Oxytocin receptors on oxytocin neurones: histoautoradiographic detection in the lactating rat

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1. The purpose of the present study was the detection at the cellular scale of the oxytocin (OT) receptors involved in the facilitatory effect of this neuropeptide on its own release during the milk ejection reflex.

2. OT binding sites were demonstrated in brain sections by using a highly selective ^{125}I-labelled OT antagonist detected by film- and histoautoradiography.

3. Film autoradiographs revealed the presence of OT binding sites in the hypothalamic magnocellular (supraoptic, paraventricular and anterior commissural) nuclei in lactating rats, suckled or not. This detection was only possible after acute I.C.V. injection of OT antagonist which probably induced an upregulation of the OT binding sites to autoradiographically detectable levels.

4. Combined application of histoautoradiographic and immunohistochemical techniques showed that the OT binding sites were concentrated on OT magnocellular neurones. Labelling concerned cell bodies and dendrites but not the axons and endings in the pituitary neural lobe.

5. The presently detected somatodendritic autoreceptors on OT neurones probably mediate the facilitatory effect of OT on its own release during the milk ejection reflex.

The neurohormone oxytocin (OT) is synthesized in the magnocellular neurones of the paraventricular (PV) and supraoptic (SO) nuclei of the hypothalamus and carried by axoplasmic transport to the neurohypophysis from where it is released into the general circulation in response to appropriate stimuli. Thus, during suckling, OT is released in a pulsatile manner and induces the milk ejection by acting on specific receptors localized on the plasma membrane of myoepithelial cells of the mammary gland. In addition to its endocrine function, OT is known to play a role in the central nervous system by affecting physiological and behavioural regulations (see Richard, Moos & Freund-Mercier, 1991). These neurotransmitter or neuromodulator effects can contribute to neuroendocrine regulations such as the control of the release of OT during milk ejection reflex. Indeed, OT injected either into the third ventricle (Freund-Mercier & Richard, 1984) or directly into the PV or SO nuclei (Moos & Richard, 1989) facilitates the milk ejection reflex by increasing the bursting activity of OT neurones. However, the OT receptors involved in this facilitatory central effect of OT on its own release have not yet been morphologically detected. Film autoradiographical studies have reported the presence of OT binding sites in various brain areas (see Freund-Mercier, Stoeckel, Palacios, Pazos, Reichhart & Richard, 1987; Tribollet, Barberis, Jard, Dubois-Dauphin & Dreifuss, 1988), but have never shown the occurrence of OT receptors in the PV and SO nuclei. The OT binding sites detected in the magnocellular nuclei by some authors (e.g. Brinton, Wamsley, Gee, Wan & Yamamura, 1984; Van Leeuwen, Van Heerikhuize, Van Der Meulen & Wolters, 1985) resulted in fact from binding of [^{3}H]OT to the neurophysin but not to a functional receptor site (Freund-Mercier, Stoeckel, Waeber, Krémarik, Palacios & Richard, 1991). Recently, Yoshimura *et al.* (1993), using human OT receptor RNA probes, showed a small group of cells expressing OT receptor mRNA in the PV and SO nuclei of female rats. However, the authors insist that there is an inconsistency between the localization of cells expressing OT receptor mRNA and that of electrophysiologically identified OT responsive cells.

In the present study we report the conditions (i.e. lactating rats receiving an I.C.V. injection of OT antagonist) under which specific OT binding sites can be detected by autoradiography in the magnocellular nuclei. In addition, by combining histoautoradiographic localization of binding sites at the cellular scale and immunocytochemistry we have identified the neural elements

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expressing the OT binding sites. A preliminary report of this study has appeared in abstract form (Freund-Mercier & Stoeckel, 1993).

METHODS

Animals

Four different groups of Wistar rats were used in these experiments (Table 1). The first group, suckled lactating rats without treatment and anaesthesia, served as control. The animals of the other groups, suckled and unsuckled lactating rats and male rats, were anaesthetized with a single dose of urethane (1·2 g kg^{-1} I.P.) and placed in a stereotaxic apparatus. A microsyringue (Hamilton) was inserted into the third ventricle and its correct position was confirmed when 1 or 2 μl of cerebrospinal fluid were drawn up to the syringe. After at least 1 h, I.C.V. injection of OT or vasopressin (VP) antagonists was performed in 1 μl of 150 mM NaCl; the rats were killed 5 or 20 min later.

All rats were killed by rapid decapitation. The brains were removed, frozen in isopentane at −40 °C and stored at −20 °C until sectioning. Serial frontal sections (20 μm) through the hypothalamus were cut on a microtome cryostat, thaw-mounted onto gelatine-coated slides and stored at −20 °C until use.

Autoradiographic detection of binding site

Ligand preparation, binding procedure and autoradiographic detection of OT and VP binding sites on films and on sections (histoautoradiography) were performed as described by Krémarik, Freund-Mercier & Stoeckel (1993).

The unlabelled OT antagonist d$(CH_2)_5$[Tyr(Me)^2,Thr^4,Tyr-$NH_2{}^9$]OVT and the linear VP antagonist Phaa,D-Tyr(Me), Phe,Gln,Asn,Arg,Pro,Arg,Tyr-NH_2, generously supplied by Dr M. Manning, were iodinated using chloramine T. The mono-iodinated compounds were purified on a reverse-phase column by high-performance liquid chromatography.

Frozen sections were pre-incubated in Tris buffer (170 mM Tris–HCl, 5 mM $MgCl_2$, 0·1% bovine serum albumin; pH 7·4) for 20 min at 15 °C. They were then incubated at 4 °C for 24 h in the same buffer containing either 30 pM ^{125}I-OT antagonist or 50 pM ^{125}I-VP antagonist. Non-specific binding was determined in the presence of 5 μM OT or VP respectively. After incubation, sections were washed twice in cold buffer, then in cold distilled water and dried with a cold air stream.

Film autoradiographs were generated by apposing labelled sections to Hyperfilms (Amersham, Aylesbury, Bucks, UK) in X-ray cassettes. After 3–5 days exposure at 4 °C, the films were developed for 5 min in Kodak D-19 at 18 °C. After exposure, the sections were postfixed with paraformaldehyde vapours at 80 °C for 3 h. The slides were then defatted and coated with K5 emulsion (Ilford, Mobberley, Cheshire, UK) for histoautoradiography. After 20–24 days exposure at 4 °C, the slides were developed for 5 min in Kodak D-19 at 18 °C, stained with thionine, mounted with Permount and observed using dark-field and bright-field light microscopy.

Immunohistochemistry

Immunoperoxidase labelling was performed on the sections processed for histoautoradiography and on untreated adjacent sections. After analysis of histoautoradiographs, the emulsion was removed from the slides by incubating them for 5 min at 37 °C in a solution containing 1·6 mg ml^{-1} trypsin, 1·6 mg ml^{-1} collagenase and 0·3 mg ml^{-1} proteinase K in phosphate-buffered saline (pH 7·3). The sections were then processed like the adjacent untreated sections. They were fixed for 20 min in 4% paraformaldehyde in 0·1 M phosphate buffer (pH 7·4) and subsequently microwave-irradiated at 750 W for 3 × 5 min, in 10 mM sodium citrate buffer (pH 6), before immunoperoxidase labelling. Antibodies were either monoclonals against OT–neurophysin (OT–NP, provided by Drs D. Pow and J. Morris, Oxford) diluted at 1:100, or VP–NP (a gift from Dr R. Acher, Paris) at 1:100000, or a polyclonal anti OT–NP (Dr R. Acher) at 1:50000. They were diluted in phosphate-buffered saline containing 0·5% Triton X-100 and applied for

Table 1. Animals and drugs used in the different experiments

Rats	Antagonists	Doses (ng)	n_{20}	n_5
Lactating, suckled	—	—	6	—
	a	500	2	—
	b	200	5	—
	b	500	4	1
	c	50	1	2
	c	100	4	3
	d	500	2	—
Lactating, unsuckled				
24 h*	c	100	2	—
1 h	c	100	2	—
20 min	c	100	2	—
0 min	c	50	2	1
Males	b	200	3	—
	c	100	3	—

Antagonists: a, d$(CH_2)_5$OVT; b, d$(CH_2)_5$[Tyr(Me)^2]OVT; c, d$(CH_2)_5$[Tyr(Me)^2,Thr^4,Tyr-$NH_2{}^9$]-OVT; d, d$(CH_2)_5$[Tyr(Me)^2]AVP. n_{20}, rats killed 20 min after the antagonist injection; n_5, rats killed 5 min after the antagonist injection. *Delay between removal of the pups and I.C.V. injection.

14 h at room temperature. Detection was performed by using the ABC-Elite kit (Vector, Burlingame, CA, USA) according to the manufacturer's instructions. After immunolabelling, some sections were slightly stained with thionine or Toluidine Blue to enable correlation with previous autoradiographic labelling of OT binding sites.

RESULTS

In lactating rats which had received I.C.V. OT antagonist injection, OT binding sites were detectable in the hypothalamic magnocellular nuclei. Film autoradiographs (Fig. 1*A*) revealed specific labelling throughout the supraoptic (SO) nuclei, whereas in the paraventricular (PV) nuclei the anterior part was mainly concerned. Among the accessory nuclei, intense labelling was constantly observed in the anterior commissural nuclei. This labelling occurred in all OT antagonist-treated lactating rats and did not depend on (1) the nature of the OT antagonist injected, (2) the delay between injection and death (5–20 min), or (3) the occurrence of the milk ejection reflex. OT binding sites were never detected in the magnocellular nuclei of male rats which received I.C.V. injections of OT antagonists. In untreated lactating rats (Fig. 1*B*), labelling of the SO and PV nuclei was generally absent; in a few cases it was hardly detectable in the PV and the SO nuclei but was never found in the anterior commissural nuclei. Specific VP binding sites were not detected in the magnocellular nuclei in either treated (I.C.V. OT or VP antagonists) or untreated male or lactating rats. The I.C.V. injection of these antagonists did not modify the distribution of the OT- and VP binding sites in the other brain areas (not shown).

Histoautoradiographic detection of the OT binding sites in the SO nucleus showed labelling of magnocellular cell bodies and of the ventral glial lamina (Fig. 2*A* and *B*). Labelling of cell bodies occurred mainly in the dorsal and lateral parts of the nucleus, whereas the area adjacent to the optic chiasma was almost unlabelled. Immunolabelling showed that the neurones exhibiting OT binding sites were also OT–NP immunoreactive (Fig. 2*A*). In the ventral glial lamina OT binding sites were highly concentrated in the area dorsal to the layer of glial cell nuclei, mainly in its lateral part (Fig. 2*B*).

In the PV nucleus, OT binding sites were mainly concentrated over the anterior medial magnocellular cell group; more caudally, they extended over the lateral group whereas the most posterior area of the nucleus

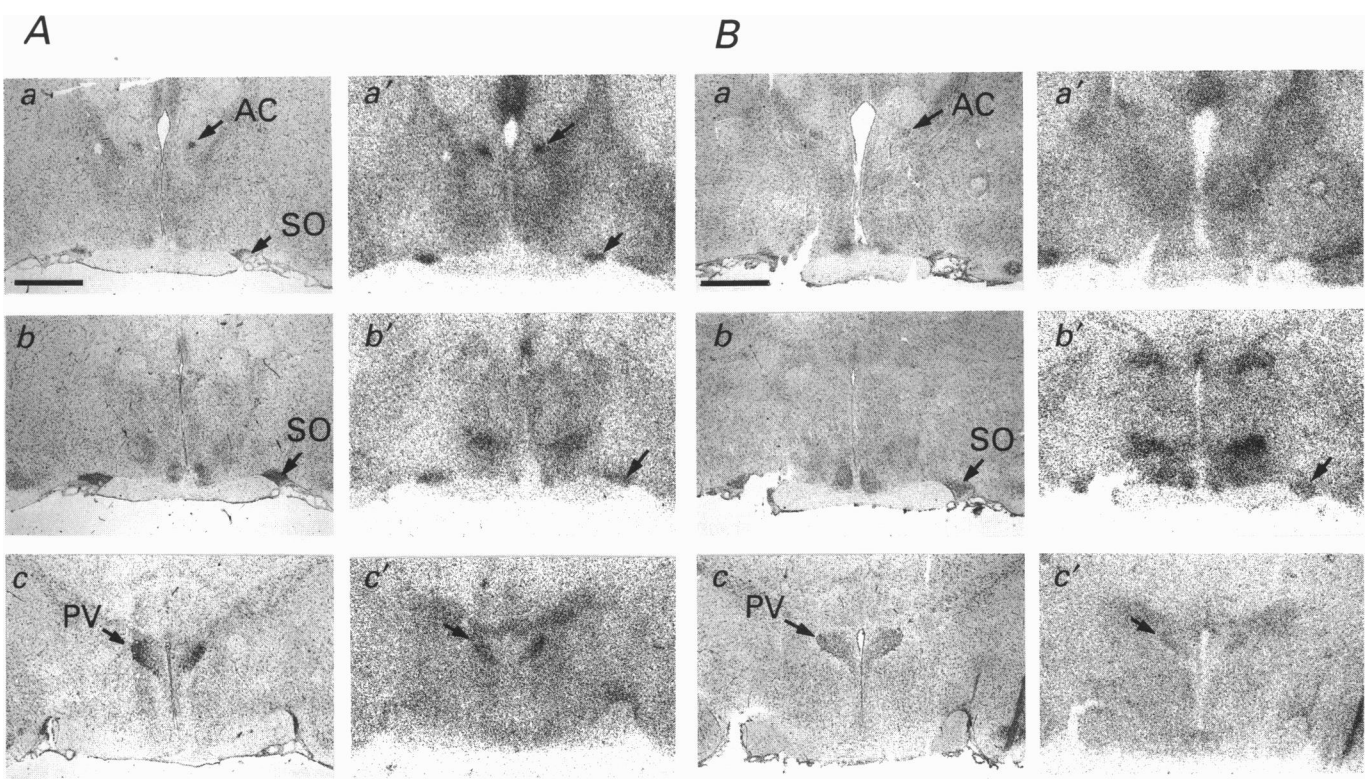

Figure 1. Localization of OT binding sites on magnocellular nuclei
^{125}I-OT antagonist-labelled, thionine-stained frontal sections (*a*, *b*, *c*) and corresponding film autoradiographs (*a'*, *b'*, *c'*) of the hypothalamus of two lactating rats. *A*, in the rat killed 20 min after I.C.V. injection of OT antagonist, OT binding sites are concentrated in the anterior commissural (AC), the SO and the PV nuclei. *B*, in the control rat (suckled, lactating, non-injected), no labelling is detectable in the AC nuclei, whereas SO and PV nuclei are faintly labelled. Scale bars, 1 mm.

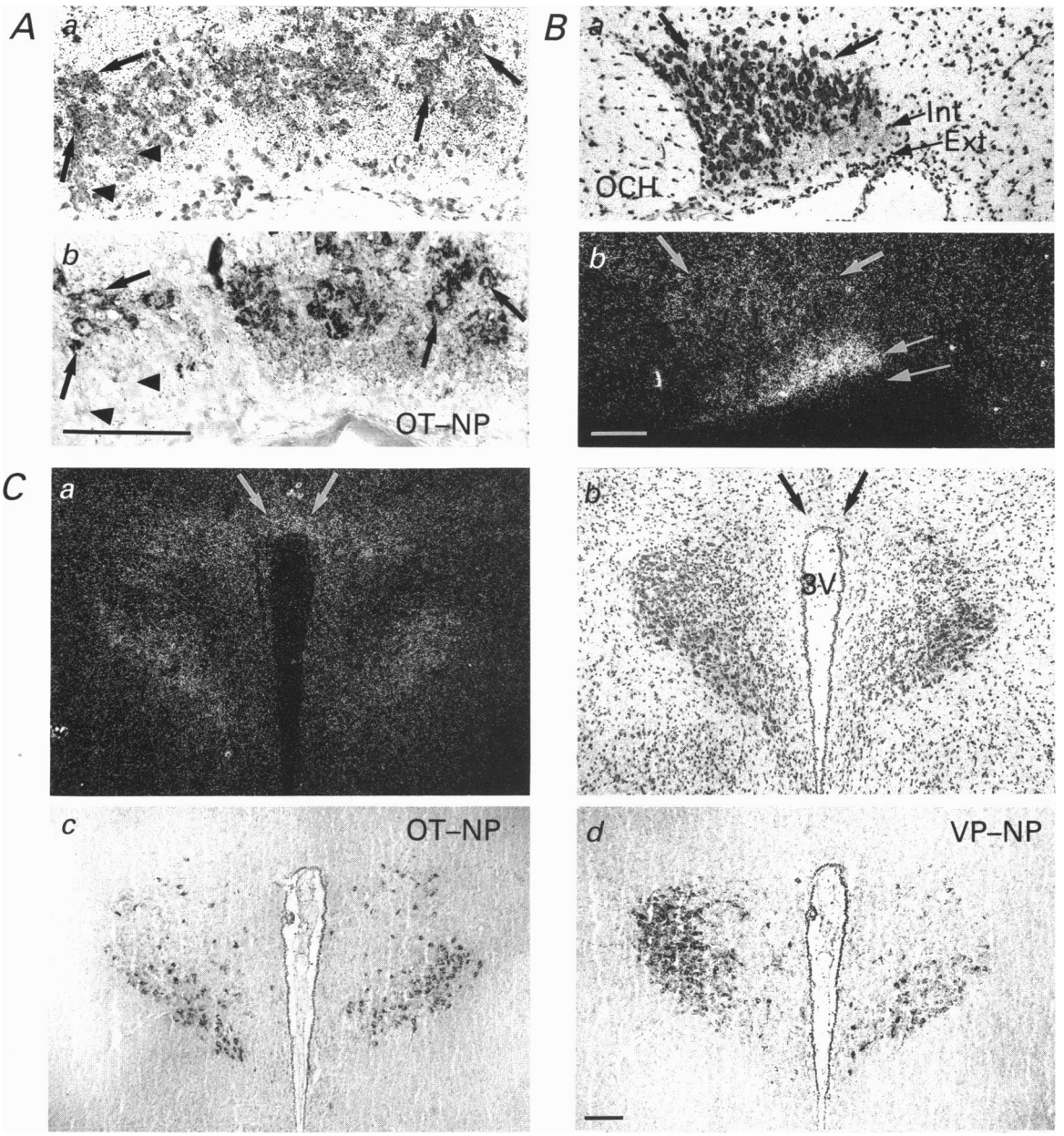

Figure 2. Comparison between distributions of OT binding sites and OT and VP neurones in the SO and PV nuclei

A, section through the anterior part of the SO nucleus successively processed for histoautoradiographic detection of OT binding sites (*A a*) and OT–NP immunolabelling with the monoclonal antibody (*A b*) showing concentration of OT binding sites on OT neurones (arrows indicate double-labelled neurones). The arrowheads indicate an area of non-immunoreactive neurones (VP cells) devoid of silver grains. *B*, bright-field (*Ba*) and dark-field (*Bb*) photomicrographs of OT binding sites on a section of the medial part of the SO nucleus. Labelling is moderate on the cell bodies of the dorsolateral area (arrows) and is much more intense on the internal layer (Int) of the ventral glial lamina which is separated from the external layer (Ext) by the alignment of the glial cell nuclei. *C*, in the PV nucleus, OT binding sites (dark-field (*Ca*) and bright-field (*Cb*) photomicrographs of the same section) exhibit the same distribution as OT–NP immunoreactive neurones labelled with the polyclonal antibody (*Cc*), but no coincidence with VP–NP immunoreactive neurones (*Cd*) detected on adjacent sections. The slightly asymmetric section includes anterior (right) and central (left) levels of the PV nuclei. Above the third ventricle (3V), the PV nuclei are linked by a labelled area deprived of cell bodies (*Ca* and *b*, arrows). OCH, optic chiasma. Experimental animals were lactating rats, 20 min after I.C.V. injection of OT antagonist. Scale bars, 100 μm.

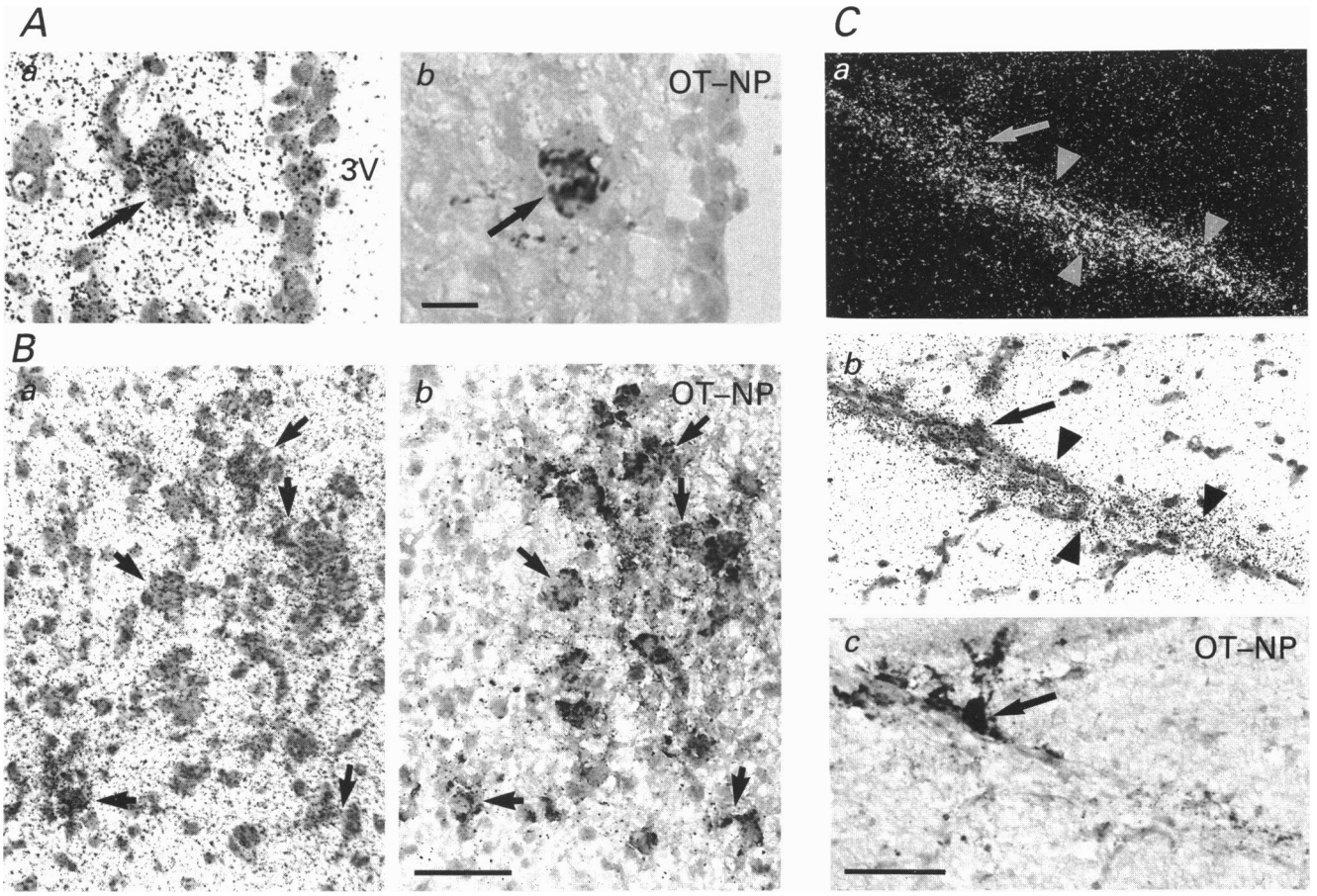

Figure 3. Detection of OT binding sites on OT neurones
OT binding sites detected by autoradiography (*A a*, *Ba*, *Ca* and *b*) and OT–NP immunoreactivity detected with the monoclonal antibody (*A b*, *Bb*, *Cc*) are co-localized on an isolated subependymal neurone (*A*, arrows) belonging to the PV nucleus, on neurones of the anterior commissural nucleus (*B*, arrows) and on a neurone of the circular nucleus (*C*). In *C*, OT binding sites (*Ca*, dark-field; *Cb*, bright-field) extend beyond the OT–NP immunoreactive cell body (*Cc*, arrows) along the underlying vascular wall (arrowheads). 3V, third ventricle. Experimental animals were lactating rats, 20 min after I.C.V. injection of OT antagonist. Scale bars: *A*, 10 μm; *B* and *C*, 50 μm.

remained unlabelled. In the labelled areas silver grains appeared concentrated on magnocellular cell bodies and scattered around them, without extending beyond the lateral borders of the nucleus (Fig. 2*C a* and *b*). Dorsal to the third ventricle, a labelled zone containing no cell bodies linked the two PV nuclei (Fig. 2*C a* and *b*). Distribution of OT binding sites clearly coincided with that of the OT–NP-positive neurones as seen on adjacent sections immunostained for OT–NP and VP–NP respectively (Fig. 2*C c* and *d*). Co-localization of OT binding sites and OT–NP immunolabelling on a subependymal PV neurone is shown on Fig. 3*A*.

In the anterior commissural nuclei, OT binding sites were highly concentrated on cell bodies which reacted with antibodies against OT–NP (Fig. 3*B*). In the circular nucleus, formed by clusters of magnocellular neurones scattered between the SO and the PV nuclei, OT–NP immunoreactive neurones exhibited high densities of OT binding sites (Fig. 3*C*). In these clusters, frequently associated with blood vessels, labelling concerned the cell bodies and extended beyond them along the vascular wall (Fig. 3*C*).

Specific OT binding sites were undetectable in the neural lobe in any experimental condition (not shown).

DISCUSSION

The present report shows for the first time the presence of specific OT binding sites on OT neurones of the magnocellular hypothalamic PV and SO nuclei. The autoradiographic demonstration of these sites was only possible in lactating rats which received I.C.V. injection of OT antagonist. These binding sites probably represent the OT receptors involved in the facilitatory action of OT on its own release during milk ejection reflex.

A combination of histoautoradiographic and immunocytochemical techniques clearly showed that OT binding sites in the hypothalamic SO, PV and accessory nuclei occur on OT magnocellular cell bodies. This was particularly obvious in the anterior commissural nucleus known to be formed by OT neurones projecting into the neural lobe (Sofroniew, 1985). The presence of OT binding sites on dendritic processes could in addition be observed in several locations. Thus, in the SO nucleus, strong labelling occurred in the dorsal layer of the ventral glial lamina, where cell bodies are absent but where dendrites of the magnocellular SO neurones are highly concentrated (Armstrong, Schöler & McNeill, 1982). In the circular nucleus, the intense perivascular labelling extending beyond the perikarya could be related to dendritic structures, which, according our ultrastructural observations, are apposed against the vascular wall. In the PV nucleus, due to the organization of the dendrites which mostly stay within the nucleus (van den Pol, 1982), dendritic labelling was less easy to demonstrate; however, the labelling dorsal to the third ventricle was probably related to the dendritic bundle linking both PV nuclei (Sofroniew & Glasmann, 1981). Taken together, our observations strongly support localization of specific OT binding sites on the cell bodies and dendrites of the OT neurones, but not on their axons and endings in the neural lobe. No observations actually support location of such sites on other neurones of these nuclei, e.g. VP neurones or interneurones. Glial OT receptors, as reported on hypothalamic astrocytes in culture (Di Scala-Guenot & Strosser, 1992), were not evident in our preparations. Thus, OT binding sites were absent from the external layer of the ventral glial lamina, which is exclusively formed by astrocytic processes.

The presence of OT binding sites on OT neurones in amounts detectable by autoradiography was probably due to an upregulation process induced by the I.C.V. OT antagonist injection. The phenomenon of antagonist-induced upregulation has been well characterized for neurotransmitter receptors. As regards the neuropeptides, available data mainly concern opiate receptor systems. Although an increase in the number of opiate receptors was mainly observed after chronic administration of opiate antagonists (Tempel, Gardner & Zukin, 1984), there are examples of rapid effects. Enhancement of brain opiate receptors occurs 5 min after peripheral naloxone administration (Pert & Snyder, 1976), a delay comparable to that with the presently detected OT binding sites on the OT neurones. The opiate receptor antagonist-induced upregulation varied, moreover, according to the localization in the brain (Tempel *et al.* 1984). This could also be true for the OT receptors, which increased to detectable levels in the SO and PV nuclei and remained apparently unchanged in other brain areas. Resistance to upregulation of the brain OT receptors has already been reported after PV nucleus lesions, whereas downregulation was, in contrast, observed after chronic administration of high concentrations of OT in the cerebrospinal fluid (Insel, Winslow & Witt, 1992). The high concentration of OT released in the magnocellular nuclei of the lactating rat (Moos, Poulain, Rodriguez, Guerné, Vincent & Richard, 1989) may possibly induce downregulation of the OT receptors on OT neurones, resulting in levels almost undetectable by the autoradiographic technique used.

The OT binding sites on the OT magnocellular neurones are probably involved in the facilitatory effect of OT on OT neurones during the milk ejection reflex, suggested by electrophysiological data (Freund-Mercier & Richard, 1984). In lactating rats, the excitatory effects of OT were restricted to OT neurones and were not detected on VP neurones or on neurones not projecting to the neural lobe. This effect was, on the other hand, specific for OT since VP did not modify the firing rates of either OT or VP neurones. This is in agreement with the absence of detectable VP binding sites in the SO or PV nuclei, even after I.C.V. injections of OT or VP antagonists. The OT binding sites described in this study cannot, however, account for all reported effects of OT in the magnocellular nuclei. Thus, in the male rat, OT has been shown to increase the firing rate of neurones in the SO and PV nuclei (see references in Richard *et al.* 1991), areas in which we could not detect specific OT binding sites, even after I.C.V. OT antagonist injections.

In conclusion, the present data show that the OT receptors mediating the facilitatory effect of OT on its own release are located on the somata and dendrites of the OT neurones. To our knowledge, these receptors represent the first example of somatodendritic autoreceptors for peptides.

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