The infrared-sensitive pit organs of pit vipers (subfamily Crotalinae) and boids (family Boidae) are innervated by branches of the trigeminal nerve (Lynn, '31; Bullock and Diecke, '56; Bullock and Barrett, '68; Molenaar, '78b). These branches project to the ipsilateral medullary nucleus of the lateral descending trigeminal tract (LTTD) (Molenaar, '74, '78a; Schroeder and Loop, '76). The LTTD in turn is connected to the optic tectum; infrared-driven activity can be recorded in the tecta of both snake families (Hartline, '72, '74; Goris and Terashima, '73; Terashima and Goris, '75; Haseltine, '78).

In a previous horseradish peroxidase (HRP) study of the pit viper (Crotalus viridis) (Gruberg et al., '79a), we found that only one circumscribed nucleus in the medulla, the nucleus reticularis caloris (RC), projects to the tectum. We proposed that the RC serves as an intermediate link between the LTTD and the tectum in this species. This hypothesis is supported by the work of Stanford and Schroeder ('79), who used degeneration techniques to demonstrate that the LTTD projects to the RC but not to the tectum in the rattlesnake. This indirect projection differs from the scheme proposed by Molenaar for boids. His degeneration study of the python (Molenaar and Fizaan-Oostveen, '78) indicated that the LTTD projects directly to the contralateral tectum in this species.

We now report the results of several additional anatomical and histochemical experiments, which define the similarities and differences between the LTTD-tectal pathways in these two groups of snakes. The results support our earlier proposal that the RC functions as a link between the LTTD and the tectum in the rattlesnake. In addition, we have recorded the electrical activity of single RC units to determine their infrared sensitivity. We have also injected HRP into the tectum of the python to study the infrared projection in this species. Preliminary results of this work have appeared elsewhere (Newman et al., '79; Gruberg et al., '79b).
METHODS

Pacific Rattlesnakes (Crotalus viridis) 40–120 cm in length were obtained from Western Zoological Supply, Monrovia, Ca. Reticulated Pythons (P. reticulatus) 80–140 cm in length were obtained from Stoneham Aquarium Supply, Stoneham, Mass. Both species were maintained at 27°C in a 12 hour-12 hour light-dark cycle.

Surgical procedures

All surgical procedures were performed under Metofane (Methoxyfluorane) anesthesia (Pitman-Moore, Inc.). The RC of the rattlesnake was approached by removing the inner ear structures and exposing the dorso-lateral medulla at the level of the VIII nerve. Snakes were cooled by packing them in ice to minimize bleeding. Once the dorsolateral medulla was exposed and the meningial layers had been removed, the RC could be reliably located from surface landmarks. The nucleus lay approximately 2 mm directly below the surface in an area just caudal to the entry of the VIII nerve root.

The tectum of one python was exposed by removing the overlying skull using a dental drill. Two meningial membranes covering the tectum were removed.

Horseradish peroxidase injections

HRP was injected into the RC of five rattlesnakes and the tectum of a single python. Both iontophoresis and pressure injection of HRP was used for rattlesnake injections. The iontophoresis method is described elsewhere (Gruberg et al., '79a). HRP was pressure-injected from micropipettes (2–5 μm tip diameters), with pressure applied to a syringe connected to the back of the pipette. In both methods, a freshly prepared, 25% solution of HRP (sigma type VI) in 0.05M Tris buffer at pH 8.6, was used. The two injection methods gave similar results but pressure injection proved more reliable. In the python, HRP was pressure injected at seven widely spaced locations in one tectal lobe at depths ranging from 200 to 800 μm below the surface—the expected location of infrared afferent terminals (Haseltine, '78). In five rattlesnakes, HRP was injected into the RC in two closely spaced locations per animal. Electrophysiological recordings were first made to ascertain the location and depth of the RC in each animal. HRP filled pipettes were then lowered through the same penetration sites to the depths indicated by the unit recordings. Multunit infrared-evoked activity was recorded from these pipettes when they were at the proper depth.

Snakes were maintained post-operatively at 27°C for times ranging from two to five days for the rattlesnakes and eight days for the python. The animals were then anesthetized, perfused first with saline and then with 2.5% glutaraldehyde and 2% paraformaldehyde in pH 7.4 phosphate buffer. The brains were removed, fixed for an additional three hours, and placed overnight in phosphate buffer with 10% sucrose. On the following day, the brains were rinsed in water, quick frozen, and cut at 40 μm in a cryostat. Sections were collected on subbed slides, thoroughly dried, and treated by the blue benzidine/sodium nitroferricyanide method (Mesulam, '76).

Cobalt

The primary afferent fibers innervating the pit organ were labelled by direct application of CoCl₂ to the pit. The external cornified layer of the pit membrane was peeled away from the membrane surface. The membrane was then lacerated at several points and covered with H₂O. The H₂O was soaked up and the external cavity of the pit filled with 300 mM CoCl₂. The pit was then sealed with petroleum jelly and tape. Following survival times of six to eight days at 27°C, animals were perfused as described above. Brains were removed, treated with ammonium sulfate (2 drops/10 ml) for 10 min, rinsed several times in H₂O, frozen and cut at 40 μm in a cryostat. The sections were collected on subbed slides and processed using Timm’s intensification technique (Tyrer and Bell, '74).

Acetylcholinesterase

Animals were anesthetized and perfused as above, using fixative containing 1% glutaraldehyde and 2% paraformaldehyde buffered to pH 7.4. Brains were removed and placed overnight in phosphate buffer with 10% sucrose. Brains were then frozen and cut at 40 μm in a cryostat. Sections were collected on subbed slides, thoroughly dried and stained using a procedure similar to that of Mesulam and Van Hoesen ('76). Sections were incubated in a solution containing 100 mg acetylthiocholine iodide (AcThChi), 75 mg glycine, 50 mg cupric sulfate, 410 mg sodium acetate (anhydrorous), and 100 ml H₂O titrated to pH 5.5 with acetic acid, until a visible white precipitate formed on the surface of the sections. Following incubation, the tissue was stained in 10% potassium ferricyanide for 1–5 min. Controls were
run by omitting the substrate (AcThChI) and by the addition of the specific acetylcholinesterase inhibitor BW 284C51. Sections were pre-incubated for 10 min in BW 284C51 and then incubated in a solution containing the substrates and the inhibitor.

The distribution of cells within the infrared nuclei in both species was studied from cresyl violet sections cut in a transverse plane at 15 μm.

Electrophysiology and lesioning

Single unit recordings were made from the RC of the rattlesnake using metal-filled micropipettes flushed with gold and plated with platinum black (Dowben and Rose, '53; Gestland et al., '59) and with standard recording techniques (Newman and Lettvin, '78). A hot tungsten filament, gated by an electromagnetic shutter (Vincent Associates) and filtered to remove visible wavelengths (Wratten filter no. 87b) was used as a stimulus. Flux of the stimulus at the pit membrane was 3 mW/cm². Electrolytic lesions were made to mark the location of single unit infrared activity by passing a 5 μA current through the electrode for 15–30 sec (electrode negative). Two to seven lesions were made in each of nine rattlesnakes. The brains were removed, fixed overnight in 100% formalin in saline, dehydrated, cleared in cedarwood oil, paraffin embedded, and cut at 15 μm. The lesions were apparent after cresyl violet staining of the sections.

RESULTS

HRP injection in rattlesnake RC

We injected HRP into the nucleus RC of the rattlesnake in order to identify its afferent input and its efferent projections. Figure 1 summarizes the projections resulting from one such HRP-RC injection. The cell groups and fiber tracts shown in the figure stained in all of our five HRP-RC injection experiments. Figure 2A shows the appearance of an HRP injection site after two days of survival. HRP injections extended over a diameter of approximately 250 μm. Because of the small size of the RC, the injections spread beyond the border of the nucleus, generally in a dorsal direction.

Cell staining

HRP injection into the RC invariably led to retrograde filling of cells in the ipsilateral LTTD (Figs. 1F, 2B). Figure 2C shows the morphology of these labeled cells, which were distributed throughout the LTTD. They are approximately 15–20 μm in diameter. In many cases, labelling was intense and spread into the dendrites. Under these conditions some labeled cells were multipolar and others bipolar. The fiber tract from these LTTD cells to the RC was clearly delineated (Figs. 1E, F). It ran in a ventro-rostral direction from the LTTD, along the lateral surface of the medulla to the RC. The stained fibers were approximately 1–2 μm in diameter.

Additional cells were stained in the caudal end of the common descending trigeminal nucleus (NDs), in the region medial and posterior to the LTTD (Figs. 1G, H). A handful of cells were stained in the dorsal horn of the spinal cord (Fig. 1I) and in a region corresponding to the motor nucleus of the trigeminal.

Fiber staining

Crossed RC-Tectal Tract (rct). Anterograde HRP transport delineated an RC projection to the contralateral optic tectum. As illustrated in Figures 1B–E, fibers from the RC crossed the midline tranversely and collected in the ventral medulla, where they coursed forward into the tegmentum. They then coursed dorsoad near the lateral surface of the tegmentum into layer 7a of Ramón (1896), in the stratum griseum centrale, where they branched into smaller diameter segments (Fig. 2D). This pathway is identical to the RC-tectal tract we described previously (Gruberg et al., 79a) on the basis of retrograde transport of HRP following tectal injections.

Descending Tracts. Large diameter fibers, approximately 3–7 μm in diameter, coursed dorsad and then caudad and mediad from the RC (Figs. 1F, G). The fibers ended abruptly in a terminal field (CRF) immediately posterior to the LTTD and ventral to the ipsilateral descending nucleus of the trigeminal (NDs) (Fig. 1G).

Abbreviations

Cb cerebellum
dCD dorsal horn
cRF central reticular field
tltd tractus descendens lateralis n. trigemini
LTTD nucleus descendens lateralis n. trigemini
NDs nucleus descendens n. trigemini
on optic nerve
RC nucleus reticularis caloris
rct nucleus RC-tectal tract
RI nucleus reticularis inferior lateralis
RM nucleus reticularis medius lateralis
teo optic tectum
ttd tractus descendens n. trigemini
V fifth nerve root
VIII eighth nerve root
XMd nucleus motorius dorsalis n. vagi
Fig. 1. Camera lucida diagrams of the distribution of HRP-stained material after injection into the rattlesnake nucleus RC. All sections are from a two-day-survival animal. Filled circles represent stained cells; dots and lines represent stained fibers. The injection site is shown in E. The level of each section within the brain is indicated in the inset.
Fig. 2. Staining following HRP injection into the rattlesnake nucleus RC. A) HRP injection site in the RC. Scale: 500 μm. B) HRP-stained cells in the LTTD (within boxed area) ipsilateral to the injection site. Section at the level of Figure 1F. Scale: 250 μm. C) Enlargement of boxed area in B, showing LTTD cells. Scale: 50 μm. D) HRP-stained fibers and terminal aborizations in the intermediate layers of the tectum contralateral to injection site. Oval cells are stained erythrocytes. Stained glial cells are distributed throughout the section. Section at approximately the level of Fig. 1B. Scale: 200 μm.
Other, thinner fibers, 1–2 μm in diameter, took a slightly more lateral route than the large diameter fibers. These fibers collected at the dorsal surface of the extreme caudal medulla and continued into the spinal cord (Figs. 1G–I).

Another discrete set of fibers coursed dorsad and slightly rostrad from the RC before coursing medioad through the ventral cerebellum, traversing to the opposite side of the medulla (Fig. 1C). We could not ascertain the termination site of this tract from our material. They can not be true commissural fibers since no cells were stained in the contralateral RC.

No HRP-stained fibers or cell bodies were found in the diencephalon following RC injections.

Cobalt

Schroeder and Loop (’76) traced the projection of one of three nerve branches innervating the pit organ in the rattlesnake, the superficial branch of the maxillary, using cobalt iontophoresis. In order to extend their results, we introduced cobalt into the terminal branches of the nerves within the pit membrane itself, thus simultaneously labelling all nerve branches innervating the pit. Following six to eight day survival times, the ipsilateral lateral trigeminal tract and nucleus showed cobalt staining. We found no evidence of a projection from the pit organ to any area other than the LTLD.

Single unit activity in the RC

We recorded the electrical activity of approximately 40 single units from the nucleus RC. These units could only be driven by infrared stimulation of the ipsilateral pit organ. They did not respond to visual, tactile, auditory, or vibrational stimulation. Figure 3A shows activity typical of the neurons sampled. Onset of a sustained infrared stimulus of moderate intensity led to a transient response, whose initial burst of spikes lasted for approximately 1.5 sec. Some units showed a low rate of spontaneous activity which was sensitive to the level of anesthesia.

Electrolytic lesions made following the recording of single unit activity allowed us to localize the recording site. These lesions (a total of 30 in nine animals) were within the RC. Figure 3B shows the lesion made following the recording illustrated in 3A.

HRP injection in the python tectum

HRP injection into the tectum of the python led to the retrograde filling of cells within the contralateral LTLD. Figure 4 shows these stained cells. They are multipolar, varying from 35 to 55 μm in diameter. They are uniformly distributed throughout the main body of the LTLD, but are not found in the caudal portion of the nucleus, which extends into the spinal cord. The small cells within the LTLD were not stained with HRP. We found no stained cells along the ventro-lateral margin of the medulla, where the nucleus RC is located in the rattlesnake.

The tract from the LTLD to the contralateral tectum in the python coursed ventrad and
mediad from the LTLD, crossing the midline in the ventral medulla. The fibers formed a discrete bundle near the lateral edge of the medulla, coursing forward into the tegmentum, then dorsad into the tectum. This pathway is similar to the one followed by the rattlesnake projection from LTLD to RC to contralateral tectum.

**Cell morphology**

Figures 5A, B, and C show Nissl-stained sections of the python LTLD, the rattlesnake RC, and the rattlesnake LTLD respectively. The python LTLD consists of small cells, approximately 5–8 μm in diameter, and large, multipolar cells, approximately 20–30 μm in diameter. The LTLD cells stained following tectal-HRP injections are presumably from the large cell population. We attribute the difference in size between the HRP-stained cells and the Nissl-stained cells to differences in tissue treatment. The large cells do not occur in the caudal tail of the nucleus.

The rattlesnake RC consists of a population of multipolar cells approximately 25–45 μm in diameter. It is these cells that stain following HRP injections into the rattlesnake tectum. The rattlesnake LTLD consists of a population of small cells, approximately 5–8 μm in diameter, similar to the small cells of the python LTLD. In addition, there is a population of medium-sized cells, approximately 15–25 μm in diameter.

**Acetylcholinesterase activity**

We investigated the distribution of acetylcholinesterase activity in the infrared system of both the python and the rattlesnake. In the python, the large, multipolar cells of the LTLD stained heavily for acetylcholinesterase activity (Fig. 6A). The noncellular background of the LTLD stained lightly as well. It is difficult to determine whether the small cells of the LTLD stain because of this background activity.

The LTLD of the rattlesnake showed a similar light stain extending over its entire extent. The medium-sized cells of the LTLD were lightly stained. The cells of the rattlesnake RC (Fig. 6B) stained heavily for cholinesterase activity; they stood out clearly against an unstained fiber tract which passes through the area.
Fig. 5. Nissl-stained material in the infrad nuclei of python and rattlesnake showing cellular sizes and morphologies. A) LTTD of the python. B) Nucleus RC of the rattlesnake. C) LTTD of the rattlesnake. All scales: 50 µm.
Fig. 6. Distribution of acetylcholinesterase activity in the LTTD of the python (A) and the nucleus RC of the rattlesnake (B). Arrows indicate some of the stained cells. Both scales: 50 μm.
Control experiments verified the specificity of the cholinesterase stain in both species. Removal of the substrate from the incubation solution eliminated all staining. The specific acetylcholinesterase inhibitor BW 284C51, when applied to the incubation solution, significantly reduced the degree of staining in the LTLD of both species and in the rattlesnake RC. Background as well as cellular staining was reduced by these procedures.

We also examined the tegmental area of the mesencephalon for acetylcholinesterase activity. A continuous band of cellular and noncellular tissue extending from the postero-lateral tegmental nucleus (PLT) to the dorso-lateral tegmental nucleus (DLT) exhibited heavy cholinesterase activity. This pattern of activity, and the connectivity and location of these nuclei (Gruberg et al., '79a), suggest that they are both a part of the nucleus isthmi. In our previous paper (Gruberg et al., '79a), we showed that the PLT projects bilaterally to the optic tectum, with its contralateral projection coursing forward into the diencephalon and crossing at the supraoptic decussation. The nucleus isthmi in both the frog (Shen et al., '55) and the pigeon (Hunt et al., '77) shows heavy acetylcholinesterase activity.

DISCUSSION

Infrared pathway in rattlesnake

The central question addressed in this paper concerns the similarities and differences between the infrared LTLD-tectal pathways in pit viper and boid snakes. In our previous paper (Gruberg et al., '79a) we investigated the connections of the optic tectum of the rattlesnake using tectal HRP injections. Retrograde HRP transport demonstrated that only one cell group in the medulla, the nucleus RC, projected to the tectum; no stained cells were found in the LTLD following HRP tectal injection. We proposed that, in the rattlesnake, the RC serves as an intermediate link between the LTLD and the tectum.

Support for this suggestion has recently come from Stanford and Schroeder ('79), who demonstrated that the LTLD of the rattlesnake projects to the RC. Following small suction lesions of the LTLD, silver staining revealed a tract of degenerating fibers that exit at the lateral boundary of the LTLD and travel rostrally along the lateral medulla to the region we identified as the RC.

The results presented here confirm our previous suggestion of this indirect trigemino-tectal pathway. Retrograde transport of HRP from the RC results in filling of cells in the LTLD. The pathway from the LTLD to the RC, marked by HRP-stained axons, corresponds to the route described by Stanford and Schroeder ('79). Anterograde HRP transport from the RC results in filling of terminal arborizations in the stratum griseum centrale of the tectum (Fig. 2D), confirming our previous findings (Gruberg et al., '79a).

Kass et al., ('78), using anatomical and physiological techniques, localized infrared responses in the optic tectum of the rattlesnake to the stratum griseum centrale, primarily in layer 7a of Ramón (1896). This corresponds closely to the localization of terminal arborizations of RC cells in the tectum which are, as we report here, confined primarily to layer 7a (Figs. 1A, B and 2D).

The electrical recording and lesioning experiments presented here demonstrate that the RC is primarily an infrared sensory nucleus. RC units respond to low level infrared stimuli, but are not driven by stimuli from other modalities. In addition, the duration of the transient response of RC neurons is intermediate between the longer responses of LTLD neurons (Stanford and Hartline, '78) and the shorter responses of tectal units (unpublished observations). These physiological results are consistent with the anatomical connections of the RC.

Our anatomical results, summarized in Figure 7A, suggest that the infrared pathway in the rattlesnake proceeds first from the LTLD to the RC and then from the RC to the tectum. The HRP experiments do not, by themselves, rule out the existence of additional neural circuitry. Some primary trigeminal fibers from the pit organ could, in a parallel pathway, project directly to the RC. This is unlikely, since our cobalt experiments and those of Schroeder and Loop ('76) identified only LTLD projections of the trigeminal fibers innervating the pit organ.

Infrared pathway in python

On the basis of a degeneration study, Molenaar and Fizaan-Oostveen ('78) reported that the LTLD of the python projects directly to the tectum. Our HRP results from python confirm this finding; tectal HRP injections in the python lead to retrograde filling of cells in the LTLD (Fig. 4). Examination of Nissl sections of the python did not reveal a cell group corresponding to the nucleus RC of the rattlesnake. Nor did we find any evidence of a projection from any other cells in the medulla to the tectum. Figure 7B summarizes the
INFRARED PATHWAY IN RATTLESNAKE AND PYTHON

Fig. 7. The infrared pathway from the pit organ to the tectum. A) Summary of pathway in the rattlesnake. B) Proposed pathway in the python. Unlike the rattlesnake, the python has multiple labial pits, only two of which are shown here. The interneuron within the python LTLD (open circle) is hypothetical and has not been demonstrated experimentally.

propose infrared pathway in the python.

Nissl-stained material reveals that the python LTLD is composed primarily of two types of cells: small neurons and large, multipolar neurons. Our observations confirm Molenaar's findings ('78a) that the large cells are confined to the main corpus of the LTLD. They do not extend into the caudal region of the nucleus. We find that the large LTLD cells that are stained following tectal HRP injections are also confined to the main body of the nucleus. This correspondence adds confidence to our identification of the large, multipolar neurons seen in Nissl sections as the ones that project to the tectum.

Comparison of infrared tectal-afferent cells

Our results demonstrate that the trigemino-tectal pathway of the rattlesnake and the python differ qualitatively in their connectivity. The two species belong to families that are distantly related phylogenetically. As Bullock and Barrett ('68) suggest, the infrared systems of these two species may have evolved independently. However, the two species show many similarities in their infrared systems. Both families have a specialized nucleus of the spinal trigeminal system, the LTLD. This nucleus is not apparent in a snake species that does not have a specialized infrared sense (Schroeder and Loop, '76). In addition, pit vipers and boid snakes have an important infrared projection to the optic tectum which is organized in a spatiotopic representation of the external world (Hartline et al., '78; Hassel et al., '77). Yet, within the medulla, the location of the cells that project to the tectum differs in the two families of snakes. In the rattlesnake, these cells lie in the RC. In the python they are located in the LTLD.

In spite of this difference, crotaline and boid infrared cells that project to the tectum share several morphological and histochemical features: 1), the cells have a similar multipolar appearance in both species; 2), both are large in size: approximately 25–45 μm in diameter in the rattlesnake and approximately 20–30 μm in diameter in the python; 3), the cells stain heavily for acetylcholinesterase activity in both species; 4), the large cells in both species share the same functional connectivity, receiving infrared input and projecting to the contralateral optic tectum.

The similarities suggest that the infrared pathways in these two species may be closely related, differing only in that the tectal afferents form a distinct nucleus in the rattlesnake but not in the python. The tectal afferent cells in both species might have a common origin and share the same pattern of connectivity. In the python, the large cells of the LTLD would function as relay neurons equivalent to the RC cells of the rattlesnake. Under this hypothesis, these neurons should receive input solely from other cells in the LTLD. Figure 7B illustrates this proposed circuitry.
Parallels of the infrared pathway in mammals

Thermosensitive single unit activity has been recorded from the marginal layer of the trigeminal subnucleus caudalis in several mammalian species, including cat (Dostrovsky and Hellon, '78; Burton et al., '79) and rat and rabbit (Dickenson et al., '79). The location of these mammalian neurons suggests that the LTTD of pit vipers and boids, which directly overlies the caudal portion of the spinal trigeminal nucleus, may be derived from the marginal zone of the reptilian homologue of the nucleus caudalis. This suggestion is strengthened by the anatomical findings of Meszler (1975), which show that the ultrastructure of the LTTD of the pit viper closely resembles that of the mammalian nucleus caudalis.

The nucleus caudalis has been shown to project to the ipsilateral lateral reticular formation in a number of mammalian species. (Carpenter and Hanna, '61; Stewart and King, '63; Dunn and Matzke, '68; Roberts and Matzke, '71; Tiwara and King, '74). In the cat, this projection originates from the marginal, thermosensitive zone of the nucleus (Burton et al., '79). In addition, Tiwara and King (1974) find that the lateral reticular formation in the squirrel monkey sends a small projection to the contralateral superior colliculus. Thus, in some mammals, there is an area in the lateral reticular formation that receives trigeminal input; a similar region, in other mammals, projects to the superior colliculus. This suggests that the LTTD-RC-tectal pathway of the pit viper may be homologous to a trigemino-reticular formation-collicular pathway in mammals. A direct projection from the nucleus caudalis to the superior colliculus has not been observed in mammals.

In summary, we have shown that the infrared trigemino-tectal pathways of the rattlesnake and the python differ qualitatively in their organization. In the rattlesnake, the pathway proceeds from the LTTD to the RC to the tectum. In the python, a direct LTTD-tectal projection occurs. The cells that project to the tectum in these two species are of similar size and shape and both exhibit cholinesterase activity. In spite of their disparate locations, they may have a common origin.

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