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Neuroanatomical Distribution of Chicken-I Gonadotropin-Releasing Hormone (cGnRH-I) in the Brain of the Male Red-Sided Garter Snake

Key Words

Terminal nerve
LHRH
GnRH
Garter snake
Thamnophis
Immunocytochemistry

Abstract

Immunocytochemistry was used to investigate the neuroanatomical distribution of the chicken-I form of gonadotropin-releasing hormone (cGnRH-I) in reproductively active, male, red-sided garter snakes (*Thamnophis sirtalis parietalis*). Cell bodies with cGnRH-I immunoreactivity (ir) were found in the terminal nerve ganglion, nucleus of the diagonal band of Broca, medial preoptic area, and the hypothalamus. Fibers containing cGnRH-Iir were distributed in the following brain areas. Within the olfactory bulb, fibers were found in the internal plexiform, mitral and glomerular cell layers, as well as in the terminal nerve; within the forebrain, fibers were observed in the diagonal band of Broca, rostral and lateral septum, lateral pallium, retrobulbar region pars dorsomedialis, nucleus accumbens, medial preoptic area, hippocampal commissure, amygdala, posterior dorsal ventricular ridge, hypothalamus, median eminence, and the thalamus; within the midbrain, fibers were found in the interpeduncular nucleus and the stratum album periventricular of the optic tectum. This study shows that the distribution of cell bodies for cGnRH-Iir in this reptile is consistent with the distribution of immunoreactivity for cGnRH-I in birds and mammalian GnRH in amphibians and mammals. Using antiserum specific to cGnRH-I, the endogenous form of GnRH, this is the first study to show that the terminal nerve in a reptile contains GnRH immunoreactivity.

Introduction

Members of the gonadotropin-releasing hormone (GnRH) family of decapeptides are found in the brains of many vertebrates, frequently with more than one form in a given species [Sherwood, 1987; Sherwood et al., 1993; Muske and Moore, 1994]. In roughskin newts (*Taricha granulosa*), mice (*Mus musculus*), and other species, studies suggest that during embryonic development, GnRH-

containing neurons migrate from the olfactory placode to specific positions in the forebrain, including the terminal nerve (TN), septal and pre-optic areas [Muske and Moore, 1988; Schwanzel-Fukuda and Pfaff, 1989; Wray et al., 1989]. Other evidence indicates that the GnRH-containing cell bodies in the midbrain do not originate embryonically in the olfactory placode but rather arise independently [Muske and Moore, 1990; Witkin, 1990; Northcutt and Muske, 1991; Muske, 1993]. There are many unanswered

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A	amygdala
AC	anterior commissure
ADVR	anterior dorsal ventricular ridge
AOT	accessory olfactory tract
DB	diagonal band of Broca
DC	dorsal cortex
DP	dorsal pallium
ep	external plexiform layer
gl	glomerular cell layer
H	hypothalamus
ig	internal granular layer
ip	internal plexiform layer
IP	interpeduncular nucleus
L	lateral pallium
LFB	lateral forebrain bundle
LPOA	lateral preoptic area
m	mitral cell layer
MC	medial cortex
ME	median eminence
MFB	medial forebrain bundle
MLF	medial longitudinal fasciculus
MOT	medial olfactory tract
mPOA	medial preoptic area
NA	nucleus accumbens
nDB	nucleus of the diagonal band of Broca
NHC	nucleus of the hippocampal commissure
NS	nucleus sphericus
OC	optic chiasm
OT	optic tract
Ot	olfactory tubercle
OV	olfactory ventricle
PDVR	posterior dorsal ventricular ridge
PT	pretectal nucleus
RDM	retrobulbar region pars dorsomedialis
SAC	stratum album centrale
SAP	stratum album periventriculare
SCN	suprachiasmatic nucleus
Sd	dorsal septum
SFGS	stratum fibrosum et griseum superficiale
SGC	stratum griseum centrale
Sl	lateral septum
Sm	medial septum
SO	stratum opticum
Sr	rostral septum
T	thalamus
TN	terminal nerve
TNg	terminal nerve ganglion
VnN	vomeronasal nerve

questions concerning the functions and neuroanatomical distributions of GnRH cells in the forebrain and midbrain.

The GnRH-containing cells found in the septal and preoptic areas send fibers to the median eminence and regulate gonadotropin release from the anterior pituitary. Release of follicle-stimulating hormone (FSH) and luteinizing hormone (LH) from the anterior pituitary, will, in turn, control a variety of reproductive functions, including the synthesis of gonadal steroids, gametogenesis, and ovulation [Hoffman et al., 1992].

Gonadotropin-releasing hormone also appears to have other functions besides regulating FSH and LH secretion. It has been demonstrated that GnRH administration can enhance the sex behavior of newts (*T. granulosa*), voles (*Microtus canicaudus*), rats (*Rattus rattus*), frogs (*Xenopus laevis*), green anole lizards (*Anolis carolinensis*), and horses [Moore et al., 1982; Boyd and Moore, 1985; Moss and McCann, 1973; Kelly, 1982; Alderete et al., 1980; McDonnell et al., 1989]. It has also been shown that GnRH levels change in specific brain areas in response to reproductive cues. The GnRH concentration in the posterior olfactory bulb of female voles (*Microtus ochrogaster*) increases in response to a male urine cue; the number of mast cells with GnRHir in the medial habenula of ring doves (*Streptopelia roseogrisea*) increases following two hours of courtship; and the GnRH concentration in the terminal nerve of *T. granulosa* increases in response to mating [Dluzen et al., 1981; Dluzen and Ramirez, 1987; Propper and Moore, 1991; Zhuang et al., 1993]. These extra-hypothalamic changes in GnRH, as well as the direct effects of GnRH on behaviors, indicate the GnRH may function as a neurotransmitter in the brain. There is evidence that in the sympathetic ganglia of the bullfrog (*Rana catesbeiana*), GnRH functions as a neurotransmitter, regulating potassium channels and the slow excitatory post-synaptic potential [Jan et al., 1979; Jones, 1987].

The distribution of GnRH in the brain is much wider than would be predicted if GnRH only controlled gonadotropin release. In general, GnRHir in the forebrain system is found in cell bodies in the terminal nerve, septal structures, preoptic area, diagonal band of Broca, and the stria terminalis [for reviews see Silverman, 1988; Kuenzel and Blasher, 1991]. In the midbrain, GnRHir cell bodies are found in the midbrain tegmentum, oculomotor complex, and the posterior tubercle of the vertebrates that have been studied [for review see Muske, 1993]. Prior to the current study, immunocytochemical studies had described the distribution of GnRHir in two species of reptiles. Using antisera for mammalian GnRH, the rat snake (*Elaphe climacophora*) was found to have GnRHir cell bodies in the me-

dial septal-preoptic region and hippocampus [Nozaki et al., 1984]. Antisera for salmon GnRH demonstrated that the chameleon (*Chameleon chameleo*) has GnRHir cell bodies in the midbrain tegmentum bordering nucleus fasciculus longitudinalis medialis and along the dorsolateral part of the oculomotor nucleus [Bennis et al., 1989]. Neither study of reptiles described GnRHir in the terminal nerve, even though the terminal nerve exists in reptiles [Johnston, 1913].

With the use of HPLC, the alligator (*Alligator mississippiensis*) has been shown to have immunoreactivity to both the chicken-I and chicken-II forms of GnRH [Lovejoy et al., 1991]. Sherwood and Whittier [1988] found that the red-sided garter snake (*T. s. parietalis*) has one major HPLC peak that elutes with the same retention time as, and cross-reacts with, antisera to chicken-I GnRH. Based on these HPLC studies in reptiles, as well as more detailed studies of GnRH's in birds, it seems likely that chicken-I and chicken-II GnRH's occur in reptiles [Silverman, 1988; Millam et al., 1991; for review see Muske, 1993]. In addition, considering that the neuroanatomical distribution of cGnRH-I in birds is consistent with the neuroanatomical distributions of salmon GnRH in teleosts and mammalian GnRH in amphibians and mammals, our working hypothesis is that the distribution of cGnRH-I in *Thamnophis sirtalis parietalis* will match that of the forebrain GnRH systems of other vertebrates [Muske and Moore, 1988; Silverman, 1988; Oka and Ichikawa, 1990; Millam et al., 1991; Muske, 1993]. We tested this hypothesis by using immunocytochemical procedures and an antiserum highly specific for chicken GnRH-I.

Materials and Methods

Reproductively active, adult, male, red-sided garter snakes (*Thamnophis sirtalis parietalis*) were collected in Manitoba, Canada during early May. Males were transported back to a field station and were housed in outdoor pens measuring 1.2 m × 1.2 m × 0.9 m. Each pen contained approximately 100 males and 3–5 unmated females. Courting males were removed from the backs of an unmated female and sacrificed by rapid decapitation within 24 hours of capture. Brains were immediately removed and fixed for 24 hours in a fixative that contained 4% paraformaldehyde, 3% sucrose, 7.5% saturated picric acid solution in 0.1 M sodium phosphate buffer. Brains were then cycled through two washes of 12 h each in 0.1 M sodium phosphate buffer. Finally, brains were washed for 12 h with, and subsequently stored in, 0.1 M sodium phosphate buffer that contained 30% sucrose and 0.1% sodium azide.

Several GnRH antisera were screened for this study (EL-14, Stienier 540, Arimura 720, King 5348, and Millam's chicken I and II antibodies). Based on sensitivity, specificity, and previous work showing that cGnRH-I exists in the brain of the red-sided garter snake,

chicken-I antibody (generously supplied by J. Millam, University of California, Davis) was chosen for these studies [Sherwood and Whittier, 1988; Millam et al., 1993]. This chicken-I antibody has a low degree of cross-reactivity for other forms of GnRH. It is 1,000 times more specific for chicken-I than chicken-II in immunocytochemistry, and has less than 0.5% cross-reactivity for mammalian, salmon, or lamprey GnRH in radioimmunoassay [Millam et al., 1989, 1993]. A series of preliminary studies to optimize the conditions for immunocytochemistry using this cGnRH-I antibody in a snake brain were performed. In addition, a chicken-II antibody (generously supplied by J. Millam, University of California, Davis) was used to determine if reproductively active males possessed immunoreactivity to this form of the peptide. Fixed brains (n = 7 for cGnRH-I and n = 2 for cGnRH-II) were frozen and embedded in Tissue-Tek® O.C.T. Compound. Sections were cut at 25 µm in the transverse (n = 6) or horizontal (n = 1) plane in a cryostat at -16 °C and thaw-mounted serially onto sets of four gelatin coated slides. Slides were stored at -20 °C until immunocytochemistry was performed using an avidin-biotin immunoperoxidase protocol (Vectastain ABC kit, Vector Laboratories, Burlingame, CA). Sections were washed twice with phosphate buffered saline (PBS) and then serially exposed to 1% H₂O₂ in PBS, 10% normal goat serum, and the primary antibody (diluted 1:4,000 in 0.1% Triton-X-100 in PBS) overnight, followed by biotinylated goat anti-rabbit IgG (diluted 1:225) for 60 min at 4 °C, and an avidin-biotin-horse radish peroxidase complex. Sections were washed twice in PBS between each of the steps. Immunoreactive GnRH was visualized with a solution that contained 0.01% H₂O₂, 0.025% diaminobenzidine, and 0.04% NiCl₂ for 5–7 min. The reaction was terminated through washing two times in dechlorinated H₂O. All antibody incubations were performed at 4 °C and other incubations at room temperature. Sections were counterstained with 1% methyl green, dehydrated, and mounted with cover slips.

Control experiments consisted of incubation of the chicken-I antibody with PBS alone or PBS with 100 µM cGnRH-I (Sigma Chemical Co., St. Louis, MO) prior to staining. Additional controls for non-specific binding were replacement of either the primary or secondary antibody with incubation buffer. Specific immunoreactive staining was completely absent when the primary antibody was preincubated with 100 µM cGnRH-I and when the primary or secondary antisera was replaced with PBS (fig. 1A, C). Nomenclature and identification of neuroanatomical structures were based on descriptions of ophidian telencephala and tract tracing studies of the visual system [Halpern, 1980; Dacey and Ulinski, 1986; see Abbreviations].

The original research reported herein was performed under the authority of Manitoba Wildlife Scientific Permits No. WSP 02-94 and Oregon State University Institutional Animal Care and Use Committee Protocol No. 1491. All research reported herein was conducted in accord with the U.S. Public Health Service 'Policy on Humane Care and Use of Laboratory Animals' and the National Institutes of Health 'Guide to the Care and Use of Laboratory Animals'.

Results

The cGnRH-Iir cell bodies and fibers were distributed widely throughout the brain of the red-sided garter snake and are described in detail below.

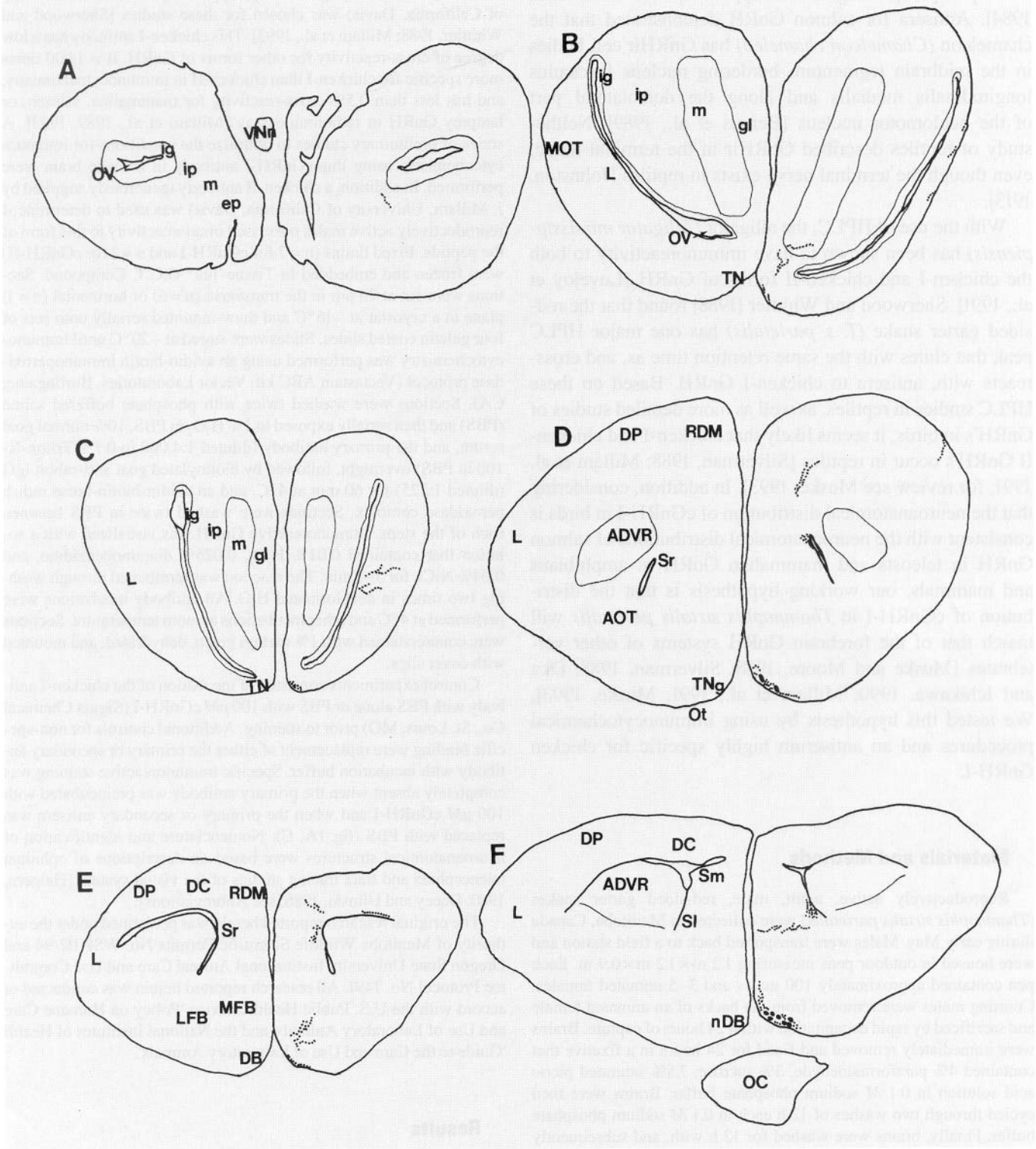
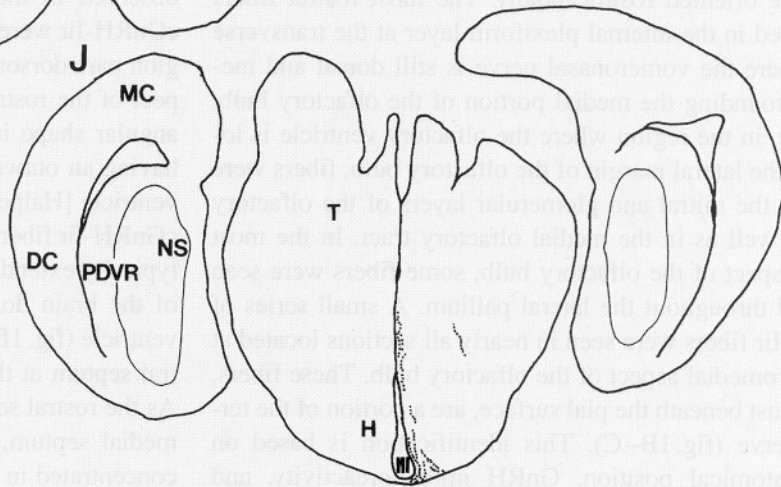
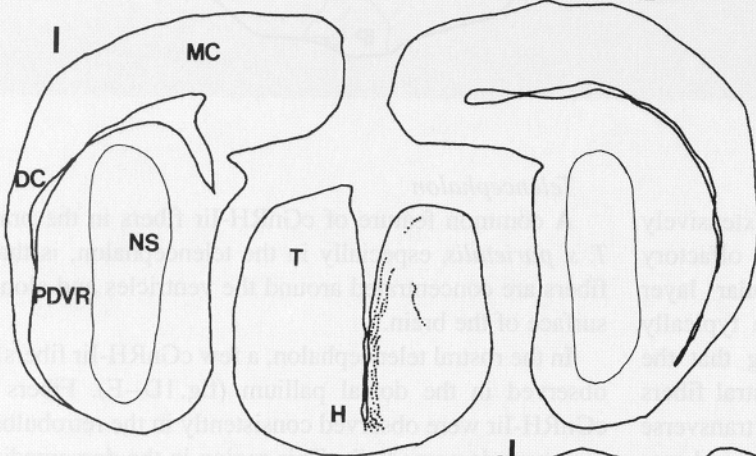
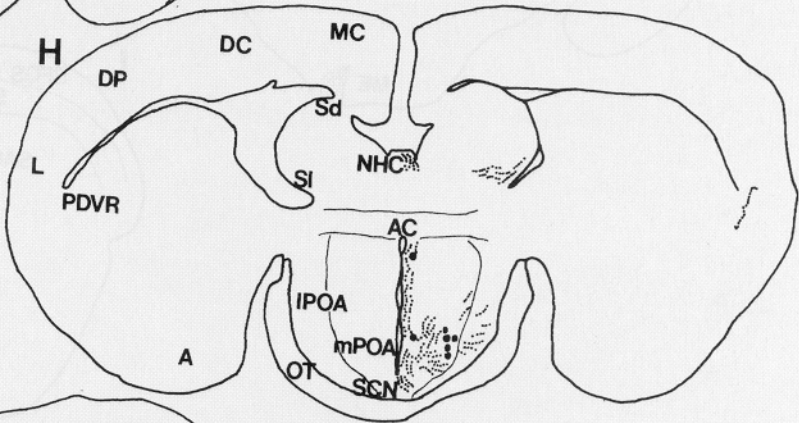
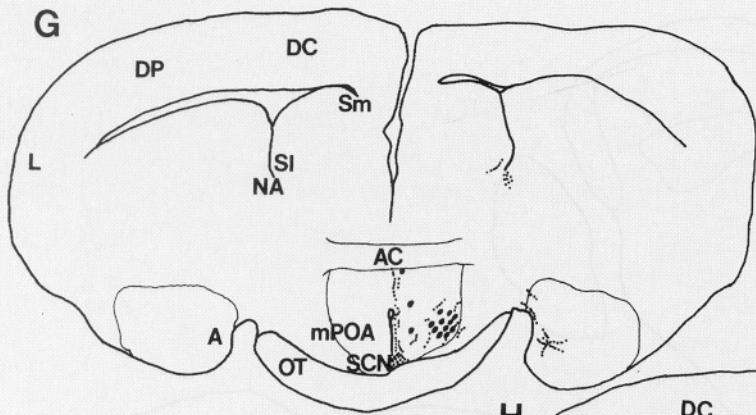
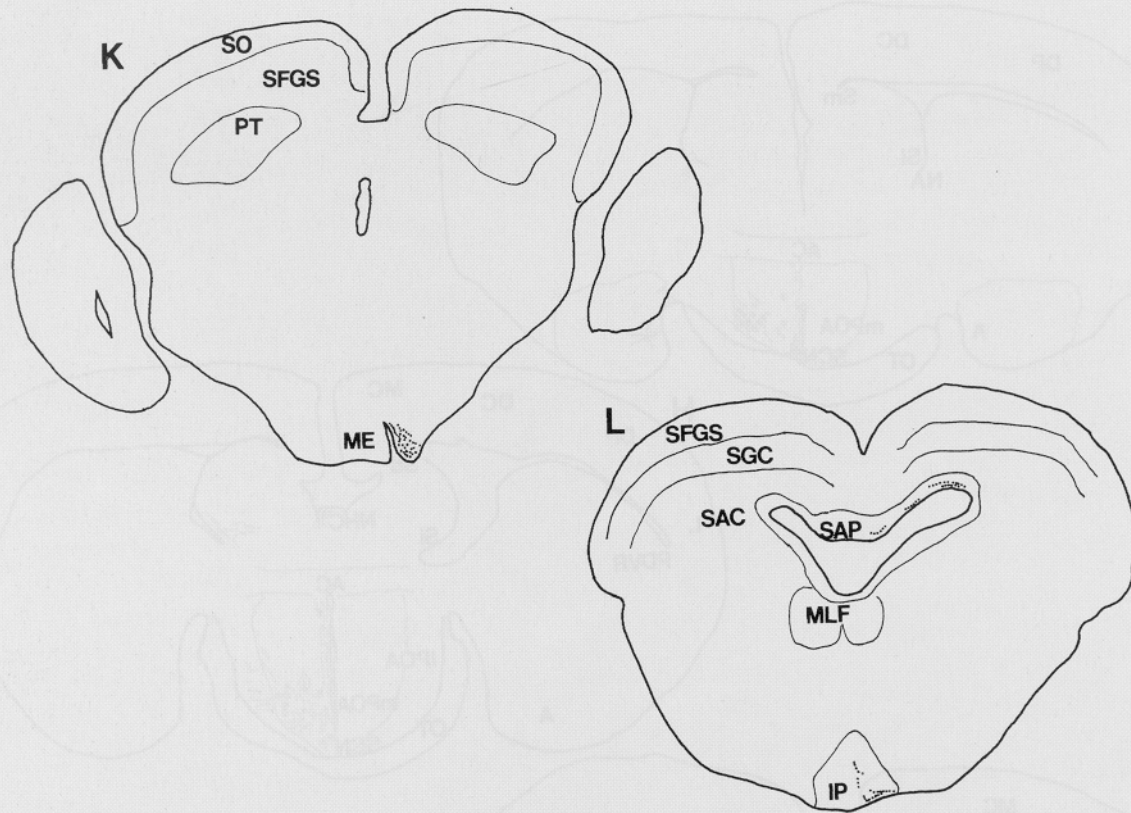


Fig. 1. Composite camera lucida drawings depict the distribution of GnRHir fibers and cell bodies in *Thamnophis sirtalis parietalis*. Cell bodies are represented by large dots, fibers by small dots.





(For legend, see p. 140.)

Olfactory Bulb

Chicken GnRH-Iir fibers appeared to be extensively beaded and diffusely scattered throughout the olfactory bulb in all layers except the internal granular layer (fig. 1A–C). Fibers in the olfactory bulb were typically short in 25 μm transverse sections, suggesting that the fibers are oriented rostrocaudally. The most rostral fibers are located in the internal plexiform layer at the transverse level where the vomeronasal nerve is still dorsal and medial, surrounding the medial portion of the olfactory bulb. Caudally, in the region where the olfactory ventricle is located at the lateral margin of the olfactory bulb, fibers were found in the mitral and glomerular layers of the olfactory bulb, as well as in the medial olfactory tract. In the most caudal aspect of the olfactory bulb, some fibers were seen scattered throughout the lateral pallium. A small series of cGnRH-Iir fibers were seen in nearly all sections located at the ventromedial aspect of the olfactory bulb. These fibers, located just beneath the pial surface, are a portion of the terminal nerve (fig. 1B–C). This identification is based on neuroanatomical position, GnRH immunoreactivity, and the forebrain sites of neuroanatomical projection.

Telencephalon

A common feature of cGnRH-Iir fibers in the brain of *T. s. parietalis*, especially in the telencephalon, is that the fibers are concentrated around the ventricles and along the surface of the brain.

In the rostral telencephalon, a few cGnRH-Iir fibers were observed in the dorsal pallium (fig. 1D–E). Fibers with cGnRH-Iir were observed consistently in the retrobulbar region pars dorsomedialis. This region in the dorsomedial aspect of the rostral telencephalon is characterized by its triangular shape in cross section, with the cGnRH-Iir fibers having an outward radiation from a point just dorsal to the ventricle [Halpern, 1980]. Also in the dorsal telencephalon, cGnRH-Iir fibers were found in the medial forebrain bundle, typically extending from just inside the ventromedial aspect of the brain dorsally to the ventral edge of the C-shaped ventricle (fig. 1E). Short, beaded fibers were seen in the rostral septum at the most rostral extent of the telencephalon. As the rostral septum separates caudally into the lateral and medial septum, the majority of the cGnRH-Iir fibers are concentrated in the lateral septum and extend from the medial preoptic area to the optic chiasm (fig. 1D–H).

Chicken GnRH-Iir fibers in the terminal nerve extend along the ventromedial aspect of the telencephalon. The terminal nerve ganglion is found just caudal to the olfactory bulb at the transition to the telencephalon (fig. 2A). This ganglion consists of two to five unipolar or bipolar cGnRH-Iir cells per section. Caudal to the terminal nerve ganglion, there is a cGnRH-Iir fiber tract that extends ventromedially to the nucleus of the diagonal band of Broca (nDB). The nDB contains the most darkly staining cGnRH-Iir cell bodies in the brain of *T. s. parietalis*. This nucleus had five to ten unipolar or bipolar immunoreactive cells per section, surrounded by many darkly immunoreactive fibers (fig. 2B).

Chicken GnRH-Iir cell bodies were also found in the medial preoptic area, which is lateral to the rostral most aspect of the third ventricle and dorsal to the optic tract (fig. 3A). Fibers with cGnRH-Iir in the medial preoptic area are darkly staining and extend into the lateral preoptic area. Ventral to the rostral end of the third ventricle is the suprachiasmatic nucleus (SCN) which extends rostrocaudally and has cGnRH-Iir fibers in a dorsoventral orientation (fig. 1G, H). This is the end point for the fiber tract that extends from the terminal nerve, through the terminal nerve ganglion, to the nucleus of the diagonal band of Broca, and caudally to the SCN. Darkly stained cGnRH-Iir fibers were seen in the median eminence at the transition between the caudal end of the nucleus sphericus and the rostral margin of the optic tectum.

The other forebrain areas that showed accumulations of cGnRH-Iir fibers were the amygdala, nucleus accumbens [see Warner, 1947] and the nucleus of the hippocampal commissure. At the level of the medial preoptic area, cGnRH-Iir fibers were found extending from near the optic tract down into the amygdala (fig. 1G). Short cGnRH-Iir beaded fibers with a rostrocaudal orientation were found in nucleus accumbens at the level of the lateral septum (fig. 1G). The nucleus of the hippocampal commissure, located above the third ventricle and medial to the lateral septum, had cGnRH-Iir fibers present in an area that has very closely spaced unstained cell bodies (fig. 1H). Finally, dark cGnRH-Iir fibers that are oriented dorsoventrally and

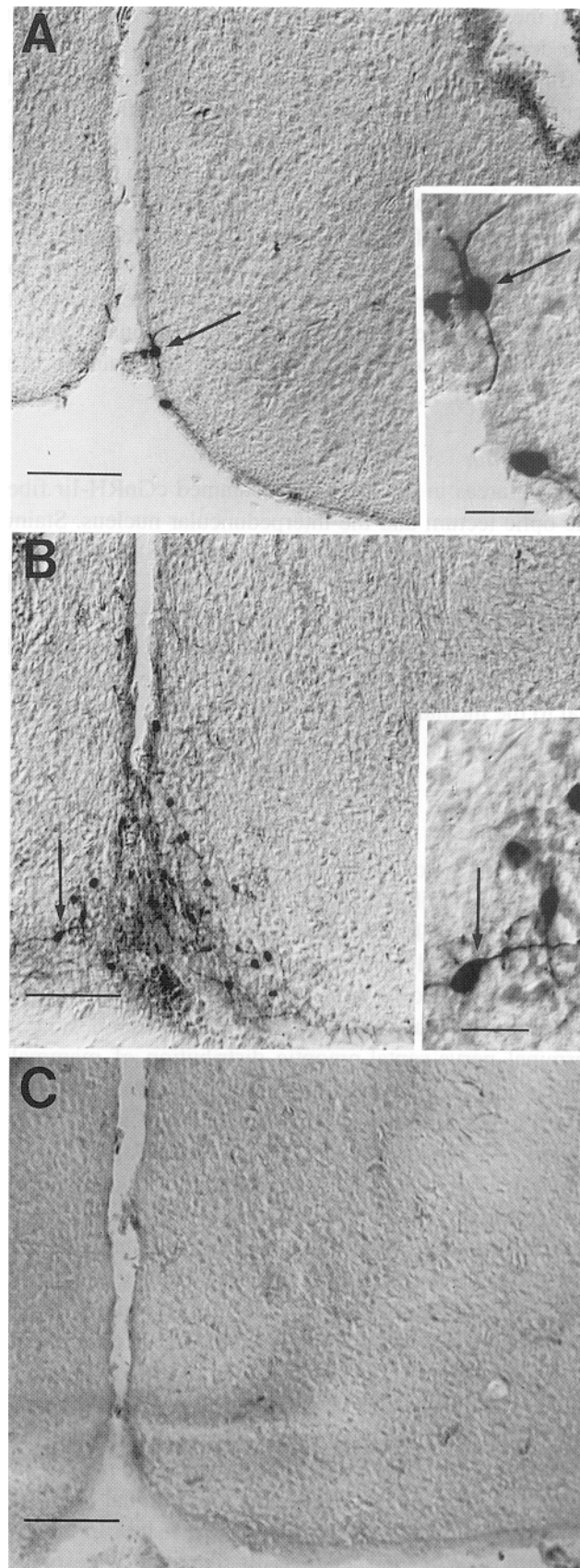


Fig. 2. Representative sections showing cGnRH-Iir cells and fibers in the terminal nerve (**A**) and the nucleus of the diagonal band of Broca (**B**) using differential interference contrast optics. Arrows in both the main figure and the insets point out the same anatomical feature at different magnifications. Control experiments demonstrate the specificity of the antibody. Photomicrograph (**C**) is a section through the nucleus of the diagonal band of Broca where the antibody was pre-absorbed with 100 μ M chicken-I. Scale bar = 100 μ m for **A**, **B**, and **C**; inset scale bar = 10 μ m.

abut the pallium were occasionally observed in the posterior dorsal ventricular ridge (fig. 1H).

In the caudal forebrain there were two main areas for dense staining: the hypothalamus and the thalamus. The hypothalamus has many cGnRH-Iir fibers distributed throughout its length in close association with the third ventricle (fig. 3B). Dense fiber staining was present along the medial aspect of the hypothalamus, and the number of fibers stained decreased caudally until the level of the optic tectum. At this level cGnRH-Iir fibers were restricted to the median eminence. The thalamus had occasional cGnRH-Iir fibers confined to the medial half of each thalamic hemisphere (fig. 1I, J).

Midbrain

Two areas in the midbrain contained cGnRH-Iir fibers: the optic tectum and the interpeduncular nucleus. Staining in the optic tectum was confined to the stratum album periventricular region, where there were several beaded fibers that ran in close proximity to and following the contours of the ventricle (fig. 1L). There were several beaded fibers in the interpeduncular nucleus, an area which is ventral to the medial longitudinal fasciculus (fig. 1L).

Discussion

This immunocytochemical study reveals the neuroanatomical distribution of cGnRH-Iir cells and fibers in the brain of the male red-sided garter snake, *T. s. parietalis*. Using an antibody directed against cGnRH-I, we found that the distribution of immunoreactive cGnRH-I parallels the forebrain distribution of GnRH in other vertebrates; i.e., the terminal nerve-septal-preoptic distribution of mammalian GnRH in mammals and amphibians, of chicken-I GnRH found in birds, and of salmon GnRH found in teleosts [for review see Muske, 1993]. This forebrain GnRH system is thought to include GnRH neurons that originate in the olfactory placode during embryonic development and then migrate into the forebrain to populate the terminal nerve, septal, and preoptic structures [for review see Muske, 1993]. To our knowledge, this is the first paper that describes GnRH staining in the terminal nerve of any reptile.

Staining in the olfactory bulb of the red-sided garter snake was diffuse and seen in many cytoarchitectonic layers. Fibers with cGnRH-Iir were seen in the internal plexiform layer, mitral layer, glomerular layer, the medial olfactory tract, the lateral pallium, and the terminal nerve. This pattern is similar to that seen in teleost fishes. In the dwarf gourami, *Colisa lalia*, cGnRH-Iir fibers are widespread in

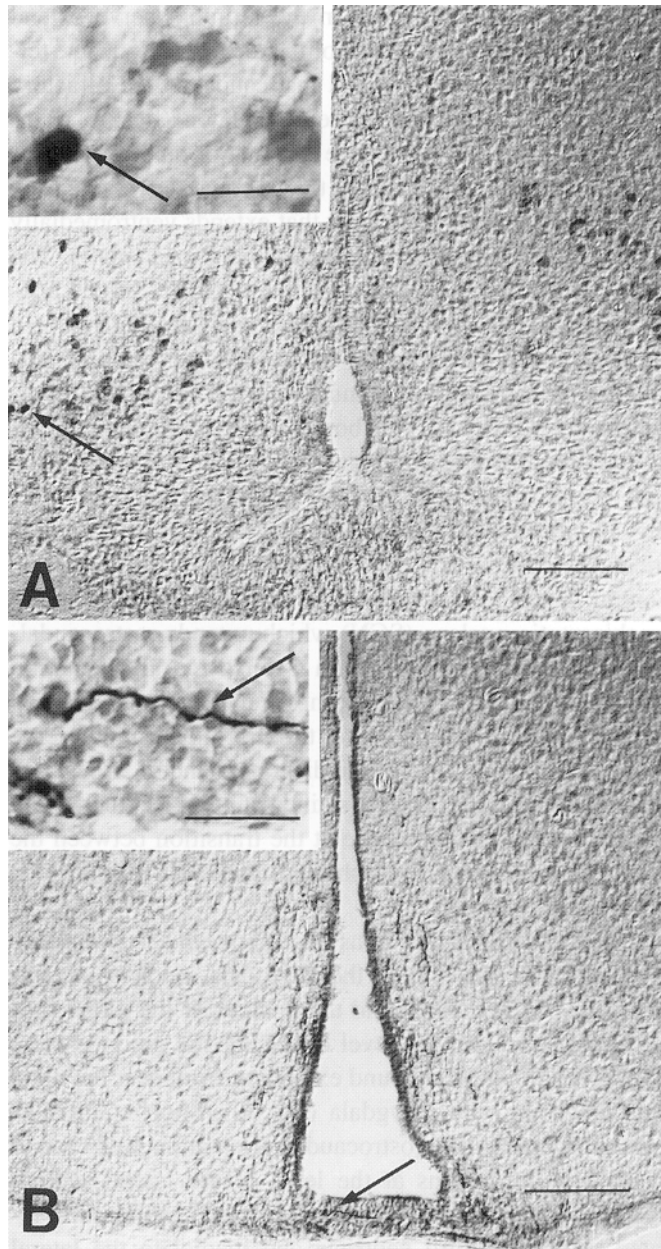


Fig. 3. cGnRH-Iir in the medial preoptic area and around the third ventricle. cGnRH-Iir in the medial preoptic area (**A**; fig. 1G) was not as intense as that seen in the nucleus of the diagonal band of Broca but was consistently present in all animals. Two regions of many immunoreactive cell bodies are located lateral to the third ventricle with fibers distributed throughout the cell bodies. The third ventricle (**B**; fig. 1J) had consistently staining fibers along its margin, from the medial preoptic area to the beginning of the optic tectum. Arrows in the main figure correspond to the arrows in the inset. Scale bar = 100 μ m for **A** and **B**; inset scale bar = 10 μ m.

the olfactory bulbs and present in all cytoarchitectonic layers [Oka and Ichikawa, 1990]. Salmon, *Oncorhynchus masou*, have a more restricted pattern of GnRHir, with staining for salmon GnRH being found in the olfactory bulb and in an area between the olfactory bulb and olfactory nerve [Amano et al., 1991]. Staining for GnRH in the olfactory bulb was reported in one other reptile, the rat snake, *E. climacophora*, but this immunoreactivity was not described in detail [Nozaki et al., 1984]. In rats, GnRHir for mammalian GnRH is seen in the accessory olfactory bulb in the form of fibers, and input of GnRHir fibers is also seen in the external plexiform layer [Witkin and Silverman, 1983; Merchenthaler, 1984]. In primates, GnRHir fibers are localized in the main olfactory bulb and lateral olfactory tract in the rhesus monkey, *Macaca fascicularis*, and in the external plexiform layer of the squirrel monkey, *Sciureus saimiri* [Witkin, 1985]. The function of GnRH in the olfactory bulb remains unknown.

The terminal nerve in *T. s. parietalis* is located on the ventral surface of the brain just below the pial layer, and extends in a rostrocaudal extent from the middle of the olfactory bulb back to the terminal nerve ganglion. The terminal nerve ganglion is located at the transition between the olfactory bulb and the telencephalic hemispheres, and, from here, terminal nerve fibers extend caudally back to the nucleus of the diagonal band of Broca. The terminal nerve ganglion (as localized by GnRHir) in some species of fish is also located at this transitional area. In the dwarf gourami, *C. lalia*, the terminal nerve ganglion is found at the transition between the olfactory bulb and the telencephalic hemispheres [Oka and Ichikawa, 1990]. In the goldfish, *Carassius auratus*, the projections of these terminal nerve fibers run peripherally from the olfactory nerve and centrally into the ventromedial forebrain, and in the platyfish, *Xiphophorus maculatus*, the nucleus olfactoretinalis, the homologue to the terminal nerve ganglion, is located at the boundary between the ventral telencephalon and the olfactory bulb [Bartheld and Meyer, 1986; Schreibmann and Margolis-Nunno, 1987]. In amphibians, the terminal nerve runs along the midline from the olfactory nerve implantation cones to the medial wall of each hemisphere before dividing into two main branches at the level of the mid-telencephalon. One branch remains on the ventral brain surface and projects medially, and the other projects dorsocaudally along the olfactory tubercle to the medial septal nucleus [Muske and Moore, 1988]. In the roughskin newt, *T. granulosa*, the terminal nerve ganglion is located caudal to the olfactory bulbs, a pattern similar to that in *T. s. parietalis* [Muske and Moore, 1988]. In mammals, terminal nerve perikarya occur as single cells or in ganglionated

clusters along the length of the terminal nerve, which extends from the vomeronasal system, along the ventromedial aspect of the brain, back to the medial septum [Schwanzel-Fukuda and Silverman, 1980].

The neuroanatomical pattern of GnRH staining in the terminal nerve appears to be similar in fish, amphibians, reptiles, and mammals, although which chemosensory nerve is associated with the terminal nerve may vary. In the male red-sided garter snake, the terminal nerve is spatially associated with the vomeronasal nerve, and the vomeronasal nerve is known to carry important reproductive information that is used in the courtship behavior in this species [Kubie et al., 1978]. The association of the vomeronasal nerve and the terminal nerve, as well as the fact that GnRH analogs have been shown to be effective in eliciting courtship behavior in male red-sided garter snakes [unpubl. observ.], raises the possibility that one of the terminal nerve's functions is the initiation or maintenance of reproductive behavior in this species by release of GnRH after reception of a pheromonal cue. The evidence concerning the terminal nerve's role in pheromonal transduction is unclear, with some studies showing that terminal nerve transection leads to mating deficits and other studies showing that there is no effect of terminal nerve lesions on parameters associated with sex behavior [Wirsig, 1987; Fujita et al., 1991; Wirsig-Wiechmann, 1993].

The telencephalon of *T. s. parietalis* had more areas with cGnRH-I immunoreactivity than any other brain sub-division examined. Like other vertebrates that have been examined, *T. s. parietalis* stained darkly for cGnRH-I fibers in the rostral septum, the nucleus of the diagonal band of Broca, the medial pre-optic area, and the median eminence, with cell bodies found in the nucleus of the diagonal band of Broca and the medial preoptic area [Nozaki and Kobayashi, 1979; Silverman, 1988; Millam et al., 1993]. The rostral septum in *Thamnophis* may be homologous to nucleus accumbens based on work showing that this region also has strong dopaminergic input in the python, *Python regius* [Smeets, 1988]. Another characteristic that appears to be shared with other vertebrates is a loose plexus of GnRHir fibers found in the periventricular region of the third ventricle at the level of the hypothalamus [Yellon et al., 1990].

Dense fiber staining was seen in the amygdala of the male red-sided garter snake; these fibers usually abutted the lateral pre-optic area and then extended down into the amygdala. Staining in the amygdala is a common feature in other vertebrates, including the guinea pig *Cavia porcellus* and the rhesus monkey *M. fascicularis*, which have mammalian GnRH localized in this area [Leonardelli and Poulain, 1977; Silverman et al., 1982]. Fibers that were

cGnRH-Iir were also seen in the nucleus of the hippocampal commissure of the red-sided garter snake. Hippocampal GnRHir has been observed in mammals for the mammalian form of the peptide and in birds for the chicken-I form of the peptide [Barry et al., 1985; Millam et al., 1993].

Chicken GnRH-I immunoreactivity in the midbrain of *T. sirtalis* was restricted to two areas: the stratum album periventricular layer (SAP) of the optic tectum and the interpeduncular nucleus. Immunoreactivity for GnRH in the optic tectum is a feature that is seen in certain fish species, but it is not confined to the SAP layer as we observed in the red-sided garter snake. Amano et al. [1991] have shown that the masu salmon has immunoreactivity to salmon GnRH seen in the deep layers of the optic tectum. Oka and Ichikawa [1990] report that the dwarf gourami has GnRH immunoreactivity in the stratum album centrale and the stratum griseum centrale. These areas both receive retinotectal terminals, and it has been proposed that GnRH may play a role in the regulation of visual processing. Merchenthaler et al. [1984], studying the distribution of mammalian GnRH in rat brains, demonstrated that rats have GnRH immunoreactivity in the interpeduncular nucleus (IPN), and Rastogi et al. [1990] have seen IPN GnRHir in the brain of the edible frog, *Rana esculenta*. Few studies have shown GnRHir in the midbrain of reptiles. Bennis et al. [1989] looked at the distribution of salmon GnRH in the brain of chameleons and showed GnRHir localized in the anterior midbrain tegmentum and the fasciculus longitudinalis medialis (MLF). Although the IPN is immediately ventral to the MLF, we observed no immunoreactivity that extended into the MLF.

It is also interesting to note what was not found in this study. Using a chicken-II GnRH antibody, we were not able to detect any cGnRH-IIir in any structure, including the midbrain. In many vertebrate species there are two populations of GnRH cells: GnRH-containing cells that are part of the terminal nerve-septal-preoptic system and cells that are found in the midbrain [for review see Muske, 1993]. As a general rule, the telencephalic structures vary in which GnRH form is present across species but cells in the midbrain are immunoreactive to chicken-II. Why we did not identify any chicken-II cells or fibers is not known, although several possibilities exist: it may be that our antibody was not sensitive enough, or that chicken-II GnRH is either not present or is present in too low a concentration to be detected.

In conclusion, this study indicates that cGnRH-Iir is widespread in the forebrains of reproductively active, male, red-sided garter snakes and that the terminal nerve in this species of reptile contains immunoreactive GnRH. We believe that the terminal nerve-septal-preoptic system that we

have seen in the male red-sided garter snake is homologous to the terminal nerve-septal-preoptic system that is seen in other vertebrate classes and that this system is most closely related to the system found in birds due to the specific presence of cGnRH-Iir.

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