

## Mapping the Auditory Central Nervous System of *Xenopus laevis* with 2-Deoxyglucose Autoradiography

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(Accepted March 18th, 1982)

**Key words:** deoxyglucose autoradiography — auditory pathway — *Xenopus laevis* — laminar nucleus of torus semicircularis

We report here on our use of 2-deoxyglucose autoradiography to identify acoustically-sensitive nuclei in the brain of the South African clawed frog, *Xenopus laevis*. The superior olive, lateral lemniscus, torus semicircularis, and posterocentral thalamus all selectively accumulated radioactively labeled deoxyglucose during acoustic stimulation. In frogs with middle ear bones removed on one side, the laminar nucleus of the torus was more heavily labeled on the side opposite the intact ear. Some cells within this area are known to concentrate sex hormones. This finding is, to our knowledge, the only example of a hormone-sensitive auditory nucleus in vertebrates.

### INTRODUCTION

The auditory system plays an important role in the breeding behavior of frogs and toads. The calls of male frogs serve as territorial markers in male–male competition, and attract sexually responsive females of the same species<sup>36</sup>. Because these interactions only occur during the breeding season, researchers have speculated that the frog auditory system might be sensitive to sex hormones<sup>1,27</sup>. Support for this possibility has come from autoradiographic studies of sex hormone localization in the brains of frogs<sup>8–12,15</sup>. In these studies, of the nuclei which concentrate hormone, one in the torus semicircularis is presumed — based on its anatomical location — to be auditory.

To confirm this tentative identification we have used the 2-deoxyglucose (2-DG) method to determine the acoustically-sensitive nuclei in the brain of *Xenopus laevis*. Radioactively labeled 2-DG, introduced systemically, accumulates in cells in proportion to their metabolic activity<sup>33</sup>, and it has been found that these changes in metabolism parallel

changes in neural activity within the brain<sup>32</sup>. Thus the auditory pathway can be mapped by comparing 2-DG accumulation in the brains of acoustically stimulated animals with 2-DG accumulation in the brains of control animals<sup>26,30–32,35</sup>.

In this paper we report that specific medullary, mesencephalic and diencephalic brain regions of intact male *Xenopus laevis* selectively accumulated radioactive 2-DG during acoustic stimulation. Among the acoustically-sensitive areas, the one that was the most sensitive — the laminar nucleus of the torus — is known to concentrate sex hormones<sup>9–11, 15</sup>. An abstract containing some of these results has been presented<sup>28</sup>.

### MATERIALS AND METHODS

#### *Subjects*

Twenty-two male *Xenopus laevis* (mean weight 47 g; range 31–61 g) were purchased from the Nasco Steinhilber Co., Fort Atkinson, WI. The experimental groups of animals are summarized in Table 1; more complete descriptions follow below.

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TABLE I

*Experimental groups*

Abbreviations: I = intact, DD<sub>s</sub> = 'deafened' with short recovery (1.5 h); DD<sub>l</sub> = 'deafened' with long recovery (>24 h); HD = 'hemi-deafened', X = present, — = absent.

<i>n</i>	<i>Operation</i>	<i>Sound</i>	<i>Perfusion</i>
6*	I	X	—
4	I	X	X
4	DD <sub>s</sub>	—	—
4	DD <sub>l</sub>	—	—
4	HD	X	—

\* One brain in this group was cut in the transverse plane and one was cut in the sagittal plane to confirm the identity of nuclei. These brains were not used for optical density measurements.

*Operation*

Twelve frogs were 'deafened', four on one side and eight on both sides, using the following procedure. Animals were anesthetized with MS222 (0.13% solution of tricaine methane sulfonate, Aldrich Chemicals) and the tympanic membrane and middle ear bones were removed through a small incision just caudal to the eye. The unilaterally deafened and 4 of the bilaterally deafened frogs were allowed 90 min post-operative recovery in tap water before injection with radioactive 2-DG. All of these animals were actively swimming and appeared recovered from anesthetic at that time. The other 4 bilaterally deafened animals were allowed to recover for 24 h. No statistical differences were seen between results from the two groups of bilaterally deafened animals, therefore the data were consolidated.

*Isotope and injection*

[<sup>14</sup>C]2-DG (337 mCi/mM; 0.048 mg/ml) was obtained from New England Nuclear. Immediately prior to injection, 50 μl of isotope was diluted with 50 μl of a 0.65% saline solution and injected into the dorsal lymph sac. As blood glucose levels for male *X. laevis* average 150 mg/dl, the amount of isotope delivered was a tracer quantity. Following the injection, frogs were placed in a glass aquarium (25 × 25 × 50 cm) filled with water at room temperature.

*Acoustic stimulation*

Conspecific vocalizations were used as the acous-

tic stimulus to ensure a strong response from the auditory system. A standard tape of conspecific vocalizations was broadcast through an underwater loudspeaker (University Sound, Model UW-30) located at one end of the aquarium. The tape lasted 110 min and contained 45 min of vocalizations recorded using a Massa Laboratories hydrophone (Model 1158) during sexual behavior of a male and a female *Xenopus laevis*. The peak sound pressure level measured at the centre of the aquarium was 30 Newtons/m<sup>2</sup> (120 dB re 20 μNewtons/m<sup>2</sup>) which approximated the sound level of the original calls. The tape was not presented to the bilaterally deafened group although they remained in the aquarium for the same time between injection and sacrifice as did the other groups.

*Tissue preparation*

Immediately following the conclusion of the tape, the frogs were sacrificed with an i.p. injection of Nembutal (1ml). Animals were perfused transcardially with 30 ml of 0.65% saline followed by 30 ml of 10% formalin. Non-perfused frogs were decapitated to reduce bleeding during brain removal. No statistical differences were seen between results from perfused and non-perfused groups, therefore the data were consolidated. In either case, the brain was removed within 15 min and frozen with powdered dry-ice.

*[<sup>14</sup>C]2-DG autoradiography*

The protocol used was similar to that originated by Sokoloff and previously described<sup>33</sup>. The tissue was sectioned in a Slee cryostat at -21 °C. All of the brains used for measurements were cut in the horizontal plane. One brain was cut in the sagittal and one in the transverse plane and these were used to confirm identifications of nuclei based on horizontal sections. The sections, 10 μm or 20 μm thick, were picked up on glass coverslips, and immediately dried on a slide warmer at 60 °C. The coverslips were then mounted on cardboard, placed in an X-ray cassette, and exposed to a sheet of X-ray film (Kodak SB5 or Dupont lo-dose mammography) for 1-3 weeks. Exposed film was developed with Kodak liquid X-ray developer, fixed, washed and dried. The source sections were retained for additional exposures or counterstained with cresyl violet acetate or luxol fast blue.

A series of [ $^{14}\text{C}$ ]-sources ([ $^{14}\text{C}$ ]-labeled methylacrylate, Amersham 196363) was used to determine the range of exposure over which optical density of autoradiographs was a linear function of [ $^{14}\text{C}$ ]-concentration. All of our experimental measurements fell within this range, that is, well below saturation of either the emulsion or the measuring method<sup>23</sup>.

#### Optical measurement of grain density

Optical density of autoradiographs was measured over 125  $\mu\text{m}$  diameter areas with a Photovolt Corporation photometer, mounted on a Zeiss standard microscope. All measurements of optical density were made relative to the optical density in an area not exposed to tissue. In order to reduce inter-animal variability due to differences in effective 2-DG dose, blood glucose levels and other factors, we normalized all measurements to a non-auditory area, the dorsal optic tectum, for each animal<sup>29</sup>. The optical density values for the darkest portions of selected brain regions with heavy accumulations of label were averaged over a minimum of 3 successive sections and then divided by the optical density of the optic tectum and the resultant ratio was used for inter-animal comparisons.

## RESULTS

Radioactive label was found throughout the brains of all animals, thus the 2-DG injected into the dorsal lymph sac was reliably transferred to the blood supply. Radioactivity was concentrated in certain areas of the brain which in autoradiographs were darker than surrounding areas. These patterns of labeling were consistent and will be described for each of the experimental groups.

#### Intact frogs

Following exposure to a 2-h tape of conspecific vocalizations, very heavy accumulations of silver grains were found in dorsal medulla, the superior olive, the interpeduncular nucleus, the lateral lemniscus, and the torus semicircularis of all intact frogs. Less dense silver grain accumulations were also seen in the anterior and posterior thalamus of all animals. In some frogs, radioactive material accumulated in the habenula, accessory olfactory

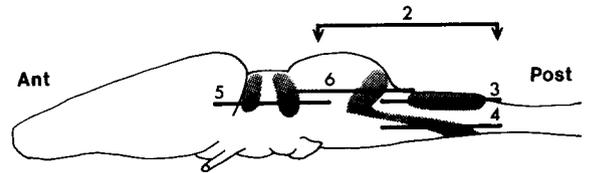


Fig. 1. Schematic side view showing areas of heavy 2-DG accumulation in the *Xenopus laevis* brain. The location of sections used in subsequent figures are displayed as a line with arrows for parasagittal sections (Fig. 2) and as lines for horizontal sections (Figs. 3-6).

bulb, medial septum, and dorsal and ventral striatum. These latter regions were not consistently labeled in all animals. Brain regions which reliably accumulated above average amounts of radioactive label are shown schematically in Fig. 1 and are described, from caudal to rostral, below.

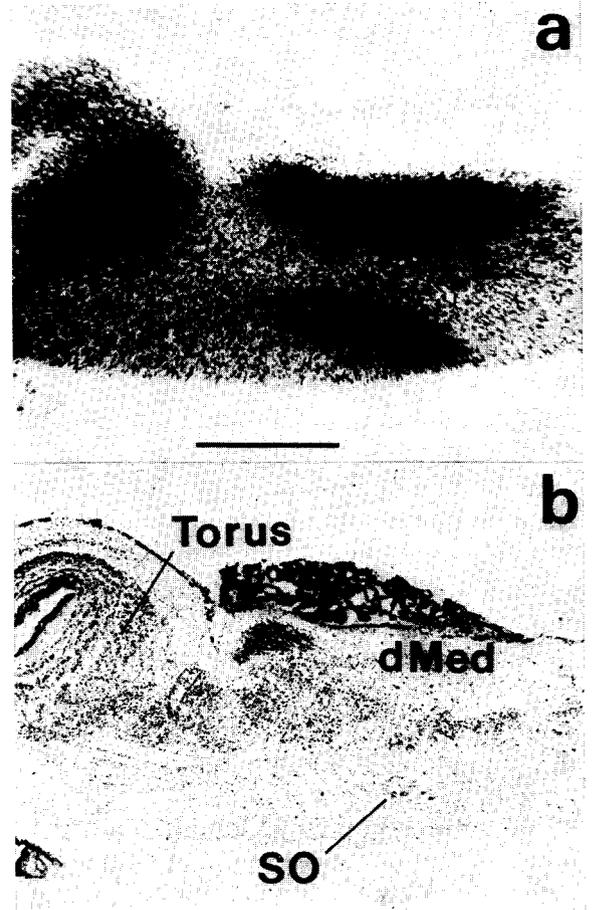


Fig. 2. Parasagittal section through the medulla and torus semicircularis. Left is anterior and top is dorsal. (a) 2-DG autoradiogram, (b) corresponding section stained with cresyl violet. Bar is 1 mm. dMed, dorsal medulla; SO, superior olive; Torus, torus semicircularis.

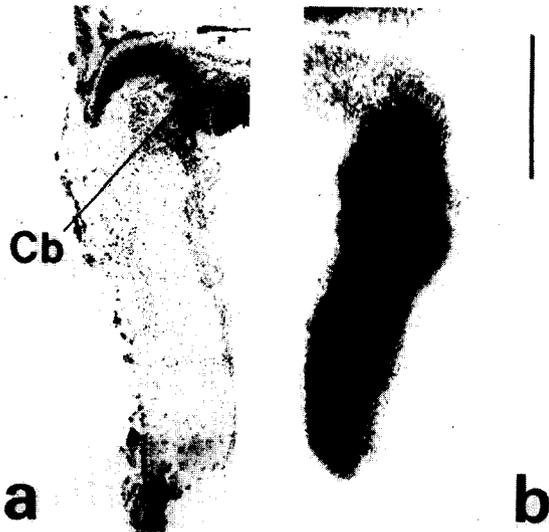


Fig. 3. Horizontal section through the dorsal medulla. Top is anterior. (a) left half of cresyl violet stained section, and (b) corresponding 2-DG autoradiogram, photographically reversed for comparison with (a). Bar is 1 mm. Cb, cerebellum.

The dorsal medulla was one of the most heavily labeled regions of the brain. Labeling occupied the dorsal third of the brainstem from the cerebellum posterior to the level of the obex (Figs. 2 and 3). The dorsal medulla contains: (1) a dorsolateral auditory nucleus; (2) a dorsomedial lateral line nucleus; and (3) ventral vestibular nuclei. The auditory nucleus was identified by its relatively small densely packed cell bodies and by its dorsolateral position. The lateral line nucleus lay adjacent to the fourth ventricle and was sparsely populated with cell bodies. The vestibular area contained large cell bodies. All three areas were heavily labeled.

The superior olivary nucleus was the most densely labeled structure in the ventral medulla. This nucleus is directly ventral to the lateral line and eighth nerve nuclei (Fig. 4) and runs diagonally from posteromedial to anterolateral. The extent of the superior olivary nucleus could be seen in cresyl violet-stained sections (Fig. 4b). Heavy 2-DG labeling extended rostrally past the boundary of the superior olive, to a cell-free region. This region was the origin of the lateral lemniscus, the pathway which projects from the superior olive to the torus semicircularis.

At the level of the cerebellum a dark spot appeared along the ventral midline (Fig. 4). In cresyl violet-stained tissue this area was a small nucleus just

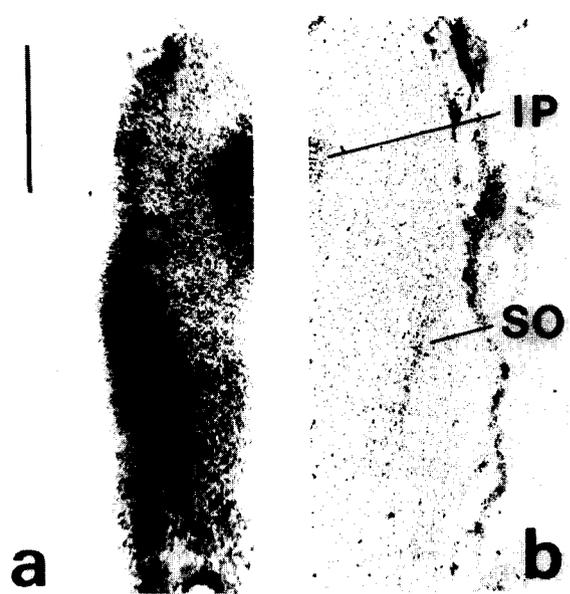


Fig. 4. Horizontal section through the ventral medulla. Top is anterior. (a) 2-DG autoradiogram of right half of section, photographically reversed for comparison with (b), and (b) cresyl violet view of same tissue used to produce autoradiogram in (a). Bar is 1 mm. IP, interpeduncular nucleus; SO, superior olivary nucleus.

posterior to the fourth motor nucleus and thus has been identified as the interpeduncular nucleus<sup>20</sup>.

The lateral lemniscus sweeps anterior and then dorsolateral at the level of the optic tecta to end in another densely labeled area, the torus semicircularis. The anatomy of the torus in the bullfrog has been described by Potter<sup>21</sup>. In *Xenopus* at least two separate dark areas could be distinguished in 2-DG autoradiograms of the torus (Fig. 6): (1) a caudal area; and (2) a rostral area just posterior to the ventricle. The caudal torus consisted of two oval areas, one on either side of the midline. In cresyl violet sections (Fig. 6, right) these corresponded to the principal nuclei of the bullfrog. The rostral area was more elongated along the mediolateral axis and corresponded to a region of alternating cell and fiber layers between the principal nucleus and the optic ventricle. Because of its laminar structure and its position we tentatively identified the rostral area as the laminar nucleus, although this region appeared to have many more layers than in the bullfrog.

Several areas in the thalamus incorporated above average amounts of 2-DG. One of these areas, the posterocentral nucleus<sup>6</sup>, was readily identified in 2-DG autoradiograms as an isolated dark area ventral



Fig. 5. Horizontal section through the dorsal thalamus. Top is anterior. (a) 2-DG autoradiogram, and (b) corresponding cresyl violet stained section. Bar = 1 mm. PL, posterolateral nucleus of the thalamus; PC, posterocentral nucleus of the thalamus.

to the posterior commissure and adjacent to the third ventricle (Fig. 5b). This region appeared in horizontal cresyl violet sections as a laminated nucleus immediately posterior to the cup-shaped laminations of the posterolateral nucleus of the thalamus (Fig. 5). Areas in the anterodorsal and anteroventral portions of the thalamus also were dark on autoradiograms but the nuclei were poorly differentiated and thus difficult to compare with similar areas in ranids.

Rostral to the thalamus the darkest areas in 2-DG autoradiograms were the habenula and the accessory olfactory bulb; however, these areas were not dark in all animals. Areas of high 2-DG uptake in the telencephalon showed marked variability from animal to animal and will not be discussed further in this paper.

#### Sound-deprived animals

The intact animals previously described were stimulated with sounds which presumably excited the auditory system and possibly the lateral line system. In the group of animals described in this section we reduced acoustic stimulation by removing the tympanic membrane and middle ear bones on both sides — which reduces sound transmission by about 20 dB in frogs<sup>5,14</sup> — and by leaving these animals in a quiet tank for the duration of 2-DG incorporation. This treatment should reduce neural activity in nuclei which process acoustic information.

In the sound-deprived animals a number of areas showed decreased labeling relative to the same areas

in the intact sound-stimulated group. The most striking example of this is the laminar nucleus, which can no longer be distinguished from the autoradiogram alone (Fig. 6, middle panel). All of the presumed auditory nuclei which were examined showed a significant decrease in label due to sound deprivation ( $P < 0.05$ , Mann-Whitney U-test; see Table II). A significant decrease was also measured in the lateral lemniscus fiber tract.

Two nuclei which are not known to respond to auditory stimuli were also examined. The nucleus isthmi did not show a significant change in label due to sound deprivation (see Table II). The interpe-

TABLE II

*Effect of sound stimulation on 2-DG uptake within the brain*

This table contains autoradiogram optical densities at a number of locations within the brain. All measurements were normalized to the optic tectum, and averaged over the sample of animals. Each entry contains this average, the corresponding standard deviation, and the sample size.

	Stimulated	Deprived	Ratio	P*
Superior olive	2.1 ± 0.4 (7)	1.4 ± 0.1 (8)	1.6	< 0.01
Lateral lemniscus	2.5 ± 0.2 (8)	1.5 ± 0.3 (8)	1.7	< 0.01
Caudal torus	2.7 ± 0.4 (8)	2.1 ± 0.3 (8)	1.3	< 0.01
Rostral torus	2.8 ± 0.3 (4)	0.87 ± 0.2 (8)	3.2	< 0.01
Posterocentral thalamus	1.5 ± 0.2 (4)	1.0 ± 0.2 (7)	1.4	< 0.05
Interpeduncular nucleus	1.7 ± 0.2 (7)	1.3 ± 0.1 (8)	1.3	< 0.05
Nucleus isthmi	1.0 ± 0.2 (4)	0.8 ± 0.1 (8)	1.2	N.S.D.

\* Mann-Whitney U-test, single-tailed.

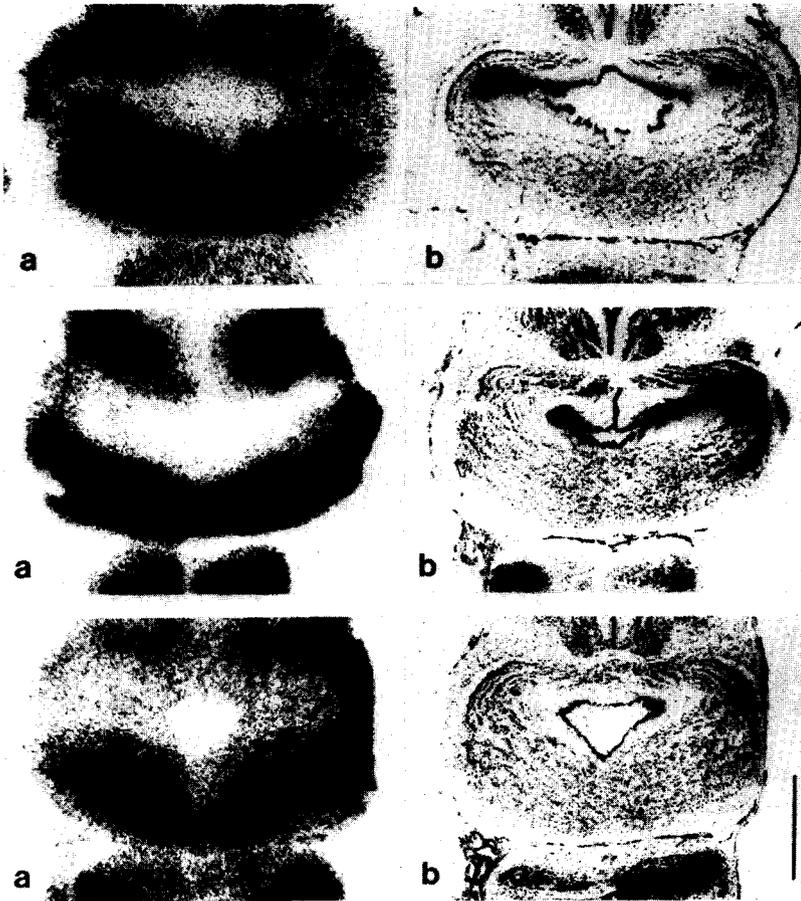


Fig. 6. Effect of deafening and hemi-deafening on 2-DG uptake within the torus semicircularis. Horizontal sections, top is anterior. (a) 2-DG autoradiogram, and (b) corresponding section, stained with cresyl violet. Top: acoustically stimulated animal. Middle: acoustically deprived animal. Bottom: hemi-deafened animal. The left tympanic membrane and middle ear bones were removed prior to acoustic stimulation. Bar is 1 mm. r, rostral torus; c, caudal torus.

duncular showed a small but significant decrease in label due to sound deprivation.

#### 'Hemi-deafened' animals

In 4 of our animals the tympanic membrane and middle ear bones were removed on one side to attenuate input to the inner ear, and then the animals were stimulated with sound. Autoradiograms were examined for differences in labeling between the two sides.

In all 4 hemi-deafened animals the laminar nucleus of the torus was asymmetrically labeled as in the example shown in Fig. 6 (bottom panel). The laminar nucleus was always lighter on the side *opposite* to the damaged middle ear. The ratio of contralateral density to ipsilateral density averaged  $1.47 \pm 0.15$  and was significantly greater than the null

hypothesis ratio of 1.0 (paired sample *t*-test). On the other hand both the caudal torus and the lateral lemniscus as it entered the torus were symmetrically labeled with differences of less than 10% between the two sides.

#### DISCUSSION

The auditory system of anuran amphibians has been studied most extensively in ranids<sup>2</sup>. In this group of frogs the auditory nerve leaves the inner ear to innervate the dorsal acoustic area of the medulla. This nucleus projects bilaterally to the superior olivary nuclei and contralaterally to the dorsal acoustic area. The superior olive projects via the lateral lemniscus to nuclei of the torus semicircularis, which project in turn to the posterocentral nucleus of the thalamus<sup>19</sup>.

All of these auditory nuclei, from the medulla to the thalamus, were heavily labeled with 2-DG in our experiments on *Xenopus leavis*. Furthermore the superior olive, lateral lemniscus, torus semicircularis, and posteroventral thalamus were more heavily labeled in sound-stimulated animals than in sound-deprived animals. These increases in labeling appear to be specific — that is, not due to general arousal or increased motor activity — since all of the measurements were normalized to a non-auditory area, the optic tectum. Furthermore, there was no significant change in label over nucleus isthmi, a visual area<sup>7</sup> which does not appear to receive input from any auditory nuclei<sup>24</sup>. It should also be noted that the vestibular and lateral line organs as well as the ear are sensitive to certain forms of acoustic energy<sup>16,25</sup> so the terms acoustic or sound sensitive are not synonymous with auditory.

These findings of sound sensitivity in *Xenopus* based on changes in metabolic activity broadly parallel those based on electrophysiological recordings in ranids, but with at least two exceptions. Acoustically-evoked potentials have been recorded in the ventral striatum of the telencephalon<sup>18</sup>, yet this area was not always labeled in sound-stimulated animals. Neurons in this higher level auditory nucleus respond to a limited range of acoustic stimuli and habituate rapidly. Thus we may have evoked changes in the telencephalon which were beyond the sensitivity or resolution of the 2-DG method. Another unexpected finding was that the label in the interpeduncular nucleus was affected by acoustic stimulation. The interpeduncular nucleus has reciprocal connections with the habenula<sup>13</sup> but neither the habenula nor the interpeduncular nucleus are known to receive auditory input. However, the interpeduncular nucleus may receive indirect auditory excitation such as that found recently in the hypothalamus<sup>34</sup>.

In addition to confirming tentative identifications of sound-sensitive nuclei, the 2-DG results offer new information on the organization of the torus semicircularis. In ranids, the primary input to the torus is from the lateral lemniscus, which strongly innervates the ipsilateral principal nucleus of the torus but not the laminar nucleus of the torus<sup>24</sup>. The laminar nucleus of the torus is a series of thin layers of cells adjacent to the rostral surface of the princi-

pal nucleus. The boundary between the laminar and principal nuclei is poorly defined; small cells in both nuclei have processes which project to the center of the principal nucleus<sup>21</sup>. Laminar nucleus cells could thus have access to auditory input, either from the lateral lemniscus or from cells of the principal nucleus. In fact, electrophysiological findings indicate that both the laminar and principal nuclei respond to acoustic stimulation<sup>4,21,22</sup>.

Based on examination of cresyl-, Golgi- and steroid-labeled tissue from *Xenopus* midbrain, and comparison with similar tissue from *Rana pipiens*, we believe that the rostral and caudal areas labeled in our autoradiograms correspond to the laminar and principal nuclei, respectively. In our 2-DG autoradiograms both areas were sensitive to sound, which agrees with electrophysiological findings in other species<sup>4,21,22</sup>. Our results further suggest that these two nuclei differ in their response properties. The labeling of the laminar nucleus was more sensitive to acoustic stimuli than any other area measured, including the principal nucleus. In addition, the laminar nucleus was exquisitely sensitive to differences in acoustic intensity at the two ears. In contrast, the principal nucleus was always symmetrically labeled when the middle ear was removed on one side.

The laminar nucleus is especially interesting because some of its cells concentrate the sex steroids estradiol<sup>15</sup> and dihydrotestosterone<sup>9,10</sup>. It seems reasonable to ask whether auditory sensitivity or the ability to localize sounds might change with hormonal level in frogs. Such effects could contribute to the reproductive success of both males and females by facilitating mate localization during the breeding season.

#### ACKNOWLEDGEMENTS

We would like to thank Barbara Goun and Christina Szmauz for technical assistance, Rosanne Kelly for secretarial services, and Dr. Donald Griffin for the loan of the hydrophone used to record *Xenopus* calls. This work was supported by PHS Fellowship 5 F32 NS05648-02 (J.A.P.), NIH Fellowship MH07670 (T.J.S.), NIH NRSA 7524 (M.L.Y.), and NIH Grant HD 12126 (D.B.K.).

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