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Carmen Díaz[a]
Luis Puelles[b]

[a] Department Cell Biology, University of La Laguna, Tenerife, Canary Islands, Spain;
[b] Department Morphological Sciences, University of Murcia, Murcia, Spain

In vitro HRP-Labeling of the Fasciculus Retroflexus in the Lizard *Gallotia galloti*

Key Words
Fasciculus retroflexus
Habenular nuclei
Nucleus interpeduncularis
Stria medullaris
In vitro labeling

Abstract
In order to test the usefulness of the *in vitro* HRP-labeling technique in the brains of small lizards, the fasciculus retroflexus was labeled in isolated brains of young specimens of the lizard *Gallotia galloti* by means of HRP applied with the tip of a micropipette located approximately midway along the descending course of the tract. Cells in the medial and lateral habenular nuclei were labeled retrogradely. Anterograde transport showed the course of the fasciculus retroflexus, first dorsoventrally into the paramedian prerubral tegmentum and then longitudinally into the isthmic nucleus interpeduncularis. Some fibers spread dorsally into the mesencephalic and isthmic tegmental fields. A substantial bundle continues caudally into nucleus raphe parvocellularis. Other fibers diverge rostralwards from the point of inflexion of the fasciculus, coursing longitudinally in the medial forebrain bundle into the basal telencephalon. Some fibers course rostrally from the nucleus habenularis lateralis in the stria medullaris and could be followed to the dorsal septum.

Introduction

Descriptive studies of the reptilian fasciculus retroflexus established its habenulo-interpeduncular course [Huber and Crosby, 1926; Tamura et al., 1955; Senn, 1968; ten Donkelaar and Niewenhuys, 1979]. Experimental analysis of this fiber system in reptiles is restricted to the study of Distel and Ebbesson [1981] in the monitor lizard, in which degeneration techniques were applied. These authors found a variety of habenular projections, closely resembling those reported by Herkenham and Nauta [1979] in rats and Kemali and colleagues [Kemali et al., 1980; Kemali and Guglielmotti, 1982; Kemali and Lázár, 1985] in frogs. The availability of these data suggested the choice of the habenulo-interpeduncular system in order to test the usefulness of *in vitro* HRP-labeling for the study of structures that are not easily approachable *in vivo* in lizard brains. A number of such *in vitro* experiments were performed, employing the lizard *Gallotia galloti*.

Luis Puelles
Dept. Morphological Sciences
Fac. Medicine
30100 Murcia (Spain)

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Materials and Methods

Animal care guidelines established by Spanish Royal Decree 223/1988 were followed. Chloroform-anesthetized lizards were briefly perfused intracardially with Tyrode solution, until the vascular system was washed out. The brains were quickly removed, freed of meninges, and sectioned through the midline. The fasciculus retroflexus could be visualized through the ventricular lining under appropriate illumination (green filter). A micropipette whose tip held dried HRP was aimed approximately at the midpoint of its dorsoventral course (fig. 1A). Either total or partial sectioning of the tract was achieved in 20 cases. Control labeling experiments were performed, depositing HRP within the interpeduncular nucleus or the habenular complex. The whole brain half was thereafter kept *in vitro* at room temperature for 12–24 h (in a few later cases for 36 h), submerged in Gibco Minimal Essential Medium, supplemented with penicilline, through which an oxygenated gas mixture (95% O_2, 5% CO_2) was continuously bubbled. Fixation of the pieces occurred in cold 1% glutaraldehyde in 0.1 *M*, pH 7.4 phosphate buffer (3–4 h). They were cryoprotected in 30% sucrose in the same buffer (overnight, or longer), and frozen sagittal sections (60 µm thick) were mounted on gelatine-pretreated slides, air dried and kept in cold buffer until the histochemical reaction was performed. A slight modification of the nickel-cobalt-diaminobenzidine procedure [Adams, 1981] was employed, by the addition of 1 drop of dimethylsulfoxide per 100 ml of incubation solution. The sections were counterstained with cresyl violet.

List of Abbreviations

ATV	area tegmentalis ventralis
ca	commissura anterior
cp	commissura posterior
DM	nucleus dorsomedialis thalami
ET	nucleus eminentiae thalami
fr	fasciculus retroflexus
HA	hypothalamus anterior
Ip	isthmic nucleus interpeduncularis
LHb	lateral habenular nucleus
mfb	medial forebrain bundle
MHb	medial habenular nucleus
R	nucleus rotundus
Rp	nucleus raphe superior
S	septum
SD	nucleus septalis dorsalis
sm	stria medullaris
SN	substantia nigra
Teg	tegmental area

Results

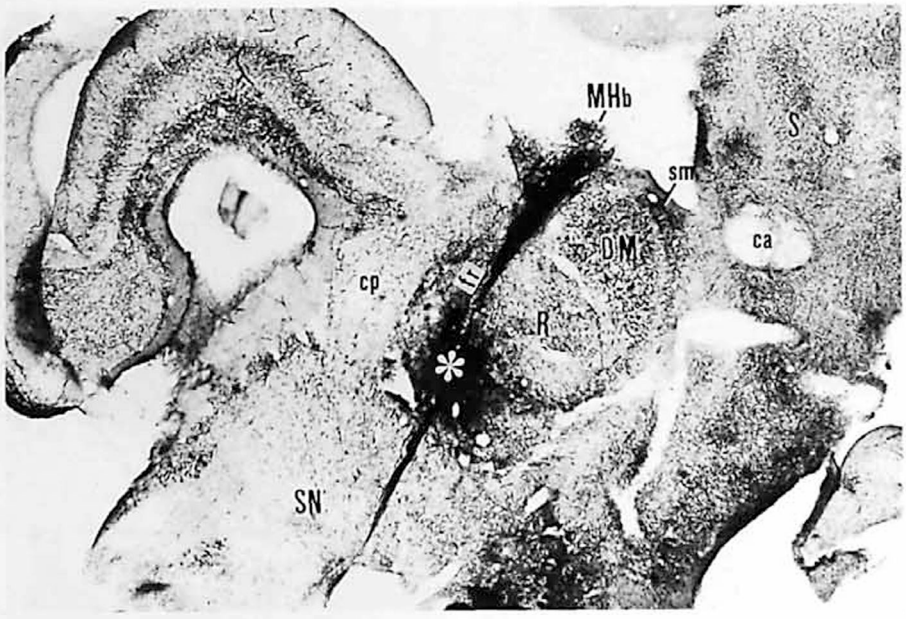
The proportion of fibers of the tract that absorbed the label could easily be ascertained by analysis of retrograde label in the habenular region. Partial labeling showed a variable number of HRP-filled cells in the medial habenular nucleus (MHb), with minor labeling of the lateral habenular nucleus (LHb). Total labeling marked the whole population of MHb and LHb (fig. 2A). The habenular commissure was never stained.

In cases of massive retrograde habenular labeling, thin fibers were observed coursing periventricularly from the habenular region into the thalamic dorsomedial nucleus, forming there a thin pericellular plexus (fig. 1B). Other fibers course rostrally in the stria medullaris (fig. 1B). Although a few of these arise from isolated labeled cells within the nucleus eminentiae thalami, most fibers pass through this nucleus, diverging at the level of the commissura pallialis anterior (fig. 2B). A few fibers descend into the anterior hypothalamus, and a sparse bundle ends in the nucleus septalis dorsalis (fig. 1B).

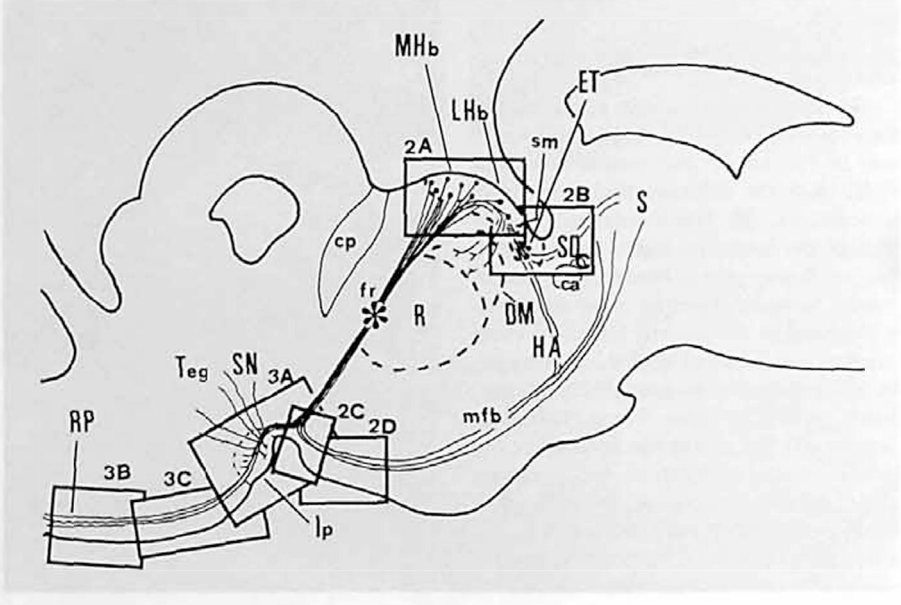
The main dorsoventral bundle of labeled fibers, constituting the fasciculus retroflexus, could be followed in all cases straight along the limit between dorsal thalamus and pretectum to the paramedian floor region (fig. 1). At this point, most fibers bend in a right angle, following a paramedian longitudinal course along the floor of pretectum and mesencephalon (fig. 1B, 3A) until they reach the isthmic region. Along this segment of the tract, collaterals of the fasciculus retroflexus densely innervate the whole area tegmentalis ventralis, which lies medial and rostromedial to the oculomotor nerve roots. In one whole-brain specimen sectioned horizontally, some fibers were seen to cross the midline here, ending sparsely within the contralateral area tegmentalis ventralis and adjacent posterior hypothalamus (fig. 3D). A few retrogradely labeled small neurons appeared in the ipsilateral area tegmentalis ventralis.

In all cases, the ipsilateral nucleus interpeduncularis was seen to receive anterogradely labeled fibers (fig. 3A, C). Fibers also cross to the other side, as observed in a control experiment in which HRP was placed in the habenular complex of a brain that was not sectioned into halves. Many fibers run more caudally, along the nucleus raphe parvocellularis (fig. 3B), practically down to the level marked by the root of the abducens nerve. These fibers were seen even in cases of partial labeling of the tract.

Massive labeling showed additional fibers that descend slightly more laterally and diverge at the angle between the vertical and longitudinal portions of the fasciculus retroflexus into either descending or ascending components (fig. 1B, 2C). Descending fibers are coarser than those ending within the nucleus interpeduncularis and diverge from the tract along its longitudinal course, entering nucleus raphe superior of the isthmus and adjacent tegmentum, including the neuropile of the substantia nigra (fig. 3).



A



B

Fig. 1. Photomicrograph (×30) (**A**) of the fasciculus retroflexus in sagittal section, showing the HRP injection site (asterisk) at the approximate midpoint of its dorsoventral course, and drawing (**B**) summarizing anterograde and retrograde transport after HRP injection. Framed zones represent areas shown in figures 2 and 3.

Ascending fibers diverge rostrally into the medial forebrain bundle (fig. 2C, D). They become sparser as they pass the anterior hypothalamus and preoptic region, but some of them could be followed into the septum (fig. 1B).

In two control cases with label placed in the interpeduncular nucleus (some label spread also into the nearby tegmentum), retrograde labeling of the medial and lateral habenular nuclei was not accompanied by any anterograde transport into the dorsomedial thalamic nucleus, nucleus eminentiae thalami or septum, even though ascending components of the medial forebrain bundle transported label as far as the area surrounding the anterior commissure. In two other control cases, injected in the interpeduncular nucleus, and with longer *in vitro* periods (36 h), a few

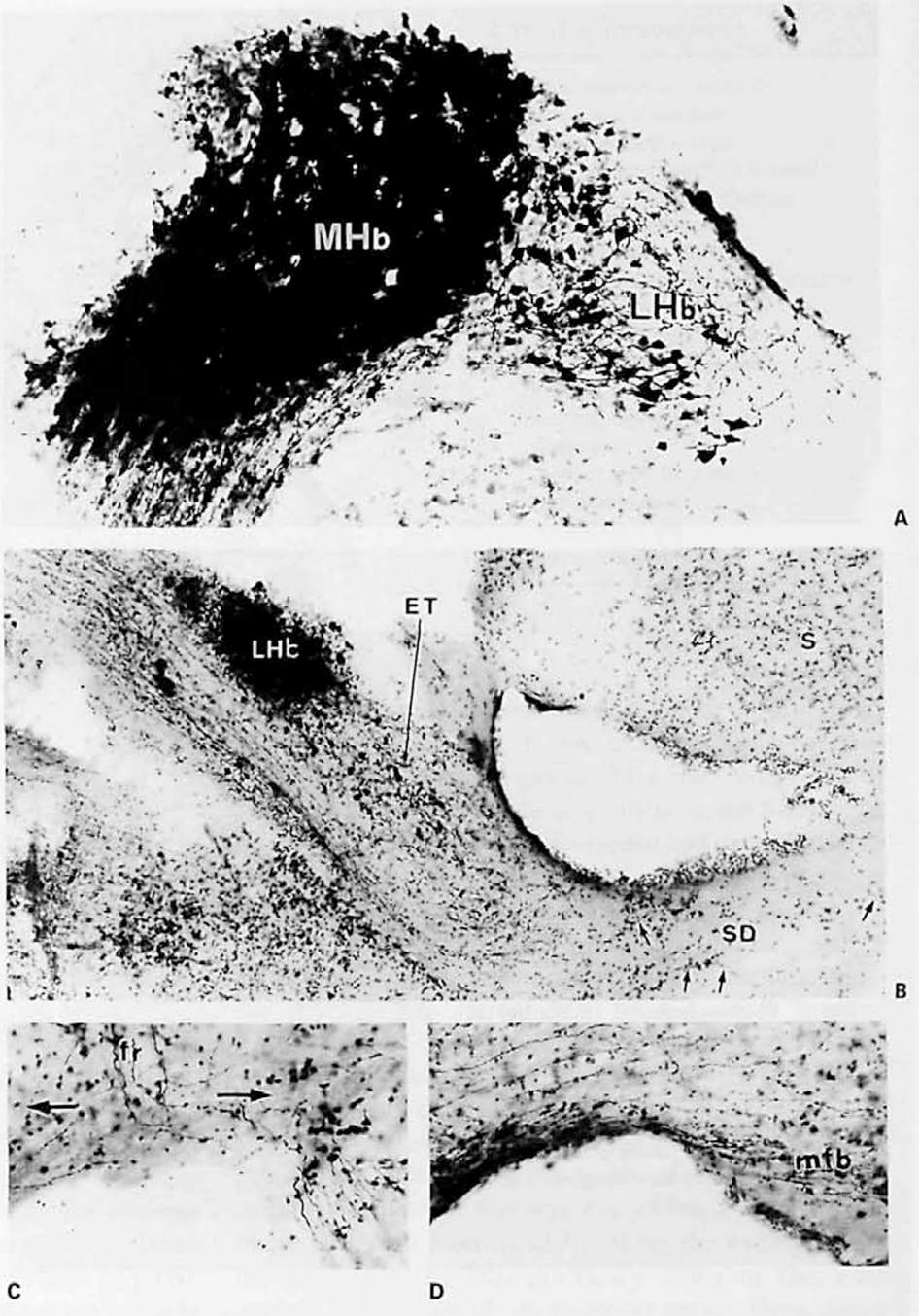


Fig. 2. Photomicrographs of sagittal sections showing (**A**) retrogradely labeled neurons in the lateral and medial habenular nuclei; note the different label intensity in each nucleus. (**B**) Fibers coursing rostrally through the habenular region at the dorsal thalamic-telencephalon border, in a case of massive habenular labeling; a few fibers can be followed to the nucleus septalis dorsalis (arrows), and scattered labeled cells are present in the nucleus eminentiae thalami, immediately rostral to the lateral habenular nucleus. (**C**) The encephalic floor where the vertical, lateral portion of the massively labeled fasciculus retroflexus bifurcates into an ascending component and a descending component. (**D**) Fibers of the ascending component coursing longitudinally in the medial forebrain bundle. **A** = × 155; **B**, **C** = × 90; **D** = × 135.

neurons appeared labeled retrogradely in the lateral preoptic/anterior hypothalamic area. Their axons were seen to course through the stria medullaris and the fasciculus retroflexus. Control injections into the habenular nuclei showed a number of telencephalic afferents, including the nucleus of the posterior pallial commissure and nucleus septalis impar. A full description of the afferents to the habenular complex is reported separately [Díaz and Puelles, 1992].

Discussion

The findings of Herkenham and Nauta [1979] in a mammal *(Rattus rattus)*, and those of Distel and Ebbesson [1981] in a lizard *(Varanus benegalensis)*, largely agree with our findings and serve to explain the double-component labeling pattern of fasciculus retroflexus in our experiments. These authors established the existence of a core

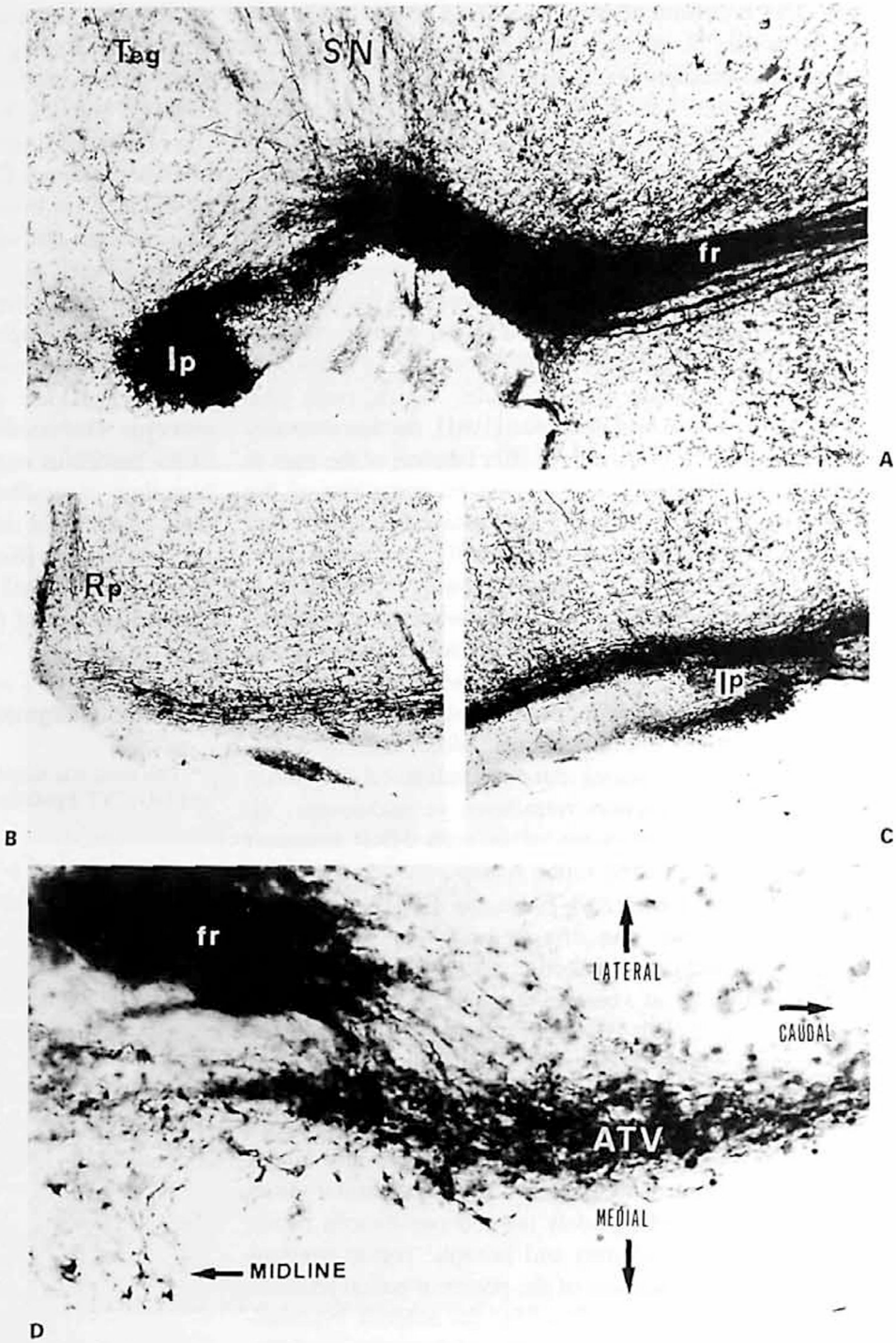


Fig. 3. Photomicrographs showing the course of the descending longitudinal component of the fasciculus retroflexus (at the paramedian ventral tegmental region). Many of the fibers end in the nucleus interpeduncularis (**A**, **C**), whereas others course dorsally into the substantia nigra (**A**) or caudally along the nucleus raphe parvocellularis (**B**). The pattern of ipsilateral and contralateral projections on the area ventralis tegmentalis is shown in a different case (**D**), sectioned horizontally after HRP application within the habenular complex. **A** = ×84; **B** = ×63; **C** = ×125; **D** = ×335.

portion of the tract, consisting of the axons of MHb neurons that project into nucleus interpeduncularis and nucleus raphe parvocellularis. A peripheral, lateral portion of the tract conducts the fibers of LHb neurons projecting into midbrain (nigral) and isthmic tegmental targets, as well as the ascending septopetal fibers that enter the medial forebrain bundle. Since our cases were labeled from the ventricular lining outwards, it may be assumed that those experiments in which the fasciculus retroflexus was only partially sectioned involved mostly the medial core por-

tion. This is consistent with the labeled cells in MHb and the terminals in nucleus interpeduncularis and nucleus raphe parvocellularis in all our cases.

Only massive, total labeling cases affected the core plus the peripheral portion of fasciculus retroflexus. This correlates with the additional labeling of tegmental descending terminals and hypothalamic, preoptic and septal ascending fibers. We observed relatively fewer fibers in the septum than did Distel and Ebbesson [1981]. This is probably due to a limited range of anterograde transport in the time-limited *in vitro* conditions employed in our study.

As regards the fibers coursing from the LHb nucleus directly into nucleus dorsomedialis, which were also described by Distel and Ebbesson [1981], the fact that they appear also in our preparations after labeling of the tract at mid-course indicates that they may be collaterals of the fibers interrupted at the lesion, which have become labeled retrogradely. The alternative possibility, that afferents to nucleus dorsomedialis from another source ascend through this tract, should not be ignored. However, we observed only a few retrogradely labeled cells within the area tegmentalis ventralis. Control injections within nucleus interpeduncularis elicited retrograde transport within nearby raphe populations but no terminals within nucleus dorsomedialis. The raphe nuclei remained unlabeled after interruption of the fasciculus retroflexus at mid-course, although the observed range of *in vitro* HRP transport encompassed the rostral raphe region.

Contrary to Distel and Ebbesson [1981], we found labeled fibers within the stria medullaris and even some labeled cells within its interstitial nucleus eminentiae thalami (the dorsalmost ventral thalamic cell mass, immediately rostral to LHb). We could follow these fibers to the anterior hypothalamus, or to nucleus septalis dorsalis, without seeing retrogradely labeled cells at either locus. Anterior hypothalamic cells were seen in two additional control cases with interpeduncular nucleus injections and longer time *in vitro*. Control injections within the habenular nuclei revealed various retrogradely labeled populations within the anterior hypothalamus and preoptic region, nucleus septalis impar and nucleus of the posterior pallial commissure. This suggests that cells within the anterior hypothalamus and septum may be the source of these stria medullaris fibers. As in mammals [Herkenham and Nauta, 1977], these fibers may bypass the habenular complex, coursing thereafter through the fasciculus retroflexus. The more limited range of retrograde transport in our other preparations may have impeded the label from reaching cell bodies in these locations.

These experiments demonstrate the usefulness of the in vitro HRP labeling procedure for studying connections in small lizard brains, within a given range of transport obtained after 24–36 h. We should note in this respect that the maximum time of adequate survival *in vitro* has not been determined. Our objective was to test the technique in young lizard brains, in order to subsequently apply the procedure to embryonic specimens, which obviously can not be labeled *in vivo*. We therefore have not tried still longer survival times. The *in vitro* HRP-labeling technique has also been employed successfully by Kriegstein et al. [1988] for short-range retrograde labeling of neurons in 300–400-μm-thick cortex slices of *Pseudemys scripta* embryos. Our results on the long-range projection pattern of the fasciculus retroflexus reveal the essential hodologic homology of reptilian and mammalian retroflexus fiber systems. Additional data on habenulo-interpeduncular connections in frogs [Kemali et al., 1980; Kemali and Guglielmotti, 1981; Kemali and Làzàr, 1985] confirm the conservative structure of this neuronal system.

Acknowledgments

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