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A proposed neural pathway for vocalization in South African clawed frogs, *Xenopus laevis*

Daniel M. Wetzel*, Ursula L. Haerter*, and Darcy B. Kelley**

Program in Neuroscience, Department of Psychology, Princeton University, Princeton, New Jersey 08544, USA, and Department of Biological Sciences, Columbia University, New York, New York 10027, USA

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Summary. 1. Vocalizations of South African clawed frogs are produced by contractions of laryngeal muscles innervated by motor neurons of the caudal medulla (within cranial nerve nucleus IX–X). We have traced afferents to laryngeal motor neurons in male and female frogs using retrograde axonal transport of horseradish peroxidase conjugated to wheat germ agglutinin (HRP–WGA).

2. After iontophoretic injection of HRP–WGA into n. IX–X, retrogradely labelled neurons were seen in the contralateral n. IX–X, in rhombencephalic reticular nuclei, and in the pre-trigeminal nucleus of the dorsal tegmental area (DTAM) of both males and females.

3. Injection of HRP-WGA into DTAM resulted in labelled cells in the striatum, preoptic area and thalamus. Posterior to DTAM, labelled cells were found in the rhombencephalic reticular nuclei as well as n. IX–X of males. Results in females were similar with the exception that n. IX–X labelled cells were only seen after very large injections of unconjugated HRP into DTAM and surrounding tegmentum. Thus, the projection of n. IX–X neurons to DTAM is not as robust in females as males.

4. These anatomical studies revealed candidate brain nuclei contributing to the generation of vocal behaviors and confirmed some features of a model for anuran vocal behavior proposed by Schmidt (1976).

5. Comparison of calling candidate brain nuclei to the location of steroid accumulating neurons (Kelley 1981) reveals that most calling nuclei contain hormone concentrating cells. Androgens may act to promote calling by influencing neural activity at multiple sites within the vocalization pathway.

Present addresses:

* Section of Neurobiology and Behavior, Cornell University, Ithaca, N.Y. 14853, USA

** To whom offprint requests should be sent: Department of Biological Sciences, Columbia University, New York, N.Y. 10027, USA

Abbreviations: *APOA* Anterior preoptic area; *c* Contralateral; *CB* Cerebellum; *CT* Central nucleus of the thalamus; *DTAM* Pretrigeminal nucleus of the dorsal tegmental area of the medulla; *GC* Griseum centrale; *i* Ipsilateral; *n.Cb* Nucleus cerebelli; *NI* Nucleus isthmi; *N. IX–X* Cranial nerves IX and X; *N. V* Cranial nerve V; *N. VIII* Cranial nerve VIII; *n. VIII* Nucleus of cranial nerve VIII; *n. IX–X* Motor nucleus of cranial nerves IX–X; *OB* Olfactory bulb; *PIT* Pituitary; *POA* Preoptic area; *PPOA* Posterior preoptic area; *Ri* Nucleus reticularis inferior; *Ridl* Nucleus reticularis inferior dorso-lateralis; *Ridm* Nucleus reticularis inferior dorso-medialis; *Riv* Nucleus reticularis inferior pars ventralis; *Rm* Nucleus reticularis medius; *Rs* Nucleus reticularis superior; *SC* Spinal cord; *SLT* Tractus solitarius; *T* Thalamus; *TECT* Tectum; *TEL* Telencephalon; *TV* Telencephalic ventricle; *V* Tectal ventricle; *VIN* Ventral infundibulum; *VLT* Ventrolateral nucleus of the thalamus; *VMT* Ventromedial nucleus of the thalamus; *VST* Ventral striatum; *VTEG* Ventral tegmental nucleus; n. *V* Motor nucleus of cranial nerve V; n. *VII* Motor nucleus of cranial nerve VII; *2°VIS* Secondary visceral nucleus

Introduction

Male South African clawed frogs, *Xenopus laevis*, emit a distinctive vocalization, the mate call, during the breeding season. Female *X. laevis* also vocalize; the female-typical vocalization, ticking, is produced by sexually unreceptive frogs in response to a clasp attempt by a male (Russell 1954; Kelley 1982). Vocalizations are produced by contractions of the laryngeal bipinnate muscles (Yaeger 1982) innervated by motor neurons in cranial nerve nucleus IX–X of the caudal medulla (Kelley 1980).

The goal of this study was to identify sources of afferent input to laryngeal neurons in order to establish neural candidates for participation in the generation of calling. Our results suggest that laryngeal afferent brain nuclei are connected in a manner with many similarities to the vocal model of Schmidt (1971, 1976). We thus suggest that these brain nuclei control the production of vocal behaviors in *X. laevis*.

In male *Xenopus laevis*, vocal behaviors are modulated by the androgens, testosterone and dihydrotestosterone (Wetzel and Kelley 1983). Androgen target neurons are located in a restricted set of diencephalic, mesencephalic and medullary brain nuclei (Kelley et al. 1975) which includes the laryngeal motor neurons (Kelley 1980). These androgen target brain nuclei appear homologous to those identified by Schmidt (1971, 1976) in other frog species as participating in the motor control of calling. Thus in this study we also compared the locations of laryngeal afferents with those of androgen-concentrating neurons and with Schmidt's (1971) neural model for anuran vocal behavior.

Female *X. laevis* do not mate call (Hannigan and Kelley, submitted). We wished to determine whether sex differences in calling reflect differences in connectivity of the brain nuclei involved in vocalization. We compared the connections of laryngeal afferent brain nuclei in male and female brains. The projection from laryngeal motor neurons to their major tegmental afferent nucleus (DTAM) is less robust in female than in male brains. This difference in connectivity could contribute to sex differences in *X. laevis* vocal behaviors.

Materials and methods

Experimental approach. Retrograde transport of horseradish peroxidase (HRP) coupled to the lectin, wheat germ agglutinin (WGA) was used to anatomically identify afferents to laryngeal motor neurons. Laryngeal motor neurons (n. IX–X) occupy a slender column approximately 200 μm wide, 300 μm in diameter and 2,500 μm long in the caudal medulla. Use of the HRP-WGA tracing method (Gonatas et al. 1979) allowed the placement of restricted but effective injection sites in this small medullary nucleus and (as identified in the course of this study) its major afferent, the pre-trigeminal nucleus of the dorsal tegmental area of the medulla (DTAM: terminology adopted from Schmidt 1974 and Kelley et al. 1975). In addition to producing very small injection sites, the HRP–WGA method produces less damage to fibers of passage in the vicinity of the injection site than does injection of HRP in agents (e.g. DMSO) that facilitate uptake of tracer (Gonatas et al. 1979). Our results using HRP–WGA in females suggested that transport from dorsal tegmentum to medulla was less robust than in males. We therefore prepared two additional females with larger injections of HRP (not conjugated to WGA) in an attempt to increase the availability of enzyme in females. Anterograde projections and fiber trajectories were usually not well visualized.

Injections. Iontophoretic injections of HRP-WGA (Miles Laboratories) were made into adult *Xenopus laevis* (Nasco Co., Fort Atkinson, Wisconsin). Frogs were anesthetized by submersion in a 0.13% solution of MS-222 (ethyl *m*-aminobenzoate, methanesulfonic acid, Aldrich Co.) and placed on ice. A head holder designed in our laboratory was used with a Kopf stereotaxic device. The head holder consisted of standard rat ear bars with small vise jaws welded to their ends. After incision of the scalp and retraction of adhering muscles the vise jaws were firmly clamped around the skull just rostral to the ocular orbits. The animal was placed in the head holder with its ventral surface up allowing access to the brain through the mouth. The ventral approach minimized bleeding and facilitated approach to n. IX–X and DTAM. The skin of the roof of the mouth was incised and retracted. Bleeding was controlled by electrocautery. A small (1 mm) hole was drilled through the ventral surface of the skull, skull sutures being used as landmarks for the approximate placement of the hole. Dura and pia membranes were teased away. Blood vessels on the surface of the brain presented a consistent pattern from animal to animal, permitting quite reliable placements of injections (see Figs. 1 and 4). Glass microelectrodes formed from 1.2 or 2 mm diameter capillary tubing were pulled on a Kopf microelectrode puller and tips broken back to a diameter of 3–20 μm. The HRP–WGA (Miles Laboratories) was received as a conjugate dissolved in 0.01 *M* sodium phosphate buffered saline pH 7.4 containing 0.02% sodium azide but no free lectin or enzyme. Upon receiving the HRP–WGA solution (shipped in dry ice) it was rapidly thawed and divided into 50 μl aliquots, then rapidly refrozen. Care was taken to keep aliquots of HRP–WGA solution frozen until approximately 5 min before use; electrodes were backfilled in this period.

Electrodes were positioned 0.6 ± 0.05 mm from the ventral surface of the medulla in the case of nucleus IX–X and 0.8 ± 0.10 mm in the case of DTAM. A constant current DC voltage source was used to pass 900–1,500 nA of current for a period of 15–60 min. After completion of the injection the skin of the mouth and head was sutured and the animal returned to its tank for recovery.

Histochemistry. All animals were maintained for 48 h before perfusion since pilot experiments revealed this time period to result in most efficient retrograde labelling of cell bodies. Frogs were deeply anesthetized by an injection of Nembutal or 1.3% MS 222 and then perfused transcardially with 20 ml of saline solution (sucrose 23 m*M*, sodium cacodylate 67 m*M*, NaCl 112 m*M*, dextrose 22 m*M*). The tissues were then fixed by transcardial perfusion of 40.0 ml of a 2.5% glutaraldehyde, 67 m*M* sodium cacodylate, 22 μ*M* Ca^{++} solution. Following perfusion, the brain was immersed in the fixative at 25 or 37 °C for 30 min, and placed in a 20% sucrose solution in 0.1 *M* phosphate buffer, pH 7.4 at 4 °C overnight. Male brains were then embedded using a fast gelatin embedding technique. Twenty ml of albumin gelatin (Snodgrass and Dorsey 1963) was rapidly mixed (<15 s) with 2 ml of 25% glutaraldehyde and poured over the brain in an embedding box. The albumin was allowed to harden for two to four hours at 25 °C. Frozen sections were then cut at forty μm in either the transverse or horizontal plane, collected in 0.1 *M* phosphate buffer, pH 7.4 at 5 °C, and reacted with the chromagen tetramethyl benzidine (TMB, Sigma) following the protocol of Mesulam (1978). All female brains were sectioned horizontally (without embedding) at 25 μm in a Bright cryostat, picked up on subbed slides and reacted, on the slide, using the Mesulam procedure. Pilot experiments com-

paring the free floating (male) versus on-the-slide sections (female) revealed no apparent diminution of the reaction in the cryostat sections. In addition, cryostat sectioned male brains yielded results equivalent to those obtained with free floating sections. All sections were reacted within 3 to 48 h of being cut and were counterstained with 0.5% neutral red solution. Exposure to alcohol was limited to less than 30 s. Sections were scanned with the aid of a Zeiss microscope using light field illumination and the locations of labelled cells noted. Brain sections and landmarks used to locate the positions of labelled cells were drawn with the aid of a microprojector. The minimum criterion for identification of a labelled cell was the accumulation of reaction product about the nucleus. Usually the reaction product filled the cell bodies and proximal dendrites.

Results

Males

Location of labelled cells after injection of n. IX–X

Histochemical reaction revealed that the injection site included n. IX–X in five (experimental) male brains (Fig. 1; right side). The smallest and most accurately placed injection was that of brain M-20 which involved a large portion of the rostrocaudal extent of n. IX–X and a limited region of anterior ventral spinal cord. The injection site in M-20 did not include the adjacent reticular formation, unlike the four larger injections. Three additional males had injections into the caudal medulla immediately adjacent to the laryngeal motor neurons (Fig. 1; left side). One control injection was ventromedial to n. IX–X, one was rostromedial and one was caudal. No labelled cells were seen in the brain of the frog which had received HRP–WGA placed on the brain surface (control for blood-borne HRP–WGA and endogenous peroxidase activity).

Comparisons of the locations of labelled cells in experimental and control brains reveal locations of labelled cells restricted to transport from n. IX–X as opposed to labelled cells resulting from HRP–WGA transport from adjacent cell groups in the ventrolateral caudal medulla. Two brain nuclei contained labelled neurons in all n. IX–X injected brains but in none of the control brains. These nuclei are the pretrigeminal nucleus of the dorsal tegmental area of the medulla (DTAM) and the contralateral n. IX–X. The nucleus reticularis inferior (Nikundiwe and Nieuwenhuys 1983) contained large numbers of labelled cells in all experimental brains; more anterior reticular nuclei also contained some labelled cells.

A detailed description of locations of labelled cells after n. IX–X injections is given below. Locations of labelled cells are shown, for the case of M-20, in Fig. 2. A photomicrograph of the injection site for M-20 is depicted in Fig. 8 B.

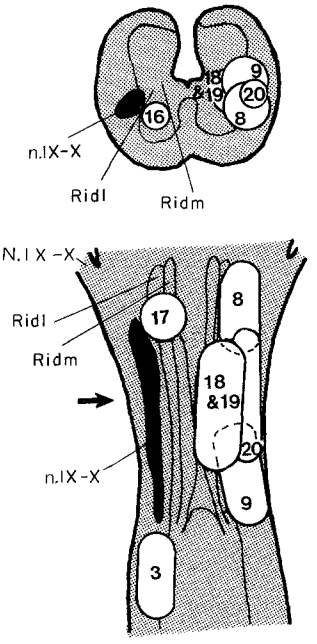

Fig. 1. Locations of HRP–WGA injection sites into 8 male brains. Control injections are shown on the left and experimental injections on the right. Injections have been mapped onto a reference horizontal (lower) and transverse (upper) section. The anterior-posterior level of the transverse section is indicated by the arrow. Five brains (8, 9, 18, 19, 20) included n. IX–X in the injection site; three others (3, 16, 17) served as controls. Injection sites in brains 3 and 17 are posterior and anterior, respectively, to the level of the transverse section. Brain 16's injection site is ventral to the level of the horizontal section. Location of n. IX–X is indicated in black on the left hand side of the figures. Upper figure: dorsal is up. Lower figure: anterior is up

1. Pretrigeminal nucleus of the dorsal tegmental area of the medulla (DTAM). The dorsal tegmental area of the medulla as described by Kelley et al. (1975) is medial and caudal to the nucleus isthmi and secondary visceral nucleus, rostral and medial to the motor nucleus of V, and ventral to the nucleus cerebelli (see Figs. 2 and 3). This nucleus corresponds in location to the pretrigeminal nucleus (pre-V) described in *Rana pipiens* by Schmidt (1974). Succinic dehydrogenase staining suggests DTAM in *X. laevis* to be homologous to pre-V in other anurans (Schmidt 1980). We have therefore modified our terminology for this nucleus adopting the combined term, pretrigeminal nucleus of the dorsal tegmental area of the medulla (DTAM). Labelled cells were found in DTAM in both the contralateral (c) and ipsilateral (i) side in all brains in which n. IX–X was injected. Labelling was heaviest in DTAM (i). A photomicrograph of HRP-labelled cells in DTAM of a male brain is shown in Fig. 9, panels A and B. None of the control brains had labelled cells in DTAM.

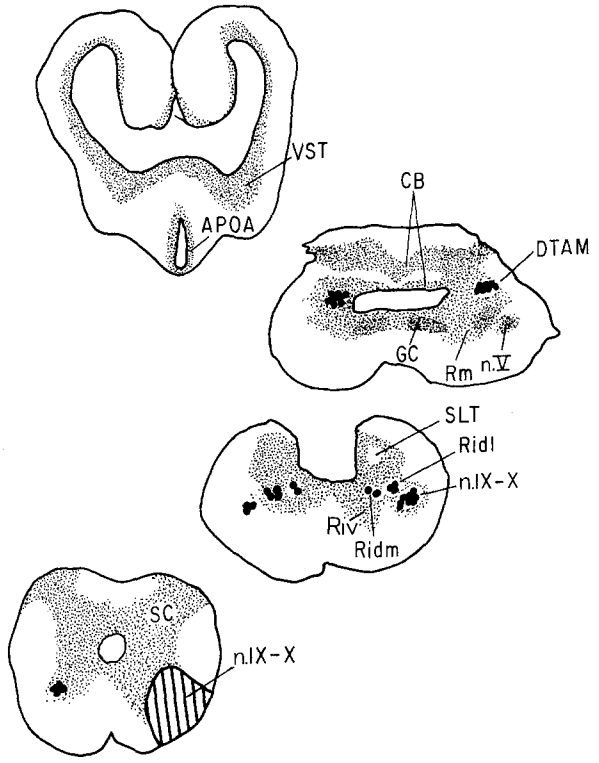

Fig. 2. Location of HRP–WGA labelled cells in transverse section after injection into n. IX–X of a male *X. laevis* (M-20). A photomicrograph of the injection site is included in Fig. 8 B. Labelled cells are found in the contralateral n. IX–X, in Ridl, in Ridm and in DTAM. No labelled cells were seen in diencephalic (APOA) or striatal nuclei (VST). The plane of section is skewed (relative to a plane perpendicular to the neuraxis) such that the dorsal half of the section is caudal to the ventral half. Each section represents the number of labelled cells (•) on one, actual 40 μm section

2. Nuclei of the rhombencephalic reticular formation. The rhombencephalic medial reticular zone is divisible into three anterior-posterior regions: the nucleus reticularis superior (Rs), the nucleus reticularis medius (Rm) and the nucleus reticularis inferior (Ri) (Nikundiwe and Nieuwenhuys 1983). The nucleus reticularis inferior is coextensive with and medial to n. IX–X. On the basis of our experimental material we have further subdivided the Ri into dorsolateral (Ridl), dorsomedial (Ridm) and pars ventralis (Riv) subnuclei (Fig. 2).

HRP labelled cells were found in both Rm and Ri after n. IX–X injection. While some labelled cells were found in all of the subnuclei of Ri in experimental brains, by far the greatest concentration was in the wedge-shaped (in transverse sections) Ridl. Labelled cells here were most numerous on the ipsilateral side and extended anteriorly and posteriorly past the limits of the injection site.

Next to Ridl, the inferior reticular nucleus with the most labelled cells in males was Riv. A few labelled cells were observed in the lateral reticular nucleus, dorsal to n. IX–X and Rm (i and c), anterior to N. IX–X. In control brain M-3 with an injection caudal to n. IX–X proper, labelled cells were seen in Ridl(i); control brains M-16 and M-17 also had some Rm and Ri labelled cells. All of the injection sites of these control brains included reticular formation itself. Since experimental injection M-20, which did not appear to encroach on adjacent reticular formation, also resulted in numerous Ri labelled cells, we suggest that Ri projects to n. IX–X.

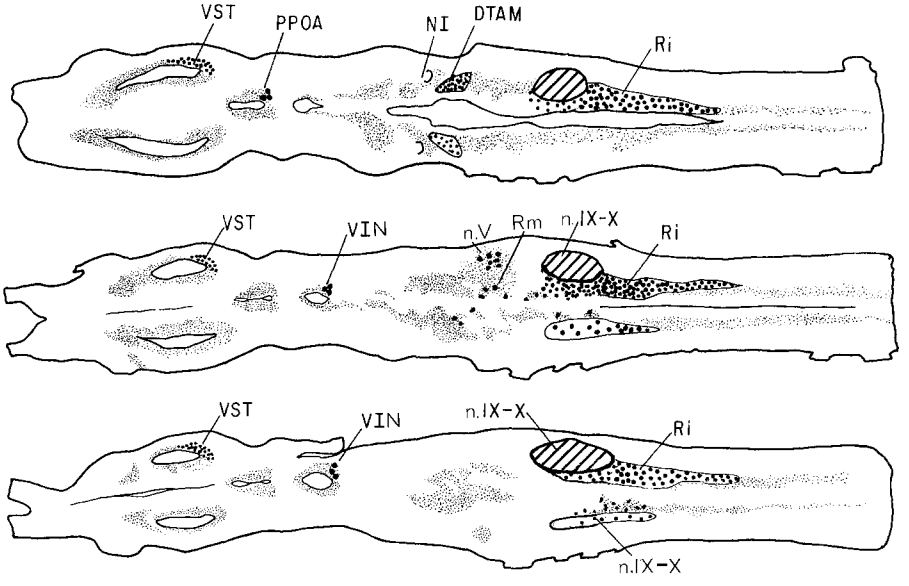

Fig. 3. Locations of labelled cells after injection of HRP–WGA into n. IX–X of a female brain (F-8). Labelled cells are seen in contralateral n. IX–X and Ri and in ipsi- and contralateral DTAM. Labelled cells are also seen in VST, PPOA and VIN. These latter nuclei only included labelled cells when the injection site extended anteriorly to n. IX–X. Anterior is to the left, the most dorsal section is the upper one

3. Cranial nerve nucleus IX–X. Cranial nerve nucleus IX–X extends from the insertion of cranial nerves IX and X past the obex and terminates at the rostral end of the spinal cord (see also Simpson et al. 1986). Motor neurons of n. IX–X are lateral to the inferior reticular nuclei of the rhombencephalon; the most lateral are found among the longitudinal fiber tracts.

In all male brains in which n. IX–X was injected, labelled cells were observed in n. IX–X on both the ipsilateral and contralateral side at the level of the injection. In one male control brain with an injection in the reticular formation ventral to n. IX–X (M-17; Fig. 1) a few cells were observed in n. IX–X (i) at the same anterior-posterior levels as the injection site. No contralateral labelled cells in n. IX–X were observed in any control brains.

4. Other nuclei containing labelled neurons. In one brain (M-8), labelled cells were seen in striatum and thalamus. These nuclei also had labelled cells in female brains F-18 and F-22. These three injection sites were in the rostral pole of IX–X and included portions of more rostral medulla. It seems likely that the ventral striatum (VST) and thalamic cells in M-8 were labelled due to enzyme transport from medulla anterior to n. IX–X.

Three of five experimental males had labelled cells in the motor nucleus of the trigeminal nerve, three had cells in the sensory nucleus of the fifth nerve and three had HRP filled cells in the nucleus tractus solitarii. In both experimental and control brains, labelled cells were found in dorsal and ventral spinal cord.

Females

Locations of labelled cells after injection into n. IX–X

Two females recieved injections into rostral n. IX–X while two additional females received control injections into Ri, medial and rostral to n. IX–X. The contralateral n. IX–X, Ri, Rm and DTAM contained labelled cells in experimental but not in control brains.

1. DTAM. In both experimental brains, labelled cells were seen in contralateral and ipsilateral DTAM. Labelled cells in DTAM of female F-18 are shown in Fig. 3.

2. Nuclei of the rhombencephalic reticular formation. Labelled cells were found in both Ri and Rm (i and c) of both experimental brains. Labelling in control brains was concentrated primarily in Ri. Control brain F-8 also had labelled cells in Rm (i and c).

3. Cranial nerve nucleus IX–X. In both experimental brains, labelling was found in ipsilateral n. IX–X (surrounding the injection site) and in contralateral n. IX–X. No n. IX–X labelled cells were seen in either control brain.

4. Other nuclei containing labelled cells. In experimental female brains, labelled cells were also found in VST and the motor nucleus of cranial nerve V. Labelled cells were found in the ventral thalamus (i and c) of brain F-22 while in brain F-18 labelled cells were found only ipsilaterally. F-18 had some labelled cells in the facial nucleus (i and c). In control brains F-8 and F-24 labelling was confined to Ri and Rm except for a few labelled cells in n. VIII of F-8.

Males

Locations of labelled cells after injection into DTAM

As described above, nucleus DTAM provides a major source of afferent input to laryngeal motor neurons in males and females. All brains with an injection into n. IX–X had labelled cells in DTAM. None of the control brains had DTAM labelled cells. After injection of HRP–WGA into DTAM labelled perikarya were found in several nuclei of the rhombencephalon, mesencephalon and diencephalon. Locations of labelled neurons unique to DTAM injections (i.e., not shared by control injections) were the ventrolateral striatum, the preoptic area (POA), the thalamus (VLT, VMT and CT), and n. IX–X. Experimental results are illustrated in Fig. 5 for the case of M-36, a brain with a small, well localized injection of HRP–WGA into DTAM. In four male brains injections hit DTAM while in seven control brains injections were close to, but not inclusive, of DTAM (Fig. 4).

1. Ventrolateral striatum. The ventrolateral striatum contained many ipsilateral HRP-filled cells in all experimental but no control brains.

2. Preoptic area. In three of four male brains with injections of HRP–WGA into DTAM, labelled neurons were observed in the ipsilateral anterior preoptic area (APOA). In control brain M-30, two labelled cells were observed in the posterior preoptic area. Labelled cells in experimental brains

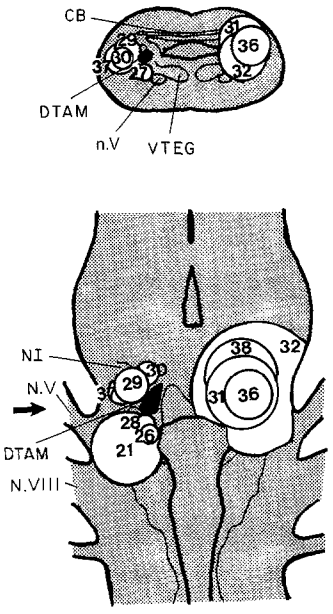

Fig. 4. Locations of experimental (31, 32, 36, 38) and control (21, 26, 27, 28, 29, 30, 37) injection sites of HRP–WGA into DTAM in male brains. Conventions as in Fig. 1

were small and relatively lightly labelled and were concentrated in APOA.

3. Dorsal tegmental area of the medulla (DTAM). In all brains with injections into DTAM, labelled neurons were seen contralaterally as well as ipsilaterally, surrounding the site of injection. In six control brains with injections close to, but not including DTAM, labelled neurons were also observed in ipsilateral DTAM. In one control brain (M-37), cells were also observed in the contralateral DTAM.

4. Cranial nucleus IX–X. Labelled neurons were observed in cranial motor nucleus IX–X in all four male brains that had received injections of HRP–WGA into DTAM (see Fig. 8A). Labelled cells were found throughout the anterior-posterior extent of the nucleus (see Fig. 5). In two cases, neurons were found in both ipsilateral and contralateral n. IX–X and in two cases only in the ipsilateral n. IX–X. No control brains had labelled cells in n. IX–X.

5. Nuclei of the rhombencephalic reticular formation. Labelled perikarya were observed in inferior reticular nuclei (Ridl, Ridm and Riv) in all experimental brains. Labelled cells in Ri were also observed in four of six control brains but the number of labelled cells was few.

Other nuclei containing labelled cells. Certain thalamic nuclei, notably the ventral (VLT, VMT) and central (terminology of Neary and Northcutt 1983), contained labelled cells in all DTAM-injected brains. In addition, both experimental and control brains had occasional labelled cells in nuclei of the Vth cranial nerve and spinal cord.

Females

Locations of labelled cells after injection into DTAM

Two female brains had well placed injections of HRP–WGA into DTAM: F-7 and F-13. Four additional brains with HRP-WGA injections placed rostral, medial and ventral to DTAM served as

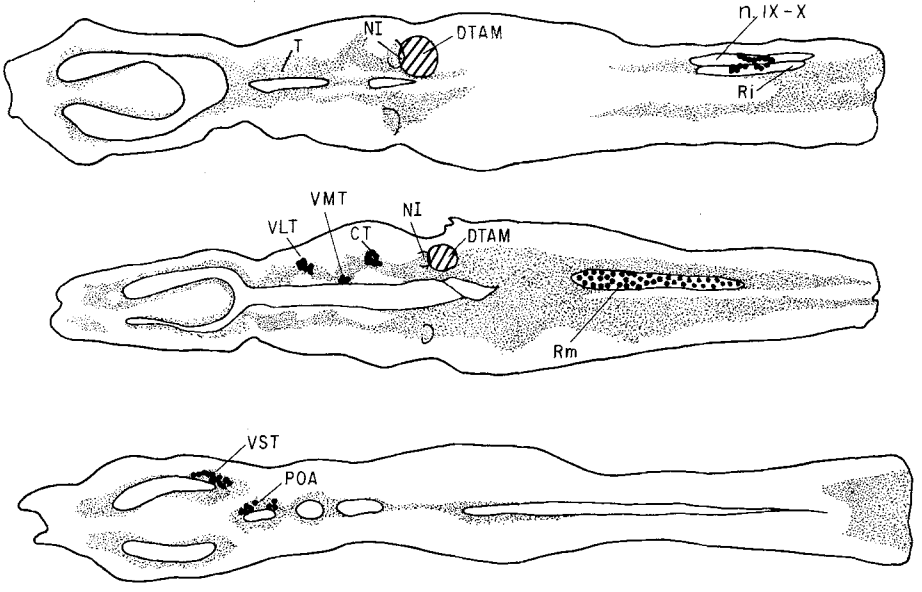

Fig. 5. Locations of labelled cells in a horizontally sectioned brain of a male *X. laevis* (M-36) after injection of HRP–WGA into DTAM. Anteriorly labelled cells are numerous in VST and POA, particularly APOA, and in thalamic nuclei (VLT, VMT, CT). Posteriorly, there are many labelled cells in n. IX–X, Ri and Rm. In this brain, there were no contralateral n. IX–X, DTAM, Rm or Ri cells; however, other brains with larger injections had both contra- and ipsilateral labelled cells in these nuclei. Anterior is to the left; the most dorsal section is at the top

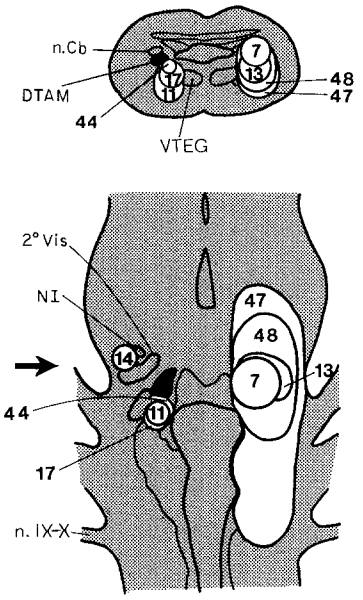

Fig. 6. Locations of injections of HRP–WGA or HRP into DTAM or adjacent medulla of 8 female brains. Conventions as in Fig. 1

controls (Fig. 6). In addition, two females received large injections of unconjugated HRP into DTAM and adjacent tegmentum (F-47 and F-48). Locations of labelled cells are illustrated in Fig. 7 and are described, anterior to posterior, below.

1. Ventrolateral striatum. Labelled cells were found in the ipsilateral VST of experimental brains F-7, F-47 and F-48 (Fig. 9, panels C and D). No VST labelling was seen in control brains.

2. Preoptic area. No labelled cells were seen in the POA of HRP–WGA injected brains, but F-47 and F-48 had labelled cells in anterior and posterior POA. Labelled cells were found in POA (i and c) of control brain F-11.

3. DTAM. In all experimental brains labelled cells were seen in contralateral DTAM. None of the control brains had DTAM labelled cells.

4. Cranial nucleus IX–X. No labelled cells were found in n. IX–X of either HRP–WGA injected female. Comparably sized injections of HRP–WGA into DTAM of male brains (see Fig. 4) would have resulted in many n. IX–X labelled cells. In an attempt to increase enzyme availability in females, large injections of unconjugated HRP were made (F-47 and F-48). The injection site in F-47 extended caudally to a region just anterior to n. IX–X itself (Fig. 6). In brain F-47 numerous labelled cells were found in ipsilateral n. IX–X and a few in contralateral n. IX–X. Brain F-48 had only seven labelled cells in ipsilateral n. IX–X and none in contralateral n. IX–X.

5. Nuclei of the rhombencephalic reticular formation. All experimental but no control brains had labelled cells in Ri (i) and Rm.

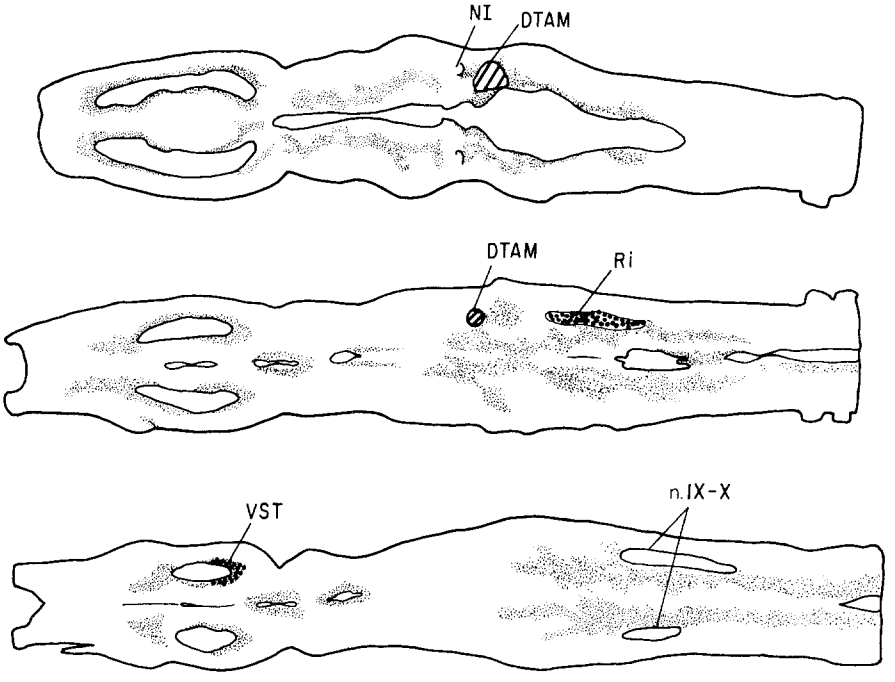

Fig. 7. Locations of labelled cells after injection of HRP–WGA into DTAM of a female brain (F-13). Numerous labelled cells are found in VST anteriorly and Ri posteriorly but not in n. IX–X

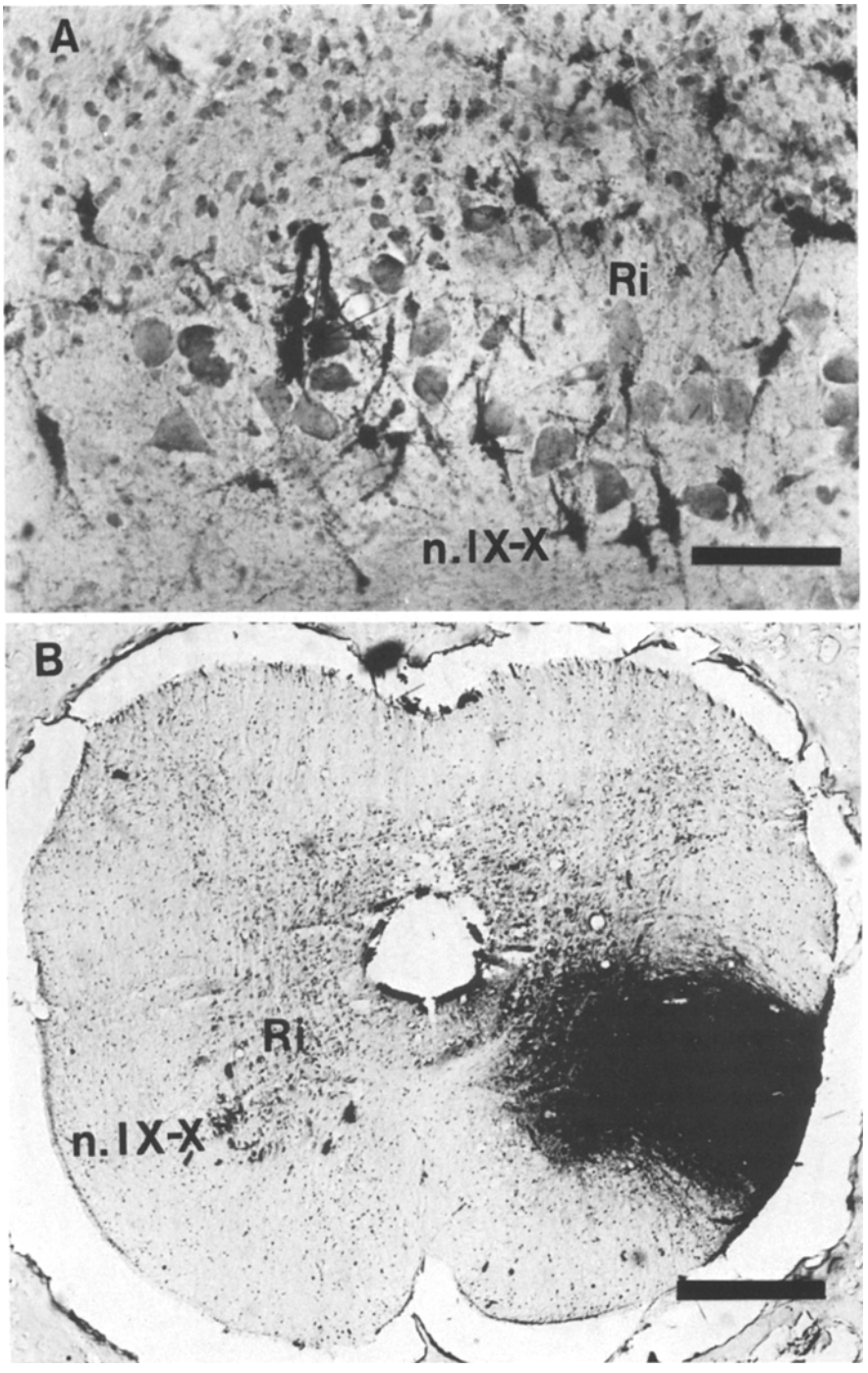

Fig. 8 A, B. Photomicrographs of HRP-labelled cells.
A Labelled n. IX–X and Ri cells after injection of HRP–WGA into DTAM of a male brain. Plane of section is horizontal, medial is up, anterior is to the left. Scale bar, 90 μm
B Injection of HRP–WGA into n. IX–X in a male; transverse section through the injection site. Note the contralateral labelled cells in n. IX–X. Scale bar, 330 μm

6. Other nuclei containing labelled cells. Occasional labelled cells were found in VST, VIN, n. V and ventral and central thalamus of experimental brains. Some control brains also had VIN and n. V labelled cells.

Discussion

Results of the present study suggest that the major afferent sources to laryngeal motor neurons (n. IX–X) arise from the medullary and tegmental reticular formations, including a specialized tegmental nucleus, the pretrigeminal nucleus of the dorsal tegmental area of the medulla (DTAM). Nucleus DTAM, in turn, receives input from the preoptic area, ventral striatum and thalamus anteriorly and from n. IX–X, Rm and Ri posteriorly. A summary of these connections is given in Fig. 10. As discussed below, these anatomical findings agree with

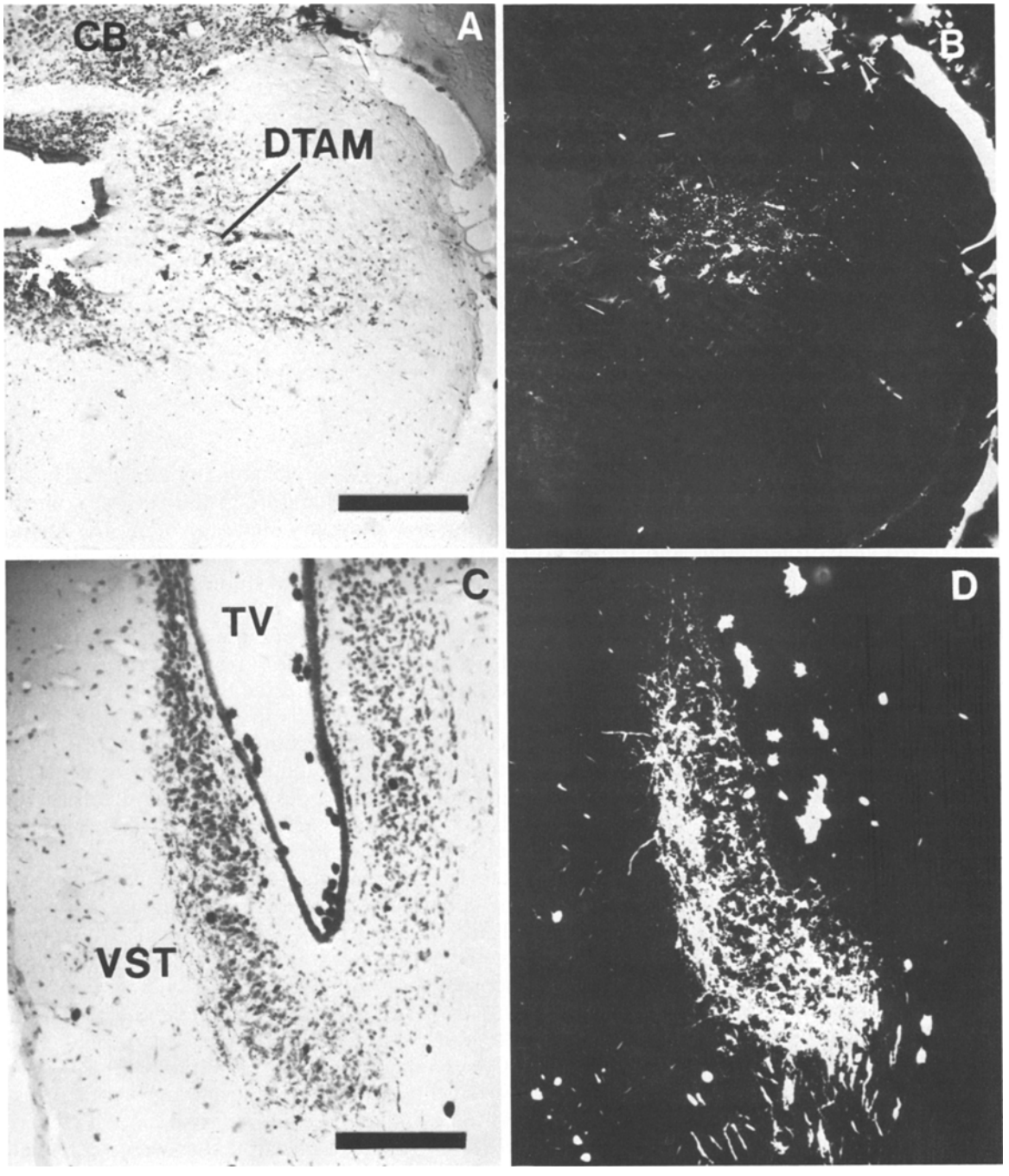

Fig. 9. **A** Bright-field photomicrograph of labelled cells in DTAM(i) of a male after injection of HRP–WGA into n. IX–X. Plane of section is transverse, medial is to the left. (Injection site in Fig. 8B.) Scale bar, 100 μm. **B** Dark-field photomicrograph of section in **A**. **C** Bright-field photomicrograph of labelled cells in VST(i) of a female after injection of HRP–WGA into DTAM. Horizontal section; anterior is up, medial is to the right. Scale bar, 200 μm. **D** Dark-field photomicrograph of **C**

results of lesion and stimulation experiments on evoked vocalization in other anurans (Schmidt 1976) and thus suggest that the pathways outlined may subserve vocalization in *X. laevis*. All of these brain nuclei in *X. laevis* contain hormone-concentrating neurons (Kelley et al. 1975; Morrell et al. 1975; Kelley 1980) and may underlie the powerful effects of steroids on vocal behaviors in this species (Wetzel and Kelley 1983).

Use of retrograde tracing techniques in general, and HRP–WGA in particular, is subject to a number of methodological considerations which

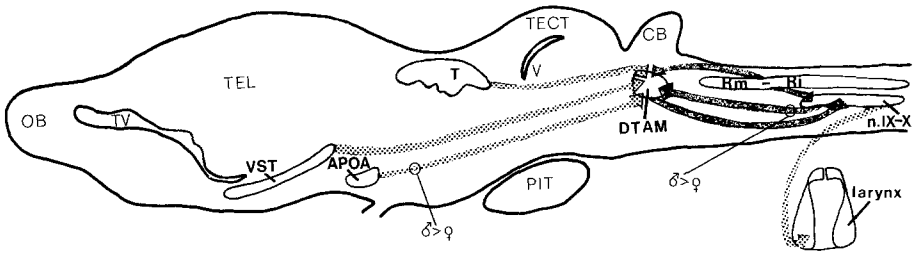

Fig. 10. Schematic representation of brain nuclei projecting to n. IX–X and DTAM in a parasagittal plane. Fiber trajectories were not visualized in the present study and thus are not literally represented on this diagram. Projections from telencephalic and diencephalic nuclei, VST, APOA, VLT and CT are ipsilateral as indicated by a light stippling. Projections from tegmental and medullary nuclei, DTAM, Rm, Ri and n. IX–X are both contralateral and ipsilateral as indicated by the dense stippling. DTAM and n. IX–X also receive input from their contralateral homologues (not shown). Projections from n. IX–X to DTAM and from APOA to DTAM are less robust in the female than in the male. Axons from n. IX–X exit the medulla in the most caudal nerve rootlet of the IX–X complex and innervate the laryngeal bipinnate muscles (Simpson et al. 1986)

must be kept in mind when data are interpreted. Experimental evidence indicates that the lectin HRP–WGA binds to receptors on the cell membrane, is internalized at axon terminals and transported to the cell bodies where the lectin-HRP complex accumulates (Gonatas et al. 1979). The number and location of retrogradely labelled cells depends on the amount of tracer injected, the injection size, the survival time and the chromagen used for visualization. The apparent size of the injection site decreases with survival time (Fahrbach et al. 1982); there is some evidence that the effective injection site size (that from which tracer can be transported) is smaller than the maximum size visualized (Mesulam 1978). Our pilot experiments indicated that a two day survival is optimal for visualizing labelled cells and that a minimum injection site size (e.g. M-26) is necessary if *any* labelled cells are to be seen. Larger injections yield more labelled cells, some of which, in the largest injections, may result from transport from brain regions adjacent to the target nucleus rather than the target itself. We have therefore adopted the conservative strategy of assigning established connections only in those cases where all (or most) experimental brains have labelled cells in a particular nucleus and none of the control brains (injections into adjacent regions) do. This strategy has the disadvantage that projections of a brain nucleus to both the target and adjacent tissue will be discounted. Thus spinal cord may project both to n. IX–X and adjacent Ri. POA may project both to DTAM and to adjacent tegmentum; Rm may also project to both regions. Particularly problematic is the projection from Ri to DTAM and/or adjacent tegmentum. A small injection site including *only* DTAM clearly results in labelled cells in Ri and Rm (Fig. 5). Similarly, a small and very lateral injection into n. IX–X yields many labelled Ri cells (Fig. 2). We have therefore included these connections on the summary diagram of n. IX–X and DTAM afferents (Fig. 10). These issues can only be resolved by additional studies using *anterograde* tracers.

A surprising result of these studies is the apparent reciprocal projection from laryngeal motor neurons to DTAM (inferred from the large number of heavily labelled cells in n. IX–X after DTAM injection). The simplest interpretation of this result is that laryngeal motor neurons send a recurrent collateral (axon or, less probably, dendrite) to DTAM, their major afferent source. Recurrent axonal collaterals of laryngeal motor neurons have been visualized in cats (Gacek 1975). Such a recurrent collateral could provide 'efference copy' to DTAM. Alternatively, labelling in n. IX–X may have been due to transynaptic transport of tracer (incorporation into DTAM cell bodies, transport to n. IX–X, release from terminals and re-uptake into n. IX–X cells), as has been reported with WGA (Ruda and Coulter 1982) and HRP–WGA (Gerfen et al. 1982). We believe transynaptic transport to be unlikely because survival times used here were short (2 days), injection sites were very small (e.g. M-36) and motor neurons were very heavily labelled (see Fig. 8), more heavily than one would expect from the loss of tracer that typically occurs during release and re-uptake (Grafstein 1971).

As far as we have been able to determine, the sole function of the *X. laevis* larynx is the production of sound. Therefore we can regard the anatomical connections demonstrated here as revealing candidate brain nuclei for the production of vocal behaviors. The connections of these nuclei in *Xenopus laevis* (Fig.10) can be directly compared to a neural model for calling behavior proposed

by Schmidt (1974, 1976; see Schmidt's 1976, Fig. 7) based primarily on lesion and electrical stimulation in *Rana pipiens*. In Schmidt's model, vocal motor neurons receive input from an inspiratory phase generator and from a vocal (expiratory) phase generator. The pretrigeminal nucleus (DTAM) participates, together with more posterior brain nuclei, in the generation of the vocal phase. The major afferent input to DTAM is the anterior preoptic nucleus. Extensive interconnections between these brain regions are postulated including input from DTAM to the contralateral vocal phase generator and reciprocal connections between vocal motor neurons and the inspiratory phase generator in each 'half' brain.

Nucleus IX–X of *X. laevis* contains laryngeal (Kelley 1980) and glottal (unpublished) motor neurons. The individual clicks which comprise *X. laevis* calls are produced by contractions of laryngeal muscles (Yaeger 1982; Tobias and Kelley 1985); glottal opening accompanies laryngeal contractions. Thus, n. IX–X motor neurons in *X. laevis* have a similar function to laryngeal and glottal muscles in *R. pipiens* (Schmidt 1971). However, *X. laevis* typically call while submerged; a silent period analogous to the 'inspiratory phase' of *R. pipiens* is not observed. The mate call of male *X. laevis*, nonetheless, consists of fast and slow trill phases (Wetzel and Kelley 1983) and it may be that these separable call components are subserved by neurons analogous to those coordinating inspiratory and vocal phases in ranids. The most likely location of these 'respiratory phase' neurons is in nuclei reticulares medii and/or inferiores. Schmidt (1976) describes the inspiratory phase generator in *R. pipiens* as being composed of small cells, medial to the hypoglossal nucleus. The location of inferior reticular nuclei providing input to n. IX–X and DTAM in *X. laevis* (especially Ridl) corresponds to Schmidt's assignment for the inspiratory phase generator. The location of the vocal or expiratory phase generator is in question. Schmidt (1976) postulates that it comprises DTAM and more posterior medullary nuclei because electrically stimulated patterned vocal activity can be disrupted by a small lesion posterior to DTAM. This more posterior brain region might be Rm. Another possibility is that recurrent collaterals of n. IX–X cells that project to DTAM are involved in the generation of vocal phase in *R. pipiens* or of different trill rates in *X. laevis*.

One characteristic of Schmidt's isolated brainstem preparation (1976) is that 'calling correlates', recorded from the laryngeal nerve can be evoked by APOA stimulation in an isolated 'hemi-brain' (transected at the midline). The result implies that the circuitry necessary for generation of the vocal pattern is present as complete ipsilateral pathways; the present study supports that interpretation. In addition, we observe extensive contralateral input to DTAM and n. IX–X from homologous nuclei and reticular formation. Activity in both laryngeal nerves must occur in order for the larynx to produce clicks (Tobias and Kelley 1985); contralateral connections should facilitate the coordination of activity in laryngeal motor neurons. We do not, however, observe contralateral input from telencephalic or diencephalic nuclei to DTAM in contrast to Schmidt's (1984) findings in *Rana*.

The present results in *X. laevis* thus agree quite closely with those of Schmidt as regards the central role of DTAM in vocal pathways. Our anatomical results indicate that DTAM has extensive reciprocal connections with n. IX–X and inferior reticular nuclei. The location of DTAM and its connections closely resemble those of a superior reticular formation nucleus in mammals, the Kolliker-Fuse nucleus. A recent experimental study in rats (Travers and Norgren 1983) revealed that injection of HRP-WGA into nucleus ambiguus (homologous to n. IX–X of anurans) results in many, heavily labelled cells in the Kolliker-Fuse nucleus. Nucleus ambiguus of rats contains motor neurons subserving ultrasonic vocalization (e.g. Wetzel et al. 1980) and it may be that homologues of DTAM play a role in vocal behavior in a variety of vertebrate species.

Nucleus DTAM in *X. laevis* also receives input from APOA, ventral striatum and thalamus. Schmidt's (1976) lesion and stimulation studies in *R. pipiens* identified only an APOA input to DTAM; however, in a later study (Schmidt 1984) stimulation near the amygdala (just caudal to VST) elicited activity correlated with the ranid vocalization chuckling. Halpern's (1972) anatomical investigations in *R. pipiens* revealed a ventral striatal projection to a restricted region of posterior tegmentum, most probably DTAM. Thus both VST and thalamic input could influence the generation of calls in *R. pipiens* and, by analogy, in *X. laevis* as well. There is reason to believe that much of this afferent input to DTAM conveys auditory information. Auditory units have been recorded in central thalamus (Mudry et al. 1977; Mudry and Capranica 1980) and ventral striatum and POA (Urano and Gorbman 1981) of *R. pipiens*. A 2-deoxy-D-glucose study in *X. laevis* revealed a number of auditory nuclei including torus semicircularis and central thalamus (Paton et al. 1982). Neurons in the vicinity of DTAM in *Rana pipiens* respond to auditory stimuli (Schmidt 1971; Aitken

and Capranica 1984). These anatomical connections could subserve acoustically-evoked or modified calling behaviors. In addition, calling in *X. laevis* is influenced by tactile stimuli and, perhaps, near field pressure changes conveyed by the lateral line system (Wetzel and Kelley 1983; Görner 1973). We did not observe any direct input from primary or secondary tactile (head, shoulders, trunk) or lateral line nuclei to DTAM or n. IX–X (see Lowe and Russell 1982; Altman and Dawes 1983; Simpson et al. 1986 for a description of these regions in *X. laevis*). The most likely sources of any specific sensory input (auditory, tactile, lateral line) to DTAM are the thalamic nuclei (CT, VMT and VLT) described here.

Mate calling of male *X. laevis* depends on the presence of circulating gonadal steroids, testosterone or dihydrotestosterone (Wetzel and Kelley 1983). The targets of these androgens include the laryngeal muscles (Segil et al. 1983) and the following CNS nuclei: n. IX–X, Ri, DTAM, CT, APOA, VIN and VST (Kelley et al. 1975; Kelley 1980). Results of the present study indicate that these hormone-concentrating brain nuclei are extensively inter-connected. With the exception of VIN, which is most probably involved in gonadotropin control (Kelley et al. 1975), all of the brain nuclei which contain androgen-accumulating neurons are also candidates for participation in the control of vocal behavior. Androgens have been found to influence biophysical events in androgen-concentrating clasping motor neurons (Erulkar et al. 1981) and more recently have been shown to alter ACh-activated channel conductances and kinetics in androgen-concentrating muscle (Erulkar and Wetzel 1985). It seems reasonable to suppose that the androgen receptor present in target neurons of 'vocal' nuclei contributes to the control of mate calling exerted by androgens in *X. laevis*.

Female *X. laevis* possess an androgen-responsive larynx (Segil et al. 1985) and androgen-accumulating neurons in 'vocal' brain nuclei (Kelley et al. 1975; Kelley 1981). They cannot, however, produce the male-typical vocal pattern when treated with androgen in adulthood (Hannigan and Kelley, submitted; Tobias and Kelley 1985). Many of the brain nuclei believed to subserve vocal behaviors in *X. laevis* are sexually dimorphic. These sex differences include fewer and smaller n. IX–X neurons (Hannigan and Kelley 1981) with less extensive dendritic trees (Kelley and Fenstemaker 1983) in females than in males. We report here that the connections of nucleus DTAM appear different in males than in females. In particular, the projection from n. IX–X to DTAM seems less robust in females as does the POA to DTAM projection. One possibility is that transport of HRP–WGA is less active in females due to a lack of neuronal activity (Ceccarelli et al. 1973) and thus these projections are more difficult to demonstrate. Another is that fewer n. IX–X cells of females send collaterals to DTAM or that the collaterals are shorter. The latter hypothesis is in keeping with recent findings that n. IX–X cell dendrites are shorter in females; androgen treatment does not increase dendritic length in adulthood (Kelley and Shih, unpublished). Thus the failure of androgens to induce mate calling in females may be due to dimorphisms in the neuroanatomical connections underlying this behavior. Comparison of neuroanatomical connections in male and female brains have yielded the general conclusion that the circuitry is present in both sexes though the cells and thus number of fibers connecting two nuclei may be reduced in females (Gurney 1981; Arnold and Gorski 1984; Konishi and Akutagawa 1985). The present results support that conclusion with the exception that one specific connection (n. IX–X to DTAM) may be absent in female *X. laevis*. If this connection plays an important role in generating the male specific vocal pattern, its absence in females could contribute to their inability to mate call.

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