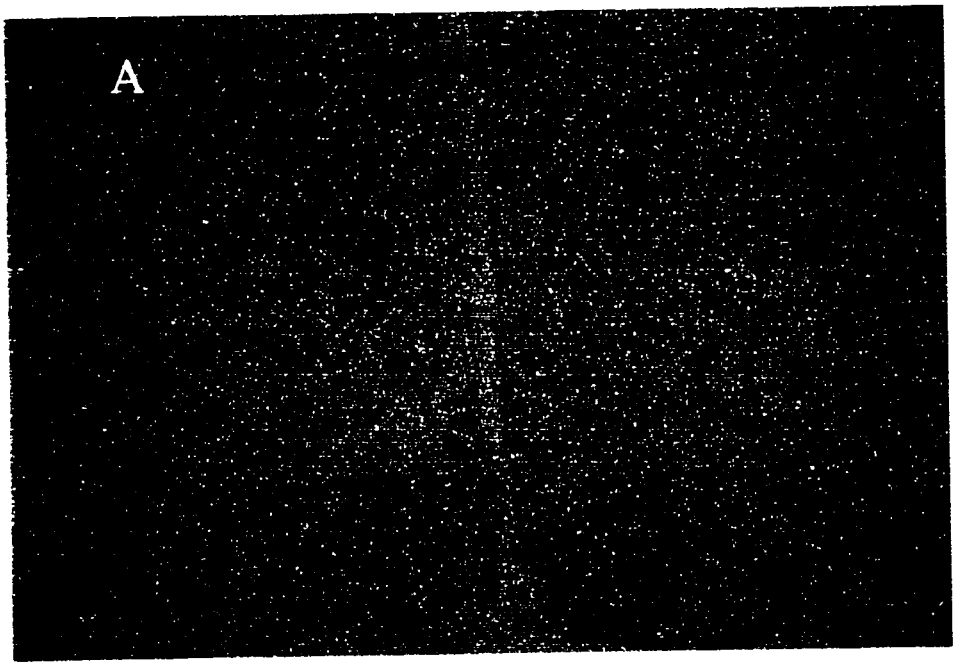


Figure 13. Validation of the *in situ* hybridization protocol. *In situ* hybridization on sections of the goldfish NPO pre-treated with 100 μg/ml RNase followed by hybridization using a digoxigenin-labelled probe for IST (A) and AVT (B) mRNAs. Labelling is abolished with RNase pre-treatment. Magnification: 85 x (A), 170 x (B).


A

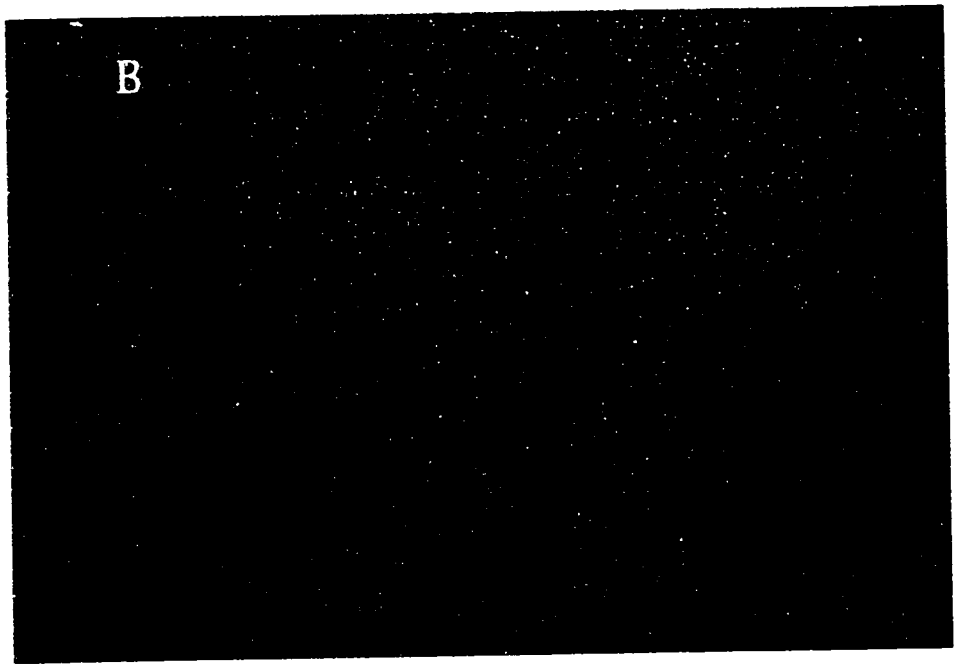

B

Figure 14. *In situ* hybridization on adjacent sections of the goldfish NPO using a digoxigenin-labelled probe for IST (A) and AVT (B) mRNAs. The neuron expressing AVT mRNA (red arrow) in B is localized between (black arrow) the two neurons expressing IST mRNA in A. The mRNAs for IST and AVT are expressed in separate neurons. Magnification: 170 x (A, B)

Figure 15. Effects of E2 administration with time on steady state levels of AVT mRNA in the goldfish NPO expressed by the number of grains per cell. Values represent mean $\pm$ SEM for each treatment group (n=4-5). Counts were completed for the entire NPO (A), parvocellular neurons (B), intermediate neurons (C), magnocellular neurons (D), and for both the intermediate and magnocellular neurons (E). *a*, $P<0.05$ compared to saline group at same time period; *b*, $P<0.05$ compared to control group.

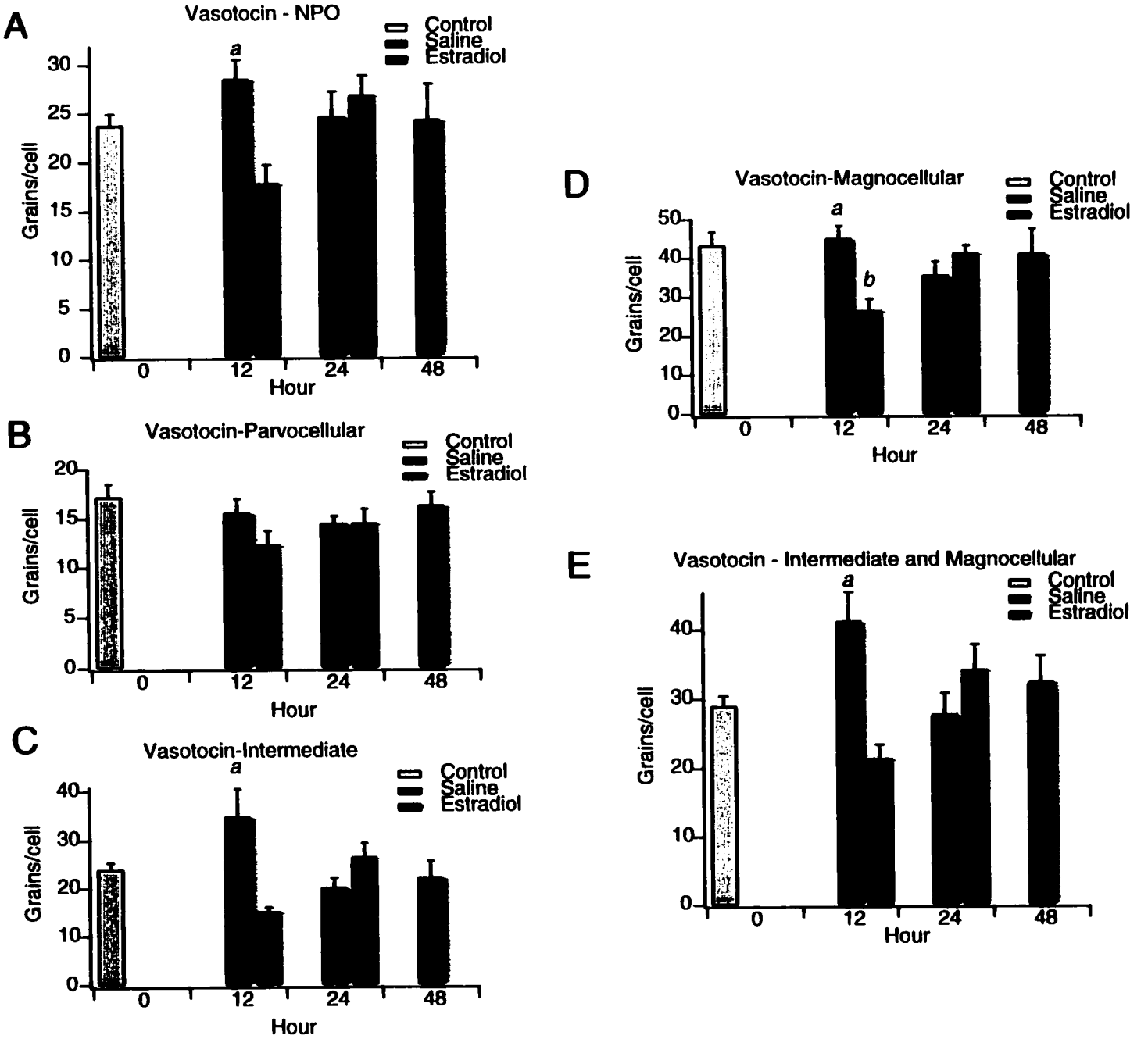

A
Vasotocin - NPO
Control
Saline
Estradiol
a
Grains/cell
30
25
20
15
10
5
0
0
12
24
48
Hour
B
Vasotocin-Parvocellular
Control
Saline
Estradiol
Grains/cell
20
15
10
5
0
0
12
24
48
Hour
C
Vasotocin-Intermediate
a
Control
Saline
Estradiol
Grains/cell
40
30
20
10
0
0
12
24
48
Hour
D
Vasotocin-Magnocellular
Control
Saline
Estradiol
a
b
Grains/cell
50
40
30
20
10
0
0
12
24
48
Hour
E
Vasotocin - Intermediate and Magnocellular
a
Control
Saline
Estradiol
Grains/cell
40
30
20
10
0
0
12
24
48
Hour

Figure 16. *In situ* hybridization of ^{35}S-labelled probe for AVT mRNA under brightfield (A, C) and darkfield (B, D) conditions from E2 (A, B) and saline (C, D) treated fish at 12 hours. A single magnocellular neuron from an E2 treated fish (arrow in A, B) is shown containing more silver grains than either of the two magnocellular neurons from the saline treated fish (arrows in C, D). Magnification: 340 x (A, B, C, D)

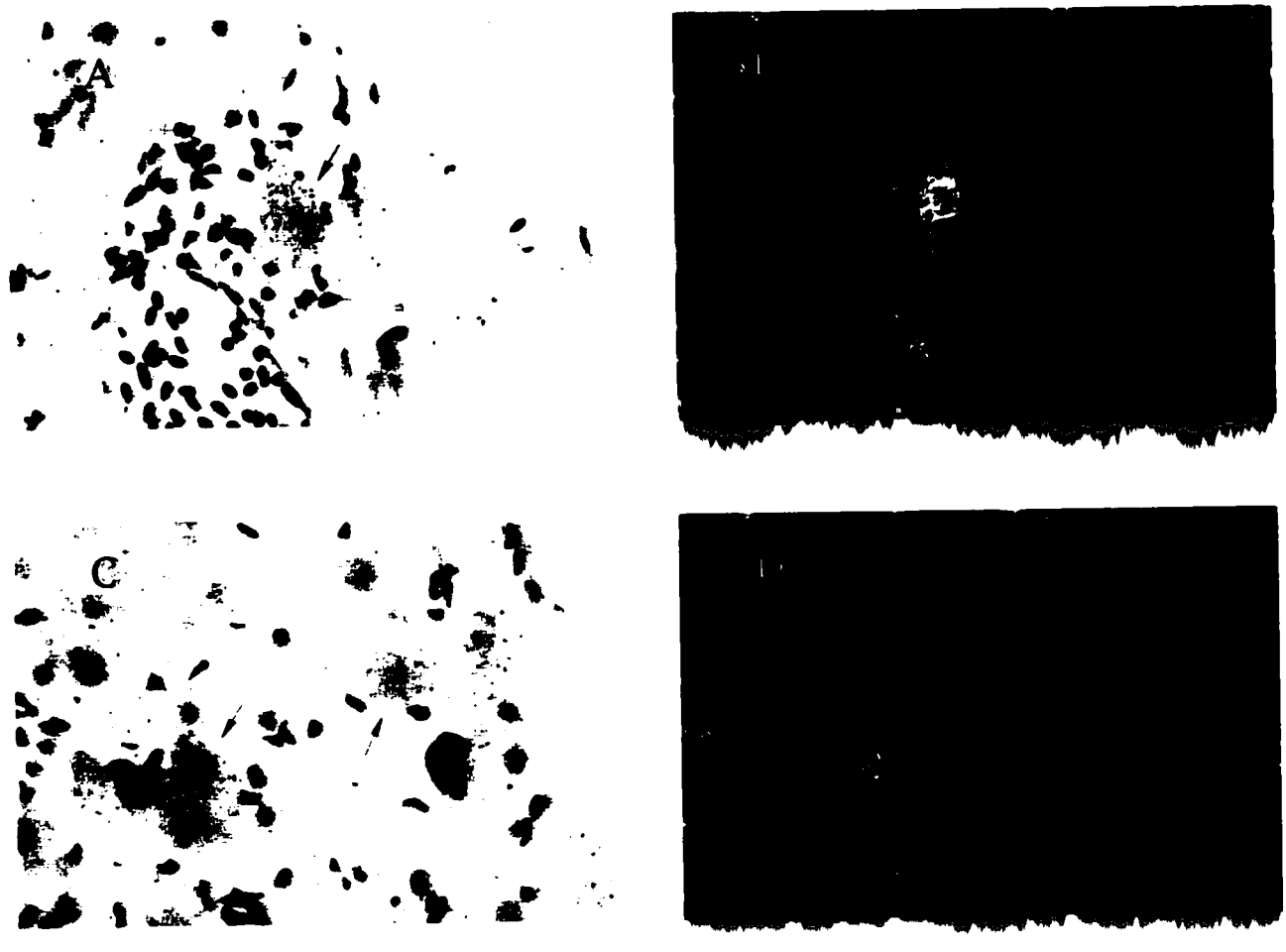
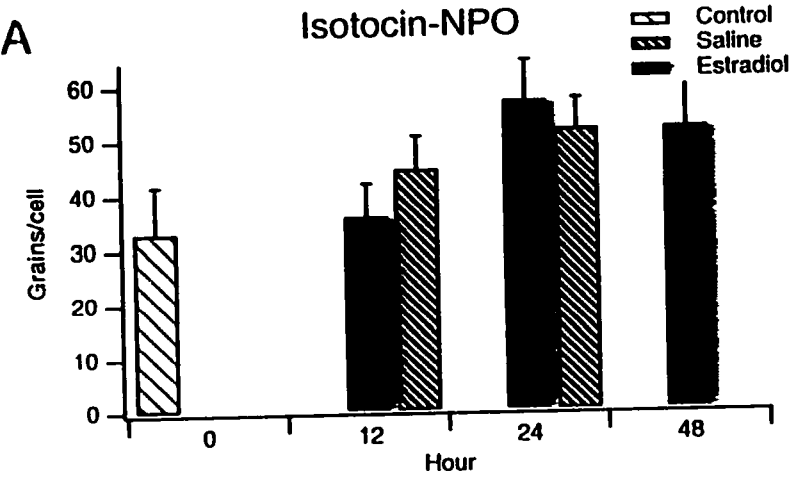
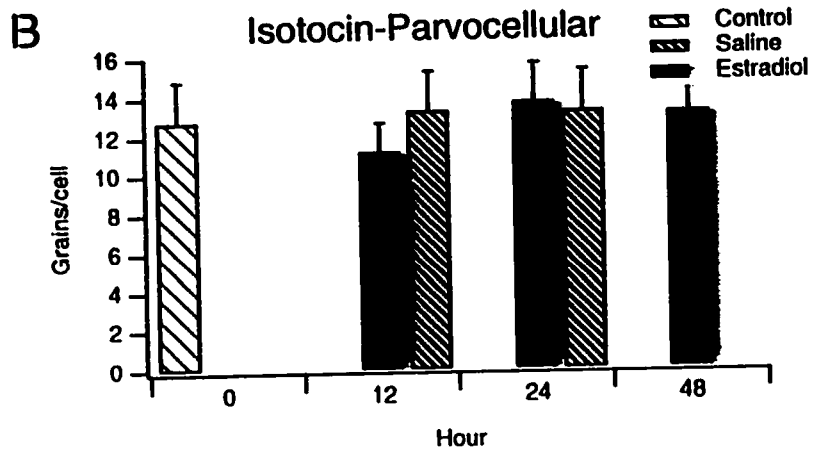
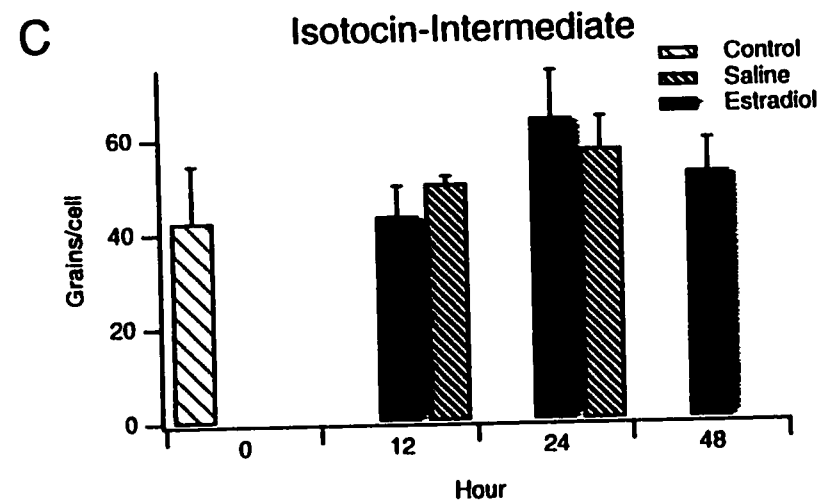
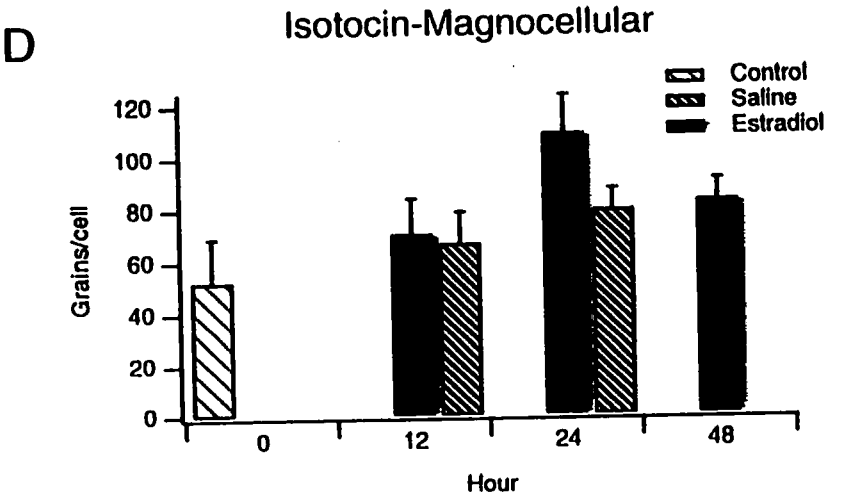

A

Figure 17. Effects of E2 administration with time on steady state levels of IST mRNA in the goldfish NPO expressed by the number of grains per cell. Values represent mean ± SEM for each treatment group (n=4-5). Counts were completed for the entire NPO (A), parvocellular neurons (B), intermediate neurons (C), magnocellular neurons (D), and for both the intermediate and magnocellular neurons (E). Statistically analysis revealed no changes in IST mRNA for any of the treatment groups.


A
Isotocin-NPO
Control
Saline
Estradiol
Grains/cell
60
50
40
30
20
10
0
0
12
24
48
Hour


B
Isotocin-Parvocellular
Control
Saline
Estradiol
Grains/cell
16
14
12
10
8
6
4
2
0
0
12
24
48
Hour


C
Isotocin-Intermediate
Control
Saline
Estradiol
Grains/cell
60
40
20
0
0
12
24
48
Hour


D
Isotocin-Magnocellular
Control
Saline
Estradiol
Grains/cell
120
100
80
60
40
20
0
0
12
24
48
Hour

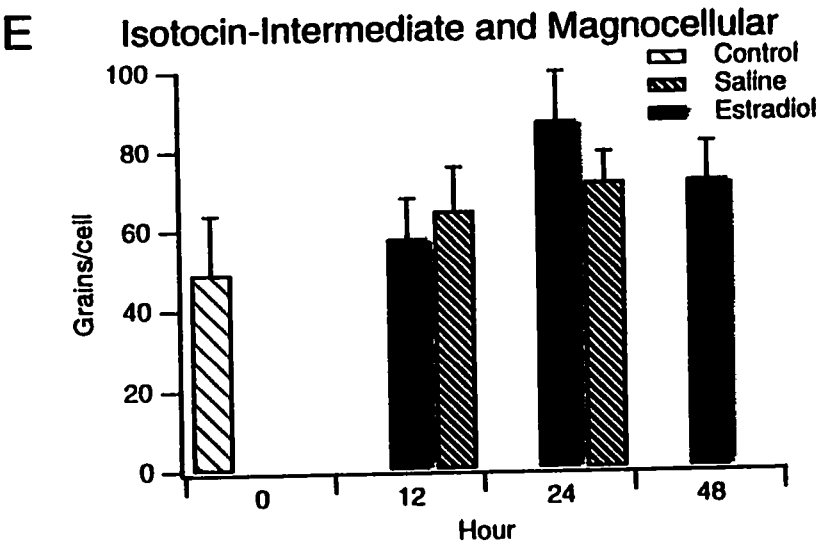
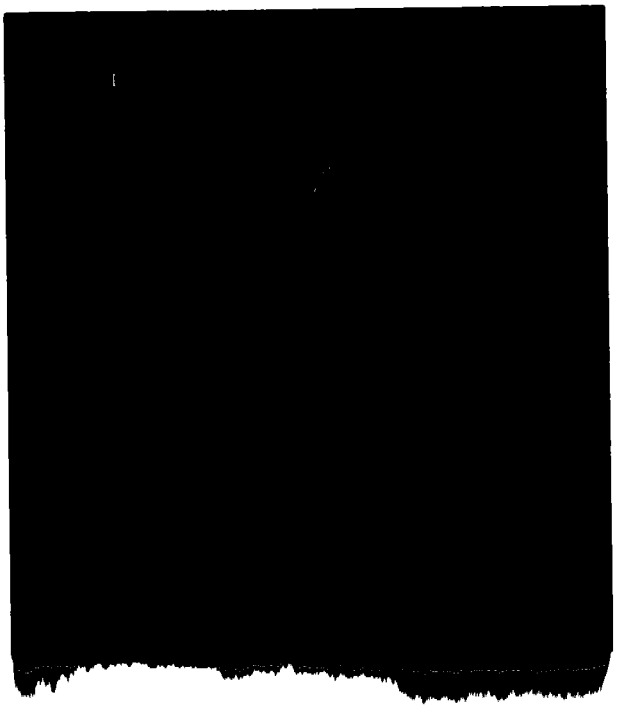
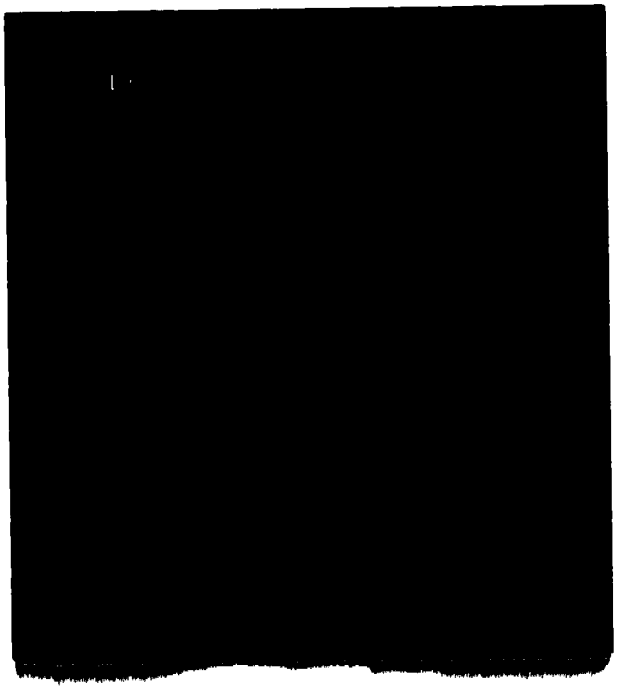

E
Isotocin-Intermediate and Magnocellular
Control
Saline
Estradiol
Grains/cell
100
80
60
40
20
0
0
12
24
48
Hour

Figure 18. *In situ* hybridization of ^{35}S-labelled probe for IST mRNA under brightfield (A, C) and darkfield (B, D) conditions from a saline treated fish (A, B) and an E2 (C, D) treated fish at 24 hours. Magnification: 250 x (A, B, C, D).


A




C



Discussion

Nucleus preopticus and cell size

The NPO of the goldfish contains a range of cell sizes. It was determined that the differences in cell sizes could be grouped, based on abundance, into three different classes. Traditionally, the smaller sized cells are called parvocellular neurons and the larger sized cells, magnocellular neurons. This nomenclature is applied here. A third population of cells that is distributed among the two other cell types has been described previously. Braford and Northcutt (1983) named the three cell populations the nucleus preopticus magnocellularis pars parvocellularis (PMp), pars magnocellularis (PMm), and pars gigantocellularis (PMg). They described the NPO as three populations localized in distinct areas whereas, in the present study, the intermediate cells were found mixed with the parvocellular and magnocellular neurons.

The regulation of NH gene expression by estradiol was examined with respect to the NPO as a whole, and then with respect to each of the three cell sizes separately. In this manner, functional differences could be distinguished between the cell sizes within the NPO (see below). Previous studies examining NH gene expression in fish during osmoregulatory adjustments have used Braford and Northcutt's (1983) PMm and PMg groupings to identify neurons involved in this aspect of fish physiology (Hyodo and Urano, 1991).

Quantitating gene expression

Interpreting results obtained using autoradiographic techniques to determine mRNA levels is difficult. Changes in mRNA levels can be due to either changes in transcription of DNA into mRNA or changes in the usable pool of mRNA by increased stability of mRNA species. *In situ* hybridization of mRNA does not explain why there is a relative increase or decrease of mRNA

species in the perikaryon of the neurons studied. Whether any changes are attributable to an increase or decrease in transcription can be answered with other techniques such as a run on assay or *in situ* hybridization with probes designed to bind to intronic sequences on heteronuclear RNA (hnRNA). This RNA species is the immediate product of transcription and is the precursor to the mature mRNA and thus better reflects gene transcription compared to mRNA.

An advantage of using *in situ* hybridization over other mRNA detecting techniques like Northern blot hybridization or dot blot hybridization is that the exact localization of the cells producing these hormones can be determined. In addition, different cell types can be distinguished such as parvocellular versus magnocellular. If more than one hormone is being considered, *in situ* hybridization can be used to determine if colocalization occurs. With the advent of synthetic oligonucleotides, very little molecular biology knowledge and materials are needed to perform *in situ* hybridization.

The oligonucleotides used in this study were derived from the published nucleotide sequences encoding AVT (Heierhorst *et al.*, 1989) and IST (Figueroa *et al.*, 1989) of the white sucker. The genes encoding goldfish AVT and IST have not yet been isolated and sequenced. Because goldfish and the white sucker are from the same family (cyprinidae), the use of the sucker sequences proved to be viable. Using the IST probe produced a robust signal with very little exposure time. On the other hand, the AVT probe produced a weaker signal (i.e. less silver grains) despite the autoradiographs being exposed for 30 days longer than the IST probe. Presumably, this weaker signal is due to more mismatches of nucleotides between the sucker AVT probe and goldfish AVT mRNA, which would have decreased the stability of the probe-mRNA hybrid. Alternatively, there may be very little AVT mRNA present in the neurons of the goldfish NPO.

In addition, as cyprinids are tetraploid, two copies of each gene are present within the genome of the goldfish and the white sucker. The sequence used to produce the IST probe is identical in both the IST-I and IST-II genes of the white sucker. On the other hand, the sequence used for the AVT probe is found in the AVT-I gene of the sucker but differs by 4 nucleotides in the same part of the AVT-II gene (Morley et al., 1990). It was determined that in the white sucker, the

AVT-I and the AVT-II mRNAs are expressed in unequal amounts (Morley *et al.*, 1990). The AVT-I mRNA represents 70% of the AVT mRNA found in the white sucker whereas AVT-II mRNA accounts for 30% of the total. It is not known whether a differential expression of AVT-I and -II mRNAs exists in goldfish. As the AVT probe has 4 mismatches with the AVT-II mRNA in the white sucker, it is possible that the probe used could have several mismatches with one of the mRNAs in goldfish. Furthermore, the number of mismatches between the goldfish and the white sucker are not known so presumably the weaker signal is a consequence of weaker binding of the AVT probe to one or both of the AVT mRNAs of the goldfish compared to the IST probe and the IST mRNAs. In preliminary experiments, an increase in washing temperatures decreased both the background and signal significantly using the AVT probe. However, the same increases in washing temperatures largely decreased the background and not the signal utilizing the IST probe, which suggests that a few mismatches exist between the AVT probe and the goldfish AVT mRNA. The washing temperatures used for the study were not very stringent, however, they were stringent enough to give a reasonable signal-to-background ratio.

Estradiol regulation of neurohypophysial hormone gene expression

The NHs appear to have both distinct and overlapping functions in vertebrates. In mammals, AVP is mainly involved with osmoregulation, cardiovascular tone and ACTH release whereas OXT is concerned with reproductive physiology (reviewed in Cunningham and Sawchenko, 1991). In fish, functional information has been limited, however, AVT and IST seem to regulate both fluid homeostasis and reproductive events (reviewed in Maetz and Lahlou, 1974). Elucidating factors that regulate biosynthesis of the NHs in teleosts will assist in determining their function.

The experiments performed for this thesis lead to the conclusion that E2 has an effect on AVT mRNA levels (expression) in the NPO of the goldfish. Looking at the NPO as a whole, from the rostral to caudal extent, there is a significant increase in AVT mRNA 12 hours after estradiol

administration compared to saline treated animals. Differences became more evident when mRNA increases were considered within individual NPO neuronal size classes. Magnocellular neurons (28-48 μm cell body diameter) displayed significant increases in AVT mRNA of both the non-injected control group and the estradiol group 12 hours after treatment compared to the saline group 12 hours after treatment. Within the intermediate neurons, differences were apparent only between the estradiol and saline groups 12 hours after treatment. The estradiol group displayed higher levels of AVT mRNA. When the combined data for the intermediate and magnocellular neurons were considered, there were higher AVT mRNA levels in the estradiol group than in the saline control group 12 hours after treatment. No differences were seen between the treatment groups in the parvocellular neurons.

The decrease of AVT mRNA levels in the saline treated group 12 hours after treatment may be attributed to two factors. Firstly, it has been shown that there are diurnal changes in plasma and brain levels of AVT in fish. In goldfish, hypothalamic and telencephalic (including the NPO and preoptic region) AVT contents are higher at night (20:00 hours) than during the daytime (10:00 hours) (Hontella and Lederis, 1985). In that study, no significant differences between the pituitary AVT content at the two times were found. The goldfish sampled for the present study had higher levels of AVT mRNA in magnocellular neurons in uninjected control animals compared to saline treated animals, 12 hours after treatment. The animals used for the control group were sampled during the daytime (between 11:00 and 12:00 hours) and those used for the 12 hour group were sampled at night (between 23:00 and 24:00 hours). Therefore, it appears there is a contradiction in the diel variation of AVT peptide and mRNA levels in the Hontella study compared to the present study, respectively. No information is available on the levels of AVT mRNA during a 24 hour period in goldfish hence it is not known whether the levels of AVT mRNA are correlated with AVT peptide in goldfish. In rainbow trout, plasma levels of AVT fluctuated over a 24 hour period (Kulczykowska and Stolarski, 1996). Fish adapted to both fresh and brackish waters showed higher levels of AVT at 16:00 hours and lower levels at 5:00 hours.

In rats, there exists a diurnal pattern of AVP peptide concentrations in the hypothalamus where levels are seen to rise during the daytime (light phases) and decrease during dark phases (Noto et al., 1983; Windle *et al.*, 1992). Parallel changes were seen in AVP plasma concentrations while pituitary contents were inversely related to both hypothalamic and plasma concentrations (Windle *et al.*, 1992). However, in the rat, no variations in AVP mRNA levels were seen in the PVN and SON over a 24-hour period (Burbach *et al.*, 1988). Therefore, in rats, AVP peptide and mRNA levels appear not to fluctuate in a parallel fashion. The same could be true of goldfish AVP peptide and mRNA levels. Although hypothalamic AVP mRNA does not vary in rats, there may be a diurnal pattern in the goldfish NPO that might cause a decrease in the AVP message during the dark hours as seen in the saline group 12 hours after treatment. In order to control for diurnal changes of NH biosynthesis, a non-injected control group should have been sampled at the various time intervals of the experiment. Due to the procedure of determining gender as outlined in the material and methods, too few animals were purchased for the study to create an adequate number of treatment groups. Also, there were not enough holding facilities available to keep additional animals.

Secondly, the stress of the injection procedure may have caused a decrease in AVT mRNA. AVT may have been released soon after the injection, as it is involved in the stress response, by stimulating ACTH release from anterior pituitary cells *in vitro* (Fryer and Lederis, 1986). *In vivo*, AVT increases the secretion of cortisol into the blood from the interrenal glands of goldfish presumably by evoking the release of ACTH (Fryer and Leung, 1982). Initially, the depleted stores of AVT may have been restored by an increase in AVT mRNA synthesis. The increased blood levels of cortisol eventually would have inhibited AVT release and/or production in the NPO to restore normal hormone levels. Indeed, cortisol has been shown to be an inhibitor of the NPO during a stress response (Fryer and Peter, 1977). This type of feedback inhibition is seen in endocrine systems throughout vertebrate classes.

Effects of estradiol on vasotocin expression

The data from the experiments in this thesis showed an increase in AVT mRNA levels within intermediate and magnocellular neurons. In fish and mammals, the effects of stress on peptide and mRNA concentrations in the hypothalamus are usually seen in parvocellular neurons rather than magnocellular neurons. Studies on the involvement of the NPO of goldfish in the stress response have shown the parvocellular neurons to be the primary regulators, however, magnocellular neurons may also be involved. In goldfish, CRF mRNA and peptide are colocalized with AVT mRNA and peptide in both parvocellular and magnocellular neurons (reviewed in Lederis *et al.*, 1994). The administration of metopirone, an adrenocortical inhibitor that reduces circulating levels of cortisol, followed by a stress, enhanced horseradish peroxidase uptake (an index of neurosecretory activity) by parvocellular neurons (Fryer and Maler, 1980). In addition, the parvocellular but not the magnocellular neurons of metopirone treated goldfish developed morphological changes indicative of activation, suggesting inhibition of these neurons by adrenocorticoids. (Fryer and Boudreault-Chateauvert, 1981). However, a decrease in AVT immunostaining and an increase in the cross-sectional area of the nucleus for both parvocellular and magnocellular neurons has been observed in metopirone treated goldfish (Fryer and Lederis, 1988). In mammals, CRF and AVP are secreted by parvocellular neurons into the median eminence where they get taken up into the hypophysial portal system to stimulate ACTH release from the corticotropes of the anterior pituitary (Linton *et al.*, 1985).

Although the parvocellular neurons are traditionally the main regulators of ACTH release and the stress response via connections to the hypophysial portal system, studies have shown that magnocellular neurons can also participate. Administration of a hypertonic solution or ether inhalation induced a significantly smaller ACTH response in rat magnocellular neurons inhibited by chronic hyponatremia, compared to normal rats (Dohanics *et al.*, 1991). This suggests a role for the magnocellular neurons in the stress pathway. Hormones secreted from magnocellular neurons projecting to the posterior pituitary can reach the anterior pituitary via short portal vessels, which

connect the posterior lobe of the pituitary to the anterior lobe (Murai and Ben-Jonathan, 1986). In goldfish, secretions of magnocellular neurons can also gain access to the anterior pituitary (pars distalis) by capillaries which loop from the neurointermediate lobe into the pars distalis (Fryer and Peter, 1983).

In the present study, the stress of removing the goldfish from their water environment, immobilizing them, and injecting them may have been physiologically significant. These types of stressors would activate the parvocellular neurons of the NPO but may also involve the intermediate and magnocellular neurons. However, no effects on AVT mRNA levels were seen in the parvocellular neurons in the present study despite a tendency for the AVT mRNA levels to be lower in the saline group compared to the estradiol group at 12 hours (Fig. 15B). Moreover, the effects of stress on AVT mRNA levels might have occurred prior to 12 hours. In response to acute, stressful stimuli in rats, AVP mRNA and hnRNA levels rise in the parvocellular neurons of the PVN (Herman, 1995; Ma *et al.*, 1997). The rise in AVP hnRNA levels occurs quickly, from 15-60 minutes after the onset of the stimulus, and precedes that of AVP mRNA which appears at 4 hours (Ma *et al.*, 1997). The hnRNA levels return to baseline measurements about 4 hours after the onset of the stimulus. No measurements were reported by Ma *et al.* beyond 4 hours so presumably the AVP mRNA levels also eventually decreased. A decrease in AVP hnRNA and mRNA levels as well as AVP immunoreactivity seen following a post-stress elevation is due to glucocorticoid feedback (Stillman *et al.*, 1977; Davis *et al.*, 1986; Herman, 1995). Therefore, in mammals, effects on AVP mRNA levels due to certain acute stresses occur rapidly. To better understand if similar effects exist in goldfish, it would be informative to sample animals at time periods prior to 12 hours such as 1, 2, 4 or 6 hours. It may be that effects of stress are seen within intermediate and magnocellular neurons prior to and up to 12 hours.

A combination of diurnal changes and stress effect may have caused lower AVT mRNA levels within intermediate and magnocellular neurons of saline treated goldfish 12 hours after treatment. Therefore, E2 administration was able to increase AVT mRNA levels offsetting the decreased AVT mRNA levels seen at 12 hours after saline treatment

Because AVT is involved with fish osmoregulation (reviewed in Urano *et al.*, 1994), the NaCl in the saline injection might have had an osmotic effect on AVT mRNA levels. This effect is probably negligible because the amount of saline injected was small (100 µl) and the saline solution was isoosmotic. Similarly, the effect of the ethanol and propylene glycol is probably negligible since the large surface area of the fish gills would have dialyzed the ethanol and propylene glycol rapidly to clear the circulation before reaching the brain.

Effects of estradiol on isotocin expression

Results from autoradiographic measurements in the IST experiments showed no differences in mRNA levels amongst the various treatment groups when looking at the NPO as a whole. There was a tendency for IST mRNA levels to increase 24 hours after estradiol treatment as compared to the non-injected, control group (P=0.051; Fig. 17D). Although mRNA levels for IST mRNA in the estradiol treatment group at 24 hours showed an increase over the saline treated group these data did not attain statistical significance. The silver grain counts reflecting IST mRNA levels for the intermediate neurons tended to increase 24 hours after estradiol treatment over the control group and its saline counterpart, but, this change was not statistically significant.

No dramatic decreases were seen in IST mRNA levels between any of the saline-injected groups and the control group as was seen with AVT mRNA levels in the present study. Similarly, no diurnal changes in plasma levels of IST were detected in the rainbow trout (Kulczykowska and Stolarski, 1996). Although IST was shown to be equipotent to AVT in increasing secretion of ACTH directly from the anterior pituitary cells of the goldfish (Fryer and Lederis, 1986), its physiological role as an ACTH releaser in fish is not known. In rainbow trout, IST was shown to be less potent than both AVT and CRF in stimulating ACTH release (Pierson *et al.*, 1996). OXT, the mammalian homologue of IST, was shown to be preferentially released over AVP in rats placed under stresses such as forced swimming or physical immobilization (Lang *et al.*, 1983). However, OXT administration failed to produce an elevation of circulating levels of ACTH in

healthy humans (Page *et al.*, 1990). Therefore, the status of OXT and IST as ACTH releasers is uncertain. Accordingly, effects on IST mRNA levels are not seen due to stress or to diurnal changes.

Studies of sex steroid regulation of gene expression in goldfish using the same dosages as in the present study found a biphasic response (Huggard *et al.*, 1996). The effect of testosterone on growth hormone gene expression produced an inhibition at 12-24 hours, followed by a stimulation of mRNA levels at 36 hours and longer time points. Using smaller doses, a stimulation of GH mRNA was seen at 24 hours. It was suggested that the biphasic response seen with the higher dose was probably due to the supraphysiological amount of testosterone injected. It is possible that the dosage used in the present study was too high, which might explain the unexpected results in the IST experiments. However, the radioimmunoassay indicated that all the estradiol treated groups had blood levels of estradiol within physiological range, according to Kagawa *et al.* (1983). Fish used in the present study were sexually immature with regressed gonads. Sexually regressed gonads are indicated by a low GSI and are accompanied by low plasma levels of E2. This approach was taken to ensure that administration of E2 produced a rise in E2 blood levels that were still within physiological range. Nevertheless, the dosage used may have had an initial effect before 12 hours that may have blunted a response that could have been, but was not seen in the present study.

Functional relevance of estradiol regulation of neurohypophysial hormone gene expression

OXT is involved in the regulation of reproductive physiology in mammals and E2 regulates OXT gene expression (Miller *et al.*, 1989; Chibbar *et al.*, 1990; Chung *et al.*, 1991), immunoreactivity (Jirikowski *et al.*, 1988) and plasma levels (Amico *et al.*, 1981; Yamaguchi *et al.*, 1979). Since IST is the teleostean homologue of OXT and has a role in teleostean reproduction, it is surprising that E2 did not stimulate IST mRNA expression. At 24 hours, IST mRNA levels tended to be higher in estradiol treated fish than their saline counterpart but were not

statistically significant mainly due to a large variation in data and a small sample size. In rats, administration of exogenous E2 increased OXT mRNA levels in the SON 48 hours after treatment (Chung *et al.*, 1991). The effects of exogenous E2 on IST mRNA levels might occur at 48 hours or beyond. The IST mRNA levels at 48 hours after E2 treatment tended to be smaller, however, they were not statistically different from levels seen at 24 hours. In addition, the IST mRNA levels at 48 hours after treatment were not statistically different from the mRNA levels of either of the two saline groups. Alternatively, effects seen at the mRNA level might have been too small to detect with the *in situ* hybridization protocol used in the present study. Performing *in situ* hybridization with probes directed toward intronic sequences to measure hnRNA levels might have revealed significant effects of E2 administration because hnRNA is the immediate product of DNA transcription and thus better reflects gene expression than mRNA. As the goldfish sequences are unknown for the NH genes, and intronic sequences being highly variable between species, this type of *in situ* hybridization is not currently feasible.

In light of the present results and previous data obtained using AVT in reproductive bioassays (reviewed in Maetz and Lahlou, 1974), AVT most likely plays an important physiological role in reproduction in teleosts. Regulation of AVT mRNA levels by E2 in teleosts probably occurs during certain physiological events rather than during a regular cycle. AVT has been shown to induce spawning and contraction of genital tracts in oviparious fish (fertilization and development of eggs occurs outside of maternal body), and premature parturition in viviparious fish (fertilization and development of eggs occurs within maternal body). Other evidence that AVT is involved with reproductive behaviour is provided by observations that AVT injections enhance the spawning reflex in the killifish (reviewed in Moore, 1993). NHs are also involved with reproductive behaviours in other vertebrates (reviewed in Moore, 1993). Egg laying in females and courtship in males in the amphibian, rough-skinned newt, are stimulated with AVT and these responses are sex steroid sensitive, including E2.

In the experiments projected in this thesis the enhancement of AVT mRNA levels because of E2 administration occurred in the intermediate and magnocellular neurons but not the

parvocellular neurons. In vertebrate physiology, the NH magnocellular neurons play a largely "classic" endocrine role whereas the parvocellular neurons are involved with ACTH secretion, neurotransmission and neuromodulation (reviewed in De Wied *et al.*, 1993; Urano *et al.*, 1994; Lederis *et al.*, 1994). The present results show that in teleosts, intermediate neurons are likely functionally more similar to magnocellular than to parvocellular neurons.

As the structure of NHs has evolved over hundreds of millions of years so have their functions. As only one NH is present in the most primitive vertebrate, the hagfish (Heierhorst *et al.*, 1992), AVT would presumably have to perform the functions of both NHs that are found in higher vertebrates. In the mollusc, *Lymnaea stagnalis*, lysine conopressin has been characterized and possesses both OXT-like reproductive functions (Van Kesteren *et al.*, 1995a) and AVP-like metabolic functions (Van Kesteren *et al.*, 1995b). In teleosts, the NHs appear to have some overlapping functions, with respect to reproduction, as well as distinct functions as AVT, but not IST, determines vascular tone in rainbow trout (Le Mevel *et al.*, 1993). Also, AVT and IST were equipotent in stimulating ACTH release in goldfish (Fryer and Leung, 1982), however, AVT was more potent than IST in stimulating ACTH release in rainbow trout (Pierson *et al.*, 1996).

In mammals, NHs have mainly distinct functions. Some overlap of function does occur as both AVP and OXT are involved with sodium excretion in the mammalian kidney (Balment *et al.*, 1986) and both have similar effects in the brain (reviewed in De Wied *et al.*, 1993). As vertebrates evolved into organisms with increasingly complex physiology, the NHs adopted functions that are more distinct. One way of achieving this distinction of function is through their target organs, as seen by the somewhat distinct localizations of the receptors for AVP and OXT (see Introduction: *NH receptors*). Selectivity of the NH receptors for one hormone over the other, despite the related structures, may also play an important role (reviewed in Acher *et al.*, 1995).

In the white sucker, IST (Hausmann *et al.*, 1995) and AVT (Mahlmann et al., 1994) receptor mRNAs are localized in similar organs such as the liver, gills, and lateral line. The IST (Hausmann *et al.*, 1995), but not the AVT, (Mahlmann *et al.*, 1994) receptor mRNA was found in female reproductive organs. However, AVT was able to activate the IST receptor *in vitro*

(Hausmann *et al.*, 1995), which suggests an alternate route for AVT to function in teleostean reproduction.

Mechanism of estradiol's actions

The mechanism by which E2 exerts its effect on NH neurons is not well understood. When E2 binds to its receptor, it is able to affect gene transcription directly as the activated receptor can bind to the promoter sequence of certain genes (reviewed in Parker, 1995). Although the mammalian OXT gene possesses a functional estrogen response element (see Introduction: *Promoter sequences of NH genes*) within its promoter sequence, there is no localization of nuclear estrogen receptors in OXT and AVP magnocellular neurons in rats (Simerly et al., 1990; Lopes *et al.*, 1995) and monkeys (Herbison *et al.*, 1995). However, there is evidence for expression of estrogen receptors in the quail (Balthazart *et al.*, 1989), guinea-pig (Warembourg and Poulain, 1991), and sheep (Voisin *et al.*, 1997).

No colocalization studies of estrogen receptors and NHs have yet been reported in fish, however, E2 binding sites have been determined in the teleost brain using steroid autoradiography. In goldfish (Kim *et al.*, 1978a) and platyfish (Kim *et al.*, 1978b), ^{3}H-estradiol was concentrated in the hypothalamus in the NPP, NLT and NPO in both the parvocellular and magnocellular neurons. In rainbow trout, both *in situ* hybridization (Salbert *et al.*, 1991) and immunocytochemistry (Anglade *et al.*, 1994) techniques have been used to visualize the estrogen receptor mRNA and peptide, respectively. Estrogen receptor mRNA and peptide localizations were similar with some differences. The mRNA was found in great abundance in the NLTp, NRL, NPP, NAPv and areas of the telencephalon. There was a weak signal found in the NLTl, NRP and NPOpc and NPOmc. Estrogen receptor immunoreactivity was concentrated in the NPP, NLTi, NLTa, and NLTp but none was found in the NPO. The differences in the steroid autoradiography studies and the

estrogen receptor localization studies, with respect to localization of estrogen receptive cells within the NPO, could be due to species variation and a greater sensitivity of the *in situ* hybridization procedure over immunocytochemistry (Anglade *et al.*, 1994).

Attempts to determine the localization of the estrogen receptor mRNA in the goldfish brain were unsuccessful during the course of the present study (data not included). *In situ* hybridization experiments using the evolutionary conserved DNA binding domain sequence of the rainbow trout estrogen receptor gene to derive an oligonucleotide probe for use in the goldfish, did not yield a detectable hybridization signal. In another laboratory, the same negative results were seen in the goldfish when a Northern Blot was performed using a riboprobe from the rainbow trout estrogen receptor (Anglade *et al.*, 1994). Also, an antibody directed toward the hormone binding domain of the rainbow trout estrogen receptor produced no immunostaining in goldfish (Anglade *et al.*, 1994). This latter result could be due to the evolutionary distance between the two species of fish (Anglade *et al.*, 1994). Whether AVT and IST cells in the goldfish and other teleosts contain estrogen receptors is still under question. Therefore, it is not known if E2 affects gene expression in a direct genomic manner or in an indirect manner, through other neurons that possess estrogen receptors. More localization studies need to be performed in order to find where estrogen receptive cells are located and to determine their chemical identity.

A recent study in rats has shown that estrogen receptors exist on neurons that project to the SON (Voisin *et al.*, 1997). Using a fluorescent retrograde tracer injected into the SON, double labelling of tracer and estrogen receptor immunolocalization occurred in approximately 15% of neurons in the OVLT, AVPv, and MPN and in 8 and 5% of neurons in the MnPO and SFO, respectively. The authors suggested that the SFO, OVLT and MnPO neurons were affecting AVP neurons as these areas are involved in fluid homeostasis and AVPv and MPN neurons are affecting OXT neurons because these neurons are involved in reproductive events. Therefore, it appears that in rats, OXT and AVP neurons are modulated by E2 indirectly through neurons found in anterior hypothalamic areas.

The chemical identity of most of the neurons projecting to the magnocellular neurons is not known. Glutamatergic and GABAergic neurons from the OVLT have been shown to respectively stimulate and inhibit SON neurons (Yang *et al.*, 1994). Receptor subunits for glutamate (Watanabe *et al.*, 1993; Van den Pol *et al.*, 1994) and GABA (Fenelon *et al.*, 1995; Fenelon and Herbison, 1995) have been localized in magnocellular neurons. Triple labelling studies of estrogen receptive neurons projecting to the SON from the brainstem showed that of these neurons very few were noradrenergic (Voisin *et al.*, 1997). In goldfish, receptors for GABA have been found in the NPO (Martinoli *et al.*, 1990). More localization studies are needed to ascertain how estradiol's effects are being mediated in the NPO.

Future directions

The NHs are an evolutionarily conserved family of peptides that have been studied extensively. The aim of this study was to determine if E2 regulates NH genes and in doing so, it was hoped to gain a better understanding of whether the NHs function in teleostean reproduction. From here, studies must be continued to ascertain whether there is any change in the E2 regulation of these genes with respect to dose response. The regulation of the NH genes by other physiological factors must also be considered. The best results for the present study would be obtained by sequencing the goldfish genes for the NHs.

Along with these physiological studies, more localization and electrophysiology work must be performed to determine the mechanisms with which E2 and/or other factors mediate their effects. Finally, characterization of receptors from other vertebrate species might shed some light on how these hormones have evolved.

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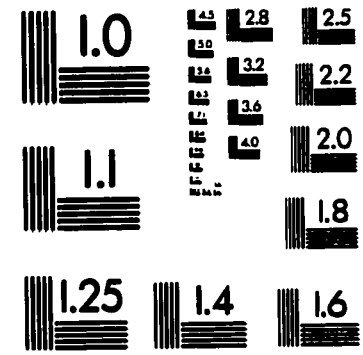
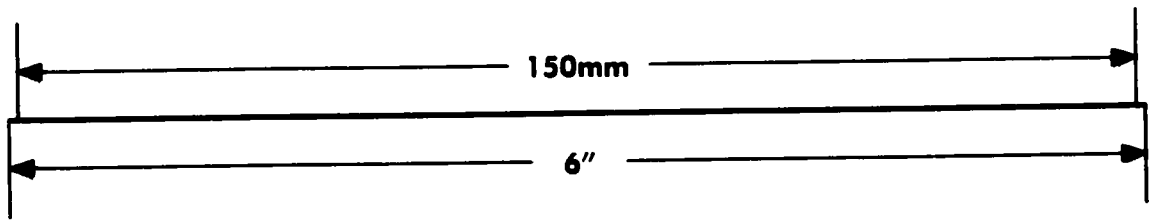
TEST TARGET (QA-3)





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