Cobalt Injected Into the Right and Left Fasciculi Retroflexes Clarifies the Organization of This Pathway

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ABSTRACT

A cobaltic lysine complex was injected separately into the right and left fasciculi retroflexes of the frog. This tracing technique labeled, in a Golgi-like manner, the neurons which initiate the fasciculi retroflexes and revealed details of their morphological pattern. The fasciculi retroflexes originate from various neurons distributed in the diencephalon and mesencephalon, but their main source is the habenular nuclei. In the frog they are dorsal and ventral habenulae which are homologous to the medial and lateral habenulae, respectively, of mammals. In the frog the dorsal habenulae are strikingly asymmetric.

Our study shows that the fasciculus retroflexus is composed of several compact separated bundles of fibers and that the fibers originating from the dorsal habenular nuclei project to the interpeduncular nucleus, while those originating from the ventral habenular nuclei project beyond the interpeduncular nucleus, but so far we have not been able to show the exact site of their termination.

The labeling of cells within the interpeduncular nucleus raises the possibility that the habenulo-interpeduncular tract is reciprocal in function. The findings support our previously reported hypothesis on the theoretical interpretation of the functional circuitry of the frog habenulo-interpeduncular system.

Key words: fasciculus retroflexus, habenulae, asymmetry, interpeduncular nucleus, cobalt tracer, frog

The fasciculus retroflexus is a complex bundle of fibers which connects the habenulae to various cerebral structures. According to Herkenham and Nauta ('79), the fibers confined to the core portion of the fasciculus retroflexus in the rat connect the medial habenula to the interpeduncular nucleus and constitute the habenulo-interpeduncular tract, while those confined to the mantle portion of the fasciculus retroflexus connect the lateral habenula mainly to the tectal, reticular formation, the raphe nuclei, and the substantia nigra.

The neuroanatomy of the habenulo-interpeduncular tract is peculiar in that the right and left tracts from the paired habenular nuclei in the epithalamus to the interpeduncular nucleus of the tegmentum mesencephali traverse this midline nucleus more than once in a zigzag wiring pattern illustrated by Cajal ('11) before terminating (Mizuno and Nakamura, '74; Leranth et al., '75; Lenn, '76; Herkenham and Nauta, '79).

Most of the literature refers to the habenular connections of mammals. In the present paper we studied the habenular connections of an amphibian, the frog Rana esculenta, after injection of cobalt into the fasciculus retroflexus. In this anuran there is one dorsal and one ventral habenula, one above the other, on each side of the epithalamus (Fig. 1a), corresponding to the medial and lateral habenular nuclei of mammals, respectively (Beccari, '43). The dorsal habenular nuclei of the frog are strikingly asymmetrical, the left one being more lobated than the right (Kemali and Braithberg, '69).

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A preliminary report of the present paper was presented at the 7th European Neuroscience Association Meeting, Hamburg (Germany), September 1983.
In previous studies using horseradish peroxidase (HRP) as a tracer (Kemali et al., '80; Kemali and Guglielotti, '82), we tried to determine whether the morphological asymmetry of the habenulae was matched by an asymmetry of their connections. HRP injected into the interpeduncular nucleus labeled neurons in the right dorsal habenula and in both portions of the left dorsal habenula, even after transection of the habenular commissure, while no labeled cells were observed in the ventral habenula (Kemali and Guglielotti, '82). Because of the small size and position of the habenular nuclei, it was not possible to independently trace with HRP the projections of the dorsal and ventral habenulae, or the two portions of the left habenula (Kemali et al., '80).

In the present study we circumvented this problem by injecting a cobaltic lysine complex (a compound which has been found to greatly improve cobalt labeling in a Golgi-like manner; see Görges et al., '79) separately into the fasciculus retroflexus of the two sides of the frog brain.

MATERIALS AND METHODS

Thirteen frogs (Rana esculenta) were used. The animals were anaesthetized with urethane (0.2 gm/100 gm body weight). The skull was opened to expose the dorsal surface of the brain. Cobaltic-lysine complex was injected iontophoretically with the aid of a micropipette into the left or right fasciculus retroflexus at the point of crossing of the coordinates Y = 2.5 and X = 1 (Kemali and Braithenberg, '80), i.e., in the middle third of the fasciculus retroflexus.

The inner diameter of the pipette was 40–50 μm, and 1 μAmp constant anodic current was passed for 25–30 minutes. The depth of the injection varied from 400 μm to 800 μm.

The site of the injection is illustrated in Figures 1e and f, which are photographs of a sagittal and a horizontal brain section respectively.

After a 24-hour survival time the animals were decapitated and the brains were processed according to the technique described by Lázár et al. (83). Serial sections 60 μm thick were cut in the horizontal, frontal, and sagittal planes.

RESULTS

The cobaltic-lysine complex injected into the fasciculus retroflexus filled the cells of origin of this pathway in a Golgi-like manner. Most of the fibers were found to originate from the habenulae and project to the interpeduncular nucleus and to regions beyond this nucleus. We were not able to trace the terminals beyond the interpeduncular nucleus.

The injection site in a sagittal section is shown in Figure 1e, and a horizontal section in Figure 1f.

Following cobalt injections we found filled cells or axons in the structures listed below.

Habenular nuclei

Both the dorsal and ventral habenular nuclei were labeled by cobalt; the asymmetric labeling of the neurons of the dorsal habenula reflects the morphological asymmetry of these structures (Fig. 1a). The left dorsal habenula is composed of a lateral and a medial portion, while the right one was a single nucleus. The different distribution of cells in the dorsal and ventral habenula is also visible in Figure 1a. The cells of the dorsal habenula form a shell around a neuropil, while the cells of the ventral habenula are distributed throughout the nucleus.

Cobalt injected into the left fasciculus retroflexus labeled cells in the left ventral habenula and the lateral portion of the left dorsal habenula (large and small arrows, respectively, in Fig. 1b). Cobalt injected into the right fasciculus retroflexus labeled neurons in the right dorsal habenula (Fig. 1c) and the right ventral habenula (Fig. 5c).

Cobalt-filled neurons of the dorsal habenula have slightly pyriform perikarya with dimensions of 12–13 × 14–15 μm. They have a single, short, stout dendrite which generally points toward the central neuropil (Figs. 1d and 2a). There are, however, several cells which have dendrites with a rectangular or hairpin-like bend parallel to the row of perikarya forming the shell of the habenula (Fig. 2b). In some cases the whole neuron had this orientation (Fig. 3a).

The peripheral part of the main dendrite is usually tortuous, and the terminal branches form glomerulus-like structures (Fig. 3a). Between the dendrites, several fine fibers, probably afferent, can be observed.

We did not find any difference in the morphology of neurons on the two sides of the dorsal habenula. There is, however, some difference in the orientation of dendrites in the lateral portion of the left habenula relative to the right habenula. The dendrites of some cells of the border separating the two subnuclei are directed toward the medial portion (Fig. 3b).

Neurons of the dorsal habenula have thin axons which originate from the perikaryon opposite to the dendritic trunk (Fig. 4, 4a–c). The arrangement of axons to form the fasciculus retroflexus is best seen in sagittal sections (Fig. 4c).

Cobalt-filled neurons of the ventral habenula are multipolar. Some of the cells have multiglomerular perikarya (Fig. 5a) with four or five main dendrites, which divide dichotomously into a few thin beaded terminal branches. Other neurons have round or pyriform perikarya with one to three thick dendrites (Figs. 5b and 5d). The axons originate from perikaryon and merge into a compact bundle (Fig. 5c).

In the habenular commissure, a few fibers were filled with cobalt. These fibers appear to contact the epiphysis (pineal) which is located above the commissure (Fig. 5e). In the same figure the arrow indicates a cobalt-filled cell with very fine, short processes within the habenular commissure.

Extra-habenular structures

In cases of larger cobalt injections, several cells were labeled among the periventricular cells of the thalamus. Some of them were located close to the third ventricle. These cells have spherical perikarya and a short dendritic

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Fig. 1. III, third ventricle. a. Frontal section of the epithalamos of the frog at the level of the dorsal (D) and ventral (V) habenulae. The asymmetry of the dorsal habenula is evident since only the left one is subdivided in a medial (M) and a lateral (L) portion. F. epiphysis. ×180. b. Cobalt-labeled cells in the left habenula after injection of cobalt into the left fasciculus retroflexus. The large and small arrows indicate labeled cells in the left ventral habenula and in the lateral portion of the left dorsal habenula, respectively. Horizontal section. ×100. c. Cobalt-labeled cells in the right habenula after injection of cobalt into the right fasciculus retroflexus. Horizontal section. ×100. d. Cobalt-labeled cells in the right dorsal habenula showing the processes of their neurons directed toward the neuropil. Horizontal section. ×540. e. Site of the cobalt injection (arrow) in a sagittal section. ×25. f. Site of the cobalt injection (arrow) on the left side of a retroflexus section. The lateral portion (L) of the dorsal habenula and the ventral (V) habenula show cobalt-labeled cells. The medial (M) portion of the dorsal habenula is devoid of cobalt-labeled cells. ×80.
Figure 1
Fig. 2. a. Cobalt-labeled neurons of dorsal left habenula after injection of cobalt into the left fasciculus retroflexus showing details of their cellular morphology. x530. b. Cobalt-labeled neurons in the right dorsal habenula with sharply bending dendrites. x530.
Fig. 3. a. Neurons in the lateral portion of the left habenula after cobalt injection into the left fasciculus retroflexus. The arrows point to the tip of the short dendritic arborization ending in a glomerulus-like fashion. Frontal section, ×600. b. The dorsal left habenula showing cobalt-labeled cells in the lateral portion after injection of cobalt into the left fasciculus retroflexus. The arrows point to some dendrites of the lateral portion (L) directed toward the neuropil of the medial portion (M). Frontal section, ×800.
Fig. 4. a and b are cobalt pyriform neurons of the dorsal habenula. Note the slender axon (arrows) arising from the pole of the cell body. Sagittal section. ×1,500. c. Group of cobalt-labeled neurons of the dorsal habenula from which the axons collect in a bundle to form a portion of the fasciculus retroflexus. Sagittal section. ×600.
Fig. 5.  a. Multipolar isolated neuron of the ventral habenula labeled by cobalt. Frontal section. ×500. b. Cobalt-labeled isolated neuron of ventral habenula showing two thick dendrites and a slender axon (arrow). Sagittal section. ×500. c. Cobalt-labeled neurons of the ventral habenula giving rise to a bundle of fibers of the fasciculus retroflexus. Horizontal section. ×500.  

d. Group of neurons of the ventral habenula labeled by cobalt. Horizontal section. ×440.  e. Cobalt-labeled fibers directed to the epiphysis or pineal (E). The arrow points to a cobalt-labeled cell with fine processes lying at the base of the epiphysis. Horizontal section. ×390.
Fig. 6. a. Horizontal section of the interpeduncular nucleus showing a fiber filled by cobalt injected into the fasciculus retroflexus. The fiber forms a loop from which various collaterals emerge (arrows). x is the middle of the nucleus. ×500. b. Frontal section of the interpeduncular nucleus showing a fiber labeled by cobalt injected into the fasciculus retroflexus which bifurcates and surrounds a blood vessel (arrow). x is the middle of the nucleus. ×500. c. Frontal section of the diencephalon showing cobalt-labeled (arrow) bundles of fiber following injection of cobalt into the left fasciculus retroflexus. ×30. The inset shows a magnification (×250) of the fasciculus retroflexus bundles. d, e. Two levels of the interpeduncular nucleus cut in frontal section. The fibers which traverse the nucleus are labeled by cobalt injected into the left fasciculus retroflexus. In d the arrow indicates a labeled neuron whose long process is directed toward the interpeduncular nucleus. ×80. f. Horizontal section of cobalt labeled neuron within the interpeduncular nucleus. ×800.
Fig. 7. a. Frontal section of the interpeduncular nucleus traversed by cobalt-labeled fibers. The arrows indicate two neurons with their processes directed perpendicularly to the direction of the fibers. ×450. b. Frontal section of the interpeduncular nucleus traversed by cobalt-labeled fibers injected into the fasciculus retroflexus of one side. The arrows indicate a fiber with several varicosities. ×450.
trunk pointing laterally. Secondary dendrites are arranged parallel to the ventricular wall. More laterally pyramidal neurons could be observed. Such neurons with widely arborizing basal dendrites may occur in the periaqueductal region of the mesencephalon.

**Interpeduncular nucleus**

The main target of the fasciculus retroflexus is the interpeduncular nucleus. Before ending in this midline nucleus or proceeding caudally to the rhombencephalic tegmentum, most of the fibers of the fasciculus retroflexus of each side cross the interpeduncular nucleus several times from one edge to the other (Ramon y Cajal, '11). Two of the fibers in the peculiar zigzag loop within the interpeduncular nucleus are shown in Figure 6a and 6b. In Figure 6a, fine beaded collaterals directed toward the central part of the nucleus were also well filled. In Figure 6b, two branches of a fiber embrace one of the large blood vessels that passes the lateral edge of the interpeduncular nucleus perpendicularly to join the subpial blood vessels on the ventral surface of the brain. The terminals of the fasciculus retroflexus fibers form a rather dense plexus in the interpeduncular nucleus, which can be recognized even under low power (Fig. 6e).

Close to the interpeduncular nucleus in the periaqueductal gray, a few neurons which send processes to the interpeduncular nucleus were filled (Fig. 6d). Along the course of the fasciculus retroflexus, fibers merge into small compact bundles (Fig. 6c, insert). They are shown in Figure 6c passing through the hypothalamus to reach the tegmentum.

Cobalt injected into the fasciculus retroflexus also labeled some neurons in the interpeduncular nucleus. One such cell is illustrated in Figure 6f at high magnification, while Figure 7a, a transverse view of the interpeduncular nucleus at low magnification, faintly shows cobalt-filled cells with their processes oriented perpendicularly to the afferent fibers of the interpeduncular nucleus.

The fibers of the fasciculus retroflexus within the interpeduncular nucleus are of different size and show varicosities along their path (Fig. 7b).

**DISCUSSION**

From our cobalt-lysine study it is clear that in the frog the main site of origin of the fasciculus retroflexus is the cells in the dorsal and ventral habenular nuclei, and the main target is the interpeduncular nucleus. Its advantage as a method is in the fine detail with which it reveals such structures as the thin axons of the dorsal habenula, the fine collaterals of the fasciculus retroflexus, and the glomerular-like endings of the short dendrites of the habenular bipolar cells. Since it also labels fibers terminating in the interpeduncular nucleus, it appears that the cobalt in the habenulo-interpeduncular tract is transported both retrogradely and anterogradely.

The method also indicates that the medial portion of the left dorsal habenula, next to the third ventricle, does not contribute to the formation of the left fasciculus retroflexus, for its cells are not filled when the fasciculus on this side is injected.

This disagrees with the results using horseradish peroxidase in which such an asymmetry of the fasciculus retroflexus was not apparent (Kemali and Guglielmotti, '82). It is probable that cells of the medial border of the lateral habenula send their dendrites to the neuropil of the medial habenula, giving the impression that HRP labels this portion of the left dorsal habenula.

In addition, the injection of cobalt into the right and left fasciculi retroflexus labeled cells ipsilaterally in the ventral habenula, whereas these nuclei were not labeled by HRP injected into the frog interpeduncular nucleus (Kemali and Guglielmotti, '82). We deduce from the cobalt injections that the bundle of fibers originating in the ventral habenula runs in the fasciculus retroflexus together with the other bundle of fibers originating in the dorsal habenula, and they subsequently divide to their separate destinations. We have seen some fibers which run caudally to the interpeduncular nucleus, but we were unable to follow their further path within the brain stem.

As the fasciculus retroflexus injections labeled a few neurons in the interpeduncular nucleus as well as the habenula, the tract appears to project in both directions.

The interpretation of the nature of neurons labeled in extra-habenular structures is not easy. In the case of larger injections, ascending mesencephalic fibers may have been damaged or intrinsic thalamic axons spontaneously may have picked up cobalt. Some extra-habenular cells, however, may belong to the habenulo-interpeduncular system because HRP injections into the interpeduncular nucleus also labeled such cells (Kemali and Guglielmotti, '82).

The fasciculus retroflexus fibers which traverse the interpeduncular nucleus are very clearly labeled by cobalt with their delicate collaterals arising from the concavity of the loops. They may represent an amplification device of the habenulo-interpeduncular system. Some of the fibers which cross the interpeduncular nucleus show varicosities, while others are in close contact with blood vessels. Electron microscopic evidence led one of us to suggest that a portion of the interpeduncular nucleus is neurosecretory (Kemali, '77), and this possibility applies also to the interpeduncular nucleus of man. The Golgi method (Kemali and Casale, '82) revealed cells in this portion which have varicose processes contacting the numerous blood vessels located on the subpial ventral surface of the interpeduncular nucleus.

The connection between the interpeduncular nucleus and the epiphysis (or pineal) agrees with the HRP studies of Kemali and Guglielmotti ('82) and provides a route by which the habenulo-interpeduncular system can be influenced by environmental light cycles through the photoreceptors of the frontal organ (see Kemali and De Santis, '83).

In a previous study (Kemali, '79), it was postulated that through the peculiar decussation of the fibers of the fasciculus retroflexus, a stimulus from only one side of the brain is sufficient to produce a massive output from the entire inter peduncular nucleus and might influence simultaneously and bilaterally the caudal motor structures which control midline organs such as the tongue. The cobalt picture of the habenulo-interpeduncular complex agrees with the architectural reconstruction of this system illustrated in that paper on the frog and supports our previous hypothesis regarding the morphological relationship established by this complex between the right and left portions of the frog brain.

**LITERATURE CITED**


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