Expression of galanin in hypothalamic magnocellular neurones of lactating rats: co-existence with vasopressin and oxytocin

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Abstract

Lactation is a physiological condition known to upregulate the expression of the hypothalamic neurohormones, oxytocin and vasopressin, in the rat supraoptic and paraventricular nuclei. Other neuropeptides such as galanin are co-localized in the same magnocellular neurones and their expression has been demonstrated to be regulated by different experimental and physiological conditions. In the present study, we investigated the possible changes in galanin expression during lactation, using *in situ* hybridization and immunohistochemistry separately or in combination. Galanin messenger RNA concentrations decreased on day 3 of lactation in both the supraoptic and paraventricular nuclei and remained low on day 7 of lactation, but no differences were observed between control and 14-day lactating rats. In parallel, immunopositive cell bodies were almost undetectable on day 7 of lactation and immunoreactivity remained weak after 14 days of lactation, whereas galanin immunoreactive profiles in the supraoptic nucleus were more numerous than in the control group. Moreover, the subcellular distribution of immunostaining changed on day 14 of lactation. Galanin immunoreactivity was confined around the nucleus in the

control females, but it became weaker and more homogenously distributed throughout the cytoplasm in the lactating rats. Electron microscopy using a pre-embedding technique confirmed that galanin immunoreactivity was no longer restricted to the Golgi complex, but was apparent throughout in the cytoplasm. Multiple labellings showed galanin and galanin messenger RNA to be co-localized with oxytocin messenger RNA in neurones of the dorsomedial part of the supraoptic nucleus during lactation. Some of those doubly labelled cells also expressed vasopressin messenger RNA in the same conditions as revealed by a triple-labelling procedure. As these co-localizations have not been observed in female control rats, lactation provided an example of a physiological condition inducing oxytocin and galanin co-synthesis in a subpopulation of magnocellular neurones.

In conclusion, we have demonstrated plasticity of galanin expression during lactation in the hypothalamic magnocellular neurones. This plasticity could be caused by changes in galanin expression or in galanin processing in magnocellular neurones.

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Introduction

The magnocellular neurones of the rat supraoptic (SON) and paraventricular (PVN) hypothalamic nuclei have been demonstrated (Bargman and Scharrer 1951) to produce neuropeptides: the antidiuretic hormone, vasopressin, and oxytocin, which regulates parturition and milk letdown. These hormones are transported in the axons through the internal layer of the median eminence to the posterior lobe of the pituitary, where they are released into the blood vessels.

In the SON and PVN of control rats, immunohistochemistry (IHC) (Vandesande & Dierickx 1975) and *in situ* hybridization histochemistry (ISH) (Mohr *et al.* 1988, Young 1992) classically described two distinct populations of magnocellular neurones synthesizing either vasopressin or oxytocin. In the same neurones, many

co-existing peptides have been demonstrated to be synthesized, stored and probably released; however, their amount as well as their rate of synthesis are lower than for vasopressin and oxytocin (Brownstein & Mezey 1986, Villar *et al.* 1990). For example, the biologically active peptide, galanin, first isolated from the pig intestine (Tatemoto *et al.* 1983), was shown to co-exist with vasopressin in normal conditions (Melander *et al.* 1986*b*, Rökaeus *et al.* 1988, Skofitsch *et al.* 1989). In contrast, the co-localization of galanin and oxytocin or oxytocin mRNA was demonstrated in some magnocellular neurones only after injection of colchicine (Gayman & Martin 1989, Landry *et al.* 1991, Levin & Sawchenko 1993) or experimental manipulations such as hypophysectomy (Meister *et al.* 1990*b*).

Different physiological or experimental stimulations can modify the neurochemical content of the magnocellular

neurones. Thus, vasopressin and oxytocin and their mRNAs have been reported to be co-localized in some magnocellular neurones of the SON and PVN after stimulation such as salt-loading (Kiyama & Emson 1990), water deprivation (Trembleau *et al.* 1993) or lactation (Mezey & Kiss 1991). Lactation induces structural plasticity of oxytocin-containing neurones in the SON (Theodosis *et al.* 1986). Moreover, oxytocin gene activity is increased by lactation (Van Tol *et al.* 1988, Zingg & Lefebvre 1988, Crowley *et al.* 1993), as is vasopressin gene expression (Van Tol *et al.* 1988, Zingg & Lefebvre 1988, Mezey & Kiss 1991, Crowley *et al.* 1993). So far, however, only a few studies have attempted to describe lactation-induced effects on co-contained galanin. Ericksson *et al.* (1996) described a decrease in galanin mRNA in the PVN after 8 days of lactation.

The aim of our study was to investigate possible changes in expression of galanin and galanin mRNA at various times of lactation, using IHC and ISH respectively. We also studied possible specific co-localizations of galanin with both main post-hypophyseal peptide mRNAs in these conditions.

## Materials and Methods

Probes

Oligonucleotide probes were synthesized by the phosphite/phosphotriester method on an automated DNA synthesizer (Applied Biosystem, Foster City, CA, USA). Six oligonucleotide probes were used for *in situ* detection of sequences belonging to vasopressin, oxytocin and galanin mRNAs.

VP-41 was a 41-mer oligonucleotide probe complementary to vasopressin mRNA sequence encoding for the glycopeptide region of the rat vasopressin gene (amino acid residues 115–128), this sequence having no counterpart in oxytocin mRNA.

OT-25 was a 25-mer oligonucleotide probe complementary to oxytocin mRNA sequence encoding for amino acid residues 97–105. Four galanin oligonucleotides complementary to several galanin mRNA sequences were used. GAL-48 was a 48-mer probe complementary to the region encoding for amino acid residues 3–19 and the three GAL-33 were 33-mer probes complementary to the region encoding for amino acid residues 33–43, 55–65 and 73–83.

The oligonucleotides were labelled with ^{35}S-dATP (>1000 Ci/mmol) (Amersham, Amersham, Bucks, UK) or digoxigenin-11-dUTP (Boehringer, Mannheim, Germany) by tailing the 3′ end using terminal transferase (Boehringer). The radiolabelling reaction was carried out by incubating in a final volume of 10 μl the following mixture: 2 pmol oligonucleotide, 20 μCi ^{35}S-dATP, 25 U terminal transferase, 1 μl 10 × CoCl$_2$ (25 mm; Boehringer) and 2 μl 5 × terminal transferase labelling

a : Galanin mRNA in the supraoptic nucleus during lactation
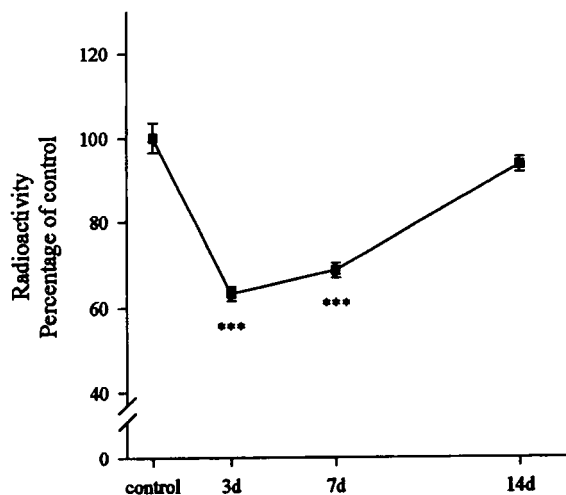
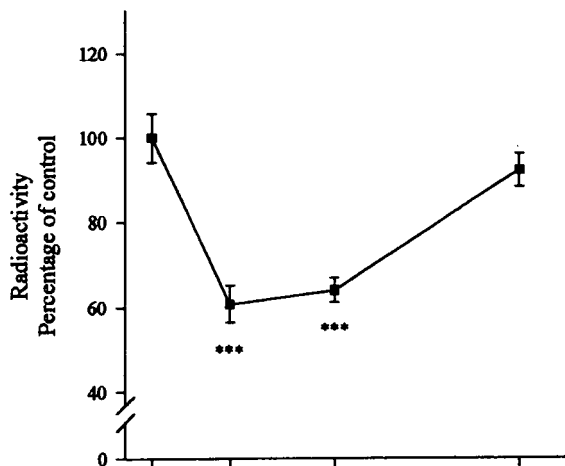**b** : Galanin mRNA in the paraventricular nucleus during lactation

Figure 1 Quantification of the effect of lactation on galanin mRNA concentrations in the SON and PVN of control female rats and rats on days 3 (3d), 7 (7d) and 14 (14d) of lactation (*n*=5 in each group). Galanin mRNA concentrations decreased on day 3 of lactation, remained low on day 7 but attained control values after 14 days of lactation in both the SON and the PVN. *** *P*<0·001 compared with control group (ANOVA followed by Dunett's test).

buffer (1 M potassium cacodylate, 125 mM Tris–HCl, 1·25 mg/ml BSA, pH 6·6; Boehringer). After 1 h at 37 °C, the reaction was stopped by the addition of 100 μl 10 mM EDTA. Finally, the radiolabelled probes were
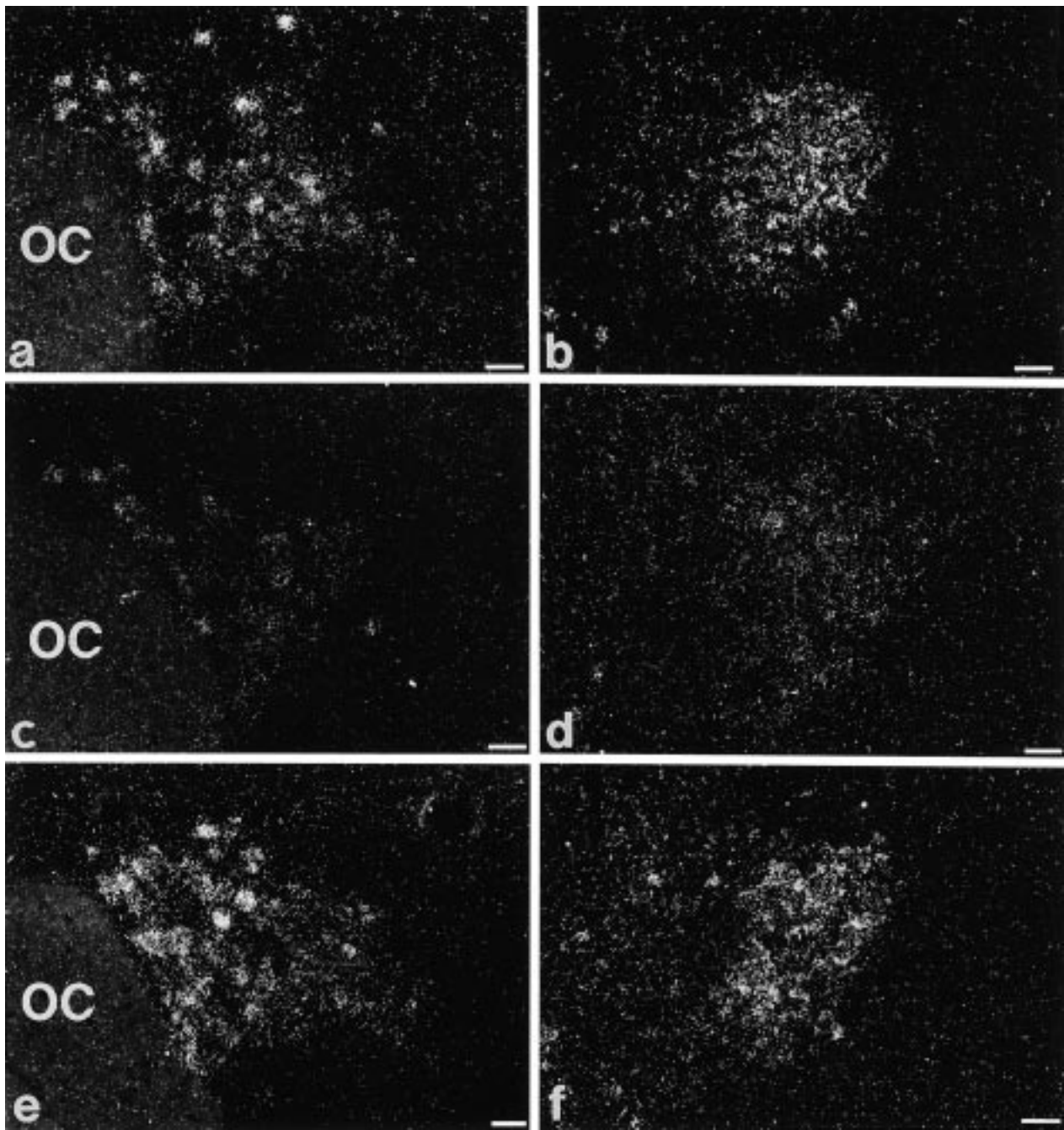
Figure 2 *In situ* hybridization of radioactive oligonucleotide for galanin mRNA to the SON (a, c, e) and PVN (b, d, f) of a control rat (a, b) and a rat on days 7 (c, d) and 14 (e, f) of lactation. The cellular intensity of the signal decreased after 7 days of lactation, but no differences were observed between control and 14-day lactating females. OC, optic chiasma. Bar represents 50 µm.

ethanol–precipitated and stored at −20 °C as described elsewhere (Trembleau *et al.* 1993). The specific activity was about 1–4 × 10⁸ c.p.m./g.

The digoxigenin-labelled probe was prepared according to a procedure described previously (Schmitz *et al.*

1991). Briefly, 100 pmol oligonucleotide were incubated in a final volume of 20 µl with 1 nmol digoxigenin-11-dUTP, 9 nmol dATP (Sigma, St Louis, MO, USA), 55 U terminal transferase, 2 µl 10 × CoCl₂ and 4 µl 5 × terminal transferase labelling buffer. After 30 min at
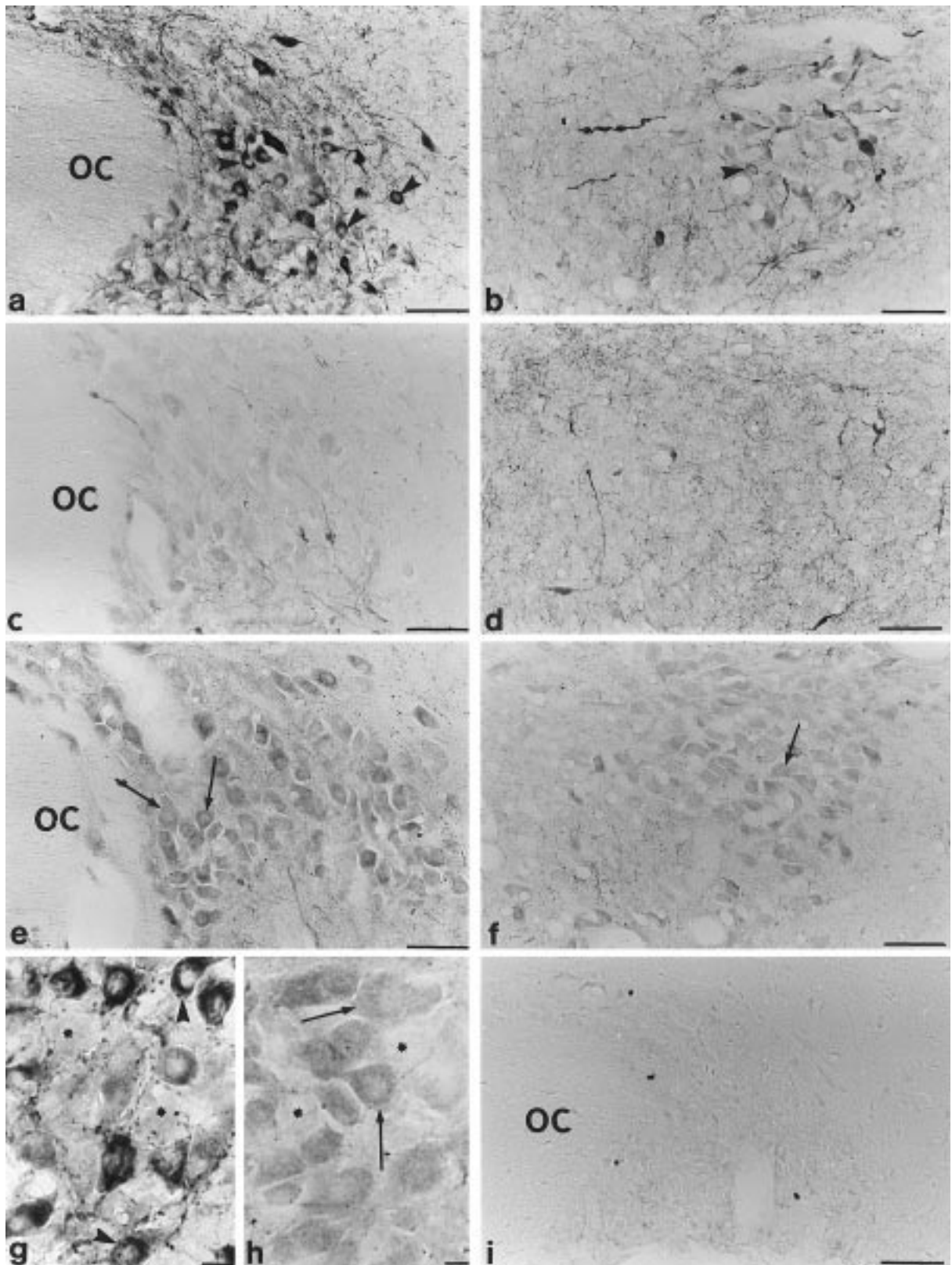
37 °C, the reaction was stopped and the probes were ethanol-precipitated and stored at − 20 °C.

Animals

Twenty-five lactating female Sprague–Dawley rats (250–350 g) on days 3 (n=5), 7 (n=7) and 14 (n=13) of lactation were studied. Control female Sprague–Dawley rats (250–350g, n=13) were at diestrus. Animals were housed in individual cages under constant light–darkness cycle (lights on between 0600 and 1800 h) in a temperature- and humidity-controlled environment. They had free access to laboratory chow and tap water. One day after delivery, the litter was limited to eight pups.

Five rats in each group were used for the quantitative ISH experiments. For the immunohistochemical and multiple-labelling experiments, two rats were killed on day 7 of lactation, six on day 14 and six control females were also killed. Control rats (n=2) and rats on day 14 of lactation (n=2) were used for the electron microscopic experiments.

In situ *hybridization with VP-41, OT-25 and GAL-33 oligonucleotides*

The animals were deeply anaesthetized with sodium pentobarbital (0·1 ml/100 g body weight) and perfused through the ascending aorta with 50 ml saline and then with 300 ml ice-cold 4% paraformaldehyde in 0·1 M phosphate buffer (pH 7·4). The brain was removed, post-fixed for 2 h in the same fixative and then immersed overnight at 4 °C in sterile PBS, pH 7·4, containing 15% sucrose. The brains were frozen in isopentane cooled to − 60 °C and stored at − 80 °C. Frontal sections (12 μm thickness) were cut in a cryostat (Reichert–Jung Frigocut 2800E) and collected in sterile culture dishes containing PBS (30 sections per well).

All the hybridization steps were carried out on free-floating sections in sterile culture dishes. The sections were rinsed several times in sterile PBS. The prehybridization step was performed by incubating the sections in 4 × SSC (1 × SSC=0·15 M NaCl, 0·015 M sodium citrate), 1 × Denhardt's solution (0·02% Ficoll, 0·02% poly-vinylpyrrolidone, 0·02% BSA) at 37 °C for 1 h, then the free-floating sections were immersed overnight at 42 °C in a hybridization buffer modified from Young (1989) (50% formamide, 600 mM NaCl, 80 mM Tris–HCl, pH 7·5,

4 mM EDTA, 0·05% disodium pyrophosphate, 0·05% tetrasodium pyrophosphate, 0·2% *N*-lauryl sarcosyl, and 10 mM dithiothreitol only when using the radioactively labelled probe; Sigma). This hybridization buffer contained 1 nM of the radiolabelled OT-25 or VP-41 probe and 10 nM of the digoxygenin-labelled OT-25 or GAL-33 probes. Finally, the sections were washed in several baths (30 min each) at room temperature (twice in 2 × SSC, once in 1 × SSC, once in 0·5 × SSC) and at 42 °C (twice in 0·1 × SSC).

In situ *hybridization with radioactively labelled GAL-48 oligonucleotide*

The animals were decapitated, the brain was quickly removed, frozen in isopentane, cooled to − 30 °C and stored at − 80 °C until frozen coronal sections (12 μm thickness) were cut in a cryostat through the hypothalamus and thaw-mounted onto Super-Frost D$^+$ slides (CML, Nemours, France). Sections were then fixed in 4% paraformaldehyde in sterile PBS for 5 min, rinsed twice for 10 min in sterile PBS, delipidized through a series of ethanols (60–100%) and then chloroform. After partial rehydration in ethanol (100–95%), they were air dried.

Without prehybridization, the sections were incubated overnight at 42 °C in the same hybridization buffer as described above, containing 1 nM of the radiolabelled GAL-48 probe. They were washed in 1 × SSC at 55 °C (four times 15 min) and then at room temperature (1 h).

Detection of the digoxigenin-labelled probes

After the post-hybridization washes, the sections were immersed for 10 min in buffer A (0·1 M Tris, pH 7·5, 1 M NaCl, 2 mM MgCl$_2$) containing 2% normal sheep serum and 0·1% Triton X-100 and then incubated overnight at 4 °C with the alkaline phosphatase-labelled anti-digoxigenin F(ab) fragment (Boehringer) (1 : 5000) in buffer A. The sections were then rinsed three times in buffer A (10 min each), in buffer B (0·1 M Tris, pH 9·5, 0·1 M NaCl, 5 mM MgCl$_2$) for 5 min, and in buffer C (0·1 M Tris, pH 9·5, 0·1 M NaCl, 5 mM MgCl$_2$) for 5 min. Alkaline phosphatase activity was developed by incubating the sections with NBT/BCIP (Gibco BRL, Gaithersburg, MD, USA) diluted in buffer C. The enzyme reaction was stopped by rinsing in buffer C. The sections were

Figure 3 Immunohistochemical detection of galanin in the SON (a, c, e, g, h) and PVN (b, d, f) of a control rat (a, b, g) and a rat on days 7 (c, d) and 14 (e, f, g) of lactation. In control rats, neurones were strongly labelled (arrow heads) and the precipitate was frequently seen in the perinuclear area (g) (arrow heads). Galanin-immunoreactive perikarya were scarcely detectable at 7 days of lactation and the signal remained weaker than in the control rats after 14 days of lactation. On day 14 of lactation, the precipitate diffused throughout the cytoplasm of the positive neurones (h) (arrows). Both in control (g) and 14-day lactating (h) females, galanin-immunoreactive neurones can be distinguished from non-immunoreactive cells (asterisks). Note that g and h are higher-power magnifications of a and c respectively; they have been rotated through 90 °. (i) No signal can be seen after pre-adsorption of the galanin antibody in the SON of a 14-day lactating female. OC, optic chiasma. Bar represents 50 μm (a–f, i) or 10 μm (g–h).

mounted onto gelatin-coated slides and air dried before being mounting in glycerol phosphate (3 : 1, v/v).

Detection of the ^{35}S-labelled probes

All the sections were air dried and covered with Amersham-β-max radiographic film (Amersham) for 2–4 h (vasopressin mRNA or oxytocin mRNA detection) or 8 days (galanin mRNA detection). The films were developed with Microdol X (Kodak, Rochester, NY, USA) for 10 min at 18 °C, rinsed and fixed for 10 min. The autoradiographic films were processed for quantitative analysis. The slides were dipped into Ilford K5 nuclear emulsion (Ilford, Mobberly, Cheshire, UK) diluted in water (1:1, v/v) and exposed for 2 days (vasopressin or oxytocin mRNA detection) or 1 month (galanin mRNA detection) at 4 °C in darkness before being developed with Kodak D19 (Kodak) for 4 min at 17 °C and fixed with sodium thiosulphate. Sections were then stained with toluidine blue. Finally, all the slides were rinsed in water and mounted in Permount. The same exposure times were used for the control and lactating groups when quantitative ISH for galanin mRNA was performed.

Whatever the technique (radioactive or non-radioactive), the distribution of the cells labelled with oxytocin probe was the same. Moreover, radioactive and non-radioactive ISH procedures for galanin mRNA detection gave similar results.

Quantitative analysis of the autoradiograms

The optical density of autoradiograms was measured using an image analysis system developed in our laboratory by P Mailly as described previously (Landry *et al.* 1995). A Lhesa LH4015 video camera equipped with a Minolta macro zoom objective was used to register the images. Each image was digitized by a IBM-AT computer to 256 × 256 matrix with 64 grey levels for each picture element, and the background was subtracted. The grey levels of eight ^{14}C-plastic standards (Amersham) (Miller 1991) exposed to the autoradiographic film together with the tissue sections, were determined and used in a third-degree polynomial approximation to construct a grey-level-to-activity transfer function. The areas of interest were identified by cresyl violet staining. The borders of the measuring fields in the SON and PVN were inter-actively defined and the average isotope concentration was calculated. In the PVN, the measurement of the isotope concentration was concentrated on the lateral magno-cellular part (Amstrong 1985). The average isotope concentration of control tissue sections was set to 100% and changes during lactation were expressed as a percentage of control ± S.E.M. All comparisons between groups were made on material hybridized as one batch, under identical conditions and exposed for the same period to the same autoradiographic film. Some autoradiographic images were computerized and coded in pseudo-colours to compare the relative intensity of the labelling after ISH for galanin mRNA.

Statistical analysis was carried out using ANOVA followed by Dunett's test. A confidence level of $P<0.05$ was considered significant.

Immunohistochemistry for galanin

The brain was dissected after perfusion as described above. Frontal sections were cut on a Vibratome (50 μm thickness) or a cryostat (Reichert–Jung) (12 μm thickness).

Free-floating sections were immersed for 30 min in PBS or buffer A containing 2% normal sheep serum (Vector, Burlingame, CA, USA) and 0·1% Triton X-100 (Sigma). They were incubated overnight at 4 °C in PBS containing the primary antibody for galanin detection (polyclonal rabbit anti-galanin, 1/1000) (Chemicon, Temecula, CA, USA). The sections were then rinsed three times for 10 min in PBS before detection of the primary antibody using the avidin–biotin complex (ABC) system (Vector). Briefly, the sections were incubated in PBS, first with a biotinylated anti-rabbit antibody (30 min at room temperature) and second with a peroxidase-labelled ABC (1 h at room temperature). After rinsing in several baths of PBS, the peroxidase activity was developed by incubating the sections in 50 mM Tris–HCl buffer containing 0·025% 3,3′ diaminobenzidine (Sigma) and 0·006% H_2O_2 (Sigma). The cryostat sections were mounted in Permount on gelatin-coated slides for light microscopy. For electron microscopy, Vibratome sections were finally post-fixed in OsO_4 (1% in phosphate buffer 0·1 M, 15 min) and embedded in Epon resin. Ultra-thin sections were obtained, contrasted with lead citrate and observed with a JEOL electron microscope. The total number of neurose-cretory granules was counted from five immunoreactive and five non-immunoreactive magnocellular cell bodies randomly chosen from each rat, these neurones displaying a similar cytoplasmic surface and a nucleus. Counting was performed directly on grids in the electron microscope. Data are presented as means ± S.D.

As control, IHC was performed both in control and lactating females after pre-adsorption with galanin peptide (10^{-6} M; Bachem, Bahbendorf, Switzerland).

Profile counts

Assessment of the number of galanin-containing profiles was obtained from the observation, under a Zeiss micro-scope, of every fourth section of three rats' SON in each group − that is control and 14-day lactating females. Eighteen sections were analysed per animal and all stained profiles that had a distinguishable nucleus were counted. Because the labelled profiles ranged in size from small to large and in shape from round to multipolar, we did not apply correction for double counting errors by the formula

Table 1 Profile counts from three control female rats and three female rats on day 14 of lactation. Immunoreactive profiles were counted on 18 sections at different levels of the supraoptic nucleus (SON)

	Rostral SON			Medial SON			Caudal SON			Whole SON		
	GAL-IR cells (No.)	Mean per rat	% of control	GAL-IR cells (No.)	Mean per rat	% of control	GAL-IR cells (No.)	Mean per rat	% of control	GAL-IR cells (No.)	Mean per rat	% of control
Control rats	209 210 275	231	100	603 494 601	566	100	230 184 238	217	100	1042 888 1114	1014	100
14-Day lactating rats	264 157 206	209	91	1135 904 835	958	169	359 449 322	376	173	1758 1510 1363	1543	152

GAL-IR, galanin-immunoreactive.

of Abercrombie (1946). In fact, this procedure is best suited to a monodispersed system of spheres or ellipsoids, but gives inaccurate estimates if the assumptions are not met (Coggeshall *et al.* 1990, Coggeshall & Lekan 1996). Furthermore, the purpose of our study was not to establish the total number of labelled cells, but to compare the apparent number of labelled profiles in a sample of sections in the SON of control and lactating females.

As control, the total number of magnocellular neurone profiles in the SON was counted in every fourth section of a sample of cresyl violet counterstained sections from non-lactating and 14-day lactating females. The numbers did not differ significantly (non-lactating 117 ± 18; 14-day lactating 121 ± 15), indicating that any changes in cell size were not affecting the counting procedures.

Multiple labellings

For double ISH on sections obtained from perfused brains, the different probes were incubated simultaneously in the hybridization buffer at the same temperature (42 °C). The alkaline phosphatase activity was developed before autoradiography. For combining ISH with IHC, all the IHC steps, including peroxidase development, were carried out after the post-hybridization washes. Digoxigenin- or radioactively labelled probes were then detected by an immunoenzymatic method, autoradiography, or both.

Sections from control ($n=3$) and 14-day lactating rats were processed for multiple labellings. Ten sections per animal were observed under a Nikon Microphot-FX microscope. Galanin-immunoreactive profiles were considered to be unequivocally double-labelled when the number of silver grains overlaying these neurones was at least five times more than the background. However, as a result of that procedure, the number of double-labelled profiles may have been underestimated. Counting was performed on a Macintosh IIx computer (Apple Computer, Cuppertino, CA, USA) equipped with a Quick Capture frame grabber board (Data Translation, Marlboro, MA, USA) and a Cage-MTI 72 CCD camera (DAGE-

MTI, Michigan City, IN, USA) connected with the Nikon microscope. However, the weak immunohistochemical signal during lactation and the average resolution of the sulphur–35 made it uneasy to decide unambiguously if a profile was double-labelled, especially in the PVN. Therefore, we did not make any attempt to quantify the absolute number of double-labelled cells in the lactating rats, but merely gave the percentage of double-labelled profiles in the medial SON.

For the multiple labellings, each marker was sufficiently distinguishable to be easily identified, especially when the two precipitates were located in different subcellular compartments, and we did not notice any artefact or interaction between the three markers as previously reported (Trembleau *et al.* 1993).

Results

In situ hybridization for galanin mRNA

In addition to many scattered cells distributed throughout the hypothalamus, the magnocellular neurones of the PVN, the SON and the nucleus circularis (data not shown) were labelled in the hypothalamus with the radioactive GAL-48 probe. Quantitation by densitometry on films (Fig. 1) showed a decrease in radioactivity after 3 days of lactation in both the SON ($P<0·001$) and the PVN ($P<0·001$). This decrease remained significant after 7 days of lactation in both nuclei ($P<0·001$ for both). In contrast, after 14 days of lactation, levels of radioactivity were not significantly different from those in control female rats in both the SON ($P>0·1$) and the PVN ($P>0·1$). The autoradiograms converted into pseudo-colour-coded images (see Fig. 6a) illustrated the absence of changes after 14 days of lactation, in both the SON and the PVN.

On the emulsion-dipped sections, the silver grains were overlaying the magnocellular neurones of the control rats in the ventral part of the SON (Fig. 2a) and in the lateral magnocellular part of the PVN (Fig. 2b). The cellular intensity of the labelling was decreased after 7 days of
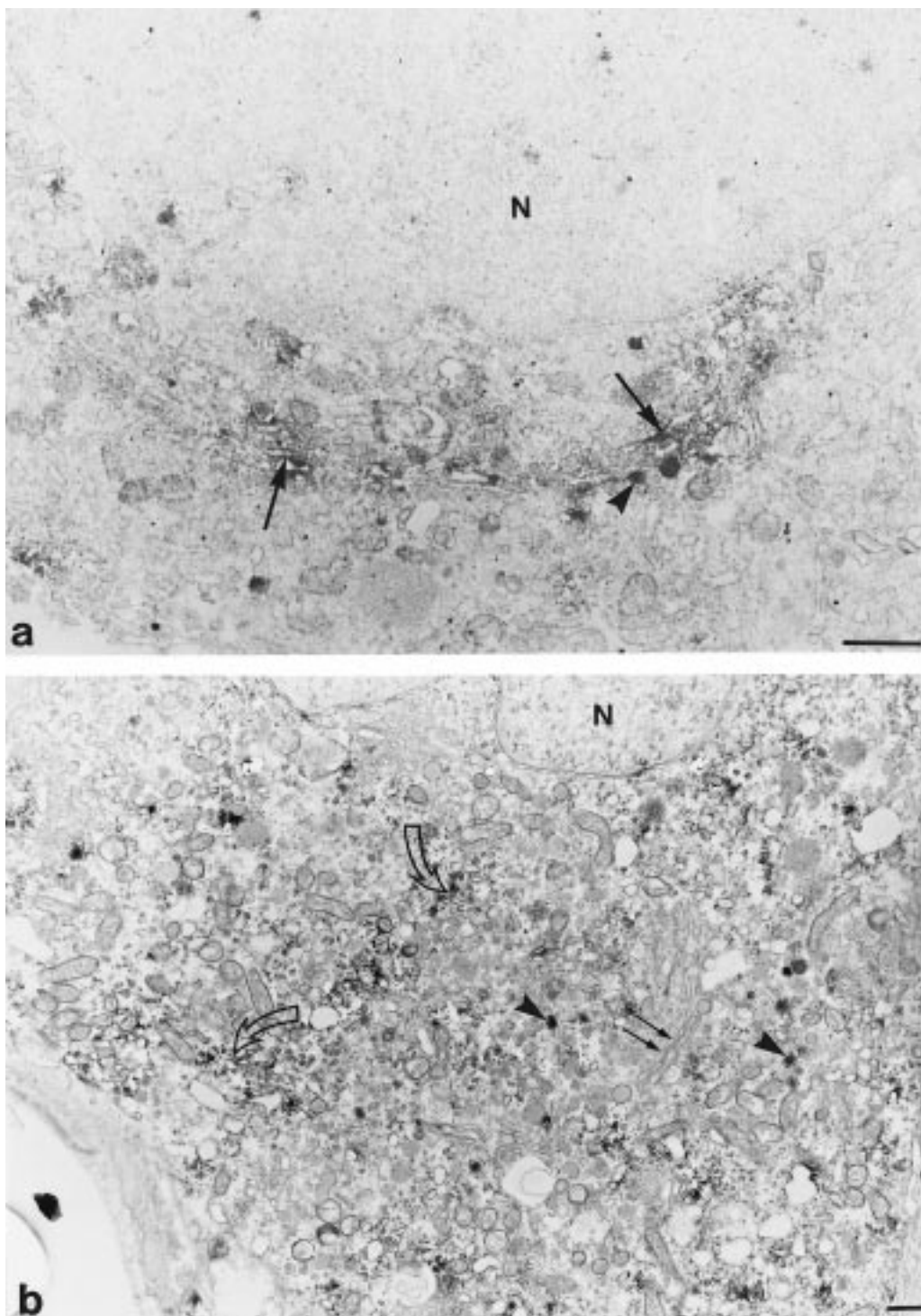
Figure 4 Immunohistochemistry for galanin at the electron microscope level using pre-embedding technique in the SON of a control rat (a) and a rat on day 14 of lactation (b). Whatever the condition, the nucleus (N) and the mitochondria remain devoid of labelling. In the control rat, the labelling is restricted to the perinuclear cytoplasm and affects the saccules (arrows) of the Golgi complex. A few neurosecretory granules (arrow heads) are also labelled in the cytoplasm. In the lactating rat, the peroxidase precipitate diffuses throughout the cytoplasm (open, curved arrows) and is no longer confined to a perinuclear area. Many neurosecretory granules (arrow heads) appear positive, but the Golgi complex is not labelled (double fine arrow). Bar represents 1 μm.

lactation in both the SON (Fig. 2c) and the PVN (Fig. 2d). In contrast, after 14 days of lactation (Fig. 2e and f), we noticed no difference in the cellular intensity of the labelling compared with that in non–lactating females.

Immunohistochemistry for galanin

Whatever the conditions, control or lactation, the PVN contained fewer stained cells than the SON and the cellular immunostaining was weaker. In control rats, galanin-immunopositive neurones were observed in the SON (Fig. 3a) and in the lateral magnocellular part of the PVN (Fig. 3b). The galanin-immunoreactive content under normal conditions was highly variable from one neurone to another, and strongly labelled cells were mixed with weakly immunoreactive ones. In addition, in most galanin-immunopositive neurones of the SON, the brown precipitate was preferentially located around the cell nucleus, where it appeared more intense (Fig. 3a and g).

After 7 days of lactation, the perikaryal staining was very weak in the SON (Fig. 3c) and almost undetectable in the PVN (Fig. 3d). However, some processes were still clearly labelled in both nuclei. On day 14 of lactation (Fig. 3e–f and h), the cellular intensity of the immunostaining was weaker than in control rats. Furthermore, the peroxidase precipitate was distributed throughout the cytoplasm of the immunopositive neurones, especially in the SON (Fig. 3e and h). However, the number of galanin-immunoreactive neurones (Table 1) increased by 152% in the SON compared with that in control rats. This increase was marked in the medial (169%) and caudal (173%) parts of the SON, but not in the rostral part of the SON (91%). Because the staining remained pale in the PVN (Fig. 3f), we did not attempt to count cell profiles in this nucleus.

The pre-adsorption of the galanin antibody abolished immunostaining in both control (data not shown) and lactating female rats (Fig. 3i).

Observations at the electron microscope level confirmed the changes in galanin immunoreactivity in the SON during lactation. In the control female rats (Fig. 4a), the peroxidase precipitate was mainly restricted to a cytoplasmic perinuclear area where the Golgi complex is typically located. The Golgi cisternae were stained, as were secretory granules in the trans-Golgi network. The nucleus was never seen to be labelled, and the peripheral parts of the cytoplasm, enriched in rough endoplasmic reticulum corresponding to the Nissl bodies, also displayed a lack of labelling. Neurosecretory granules were immuno-positive in control rats, but the number of neurosecretory granules in the perikaryal cytoplasm was lower in galanin-immunopositive magnocellular neurones than in non–immunoreactive neurones (Table 2).

After 14 days of lactation, the intensity of the labelling became weaker than that in the control rats and the intracellular distribution of the reaction product was modified (Fig. 4b). The peroxidase precipitate was

Table 2 Number of neurosecretory granules in five galanin (GAL)-immunoreactive and five non-immunoreactive magnocellular neurones from the supraoptic nuclei of two control female rats and two female rats on day 14 of lactation. Data are expressed as means ± S.D. Note the comparatively low number of granules in galanin-immunoreactive neurones from the control rats

|	GAL-immunoreactive neurones	Non-immunoreactive neurones
Control rats	15 ± 12	32 ± 6
14-Day lactating rats	41 ± 18	45 ± 10

no longer confined to a perinuclear area, but was spread throughout the cytoplasm of the immunopositive magnocellular neurones of the SON. The patches of immunostaining were generally not related to the rough endoplasmic reticulum, but the labelling was diffuse in the cytoplasm. Moreover, in contrast to the observation in control rats, the Golgi cisternae remained devoid of labelling and many neurosecretory granules, among which most were immunoreactive, were observed. The number of neurosecretory granules was similar in immunoreactive and non–immunoreactive neurones (Table 2).

Multiple labellings

Coupling IHC for galanin and ISH with oligonucleotide probes did not alter the distribution of the different labellings, either in the control or in the lactating groups. Thus the localization of the galanin-immunoreactive neurones and of the cells overlain with silver grains or alkaline phosphatase-labelled was similar to that observed after single labellings.

In control rats, no clearly doubly labelled cell could be detected either in the SON (Fig. 5b) or in the PVN (data not shown) after combining immunohistochemical detection of galanin and radioactive ISH for oxytocin mRNA. All the hypothalamic magnocellular positive neurones were singly labelled cells and displayed either silver grains or brown precipitate. In contrast, on day 14 of lactation, the same technique led to visualization of three kinds of labelled neurones in the SON (Fig. 5c–g). Many of them were singly labelled neurones, positive either for IHC or for ISH, but some magnocellular neurones exhibited both brown precipitate and silver grains overlaying the cytoplasm (Fig. 5c–g). These double-labelled profiles were located in the dorsal part of the medial SON. The rate of double-labelled profiles was about 15% of the total number of galanin–immunoreactive profiles in the medial SON of three lactating rats (254 of 1710). Galanin immuno-reactivity was too weak in the PVN to permit clear detection of any double labelled neurones.

Double ISH experiments were performed on the same tissue sections with the mixture of three digoxigenin-labelled GAL-33 oligonucleotides and the radioactively
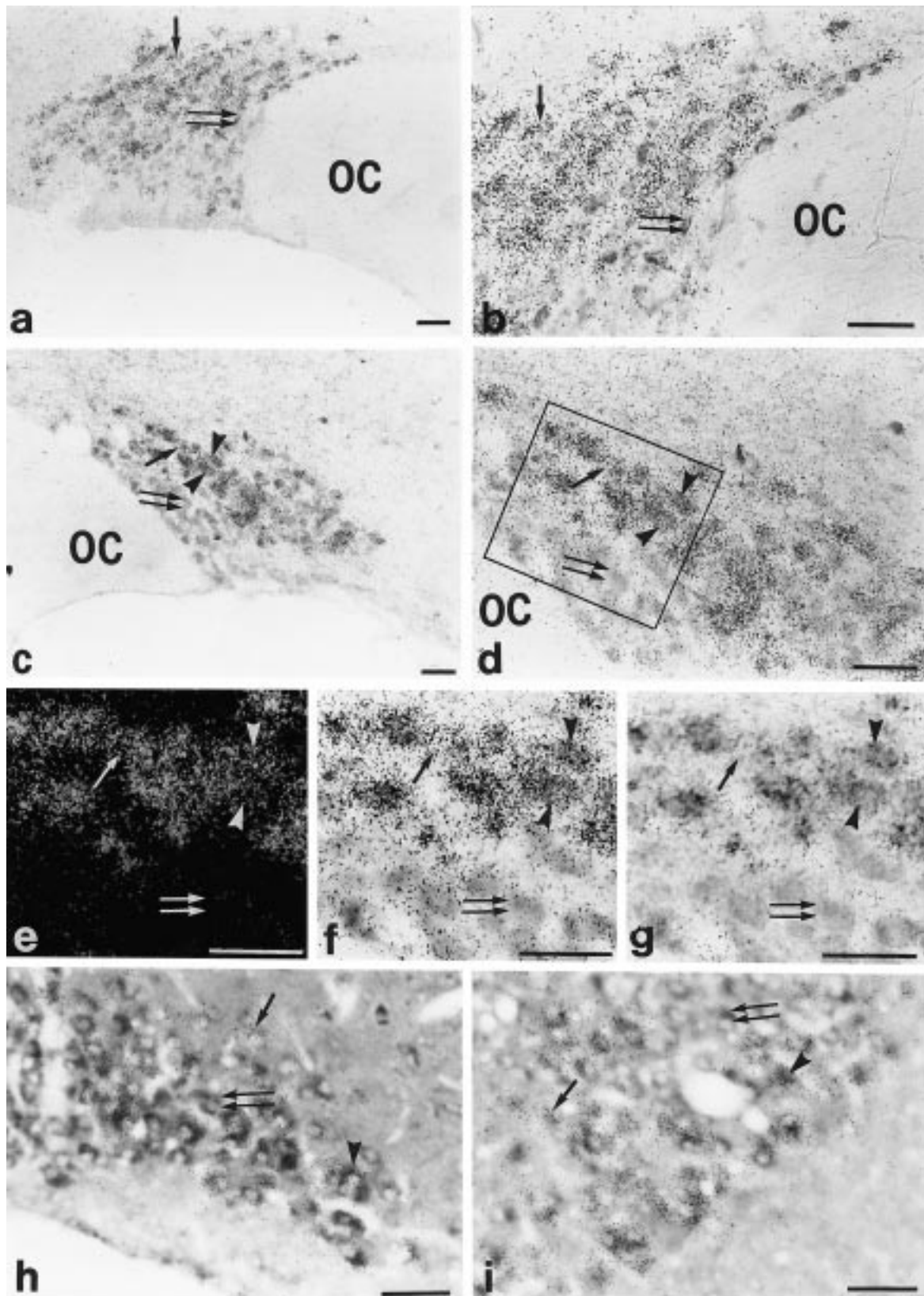
labelled OT-25 oligonucleotide (Fig. 5h and i). Within the neurones labelled by the GAL-33 probes, the alkaline phosphatase precipitate was only found in the perikaryal cytoplasm. Three kinds of positive cells were observed in the SON and the PVN of the 14-day lactating rats. They contained alkaline phosphatase precipitate, silver grains, or both. Double-labelled neurones were also detected in the PVN when the double ISH procedure was used.

Triple labelling was performed in 14-day lactating female rats by combining IHC for galanin with radioactive ISH using the VP-41 oligonucleotide probe and non-radioactive ISH using the OT-25 oligonucleotide probe. Then, we observed three kinds of labelled cells. Many of them only contained blue precipitate, or were double-stained by both silver grains and brown precipitate. The distribution of each type of labelled cells was consistent with the above observations after single and double labellings. However, neurones displaying the three markers were also observed in the dorsal part of the medial SON. At a high magnification (Fig. 6b), the alkaline phosphatase precipitate could be distinguished from the immunoperoxidase reaction product within the cytoplasm. Silver grains were visible above the perikaryal cytoplasm of those magnocellular neurones that were triple-labelled (Fig. 6c).

Discussion

Technical considerations

ISH procedure The specificity of our oligonucleotides complementary to galanin, vasopressin or oxytocin mRNA has been demonstrated previously (Kawata *et al.* 1988, Cortés *et al.* 1990*a*,*b*, Trembleau *et al.* 1990, 1993, Landry *et al.* 1991, Trembleau & Bloom 1995). In the available literature, good similarity exists between results obtained after ISH with the probes used in this study and after IHC using specific antibodies (Meister *et al.* 1990*a*, Villar *et al.* 1990, Trembleau *et al.* 1993). Finally, the distribution of the three transcription products within the magnocellular nuclei is in agreement with findings of previous studies (Kawata *et al.* 1988, Meister *et al.* 1990*a*,*b*, Mohr *et al.* 1988, Jacobowitz & Skofitsch 1991, Landry *et al.* 1991). The mixture of the three GAL-33 oligo-

nucleotides was necessary in order to visualize galanin mRNA-containing neurones by non-radioactive ISH. Thus our multi-oligoprobe enhanced sensitivity for galanin ISH without loss of specificity as previously reported (Trembleau & Bloom 1995).

IHC procedure In the non-lactating animals, the distribution of the galanin-immunoreactive cells closely corresponded to that observed in earlier studies (Melander *et al.* 1986*a*, Skofitsch & Jacobowitz 1985, Arai *et al.* 1990, Meister *et al.* 1990*a*,*b*, Villar *et al.* 1990, Jacobowitz & Skofitsch 1991, Landry *et al.* 1991). Moreover, in those animals, there was a good overlapping between the neuronal populations stained after IHC for galanin and ISH for galanin mRNA. Finally, the results of the pre-adsorption control experiments confirm the specificity of our galanin immunohistochemical detection in the hypothalamo–neurohypophyseal system.

In almost all the previous studies, colchicine was injected before the galanin immunohistochemical procedure to increase the intensity and the number of cell bodies expressing galanin immunoreactivity, by inhibiting the axonal transport of the granules (Hökfelt & Dahlström 1971). However, colchicine has been shown also to induce changes in gene expression (Cortés *et al.* 1990*b*, Boyer *et al.* 1994). Our highly sensitive procedure using free-floating sections did not require such pretreatment to obtain clear visualization of galanin-immunoreactive neurones, and therefore both distribution of and variations in galanin immunoreactivity under different physiological conditions could be studied without any colchicine-induced side-effects on expression of galanin in magnocellular neurones.

At the electron microscopic level, the diffusion of the peroxidase precipitate prevented assessment of the precise localization of the labelling, but allowed characterization of galanin-containing subcellular areas. Such diffusion may also explain our finding that the immunoreactive product was often not associated with an organelle in neurones from rats on day 14 of lactation.

Multiple labellings The sensitivity of the ISH procedures remained high and consistent with multiple detections, and the specificity of our reactions was not altered.

Figure 5 (a)–(g) Double labelling combining *in situ* hybridization for oxytocin mRNA (silver grains) and immunohistochemistry for galanin (dark precipitate) in the SON of a control rat (a, b) and a rat on day 14 of lactation (c–g). The low magnification (a, c) shows the general distribution of the two labellings. Cells containing oxytocin mRNA (arrow) are preferentially localized in the dorsal part of the SON and cells containing galanin (double arrow) are mainly detected in its ventral part. At higher magnification (b, d–g), only two kinds of labelled neurones containing either oxytocin mRNA (arrow) or galanin (double arrow) are observed in the control rat (b) whereas some cells contained both markers (arrow heads) after 14 days of lactation (d–g). Micrographs (e) to (g) are higher magnifications of micrograph (d), with different exposures. (h), (i) Double *in situ* hybridization combining the detection of oxytocin mRNA with a radioactive probe (silver grains) and the detection of galanin mRNA using a digoxigenin-labelled multi-oligoprobe (dark precipitate). In addition to cells containing only oxytocin mRNA (arrow) and cells containing only galanin mRNA (arrow head), double-labelled neurones (double arrow) were detected in the SON (h) and the PVN (i) of a 14-day lactating rat. OC, optic chiasma. Bar represents 50 μm.
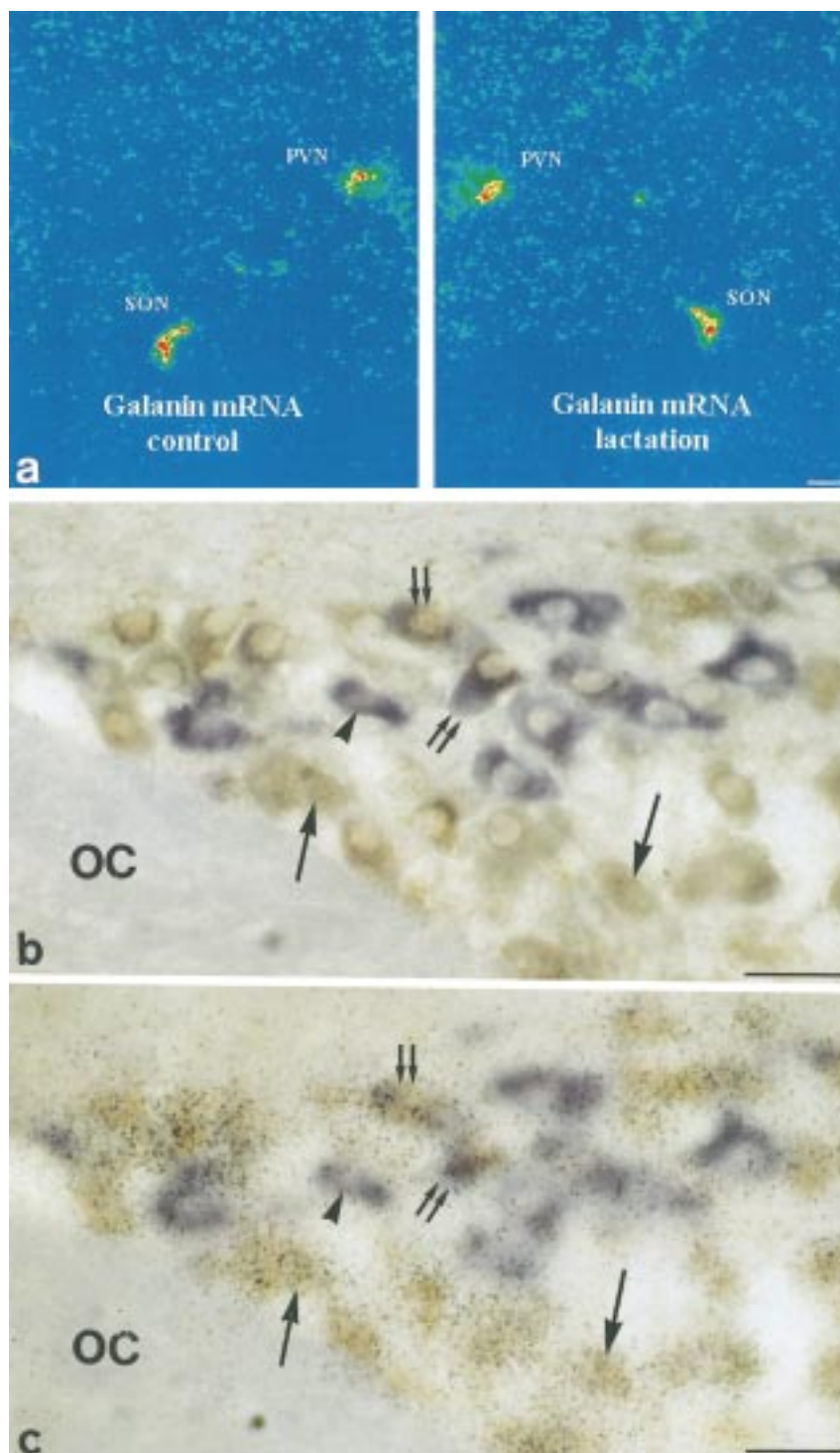
Figure 6 (a) Pseudo-colour-coded image showing half frontal sections of a control rat (left) and a 14-day lactating rat (right). No modification of the intensity of the labelling was observed in the PVN and in the SON. (b), (c) Triple labelling combining radioactive ISH for vasopressin mRNA (silver grains) and non-radioactive ISH for oxytocin mRNA (blue precipitate) with IHC for galanin (brown precipitate) after 14 days of lactation. Focusing on the immunoenzymatic precipitate (b) showed three kinds of positive neurones containing oxytocin mRNA (arrow head), galanin (arrows), or both (double arrows). The last was located in the dorsal part of the medial SON. When silver grains were in focus (c), they were mainly overlaying the single galanin-immunoreactive neurones (arrows), but also cells co-containing oxytocin mRNA and galanin (double arrows). OC, optic chiasma. Bar represents 200 µm (a) or 50 µm (b, c).

Galanin expression

Galanin mRNA concentrations decreased by day 3 of lactation, remained low after 1 week lactation and then increased to control values by day 14 of lactation. In contrast, previous studies (Van Tol *et al.* 1988, Zingg & Lefebvre 1988, Ericksson *et al.* 1996) have shown an increase in vasopressin and oxytocin mRNA concentrations in the hypothalamus with a peak on day 7 of lactation. Thus accumulation of galanin mRNA was changed in the opposite direction, in agreement with findings in the PVN after 8 days lactation in an earlier study (Ericksson *et al.* 1996).

During lactation, the individual cellular immunoreactivity was scarcely detectable in the cell bodies after 1 week of lactation and remained weak in all the galanin-immunoreactive magnocellular neurones after 2 weeks of lactation.

The count of immunoreactive profiles suggested that the number of galanin-containing neurones may increase in the SON during lactation. Moreover, in the lactating rats, galanin was homogenously distributed throughout the cytoplasm of neurones in the PVN and SON, as demonstrated at both light and electron microscope levels. The low number of neurosecretory granules in the galanin-immunoreactive magnocellular cytoplasm of control rats has also been reported for the PVN by Sawchenko & Pfeiffer (1988). When compared with non-immunoreactive neurones, it suggests that galanin immunoreactivity may occur in a particular subset of cells, possibly involved in active release processes. However, the paraformaldehyde fixation used in this study is not best suited to the preservation of the neurosecretory granules and could therefore have led to underestimation of their number. In contrast, no differences were noticed between galanin-immunoreactive and non-immunoreactive neurones on day 14 of lactation.

To explain changes in the expression of galanin during lactation, different mechanisms such as a decrease in galanin synthesis in the cell bodies or changes in galanin processing, sorting, or both, may be considered.

Expression of galanin could be decreased during lactation, being thus in contrast to expression of oxytocin and vasopressin (Young 1992). Decrease in the expression of galanin has been reported previously in gonadotrophin-releasing hormone neurones during lactation (Marks *et al.* 1993). Studies have shown that galanin gene expression in the anterior pituitary (Vrontakis *et al.* 1989) and in the magnocellular vasopressin neurones (Levin & Sawchenko 1993) is increased by oestrogen. If galanin expression also depends on oestrogen concentrations in magnocellular neurones, a decreased expression may be related to the reduced concentrations of oestrogen during lactation (Crowley *et al.* 1993, Ericksson *et al.* 1996). Alternatively, changes in mRNA stability may play a part in altered galanin content during lactation, and the rapid decrease in the expression of galanin could reflect increased degradation of that mRNA, as previoulsy hypothesized for oxytocin mRNA in early lactation (Crowley *et al.* 1993).

In addition, galanin may normally be present in many hypothalamic magnocellular neurones, but not in an immunoreactive form, and lactation might then make the detection of this peptide possible in a large number of cells by changing galanin processing. Such changes have been proposed to interpret discrepancies between immunohistochemical data in the literature (Skofitsch & Jacobowitz 1985, Gayman & Martin 1989, Meister *et al.* 1990*a*). Alternatively, as suggested for other peptides such as neuropeptide Y in axotomized large dorsal root ganglia neurones (Zhang *et al.* 1995), stimulation of neuronal activity may induce galanin mis-sorting and thereby modify galanin distribution at 14 days lactation by diverting some galanin from the secretory pathway.

Co-localizations

On day 14 of lactation, we detected double-labelled cells containing both oxytocin mRNA and galanin or galanin mRNA. The co-existence of oxytocin mRNA and galanin mRNA was also seen in the SON and the PVN. The triple-labelling experiments showed, as expected, that nearly all the galanin-immunoreactive cells contained vasopressin mRNA; however, in addition, some neurones that co-contained galanin and vasopressin mRNA also expressed oxytocin mRNA.

Galanin has been demonstrated to co-exist with vasopressin in normal conditions (Melander *et al.* 1986*b*, Rökaeus *et al.* 1988, Skofitsch & Jacobowitz 1985), whereas the co-localization between oxytocin or oxytocin mRNA and galanin was documented in some hypothalamic magnocellular neurones only after experimental manipulations such as hypophysectomy (Villar *et al.* 1990) or intracerebroventricular injection of colchicine with (Gayman & Martin 1989) or without (Landry *et al.* 1991) pretreatment of the sections by trypsin. In ovariectomized females, cells containing both galanin and oxytocin were exceptional findings in the absence of colchicine injection (Levin & Sawchenko 1993). In the absence of any pretreatment, galanin and oxytocin mRNA were contained within two distinct neuronal populations in the control female rats. In contrast, the present study strongly suggests that the galanin gene is transcribed and also translated in some oxytocin mRNA-containing neurones during lactation. Thus lactation represents a physiological condition that could stimulate the expression of galanin in oxytocinergic magnocellular neurones.

The co-existence of vasopressin mRNA with oxytocin mRNA in the triple-labelled cells was in accordance with the findings of a study by Mezey & Kiss (1991). The present study also demonstrated that galanin was also present in cells co-expressing vasopressin and oxytocin mRNAs. The multiple-labelled neurones were detected in

the dorsal part of the medial SON where oxytocinergic cells are known to be abundant (Hou-Yu *et al.* 1986, Mohr *et al.* 1988). Such a localization suggests that these multiple-labelled neurones are basically oxytocinergic cells. Alternatively, galanin may also be present at an undetectable level in oxytocinergic cells and lactation-induced changes in galanin processing could result in galanin immunoreactivity within the cytoplasm of some oxytocinergic neurones; or galanin could be co-expressed with a vasopressin–oxytocin hybrid transcript in those multiple-labelled cells. Indeed, data exist (Mohr *et al.* 1994) that demonstrate somatic non-homologous crossing-over between vasopressin and oxytocin in the magnocellular neurones of the rat SON. Moreover, the recombination is likely to affect only one of the two alleles, so that both kinds of mRNA can be detected within a single neurone. The number of hybrids apparently increases when the animals are exposed to stress conditons, as a possible consequence of the increased rate of gene transcription (Mohr *et al.* 1994), and lactation could induce hybrid formation.

The functional role of the co-packaged galanin is not yet well established. Galanin could be released in the blood vessels and have a role in milk ejection in its own right. Galanin has also been proposed to have an inhibitory role in neurohormone gene expression in the magno-cellular neurones (Kondo *et al.* 1991, Björkstrand *et al.* 1993, Landry *et al.* 1995, Ericksson *et al.* 1996). In particular, despite the fact that galanin has no effect on oxytocin release from pituitary nerve endings (Gayman & Falke 1990), injections of galanin decrease plasma concentrations of oxytocin (Björkstrand *et al.* 1993). Thus the putative inhibitory effect of galanin on the expression of oxytocin could be attenuated by galanin downregulation.

In summary, the present results demonstrate the plasticity of galanin expression during lactation. Both peptide and mRNA concentrations decreased in the magnocellular neurones in the early stages of lactation. After 2 weeks of lactation, the rate of expression of galanin mRNA increased and attained control values. In contrast, galanin immunoreactivity remained weak, and its distribution pattern was modified. The study has also provided evidence for co-expression of galanin, vasopressin and oxytocin in a subpopulation of magnocellular neurones under physiological conditions. However, the physiological significance of galanin plasticity during lactation remains unclear.

Acknowledgements

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