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The Serotoninergic System of the Brain of the Viper, *Vipera aspis*. An Immunohistochemical Study

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ABSTRACT

Serotoninergic cell bodies and fibers in the brain of the viper, *Vipera aspis*, were visualized by immunohistochemistry. Immunoreactive cell bodies were observed in the diencephalic hypothalamic periventricular organ and in the dorsal wall of the infundibular recess, in the nuclei raphe superior and inferior of the midbrain and hindbrain, and to a lesser extent in the nuclei reticularis superior, reticularis inferior and reticularis lateralis. In contrast to other reptilian species, serotoninergic cells were also observed in the central gray matter of the midbrain in the neighbourhood of the nucleus of the trochlear nerve. Immunoreactive fibers are widely distributed throughout the brain of the viper. In the olfactory bulb, fibers were observed in the internal plexiform layer and mitral cell layer. The cerebral cortex contains the highest density of fibers in the dorsal region. The distribution of immunoreactive fibers in the dorsal ventricular ridge is extremely heterogeneous, and five subcomponents of this structure can be distinguished. The majority of diencephalic and mesencephalic structures that contain immunoreactive fibers are also primary visual centres: the nuclei geniculatus lateralis pars dorsalis, the n. posterodorsalis and n. opticus tegmenti, and the optic tectum. Serotoninergic fibers in the nuclei of the oculomotor and motor crainal nerves (III, IV, V, VII, X) are disposed in a tightly woven basket around the non-immunoreactive cell bodies of the motoneurons. These findings, together with the available literature, suggest that the serotoninergic system in snakes is comparable to that in lizards, with a massive ascending projection of fibers from the n. raphe superior to mesencephalic and prosencephalic structures, and a descending projection from the n. raphe inferior to the spinal cord.

KEY WORDS: Serotonin Central nervous system Ophidians Immunohistochemistry

INTRODUCTION

The immunochemical techniques that have become available for the study of serotoninergic systems (Steinbusch *et al.*, 1978; Takeuchi *et al.*, 1982) have a number of advantages over the formaldehyde-induced fluorescence technique (FIF; Falck, 1962; Falck *et al.*, 1962) that was used for the last two decades to study the distribution of biogenic amines in the vertebrate central nervous system (see, e.g. Parent, 1984 and Wolters *et al.*, 1985 for review). The fluorescent derivative of serotonin (β-carboline) produced by the FIF procedure is photolabile, and the freeze-drying necessary for the procedure degrades the quality of the final histological preparation. Thus the procedure is likely to lead on the one hand to an underestimate of the density of serotoninergic cell bodies and fibers, and on the other hand the precision with which fluorescent structures can be located may be somewhat questionable.

While immunohistochemical techniques have been recently used to study the distribution of serotoninergic structures in a variety of vertebrates, the greatest number of these studies concern mammals (see Steinbusch and Nieuwenhuys, 1983 and Wolters *et al.*, 1985 for review). It is evident that a comprehensive account of the evolution of the serotoninergic system in tetrapods will necessarily involve an exhaustive comparative analysis of this system in reptiles, given their critical position in vertebrate phylogeny. Unfortunately, the available data are extremely limited; the serotoninergic system has been described in but two species of turtle (by the FIF technique in *Chrysemys picta*, Parent and Poirier, 1971, Parent and Poitras, 1974, and immunohistochemically in *Clemmys japonica*, Ueda *et al.*, 1983), and among the squamates the data are provided

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entirely by lizards: *Chameleo chameleo* (Bennis *et al.*, 1990), *Gekko gecko* (Smeets and Steinbusch, 1988), *Lacerta agilis* (Petko and Ihionvien, 1989), *L. muralis* and *L. sicula* (Marschall, 1980), *Ophisaurus apodus* (Pierre *et al.*, 1990), *Psammodromus algirus* and *Podarcis hispanica* (Guirado *et al.*, 1989), and *Varanus exanthematicus* (Wolters *et al.*, 1985). We therefore present below the first study of the serotoninergic system in an ophidian, *Vipera aspis*.

METHOD

Antibodies to the serotonin–bovine serum albumin complex formed by fixation with paraformaldehyde (type 601A) or glutaraldehyde (type 601B) were obtained from a commercial supplier (Immunotech, Marseille).

Snakes were anesthetized with Nembutal (25–30 mg/kg), perfused with physiological (0.9%) saline followed by a fixative composed of either 4% paraformaldehyde or 1% paraformaldehyde–1% glutaraldehyde in 0.12 M-phosphate buffer (pH 7.4). Brains were dissected into fresh fixative and stored for 12 h before being washed in several changes of phosphate buffer. Washed brains were subsequently dehydrated in a graded series of ethanol, impregnated with polyethylene glycol (PEG; molecular weight 400) and finally embedded in PEG 1000–1500. Blocks were stored over silica gel at 4°C.

Sections were cut at room temperature, at 12–18 μm on a rotary microtome and transferred to phosphate-buffered saline (PBS), washed in several changes of PBS–Triton, treated with 0.1 M-lysine or 0.3% sodium borohydride to inactivate the residual free aldehyde groups of the fixing agent and finally washed in PBS–Triton containing 0.2% gelatin and 0.1% sodium azide prior to immunochemical treatment.

Washed sections were incubated for 12 h in a solution of the primary antibody, washed in PBS–Triton, incubated for 1 h in a solution of the second antibody (biotinylized goat anti-rabbit, Vector), washed again in PBS–Triton and transferred to a solution of avidine-biotin-horseradish peroxidase (HRP). Peroxidase was visualized by the diaminobenzidine reaction, and sections were finally gathered on gelatinized slides, dehydrated, cleared and mounted in Eukitt. The specificity of the immunochemical procedure was controlled by replacing the primary antibody with normal rabbit serum; in no such case was any labelling observed. In no case did we observe any differences that could be ascribed to the use of two different antibodies, each of which was associated with a different fixative.

The anatomical nomenclature that we use below relies as far as possible on the following studies of the ophidian brain: for the telencephalon, Ulinski (1974, 1983), Halpern (1980); for the diencephalon, Repérant (1973); for the mesencephalon, Repérant (1973), Ten Donkelaar and Nieuwenhuys (1979); and for the rhombencephalon. Molenaar (1977), Ten Donkelaar and Nieuwenhuys (1979).

RESULTS

Telencephalon (Fig. 1A–E)

The telencephalon contains many immunoreactive fibers and terminal arborizations, but no immunoreactive cell bodies.

In the principal olfactory bulb (Fig. 1A) both the mitral cell layer and the internal plexiform layer contain numerous serotoninergic fibers which are absent in the fibrous and glomerular layers; in the accessory olfactory bulb (Fig. 1B, Fig. 2) an abundant plexus of reactive fibers is seen in the external and internal plexiform layers together with the mitral cell layer. The lateral olfactory tract also contains a large number of fine immunoreactive fibers.

The labelling in the cortex (Fig. 1C–E) is extremely heterogeneous. The greatest density of immunoreactive fibers is found in the dorsal cortex (Fig. 4), within which the fibers are observed primarily in the external plexiform layer and in the posteromedial region of the internal plexiform layer, the cellular layer containing but a very small proportion of labelled fibers. The dorsomedial cortex shows an appreciable number of terminals which, for the most part, are situated at the periphery of the external plexiform layer. In the medial cortex, the much smaller number of labelled fibers are located on either side of the cellular layer. The lateral cortex contains rostrally a thin superficial layer of labelled fibers, but elsewhere shows only a few scattered immunoreactive axons.

The serotoninergic innervation of subcortical structures (Fig. 1C–E) is extremely variable. The greatest density of serotoninergic fibers is found in the nuclei accumbens and septalis dorsalis (Fig. 3) and the partes angulolateralis and angulomedialis of the dorsal ventricular ridge, each of which contains an ovoid plexus of intensely reactive fibers. A lower density of labelled fibers is found in the pars medialis of the dorsal ventricular ridge (Figs 4, 5), the nucleus of Broca's band, the amygdala complex, the corpus striatum and the n. commissurae hippocampi. Immunoreactivity is relatively weak in the partes rostralis and caudalis of the dorsal ventricular ridge, the nn. septalis lateralis and medialis, the olfactory tubercle and the n. sphericus. Among the fiber tracts, the medial longitudinal fascicle is heavily labelled (Fig. 5), the anterior commissure contains a few immunoreactive fibers, while the accessory olfactory tract and the lateral forebrain bundle are practically devoid of label.

Diencephalon (Fig. 1F, G)

The hypothalamic periventricular organ (OPH; Fig. 1G, Fig. 8) forms the most rostral group of

immunoreactive neurons. The majority of labelled cell bodies, which are either globular or elongated, give off two prolongations perpendicular to the ventricular wall; the medial, internal prolongation emerges into the ventricular lumen and is bathed by the cerebrospinal fluid, while the lateral, external prolongations extend in the opposite direction towards the n. periventricularis hypothalami, forming a dense plexus of thin fibers. The dorsal wall of the infundibular recess also contains a small number of immunoreactive cell bodies in contact with the cerebrospinal fluid.

With the exception of the optic nerve and chiasma, the marginal optic tract and the subcommissural organ, all of which are totally free of label, the diencephalon is generally well endowed with serotoninergic fibers. The most heavily labelled nuclei are the nuclei geniculatus lateralis pars dorsalis (Fig. 1F, Fig. 6) and posterodorsalis (Fig. 1G, Fig. 7), the hypothalamic region lateral to the OPH, and a nucleus in the posteroventral diencephalon lying laterally against the tectothalamic tract which most likely corresponds to the subpretectal nucleus described in *Crotalus viridis* by Berson and Hartline (1988). The nn. geniculatus lateralis pars ventralis and pretectalis are slightly less intensely labelled. The remaining diencephalic structures (the epithalamic nn. habenulares medialis and lateralis, the thalamic nn. lentiformis mesencephali, geniculatus pretectalis, lentiformis partes extensa and plicata, rotundus, ventromedialis and ventrolateralis thalami, and the centrifugal optic nucleus, together with the hypothalamic nn. periventricularis hypothalami and ventralis hypothalami, the area preoptica and the ventral peduncle of the lateral forebrain bundle) are considerably less well endowed with serotoninergic fibers.

Mesencephalon (Fig. 1H–J)

Immunoreactive cell bodies are found in the partes medialis and lateralis of the n. raphe superior at the level of the oculomotor nucleus (Fig. 1I, J, Fig. 10), the labelled cells of the pars medialis extending further caudally to the level of the isthmus (Fig. 10). The cell bodies are relatively small and variable in shape, most frequently ovoid but sometimes fusiform, with poorly distinguishable neurites. More laterally, the n. reticularis superior (Fig. 1I, J, Fig. 11) contains a sparse population of reactive cells in both the partes medialis and lateralis, which give off prolongations running parallel to the ventral surface of the brainstem. At the level of the trochlear nucleus (Fig. 1J) a few immunoreactive, fusiform cells, oriented dorsoventrally, can be observed around the medial longitudinal fascicle, as well as a few multipolar cells in the ventrolateral zone of the substantia grisea centralis (Fig. 1J, Fig. 12).

High densities of serotoninergic fibers are only found, in the midbrain, in the nn. opticus tegmenti, interpeduncularis, raphe superior pars medialis, ventralis mesencephali and the caudal part of the n. isthmi (Fig. 1H–J). Smaller quantities of labelled fibers are found in the n. profundus mesencephali, the torus semicircularis and the substantia grisea centralis and the nn. nervi oculomotorii and trochlearis. In the two latter nuclei the serotoninergic fibers form baskets around non-reactive motoneurons (Fig. 13). Immunoreactive fibers are also scattered throughout the optic tectum (Fig. 9) with no tendency to be grouped in any particular layer, but are rarer in the strata opticum and zonale.

Rhombencephalon (Fig. 1K–M)

Serotoninergic cell bodies are found throughout the length of the n. raphe inferior (Fig. 14), with particular clustering at the level of the nuclei of the VIth and VIIth cranal nerves. Labelled cells are also found in the nn. reticularis superior pars medialis (Fig. 1K), reticularis lateralis and reticularis inferior partes ventralis and lateralis (Fig. 1M). These medium- to large-sized cells give off processes which are oriented most frequently parallel to the ventral surface of the brainstem. Further caudally, a small number of immunoreactive cells are found on the internal surface of the medial longitudinal and predorsal fascicles.

Two rhombencephalic structures in particular receive a pronounced supply of serotoninergic fibers, the nn. motorii trigemini and nervi facialis, in which the fibers are disposed in baskets around unlabelled motoneurons. A large population of immunoreactive fibers is also found lying ventrolaterally to these nuclei.

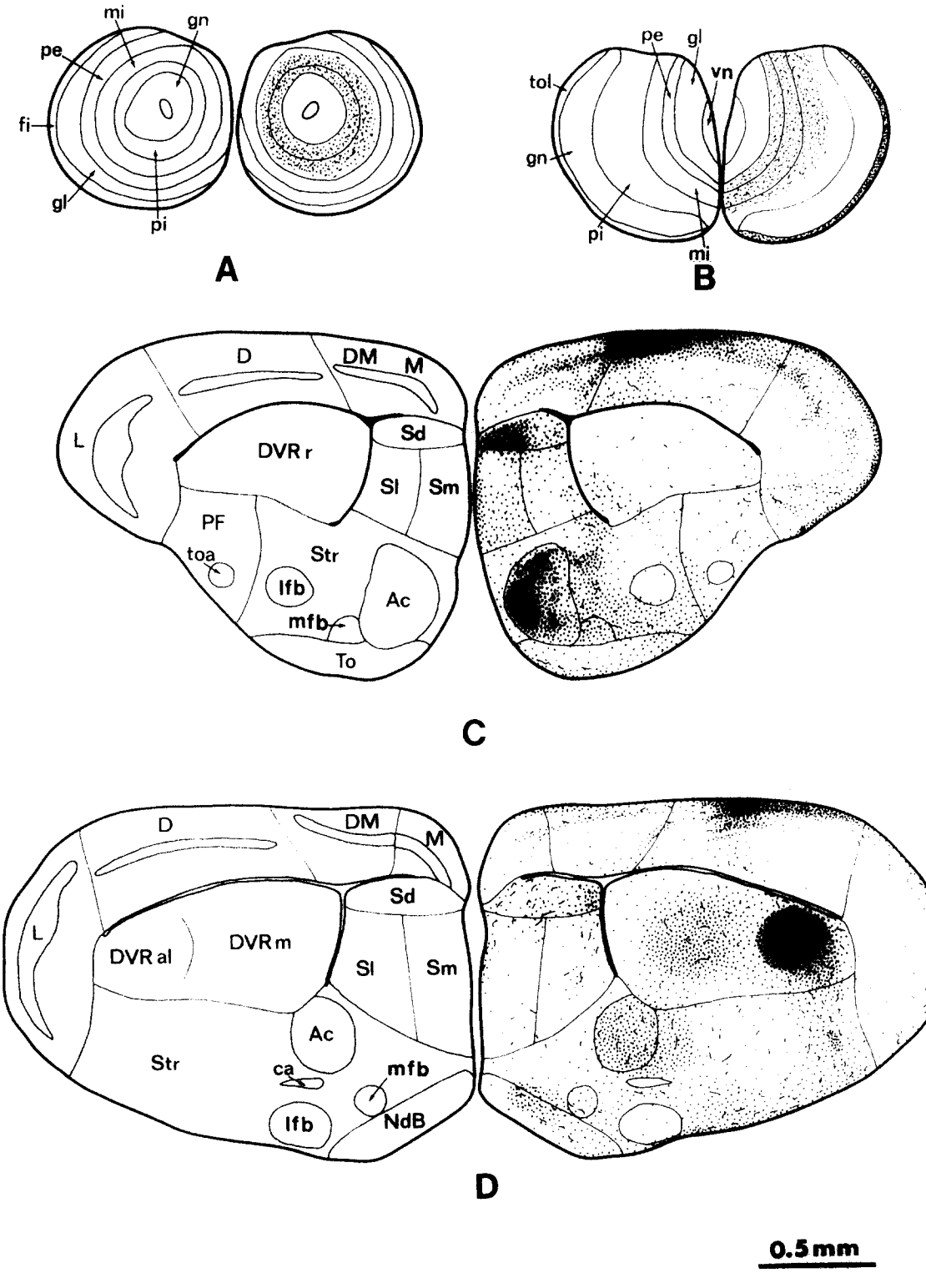
Appreciable quantities of serotoninergic fibers are also found in the granular layer of the cerebellar cortex, the substantia grisea centralis, the nuclei of the VIth, VIIIth and XIIth nerves, and to a lesser extent the nuclei of the Xth nerve, the n. tractus solitarius and the nucleus of the funiculus dorsalis. The immunoreactivity of the remaining rhombencephalic structures is extremely weak.

DISCUSSION

We have pointed out above that the available data concerning the serotoninergic system of the reptilian brain are somewhat fragmentary, and in the absence of findings in a wider and more representative set of species, any conclusions will thus be necessarily somewhat tentative. Nevertheless, in the discussion which follows we attempt to compare our results, obtained in an ophidian, with those obtained in other reptilian groups, and we point out that there does appear to be a general tendency underlying the evolution of the serotoninergic system in vertebrates.

The distribution of serotoninergic neurons

In *Vipera aspis*, as in anamniotes and in the majority of other reptilian species, the most rostral immuno-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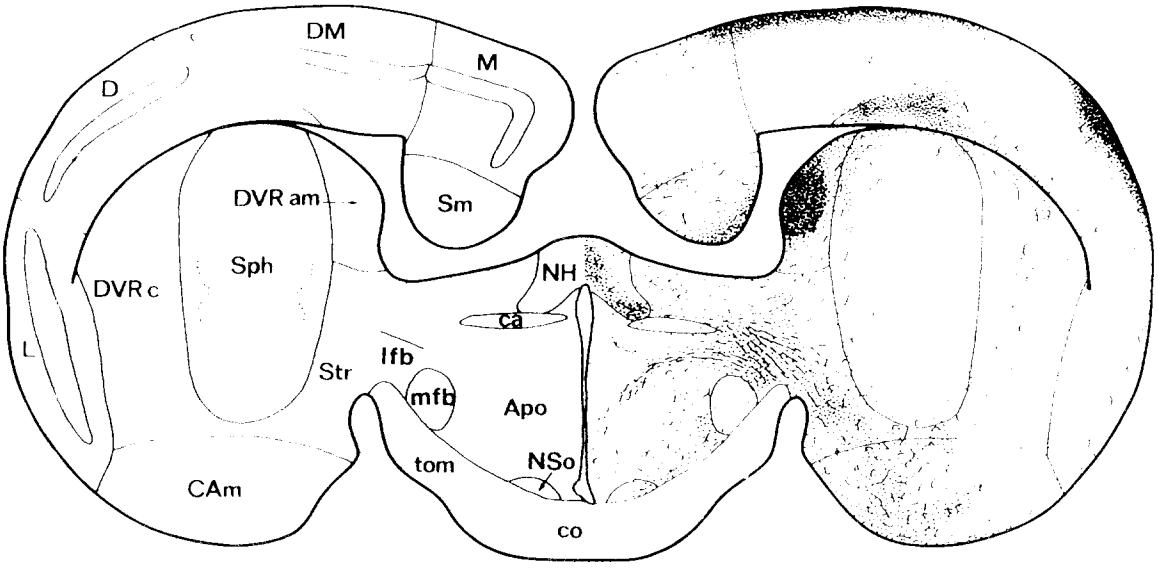
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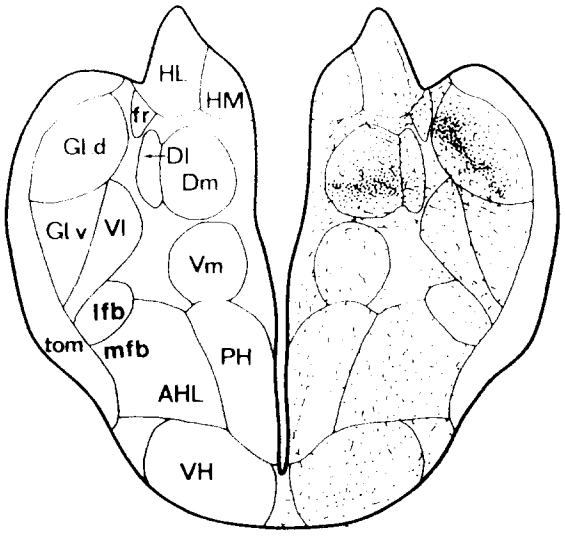
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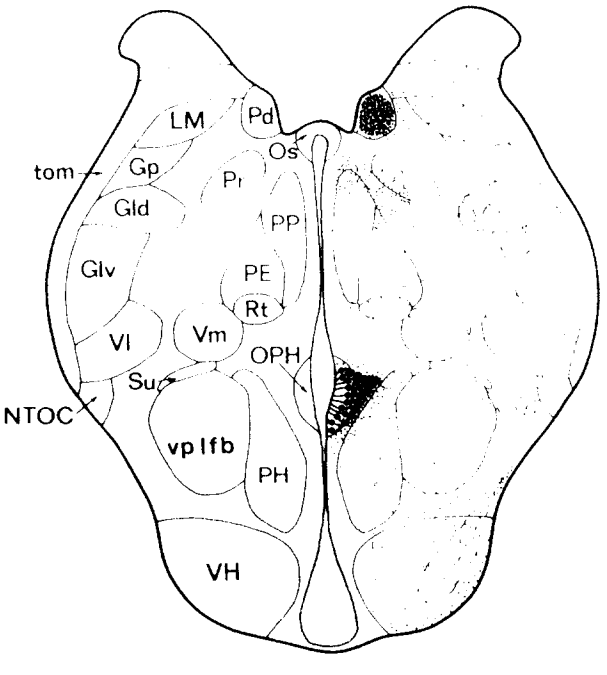
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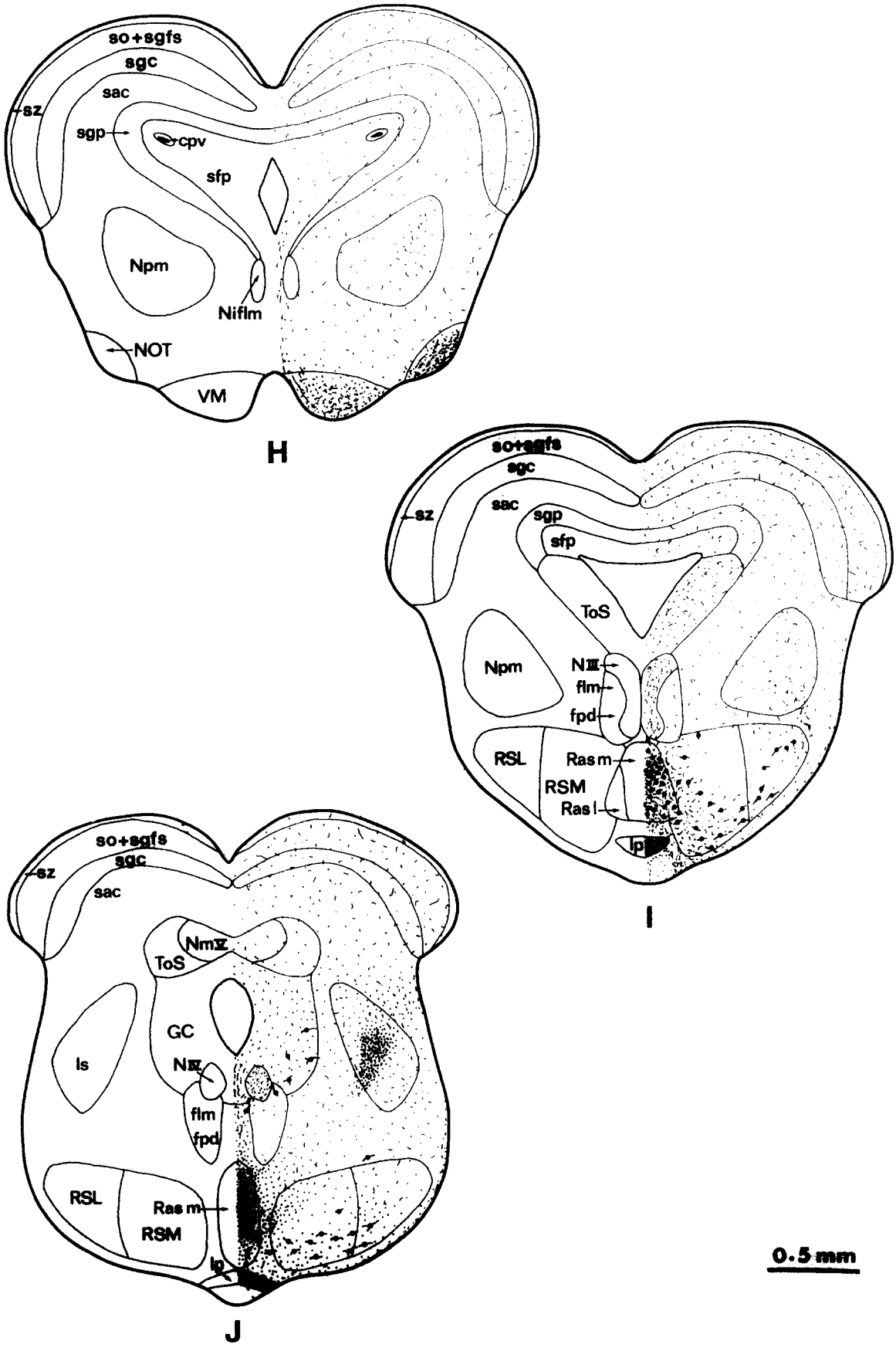
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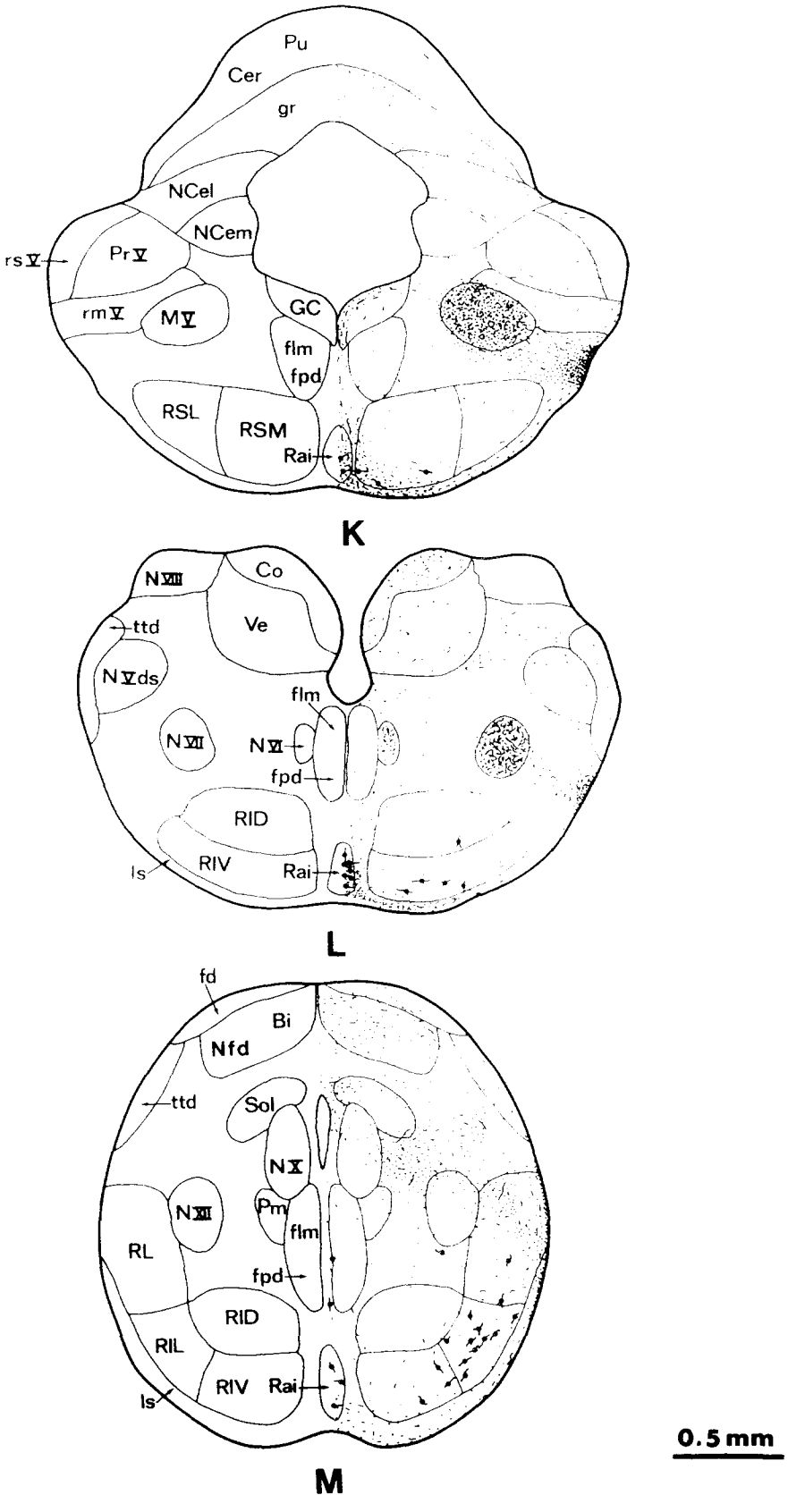
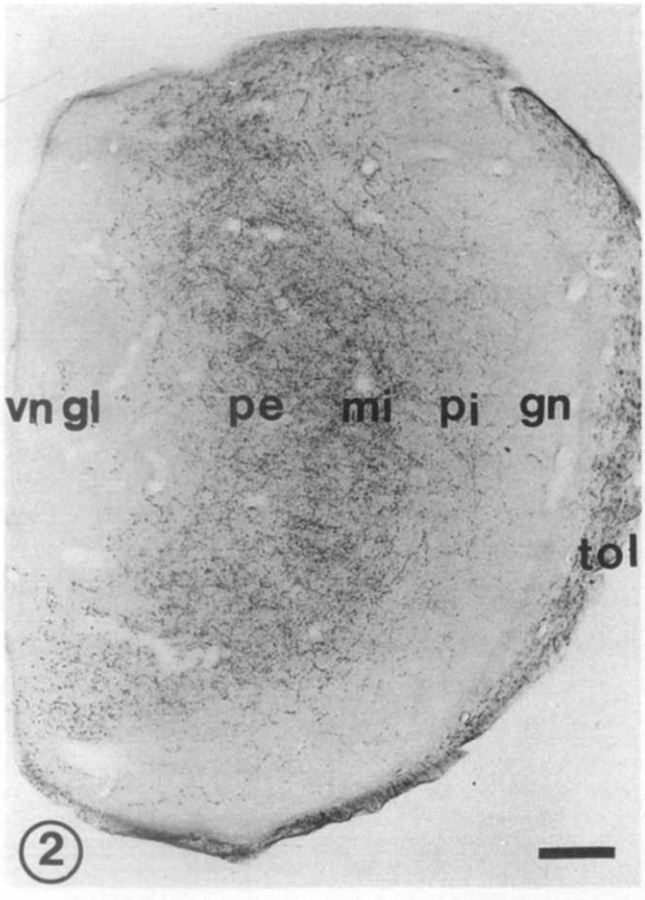


Fig. 1 k–m.

Fig. 1A–M. Camera lucida drawings of transverse sections of the viper brain, showing the distribution of serotonin-immunoreactive cell bodies (large dots) and fibers and terminals (fine stippled areas). (A) Principal olfactory bulb; (B) accessory olfactory bulb; (C,D) telencephalon; (E) telencephalo-diencephalic junction; (F,G) diencephalon; (H–J) mesencephalon; (K) cerebellum and rhombencephalon; (L,M) rhombencephalon.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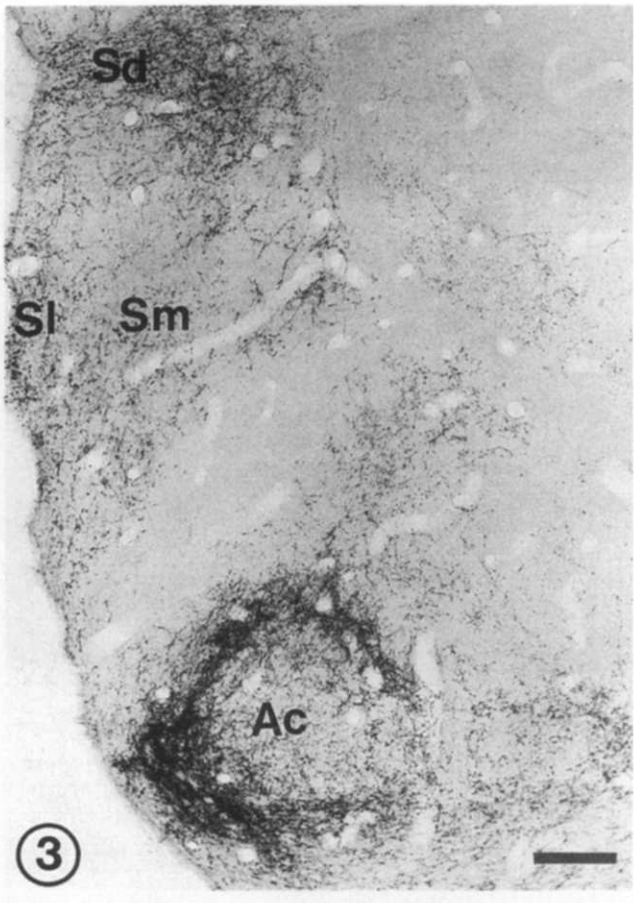
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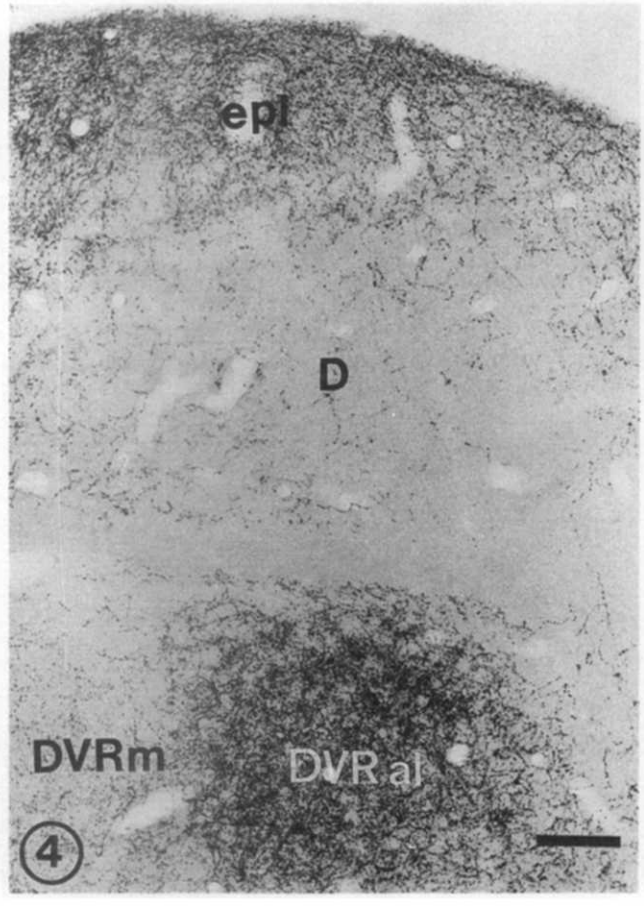
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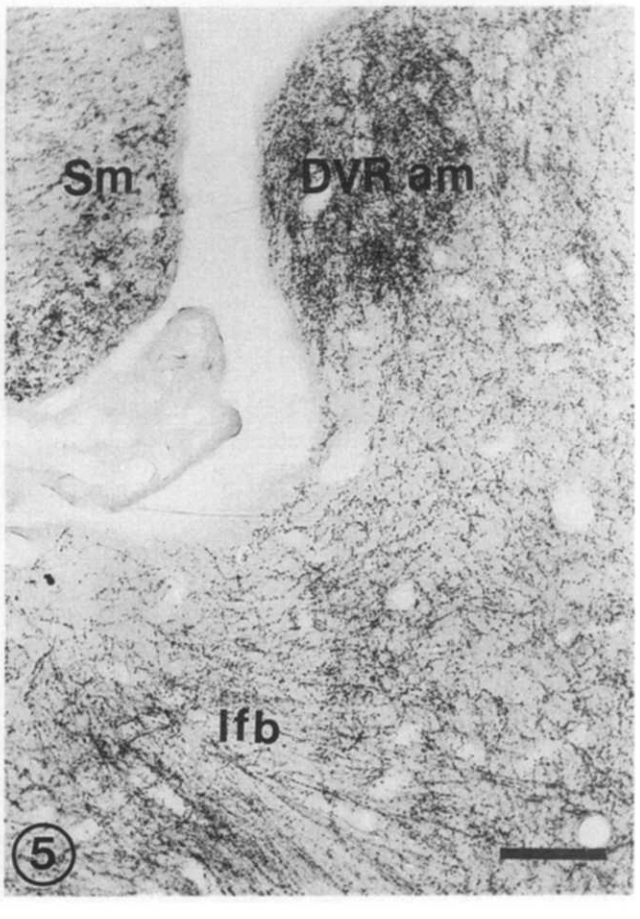
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reactive cells are found in the periventricular organ in the wall of the third ventricle; this structure, referred to as the paraventricular organ by some authors, has also been shown to contain serotoninergic cells in a variety of lizards (*Gekko gecko*, Smeets and Steinbusch, 1988; *Lacerta agilis*, Petko and Ihionvien, 1989; *L. muralis*, Braak *et al.*, 1968; Marschall, 1980; *L. sicula*, Marschall, 1980; *Ophisaurus apodus*, Pierre *et al.*, 1990; *Varanus exanthematica*, Wolters *et al.*, 1985) and in the turtle *Clemmys* (Sano *et al.*, 1983; Ueda *et al.*, 1983), whereas no serotoninergic cells were described in the periventricular organ of *Chameleo* (Bennis *et al.*, 1990) or of *Chrysemys* (Parent and Poirier, 1971; Parent and Poitras, 1974; Parent, 1979).

These results were obtained either with the FIF technique (Braak *et al.*, 1968; Marschall, 1980; Parent and Poirier, 1971; Parent and Poitras, 1974; Parent, 1979) or by immunochemistry (Bennis *et al.*, 1990; Petko and Ihionvien, 1989; Pierre *et al.*, 1990; Sano *et al.*, 1983; Ueda *et al.*, 1983; Smeets and Steinbusch, 1988; Wolters *et al.*, 1985) and thus the contradictory findings cannot be ascribed to the choice of method used. According to Marschall (1980) the concentration of monoamines in the OPH fluctuates considerably throughout the reproductive cycle and is greatest during the period of sexual activity. It is thus conceivable that the studies of *Chameleo* and *Chrysemys* were made during the period of sexual inactivity and hence that the concentration of serotonin in this structure was too low to be detected by either of the two methods.

The serotoninergic cells situated in the dorsal wall of the infundibular recess of the viper (present results) have also been described in *Ophisaurus* (Pierre *et al.*, 1990), *Gekko* (Smeets and Steinbusch, 1988) and *Clemmys* (Ueda *et al.*, 1983).

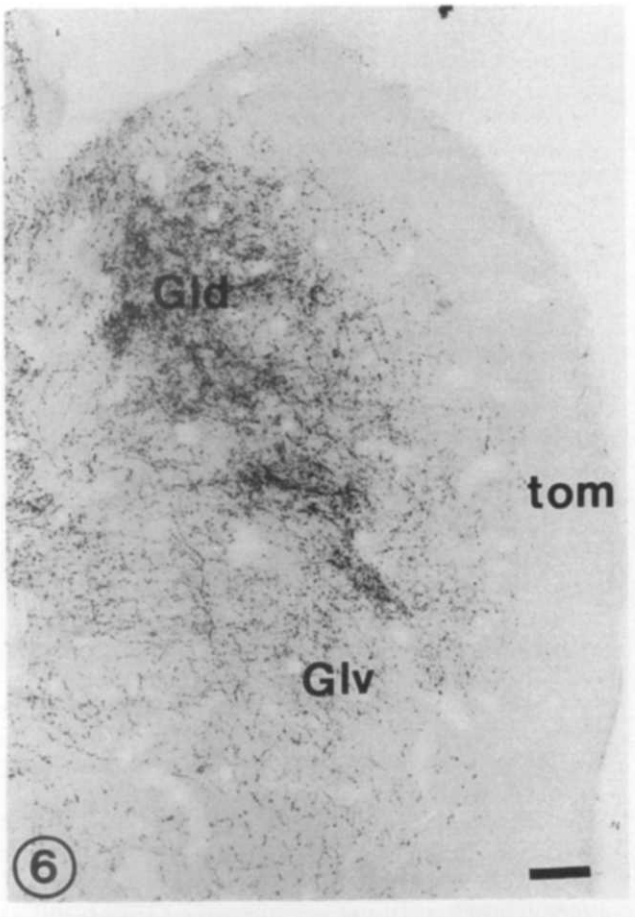
The majority of serotoninergic cell bodies in the viper brain are, however, distributed longitudinally throughout the ventral brainstem. With a few exceptions that we note below, the distribution of these cells corresponds to that described in the lizard *Varanus* by Wolters *et al.* (1985) and in the turtle *Clemmys* by Ueda *et al.* (1983). This state of affairs suggests that in reptiles in general the serotoninergic system of the brainstem is organized into two distinct ventromedial nuclei of the raphe, together with a sparse population of cells situated in the ventrolateral brainstem. The departures from this general plan of organization that we observe in *Vipera* are (i) an appreciable number of immunoreative cells in the mesencephalic and rhombencephalic reticular formation, and (ii) a pronounced cluster of serotoninergic cells in the ventrolateral central gray matter of the midbrain, dorsolateral to the nucleus of the trochlear nerve. This population of dorsally situated, small ovoid cells does not appear to have been described in any non-ophidian reptile, although a small number of serotoninergic cells were observed in *Gekko* lying around the trochlear nucleus (Smeets and Steinbusch, 1988). We note that the serotoninergic cells described in the n. mesencephali profundus pars caudalis of *Clemmys* (Ueda *et al.*,1983) and of *Ophisaurus* (Pierre *et al.*, 1990) do not appear to exist in *Vipera*. Finally, we point out that the serotoninergic cells observed in *Lacerta agilis* in the perventricular region of the torus semicircularis (Petko and Ihionvien, 1989) and described as a nucleus raphe superior, and in the telencephalon, do not appear to exist in our material and have not been described in any other reptilian species.

The existence of two distinct populations of serotoninergic cell bodies in both *Vipera* and other reptiles, one in the hypothalamic region of the diencephalon and a larger one in the ventral brainstem, is in general reminiscent of the state of this system in anamniotes (Steinbusch *et al.*, 1981; Kah and Chambolle, 1983; Meek and Joosten, 1989; Ueda *et al.*, 1984; Corio, 1989). In cyclostomes and some teleosts, however, serotoninergic cell bodies are also found in the pretectal region of the thalamus (Steinbusch *et al.*, 1981; Kah and Chambolle, 1983; Meek and Joosten, 1989). In birds, while some serotoninergic cells are found in the OPH (Fuxe and Ljunggren, 1965; Yamada *et al.*, 1984), the overwhelming majority of these neurons are found in the rostral brainstem. In mammals serotoninergic cell bodies are concentrated within the brainstem and the presence of serotoninergic cells elsewhere is controversial. If such cells can be demonstrated in the hypothalamus by autoradiography after injection of [^{3}H]serotonin or pharmacological inhibition of monoamine oxidases (Kent and Sladek, 1978; Beaudet and Descarries, 1979), they are not revealed by standard immunohistochemical methods (Steinbusch, 1981). It thus appears that the evolution of the phylogenetically ancient serotoninergic system has involved the regression of the rostral components of an initially diffuse system and the concentration of serotoninergic cell bodies within clearly delimited areas of the caudal region of the brain.

Distribution of immunoreactive fibers

Serotoninergic fibers are widely distributed throughout the viper brain. In the olfactory bulb, immunoreactive terminals are found in the internal plexiform

Figs 2–5. Immunoreactive fibers and terminals in different regions of the brain of *Vipera*. Fig. 2 (cf. Fig. 1B). Cross-section of the accessory olfactory bulb. Fig. 3 (cf. Fig. 1C). A section through the anterior telencephalon in which a high density of immunoreactive fibers can be seen in the nuclei accumbens (Ac) and septalis dorsalis (Sd). Fig. 4 (cf. Fig. 1D). A Section through the central telencephalon showing immunopositive fibers in the dorsomedial cortex (DM) and the dorsal ventricular ridge (DVRal). Fig. 5 (cf. Fig. 1E). In the posterior telencephalon intense immunoreactivity is seen in the n. septalis medialis (Sm) and many fibers in the lateral forebrain bundle (lfb) are immunopositive. Scale bars, 100 μm.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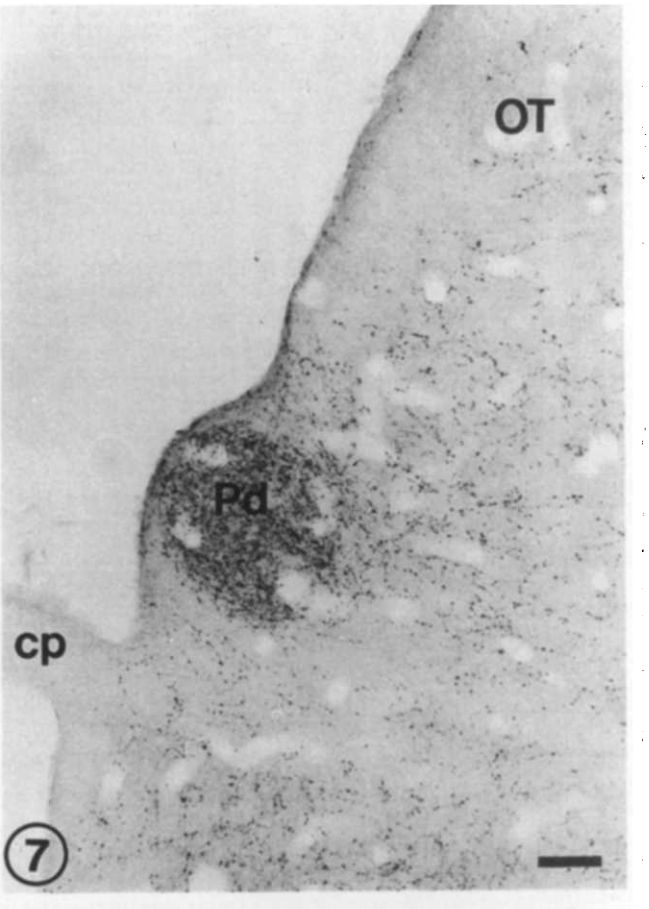
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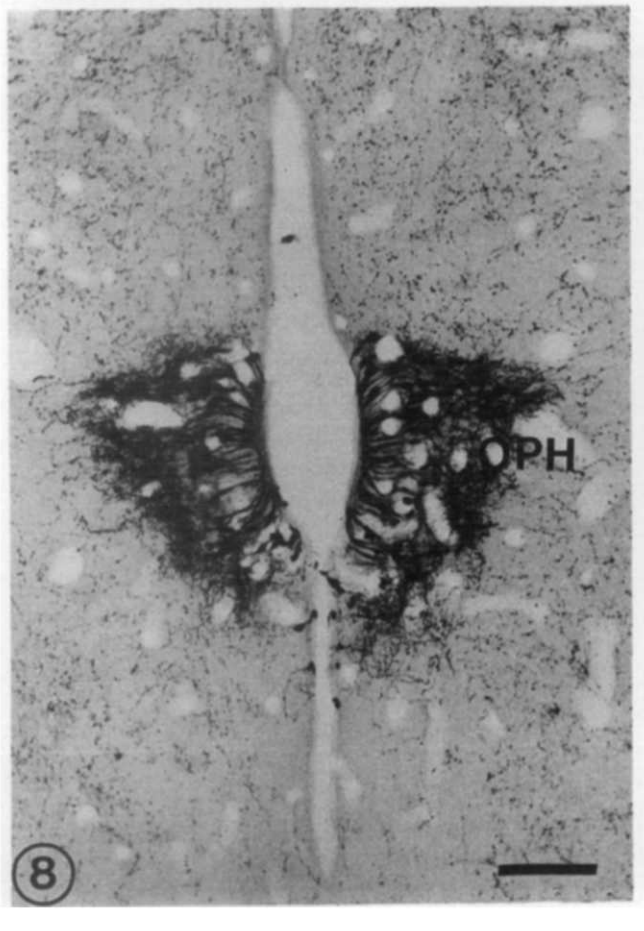
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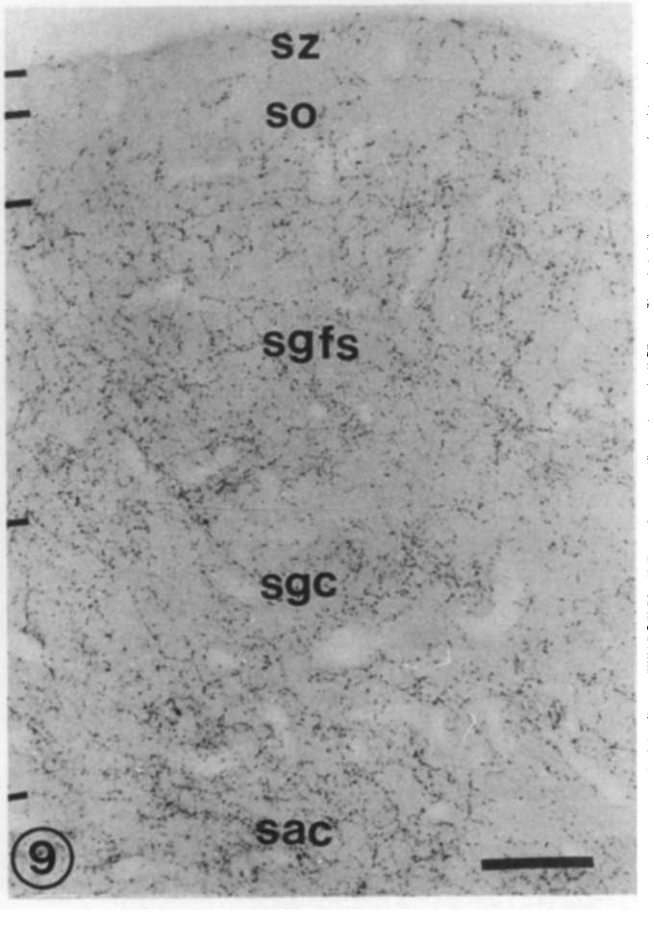
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layer and in the mitral cell layer; serotoninergic innervation of the olfactory bulb has been described in *Ophisaurus* (Pierre *et al.*, 1990), *Gekko* (Smeets and Steinbusch, 1988), *Chameleo* (Bennis *et al.*, 1990) and *Clemmys* (Ueda *et al.*, 1983), the precise location of the labelling depending on the species in question.

The distribution of serotoninergic terminal arborizations within the cortex of *Vipera* appears similar to that observed in lizards (Guirado *et al.*, 1989; Bennis *et al.*, 1990) and differs from that described in *Clemmys japonica* (Ueda *et al.*, 1983). The major difference concerns the innervation of the cellular layer, which is rich in the cortex of *Clemmys* but poor in the squamates.

In *Vipera* the medial cortex contains only a few fibers in the two plexiform layers; this organization is also seen in the lizards *Psammodromus* and *Podarcis* (Guirado *et al.*, 1989) and in *Gekko* (Smeets and Steinbusch, 1988), while in *Clemmys* the medial cortex receives a very strong serotoninergic innervation in the cellular layer (Ueda *et al.*, 1983).

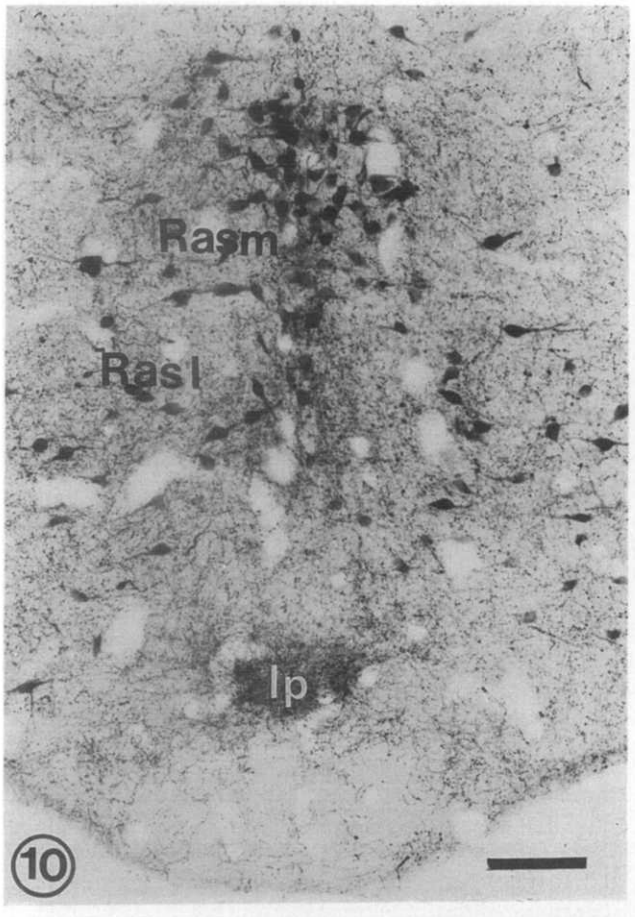
The dorsomedial cortex of the viper, anteriorly, shows a serotoninergic innervation essentially the same as that of the medial cortex, while caudally a high density of immunoreactive fibers is seen in the superficial plexiform layer. Guirado *et al.* (1989) found similar results in *Psammodromus* and *Podarcis*; in the gecko the dorsomedial cortex contains a low density of serotoninergic fibers, in the two plexiform layers, throughout its rostrocaudal extent. In contrast, the serotoninergic innervation of the dorsomedial cortex of the turtle *Clemmys* differs considerably from that of squamates; in this turtle this region of cortex shows a very dense plexus of immunoreactive fibers in the plexiform and cellular layers.

In comparison with other cortical regions, the dorsal cortex of the viper shows the highest density of serotoninergic fibers, principally in the external plexiform layer and to a lesser extent in the posteromedial region of the internal plexiform layer. In *Psammodromus, Podarcis* (Guirado *et al.*, 1989) and *Gekko* (Smeets and Steinbusch, 1988) it is also the dorsal cortex which shows the greatest serotoninergic innervation; however, in these three lacertilians the highest concentration of fibers is seen in the medial part of the dorsal cortex, whose lateral portion contains fewer fibers. The dorsal cortex of *Clemmys*, on the other hand, is poorly endowed with serotoninergic fibers that are distributed essentially in the external plexiform layer (Ueda *et al.*, 1983). Concerning this region of cortex, we point out that the dorsal cortex of squamates (lizards and snakes) is probably not equivalent to that of turtles; by virtue of its reciprocal connections with the nucleus geniculatus lateralis pars dorsalis, the dorsal cortex of turtles has been compared to the mammalian visual cortex, whereas the dorsal cortex of squamates has been considered to be a limbic structure (Butler, 1980; Guirado *et al.*, 1989).

In *Vipera* the lateral cortex contains very few immunoreactive terminals, with the exception of a thin superficial layer of fibers in the most rostral portion. The serotoninergic innervation of the lateral cortex in lizards shows some degree of interspecific variation; while this region, as in the viper, is virtually devoid of fibers in *Psammodromus* and *Podarcis* (Guirado *et al.*, 1989), the lateral cortex of *Gekko* shows a weak serotoninergic innervation of all three layers (Smeets and Steinbusch, 1988). In *Clemmys*, the lateral cortex, on the other hand, shows a massive serotoninergic innervation throughout its entire extent (Ueda *et al.*, 1983).

The serotoninergic innervation of the dorsal ventricular ridge (DVR) of *Vipera* shows pronounced differences from other reptilian species. Because of the fragmentary, and somewhat contradictory, nature of the available data, any attempt to discuss in detail interspecific differences in the serotoninergic innervation of the DVR will be somewhat speculative if not unduly premature. Whether the extreme heterogeneity of this innervation that we described in *Vipera* is typical of ophidians, and whether the few scattered fibers described in *Clemmys* (Ueda *et al.*, 1983) are typical of chelonians, remain two open empirical questions. In addition, it is not clear to what extent Northcutt's (1978) distinction between Type I lacertomorph lizards and Type II dracomorph lizards applies to the serotoninergic system. While the cytoarchitecture of the DVR in Type II lizards resembles that in snakes (Ulinski, 1983), this structure is totally devoid of immunoreactivity in the dracomorph *Chameleo* (Bennis *et al.*, 1990), in contrast to the marked heterogeneity that we describe in *Vipera*. In addition, the distribution of immunoreactive fibers differs considerably among the three Type I lizards *Gekko, Lacerta* and *Ophisaurus*. In *Gekko* (Smeets and Steinbusch, 1988) the concentration of serotoninergic fibers is lower than in *Vipera* and the heterogeneity of their distribution is less marked; in the rostral DVR the density of fibers is low medially and moderate laterally, this distribution being reversed in the caudal region. In *Lacerta* (Petko and Ihionvien, 1989), on the other hand, immunoreactivity is limited to the

Figs 6–9. Cross-sections of the diencephalon and mesencephalon. Fig. 6 (cf. Fig. 1F) showing serotoninergic fibers in n. geniculatus lateralis pars dorsalis (Gld) and the absence of label in the marginal optic tract (tom). Fig. 7 (cf. Fig. 1G) shows, to the right, a plexus of immunoreactive fibers in the n. posterodorsalis (Pd), while the subcommissural organ (Os) at the left of the figure is devoid of label. Fig. 8 (cf. Fig. 1G) illustrates immunoreactive cell bodies in the hypothalamic periventricular organ. Fig. 9 (cf. Fig. 1H), a section through the anterior mesencephalon showing serotoninergic fibers diffusely distributed in the optic tectum. Scale bars, 50 μm in Figs 6 and 7, 100 μm in Figs 8 and 9.



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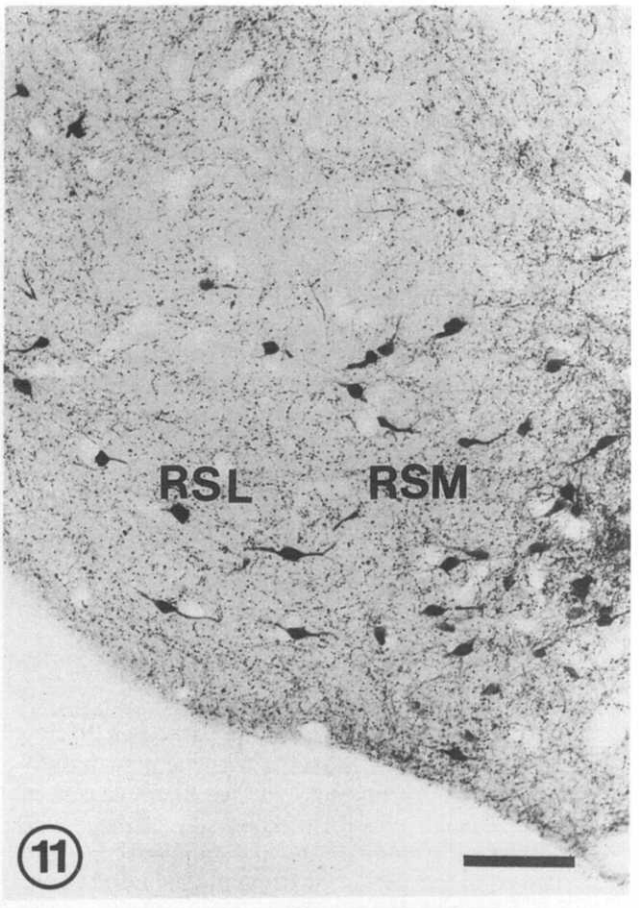
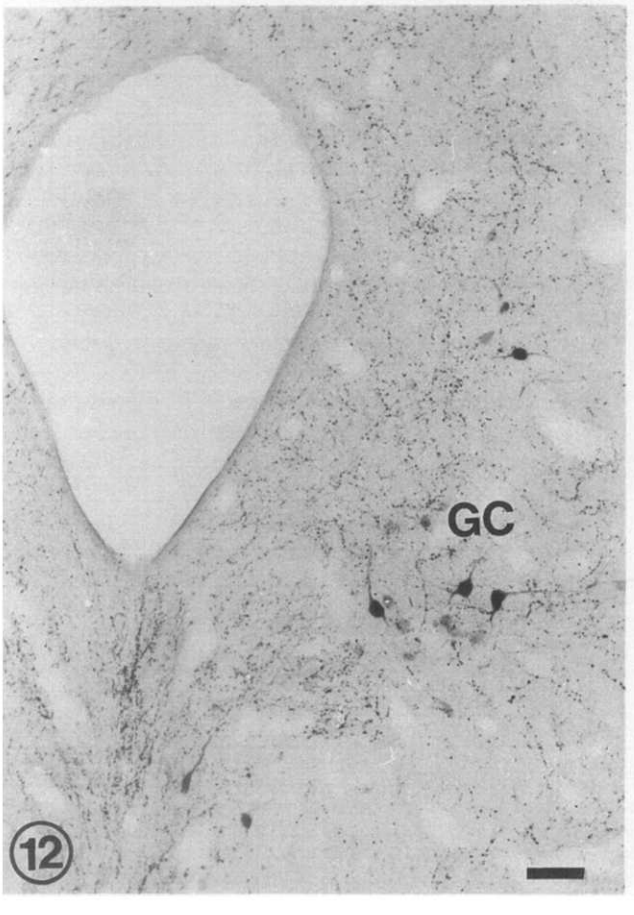
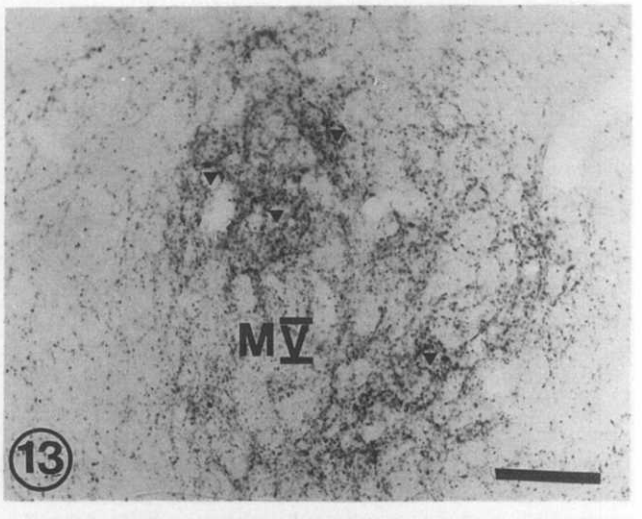


11



12



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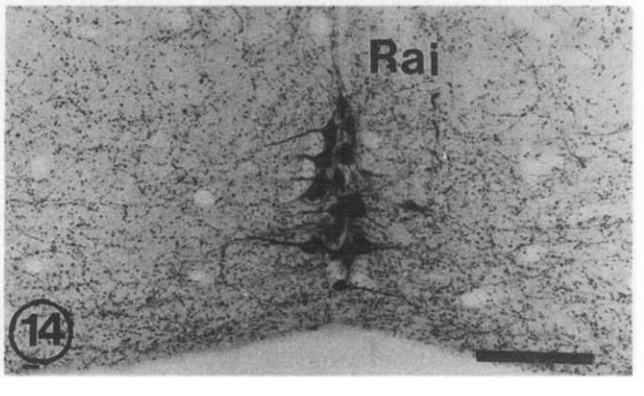

14

medial DVR, while in *Ophisaurus* (Pierre *et al.*, 1990) the immunoreactivity of the DVR is generally weak.

The rich serotoninergic innervation of the nucleus accumbens that we find in the viper has also been described in *Ophisaurus* (Pierre *et al.*, 1990) and *Gekko* (Smeets and Steinbusch, 1988), while in the chameleon this nucleus is only very weakly innervated (Bennis *et al.*, 1990).

The septum is generally recognized as receiving a serotoninergic innervation in reptiles, but the location of the most intense immunoreactivity varies from species to species: the nucleus septalis dorsalis in *Vipera* (present results) and *Lacerta* (Petko and Ihionvien, 1989), the n. septalis medialis in *Ophisaurus* (Pierre *et al.*, 1990) or n. septalis lateralis in *Gekko* (Smeets and Steinbusch, 1988), the ventromedial septal area in *Clemmys* (Ueda *et al.*, 1983) and the lateral septum in *Chameleo* (Bennis *et al.*, 1990).

The majority of serotoninergically innervated structures in the diencephalon and mesencephalon of *Vipera* have been described in other reptiles, but with some interspecific variation. The most intensely labelled structure in lizards and in *Clemmys* corresponds to the nucleus pretectalis dorsalis (Ueda *et al.*, 1983; Smeets and Steinbusch, 1988; Bennis *et al.*, 1990; Pierre *et al.*, 1990), whereas in *Vipera* the structure which corresponds topographically to this nucleus is very weakly labelled. On the other hand, the viper shows intense immunoreactivity in a more ventral pretectal nucleus, the n. subpretectalis. Among the diencephalo-mesencephalic structures that are strongly labelled are the primary visual centres. In the viper the n. geniculatus lateralis pars dorsalis is strongly labelled (present results), whereas in *Ophisaurus, Gekko* and *Clemmys* it is rather the pars ventralis of this nucleus which has a rich serotoninergic supply (Ueda *et al.*,1983; Smeets and Steinbusch, 1988; Pierre *et al.*, 1990). The pretectal visual nuclei (nuclei lentiformis mesencephali, geniculatus pretectalis, griseus tectalis and posterodorsalis) are moderately labelled in the lizards and *Clemmys* (Ueda *et al.*, 1983; Smeets and Steinbusch, 1988; Bennis *et al.*, 1990; Pierre *et al.*, 1990), while in the viper the first three of these structures are very weakly innervated while the n. posterodorsalis is extremely immunoreactive. In *Vipera* as in all other reptilian species the nucleus opticus tegmenti is richly endowed with serotoninergic arborizations.

The optic tectum of the majority of reptiles (*Chameleo, Chrysemys, Clemmys, Gekko, Ophisaurus* and *Varanus*) shows a typically laminar distribution of serotoninergic fibers and terminals, the details of which vary from species to species. For example, in *Ophisaurus* (Pierre *et al.*, 1990) the stratum fibrosum et griseum superficiale (sfgs) is strongly innervated, the stratum album et griseum perventriculare less so, while the strata griseum centrale (sgc) and album centrale are virtually devoid of terminals. In *Chameleo*, on the other hand (Bennis *et al.*, 1990), as in *Varanus* (Wolters *et al.*, 1985), the higest density of fibers is seen in the sfgs and sgc, the other tectal layers being more weakly labelled. This typical stratified organization is not found in the tectum of *Vipera*, which shows a weak, sparse serotoninergic innervation with no particular regions of concentration.

The differences between the serotoninergic innervation of the primary visual centres of *Vipera* and of other reptiles may be considered in the light of the other structural peculiarities of the ophidian visual system, notably the poorly stratified organization of the optic tectum and the enlargement of the thalamofugal visual relay, the n. geniculatus lateralis dorsalis, in snakes (see Repérant *et al.*, 1991 for a review).

The total absence of immunoreactivity in the optic nerves, in the viper as in other reptilian species, rules out the possibility that serotoninergic fibers arise in the ganglion cell layer of the retina. Nevertheless, Weiler and Ammermüller (1986) have demonstrated serotoninergic retinal ganglion cells and serotoninergic axons in the optic nerve of the turtle *Pseudemys scripta*. In the same chelonian species, Schütte and Weiler (1988) describe a single serotoninergic neuron situated in the mesencephalic tegmentum, which projects to the retina; the centrifugal fiber ramifies in the temporal hemiretina and its arborizations cover about a third of the total retinal surface (see Repérant *et al.*, 1989 for a review of the reptilian centrifugal visual system). In *Vipera*, the cells of origin of the retinopetal fibers are situated in the thalamic optic centrifugal nucleus (Repérant *et al.*, 1980); the present results show no immunoreactive cells in this nucleus of the ventral thalamus, which rules out the possibility that serotonin is the neurotransmitter of the centrifugal visual system in the viper.

The most strongly immunoreactive structures in the brainstem of the viper are the nuclei of the oculomotor (III, IV, VI) and motor (V, VII, X) cranial nerves, most particularly the n. motorius trigemini. In each of these structures the immunoreactive

Figs 10–14. Immunoreactive cell bodies and fibers in the brainstem. Fig. 10 (cf. Fig. 11). Serotoninergic cell bodies in the partes medialis (Ras m) and lateralis (Ras l) of the n. raphe superior, and numerous immunopositive fibers in the n. interpeduncularis. Fig. 11 (cf. Fig. 11). Immunoreactive cell bodies in the partes lateralis (RSL) and medialis (RSM) of the n. reticularis superior. Fig. 12 (cf. Fig. 11). Serotoninergic cell bodies in the ventrolateral region of the substantia grisea centralis. Fig. 13 (cf. Fig. 1K). In the n. motorius nervi trigemini immunoreactive fibers are disposed in baskets around unlabelled notoneurons (arrows). Fig. 14 (cf. Fig. 1L). Cell bodies in the n. raphe inferior (Rai). Scale bars, 50 μm in Fig. 12, 100 μm elsewhere.

ABBREVIATIONS USED IN FIGURES

Ac	nucleus accumbens
Apo	area preoptica
AHL	lateral hypothalamic area
Bi	Bischoff's nucleus
ca	commissura anterior
CAm	Amygdaloid complex
Cer	Cerebellum
Co	nucleus cochlearis
co	chiasma opticum
cp	commissura posterior
cpv	periventricular layer of the optic tectum
D	dorsal cortex
Dl	nucleus dorsolateralis thalami
DM	dorsomedial cortex
Dm	n. dorsomedialis thalami
DVRal	dorsal ventricular ridge, pars angulolateralis
DVRam	dorsal ventricular ridge, pars angulomedialis
DVRc	dorsal ventricular ridge, pars caudalis
DVRm	dorsal ventricular ridge, pars medialis
DVRr	dorsal ventricular ridge, pars rostralis
epl	external plexiform layer
fd	funiculus dorsalis
fi	fibrous layer of the olfactory bulb
flm	fasciculus longitudinalis medialis
fpd	fasciculus predorsalis
Fr	fasciculus retroflexus
GC	substantia grisea centralis
gl	glomeruler layer of the olfactory bulb
Gld	nucleus geniculatus lateralis pars dorsalis
Glv	nucleus geniculatus lateralis pars ventralis
gn	granular layer of the olfactory bulb
Gp	nucleus geniculatus pretectalis
gr	granular layer of the cerebellum
HL	nucleus habenularis lateralis
HM	nucleus habenularis medialis
Is	nucleus isthmi
Ip	nucleus interpeduncularis
L	lateral cortex
lfb	lateral forebrain bundle
LM	nucleus lentiformis mesencephali
ls	lemniscus spinalis
M	medial cortex
mfb	medial forebrain bundle
mi	mitral cell layer of the olfactory bulb
MV	nucleus motorius nervi trigemini
N III	nucleus nervi oculomotorii
N IV	nucleus nervi trochlearis
N Vds	nucleus descendens nervi trigemini
N VI	nucleus nervi abducentis
N VII	nucleus nervi facialis
N VIII	nucleus vestibulocochlearis
NX	nucleus nervi vagi
N XII	nucleus nervi hypoglossi
NCel	nucleus cerebellaris lateralis
NCem	nucleus cerebellaris medialis
NdB	nucleus of the diagonal band
Nfd	nucleus funiculi dorsalis
NH	nucleus commissurae hippocampi
Niflm	nucleus interstitialis fasciculi longitudinalis medialis
Nm V	nucleus mesencephalicus nervi trigemini
NOT	nucleus opticus tegmenti
Npm	nucleus profundus mesencephali
NSo	nucleus supraopticus
NTOC	thalamic centrifugal optic nucleus
OPH	periventricular hypothalamic organ
Os	subcommissural organ
OT	optic tectum
Pd	nucleus posterodorsalis
PE	nucleus lentiformis, pars extensa
pe	external plexiform layer of the olfactory bulb
PF	nucleus perifascicularis
PH	nucleus periventricularis hypothalami
pi	internal plexiformlayer of the olfactory bulb
Pm	nucleus parvocellularis medialis
PP	nucleus lentiformis, pars plicata
Pr	nucleus pretectalis
Pr V	nucleus princeps nervi trigemini
Pu	Purkinje cell layer of the cerebellum
Rai	nucleus raphe inferior
Ras l	nucleus raphe superior, pars lateralis
Ras m	nucleus raphe superior, pars medialis
RID	nucleus reticularis inferior, pars dorsalis
RIL	nucleus reticularis inferior, pars lateralis
RIV	nucleus reticularis inferior, pars ventralis
RL	nucleus reticularis lateralis
rm V	motor root of the trigeminal nerve
rs V	sensory root of the trigeminal nerve
RSL	nucleus reticularis superior, pars lateralis
RSM	nucleus reticularis superior, pars medialis
Rt	nucleus rotundus
sac	stratum album centrale (optic tectum)
Sd	nucleus septalis dorsalis
sfp	stratum fibrosum periventriculare optic tectum)
sgc	stratum griseum centrale (optic tectum)
sgfs	stratum griseum et fibrosum superficiale (optic tectum)
sgp	stratum griseum periventriculare (optic tectum)
Sl	nucleus septalis lateralis
Sm	nucleus septalis medialis
so	stratum opticum (optic tectum)
Sol	nucleus tracti solitarii
Sph	nucleus sphericus
Str	corpus striatum
Su	nucleus suprapeduncularis
sz	stratum zonale (optic tectum)
To	tuberculum olfactorium
toa	accessory olfactory tract
tol	lateral olfactory tract
tom	tractus opticus marginalis
toS	torus semicircularis
ttd	tractus descendens nervi trigemini
Ve	nucleus vestibularis
VH	nucleus ventralis hypothalami
Vl	nucleus ventrolateralis thalami
VM	nucleus ventralis mesencephali
Vm	nucleus ventromedialis thalami
Vn	vomeronasal nerve
vp lfb	ventral peduncle of the lateral forebrain bundle

fibers are disposed as a tight basket around the non-reactive cell bodies of the motorneurons. According to Smeets and Steinbusch (1988) the numerous varicosities of the serotoninergic fibers may represent axosomatic contacts. This characteristic pattern of innervation is found in other reptilian species, although the nuclei of the third and fourth cranial nerves are not immunoreactive in turtles (Parent, 1979; Ueda *et al.*, 1983) nor in *Ophisaurus* (Pierre *et al.*, 1990). The pronounced serotoninergic supply to the n. motorius trigemini in the viper is very possibly related to the extreme degree of sophistication of the masticatory apparatus and its motor control in ophidians (Moody and Meszler, 1980a,b).

Experimental pathway-tracing studies in a number of reptilian species have shed light on the projections of various structures which contain serotoninergic neurons. The combined use of HRP as an axonal tracer and histofluorescence by Ulinski (1981) showed that, in the two snakes *Thamnophis sirtalis* and *Natrix sipedon*, the nucleus raphe superior sends a monoaminergic projection to the dorsomedial cortex. Other studies, using only the HRP technique and carried out in a variety of lizards (*Ophisaurus apodus*, Belekhova and Kenigfest, 1983; *Tupinambis nigropunctatus*, Lohman and Van Woerden-Verkley, 1978; *Iguana iguana* and *Gekko gecko*, Bruce and Butler, 1984) and the turtle *Pseudemys scripta* (Ouimet *et al.*, 1985) have shown that the medial and dorsomedial cortex is supplied by the n. raphe superior in these species, and it has also been shown that this nucleus projects to the n. dorsolateralis anterior thalami and to the nn. habenularis (Belekhova and Nemova, 1987; Hoogland, 1982) as well as to the septum (Belekhova and Nemova, 1988; Nemova, 1988). In the snakes *Python reticulatus* (Welker *et al.*, 1982) and *Thamnophis sirtalis* (Dacey and Ulinski, 1986), and in the lizard *Varanus exanthematus* (Ten Donkelaar *et al.*, 1983) a bilateral projection has been demonstrated from the reticular nuclei of the brainstem, a region in which a considerable number of serotoninergic neurons has been detected, to the optic tectum.

The zones of projection of the hypothalamic periventricular organ, however, are somewhat controversial. According to Marschall (1980) it mainly

innervates the n. ventrolateralis hypothalami, while Bruce and Butler (1984) found labelled cell bodies in this organ after injection of HRP into the dorsal or dorsomedial cortex of a lizard. Neither of these results was confirmed by similar experiences in other species (Lohman and Van Woerden-Verkley, 1978; Belekhova and Kenigfest, 1983).

These studies provide a certain amount of information about the projections of structures which are known to contain monoaminergic neurons. However, the fact that it is indeed the serotoninergic neurons which are involved in these projections awaits direct demonstration by double-labelling.

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