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Identification and Localization of Neurohypophysial Peptides in the Brain of a Caecilian Amphibian, *Typhlonectes natans* (Amphibia: Gymnophiona)

CARYL HILSCHER-CONKLIN,[1] J. MICHAEL CONLON,[2] AND SUNNY K. BOYD[1]*
[1]Department of Biological Sciences, University of Notre Dame, Notre Dame, Indiana 46556
[2]Regulatory Peptide Center, Department of Biomedical Sciences, Creighton University School of Medicine, Omaha, Nebraska 68178

ABSTRACT

The amphibian order Gymnophiona contains more than 150 different species of caecilians. The characterization and distribution of neurohypophysial peptides, however, has not been described for any member of this order. By using high-performance liquid chromatography, radioimmunoassay, and mass spectrometry, we identified the peptide arginine vasotocin (AVT) in brain and pituitary extracts from the caecilian *Typhlonectes natans*. By using immunocytochemistry, we found five populations of AVT-immunoreactive (AVT-ir) cells in the brain of *T. natans*. AVT-ir cell bodies were located in the preoptic area, amygdala pars medialis, ventral thalamus, dorsal hypothalamic nucleus, and nucleus of the solitary tract. AVT-ir fibers and terminal fields were widespread. We also identified a mesotocin-like peptide. The distribution of this peptide in the brain of *T. natans* was more restricted than the distribution of AVT. Mesotocin-like-immunoreactive cell bodies were located almost exclusively in the preoptic area, with only a few other cells located in the amygdala pars medialis. This caecilian species, therefore, possesses neurohypophysial peptides that are similar in their structure and distribution to the peptides found in anuran and urodele amphibian orders. J. Comp. Neurol. 394:139–151, 1998. © 1998 Wiley-Liss, Inc.

Indexing terms: vasotocin; mesotocin; vasopressin; high-performance liquid chromatography; immunocytochemistry

The class Amphibia includes three extant orders, two of which (Anura and Urodela) have received much attention. The third amphibian order, Gymnophiona, is a little-recognized group that consists of five families and 154 known species of caecilians (Nussbaum and Wilkinson, 1989; Hedges et al., 1993). Caecilians are elongate, legless, fossorial or aquatic animals that are exclusively tropical in distribution. Their eyes serve only to detect light and are covered either by skin or by skin and bone (Fritzsch et al., 1985; Himstedt and Manteuffel, 1985; Wake, 1985; Himstedt, 1995). Although the three living orders of amphibians have similarities, their fossil histories are long and separate (Carroll and Currie, 1975). Based on morphology, caecilians are considered to be a monophyletic lineage that is separated evolutionarily from urodele and anuran amphibians by at least 200 million years (Duellman and Trueb, 1986). Caecilians differ significantly from anurans and urodeles in many regards, including body form (legless; Taylor, 1968), sensory systems (e.g., chemosensory tentacle; Wake, 1993), and reproductive physiology (e.g., viviparity; Wake, 1992). Thus, there is reason to expect that the neuroanatomy and neurochemistry of caecilians will differ from those of other amphibians, but information is scant. Because neurohypophysial peptide pathways are among the best described of neurochemical pathways in anuran and urodele brains (see, e.g., Follett and Heller, 1964; Vandesande and Dierickx, 1976; Conway and Gainer, 1987; Jokura and Urano, 1987; Nojiri et al., 1987; Gonzalez and Smeets, 1992a,b; Boyd, 1997), we investigated this system in the caecilian, *Typhlonectes natans*.

All vertebrates examined to date have at least two types of neurohypophysial peptides, except the primitive hagfish

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*Correspondence to: Sunny K. Boyd, Ph.D., Department of Biological Sciences, University of Notre Dame, Notre Dame, IN 46556.
E-mail: boyd.1@nd.edu

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and the lamprey, which possess only arginine vasotocin (AVT; Urano et al., 1992; Acher, 1993). Due to its presence in all vertebrates, AVT is considered the ancestral precursor of peptides in this family (Acher, 1993). A duplication of the AVT gene likely gave rise to the two groups of neurohypophysial peptides (basic and neutral) found in extant vertebrates. The basic peptide lineage includes arginine vasopressin (AVP) in most mammals, lysine vasopressin in pigs and some marsupials, and AVT in all other vertebrates. The neutral lineage consists of oxytocin in mammals, mesotocin (MT) in nonmammalian tetrapods, isotocin in most teleosts, and a variety of other peptides in cartilaginous fishes (Urano et al., 1992; Acher, 1993, 1996; Hiraoka et al., 1993; Suzuki et al., 1995). A striking feature of these structural shifts is the first appearance of MT in lungfish and its persistence in anuran and urodele amphibians, reptiles, birds, and marsupials. Parts of this critical transition (between fish isotocin and tetrapod MT) are not completely documented, however. Although isolation and characterization of neurohypophysial peptides in lungfish (four species; Follett and Heller, 1964; Acher et al., 1970; Michel et al., 1993) and anuran amphibians (11 species; Sawyer et al., 1959; Nojiri et al., 1987; Rouille et al., 1989; Michel et al., 1990; Chauvet et al., 1991; Ouedraogo and Sawadogo, 1994) are broadly representative, the other two amphibian orders are either not represented at all (caecilians) or are poorly represented (urodeles; AVT found in two species and MT in only one species; Chauvet et al., 1991). One goal of this study, therefore, was to isolate and characterize the neurohypophysial peptides of a representative caecilian by using a combination of high-performance liquid chromatography (HPLC), radioimmunoassay, and mass spectrometry.

Immunocytochemistry has been used to locate cells and fibers containing neurohypophysial peptides in brains of a variety of amphibians. Distribution of immunoreactive AVT and MT has been well described for eight anurans (*Rana temporaria*, *R. esculenta*, *R. ridibunda*, *R. catesbeiana*, *R. sylvatica*, *Bufo bufo*, *B. japonicus*, and *Xenopus laevis*; Vandesande and Dierickx, 1976; Jokura and Urano, 1985, 1987; Conway and Gainer, 1987; Boyd et al., 1992; Gonzalez and Smeets, 1992a, b; Boyd, 1994a; Mathieson, 1996) and for one urodele amphibian (*Pleurodeles waltlii*; Gonzalez and Smeets, 1992a). The distribution of AVT and MT has never been described in any caecilian species. Therefore, anatomical locations of peptide cells and fibers in the brain of *T. natans* were also mapped by using immunocytochemistry.

Neuroanatomical Abbreviations

AOL	area octavolateralis
APL	amygdala pars lateralis
APM	amygdala pars medialis
DH	dorsal hypothalamus
DP	dorsal pallium
H	habenula
LC	locus coeruleus
LP	lateral pallium
LS	lateral septum
M	mesencephalon
MO	medulla oblongata
MP	medial pallium
MS	medial septum
NA	nucleus accumbens
OB	olfactory bulb
OT	optic tectum
PAL	pallium
POA	preoptic area
S	spinal cord
SC	suprachiasmatic nucleus
SOL	solitary tract
ST	striatum
TE	thalamic eminence
TEG	tegmentum
ThD	dorsal thalamus
ThV	ventral thalamus
VH	ventral hypothalamus
Vm	trigeminal motor nucleus

MATERIALS AND METHODS

Isolation of neurohypophysial peptides

Peptides were isolated separately from caecilian pituitaries and from brains. Pituitaries (whole glands; n = 22; weight = 39.2 mg) and brains (n = 24; weight = 1.147 g) were collected from anesthetized adults of both sexes, frozen on dry ice, and stored at -80°C. All methods were approved by the institutional animal care and use committee. Tissues were homogenized with a Polytron (Brinkmann Instruments, Westbury, NY) in 20 ml of 1 M HCl containing 15% (v/v) trifluoroacetic acid (TFA), 5% (v/v) formic acid, and 1% (w/v) NaCl. Homogenates were incubated for 1 hour at 0°C then centrifuged for 20 minutes (1,200g) at 4°C. Each supernatant was passed twice at a flow rate of 2 ml/minute through a Sep-Pak cartridge (Millipore Corp., Milford, MA) equilibrated with 0.1% TFA. For each extract, the column was washed with 4 ml 0.1% TFA and then with 4 ml acetonitrile/water/TFA (70.0/29.9/0.1, v/v/v) to elute peptides. Eluate volume was reduced in a refrigerated vacuum centrifuge (Savant Speedvac, Holbrook, NY) to approximately 1 ml.

Peptides were purified by using reversed-phase HPLC. After partial purification on Sep-Pak cartridges, as described above, extracts were injected onto a Vydac 218TP510 C-18 (1 / 25 cm) semipreparative column (Separations Group, Hesperia, CA) that had been equilibrated with 0.1% TFA/water (v/v) at a flow rate of 1.5 ml/minute. Concentration of acetonitrile in eluting solvent was raised to 49% (v/v) over 70 minutes, with a linear gradient. Absorbance was measured at 214 nm, and fractions were collected every 0.5 minute (pituitary extract) or 1 minute (brain extract) and stored at -20°C. After chromatography was complete, neurohypophysial peptide standards (5 µg or 10 µg each of AVT, AVP, hydrin 2, MT, oxytocin, and isotocin; Bachem, Torrance, CA) were injected under conditions identical to those described above, and elution times were compared with peaks of endogenous peptides. Fractions of chromatographic effluent eluting between 15 and 40 minutes were assayed for neurohypophysial peptide-like immunoreactivity by using the radioimmunoassay (RIA) described below. Immunoreactive peaks were purified to near homogeneity by further chromatography on a Vydac 214TP54 (C-4) column (0.46 / 25 cm) equilibrated the same as the C-18 column described above. Concentration of acetonitrile in the eluting solvent was raised to 40% (v/v) over 40 minutes, with a linear gradient. The fraction from the brain extract that coeluted with MT was further purified by chromatography on a C-18 Vydac (218TP54) analytical column (25 / 0.46 cm).

AVT RIA was performed by using a procedure that was validated previously for amphibian plasma and brain (Zoeller and Moore, 1986; Boyd and Moore, 1992; Boyd, 1994b). The antibody (R-70; provided by D. Fisher; Rosenbloom and Fisher, 1974) was raised in rabbits to lysine

vasopressin and was used at a final dilution of 1:75,000. Synthetic AVT (Bachem) was used as the standard in the range of 1–200 pg. Iodinated AVP ([^{125}I-AVP]; Amersham, Arlington Heights, IL; SA = 2,000 Ci/mmol) was used as the tracer. Minimum detectable concentration of AVT was 1 pg/tube. Cross reactivity of related neurohypophysial peptides under these conditions was 100%, 24%, and 5% for AVP, MT, and oxytocin, respectively. The antibody did not cross react with isotocin or hydrin 2, even at peptide concentrations as high as 200 pg/tube. Note that this antibody (used for the RIA only) did cross react with AVT and MT. Different antibodies that were specific for each peptide were used for immunocytochemistry.

Mass spectrometry of purified peptide was performed by Dr. Per F. Nielsen at the Novo/Nordisk Research Institute in Bagsvaerd, Denmark by using an API III LC/MS/MS system (Sciex; Thornhill, Ontario, Canada), as described previously (Conlon et al., 1992). Accuracy of mass measurement was ±0.02%.

AVT and MT immunocytochemistry

Sexually mature caecilians (13 of each sex; n = 26; length 25–42 cm) were obtained from Wilson Pet Supply (Woodale, IL). Caecilians were deeply anesthetized in benzocaine (0.02% solution) and perfused through the truncus arteriosus with about 100 ml 0.9% saline followed by about 150 ml 5% acrolein (Aldrich, Milwaukee, WI) in a 0.1 M phosphate buffer, pH 7.2. Brains were postfixed in situ in fresh acrolein solution for 2 hours, then removed from the skull, and placed in fresh fixative for an additional 2 hours. Brains were stored overnight in 30% sucrose in phosphate buffer at 4°C, embedded in butter, and 75-μm Vibratome sections were cut (the Vibratome contained 0.9% NaCl in 0.05 M Tris-HCl, pH 7.6, at 0°C). Brains were sectioned in horizontal, sagittal, and frontal (transverse) planes.

Free-floating sections were processed for AVT or MT immunoreactivity with a procedure that was validated previously for amphibian brains (Boyd et al., 1992). Briefly, alternate sections from the same brain were treated with either rabbit antivasopressin serum (ICN, Lisle, IL; 1: 2,000 dilution) or rabbit anti-MT serum (affinity-purified rabbit polyclonal VA-10; 1:200 dilution; donated by Harold Gainer; Conway and Gainer, 1987). The antivasopressin serum cross reacted with AVP and AVT but not with MT or isotocin (Boyd et al., 1992). After affinity purification, the VA-10 antibody was specific for MT and did not cross react with AVT (based on immunohistochemistry, differential absorption assays, and radioimmunoassays; Conway and Gainer, 1987; Altstein et al., 1988). Immunoreactivity was visualized with the peroxidase-antiperoxidase technique and diaminobenzidine. Specificity of the antiserum and technique was also tested on alternate sections from some brains. Sections were processed with 2% normal goat serum replacing the primary antibody or with primary antiserum that had been preadsorbed with 50 μM AVT, MT, or isotocin.

Neuroanatomy

Cell size was determined with the NTS hardware and software system (from Eutectics Electronics, Inc., Raleigh, NC). Immunoreactive cell bodies were drawn with a camera lucida, digitized by the system, and soma area was computed based on internal size calibration. All immunoreactive cell bodies in a single thick (75 μm) section were drawn for each animal in each brain area. This prevented drawing cells more than once, exclusion of large cells that might have been spread across multiple thin sections, and possible bias of the drawer toward larger cells. Determination of neuroanatomical areas was based primarily upon Northcutt and Kicliter (1980) for the telencephalon; on Kuhlenbeck (1922), Fritzsch et al. (1985), and Clairambault et al. (1980) for the diencephalon and mesencephalon; and on Gonzalez and Smeets (1994) and Wicht and Himstedt (1990) for the metencephalon and myelencephalon.

RESULTS

Isolation of peptides

The elution profile of caecilian pituitary extract from a semipreparative Vydac C-18 column contained only one immunoreactive peak (Fig. 1A). That peak coeluted with AVT. Further purification of this material on an analytical Vydac C-4 column resulted in clear separation of a single, immunoreactive peak (Fig. 1C). Mass spectrometry analysis showed a monoisotopic molecular weight of 1,049.4 atomic mass units (amu) for this extract. The theoretical value for AVT is 1,049.5 amu.

Brain extract elution profiles from a C-18 column contained two immunoreactive peaks. One peak coeluted with AVT, and the other coeluted with MT (Fig. 1B). Fractions coeluting with MT were further purified on analytical C-4 and analytical C-18 columns. On both column types, endogenous material coeluted with synthetic MT and was immunoreactive. However, we were not able to separate this peptide in a form that was sufficiently pure for edman degradation or mass spectrometry analysis.

Immunocytochemical localization of peptides

Distribution of AVT immunoreactivity. The most rostral AVT-immunoreactive (-ir) cells were found in the anterior portion of the preoptic area (POA; Fig. 2, rostrocaudal orientation; immunoreactivity in Figs. 3D–F, 4A, 5A,B, 6A,C,E). Darkly stained cells first appeared coincident with the appearance of the third ventricle (in frontal section). This population rapidly expanded into dense, periventricular columns of cells at more caudal levels. At the same level where POA cells first appear, a lateral population of more lightly stained cells appeared in the amygdala pars medialis (Figs. 3D, 7A). The amygdala cell population extended caudally within the pars medialis and perhaps the rostral amygdala pars lateralis. AVT-ir cell bodies were not present in the caudal amygdala pars lateralis (see, e.g., Fig. 3E,F). A third population of AVT-ir cells was found in the ventral thalamus (Figs. 4B, 7B). This small population (about 10–20 cells per side) was displaced laterally from the ventricle and was separated from dorsal POA cells by a clear region that lacked immunoreactive cells. Most of these cells extended processes directly laterally. Slightly more caudally, a large compact population of darkly stained AVT-ir cells was observed in the dorsal hypothalamic nucleus (Figs. 4D, 7C). There were no AVT cells in ventral hypothalamic or suprachiasmatic nuclei. The most caudal population of AVT-ir cells was located in the nucleus of the solitary tract (Figs. 4G, 7D; location based on Gonzalez and Smeets, 1994). This small population (five to ten cells per side) consisted of large, lightly stained cells. This population was not observed in every brain.

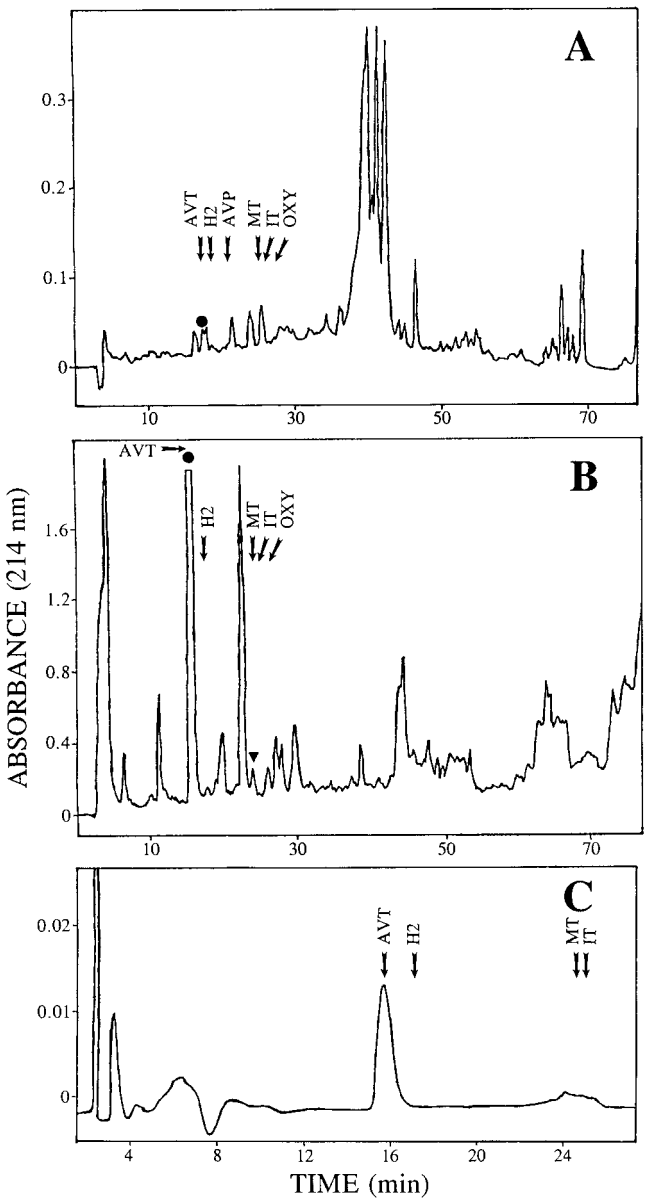

Fig. 1. Purification of caecilian neurohypophysial peptides by reversed-phase high-performance liquid chromatography (HPLC). Arrow and large dots denote the peak(s) containing neurohypophysial peptide-like immunoreactivity, as determined by using an antiserum that reacted with both arginine vasotocin (AVT) and mesotocin (MT). **A:** Elution profile of pituitary extract on a Vydac C-18 column. **B:** Elution profile of brain extract on a C-18 column. **C:** Elution profile of the peak of AVT-like immunoreactivity from pituitary repurified on C-4 column. AVP, arginine vasopressin; IT, isotocin; OXY, oxytocin; H2, hydrin 2.

The distribution of AVT-ir fibers in this caecilian brain was much more extensive than the distribution of MT-ir fibers. AVT-ir fibers were present at virtually every level, with the exception only of the most rostral areas. In the telencephalon, AVT-ir fibers were strikingly associated with subpallial structures and were missing almost entirely from the pallium. Rare fibers were observed in the accessory olfactory bulb (mitral layer), but significant staining first appeared in the nucleus accumbens and striatum (Fig. 3A,B). Immunoreactivity in these regions consisted of thin varicose fibers and terminal fields. As the amygdala pars medialis replaced the nucleus accumbens more caudally, the distribution and density of AVT-ir fibers continued with no obvious changes in pattern. In the rostral telencephalon of caecilians, the medial septum is the most prominent feature of the medial wall (Northcutt and Kicliter, 1980). The medial pallium is small and is not well developed dorsally, and the lateral septum has not yet appeared. Significantly, we found no AVT immunoreactivity in the well-developed medial septum of *T. natans*. On the other hand, dense AVT-ir fibers were found more caudally in the lateral septum (Fig. 3B). AVT-ir cells were not found in either septal area. Finally, in the most caudal telencephalon, AVT immunoreactivity was located in pallial fields for the first time. Dense terminal fields were present in the amygdala pars lateralis (Fig. 3E,F) and the lateral pallium (Figs. 3E,F, 4A,B).

The densest area of AVT-ir fibers was located in association with the POA (Figs. 3E,F, 4A, 6A,C,E). Most fibers were thick, were without varicosities, and coursed laterally or ventrolaterally. A few, similar thick fibers also extended dorsally into the thalamic eminence (Fig. 3D–F). The entire thalamus, in fact, contained dense AVT-ir fiber staining (Fig. 4A–D). In the ventral thalamus, most fibers were oriented in a distinct, vertical (dorsoventral) position within the neuropil (see, e.g., Fig. 4C). In the dorsal thalamus, on the other hand, thin varicose fibers and terminal fields were found. Staining in the habenula was absent in rostral regions (Fig. 3E,F) and was moderate in more caudal regions (Fig. 4A,B). Dense fiber staining was found in both dorsal and ventral hypothalamic nuclei (Fig. 4C,D).

Moderate densities of AVT-ir fibers were found throughout the hindbrain, including the optic tectum and tegmentum (Fig. 4E,F). Tectal staining was most prevalent in rostral regions and disappeared caudally, in areas where tegmental staining was most dense. At the level of the solitary tract AVT-ir cell population, scattered fibers were located in dorsal and lateral regions (Fig. 4G).

Size of AVT-ir cell bodies. Area of AVT-ir cell somata varied significantly across different populations, with the smallest cells in the dorsal hypothalamus and the largest cells in the solitary tract of the brainstem (Table 1). Within the POA, there was no obvious bimodal distribution in cell somal area (Figs. 6A,C,E, 8). Instead, cell size showed a unimodal, asymmetric distribution, and log transformation of the data resulted in a normal distribution (Kolmogorov-Smirnov $Z = 1.087$; $P = 0.188$). Although the very large POA AVT-ir cells were located in the caudal and dorsal portions of the nucleus, no nucleus directly comparable to the magnocellular POA of anurans (e.g., bullfrogs; Boyd et al., 1992) was found in *T. natans*. Posterior POA cells were not displaced laterally from the ventricle and were not consistently larger.

Distribution of MT-like immunoreactivity. Distribution of MT-ir cells and fibers was much more restricted than that of AVT. MT-ir cell bodies were most abundant in the anterior POA (Figs. 3D–F, 5, 6B,D,F), where they exhibited a somewhat laminar arrangement along the third ventricle. This group of cells extended laterally and graded into the amygdala (primarily the pars medialis). Thick and thin fibers were present in the POA oriented in a ventrolateral position, but they were much less dense than AVT-ir fiber projections. Scattered fibers and terminal

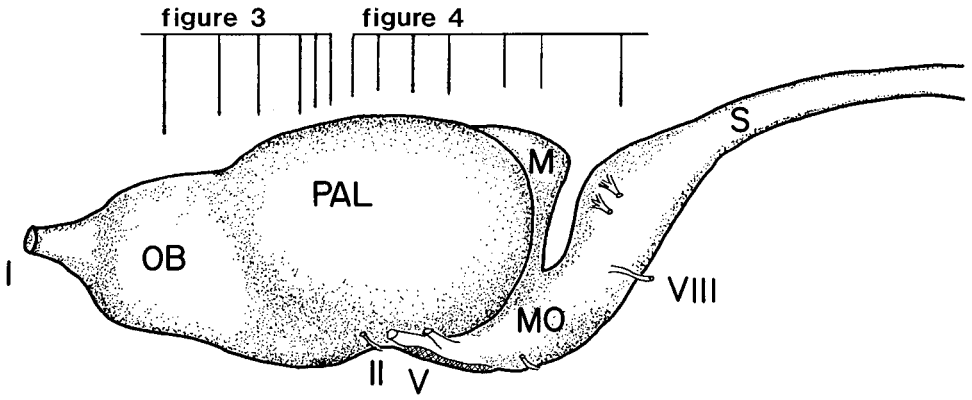

Fig. 2. Side view of the brain of the caecilian *Typhlonectes natans* (approximate levels of drawings in Figs. 3 and 4 are indicated by vertical lines). Roman numerals label cranial nerves. For abbreviations, see list.

fields were also found in the ventral thalamus, lateral suprachiasmatic nucleus (SCN), ventral hypothalamus, optic tectum, tegmentum, and dorsolateral hindbrain (Fig. 4A–G)

Control staining. Staining in tissue was abolished when each primary antibody was preadsorbed with its respective antigen (AVT antiserum + 50 μM AVT; MT antiserum + 50 μM MT). When the antiserum was adsorbed with a neurohypophysial peptide for which it was not specific (AVT antiserum + 50 μM MT; MT antiserum + 50 μM AVT), normal staining of cells occurred (Fig. 6C,D). Regional differences in staining were further evidence of antibody specificity (e.g., MT antibody stained no cells in the ventral thalamus or hindbrain). Isotocin preadsorption had no effect on staining by either antibody (Fig. 6E,F); thus, neither antibody used for immunocytochemistry would have recognized isotocin cells. Replacement of primary antibody with normal serum resulted in no specific staining.

DISCUSSION

The structure and distribution of neurohypophysial peptides in the brain of this caecilian, *T. natans*, support the hypothesis of a monophyletic origin for all three orders of extant amphibians. This species possessed AVT and an MT-like peptide (see below), similar to the peptides found in all anuran and urodele amphibians studied to date. In addition, AVT-ir cells in this caecilian brain are located in substantially similar neuroanatomical areas compared with other amphibians. MT-like immunoreactivity was also distributed in densities and locations similar to other amphibians. Thus, it appears that the neurohypophysial peptide systems of the order Gymnophiona, as described here for the first time, are more similar to those of anuran and urodele orders of amphibians than to those of teleost fish.

The presence of AVT in caecilians was determined unambiguously by mass spectrometry analysis. Both brain and pituitary extracts contained material that coeluted with AVT standards and was immunoreactive toward an antiserum recognizing AVT. This peptide has been found previously in 11 anuran species (including Ranids, Bufonids, and Pipids; Sawyer et al., 1959; Nojiri et al., 1987; Rouille et al., 1989; Michel et al., 1990; Chauvet et al., 1991; Ouedraogo and Sawadogo, 1994) and in two urodele species (*P. waltlii* and *Ambystoma mexicanum*; Chauvet et al., 1991). AVT has also been isolated from fish and from representatives of virtually every other vertebrate class, although the presence of AVT in mammals has been disputed (Acher and Chauvet, 1995; Acher, 1996).

Caecilian brain extracts also contained material that coeluted with synthetic MT and was immunoreactive in our RIA. MT has been identified previously in four anurans (*R. esculenta*, *B. marinus*, *B. japonicus*, and *X. laevis*; Nojiri et al., 1987; Rouille et al., 1989; Michel et al., 1990) and in one urodele species (*P. waltlii*; Chauvet et al., 1991). It is therefore most likely that the second neurohypophysial peptide present in this caecilian brain is MT. Certainly, due to differences in retention time and/or lack of immunoreactivity, this second peak is unlikely to be hydrin 2, oxytocin, or vasopressin (Fig. 1). It is also unlikely that this peak is entirely isotocin, because our RIA antibody did not recognize isotocin. It is possible, however, that this caecilian brain contains isotocin in addition to an immunoreactive peptide. Because MT and isotocin elute near each other, the peak we identified could have contained both peptides. This peak may also represent a novel peptide with a retention time similar to that of MT and cross reactivity with our RIA antibody. One candidate is a variant of MT found in the African toad *B. regularis* (Chauvet et al., 1995). This peptide is identical in structure to MT, but with a substitution of serine at position 5. This peptide was not available, so we could not determine its possible cross reactivity with our RIA antibody.

The brain of this caecilian contained a prominent population of intermingled AVT-ir and MT-like-ir cells in the POA. A similar population is present in all vertebrates thus examined, including anuran and urodele amphibians (e.g., *R. catesbeiana*: Boyd et al., 1992; *P. waltlii*: Gonzalez and Smeets, 1992a). Dense fibers from these cells likely project to the posterior pituitary and correspond to neurosecretory cells in the paraventricular and supraoptic nuclei of reptiles, birds, and mammals (see, e.g., Swaab and Pool, 1975; Stoll and Voorn, 1985; Kiss et al., 1987; Smeets et al., 1990; Propper et al., 1992). The histological structure of caecilian pituitary is similar to that of other amphibians (Schubert et al., 1977; Doerr-Schott and Zuber-Vogeli, 1984; Masood-Parveez et al., 1994), and we isolated very large concentrations of AVT from the pituitary of

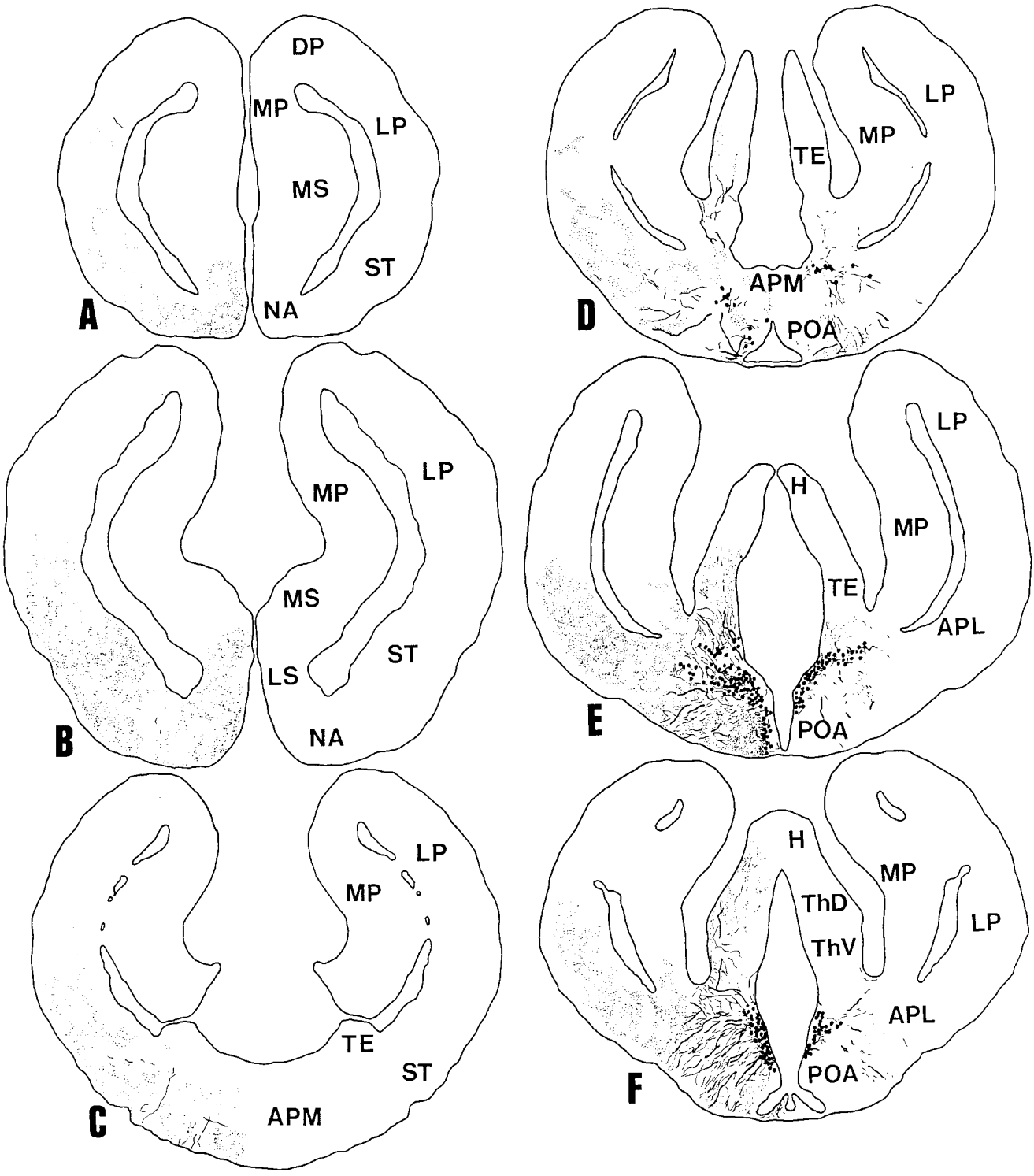

Fig. 3. **A–F:** Distribution of arginine vasotocin immunoreactive (AVT-ir) and mesotocin immunoreactive (MT-ir) cells and fibers in representative frontal sections through the caecilian brain (from rostral to caudal). The left side of each drawing shows camera lucida drawings of sections stained for AVT immunoreactivity; the right side of each drawing shows MT-ir distribution. For abbreviations, see list.

T. natans. In contrast to other vertebrates, however, there was no bimodal distribution of AVT-ir cell sizes in the caecilian POA and no indication of a separate "magnocellular" nucleus. In rats (Swanson and Sawchenko, 1983) and fish (Holmqvist and Ekstrom, 1995), for example, magnocellular neurons are the specific cells that project primarily

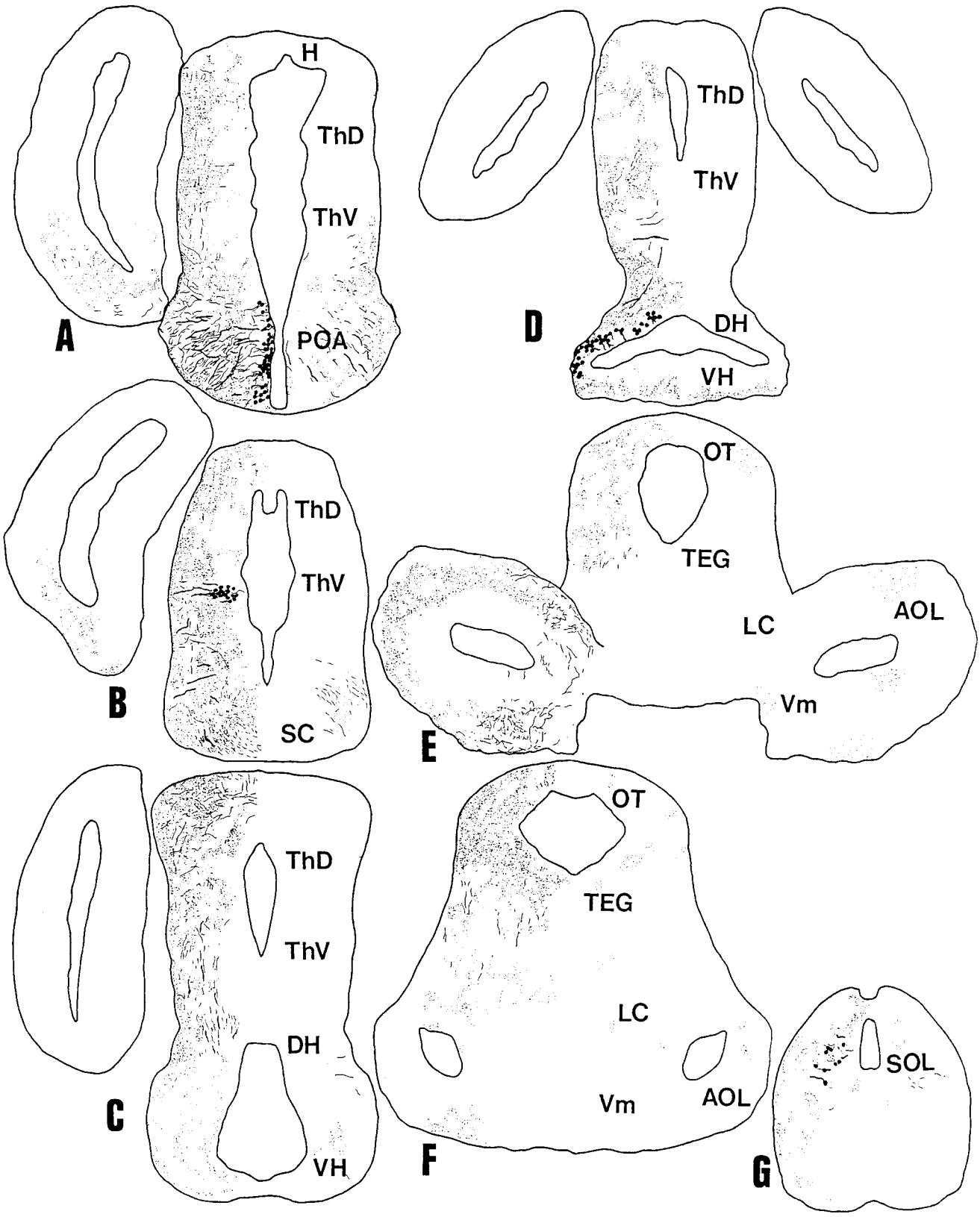

Fig. 4. **A–G:** Continuation of Figure 3 at more caudal levels. Right telencephalic hemisphere drawings are not displayed (A–C), because mesotocin (MT) immunoreactivity was never observed in these regions. For abbreviations, see list.

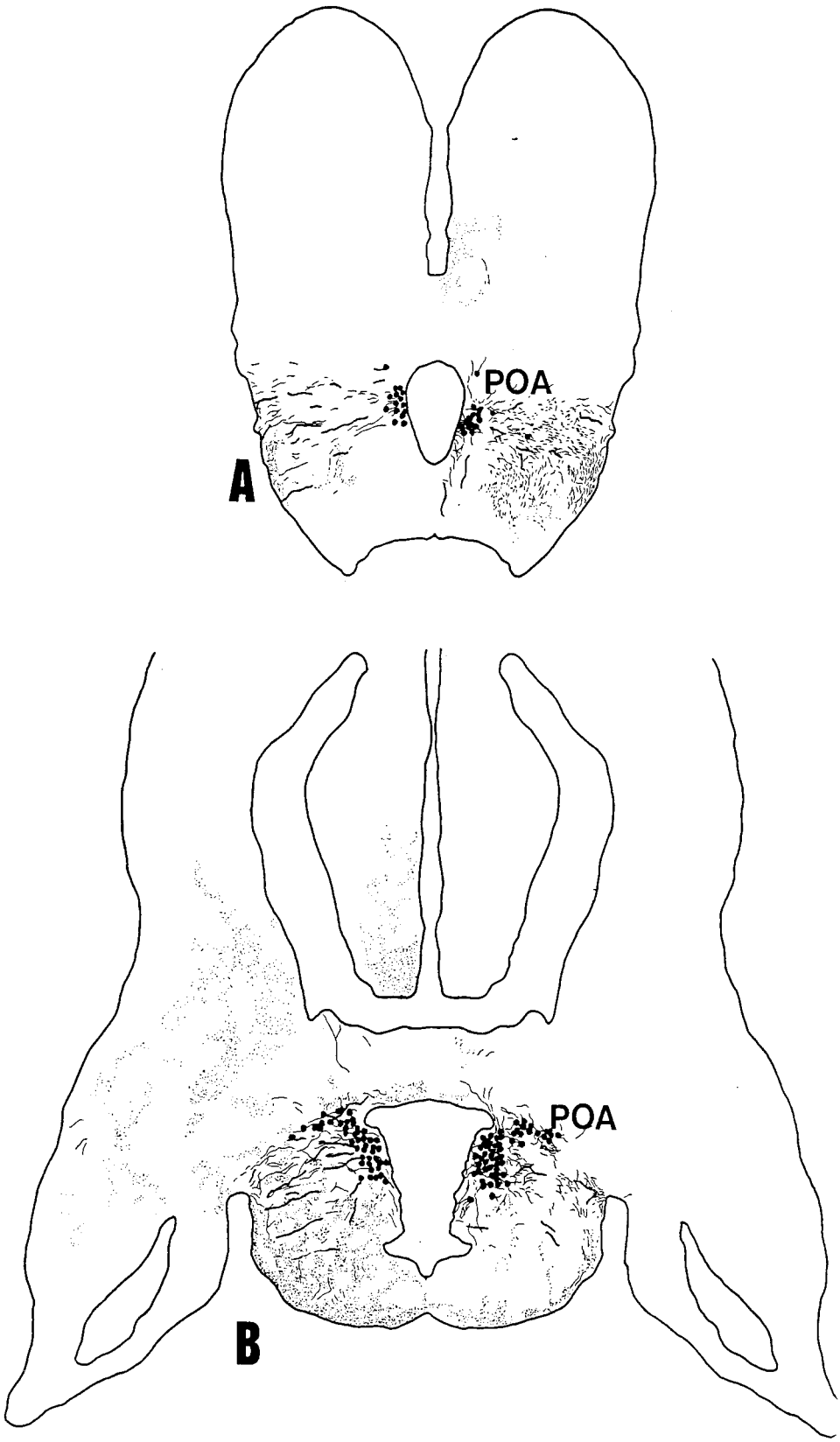

Fig. 5. Camera lucida drawings of the distribution of arginine vasotocin immunoreactive (AVT-ir; left) and mesotocin immunoreactive (MT-ir; right) cells and fibers in horizontal sections through the caecilian brain. Anterior is at top. The top section (**A**) is at a level ventral to the bottom section (**B**). POA, preoptic area.

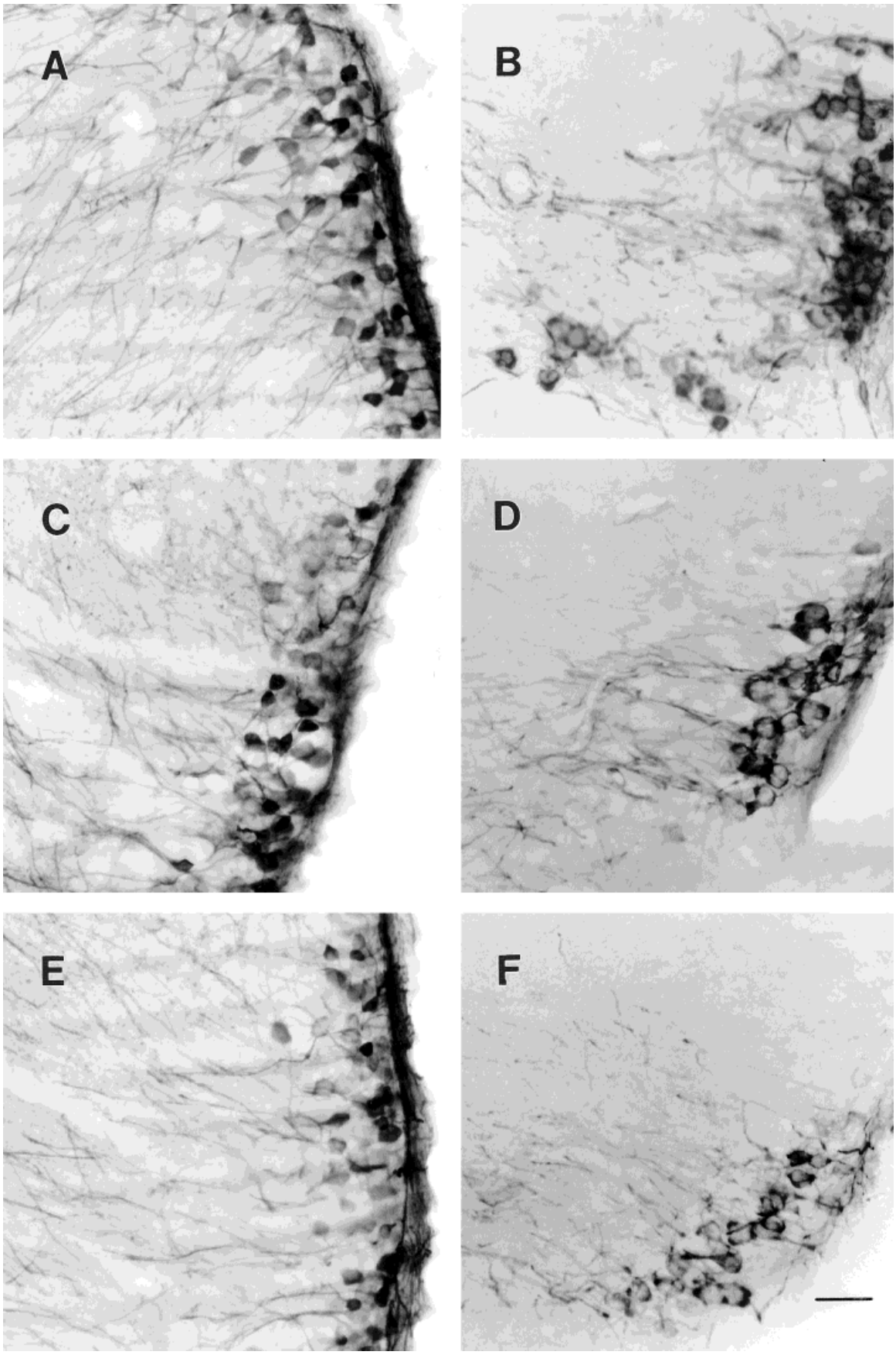

Fig. 6. Distribution of arginine vasotocin (AVT) and mesotocin (MT) immunoreactivity in the preoptic area (third ventricle is to the right; horizontal sections). **A:** Staining with AVT antibody alone. **B:** Staining with MT antibody alone. **C:** Staining when AVT antiserum is preadsorbed with MT. **D:** Staining when MT antiserum is preadsorbed with AVT. **E:** Staining when AVT antiserum is preadsorbed with isotocin. **F:** Staining when MT antiserum is preadsorbed with isotocin. Scale bar = 50 µm.

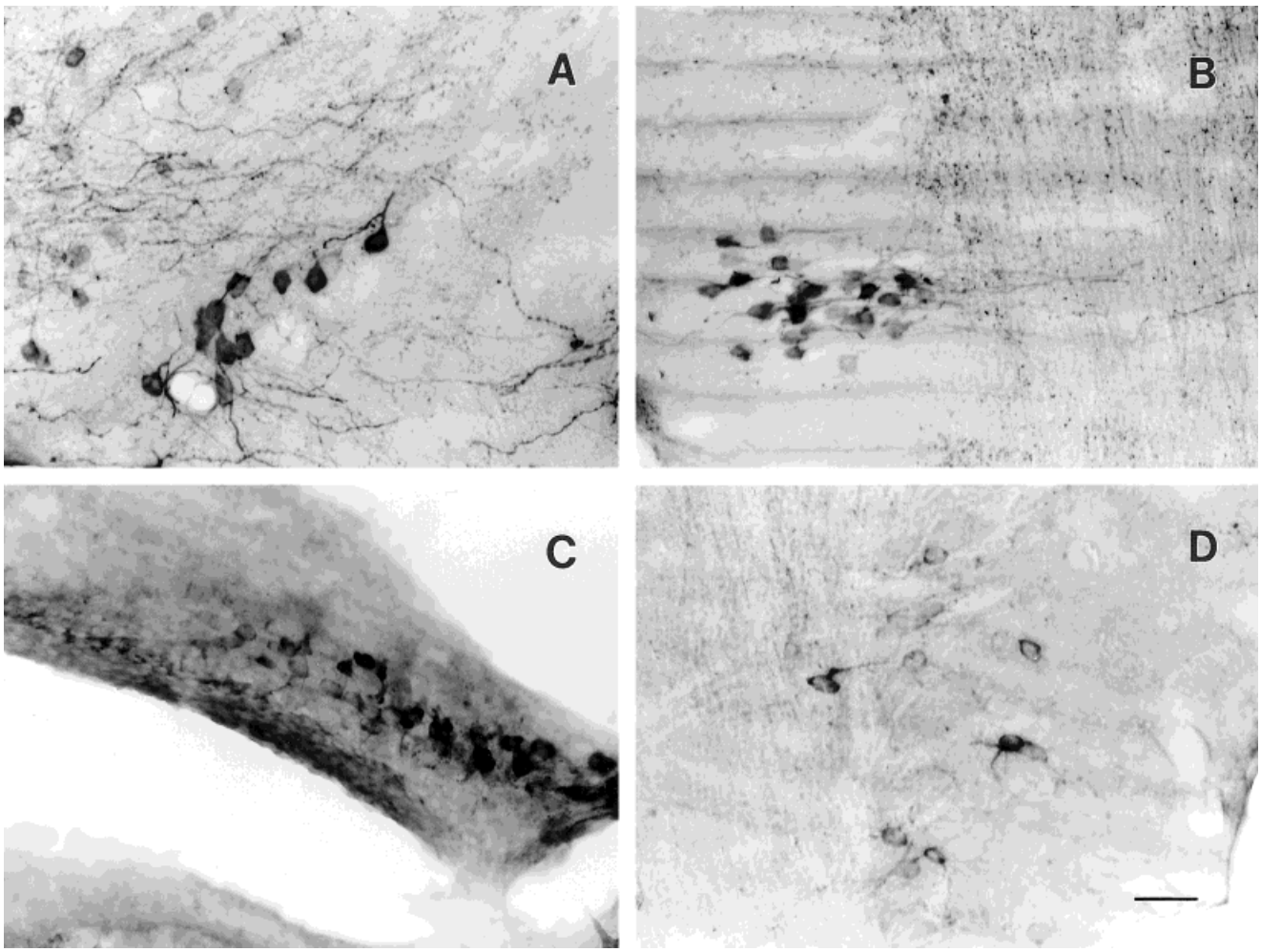

Fig. 7. Arginine vasotocin immunoreactive (AVT-ir) cells and fibers in the amygdala (**A**), ventral thalamus (**B**), dorsal hypothalamic nucleus (**C**), and solitary tract (**D**). Horizontal sections. Scale bar = 50 μm.

TABLE 1. Area of Arginine Vasotocin Immunoreactive Cell Bodies in the Brain of *Typhlonectes natans*

Brain area	Number of animals	Number of cells	Soma area (μm²) (mean ± S.E.M.)[1]
Amygdala pars medialis	5	60	220 ± 7[a,b*]
Preoptic area	19	452	171 ± 3[c]
Dorsal hypothalamus	5	50	155 ± 7[c]
Ventral thalamus	2	16	202 ± 10[a]
Solitary tract	1	8	255 ± 15[a,b]

[1]One-way analysis of variance results: F = 14.03; d.f. = 4, 581; $P < 0.0001$.
*Groups with the same superscript letter are not significantly different from each other but are significantly different (Fisher's least significant difference test; $P < 0.05$) from all other groups without that letter.

to the posterior pituitary. Differences in neurosecretory cell size are also likely associated with cell activity and not solely with efferent projections, however (e.g., rats: Kalimo, 1975; chickens: Chaturvedi et al., 1994; frogs: Subhedar and Krishna, 1990). Thus, the projection from the POA to the posterior pituitary likely exists in this caecilian despite the absence of a distinct magnocellular nucleus, and smaller cell sizes may reflect less active neurosecretory cells in this aquatic species.

Based on their location close to the median eminence and pituitary, AVT-ir cells in the dorsal hypothalamus of caecilians may also be neurosecretory. Cells in the dorsal hypothalamus did not differ significantly in size from presumptive neurosecretory cells in the POA. Furthermore, neurosecretory cells have been identified in this region in the brains of two other caecilians (*Chthonerpeton indistinctum*, *Ichthyophis paucisulcus*) by using classic histological and enzyme histochemical techniques (Welsch et al., 1976). Anuran and urodele amphibians also possess hypothalamic AVT cells (e.g., *R. catesbeiana*, *R. ridibunda*, *R. sylvatica*, *X. laevis*, and *P. waltlii*; Boyd et al., 1992; Gonzalez and Smeets, 1992a,b; Mathieson, 1996). This population is more variable in other vertebrates, however. It was not found in rainbow trout, but a possible homologous population is present in the nucleus periventricularis hypothalami of the cartilaginous fish *Scyliorhinus canicula* (Van Den Dungen et al., 1982; Vallarino et al., 1990). In reptiles and birds, AVT-ir cell populations are present in the infundibulum or hypothalamus of several species (Viglietti-Panzica and Panzica, 1991; Propper et al., 1992; Sugita, 1994; Aste et al., 1996; and references therein). An

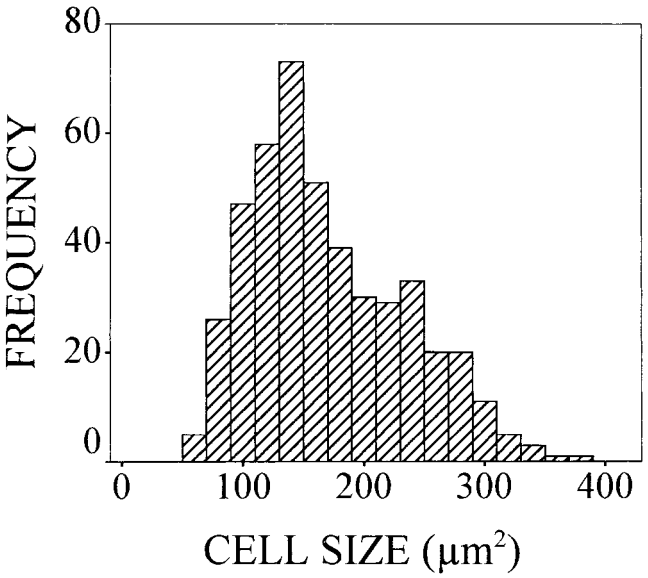

Fig. 8. Cell-size histogram showing the distribution of arginine vasotocin immunoreactive (AVT-ir) cell areas within the POA of caecilians (452 cells). Bin width was 20 μm².

AVP-ir population in the dorsomedial hypothalamic nucleus of rats may also be homologous (Caffe and Van Leeuwen, 1983).

AVT-ir cells were not observed in two brain regions that contained such cells in some other amphibians. First, no AVT-ir cells were found in the septal nucleus of *T. natans*. Septal AVT-ir cells have been found in the bullfrog (*R. catesbeiana*) brain (Boyd et al., 1992; Mathieson, 1996) but not in any other amphibian described so far. Second, suprachiasmatic AVT-ir cells also were not found in this caecilian. Although the eyes of caecilians are much reduced, they are functional in at least several species, including *Typhlonectes* (Wake, 1980, 1985; Himstedt and Manteuffel, 1985). The precise location of the caecilian SCN is difficult to discern, however, due to reduced size of the optic nerves and absence of an external optic chiasm. The location where optic nerves enter the brain of the caecilians *Ichthyophis kohtaoensis* and *Typhlonectes compressicauda* has been described (Clairambault et al., 1980; Fritzsch et al., 1985; Himstedt and Manteuffel, 1985). AVT-ir cells were not present in these locations in *T. natans*. AVP and AVT cells are located in the SCN of rats (Swaab and Pool, 1975; Vandesande et al., 1975) and frogs (*R. catesbeiana*, *R. sylvatica*, *R. ridibunda*, and *X. laevis*; Boyd et al., 1992; Gonzalez and Smeets, 1992a,b; Mathieson, 1996) and in an anatomically similar area in fish (Van Den Dungen et al., 1982) but not in the salamander *P. waltlii* (Gonzalez and Smeets, 1992a). The SCN of most birds and reptiles, in addition, does not contain AVT or MT-ir cells (Stoll and Voorn, 1985; Kiss et al., 1987; Smeets et al., 1990; Propper et al., 1992; but see Panzica, 1985).

A small population of AVT- and MT-ir cells was also located in the amygdala pars medialis. No positive staining for either peptide was observed in the amygdala pars lateralis, where AVT cells are located in the bullfrog (Boyd et al., 1992; Mathieson, 1996). A population of AVT-ir cells in the pars medialis portion of the amygdala is also typical of other amphibians studied, including *Xenopus*, *R. sylvatica*, *R. ridibunda*, and *P. waltlii* (Gonzalez and Smeets, 1992a,b; Mathieson, 1996). In other amphibians, the amygdala receives projections from the olfactory bulb (Northcutt and Royce, 1975), so an association with the well-developed olfactory system in caecilians is possible.

A discrete group of AVT-ir cells and fibers was found in the anteriormost regions of the ventral thalamus in *T. natans*. In anuran amphibians (e.g., bullfrogs; Boyd et al., 1992), AVT-ir cells are present in the same location. These cells are similar morphologically to caudal POA cells and appear to be related to the POA group in sagittal sections. The ventral thalamus cells in both bullfrogs and this caecilian, however, are separated from the POA by a clear zone that is lacking in AVT-ir cell bodies. The functions and connections of this population are currently unknown.

Hindbrain populations of AVT-ir or AVP-ir cells have been described in a variety of vertebrates, but the location of these populations seems to be the most variable across species. No MT-ir cells were present in the hindbrain of the caecilian, but AVT-ir cells and fibers were present in the presumed solitary tract of the myelencephalon. In the rhombencephalon of *R. ridibunda* and *P. waltlii*, AVT-ir cells have been observed in the nucleus of the solitary tract and near the locus coeruleus, respectively (Gonzalez and Smeets, 1992a). AVT-ir cells are also located in the pretrigeminal nucleus of the bullfrog and in a similar area in the lizard *Gecko gecko* (Stoll and Voorn, 1985; Boyd et al., 1992). Determining anatomical overlap is especially difficult for caecilians, however, due to the pronounced pontine flexure in caecilian brain. An AVP-ir population in the rat locus coeruleus (Caffe and Van Leeuwen, 1983) may be homologous to the caecilian AVT-ir hindbrain cell group. Specifically, the locus coeruleus AVP-ir cells in rats also contain noradrenaline (Caffe et al., 1985). Although coexistence of catecholamines in AVT-ir cells has not been shown in caecilians, tyrosine hydroxylase-ir cell bodies are found in the locus coeruleus and solitary tract of the caecilian *T. compressicauda* (Gonzalez and Smeets, 1994). These cells may be involved in the control of heart rate or blood pressure (Matsuguchi et al., 1982; Berecek et al., 1984).

These results support the hypothesis that the tetrapod pattern of neurohypophysial peptide neurochemistry and neuroanatomy first arose before divergence of orders within the Lissamphibia. Specifically, the general teleost pattern consists of cells that synthesize the peptide pair of AVT and isotocin. There are no extrahypothalamic cell populations, and extrahypothalamic fiber projections are rare. In contrast, this caecilian species possesses the general tetrapod pattern of neurohypophysial peptide production (the vasotocin and MT pair), and extrahypothalamic cells and fibers are common.

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