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Immunocytochemical Study on the Extrahypothalamic Projections of Oxytocinergic and Vasopressinergic Neurons in the Mouse[1]

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= Abstract = An immunocytochemical study on the pathways and distribution of extrahypothalamic projections of oxytocinergic and vasopressinergic neurons was performed. Monoclonal antibodies to oxytocin and vasopressin, avidin-biotin-peroxidase complex, vibratomy, and free floating methods were applied in the immunocytochemical procedure of the mouse brain.

Oxytocinergic fibers arose from the paraventricular nucleus and bed nucleus of the stria terminalis and projected mainly to the brain stem. The fibers to the brain stem followed two major courses and reached the central gray area, lateral reticular nucleus, dorsal motor nucleus of vagus, locus coeruleus, etc. Other fibers projected to the various regions of the forebrain such as the amygdala, and the septal nuclei. Vasopressinergic fibers were present mainly in the amygdala, and a few fibers were observed in the lateral habenula and lateral reticular nucleus. In certain nuclei, the oxytocingergic and vasopressinergic fibers ramified and formed the perineuronal punctate structures.

The extensive pathway in the central nervous system and synaptic-like localization of oxytocinergic and vasopressinergic fibers revealed in this study support their role as neuromodulators or neurotransmitters and a wide variety of their functions in the behavioral process, memory, and modulation of autonomic nervous system. In view of its localization, oxytocin is thought to play a dominant role in the regulation of the autonomic nervous system.

Key words: ***Oxytocin, Vasopressin, Extrahypothalamic projections, Immunocytochemistry, Mouse***

INTRODUCTION

Vasopressin(VP) and oxytocin(OT) have been thought of primarily as peptide hormones of the pituitary which are produced at the paraventricular nucleus and supraoptic nucleus of the hypothalamus, and released into the blood vessels of the neurohypophysis. Thus. their functions as hormones have dominated considerations about these peptides for many years.

But, during the past decade, it has been reported the VP and OT may be involved in behavioural processes, memory, and modulation of the autonomic nervous system(Gah and Thomas 1985; Sofroniew 1985; Gardiner and Bennett 1986), and that they may act as neuromodulators or putative neurotransmitters, and not only as simple hormones(Buijs and Van Heerikhuize 1982; Voorn and Buijs 1983; Schmitt 1984). Moreover, the demonstration of the presence of VP and OT in the extrahypothalamic central nervous system by immunocytochemistry and radio-immunoassay(Nilaver *et al.* 1980; Van Leeuwen and Caffe 1983; Caffe and Van leeuwen 1983; De Vries *et al.* 1985) has raised the interest in their functions as neuromodulators or neurotransmitters. However there has been no report about their exact functions and mechanism of action as neuropeptides, because of the complexity of the synaptic events between the

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distant neurons throughout the whole central nervous system.

Therefore, to obtian an access to those problems, it is quite reasonable that the normal anatomy of the extrahypothalamic projections of VP and OT neurons must be clarified, but details concerning the exact pathways of their extrahypothalamic projections are not yet fully understood because of the limitations of classical antisera to VP and OT used in immunocytochemistry(Gorbman *et al.* 1983). The antisera, which were raised by the repetitive innoculation of antigen(VP or OT) to animal, do not exclude the cross reaction and non-specific reaction completely.

Recently, to solve these problems of antisera, the monoclonal antibody technic has been introduced into the field of immunocytochemistry and has proved to have a high sepcificity in antigen-antibody reaction. Hou-Yu *et al.* (1982) first developed a monoclonal antibody to VP. Also in our laboratory, Cho *et al.* (1984) and Lee and Cho(1986) developed monoclonal antibodies to VP and OT, and immunohistochemical studies on the VP and OT neurons in the hypothalamus-hypophysis system using monoclonal antibodies have been performed.

In this paper the pathway of the extrahypothalamic projections of VP and OT neurons and their relationship with the other systems were studied in the brain with the tools of monoclonal antibodies, vibratomy and the free floating method.

MATERIALS AND METHODS

30 adult Balb/C mice weighing about 25 gm a piece were used in this study. Each mouse was anesthetized with diethyl ether and perfused through the heart with 0.9% saline, followed by 10% neutral buffered formalin or 5% glutaraldehyde. The whole brain was dissected and immersed in the same fixative for 24 hours. Subsequently, 50 or 100 μ-thick serial sections were cut with a vibratome(Microslicer DTK-2,000). The free-floating sections were incubated with solutions by the following procedures.

1) 0.05 M phosphate buffered saline(pH 7.4) containing 0.5% Triton X-100(PBS-TX), 30-minute rinse.

2) Monoclonal anti-VP or OT antibody in PBS-TX, overnight at 4°C.

3) Biotinylated anti-mouse IgG(Vector) in PBS-TX, 90 minutes.

4) Avidin-biotin-peroxidase complex(Vector) in PBS-TX, 90 minutes.

5) 0.05% 3-3'-diaminobenzidine(Sigma) in PBS with 0.01% hydrogen peroxide, 10 minutes.

Following the steps as above, the tissue preparations were mounted on gelatin-coated slides and coverslipped after dehydration and clearing. The adjacent section was stained with cresyl violet for identification and referenece of the brain area

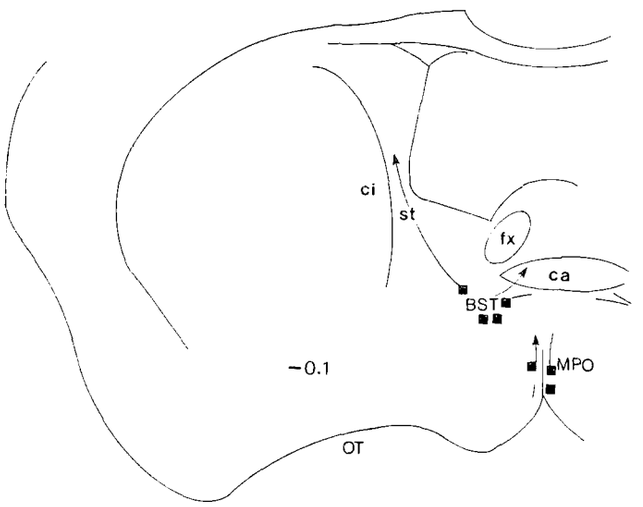

Fig. 1. Drawing illustrating OT cell bodies (squares) and direction of their fibers (arrows) at the plane 0.1 mm caudal to reference plane (the plane where anterior commissure shows its greatest size at midpoint). See Table 2 for abbreviations.

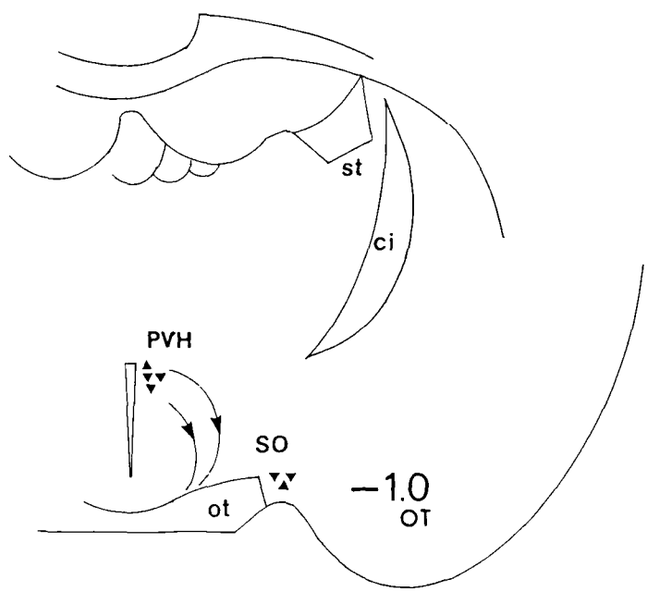

Fig. 2. OT cell bodies (triangles) and their fibers at plane 1.0 mm caudal to reference plane.

according to the atlas (Slotnick and Leonard 1975).

The preparations were observed with light microscope and vasopressinergic or oxytocinergic fibers were traced serially and mapped.

RESULTS

1) Oxytocinergic cell bodies and fibers

Oxytocinergic cell bodies were observed in the paraventricular nucleus(PVH), supraoptic nucleus (SO), bed nucleus of stria terminalis(BST), and small islands scattered over the hypothalamus (Plate 1, 2). The shape of the oxytocinergic neuron was bipolar or multipolar. The diameter of the cell body was about 20 to 25 μ m, belonging to the magnocellular group. In the horizontal sections, the distribution of oxytocinergic cell bodies showed a star-shaped appearance around the third ventricle, suggesting their multiple extrahypothalamic projections(Plate 1). Especially from the PVH and BST, massive extrahypothalamic projections were traced.

In general, the fibers to the brain stem originated from the PVH while the BST was a main source of the fibers to the forebrain(Fig. 6).

Oxytocinergic fibers from the BST course rostrally, pass the anterior commissure ventrally, and reach the lateral septal nucleus. The PVH also sends a few fibers to the nucleus (Fig. 1, 6).

Other fibers from the BST follow the stria medullaris along the dorsal margin fo the thalamus and reach the lateral habenula(Fig. 6).

Another tract from the BST follows the stria ter-

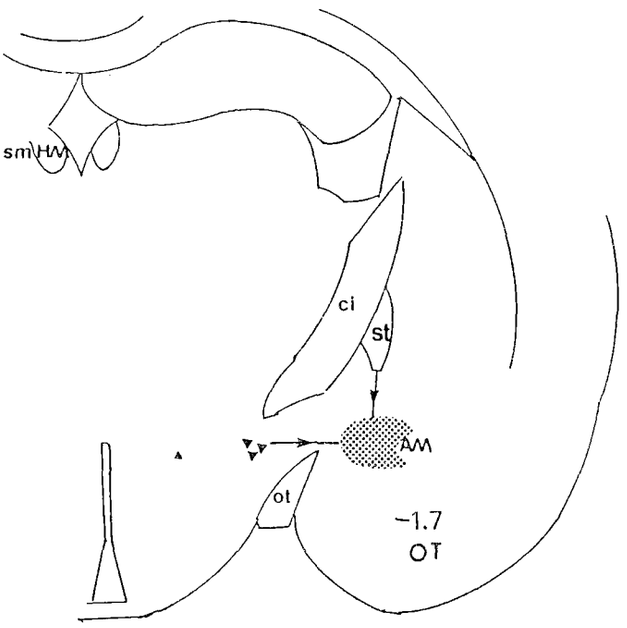

Fig. 3. OT cell bodies (tringles) and fibers at plane 1.7 mm caudal to reference. Small dots mark the area where fibers terminate.

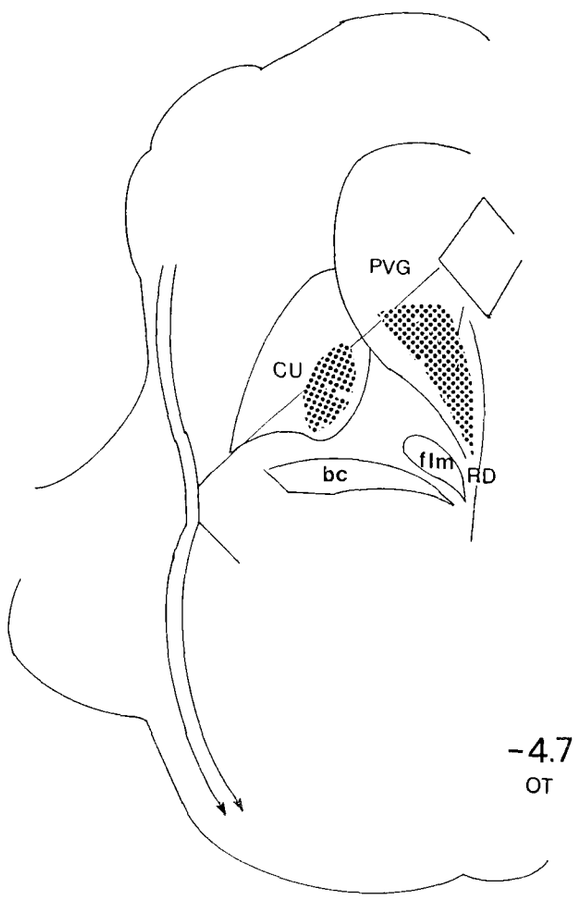

Fig. 4. OT fibers at upper pons. Small dots mark the area where fibers terminate.

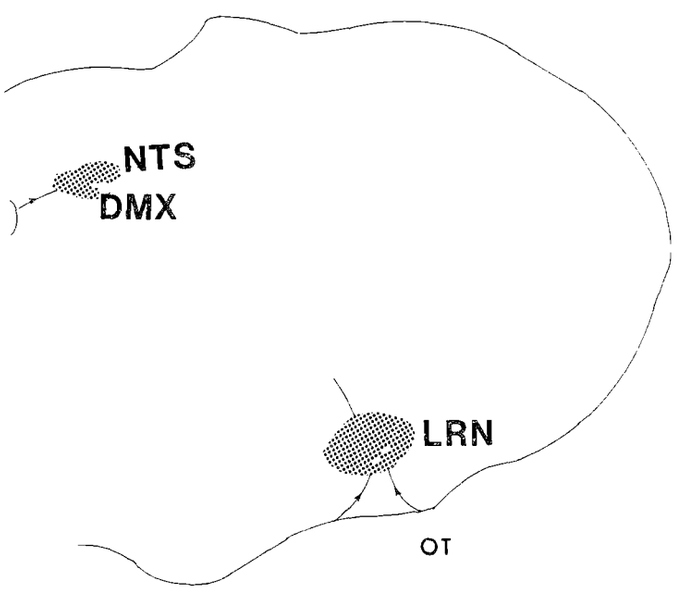

Fig. 5. OT fibers at medulla oblongata.

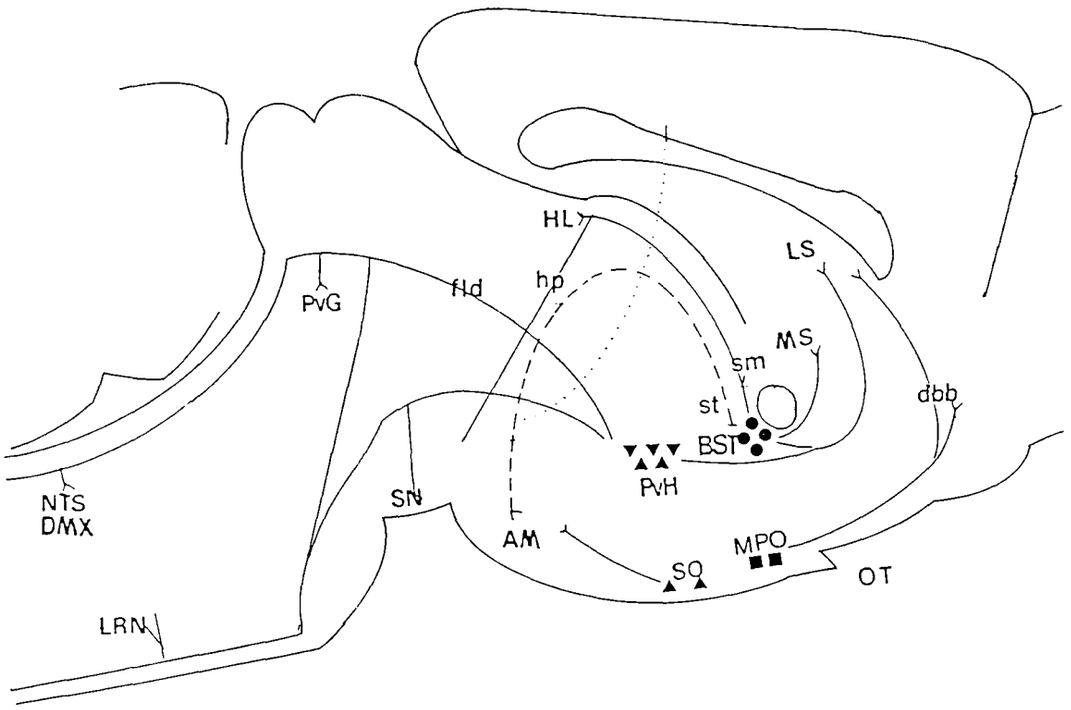

Fig. 6. Drawings illustrating major extrahypothalamic pathways of OT.

minalis along the ventral side of the lateral ventricle, and the most of the fibers reach the amygdala. A few fibers passing through the stria terminalis can be followed via the tapetum into the cortex(Fig. 1, 3, 6).

At the diagonal band of Broca, some fibers run dorsally. Their original cell bodies were probably in the small islands at the medial preoptic area(Fig. 6, Plate 3).

The fibers from the PVH were divided into two major groups. One group of the fibers run caudally via the fasciculus longitudinalis dorsalis along the ventricle and send the branches to the nuclei around the ventricle at the various levels of the brain stem: to the central gray area, the cuneiform nucleus, locus coeruleus, solitary nucleus, and dorsal motor nucleus of vagus, and so on(Fig. 4, 5, 6, Plate 4).

Another group of the fibers from the PVH run in a caudo-lateral direction. The fibers run along the medial lemniscus, sending some of the fibers to the substantia nigra.

Subsequently at the lateral portion of the upper pons, the massive bundle of the thin fibers runs ventrally to the ventral margin and descends to the lower brain stem. But the considerable fibers of the previous group via the central gray area join this bundle(Fig. 4, 6). As the fiber bundle descends, its position in the ventrolateral tegmentum moves laterally. During its medullary trajectory, some fibers are seen emanating from the main bundle to run transversely. Subseuquently they reach the

Table 1. Distribution of OT and VP fibers in the mouse central nervous system

Area	Density of fibers	
	OT	VP
I. Forebrain		
Cortex	+	
Lateral septal nucleus	+	
Medial septal nucleus	+	
Diagonal band of Broca	+	
Amygdala	++	++
Lateral habenula	+	+
II. Brain stem		
Substantia nigra	++	
Central gray area	+++	
Cuneiform nucleus	++	
Dorsal raphe nuclesus	+	
Locus coeruleus	++	
Solitary nucleus	++	
Dorsal motor nucleus of vagus	++	
Lateral reticular nucleus	+++	+

+-+++ Indicates density of fibers

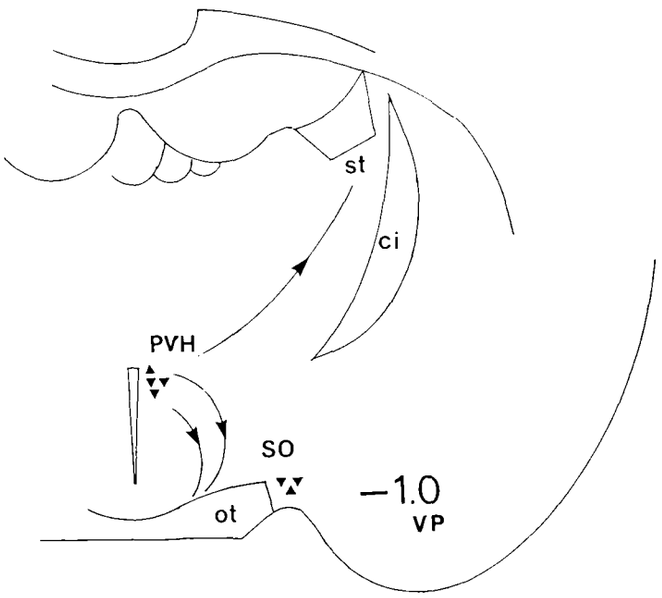

Fig. 7. VP cell bodies (triangles) and fibers at plane 1.0 mm caudal to reference plane.

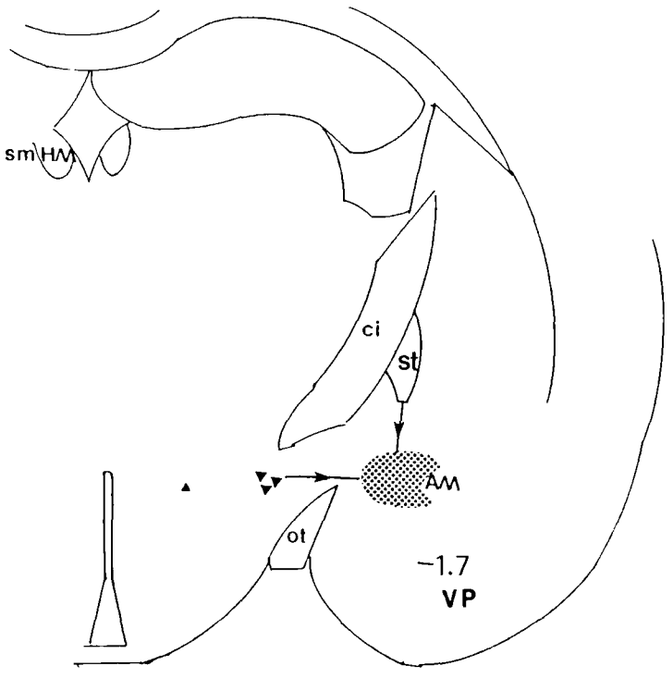

Fig. 8. VP cell bodies (triangles) and fibers at plane 1.7 mm caudal to reference plane.

lateral reticular nuclei. Some of them appear to terminate at the nuclei, other fibers merely pass through the nuclei and run in the reticular formation(Fig. 5, Plate 6, 7).

The density of the oxytocinergic fibers in the various region is summarized in Table 1. The fibers to the brain stem predominated. Especially in the central gray area and lateral reticular nucleus, the oxytocinergic fibers showed the highest density.

2) Vasopressinergic cell bodies and fibers

Vasopressinergic cell bodies were observed in the paraventricular nucleus, supraoptic nucleus and other accessory nuclei, but not in the bed nucleus of stria terminalis. The shape and size of vasopressinergic cell bodies were similar to those of oxytocinergic cell bodies(Plate 8).

However the extrahypothalamic projections of vasopressinergic neurons were distinct only in the amygdala, and a few fibers were observed in the lateral habenula and lateral reticular nucleus. The fibers from the paraventricular nucleus, in which vasopressinergic neurons were grouped more laterally than oxytocinergic neurons, follow the stria terminalis to reach the amygdala. The additional fibers reached the amygdala from the cell bodies scattered in the laterla hypothalamus, not through the stria terminalis(Fig. 7, 8, Plate 9, 10). The fibers in the laterla habenula were derived from the paraventricular nucleus, but the pathway of the fibers to the lateral reticular nucleus were not identified. Probably, they followed the same course as the oxytocinergic fibers.

Table 2. Abbreviation of nomenclature used in this paper (based on Slotnick & Leonard 1975)

AM	amygdala	LRN	lateral reticular nucleus
bc	brachium conjunctivum	LS	lateral septal nucleus
BST	Bed nucleus of stria terminlais	MPO	medial preoptic area
ca	anterior commissure	MS	medial septal nucleus
ci	internal capsule	NTS	solitary nucleus
dBB	diagonal band of Broca	OT	oxytocin
		ot	optic tract
DMX	dorsal motor nucleus of vagus	PVG	central gray area
		PVH	paraventricular nucleus
fld	fasciculus longitudinalis dorsalis	RD	dorsal raphe nucleus
		SO	supraoptic nucleus
flm	medial longitudinal fasciculus	sm	stria mediullaris
fx	fornix	st	stria terminalis
HL	lateral habenula	SN	substantia nigra
HM	medial habenula	VP	vasopressin
hp	fasciculus retroflexus		

The typical immunoreactive fibers showed a bead-like appearance, but the pattern of the fibers varied according to their position. At the stria terminalis and the lateral part of the upper pons the

fibers were much thinner and rarely ramified(Plate 9). In the nuclei, however, there were thick beaded fibers which ramified. Especially in the central gray area, the fibers ramified extensively and formed numerous perineuronal punctate structure. Also in the lateral reticular nucleus and solitary nucleus, perineuronal punctate structures were observed-(Plate 4, 6, 7, 10)

DISCUSSION

The choice of the immunocytochemical technique for demonstrating neuronal pathways is dependent on the availability of the specific antibody as well as on the necessity for demonstrating the chemical nature of a pathway as opposed to anatomical connections. Fortunately, the monoclonal antibodies to VP and OT have been developed in our laboratory and their specificity has been proved (Cho *et al.* 1984; Lee and Cho 1986). The other neuroanatomical tract-tracing methods include the Golgi method, lesion making method, and the use of axonal trasport of macromolecules, such as HRP. But their inability to demonstrate the neuronal pathways containing the specific substances or neurotransmitters makes the immunocytochemical technique chosen as the major tool in this study.

The immunocytochemical technique, however, also has some disadvantages. For example, it can not demonstrate the distinct nerve terminal and the fibers containing the very small amount of antigen are difficult to qualify. Therefore, the study could have obtained better results if the other tract-tracing methods were combined.

The immunocytochemical methods alone made a partial distinction between the fibers in the various regions in this study. The thin and rarely ramifing fibers in the stria terminalis and lateral portion of the upper pons, were probably 'fibers of passage', which suggest that they are of the rapid axoplasmic transport. The fibers which ramified extensively and/or formed the perineuronal punctate structures similar to synapse in morphology might be neurosecretoy in nature or terminals(DeVries *et al.* 1985).

The first reports on the extrahypothalamic pathways of oxytocinergic and vasopressinergic neurons were based on the immunohistochemical studies of paraffin-embedded sections (Buijs 1978; Buijs *et al.* 1978). More recently, the sensitivity of the immunocytochemical procedure has considerably increased by using vibratome or forzen sections (De-Vries *et al.* 1985). The vibratomy, sectioning the tissue directly after fixation without tissue processing, minimizes the chemical or physical insult which may change the antigenicity. In addition to the vibratomy procedure, te free floating method was applied to improve the antigen-antibody reaction and a membrane detergent (Triton X-100) was used to improve the exposure of antigen.

In this study, the results of oxytocin pathways generally conform to those of other reports in rat-(Buijs 1978; Buijs *et al.* 1978; Nilaver *et al.* 1980). But Buijs(1978) did not describe the extrahypothalamic proections from the BST, which were observed in this study, and Nilaver *et al.* (1980) did not observe the fibers through the fasciculus longitudinalis dorsalis to the brain stem, which were also described in this study. These discrepancies may be attributed to the low sensitivity of the immunocytochemical technique which they applied, such as paraffin-embedding and polyclonal antisera. In favor of this study, the contribution of the BST to the extrahypothalamic projections was described in the study using vibratomy (De Vries and Buijs 1983). More recently, Lutein *et al.* (1985) reported on the course of paraventricular eferents to the brain stem by application of the anterograde transport technique of leuco-agglutinin, where they obtained results very similar to the results of this immunocytochemical study. They described two distinct descending axon pathways which are comparable to two groups of the oxytocinergic pathways to the brain stem.

However, this study obtained results of the vasopressinergic system which differ from those of the previous reports in many ways. Caffe and Van Leeuwen(1983) and Van Leeuwen and Cafe(1983) reported that pretreatment of rats with clochicine enabled the detection of vasopressinergic cell bodies within the BST, the medial amygdaloid nucleus, and the locus coeruleus. More recently, the vasopressinergic cell bodies in the BST and the medial amygdaloid nucleus could be visualized without colchicine pretreatment in the rat fixed by 5% glutaraldehyde. In this study, however, no vasopressinergic neuron was observed in those nuclei even in the mice fixed by 5% glutaraldehyde. Also there is a discrepancy about the extrahypothalamic vasopressinergic fibers. Buijs(1978) and De Vries *et al.* (1985) reported that the vasopressinergic fibers were distributed widely from the forebrain to the brains tem, preferentially to the

limbic system.

Therefore, the cause of this discrepancy should be discussed. The first possibility is that the vasopressinergic systems of the mice may be more restricted than those of other species. The second possiblity might be the matter of methodology. As mentioned above, the colchicine pretreatment was reported to enhance the sensitivity of the immunocytochemistry technique in detecting the vasopressinergic system: therefore the methods used in this study may be not sensitive enough to detect the vasopressinergic system completely. Nevertheless, it is apparent that the extrahypothalamic vasopressinergic fibers are projected chiefly to the amygdala, which is the part of the limbic system.

In summary, the oxytocinergic fibers predominate in the brain stem, while the vasopressinergic fibers terminate mainly in the limbic system. In some nuclei they appeared to contact neuronal perikarya, which suggests that vasopressin and oxytocin may act as the putative neurotransmitters or neuromodulators. In this study, the oxytocinergic fibers reached the dorsal motor nucleus of the vagus which gives rise to preganglionic parasympathetic fibers, and the vasopressinergic and oxytocinergic fibers were present in the limbic system which is known to be related to the memory and behavior. Also in the solitary nucleus where baroreceptor afferent fibers in the glossopharyngeal and vagus nerves terminate, and in the lateral reticular nucleus at the ventrolateral medulla which has effects on cardiovascular control(Dampney *et al.* 1982), the oxytocinergic fibers were predominant. The predominance of the oxytocinergic fibers in the brain stem was reported in the earlier anatomical studies(Buijs 1978; nilaver *et al.* 1980). Also in an in vitro study on the synaptic release of the peptides in the solitary nucleus, oxytocin was released in measurable amounts when the preparation was stimulated, but there was no measurable release of vasopressin (Buijs and Heerikhuize 1982). Therefore, the oxytocinergic system appears to play a major role in the autonomic nervous system.

In conclusion, this study supports previous reports that oxytocin and vasopressin are neuromodulators or putative neurotransmitters which may play a role in the behavioral process, memory, and the modulation of the autonomic nervous system. In respect to the autonomic system, oxytocin is thought to dominate vasopressin.

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= 국문초록 =

생쥐 Oxytocin 및 Vasopressin 신경세포의 시상하부외로성 투사섬유에 관한 면역세포화학적 연구

서울대학교 의과대학 해부학교실

박경한 · 조사선 · 이광호

정상 성숙 생쥐의 뇌에서 oxytocin과 vasopressin 신경세포의 시상하부외로성 투사섬유의 경로와 분포상태를 규명하기 위하여 oxytocin과 vasopressin에 대한 단세포군 항체, avidin--biotin-peroxidase complex, vibratomy, fee floating방법 등을 이용한 면역세포화학적 염색을 시행하였던 바 다음과 같은 결론을 얻었다.

Oxytocin 신경섬유는 실방핵(paraventricular nucleues), bed nucleus of stria terminlais 등에서 유래하여 뇌간과 전뇌에 투사되었다. 뇌간에 투사된 섬유는 2가지의 주된 경로를 통해 중심회백질(central gray area), 측망상핵(lateral reticular nucleus), 고속핵(solitary nucleus), 미주신경배측핵(dorsal motor nucleus of vagus), 청반(locus coeruleus), 흑질(substantia nigra) 등에 분포하였고 전뇌로 투사된 섬유는 편도체(amygdala), diagonal band of Broca 등 변연계에도 분포하였다. Vasopressin 신경섬유는 편도체에서만 뚜렷하게 관찰되었고 기타 외측고삐핵(lateral habenula), 측망상핵에서 소수가 분포하였다. 이들 신경섬유들은 이상의 신경핵 내에서 신경세포체 주위에서 분지하거나 점상의 구조로 되어 있어 형태학적으로 신경연접의 특징을 보였다.

이상과 같은 oxytocin과 vasopressin의 중추신경내 분포양상으로 볼 때 이들이 자율신경조정 등의 다양한 기능과 연관되어 있음을 시사하는 것으로 생각되며, 특히 자율신경의 조정에 관해서는 oxytocin이 vasopressin보다 주된 역할을 할 것으로 사료된다.

LEGENDS FOR PLATES

Plate 1. Horizontal section through the hypothalamus. OT cell bodies are arranged in star-shape (only a half of the shape is seen) around third ventricle(V), suggesting various directions of their fibers. x100.

Plate 2. OT cell bodies and fibers in the BST. Note their bead-like fibers(arrowheads). x250.

Plate 3. Sagittal section at the dBB. OT fibers with ramification(arrowheads). x250.

Plate 4. PVG at upper pons level. OT fibers ramify extensively and form perineuronal punctate structures. x400.

Plate 5. Sagittal section through medulla oblongata. Transverse OT fibers reach LRN (triangle). x400.

Plate 6. LRN at medulla. OT fibers. x400.

Plate 7. Perineuronal punctate terminals(arrowheads) of OT fibers at LRN. x1,000.

Plate 8. Transverse section through hypothalamus. VP cell bodies and fibers in PVH. x125.

Plate 9. VP fibers in st. x400.

Plate 10. VP fibers in AM. x250.


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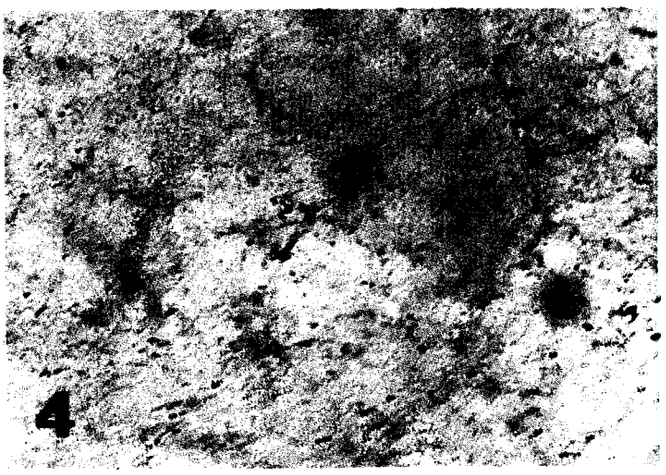
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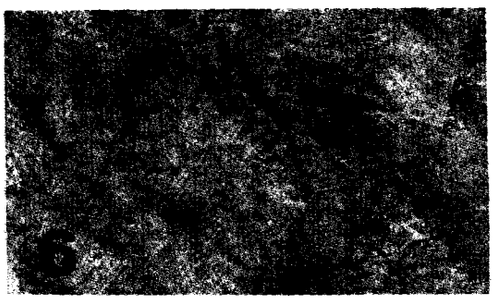
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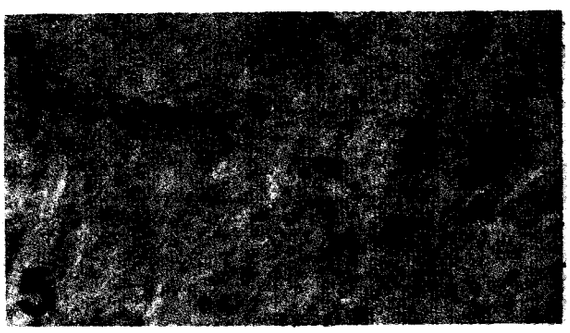
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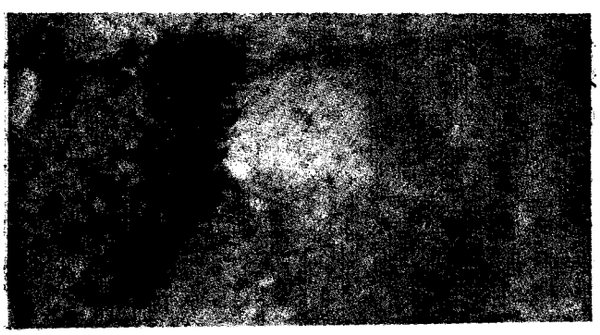


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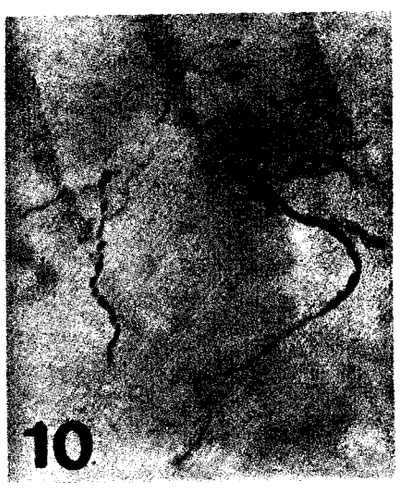
10