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The Afferent Projections to the Anterior Part of the Preoptic Nucleus in Japanese Toads, *Bufo japonicus*

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ABSTRACT—The afferent innervations of the anterior part of the preoptic nucleus (APON), which is a presumed center for triggering reproductive behavior in anuran amphibians, were studied by use of a retrograde horseradish peroxidase (HRP) method in Japanese toads, *Bufo japonicus*. HRP was introduced into the APON through a bipolar theta-style glass microcapillary. Evidence of transported enzymatic activity was observed in perikarya and neuropil of the limbic cortex, the posterior part of the preoptic nucleus including the magnocellular part, the thalamic area, and the subtectal and tegmental regions including the reticular formation. Neurons in these regions appear to send their axons to the APON mainly *via* the medial and the lateral forebrain bundles, since HRP activity was distributed in these fiber structures in a continuous pattern from the APON to the region mentioned above. Localization of some HRP-labeled perikarya and fibers coincides with that of immunoreactive perikarya and fibers containing either luteinizing hormone-releasing hormone, vasotocin or thyrotropin-releasing hormone which have been considered to project to the APON.

INTRODUCTION

The preoptic area is an important locus for initiating sexual behavior in many vertebrate species, such as teleosts [1], amphibians [2, 3], reptiles [4], birds [5], and mammals [6, 7]. In the anuran brain, the region concerned with male mate calling has been localized experimentally in the anterior part of the preoptic nucleus (APON). Sexual calling has been induced by implantation of testosterone pellets in or close to the APON [8] or by electrical stimulation of this region [9, 10]. Since there were many sex steroid-accumulating neurons in the APON [11], neuronal activity in this locus can be regulated by circulating sex steroids, plasma levels of which are probably determined by luteinizing hormone-releasing hormone (LHRH) through the hypothalamo-hypophysial-gonadal axis [12]. Moreover, many neurons which contain hypothalamic neurohormones, such as LHRH and vasotocin [13] and thyrotropin-releasing hormone (TRH; Fujita and Urano, unpublished), send their immunoreactive axons to the APON. Significant seasonal changes were found in their immunological stainability, especially of LHRH [14] and TRH (Fujita and Urano, unpublished). Therefore, the APON can play an important role in initiation of seasonal breeding activity under modulatory control by these neurohormones [15], in addition to the influence of sex steroids.

Neural activity of the APON neurons, however, seems to be controlled primarily by neural signals, such as a conspecific acoustic signal which excited a considerable number of APON neurons in *Rana pipiens* [16]. Neurohormones may have a role to modulate such neural signals, as has been discussed previously [13]. Then information on the APON afferents would permit better understanding of the sensory modalities and activating or inhibitory pathways that might trigger or modulate sexual behavior through the APON. We therefore examined the afferent connections of the APON with other parts of the brain in Japanese toads (*Bufo japonicus*) by use of a retrograde horseradish peroxidase (HRP) method. In the present study, special attention was paid to confirm whether perikarya in the regions which contain either LHRH, TRH or vasotocin neurons can be actually labeled with HRP.

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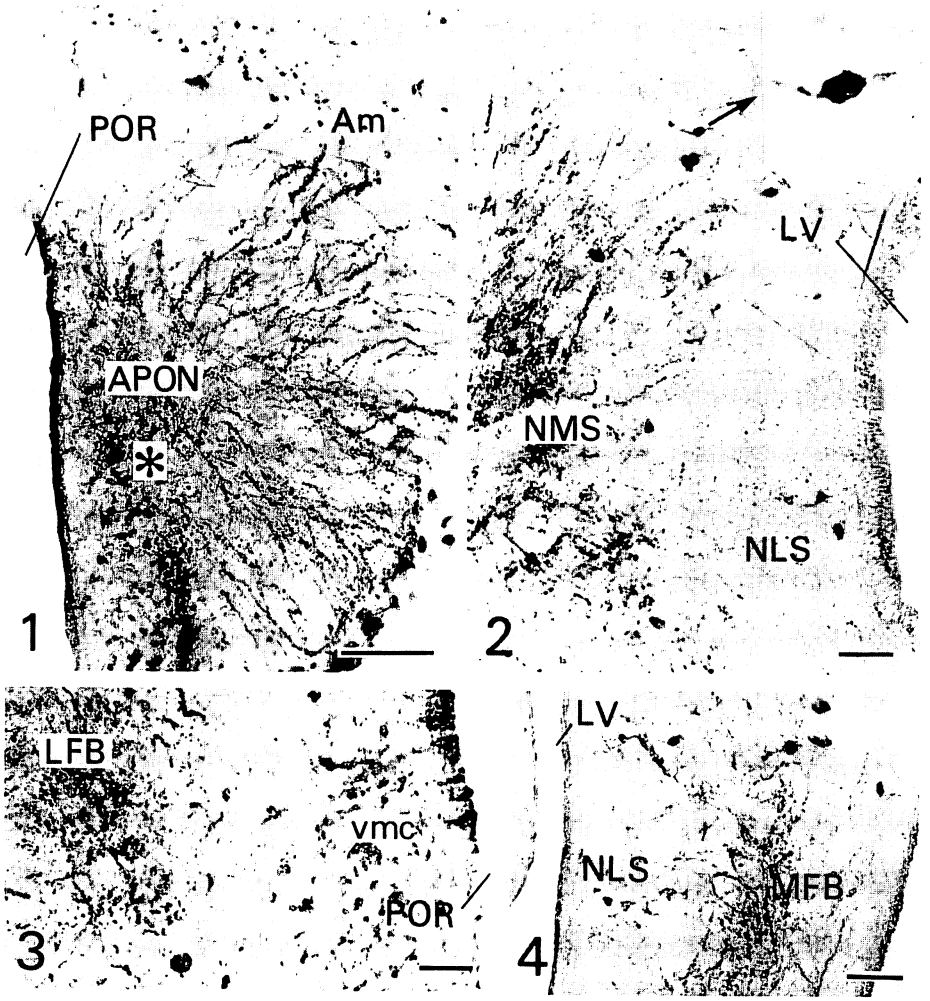

FIG. 1. HRP reaction product at the injection site in the APON (*). Note HRP-labeled fibers which connect mutually the APON with the amygdala pars medialis to form the medial amygdala-APON complex. Scale, 100 μm.

FIG. 2. HRP-labeled perikarya and fibers in the nuclei medialis septi and lateralis septi. Inlet shows higher magnification of the labeled neuron indicated by the arrow. Scale, 100 μm.

FIG. 3. HRP-labeled neurons in the ventral magnocellular part of the preoptic nucleus, and HRP-labeled fibers in the lateral forebrain bundle. Scale, 100 μm.

FIG. 4. HRP-labeled fibers in the medial forebrain bundle at the level of the nucleus accumbens septi. Scale, 100 μm.

Abbreviations for FIGS. 1–10. Al: amygdala, pars lateralis. Am: amygdala, pars medialis. APON: anterior part of the preoptic nucleus. AVA: area ventralis anterior thalami. AVL: area ventrolateralis thalami. CBL: cerebellum. dpv: dorsal periventricular part of the preoptic nucleus. GC: griseum centrale rhombencephali. IR: infundibular recess. LV: lateral ventricle. LFB: lateral forebrain bundle. MFB: medial forebrain bundle. NAD: nucleus anterodorsalis tegmenti mesencephali. NAS: nucleus accumbens septi. NAV: nucleus anteroventralis tegmenti mesencephali. NCER: nucleus cerebelli. NDB: nucleus of the diagonal band of Broca. NDMA: nucleus dorsomedialis anterior thalami. NI: nucleus isthmi. NID: nucleus infundibularis dorsalis. NIP: nucleus interpeduncularis. NIV: nucleus infundibularis ventralis. NLS: nucleus lateralis septi. NMS: nucleus medialis septi. NOA: nucleus olfactorius anterior. NPC: nucleus posterocentralis thalami. NPD: nucleus posterodorsalis tegmenti mesencephali. NPL: nucleus posterolateralis thalami. NPV: nucleus posteroventralis tegmenti

MATERIALS AND METHODS

Animals Twenty-seven adult Japanese toads of both sexes (snout to vent, 11–16 cm; body weight, 80–417 g), captured by an animal collector in June, were used in this study. Toads were anesthetized by injecting MS222 (tricaine methanesulfonate, 0.1 mg/g body weight) into the dorsal lymph sac, and were positioned supine in a stereotaxic apparatus. HRP was injected into the APON by ventral approach.

Injection of HRP Theta-style glass microcapillaries were used for electrophoretic application of HRP. Both of the two barrels of the microcapillary were filled with a 5% solution of HRP (Sigma, type VI) in 0.05 M phosphate buffer (pH 7.4) containing 0.16 M NaCl. The tips of the capillaries were then broken to yield a tip diameter of 20 to 30 μm, and were bevelled with a settled slurry of 0.5 μm alumina powder in saline. The glass capillaries were introduced into the APON through a small trephined hole in the parasphenoid bone under stereo-microscopic guidance. The electrode tip was located at a point 500 μm anterior to the anterior margin of the optic chiasma, 150 to 200 μm lateral to the preoptic recess, and 300 to 500 μm deep under the ventral surface of the lamina terminalis. HRP was applied electrophoretically by use of negative 0.5 sec rectangular pulses of 10 μA between the tips of the adjacent barrels; currents were given at intervals of 1 sec for 30 min. The capillaries were left in place for 15 min after completion of the HRP injection.

Preparation of tissue sections Animals were anesthetized with MS222 at times from 6 hr to 48 hr after HRP injection. They were perfused through the heart with 30 ml of frog Ringer's solution, and then with 60 ml of an ice-cold fixative which contained 1% paraformaldehyde and 1.25% glutaraldehyde in 0.05 M phosphate buffer (pH 7.4). Immediately after the completion of the perfusion, the brains were taken out and were further fixed by immersion in the fixative at 4°C for 1 to 3 hr. After the fixation, they were washed in cold 0.05 M phosphate buffer (pH 7.4) which was changed two times in 1 hr. The brains were then frozen, and were cut into 50 μm thick transverse sections on a frozen microtome. Tissue sections were floated in 0.05 M phosphate buffer (pH 7.4).

Histochemical procedure The distribution of HRP activity in the tissue sections was visualized by a histochemical procedure using tetramethyl benzidine as chromogen [17]. The sections were first preincubated at room temperature in a freshly prepared medium which contained 0.01% 3, 3′, 5, 5′-tetramethyl benzidine (TMB, Sigma), 0.1% sodium nitroferricyanide, 30% ethanol and HCl-acetate buffer (pH 3.3; final concentration, 0.01 M). After 20 min of preincubation, hydrogen peroxide (to a final concentration of 0.01%) was added to the medium, and the tissue sections were incubated at room temperature for 5 min. Reaction product in the brain sections was then stabilized at 0°C in a bath containing 9% sodium nitroferricyanide, 50% ethanol and acetate buffer (pH 5.0; final concentration, 0.01 M). Following stabilization for at least 20 min, the brain tissue sections were rinsed in distilled water, and were mounted on gelatinized glass slides. Afterward, the sections were counterstained in a 1% solution of neutral red in acetate buffer (pH 5.0) for 5 min, washed, dehydrated through graded alcohols, cleared in xylene and coverslipped with Permount (Fisher Sci. Co.).

Nomenclature The nomenclatorial usage in this paper is basically those of Wada *et al.* [18] and of Takami *et al.* [19].

RESULTS

The toad preoptic nucleus (PON) is clearly divided into anterior and posterior parts by a thin

mesencephali. NRIS: nucelus reticularis isthmi. NRM: nucleus reticularis medius. NRS: nucleus reticularis superior. OC: optic chiasma. PD: pallium dorsale. PL: pallium laterale. PLd: pallium laterale, pars dorsalis. PLv: pallium laterale, pars ventralis. PM: pallium mediale. POR: preoptic recess. PPON: posterior part of the preoptic nucleus. SGC: stratum griseum centrale tecti. SGP: stratum griseum periventricularis tecti. SGRN: stratum granulare cerebelli. SGS: stratum griseum superficiale tecti. ST: striatum. STv: striatum pars ventralis. TS: torus semicircularis. V: motor nucleus of the trigeminal nerve. VN: vomeronasal nerve. vmc: ventral magnocellular part of the preoptic nucleus. vpv: ventral periventricular part of the preoptic nucleus.

cell-poor zone [19]. The anterior part (APON) surrounds the preoptic recess as a packed mass of small neurons. After HRP injection, enzymatic reaction product could be traced in a continuous pattern from the APON to a series of presumed afferents, mainly through the medial and lateral forebrain bundles.

Reaction product of HRP activity was very dense at the injection site in the APON 6 hr through 48 hr after the electrophoretic injection. Figure 1 shows that diffusion of HRP was limited within the APON and the adjacent white matter. Even in the animal killed immediately after injection, reaction product was not found beyond the amygdala pars medialis. Meanwhile, enzyme activity in particular neuronal structures distant from the injection site increased gradually up to 48 hr after the injection.

Time course of changes in HRP labeling Six hours after the injection, many unipolar and bipolar neurons in the APON and the amygdala pars medialis at or adjacent to the HRP injection site were labeled heavily with the enzymatic reaction product (Fig. 1). Labeled processes of APON neurons projected to the amygdala pars medialis, while amygdala neurons sent their processes to the APON. The present HRP study thus confirmed the previous result by rapid Golgi method that these sexually dimorphic nuclei are connected each other to form a functional and

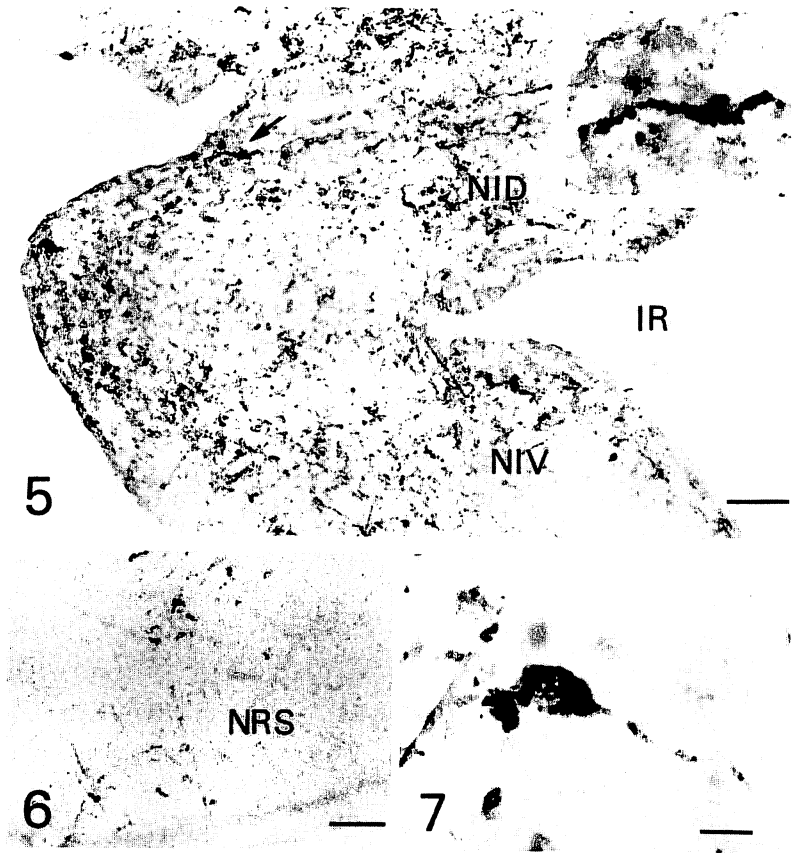

FIG. 5. HRP-labeled perikarya and neuropil in the infundibular region. Note that HRP-labeled neurons are localized in both the nuclei infundibularis dorsalis and ventralis. Inlet shows higher magnification of the neuron indicated by the arrow. Scale, 100 μm.

FIG. 6. HRP-labeled fibers in the nucleus reticularis superior. Scale, 100 μm.

FIG. 7. HRP-labeled perikaryon in the griseum centrale rhombencephali at higher magnification. Scale, 10 μm.

morphological complex [20, 24]. Rostrally, labeled perikarya and neuropil were found in the caudal region of the nucleus lateralis septi (Fig. 2), accumbens septi, and the nucleus of the diagonal band of Broca. Caudally, perikarya in the ventral magnocellular part of the PON were labeled with HRP (Fig. 3). A considerable number of labeled fibers were found in the medial and lateral forebrain bundles (Fig. 4). In brains of animals killed 12 hr after the injection, HRP activity spread further to the rostral region of the nucleus medialis septi, the pallium mediale, and the nuclei infundibularis dorsalis and ventralis (Fig. 5). In animals survived for 24 to 48 hr after the HRP injection, labeled perikarya and fibers were found throughout in many discrete brain loci, such as the nucleus olfactorius anterior, the area ventrolateralis and ventromedialis thalami, the mesencephalic reticular nuclei (Fig. 6), and the griseum centrale rhombencephali (Fig. 7). Most of the labeled perikarya were found ipsilaterally to the injection site. Notable diminution in magnitute of HRP labeling was not observed up to 48 hr after the injection. However, no heavily labeled neurons were observable even at the injection site in brains of animals sacrificed as long as 72 hr after the injection.

Distribution of HRP-labeled perikarya and fibers mentioned above indicates that afferent pathways to the APON may have origins in the limbic cortex, the thalamus, the brain stem, and the hypothalamus, and that afferent fibers may travel mainly through the medial and lateral forebrain bundles (Figs. 8 and 9). Further precise description for the distribution of HRP-labeled perikarya is given below. Since it is difficult to discriminate anterogradely labeled fibers from retrogradely labeled ones, the description for HRP-labeled fibers was limited mostly to those directly associated with the labeled perikarya.

The limbic cortex As shown in Figure 8, HRP-labeled perikarya in the telencephalon were dividable into dorsal and ventral groups according to their location and fiber pathways. The dorsal group was composed of neurons in the pallium dorsale and mediale, the rostral region of the nucleus medialis septi, a part of the nucleus lateralis septi, and the amygdala pars lateralis and medialis. HRP-labeled fibers continuous into these cell masses (Fig. 9) were closely associated with those in the medial forebrain bundle. Many HRP-labeled fibers were found in the white matter over the pallium dorsale and laterale. These fibers may be the efferents of the APON to these loci, since the enzyme reaction product in perikarya associated with these fibers was not found.

In the ventral region of the telencephalon, HRP-labeled perikarya were observed in the nucleus olfactorius anterior, the striatum, the nucleus of the diagonal band of Broca, the nucleus accumbens septi, and a part of the nucleus lateralis septi (Fig. 9). Labeled fibers associated with these structures seemed to converge with the fibers of the dorsal group in the medial forebrain bundle at the anterior margin of the preoptic area, and then projected posteriad to the APON (Fig. 8).

Immunoreactive LHRH neurons were localized

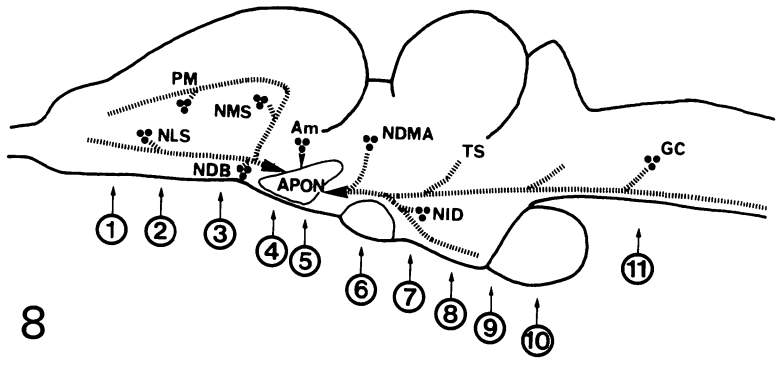

FIG. 8. Schematic diagram of the lateral view of the toad brain showing neurons (solid circles) and pathways (broken lines) labeled with HRP reaction products. Each number with an arrow shows the level of each frontal section in Figs. 9 and 10.

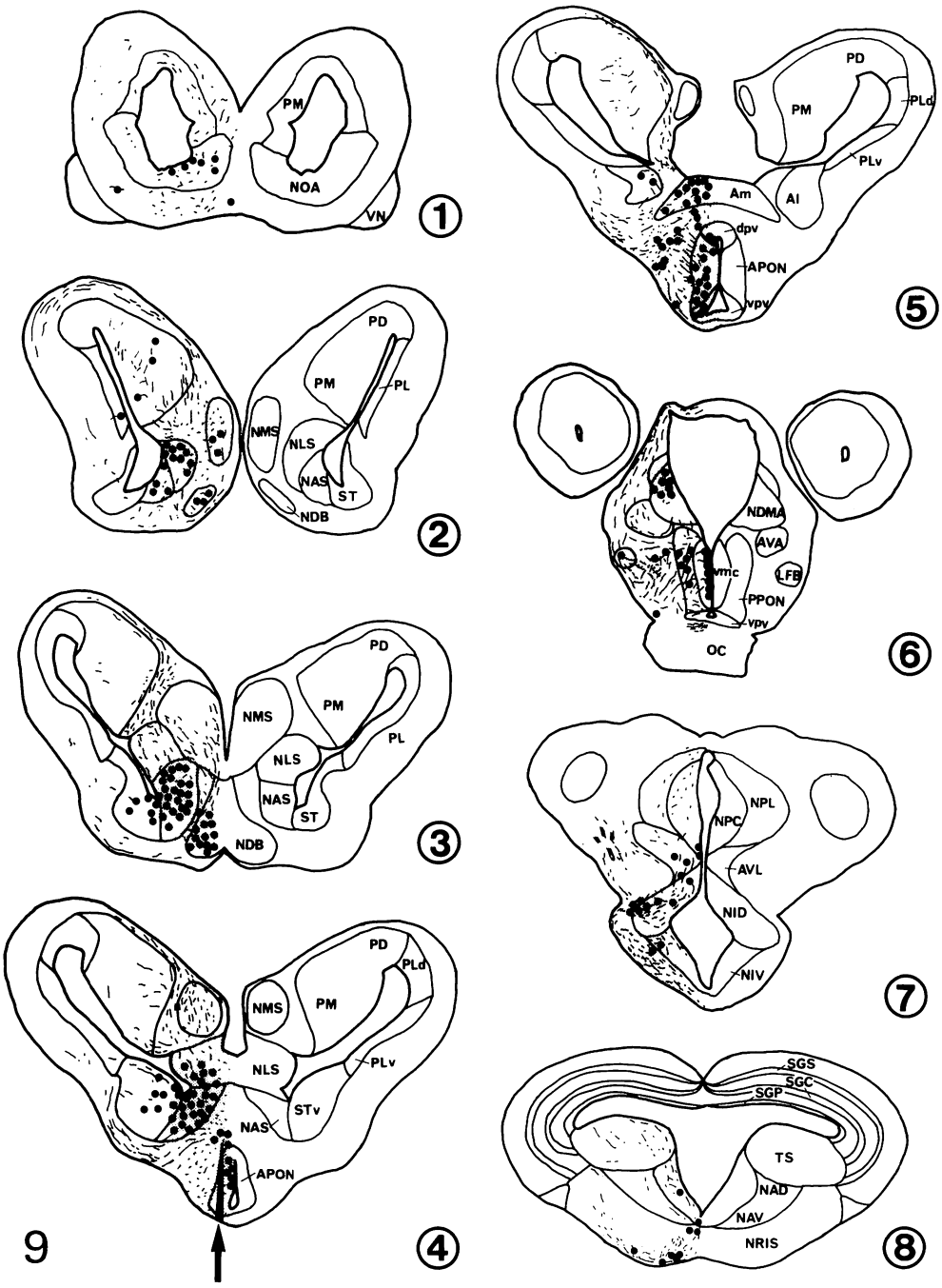

FIG. 9. Diagrams showing the distributions of HRP reaction product 48 hr after the injection of HRP into the APON. The number of each diagram indicates the level of the tissue section shown in Fig. 8. Solid circles indicate HRP-labeled perikarya, and fine lines HRP-labeled fiber structures. Shaded areas indicate the regions in which very many perikarya and fibers are heavily labeled with HRP reaction products. Continuation of Fig. 9 is placed in facing page as Fig. 10.

in the nucleus medialis septi and the nucleus of the diagonal band of Broca. They projected their immunoreactive fibers to the APON [14]. The localization of HRP labeled neurons in the nucleus medialis septi and the nucleus of the diagonal band of Broca seems to be comparable with the loci rich in immunoreactive LHRH perikarya.

The thalamus A few thalamic nuclei included HRP-labeled perikarya whose enzyme activity was mainly associated with that in the lateral forebrain bundle. Perikarya labeled with HRP reaction product were localized in the dorsal part of the nucleus dorsomedialis thalami, and the area ventromedialis and ventrolateralis thalami (Fig. 9).

The brain stem Although the number of labeled cells and fibers was not conspicuous in the brain stem, HRP activity was found in the tegumental white matter including the reticular formation (Fig. 6), and in the following mesencephalic and rhombencephalic nuclei (Figs. 9 and 10): the torus semicircularis, the nucleus anterodorsalis tegmenti mesencephali, the nuclei reticularis superior, isthmi and medius, and the griseum centrale rhombencephali. HRP-labeled fibers which ran through the white matter of the mesencephalon were continuous to the lateral forebrain bundle.

The hypothalamus Many APON neurons just adjacent to the injection site of HRP were heavily stained with the reaction product which filled their perikarya and processes. As was mentioned above, heavily labeled processes ran across the medial forebrain bundle to innervate into the amygdala pars medialis. Further, HRP-labeled processes arising from the APON proceeded toward the neurohypophysis along the preoptico-hypophyseal tract. The injected HRP presumably was taken up by somata and dendrites of these neurons, as in other vertebrates [21]. HRP taken up by the APON neurons then may be transported anterogradely to their nerve endings, e.g., in the amygdala pars medialis and the neurohypophysis.

In the posterior part of the PON, some HRP-labeled neurons were contiguous with the heavily labeled APON neuronal mass. The number of labeled neurons was higher in the ipsilateral side than in the contralateral side relative to the injection site. The region where these perikarya were observed is comparable with the magnocellular part rich in neurosecretory neurons, e.g., vasotocinergic cells [19] and mesotocinergic cells. HRP activity which was continuous with neuronal structures from the medial forebrain bundle was also observed in a small number of neurons in this region.

In both dorsal and ventral infundibular nuclei, HRP activity was found in a considerable number of perikarya. HRP-labeled fibers in this region were associated mainly with the preoptico-infundibular tract. The loci where HRP-labeled perikarya were found coincide with the region rich in immunoreactive TRH neurons (Fujita and Urano, unpublished).

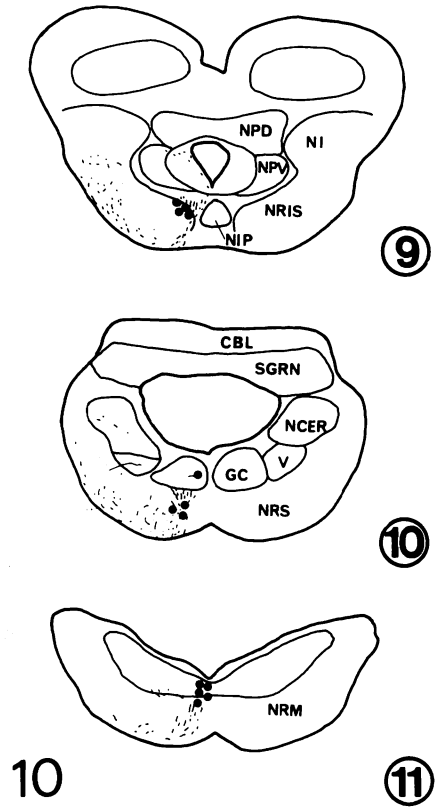

FIG. 10. Continued from Fig. 9.

DISCUSSION

The brain structures in which HRP activity was demonstrable after the injection of HRP into the

APON can be categorized into: the limbic system, the thalamo-preoptic system, the tegmento-preoptic system, the reticular system, and the intrahypothalamic connections. Traceable fiber pathways in these structures proceeded to the APON mainly *via* the medial and the lateral forebrain bundles.

In the present study, diffusion of electrophoretically applied HRP was limited within the APON and the adjacent white matter. It is then highly probable that the enzymatic reaction product found in perikarya in many regions distant from the APON represents localization of HRP retrogradely transported from the injection site. Since the tips of the glass capillaries were bevelled so as to minimize damage of brain tissues, the uptake of HRP by axons of passage may be negligible. Thus, major inherent sources of error in the retrograde HRP method [22] were circumvented by injecting HRP electrophoretically through fine glass microcapillaries, and had little bearing on interpretation of the present results.

Our study of HRP distribution confirmed the presence of mutual innervations between the amygdala pars medialis and the APON [20], showing that they form a morphological and probably functional complex. The presence of the amygdala-preoptic tract has been suggested in all vertebrate classes from cyclostomes to mammals [23]. Although the volume of the toad medial amygdala-APON complex is sexually dimorphic [24], we did not attempt to find sexual differences in their fiber connections, because of technical limitations. The septal projection to the preoptic area in the toad has an apparently homologous relationship to a similar pattern in the lizard [25] and the rat [26, 27]. A degeneration study by Halpern [28] had provided experimental evidence of the axonal connections between the medial part of the telencephalon and the preoptic area in *Rana pipiens*. The present study shows more precisely the origin of the telencephalic afferents to the APON, indicating that there is retrograde continuity from the APON to the septal nuclei *via* the medial forebrain bundle.

Continuity of HRP activity from the APON to the thalamic nuclei suggests thalamo-preoptic projections in the toad brain. These projections may coincide with the connection between the dorsal thalamic area and the hypothalamus which was referred to as the tractus pretecto-hypothalamicus and the tractus tecto-hypothalamicus anterior [29] and the thalamo-hypothalamic fibers [23, 30] in amphibian brains. Little or no functional value can be attributed to the thalamo-hypothalamic system in any vertebrate group [31].

Mesencephalic projections to the anterior hypothalamus are well known in amphibian brains [23, 29, 30, 32] as well as in other vertebrate classes [23, 33, 34]. In the present study, the probable origins of the mesencephalic projections to the APON of *Bufo japonicus* were localized in the subtectal area, the tegmental gray, and the mesencephalic reticular formation. In the mammalian brain, the preoptic area is directly continuous with a vast nonspecific neuronal apparatus of the brain stem reticular formation [35]. The ascending reticular activating system is in excellent position to exert an influence on sexual behavior [36]. In anuran brains, afferents to the mesencephalic reticular system arise from various parts of the brain, such as the telencephalon [28, 37], the optic tectum [38] and the superior olivary nucleus [39]. These multimodal inputs suggest a nonspecific or generalized character of function of the anuran reticular formation, as well as a possible activating or inhibitory regulatory system which may influence the neural substrates for mating behavior as in mammalian brains.

HRP reaction product was continuous with neuronal structures from the injection site in the APON to the other parts of the hypothalamus: the magnocellular part of the PON, and the nuclei infundibularis dorsalis and ventralis. The regions where HRP-labeled neurons were observed are rich in vasotocinergic and mesotocinergic neurosecretory neurons and TRH neurons, respectively. Meanwhile, HRP labeled perikarya were found in the nucleus medialis septi and the nucleus of the diagonal band of Broca where many immunoreactive LHRH neurons which project to the APON were localized [13]. These facts strongly support the idea that afferents from LHRH, TRH and vasotocin neurons project to the APON neurons to modulate their neuronal activity for intiation of sexual bahavior [15].

The present results further indicate that the APON neurons may be influenced by various kinds of sensory inputs other than neurohormonal signals. The septal nuclei receive olfactory inputs through the medial olfactory tract [40, 41], and the amygdala is innervated by projections from the accessory olfactory bulb [42]. These limbic nuclei, from which afferents to the APON arise, may relay olfactory signals to the APON neurons, while visual, acoustic and tactile signals can be conveyed by the afferents from the brain stem. Physiological roles of these sensory afferents to the APON on anuran mating behavior remain to be solved.

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