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Characterization and ontogeny of cholecystokinin and estrogen receptor immunoreactive circuits in the brain

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Characterization and ontogeny of cholecystokinin and estrogen receptor immunoreactive circuits in the brain

Fox, Charles Allan, Ph.D.

Iowa State University, 1991
Characterization and ontogeny of cholecystokinin and estrogen receptor immunoreactive circuits in the brain

by

Charles Allan Fox

A Dissertation Submitted to the Graduate Faculty in Partial Fulfillment of the Requirements for the Degree of DOCTOR OF PHILOSOPHY

Department: Veterinary Anatomy
Interdepartmental Major: Molecular, Cellular, and Developmental Biology

Approved:

In Charge of Major Work

For the Major Department

For the Interdepartmental Major

For the Graduate College

Iowa State University
Ames, Iowa

1991
To my wife, Sharon, and my parents, Allan and Harriet.

Without their support this would not be possible.
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GENERAL INTRODUCTION

Originally purified from porcine gut as a 33 amino acid peptide (Mutt and Jorpes, 1968), cholecystokinin (CCK) had been known since 1928 as a gastrointestinal factor which was capable of causing gall bladder contraction (Ivy and Oldberg, 1928). In 1943, the same factor in small intestine extracts, then referred to as pancreozymin, was found to stimulate secretion of pancreatic enzymes (Harper and Raper, 1943). Ironically, the first description of CCK in the central nervous system was as gastrin-like immunoreactivity. In 1975, Vanderhaeghen and coworkers described a gastrin-like immunoreactivity in the brain (Vanderhaeghen et al., 1975). It was later determined that the peptide responsible for the gastrin-like immunoreactivity described by Vanderhaeghen et al. was, in fact, CCK (Dockray, 1976; Dockray et al., 1978; Muller et al., 1977; Rehfeld 1978a, b; Robberecht et al., 1978).

Now it is well known that CCK is one of the most abundant neuropeptides in the mammalian central nervous system. Cholecystokinin is found in a variety of molecular forms within the brain. Specifically, CCK molecules of 58, 39, 33, 8, 5, and 4 amino acids in length have been isolated from the brain. However, the tyrosine-O-sulfated, C-terminal amidated octapeptide is the most abundant form (Rehfeld et al., 1985). The family of cholecystokinin molecules are all translated from one mRNA that is approximately 0.8kb in size (Deschenes et al., 1984) and the variation in sizes of the peptide itself arise from posttranslational processing by peptidases in the cell (Reeve et al., 1989).

The brain contains a wide distribution of CCK containing somata and fibers (Beinfeld et al., 1981a, b; Crawley, 1985; Fallon and Seroogy, 1985; Gall, 1984; Larsson and Rehfeld, 1979; Miceli et al., 1987; Vanderhaeghen et al., 1980, 1981). In the rat, CCK is found in the cerebral cortex, caudate nucleus, amygdaloid complex (Micevych et al., 1988a), hippocampus (Greenwood...
et al., 1981; Handelmam et al., 1981), olfactory bulb (Matsutani et al., 1988; Seroogy et al., 1985), septum, thalamus, hypothalamus (Micevych et al., 1987), midbrain, and brainstem. Within the overwhelming distribution of CCK within the brain, some distinct CCK containing circuits can be dissected.

One major source of CCK in the brainstem arises from gastric branches of the vagus nerve which send afferent input to the solitary tract (sol) and nucleus of the solitary tract (Sol; Kubota et al., 1983; Norgren and Smith, 1988; Palkovits et al., 1982; Shapiro and Miselis, 1985). At least some of the neurons in the Sol that receive this CCKergic input send CCK containing efferents to the parabrachial nucleus (PB) in the rostral brainstem (Herbert and Saper, 1990). Cholecystokinin containing fibers then project from the PB to the ventromedial hypothalamus of the rat (Fulwiler and Saper, 1985). It is likely that this CCK containing circuit in the rat brain may carry information from the gut to the brain and thus modulate feeding behavior (Crawley and Schwaber, 1984; Smith et al., 1981b; van der Kooy, 1984).

Another important CCK containing circuit in the rat brain is located in the limbic regions of the forebrain. Cholecystokinin immunoreactive cells are found in the medial amygdala, bed nucleus of the stria terminalis, and the medial preoptic area (MPA). Both the amygdala and the bed nucleus are important afferent nuclei of the medial preoptic area (Simerly and Swanson, 1986). Interestingly, both systemically administered estrogen and testosterone are able to regulate the numbers of CCK immunoreactive neurons in these three regions coordinately (Micevych and Bloch, 1989; Oro et al., 1988). This circuit may mediate reproductive function in the rat (Micevych and Bloch, 1989).

In situ hybridization reveals an extensive distribution of CCK mRNA in the rodent CNS which is consistent with the distribution of CCK immunoreactive somata (Savasta et al., 1988;
Ingram et al., 1989). Cholecystokinin's broad anatomical distribution suggests that it is involved in a wide variety of functions carried out by the brain.

Cholecystokinin modulates several behaviors and physiological functions that are controlled by the brain. One of its roles is in feeding behavior where it is thought to act as a satiety factor. Administration of CCK intraperitoneally or into the cerebral ventricles decreases meal size in fasted animals (Baile and Della-Fera, 1985; Smith et al., 1981a). There is currently debate on systemically released CCK's mode of action on feeding behavior. Central administration studies indicate that CCK's presence in the brain can induce satiety. However, it is unlikely that blood born CCK is able to cross the blood brain barrier (Passaro et al., 1982).

One mechanism for CCK's action in the periphery involves CCK receptors of the gastric branches of the vagus nerve. Cholecystokinin released from the gut may bind these vagal receptors which then excites fibers of the vagus nerve sending a signal to the brain (Smith et al., 1981b). While it appears that the gastric vagus is critical for CCK's effects on satiety in the normal individual (Smith et al., 1981b), it is clear that CCK is an important factor in normal feeding behavior.

Cholecystokinin also acts on reproductive behavior. In rats, lordosis behavior is modulated by peripheral administration of CCK. In females rats, the degree of receptivity determines the direction and intensity of CCK's modulatory effect (Bloch et al., 1987). In addition, after gonadectomy and estrogen priming, lordosis behavior in male rats can be increased by injection of CCK into the lateral ventricle (Bloch et al., 1988).

There is also evidence that CCK has a role in gonadotropin secretion. Cholecystokinin injected into the third ventricle of ovariectomized rats affects secretion of luteinizing hormone (LH), prolactin, growth hormone, and thyrotropin (Vijayan et al., 1979). In addition, implantation of CCK into the MPA increases plasma LH levels (Kimura et al., 1983). Conversely, CCK injected into the third ventricle decreased plasma LH levels. This disparity is
probably due to the location of the CCK administration. Cholecystokinin injected into the third ventricle is likely to act on the median eminence directly, whereas CCK placed in the MPA will likely modulate the activity of cells locally in the MPA (Kimura et al., 1983).

Since CCK modulates sexual behavior and gonadotropin secretion, it is not surprising that specific areas of the brain contain sex differences in CCK concentration (Frankfurt et al., 1985; Micevych et al., 1988b; Siegel et al., 1985). Radioimmunoassay techniques have shown that in the rat MPA, CCK concentrations are higher in males than in females (Frankfurt et al., 1985; Micevych et al., 1988b). Immunohistochemistry has revealed with greater anatomical resolution the nature of the CCK sex difference. Micevych and coworkers (1987) have shown that the periventricular preoptic nucleus and the dorsomedial preoptic area of the female rat contain more CCK immunoreactive perikarya than that of the male. However, the CCK immunoreactive cells in these nuclei were larger in the male than the female. Conversely, male rats had more CCK immunoreactive cells in the medial nucleus of the amygdala (MeA), bed nucleus of the stria terminalis (BST), central division of the medial preoptic nucleus (MPNc) and posterior magnocellular paraventricular nucleus than found in the females. The difference in the number of CCK immunoreactive cells in the MPNc would explain the sex difference in the MPA radioimmunoassays, since a major component of the MPA is the MPNc. This data also indicates that two important afferent regions of the MPA, the MeA and the BST, also contain sexually dimorphic distributions of CCK. Cholecystokinin synapses have been localized in the sexually dimorphic MPNc (Larriva-Sahd et al., 1986), more evidence indicating that the CCK containing inputs to the MPNc are sexually dimorphic.

In addition to being sexually dimorphic, several studies have revealed that circulating gonadal steroids can influence the levels of CCK in specific circuits in the brain. Radioimmunoassays have shown that CCK concentrations vary with the stage of the estrus cycle
in female animals (Frankfurt et al., 1986; Micevych et al., 1988b). Simerly and Swanson (1987) demonstrated that castration reduced CCK immunoreactivity within the MPNc of the male rat. Cholecystokinin immunoreactivity was concomitantly reduced in the BST and the MeA (Simerly and Swanson, 1987). Estrous cycle variations in the number of CCK immunoreactive somata were also discovered in these same three nuclei in female rats (Oro et al., 1988). There is also evidence that estrogen can facilitate CCK release in the medial basal hypothalamus of the rat (Micevych et al., 1988b).

It is generally agreed that estrogen exerts its effects on neurons by binding to a estrogen receptor within its target cell (for review see: Blaustein and Olster, 1989). Although there is evidence for rapid membrane effects of estrogen that are not likely mediated by the classical estrogen receptor (Kelly et al., 1977a,b; Nabekura et al., 1986), the intracellular estrogen receptor is thought to modulate most behaviors and physiological responses to estrogen (Blaustein and Olster, 1989).

The estrogen receptor belongs to a family of steroid receptors that is characterized by a location in the cytoplasmic or nuclear compartment of a receptive cell. Because the ligand for the receptor, in this case estrogen, is lipid soluble, it can freely diffuse across cell membranes to gain access to its receptor. When the receptor binds estrogen, it is transformed. Transformation allows the receptor to bind to the cell's DNA and regulate expression of estrogen sensitive genes (Parker et al., 1990).

Estrogen receptors are located in cells in the mammalian brain. Pfaff and Keiner (1973) mapped the location of estrogen concentrating cells in the brain of the rat using tritiated estrogen in an autoradiographic procedure. Several biochemical studies have also confirmed the presence of estrogen receptors in the brain (Gerlach et al., 1983; Handa et al., 1991; Vito et al., 1983). All of these studies agree on the presence of high amounts of estrogen receptors in the limbic
system and hypothalamus. Anatomically, this indicates that estrogen receptors are in positions to modulate a variety of "lower" or "limbic" behaviors.

In addition to a role in gonadal and reproductive function, estrogen has a vital role in the control of reproductive behavior (Barfield and Chen, 1977; Davis et al., 1979, 1982; Howard et al., 1984; Meisel et al., 1987; Meisel and Pfaff, 1985; Rainbow et al., 1982; Rubin and Barfield, 1980; Yahr, 1981), cyclic gonadotropin secretion (Barraclough et al., 1979), and feeding behavior (Beatty et al., 1974; Jankowiak and Stern, 1974; Wade and Zucker, 1970). Estrogen modulation of these functions may involve regulation of the levels of specific neuroactive, signal molecules in the brain. As mentioned above, evidence exists indicating that CCK levels are regulated by circulating estrogen levels. Other important neuropeptides including substance P and methionine enkephalin have sexually dimorphic distributions and their levels fluctuate during the estrus cycle (Frankfurt et al., 1986; Micevych et al., 1988; Watson et al., 1986a), factors that point to regulation of their expression by estrogen.

Recently, direct evidence was presented for estrogen’s ability to regulate neurotransmitter levels. Romano and coworkers (1990) demonstrated that in the ventrolateral portion of the ventromedial hypothalamic nucleus, estrogen regulated proenkephalin gene expression differently in male and female rats.

The cholecystokinin and estrogen receptor systems in the adult brain have been well studied and although several questions, like that of colocalization still exist, much detail is known about both systems. Less well studied is the ontogeny of both cholecystokinin and estrogen receptors in the central nervous system.

In addition to its role in many centrally controlled functions, CCK may have an important contribution to the development of the brain (Cho et al., 1983). Cholecystokinin is present in the prenatal rat brain implicating its possible role in developmental events (Cho et al., 1983).
Furthermore, CCK levels increase in the rat brain at the time of synaptogenesis indicating that it may be an important neuromodulator in the early functions of the brain (Cho et al., 1983). There is no direct evidence to support a role for CCK in development. However, CCK's presence in the developing brain indicates that it could have a role in developmental events.

Likewise, estrogen receptors may also have an important role in brain development. Estrogen receptors first appear shortly after completion of neurogenesis in most areas of the limbic system (Friedman et al., 1983; Gerlach et al., 1983; Vito and Fox, 1979) indicating that estrogen may influence many major events in brain development including neuronal migration, neurite outgrowth, and synapse formation.

The aim of the studies in this dissertation was to use a novel mammalian animal model, the Brazilian short-tailed opossum (Monodelphis domestica), to study the ontogeny of cholecystokinin and estrogen receptor containing circuits in the brain. Monodelphis domestica is a small, pouchless marsupial that breeds well in the laboratory (Krause and Fadem, 1987; Vandeberg, 1983). Its young are born after a 14 day gestation period in an extremely immature and sexually undifferentiated state (Jacobson, 1984). Thus Monodelphis is an ideal animal model in which to carry out developmental studies of the mammalian brain (Dore et al., 1990; Rivkees et al., 1988; Schwanzel-Fukuda et al., 1988). As a prelude to the developmental studies, anatomical characterization of CCK and ER in the adult opossum was necessary because the neuroanatomy of this marsupial differs significantly from that of more popular rodent animal models.

Explanation of Dissertation Format

This dissertation is submitted to the graduate faculty as an alternate format dissertation. It includes four chapters. The first two have been accepted for publication in international journals in
the field of neuroscience, and the last two have been submitted for publication. All of the experiments described in this dissertation were performed by myself in Dr. Carol D. Jacobson's laboratory with her supervision and support.
SECTION I. LOCALIZATION OF CHOLECYSTOKININ-LIKE IMMUNOREACTIVITY IN THE MEDIAL PREOPTIC AREA AND ANTERIOR HYPOTHALAMUS OF THE BRAZILIAN GRAY SHORT-TAILED OPOSSUM: A SEX DIFFERENCE
INTRODUCTION

Cholecystokinin (CCK), a gastrointestinal hormone, is one of the most abundant peptides found in the central nervous system (CNS; Beinfeld et al., 1981a; Crawley, 1985; Innis et al., 1979; Larsson and Rehfeld, 1979). Its anatomical distribution and physiological properties suggest that it has an important role in many centrally controlled functions including feeding behavior (Baile and Della-Fera, 1985; Della-Fera and Baile, 1980; Gibbs et al., 1973; Smith et al., 1981a), nociception (Baber et al., 1989; Faris et al., 1982, 1984), reproductive behavior (Bloch et al., 1987, 1988), and control of gonadotropin as well as other pituitary hormone secretion (Hashimoto and Kimura, 1986; Kimura et al., 1983; Vijayan et al., 1979).

The mammalian CNS contains a wide distribution of CCK containing somata and fibers (Beinfeld et al., 1981a,b; Crawley, 1985; Fallon and Seroogy, 1985; Gall, 1984; Larsson and Rehfeld, 1979; Miceli et al., 1987; Vanderhaeghen et al., 1980, 1981). In the rat, CCK is found in the cerebral cortex, caudate nucleus, amygdaloid complex (Micevych et al., 1988a), hippocampus (Greenwood et al., 1981; Handelmann et al., 1981), olfactory bulb (Matsutani et al., 1988; Seroogy et al., 1985), septum, thalamus, hypothalamus (Micevych et al., 1987), midbrain and spinal cord. In situ hybridization also reveals an extensive distribution of CCK mRNA in the rodent CNS which is consistent with the distribution of CCK immunoreactive somata (Ingram et al., 1989; Savasta et al., 1988).

In the rat, several areas of the brain have sex differences in CCK concentration (Frankfurt et al., 1985; Micevych et al., 1988b; Siegel et al., 1985). Within the medial preoptic area (MPA), radioimmunoassay has shown that CCK concentrations are higher in males than in females (Frankfurt et al., 1985; Micevych et al., 1988b). In addition, CCK concentrations vary with the stage of the estrus cycle in female animals (Frankfurt et al., 1986; Micevych et al., 1988a, 1988b).
Estrogen may facilitate CCK release in the medial basal hypothalamus of the rat (Mivevych et al., 1988b).

Immunohistochemistry has also revealed sexually dimorphic CCK containing systems in the rat hypothalamus. Cholecystokinin synapses have been localized in the sexually dimorphic central part of the medial preoptic nucleus (MPNc; Larriva-Sahd et al., 1986). Furthermore, Simerly and Swanson (1987) demonstrated that castration reduced CCK immunoreactivity within the MPNc of the male rat. Cholecystokinin immunoreactivity was concomitantly reduced in the encapsulated part of the bed nucleus of the stria terminalis (BST) and the medial nucleus of the amygdala (MeA; Simerly and Swanson, 1987). Estrous cycle variations in the number of CCK immunoreactive somata were also discovered in these same three nuclei in female rats (Oro et al., 1988).

Micevych and coworkers (1987) have shown that the periventricular preoptic nucleus and the dorsal medial preoptic area of the female rat contain more CCK immunoreactive perikarya than those of the male. However, the CCK immunoreactive cells in these nuclei were larger in the male than the female. Conversely, male rats had more CCK immunoreactive cells in the MeA, BST, MPNc and posterior magnocellular paraventricular nucleus than the females.

We have begun a series of experiments on the Brazilian gray short-tailed opossum, *Monodelphis domestica*, a small, pouchless marsupial which breeds well under laboratory conditions (Krause and Fadem, 1987; Vandeberg, 1983). The young are born after a 14 day period of gestation in a sexually undifferentiated, immature state (Jacobson, 1984). Since marsupials minimize gestation and have an extended postnatal developmental period, they are becoming an increasingly attractive model for those studying mammalian development (Martin et al., 1989; Renfree, 1981; Rivkees et al., 1988; Schwanzel-Fukuda et al., 1988; Shaw et al., 1988; Walker and King, 1989; Xu and Martin, 1989). In the present study, we have used
immunohistochemistry to characterize the anatomical distribution of cholecystokinin-like immunoreactive (CCK LI) neuronal fibers and cell bodies in the MPA and anterior hypothalamus (AH) of the Brazilian short-tailed opossum. Preliminary results have been presented at the Society for Neuroscience annual meeting (Fox et al., 1988; Hoffman et al., 1987).
MATERIALS AND METHODS

Animals

Adult male and female Brazilian gray short-tailed opossums from a colony at Iowa State University were used in this study. The initial animals were obtained from the Southwest Foundation for Research and Education (San Antonio, Texas). The animals were housed individually in plastic rat cages and maintained at a constant temperature (26°C) on a 14:10 light-dark cycle. Water and food (Reproduction Fox Chow; Milk Specialties Products, Madison, WI) were available ad libitum.

Tissue Preparation

Sixteen male (80-120g) and sixteen female (50-100g) adult opossums were examined in these studies. Forty-eight to seventy-two hours prior to sacrifice, four males and four females were given intracerebroventricular (i.c.v.) injections of colchicine (30μg in 6μl 0.9% saline; Calbiochem; Micevych et al., 1987) while under metofane (Pitman-Moore) anesthesia. Colchicine was used to enhance cholecystokinin-like immunoreactivity within neuronal somata. Sixteen other animals (eight males and eight females) were perfused without receiving colchicine treatment. At the time of perfusion, the animals were deeply anesthetized with ether, injected with 1ml of heparinized saline in the left ventricle, and perfused transcardially with 0.9% saline (until the perfusate was free of blood). Zamboni’s fixative was then perfused at a constant rate for 15 minutes. The brains were removed from the calvaria, postfixed for 24 hours in Zamboni’s fixative, allowed to sink in a 30% sucrose solution, and stored in a cryoprotectant solution (Watson et al., 1986) at 4°C prior to sectioning.
After sinking the brain in buffered 30% sucrose to remove cryoprotectant, 30μm thick coronal sections approximating the transverse planes described in an atlas of the brain of the North American Opossum (Oswaldo-Cruz and Rocha-Miranda, 1968) were cut on a freezing microtome. Sections containing the MPA and/or hypothalamus were selected for immunohistochemistry.

**Immunohistochemistry**

The protocol utilized for immunohistochemistry was a modification of that reported by Watson, Hoffman, and Wiegand (1986). Briefly, the sections were rinsed with 50mM potassium phosphate buffered saline (KPBS), exposed to normal goat serum (Vector; 1:50), and then incubated in anti-cholecystokinin octapeptide IgG (Immunonuclear; 1:6000) for 20 hours at 4°C. Following adequate washing, tissue sections were incubated in goat anti-rabbit IgG (Vector; 1:600) for 1 hour at room temperature, and then reacted with avidin-biotin complex (Vector; 1:200) for an additional hour at room temperature. After rinsing, the sections were exposed to 0.04% diaminobenzidine tetrahydrochloride (DAB; Sigma), 2.5% nickel sulfate (Fisher Scientific) and 0.01% hydrogen peroxide dissolved in 0.1M sodium acetate. This reaction was terminated after 6-8 minutes with two successive rinses in 0.9% saline.

Preabsorption controls were run by incubating the CCK antisera with 50μM concentrations of CCK-8 (sulfonated), gastrin I, pentagastrin, substance P, oxytocin, vasopressin, neurotensin, bombesin (all from Sigma) or calcitonin gene related peptide (Cambridge Research Biochemicals) for 24 hours at 4°C prior to processing the tissue for immunohistochemistry. Sulfonated CCK-8, gastrin I, and pentagastrin blocked specific staining of the anti-CCK-8, while none of the other peptides tested affected the antibody’s efficacy. Negative controls were also run in which incubation of the tissue in primary antibody was replaced by incubation in normal goat
serum. This process produced no specific staining of the tissue. Tissue sections from males and females were processed in parallel to allow analysis for sexual dimorphisms.

Following immunohistochemical processing, the sections were mounted onto subbed slides, dried 24-48 hours at 35°C, dehydrated in alcohol, cleared in xylene, coverslipped, and observed under the light microscope.

**Quantification of CCK LI Somata**

Cholecystokinin immunoreactive somata within the MPA of colchicine treated animals were recorded utilizing a Zeiss Universal microscope with a camera lucida attachment. From these recordings, the cells were counted. The area analyzed was defined rostrally by the anterior extent of the third ventricle, posteriorly by the point where the third ventricle contacts the anterior commissure, medially by the third ventricle, and ventrally by the base of the brain. An overlay was used to delimit the dorsal and lateral boundaries. The overlay defined an area slightly larger than the MPA in all animals, but excluded other areas containing CCK LI cells. The same overlay was used for male and female animals, however, the rostrocaudal distance analyzed was somewhat shorter in the females than the males. Only cells located in the right half of the brain were counted.

**Quantification of CCK LI Fibers**

To quantify the amount of CCK LI elements in the MPA of non-colchicine treated opossums, a microdensitometrical process employing a Zeiss SEM-IPS image analysis system (Zeiss-Kontron) was used (Mize et al., 1988; Zoll et al., 1988). For each animal, three immunohistochemically stained sections at the rostral, middle, and caudal levels within the MPA
were analyzed. The sections were placed on a Zeiss Axiophot microscope, trans-illuminated using the microscope’s light source, and viewed with a Sony 3CCD color video camera at 40x.

In order to insure constant intensity of illumination for analysis of each tissue section, the following procedure was implemented. The amount of light passing through a blank slide was measured after each section was analyzed. If the light intensity deviated from a previously defined set point more than 0.5 gray levels (based on an average grey level from all pixels in the analyzed field; out of 256 total gray levels/pixel) the light source was readjusted and the section reanalyzed. Percent transmitted light was obtained by determining the average gray level of the field under study and converting this information to a percentage using a transformation table. Zero percent transmitted light was set by blocking the light path to the video camera. One hundred percent transmitted light was defined as the amount of light transmitted though a negative control section (a section which was processed for immunohistochemistry except that the primary antibody step was replaced by incubation in normal goat serum; Mize et al., 1988). This definition took into account the amount of light absorbed by unstained tissue or non-specific background staining.

Seven rectangular fields (156µm x 134µm each) were analyzed per section. Each field covered approximately half the width of the MPA on one side and slightly less than one-third the distance from the optic chiasm to the anterior commissure. In the MPA, three fields on each side of the third ventricle (or midline in the rostral sections where the third ventricle did not ascend to the anterior commissure dorsally) were examined. In addition, one field in the cerebral cortex dorsal to the lateral ventricle was studied. Field one was the ventral most field on the right side of the third ventricle, field two was half way up the right side and field three was located immediately ventral to the anterior commissure on the right side. Fields four through six descended ventrally along the left side of the ventricle or midline. Areas with percent transmitted
light higher than 100% (i.e. blood vessels or tears in the tissue) were automatically subtracted from the field before a value was obtained. The first measurement taken for a particular field was the overall percent transmitted light. Each field in the MPA was then divided into three equal, 52μm x 134μm sectors by the computer. The sector nearest the third ventricle was labeled the near sector, and the sector farthest from the ventricle was called the far sector. Percent transmitted light scores were then obtained for each of the sectors. These values were then converted to percent blocked light (100 - percent transmitted light) to allow for a more clear description of the data. All values were stored in a data base, and statistically analyzed using SAS. One and two-way analysis of variance were performed in order to determine if there were main effects of sex and/or distance from ventricle (near vs. far sectors) or a sex by distance interaction. A P ≤ 0.05 was accepted as statistically significant.

**Atlas Drawings**

Four adult male and four female Brazilian opossums were deeply anesthetized with ether and perfused transcardially with neutral formalin. Their brains were removed, postfixed for 24 hours in formalin, and embedded in paraffin. The brains were sectioned at a thickness of 6μm, mounted onto slides and stained with cresyl violet. These sections were utilized to make anatomical maps of specific levels within the Brazilian short-tailed opossum forebrain.

Photomicrographs of specific brain sections were obtained and digitized using Autocad, a computer aided design program. The digitized drawings of the left halves of the brain sections were matched to the respective photomicrographs of the right halves and the nuclear groups named using two North American opossum brain atlases (Loo, 1931; Oswaldo-Cruz and Rocha-Miranda, 1968; see Fig. 1). The nomenclature was revised, when possible, using the atlas of the
rat brain by Paxinos and Watson (1986) to allow direct comparison of the Brazilian opossum to the current literature for the laboratory rat.
RESULTS

Atlas Drawings

Since no atlas of the forebrain for *Monodelphis domestica* exists, drawings of four levels from the MPA and AH were constructed. The MPA and AH of the Brazilian opossum are somewhat different from these regions in the rat. The MPA and AH are poorly differentiated cytoarchitechtically. In fact, some cytoarchitechtically defined regions in the rat MPA are indistinguishable in the Brazilian opossum. Most importantly, no medial preoptic nucleus is seen in the MPA of *Monodelphis*. The MPA is composed of a large region of increased cell density adjacent to the third ventricle. No gross morphological sex differences were observed in the MPA or AH of these animals.

Cholecystokinin Immunohistochemistry

Our results indicate that cholecystokinin-like immunoreactivity was present in neuronal fibers and cell bodies within the MPA and AH. Although the CCK antiserum utilized did not differentiate CCK from gastrin I or pentagastrin, it has been shown utilizing gel filtration chromatography, sequence specific antibodies for the amino terminus of gastrin, and high pressure liquid chromatography that gastrins are not present in the rat hypothalamus (Micevych et al., 1987). Experiments are currently underway to test for the presence of gastrins in the opossum MPA and hypothalamus. For the remainder of this paper, we will refer to cholecystokinin-like immunoreactivity as CCK LI.
CCK LI Somata

A group of CCK LI cell bodies was located in the opossum MPA (Figs. 1 and 2C). On average, the MPA of the colchicine treated male opossums possessed a larger number of CCK LI cell bodies than the MPA of the colchicine treated females (males: 2385 ± 470 vs. females: 1600 ± 172; mean ± standard error of the mean). However, this difference in cell number was not statistically significant.

Cholecystokinin immunoreactive cell bodies were also found in the caudomedial portion of the suprachiasmatic nucleus (SCh; Figs. 1 and 3A) and in the periventricular hypothalamic nucleus (Pe; Figs. 1 and 3B). There was no apparent sex difference in the number of CCK LI perikarya in the SCh or Pe, thus CCK LI cells in these regions were not quantified.

CCK LI Fibers

A CCK LI fiber plexus was observed within the periventricular region of the MPA (Figs. 1, 4, and 2A,B). The majority of CCK LI fibers in the MPA were located within this plexus. By microdensitometry we determined that the percent blocked light for fields measured in the periventricular region of the MPA of male opossums was significantly higher than fields in the same location measured in the female (50.17% vs. 35.56% respectively; F=17.09; P=0.001). There was no sex difference in the cortical field values (18.47 in the male vs. 21.22 in the female; F=0.4534; P=0.5117).

When the fields were subdivided into sectors based on proximity to the third ventricle (Fig. 5), statistically significant differences were present between males and females for the near sector percent blocked light analysis (F=21.86; P=0.0004), and the far sector percent blocked light analysis (F=13.51; P=0.0025). The near sector measurement, for each sex, was
Figure 1: Photomicrograph/line drawings of coronal sections from the brain of the Brazilian short-tailed opossum at a level immediately rostral to the medial preoptic area (MPA; A), at a level midway through the rostrocaudal extent of the MPA (B), at the level of the caudal MPA/rostral anterior hypothalamus (AH; C) and the caudal AH (D). The left half of the figure is an Autocad generated tracing of the left half of the brain at this level. The right half of the figure is a photomicrograph of a cresyl violet stained section showing the right half of the brain at this level. See appendix A for a list of abbreviations.
Figure 2: Photomicrographs of the periventricular medial preoptic area of the Brazilian opossum. The periventricular preoptic area from a female opossum is represented in A and from a male in B. Cholecystokinin-like immunoreactive cell bodies in the medial preoptic area of a colchicine treated female Brazilian opossum are shown in C. 3V indicates the third ventricle. A sex difference was observed in this region in the density of CCK LI fibers in non-colchicine treated animals (A and B). No sex difference was observed in the number of CCK LI cell bodies in this region (C).
Figure 3: Four photomicrographs showing cholecystokinin-like immunoreactive elements in the anterior hypothalamus. Cholecystokinin immunoreactive elements in the suprachiasmatic nucleus of a female (A) and periventricular hypothalamic nucleus of a male (B) colchicine treated Brazilian opossum. Cholecystokinin immunoreactive elements in the suprachiasmatic nucleus of a female (C) and periventricular hypothalamic nucleus of a male (D) Brazilian opossum that was not cholchicine treated. 3V indicates the third ventricle. No sex difference was observed for CCK LI in the suprachiasmatic or periventricular hypothalamic nuclei.
Figure 4: Cholecystokinin-like immunostained sections from the medial preoptic area of male and female Brazilian opossums. Photomicrographs of coronal sections in the rostral, middle and caudal medial preoptic area of the female (A-C) and male (D-F). 3V indicates the third ventricle. The anterior commissure is the white area located at the top of each panel. A sex difference was observed in the density of CCK LI fibers in the medial preoptic area (see Fig. 5).
Figure 5: The percent blocked light values (100 - percent transmitted light) for the near and far sectors of eight female and eight male Brazilian opossums. Solid bars represent values obtained from the females. Hatched bars represent values obtained in the males. The standard error of the mean for each group is indicated on the respective bar.
consistently higher than the far sector measurement, resulting in a significant main effect of
distance from the ventricle (F=36.71; P=0.0001). Two-way analysis of variance revealed a sex
by distance interaction (F=20.29; P=0.0005) indicating that there was a significantly larger
difference between the near and far sector percent blocked light measurements in the males than
in the females.

Within the AH there was a dense accumulation of CCK LI fibers within the caudomedial
portion of the SCh (Figs. 1 and 3C). Cholecystokinin immunoreactive fibers were also found
within the Pe (Figs. 1 and 3D). Other areas of the MPA and AH contained little or no CCK LI
fibers. No sex differences were observed in the amount of CCK LI fibers in the SCh or Pe, thus
quantitation of these regions was not conducted.
DISCUSSION

Neuroanatomical and physiological evidence indicates that cholecystokinin is important in several sexually differentiated functions in the rat (Bloch et al., 1987, 1988; Frankfurt et al., 1985, 1986; Micevych et al., 1987, 1988b; Oro et al., 1988; Simerly and Swanson, 1987). The immunohistochemical data presented in this study reveal that CCK is found within the Brazilian short-tailed opossum MPA and AH. Moreover, there is a sexually dimorphic distribution of CCK LI elements within the Monodelphis MPA.

Although the mean number of CCK LI perikarya in the male MPA was greater than the female, this difference was not statistically significant due to a high degree of variability in the number of stained cells per animal. This could be attributed to a natural instability in the number of CCK cell bodies in the Brazilian opossum MPA, or may be due to inaccuracies in our detection methods. The animals that were utilized for the cell count study were treated with colchicine via an i.c.v. injection. Colchicine must be used in order to visualize maximal numbers of CCK LI somata with our immunohistochemical technique. Although the animals were given equivalent amounts of colchicine, variability in the way the animals responded to the drug may have contributed to the inconsistency of our cell counts.

The Brazilian opossum MPA contains a sex difference in percent blocked light measurements obtained from CCK immunostained sections. The 0% blocked light reading was defined as the amount of light passing through the MPA of a negative control section. Therefore, values for the percentage of blocked light reflect the density of immunoreactive elements within the tissue sections analyzed (Mize et al., 1988; Zoll et al., 1988). Since the animals utilized for this analysis were not colchicine treated, and few cell bodies were visible in the MPA, the percent blocked light measurements are proportional to the density of CCK LI fibers in the sections.
analyzed. However, this method provides a relative measurement and does not reflect absolute amounts of CCK in the tissue.

In the opossums we examined, there is a higher mean percentage of blocked light in the male MPA than in that of the female. In contrast, percent blocked light values in the cortical samples indicate no sex difference in this region. Therefore, the sex difference reported is not a general disparity in staining between males and females. Males have higher percentages of blocked light in both the near and far sectors, while lower values are found in the female. A distance effect is also noted with the near sector having significantly higher percent blocked light readings than the far sector. Furthermore, the significant interaction of sex by distance indicates that the distance effect is greater in males than in females.

Taken together, these data indicate that the males not only contain more CCK LI elements in the MPA, but that the density of CCK LI elements in the MPA is more heterogeneous in males than in females. Males had a higher concentration of CCK LI elements (mainly fibers) adjacent to the third ventricle; the density of these elements decreased as the distance away from the ventricle increased. Females, on the other hand, had a more homogeneous distribution of CCK LI elements in their MPA.

Alternatively, this data may indicate that there is a sexual dimorphism in the size of neuronal fibers in the MPA. It is possible that male opossums have larger CCK LI fibers than the females resulting in the higher percent blocked light values in the males. More detailed morphometric analyses of neuronal fibers in the MPA will be necessary to determine if the sex difference in percent blocked light is due to a higher density of CCK LI fibers or larger CCK LI fibers in the male than in the female.

Presently, the location of perikarya which contribute fibers to the sexually dimorphic plexus in the Brazilian opossum is unknown. It is possible that these periventricular fibers arise
from the CCK LI perikarya located in the MPA. However, the data in this report does not support a sex difference in the number of CCK LI somata in the MPA. The lack of a difference may be due to a failure of our detection methods for CCK LI somata, or it may imply that CCK LI cells in the male opossum MPA have more CCK LI processes than do the CCK LI cells in the female opossum MPA. It is also possible that perikarya outside the MPA and AH contribute fibers to the CCK LI plexus in the MPA. Micevych and coworkers (1987; 1988a) have shown sex differences in the number of CCK LI cell bodies in several nuclei of colchicine treated rats. The medial amygdaloid nucleus, bed nucleus of the stria terminalis, and posterior magnocellular portion of the paraventricular hypothalamic nucleus all contain more CCK LI cell bodies in male rats. Sexually dimorphic amounts of CCK have also been found in the midbrain of the rat with the male having more CCK than the female (Frankfurt et al., 1985). Cholecystokinin containing cells from these areas may send fibers to the preoptic area in the opossum, and may be responsible for the observed sex difference. Experiments are currently underway to locate the somata of origin of the sexually dimorphic CCK LI fiber plexus.

A caveat to this study remains. This sex difference is reported in gonadally intact opossums. Several groups have shown using RIA and immunohistochemistry that both the number of CCK LI cells and the density of CCK LI fibers in the MPA are regulated by circulating gonadal hormones (Frankfurt et al., 1986; Malsbury and Nance, 1988; Micevych et al., 1988b; Oro et al., 1988; Simerly and Swanson, 1987). Whether this is the case in the Brazilian opossum is still unknown. Studies are currently in progress to investigate the role of gonadal hormones in expression of this sexually dimorphic CCK LI system in the Brazilian opossum.

In summary, the sex difference in CCK LI fibers in the MPA of the Brazilian opossum provides a unique opportunity to study the ontogeny of sexual dimorphisms in the preoptic area,
since neurogenesis and morphogenesis of the MPA occurs postnatally in this species (Jacobson, 1984; Larsen and Jacobson, 1986). Using this animal model, organizational and activational aspects of the sexually dimorphic CCK LI system are currently being explored.
SECTION II. LOCALIZATION OF CELLS CONTAINING ESTROGEN RECEPTOR-LIKE IMMUNOREACTIVITY IN THE BRAZILIAN OPOSSUM BRAIN
INTRODUCTION

Estrogen is one of the important signal molecules in the body. In addition to controlling reproductive function and behavior in adult animals, it also has a vital role in development. Many reproductive behaviors (Yahr, 1981), cyclic release of gonadotropins (Barraclough et al., 1979), and feeding behavior (Beatty, et al., 1974; Jankowiak and Stern, 1974; Wade and Zucker, 1970) are modulated by estrogen. Estrogen modulation may involve the regulation of the levels of specific neuroactive molecules in the brain. Cholecystokinin (Fox et al., 1990; Micevych et al., 1988; Siegel et al., 1985), substance P (Micevych et al., 1988), and methionine enkephalin (Watson et al., 1986a) all have sexually dimorphic distributions in the brain. In addition, the levels of these compounds fluctuate during the estrus cycle of female rats.

Estrogen’s modulatory effects on feeding and reproductive behavior, as well as on gonadotropin secretion are thought to occur through direct action of estrogen on cells in the brain. Biochemical determination of estrogen binding and steroid autoradiography have helped investigators demonstrate that the limbic system and several nuclei within the hypothalamus contain high numbers of estrogen concentrating cells (Handa et al., 1991; Pfaff and Keiner, 1973; Vito et al., 1983). With the advent of more sophisticated biochemical and molecular techniques, the estrogen receptor has been isolated, characterized, and more sensitive tools for its detection have been developed.

For example, using antibodies with high affinity for the estrogen receptor, investigators determined cellular and anatomical distribution of estrogen receptors in the brain (Balthazart et al., 1989; Blaustein and Turcotte, 1989; Cintra et al., 1986; DonCarlos et al., 1990). The regions of the brain with a high density of estrogen receptor immunoreactivity correspond to those areas having a large amount of estrogen binding activity, as demonstrated by biochemical
determination of steroid binding (Handa et al., 1991; Vito et al., 1983) or with steroid autoradiography (Pfaff and Keiner, 1973).

We have begun a series of studies investigating the anatomical distribution of estrogen receptor immunoreactivity in the brain of the Brazilian short-tailed opossum. The Brazilian gray short-tailed opossum, *Monodelphis domestica*, is a small, pouchless marsupial which breeds well under laboratory conditions (Krause and Fadem, 1987; Vandeberg, 1983). Its young are born after a 14 day gestation period in an immature, sexually undifferentiated state (Jacobson, 1984). Thus, *Monodelphis*, as well as some other marsupials are ideal animal models for studying the development of the mammalian brain (Dore et al., 1990; Martin et al., 1989; Renfree, 1981; Rivkees, et al., 1988; Schwanzel-Fukuda et al., 1988; Shaw, et al., 1988; Walker and King, 1989; Xu and Martin, 1989). As a prelude to the developmental studies, in the present study, we describe the distribution of estrogen receptor-like immunoreactivity (ER LI) in the brain of the gonadectomized adult male and female Brazilian short-tailed opossum. Gonadectomies were performed to minimize endogenous estrogen in the animals, thus to insure maximal staining of estrogen receptors.
MATERIALS AND METHODS

Animals

Adult male and female Brazilian short-tailed opossums from a colony at Iowa State University were used for this study. The initial animals used to start the breeding colony were obtained from the Southwest Foundation for Research and Education in San Antonio, Texas. The animals were individually housed in plastic rat cages, maintained at a constant temperature (26°C) on a 14:10 light-dark cycle and provided water and food ad libitum (Reproduction Fox Chow; Milk Specialties Products, Madison, WI). Four male and four female adult (9-15 months of age) opossums were used. Animals were gonadectomized while under metofane anaesthesia (Pitman-Moore). Nine to thirteen weeks later, animals were killed by over-etherization.

Tissue Preparation

Following injection of heparin into the left ventricle, animals were perfused transcardially with 0.9% saline followed by Zamboni’s fixative for 10 minutes. The brains were removed from the calvaria and cut at the border between the superior and inferior colliculus into two blocks (forebrain and hindbrain). Each block was postfixed for 24 hours in Zamboni’s fixative and subsequently infiltrated with a 30% buffered sucrose solution. After infiltration, 30 μm thick coronal sections, approximating the transverse planes as described previously (Fox et al., 1990), were cut on a freezing microtome. The sections were placed in a cryoprotectant solution (Watson et al., 1986b) at -15°C for storage until immunohistochemistry was conducted. Approximately every sixth section of each brain was processed for immunohistochemistry with the estrogen receptor antibody.
**Immunohistochemistry**

The protocol utilized for immunohistochemistry was a modification of that reported previously (Fox et al., 1990). The sections were rinsed with 50mM potassium phosphate buffered saline, incubated with a 0.3% H₂O₂ solution to remove endogenous peroxidase activity, exposed to normal rabbit serum as a blocking agent (Vector; 1:67) and then incubated in estrogen receptor (ER) primary antibody (a gift of Abbott Labs; Abbott H222; 1\(\mu\)g/ml) for 20 hours at 4°C. After adequate washing, the tissue sections were incubated in rabbit anti-rat IgG (Vector; 1:200) for 1 hour at room temperature, rinsed, and reacted with avidin-biotin complex (Vector Elite Kit; 1:50) at room temperature for an additional hour. After washing, the tissue sections were stained by exposing them to a substrate composed of 0.04% 3,3’ diaminobenzidine tetrahydrochloride (DAB; Sigma), 2.5% nickel sulfate (Fisher Scientific) and 0.01% hydrogen peroxide, dissolved in 0.1 M sodium acetate. After staining for 6 minutes the reaction was terminated by placing the sections into two successive rinses of 0.9% saline. At this time the tissue sections were mounted onto subbed slides (1% gelatin coated), air dried, dehydrated in alcohol, cleared in xylene, coverslipped with permount, and analyzed with a light microscope.

**Immunohistochemical Control Procedures**

Preabsorption controls were run by incubating the H222 antibody with an estrogen receptor enriched cell-free lysate (approximately 50 nM; preparation described below) or with the vehicle of the estrogen receptor lysate (0.05 M KPBS with 10% glycerol) for 4 days at 4°C prior to processing the tissue sections for immunohistochemistry. Negative and positive controls were run simultaneously with the preabsorption controls. The negative control consisted of replacement of the primary antibody incubation with an incubation in normal rabbit (blocking) serum. The positive control tissue was run according to normal protocol.
The estrogen receptor enriched cell-free lysate was prepared from rat uterus. Twelve female Sprague-Dawley rats (250-300 grams; Sasco Inc., Omaha, NE) were ovariectomized 1 week prior to tissue collection. Immediately following death, uteri were removed and homogenized in ice-cold TEGMD buffer (10 mM TRIS; 25 mM EDTA, 25 mM Molydate, 10% glycerol, 1 mM Dithiotreitol; pH = 7.4) with a glass-teflon homogenizer. The homogenate was centrifuged at 106,000 x g for 30 min. at 4°C in a Dupont-Sorvall OTD 55B Ultracentrifuge using a TFT-80.4 rotor. The resulting crude cytosol receptor preparation was further purified by precipitation at 40% saturation with ammonium sulfate. Saturated ammonium sulfate was added dropwise to the stirred cytosol on ice. After 30 min., the precipitate was collected by centrifugation at 1,000 x g for 30 min. The resulting pellet was dissolved in 0.05 M phosphate buffered saline containing 10% glycerol (PBS-g; pH = 7.4) and dialyzed extensively in 3 liters of 0.05 M PBS-g to remove residual ammonium sulfate. This preparation yielded an enriched estrogen receptor lysate (535 fmol/mg protein) as determined by an in vitro binding assay. Binding analysis used \(^{3}H\)-estradiol (5 nM) as the labelled ligand. Radioinert moxestrol (1 \(\mu\)M) was used to determine nonspecific binding. Following incubation for 3 hours at 25°C, bound and free ligand were separated by Sephadex LH-20 column chromatography. Specific binding was determined by subtracting binding in the presence of moxestrol from that in its absence. Soluble protein was determined by the method of Lowry and coworkers (Lowry et al., 1951).

Analysis of Tissue

Sections at 150 \(\mu\)m intervals from immediately caudal to the olfactory bulbs to the caudal extent of the brain stem were analyzed from each animal. Sections were observed with a Zeiss Axiophot microscope and regions containing ER LI were identified on maps of coronal sections of the opossum brain made from nissl sections (see Fig. 6). The density of immunoreactive cells in
brain regions were subjectively scored by the observer as high, low, or none. Areas with no ER LI cell were scored as none. Areas containing scattered ER LI cells were scored as low. Regions with many ER LI cells were scored as high. No attempts were made to quantitate the specific number of ER LI cells in each region or in the brain as a whole.
RESULTS

Estrogen receptor immunoreactivity was found in many distinct cell groups within the Brazilian opossum brain. This immunoreactivity was completely abolished by incubating the H222 antibody in an estrogen receptor rich, cell-free lysate for four days prior to using it in the immunohistochemical procedure. Specific staining was not interfered with when the H222 antibody was incubated in the vehicle of the lysate for four days prior to applying it to the tissue sections.

A high number of ER LI cell nuclei were observed in the medial preoptic area (MPA) of Monodelphis (Figs. 6B, C, and 7). The ventral part of the lateral septal nucleus (LSV) also contained large numbers of ER LI cell nuclei (Figs. 6B and 8), and high numbers of ER LI cells were observed in the medial division of the bed nucleus of the stria terminalis (BSTM; Fig. 6C). The caudal hypothalamus contained three regions with high numbers of ER LI cell nuclei, namely the lateral portion of the ventromedial hypothalamic nucleus (VMH; Figs. 6E and 9A), the arcuate nucleus (Arc; Figs. 6E, and 9A), and the ventral premammillary nucleus (PMV). The posterior amygdaloid area (PAA) also contained a high density of ER LI cells (Fig. 6E). In addition to these regions of the forebrain, the central grey (CG) contained a high number of ER LI cell nuclei, especially in the region of the mesencephalic nucleus of the trigeminal nerve (Me5; Figs. 6F and 10).

Lower densities of ER LI cells were observed in the intermediate subdivision of the lateral septal nucleus (LSI; Figs. 6A and 8) and in the anterior, medial, and posterior cortical amygdaloid nuclei (ACo, MeA, and PCo respectively; Fig. 6C, D, E). A low density of ER LI cells was also observed in the central amygdaloid nucleus and the magnocellular division of the basal amygdaloid nucleus (CA and BAM; Fig. 6D). The dorsomedial hypothalamic nucleus
(DM) contained a low density of ER LI cells (Fig. 6E) as well as the dorsal prefrontal nucleus (PMD). A low number of ER LI cells were also located in an area ventral to the internal capsule (Fig. 6C). This area may be a caudal continuation of the ventral division of the bed nucleus of the stria terminalis (BSTV).

With this antibody (H222), the majority of the immunoreactivity was localized in the nuclear compartment of the cells. In the VMH and the lateral septal area however, staining was observed in the cytoplasm and some cytoplasmic processes of cells as well as in the nucleus (Fig. 9B).

There were no apparent sex differences in the density or distribution of estrogen receptor containing cells in the brains of the animals analyzed. In addition, there were no apparent sex differences in the patterns of nuclear and cytoplasmic staining in the brains of the animals in this study.
Figure 6: Drawings of selected coronal sections from rostral to caudal (A-F) of the Brazilian opossum brain showing regions with no, low (low density stippling), or high (high density stippling) numbers of cells containing estrogen receptor-like immunoreactivity. See appendix A for a list of abbreviations. No sex difference was observed in ER LI in any regions of the brain analyzed in this study.
Figure 7: A photomicrograph showing estrogen receptor-like immunoreactive cells in the medial preoptic area of a gonadectomized female *Monodelphis domestica*. The third ventricle is the space located on the right side of the photomicrograph and the optic chiasm is on the lower right. No sex difference was observed in ER LI cells in the opossum medial preoptic area.
Figure 8: A photomicrograph showing estrogen receptor-like immunoreactive cells in the septum of a gonadectomized female *Monodelphis domestica*. The lateral ventricle is the space on the left side of the photomicrograph and the anterior commissure runs across the bottom of the photograph. No sex difference was observed in ER LI cells in the opossum septum.
Figure 9: Photomicrographs of estrogen receptor-like immunoreactive cells in (A) the ventromedial hypothalamic nucleus (VMH) and the arcuate nucleus of a gonadectomized female Monodelphis domestica. B is a higher magnification picture taken from the region indicated by the box in A. Note the staining in the cytoplasmic processes of many labeled cells. The third ventricle is the space on the right side of the photomicrograph. No sex difference was observed in the pattern of ER LI cells or the pattern of ER LI cellular staining in the opossum VMH or arcuate nucleus.
**Figure 10:** A photomicrograph showing estrogen receptor-like immunoreactive cells in the midbrain central grey of a gonadectomized male *Monodelphis domestica*. The aqueduct is the space at the lower left of the photomicrograph. No sex difference was observed in ER LI cells in the opossum central grey.
DISCUSSION

The anatomical distribution of ER LI in the *Monodelphis* brain is quite similar to that for estrogen concentrating cells in the rat brain following autoradiography as described first by Pfaff and Keiner (1973). Studies utilizing estrogen receptor immunohistochemistry with the H222 antibody in the rat (Cintra et al., 1986) and in the Guinea pig (DonCarlos et al., 1990) have also yielded similar anatomical distributions.

Differences between the distribution of estrogen concentrating cells in the rat and ER LI cells in *Monodelphis* are few. One difference exists in the olfactory tubercle. The rat shows a high density of estrogen concentrating cells in the Islands of Calleja (Pfaff and Keiner, 1973). However, the Brazilian opossum contains no ER LI cells within this region. Other differences appear to be small deviations in the density of ER LI compared to that for estrogen concentrating cells in specific regions of the brain.

Previously, we have described the distribution of estrogen receptors in the forebrain of the opossum using an in vitro estradiol binding assay (Handa et al., 1991). In that study we demonstrated that the estrogen receptor isolated from the opossum forebrain has very similar characteristics as that isolated from the laboratory rat. Furthermore, the estrogen receptors were mainly localized within the medial preoptic area, ventromedial hypothalamus, medial amygdala and arcuate nucleus. Results presented in this study indicate that the monoclonal antibody generated against the human estrogen receptor (H222) is capable of recognizing the opossum estrogen receptor as well. These results also confirm the regional localization of the receptors to that which we and others have determined biochemically (Etgen and Fadem, 1987; Handa et al., 1991). As presented in the biochemical study, we did not find any apparent sex differences in the density of cells containing ER LI.
The commonality of estrogen receptor distributions across species as obtained from biochemical analysis, steroid autoradiography and now immunohistochemistry suggests that several regions of the brain serve as important targets for the action of estrogenic hormones. The MPA of rodents is involved in the control of several sexually differentiated neuroendocrine functions, including cyclic release of gonadotropins (Barraclough et al., 1979), maternal behavior (Jacobson et al., 1980), masculine sexual behavior (Arendash and Gorski, 1983), and scent marking behavior (Yahr, 1981). It is possible that estrogens may have a role in any or all of these functions in the Brazilian opossum as well. The VMH also has a role in estrogen modulated sexual behavior in rodents (Barfield and Chen, 1977; Rubin and Barfield, 1980, 1983). In addition, the VMH has an important role in feeding behavior in rats, a behavior which may also be regulated by estrogens (Beatty et al., 1974; Jankowiak and Stern, 1974; Wade and Zucker, 1970). It has also been demonstrated by Pfaff and coworkers (Pfaff et al., 1973a,b; Pfaff and Keiner, 1973) that pathways carrying sensory information for lordosis behavior in rats include the lateral portion of the central grey.

It is interesting to note that the distribution of ER LI cells in the Brazilian opossum brain parallels the distribution of cholecystokinin (CCK), a neuropeptide whose anatomical distribution has been described in *Monodelphis* (Fox et al., 1990). High concentrations of CCK like immunoreactive (CCK-LI) fibers and somata are found in the opossum MPA and anterior hypothalamus. In addition, there is a sex difference in the density of CCK-LI fibers in the MPA of *Monodelphis* (Fox et al., 1990). Whether this sexual dimorphism is controlled by estrogen in the adult or is organized by gonadal hormones during development is currently under investigation.
Several other regions of the Brazilian opossum forebrain that contain ER LI cells also contain CCK-LI structures. These include the bed nuclear system, medial amygdaloid nuclei, medial preoptic area and hypothalamus (Fox and Jacobson, unpublished observations).

The results from this study also indicate that the cellular localization of ER LI differed in various regions of the brain. In most areas of the brain which contained ER LI, the immunoreactivity was located in the cell nucleus. The ventromedial hypothalamic nucleus, however, contained cells with staining in their somatic cytoplasm and in cytoplasmic processes (Fig. 8B). This phenomenon has also been observed in the guinea pig (Blaustein and Turcotte, 1989; Silverman et al., 1990). Whether this staining represents cytoplasmic estrogen receptors, another protein that binds H222, or some other nonspecific staining is not known.

There were no apparent sex differences in the density of ER LI cells in the Brazilian opossum brain. Furthermore, no sex differences were observed in the nuclear or cytoplasmic staining patterns in the opossum brain.

In summary, the anatomical distribution of ER LI in the Brazilian opossum is very similar to the distribution of estrogen receptors in other animals as characterized by a variety of techniques. The ER LI cells are located in regions of the brain that have critical roles in reproductive behavior, cyclic gonadotropin secretion, as well as feeding behavior, all of which are regulated by circulating estrogen levels. These results are supportive of the use of Monodelphis for investigating the role of estrogen in the adult animal. Moreover, due to its protracted extrauterine development, the Brazilian opossum may provide a unique opportunity to study the organizational effects of estrogen on the developing central nervous system.
SECTION III. ONTOGENY OF CELLS CONTAINING ESTROGEN RECEPTOR-LIKE IMMUNOREACTIVITY IN THE BRAZILIAN OPOSSUM BRAIN
INTRODUCTION

Early in the development of the central nervous system, estrogen and other steroid hormones induce morphological changes in the brain. Specifically, during critical periods of early brain development, estrogen and aromatizable androgens play an important role in the development of the sexually dimorphic nucleus of the preoptic area (SDN-POA; Döhler et al., 1984; Jacobson et al., 1981) as well as, the synaptic connections in the arcuate nucleus (Matsumoto and Arai, 1980). Other sexually dimorphic regions of the brain, like the ventromedial hypothalamic nucleus (Matsumoto and Arai, 1983), amygdala (Mizukami et al., 1983), bed nucleus of the stria terminalis (Hines et al., 1985), hippocampus (Juraska et al., 1989; Juraska, 1990), and cerebral cortex (Juraska, 1984) may also be under estrogen’s influence during early development.

Estrogen can modulate the expression of signal molecules in the developing brain. Watson and coworkers (1988) have demonstrated that estrogen treatment of female rats as young as 15PN caused precocious expression of methionine enkephalin immunoreactive (m-ENK IR) fibers in a sexually dimorphic fiber plexus in the preoptic area.

It is generally thought that estrogen exerts its effects on the brain by acting on a specific intracellular receptor protein. Once estrogen is bound to its receptor, the receptor is transformed, allowing it to bind DNA and alter the expression of a family of estrogen sensitive genes (Parker, 1990; Walters et al., 1985). The estrogen receptor has been isolated and characterized from many species. These data indicate that the estrogen receptor is conserved in structure and function (Lubahn et al., 1985).

Estrogen receptors are present in the marsupial brain (Etgen and Fadem 1987; Fox et al., 1991; Handa et al., 1990). Biochemical evidence indicates that the estrogen receptor found in the
opossum brain has characteristics identical to that found in the rodent brain (Handa et al., 1990). The anatomical distribution of estrogen receptors also has been characterized in the Brazilian opossum using immunohistochemistry (Fox et al., 1991). Based on biochemical and anatomical data, the distribution of estrogen receptors in the opossum brain is similar to distributions described for the brain of other mammals.

In this study, we have used the Brazilian short-tailed opossum as a model to study the ontogeny of estrogen receptor expression. The Brazilian gray short-tailed opossum, *Monodelphis domestica*, is a small, pouchless marsupial which breeds well under laboratory conditions (Krause and Fadem, 1987; Vandeberg, 1983). Its young are born after a 14 day gestation period in an immature, sexually undifferentiated state (Jacobson, 1984). Thus, *Monodelphis*, as well as some other marsupials are ideal animal models for studying the development of the mammalian brain (Dore et al., 1990; Martin et al., 1989; Renfree, 1981; Rivkees et al., 1988; Schwanzel-Fukuda et al., 1988; Shaw et al., 1988; Walker and King, 1989; Xu and Martin, 1989). The Abbott H222 monoclonal estrogen receptor antibody was utilized to immunohistochemically characterize the distribution of estrogen receptor immunoreactivity (ER LI) in the developing Brazilian opossum brain.
MATERIALS AND METHODS

Animals

Developing male and female Brazilian short-tailed opossums from a colony at Iowa State University were used for this study. The initial animals used to start the breeding colony were obtained from the Southwest Foundation for Research and Education in San Antonio, Texas. The animals were housed in plastic cages, maintained at a constant temperature (26°C) on a 14:10 light-dark cycle and provided water and food ad libitum (Reproduction Fox Chow; Milk Specialties Products, Madison, WI). For breeding, male and female animals were paired for 14 days. Animals were then separated and the dam checked daily at 1500 for the presence of pups (day of birth = 1PN). The gestation period for the Brazilian opossum is 14-15 days (Fadem et al., 1982), and birth generally occurs 5 days after the termination of pairing.

Tissue Collection

Forty eight animals from 1-60 days postnatal age (PN) were used in this study. Four animals were collected on days 1 and 5 PN each. At these ages, the gender of the neonates cannot be determined grossly or histologically, and thus were considered sexually undifferentiated. The gender of the animals can be determined by 10PN by visual inspection of the external genitalia. Four female and four male animals were collected for each of the following time points: 10, 15, 25, 35, and 60 PN. No more than two animals were taken from a litter at each time point.

The brain was collected from 1 to 15 PN opossums by cooling the animals in a -15°C freezer until anesthetized. The animals were then decapitated and the heads placed in Zamboni’s fixative for 48 hours. After fixation, the heads were infiltrated with 30% sucrose overnight, and
then cut into 20μm thick coronal sections on a cryostat (Reichert Instruments). The sections were
thaw mounted onto poly-L-lysine coated slides and stored at 4°C until processed for
immunohistochemistry.

Twenty five and thirty five day old opossums also were anesthetized by cooling. These
animals then were perfused transcardially with 15 ml of Zamboni's fixative. The brains were
isolated and postfixfixed in Zamboni's fixative for 48 hours. After postfixivation, the brains were
infiltrated with 30% buffered sucrose overnight, and processed as described above.

Sixty day old animals were killed by over-etherization. After injection of heparin into the
left ventricle, the animals were perfused transcardially with 0.9% saline followed by Zamboni's
fixative for 10 minutes. Brains were removed from the calvaria, postfixed for 48 hours in
Zamboni's fixative, and processed as described above. Twenty micrometer thick coronal
sections, approximating the transverse planes as described previously (Fox et al., 1990), were
obtained. Two of the brains of the 60 PN animals (one male and one female) were cut at 30μm.
The sections were collected in a cryoprotectant solution (Watson et al., 1986b), and stored at
-15°C until immunohistochemistry was conducted.

Immunohistochemistry

The protocol utilized for immunohistochemistry was a modification of that reported
previously (Fox et al., 1990). The slide mounted sections were rinsed with 50 mM potassium
phosphate buffered saline, incubated with a 0.3% H₂O₂ solution to remove endogenous peroxidase
activity, exposed to normal rabbit serum as a blocking agent (Vector; 1:67) and then incubated in
estrogen receptor (ER) primary antibody (a gift of Abbott Labs, Abbott Park, IL; Abbott H222;
1:1000) for 20 hours at room temperature. After adequate washing, the tissue sections were
incubated in rabbit anti-rat IgG (Vector; 1:200) for 2 hours at room temperature, rinsed, and
reacted with avidin-biotin complex (Vector Elite Kit; 1:50) at room temperature for an additional
hour. After washing, the tissue sections were stained by exposing them to a substrate composed
of 0.04% 3,3’ diaminobenzidine tetrahydrochloride (DAB; Sigma), 2.5% nickel sulfate (Fisher
Scientific) and 0.01% hydrogen peroxide, dissolved in 0.1 M sodium acetate. After staining for 6
minutes, the reaction was terminated by placing the slides into two successive rinses of 0.9%
saline. The sections were then dehydrated in graded alcohols, cleared in xylene and coverslipped
with permount mounting media and analyzed with a light microscope.

Unmounted tissue sections were immunohistochemically processed using a floating tissue
technique described previously for adult tissue (Fox, et al., 1991). This allowed comparison of
mounted tissue to floating tissue immunohistochemistry.

**Immunohistochemical Control Procedures**

Both negative and preabsorption control procedures used with the H222 antibody have
been described previously (Fox et al., 1991).

**Tissue Analysis**

Drawings of seven cranio-caudal levels of the opossum brain were made for each age
from either cresyl violet or neutral red stained sections from a collection in our laboratory.
Twenty micrometer thick sections from a 1 in 3 series were observed with a Zeiss microscope.
Regions of the brain containing ER LI nuclei were indicated on the prepared illustrations. The
number of ER LI nuclei in each region was estimated from counts made on that region by one of
the investigators.
RESULTS

No ER LI structures were seen in 1 or 5PN brains. The H222 antibody readily detected ER LI structures in opossum brains older than 10PN. The earliest expression of ER LI cell nuclei occurred at 10PN in three out of eight animals in the dorsomedial (Fig. 11B) and ventromedial hypothalamic regions. These regions contained lightly stained nuclei at this age.

Most regions that were characterized as having ER LI in the adult contained ER LI nuclei at 15PN. This included the lateral septum (LSI; Fig. 12A), medial preoptic area (MPA; Figs. 12B and 13C), medial subdivision of the bed nucleus of the stria terminalis (BSTM; Fig. 12C), periventricular preoptic area and hypothalamus (Pe; Figs. 12C and D), posterior cortical amygdaloid nucleus (PCo; Figs. 12D,E,F, and 14A), dorsomedial hypothalamic nucleus (DM; Figs. 12E and 11A), ventromedial hypothalamic nucleus (VMH; Figs. 12E and 15A), arcuate nucleus (Arc; Figs. 12E,F, and 15A), ventral premammillary nucleus (PMV; Fig. 12F), and midbrain central grey (Figs. 12G and 16C). All regions expressing ER LI at 15PN contained lower numbers of ER LI nuclei than were observed at later points in development. The dorsomedial and ventromedial hypothalamic nuclei both had increased numbers of ER LI nuclei from 10 to 15PN.

The MPA had an increase in the number of ER LI cells from 15PN to 60PN with an adult-like pattern of staining observed at the 60PN time point (Fig. 13).

Multiple patterns of onset of ER LI were observed in the amygdala. The PCo contained ER LI cells at 15PN and had an increase in the number of ER LI cells to 60PN (Fig. 14). However, the anterocortical amygdaloid nucleus (ACo), the cortical amygdaloid nucleus (CA), and the posterior amygdaloid area (PAA; Fig. 12D and E) do not contain ER LI cells until 25PN.
Figure 11: Photomicrographs of estrogen receptor-like immunoreactive (ER LI) cells in the dorsomedial hypothalamus of (A) a postnatal day 15 male opossum and (B) a postnatal day 10 female opossum. No sex difference was observed in ER LI cells in the developing *Monodelphis* dorsomedial hypothalamus. The bars represent 100μm.
Figure 12: Drawings of selected coronal sections from rostral to caudal of the Brazilian opossum brain. The drawing on the left is a postnatal day 60 section, in the middle is a postnatal day 35 section, and on the right is a postnatal day 15 section. Regions with no, scattered (1-50 estrogen receptor-like immunoreactive (ER LI) cells per section; lowest stippling), low (50-100 ER LI cells per section; low stippling), moderate (100-200 ER LI cells per section; medium stippling), and high (>200 ER LI cells per section; high stippling) are represented on the right side of each section. See appendix A for a list of abbreviations.
Figure 13: Photomicrographs of estrogen receptor-like immunoreactive (ER LI) cells in the medial preoptic area of (A) a postnatal day 60 female opossum, (B) a postnatal day 35 female opossum, and (C) a postnatal day 15 male opossum. No sex difference was observed in ER LI cells in the developing *Monodelphis* medial preoptic area. The bars represent 100μm.
Figure 14: Photomicrographs of estrogen receptor immunoreactive (ER LI) cells in the posterior cortical amygdaloid nucleus of (A) a postnatal day 15 opossum, (B) a postnatal day 35 opossum, and (C) a postnatal day 60 opossum. No sex difference was observed in ER LI cells in the developing posterior cortical amygdaloid nucleus. The bars represent 100μm.
Figure 15: Photomicrographs of estrogen receptor immunoreactive (ER LI) cells in the arcuate nucleus and ventromedial hypothalamic nucleus of (A) a postnatal day 15 opossum, (B) a postnatal day 35 opossum, and (C) a postnatal day 60 opossum. No sex difference was observed in ER LI cells in the developing arcuate or ventromedial hypothalamic nucleus. The bars represent 100μm.
Figure 16: Photomicrographs of estrogen receptor-like immunoreactive (ER LI) cells in the midbrain central grey of (A) a postnatal day 60 male opossum, (B) a postnatal day 35 female opossum, and (C) a postnatal day 15 male opossum. No sex difference was observed in ER LI cells in the developing Monodelphis central grey. The bars represent 100μm.
The ACo and CA only contain low numbers of ER LI cells at 60PN. Alternatively, the PAA has a higher number of ER LI cells at 60PN than when they were first observed at 25PN.

Both the Arc and VMH contained ER LI cell nuclei at 15PN. The number of ER LI cells increased in the Arc and VMH from 15PN to 60PN (Figs. 12E, F, and 15).

Cells containing ER LI were also found in the CG on 15PN. The number of ER LI cells increased from 15PN to 60PN in the CG (Figs. 12G and 16).

Labeling of ER LI in the area of the subiculum is shown in figure 12F. It is likely that the ER LI cell nuclei in this region are part of a caudal continuation of the PCo.

Numbers of ER LI cells were characterized as follows: Areas represented by scattered ER LI nuclei contained up to 50 labeled cells in an average section. Areas characterized as having low numbers of ER LI cells contained 50-100 labeled cells on a typical section. Regions containing a moderate number of ER LI cells had 100-200 labeled cells on an average section through that area. High numbers of ER LI cells were found in areas containing 200 or more labeled nuclei in a representative section. The immunohistochemical staining of estrogen receptors was limited to the nuclear compartment of the neurons in all ages observed in this study.

No sex differences were noted in the number of nuclei containing ER LI at any of the ages analyzed in this study.
DISCUSSION

Immunohistochemical localization of estrogen receptors using the H222 antibody reveals ER LI as early as 10PN in *Monodelphis*. By 15PN most areas that contain ER LI cells in the adult opossum (Fox et al., 1991) contain ER LI cells. These nuclear groups include the lateral septal nucleus, medial preoptic area, periventricular preoptic area and hypothalamus, lateral ventromedial hypothalamus, ventral premammillary nucleus, medial subdivision of the bed nucleus of the stria terminalis, dorsomedial hypothalamic nucleus, posterior cortical amygdaloid nucleus, and midbrain central grey. From 15PN to 60PN, the number of ER LI cells increases in all regions that contain ER LI. Although absent at 15PN, at 25PN the anterior cortical amygdaloid area, posterior cortical amygdaloid area, and cortical amygdaloid nuclei contain ER LI cells.

Most neurons in the preoptic area and hypothalamus of the Brazilian opossum undergo neurogenesis until 9-11PN (Larsen and Jacobson, 1986; Rivkees et al., 1988). Estrogen receptor like immunoreactivity first appears in these regions between 10PN and 15PN, one to six days after neurogenesis is completed. Similar observations have been made in other species. Biochemical estrogen receptor identification shows that these receptors first appear after the end of neurogenesis in the mouse, rat, ferret and rhesus monkey (Friedman et al., 1983; Gerlach et al., 1983; Holbrook and Baum, 1983; Pomerantz et al., 1985; Vito et al., 1985; Vito and Fox, 1979, 1982). The time course for estrogen receptor expression in the opossum brain may indicate the beginning of a critical period for sexual differentiation of the brain.

The adult Brazilian opossum displays several sexually dimorphic behaviors. Scent marking and aggressive behavior are both sexually dimorphic in *Monodelphis* (Fadem, 1989; Fadem and Cole, 1984). Furthermore, in *Monodelphis*, estrous cycles are triggered by
pheromones contained in the male's suprasternal scent gland (Fadem, 1987). Although these sexually dimorphic behaviors indicate that there may be sexually dimorphic circuits in the opossum brain, to date, no morphologic sex differences have been found.

A neurochemical sex difference does occur in the *Monodelphis* brain. Cholecystokinin immunoreactive (CCK IR) cells and fibers are located in high concentrations in the Brazilian opossum MPA. The concentration of periventricular CCK IR fibers in the adult opossum MPA is higher in males than in females (Fox et al., 1990). Cholecystokinin also has a sexually dimorphic distribution in the preoptic area and hypothalamus of the rat (Frankfurt et al., 1985; Micevych et al., 1987). Since CCK is involved in gonadotropin secretion (Kimura et al., 1983; Vijayan et al., 1979), and lordosis behavior (Bloch et al., 1987, 1988) in the rat, it is possible that it may be involved in sexually dimorphic opossum behavior and function. Whether the CCK IR system in the opossum is under the control of estrogen is currently being investigated.

A difference in the time course for the onset of estrogen receptor expression in the opossum and in the mouse and rat, is the length of time over which the ER LI or estrogen binding activity increases to a maximal value. The time span from the first detectible estrogen receptors to maximal expression of receptors is approximately 20 days for the mouse (Friedman et al., 1983; Gerlach et al., 1983) and 7 days for the rat (Vito and Fox, 1979, 1982). The increase in ER LI expression in the opossum occurs over approximately a 50 day period of development. This prolonged period of development of ER LI expression is not suprising since *Monodelphis* has a retarded rate of development when compared to rodents. Morphogenesis and neurogenesis of the preoptic area, suprachiasmatic nucleus, and the cerebellum are prolonged when compared to the rat (Dore et al., 1990; Larsen and Jacobson, 1986; Rivkees et al., 1988). Furthermore, *Monodelphis* does not reach sexual maturity until 4 months of age (Krause and Fadem, 1987).
The time course for ER LI expression in *Monodelphis* does differ from existing data on the developmental expression of estrogen binding activity in this species. Binding studies performed on brain cytosols from developing Brazilian opossums revealed binding activity as early as 4PN. The levels of binding increased to 63PN when it was half of the adult level of binding (Etgen and Fadem, 1989). The disparity in ER LI expression and estrogen binding studies could be caused by several factors. First, the immunohistochemical techniques used in the current study may not be as sensitive as estrogen binding studies. Second, a molecule with estrogen binding activity, but not H222 immunoreactivity may be expressed by brain cells prior to 10PN in *Monodelphis*. More detailed work will be needed to determine the reason for this disparity.

Anatomical differences in estrogen receptor expression between the rodent and the opossum are few within the limbic system. However, no ER LI staining was seen in the cortex of the opossum at any developmental age. It is possible that the cortical receptor is a different protein which concentrates estrogen in binding studies but is not recognized by the H222 antibody. However, this is unlikely because *in situ* hybridization studies using probes complementary to sequences that encode the H222 epitope, readily recognize estrogen receptor mRNA in the developing rat cerebral cortex (Miranda and Toran-Allerand, 1990; Toran-Allerand et al., 1990). It is more probable that *Monodelphis* lacks cortical estrogen receptors. More sensitive techniques will have to be utilized to further explore this question in the opossum.

Although the immunohistochemical results of this study are in no way quantifiable on a per cell basis, the increase in ER LI expression appears to involve both an increase in the number of ER LI cells and an increase in nuclear staining intensity. Using more quantitative techniques, Gerlach and coworkers (1983) observed that the increase in estrogen receptor expression in the
pre and postnatal mouse involves an increase in both the number of cells binding estrogen and the amount of estrogen bound per cell.

The appearance of ER LI in the opossum brain between 10 and 15PN correlates well with the onset of high levels of aromatase activity in this species (Fadem et al., 1990). Aromatase converts testosterone to estrogen in the brain. In perinatal rodents, testosterone is not bound by the estrogen sequestering alpha-fetoprotein and allows for the presence of estrogen during early development of the male brain. Estrogen acts to masculinize and defeminize a brain which is inherently female (for review see: Blaustein and Olster, 1990). It is presently unknown whether the neonatal opossum has a circulating estrogen binding molecule. Fadem and coworkers (1990) have recently demonstrated aromatase activity at adult male levels in the brains of 16PN Brazilian opossums. These results, combined with the data in this study indicate that not only is the opossum able to convert testosterone to estrogen at this early age, but the receptive machinery is also present. These data indicate that 15 to 16PN may be the beginning of a critical period for sexual differentiation of the Brazilian opossum brain.

Without exception, ER LI was located within cell nuclei. The strong cytoplasmic staining seen in adult Monodelphis (Fox et al., 1991) and guinea pig (Blaustein and Turcotte, 1989) was absent at all ages analyzed in this study. Although intensity of immunoreactivity at different ages varied from light at early ages to intensely stained nuclei at 60PN, no cytoplasmic staining was observed. This indicates that the cytoplasmic staining patterns seen in adult opossums and guinea pigs is probably not an "overflow" of ER LI into the cytoplasm of intensely staining cells but likely represents a physiologic phenomenon that is not yet fully understood.

In summary, ER LI cells in Monodelphis appear between 10 and 15PN and increase in number in all regions that contain ER LI cells to the highest number observed at 60PN. This indicates that cells in the opossum brain may be sensitive to estrogen early in its development.
Since the entire time course for expression of estrogen receptors occurs postnatally in the opossum, it provides an excellent model on which to examine the role of steroids in the early development of sexually differentiated circuits in the brain.
SECTION IV. ONTOGENY OF CHOLECYSTOKININ-LIKE IMMUNOREACTIVITY IN THE BRAZILIAN OPOSSUM BRAIN
INTRODUCTION

Cholecystokinin (CCK) is an abundant neuropeptide in the mammalian central nervous system (Beinfeld et al., 1981; Crawley; 1985; Innis et al., 1979; Larsson and Rehfeld, 1979). Originally isolated from the gastrointestinal tract, CCK has many important roles in the brain. In adult mammals, CCK acts as a feeding satiety factor (Baile and Della-Fera, 1985; Della-Fera et al., 1980; Gibbs et al., 1973; Smith et al., 1981), has antinociceptive properties (Baber et al., 1989; Faris et al., 1982, 1984), is involved in thermoregulation (Shian and Lin, 1985), and modulates gonadotropin secretion (Hashimoto and Kimura, 1986; Kimura et al., 1983; Vijayan et al., 1979) and sexual behavior (Bloch et al., 1987, 1988). Cholecystokinin is present in the developing brain. However, its role during differentiation is still unclear (Beinfeld et al., 1983; Cho et al., 1983; Hayashi et al., 1989; Matsutani et al., 1988; Scalise et al., 1988; Westenbroek et al., 1987; Yamano et al., 1984).

Immunohistochemistry has demonstrated CCK immunoreactive cells and fibers in the brains of day 15 rat fetuses (Cho et al., 1983). In general, the number of CCK immunoreactive cells and fibers steadily increases until adulthood (Cho et al., 1983). Radioimmunoassay has shown that in the rat, CCK is present at birth and increases in concentration to adulthood (Varró et al., 1983).

Cholecystokinin binding sites develop postnatally in the rat (Hays et al., 1981; Pelaprat et al., 1988). In situ binding studies show an increase in the number of binding sites from birth to day 17 of postnatal life, followed by a decrease to adult levels (Pelaprat et al., 1988).

The adult marsupial brain contains cholecystokinin-like immunoreactive (CCK LI) cells and fibers (Fox et al., 1990). The distribution of CCK LI cells and fibers has been described for the medial preoptic area and anterior hypothalamus in the Brazilian opossum (Fox et al., 1990). Like
rodent animal models, the opossum contains a sex difference for CCK LI structures in the medial preoptic area, with the male having more CCK LI fibers in the periventricular preoptic area than that in the female (Fox et al., 1990).

In this study we have used the Brazilian short-tailed opossum, *Monodelphis domestica*, as a model to study the ontogeny of CCK expression. *Monodelphis* is a small pouchless marsupial which breeds well under laboratory conditions. Its young are born after 14 days of gestation in an immature, sexually undifferentiated state. These properties make marsupial animal models like *Monodelphis* ideal for studying development of the mammalian brain (Dore et al., 1990; Martin et al., 1989; Renfree, 1981; Rivkees et al., 1988; Schwanzel-Fukuda et al., 1988; Shaw et al., 1988; Walker and King, 1989; Xu and Martin, 1989). Immunohistochemistry for CCK was utilized to determine the anatomical distribution of CCK LI structures in the brain of adult and developing Brazilian opossums.
MATERIALS AND METHODS

Animals

Adult and developing male and female Brazilian short-tailed opossums from a colony at Iowa State University were used for this study. The initial animals used to start the breeding colony were obtained from the Southwest Foundation for Research and Education in San Antonio, Texas. The animals were housed in plastic cages, maintained at a constant temperature (26°C) on a 14:10 light-dark cycle and provided water and food ad libitum (Reproduction Fox Chow; Milk Specialties Products, Madison, WI). For breeding, male and female animals were paired for 14 days. Animals were then separated and the dam checked daily at 15:00 for the presence of pups (day of birth = 1PN). The gestation period for the Brazilian opossum is 14-15 days (Fadem et al., 1982), and birth generally occurs 5 days after the termination of pairing. Young animals up to 60PN were housed with their mothers.

Tissue Collection

Fifty six animals from 1-180 days postnatal age (PN) were used in this study. Four animals were collected on both day 1 and 5 PN. At these ages the gender of the neonates cannot be determined grossly or histologically, and thus animals were considered sexually undifferentiated. The gender of the animals can be determined by 10PN by visual inspection of the external genitalia. Four female and four male animals were collected for each of the following time points: 10, 15, 25, 35, 60, and 180PN.

The brains were collected from 1 to 15 PN opossums by cooling the animals in a -15°C freezer until anesthetized. The animals were then decapitated and the heads placed in Zamboni’s fixative for 48 hours. After fixation, the heads were infiltrated with 30% sucrose overnight, and
then cut into 20μm thick coronal sections on a cryostat (Reichert Instruments). The sections were.
thaw mounted onto slides and stored at 4°C until processed for immunohistochemistry.

Twenty five and 35 day old opossums also were anesthetized by cooling. These animals
then were perfused transcardially with 15 ml of Zamboni’s fixative. The brains were isolated and
postfixed in Zamboni’s fixative for 48 hours. After postfixation, the brains were processed as
described above.

Sixty and 180 day old animals were killed by over-etherization. After injection of heparin
into the left ventricle, the animals were perfused transcardially with 0.9% saline followed by
Zamboni’s fixative for 15 minutes. Brains were removed from the calvaria, postfixed for 48
hours in Zamboni’s fixative, and processed as described above. Two of the brains of the 60 PN
animals (one male and one female) and six 180PN animals (three males and three females) were
cut at 30μm. These sections were collected in a cryoprotectant solution (Watson et al., 1986),
and stored at -15°C until immunohistochemistry was conducted.

**Immunohistochemistry**

The protocol utilized for immunohistochemistry was a modification of that reported
previously (Fox et al., 1990). The slide mounted sections were rinsed with 50mM potassium
phosphate buffered saline, incubated with a 0.3% H₂O₂ solution to remove endogenous peroxidase
activity, exposed to normal goat serum as a blocking agent (Vector; 1:67) and then incubated in
CCK primary antibody (rabbit; INC; 1:6000) for 20 hours at room temperature. After adequate
washing, the tissue sections were incubated in goat anti-rabbit IgG (Vector; 1:200) for 2 hours at
room temperature, rinsed, and reacted with avidin-biotin complex (Vector Elite Kit; 1:50) at room
temperature for an additional hour. After washing, the tissue sections were stained by exposing
them to a substrate composed of 0.04% 3,3' diaminobenzidine tetrahydrochloride (DAB; Sigma),
2.5% nickel sulfate (Fisher Scientific) and 0.01% hydrogen peroxide, dissolved in 0.1 M sodium acetate. After staining for 6 minutes, the reaction was terminated by placing the slides into two successive rinses of 0.9% saline. The sections were then dehydrated in graded alcohols, cleared in xylene and coverslipped with permount mounting media and analyzed with a light microscope.

Unmounted tissue sections were immunohistochemically processed using a floating tissue technique described previously for adult tissue (Fox, et al., 1990). This allowed comparison of slide mounted tissue with floating tissue immunohistochemistry.

**Immunohistochemical Control Procedures**

Both negative and preabsorption control procedures used with the CCK antibody have been described previously (Fox et al., 1990).

**Tissue Analysis**

Drawings of ten cranio-caudal levels of the opossum brain were constructed using Autocad, a computer aided design program, for each age from cresyl violet or neutral red stained coronal sections from a collection in our laboratory. Twenty micrometer sections from a 1 in 3 series were observed with a Zeiss microscope. Regions of the brain containing CCK LI cells and fibers were indicated on the prepared illustrations.
RESULTS

CCK LI somata in the adult opossum brain

Although none of the animals in this study were treated with colchicine, CCK LI cell bodies were observed in several regions of the brain.

Brainstem

The brainstem contained a population of intensely immunoreactive cell bodies in the dorsolateral portions of the nucleus of the solitary tract (Sol; Fig. 17I; Fig. 18H; Table 1).

Midbrain

In the midbrain, scattered CCK LI soma were observed in the dorsal raphe (DR) just ventral to the central canal (Fig. 17F; Table 1).

Hypothalamus

The hypothalamus contained intensely staining soma in the periventricular preoptic area (Pe) and suprachiasmatic nucleus (SCh; Fig. 17C; Table 1). No CCK LI cells were observed in the medial preoptic area (MPA).

Thalamus

In the thalamus, lightly staining cell bodies were located in many nuclei (see Table 1; Fig. 17C-E).
TABLE 1: CCK LI structures in the developing opossum brain

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### TABLE 1: continued

#### Thalamus:

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Here, the symbols +/- and -/- indicate the presence or absence of specific characteristics in the Thalamus and Hypothalamus regions.
### TABLE 1: continued

#### Midbrain:

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#### Brainstem:

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* C indicates cholecystokinin-like immunoreactive (CCK LI) cells. F indicates CCK LI fibers.
+ indicates the presence of CCK LI cells or fibers. - indicates no CCK LI cells or fibers were observed

** A sex difference was observed in the MPA with males having more CCK LI structures in this region than the female animals in this study. See text and figure 21.
Figure 17: A series of line drawings of selected coronal sections from rostral to caudal (A-J) of the Monodelphis brain. Drawings from brain sections of postnatal day 180, 60, 35, and 10 are represented from left to right. Black triangles indicate regions of the brain that contain cholecystokinin-like immunoreactive (CCK LI) cells. Regions containing no, low, or high concentrations of CCK LI fibers are indicated by no, low, or high densities of stippling respectively. See appendix A for a list of abbreviations.
**Cortex/Basal Ganglion**

Cholecystokinin immunoreactive perikarya were located throughout the cortex (Fig. 17A-E; Table 1).

**Limbic System**

Cholecystokinin immunoreactive soma were located in the entire rostro-caudal extent of the hippocampus. These cells were scattered throughout the dentate gyrus (DG), and CA1, CA2, and CA3 regions (Figs. 17A-F, 19B; Table 1).

**CCK LI fibers in the adult opossum brain**

Cholecystokinin immunoreactive fibers were found to be widely distributed throughout the opossum brain. A spectrum of CCK LI fiber concentrations were observed from none to very high. For the purpose of discussion, areas have been classified as containing no, low, or high amounts of immunoreactive fibers.

**Brainstem**

In the rostral brainstem, high densities of CCK LI fibers were located in the dorsal nucleus of the lateral lemniscus (DLL) and in the dorsal portion of the parabrachial nucleus (PB; Fig. 17H). More caudally in the brainstem, high concentrations of CCK LI fibers were found in the solitary tract (sol) and nucleus (Sol; Figs. 17I,J, 18A-H). In addition, the brainstem contained low densities of CCK LI fibers coursing from the ventrolateral brainstem to the sol (Fig. 17I,J). Other regions of the brainstem containing CCK LI fibers are listed in Table 1 and illustrated on Figure 17I-J.
Figure 18: A photomontage of cholecystokinin-like immunoreactive (CCK LI) structures in the caudal solitary complex of (A) a 10PN male opossum, (C) a 35PN male opossum, (E) a 60PN female opossum, and (G) a 180PN male opossum. B, D, F, and H are photomicrographs of CCK LI structures in the rostral solitary complex of 10PN male, 35PN male, 60PN female, and 180PN male opossums respectively. No sex difference was observed in CCK LI cells or fibers in the opossum solitary complex. The bars indicate 100μm.
Figure 19: Photomicrographs of hippocampal cells that contain CCK-like immunoreactivity. A is hippocampal cells in a 25PN female opossum. B is hippocampal cells in a 180PN male opossum. No sex difference was observed in CCK LI cells or fibers in the opossum hippocampus. The bar indicates 50μm.
Midbrain

The midbrain contained several nuclei low amounts of CCK LI fibers. These nuclei include the central grey (CG), dorsal raphe nucleus (DR), the ventral and dorsal parts of the ventral nucleus of the lateral lemniscus (VLLv and VLLd respectively), and the ventral tegmental area (VTA; Figure 17F-H).

Hypothalamus

The MPA of male animals in this study contained a dense collection of fibers adjacent to the third ventricle (Figs. 17B, 20A). The female animals in this study had a low density of fibers in this region (Fig. 20E). In the anterior hypothalamus, high densities of CCK LI fibers were located in the SCh (Fig. 17C) and the periventricular hypothalamus (Pe; Fig. 17D). In the posterior hypothalamus, high concentrations of CCK LI fibers were found in the ventral parts of the dorsomedial hypothalamic nucleus (DM; Fig. 17E, 21A) and the dorsal one-half of the arcuate nucleus (Arc; Fig. 17F).

Thalamus

Cholecystokinin immunoreactive fibers were mainly located in the midline thalamic nuclei. However CCK LI fibers were also observed in the mediodorsal thalamic nucleus (MD; Fig. 17C) as well as the dorsolateral geniculate nucleus (DLG; Fig. 17D), and the posterolateral thalamic nucleus (PL; Fig. 17E). For a summary of CCK LI fibers in the thalamus see Fig. 17C-E and Table 1.

Cortex/Basal Ganglion

No CCK LI fibers were observed in the cortex or the basal ganglion of the adult opossum.
Figure 20: A photomontage of cholecystokinin-like immunoreactive (CCK LI) structures in the medial preoptic area (MPA) of male and female opossums. A-D are photomicrographs of CCK LI structures in the MPA of 180, 60, 35, and 25PN male opossums. E-F are photomicrographs of CCK LI structures in the MPA of 180, 60, 35, and 25PN female opossums respectively. A sex difference was observed in the onset of CCK LI in cells and fibers in the *Monodelphis* medial preoptic area. The bars indicate 50μm.
Figure 21: Photomicrographs of cholecystokinin-like immunoreactive fibers in the dorsomedial hypothalamic nucleus of (A) a 180PN male opossum, (B) a 60PN female opossum, and (C) a 35PN male opossum. No sex difference was observed in CCK LI in the developing Monodelphis dorsomedial hypothalamic nucleus. The bar indicates 100μm.
Limbic System

The amygdala contained high concentrations of CCK LI fibers in the magnocellular basal amygdaloid nucleus (BAM; Figs. 17D, 22D). In the olfactory tubercle, heavy staining of CCK LI fibers was observed in the Islands of Calleja (ICj; Figs. 17A) and the nucleus of the lateral olfactory tract (LOT, Fig. 17C).

CCK LI cells in the developing opossum

No CCK LI cell bodies were seen in the Monodelphis brain on 1PN or 5PN.

Brainstem

The only region of the brain that contained CCK LI soma at 10PN was the Sol (Figs. 17I, 18A,B). These were mainly located in the dorsal portion of the Sol. Cholecystokinin immunoreactive cells were seen in the Sol throughout the rest of development and into adulthood (Fig. 17J, 18D,F,H). Somata in the dorsal portion of the parabrachial nucleus transiently expressed CCK LI on day 60PN (Fig. 17H).

Midbrain

On postnatal day 15, several regions of the midbrain contained CCK LI cell bodies for the first time. These regions were CG, DR, VLLv, and VTA (Fig. 17F-H). With the exception of 60PN, CCK LI somata were observed in the DR through the adult time point in this study. In contrast, CCK LI cells were present in the CG only through 25PN and are found in the VLLv only on day 15.
Figure 22: Photomicrographs of cholecystokinin-like immunoreactive fibers in the magnocellular basal amygdaloid nucleus of (A) a 15PN female opossum, (B) a 35PN male opossum, (C) a 60PN female opossum, and (D) a 180PN male opossum. No sex difference was observed in CCK LI fibers in the opossum magnocellular basal amygdaloid nucleus. The bars indicate 100µm.
Hypothalamus

Cholecystokinin immunoreactive perikarya were observed in the MPA of the male animals in this study from 25PN to 35PN (Figs. 17B, 20D, C). At no time were CCK LI cells observed in the female opossum MPA. Cells in the suprachiasmatic nucleus (SCh) first expressed CCK LI on day 60PN. Similar staining was also observed at 180PN (Fig. 17C). Cholecystokinin immunoreactivity was transiently expressed in cells in the caudal hypothalamus on day 35. These cells were located in the region of the dorsal premammillary nucleus (PMD; Fig. 17F).

Thalamus

Somata containing CCK immunoreactivity were first observed in most of the thalamic nuclei on 35PN (Fig. 17C-E). The perifornical thalamic nucleus (PF) contained CCK LI cells from 25PN through 180PN (Fig. 17E). Most regions maintained a population of CCK LI cells into adulthood.

Cortex/Basal Ganglion

Cholecystokinin immunoreactive cell bodies were located throughout the neocortex of developing opossums from 15PN through adulthood. Transient expression of CCK LI was observed in cells in the striatum (caudate putamen {CPu} and globus pallidus {GP}) on day 35PN (Fig. 17C).

Limbic System

Cholecystokinin immunoreactive somata were first observed on 25PN in the olfactory tubercle and hippocampus. In the olfactory tubercle, CCK LI cells were observed in the LOT
from 25PN through 60PN (Fig 17C). The hippocampus contained scattered CCK LI cells in CA1-3 and the dentate gyrus (DG). These cells were observed at all time points from 25PN through 180PN (Figs. 17A-F, 19).

CCK LI fibers in the developing opossum

No CCK LI fibers were observed in the opossum brain at day 1PN.

Brainstem

Cholecystokinin immunoreactive fibers were first observed in the brainstem at 5PN. These fibers were located in the dorsolateral portion of the caudal brainstem (Fig. 23). At day 10PN, CCK LI fibers were located in the sol, PB, inferior olivary nucleus (OI), and hypoglossal nucleus (12). Fibers in the sol, PB, and OI persist into adulthood, in contrast to fibers in 12 which are not present after 15PN (Figs. 17H-J, 18). For staining in other regions of the brainstem see Table 1 and Fig. 17H-J.

Midbrain

The earliest CCK LI fibers in the midbrain are located in the VLLd and VLLd at 10PN. Cholecystokinin immunoreactive fibers were observed in the DR and SC from day 35PN to 180PN. A group of fibers was observed adjacent to the medial geniculate body in the centraltegmentum (ctg) at 60PN. For staining in the remainder of the midbrain see Table 1 and Fig. 17F and G.
Figure 23: A photomicrograph of cholecystokinin-like immunoreactive fibers in the dorsal brainstem of a 5PN opossum. The gender of the animal could not be determined at this age. The space in the upper right of the photomicrograph is the fourth ventricle. No sex difference was observed in the onset of CCK LI expression in fibers in the developing Monodelphis brainstem. The bar indicates 100μm.
Hypothalamus

Most areas of the hypothalamus that contained CCK LI fibers first had immunoreactive fibers at 35PN and maintained these fibers into adulthood. Exceptions to this was the MPA (Fig. 20) and zona incerta which first contained CCK LI fibers at 25PN and 15PN respectively, and the median preoptic nucleus (MnPO), lateral hypothalamus (LH), PMD, and PMV which did not contain CCK LI fibers until 60PN (Fig. 17B-D).

The developmental pattern of CCK LI expression in the MPA of the male and female opossums differed in this study. While male animals contained a low number of CCK LI fibers at 25PN and the amount of fibers increased into adulthood (Fig. 20A-D), female animals did not contain CCK LI fibers until 35PN. At 35PN an extremely low number of fibers was observed in the female MPA. The number of CCK LI fibers did increase to the low number of fibers seen in the adult females in this study (Fig. 20E-H).

Thalamus

Most of the thalamic nuclei which contained CCK LI fibers first had them at 60PN. Exceptions to this pattern included the DLG which contained CCK LI fibers on 35PN and the PL which contained CCK LI fibers on 10PN.

Cortex/Basal Ganglion

Cholecystokinin immunoreactive fibers were observed in the CPu only on 15PN. The GP contained CCK LI fibers only on 10PN.
Limbic System

Many regions of the limbic system first contained CCK LI fibers on 60PN. These areas included the ICj, Pir, hippocampus, and septofimbrial nucleus (Fig. 17A-F). The LOT first contained CCK LI fibers on 25PN. The BAM expressed CCK LI fibers from 15PN into adulthood (Figs. 17D, 22). The posterior amygdaloid area (PAA) did not contain CCK LI fibers until 180PN. The ventral division of the bed nucleus of the stria terminalis (BSTV) contained a high concentration of CCK LI fibers from 15PN to adulthood. In the septum, the LSI only expressed low amounts of CCK LI fibers on 180PN, however the LSV contained CCK LI fibers as early as 35PN. Cholecystokinin immunoreactive fibers were found in the ventral nucleus of the diagonal band of broca (VDB) from 10PN to 180PN.

For a summary of regions containing CCK LI fibers in the developing opossum brain see Table 1 and Figure 17A-J.
DISCUSSION

In general, the distribution of CCK LI fibers in the adult Brazilian opossum was similar to that observed in the adult rodent (Fallon and Seroogy, 1985; Innis et al., 1979; Vanderhaeghen et al., 1980). Cholecystokinin immunoreactive structures were widely distributed throughout the *Monodelphis* brain. The only region of the brain that lacked CCK LI cells or fibers was the cerebellum.

Regions of the adult *Monodelphis* brain which contain CCK LI soma are the Sol, DR, Pe, SCh, cortex, and hippocampus. However, it is important to note that none of the animals in this study were colchicine treated. Therefore, it is likely that many regions that contain CCK synthesizing cell bodies may not have been detected in this study. Colchicine does enhance CCK LI in neuronal cell bodies in the opossum MPA (Fox et al., 1990) and this group of perikarya was not detected in the present study. This may be due to rapid transport of CCK out of the somata in the MPA, a process which is blocked by colchicine. It may also be due to low levels of synthesis of CCK in the MPA which is stimulated by colchicine.

Cholecystokinin immunoreactive fibers are found in very high concentrations in the Sol and the Sol. This observation should be viewed with caution, though, since gastrin has been localized in the vagus nerve, a major contributor of fibers to the solitary complex (Uvnäs-Wallenstein et al., 1977). The antibody used in this study does cross react with gastrin (Fox et al., 1990). Thus, at least part of the immunoreactivity that is observed in the solitary complex with this antibody could be due to its cross reactivity with gastrin.

Some of the fibers of the vagus nerve that innervate the Sol contain CCK. These projections arise primarily from branches of the vagus nerve which innervate the stomach and are believed to carry information about gastric filling (Avery and Livosky, 1986; Gibbs et al., 1973;
Kubota et al., 1983; Palkovits et al., 1982). Furthermore, cholecystokinin containing fibers project from the Sol to the parabrachial nucleus. In the rat, this projection may relay gastrointestinal information (Herbert and Saper, 1990). The PB sends CCK LI fibers to several regions of the forebrain including the ventromedial hypothalamus, a projection which may also be involved in carrying gastrointestinal information (Fulwiler and Saper, 1985). In Monodelphis, CCK LI fibers and soma are present in the solitary complex and CCK LI fibers are present in the PB. Although the VMH of the opossum does not contain CCK LI fibers, several other regions of the hypothalamus such as the DM and the Pe do contain CCK LI fibers and may be targets of CCK LI output of the PB. Experiments to test this hypothesis are underway.

High amounts of CCK LI fibers are also observed in the preoptic area, amygdala, and the bed nucleus of the stria terminalis. These three interconnected loci contain CCK LI in the rat. Variations in the number of CCK LI cells in these nuclei are found during the estrus cycle, and the ability of estrogen implants to regulate the number of cells in these nuclei that express CCK LI indicates that estrogen may regulate the level of CCK expression in cells in this interconnected circuit in the rat (Oro et al., 1988). A sex difference has been characterized in the density of CCK LI fibers in the MPA of Monodelphis (Fox et al., 1990), however no sex difference has been characterized in the amygdala or bed nucleus of the stria terminalis of this animal model. Careful morphometric studies are underway to thoroughly examine the CCK LI components of these areas in the opossum. However, it is possible that this estrogen regulated circuitry may exist in Monodelphis. Like the rat, Monodelphis has estrogen receptor containing cells in the MPA, amygdala, and bed nucleus of the stria terminalis (Fox et al., 1991a; Handa et al., 1991).

Another region that contains CCK LI fibers in the adult Brazilian opossum is the ventral tegmental area (VTA). In the rat, CCK is colocalized with dopamine in a large percentage of neurons in the VTA that project to the nucleus accumbens, olfactory tubercle, and septum (Fallon...
et al., 1983; Hökfelt et al., 1980; Seroogy et al., 1988). Whether CCK is colocalized with dopamine in the *Monodelphis* midbrain is unknown. However, only low amounts of CCK LI fibers are found in the opossum VTA and no CCK LI fibers are observed in the adult striatum. This may indicate that CCK is not an important neuromodulator in the *Monodelphis* mesolimbic and mesostriatal pathways as it is in the rat.

The earliest expression of CCK immunoreactivity in the opossum brain is in the brainstem. At 5PN, CCK LI fibers are located in the dorsolateral brainstem in the region of the Sol. It is likely that many of these fibers are of vagal origin, since they can be traced to the ventrolateral portion of the brainstem, however the source of these fibers has not been experimentally confirmed. During development the density of CCK LI fibers increases in the sol from 5PN to 60PN. At 60PN CCK LI fibers have reached their highest levels and decrease in density slightly from 60PN to 180PN (Fig. 19A,C,E,G). A peak in CCK LI fibers followed by a slight decline into adulthood may represent some fine tuning of the system during late development.

In addition to fibers in the dorsal brainstem, at 10PN CCK LI fibers are seen in other regions that may be intrinsic to the developing brain. Cholecystokinin immunoreactive fibers in the PB may come from CCK LI cell bodies that are located in the dorsal brainstem at 10PN. Areas in the hypothalamus that may serve as targets for brainstem CCK LI efferent pathways, namely the DM and the Pe do not contain CCK LI fibers until 35PN.

The amygdala and bed nucleus of the stria terminalis first contain CCK LI fibers on 15PN, the same time that estrogen receptor immunoreactive (ER LI) cells were first observed in these regions of the opossum brain (Fox et al., 1991b). Cholecystokinin IR fibers and cells were first observed in the medial preoptic area at 25PN in male opossums. This data indicates that these estrogen receptor rich regions of the brain may communicate via CCK LI projections, and that the CCK LI fibers project from the amygdala and the bed nucleus of the stria terminalis between day
15 and day 25 in the male and day 25 and 35 in the female. Furthermore, CCK LI fibers the
medial preoptic area continue to increase to 180PN. Whether this is an increase in the number of
fibers expressing CCK or an actual increase in the number of fibers with the potential to contain
CCK is not known. However in the rat, when the female is given estradiol implants the number
of CCK LI cells in the amygdala, bed nucleus of the stria terminalis, and the medial preoptic area
increase to the level observed in the male. This data indicates that it may be a difference in CCK
expression, rather than an anatomical difference in the actual number of fibers in the medial
preoptic area. More detailed studies need to be done in the opossum to determine the
morphological development of this circuit, and to determine if CCK is under estrogen regulation
in early development.

It is interesting to note that a sex difference in CCK LI structures exists in the MPA from
the time that CCK LI is first observed there. Because ER LI is present in the MPA, amygdala,
and bed nucleus of the stria terminalis at or prior to CCK immunoreactivity in these regions (Fox
et al., 1991b) it is possible that estrogen influences the sexually dimorphic expression of CCK LI
structures in the MPA.

The midbrain also contains CCK LI structures early in development of the Monodelphis
brain. The VTA contains CCK LI cells on 15PN and CCK LI fibers are located in the VTA from
25PN to 180PN. However, only low numbers of weakly CCK LI cells were seen at 15PN and
only low amounts of CCK LI fibers were seen in the opossum VTA. The nucleus accumbens a
major target of the VTA in the rat, only contains high amounts of background staining which
cannot be localized in fibers or cells. It is possible that the CCK LI input to this region is in the
form of very small fibers that release CCK at a high rate thus causing the observed pattern of
staining. On the other hand, it may be that CCK is not an important neurotransmitter in the
VTA-nucleus accumbens projection as it is in other mammalian species. The DR also expressed
CCK LI cells at 15PN. However, unlike the VTA the DR contained CCK LI into adulthood. This indicates that the dorsal raphe cells are under a different system of regulatory control than the VTA neurons, possibly synthesizing CCK at a continuous high level from the onset of CCK expression.

Some hippocampal neurons also contain CCK LI from the onset of expression at 25PN through the 180PN time point. In the rat there may be two sources of CCK LI in the hippocampus. An intrinsic source from cells in the hippocampus. These CCK LI cells may give rise to intrinsic fibers and efferent fibers to the lateral septal nucleus, and possibly an afferent set of CCK LI fibers that comes from CCK LI cells in the entorhinal cortex and septum (Greenwood et al., 1981). The CCK LI terminals in the rat hippocampus may all be intrinsic since lesions involving the entorhinal cortex does not reduce the amount of CCK LI in the hippocampus (Handelmann et al., 1981). In Monodelphis we observed two patterns of CCK LI onset in the hippocampus, the appearance of CCK LI in cells at 25PN, and in fibers at 60PN. It is possible that the cells in the opossum hippocampus are part of intrinsic or efferent CCK LI circuitry and that at least some of the fibers arise from cells outside the hippocampus that may express CCK immunoreactivity later than cells in the hippocampus.

It should be kept in mind that none of the animals in this study were treated with colchicine. Although we did observe cells that contained CCK immunoreactivity in several regions, many other regions may contain CCK LI cells, but because of low levels of synthesis or fast transport of CCK out of the cell body they may not have been detected in this study. In addition, regions that contained CCK LI cells transiently, like the medial preoptic area, central grey, and parabrachial nucleus do not indicate that these cells stopped synthesizing CCK. It is possible that synthesis levels drop after a period of high synthesis and maintain lower undetectable levels, or that transport systems develop later than the ability of the cell to synthesize CCK.
A possibility still exists for sex differences in CCK LI in regions of the brain outside the MPA. The sex difference for fibers in the MPA of the opossum is not paralleled by a sex difference in CCK LI cells in this region in the adult (Fox et al., 1990). Thus the sex difference may arise from CCK LI somata in the MPA that contain more CCK LI processes in the male than in the female. It is more likely that these fibers arise from perikarya outside the MPA. Since the animals in this study were not colchicine treated, we were unable to evaluate this second possibility. It is also possible that small sex differences from multiple regions of the brain converge on the MPA. We observed no sex differences in CCK LI fibers outside of the medial preoptic area. However, to detect subtle sexual dimorphisms in CCK immunoreactivity more sophisticated morphometric analysis or more quantitative techniques such as radioimmunoassay may be required.

Another aspect of CCK’s early expression in the opossum brain is what influence CCK may have on developmental events in the brain. Neurogenesis of the suprachiasmatic nucleus (SCh) is not completed in Monodelphis until 8PN. It is not until 17PN that the SCh could be identified morphologically using light microscopy. On day 20 a clear day-night rhythm in metabolic activity was observed (Rivkees et al., 1988). Cholecystokinin immunoreactivity is not present in the SCh of Monodelphis until 35PN in fibers and 60PN in cells. This indicates that CCK immunoreactivity is not necessary for the morphogenesis or basic function of this nucleus, however, it may be important in fine tuning the output of the SCh.

It is also possible that CCK functions in roles other than neurotransmission or neuromodulation during development. Unfortunately, little is known about when connections are made in particular nuclei of the opossum brain. Therefore, it is impossible to make correlations between the timing of synapse formation and CCK expression in the Monodelphis brain.
Experiments combining synapse specific markers and CCK immunohistochemistry may provide an interesting correlation about the role of CCK in this important developmental event.

In summary, CCK LI somata and fibers were found to be widely distributed throughout the adult *Monodelphis* brain. The cerebellum being the only major region of the brain that did not contain CCK LI structures. In addition to its widespread distribution, a wide range of onset patterns were observed indicating that CCK may have many roles in brain circuitry, which become functional at different stages of development.

For example, *Monodelphis* first expresses CCK-like immunoreactivity in the brainstem at 5PN, very early in opossum brain development. Consistent with this pattern of expression is the fact that the marsupial neonate feeds from the time of birth. However, CCK LI structures are not present at birth. This may be because it is below the limits of detectibility in our immunohistochemical procedure, or CCK may not be regulating feeding in the newborn opossum. Of course gut released CCK may act as a hormone and not as a central neurotransmitter before 5PN. This early expression of CCK LI structures in regions of the brain that are important in the regulation of feeding provides an excellent model to study the role of CCK in the onset of feeding patterns.

On the other extreme, CCK LI fibers are not found in the SCh until 35PN indicating that CCK may not have a role in the early development of the SCh. However, CCK’s later appearance in the SCh indicates that it may have a function in the more mature SCh.

Finally, CCK LI structures are found in several regions of the *Monodelphis* brain that contain ER LI cells at or later than the time of onset of ER immunoreactivity. Thus, it is possible that estrogen may modulate CCK expression in several regions of the opossum brain. Experiments are currently underway to attempt to understand estrogens organizational role in these circuits. Since estrogen receptor expression occurs entirely during postnatal development in
Monodelphis (Fox et al., 1991b), it will be an excellent model for studying the role of estrogen in the development of CCK LI circuitry.
SUMMARY

In the first study, the anatomical localization of cholecystokinin-like immunoreactivity (CCK LI) was examined in somata and fibers in the MPA and AH of the Brazilian gray short-tailed opossum, *Monodelphis domestica*. With the aid of an avidin-biotin, nickel enhanced, immunohistochemical technique, CCK LI neuronal elements were found within the MPA and AH. A large number of CCK LI cell bodies were located in the MPA of colchicine treated opossums. The MPA also contained a CCK LI fiber plexus. Quantitative image analysis revealed that the periventricular preoptic area of non-colchicine treated male opossums had significantly higher percent blocked light measurements than that of the non-colchicine treated females, indicating a higher density of CCK LI neuronal elements in the males. Neuronal fibers and somata containing CCK LI were also found within the periventricular hypothalamic nucleus (Pe), and the suprachiasmatic nucleus (SCh). These results show that CCK LI neuronal elements are found within the MPA and AH of the Brazilian short-tailed opossum. Furthermore, there is a sexually dimorphic distribution of CCK LI elements within the MPA of this small marsupial.

In the second study, we examined the distribution of estrogen receptor-like immunoreactive (ER LI) cells in the brains of gonadectomized male and female Brazilian opossums using Abbott H222 rat monoclonal estrogen receptor antibody (H222 is a gift of Abbott labs). An indirect immunohistochemical procedure similar to the one described above was used. A large number of ER LI cell nuclei were observed in the medial preoptic area, ventral septal nucleus, medial division of the bed nucleus of the stria terminalis, lateral part of the ventromedial hypothalamus, premammillary nucleus, arcuate nucleus, posterior amygdaloid nucleus, and the midbrain central grey. Lower numbers of ER LI cell nuclei were observed in the intermediate subdivision of the lateral septal nucleus, and in the anterior, medial, and posterior cortical
amygdaloid nuclei. The anatomical distribution of ER LI in the Brazilian opossum brain is similar to that which has been reported for estrogen binding sites following biochemical analysis (Handa et al., 1991; Etgen and Fadem, 1987). Based on these findings, we believe specific regions of the Brazilian opossum brain may serve as substrata for the action of estrogen in the adult. In addition, these results are supportive of the use of this animal model to investigate the organizational effects of estrogen on the developing central nervous system.

As a continuation of study two, in study three, the Brazilian short-tailed opossum was utilized as a model to study the ontogeny of estrogen receptors in the mammalian brain. The Abbott H222 monoclonal rat estrogen receptor antibody (gift of Abbott Laboratories) was utilized in an indirect immunohistochemical procedure to detect estrogen receptors in the developing opossum brains. Estrogen receptors were first expressed in the dorsomedial and ventromedial hypothalamus of the opossum ten days after birth (10PN). Most regions that contained ER LI cells in the adult opossum contained ER LI at 15PN. These areas include the lateral septum, medial preoptic area, bed nucleus of the stria terminalis, periventricular preoptic area and hypothalamus, amygdala, dorsomedial and ventromedial hypothalamic nuclei, arcuate nucleus, ventral premammillary nucleus, and the midbrain central grey. In each of these areas, the number of ER LI cells increased to the 60PN time point. This pattern of expression indicates that estrogen receptors are present in early development of the Monodelphis brain and may indicate the beginning of a critical period for sexual differentiation of the opossum brain.

In the fourth and final study, the anatomical distribution of CCK LI somata and fibers in the brain of the adult and developing Brazilian short-tailed opossum was investigated. An indirect immunohistochemical technique was used to identify CCK LI structures. Cholecystokinin immunoreactive cell bodies were located throughout the cerebral cortex and hippocampus of adult opossums. Somata containing CCK immunoreactivity were also observed in the hypothalamus,
thalamus, midbrain, and brainstem. Cholecystokinin immunoreactive fibers had a wide distribution in the adult *Monodelphis* brain. The only region of the brain that did not contain CCK LI fibers was the cerebellum. The earliest expression of CCK immunoreactivity was in fibers in the dorsal brainstem of 5PN opossum pups. Cholecystokinin immunoreactive somata were observed in the brainstem on 10PN. A broad spectrum of patterns of onset of CCK expression were observed in the opossum brain. The wide distribution and varied patterns of onset of expression of CCK LI structures indicates CCK may have many functions in the opossum brain.
DISCUSSION

These studies provide a body of information which promotes the use of the Brazilian opossum for the study of brain development. Both CCK LI and ER LI systems are similar to those observed in the laboratory rat (Cho et al., 1983; Pfaff and Keiner, 1973; Vanderhaeghen et al., 1980), mouse (Friedman et al., 1983; Gerlach et al., 1983), hamster (Miceli et al., 1987), guinea pig (Blaustein and Turcotte, 1989), and other species in which these systems have been studied. The conservation of the basic patterns of CCK LI in these species emphasizes its important role in the functional output of this circuitry. Furthermore, the developmental pattern of CCK LI onset in the brain adds more evidence for a role in feeding and reproducitively relevant circuitry, as well as possible function in developmental events in the brain. The highly similar pattern of estrogen receptor distributions in the adult and developing rat and opossum also suggest important roles for estrogen receptors in the modulation of reproductive behavior and gonadotropin secretion, and participation in important early events in brain development. Finally, the temporal and spatial properties of both ER LI and CCK LI expression suggests the possibility of steroid regulation of neuropeptide expression.

Cholecystokinin has a very broad distribution in the adult opossum brain being found in all major divisions of the brain except the cerebellum. Anatomical localization of CCK in the opossum brain indicates that it may be involved in feeding behavior, reproductive behavior and gonadotropin secretion, as shown for the rat and other eutherian species (Baile and Della-Fera, 1985; Bloch et al., 1987, 1988; Della-Fera, et al., 1980; Gibbs et al., 1973; Hashimoto et al., 1986; Kimura et al., 1983; Smith et al., 1981a; Vijayan et al., 1979). Cholecystokinin immunoreactive somata and fibers are found in the highest concentrations in the solitary tract and nucleus. This region of the brainstem is important in feeding as well as other autonomic
functions (Shapiro and Micelis, 1985). Interestingly, CCK is located in other regions of the brain that are involved in feeding behavior. These regions include the parabrachial nucleus, and hypothalamus. Cholecystokinin-like immunoreactivity is located in the dorsomedial hypothalamic nucleus and the periventricular hypothalamus and may regulate feeding behaviors via these loci.

It is also interesting to note that the first CCK LI observed in the opossum brain is seen in the dorsal brainstem in the area of the presumptive Sol at 5PN. Consistent with this early pattern of CCK expression is the fact that the *Monodelphis* neonate must feed from the time of birth. This early expression of CCK in a region of the brain involved in the control of feeding indicates that peptides like CCK may have important roles in the development of circuitry in which they will later function as neuromodulators.

Cholecystokinin immunoreactivity was observed at later points in development in other regions of the brain. An example of a nucleus which doesn't contain CCK LI structures until later in brain ontogenesis is the suprachiasmatic nucleus (SCh). This nucleus is thought to serve as the circadian pacemaker in the mammalian brain and has been well studied in many species including *Monodelphis*. Neurogenesis of the SCh is not completed in *Monodelphis* until 8PN and its morphogenesis is not completed until 17PN (Rivkees et al., 1988). On day 20PN a clear day-night rhythm in SCh metabolic activity is detected indicating that it may be functioning as a circadian pacemaker at this point (Rivkees et al., 1988). Cholecystokinin immunoreactivity is not present in the SCh of *Monodelphis* until 35PN in fibers and 60PN in cells. This late onset of CCK LI in the SCh indicates that CCK may not be involved in the morphogenesis or basic function of this nucleus, but may have a role in fine tuning the output of the SCh.

In adult Brazilian opossums, CCK LI cells and fibers and ER LI cells are located in the medial preoptic area in high concentrations. Furthermore, a sex difference is present for CCK LI fibers in the MPA. These CCK LI fibers may arise from cells in the MPA, or may come from
cells outside the MPA. Two areas that send CCK LI fibers to the MPA of the rat are the amygdala and the bed nucleus of the stria terminalis (Oro et al., 1988). Both of these nuclear groups contain CCK LI fibers in the adult opossum, whether they contain CCK LI cells has not been determined. The medial preoptic area, amygdala, and bed nucleus contain ER LI cells in the opossum. It is also known that in the rat, estrogen regulates the number of CCK LI cells, and the levels of CCK in the amygdala, bed nucleus, and MPA (Frankfurt et al., 1986; Micevych et al., 1988b; Oro et al., 1988). Whether estrogen regulates CCK LI levels in the opossum is unknown.

There is no sex difference in the number of ER LI cells in the medial preoptic area, amygdala, or bed nucleus of the stria terminalis in the gonadectomized adult or developing intact opossum. Thus, this data does not support the hypothesis that estrogen may drive a sex difference in CCK LI observed in the Monodelphis medial preoptic area. Alternative hypotheses include the possibility that the sex difference in CCK LI is genetically predetermined. On the other hand, it may be a sex difference which is organized during development by a factor other than estrogen. This could be a hormonal factor, such as testosterone, or a transcriptional regulator that modulates CCK gene expression and has a sexually differentiated expression in cells that are afferent to the medial preoptic area. It is also possible that there is sexually dimorphic neuronal circuitry that inputs on CCK LI afferents to the medial preoptic area. This type of input could drive the sex difference in CCK LI fibers.

There is evidence that the sex difference in CCK LI fibers may be organized quite early in opossum brain development. In Monodelphis, a sex difference is observed in CCK LI fibers when CCK first appears in the MPA at 25PN. This sex difference is maintained into adulthood. However, whether this sex difference remains in the absence of the normal gonadal hormone milieu is not known. The effect of gonadectomy on CCK LI systems has not been examined. In
the adult rat, CCK levels are always higher in the male MPA than in the female, regardless of
what stage of the estrus cycle the female is in (Micevych et al., 1988b; Siegel et al., 1985). On
the other hand, when female rats were gonadectomized and treated with 17β-estradiol implants,
the numbers of CCK LI neurons in the medial preoptic nucleus, medial amygdaloid nucleus, and
bed nucleus of the stria terminalis reached numbers equivalent to those seen in intact male animals
(Oro et al., 1988). This data indicates that neurons in these regions in the female have the same
capacity for CCK expression as in the male, this capacity is simply not realized in the hormone
environment of the normal animal.

To investigate the organizational and activational effects of estrogen on CCK expression,
detailed morphometric studies need to be performed to determine if the higher density of CCK LI
fibers in the opossum MPA is due to a higher survival rate of CCK LI neurons in the developing
male, or if it is driven by circulating gonadal steroid levels in the adult. Before these experiments
can be carried out, preliminary studies need to be done to determine the location of cells giving
rise to the CCK LI fibers in the sexually dimorphic MPA plexus. Finally, the possibility of CCK
LI cells in the male opossum giving rise to more or larger CCK LI processes cannot be ignored.
Morphometric studies employing the Golgi technique for examining neuronal processes should be
used to examine the Monodelphis medial preoptic area.

It is also possible that CCK LI cells are regulated by another hormonal signal in the brain.
The distribution of androgen receptors has not been investigated in Monodelphis and may shed
some light on the role of androgens in the brain. A sexually dimorphic distribution in the number
of cells containing androgen receptor-like immunoreactivity and a colocalization of androgen
receptors in CCK LI cells in the opossum forebrain would implicate an androgen supported sex
difference in CCK LI in the MPA.
If the levels of estrogen in the brain differ between male and female opossums, then estrogen may drive the sex difference observed for CCK LI in the Monodelphis MPA. One possible mechanism for estrogen's action on CCK LI neurons involves estrogen having a direct effect on that cell's ability to synthesize CCK. In this scenario, a CCK synthesizing cell must contain ER. Estrogen can then enter the cell, bind to its nuclear receptor, causing ER transformation. Receptor transformation allows the estrogen receptor to bind to the neuron's DNA acting as a transcriptional regulator for a variety of genes. One of these genes could be the CCK gene, but it is more likely that transcriptional regulators are transcribed, some of which may regulate expression of the CCK gene. This may be up or down regulation of the CCK gene, depending on the structure of the regulatory portion of the gene and the particular transcriptional regulator. However, direct evidence is lacking for the localization of ER in CCK synthesizing cells.

Immunohistochemical double labeling experiments carried out in our lab have not colocalized ER LI and CCK LI. Both immunoreactivities can be demonstrated in the same tissue section, but not the same cells. To date, no one has reported colocalization of estrogen receptors and CCK in the brains of other species. Technically, this is not a trivial question to answer. Normally, neuropeptides are localized in neuronal fibers. Estrogen receptors are located in the cell nucleus. Thus, ER and CCK may be in the same cell, however the asymmetry of the neuron allows them to be in quite different regions of the brain rendering conventional double label immunohistochemistry less than useful. Colchicine techniques may be used to attempt to maximize the number of neuropeptide immunoreactive cell bodies, but one must always consider the fact that working with colchicine treated animals is less than ideal due to its severe side effects. The best approach to the question of estrogen receptor CCK colocalization is to combine estrogen receptor immunohistochemistry with in situ hybridization for CCK mRNA. Since both
estrogen receptors and CCK mRNA are located in the neuronal somata, if colocalized this technique would allow the detection of both target molecules in an unperturbed system.

An alternative hypothesis suggests that estrogen may have a transsynaptic effect on CCK expression (Fig. 24). In this model, estrogen receptive "interneurons" could alter their output in response to circulating estrogen. These interneurons could respond to changes in circulating estrogen by either changing their firing rate, altering their neurotransmitter/neuromodulator output, or a combination of these factors. A change in the firing rate of an estrogen sensitive neuron that synapses onto a CCK LI neuron could alter the sensitivity of the postsynaptic neuron, facilitating or inhibiting the release of CCK, thereby altering the observed CCK levels in the cell. Alternatively, if the interneuron changes its neurotransmitter/neuromodulator output in response to changing estrogen levels it could activate a second messenger system, and alter the expression of the CCK gene in the postsynaptic cell. This would allow for up or down regulation of the cellular CCK levels.

Finally, one cannot rule out the possibility of direct membrane effects of estrogen on CCK LI neurons. Estrogen has been shown to have electrophysiological effects on neurons in the septum, preoptic area, and amygdala (Kelly et al., 1977; Nabekura, et al., 1986). It is possible that estrogen is having rapid effects on these neurons that does not occur through the classical intracellular receptor. However, the long periods of estrogen exposure needed to observe changes in the rate of CCK release indicates that these rapid effects may not have a significant effect on CCK release.

To test these hypotheses in the opossum, experiments comparing CCK levels in gonadectomized and intact opossums as well as estrogen replacement experiments need to be done. In addition, measurement of CCK mRNA expression with Northern blot analysis and quantitative in situ hybridization in estrogen altered opossums may yield more evidence for the
Figure 24: An illustration of the hypothesized estrogen regulated cholecystokinin immunoreactive circuitry connecting the amygdala (AMY), bed nucleus of the stria terminalis (BST), and medial preoptic area (MPA). In this circuit cholecystokinin (CCK) is at least one of the neurotransmitters/neuromodulators of neurons in this circuit. Estrogen receptors (ER) are found in interneurons in this regions that synapse onto the CCK and possibly other neurons in these regions. In this model estrogen transsynaptically modulates the expression of CCK in this circuit changing its output, thus affecting feeding behavior, gonadotropin secretion, and sexual behavior.
other neurons?

neurons controlling feeding
neurons controlling gonadotropin secretion
neurons controlling sexual behavior

olfactory bulb
regulation of CCK by estrogen. Detailed experiments using in situ hybridization to monitor CCK gene expression in response to estrogen treatment and ER immunohistochemistry to confirm or rule out the presence of ER in CCK expressing cells would also discriminate between direct and indirect modes of regulation.

Although it is technically difficult to rule out rapid membrane effects of estrogen, in the future markers may be discovered that will indicate a cell's ability to respond to estrogen outside of the classical intracellular receptor. Experiments measuring the time course of immediate early gene responses to estrogen treatment may shed some light on this question. If genes like c-fos are expressed in CCK LI cells in the amygdala and bed nucleus within an hour or two after estrogen treatment, rapid membrane effects may be occurring in these regions. However, lack of an immediate early gene response would not rule out membrane effects on CCK in these regions nor would a positive result indicate that rapid estrogen responses are linked to CCK levels in this circuit. Temporal data from the developmental studies indicates that ER immunoreactivity is present in the amygdala, bed nucleus of the stria terminalis, and MPA earlier than CCK LI structures are found there. Interestingly, a sex difference in CCK LI structures is present when CCK immunoreactivity is first expressed in the MPA at 25PN. During development, estrogen may induce temporary, or activational changes in CCK expression via mechanisms described above in the adult (see Fig. 24). However, it is possible that estrogen may also induce more permanent organizational changes in some CCK LI systems in the developing opossum.

To summarize, CCK LI cells and fibers have a widespread distribution in the adult opossum brain and have distinct patterns of developmental expression in many regions. Estrogen receptor-like immunoreactive cells are also found in the adult and developing Monodelphis brain. Although we now understand the anatomy of CCK LI cells and fibers and ER LI cells in the developing brain, the functions of CCK and ER during development remain elusive. Monodelphis
will be an excellent model for studying the roles of these important molecules in brain development.
LITERATURE CITED


ABBREVIATIONS USED IN FIGURES 1, 6, 12, 18, AND TABLE 1

AAA anterior amygdaloid area
ac anterior commissure
ACo anterior cortical amygdaloid nucleus
AD anterodorsal thalamic nucleus
AM anteromedial thalamic nucleus
Amb ambiguus nucleus
Arc arcuate nucleus
AV anteroventral thalamic nucleus
BAA basal amygdaloid nucleus, accessory division
BAM basal amygdaloid nucleus, magnocellular division
BAP basal amygdaloid nucleus, parvocellular division
BSTD bed nucleus of the stria terminalis, dorsal division
BSTM bed nucleus of the stria terminalis, medial division
BSTV bed nucleus of the stria terminalis, ventral division
CA central amygdaloid nucleus
CA1 CA1 field of Ammon’s cortex
CA2 CA2 field of Ammon’s cortex
CA3 CA3 field of Ammon’s cortex
CeS central superior nucleus
CG central gray
CGD central grey, pars dorsalis
CGV  central grey, pars ventralis
CIC  central nucleus of the inferior colliculus
Cl  claustrum
CL  centrolateral thalamic nucleus
CM  central medial thalamic nucleus
CnF  cuneiform nucleus
Cor  cerebral cortex
cp  cerebral peduncle
CP  nucleus of the posterior commissure
CPu  caudate putamen (striatum)
csc  commissure of the superior colliculus
ctg  central tegmental tract
Cu  cuneate nucleus
DA  dorsal hypothalamic area
DG  dentate gyrus
Dk  nucleus of Darkschewitch
DLG  dorsal lateral geniculate nucleus
DLL  dorsal nucleus of the lateral lemniscus
DM  dorsomedial hypothalamic nucleus
DPC  dorsal nucleus of the posterior commissure
DR  dorsal raphe nucleus
DTg  dorsal tegmental nucleus
ec  external capsule
ECIC  external cortex of the inferior colliculus
ECu  external cuneate nucleus
EF   endorhinal fissure
EnP  endopiriform nucleus
Ent  entorhinal cortex
f    fornix
F    nucleus of the fields of Forel
fi   fimbria of the hippocampus
fr   fasciculus retroflexus
GP   globus pallidus
Gr   gracile nucleus
hc   hippocampal commissure
I    intercalated nuclei of the amygdala
IAD  interanterodorsal thalamic nucleus
ic   internal capsule
IC   inferior colliculus
ICj  islands of Calleja
IMD  intermediodorsal thalamic nucleus
IO   inferior olive
IPC  interstitial nucleus of the posterior commissure
IPD  interpeduncular nucleus
La   lateral amygdaloid nucleus
LC   locus coeruleus
LD   laterodorsal thalamic nucleus
LH   lateral hypothalamic area
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Name</th>
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<tbody>
<tr>
<td>LHb</td>
<td>lateral habenular nucleus</td>
</tr>
<tr>
<td>lo</td>
<td>lateral olfactory tract</td>
</tr>
<tr>
<td>LOT</td>
<td>nucleus of the lateral olfactory tract</td>
</tr>
<tr>
<td>LPAM</td>
<td>lateral preoptic area, magnocellular division</td>
</tr>
<tr>
<td>LPO</td>
<td>lateral preoptic area</td>
</tr>
<tr>
<td>LRt</td>
<td>lateral reticular nucleus</td>
</tr>
<tr>
<td>LSD</td>
<td>lateral septal nucleus, dorsal part</td>
</tr>
<tr>
<td>LSI</td>
<td>lateral septal nucleus, intermediate part</td>
</tr>
<tr>
<td>LSV</td>
<td>lateral septal nucleus, ventral part</td>
</tr>
<tr>
<td>LV</td>
<td>lateral ventricle</td>
</tr>
<tr>
<td>MD</td>
<td>mediodorsal thalamic nucleus</td>
</tr>
<tr>
<td>MdD</td>
<td>medullary reticular nucleus, dorsal part</td>
</tr>
<tr>
<td>MdV</td>
<td>medullary reticular nucleus, ventral part</td>
</tr>
<tr>
<td>ME</td>
<td>median eminance</td>
</tr>
<tr>
<td>Me5</td>
<td>mesencephalic trigeminal nucleus</td>
</tr>
<tr>
<td>MeA</td>
<td>medial amygdaloid nucleus, anterior part</td>
</tr>
<tr>
<td>MG</td>
<td>medial geniculate nucleus</td>
</tr>
<tr>
<td>MHB</td>
<td>medial habenular nucleus</td>
</tr>
<tr>
<td>ml</td>
<td>medial lemniscus</td>
</tr>
<tr>
<td>mlf</td>
<td>medial longitudinal fasciculus</td>
</tr>
<tr>
<td>MnPO</td>
<td>median preoptic nucleus</td>
</tr>
<tr>
<td>MnR</td>
<td>median raphe nucleus</td>
</tr>
<tr>
<td>Mo5</td>
<td>motor trigeminal nucleus</td>
</tr>
<tr>
<td>MPA</td>
<td>medial preoptic area</td>
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</table>
MVe  medial vestibular nucleus
OI  olivary nucleus, inferior
opt  optic tract
OS  olivary nucleus, superior
ox  optic chiasm
Pa  paraventricular hypothalamic nucleus
PAA  posterior amygdaloid area
PB  parabrachial nucleus
pc  posterior commissure
PCo  posterior cortical amygdaloid nucleus
Pe  periventricular hypothalamic nucleus
PF  parafascicular thalamic nucleus
PFI  paraflocculus
PH  posterior hypothalamic area
Pir  piriform cortex
PL  posteriolateral thalamic nucleus
PMD  premammillary nucleus, dorsal part
PMV  premammillary nucleus, ventral part
Pn  pontine nuclei
PnV  pontine reticular nucleus, ventral part
Po  posterior thalamic nucleus
Pr5  principal sensory trigeminal nucleus
PrH  prepositus hypoglossal nucleus
PrT  pretectal nucleus
PT  paratenial thalamic nucleus
PV  paraventricular thalamic nucleus
PVA paraventricular thalamic nucleus, anterior part
py  pyramidal tract
Re  reuniens thalamic nucleus
RF  rhinal fissure
Rh  rhomboid thalamic nucleus
ROb raphe obscurus nucleus
RPn  reticular pontine nucleus
Rt  reticular thalamic nucleus
RtTg  reticulotegmental pontine nucleus
S  subiculum
SC  superior colliculus
SCh  suprachiasmatic nucleus
SFi  septofimbrial nucleus
sm  stria medullaris of the thalamus
SO  supraoptic nucleus
sol  solitary tract
Sol  nucleus of the solitary tract
sp5  spinal trigeminal tract
Sp5C  spinal trigeminal nucleus, caudal part
SPF  subparafascicular thalamic nucleus
SPT  subparatenial nucleus
st  stria terminalis
STh  subthalamic nucleus
TS  triangular septal nucleus
Tu  olfactory tubercle
Tz  nucleus of the trapezoid body
VDB  nucleus of the vertical limb of the diagonal band
VL  ventrolateral thalamic nucleus
VLG  ventrolateral geniculate nucleus
VLLd  ventral nucleus of the lateral lemniscus, dorsal part
VLLv  ventral nucleus of the lateral lemniscus, ventral part
VM  ventromedial thalamic nucleus
VMH  ventromedial hypothalamic nucleus
VTA  ventral tegmental area
VTg  ventral tegmental nucleus
ZI  zona inserta
10  dorsal motor nucleus of vagus
12  hypoglossal nucleus
2n  optic nerve
3V  third ventricle
4v  fourth ventricle
5  trigeminal nerve
5n  trigeminal nerve
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