DISTRIBUTION OF OXYTOCIN IN THE SPINAL CORD AND ITS PRESENCE IN THE PERIPHERAL NERVOUS SYSTEM

Ph. D. Thesis

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List of abbreviations

b. wt.	body weight
HPLC	high-performance liquid chromatography
PAP	peroxidase-antiperoxidase method
RIA	radioimmunoassay
RT	room temperature
TFA	trifluoroacetic acid

1. Introduction

Oxytocin is a neuropeptide synthesized mainly in the paraventricular and supraoptic nuclei of the hypothalamus. As one of the classical neurohormones, oxytocin is transported to the neurohypophysis where it is stored and released to the blood circulation. It plays an important role in neuroendocrine regulation of parturition and lactation. Oxytocin is present not only in the hypothalamo-neurohypophyseal system, but is also located in various areas of the central and peripheral nervous system. Furthermore, numerous studies have shown that oxytocin mRNA is present in peripheral tissues such as decidua, chorion, amnion, ovary, testis and heart. Locally synthetized oxytocin can stimulate uterine contractions in the time of parturition in a paracrine fashion without increasing circulating maternal oxytocin concentrations. Actions of local oxytocin in the ovary are linked to luteinization, luteolysis and steroidogenesis. It may be involved in the local control of androgen biosynthesis and spermatogenesis in the testis. The heart and large vessels like aorta and vena cava are also sites of oxytocin synthesis and the locally produced oxytocin may have important regulatory functions within the heart and the vascular system.

1.1 Oxytocin in the spinal cord

A growing body of knowledge has accumulated on the location, origin, putative roles and regulation of oxytocin in the spinal cord. The majority of oxytocin-containing fibers in the spinal cord were shown to arise from the hypothalamic paraventricular nucleus. Oxytocinergic fibers descending from the paraventricular nucleus make contacts with preganglionic neurons in the rat spinal cord, as demonstrated by a combination of retrograde cell body labelling and immunocytochemistry. Oxytocinergic terminals were found in the dorsal horn, central gray and intermediolateral column by means of electron microscopic immunocytochemistry. The distribution of the oxytocinergic hypothalamo-spinal neuronal projections shows a good overlap with that of the spinal oxytocin receptors, supporting the view that oxytocin acts as a neurotransmitter in the central nervous system. Several lines of evidence suggest that central oxytocin is an endogenous modulator of various spinal functions. It has been shown to induce spinal antinociception through activation of oxytocin receptors. In addition, several studies indicate the involvement of oxytocin in the regulation of spinal autonomic functions: it can inhibit sympathetic preganglionic neurons of the spinal cord, plays an important role in the control of penile erection and male sexual behaviour, mediates the pupillary dilatation response to vaginocervical stimulation, modulates the

micturition reflex as well as uterine motility, and it can increase the heart rate and renal sympathetic activity. Experiments have also been directed at defining physiological and pharmacological stimuli that can alter the spinal levels of oxytocin. Spinal oxytocin displays cyclic variations during the stages of the rat estrous cycle, suggesting that it may be regulated by ovarian hormones. Evidence has been provided for a direct estrogen-dependent release of oxytocin within the spinal cord in response to vaginocervical stimulation. Various other stimuli, such as immobilization stress, isotonic or hypertonic saline also affect the oxytocin content in the spinal cord. In addition, oxytocin release from spinal cord synaptosomes seems to be under inhibitory control of opioid peptides.

1.2 Oxytocin in the peripheral nervous system

Data available on the occurrence, origin and role of oxytocin in the peripheral system is yet scanty. Oxytocin immunoreactivity was localized by immunohistochemistry in neurons of rat sensory ganglia and characterized by high-performance liquid chromatography. Immunoreactive oxytocin detected in extracts of human lumbar sympathetic paravertebral ganglia was identified chromatographically as being authentic oxytocin. Occurence of oxytocin has been also revealed in peripheral nerves. Recently, transcripts for oxytocin and its receptor was found in ganglion cells of myenteric and submucosal plexuses. In addition, oxytocin and oxytocin receptor proteins have been demonstrated by immunohistochemistry in the enteric nervous system of the rat.

1.3 Rationale and objectives

I. Although several studies have reported the localization of oxytocin- and/or neurophysin-immunoreactive fibers in the rat spinal cord, most of them lack a detailed analysis of their segmental distribution. The only truly detailed and comprehensive study demonstrated exclusively neurophysin I (the carrier protein of oxytocin) staining, presumably due to more intense staining obtained for neurophysin as compared to oxytocin. We have therefore sought to examine the distribution of oxytocin-immunoreactive neuronal elements in the rat spinal cord, using a specific oxytocin antibody for light and electron microscopic immunocytochemical localization and quantitation by RIA.

II. Central histaminergic neurons have been shown to be involved in the control of oxytocin secretion under various physiological conditions including parturition and lactation, and both central and peripheral administration of histamine increases plasma oxytocin level. However, the literature falls short of providing sufficient data about the effect of peripheral histamine administration on the oxytocinergic neuronal systems of the central nervous system. Our objective was to examine whether oxytocin levels change in the spinal cord after peripheral administration of histamine.

III. There is scanty information available on the occurrence of oxytocin in the peripheral nervous system. In order to obtain data in the human, sensory and sympathetic ganglia as well as peripheral nerves have been examined for the presence of oxytocin using high-performance liquid chromatography and radioimmunoassay.

2. Subjects and methods

2.1 Identification of immunoreactive oxytocin in rat spinal cord

2.1.1 Light microscopic immunohistochemistry

The experiments were carried out on 12 male CFY rats weighing 200 - 310 g. The animals were anaesthetized with sodium pentobarbital and perfused through the heart with isotonic saline solution followed by 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). The spinal cords were then removed and kept in the same fixative for 4-5 hours. Transverse and horizontal sections 50-70 µm thick were cut on a Vibratome (Oxford Instruments). Oxytocin was localized by means of the peroxidase-antiperoxidase (PAP) method, using a rabbit antiserum to oxytocin.

2.1.2 Electron microscopic immunocytochemistry

Four male CFY rats weighing 200-310 g were anaesthetized with sodium pentobarbital and perfused through the heart with isotonic salt solution followed by a fixative containing 4% paraformaldehyde and 0.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4). Subsequently, the spinal cords were removed and kept in the same fixative for 4-5 hours. For preembedding staining, 40-50 µm thick cross-sections were cut with Vibratome. The sections were infiltrated with 30% sucrose, rapidly frozen in liquid nitrogen and thawed in 0.1 M phosphate buffer. Oxytocin immunoreactivity was localized by means of the PAP method. The Vibratome sections were osmicated, dehydrated and embedded. Finally, ultrathin sections were made and examined under the electron microscope.

2.1.3 Radioimmunoassay (RIA)

Twenty male CFY rats of 220-250 g body weight were decapitated and their spinal cords were quickly dissected into cervical, thoracic, lumbar, sacral and coccygeal regions. Tissue samples were sonicated in ice-cold 1.0 M HCl. Subsequently, phosphate buffer was added and the pH was adjusted to 4.0. An aliquot of the homogenate was kept for determination of the protein content. The remainder was centrifugated, and oxytocin was extracted from the supernatant using thermally activated Vycor glass powder. The evaporated residues of the extraction were redissolved in RIA buffer. Oxytocin was determined in duplicate by RIA. Results were calculated statistically by two-way ANOVA without interaction, followed by Scheffe’s test for multiple comparison.

2.1.4 Measurement of oxytocin after histamine treatment

The experiments were performed in male Wistar rats weighing 180-220 g. The animals were maintained on a 14 h light/10 h dark cycle (lights on between 06.00 and 20.00 h) and allowed to consume standard food and tap water ad libitum. They were injected intraperitoneally (i.p.) with an aqueous solution of histamine dihydrochloride in a dose of 20 mg/kg b. wt. The controls received physiological saline in the same volume. Fifteen min after the treatment the animals were killed by decapitation. Subsequently, the spinal cords were removed and their oxytocin contents were determined by RIA. The statistical evaluation was performed with the Student's t-test.

2.2 Chromatographical characterization of oxytocin in human ganglia and peripheral nerves

Four pieces of Gasserian ganglia, two pieces of L_5 ganglia, two pieces of celiac ganglia and four pieces of vagal nerves (cumulative specimens from cervical and thoracic portions) were obtained at routine post-mortem dissection from males who died without a history of any neuropsychiatric and endocrine illness. The age of the subjects ranged from 50 to 71 years, and the time elapsed from death to removal of the ganglia was 10-42 h. After removal, the samples were stored on dry ice until homogenization. They were cut into small pieces and sonicated in 1 M HCl (1 ml HCl/100 mg wet weight tissue). The acid extracts were centrifugated and the supernatants were applied to SEP-PAK C_{18} cartridges (Waters Assoc., U.S.A.) which were then washed with 0.1% trifluoroacetic acid (TFA). The immunoreactive material was eluted from the cartridges with aqueous methanol containing 0.1% TFA. An aliquot of each eluted fraction was evaporated to dryness under a nitrogen stream and subjected to RIA analysis for oxytocin. The eluted immunoreactive material was characterized using a high-performance liquid chromatography (HPLC) system.

3. Results

3.1 Immunoreactive oxytocin in the rat spinal cord

3.1.1 Light microscopic distribution of oxytocin-immunoreactive fibers

Each segment of the spinal cord contained oxytocin-immunoreactive fibers. However, no cell bodies were labelled. In the gray matter, the most intense immunoreactivity was observed at C_8-T_4 and T_{13}-L_4 levels. The superficial dorsal horn (Rexed laminae I and II) displayed moderate to intense immunoreactivity throughout the entire spinal cord. Immunoreactive fibers were found mainly in the marginal zone (Rexed lamina I) with extensions into the subjacent laminae II and III. A longitudinally oriented network of labelled fibers was detected around the central canal. The strongest labelling was observed in the upper and lower thoracic, and lumbar segments. Some fibers were seen to closely appose the ependymal layer. A preferential accumulation of labelled fibers was observed in the dorsal gray commissure in the thoracic, lumbar and upper sacral spinal cord. At the same levels, strong patchy accummulations of fibers were found in the intermediolateral column. These immunoreactive patches were interconnected by a network of horizontally running fibers. The perikarya of the preganglionic autonomic areas seemed to be surrounded by immunoreactive fibers. In the lamina VII of segments C_8-T_4 and T_{13}-L_4, strong immunoreactive bundles at irregular intervals coursed between the dorsal gray commissure and the intermediolateral column. This location corresponded to the area of nucleus intercalatus spinalis; for designation of specific autonomic nuclei. Fibers around motoneurons of the anterior horn were labelled mostly in the C_5-C_7 and L_4-L_6 segments. The rest of the gray matter contained very few labelled fibers.

In the white matter, the majority of labelled fibers were detected in the dorsal part of the lateral funiculus in the entire spinal cord. We frequently observed stained fibers in the Lissauer's tract in all spinal segments. Some of these fibers were found to enter the marginal zone of the dorsal horn. Fibers were often extending between the dorsal part of the lateral funiculus and superficial dorsal horn, Lissauer's tract, intermediolateral column and dorsal gray commissure. In the lateral funiculus, reactive fibers were seen to leave the intermediolateral column and appear around the preganglionic neurons of the pars funicularis of nucleus intermediolateralis.

Numerous fibers formed a longitudinally oriented network observed in horizontal sections. This network was the densest below the pia mater, and some fibers did enter it.

Oxytocin-immunoreactive fibers were also seen close to the subarachnoid space at the Lissauer's tract and dorsal part of the lateral funiculus. Occasional fibers were labelled in the anterior funiculus, mostly in the lumbar segments. These fibers entered the anterior funiculus from the network around the central canal, and a few of them run to the surface of the ventral median fissure. The posterior funiculus contained only a few scattered reactive fibers.

In horizontal sections of the spinal cord longitudinally oriented oxytocin-immunoreactive networks were detected around the central canal, in the dorsal gray commissure, superficial dorsal horn, Lissauer's tract, dorsal part of the lateral funiculus and lateral funiculus.

3.1.2 Electron micropscopic identification of oxytocin-immunoreactive terminals

Oxytocin-immunoreactivity was seen in unmyelinated fibers of small diameter and axon terminals in the laminae I, II, VII and X of the spinal gray matter. No immunoreactive cell bodies were labelled. Oxytocin-containing axon terminals formed mainly axo-dendritic synapses. We also found a few axo-somatic synaptic contacts with presumptive neuronal cell bodies located in the ependymal lining of the central canal. In most cases, the electron dense peroxidase immunoprecipitate completely filled the cytoplasm of the axon terminals and masked the subcellular organelles and synaptic densities. When the reaction product was lightly distributed, it was possible to see that the immunoreactive terminals contained numerous small clear vesicles and a few large dense-cored vesicles.

3.1.3 Quantitative distribution of oxytocin

Our RIA measurements revealed that oxytocin was concentrated in the caudal regions of the rat spinal cord. The oxytocin content in the cervical spinal cord was significantly lower than those found in the thoracic, lumbar, sacral and coccygeal regions. The highest quantity was measured in the lumbar spinal cord

3.1.4 Acute effect of peripheral histamine administration on spinal oxytocin content

The i.p. injection of histamine induced a 36% decrease in oxytocin content in the rat spinal cord.

3.2 Chromatographically identified oxytocin in the human peripheral nervous system

Oxytocin-like immunoreactivity was detected in extracts of human sensory (spinal L5, Gasserian) ganglia, sympathetic (celiac) ganglia and peripheral nerves (nervus vagus) by

radioimmunoassay (RIA) specific to oxytocin and was characterized by high-performance liquid chromatography (HPLC). HPLC-RIA measurements demonstrated that the Gasserian, L5 ganglia and the celiac ganglia of humans contained significant levels of immunoreactive oxytocin. However, our results showed that the immunoreactive oxytocin migrated as two individual peaks. The first immunoreactive peak was eluted at the same position as the authentic oxytocin standard. The identity of the second immunoreactive peak is not known, but it might be a degradation product of oxytocin.

4. Discussion

This study was undertaken to examine the distribution of oxytocin in the spinal cord and reveal its occurence in various parts of the peripheral nervous system. Our results show that oxytocin is localized exclusively in axons, and the distribution of oxytocin-immunoreactive fibers in the spinal cord correlates with structures related to nociceptive, autonomic and motor functions. The oxytocin-immunoreactive fibers clearly display segmentally uneven distribution. The significant rise in oxytocin content in the thoracic, lumbar and sacral segments is associated with the dense innervation in the spinal autonomic centers. We conclude that the preferential sites of accumulation of oxytocin-containing fibers in the spinal cord bear close resemblance to the locations of sympathetic and parasympathetic preganglionic neurons, suggesting oxytocinergic modulation of autonomic functions. An important finding of this study is that oxytocin-containing fibers innervate the nucleus intermediolateralis pars funicularis in the lateral funiculus, which has not been noticed by previous investigators. Our results demonstrate that oxytocin-immunoreactive fibers get close to the internal and external liquor spaces of the spinal cord. Although at the light microscopic level, many fibers seemed to appose the ependyma of the central canal, the electron microscopic immunocytochemistry revealed only occasional synaptic contacts with presumptive neuronal perikarya in the ependymal layer. Our experiments also showed that some fibers from the lateral funiculus entered the pia mater. The close proximity of oxytocin-containing fibers to the spinal liquor spaces suggests that their terminals may release oxytocin into the cerebrospinal fluid. This possibility is supported by physiological experiments in which electrical stimulation of the hypothalamic paraventricular nucleus yielded increased concentration of oxytocin in perfusates obtained from the subarachnoid space of the spinal cord. In accordance with our light microscopic results, in ultrathin electron microscopic

sections we found numerous oxytocin-immunoreactive axon profiles in the gray matter of the spinal cord, but could not detect labeled cell bodies. Our electron microscopic findings confirm the literature data that many labelled axon terminals made synaptic contacts with dendrites in the posterior horn, around the central canal and the autonomic areas of the spinal gray matter. Moreover, we also found a few axo-somatic synaptic contacts in the area of the central canal. Since the pre-embedding immunoperoxidase technique that we applied is not suitable for a more precise analysis of the subcellular structure of oxytocin-labeled synapses, we could not confirm the results of previous investigators showing that the immunolabelled presynaptic vesicles belong to the electron dense type.

Several studies indicate the role of the central histaminergic system in the control of oxytocin and vasopressin secretion. However, the effect of peripheral histamine on the oxytocin and vasopressin content of the central nervous system has been less studied. Our results show that peripheral histamine administration can produce a significant decrease in the spinal content of oxytocin. The mechanism of the action of peripheral histamine administration on the spinal oxytocin system is not clear. Histamine may exert its effect through stimulation of histamine receptors in sensory ganglia and nerve endings, and/or through baroreceptor and chemoreceptor activity alterations in the vascular system. As a result, the neuronal impulses generated, we assume, can ultimately reach oxytocinergic neurons in the paraventricular nucleus projecting to the spinal cord. Alternatively, histamine may act on the paraventriculo-spinal oxytocinergic system via the circumventricular organs.

Our RIA measurements revealed significant levels of immunoreactive oxytocin in selected samples of the human peripheral nervous system, including sensory (spinal L5, Gasserian) and sympathetic (celiac) ganglia as well as peripheral (vagal) nerves. HPLC showed that the immunoreactive oxytocin was eluted at the same position as the authentic oxytocin standard. Our results provide the first evidence for the presence of oxytocin in the human peripheral nervous system. The presence of oxytocin was later confirmed in human sympathetic paravertebral ganglia, too. The occurrence of neurohypohysial peptides has been detected in several components of the peripheral nervous system of mammals. It was found that a bioactive and immunoreactive vasopressin-like peptide is present in the sympathetic nervous system of the rat. Authentic arginine vasopressin and oxytocin were identified by chromatography in sensory neurones and peripheral nerves. Oxytocin was reported to be present in both the small dark and the large light sensory neurones of L5 dorsal root ganglia and to coexist with arginine vasopressin in the rat. Arginine-vasopressin-like immunoreactivity was axonally transported in the dorsal root and sciatic nerve. Furthermore,

observations obtained in animals neonatally treated with capsaicin suggests that vasopressin is located in primary afferent terminals in upper dorsal horn of the spinal cord. Finally, oxytocin and oxytocin receptor transcripts and protein have been recently found in the enteric nervous system. The origin and functional significance of oxytocin in the peripheral nervous system are not clear yet. Although it is very likely that oxytocin is synthesized locally in neurons of sensory and autonomic ganglia, further studies should be performed to confirm this assumption. The axonal transport of oxytocin from ganglion cells also remain to be studied. Although the great majority of oxytocin in the spinal cord has been shown to stem from the hypothalamic paraventricular nucleus, there is a minor amount the source of which is unclear. The possibility that it originates from dorsal root ganglion cells should thus be tested. There are only scarce data available on the role of oxytocin in the peripheral nervous system. Oxytocin is thought to depress the ganglionic transmission in superior cervical ganglia. In other experiments it has been shown to activate intrathoracic ganglionic neurons involved in efferent sympathetic cardiac regulation. Further studied are obviously needed to elucidate the precise role and functional significance of oxytocin in different parts of the peripheral nervous system.

5. Summary

We investigated the distribution of immunoreactive oxytocin in the rat spinal cord using immunocytochemistry and RIA. The presence of authentic oxytocin in the human peripheral nervous system was demonstrated by means of HPLC and RIA.

By light microscopic immunohistochemistry, each segment of the spinal cord from cervical to coccygeal ones contained oxytocin-immunoreactive fibers. The Rexed laminae I and II of the dorsal horn showed moderate to intense immunoreactivity. A dense network was found around the central canal where some fibers apposed the ependyma. The autonomic centers of the spinal cord at the thoracolumbar and sacral segments were heavily innervated. Few fibers were seen around the motoneurons. In the white matter, the immunoreactivity was localized mainly in the dorsal part of the lateral funiculus, in the pars funicularis of the nucleus intermediolateralis and in a longitudinal network of the lateral funiculus below the spinal cord surface. Some fibers from this network were found to enter the pia mater.

Our electron microscopic investigations showed that the oxytocin-immunoreactive fibers were unmyelinated and most of the immunoreactive axon terminals made synaptic contacts with dendrites. A few oxytocin-containing axon terminals were also observed synapsing with neronal somas located in the ependymal lining of the central canal.

RIA measurements revealed that the oxytocin content was preferentially accumulated in the caudal regions of the rat spinal cord. The cervical spinal cord had lower oxytocin content than that found in either the thoracic, lumbar, sacral or coccygeal region.

Experiments made with peripheral administration of histamine demonstrated that this agent can bring about a significant decrease in the oxytocin content of the rat spinal cord.

Oxytocin immunoreactivity was detected in extracts of human spinal L_5, Gasserian and celiac ganglia as well as vagus nerve by RIA and characterized by HPLC. HPLC/RIA examinations showed that a major part of the immunoreactive material co-eluted with a reference synthetic oxytocin, but in the extracts of spinal L5, Gasserian and celiac ganglia a second immunoreactive peak was also observed. The identity of the latter material is unknown, but it might well be a degradation product of oxytocin.

In conclusion, our results demonstrate that the distribution of oxytocin in the rat spinal cord correlates with anatomic locations related to nociceptive, autonomic and motor functions. Oxytocin-containing axon terminals shown to make synaptic contacts with dendrites and cell somas are thought to be the structures through which oxytocin modulates

spinal functions. We assume that oxytocin-containing axon terminals situated in close proximity of spinal liquor spaces play a role in secreting oxytocin into the cerebrospinal fluid. Our experiments also showed that peripherally administered histamine alters oxytocin content in the spinal cord which may have relevance under physiological and/or pathological conditions. Finally, this study revealed the occurrence of oxytocin in various parts of the human peripheral nervous system. The morphological aspects and functional significance of this finding remain to be elucidated.

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Publications related to the thesis

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