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Immunocytochemical Localization of Gonadotropin-Releasing Hormones in the Brain of a Viviparous Caecilian Amphibian, *Typhlonectes natans* (Amphibia: Gymnophiona)

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Key Words

LHRH · Neuropeptide · Reproduction · Hypothalamus · GnRH · Caecilian · Viviparous · Gymnophiona · Amphibian

Abstract

The molecular forms and brain distribution of gonadotropin-releasing hormone (GnRH) have been well studied in the amphibian orders Urodela (salamanders and newts) and Anura (frogs and toads). In the order Gymnophiona (caecilians), however, few species have been investigated. Antibodies against different molecular forms of GnRH were used to immunohistochemically localize the GnRH-containing neurons in the brain of the caecilian, *Typhlonectes natans* which differs from most other amphibians in that it is viviparous. An antibody selective for mammalian GnRH recognized cell bodies predominantly in the septo-preoptic area but only with occasional cell bodies in the lateral hypothalamus and ventral thalamic eminence. Thick, prominent fibers in the septal region and fibers within the terminal nerve were also labeled. An antibody selective for chicken-II GnRH labeled a population of cell bodies in the dorsal hypothalamus, ventral thalamus and midbrain tegmentum. Thin fibers projected laterally from these cells. An antibody specific for salmon GnRH did not label cell bodies but did show intense terminal field immunoreactivity. The brain of this caecilian, therefore, contains three antigenically distinct forms of GnRH. The mammalian and chicken-II GnRH peptides have been shown in other amphibians but the distribution of cells and fibers was unique in this caecilian.

Introduction

Vertebrate reproduction is controlled primarily by the neuropeptide gonadotropin-releasing hormone (GnRH). Gene duplication and several structural modifications from a putative ancestral peptide have resulted in at least nine different molecular forms of GnRH including mammalian [Matsuo et al., 1971; Burgus et al., 1972], chicken-I [King and Millar, 1982], salmon [Sherwood et al., 1983], chicken-II [Miyamoto et al., 1984], lamprey-I [Sherwood et al., 1986a], dogfish [Lovejoy et al., 1992], catfish [Ngamvongchon et al., 1992], lamprey-III [Sower et al., 1993] and sea bream [Powell et al., 1994] forms. The chicken-II GnRH form (cIIGnRH) is found in all classes of gnathostomes [King and Millar, 1995]. Most vertebrates have at least one other form of GnRH in addition to cIIGnRH.

Multiple peptide forms of GnRH have also been described in anuran and urodele amphibians. Within these two orders, strong evidence exists for the presence of mammalian GnRH-like (mGnRH) immunoreactivity [e.g. Doerr-Schott and Dubois, 1975; Alpert et al., 1976; Doerr-Schott, 1976; Goos et al., 1976; Kubo et al., 1979; Nozaki and Kobayashi, 1979; Jokura and Urano, 1986; Murakami et al., 1992] and cIIGnRH-like immunoreactivity [e.g. Muske and

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Abbreviations

APM	amygdala pars medialis	Med	medulla	TEG	tegmentum
APL	amygdala pars lateralis	MP	medial pallium	ThD	dorsal thalamus
DH	dorsal hypothalamus	MS	medial septum	ThV	ventral thalamus
DP	dorsal pallium	OT	optic tectum	TN	terminal nerve
H	habenula	POA	preoptic area	VH	ventral hypothalamus
LP	lateral pallium	ST	striatum		
LS	lateral septum	TE	thalamic eminence		

Moore, 1990, 1994]. The presence of mammalian and cII GnRH forms in amphibian brains is also supported by physicochemical evidence including high performance liquid chromatography (HPLC), radioimmunoassay (RIA) and amino acid sequencing studies [Rivier et al., 1981; Eiden et al., 1982; King and Millar, 1986; Sherwood et al., 1986b; Conlon et al., 1993; King et al., 1994; Licht et al., 1994; Rastogi et al., 1996]. In addition, some species have a proposed salmonid-like GnRH form [Eiden et al., 1982; Sherwood et al., 1986b; King et al., 1994] or a post-translationally modified mammalian form, hydroxyproline mGnRH [Gautron et al., 1991; King et al., 1994; Iela et al., 1996]. The two main amphibian forms of GnRH, mammalian and chicken-II, are distributed in distinctly different neuronal populations in anuran and urodele brain. The 'anterior group' is located in the nervus terminalis and septo-preoptic area and includes cells and fibers immunoreactive for mGnRH. The 'posterior group', which is located in the posterior diencephalon and anterior midbrain, contains cIIGnRH immunoreactivity [Muske and Moore, 1988]. Anuran and urodele amphibians thus possess multiple forms of GnRH and each form has a unique distribution.

Despite these general similarities, there are interesting differences in the distribution of GnRH forms across amphibian species [e.g. Rastogi et al., 1998]. For example, the salamander *Ambystoma mexicanum* has mGnRH cells in the ependymal lining which could secrete peptide into the cerebral spinal fluid. Additionally, the newt *Triturus vulgaris* has two populations of cIIGnRH cells, whereas most species have only one population [Rastogi et al., 1998]. It has thus far been impossible to detect phylogenetic patterns in the distribution of GnRH forms in the amphibian brain. Study of the third order of amphibians, Gymnophiona, could be useful in this context. The order Gymnophiona has been separated from Anura and Urodela for at least 200 million years [Duellman and Trueb, 1994] and contains about 150 species [Nussbaum and Wilkinson, 1989; Hedges et al., 1993]. These animals (commonly called caecilians) are noteworthy in that more than half the species are viviparous, a life history which is rare in anuran and urodele amphibians [Wake, 1993]. To date, brain distribution of GnRH has been studied in only two other caecilians [Pinelli et al., 1997; Rastogi et al., 1998]. Study of the forms and distribution of GnRH in other caecilian amphibians will allow comparison with other amphibians and provide insight into conserved patterns in GnRH neurochemistry.

Materials and Methods

Adult male (n = 18) and female (n = 12) caecilians (mean ± SEM weight = 27.6 ± 2.04 g; mean ± SEM length = 34.3 ± 0.88 cm) were obtained commercially. In addition, two pregnant females (one with five fetuses of mean length 4.6 cm and one with three fetuses of mean length 12.3 cm) and one fetus were used. Animal care and use procedures were approved by the institution IACUC and conformed to NIH guidelines. Animals were anesthetized (0.02% benzocaine) and decapitated. Brains were fixed in situ (4% paraformaldehyde, 3% sucrose and 7.5% saturated picric acid in 0.1 *M* phosphate buffer, pH = 7.4) for 2–3 h, then removed from skulls and placed in fresh fixative overnight at 4 °C. Frozen sections (50 µm; frontal, sagittal or horizontal) were placed into vials of buffer and stored at 4 °C. Sections were placed alternately between two vials, to yield two matched sets of sections from each brain.

Free-floating sections were stained using procedures of Oka and Ichikawa [1990] and Boyd et al. [1992]. Primary antisera used included: mammalian GnRH antiserum (n = 21 brains, IncStar), chicken-II GnRH antiserum [n = 22 brains, #675 from J. King; King et al., 1994] and salmon GnRH antiserum [n = 3 brains, #1668 from J. King; King et al., 1994]). Sections were treated sequentially as follows (room temperature unless otherwise noted): 1% H_2O_2 in phosphate-buffered saline (PBS; 0.1 *M* phosphate buffer, 0.9% sodium chloride for 15 min); PBS rinse (5 min); 0.1% $NaBH_4$ (15 min); PBS rinses (3×5 min); 20% normal goat serum (NGS for 10 min); incubation in primary antibody (in 0.1 *M* PBS and 0.3% Triton X-100; PBST) at 1:800 dilution. After incubation for 40–48 h at 4 °C, sections were rinsed in PBST (3×10 min); incubated with goat-antirabbit biotinylated secondary antibody (1:200, Vector) for 1 h; rinsed in PBST (3×10 min) and placed in avidin-biotin complex (Vectastain ABC Elite Kit) for 1 h. Sections were rinsed with PBST (3×10 min) and staining visualized with 0.05% diaminobenzidine tetrachloride (Sigma Chemical Co.; in 0.1 *M* phosphate buffer with 0.1% H_2O_2) for 15–17 min followed by subsequent buffer rinses (2×10 min). Sections were mounted, dried (37 °C) and coverslipped with Permount. Specificity controls included

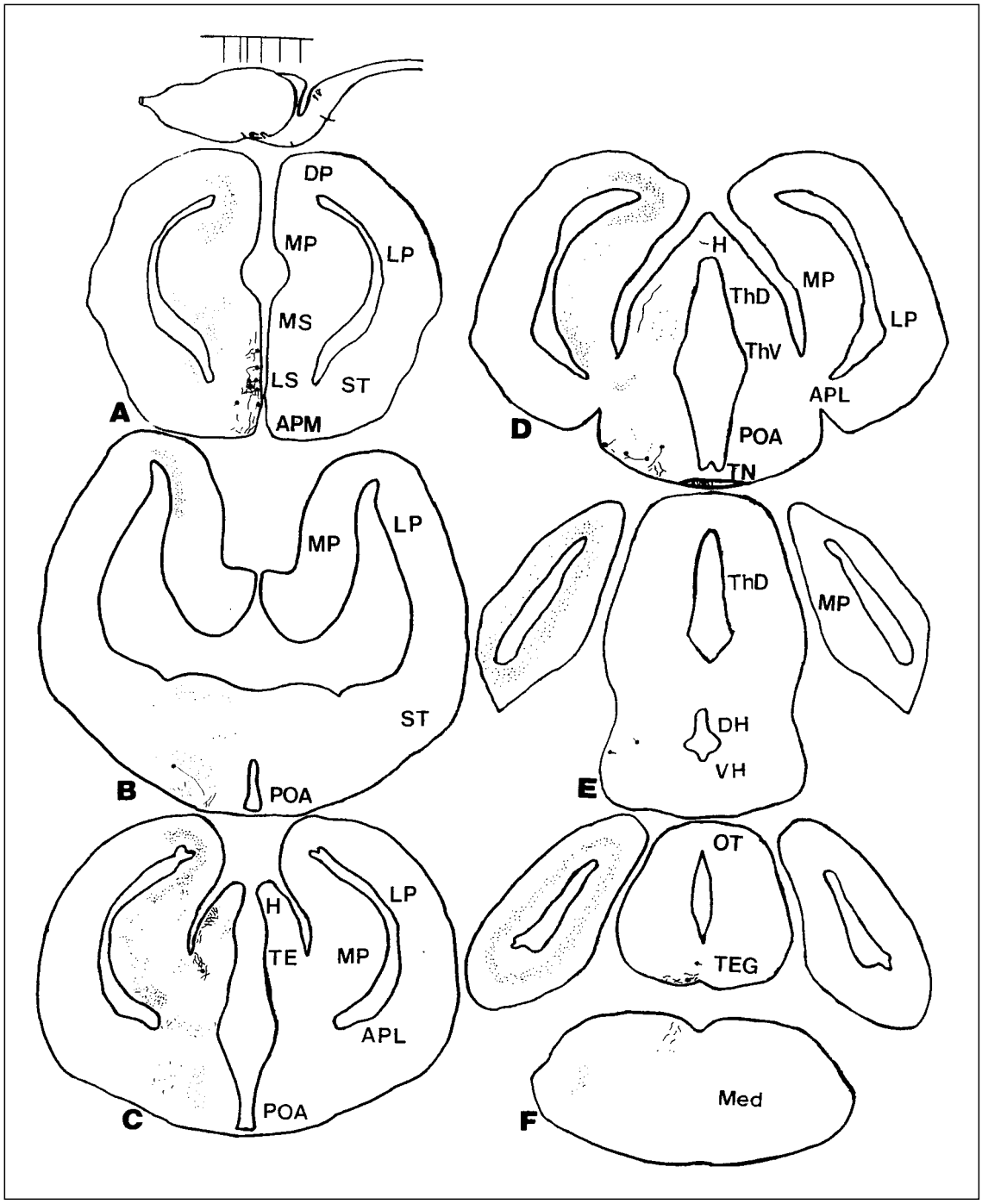


Fig. 1. Camera lucida drawings of the distribution of mammalian GnRH-like immunoreactive cells and fibers (left side) in frontal sections through the caecilian brain (rostral to caudal); neuroanatomy defined on right side (for abbreviations see list). Approximate level of sections is indicated in upper left drawing.

(1) eliminating the primary antibody or (2) incubation with primary antibody preadsorbed for 90 min with 50 μ*M* synthetic GnRH peptides.

Mammalian GnRH-ir cell bodies (n = 68; 6 animals; 4 females: mean ± SEM weight = 23.6 ± 1.94 g and 2 males: 37.0 and 52.5 g) and chicken-II GnRH-ir cell bodies (n = 88; 6 animals; 5 males: mean ± SEM weight = 34.6 ± 5.52 g and 1 female: 28.5 g) were traced using a computerized neuron tracing system (NTS; Eutectic Electronics, Inc.). Cell bodies with an easily identifiable outline and a darkly stained cytoplasm were selected from the septal-preoptic area or midbrain tegmentum. In most cases, the clearest 10–15 cells were selected from one or two 50 μm sections per animal. This sample size represented between 15 and 30% of the total number of stained cells in each animal in each brain area. Mean soma area was computed for each animal. Mammalian GnRH and cIIGnRH soma areas were compared using a t test. There was no significant correlation between cell size and either body length or body weight (Pearson's correlation; mGnRH cell size vs. body length, $r = -0.427$, $n = 6$; mGnRH cell size vs. body weight, $r = -0.196$, $n = 6$; cIIGnRH cell size vs. body length, $r = -0.280$, $n = 5$; cIIGnRH cell size vs. body weight, $r = -0.578$, $n = 5$, all $p > 0.05$). Therefore, absolute cell sizes were used. To determine the number of stained cells, all immunoreactive cells were counted and the number was doubled because only every other section through the brain was used. Neuroanatomy was based primarily on Kuhlenbeck [1922], Northcutt and Kicliter [1980], Wicht and Himstedt [1990], Gonzalez and Smeets [1994] and Hilscher-Conklin et al. [1998].

Results

Mammalian GnRH-Like Immunoreactivity

The septal area of the telencephalon contained the most prominent population of cells that stained with an antibody to mGnRH (fig. 1A, 2A). Cells were located predominantly in lateral septum (approximately 30–40 cells per animal) but a few were found in the dorso-caudal portions of the medial septum [septal terminology of Northcutt and Kicliter, 1980]. A smaller population of cells (approximately 10–15 per animal) was present in the preoptic area (fig. 1B, D, 2E). In the posterior POA, some cells were located more laterally (fig. 2F). A few cells in this group were located along the ventral surface adjacent to the terminal nerve. These forebrain septal and POA populations were clearly observed in all animals. In a few caecilians, one or two cells with mGnRH-like immunoreactivity (mGnRH-ir) were observed in the ventralmost portion of the thalamic eminence (fig. 1C), the lateral hypothalamic area (fig. 1E) and within the midbrain tegmentum (fig. 1F). These cells were morphologically similar to mGnRH immunoreactive cells observed in the septo-preoptic region, and they were not found when antiserum was preadsorbed with mGnRH.

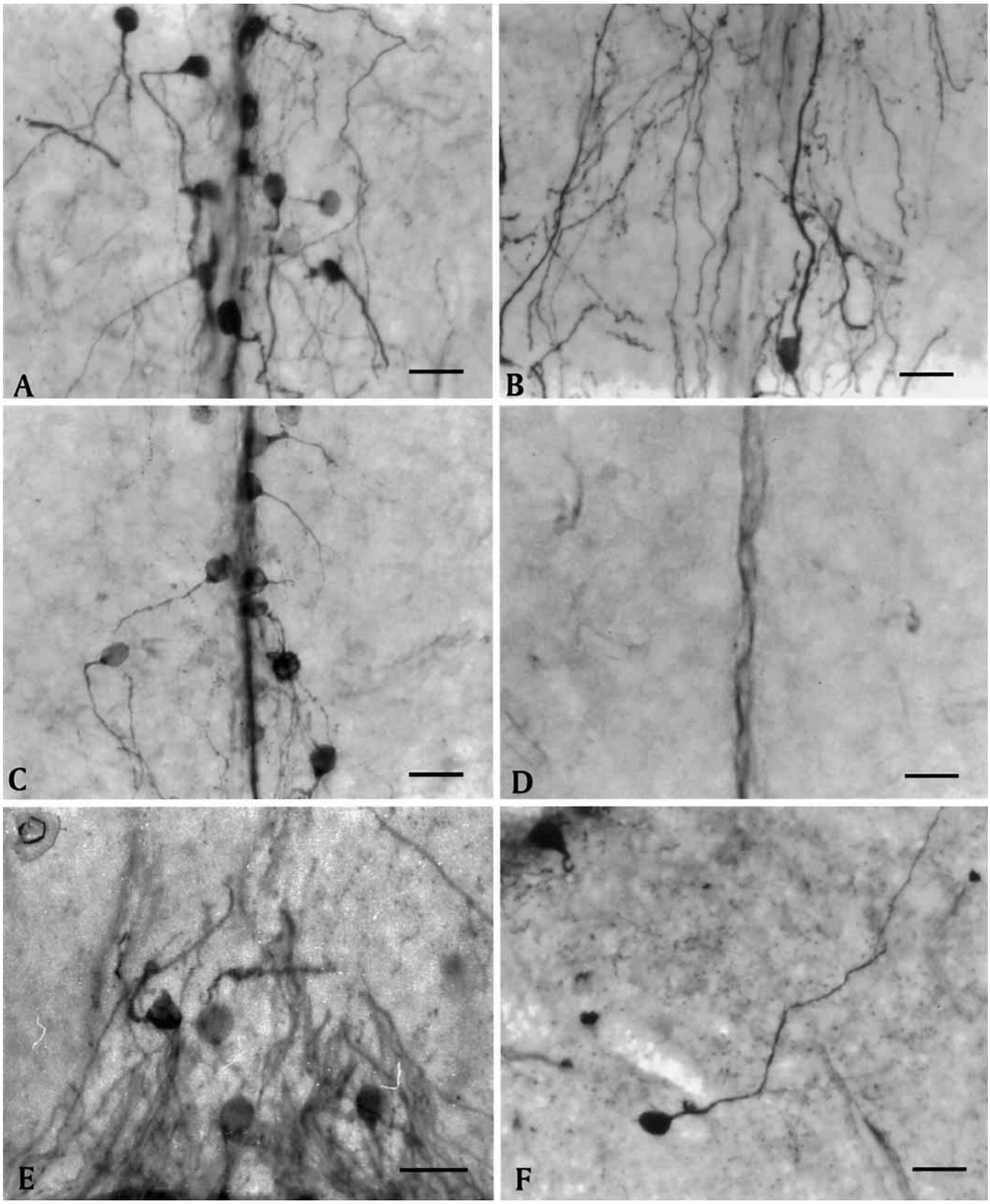
Fibers possessing mGnRH-like immunoreactivity were sparsely distributed in the rostral medial septum but increased dramatically in the lateral septum (fig. 1A). These fibers were thick and oriented in a dorso-ventral pattern toward the median eminence (fig. 2B). Within the anterior POA, a group of fibers projected ventrally, filling a small triangular-shaped area (fig. 2E). Caudally, many fibers were distributed along the ventral surface of the brain adjacent to the terminal nerve. Within the terminal nerve, many small darkly-stained fibers were oriented perpendicular to the nerve's main axis. The terminal nerve entered the brain at the ventral preoptic area (fig. 1D).

Terminal fields with mGnRH-like immunoreactivity were located at most levels of the brain. Rostrally, this punctate staining was present in the medial pallium, lateral portions of medial septum, and posterior portions of the nucleus accumbens (fig. 1A). Medial pallium fields continued throughout the entire telencephalon along with lateral pallium fields in the caudal telencephalon. Sparse terminal field staining was also found in the lateral septum, dorsal pallium, rostral thalamic eminence, habenula, thalamus, and throughout the preoptic area (fig. 1C). A band of immunoreactivity located dorsally to the preoptic area was observed (fig. 1B). Within the posterior midbrain and anterior hindbrain region, dense fields were located in the dorsal nucleus of the area octavolateralis, whereas sparse fields were observed in the trigeminal motor nucleus, medial tegmentum, ventral optic tectum, and dorsal to the raphe nuclei. The qualitative pattern of staining with mGnRH antibody was the same in the pregnant caecilians and fetus. In the fetus [stage 34; Sammouri et al., 1990], however, cell body staining was light and immunostained fibers and terminal fields were rare.

Staining remained robust when mammalian GnRH antiserum was preadsorbed with chicken-II GnRH (fig. 2C), chicken-I GnRH and salmon GnRH (not shown). Staining disappeared when the antibody was preadsorbed with mammalian GnRH (fig. 2D).

Chicken-II GnRH-Like Immunoreactivity

A large population of cells (approximately 50–60 per animal) stained with an antibody to chicken-II GnRH. This population of cells formed a continuum entirely within the caudal diencephalon and anterior midbrain. Within this population, cells were located in the dorsal hypothalamus bordering the third ventricle (fig. 3A). This same population continued caudally where cells were found in the ventral thalamus (fig. 3B, 4A) and finally in the midbrain tegmentum surrounding the mesencephalic ventricle (fig. 3C, 4B). These cells, unlike mGnRH-ir cells, were lightly stained and were present in tight clusters bordering ventricular areas. The soma size of cIIGnRH-ir cell bodies (mean ± SEM area = 314 ± 61.3 μm^2) was significantly greater (t test; $p < 0.05$) than mGnRH-ir cell bodies (mean ± SEM area = 238 ± 19.7 μm^2).


A
B
C
D
E
F

Chicken-II GnRH-like immunoreactive (cIIGnRH-ir) fibers were thinner and possessed more varicosities than mGnRH fibers. They were sparsely distributed in the septal region of the telencephalon. Within the midbrain, fibers that extended from cell bodies coursed laterally away from the ventricles. Terminal field distribution overlapped with that of mGnRH-like staining. Staining remained robust when chicken-II GnRH antiserum was preadsorbed with mammalian GnRH (fig. 4C), chicken-I GnRH and salmon GnRH (not shown). Staining was eliminated when the antibody was preadsorbed with chicken-II GnRH (fig. 4D).

Salmon GnRH-Like Immunoreactivity

Specific salmon GnRH-like immunoreactivity (sGnRH-ir) was not observed in any cell bodies. However, sGnRH-ir fibers were abundantly distributed in septal areas. The morphology of these fibers more closely resembled cIIGnRH-like fibers than mGnRH-like fibers. The distribution of sGnRH-like immunoreactive terminal fields was broad and very similar to the field distributions of mGnRH and cIIGnRH, especially in the forebrain (fig. 5). Preadsorption of the sGnRH antiserum with synthetic mGnRH and cIIGnRH antigens resulted in slightly reduced staining and this was more apparent with cIIGnRH preadsorption than with mGnRH preadsorption. When the sGnRH antibody was preadsorbed with mGnRH, only staining in the POA disappeared. Preadsorption with salmon GnRH eliminated all staining.

Discussion

The brain of this viviparous caecilian amphibian, *T. natans*, contained clear immunoreactivity for two different forms of GnRH and also a possible third form. The distribution of mGnRH-like immunoreactivity and cIIGnRH-like immunoreactivity was broadly similar to the pattern found in other amphibians, and in vertebrates in general, but the specific distribution was unique to this species. This is the first

Fig. 2. Photomicrographs of mammalian GnRH-like immunoreactivity in the caecilian brain (frontal sections). **A** mGnRH-ir cell bodies in the septal region; **B** mGnRH-ir fibers coursing in a dorso-ventral orientation in the septum; **C** staining in the septal region when mammalian GnRH antiserum was preadsorbed with chicken-II GnRH; **D** background staining in the septal region when mammalian GnRH antiserum was preadsorbed with mammalian GnRH; **E** mGnRH-ir cell bodies in the anterior preoptic area; **F** mGnRH-ir cell bodies in the lateral preoptic area. Scale bar = 50 µm.

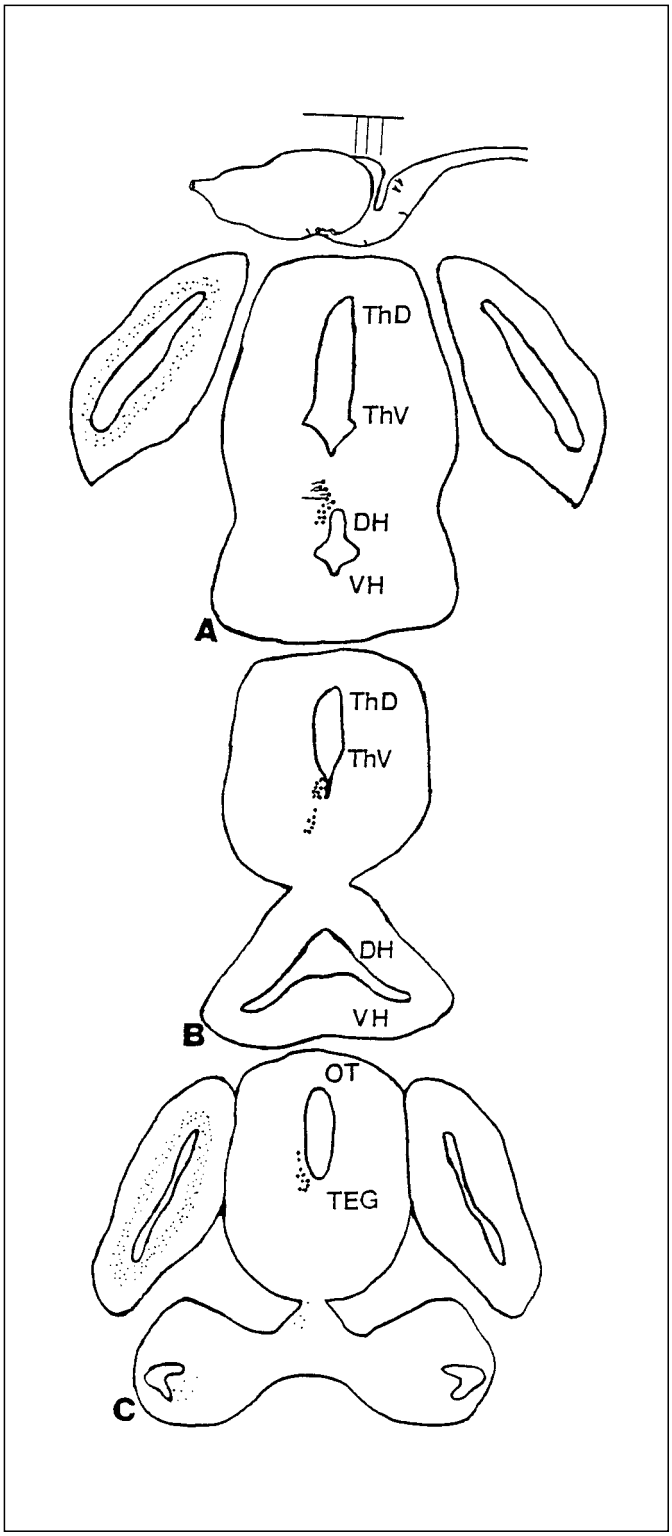


Fig. 3. Camera lucida drawings of the distribution of chicken-II GnRH immunoreactive cells and fibers (left side) in frontal sections through the caecilian brain (rostral to caudal); neuroanatomy defined on the right side (for abbreviations see list). Approximate level of the sections is indicated in the side-view drawing at the upper center.

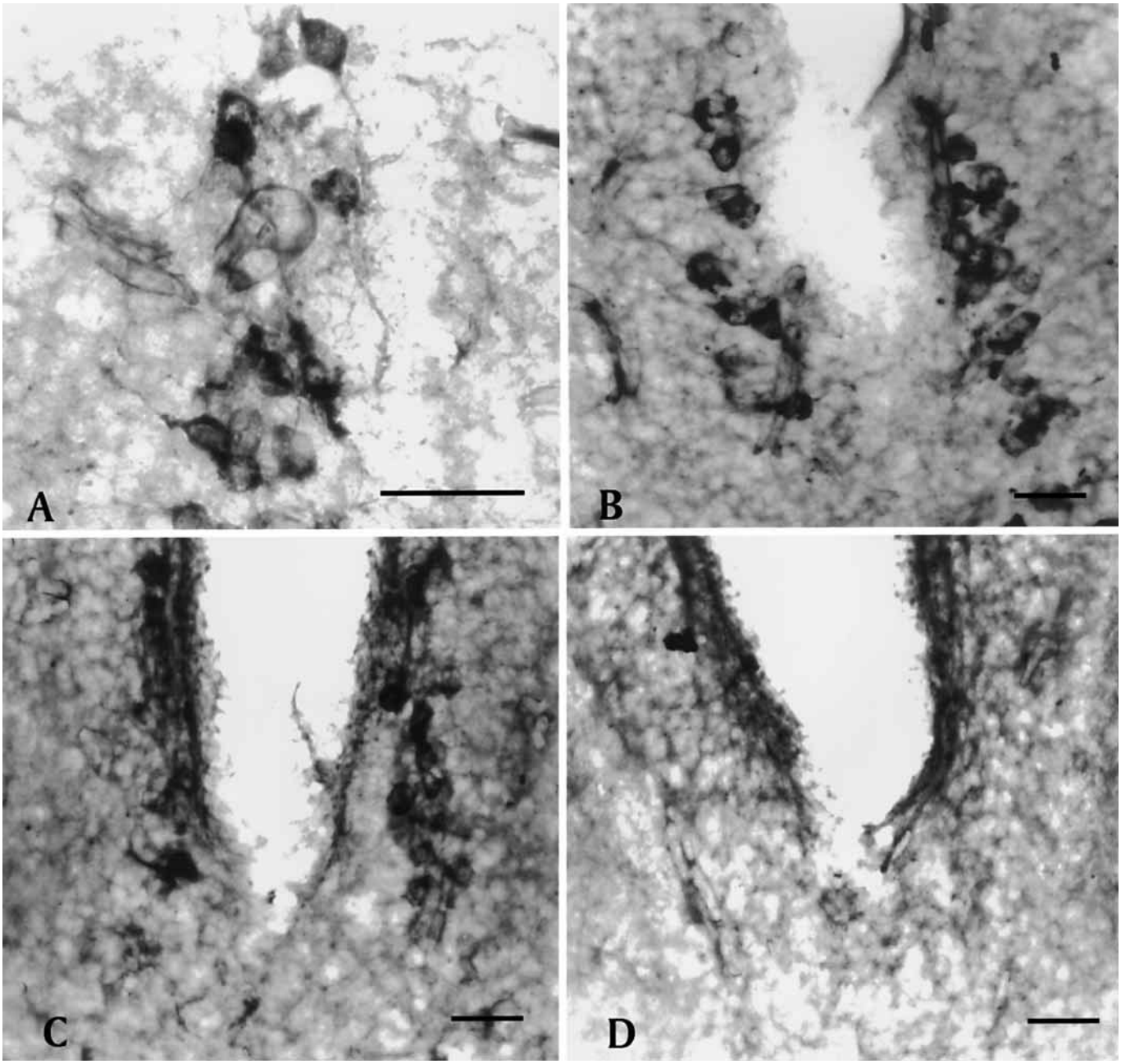


Fig. 4. Photomicrographs of chicken-II GnRH immunoreactivity in the caecilian brain. **A** cIIGnRH-ir cell bodies in the ventral thalamus of the midbrain; **B** cIIGnRH-ir cell bodies in the midbrain-tegmental region; **C** staining in the ventral thalamus when chicken-II GnRH antiserum was preadsorbed with mammalian GnRH; **D** background staining in the ventral thalamus when chicken-II GnRH antiserum was preadsorbed with chicken-II GnRH. Scale bar = 50 µm.

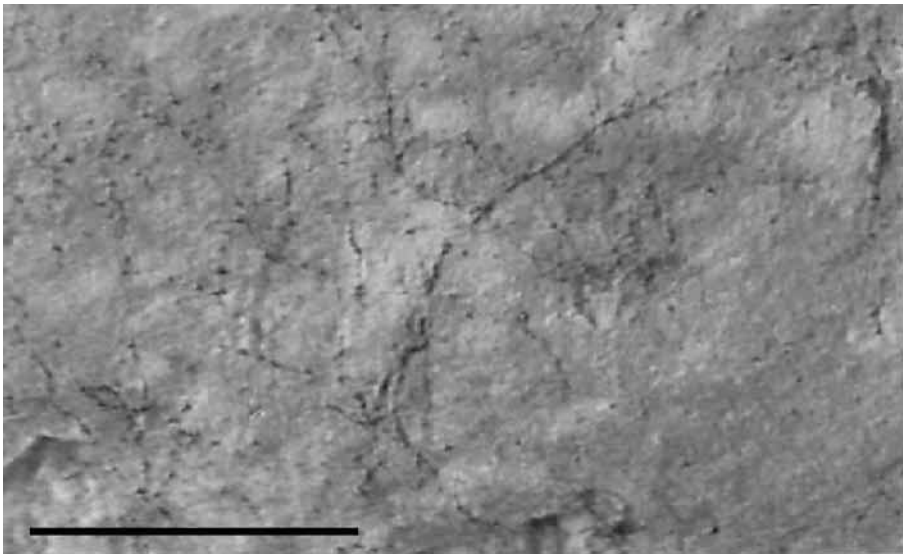


Fig. 5. Photomicrograph of sGnRH-like immunoreactive fibers and terminal fields in the medial pallium of the caecilian forebrain. Scale bar = 50 µm.

reported evidence for salmon GnRH-like immunoreactivity in a caecilian amphibian and the first time that the distribution of sGnRH terminal fields has been described for any amphibian.

The most prominent population of stained cells in this caecilian contained mGnRH-like immunoreactivity. This form of GnRH has been found in many anuran and urodele amphibian species using HPLC and RIA techniques or immunocytochemistry [Rastogi et al., 1998]. The gene for mGnRH has been cloned in *Xenopus laevis* [Hayes et al., 1994]. Evidence for the presence of this form in amphibian brain is thus strong. Mammalian GnRH is, of course, also found in mammals [review: Chieffi et al., 1991] and fish [Leischeid et al., 1995]. It has never been identified chemically in agnathans, reptiles or birds.

The distribution of mGnRH-ir in *T. natans* was similar to other amphibians with a few notable differences. The common pattern in anurans and urodeles, with cell bodies primarily located in the septo-preoptic regions [e.g. Doerr-Schott, 1976; Crim, 1985; Muske and Moore, 1994; Rastogi et al., 1998], was also found in this caecilian. Additionally, fibers projecting toward the median eminence and associated with the terminal nerve were observed in *T. natans*, as they have been in other amphibians. This ventral forebrain distribution is also found in other vertebrates possessing mGnRH [Chieffi et al., 1991]. The brain distribution of mGnRH-like immunoreactivity has been described for two other caecilian amphibians, the oviparous species *Ichthyophis beddomei* [Pinelli et al., 1997] and the viviparous species *T. compressicauda* [Rastogi et al., 1998]. In both of those species, an extremely broad rostro-caudal distribution of mGnRH cell bodies was observed. We observed a more restricted distribution in *T. natans* and did not find mGnRH cell bodies in the olfactory bulb and rostral telencephalon. Instead, in *T. natans* cells were found predominantly in the lateral septum. Our designation of this area as 'lateral septum' is based on the terminology of Northcutt and Kicliter [1980], who specifically describe the unique anatomy of the caecilian septal nuclei. This same region has been called the medial septum in the other two caecilian species [Pinelli et al., 1997; Rastogi et al., 1998]. We found scattered mGnRH cell bodies in the amygdala pars medialis and anterior preoptic area, as observed in the other two caecilian species. Finally, we found mGnRH cell bodies in three areas where such cells have not been described in caecilians: thalamic eminence, lateral hypothalamic area and midbrain tegmentum. Cells in the thalamic eminence of this caecilian might be homologous to the habenular cells of X. laevis [Rastogi et al., 1998]. Hypothalamic mGnRH cells have been occasionally described in other amphibians [Hayes et al., 1994; Somoza et al., 1996; Rastogi et al., 1998]. This is the first demonstration of mGnRH-ir cells in the midbrain for any amphibian. In summary, the distribution of mGnRH-like immunoreactive cells in this caecilian has a more caudal bias than previously described for caecilians or most other amphibians. The location of cells in diverse areas (e.g. septum and tegmentum) makes it unlikely that afferent input to all these cells is the same, but each area where we found mGnRH cells has been shown to receive terminal nerve input in other amphibians [Hofmann and Meyer, 1989; Schmidt and Wake, 1990].

This caecilian also possessed a population of cells immunoreactive for cIIGnRH. This molecular form of GnRH is found in most anuran [King and Millar, 1986; Conlon et al., 1993; King et al., 1994; Rastogi et al., 1996, 1998] and urodele species [Muske and Moore, 1994; Battisti et al., 1997]. All gnathostomes examined have cIIGnRH, which is considered the most conserved form in GnRH evolution [review: Muske, 1993]. Chicken-II GnRH immunoreactive cell bodies are usually found almost exclusively within the midbrain region of other vertebrates, including mammals [Millam et al., 1989; Amano et al., 1991; Dellovade et al., 1993; Lescheid et al., 1997; Urbanski et al., 1999]. The presence of a second form of GnRH, the likely identity of that form as chicken-II GnRH, and the general midbrain distribution of cell bodies that we found in the caecilian *T. natans,* was thus typical for gnathostomes. On the other hand, our finding of cIIGnRH-like immunoreactive cell bodies in the diencephalon was unusual. Such cells were not found in the other two caecilian species, *I. beddomei* and *T. compressicauda,* previously studied [Pinelli et al., 1997; Rastogi et al., 1998] and have only been reported in one anuran, *R. ridibunda* [Conlon et al., 1993; Collin et al., 1995]. We found cIIGnRH-ir cell bodies in a continuum from the dorsal hypothalamus, through the ventral thalamus, and into the midbrain ventral tegmentum. This broader distribution, including cIIGnRH-ir cells in the infundibular hypothalamus, paraventricular organ, and posterior tubercle, has been seen in several urodele amphibians [Muske and Moore, 1994; Northcutt and Muske, 1994; Rastogi et al., 1998]. Therefore, the distribution of cIIGnRH cell bodies in this caecilian is more similar to the distribution in urodeles than in other caecilians or in anurans.

Finally, we found immunologic evidence for a possible third form of GnRH in the brain of this caecilian amphibian. Salmon GnRH-like immunoreactivity was observed in several brain regions. This form has not been previously found in any caecilian brain. There is HPLC and RIA evidence for the presence of sGnRH in several anuran species [*R. catesbeiana,* Eiden et al., 1982; *R. esculenta,* Cariello et al., 1989; Fasano et al., 1993; *R. pipiens* and *H. regilla,* Sherwood et al., 1986b; *X. laevis,* King and Millar, 1986; King et al., 1994] and urodeles species [*T. granulosa* and *A. gracile,* Sherwood et al., 1986b]. This form predominates throughout the telencephalon of osteichthyes [Amano et al., 1991] and has also been identified in cartilaginous fish [Wright and Demski, 1991] and reptiles [D'Aniello et al., 1994]. In amphibians, the brain distribution of salmon GnRH-immunoreactive cells, fibers, or terminal fields has not been previously reported. In this caecilian brain, we found prominent fibers and terminal fields at most brain levels. The distribution of sGnRH-ir terminal fields overlapped substantially with mGnRH and cIIGnRH terminal fields. It is unlikely that this overlap resulted entirely from cross-reactivity of our antibodies because each was highly specific. The sGnRH antibody (King #1668), in particular, shows less than 1% cross-reactivity with mGnRH or cIIGnRH [King et al., 1994]. The sGnRH antibody did not specifically stain cell bodies in the caecilian septum or tegmentum where high levels of expression of mGnRH and cIIGnRH were seen with their respective specific antibodies. The level of cross-reactivity of the sGnRH antibody must thus be minimal. Although the sGnRH antibody was specific, in that it did not recognize mGnRH and cIIGnRH appreciably, the immunoreactive material in the caecilian brain might not be identical to sGnRH. In *X. laevis* brain, a form of GnRH which is similar but not identical to sGnRH has been found [King et al., 1994]. Interestingly, we found no cell bodies stained with sGnRH antibody. Staining of terminal fields, but not cell bodies, could reflect a peptide processing event that occurs only in terminals or it could be that levels of sGnRH synthesis are so low that the peptide is detected only with a buildup in the terminals. The finding of a possible third GnRH form within a single species' brain has been shown previously in such nonmammals as the amphibian *X. laevis* [King et al., 1994], gilthead sea bream [Gothilf et al., 1996] and African cichlid [White et al., 1995]. Molecular cloning of GnRH forms in the caecilian brain would establish the number and identity of those peptides conclusively.

The septo-preoptic population of mGnRH-ir cells is clearly linked to control of gonadotropin release in many vertebrates [review Chieffi et al., 1991]. Caecilian amphibians possess a pituitary gland with histological structure similar to other amphibians and cells that contain mammalian LH-like immunoreactive material [Schubert et al., 1977; Doerr-Schott and Zuber-Vogeli, 1984; Masood-Parveez et al., 1994]. It is thus likely that diencephalic GnRH in caecilians controls pituitary gonadotropin production, although neither the identity of gonadotropins nor hypothalamic control of the pituitary has been shown in any caecilian. Further, the distribution of tyrosine hydroxylase immunoreactivity and the presence of a cholinergic input to the POA in other caecilian species supports homology between caecilian gonadotropin control mechanisms and those in other vertebrates [Welsch et al., 1976; Peter et al., 1990; Gonzalez and Smeets, 1994]. Although evidence for mGnRH involvement in control of the pituitary of amphibians is strongest, there is also evidence that cIIGnRH acts at the pituitary level in frogs [Licht et al., 1994] and that reproductive functions can be exclusive of terminal nerve GnRH neurons in fish [Oka and Matsushima, 1993; Kobayashi et

al., 1994]. The contributions of the three forms of GnRH to pituitary control in caecilians thus remains to be determined.

In this caecilian, cIIGnRH-ir cell body size was significantly larger than mGnRH-ir cell body size. Chicken II GnRH-ir cells are proposed to play a role in reproductive behaviors in some vertebrates [King and Millar, 1995], but there is no evidence for this in amphibians. In the frog *R. esculenta*, concentrations of mGnRH and cIIGnRH vary according to reproductive status with the highest levels of both in the prebreeding period [Rastogi et al., 1996]. In several teleosts as well, GnRH cell size correlates with reproductive status. For example, in plainfin midshipman, GnRH cell size differences in the POA are related to the onset of sexual maturation [Grober et al., 1994]. In *Haplochromis burtoni*, GnRH soma sizes for cells projecting to the pituitary increase in territorial males [Bushnik and Fernald, 1995]. Differences in cell body size are often associated with differences in cell activity for neuroendocrine cells [Kalimo, 1975; Subhedar and Krishna, 1990; Chaturvedi et al., 1994]. Differences in the size of mGnRH cells and cIIGnRH cells in this caecilian could therefore reflect variability in cell activity and indicate different functions for the two forms of the peptide. Our estimates of GnRH-ir cell numbers also differ from published reports. We found 50–60 mGnRH cells in *T. natans* whereas Pinelli et al. [1997] report between two and three times more mGnRH cells in the caecilian *I. beddomei*. However, *T. natans* possessed three to four times more cIIGnRH cells than in *I. beddomei*. The frog *X. laevis* has been reported to have 200–300 mGnRH-ir cells [Hayes et al., 1994; Setalo, 1996]. Although reproductive condition of the animal and technique affect the number of stained cells, caecilians, in general, might have fewer GnRH neurons than other amphibians.

This caecilian amphibian, *T. natans*, is viviparous and it shares this characteristic with about half of all caecilian species [Wake, 1993]. Viviparity is otherwise rare in amphibians. Because the function of different forms of GnRH is poorly understood in all amphibians, the importance of GnRH in the evolution of viviparity is unclear. There were no large differences in the GnRH system of this viviparous species compared to oviparous amphibians which could obviously account for the difference in reproductive mode. It is likely, however, that viviparous caecilians will have unique mechanisms for the control of GnRH synthesis and secretion, compared to oviparous species. We are currently investigating, for example, whether caecilian fetuses produce hormones that could inhibit the GnRH system of pregnant females.

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