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Extrahypothalamic Projection of Immunoreactive Vasotocin Fibers in the Brain of the Toad, *Bufo japonicus*

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ABSTRACT—Extrahypothalamic projection of vasotocin (AVT) fibers in the brain of the toad (*Bufo japonicus*) was examined immunohistochemically by the avidin-biotin-peroxidase complex (ABC) method. Immunoreactive AVT perikarya are localized in the nucleus preopticus pars magnocellularis. The AVT neurons send their immunoreactive varicose fibers to many discrete brain regions, such as the limbic cortex, the thalamus, the optic tectum and the lower brain stem, in addition to the neurohypophysis. A dense network of AVT fibers was found in the septal nuclei and the anterior part of the preoptic nucleus. AVT fibers which run postero-dorsad project to the nucleus posterocentralis thalami, the nucleus posterodorsalis tegmenti mesencephali, and the nucleus isthmi. Meanwhile, AVT fibers which run through in the dorsal infundibular region and then the mesencephalic reticular formation are distributed in the medulla oblongata. These findings suggest that AVT acts as a neuromodulator or a local hormone in the toad brain.

INTRODUCTION

It is well established that, in anuran amphibians, vasotocin (AVT) has both antidiuretic and vasopressor effects. In addition, AVT shows pronounced effects on reproductive behavior in *Rana pipiens* [1] and *Taricha granulosa* [2]. In *T. granulosa,* AVT may be involved in control of sexual behavior by acting neurons in the central nervous system [3]. We have shown in the toad brain that vasotocin neurons project their varicose axons into the anterior part of the preoptic nucleus (APON) which is considered to be the triggering center for male mate calling behavior in anuran amphibians [4]. These results suggest that AVT neurons transmit APON neurons peptidergic information concerned with initiation of sexual behavior.

In mammalian brains, the distributions of various neurohormones including arginine-vasopressin (AVP) are not confined only in the hypothalamo-neurohypophyseal system. They are widely distributed throughout in discrete brain loci [5]. Ultrastructural studies showed that axon endings of immunoreactive (ir) luteinizing hormone-releasing hormone (LHRH) fibers [6] and ir-AVP ones [7] form synapses and synaptoid contacts with other neurons. Further, varicose LHRH fibers form en passant synapses in the preoptic area of the guinea pig [6]. Therefore, it is probable in amphibian brains that, in addition to the APON and the neurohypophysis, AVT neurons send their fibers to many extrahypothalamic regions.

In this study, we examined immunohistochemically the extrahypothalamic distribution of AVT fibers in the toad brain to learn whether AVT neurons project their axons to the loci which are related to control of mating behavior. Further, we have tried to elucidate phylogenetically fundamental distributional pattern of AVT in the vertebrate brain, since the amphibian brain is considered to show fundamental structural organization of the vertebrate brain [8].

MATERIALS AND METHODS

Adult toads (*Bufo japonicus*) of both sexes, body weight ranging from 117 to 303 g, and body

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length (snout to vent) from 11.9 to 14.1 cm, were used. These animals were either obtained from an animal supplier in late September or were captured at the breeding season. Three animals, which were obtained from an animal supplier in late November, were used for immunohistochemical staining of thick frozen sections.

Immunohistochemical procedure

Distribution of AVT was immunohistochemically localized in paraffin sections and thick frozen sections that were cut either transversally, sagittally or horizontally to determine exact loci where ir-AVT fibers were found. As for immunohistochemical staining of vasotocin in the paraffin sections of the toad brain, details of fixation, tissue preparation and immunohistochemical procedure, in which the Vectastain ABC kit (Vector) was used, have been described previously [9, 10].

For the staining of thick frozen sections, the brains were fixed by transcardial perfusion with a fixative solution containing 1% glutaraldehyde, 2% paraformaldehyde and 4% sucrose in 0.1 M phosphate buffer (pH 7.4). The brains were removed, postfixed in the same fixative at 4°C overnight, and were washed in 0.1 M phosphate buffer. Frozen sections were cut at 50 μm, and were washed in 0.1 M phosphate buffered saline (pH 7.4) at room temperature for 30 min. Then, they were stained immunohistochemically by use of the Vectastain ABC kit. The sections were first incubated with the primary antiserum in a reaction medium at 4°C for 12–16 hr. The solution for incubation contained anti-arginine-vasopressin

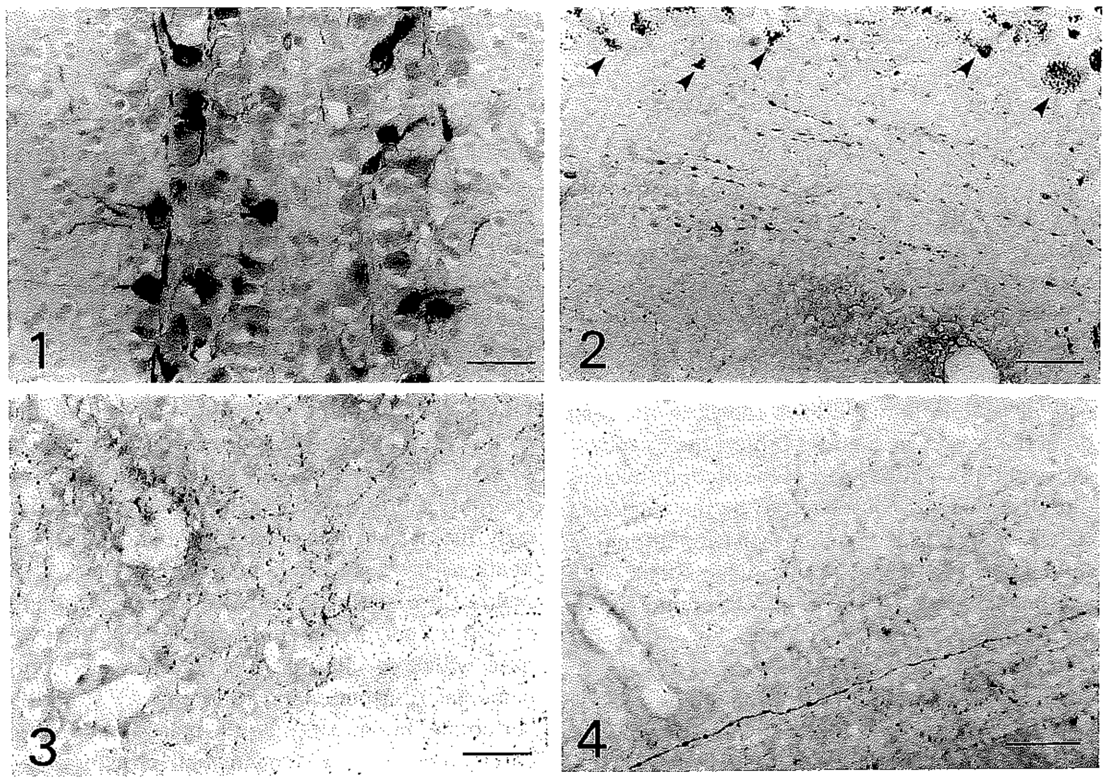


FIG. 1. Ir-vasotocin perikarya in the nucleus preopticus pars magnocellularis. Transversal paraffin section. Scale, 40 μm.

FIG. 2. Ir-vasotocin fibers travelling in the nucleus infundibularis dorsalis. Arrowheads indicate melanin granules. Sagittal frozen section. Scale, 40 μm.

FIG. 3. Plexus of ir-vasotocin fibers in the region anterior to the nucleus isthmi. Thick sagittal frozen section. Scale, 40 μm.

FIG. 4. Ir-vasotocin fibers in the floor of the medulla oblongata. Thick sagittal frozen section. Scale, 40 μm.

serum (1 : 8000 dilution, Bioproducts), which cross-reacts completely with vasotocin but not with mesotocin (cross reactivity, 0.3%), in 0.1% Triton X-100 dissolved in 0.1 M phosphate buffered saline (PBS–T). After the incubation with the primary antiserum, the sections were washed in PBS–T at room temperature for 30 min, incubated with biotinylated anti-rabbit Ig-G for 1 hr, and were washed in PBS–T. Afterward, the tissue sections were incubated in the avidin-biotin-peroxidase complex in PBS–T for 30 min. After a few rinses, they were incubated in DAB solution including 0.05% 3, 3′-diaminobenzidine (Sigma) and 0.01% hydrogen peroxide for 10 min, washed briefly in phosphate buffer, and were mounted on slide glasses with 40% ethanol containing 0.75% gelatin. They were then dehydrated, and were cover-slipped with Permount (Fisher). The tests for specificity of immunohistochemical staining followed the previous study [4, 9].

Nissl stained tissue sections were refered to for describing precise localization of ir-AVT. Nomenclatorial usage in this paper is basically those in *Rana pipiens* [11] and *Bufo japonicus* [9].

RESULTS

Distribution of ir-AVT perikarya and fibers

As was described previously [4, 9], ir-AVT perikarya are localized in the ventral (VMC) and dorsal (DMC) magnocellular parts of the preoptic nucleus (Figs. 1 and 5). Beaded or varicose ir-AVT fibers were widely distributed among the discrete extrahypothalamic loci in the limbic system and the brain stem (Figs. 2–4). They were not found in the dorsal and anteroventral regions of the telencephalon. The extrahypothalamic ir-AVT projections can be classified roughly into three groups according to their destinations (see Fig. 5). Neither notable seasonal variation nor sexual difference was found in the distribution of ir-AVT fibers in this study.

Projection to the telencephalon (Figs. 5–8)

A part of ir-AVT fibers emanating from the VMC project to the posteromedial region of the telencephalon, principally to the nuclei medialis septi and lateralis septi. Ir-AVT fibers are sent out to these loci mainly through the white matter including the medial forebrain bundle which surrounds the neuronal cell mass of the APON. A

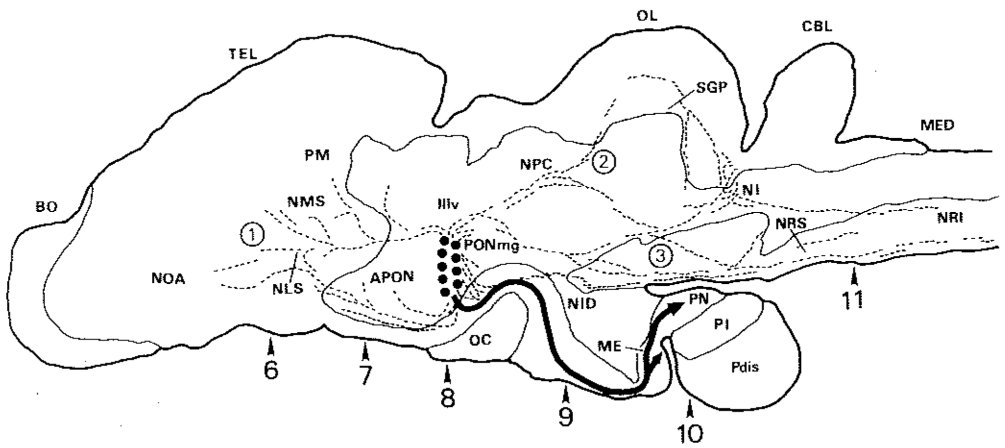
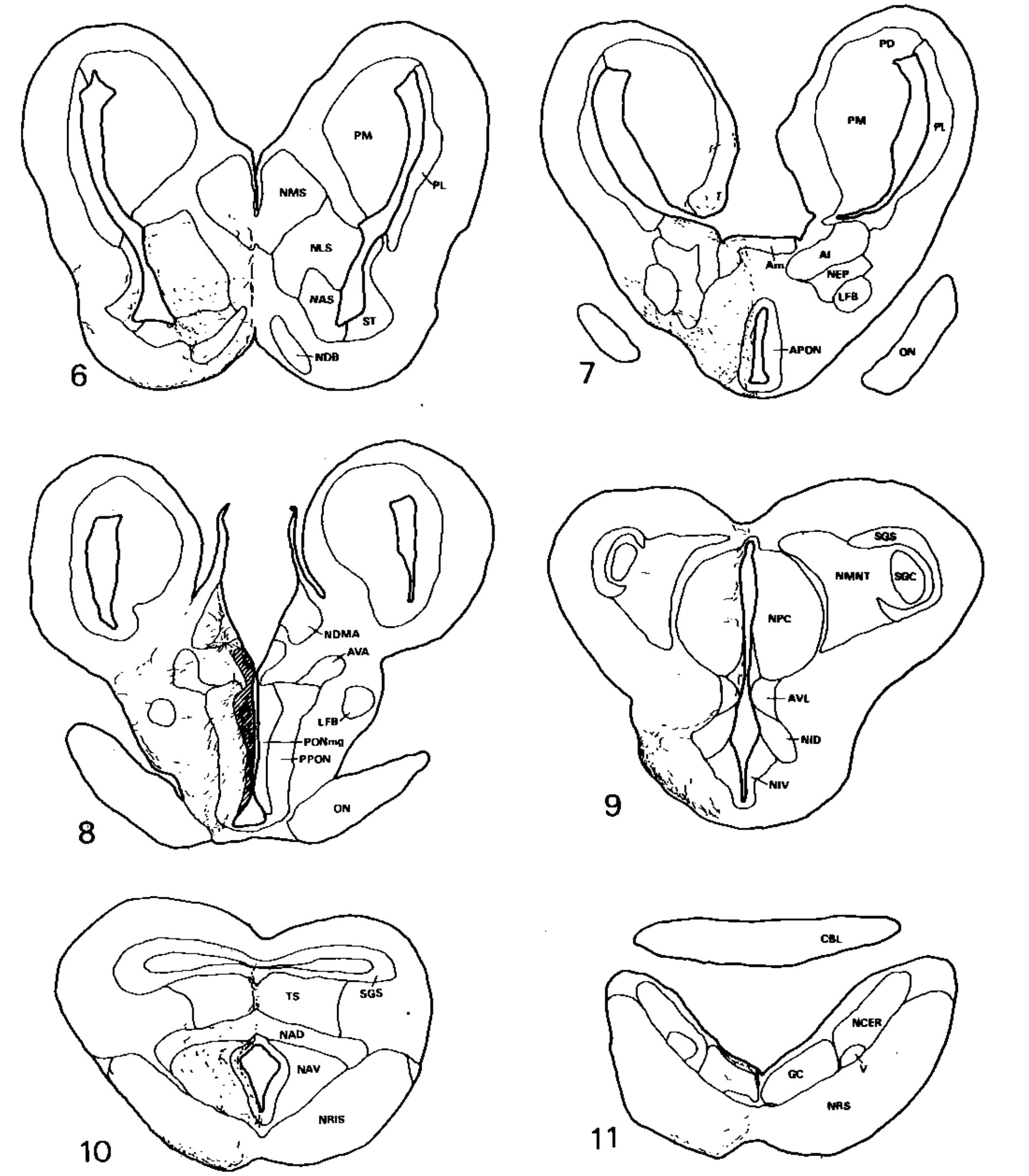


FIG. 5. Diagram of the mid-sagittal plane of the toad brain illustrating the distribution of ir-vasotocin fibers (broken lines) and ir-vasotocin perikarya (filled circles). The thick line shows the hypothalamo-neurohypophyseal vasotocinergic tract. Each numbered arrowhead shows the level of drawings (Figs. 6–11) illustrating the distribution of ir-vasotocin fibers and perikarya. Ir-vasotocin fibers are assembled into three projection groups: 1, projection to the telencephalon; 2, to the thalamus and the tectum; and 3, to the brain stem.

considerable number of varicose ir-AVT fibers that diverge from the projection to the telencephalon innervate into the APON and the amygdala medialis. Scattered ir-AVT fibers were often found in the pallium mediale and the nucleus of the diagonal band of Broca. The ir-AVT fibers found in the telencephalon have a varicose form, however, they rarely show Herring bodies which are frequently observed in the magnocellular preopticoneurohypophyseal neurosecretory system.

Projection to the thalamus and the tectum (Figs. 5, 8–9).



FIGS. 6–11. Diagrammatic camera lucida drawings illustrating the distribution of ir-vasotocin fibers and perikarya (filled circles). The hatched area in Fig. 8 shows the region in which dense ir-vasotocin fibers were observed.

Many fibers arising from both the VMC and DMC run posterodorsad to the dorsomedial thalamic region, and are distributed mainly in the nuclei dorsomedialis anterior thalami and posterocentralis thalami. A few ir-AVT fibers further proceed posteriad to terminate in the optic tectum.

Projection to the brain stem (Fig. 5, 10–11).

Ir-AVT fibers which project to the brain stem arise from ir-AVT neurons in the VMC. They initially proceed laterad into the white matter in the preoptic region. Then, they turn their destination caudad to the direction of the lower brain stem with many ir-AVT fibers that project to the neurohypophysis. Thereafter, the fibers projecting to the brain stem diverge from the preoptico-neurohypophyseal tract around the dorsal infundibular region. A few beaded ir-AVT fibers are localized in the nucleus infundibularis dorsalis (Fig. 2). Many ir-AVT fibers run down to the mesencephalic tegmentum. They project to the nuclei posterodorsalis tegmenti mesencephali and isthmi, and further to the griseum centrale rhombencephali. A considerable number of fine beaded fibers gather together to form a plexus in the region anterior to the nucleus isthmi (Fig. 3). Some fibers which emanate from this plexus proceed dorsad to the stratum griseum tecti. Many ir-AVT fibers which run posteriad through the white matter in the floor of the mesencephalon were also found (Fig. 4). These fibers, which diverge from the fiber group destining to the isthmic region at the level caudal to the dorsal infundibular region, reach the rhombencephalic reticular formation, and proceed further toward the spinal cord.

DISCUSSION

The present study showed that ir-AVT fibers are distributed widely among many extrahypothalamic loci in the toad brain. Such regions are the limbic cortex, the thalamus, the optic tectum, the isthmic region and the lower brain stem.

The distributional pattern of extrahypothalamic ir-AVT fibers in the toad brain seems to be homologous to those described in the brains of other vertebrate classes. In the rat and the monkey, vasopressin neurons, the mammalian counterpart of AVT neurons, project their immunoreactive fibers to the hippocampus, the septum, the amygdala and the preoptic area. They

Abbreviations for Figs. 5–11.

Al	amygdala, pars lateralis	NOA	nucleus olfactorius anterior
Am	amygdala, pars medialis	NPC	nucleus posterocentralis thalami
APON	anterior part of the preoptic nucleus	NRI	nucleus reticularis inferior
AVA	area ventralis anterior thalami	NRIS	nucleus reticularis isthmi
AVL	area ventrolateralis thalami	NRS	nucleus reticularis superior
BO	bulbus olfactorius	OC	optic chiasma
CBL	cerebellum	OL	optic lobe
GC	griseum centrale rhombencephali	ON	optic nerve
LFB	lateral forebrain bundle	PD	pallium dorsale
ME	median eminence	Pdis	pars distalis hypophysis
MED	medulla oblongata	PI	pars intermedia hypophysis
NAD	nucleus anterodorsalis tegmenti mesencephali	PL	pallium laterale
NAS	nucleus accumbens septi	PM	pallium mediale
NAV	nucleus anteroventralis tegmenti mesencephali	PN	pars nervosa hypophysis
NCER	nucleus cerebelli	PONmg	preoptic nucleus, pars magnocellularis
NDB	nucleus of the diagonal band of Broca	PPON	posterior part of the preoptic nucleus
NDMA	nucleus dorsomedialis anterior thalami	SGC	stratum griseum centrale tecti
NEP	nucleus entopeduncularis	SGP	stratum griseum periventricularis tecti
NI	nucleus isthmi	SGS	stratum griseum superficiale tecti
NID	nucleus infundibularis dorsalis	ST	striatum
NIV	nucleus infundibularis ventralis	TEL	telencephalon
NLS	nucleus lateralis septi	TS	torus semicircularis
NMNT	nucleus mesencephalicus nervi trigemini	IIIv	third ventricle
NMS	nucleus medialis septi	V	motor nucleus of the trigeminal nerve

project further to the thalamus, the superior colliculus, and the several pontine and medullary nuclei [12]. A similar immunohistochemical distributional pattern of ir-AVT fibers was observed in the brain of the lizard *Gekko gecko* [13] and the eel *Anguilla japonica* (Fujiwara *et al.*, unpublished). A radioimmunoassay study of microdissected brain areas of rough-skinned newts also showed a similar distributional pattern of AVT [14]. These results indicate that the patterns of extrahypothalamic projections of AVT and vasopressin are fundamentally homologous in all vertebrate classes.

Immunoelectron microscopic studies demonstrated the presence of synapses containing neurophypopphysial hormones in the rat brain [7, 15]. In the previous study, we showed that LHRH and AVT fibers may contact synaptically with APON neurons [4]. It is therefore highly probable that, in the toad brain, the extrahypothalamic AVT fibers form ordinary and/or en passant synapses with neurons in the loci where AVT fibers were localized. As was discussed in our previous paper [4], beaded or varicose ir-AVT fibers traveling through the white matter may form such synapses with dendrites of many central neurons, because in the amphibian brain many neurons located in the medial cell mass develop their dendritic fields in the adjacent white matter [8, 16]. Since neurohypophysial hormones could excite unit-spike activity of neurons in the rat supraoptic and paraventricular nuclei [17, 18], the eel preoptic nucleus [19] and the toad APON (Fujita and Urano, unpublished), vasotocin may facilitate activity of many central neurons as a neuromodulator or a local hormone. The latter possibility is supported by the fact that AVP of 10^{-9} M, comparable to effective doses of AVP for peripheral targets, could excite rat paraventricular neurons [18]. However, physiological roles of vasotocinergic transmission in the anuran brain are not clear at present, although an involution in reproductive behavior has been suggested as is described in the introduction.

The present study showed that vasotocin fibers are distributed in the loci concerned with reproductive behavior. Such regions are the limbic cortex, the preoptic area, the optic tectum and the central gray. In the limbic cortex, a considerable number of ir-AVT fibers were found in the septal nuclei, and also in the nucleus of the diagonal band of Broca. These regions contain the majority of ir-LHRH neurons in the toad brain [4, 10]. A similar projection of vasopressin fibers was found in the organum vasculosum lamina terminalis in the mammalian brain where many ir-LHRH perikarya are localized [12]. In the eel, vasotocin fibers were found in the preoptic region (Fujiwara *et al.*, unpublished). Meanwhile, ir-LHRH fibers project to the VMC in the toad brain [20]. These observations suggest that the LHRH-ergic and vasotocinergic neurosecretory systems are mutually connected, and that they interact on each other for controlling sexual behavior.

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