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APOPTOSIS IN THE ADULT GOLDFISH PITUITARY

By

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Cette Thèse est dédiée à la mémoire de Martha Sewell, Rita Thériault, Gustave Thériault, Jaques Beaulieux et Madeleine Therriault-Beaulieux.

ABSTRACT

This thesis research characterized apoptosis in the adult goldfish pituitary. Apoptosis is a form of programmed cell death that plays key roles in many biological processes by selectively removing old or unwanted cells. Morphological and biochemical signs of apoptosis established its presence within the gland. The pituitary gonadotrophs secrete gonadotropin-II to regulate sex steroid production, ovulation and sperm production.

It was possible to combine TUNEL with immunocytochemistry and demonstrate apoptosis in gonadotropin-II positive cells in the pars distalis of the pituitary, allowing for the first time the identification of apoptotic gonadotrophs *in situ*. Other studies suggest that pituitary cell populations and function fluctuate through the seasonal reproductive cycle. Apoptosis could play a role in regulating secretion by regulating the cell number. Treatments with the dopamine agonist apomorphine, and with a gonadotropin-releasing hormone agonist failed to modulate the apoptosis levels measured by DNA laddering or by TUNEL. Finally, this study demonstrated that the calcium ionophore A23187, significantly increases apoptosis in whole pituitary gland cultures. These studies established a new method to identify apoptotic hormone-secreting cells.

Cette recherche de thèse caractérise l'apoptose dans l'hypophyse du poisson rouge adulte. L'apoptose est une forme de mort cellulaire programmée qui joue un rôle important dans plusieurs processus biologiques par l'élimination sélective de cellules vieilles ou indésirables. Des signes biochimiques et morphologiques indicateurs d'apoptose ont confirmé sa présence dans la glande. Les gonadotropes de l'hypophyse secrètent la gonadotropine-II pour réguler la production de stéroïdes sexuel, l'ovulation et la spermatogenèse.

Il fut possible de combiner le marquage TUNEL avec l'immunocytochimie et démontrer l'apoptose chez des cellules gonadotropine-II positives dans la pars distalis de l'hypophyse, permettant pour la première fois l'identification de gonadotropes apoptotiques *in situ*. Les traitements avec l'agoniste de la dopamine apomorphine ou avec un agoniste du GnRH n'ont pas démontré d'effet sur le niveau de l'apoptose mesuré par fragmentation d'ADN sur gel ou par TUNEL. Finalement, l'étude a démontré que l'ionophore calcique A23187, communément utilisé lors d'études sur la sécrétion hormonale, augmente de manière significative le niveau d'apoptose dans une culture de glande entière. Ces études ont établie une nouvelle méthode d'identification des cellules hormonales apoptotiques.

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LIST OF ABBREVIATIONS

AIF	apoptosis initiation factor
ANOVA	analysis of variance
Apaf-1	apoptosis protease activating factor-1
Apo	apomorphine
ATP	adenosine triphosphate
Bad	BCL2-antagonist of cell death
Bax	Bcl-2 associated protein X
Bcl-2	first identified in B cell lymphoma
Bcl-X	B-cell leukemia/lymphoma x
Bcl-Xl	Bcl-X long
Bcl-Xs	Bcl-X short
Bid	BH3 interacting domain death agonist
Bik	BCL2-interacting killer
Bp	base pair, a measure of DNA length
BrdU	bromodeoxyuridine
BSA	bovine serum albumin
Ced 3, 4, 9	from the <i>C. elegans</i> gene cell death defective
CAD	caspase-activated-Dnase
CCD	charge coupled device
D1	dopamine receptor 1
D2	dopamine receptor 2

DA	dopamine
DAPI	4'6-diamidino-2-phenylindole
DED	death effector domain
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
dUTP	deoxyuridine triphosphate
E ₂	estradiol
EDTA	ethylenediamine tetra-acetic acid
FADD	Fas associated death domain
FSH	follicle stimulating hormone
FKBP12	FK506 binding protein
FRAP	FKBP12-Rapamycin associated protein
GABA	gamma amino-butyrlic acid
GH	growth hormone
GnRH	gonadotropin-releasing hormone
sGnRH, cGnRH	Salmon and Chicken GnRH
GTH	gonadotropin
ICAD	CAD inhibitor
IGF-1	insulin-like growth factor 1
IgG	immunoglobulin G
ISEL	<i>in situ</i> end labeling
kbp	kilo base pair, a measure of DNA length

Mcl-1	myeloid leukemia cell differentiation protein
Met	metoclopramide
mRNA	messenger RNA
MS-222	tricainemethanesulfonate
NIL	neurointermediate lobe
NO	no treatment (i.e. control)
PD	pars distalis
PIT	pituitary (implies whole gland)
PBS	phosphate buffered saline
RNA	ribonucleic acid
RNase	ribonuclease
SDS	Sodium Dodecyl Sulfate
SAL	saline injection treatment
Serneg	lack of serum treatment
TAE	tris acetate EDTA buffer
TE	tris EDTA buffer
TdT	terminal desoxynucleotidyl transferase
TNF	tumor necrosis factor
TUNEL	terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling
zfBLP1	zebrafish Bcl like protein 1

INTRODUCTION

Apoptosis is a fundamental biological process involved in several biological events, and is known to be present in the pituitary. The focus of this thesis is to morphologically characterize apoptosis in the goldfish (*Carassius auratus*) pituitary. Moreover, the goldfish model has been shown to be useful in the study of cell-type specific apoptosis because pituitary hormones can still be detected immunocytochemically late into the apoptotic process, allowing the specific identification of dying cells.

Apoptosis

Apoptosis is a form of programmed cell death characterized by cell shrinkage, a condensation of the chromatin, DNA cleavage and membrane blebbing (Wyllie, 1997; Tilly and Robles, 1999; Kerr *et al*, 1972). The cell breaks up into membrane-bound bodies that are, *in vivo*, phagocytosed by neighboring cells and macrophages with no inflammation. This is different from necrosis, a form of passive cell death resulting from massive cellular trauma, that results in the lysis of the dying cell and release of its content into the intercellular environment, triggering inflammation (Steller, 1995). It has been proposed that one of the fundamental functions of apoptosis is to allow the safe and non-inflammatory removal of dead cells (Leist and Jaattela, 2001).

Apoptosis can be triggered by DNA injury, cytotoxic cells (Willie, 1997), withdrawal of growth factors and by the presence of chemical messengers such as cytokines (Bowen *et al*, 1998) or neurotransmitters (Drewett *et al*, 1993). Apoptosis can be a transcriptional or a non-transcriptional event, since inhibitors of transcription or translation can stop cell death in certain conditions, but they fail to do so (or even induce apoptosis) in others. This suggests that, in

most cells, the apoptotic machinery is fully present and that the cell is ready to commit suicide following appropriate signals (Steller, 1995). Apoptosis plays an important role in embryogenesis by removing excess cells during tissue remodeling, in homeostasis of tissues by maintaining the cellular population (Bowen *et al*, 1998), in tumor suppression by removing damaged cells that might have become malignant (Lyons and Clarke, 1997) and in the immune system by killing infected cells and by eliminating lymphocytes that are no longer necessary. Inappropriate control of apoptosis can lead to cancer (Evan and Littlewood, 1998), autoimmune disease (Fadeel *et al*, 1999) or can play a part in various degenerative pathologies such as Parkinson's disease (Willie, 1997). Apoptosis also plays a role in the loss of cells due to diverse traumas such as strokes (Snider *et al*, 1999)

The apoptotic machinery

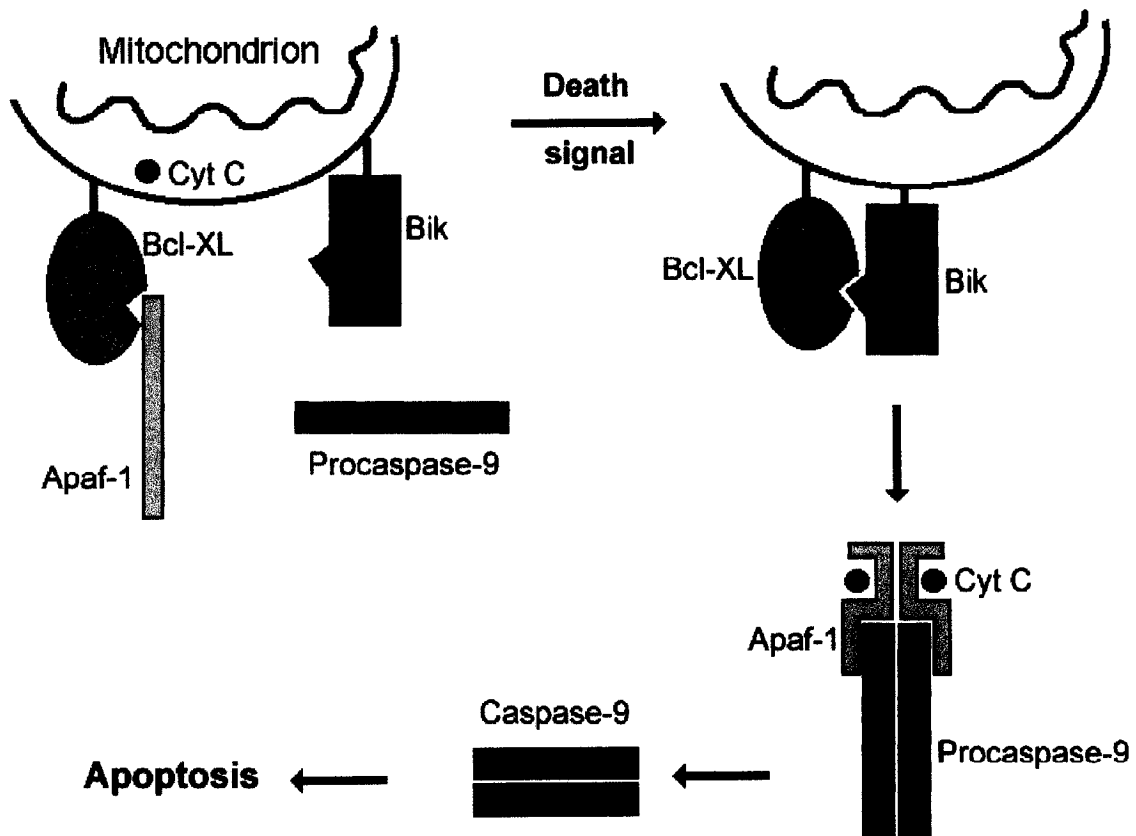
The apoptotic machinery is evolutionary conserved in metazoans. Studies in the nematode *Caenorhabditis elegans* have revealed 3 central components of the cell death program: the genes *ced-3*, *ced-4* and *ced-9* (Song *et al*, 1999). *Ced-4* acts as an activator of *Ced-3*, while *Ced-9* inhibits *Ced-4*. *Ced-3* is a cysteine protease that executes apoptosis in *C. elegans* (Golstein, 1997). Functional homologues of *ced-3*, *ced-4* and *ced-9* have been found in mammals. The cysteine protease family called caspase is the mammalian homologue of *Ced-3* and *Ced-9* is a structural and functional homologue of *Bcl-2*. The functional homologues of *Ced-4* have been recently discovered, *Apaf-1* (Adams and Cory, 1998) and *Apaf-2*. *Apaf-2* is a dual function protein and turned out to be cytochrome *c* (Bowen *et al*, 1998). The apoptotic machinery is far more complex in vertebrates than in invertebrates, involving more genes and pathways (Aravind *et al*, 2001)

The Bcl-2 family:

The Bcl-2 gene family contains both pro- and anti-apoptotic members and is central to the control of apoptosis. Bcl-2, Bcl-x_L, Mcl-1, Bcl-w are proteins of the Bcl-2 family that inhibit or delay apoptosis, while Bax, Bak, Bad, Bik, Bid and Bcl-x_S are members that facilitate or induce apoptosis (Tilly and Robles, 1999). Over-expression of Bcl-2 itself protects cells from apoptosis, although certain apoptotic pathways, such as activation of the Fas receptor, a death receptor involved in immune-triggered apoptosis, are not inhibited by Bcl-2. All members of the Bcl-2 family have at least one of four conserved homology domain (BH1 to BH4) (Adams and Cory, 1998). Bcl-2 is located on the outer membrane of the mitochondria, nucleus (Antonsson and Martinou, 2001) and endoplasmic reticulum (Annis *et al*, 2001).

The mechanisms by which the pro- and anti-apoptotic members of the Bcl-2 family regulate apoptosis are still not fully understood. It is known that they can homodimerize and heterodimerize and one current model proposes that the balance between the pro- and anti-apoptotic proteins will determine cell fate. It is hypothesized that certain pro-apoptotic members of the Bcl-2 family can create channels in the mitochondria, further releasing pro-apoptotic molecules, such as cytochrome *c*. The anti-apoptotic members would inhibit this channel creation. An alternate role for the family would be the regulation of existing channels, the anti-apoptotic members keeping them closed, and the pro-apoptotic members opening them (Gross, 2001; Bowen *et al*, 1998). However, some reports have shown that initiation or inhibition of cell death is not dependent on such interactions in some systems (Song *et al*, 1999). Whether this means that the model is incomplete, or that some apoptotic pathways circumvent the Bcl-2 family altogether, remains to be determined.

It is known that one of the principal effects of the Bcl-2 protein is to inhibit release of cytochrome *c* from the mitochondria. The cytochrome *c*, once released, interacts with Apaf-1 and allows it to dimerize procaspase-9, forming a structure called the apoptosome. Procaspase-9 autocatalyses and the activated caspase-9 activates other caspases to engage the apoptotic cascade (Green and Reed, 1998). It has been reported that anti-apoptotic members of the Bcl-2 family such as Bcl-x_L or Boo could bind Apaf-1 and stop it from interacting with caspase-9 (Adams and Cory, 1998; Song *et al*, 1999) (see Fig. 1) but other reports claim that this is not the case (Johnson, 1999).



Adapted from Addams and Cory, 1998

Fig. 1. A model of the regulatory effects of the Bcl-2 family members, in which an anti-apoptotic member (Bcl- x_L) binds Apaf-1 and prevents it from activating procaspase-9. A death signal causes a pro-apoptotic member (Bik) to displace Apaf-1 from the anti-apoptotic protein. The release of cytochrome c (also controlled by Bcl-2s) allows Apaf-1 to dimerize procaspase-9, leading to auto-proteolysis and activation of caspase-9. Caspase-9 will then activate other caspases and cleave different target molecules, triggering apoptosis.

The caspases:

Caspases are a family of cysteine proteases that cleave after an aspartic acid. They are the mammalian equivalent of ced-3. Caspases are the effectors of apoptosis, acting by cleaving inhibitors of the nucleases that degrade DNA, cleaving anti-apoptotic proteins of the Bcl-2 family, activating other caspases, destroying the nuclear lamina and indirectly reorganizing the cellular skeleton. They are normally found in an inactive form and need to be cleaved to be activated. This cleavage will be accomplished by auto-proteolytic means via the apoptosome or by already activated caspases (Thornberry and Lazebnik, 1998).

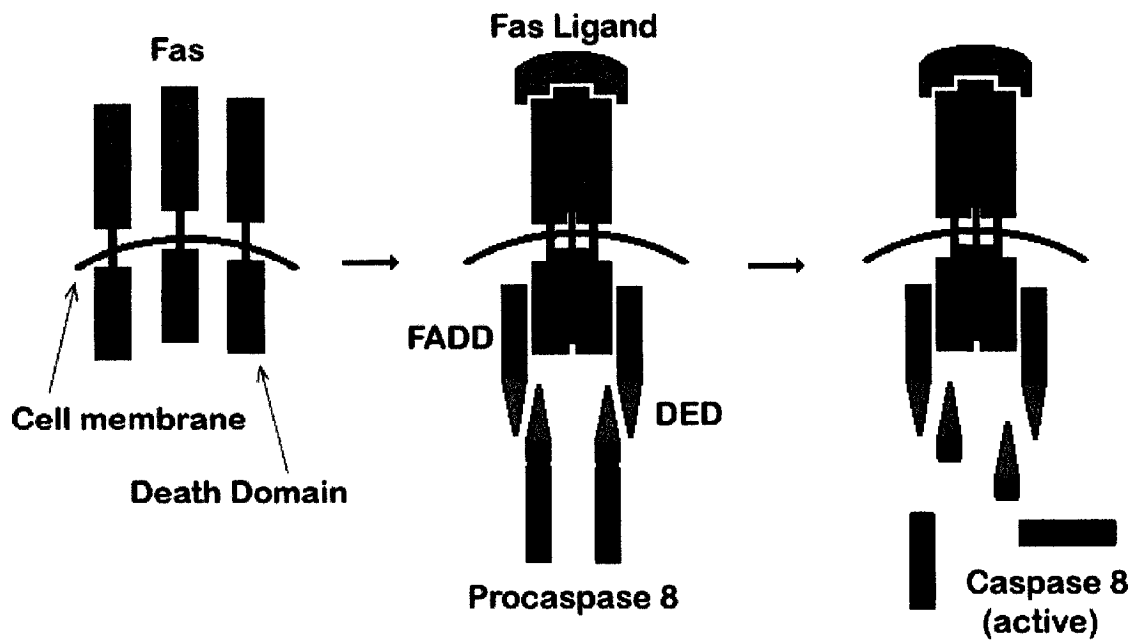
Death receptors:

Apoptotic death can also be triggered by receptors of the tumor necrosis factor (TNF) receptor superfamily, defined by their homologous cysteine-rich extracellular domains. These cell surface receptors that transmit apoptotic signals are called death receptors, and they are activated by specific “death ligands” (Ashkenazi and Dixit, 1998). This method of apoptosis initiation allows the organism to directly induce death in a specific cell. It is mostly used within the immune system, but it can also play a role in the reproductive system. It has been shown that epithelial cells of the prostate undergo apoptosis and that this process is mediated by a member of the TNF family, Fas (Kiess and Gallaher, 1998). Fas is the best-studied death receptor and is also called CD95 or Apo1. When activated by its ligand, Fas trimerizes and recruits the adaptor protein FADD (Fas-associated death domain, also known as Mort-1) in the cytoplasm. FADD will recruit pro-caspase 8, initially called FLICE, by binding to the death effector domain (DED) (Medema *et al*, 1997). Caspase-8 will activate through self-cleavage and activates other downstream effector caspases, such as caspase-9 (see Fig. 2). Various cell types

will express various death receptors, and some other cell types seem to lack death receptors. The Fas pathway is an example of transcription-independent apoptosis, since all the elements to initiate and complete the apoptotic cascade are present. The function of Fas and other death receptors has been reviewed by Ashkenazi and Dixit (1998).

p53:

The p53 gene product is a transcription factor that is activated by the presence of DNA damage in the cell. It is believed that p53 can trigger apoptosis by upregulating Bax, a pro-apoptotic gene. However, p53 can also lead to cell differentiation, cell repair or growth arrest, and its roles and regulation are highly complex and not yet fully understood (White, 1996). Part of the complexity of p53 function may be due to the fact that it possesses two DNA binding areas. The central area of the protein contains a specific DNA binding area, while the C-terminus section has a binding area that is not sequence specific but rather strongly binds to a variety of DNA structures such as short single strands or insertions/deletions. While the mechanisms involved remain unclear, the binding at the C-terminus affects the ability of the central binding domain to bind to its target DNA sequence (Ahn and Prives, 2001). It is believed that p53 plays a key role in tumor suppression, since about 50% of human cancers have one or more mutation in the p53 gene (Green *et al*, 1997). This activity could be explained by the fact that p53 can induce the transcription of Bax and Fas, and also suppress Bcl-2 expression, therefore promoting the death of damaged cells. Cells with p53 mutations would be more resistant to apoptosis and more prone to become cancerous (White, 1996). The p53 pathway is an example of a transcription-dependent form of apoptosis.



Adapted from Ashkenazi and Dixit, 1998

Fig. 2: Basic mechanism of action of the Fas death receptor. When the Fas receptor is trimerized by its ligand, the intracellular death domains are also trimerized. This allows the binding of FADD (Fas-associated Death Domain). FADD acts as an adapter, and recruits several procaspase 8 molecules through homophilic interactions of the shared death effector domain (DED). The close proximity allows for self-cleavage of caspase 8, which will then activate downstream effectors and initiate the apoptotic cascade.

DNA fragmentation:

The cleavage of DNA in a specific pattern is one of the hallmarks of apoptosis. The DNA fragmentation first occurs at a large scale (Filipski *et al*, 1990; Walker *et al*, 1999), generating 300 kbp and 50 kbp fragments. The fragments are then further cut at an internucleosomal level, resulting in DNA strands of approximately 180-200 bp in length. Caspase-activated-DNase (CAD) catalyzes this smaller-scale degradation. An inhibitor (ICAD) normally inactivates CAD and this inhibitor is cleaved by caspases during apoptosis (Susin *et al*, 2000). It has been suggested that the function of DNA fragmentation is to protect the neighboring cells from transformation by oncogenes or viral DNA that could be contained in the dying cell. DNA is also a strong autoantigen. Its presence outside of the cell nucleus can trigger strong autoimmune responses. DNA fragmentation could help reduce inflammation in cases of large-scale cellular deaths overwhelming the capacity of phagocytes to ingest the dead cells (Nagata, 2000). It has been suggested that there are redundant pathways leading to the large-scale fragmentation of DNA that are caspase and CAD-independent. The apoptosis initiation factor (AIF) is believed to play such a role (Susin *et al*, 2000) (see Fig. 3). AIF is a large protein that normally has NADH-oxidase activity and its release from the mitochondria during apoptosis leads to large scale DNA degradation (Gross, 2001)

The exact definition of apoptosis still remains to be firmly established. Complicating the issue are morphological and biochemical differences between certain cell types. These differences can lead to different apoptotic pathways being employed, resulting in different “apoptotic signatures”. Some research groups view necrosis and apoptosis as a continuum (Murphy, 1999), in which intermediate forms of death may exist. For instance, a cell could initiate apoptosis but run out of ATP, then switch to necrosis. ATP is needed for the activation

of caspase-9 via cytochrome c and Apaf-1 (Egushi *et al* 1999). Cell death can occur despite the inhibition of caspases whether by gene knockout (Susin *et al*, 2000) or pharmacological inhibitors (Blagosklonny, 2000). Such cell deaths often have different morphological and biochemical characteristics, notably a reduced degree or absence of chromatin condensation and modified DNA cleavage. Some groups have defined apoptosis as caspase-dependent cell death (Blagosklonny, 2000; Samali *et al*, 1999) while other groups study “caspase-independent apoptosis”, such as the AIF pathway (Susin *et al*, 2000). Others have made definitions dependent uniquely on somewhat ill-defined morphological features (Leist and Jaattela, 2001). Certain proteins involved in necrosis, such as calpains, a group of cysteine proteases with a different preferred cleavage site, can also play a role in apoptosis in certain cell types (Wang, 2000). The great variety of cell types, of apoptotic triggers and the fact that the observed apoptotic morphology depends in part on the timing of the observations will make the formulation of an exact definition rather difficult.

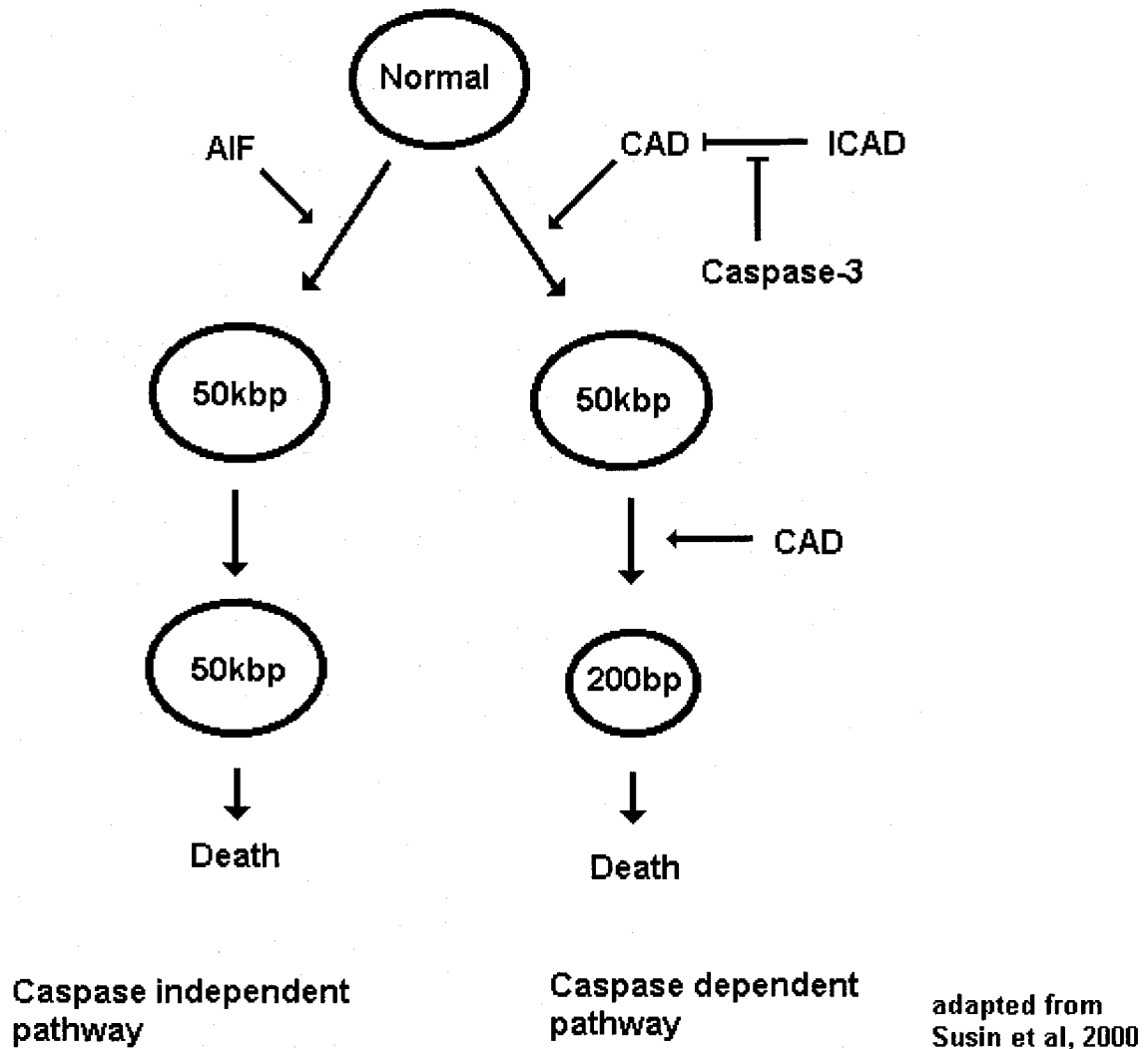


Fig. 3: Caspase-dependent vs. caspase-independent pathway. The Apoptosis Initiation Factor (AIF) can migrate from the mitochondria and mediate a caspase-independent pathway. The changes in nuclear morphology and the scale of the DNA degradation is greatly reduced when compared to the caspase-dependent, caspase-activated DNase (CAD) mediated pathway. CAD acts in the nucleus to cleave chromosomal DNA. The CAD inhibitor (ICAD) is cleaved by caspases during apoptosis.

Apoptosis in teleosts

Several studies have demonstrated the presence of apoptosis in diverse fish species and tissues. Apoptosis seems to play critical roles in the development of the fish, its reproductive and immune systems, or as a mean to eliminate cells damaged by various traumas. These roles are very similar to those played by apoptosis in mammals. However, there are notable differences.

The cavefish *Astyanax mexicanus* has sub-populations with and without eyes, depending on the presence of light in their respective environments. Apoptosis, detected via the terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) technique, was shown to play a significant role in the degradation of the eye during development of the eyeless population (Jeffery and Martasian, 1998). In a study using the goldfish as an animal model to study excitotoxicity-induced neuronal damage, kainic acid, a glutamate agonist, has been shown to trigger neuronal apoptosis in goldfish retina. The TUNEL technique was used and morphological features typical of apoptosis were observed. Low levels of apoptosis were also found to be part of the normal development of the goldfish retina (Villani *et al*, 1997). Apoptosis also occurs during the development of the zebrafish embryo and was detected by DNA fragmentation and nuclear spread (Chan and Yager, 1998). These studies indicate the role of apoptosis in teleost development in general and of the eye in particular.

Studies in the brown ghost *Apteronotus leptorhynchus* cerebellum have shown that teleost fish can regenerate brain injuries. In mammals, mechanical injuries to the brain typically lead to scarring and cavitations. However, teleosts can heal such injuries with no such damage and seem to restore functionality to the area, with generation of new neural connections and the establishment of new neurons. This is perhaps due in part to the greater role of apoptosis in the

death of the injured teleost neurons, which reduces inflammation and scarring and also because of the constant generation of new neurons ready to replace injured ones. New neurons that are not needed will die by apoptosis. The neuropeptide somatostatin is present in the area of proliferation and the area of injury and is believed to play an important role. Apoptosis was detected using the TUNEL technique and by observation of the cell morphology via electronic microscopy (Zupanc, 1999a; Zupanc, 1999b; Zupanc *et al*, 1998). In the angelfish *Pterophyllum scalare*, large-scale death of motoneurons occurs naturally at specific developmental stages. Morphological observation and the TUNEL technique were used to achieve these observations. It is hypothesized that this mimics the death of neurons in mammals during development, where neurons that have not established proper connection are eliminated by apoptosis due to lack of paracrine support (Sakamoto *et al*, 1999).

In the medaka *Oryzias latipes*, skin pigment cells will die by apoptosis as a means of adaptation to changes in the color of the environment. Annexin V binding assay was used to detect early changes in the cell membrane caused by apoptosis, and both TUNEL and DNA laddering indicated the presence of DNA fragmentation. Sympathetic innervation plays a role, as norepinephrine increases apoptosis in melanophore cultures (Sugimoto *et al*, 2000). This is an example of neuroendocrine control of apoptosis in response to environmental changes.

Apoptosis has been detected in the goldfish thymus, mainly in thymocytes. It is highly probable that, like in mammals, these thymocytes were auto-reactive and thus eliminated (Ottaviani *et al*, 1997). Cultured carp lymphocytes can die by apoptosis, both from exposition to elevated levels of cortisol and from lack of survival signals. Apoptosis was measured using the TUNEL technique and a flow cytometry cell counting system (Weyts *et al*, 1997). Peripheral blood leukocytes in channel catfish utilize an apoptotic killing mechanism on target cells. This

effect was observed via TUNEL and electronic microscopy. While the target cells were mammalian, it was proposed that fish cells would be killed in a similar fashion (Hogan *et al*, 1999). These studies indicate that, like in mammals, apoptosis plays an important role in the regulation of the immune system in teleosts.

Apoptosis has been found in the fish gonad. In the rainbow trout *Oncorhynchus mykiss*, apoptosis occurs both in preovulatory (Janz and Van Der Kraak, 1997), detected by DNA laddering, and postovulatory follicles (Wood and Van Der Kraak, 2001), detected both by DNA laddering, and cell morphology combined with the TUNEL assay, and it seems to play a role in ovarian growth and postovulatory regression. Unlike in mammals, however it does not seem to be an early event in follicular atresia. This may be due to differences in the reproductive strategy. In mammals, only a small portion of the follicles are recruited for ovulation. The rest undergo follicular atresia, a process involving apoptosis. It is hypothesized that this limited recruitment constitutes a selection process of some sort, in which only the best ova are selected (Wood and Van Der Kraak, 2001). Whether ovum fitness confers a greater resistance to apoptosis, making survival itself the selector, or whether apoptosis is only used to dispose of unselected ova is unknown (Tilly, 2001). Some teleosts will typically recruit thousands of oocytes at a time, the majority of them successfully, probably eliminating the need for large-scale atresia (Wood and Van Der Kraak, 2001). Similar findings to those made in trout were made in the goldfish using similar detection techniques (Wood and Van Der Kraak, 2001). Light and electronic microscopic studies observing cell morphology in the *Astyanax bimaculatus lacustris* indicate that apoptosis is the major mechanism responsible for the resorption of postovulatory follicles (Drummond *et al*, 2000). These results suggest that apoptosis plays a role in normal teleost ovarian growth and post ovulation recovery, but not in follicular atresia.

Apoptosis is also present in the goldfish testes. It was found that the neuropeptide gonadotropin-releasing hormone (GnRH) increased DNA fragmentation, a hallmark of apoptosis, in cultured testicular slices. The fragmentation was detected using a cell-death ELISA detection kit. It was also found that human chorionic gonadotropin reduced both the basal level of apoptosis and the GnRH-induced apoptosis (Andreu-Vieyra and Habibi, 2001).

Apoptosis has also been found in rainbow trout hepatocytes, via DNA laddering. Tributyltin, a water pollutant found in paint, increases apoptosis in rainbow trout hepatocytes, via a pathway that is Ca^{2+} -dependent and seems to involve cysteine proteases, perhaps calpains (Reader *et al*, 1999). Exposure to Bleach Kraft pulp mill effluents, known to contain endocrine-disrupting chemicals, results in elevated levels of ovarian follicular apoptosis and a reduction in reproductive fitness in the white sucker, *Catostomus commersoni* (Janz *et al*, 1997). DNA laddering was observed in that study and used to determine the apoptosis level. Exposure to increased ultraviolet radiation has been shown to increase embryonic death, oxidative stress, DNA damage and up-regulation of the p53 gene in the Atlantic cod *Gadus morhua* larvae. Apoptosis was hypothesized as the mechanism behind the increased mortality. This phenomenon is suggested as a possible cause for the decline of this species (Lesser *et al* 2001).

Apoptosis has also been found in fish cell lines. The Rhabdovirus, Spring Viremia, of carp, which can be lethal for carp, triggers cell death in a carp epithelial papilloma cell line. Apoptosis is involved, as shown by nuclear morphology and DNA laddering, and a human endogenous cysteine protease inhibitor will inhibit this apoptosis (Bjorklund *et al*, 1997). The infectious Pancreatic Necrosis Virus has been shown to trigger apoptosis in a Chinook salmon embryonic cell line. Apoptosis was detected via cell observation with electronic microscopy and by the observation of DNA fragmentation via DNA laddering. The virus caused the increase in

apoptosis by down-regulating the expression of the anti-apoptosis regulator Mcl-1, a member of the Bcl-2 family (Hong *et al*, 1999).

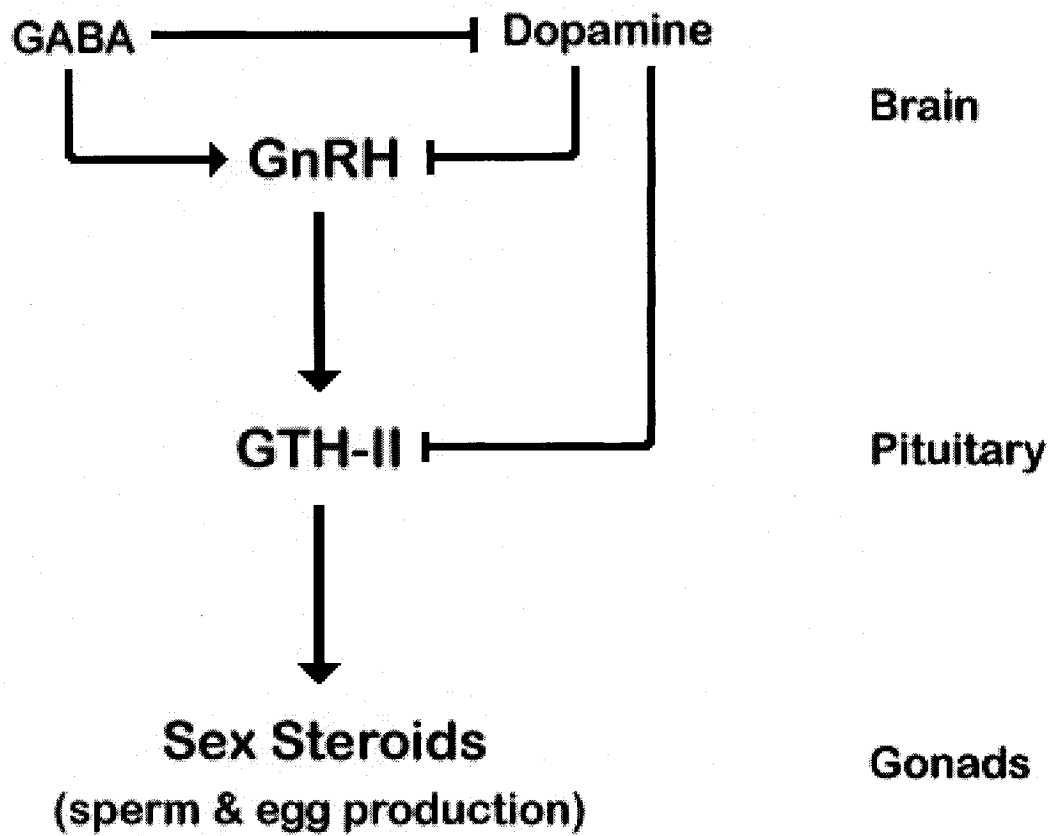
The evidence suggests that the basis of the apoptotic machinery is conserved in fish. Several genes with homology to various mammalian regulators and effectors of apoptosis were recently found in the zebrafish, including caspases, Bcl-2 family members, death receptors and others (Inohara and Nunez, 2000). One of these genes, zfBLP1, a Bcl-x_L homologue found in the zebrafish endoplasmic reticulum, produces four mRNA species that have varying expression patterns during embryogenesis (Chen *et al*, 2001). The presence of Bcl-x_L, a Bcl-2 family member with anti-apoptotic properties, has been reported in trout hepatocytes, via western blot using anti-human Bcl-x_L antibodies (Reader *et al*, 1999). However, because of the technique used, it is possible that this trout protein is simply a Bcl-2 fish family member and not Bcl-x_L itself.

Pituitary function

The pituitary gland secretes several hormones, such as growth hormone (GH), luteinizing hormone (LH, or gonadotropin-II (GTH-II) in fish) and follicle stimulating hormone (FSH, or GTH-I in fish). These hormones play important roles in the regulation of reproduction and growth. A specific cell type secretes each hormone in teleosts (Nozaki *et al*, 1990). In the goldfish, these cell types are not homogeneously distributed but rather are found in clumps and are segregated to different parts of the gland. The pars distalis (PD, or anterior pituitary) is composed mostly of gonadotrophs which secrete the GTHs, and somatotrophs which secrete GH. Also found in the PD are prolactin cells (Follenius and Doeer-Schott, 1978). The gonadotroph cell has an average diameter of approximately 16 µm in culture and possesses a relatively small nucleus when compared to somatotrophs or lactotrophs (Van Goor *et al*, 1994).

Goldfish spawn once a year followed by a period of somatic growth. Serum levels of GTH-II control the annual cycle of gonadal function and growth. GH is also believed to play a role. The hypothalamus controls the pituitary gland by secreting various neurotransmitters and neuropeptides (Trudeau, 1997). Teleosts lack a hypothalamo-pituitary portal system. However, part of the anterior pituitary is directly innervated by neurons that secrete neuropeptides and neurotransmitters. This anatomical arrangement provides a convenient model to study the interaction between endocrine cells and neurotransmitters (Blazquez *et al*, 1998).

Gonadotropin-releasing hormone (GnRH) is the main stimulatory peptide for the release of GTH-II (Peter, 1986; Jobin *et al*, 1996). In goldfish, two forms of this decapeptide are found: (salmon) sGnRH and (chicken) cGnRH-II. Both stimulate GTH-II release by binding to the same class of receptor on gonadotrophs. However, their mechanisms of action are different. The stimulation of GTH-II release by sGnRH relies on mobilization of intracellular calcium and arachidonic acid, while the effect of cGnRH-II is more dependent on external sources of calcium, via voltage-sensitive calcium channels (Chang *et al*, 1993). It has been shown that dopamine (DA) is an inhibitor of GTH-II release via the D2 receptor in goldfish (Omeljaniuk *et al*, 1989) and in trout (Linard *et al*, 1995). A D2 agonist inhibits the GnRH-induced increase of intracellular calcium, and inhibition of GTH-II release can be reversed with a calcium ionophore (Chang *et al*, 1993). Dopamine can also inhibit GnRH release via the D1 receptor, both in pituitary nerve terminals and in the preoptic-hypothalamic area (Yu and Peter, 1990). The neurotransmitter gamma aminobutyric acid (GABA) plays an important role in modulating GTH-II release and can have positive long-term effects on its release (see fig. 4). Negative and positive feedback loops from the gonads via sex steroids also play important roles (Trudeau, 1997).



Adapted from Trudeau, 1997

Fig. 4: Basic control of the fish hypothalamic-pituitary-gonadal axis. Dopamine acts as an inhibitor of GTH-II release both at the brain and pituitary level. The neurotransmitter GABA provokes GTH-II release by stimulating GnRH release and by inhibiting dopamine neurons.

The pituitary and apoptosis

Apoptosis has been detected in the pituitary gland in mammals (Drewett *et al*, 1993; Spengler *et al*, 1997; Ahlbom *et al*, 1998; Kulig *et al*, 1999), in birds (Chowdhury and Yoshimura, 2002) and in fish (Melamed and Yaron, 1999; Melamed *et al*, 1999). The mammalian anterior pituitary also shows mitotic activity (Levy, 1999; Nolan *et al*, 1998; Pagotto *et al*, 1999). This activity is age-dependent, and is greatly reduced in older animals (Kolopp *et al*, 1992; Nolan *et al*, 1999). The presence of mitosis and apoptosis indicates that a cellular renewal process is present.

Conflicting studies investigated the prevalence of apoptosis in normal and cancerous pituitaries and the expression of apoptosis regulators in those glands. For instance, one study found that Bcl-2 was expressed at similar levels in healthy human pituitary and pituitary adenoma, but was decreased in pituitary carcinoma (Kulig *et al* 1999). On the other hand, another study showed that Bcl-2 was not expressed in normal human pituitary, and that its expression was increased in cancerous pituitary adenoma, especially in non-recurrent tumors (Turner *et al*, 2000). The study by Klugig *et al* (1999), using the TUNEL technique, also showed low levels of apoptosis in normal human pituitary and pituitary adenoma, as well as elevated levels in pituitary carcinoma. Another study, using an *in situ* end labeling (ISEL) technique, failed to detect apoptosis in normal human pituitary but found elevated levels in pituitary adenoma (Green *et al*, 1997). These differences may be explained by differences in the methods used for tissue preparation and apoptosis detection.

In rats treated for 6 weeks with estrogen (subcutaneous implants of diethylstilbestrol dipropionate), estrogen withdrawal combined with treatment with bromocriptine, a dopamine receptor agonist, caused a significant increase of apoptosis in the pituitary. Light and electron

microscopy was used to observe morphological changes in cells indicative of apoptosis (Drewett *et al*, 1993). Somatostatin can also induce apoptosis in a mouse pituitary cell line (AtT-20) in a cell cycle-dependent manner. Flow cytometry analysis of the DNA content of the cells was used, as well as a TUNEL label (Strikant, 1995). In rats, adrenalectomy followed by a replacement dexamethasone treatment will cause a burst of apoptotic activity in the pituitary, detectable by TUNEL and morphological cellular features (Nolan *et al*, 1998). However, some reports failed to detect apoptosis in the non-cancerous pituitary (Green *et al*, 1997) using the ISEL technique. Even in studies where apoptosis is detected, identification of the cell type can be difficult since at the late stage at which apoptosis becomes visible, the hormonal content of mammalian pituitary cells has been depleted, making immunological identification of the cell types very difficult if not impossible (Nolan *et al*, 1998). It has been shown that a transient fluctuation in the detectable levels of apoptosis can have a considerable impact on the pituitary cell population. Because apoptosis is only histologically detectable for a short period of time (44 minutes in the rat pituitary), an increase of a fraction of 1% would be sufficient to kill hundred of thousands of cells in 48 hours (Nolan *et al*, 1998).

The expression of Bcl-2 family members in conjunction with increased apoptosis has been studied in mammals. In the rat pituitary, end of lactation has been shown to cause a decrease in Bcl-2 immunoreactivity, but no change in Bcl-2 mRNA levels and an increase in Bax immunoreactivity and mRNA levels (Ahlbom *et al*, 1998).

In the white leghorn laying hen, starvation inhibits egg laying. TUNEL and bromodeoxyuridine (BrdU) staining of paraffin sections showed that resumption of laying was accompanied by an increase in both apoptosis and cell proliferation in the anterior pituitary. This

suggests that the hormonal changes associated with starvation and resumption of laying can have a remodeling effect on the anterior pituitary (Chowdhury and Yoshimura, 2002).

In teleosts, only a few studies concerning apoptosis in the pituitary have been published. The calcium ionophores A23187 and ionomycin induce both morphological and biochemical apoptotic changes in cultured tilapia pituitary cells, specifically nuclear condensation, cell shrinkage and DNA fragmentation in 200 bp multiples (Melamed and Yaron, 1999). Increased concentration of calcium from extracellular (Chang *et al*, 1993; Jobin *et al*, 1996b) or multiple intracellular (Johnson *et al*, 2000) sources is involved in the GnRH-stimulated secretion of GTH-II. Calcium has multiple functions in endocrine cells, but the fact that a calcium ionophore triggers apoptosis raises the possibility that apoptosis and secretion may be somehow linked. It may also indicate that some of the calcium and secretion studies could have resulted in large-scale death in the pituitary, since many employ ionophores.

Another study showed that there is a probable paracrine relationship between gonadotrophs and somatotrophs in the fish pituitary. Isolated tilapia somatotrophs in culture show nuclear condensation and fragmentation typical of apoptosis. Those symptoms were decreased by the addition of insulin-like growth factor 1 (IGF-1) and estradiol (E_2) to the growth medium. Somatotrophs in mixed culture also had lower apoptosis levels. It appears that the presence of gonadotrophs is needed for the production of IGF-1, although they are not believed to produce it themselves. The gonadotroph cell fraction also had aromatase activity, suggesting that gonadotrophs, or another type of cell present in the gonadotroph fraction, are the source of the pituitary E_2 . Gonadotrophs, along with other cells, therefore act in a paracrine fashion to suppress apoptosis in somatotrophs (Melamed *et al*, 1999). A study in Trudeau's lab using the differential display technique showed that GABA up-regulates FKBP12-Rapamycin-associated

protein (FRAP) in the goldfish pituitary (Blazquez *et al*, 1998b). This protein is involved in cell cycling, Ca²⁺ signaling and apoptosis (Chen *et al*, 1995). Its presence in the goldfish suggests that these processes are also regulated by the neuroendocrine system.

Hypothesis and goals

The pituitary is composed of a dynamic cell population that undergoes apoptosis both in mammals and in fish. Studies have already shown that gonadotrophs can vary in number with the reproductive state of rainbow trout (Nozaki *et al*, 1990; Naito *et al*, 1991). We therefore hypothesize that apoptosis plays an important role in the regulation of hormonal secretion, by regulating the various populations of pituitary endocrine cells. Such a process could be regulated by various neurotransmitters. Since DA is a strong inhibitor of GTH-II secretion and DA agonists increase levels of apoptosis in the rat pituitary, it is a strong candidate for apoptosis up-regulation.

To begin addressing this question, the goals of my study were to first determine if apoptosis is present in the goldfish pituitary and the gonadotrophs in particular. To do so, a double-labeling technique that allows the visualization of both apoptosis and gonadotrophs simultaneously was developed. A second goal was to determine whether DA, GnRH and the calcium ionophore A23187 affect apoptosis in the goldfish pituitary, and more specifically if they do so in gonadotrophs. Finally, to determine the effect of *in vitro* incubation on apoptosis levels, whole pituitary gland samples were dissected, incubated in culture media, examined and compared to non-incubated samples.

MATERIALS AND METHODS

***In vivo* experiments:**

Goldfish were purchased from Mount Parnell Fisheries, Inc. (PA, USA) and immediately sexed using fin characteristics as a sexual marker. The 2 sex were kept separately. Fish were fed daily and maintained at 18°C in a natural light cycle for at least 2 weeks to acclimatize.

To evaluate the effect of the neurotransmitter dopamine on apoptosis in the goldfish, female fish (160) were placed into 4 experimental groups (n = 40) under the following conditions: control (fish left alone untreated until dissection); saline (fish injected with a saline sham); 50 mg of apomorphine per gram of fish (dopamine agonist, Sigma) and 50 gm of metoclopramide per gram of fish (dopamine antagonist, Sigma). All solutions were prepared on the same day as the injections. Injection vehicle consisted of 0.6% saline with 5% dimethyl sulfoxide (DMSO), needed to dissolve the apomorphine.

Goldfish were anaesthetized in MS-222 (tricainemethanesulfonate, Sigma) prior to injection. Goldfish were subsequently weighed and injected intraperitoneally with 5 µl of injection vehicle per gram of fish. Fish were allowed to recover. After a 24 hour period, the fish were anaesthetized and re-injected with the same treatment. Four hours after the second injection, the fish were anaesthetized again and re-weighed. Fish were then sacrificed by sectioning of the spine at the base of the skull. Gonads were removed and weighed, and the sex of the fish was confirmed upon inspection of the gonads. The pituitary was removed and dissected into two sections, the neurointermediate lobe and the pars distalis, using a dissection microscope and a small scalpel. These sections were pooled in groups of five and stored on dry ice prior to further analysis (DNA extraction). Due to initial sexing errors, not all fish were

analyzed. Therefore, the number of pooled samples varied among treatments: control (n = 7); saline (n = 6); apomorphine (n = 7); and metoclopramide (n = 8). Each n represents a group of 5 pars distalis gland section and a group of 5 neurointermediate lobe gland section.

DNA extraction

To analyze the levels of apoptotic DNA fragmentation from the goldfish pituitary samples, DNA had to be extracted and purified. The protocol was adapted from Gross-Bellard *et al.* (1973), Tilly and Hsueh (1993), and Janz and Van Der Kraak (1997a). Sample tissue was gently homogenized by hand with a sterile plastic mortar in a 1.5 ml microfuge tube with 400 μ l of cell homogenization buffer (0.1 M NaCl, 0.01 M ethylenediamine tetra-acetic acid (EDTA), 0.3 M Tris-HCl pH 8.0, 0.3 M sucrose). 25 μ l of 10% sodium dodecyl sulfate (SDS) was added and the sample was incubated at 65°C for 1 hour. Subsequently, 75 μ l of 8 M potassium acetate was added and the sample was incubated on ice for 1 hour. The sample was then centrifuged (Eppendorf 54151) at 8000 rpm for 10 minutes at 4°C and the supernatant (about 400 μ l) transferred to a new 1.5 ml tube.

One volume of water-saturated phenol (pH 8.0, Sigma) was added to the supernatant and the sample was gently inverted. The sample was then centrifuged at 4°C for 5 min at 6000 rpm and the aqueous layer was collected. The extraction was repeated twice with phenol : chloroform : isoamylalcohol (25:24:1, pH 8.0, Sigma) and once with chloroform. The protein layer found at the interface between the aqueous and the organic phase was also collected, since DNA can be trapped in that layer. The DNA was re-extracted, and added to the final sample.

DNA was precipitated with the addition of 0.1 x supernatant volume of 3 M sodium acetate and 2.5 x supernatant volume of 95% ethanol and the tube was left at -20°C overnight.

The next day, the sample tube was centrifuged at 14 000 rpm for 30 minutes at 4°C. The ethanol was removed, and the sample air-dried. The sample was resuspended at 4°C for 2 hours in 40 µl TE (10 mM Tris-HCL, 1mM EDTA). 2 µl of DNase-free RNase (Boehringer Mannheim) was added to the sample and incubated at 37°C for 1 hour. DNA was extracted with an equal volume of 25:24:1 phenol : chloroform : isoamylalcohol once and with chloroform once, as previously described. DNA was precipitated again with 3 M sodium acetate and 95% ethanol. The sample was then centrifuged at 4°C. The ethanol was discarded and the DNA pellet was washed twice with 80% ethanol and air dried for 20 minutes. The DNA pellet was resuspended in 40 µl TE, quantified by spectrophotometry (Gene Quant, Pharmacia Biotech), and then stored at -20°C until needed.

Radiolabeling

To detect DNA fragmentation, the DNA had to be labeled, allowing visualization on an electrophoresis gel. The method was adapted from Tilly (1994) and Janz and Vanderkraak (1997a). Two µg of the DNA sample were radiolabeled with 2 µl of [$\alpha^{32}\text{P}$]ddATP (Amersham) using the TdT (Roche Molecular) enzyme at 37°C for 1 hour. Five hundred ng of a 123 bp DNA ladder (Gibco BRL) were also radiolabeled. The reaction was terminated with 0.5 M EDTA. The sample was purified with a ProbeQuant G-50 micro-column (Amersham Bioscience) and centrifuged at 750 g for 2 min at room temperature to remove unbound radioactive nucleotides.

Quantification of DNA fragmentation

To visualize the DNA laddering, a 2% agarose (Biobasics) gel was prepared in an Owl gel apparatus (model B3) with 100 ml of TAE (40 mM Tris-actetate, 1 mM EDTA). DNA

loading buffer (Gibco BRL) was added to the samples. Samples were split in half and loaded into the gel into 2 separate wells per sample. The gel was run at 70 V for 4 hours.

Drying the agarose gel was necessary to reduce its thickness and to avoid moisture coming in contact with the phosphor screens (see below) used to quantify the radiolabeling. Three layers of drying paper were put onto the vacuum dryer (Biorad model 583), as well as a sheet of Hybond N+ nylon membrane (Amersham Pharmacia Biotech). The Hybond membrane was equilibrated in TAE buffer for 10 min prior to use. The membrane hinders DNA migration out of the gel due to buffer displacement during drying. The drying process causes the gel and membrane to adhere strongly together, eliminating risk of misalignment between the two. The gel was removed from the tray and excess buffer was allowed to drain. The gel was put on top of the membrane on the vacuum dryer, and any bubbles were gently pushed away. The gel was heated at 50°C under vacuum for 2 hours. The drying paper was carefully replaced afterwards, and the drying process repeated. Dried gels were stored at 4°C.

The gel and the membrane were scanned together using a phosphor screen (Kodak Imaging screen-K) and a phosphor-imager (Biorad Molecular Imager FX). Exposure time varied between 2 and 12 hours. Gel images were analyzed using Quantity One (V. 4.1.1 Biorad) software. The 246 bp ladder was used as a standard.

Observations of DNA gels revealed the presence of a large-sized fragment of unknown size that seemed to be regulated (see Figs. 6 and 13). To determine the size of the fragment, a series of preliminary experiments were made on low-density agarose gels in an Owl gel apparatus (model B3). It was found that running a 0.3% agarose (Biobasics) gel at 25V for 24 hours was needed for good resolution. A 2% basal layer was used to solidify the gel. A large

molecular weight (digested and undigested lambda phage DNA, Gibco BRL) was used as a size marker.

***In Vitro* experiments:**

Preliminary *in vivo* experiments were made to determine the apoptosis levels in whole goldfish pituitary using the TUNEL technique (see below). Apoptosis levels were found to be extremely low and apparently unresponsive to treatments. It was thought that direct application of the treatments in a culture medium would improve response.

Pituitary whole gland culture

To determine the effect of neurotransmitters, lack of serum and of calcium ionophore, whole goldfish pituitary glands were put in culture medium with the appropriate treatments. Fifteen male fish were used for this experiment. The fish were sacrificed as described above and the pituitary gland excised with great care, briefly washed in phosphate-buffered saline (PBS, 0.02 M NaH₂PO₄, 0.08 M Na₂HPO₄, 0.6% NaCl, pH 7.4) and suspended in 5 ml of culture medium (M199, Gibco BRL) with 1% horse serum. Treatments consisted of 5 groups of 3 glands: control, no serum, 1 μM apomorphine (Sigma), 100 nM salmon GnRH analogue ([Des-Gly¹⁰ D-Arg⁶ Trp⁷ Leu⁸ Pro⁹]-LH-RH ethylamide, Bachem AG) and 10 μM calcium ionophore A23187 (Sigma). The use of DMSO was needed to dissolve the apomorphine; therefore DMSO (0.01%) was added to the culture media in all treatments. Glands were incubated at room temperature for 6 hours and then fixed overnight with paraformaldehyde for TUNEL labeling. This *in vitro* culture experiment was carried out in August, and was repeated with 25 male fish

under the same conditions and treatments (n = 5) in November. To see the basal *in vivo* level of apoptosis, 4 glands were not incubated but fixed immediately after dissection.

Tissue processing

Pituitary glands were left in 4% paraformaldehyde overnight (16 hours) at 4°C. Glands were then washed for 15 minutes on ice with PBS followed by 15 minutes immersions in an ethanol gradient consisting of 25%, 50% 70% 90%, 95% and finally 3 times with 100% ethanol. Samples were then washed 4 times in Stoddart's solvent (Harleco) at room temperature for 15 minutes per wash. Samples were put into melted paraplast (X-TRA, Oxford) at 60°C overnight (16 hours) and then transferred to a second wash of paraplast for 24 hours. The sample was put in an embedding mold filled with melted paraplast and left at 60°C for 15 minutes, then left at room temperature for hardening. When possible, samples were oriented so that the resulting paraffin sections would result in both the par distalis and the neuro-intermediate lobes being visible on the same section, creating a "mushroom profile" (see Fig. 7)

Samples were sectioned using a microtome (Spencer, American Optical Company) and MB35 (Shandon) blades into 6 µm thick sections. One in every 15 sections was wet-mounted on a Superfrost Plus (WVR) slide with the help of a heated water bath. This process usually generated 9 sections per gland for the *in vitro* samples. Since apoptosis levels in the non-incubated glands were low, it was felt that more observations would be beneficial and one in every 10 sections was processed instead. This process usually generated 14 sections for the non-incubated *in vivo* samples. The sections were heated at 41°C overnight to evaporate all traces of water.

TUNEL labeling

TUNEL labeling allows the visualization of nuclei containing high levels of DNA fragmentation due to apoptosis. Apoptotic cells have a high number of single and double-strand DNA breaks due to CAD and IAF activity. The ApopTag Fluorescein *In Situ* Apoptosis detection kit (Intergen) is based on TUNEL and uses the TdT enzyme to add digoxigenin-labeled nucleotides to the 3'-OH end of single and double strands of DNA. This protocol was adapted from the one designed for the ApopTag kit and the TUNEL assay developed by Gavrieli *et al.* (1992).

Samples were immersed in Stoddart's solvent for 15 min 3 times to ensure complete wax removal. Samples were then re-hydrated using an ethanol gradient consisting of two 5 minutes wash in 100% ethanol, followed by 3 minute immersions in 95%, 90%, 70% and 50% ethanol. Samples were then washed in PBS for 5 minutes. After deparaffinization, the tissue was pre-treated by applying the detergent 0.5% TRITON X-100 (Sigma) in PBS with 0.1% bovine serum albumin (BSA, Sigma) for 30 minutes at room temperature. Samples were then washed in PBS.

Equilibration buffer (Intergen) was applied to the samples, followed by working strength terminal deoxynucleotidyl transferase enzyme (70% reaction buffer and 30% stock TdT enzyme, Intergen). The samples were incubated in a humidified chamber at 37°C for 1 hour. The reaction was stopped by application of the stop/wash buffer followed by several washes in PBS. Working strength anti-digoxigenin conjugate (53% blocking solution, 47% stock anti-digoxigenin conjugate, an affinity purified polyclonal sheep antibody, Intergen) was added to the tissue at room temperature. This conjugate contains the fluorescein dye. The samples were incubated in a humidified chamber for 30 minutes at room temperature while avoiding exposure to light. The samples were washed in PBS and were then ready for GTH-II labeling.

GTH-II immunocytochemistry

An anti-GTH-II antibody was previously developed in rabbit (Breton *et al*, 1984). Combined with a donkey anti-IgG-rabbit labeled with the red fluorochrome Cy3, this allowed the identification of gonadotrophs within the pituitary, since only those cells contain the GTH-II hormone. The labeling was done immediately after the end of the Apoptag protocol. The TUNEL method uses sheep antibodies and no cross-labeling was observed. This allows the detection of apoptosis in gonadotrophs.

Following the TUNEL labeling, the excess liquid was blotted off, and 1:2500 anti-carp-GTH-II polyclonal antibody, diluted in 0.1 % BSA (Sigma) in PBS, was added. Samples were incubated in a dark humidified chamber for 1 hour at room temperature. After washes in PBS, samples were incubated with 1:500 donkey whole affinity purified anti-rabbit antibodies IgG (H+L) conjugated with Cy3 (Jackson ImmunoResearch Laboratories, Cy3 is a cyanine dye produced by Amersham Biosciences) for 30 minutes in a dark humidified chamber at room temperature. Specimens were washed in PBS. The excess liquid was blotted off and the mounting medium Vecta-shield (Vector Labs) containing the nuclear stain 4'6-diamidino-2-phenylindole (DAPI) was added. Samples were stored at -20C away from light.

Microscopy

Samples were examined using an epifluorescence microscope (Axiophot by Zeiss) using an oil immersion 63x/1.25 objective. A G 365 nm excitation filter/ LP 420 nm barrier filter was used to visualize the DAPI stain, a BP 450-490 nm excitation filter/ BP 515-565 nm barrier filter was used to visualize TUNEL and a BP 546 nm excitation filter/ LP 590 nm barrier filter was used to visualize the GTH-II labeling. Images were taken using a Hamamatsu integrating fluorescence charge coupled device (CCD) camera and analyzed using Metamorph v. 2.75

software (Universal Imaging) and Adobe Photoshop 4.0 software. Fields of view were chosen to cover the most surface area of the section possible. Care was taken that they did not intersect an edge, did not contain large areas void of tissue, and were not overlapping. Depending on the size of the section, between 1 and 5 fields of view were observed, each with an area of 125 000 μm^2 . The field of view area was calibrated using a micrometer. The number of apoptosis-positive nuclei was counted in the optical field of view. An average of 21 fields of view were counted per gland. Because of the shape of the gland, and of the GTH-II labeling, it was possible to determine which area of the gland was observed. Each field of view was classified as being pars distalis (gonadotrophs present in the majority of the field), neurointermediate lobe (no gonadotrophs) or indeterminate (half or less of the field has gonadotrophs). Intermediate fields were counted for the whole gland data, but not for the PD or NIL data.

Statistical analysis:

Data collected for the *in vivo* DNA laddering experiments were analyzed by one-way ANOVA and the Fisher's Least-Significant-Difference Test. Data collected in the 2 *in vitro* TUNEL labeling experiments were analyzed using a 2-way non-parametric ANOVA. Although the second experiment had a greater baseline, no significant interactions were found between the 2 experiments and treatment. This indicates that the pituitary glands responded in a similar fashion at the two time periods. Data were therefore pooled and re-analyzed using a one-way ANOVA and the Dunnett's 2-sided Test. Data were expressed as a ratio of treatment/control, i.e. the results for each treatment were the average of the ratio between each treatment value and the average value for the control. The data were analyzed using the SYSTAT 9.0 (Systat software inc.) and the G*POWER 2.0 software by Faul, F. and Erdfelder, E.

RESULTS

Evidence and characteristics of apoptosis

DNA Laddering

DNA extracted from the goldfish pituitary gland, when separated by electrophoresis on a 2% agarose gel, revealed a faint ladder pattern, characteristic of apoptotic internucleosomal DNA fragmentation (Fig. 6). Using the 123 bp ladder as a size standard, it was determined that these fragments were 198 ± 1.4 bp multiples (mean \pm standard error of the mean; see Fig. 5). Seven fragments were measured. A smaller fragment of approximately 80 bp was detected also; furthermore, a fragment of larger size, outside the linear range of the standards was detected (see below).

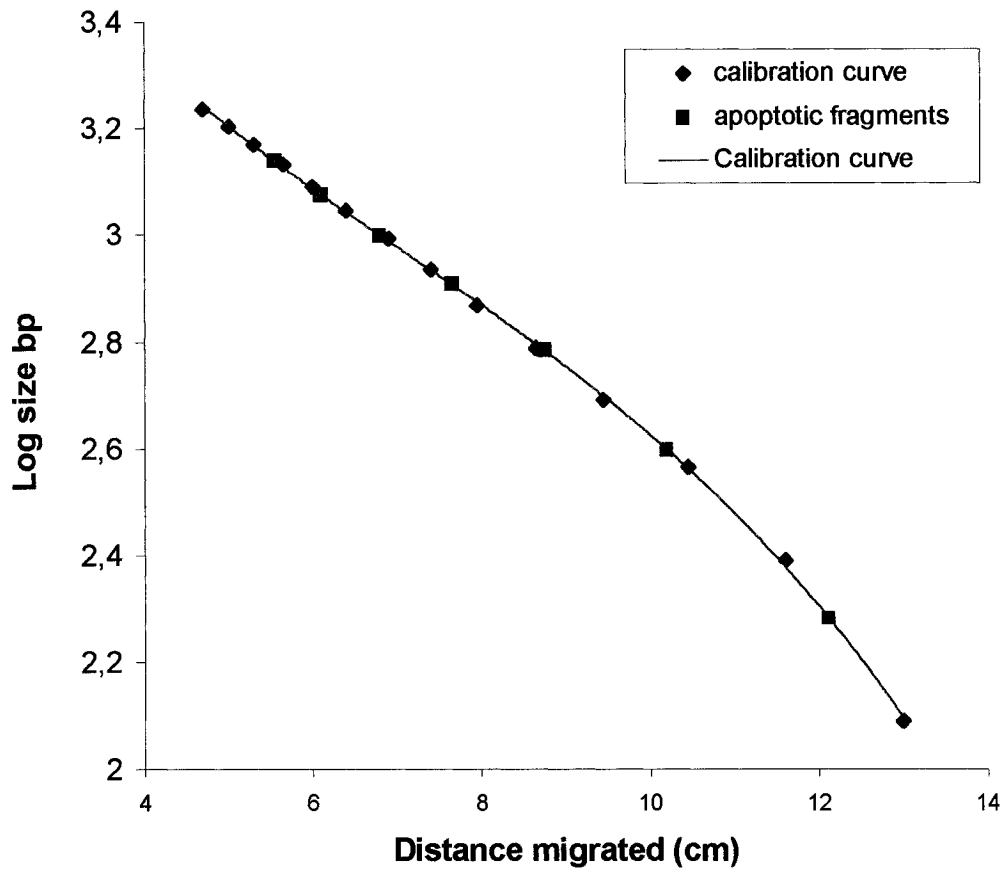


Fig. 5: Determination of the size of the DNA fragments, using the 123 bp ladder as a standard. A 3rd degree equation was determined to be the best fit for the calibration curve ($R^2 = 0.9997$). Using this equation, the fragments appear to be approximately multiples of 198 ± 1 bp

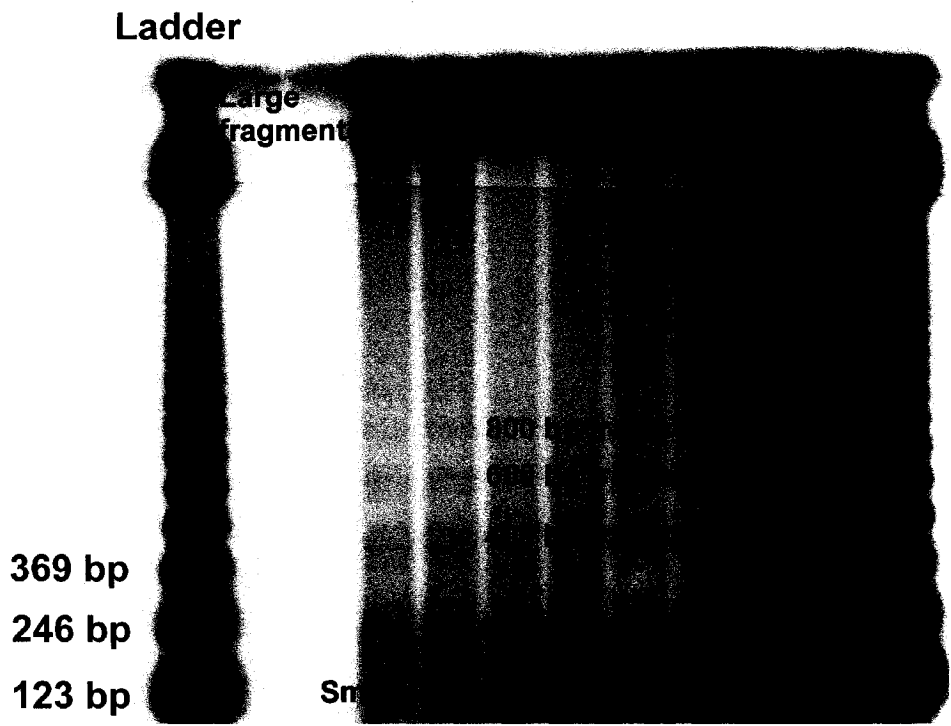


Fig. 6: 2% Agarose gel of radioactively labeled goldfish pars distalis DNA. A 123 bp DNA ladder was used on each side of the gel as a size marker and used to determine the size of the sample fragments (see fig 5). The size of these fragments corresponds to apoptotic DNA fragmentation. Note the small and large fragments of unknown size.

Epifluorescence Microscopy

Observation of 6 μm -thick paraffin sections stained with the TUNEL technique revealed that the level of apoptosis *in vivo* is very low, with 0.27 ± 0.09 TUNEL-positive nuclei per 125 000 μm^2 field of view (N=4), or about 1 per 500 000 μm^2 . *In vitro* levels were much higher: incubation for 6 hours at room temperature in M199 media significantly increased the level of detectable apoptosis (67 ± 23 TUNEL-positive nuclei per field of view, N=8).

To detect gonadotrophs, an anti-GTH-II antibody was used. It was determined that a dilution of 1:2 500 of the primary antibody and of 1:500 of the secondary antibody yielded the best results. This allowed for the localization of the gonadotrophs (Fig. 7) within the pars distalis of the gland.

This GTH-II labeling was combined with the TUNEL technique and the general nuclear stain DAPI, allowing the detection of apoptotic gonadotrophs in the goldfish pituitary gland *in vivo* (Fig 8). It therefore has been possible to observe apoptotic gonadotrophs *in vivo* several times (6 observations were made). However, compared to the number of non gonadotropic TUNEL-positive nuclei, the observation of an apoptotic gonadotroph is a very rare event *in vivo*.

Since the rate of detectable apoptosis *in vitro* was much higher than *in vivo*, double-labeled apoptotic gonadotrophs were much more common and more detailed observations were possible. Nuclear fragmentation was observed in the majority of sections (Fig. 9) both in treated and non-treated glands and in both gonadotrophs and non-gonadotrophs. Most TUNEL-positive nuclei were of a smaller, rounder size and showed signs of chromatin condensation. The GTH-II label can act as a cytoplasmic marker for gonadotrophs, since GTH-II is fairly evenly distributed. This allowed the observation of blebbing in TUNEL-positive gonadotrophs (Fig. 10) on two occasions.

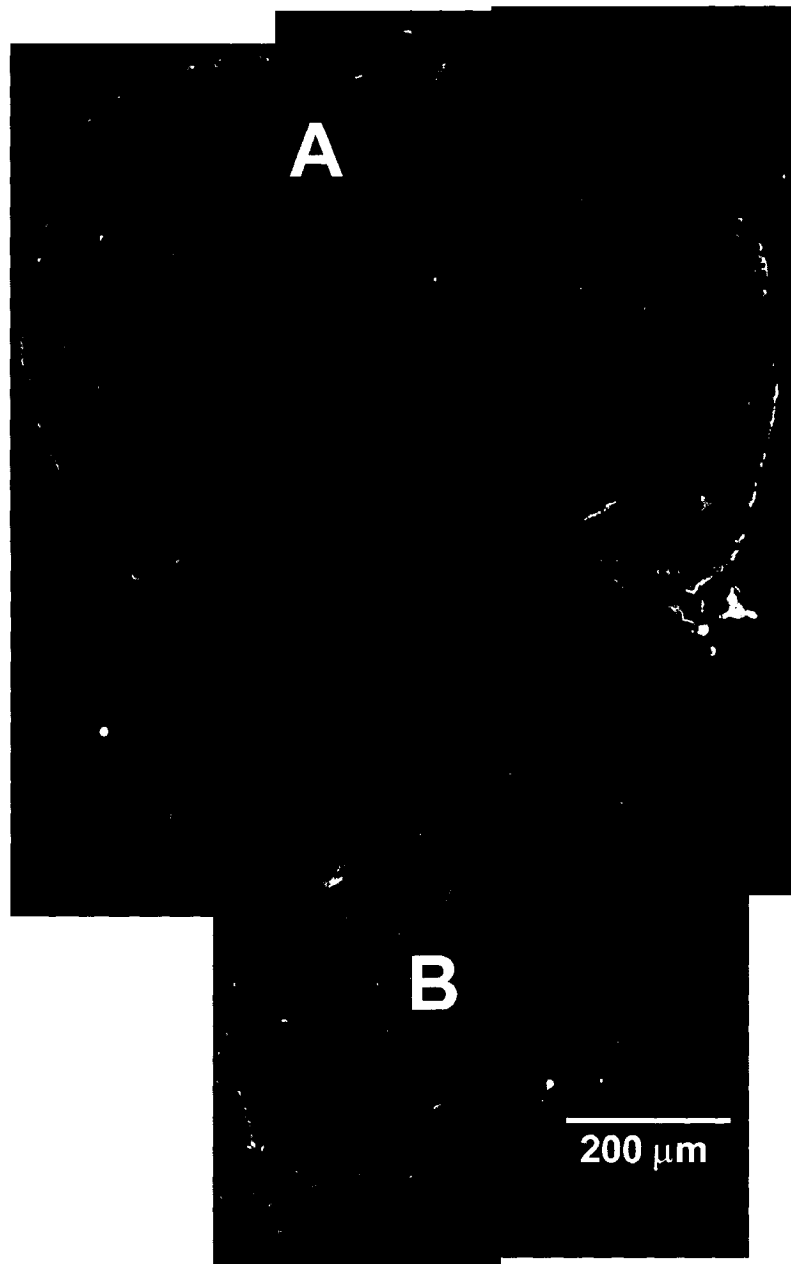


Fig. 7: Composite image of a transversal, 6 μm thick goldfish pituitary section, stained with a GTH-II antibody. The pars distalis (PD) is the " mushroom cap " (A) and the neurointermediate lobe (NIL) is the " stem " (B). The gonadotrophs are only found in the pars distalis and are disposed in clumps. The bright spots found in the NIL are probably due to auto-fluorescing goldfish erythrocytes. The image was made of 8 digital pictures taken at a x100 magnification joined together using Photoshop 4.0 (Adobe).

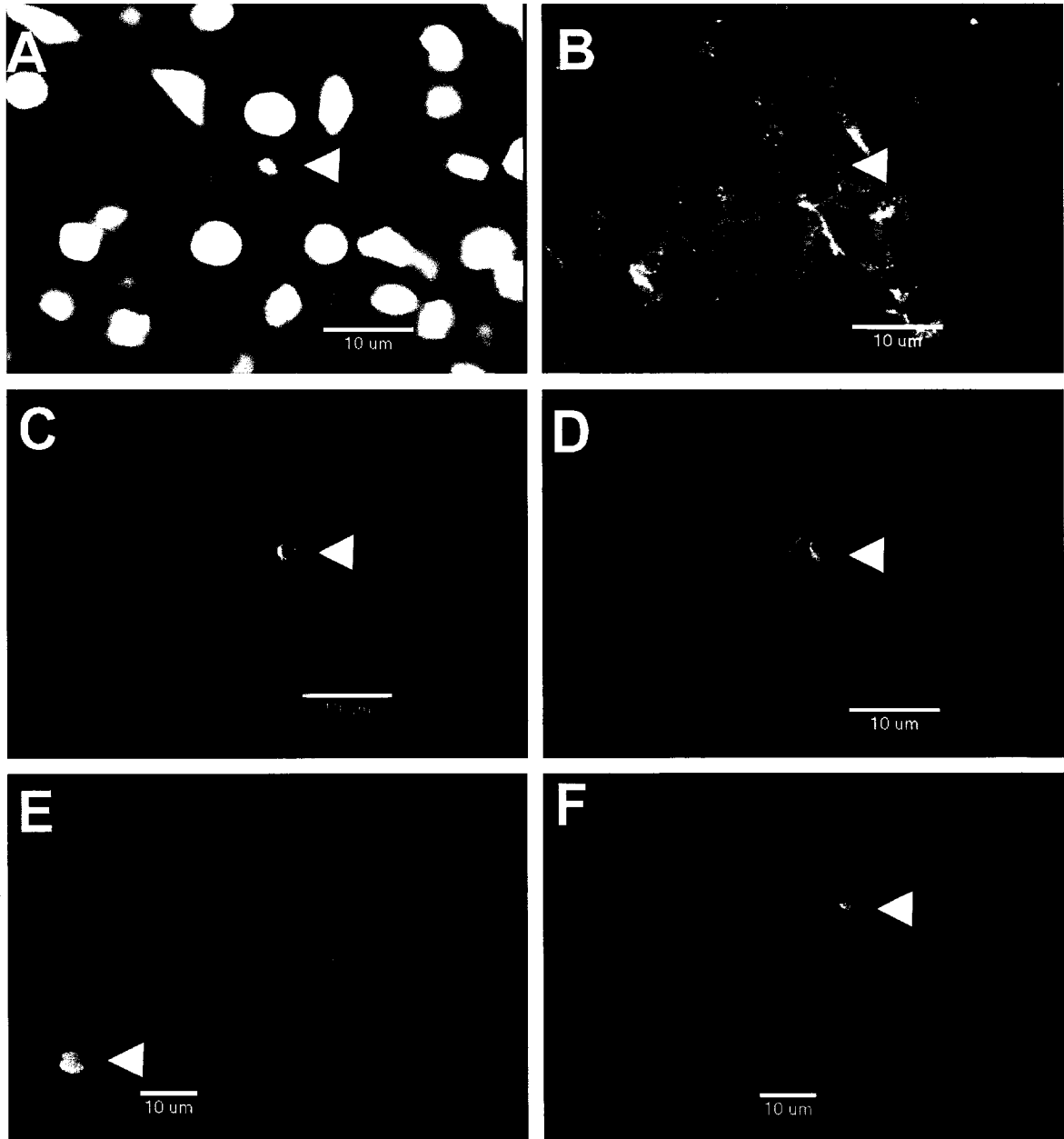


Fig. 8: Evidence of apoptosis in goldfish gonadotrophs. A: DAPI staining of an *in vivo* 6 μm thick paraffin pituitary section. DAPI stains DNA and reveals the nuclei. B: GTH-II labelling of the same area, taken using a different filter to block the DAPI staining. Note the black spots left by the unlabeled nuclei. C: TUNEL staining of the same area, using another filter so only the TUNEL labelling is visible. D: Computer generated overlay of A (in blue), B (in red) and C (in green). E, F: Overlay of other *in vivo* pituitary sections with apoptotic gonadotrophs. Arrowheads indicate apoptotic nuclei.

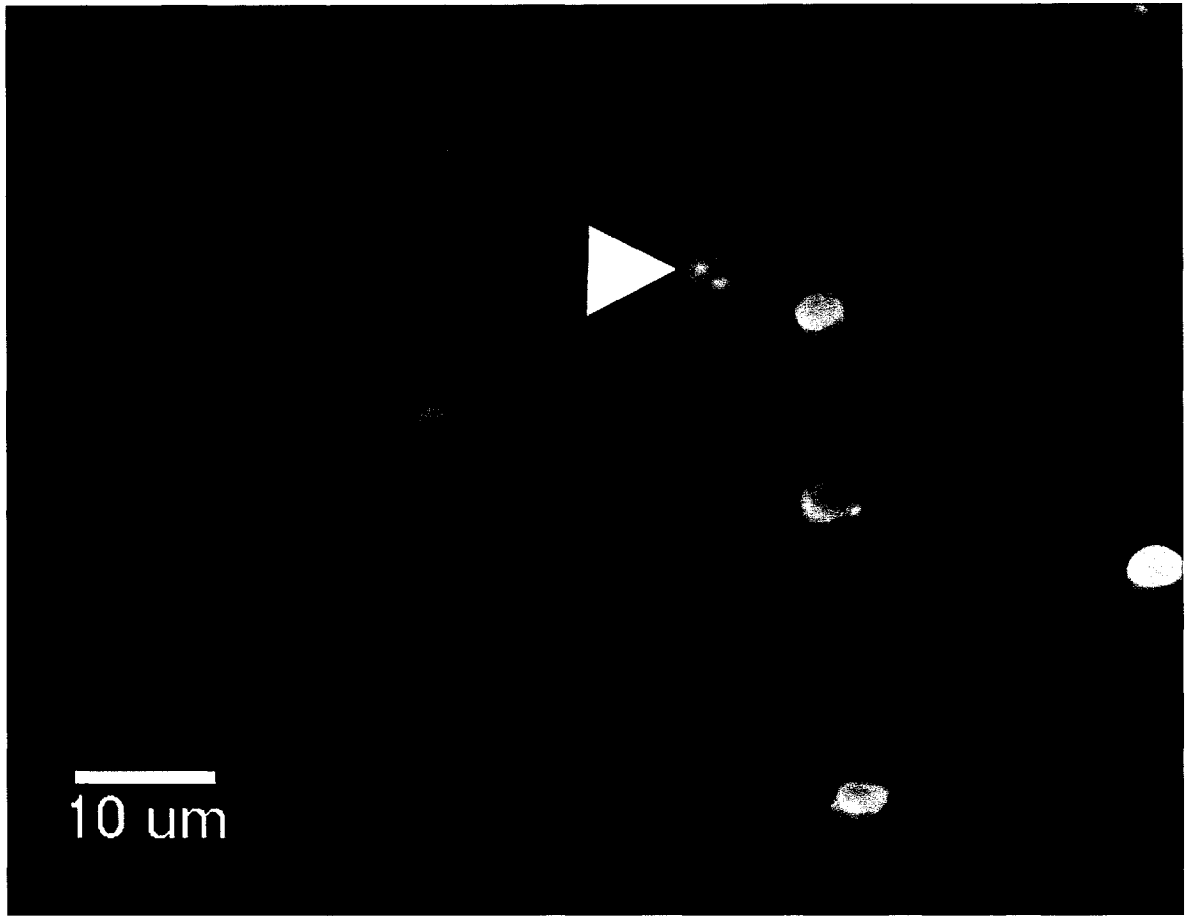


Fig. 9: Evidence of nuclear fragmentation in apoptotic gonadotrophs. Computer generated overlay of DAPI staining in blue (showing all nuclei), TUNEL in green (showing apoptotic nuclei) and anti-GHT-II labelling in red (revealing gonadotrophs) of an *in vitro* 6 μm thick paraffin pituitary section. The arrowhead indicates the fragmenting nucleus. The whole glands were incubated at room temperature for 6 hours in M199 medium with 1% horse serum.

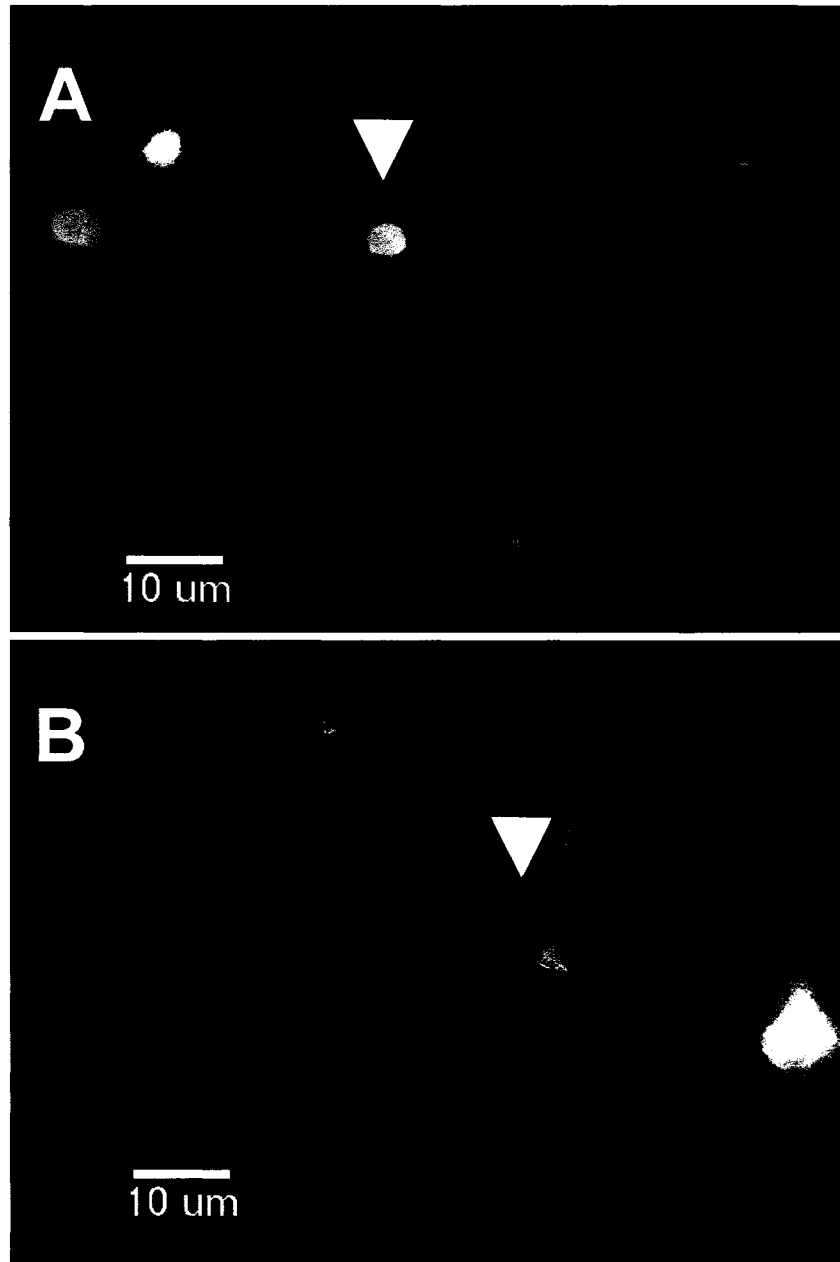


Fig. 10: Evidence of cellular blebbing *in vitro* in A: control and B: A23187-treated apoptotic gonadotrophs (see arrowheads). These images are computer-generated overlays of DAPI nuclear staining in blue, TUNEL (apoptotic nuclei) in green and anti-GTH-II labelling in red (revealing gonadotrophs) of *in vitro* 6 µm thick paraffin pituitary sections. The GTH-II labelling enables the visualisation of the cytoplasm. The whole glands were incubated at room temperature for 6 hours in M199 medium with 1% horse serum.

***In vivo* experiments**

In the goldfish pituitary, apoptosis was detected using a DNA laddering technique (see Figs. 5 and 6). The levels of apoptosis were often low, with only a few fragments visible. Increasing the gel exposure time to the phosphor screen did not improve fragment visibility. A high variability between samples was observed (see Fig. 11). The relative level of fragmented DNA was quantified using the phosphor-imager and Quantity-One software by comparing it to the intensity of the second band (246 bp) of the 123 bp ladder. Analysis was performed on the first apoptotic fragment (of approximately 200 bp size), on the second one (approximately 400 bp) and on the large fragment (of approximately 71 kbp). At the time of the analysis, the size of the large fragment was unknown.

Statistical analysis revealed that the treatments had no significant effects on the intensity of the apoptotic bands for either the 200 bp or the 400 bp apoptotic fragments (Fig. 12) when compared to the control (non treated fish). The saline sham treatment also had no effects. The NIL tended to have more intense bands, and therefore more apoptosis, when treated with apomorphine when compared with the pars distalis. This trend was reversed with the metoclopramide treatment. However, these trends were not statistically significant.

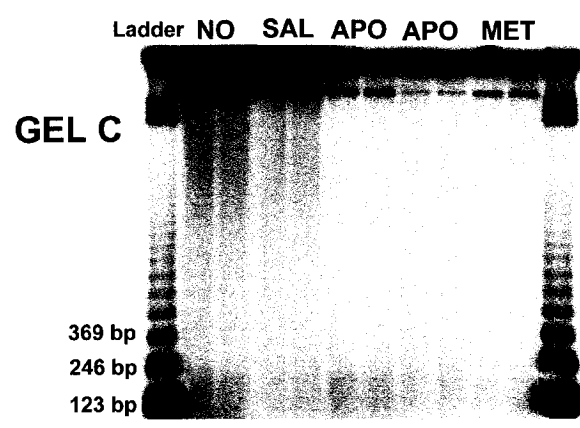
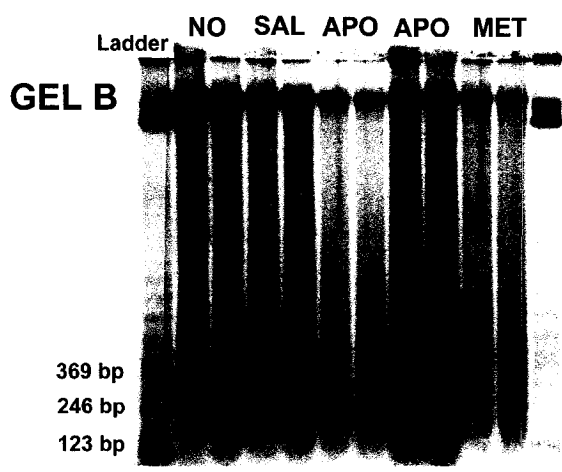
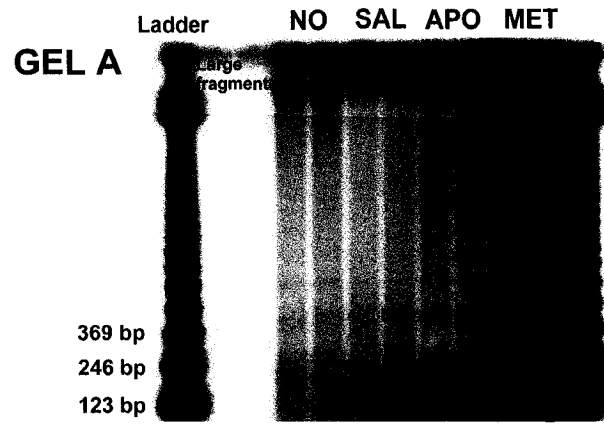
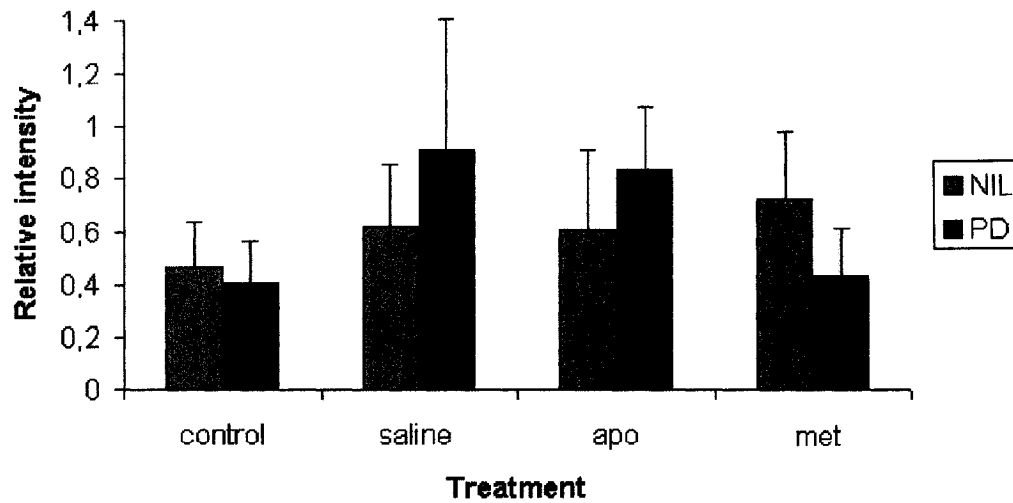


Fig 11: 2% Agarose gel of radioactively labeled goldfish pars distalis DNA. A 123 bp DNA ladder was used on each side of the gel as a size marker. NO = no treatment, SAL = sham injection with saline, APO = apomorphine treatment and MET = metoclopramide. The 246 bp band of the ladder was used as a standard. Each gel had different samples. Gel A represent the best case obtained, gel B was typical with faint but measurable fragments and gel C illustrates a gel where the fragments were not quantifiable.

200 bp apoptotic fragment



400 bp apoptotic fragment

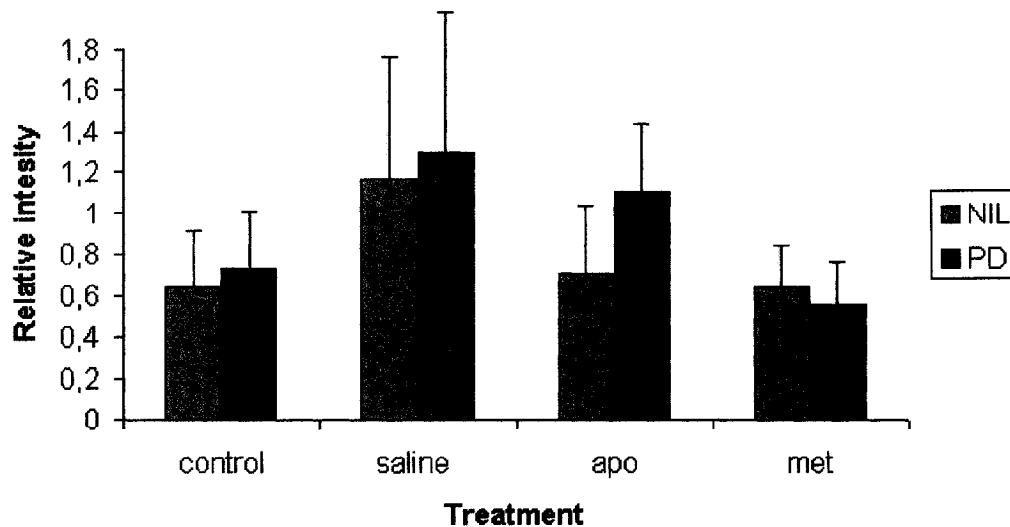


Fig. 12: Analysis of the degree of apoptotic fragmentation *in vivo*, using the 200 and 400 bp fragments. The value expressed is of the intensity of the band relative to the 246 bp band of the 123 bp ladder. The error bar displayed is the standard error. No significant differences were found between the control and treatments or between the 200 and 400 bp fragments. Control = untreated fish, saline = injection with saline, apo = apomorphine treatment and met = metoclopramide. PD = pars distalis, NIL = neurointermediate lobe. N varies between 5 and 7 pools of DNA extracted from 5 pituitaries per pool.

The large fragment, due to its constant presence and sharp definition, was also investigated. A one-way ANOVA revealed that the intensity of the band in the 2 tissues was significantly different, being much higher in the neurointermediate lobe. The treatments had no statistically significant effect on the intensity of the large fragment, except for the apomorphine, which significantly increased the intensity of the large fragment in the pars distalis (Fig. 13)

To further characterize the nature of the large fragment, a series of low-density agarose gel electrophoresis trials were undertaken. It was found that the large fragment grew fainter and more diffuse when the density of the agarose decreased. It was found that running a 0.3% agarose gel at 25V for 24 hours was needed to get good separation. A large molecular weight DNA ladder, composed of digested and undigested lambda phage DNA (Gibco BRL) was used (see Fig. 14). The fragment was found to be of approximately 71 kbp in length (see Fig. 15). This value lies outside of the calibration curve and thus can only be considered a rough estimate. Extraction of fish DNA often results in genomic DNA of roughly 70 kbp in length (C.R. Martin, University of Ottawa, personal communication). It is therefore likely that this is genomic DNA rather than apoptotic fragments. Further investigation of the large fragment was not pursued.

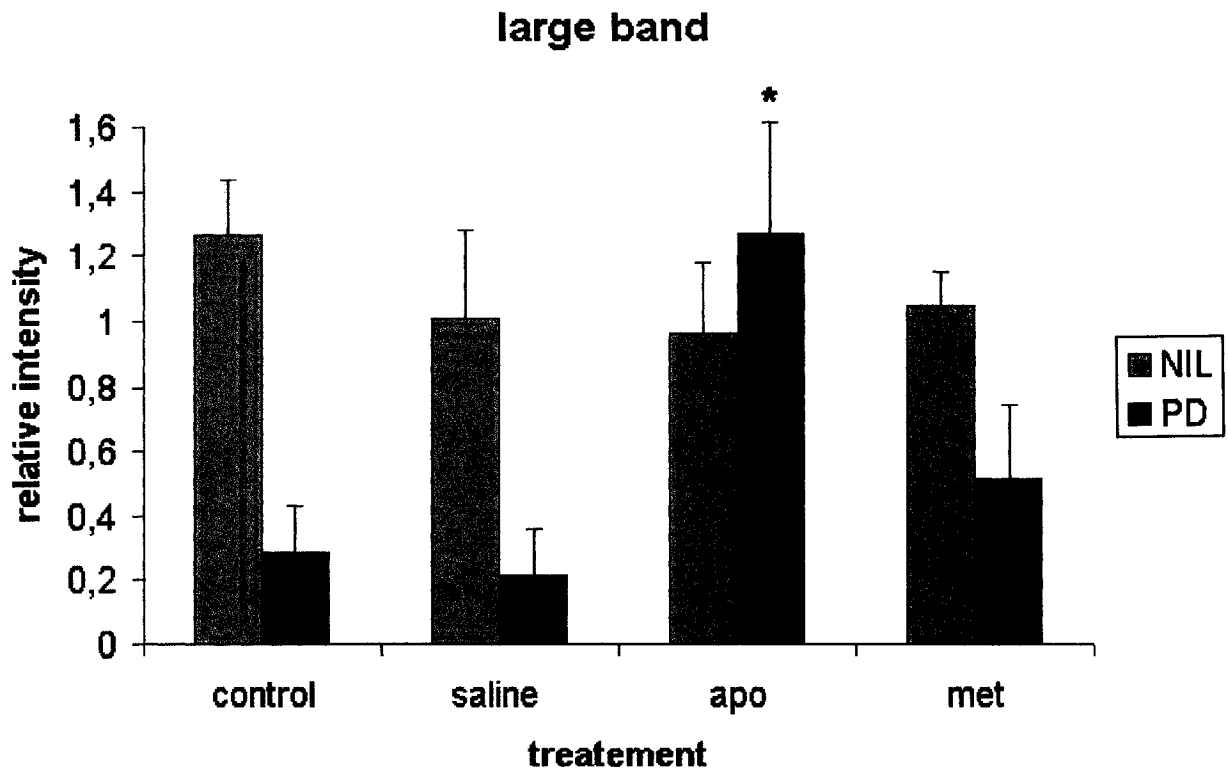


Fig. 13: Analysis of the levels of the large fragment *in vivo*. The value expressed is of the intensity of the band relative to the 246 bp band of the 123 bp ladder. The error bar displayed is the standard error. It was found that apomorphine significantly increases the intensity of the large fragment in the pars distalis (* = $p = 0.002$, using the Fisher's Least-Significant-Difference Test.). Control = non-treated fish, saline= injection with saline, apo = apomorphine treatment and met = metoclopramide. PD = pars distalis, NIL = neurointermediate lobe. N varies between 5 and 7.

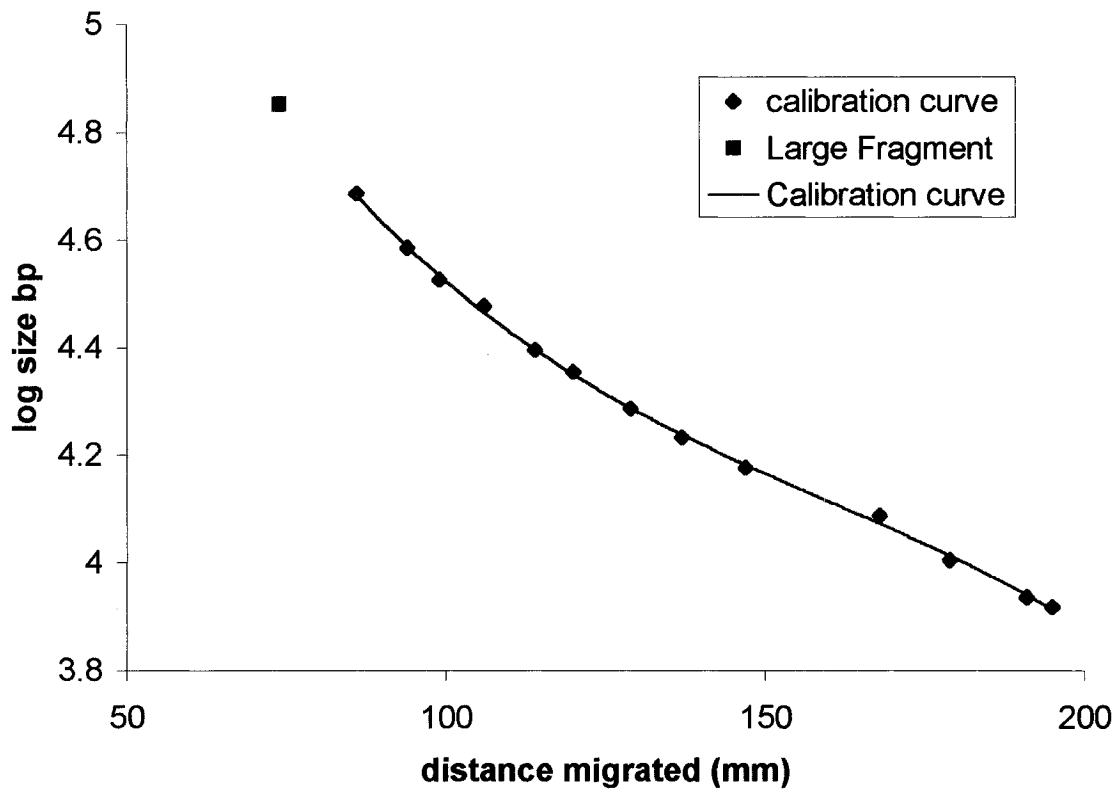


Fig. 14: Determination of the size of the large unknown fragment, using the high molecular weight lambda DNA digest as a standard. The gel was run for 24 hours at 25 volts. A 3rd degree equation was determined to be the best fit for the calibration curve. Using this equation, the fragment's size appears to be of approximately 71 kbp in size.

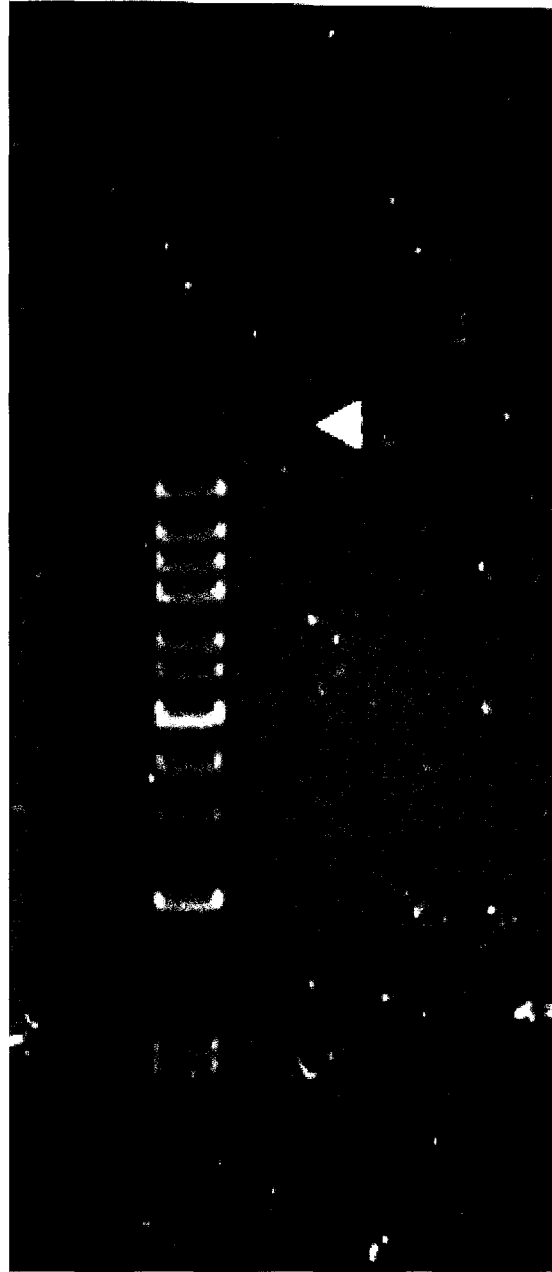


Fig. 15: Determination of the size of the large DNA fragment. 0.3% agarose gel of goldfish pituitary DNA ran at 25 volts for 24 hours and stained with ethidium bromide. The arrowhead indicates the position of the fragment, which is diffuse and barely visible, probably due to lack of compression. A high molecular weight lambda DNA digest was used as the ladder. Sizes of the digested fragments are indicated to the left of the ladder.

***In vitro* experiments**

Using the TUNEL technique and GTH-II labeling, the levels of apoptosis were studied using *in vitro* culture of whole goldfish pituitaries. A pilot experiment was undertaken (n=3), followed by a repeat (n=5) to increase the number of observations. However, it was found that the first experiment (with n=3) had a much lower baseline (control = 24.5 ± 1.2 TUNEL-positive nuclei per 125 000 μm^2 field of view, in the whole gland) than the second (control = 94 ± 29 for the whole gland). This is perhaps because the two experiments were performed at different times of the year. It became necessary to determine whether these two experiments could indeed be pooled. A two-way non-parametric ANOVA revealed that while the levels of apoptosis were significantly lower in the first experiment as opposed to the second ($p = 0.006$ for the whole gland), there was no significant interaction between the experiment and the treatment. This meant that the treatments have similar effects on a relative scale in both experiments. The data were therefore pooled and normalized, expressed as a value relative to the control. The average value of the control over the two experiments was 67 ± 23 TUNEL-positive nuclei per 125 000 μm^2 field of view (N=8). This is much higher than the levels found *in vivo* (0.27 ± 0.09 TUNEL-positive nuclei per 125 000 μm^2 field of view, N=4)

Using GTH-II labeling, it was possible to distinguish the pars distalis and the neurointermediate lobe because gonadotrophs are found only in the pars distalis. This allowed for the quantification of the levels of apoptosis in these two areas. The data were statistically treated the same way as the whole gland data. The whole gland data contained data from fields of view both in the pars distalis and the neurointermediate lobe, as well as from fields that were indeterminate (fields overlapping both areas of the gland).

The experiment revealed that the *in vitro* basal level of apoptosis is higher in the pars distalis (76 ± 28 TUNEL-positive per field of view) than in the neuro-intermediate lobe (53 ± 16). However, this difference was not statistically different. There was a tendency for the absence of serum in the culture medium to affect the pars distalis more than the neurointermediate lobe. Also, the addition of apomorphine reduced the level of apoptosis in the whole gland by about 40%. However, these tendencies were not statistically significant. GnRH increased the number of TUNEL-positive nuclei by 50% in the pars distalis, but not in a statistically significant manner. It was found that the addition of the calcium ionophore A23187 significantly increased apoptosis both in the pars distalis and the neurointermediate lobe by over 3 fold (see Fig. 16). This change was statistically significant, using a 2-sided Dunnett's Test. This effect was noticeable under the microscope (Fig. 17).

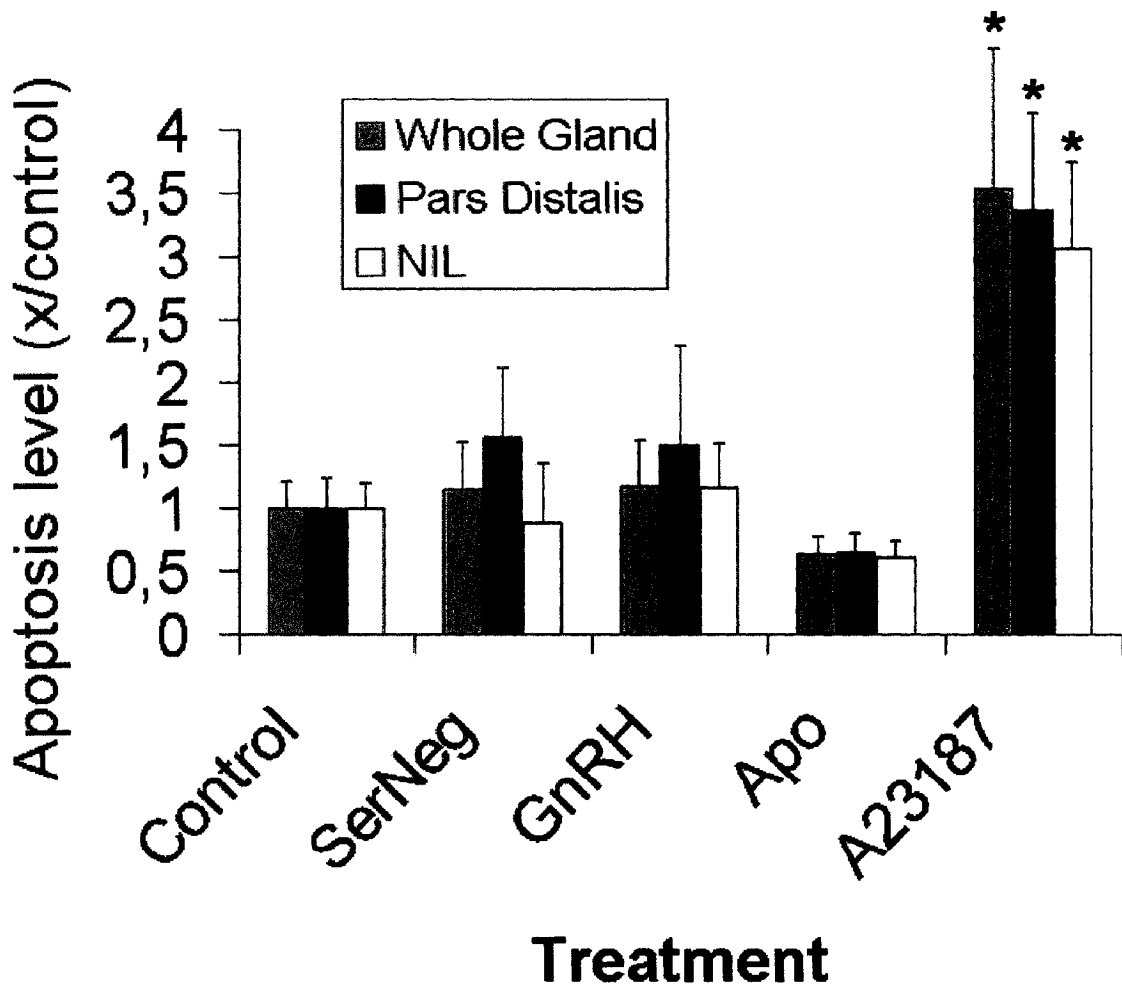


Fig. 16: Effects of GnRH and apomorphine on the level of pituitary apoptosis *in vitro*. The means plus the standard error are presented. * = $p < 0.05$, significantly different from control. These results are normalized, and expressed as a ratio of treatment results on the mean of the control. See text for more details. The absolute value for the whole gland was 67 ± 23 TUNEL-positive nuclei per $125\ 000\ \mu\text{m}^2$ field of view, 76 ± 28 for the pars distalis and 53 ± 16 for the neuro-intermediate lobe (NIL). N=8. Control = 6 hour incubation in M199, SerNeg = control with no serum, GnRH = control treated with 100 nM salmon GnRH analogue, A23187 = control with 10 μM calcium ionophore A23187.

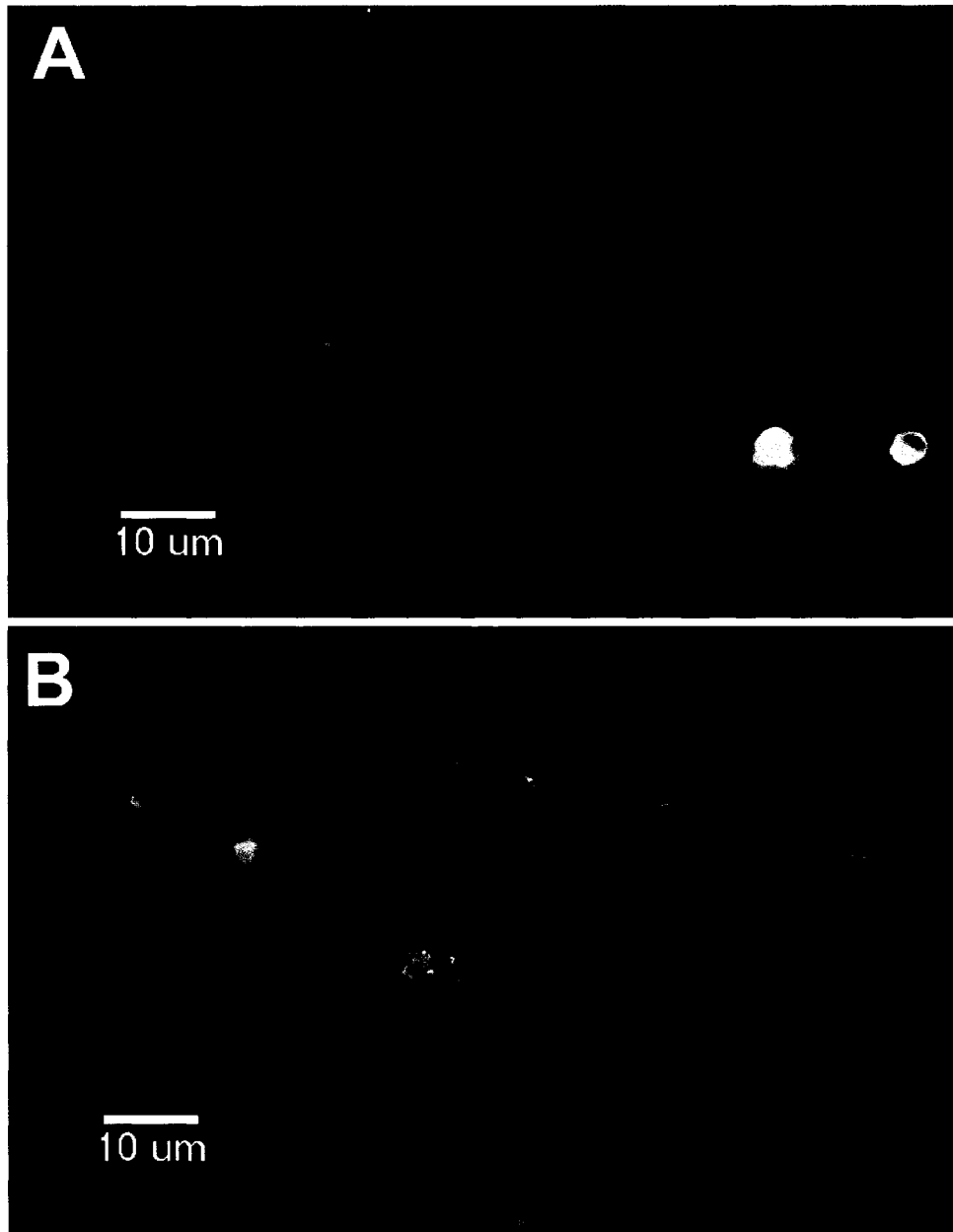


Fig. 17: Effect of the calcium ionophore A23187 on apoptosis *in vitro*: These images are computer-generated overlays of DAPI staining in blue (showing all nuclei), TUNEL in green (showing nuclei with DNA fragmentation) and anti-GHT-II labelling in red (showing gonadotrophs) of *in vitro* 6 μm thick paraffin pituitary sections. A: Overlay of a section of a non-treated, pituitary gland. B: Overlay of a section of a A23187-treated pituitary gland. Note the increase in TUNEL-labelled nuclei. These images were taken in the pars distalis. Similar effects can be seen in the neurointermediate lobe. The whole glands were incubated at room temperature for 6 hours in M199 medium with 1% horse serum.

DISCUSSION

Apoptosis is present in the goldfish gonadotroph

The process of cellular apoptosis has both morphological and biochemical characteristics which allow for its detection. The fragmentation of DNA into approximately 200 bp multiples is considered a hallmark of apoptosis (Wyllie, 1980; Nagata, 2000) and was observed *in vivo* in the goldfish pituitary. This DNA fragmentation was also detected using the TUNEL technique. A double labeling using a GTH-II antibody revealed the apoptotic process specifically in the gonadotrophs of adult goldfish, both *in vivo* and *in vitro*.

Although the TUNEL technique allows for the detection of DNA fragmentation (Gavrieli *et al*, 1992) caused by apoptosis, some forms of necrosis do have a certain amount of non-specific, single strand DNA breaks that can be also detected by TUNEL (Charriaut-Marlangue and Ben-Yari, 1995). Observation of hallmark morphological features is therefore necessary to establish true apoptosis. Nuclear and chromatin condensation was observed *in vivo* (see Fig 8) in gonadotrophs and non-gonadotrophs throughout the pituitary. Because apoptosis was much higher *in vitro*, apoptotic morphological features were more easily observed, including nuclear fragmentation in gonadotrophs and non-gonadotrophs and evidence of cellular membrane blebbing in gonadotrophs. Nuclear condensation, fragmentation, and membrane blebbing are all features of apoptosis (Kerr *et al*, 1972). Together these observations make for a very strong case for the presence of apoptosis in the goldfish pituitary in general and in the gonadotrophs in particular.

The GTH-II labeling clearly indicates that the gonadotrophs are found in clumps, exclusively in the proximal pars distalis, which was expected (Follenius and Doer-Schott,

1978). The teleost pituitary is regionalized, thus facilitating the localization of gonadotrophs within the whole gland. Glycoprotein hormones like GTH-II are not present within the nucleus; a darker “hole”, corresponding to the nucleus, appears in gonadotrophs stained for GTH-II. By overlaying images taken at the same spot using different filters on the microscope, the matching of a specific gonadotroph with a specific nucleus is possible. A double-labeling protocol for GTH-II and TUNEL was optimized, allowing the identification of apoptotic gonadotrophs *in situ* (Fig 8). To the best of our knowledge, this is the first time this has been achieved in teleost pituitary cells and in gonadotrophs in general. It had been reported that by the time a rat pituitary cell was histologically detectable as apoptotic by light microscopy, the hormonal content was depleted, making double labeling and thus identification of the apoptotic pituitary cells impossible to determine (Nolan *et al*, 1998). This does not seem to be the case with this goldfish model.

***In vivo* measurement of apoptosis**

Analysis of the levels of DNA fragmentation revealed a high variability between samples. Levels of fragmentation showed variations between treatments, but no effects of *in vivo* treatments with apomorphine and metoclopramide were found. It was expected that apomorphine, a dopamine agonist and inhibitor of GTH-II secretion and release (Omeljaniuk *et al*, 1989), would increase apoptosis in the pars distalis. It was also expected that the dopamine inhibitor metoclopramide would inhibit apoptosis in the pars distalis. While this trend was visible, it was not statistically significant. Because of the high variability, it is impossible to rule out a small effect. The levels of apoptosis detected by DNA laddering are low. Certain samples had barely detectable fragments, making quantification somewhat difficult. Because of this and

of the high variability, *in vivo* levels of pituitary apoptosis appear to be at the lower limit of detection of the DNA laddering method at the times of year studied.

Electrophoresis of the extracted DNA revealed the presence of two fragments of sizes not matching the 200 bp multiples (Fig 6). A small, approximately 80 bp-sized fragment was found, of an unknown nature. A fragment of similar size was present in other studies on fish ovary using the same extraction and radiolabeling method (Janz and Van Der Kraak, 1997; Wood and Van Der Kraak, 2001). A second, larger fragment was also detected in the pituitary. Again, a fragment of similar size was present in others studies that used the same methodology in fish ovary (Janz and Van Der Kraak, 1997; Wood and Van Der Kraak, 2001). Low-density agarose electrophoresis revealed that it was of approximately 71 kbp in size. This fragment was significantly upregulated in the pars distalis by the apomorphine treatment and was generally more intense in the neurointermediate lobe. The nature of this fragment is unknown at this time. If it is genomic DNA, the varying levels could suggest a problem in the DNA extraction procedure. A 50 kbp fragment is generated in the earlier stages of fragmentation (Walker *et al*, 1999), corresponding to one chromatin loop (Filipski *et al*, 1990). Since these measurements were made on mammalian DNA and that the approximately 50 kbp band has a rather variable size, it is plausible that the 71 kbp fragment is due to early stage apoptotic degradation. If this were the case, the increase in apoptosis in the pars distalis following application of a dopamine agonist would meet the hypothesis that neurotransmitters control secretion by modulating cell populations. Since dopamine inhibits GTH-II release, it could also be triggering apoptosis. Further inquiries, using pulse field electrophoresis and extraction methods for high molecular weight DNA, are needed to determine if this fragment is a product of early stage apoptosis. Until

this hypothesis is tested, the results concerning the large DNA fragments should be interpreted with caution.

***In vitro* measurement of pituitary apoptosis**

Effects of *in vitro* culture

The *in vitro* levels of apoptosis are much higher than those detected *in vivo*. The higher levels *in vitro* can be perhaps explained by withdrawal of several neuroendocrine inputs. The teleost pituitary is directly innervated by a multitude of neuropeptide and neurotransmitter producing neurons from the hypothalamus (Blazquez *et al*, 1998) and the dissection of the gland would have cut the axons going to the pituitary, creating the neuroendocrine withdrawal. Since the glands were not sectioned, proper penetration of the nutrients of the culture media into the middle of the gland was also a concern. However, no increase in apoptosis was observed in the center of the pituitary as opposed to the outer area, which would have been expected had the center of the gland been starved of nutrients. Levels of apoptosis were highly variable, both within a gland and between samples. This required the observation of a large number of fields of view per gland (average of 21 field per gland).

Not all pituitary whole gland cultures resulted in such a drastic (over a hundred fold) increases in apoptosis levels when compared to the *in vivo* levels. Culture of juvenile rat pituitaries at room temperature showed no increase in apoptosis for up to 24 hours (Nolan *et al*, 1999). This discrepancy might be explained by differences between the juvenile rat and the goldfish (such as the rat pituitary is not directly innervated by the hypothalamus and is more vascularized), by differences in the culture methods (10% calf serum was used for the rat as opposed to 1% horse serum, the rat pituitary culture was not done at its normal body temperature

while the goldfish culture was) or by difference in age (the rats were 6 week old juveniles while the goldfish were adults).

A statistically significant difference was found between the apoptosis levels of the first and the second experiments. This difference may be related to a difference in the endocrine and reproductive state of the goldfish in the two different times of year examined. The first experiment was done in August, which is the end of the period of gonadal regression, while the second experiment was done in November, which corresponds to the beginning of the gonadal recrudescence period when both GH and GTH-II secretions are increasing (Trudeau, 1997). It is possible that, if there is cellular proliferation in the pituitary gonadotrophs in conjunction with this increase in pituitary activity, these new cells increase the requirements for neurohormonal support from the hypothalamus. The pituitary glands collected in November would therefore be more affected by the dissection than the glands removed in August and more vulnerable to apoptosis. It is also possible that there is an actual increase in the apoptosis level, caused by a greater rate of cellular renewal of the pituitary cells at the beginning of the recrudescence, to better meet the increased secretory requirements by disposing of older, less productive cells. However, the pituitary gland responded generally the same way to treatments in both periods, since a two-way ANOVA failed to demonstrate an interaction between the experiment time and the treatments. This allowed the pooling of the 2 experiments and the standardization of the data, expressed at the ratio between the mean observed of the treatment and the mean of the control.

Effects of serum starvation

Apoptosis can be induced by serum starvation in several cell types (Sastry and Rao, 2000). The absence of serum did not significantly increase the levels of apoptosis in the goldfish

pituitary. This could indicate that the serum level used in the culture medium was insufficient, or perhaps that horse serum is inappropriate. If this is the case, it may help explain the hundred-fold increase in TUNEL-positive nuclei seen *in vitro* in the goldfish pituitary when compared to *in vivo* levels, the control conditions being similar to serum starvation.

The culture conditions for this experiment were similar to those used in perfusion goldfish pituitary fragment culture experiments and in cell culture experiments (Jobin and Chang, 1996b; Johnson and Chang, 2000; Chang *et al*, 1992; Van Goor *et al*, 1994; Johnson *et al*, 2000). While those conditions are optimized for hormonal responses, it is possible that they might increase the level of apoptosis in a more sensitive cell sub-population, resulting in a cell culture that does not represent the entirety of the whole gland cell population. Teleost cells do not always undergo apoptosis under serum starvation, however. A 24 hour incubation in a serum free environment did not increase the levels of apoptosis in goldfish ovary follicles (Wood and Van Der Kraak, 1997). Because of the significant difference between ovary follicles and pituitary, it is uncertain whether this resistance should be expected in teleost pituitary as well.

Effects of neurotransmitters

My experiments failed to show any significant difference between the control and the GnRH and the dopamine agonist apomorphine treatment. Apomorphine caused a 35% decrease in apoptosis in the pituitary, while GnRH caused a 49% increase in the gonadotroph-rich pars distalis. These trends, if they were statistically significant, would have been surprising and contrary to our predictions. Dopamine is a strong inhibitor of GTH-II secretion (Blazquez, 1998; Omeljaniuk *et al*, 1989; Chang *et al*, 1993) and can increase apoptosis in the rat pituitary under certain circumstances (Drewett *et al*, 1993). GnRH stimulates GTH-II secretion (Peter, 1986; Jobin *et al*, 1996a). These trends would also be very significant biologically speaking, since only

a small increase in apoptosis can have a profound impact on cellular populations (Nolan *et al*, 1998). Because of the low N number and the high variability, the statistical test performed has a low power (24%). This means that the chance of a type II error, which is concluding that there is no effect when there is an effect, is high (76%). More investigation would be warranted before claiming that apomorphine and GnRH do not affect apoptosis levels in the goldfish pituitary gland.

Effects of calcium ionophore

Treatment of the pituitary goldfish with the calcium ionophore A23187 has a statistically significant impact on the levels of apoptosis, increasing it by over three fold. The difference was high enough that it was observable at a glance. This result was expected. High levels of intracellular calcium can trigger apoptosis (Kiess and Gallaher 1998; Sastry and Rao, 2000; Kroemer *et al* 1995). An increase in intracellular calcium was proposed as part of a mechanism for the increase in apoptosis triggered by tributyltin in trout hepatocytes (Reader *et al*, 1999). A23187 has been shown to cause an increase of apoptosis in unidentified cells in pituitary cell cultures from the bony fish tilapia (Melamed and Yaron, 1999). However, calcium is also very important for the secretion of GTH-II. GnRH induces an increase in intracellular levels of calcium, and this increase is necessary for the release of GTH-II in the goldfish (Chang *et al*, 1993; Jobin *et al*, 1996; Johnson *et al*, 2000). Calcium is also believed to be involved in the secretion of GH (Johnson and Chang, 2000). Since dopamine can inhibit the GnRH-induced increase of intracellular calcium, it is possible that it could act as a protective agent, which might explain the trend observed (Fig 16). It was found that the levels of calcium ionophore necessary to trigger a GTH-II or GH secretory response also triggered apoptosis and inhibited mRNA synthesis of GTH-II and GH in tilapia pituitary cells (Melamed and Yaron, 1999). The fact that

A23187 also increases apoptosis in the goldfish not only confirms that the experimental protocol had some validity (since this result was expected), but also raises question about the studies on hormonal secretion that use calcium ionophores. Finally, there could be a direct link between apoptosis and secretion. The hormonal content of the gonadotrophs is not depleted, even at advanced stages of apoptosis (see Figs 8 and 9). Whether the GTH-II is degraded by phagocytes or is released later during the apoptotic process remains to be determined.

CONCLUSION

The current study has demonstrated the presence of apoptosis in the goldfish pituitary gland in general, and in gonadotrophs in particular. Both morphological and biochemical features of 'classical' apoptosis were observed. It was found that GnRH and dopamine agonists and antagonists have no statistically significant effects on apoptosis levels *in vivo* or *in vitro*, although the high variability makes it impossible to positively conclude that these neurohormonal factors have no effects at all. A seasonal difference was found in the basal level of cultured whole pituitary gland, corresponding to different period in the goldfish reproductive cycle. The calcium ionophore A23187 was shown to have a strong and significant effect on *in vitro* whole gland apoptosis levels, demonstrating that the experimental model could detect large effects and raising questions on certain other studies using this ionophore to stimulate hormonal secretion. The increase in apoptosis could have reduced certain cells populations and lead to inappropriate conclusions being drawn. Finally, the current study has demonstrated the possibility of labeling TUNEL-positive cells as gonadotrophs or non-gonadotrophs, allowing for the detection of apoptosis in the intact pituitary and in specific endocrine cells. Indeed, this has not been done in the pituitary of other animal models, thus making the goldfish attractive for future analysis of apoptosis in the pituitary gland.

Future studies

The presence of apoptosis has been established in the goldfish pituitary, yet the exact function of apoptosis in the adult pituitary is unknown. It remains to be established if apoptosis plays a role in the physiological regulation of seasonally varying levels of GTH-II and other pituitary hormones by disposing of excess cells. Apoptosis may also play other roles, such as playing part in cell renewal to prime the gland for increased secretion by disposing of older cells,

or tissue maintenance and homeostasis. Investigations with pulse field electrophoresis could reveal if the increase in the presence of high molecular weight DNA fragments in conjunction with a dopamine agonist is linked to an increase in early stage apoptotic DNA fragmentation. The ability to measure apoptosis in the pituitary can lead to establishment of improved conditions for both whole gland and cell culture, favoring cell survival. Such conditions might allow for the detection of smaller effects and the study of the effects of physiologically relevant levels of hormones, growth factors and neurotransmitters on pituitary apoptosis. Cell cultures, combined with double labeling, will also allow the study of cell-type specific apoptosis in the goldfish gland. Finally, further studies could establish if other hormone-secreting pituitary cells can be positively identify as being apoptotic.

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