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Septal Complex of the Telencephalon of the Lizard *Podarcis hispanica*. II. Afferent Connections

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ABSTRACT

The afferent connections to the septal complex were studied in the lizard *Podarcis hispanica* (Lacertidae) by means of a combination of retrograde and anterograde tracing. The results of these experiments allow us to classify the septal nuclei into three main divisions. The central septal division (anterior, lateral, dorsolateral, ventrolateral, and medial septal nuclei plus the nucleus of the posterior pallial commissure) receives a massive, topographically organized, cortical projection (medial, dorsal, and ventral areas) and widespread afferents from the tuberomammillary hypothalamus and the basal telencephalon. Moreover, it receives discrete projections from the dorsomedial anterior thalamus, the ventral tegmentum, the midbrain raphe, and the locus coeruleus. The ventromedial septal division (ventromedial septal nucleus) receives a massive projection from the anterior hypothalamus, dense serotonergic innervation, and a faint amygdalohypothalamic projection, but it is devoid of direct cortical input. The midline septal division (nucleus septalis impar and dorsal septal nucleus) receives a nontopographic cortical projection (dorsomedial and dorsal cortices) and afferents from the preoptic hypothalamus, the dorsomedial anterior thalamus, the midbrain central gray, and the reptilian A8 nucleus/substantia nigra.

Our results indicate that the cortex provides a physiologically complex, massive input to the septum that terminates over the whole dendritic tree of septal cells. In contrast, most of the ascending afferents make axosomatic contacts by means of pericellular nests. The chemical nature of the main septal afferents and the comparative implications of the available hodological data on the organization of the septal complex of tetrapod vertebrates are discussed. J. Comp. Neurol. 383:489–511, 1997.

Indexing terms: limbic system; reptiles; hypothalamus; hippocampal cortex; comparative neuroanatomy

The accessibility of the sensory organs has attracted many neuroanatomists to the study of the organization and connections of the sensory systems. Consequently, most of the current hypotheses on the evolution of the vertebrate telencephalon are based on comparisons of the sensory projections to the telencephalon among extant vertebrates (Ebbesson, 1980; Northcutt, 1981; Butler, 1994a,b). On the other hand, neural centers that are not directly involved in the processing of sensory information have received only minor attention from a comparative point of view, with the remarkable exceptions of a few nicely organized cortical centers, such as the hippocampus.

However, understanding the function and organization of these "nonsensory" neural centers is of great interest to comparative neurobiology. A paradigmatic example of this fact is the limbic telencephalon. The limbic centers of the telencephalon are usually considered to be ancient structures that were already present in ancestral vertebrates

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(Northcutt, 1981). Therefore, it is impossible to understand the evolution of the telencephalon in vertebrates without having a clear picture of the structure of the limbic telencephalon in representatives of the main vertebrate taxa. The limbic telencephalon of vertebrates includes not only cortical structures, such as the hippocampal formation (which has been relatively well studied), but also subcortical structures, namely, the amygdala, the septal complex, and the limbic striatum.

The septal formation of the telencephalon of tetrapods is a cytoarchitectonically complex structure (mammals: DeFrance, 1976; Swanson and Cowan, 1979; birds: Krayniak and Siegel, 1978a; reptiles: Halpern, 1980; Smeets et al., 1986a; Font et al., 1995; amphibians: Northcutt and Kicliter, 1980; Neary, 1990) that is composed of poorly delimited nuclei, which are crossed by several fiber bundles (the medial forebrain bundle, the fornix, and the hippocampal or pallial commissures). Complete knowledge of the afferents of the septal complex is needed to identify the sources of the sensory information that the septum uses to perform its physiological role and the neural systems that modulate its functioning. However, the above-mentioned structural complexity of the septal formation and the limitations of most current neuroanatomical methods have hindered the study of the septal fiber connections. In fact, although the afferents of the septum are relatively well studied in some mammals (for review, see Jackab and Leranth, 1995), data in nonmammals are scarce and incomplete.

Knowledge of the organization of the reptilian limbic telencephalon, including the septum, is especially important in order to understand the evolution of the vertebrate brain, because reptiles were the stem taxon from which birds and mammals evolved. Moreover, volumetric studies on the telencephalon of reptiles (Platel, 1980) indicate that some species of lizards and snakes (squamate reptiles) display a relatively big septohippocampal system that represents more than 50% of the telencephalic volume. This fact and the relatively simple organization of the brain of reptiles (compared with that of birds and mammals) make reptiles good experimental subjects for the study of the anatomofunctional relationships of the vertebrate septohippocampal system. Despite these advantages, the reptilian subcortical limbic centers have been neglected by comparative neuroanatomists, and our knowledge of their organization and connections is based on a few fragmentary studies in different species. In fact, the only study specifically devoted to the analysis of the afferents to the reptilian septum (Belekhova and Nemova, 1988) does not pay attention to the cytoarchitectonic complexity of this telencephalic structure (Font et al., 1995).

Therefore, we have undertaken a study of the afferent connections of the septal complex in the Old World lizard *Podarcis hispanica* (Lacertidae), for which a detailed description of its cytoarchitecture is available (Font et al., 1995). To study the afferent connections, we combined retrograde and anterograde tracing techniques in order to identify the cells of origin of the main septal afferents and their terminals within the different septal nuclei.

MATERIALS AND METHODS

Tracing the connections of each septal nucleus would require a huge number of small injections of powerful, nondiffusable neuroanatomical tracers that are not taken up by fibers of passage. Although some modern tracers fulfill the first requirement [*Phaseolus vulgaris*-leucoagglu-

Abbreviations

5-HT	serotonin
ac	anterior commissure
Acc	nucleus accumbens
AH	anterior hypothalamus
Bst	bed nucleus of the stria terminalis
CG	central grey
DA	dopamine
DB	diagonal band nucleus
DC	dorsal cortex
DHA	dorsal hypothalamic area
DLA	dorsolateral anterior thalamic nucleus
DM	dorsomedial cortex
DMA	dorsomedial anterior thalamic nucleus
DVR	dorsal ventricular ridge
H	habenula
III	nucleus nervi oculomotorii
LC	lateral cortex
LCo	locus coeruleus
lfb	lateral forebrain bundle
LHA	lateral hypothalamic area
Lp	lentiforme thalamic nucleus pars plicata
LPA	lateral preoptic area
MAM	mammillary nucleus
MC	medial cortex
MP	medial preoptic nucleus
NA	noradrenaline
Nac	nucleus of the anterior commissure
Nmfb	bed nucleus of the medial forebrain bundle
Nppc	nucleus of the posterior pallial commissure
NPY	neuropeptide Y
NS	nucleus sphericus
PA	preoptic area
PM	posteromedial thalamic nucleus
PO	paraventricular organ
PP	periventricular preoptic nucleus
psfx	postcommisural fornix
PV	periventricular hypothalamic nucleus
R	raphe nucleus
RA8	reptilian A8 nucleus
RC	retrochiasmatic nucleus
Rot	nucleus rotundus
Sa	anterior septal nucleus
SC	suprachiasmatic nucleus
Sd	dorsal septal nucleus
Sdc	central part of the dorsal septal nucleus
Sdd	dorsal part of the dorsal septal nucleus
Sdl	dorsolateral septal nucleus
Si	nucleus septalis impar
Sl	lateral septal nucleus
Sm	medial septal nucleus
Sn	substantia nigra
SO	supraoptic nucleus
SP	substance P
St	striatum
SUM	supramammillary nucleus
Svl	ventrolateral septal nucleus
Svm	ventromedial septal nucleus
TM	tectum mesencephali
TO	olfactory tubercle
TSc	torus semicircularis pars centralis
TSl	torus semicircularis pars laminaris
VC	ventral cortex
VMH	ventromedial hypothalamic nucleus
VPA	ventral posterior amygdala
VTA	ventral tegmental area

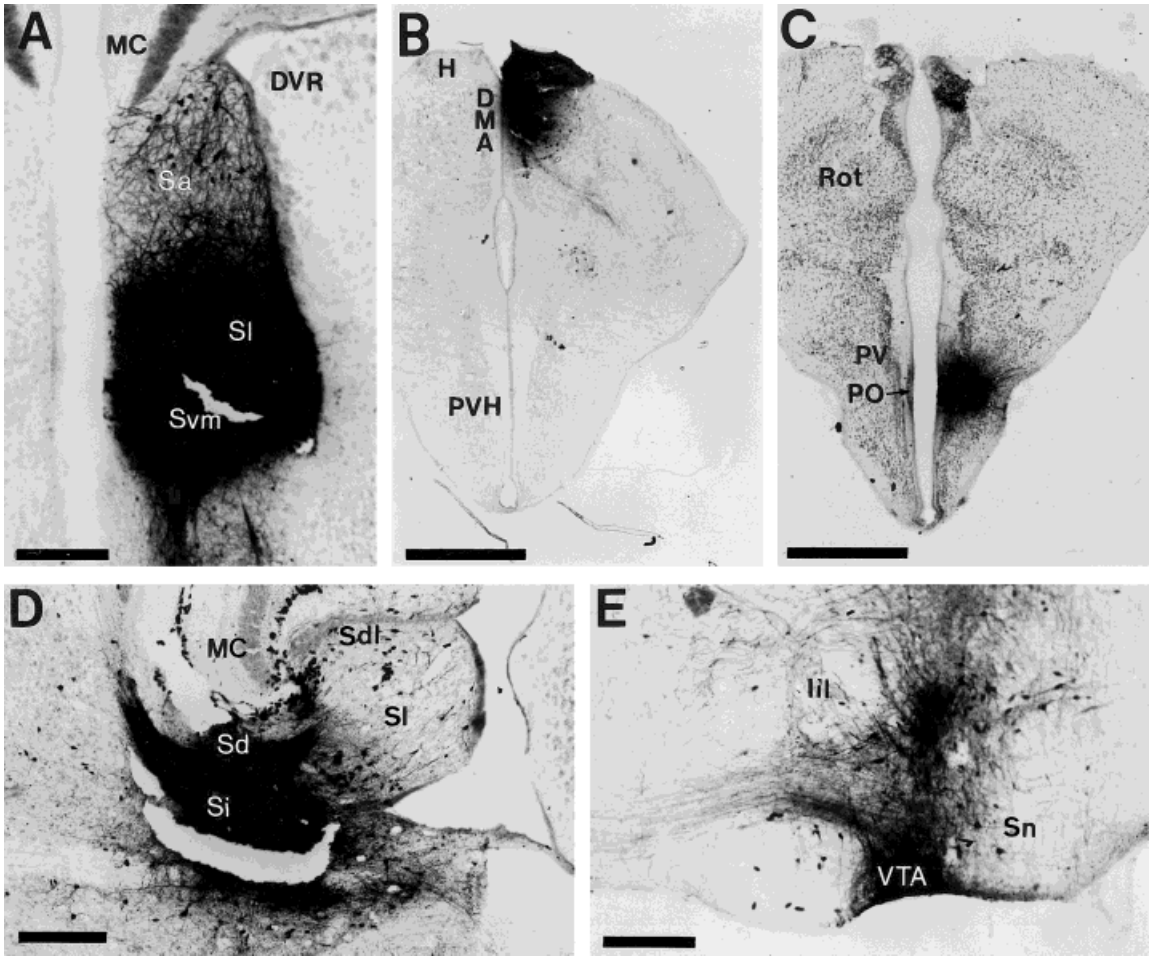

Fig. 1. Photographs of intraseptal (A,D) and extraseptal (B,C,E) injection sites with different tracers. **A:** Biotinylated dextranamine (BDA) injection into the ventromedial (Svm) and lateral (Sl) septal nuclei (case B9519). **B:** Horseradish peroxidase (HRP) injection into the dorsomedial anterior thalamic nucleus (DMA) and habenula (case H9298). **C:** BDA injection into the periventricular hypothalamic nucleus and paraventricular organ (case B9457). **D:** HRP injection into the nucleus septalis impar (Si) and dorsal septal nucleus (Sd; case H9271). **E:** HRP injection into the ventral tegmental area (case H9267). For other abbreviations, see list. Scale bars = 150 µm in A,D,E, 500 µm in B,C.

tinin (PHA-L); Gerfen and Sawchenko, 1984; biotinylated dextranamine (BDA); Veenman et al., 1992], all of them are (more or less) taken up by passing fibers.

A practical solution to overcome these technical problems is to perform a two-step connectional study. In the first step, iontophoretic injections of tracers are placed in the septal complex to study the retrograde transport to its afferent neurons. In the second group of experiments, some of the areas that are retrogradely labeled after septal injections are then injected, and the resulting anterograde labeling in the septum is analyzed to determine where each afferent terminates within the septal complex.

Surgery and application of tracers

Adult (48–54 mm snout-vent) specimens of the lizard *P. hispanica* of both sexes were used for this study. The animals considered here were processed between 1986 and 1995. They were treated throughout according to the guidelines of the European Community and Spanish Ministry of Agriculture on the use, handling, and care of experimental animals.

Surgery was always carried out under anesthesia. In the first experiments, animals were first anesthetized with ethylic ether and were then put on a bed of ice during surgery. Since 1991, animals were preanesthetized with halothane vapours (Aldrich, Steinheim, Germany), and then they received i.m. injections of 6.5 µl/g of Ketolar (ketamine 50 mg/ml; Parke-Davis, El Prat del Llobregat, Spain), which provided a reliable, deep anesthesia for the whole surgical operation.

For all tracer injections, animals were fixed on a stereotaxic device (Stoelting, Standard Laboratories, Wood Dale, IL). Injections were placed in the septum (n = 10), the cerebral cortex (n = 14), the basal telencephalon (n = 2), the hypothalamus (n = 10), the dorsomedial thalamus and epithalamus (n = 2), and the midbrain (n = 4) by using the anterior vertex of the pineal scale as a reference point for stereotaxic coordinates. The location and extent of the injection site of the most representative cases (Fig. 1) and a semiquantitative report of the retrograde or anterograde labeling found after these injections are shown in Tables 1 (septal injections) and 2 (extraseptal injections).

TABLE 1. Retrograde Labeling After Tracer Injection Into the Septum[1]

Case	Injection site	Bst/ VPA	Basal tel.	Preop area	AH	PVH	LHA	SUM/ MAM	DMA	DLA	VTA	Sn/ RA8	CG	LCo	R	MC	DMC	DC	VC
H8812	Sa, Sdl, (*)	0	++	0	0	++	0	+++	0	+++	++	0	0	++	++	–	–	–	–
H8697, H8734	Sa, Sdl, MC	0	++	0	0	++	0	+++	0	+++	++	0	0	++	++	++ (cl)	++ (cl)	0 (cl)	0 (cl)
B9519	Svm, Sl (r)	+	+++	++	++++	+++	+	++	0	++	0	0	0	0	+	+	0	+++	++++
P9226	Sm, Sdd, Sa (c)	0	++	++	0	+	0	++	+++	+	++	+	+	+	++	++	++	+++	0
H9271	Si, Sd	0	++	+++	++	++	++	++	++	+	0	+++	++	0	++	0	++	+++	0
P9249	Sm, Sa, Sl, Sdl, (*)	0	++	++	0	+	0	++	++	+	0	0	0	0	0	–	–	–	–
H9216	Sa, Sl, Svm, Sdl, (*)	+	+	++	++	+	+	++	+	++	+	+	+	+	+	+ (cl)	+ (cl)	0 (cl)	0 (cl)
P9259	Sm, Sd, Sa, Sl, MC	0	++	+	0	+	+	++	++	++	+	0	0	0	++	+ (cl)	++ (cl)	0 (cl)	0 (cl)
H92100	Sa, Sdl, Sl, Nac, MC	0	++	++	0	++	++	+++	0	+++	++	+	0	+	++	+ (cl)	++ (cl)	0 (cl)	0 (cl)

[1]*, The medial cortex was removed to gain access to the septum; 0, no labeling; c, caudal; cl, contralateral; d, dorsal; r, rostral. For abbreviations, see list.

TABLE 2. Anterograde Labeling in the Septum After Extraseptal Injections[1]

Case	Injection site	Sa	Sl	Sm	Sdd	Sdc	Sdl	Svm	Svl	Si	Nppc
H8897/H8938	MC (r)	+++	0	0	+	0	++	0	0	+	0
H8934	MC (vt)	+++	0	0	+	0	++	0	0	+	0
H8935	MC (h)	0	++ (d)	0	0	0	0	0	0	0	0
H8628	DC (r)	0	++ (d)	++	++	0	0	0	0	+	0
H8648/H8982/H8650	DC (c)	0	++ (i)	++	++	0	0	0	0	+	0
H8974/H9032/H9047	DC	0	+++	++	++	0	0	0	0	+	0
H9250	VC	0	++ (v)	0	++	0	0	0	+	+	+
P9227/H9231	VC, DC (c)	0	++ (i, v)	0	++	0	0	0	+	+	+
P9242	Nmfb, DB	+	+	+	+	+	+	+	+	+	+
H9288	Nac	+	+	+	+	+	+	+	+	+	+
H9298	DMA, H	0	+ (v, r)	++	++	+	0	0	0	++	+
H9303	H	0	0	0	0	0	0	0	0	0	0
B9521	Preoptic area	+	+	+	++	++	0	0	++	0	0
H9289	Preoptic area, AH	+	+	0	+	+	+	+++	+	+	+
B9520	AH	+	+	+	+	+	+	+++	+	+	+
B9457	PVH	+ (r)	+ (r)	+	+	+	0	0	0	0	0
B9421	PVH (d), DHA, AH	+	+	+	++	++	+	++	+	+	+
B9416	PVH (v), VMH	0	+	++	++	++	+	0	+++	+	+
H9204	LHA, MAM	+	+	0	+	+	+	0	+	+	+
H9322	SUM, MAM	+	+	+	+	+	+	0	+	+	+
H8801/H9205	MAM	+	+	0	+	+	+	0	+	+	+
H9267	VTA	0	+ (v)	+	0	0	+	0	+	+	0
H9340	Sn/RA8, red nucleus	0	0	0	+	+	0	0	0	0	0
H9150	Sn/RA8, LCo	0	0	0	+	+	+	0	0	0	0
H9268	Tegmentum (c), CG	0	0	0	+	0	0	0	0	+	0

[1]0, No labeling; c, caudal; d, dorsal; h, horizontal; i, intermediate; r, rostral; v, ventral; vt, vertical. For abbreviations, see list.

Different tracers have been used throughout the present work: horseradish peroxidase (HRP; Sigma type VI, St. Louis, MO), PHA-L (Vector, Burlingame, CA), and BDA (10,000 m.w., lysine fixable; Molecular Probes, Eugene, OR). Because some lizards received injections of two different tracers, each injection was named with the number of the animal preceded by a letter indicating the tracer employed: P for PHA-L, H for HRP, and B for BDA.

Peroxidase (10% in 0.05 M Tris buffer, pH 8.6) was iontophoretically delivered from 30–50 µm (inner tip diameter) micropipettes by means of positive current pulses (2–5 µA; 7 seconds ON, 7 seconds OFF) using a current generator (Direlec, Madrid, Spain). PHA-L and BDA were injected from 2.5% (PHA-L) or 5–10% (BDA) solution in 10 mM phosphate buffer (PB), pH 7.4, through 15–20 µm inner-diameter micropipettes by using the same pattern of positive current. In a few experiments during the first part of this study, HRP/saponin crystals (obtained by desiccation of a 25% HRP-5% saponin solution) were placed directly on the cortical surface under visual guidance or in the dorsal aspect of the septum after removal of the overlying cerebral cortex.

Histochemical procedures

HRP injections. Seven days after the injection, animals were deeply anesthetized and transcardially perfused with Karnovsky's fixative (1.25% glutaraldehyde, 1% paraformaldehyde in 0.1 M PB, pH 7.4). Their brains were carefully removed, postfixed for 4 hours in the same fixative, and cryoprotected by immersing them in 30% buffered sucrose until they sank. Then, 30–50 µm-thick frontal sections were obtained with a freezing microtome and collected in two or three matching series that were reacted for peroxidase histochemistry. Some series were processed according to the tetramethyl benzidine procedure (TMB-AHM) of Olucha et al. (1985), and others were processed with the nickel-enhanced diaminobenzidine (DAB) procedure (DAB-Ni, DAB 0.04%, 0.4% nickel ammonium sulphate in Tris buffer, pH 8.0, plus 0.008% of H_2O_2). A few of the series were counterstained with neutral red (TMB-AHM) or with acidic toluidine blue (DAB-Ni).

PHA-L injections. After 12 days of survival, animals were perfused with a fixative containing 4% paraformadehyde and 0.5% glutaraldehyde in 0.1 M PB, and brains were postfixed overnight. Freezing microtome frontal sections were processed for the avidin-biotin complex (ABC) indirect immunoperoxidase detection of PHA-L (1:2,000 goat anti-PHA-L, for 24 hours; Vector; 1:200 biotinylated rabbit anti-goat IgG and ABC; Vectastain kit, Vector) by using DAB as a chromogen. Two or three matching series were processed, one of which was usually counterstained with toluidine blue.

BDA injections. Twelve days after the BDA injection, animals were perfused with either PB-buffered 4% paraformaldehyde or Karnovsky's fixative. After postfixation (4–24 hours) and cryoprotection, frontal sections were obtained on a freezing microtome. Sections were incubated in ABC complex for 3 hours at room temperature. The resulting peroxidase label was then developed with either DAB

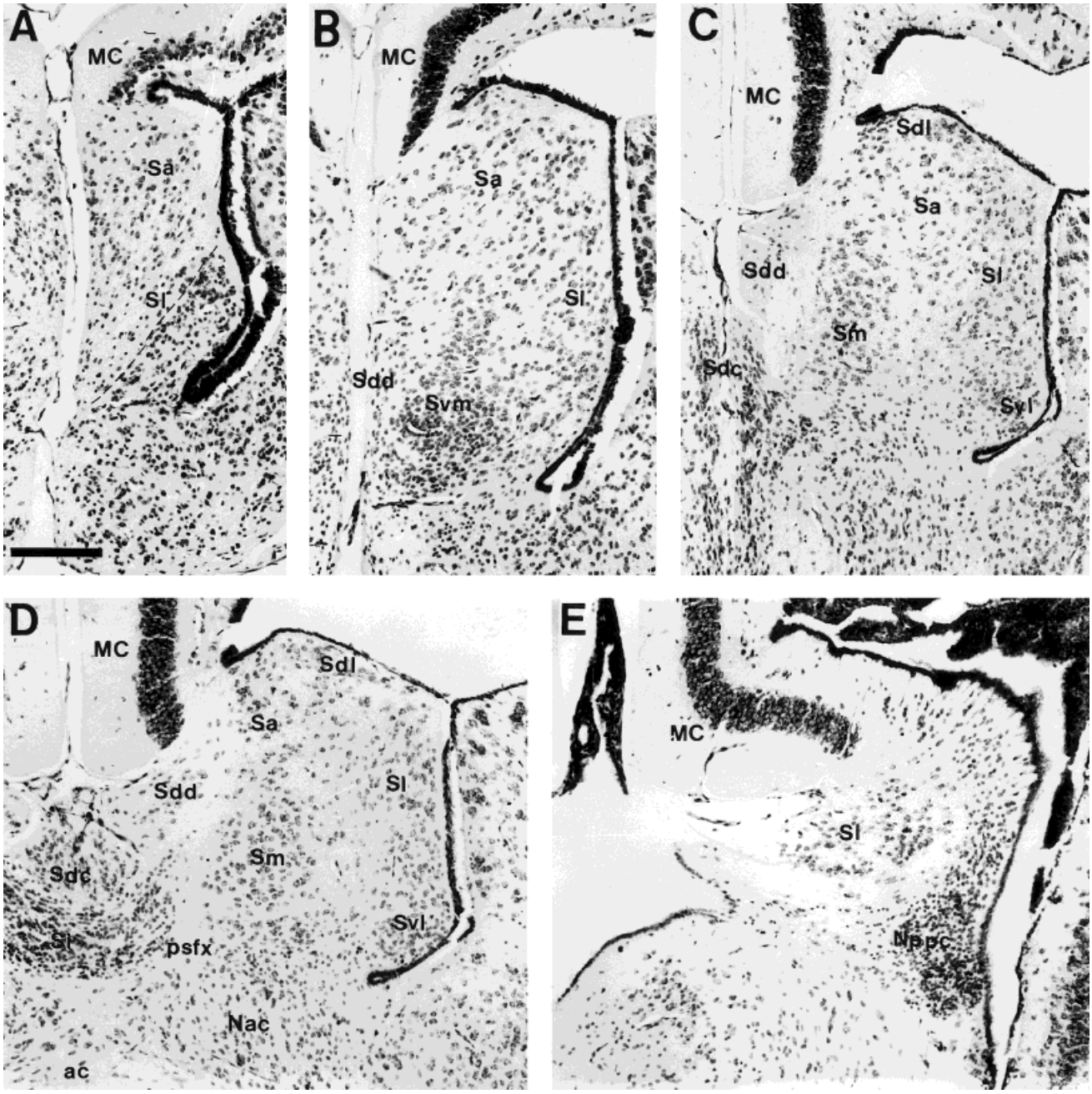

Fig. 2. **A–E:** Nissl-stained transverse sections through the septal complex of the telencephalon of *Podarcis hispanica* from rostral (A) to caudal (E) levels showing the location of the different septal nuclei. For abbreviations, see list. Scale bar = 100 μm.

alone (0.05% in Tris buffer, pH 7.4) or DAB-Ni. Sections were mounted on gelatin-coated slides and counterstained with acidic toluidine blue.

RESULTS

First, we provide a short account of the cytoarchitecture of the septum of *P. hispanica* that can be helpful in following the description of the labeling found after the application of tracers. Then, following the experimental design described above, the retrograde labeling that results from septal injections is described. Finally, the anterograde transport to the septal nuclei that follows extraseptal injections is reported.

Notes on the cytoarchitecture of the septum of *P. hispanica*

The septal complex of *P. hispanica* is composed of nine nuclei (Fig. 2) that are characterized by a distinct distribu-

tion of several neurotransmitters and neurotransmitter-related histochemical markers (Font et al., 1995). These nuclei have been named by using a pure topographical nomenclature. The anterior (Sa), lateral (Sl), medial (Sm), and ventromedial (Svm) nuclei occupy the center of the septal formation. Next to these nuclei and in the caudal half of the septal complex, cells near the lateral ventricle are arranged in a characteristic way, thus composing two distinct cell groups known as the ventrolateral (Svl) and the dorsolateral (Sdl) septal nuclei.

The midsaggital aspect of the septum is occupied by two more nuclei: the dorsal septal nucleus (Sd) and the nucleus septalis impar (Si). The Si is crossed by the fibers of the anterior pallial commissure. The Sd can be divided, in turn, into two subnuclei that display differential histochemical features: The dorsal subdivision (Sdd) is crossed by fimbrial fibers (named by some authors the septofimbrial nucleus; Halpern, 1980). The central division of the Sd (Sdc), dorsally adjacent to the anterior pallial commissure, shows caudal continuity with the subfornical organ. Finally, the nucleus of the posterior pallial commissure (Nppc) is composed of small cells associated with the fibers of this commissure.

Despite it's intention to be objective (from a comparative point of view), the topographical nomenclature can also be misleading. Therefore, it should be stressed that the names *medial* and *lateral* applied to the nuclei of the reptilian septum are indicative of neither homology nor analogy with the medial and lateral septal nuclei of the mammalian telencephalon. In fact, as discussed by Font et al. (1995), the use of histochemical criteria reveals that the Sm of reptiles is comparable to the intermediate part of the lateral septum of rats. Furthermore, the medial septum of mammals has no counterpart in the reptilian septum, but it seems to be comparable to a cell group of the basal telencephalon of lizards and turtles that is closely associated with the diagonal band nucleus (DB). Comparison of the reptilian septal complex with the septum of other tetrapods awaits detailed studies of its fiber connections and histochemical features in birds and amphibians.

Retrograde labeling after septal tracer injections

Retrograde labeling was found in several areas of the telencephalon (including cortical and subcortical areas), diencephalon, and midbrain after all tracer injections in the septum. First, the labeling found in the midbrain and extracortical forebrain is reported. This is followed by a description of the location and morphology of retrogradely labeled cells in the cerebral cortex.

Extracortical labeling

Common labeling found after all septal injections. In the telencephalon, retrograde labeling was always found in the DB, in part of the bed nucleus of the medial forebrain bundle (Nmfb; Figs. 3–6, 7A), and, as a caudal continuation, in the nucleus of the anterior commissure (Nac), where labeling became bilateral. In the hypothalamus, retrograde labeling was observed after all septal injections at tuberal and mammillary levels. At tuberal levels, retrogradely labeled neurons were seen in the periventricular nucleus, most of them in the vicinity of the paraventricular organ (Fig. 7B). This group of labeled cells merged caudally with labeling in the supramammillary nucleus. Retrograde labeling was also present in all cases in the mammillary nucleus (Fig. 7C), where it usually became bilateral. At mesencephalic levels, labeled cells were always seen in the raphe nucleus.

Specific labeling after restricted septal injections. In addition to the labeling described above, small tracer injections restricted to a few septal nuclei gave rise to specific retrograde labeling in several extracortical forebrain and midbrain areas. In this section, this specific labeling is described for four injections (Figs. 3–6).

The first case was an HRP injection into the precommissural Sa that also affected the rostral edge of the Sdl (case H8812; Fig. 3). This case showed retrogradely labeled neurons in the dorsolateral anterior nucleus of the thalamus (DLA) and in the ventral tegmental area (VTA) as well as the labeling common to all septal injections. In addition, a small group of faintly labeled cells was observed on the mesorhombencephalic boundary, the location of which coincided with the locus coeruleus (LCo), as defined by Smeets and Steinbush (1989) by the presence of noradrenaline immunoreactive cells in the *Gekko.* A similar cell group displayed tyrosine hydroxylase- but no dopamine-like immunoreactivity in the brain of *P. hispanica* (unpublished results).

Injection B9519 was restricted to the Svm and the rostralmost part of the Sl (Fig. 4). After this injection, specific retrograde labeling was observed in the bed nucleus of the stria terminalis (Bst) and the ventral posterior amygdala (VPA), in the thalamic DLA, and in the medial preoptic nucleus and lateral preoptic area. However, the most remarkable group of retrogradely labeled neurons was observed in the dorsal zone of the anterior hypothalamus, medial to the lateral forebrain bundle (Figs. 4D,E, 7D), where labeling was bilateral with an ipsilateral predominance. Some BDA^+ cells were also seen dorsally within the periventricular hypothalamic nucleus.

Case P9226 (Fig. 5) was a PHA-L injection centered in the Sm that also involved the dorsal part of the Sdd and the caudal edge of the Sa. At preoptic levels, labeled cells were observed in the medial and lateral areas. Thalamic labeling differed from that of other cases: along with a few labeled cells scattered in the DLA, many PHA-L-immunoreactive neurons were found bilaterally in the dorsomedial thalamic nucleus (DMA; Fig. 7E). In the midbrain, retrograde labeling was present in the VTA. A few labeled cells were distributed within a lateral area of the midbrain tegmentum that apparently coincided with both the substantia nigra (Sn) and the reptilian aminergic group 8 (RA8; Smeets et al., 1986b) due to its location and to the presence of tyrosine hydroxylase-immunoreactive cells (unpublished results). Retrograde labeling was also observed in the ipsilateral LCo and CG.

A caudal HRP injection was restricted to the Sd and the Si (H9271; Fig. 6), although the dorsal part of the Nac and the rostral edge of the thalamic DMA were probably affected by diffusion of the tracer. In addition to the labeling common to all septal injections, this case also showed labeled neurons in the nucleus accumbens. In the thalamus, labeled cells were found in the caudal DMA (outside the injection site), and a few cells were seen in the DLA and in several nuclei of the posterior dorsal thalamus (see Fig. 6F). The hypothalamus showed bilateral labeling in the medial and lateral preoptic areas that was especially noticeable in the medial and periventricular preoptic nuclei (Fig. 7F), and in the anterior hypothalamus (the retrochiasmatic and periventricular nuclei and the lateral

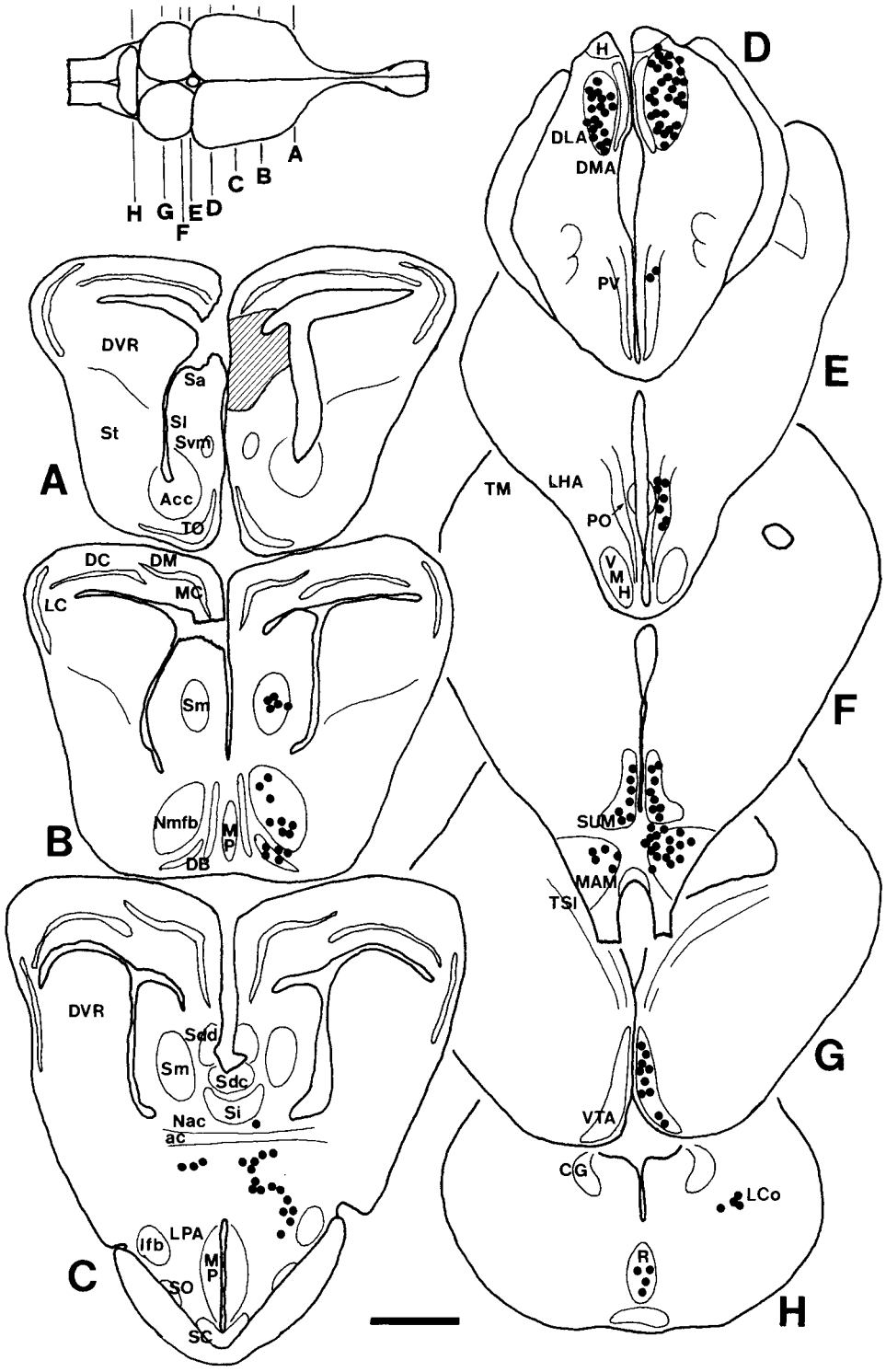

Fig. 3. **A–H:** Semischematic camera lucida drawings of frontal sections through the brain of *P. hispanica* showing the retrograde labeling (solid circles) that resulted from deposition of an HRP crystal in the anterior septal nucleus (case H8812) and the rostral edge of the dorsolateral septum. Because the cerebral cortex was partially removed to allow direct access to the septum, labeling in the cerebral cortex could not be studied. For abbreviations, see list. Scale bar = 500 μm (also applies to Figs. 4–6).

hypothalamic area). More caudally, the dorsolateral hypothalamic nucleus and the lateral hypothalamic area showed ipsilateral retrograde labeling. Mesencephalic labeling was more extensive than in other septal injections. A high number of retrogradely labeled neurons were present in the Sn/RA8, the raphe nucleus (R), and the midbrain

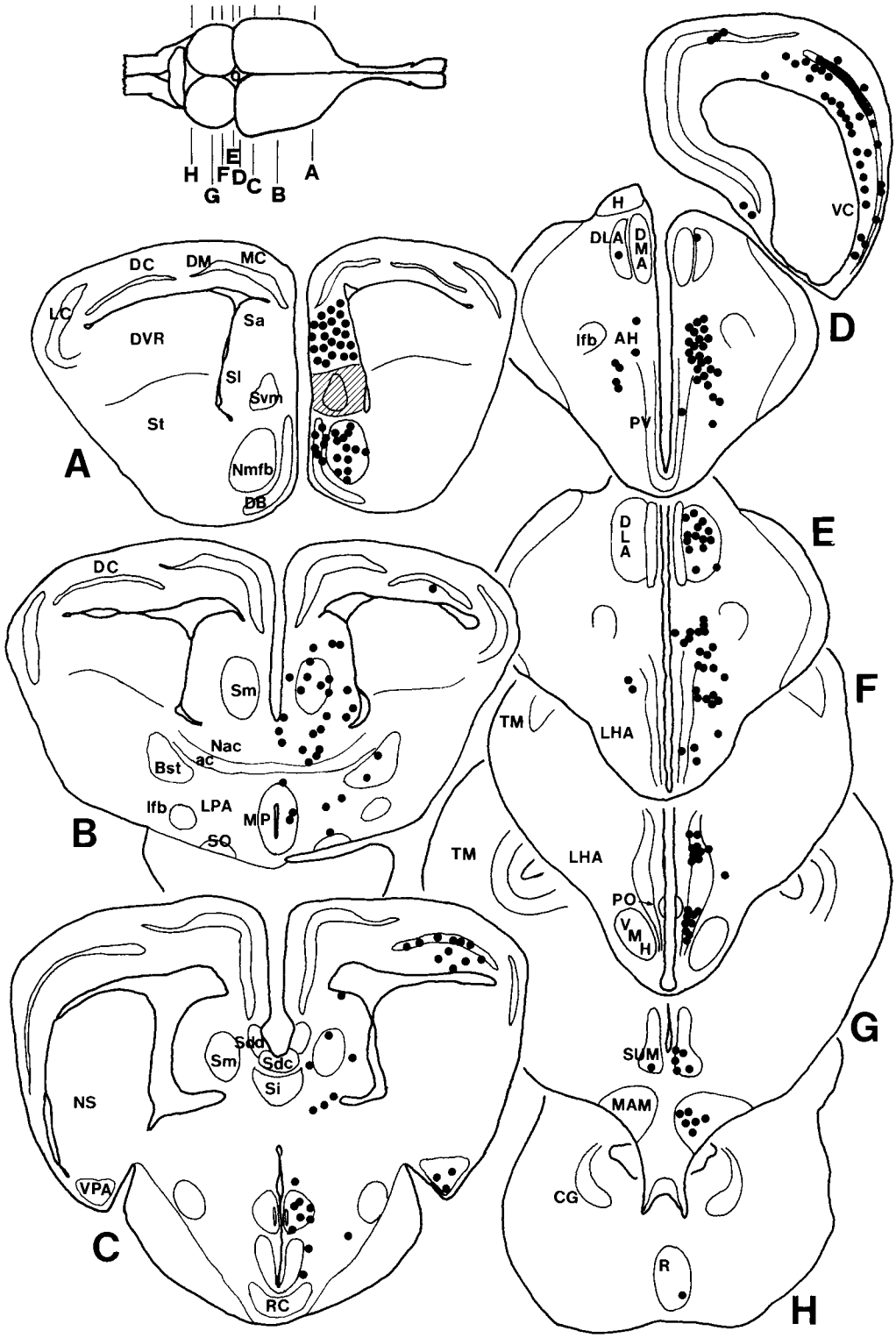

Fig. 4. **A–H:** Retrograde labeling in the brain of a lizard that received an iontophoretic injection of BDA into the ventromedial septal nucleus and the rostral edge of the ventral Sl (case B9519). For abbreviations, see list.

central gray (CG). A few cells were also seen in the laminar part of the torus semicircularis (Fig. 6G).

Retrogradely labeled somata were absent in all of these areas after HRP injections restricted to the DMA and the habenula (case H9298), except for the dorsolateral hypothalamic nucleus, the posterior dorsal thalamus, the torus semicircularis, and the midbrain raphe nucleus (see open circles, Fig. 6). Therefore, it can be assumed that retro-

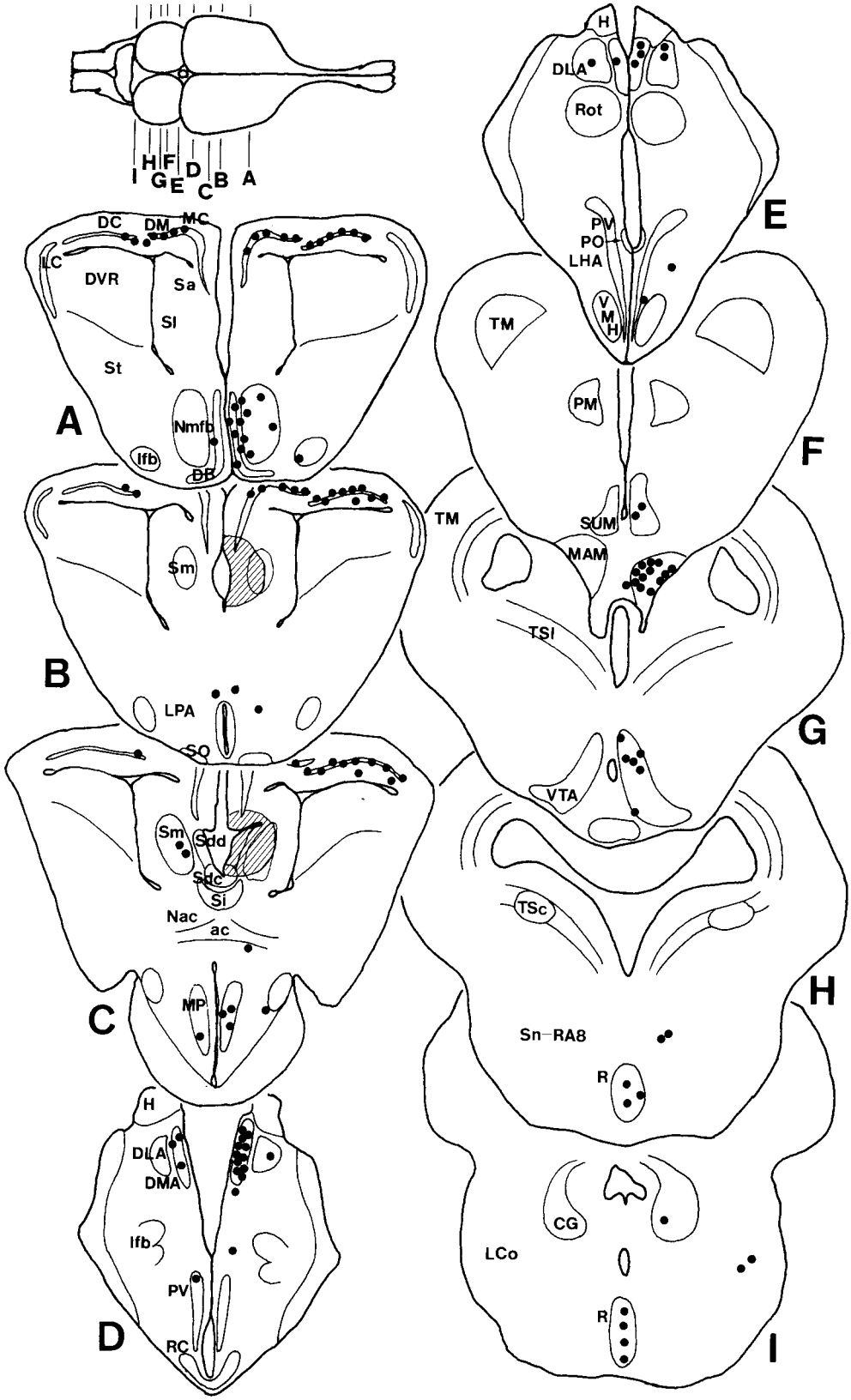

Fig. 5. **A–I:** Retrograde labeling resulting from a Phaseolus vulgaris-leucogglutinin (PHA-L) injection (case P9226) centered in the medial septal nucleus (Sm) and the dorsal part of the Sd. A small portion of the medial cortex was also affected by diffusion of the tracer. For other abbreviations, see list.

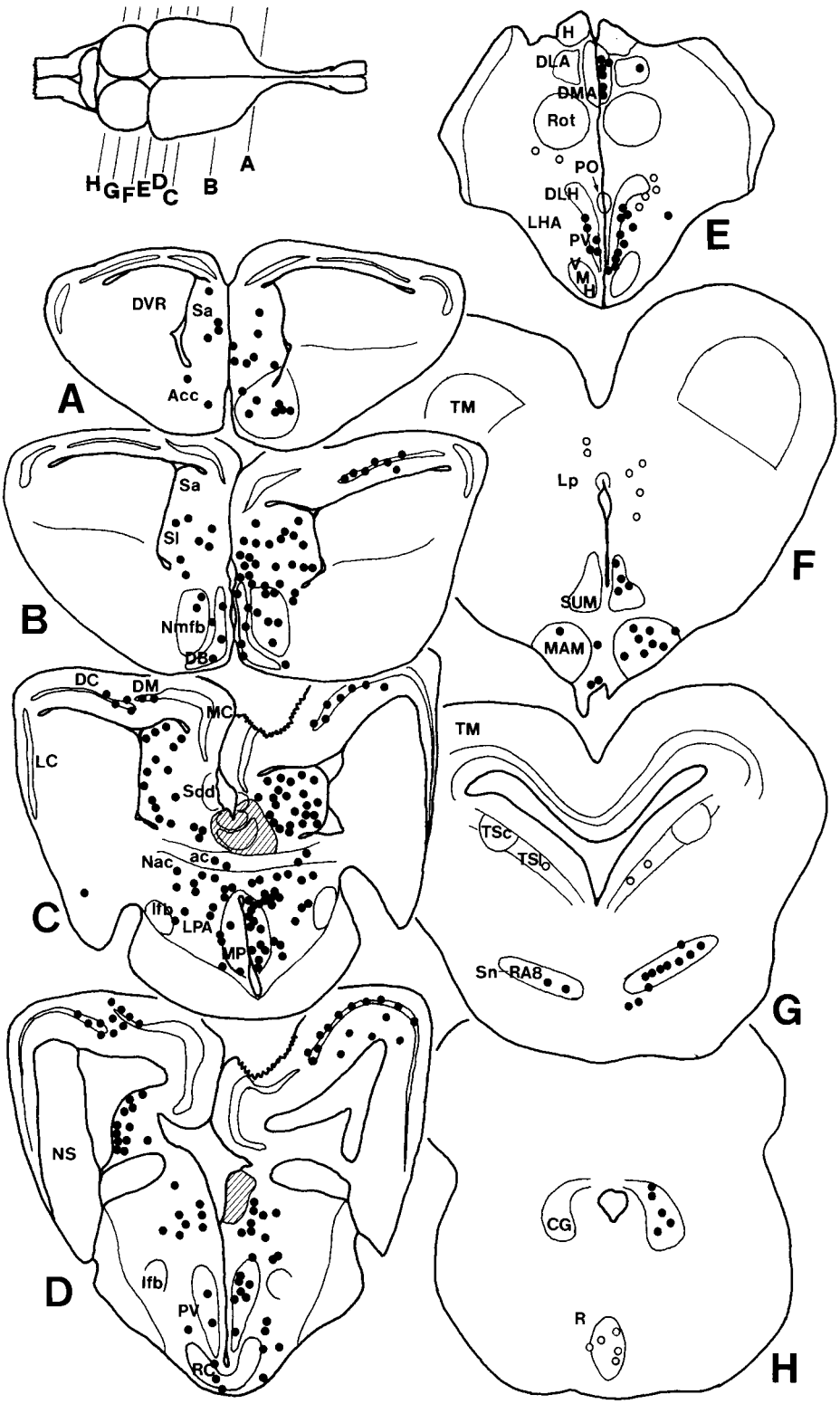

Fig. 6. **A–H:** Cells retrogradely labeled by an HRP injection (case H9271) into the posterior septum affecting the Si and the central part of the dorsal septal nucleus, which apparently also affected the rostral rim of the dorsomedial anterior thalamic nucleus (DMA). Open circles indicate labeling that was observed in this specimen and also after a control injection into the DMA and the habenula (case H9298). Therefore, labeling indicated by solid circles is interpreted as being due to retrograde transport from the septal nuclei affected by the injection. For other abbreviations, see list.

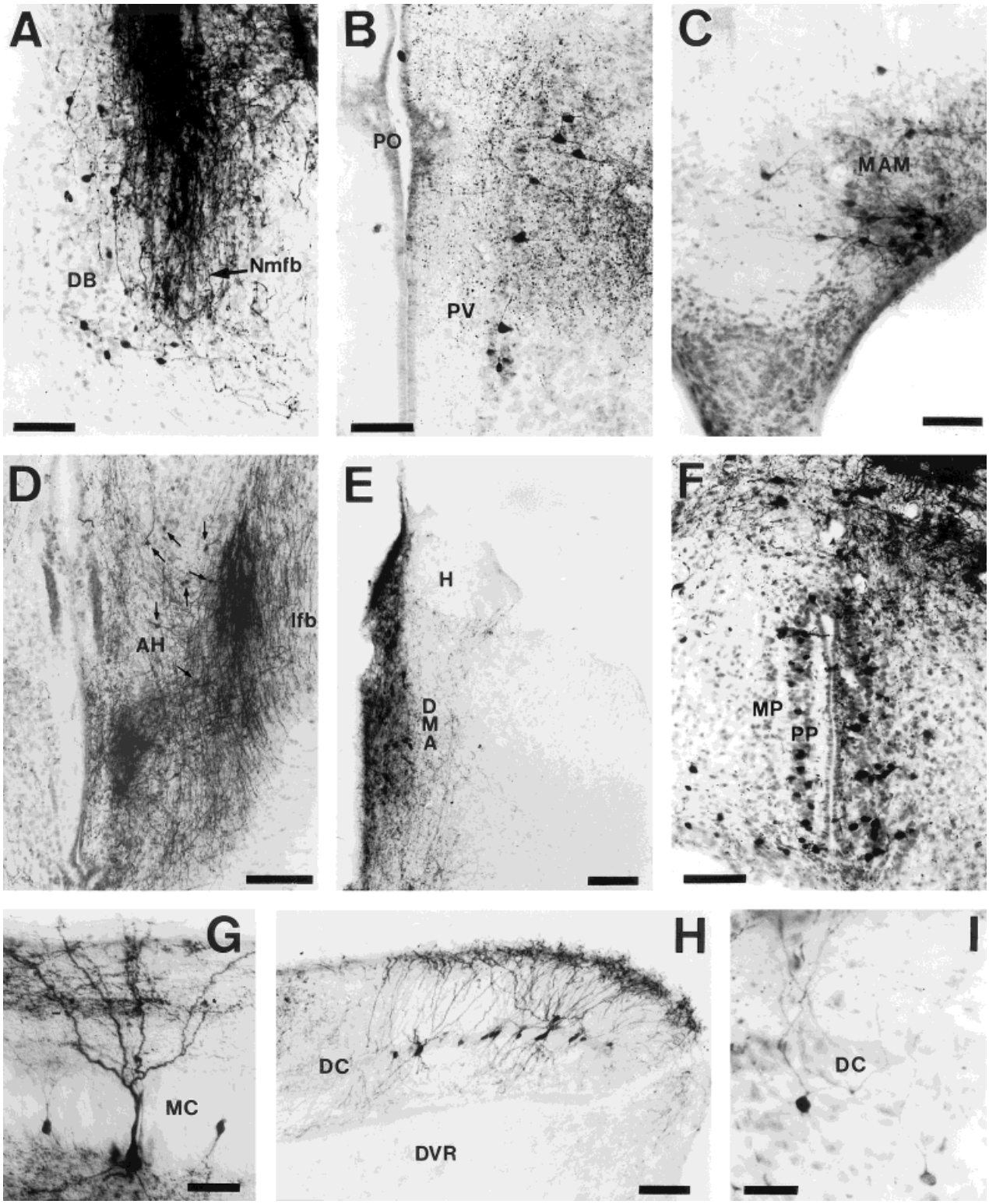

Fig. 7. Photomicrographs of frontal section showing the retrograde labeling in the different parts of the brain of *P. hispanica* after septal injections. **A:** Labeled cells in the diagonal band nucleus (DB) and the bed nucleus of the medial forebrain bundle (Nmfb). **B:** Retrogradely labeled neurons in the periventricular hypothalamic nucleus (PVH) adjacent to the paraventricular organ (PO). **C:** Labeled perikarya in the mammillary bodies. **D:** BDA-labeled cells (arrows) in the anterior hypothalamus after Svm/Sl injection (case B9519). **E:** Retrogradely labeled neurons in the DMA after a PHA-L injection centered in the medial septal nucleus (Sm). **F:** Labeled neurons in the preoptic area following an HRP injection in the Si/Sd (H9271). **G:** Labeled pyramidal neurons in the cell layer of the dorsomedial and dorsal contralateral cortex. **H:** Labeled pyramidal neurons in the cell layer and mainly nonpyramidal neurons in the inner plexiform layer (I) of the dorsal cortex after BDA injection in the Svm/Sl. For other abbreviations, see list. Scale bars = 100 μm in A–F,H, 30 μm in G,I.

grade labeling in the remaining areas was due to tracer uptake by axonic terminals in the injected septal nuclei.

Cortical retrograde labeling after septal injections. Some of the tracer injections described above also involved a small part of the overlying medial cortex (MC; Table 1). In some other cases, the cerebral cortex was partially removed to gain access to the septum, where HRP crystals were directly deposited (e.g., case H8812; Fig. 3). In these cases, cortical labeling could not be studied. In the remaining injections (Table 1), beside the labeling described in the preceding section, retrograde labeling of cortical cells was also observed, the location of which depended on the injection site within the septum.

Rostral injections into the anterior septum (cases H8697 and H8734) gave rise to bilateral neuron labeling in the cell layer of the MC and the dorsomedial cortex (DM). Although labeling in the DM can be due to uptake of the tracer by passage fibers on their way to the opposite cortex (the DM is the main source of commissural fibers; Martínez-García et al., 1990), this result indicates a bilateral projection of the MC to the Sa (further supported by anterograde transport experiments; see below).

However, restricted injections into the rostral lateral septum (case B9519) led to cell labeling that was restricted to the caudal dorsal cortex, with the exception of its most medial part. Labeled cells were also found in a cytoarchitecturally distinct area of the caudal edge of the cortex, characterized by the presence of a well-defined inner cell plate, named by some authors the ventral cortex (VC; Voneida and Ebbesson, 1969). The results revealed a projection from the dorsal cortex (DC) and the VC to the Sl.

Finally, a restricted HRP injection into the Si and Sd (case H9271) showed retrogradely labeled cells bilaterally in the DM and in the medial subfield of the DC. Because the Si is crossed by the fibers of the anterior pallial commissure, this labeling can be attributed in part to uptake of the tracer by commissural fibers arising from these two cortical areas (Martínez-García et al., 1990). However, as we will see, commissural fibers seem to give rise to boutons en passant at the level of the Si (see below). Moreover, the central and lateral subfields of the DC showed labeled neurons only ipsilateral to the injection, whereas no labeled cells were present in the MC. These results suggest the presence of a projection from the DC to the Si and/or the Sd, which was confirmed by anterograde tracing experiments (see below). Cortical labeling after less restricted injections involving several septal nuclei was coherent with the labeling pattern described here.

Labeled neurons in the MC and the DM were always seen in the cell layer. Two types of corticoseptal cells were found in the MC (Fig. 7G). The most common type displayed big cell bodies with well-developed apical dendritic trees that reached the pial surface and bore a high density of spines. Smaller cells were also labeled in the MC. Their apical dendrites did not reach the pial surface and were apparently nearly devoid of spines. Labeled cells in the DM always showed the typical bipyramidal morphology, with both apical and basal dendritic trees (although the latter were relatively reduced) that bore a high density of dendritic spines.

Labeling in the DC differed from that in more medial cortical areas: Although most of the cells were located in the cell layer, some labeled neurons were also present in the inner plexiform layer, especially at caudal levels (Figs. 4C,D, 5C, 6D). Labeled neurons in the cell layer showed bipyramidal morphology similar to DM neurons (Fig. 7H), but cells in the inner plexiform layer were morphologically heterogeneous. Most of these inner cells were sparsely spiny stellate neurons that belong to the cell clusters typical of this layer (Fig. 7I). Septal injections of tracers also gave rise to retrograde labeling of juxtaependimal cells of the DC that displayed reduced dendritic trees, which consisted of single, aspiny dendrites that branches only once or twice within the inner plexiform layer.

Studies in other lizards (Lohman and van Woerden-Verkley, 1978; Hoogland and Vermeulen-VanderZee, 1989a) and our own unpublished data in *P. hispanica* indicate that the DC projects to the hypothalamus, thalamus, and midbrain via the fornix. However, septal injections that spared the fornix (cases B9519 and H9271) gave rise to many labeled cells in both the cell layer and the inner plexiform layer of the ipsilateral DC, so that we are confident that the labeled neurons in these cases were corticoseptal cells.

Fiber labeling in the septum after extraseptal injection of anterograde tracers

In this section, we will start by describing the intraseptal fiber labeling that followed cortical tracer injections. Then, we will go on to describe the labeling that resulted from basal telencephalic, thalamic, hypothalamic, and midbrain injections.

Injections into the cerebral cortex. HRP injections into different cortical areas resulted in intense anterograde labeling in different septal nuclei (Fig. 8). The results of injections into the MC have already been published (Martínez-García and Olucha, 1988; Olucha et al., 1988) and will not be described here in detail. Briefly, different parts of the MC projected to different septal areas: A bilateral projection terminating in the Sa arose from the rostral and vertical MC (Fig. 8A), and a strictly ipsilateral projection that reached the Sl (dorsal part) originated in cells of the horizontal portion of the caudal MC (Fig. 8B). We were not able to perform restricted injections into the DM of *P. hispanica*. However, the results of injections involving the DM and some of the neighboring cortical areas (MC or DC) were congruent with the view that, like in *Gallotia* (Martínez-García et al., 1990), the DM area of *P. hispanica* projects bilaterally to the Sdl. Some of the commissural fibers arising from the DM seemed to give rise to a termination field as they crossed the Si, where they displayed a high density of boutons en passant.

Injections in the DC (cases H8628 and H8648) resulted in ipsilateral terminal-like labeling in the Sl, Sm, Sdd, and Si (Fig. 8C). The VC received a restricted injection in one animal (case H9250), and, in two lizards that received larger injections, which also involved other cortical areas (cases P9227 and H9231). In these cases, varicose labeled fibers entered the caudal septum via the Nppc and ran rostralward in the corner of the ventral sulcus of the lateral ventricle, thus crossing the Svl and the ventral aspect of the Sl (Fig. 8D, bottom). At more rostral levels, labeling seemed to avoid the Svm but occupied most of the ventral half of the Sl (Fig. 8D, top). Additional terminal labeling was seen bilaterally in the Sdd. Moreover, labeled fibers crossed the anterior pallial commissure to reach the opposite Svl and ventral Sl, where terminal labeling was also found.

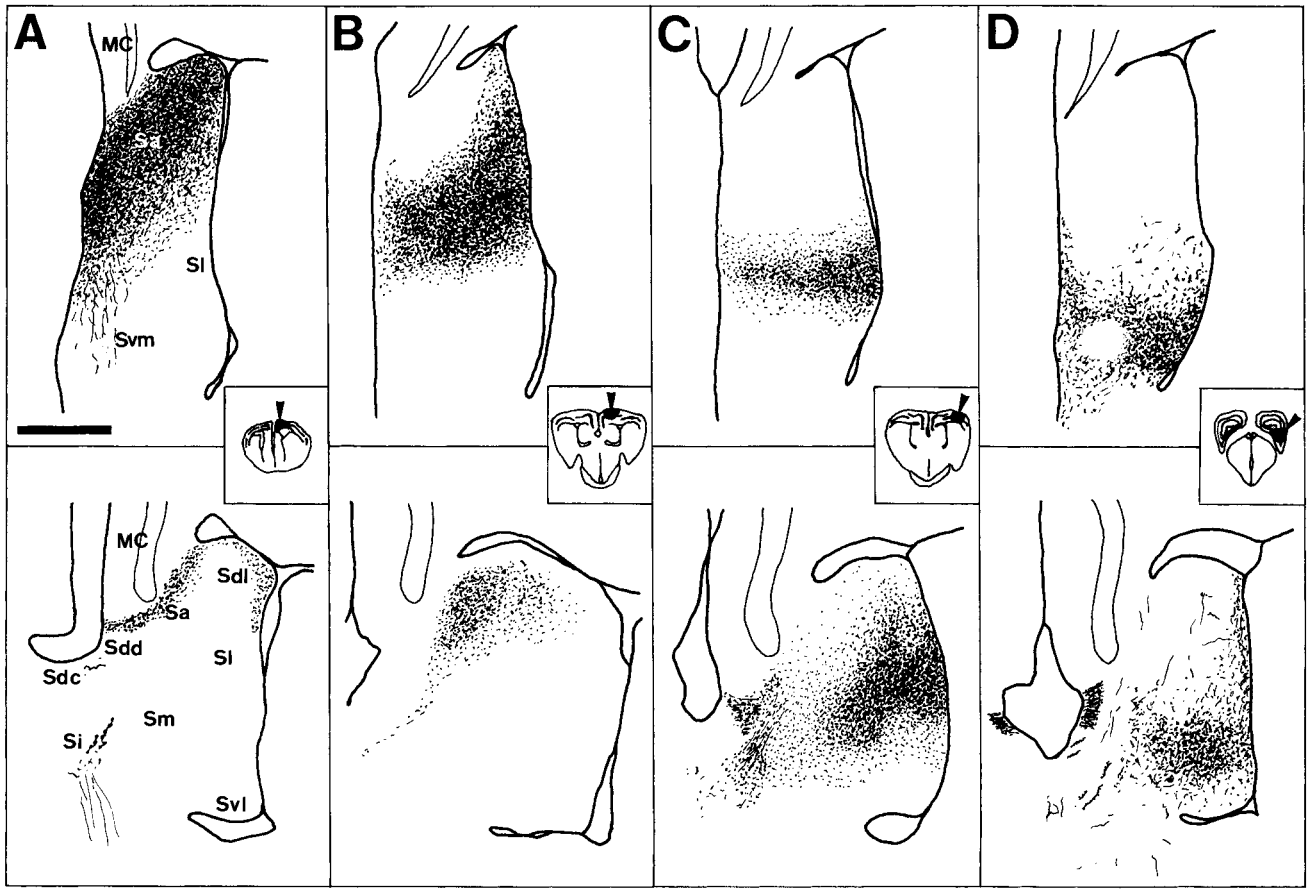

Fig. 8. Semischematic camera lucida drawings of two frontal sections through the septal complex (top, precommissural level; bottom, commissural level) showing the anterograde labeling that resulted from different cortical injections (see **insets**). **A:** Injection into the rostral part of the medial cortex (case H8897). **B:** Injection into the caudal part of the medial cortex (case H8935). **C:** Injection into the dorsal cortex (case H8648). **D:** Injection into the ventral cortex (case H9250). For abbreviations, see list. Scale bar = 200 μm (also applies to Fig. 9).

Rostral injections into the DC resulted in terminal-like labeling in the dorsal aspect of the Sl, whereas caudal DC and VC injections anterogradely labeled the ventral Sl. Therefore, it seems that the DC-VC complex projects to the Sl in a topographical fashion.

Injections in the basal telencephalon. In two cases, tracer injections were placed in the basal telencephalon, thus involving the Nmfb and the DB (P9242) or their caudal continuation in the nucleus of the anterior commissure (Nac; case H9288). In both cases, thin and varicose labeled fibers were observed in the whole septal complex, but intense retrograde labeling in several septal nuclei made this labeling difficult to interpret. However, some labeled fibers displayed a characteristic morphology: in the Sdd, thick and smooth labeled fibers coursed dorsally to enter the cortex, whereas thick and varicose fibers were frequent in the Si and Nppc.

Injections in the anterior thalamus and habenula. Injection H9298 was restricted to the DMA and the habenula (Fig. 9A). Beside the presence of dense anterograde labeling in the nucleus accumbens and striatum and of some labeling in the DVR (also reported in *Gekko;* González and Russchen, 1988; González et al., 1990; Henselmans, 1992), the septum of this lizard showed thin, labeled, varicose fibers in several septal nuclei (Fig. 9A). Labeled fibers were seen in the ipsilateral Sd and bilaterally, with ipsilateral predominance, in the Sm. A few labeled fibers were also observed ipsilaterally in the ventral part of the rostral Sl (Fig. 9A, top) and in the Nppc. The Si (Fig. 9A, bottom; Fig. 10A,B) displayed two kinds of labeled fibers: thick fibers bearing big swellings, which apparently arose from the high numbers of retrogradely labeled somata present in the Si itself, and thin fibers similar to those present in the Sm, which were probably due to anterograde transport from the injection site. A restricted PHA-L injection in the lateral habenular nucleus (case P9303) resulted in the total absence of labeling in the septum, which indicates that labeled fibers found in case H9298 (see above) actually arose from DMA neurons.

Injections into the hypothalamus. Tracer injections into different parts of the hypothalamus resulted in different patterns of anterograde labeling in the septum. In this section, we will describe the results of injections into the preoptic, anterior, tuberal, and mammillary hypothalamus.

Large injections affecting the preoptic area (case B9521) resulted in anterograde labeling of fibers that left the injection site and terminated in the Sd (Sdd and Sdc) and the Svl, where intensely labeled terminal fields were observed. Scarce fiber labeling was also present in other

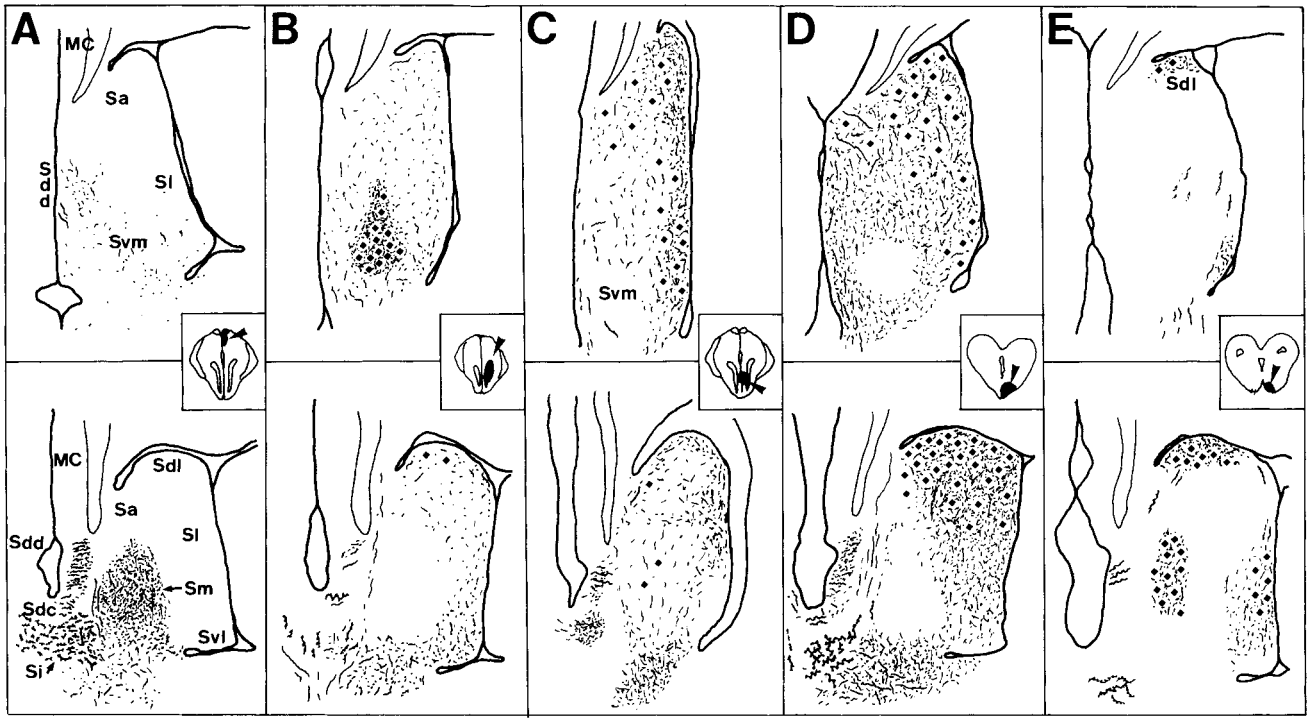

Fig. 9. Semischmatic camera lucida drawings of two frontal sections through the septal complex (top, precommisural levels; bottom, commisural levels) showing anterograde labeling after different subcortical injections. In each case, the injection site is indicated in small drawings of frontal sections through the diencephalon or midbrain (see **insets**). Diamonds represent pericellular nests. **A:** Injection of HRP into the dorsomedial anterior thalamus and habenula (case H9298). **B:** HRP injection into the anterior hypothalamus (case H9289). **C:** BDA injection into the periventricular hypothalamic nucleus near the paraventricular organ (case B9457). **D:** HRP injection into the mammillary complex (case H8801). **E:** HRP injection into the ventral tegmental area (case H9267).

septal nuclei (Sa, Sl, and Sm), but it was difficult to interpret due to the intense retrograde labeling of septal neurons.

Injection H9289 (Fig. 9B) was confined to the border zone between the anterior and the preoptic hypothalamus. The injection was laterally adjacent to the periventricular nucleus and medial to the lateral forebrain bundle. Labeling was seen in all of the septal nuclei, with the exception of the Sm. The Sd showed thick, varicose fibers in both the dorsal (Sdd) and central (Sdc) parts, although their course and appearance differed. The Si displayed a moderate innervation by thick fibers bearing frequent swellings, which crossed the midsaggital plane to innervate also the contralateral half of the nucleus (Fig. 10E). Similar labeling was seen in the Nppc (Fig. 10F), but the most characteristic labeling was found bilaterally, with clear ipsilateral dominance, in the Svm (Figs. 9B, 10C). This labeling was composed mainly of juxtasomatic boutons (Fig. 10D). Another lizard received a BDA injection restricted to the anterior hypothalamus, just medial to the lfb (case B9520): Anterograde labeling of the Svm was so massive and intense that the nucleus appeared as a black spot within the rostral septum that was visible even to the naked eye.

Another BDA injection (case B9457; Fig. 9C) was restricted to the periventricular nucleus at tuberal levels, next to the paraventicular organ. In this case, anterograde labeling was found in several septal nuclei. Scattered fibers with some varicosities were seen in the Nac, in the Sm, where a few basket-like figures were observed, and in both parts of the Sd (Sdd and Sdc). However, most of the labeled fibers crossed the caudal septum and ran farther rostrally to reach the anterior aspect of the Sa and the Sl. In both areas, huge numbers of labeled pericellular baskets surrounded most of the septal cells, especially those located near the lateral ventricle (Fig. 9C).

Injection H8801 was restricted to the mammillary nucleus. In this case, bilateral fiber labeling was present in all septal nuclei with the exception of the Sm and the Svm (Fig. 9D). In the Sa, Sl, and Sdl, this labeling was composed mainly of pericellular baskets. Fiber labeling was also very dense in the Sd and Sdl.

Injections into the midbrain tegmentum. A restricted HRP injection centered in the ventral tegmental area and a small part of the lateral tegmental area (case H9267; Fig. 9E) showed labeled fibers in the Sdl (Fig. 10G), Sm, and Svl, which extended rostrally into the ventrolateral aspect of the Sl. Most of these labeled fibers formed basket-like figures around unlabeled cell bodies. Thick fibers with big enlargements were also present in the Si.

Another HRP injection (case H9340) affecting the Sn/RA8 complex and the rubral tegmentum showed anterograde labeling in the Sd. A bigger injection that involved the whole caudal tegmentum and the locus coeruleus (case H9150) showed labeled fibers not only in the Sd but also in the Sdl.

Finally, case H9268 received an HRP injection into the caudal tegmentum that also involved the midbrain CG and the lateral tegmental nucleus. In the septum of this animal, terminal-like labeling was visible only in the Sdd and the Si.

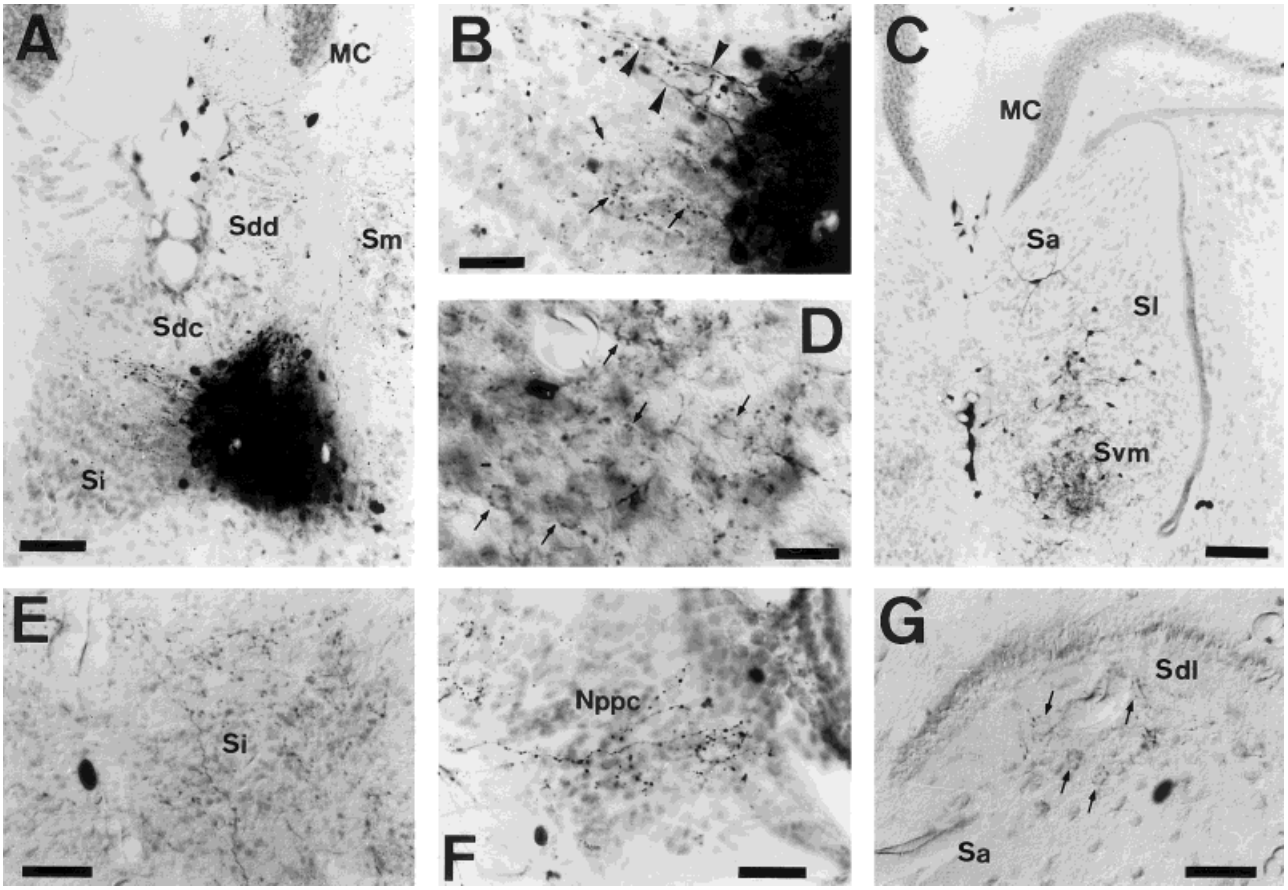

Fig. 10. Photomicrographs of frontal sections through the septal complex showing the anterograde labeling in the septum after subcortical injections. **A:** Labeling in the Si and Sm following an injection into the DMA and habenula. **B:** Higher magnification of the same section showing the two kinds of labeled fibers that appear in the Si (arrowheads and arrows) after thalamic injections. **C:** Labeled fiber plexus in the Svm after an HRP injection into the anterior hypothalamus. **D:** Higher magnification of the same section showing the presence of labeled boutons aposed on cell bodies (arrows) of the Svm. Labeled thick, varicose fibers in the Si (**E**) and the nucleus of the posterior pallial commissure (Nppc; **F**) following an HRP injection into the periventricular hypothalamic nucleus at tuberal levels. **G:** Labeled basket-like figures (arrows) in the dorsolateral septal nucleus (Sdl) after an HRP injection restricted to the ventral tegmental area (VTA; Nomarski interferential microscopy). For other abbreviations, see list. Scale bar = 50 μm in A, 20 μm in B,D, 100 μm in C, 30 μm in E–G.

DISCUSSION

On the method

The results of retrograde transport after tracer injections in the septum and of anterograde transport to the septum of tracers injected in its putative afferents give a solid picture of the main septal afferents that includes the location of their cells of origin and their termination pattern within the septum. However, our experimental work has not been free from technical problems.

All of the tracers, even those that were originally reported as good anterograde tracers (PHA-L: Gerfen and Sawchenko, 1984; BDA: Veenman et al., 1992), gave rise to intense retrograde labeling in most of our injections, as sometimes happens in the mammalian and avian brain (see, e.g., Shu and Peterson, 1988; Veenman et al., 1992). In fact, in this work, we have used all three substances (HRP, PHA-L, and BDA) as both anterograde and retrograde tracers. In some cases, this made it difficult to interpret the origin of the resulting fiber and terminal labeling, because it can arise either from the injection site or from retrogradely labeled cells.

Another important problem was the extent of our injections. Given the small size of the brain of *P. hispanica*, most of our injections into the septum involved several nuclei and, in some cases, part of the overlying MC was affected by the micropipette track (see Table 1). Interpretation of the results of retrograde transport in these cases, again, was problematic.

Most of these problems were overcome by using confirmative injections in the second step of our experimental work. For instance, the low number of septal injections in which the cerebral cortex was not affected by the micropipette track was clearly insufficient to perform a detailed study of the origin of the corticoseptal projections. However, the results of anterograde tracing after injections into the cortex together with the cortical retrograde labeling that followed restricted septal injections allow for a characterization of the cells of origin and the termination way of the corticoseptal projections.

Even the problems of passage fibers, which are very serious in areas that, like the septum, are crossed by many fiber tracts, can be solved by using our experimental

approach. For instance, a projection from the DM to the Si is suggested by retrograde tracing and is confirmed by injections in the cortex, which show commissural fibers giving rise to boutons en passant at the level of the Si.

During the first years of our experimental work, we only used HRP as a tracer. This, again, may pose some limitation to our work: HRP was originally used as a pure retrograde tracer, but we used it as an anterograde tracer in some of our confirmatory extraseptal injections. Therefore, it is necessary to note that HRP gives excellent results as an anterograde tracer in the reptilian brain, especially when injected by iontophoresis (for instance, see Dacey and Ulinski, 1986). In fact, in our material, HRP anterograde labeling allowed for a detailed study of the morphology of labeled fibers and boutons (see Fig. 10).

The results obtained with the three neural tracers (BDA, PHA-L, and HRP) appear to be directly comparable regarding both the anterograde and the retrograde transport. In the present paper, we show two similar injections into the VC with PHA-L (case P9227) and HRP (case H9231). The anterograde (see Table 2) and retrograde (not shown) labeling are virtually the same, although PHA-L, as expected, yields a clearer morphological definition of anterogradely labeled axons and retrogradely labeled somata than HRP (Gerfen and Sawchenko, 1984). Similar injections of HRP and BDA in other neural centers, such as the optic tectum or the dorsal ventricular ridge (not included in this paper), show that BDA and HRP also give rise to comparable anterograde and retrograde labeling (unpublished observations): Although BDA gives rise to better quality labeling than HRP (Veenman et al., 1992), anterograde and retrograde labeling are found in the same areas, irrespective of the tracer employed.

Afferent connections of the septal nuclei of lizards

The only reports on the afferents of the septum in reptiles, as far as we known, are those by Belekhova and Nemova (1988) in the lizard *Ophisaurus apodus* and the study by Nemova (1988) on the septal afferents in turtles. Some data on the afferent connections of the reptilian septum can also be found in studies of the cortical efferents in several species of lizards and snakes (see references below). However, neither of these studies paid attention to the nuclear heterogeneity of the septal complex. Our results are the first to give a relatively complete account of the afferents to the different septal nuclei in a reptilian species.

Descending projections to the septum. Our results clearly show that an important afferent of the septal complex arises from the *cerebral cortex*. Corticoseptal projections have been described previously in the lizards *Gekko* (Bruce and Butler, 1984; Hoogland and Vermeulen-VanderZee, 1989b), *Iguana* (Bruce and Butler, 1984), *Tupinambis* (Lohman and Mentink, 1972; Lohman and van Woerden-Verkley, 1976), *Podarcis*, and *Gallotia* (Martínez-García and Olucha, 1988; Olucha et al., 1988) and in several snakes (Ulinski, 1975; Halpern, 1980). Our results indicate that some of the cortical projections to the septum are arranged in a topographical fashion, whereas other corticoseptal projections apparently lack any topography.

The projections from the different parts of the cortex clearly delineate some of the septal nuclei, further supporting the cytoarchitectonical classification of the septal nuclei by Font et al. (1995). Different parts of the MC project to the Sa and the Sl (Martínez-García and Olucha, 1988; Olucha et al., 1988; Hoogland et al., 1994), whereas the DC projects to the Sd and the Sl/Sm complex. The latter projection shows a clear topographic organization (Ulinski, 1975): The rostral DC projects to the dorsal Sl and Sm, the caudal DC projects to the ventral aspect of both nuclei, and the caudalmost aspect of the DC, namely, the VC, projects (bilaterally) farther ventrally to the most ventral aspect of the Sl, to the Svl, and to the Nppc. Despite their topography, the corticoseptal pathways are not organized in a point-to-point fashion, but they are quite divergent. In fact, small restricted injections of tracers into any cortical area result in terminal-like labeling of a band of the corresponding septal nuclei (Sa, Sl, Sm, Svl, and/or Nppc) throughout its anteroposterior axis.

The coincidence of the intense Timm reactivity of the Sa, Sl, and Sm (Olucha et al., 1988; Pérez-Clausell, 1988; Font et al., 1995) with the massive projection of the cortex to these nuclei suggests that at least some of the axonic terminals of cortical origin are rich in heavy metals, as are some of the intracortical axonic systems (López-García and Martínez-Guijarro, 1988; Martínez-García and Olucha, 1988). Experimental evidence obtained in both reptiles (Martínez-Guijarro et al., 1991) and mammals (Beaulieu et al., 1992) indicates that zinc-rich ($Timm^+$) boutons are glutamatergic and are therefore mainly excitatory. However, other neurotransmitters may be present in some of the corticoseptal projections. In fact, the DC shows a specific projection to the Sdd, a characteristically $Timm^-$ nucleus, which indicates that at least part of the DC projection to the septum is $Timm^-$. Likewise, it is interesting to note that the Sm, Svl, Nppc, and Si, which only show moderate Timm reactivity, also receive a substantial projection from the DC. This fact suggests that the projection from the DC is heterogeneous with respect to the neurotransmitter employed; therefore, its influence on septal cells may also be diverse. This view is supported by the morphological heterogeneity shown by DC corticoseptal cells (Fig. 7H,I). Whereas most of them are principal neurons (bipyramidal cells located in the cell layer), stellate cells located in the inner plexiform layer and unipolar juxtaependymal neurons are also labeled after septal injections. Nonpyramidal cells showing a similar morphology and laminar location display γ-aminobutyric acid (GABA) immunoreactivity in the DC of *P. hispanica* (Teruel et al., 1990), thus suggesting that some of the DC corticoseptal cells may be GABAergic.

Ascending projections to the septum. Results from the retrograde transport of tracers after injections into the septum and from the anterograde transport from injections into several diencephalic and mesencephalic areas indicate that projections from several telencephalic and extratelencephalic centers meet in the septum. Some of these projections seem to be widespread, terminating diffusely in most of the septal nuclei. In contrast, some other areas project quite specifically to a few septal nuclei.

Widespread ascending projections to the septum. Every injection into the septum resulted in the appearance of labeled somata in two locations: the basal telencephalon and the caudal hypothalamus. In the basal telencephalon, labeled cells were seen in the DB, the nucleus of the mfb, and the nucleus of the anterior commissure. The labeled cell group of the caudal hypothalamus consisted of the tuberal periventricular nucleus adjacent to the paraventricular organ and the supramammillary and mammillary

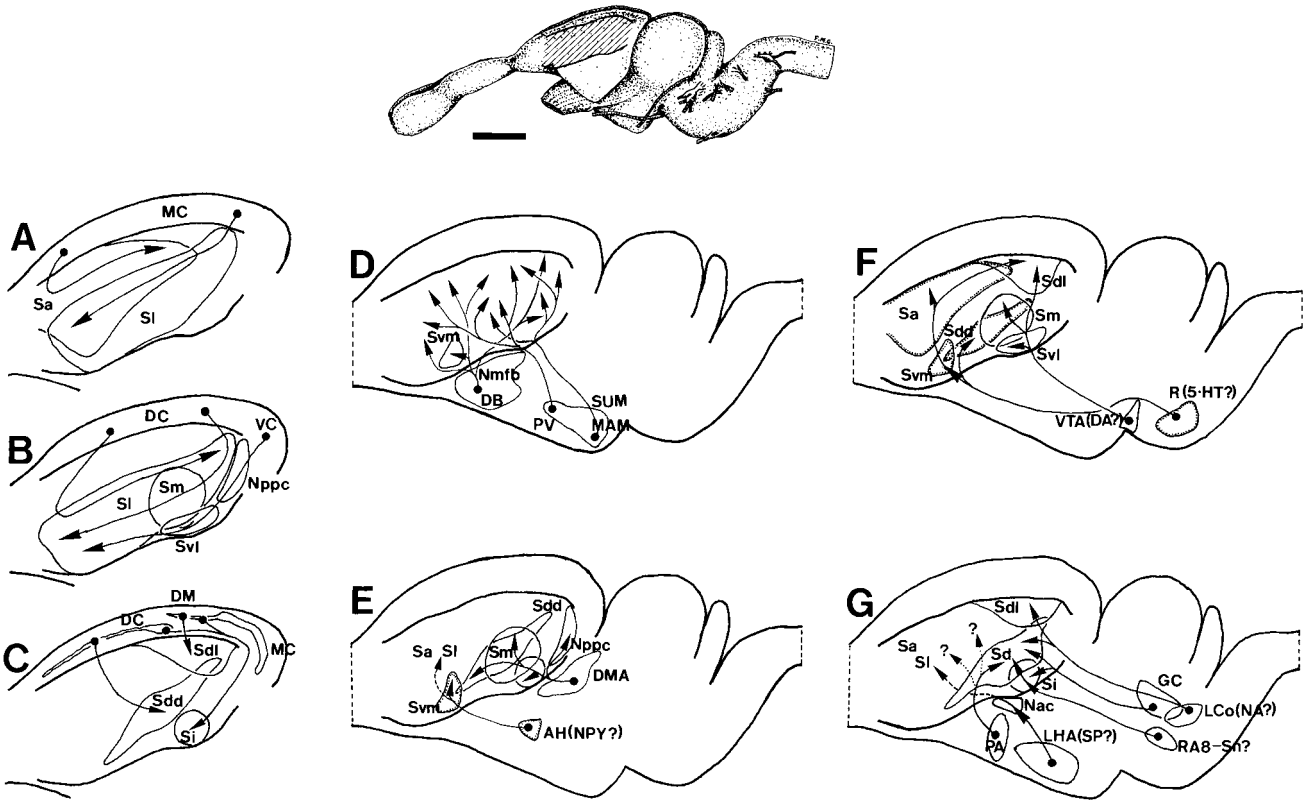

Fig. 11. **A–G:** Schematic drawings summarizing the afferent projections to the septum of *P. hispanica* according to our results of anterograde and retrograde tracing. Corticoseptal projections (left column) are represented in three different drawings: the topographic projections from the medial (A) and dorsal cortex (B) and the nontopographic projections from the dorsomedial and dorsal cortices (C). The topography, in fact, is simplified (see text). Ascending pathways to the septum are represented in four figures (D–G) to avoid overcrowding. The widespread projection from the caudal hypothalamus seems to exclude the Svm, whereas the projection from the basal telencephalon apparently reaches every septal nucleus (D). The rest of the afferents terminate in specific septal nuclei, and their origin and possible way of termination in the septum are represented in three additional diagrams (E–G). The actual position and size of the septum is indicated in a lateral view of the brain of *P. hispanica* (top). For other abbreviations, see list. Scale bar = 1 mm.

nuclei. These diffuse septal afferents were confirmed by anterograde tracing: Restricted injections of PHA-L or HRP into the basal telencephalon and HRP injections restricted to or involving the mammillary nuclei or the tuberal periventricular hypothalamus resulted in the anterograde labeling of fibers in most of the septal nuclei. The only exception was the Svm, which appeared to be free of labeling after caudal hypothalamic injections (Figs. 9C,D, 11D).

According to previous studies on the reptilian septal efferents (Hoogland et al., 1978; Sligar and Voneida, 1981; Belekhova and Nemova, 1988) and our own results (next paper in this series), these two structures are among the main targets for the septal output. Therefore, their projection back to the septum can subserve a feed-back control of the septal response. Moreover, both, the basal forebrain/diagonal band complex and the mammillary bodies project diffusely not only to the septum but also to the cerebral (hippocampal) cortex (Bruce and Butler, 1984; Martínez-García and Olucha, 1988), which suggests that these centers convey basic information for the whole septohippocampal system (e.g., acting as a level setter). The chemical nature of these projections is difficult to ascertain. Immunohistochemical data suggest that the ascending projection from the caudal hypothalamus may consist of several components that use different neurotransmitters, such as histamine (Inagaki et al., 1990), cholecystokinin (Reiner and Beinfeld, 1985; Pérez-Clausell and Fredens, 1988), or dopamine (Smeets et al., 1986b). In fact, anterograde tracing of the caudal hypothalamus-septal projection gives rise to anterograde labeling of fibers that show different morphologies, courses, and terminations, within the septum. The projection from the caudal PVH seems to be composed of pericellular nests to the Sa and the rostral Sl plus scattered fibers in the rostral part of the Sl and the Sdl (Fig. 9C), whereas the projection from the mammillary bodies is probably more complex and extensive (Fig. 9D). The only septal nucleus that was devoid of a direct afferent from the tuberal and mammillary hypothalamus was the ventromedial septum (Fig. 11).

Concerning the basal forebrain afferent, the distribution of acetylcholinesterase (Font et al., 1995) and choline acetyltransferase (Hoogland and Vermeulen-VanderZee, 1990; Medina et al., 1993) suggest that this projection can be partially cholinergic. A recent study of the ascending projection from the basal telencephalon to the cerebral cortex in *P. hispanica* (Martínez-Guijarro and Freund, 1992a) demonstrates the GABAergic nature of this projection, and a similar situation might be found for the

projection to the septum, where an important GABAergic innervation is present (Font et al., 1995).

Specific ascending afferents. The results of anterograde and retrograde transport experiments indicate that several diencephalic and mesencephalic centers project specifically to some nuclei of the septal complex. In the thalamus, the DMA is well known to project to the nucleus accumbens, the striatum, and the DVR (González and Russchen, 1988; González et al., 1990; Henselmans, 1992; our unpublished findings). Our results clearly indicate that the DMA also displays a restricted projection to the caudal septum (retrograde tracing) that terminates in the Sm, Sd, Si, and Nppc (anterograde tracing). Tracer injections in the septum never lead to retrograde labeling in the habenular nuclei, contrary to the results of lesion-degeneration experiments by Distel and Ebbesson (1981) in the monitor lizard, *Varanus benegalensis*. Those authors reported an important habenular projection to a region of the septal complex that coincided with the Sm of *P. hispanica*. Our results of retrograde and anterograde labeling suggest that the septal degeneration debris reported by Distel and Ebbesson after habenular lesions may have been due to sectioning of DMA fibers on their way to the septum. However, the presence of interspecific variation or unknown technical problems can also account for this discrepancy.

Belekhova and Nemova (1988) also reported a DLA-septal projection in the lizard *Ophisaurus*. Most of our tracer injections into the septum also gave rise to retrograde labeling in the DLA. However, because the DLA is well known to project to the cortex (Lohman and van Woerden-Verkley, 1978; Bruce and Butler, 1984; Martínez-García and Lorente, 1990), it seems likely that this labeling was due to uptake by passage fibers. Some data support this view: First, only rostral injections into the septum that affect the fornix at its entrance into the cortex show substantial DLA labeling (see Figs. 3, 4). Second, restricted HRP injections into the DLA of the gekko (Martínez-García et al., 1993) clearly indicate that DLA-cortical fibers do not terminate in the septum.

In the hypothalamus, several nuclei project to the septum in addition to the mammillary and premammillary periventricular nuclei discussed above. The medial preoptic nucleus and the lateral preoptic area showed labeled cells after several injections in the septum (Figs. 4–6), but no labeling was found in other injections (Fig. 3). This fact suggests that this area only projects to some septal nuclei. All of the injections that resulted in this kind of retrograde labeling were centered in the ventral aspect of the septum. Moreover, a large injection in the preoptic area (case B9521) showed intense terminal labeling in both parts of the Sd (Sdd and Sdc) as well as in the Svl, thus giving support to the view of a specific preoptic projection to at least these two nuclei.

Another hypothalamic center that was retrogradely labeled after some septal injections was located in the anterior hypothalamus between the lfb and the dorsal tip of the periventricular nucleus. Retrograde labeling was found here only after septal injections involving the Svm. This projection was confirmed by the characteristic terminal labeling (mainly composed of pericellular nests) found in the Svm after injections affecting this anterior hypothalamic nucleus. The Svm of *P. hispanica* shows a dense neuropeptide-Y (NPY)-like immunoreactive innervation (see Fig. 1 in Salom et al., 1994). An NPY-immunoreactive cell group is present in the anterior hypothalamus of several lizards (Medina et al., 1992; our unpublished results), the location of which coincides with that of retrogradely labeled cells after septal injections (present study). Therefore, it seems likely that this anterior hypothalamic-septal projection is NPYergic (Fig. 11E). Because NPY seems to be involved in feeding and sexual behaviors and in the control of reproduction physiology (for review, see Kalra et al., 1988; Danger et al., 1990), the Svm may well be involved in such biological functions.

A third area that displayed retrograde labeling after some septal injections was the lateral hypothalamic area (LHA). The number of LHA labeled neurons was always low when tracer injections were confined to the septum, but they increased (mainly to rostral levels) when the Nac was also involved (case H92100). These results and those from anterograde transport of HRP after injections into the LHA (case H9205) or the preoptic area (case B9521) are congruent with the view that LHA neurons send their axons to the Nac, where they give rise to a terminal arborization, but that some fibers run farther rostrally to give a sparse terminal field in the Sl, Sa, and Svl (dashed arrow, Fig. 11G). Several lines of evidence suggest that this projection could have a substance P(SP)-ergic component. In *P. hispanica*, an important, relatively diffuse, SP-like-immunoreactive septal innervation is found in the rostral septum (Font et al., 1995), and SP-like-immunoreactive cells are found in the LHA of lizards (Petkó and Ihionvien, 1989) and turtles (Reiner et al., 1984; Reiner, 1992). In our material (see Font et al., 1995), the SP-like-immunoreactive fibers innervated the Sl after crossing the dense termination field in the Nac.

Our anterograde and retrograde tracing experiments confirm that the septum also receives afferents from brainstem nuclei, thus extending the findings by Belekhova and Nemova (1988) in the lizard *Ophisaurus apodus*. After our septal injections, retrogradely labeled cells were seen not only in the VTA and raphe nucleus (R) but also in the Sn/RA8 group, the midbrain CG, and the LCo. Immunohistochemical studies in several reptiles (see Smeets, 1988), including *P. hispanica* (our unpublished results), indicate that some of these midbrain nuclei show a dense population of monoaminergic neurons: dopaminergic (VTA, RA8/Sn), serotonergic (R), and noradrenergic (LCo). The presence of axonic nests that are immunoreactive for all three monoaminergic systems around septal cells (Smeets, 1988; Font et al., 1995) strongly suggests that the VTA, R, and LCo give rise to monoaminergic projections to specific septal nuclei.

Our results also indicate the presence of an ascending projection from the midbrain CG that terminates mainly in the Sd and the Si. Although such a projection has not previously been reported in any reptile, both anterograde (case H9268) and retrograde (case H9271) labeling indicated that such a projection does exist in *P. hispanica* and may subserve a feedback control for the descending septal (our unpublished results) and cortical (Hoogland and Vermeulen-VanderZee, 1989a) projection to the same area.

Although, in our septal injections, labeled neurons were not found in nuclei caudal to the LCo, neurons in the caudal medulla or even in the spinal cord may still give rise to a diffuse projection to the septum that was not detected in our tract-tracing experiments. In fact, parvalbumin-like-immunoreactive cells are restricted to the spinal cord in the diurnal geckonid *Phelsuma* (Hoogland,

unpublished observations), and their axons can be followed up to the caudal septum. Moreover, when phenylethanolamine-N-methyltransferase immunohistochemistry is applied to the brain of *P. hispanica*, reactive fibers are seen in the Sdc and the caudal Sl (our unpublished results), whereas the most rostral reactive cell group is found in the ventrolateral medulla (Smeets and Jonker, 1990).

In conclusion, our results indicate that the septal complex of *P. hispanica* can be subdivided into three domains relative to their main afferents. The first one, the central septal division, is composed of several nuclei that occupy the center of the septal complex: Sa, Sl, Sdl, Sm, Svl, and Nppc. It receives a complex, topographically organized cortical input and widespread afferents from the caudal hypothalamus and the basal telencephalon plus some specific inputs from discrete thalamic, hypothalamic, and midbrain centers. Another septal domain, the ventromedial septal division, is composed of the Svm, which receives neither direct cortical input nor ascending projection from the caudal hypothalamus. This septal nucleus seems to be dominated by a specific projection from the anterior hypothalamus (likely to be NPYergic) and by an important serotonergic input (probably arising from the midbrain raphe nucleus; Smeets and Steinbusch, 1988). Retrograde tracing suggests that the Svm receives a direct input from the amygdala. The third septal domain, the midline septal division, is composed of the Si and the Sd. Both nuclei receive a nontopographic input from the cerebral cortex as well as several ascending afferents that include the preoptic hypothalamus, the dorsomedial thalamus, and several midbrain nuclei, such as the CG and some tegmental neurons, which may belong to the Sn/RA8 nucleus.

The differences in their afferent connection patterns suggest that these three septal divisions are involved in different physiological and behavioral processes. For instance, the presence of a specific preoptic projection to the midline septal division suggests that it is involved in sexual behavior (Wade and Crews, 1991) and/or hydrosaline homeostasis (Simerly, 1995).

Comparative overview of the septal afferents

Although studies on the septal afferents in nonmammalian vertebrates are very scarce, the fragmentary data available suggest that the pattern of septal afferents is similar in all tetrapods. A descending projection from the hippocampal complex to the main part of the septum is present in mammals (Swanson and Cowan, 1977), birds (Krayniak and Siegel, 1978b), and amphibians (Neary, 1990; Northcutt and Ronan, 1992). In all cases, the cortical afferent constitutes a massive projection that terminates in a series of septal nuclei that do not project back to the hippocampus. On the contrary, the ventral septum (medial septum of mammals) is nearly devoid of a direct hippocampal influence and displays an ascending projection to the hippocampus.

In mammals, the main hippocamporecipient septal field is called the lateral septum, and it is usually divided into different subnuclei that differ in their relative position, cytoarchitecture (Swanson and Cowan, 1979), and distribution of neurotransmitters and neurotransmitter-related markers (see, e.g., Gall and Moore, 1984). In other tetrapod vertebrates (like birds and amphibians), this septal area receives different names, such as lateral and/or medial septal nuclei (Krayniak and Siegel, 1978b; Neary, 1990; Northcutt and Ronan, 1992). Our anatomical data together with histochemical criteria (Font et al., 1995) indicate that, in lizards, the same hippocamporecipient septal central mass is composed by the Sl, Sdl, Svl, and Sm. In both reptiles (this study) and mammals (Siegel et al., 1974), this hippocamposeptal projection shows a topographical arrangement.

The presence of a rostral septal nucleus in some lizards, known as the Sa, that receives a descending projection from the MC seems to be a specific feature of squamate reptiles. The MC of lizards gives rise to an intracortical zinc-rich projection that is usually compared to the mossy fiber system of the mammalian hippocampus (López-García and Martínez-Guijarro, 1988; Martínez-García and Olucha, 1988; Olucha et al., 1988). On the basis of this evidence and of similarities in cytoarchitecture, hodology, and cellular typology between the reptilian MC and the mammalian fascia dentata, both structures have been proposed to be homologous (see Schwerdtfeger and Germroth, 1990).

However, the mammalian fascia dentata does not project to the septum, whereas the reptilian MC does. Two alternative explanations for this difference can be proposed. On the one hand, a relative expansion of the "mossy fibers" might have occurred during evolution to squamate reptiles, a process that would have been parallelled by an anterodorsal expansion of the "lateral" septum, the Sa, which specifically receives the projection from the MC. In fact, the relative expansion of the Sa found in geckonids is accompanied by an increase in the size of a the part of the MC projecting to it (Hoogland and Vermeulen-VanderZee, 1993). On the other hand, this difference between mammals and reptiles can be explained by refusing the homology between the MC of lizards and the mammalian dentate gyrus. However, there is an important body of evidence to support this homology that relies on histochemical (Dávila et al., 1988, 1991; Pérez-Clausell, 1988; Schwerdtfeger and Lorente, 1988; Martínez-Guijarro et al., 1991; Martínez-Guijarro and Freund, 1992a,b) and hodological (Lohman and van Woerden-Verkley, 1978; Martínez-García and Olucha, 1988; Olucha et al., 1988; Martínez-García et al., 1990; Hoogland and Vermeulen-VanderZee, 1993) criteria as well as on comparison of cellular types (Lacey, 1978; Martínez-Guijarro et al., 1987).

An additional, more restricted hippocamposeptal projection seems to terminate in several midline septal nuclei that are closely associated with fiber bundles. In mammals, the septofimbrial and triangular nuclei receive a distinct hippocampal afferent (Swanson and Cowan, 1979), similar to the lacertilian Sdd and Si. The latter nucleus is associated with the hippocampal commissure, and a projection to a septal midline nucleus closely associated with the hippocampal commissure also seems to be present in birds and amphibians (see references, above). These distinct hippocampal projections apparently lack a topographical organization.

The Svm is a peculiar area of the lacertilian septum that is free of direct cortical influence, although a cortical input to the distal dendrites of its neurons cannot be discarded. Injections into other septal areas indicate that the Svm is only scarcely interconnected with other septal nuclei. The main input to the Svm arises, in fact, from an NPY-immunoreactive cell group of the anterior hypothalamus. Thus, the Svm seems to constitute a peculiar septal nucleus from both a histochemical (Salom et al., 1994; Font et al., 1995) and connectional viewpoint. The role of

this septal nucleus is unknown, although the dense NPYergic afferent suggests that it is involved in some of the functions attributed to this neuropeptide. The Svm also shows an intense serotonergic innervation (see Font et al., 1995). Data on the distribution of NPY, serotonin, and other markers do not show a similar cell group in the rostral septum of other vertebrates. However, our results indicate that a faint amygdaloseptal projection terminates in the Svm. In mammals, a similar projection terminates in the ventralmost part of the lateral septum, which is also innervated by NPYergic fibers (Allen et al., 1984; Staiger and Nürnberger, 1989). Although it is indirect, this evidence suggests that the reptilian Svm may well have a counterpart in a poorly defined area of the rostral and ventral lateral septum of mammals.

With regard to other ascending projections to the septal complex, our results indicate that a widespread afferent to the septum arises from the DB complex and neighboring cells in the basal telencephalon (bed nucleus of the mfb). Such a projection seems to exist in mammals (Staiger and Nürnberg, 1989), in birds (Balthazart et al., 1994), and perhaps in amphibians (Neary, 1990). The location of the Nmfb in the lacertilian basal forebrain and its main neurochemical and hodological features (cholinergic cells: Hoogland and Vermeulen-VanderZee, 1990; Medina et al., 1993; projections to the hippocampal and olfactory cortices: Bruce and Butler, 1984; Martínez-García et al., 1986; Martínez-García and Olucha, 1988; GABAergic nature of at least a part of this ascending projection: Martínez-Guijarro and Freund, 1992a) strongly suggest its homology with the mammalian medial septum/DB complex (for review, see Swanson et al., 1987). Therefore, the important basal forebrain-septal projection that we have found in lizards may represent the medial septum-lateral septum projection found in mammals (Meibach and Siegel, 1977; Staiger and Nürnberger, 1989).

The other widespread afferent to the reptilian septal complex arises from a cell group in the caudal hypothalamus, which comprises the mamillary and supramammillary nuclei, and its rostral continuation in the premammillary hypothalamus. Several studies in mammals (Veazey et al., 1982; Köhler et al., 1985; Shibata, 1987; Staines et al., 1987; Canteras et al., 1992; Vertes, 1992) and birds (Berk and Hawkin, 1985) indicate that a complex and widespread septal afferent arises in both groups from the mammillary bodies. Whereas this projection in mammals arises from the supramammillary nucleus, in birds and lizards, this projection originates from both the supramammillary and the mammillary nuclei. The same is true for the mammilohippocampal projection.

The only thalamic nucleus that projects to the septum of lizards is the DMA. The widespread ascending projection from the reptilian DMA to the DVR, striatum, and nucleus accumbens has been compared to the unspecific projection from the intralaminar nuclei of the mammalian thalamus (Lohman and van Woerden-Verkley, 1978; González et al., 1990). Our finding of a projection from the DMA to the septum does not tie in with this comparison. On topographical and hodological grounds, the reptilian DMA better resembles the mammalian paramedial thalamus (nuclei parataenial, paraventricular, and reuniens). In fact, both the reptilian DMA and the paramedial thalamus of mammals project not only to the septum (reptiles: this study; mammals: Swanson and Cowan, 1975; Herkenham, 1978) but also to the nucleus accumbens and the olfactory tubercle (reptiles: González and Russchen, 1988; mammals: Newman and Winans, 1980a,b) and receive a descending projection from the septum (reptiles: Hoogland et al., 1978; Sligar and Voneida, 1981; our unpublished results; mammals: Meibach and Siegel, 1977; Swanson and Cowan, 1979).

The lack of detailed tract-tracing studies in other vertebrates makes it difficult to compare the afferents to specific septal nuclei of *P. hispanica* with those of other tetrapods. However, immunohistochemical data can be helpful in revealing the innervation of the some septal nuclei by different chemically identified cell groups (see Font et al., 1995). For example, a dopaminergic innervation of a septal area is found in both reptiles and mammals. Our data in lizards strongly suggest that this projection may arise from the VTA (see above). Double-labeling experiments in rats (Swanson, 1982) indicate that about 70% of the VTA neurons projecting to the lateral septal nucleus are dopaminergic but that a part of the dopaminergic innervation of the lateral septum arises from the caudal supramammillary nuclei. As discussed above (see unspecific afferents), a similar situation can be found in lizards, where the supramammillary nuclei also show dopamine-immunoreactive cells (Smeets et al., 1986b; our unpublished results in *P. hispanica*) and project to the septum (see Results). In lizards, the termination area of these putative dopaminergic projections consists of the Sm and neighbouring nuclei of the caudal septum. A patch of tyrosine hydroxylase-immunoreactive fiber nests, around unreactive somata, is found in the caudal aspect of the lateral septum of birds (Bailhache and Balthazart, 1993) and amphibians (González et al., 1993; González and Smeets, 1994), thus suggesting that the presence of a dopaminergic projection from the VTA (and maybe supramammillary nucleus) to the caudal lateral septum is a common feature in tetrapods.

Studies in mammals indicate that other monoaminergic afferents (serotonergic and adrenergic) also arise from midbrain/brainstem cell groups (Köhler et al., 1982; Luiten et al., 1982), as is suggested by the correlation of our connectional studies in lizards and the available data on the distribution of monoamines and their synthetic enzymes (see Smeets, 1988).

Our results together with the data available in the literature suggest that the septal complex of all tetrapod vertebrates studied shows a similar pattern of afferents. Thus, the septum seems to play a similar role within the limbic system of most vertebrates. Detailed studies on the afferent and efferent connections of the septum in representatives of the different vertebrate taxa are needed to shed some light on the roles that the different septal aggregates play on the complex functions of the vertebrate limbic system.

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