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Increased Colocalization of Corticotropin-Releasing Factor and Arginine Vasopressin in Paraventricular Neurones of the Hypothalamus in Lactating Rats: Evidence from Immunotargeted Lesions and Immunohistochemistry

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Abstract

In lactating female rats, tonically elevated glucocorticoid secretion is accompanied by blunted stress responsiveness, reduced expression of hypothalamic corticotropin-releasing factor (CRF) mRNA and modest increases in arginine vasopressin (AVP) expression in the paraventricular nucleus (PVN). To determine the relative contribution of CRF and AVP to parvocellular function, we performed selective CRF (CRF-Tx) or AVP (AVP-Tx) lesions in the PVN neurones of ovariectomized virgin or lactating females (day 2 of lactation) by using ricin A associated with monoclonal antibodies directed towards CRF or AVP. We also performed double immunohistochemical labelling of CRF and AVP in the PVN of control rats injected with immunoglobulin (Ig)Gs associated with the ricin A (IgG-Tx). Brains were collected 12 days after the lesion and processed for *in situ* hybridization of CRF and AVP mRNA or for double fluorescence CRF and AVP immunohistochemistry. We found that lactating females exhibit a high degree of CRF and AVP colocalization in parvocellular PVN neurones, hypothalamic processes and median eminence terminals compared to virgins. While CRF mRNA is significantly reduced in lactating rats, AVP mRNA and protein levels are greatly enhanced in parvocellular PVN neurones during lactation. Hypothalamic CRF or AVP ricin-A lesions significantly reduced both CRF and AVP expression (15–35% decrease) as well as peptide immunoreactivity in PVN neurones in both groups of females. The specificity of the lesions varied between virgins and lactators since in virgin females, AVP-Tx did not affect CRF mRNA expression whereas in lactating females, this same lesion significantly reduced CRF mRNA expression, suggesting that parvocellular PVN neurones are more sensitive to the effects of the lesions during lactation. In both virgins and lactators, lesion with CRF-Tx tended to increase AVP mRNA expression; however, in virgins, parvocellular PVN neurones were possibly compensating for the loss of CRF synthesis by increasing AVP expression and immunoreactivity. We conclude that lactation is associated with a high degree of CRF and AVP colocalization in parvoPVN neurones and that the increased AVP production in these neurones increases their sensitivity to immunotargeted lesions. The opposite regulation of CRF and AVP gene expression during lactation might provide a useful model to study differential sensitivity to glucocorticoid feedback or hypothalamic activation of transcription factors.

Corticotropin-releasing factor (CRF) and arginine vasopressin (AVP) produced by parvocellular neurosecretory neurones of the hypothalamic paraventricular nucleus (PVN) are potent modulators of the activity of the adrenocortical axis in most mammalian species (1–3). However, the precise role of each peptide in regulation of hypothalamic-pituitary-adrenal-axis (HPA) function depends on the species (4) and the conditions examined. The contribution of AVP to the control of pituitary adrenocorticotropic hormone (ACTH) secretion becomes particularly important when the parvocellular neurones are

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subject to continuous or repeated stimulation (5–7), or when glucocorticoid feedback is removed. Following adrenalectomy, there is an increase in the production of AVP in parvocellular CRF neurones (8, 9), an increased AVP : CRF ratio in the hypophysial portal circulation (10) and an increase in AVP-immunoreactive vesicles containing CRF in the external zone of the median eminence (11). Similar to adrenalectomy, tonic elevations of glucocorticoid production during chronic stress also appear to be associated with an increased production and release of AVP (12, 13). It has been suggested that the long-term changes in HPA regulation induced by chronic stress might be preferentially mediated by alterations in the expression of AVP rather than CRF in neurones of the paraventricular nucleus (6, 14, 15).

Lactation represents a different physiological condition of tonic increase in glucocorticoid secretion that can be directly linked to the presence of the suckling stimulus (16–18). However, in contrast to the situation of chronic stress, stress-induced CRF mRNA production (19, 20) and ACTH secretion (21–24) are greatly reduced during lactation without significant changes in glucocorticoid feedback sensitivity (22). In addition, we recently demonstrated that lactating females exhibit a shift in pituitary sensitivity towards AVP and away from CRF (25), suggesting that the efficacy of AVP might be enhanced during this period. Apart from the well-characterized stimulation of AVP production in magnocellular neurones of the hypothalamus during lactation (26), recent data have also demonstrated an increase in AVP expression in the parvocellular region of the PVN (20). However, it is unclear whether this increase is due to an increase within neurones that express AVP alone or an increased synthetic activity of neurones coexpressing both CRF and AVP.

These studies were designed to compare the relative contribution of AVP and CRF from the parvocellular region of the PVN in regulating basal activity of the HPA axis between virgin and lactating females. Furthermore, we wanted to determine whether modifications in CRF and AVP expression during lactation are confined to neurones that normally express both peptides, or whether additional neurones are recruited to express AVP during lactation. To this end, we took advantage of the selective targeting of a neurotoxin, ricin A, by monoclonal antibodies directed towards CRF or AVP expressing neurones in the PVN (27, 28). We show that treatment of virgin or lactating females with the anti-CRF antibody associated with the toxin eliminates 15–35% of parvocellular neurones and that both parvocellular and magnocellular neuronal populations are reduced with the toxin associated with an antibody directed towards AVP. However, the neurotoxin directed against AVP produced more cell loss in lactating than in virgin females suggesting that during lactation, a greater number of parvocellular CRF neurones coexpress AVP. This enhanced colocalization was further demonstrated by double immunohistochemical labelling of PVN neurones and terminals in the median eminence.

Materials and methods

Animals

Sprague-Dawley virgin or pregnant females on day 16–17 of gestation (Charles River, St Constant, Quebec, Canada) were received in our animal facility and maintained under controlled conditions of light (12 h : 12 h light : dark, lights on at 06.00 h), temperature (22–25°C) and humidity (70–80%). All rats were fed a rat chow diet and water *ad libitum*. The day of birth was designated as day 0 and on day 2, litters were culled to 10–11 pups per mother. Mothers were singly housed with their litters throughout the experimental procedure while virgin females were group housed until treated to induce the central lesions and then housed singly. All procedures were approved by the Committee on Animal Care at McGill University and followed guidelines edited by the CCAC.

Administration of the immunolesions, ovariectomy and tissue collection

Virgin females (240.4 ± 3.4 g) or nursing females (328.9 ± 7.1 g) on day 2 of lactation were rapidly anaesthetized with sodium brietal (0.5 ml/100 g body weight, i.p., 50 mg/ml, Eli Lilly, Indianapolis, Indiana, USA), placed in the stereotaxic frame and the skull exposed for determination of the bregma coordinates. A double 33G injector with a spacing of 1.6 mm was lowered over the PVN using the following coordinates with reference to the bregma: A/P = −1.0 mm, L = 0.8 mm, V = 6.8 mm. We directed injections to the dorsal limit of the PVN rather than within the nucleus to avoid cellular damage caused by the injector. Solutions of the purified ricin A chain (100 nM, Tx, Sigma, St Louis, MO, USA), and monensin (50 nM, Calbiochem, Paris, France) associated with either nonspecific IgGs, a CRF or an AVP monoclonal antibody were prepared fresh on the day of the experiment as described in detail previously (29). All treatments were slowly infused using a micropump at a volume of 0.25 µl/site of injection over 4 min (0.063 µl/min). Once the injection was completed, the injector was left in place for 4 min to prevent backflow into the injector and to allow for adequate diffusion. After removal of the injector, the skin wound was sutured and the rat was taken out of the stereotaxic frame to be prepared for the ovariectomy. To avoid the confounding effects of fluctuating gonadal steroid concentrations in virgin compared to lactating females, bilateral ovariectomy was performed by the dorsal approach with two incisions made slightly ventral to the intersection between the ribcage and the vertebral column. Each ovary was gently excised from the peritoneum and the uterine horn ligated prior to total removal of the ovary. The peritoneum and the overlying skin were sutured with surgical silk. All females were left to recover from surgery on a warming pad and returned to individual cages as soon as they showed adequate orientation and locomotion. Virgins or lactating females were assigned to three experimental groups receiving either IgGs-Tx, CRF-Tx or AVP-Tx delivered over the PVN. Three days later, rats were implanted with jugular catheters under light metofane anaesthesia. Blood sampling was performed 48 h later for ACTH and corticosterone measurements (data not shown).

All females were killed 12 days after administration of the central treatments (day 14 of lactation). Half of the rats in each experimental group were decapitated and the brains rapidly frozen in isopentane for determination of CRF and AVP mRNA levels by *in situ* hybridization. The remaining rats from each experimental group were treated with colchicine (50 µg/50 µl CSF) injected into the cisterna magna under light metofane anaesthesia and lactating females had their pups removed after colchicine injection. After 24 h, the rats were deeply anaesthetized with sodium pentobarbital (60 mg/kg body weight, i.p.) and perfused transcardially with a solution of 4% paraformaldehyde containing picric acid and sucrose as described previously (30). Brains were rapidly removed and fixed in the same fixative for 24 h at 4°C. After rinsing in tap water, brains were immersed in PBS containing sucrose (25 M) for 24–48 h at 4°C, followed by rapid freezing in liquid nitrogen. Brains were kept at −75°C until processed for immunocytochemistry.

Immunocytochemistry for CRF and AVP

Twenty µm coronal brain sections were collected onto gelatin-coated slides and each slide included sections from one control (non specific IgG injection) and one treated (AVP-Tx or CRF-Tx injection) rat to enable direct comparison between treatments. All sections were kept at −20°C before immunostaining, which was performed simultaneously for all sections in order to reduce variability due to successive protocols. Vasopressin immunoreactivity was detected by immunofluorescence using a mouse monoclonal antibody (C2.23) (31), that has been previously prepared and characterized in our laboratory. Immunoreactive CRF was detected using a rabbit polyclonal antibody generously provided by Dr Barbanel (UPRESA, Montpellier, France). Double immunostaining of AVP and CRF on the same brain sections was performed by the immunofluorescence method. Briefly, after 30 min treatment with normal sheep serum mixed with 0.05% Triton X-100, the sections were incubated with a mixture of both specific antibodies (monoclonal AVP

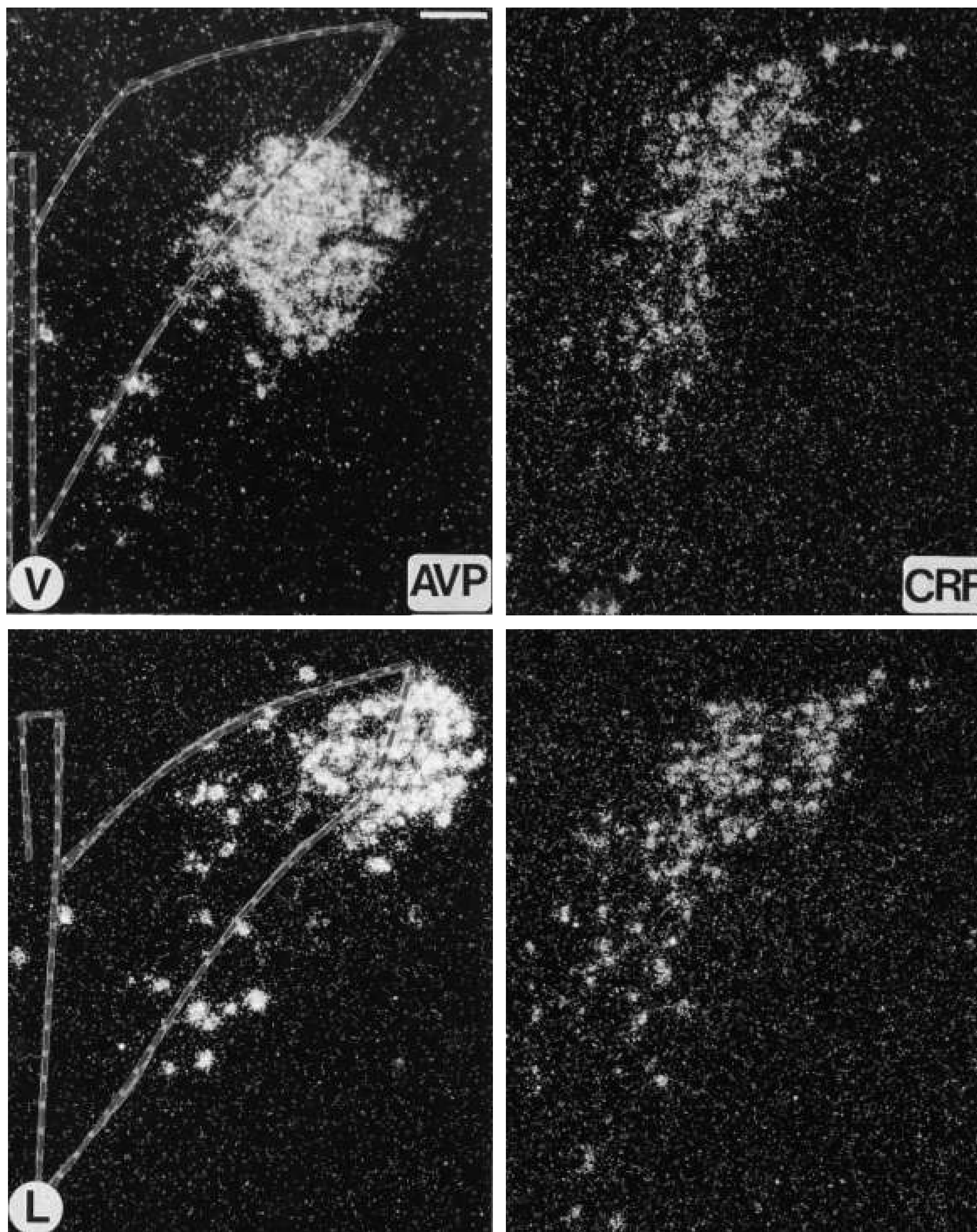

FIG. 1. Expression of arginine vasopressin (AVP) mRNA (left) and corticotropin-releasing factor (CRF) mRNA (right) by *in situ* hybridization in virgins (top) and lactating (bottom) females on day 14 of lactation. Two successive serial sections were hybridized either for CRF mRNA or AVP mRNA. The outline shown on the left panel represents the area used for the analysis of CRF hybridization. The surface of AVP mRNA that overlapped with CRF mRNA was considered to be the parvocellular subset of paraventricular nucleus (PVN) neurones and the remaining surface was identified as the magnocellular subset of PVN neurones. Dark field illumination of coronal sections is depicted. Bar = 100 μm.

antibody 1 : 1000 and polyclonal CRF antibody 1 : 2000) for 24 h at 4°C. The sections were rinsed in PBS and incubated for 2 h at room temperature with a mixture of sheep antirabbit IgGs labelled with fluorescein isocyanate (1 : 100, Jackson ImmunoResearch,West Grove, PA, USA) and monkey antimouse IgGs labelled with rhodamine (1 : 200, Applied Biosystems, Paris, France). The sections were rinsed with PBS and mounted. Each relevant section was observed under specific illumination for fluorescein and rhodamin fluorescence, successively. The presence of both fluorochromes in the same neurones

was recorded and the number of double stained neurones was measured in several sections throughout the PVN. As documented earlier (27), staining for CRF and AVP was specific and we failed to detect any signal when the specific antibodies were replaced by a normal rabbit or mouse serum.

In situ *hybridization for CRF and AVP*

In situ hybridization for CRF and AVP mRNAs was performed according to procedures described previously (29). The AVP probe was a 27-bp oligomer complementary to bases 964–990 of the exon C of the AVP gene (32) and the CRF probe was a 45-bp oligomer, directed against bases 523–567 of the 2nd exon of the CRF gene (33). The probes were 3′-end labelled with ^{35}S and terminal deoxynucleotidyl-transferase before being purified on Sephadex G25 columns (Tebu, LePerray-en-Yvelines, France). Four rats per group were used and 12 µm brain coronal sections were collected onto gelatin-coated slides, exposed to 4% paraformaldehyde for 10 min and then dehydrated in graded ethanol prior to storage at −80°C. Similar to the procedure for immunocytochemistry, each slide included sections from one control (non specific IgG injection) and one treated (AVP-Tx or CRF-Tx injection) animal in order to allow direct comparison between treatments. All manipulations were performed under RNAse-free conditions, carefully respecting the requirements for quantification (homogenous thickness of sections, simultaneous treatment of control and experimental brain sections, large experimental batches).

The specificity of the radioactive signal was checked by standard methods, i.e. the signal did not appear when the labelled probe was omitted or when a 100-fold amount of unlabeled probe was used. The autoradiographic silver grains were quantified under dark-field illumination and the morphometric data included the total size of the labelled area, which correlated with the number of neurones and the total grain area per surface unit as a measure of the intensity of labelling. The measures were taken in the median part of the PVN in the rostro–caudal direction, from four consecutive serial sections (right and left nuclei) collected from different rats (n = 4) belonging to the same experimental group. The hybridization signal for AVP mRNA was semiquantified in the magno-and the parvocellular subdivisions of the PVN. To achieve adequate subdivision of the PVN, two adjacent serial sections, respectively, hybridized for CRF mRNA and AVP mRNA were used. The hybridized section area for CRF mRNA was registered on the hybridized area for AVP, using the third ventricle to check for the dorso–ventral position of the structure. As an example, the outline shown in the left panel of Fig. 1 represents the area used for the analysis of CRF hybridization. The area of AVP mRNA that overlapped with CRF mRNA was considered to be the subset of parvocellular PVN neurones and the remaining area was identified as the magnocellular subset of PVN neurones. Although this method allowed to determine the intensity of labelling of each subset of PVN neurones, it was considered too imprecise to measure the size of the labelled area of each subset of PVN neurones. Thus, to avoid selection of only few isolated magnocellular neurones in our determination of labelling intensity, we restricted our measure to the parvocellular division of the PVN as outlined by the distribution of CRF neurones (Fig. 1). The objective magnification for the analysis is 10 (total magnification 10 × 10 = 100) and all measurements were made by an experimenter blind to the treatments.

Statistical analysis

All results are presented as means ± SEM and data are subjected to one way analysis of variance (ANOVA) followed by post-hoc Student's t-test or Newman–Keuls tests. To minimize variability due to successive hybridization protocols, the size of the labelled areas measured in the PVN of AVP-Tx or CRF-Tx treated rats were expressed as the percentages of the size of the labelled areas measured in each control group injected with the non specific IgG-toxin mixture. For these results presented as percentage values, we used a non parametric Kruskal Wallis post hoc test followed by a Mann–Whitney test. $P < 5\%$ was considered statistically significant.

Results

The toxin lesions of the PVN in either lactating or virgin females did not significantly alter body weight gain for the 3–4 days following central ricin-A conjugate injection and ovariectomy. In all experimental groups, virgin females gained significantly more weight than lactating females (Virgins: IgG-Tx = 21.5 ± 6.8 g, CRF-Tx = 20.9 ± 4.2, AVP-Tx = 13.4 ± 5.6; Lactating: IgG-Tx = −0.22 ± 5.8 g, CRF-Tx = 0.17 ± 3.6, AVP-Tx = 1.2 ± 3.2; $P < 0.05$ for all experimental groups). This is likely due to the fact that they are not experiencing as much caloric drain as the nursing females. Hypothalamic CRF or AVP lesions did not result in significant changes in basal plasma ACTH or corticosterone secretion in either virgin or lactating females (not shown).

Effect of CRF lesions on CRF and AVP expression in the PVN

For both virgin and lactating females, the effect of lesions are normalized to the corresponding level of expression in control, IgG-toxin injected rats and all values are expressed as percentage of the IgG control group. Injection of CRF toxin significantly reduced the surface hybridized for CRF neurones (Fig. 2, top left) and the intensity of labelling (top, right) for CRF mRNA levels in both virgin and lactating females. The efficiency of the CRF lesion was greater in lactating than in virgin females (surface: $P = 0.008$; intensity: $P = 0.001$). In virgin females, the reduction in size of the labelled area was 16% compared to controls (IgG group), while it reached 33% in lactating females. Similarly, labelling intensity was reduced by 18% and 58% in virgin and lactating females, respectively.

The effect of the CRF lesion on AVP mRNA expression is represented in the bottom panel of Fig. 2. The size of the labelled area for AVP represents the total surface including both magnocellular and parvocellular sets of neurones. In contrast to the measure of size of the labelled area, labelling intensity was measured only for the parvocellular division of the PVN (excluding magnocellular neurones). While injection of CRF toxin did not affect the surface or intensity of hybridization for AVP mRNA in virgin females, it significantly reduced the AVP labelling intensity by 54% in parvocellular neurones of the lactating females ($P < 0.001$), suggesting the increased colocalization of CRF and AVP in lactating compared to virgin females.

Effect of AVP lesions on CRF and AVP expression in the PVN

As for the effects of CRF lesions, changes in the expression of CRF and AVP mRNAs after AVP lesions are expressed as a percentage of control, ricin-A-IgG-injected animals for each experimental group. Injection of AVP toxin significantly reduced AVP mRNA expression in virgin and lactating females and for both groups, the size of the labelled area for AVP mRNA was reduced by 15–18% (Fig. 3, bottom, left panel). This reduction in size of the labelled area represents the total surface including both magnocellular and parvocellular sets of neurones. As a better estimate of the lesion effect in parvocellular neurones, we observed a large reduction in the intensity of AVP mRNA expression following AVP toxin injection in virgin (76%) and lactating (58%) females (virgin versus lactating females, $P > 0.05$; Fig. 3 bottom, right panel). In addition, AVP lesion produced a small reduction in AVP mRNA expression (grains) in magnocellular neurones (data not shown) of virgin females (27%, $P < 0.001$ compared to

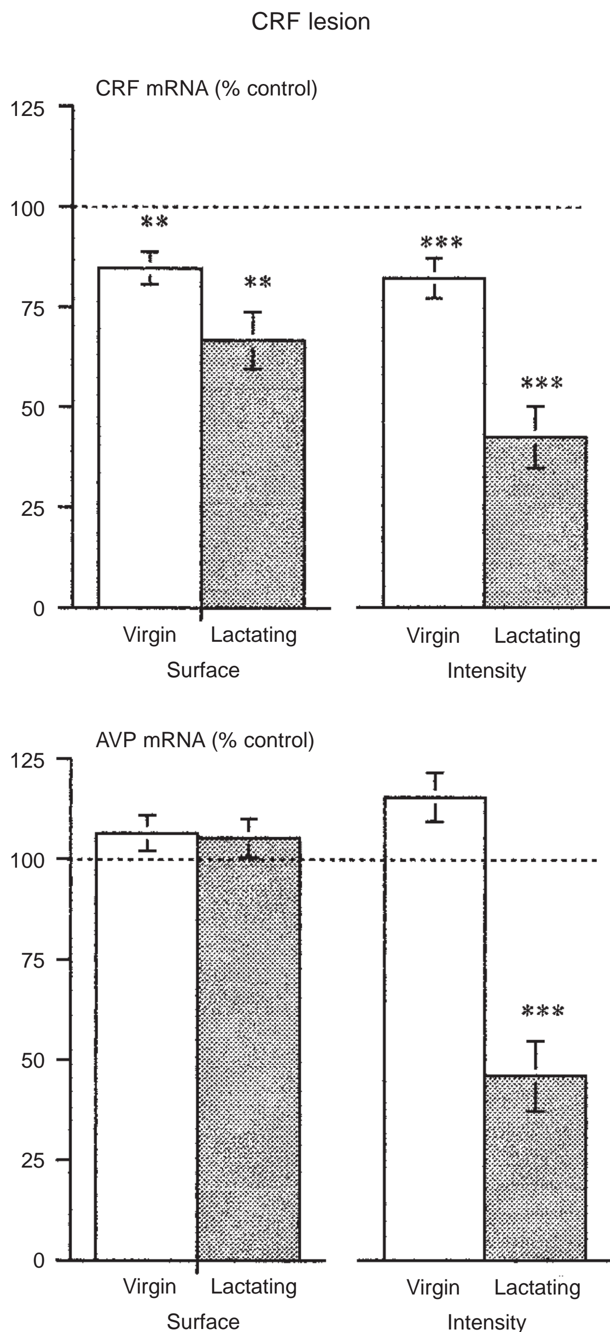

FIG. 2. Effects of corticotropin-releasing factor (CRF) lesions (CRF-Tx) of the paraventricular nucleus (PVN) on the expression of CRF mRNA (top panel) in the parvocellular portion of the hypothalamic PVN and arginine vasopressin (AVP) mRNA (bottom panel) in the magnocellular and parvocellular portions of the hypothalamic PVN (surface). To determine the intensity of AVP mRNA labelling, only the parvocellular portion of the PVN was considered. Females received lesions on day 2 of lactation and were sacrificed on day 14 of lactation (grey bars). A similar time course was followed for virgin females (open bars). Results are expressed as percentage of the control, immunoglobulin (Ig)G-Tx injected animals for the size of the labelled area as well as for the intensity of labelling (grains). Statistical differences compared to IgG-Tx injected females are shown on top of the bars. Values from the IgG-Tx group are shown in Table 1. Data are presented as mean ± SEM and for each female group, 24–37 sections from 5–6 females were used. $^{**}P<0.01$; $^{***}P<0.001$ compared to the IgG-Tx group for virgin and lactating females, based on the number of sections analysed.

controls), but not in lactating females (7% reduction, not significant).

The effect of AVP lesion on CRF mRNA expression is represented in the top panel of Fig. 3. In virgin females, administration of AVP toxin did not affect the size of the labelled area for CRF mRNA; however, in lactating females, the lesion significantly reduced CRF mRNA area in the parvocellular portion of the PVN ($P=0.02$, 22% reduction). When intensity of labelling was considered, the reduction in CRF mRNA following AVP lesion was even more drastic in lactating females (60% reduction, $P=0.001$). In contrast, the intensity of labelling for CRF mRNA was increased in virgin females following AVP lesion (13% increase, $P<0.05$), suggesting a small compensatory change in parvocellular neurones that was unique to virgin females.

Increased colocalization of CRF and AVP in PVN neurones of lactating females

In agreement with earlier studies (19) and as shown in Table 1 and Fig. 1, size of the labelled area and labelling intensity for CRF mRNA in the control (IgG-injected) females was significantly lower in lactating females than in virgins ($P<0.01$). In contrast, the total size of the labelled area for AVP mRNA (including magnocellular and parvocellular portions) in the control females was significantly greater in lactating females than in virgin rats ($P<0.01$). Similarly, the intensity of AVP labelling in either magnocellular or parvocellular neurones of the PVN was greatly increased in lactating females, suggesting a larger number of parvocellular neurones expressing AVP and an increased synthesis of AVP in these neurones during lactation.

In concordance with increased AVP mRNA expression in lactating females, AVP immunoreactivity (IR) in paraventricular neurones was greatly enhanced both in the magnocellular portion of the PVN and in the parvocellular portion of the nucleus (Fig. 4, left). Interestingly, this increase in AVP-IR was particularly marked in the dorsomedial region of the parvocellular division, a region immediately adjacent to clearly defined magnocellular neurones. For cell bodies, colocalization of AVP in CRF-positive neurones was very extensive in lactating females (Fig. 5). In the PVN, some parvocellular neurones contain exclusively CRF-IR (open arrows, right panel) while others demonstrate double labelling for CRF and AVP (large filled arrows). Cell bodies expressing AVP only are found in both female groups as indicated by the long thin arrows.

In the median eminence, increased AVP-IR was seen in the internal and external zone during lactation Fig. 6, left panel). Using confocal microscopy, colocalization with CRF was observed in the external layer only and was much enhanced in lactating compared to virgin females (Fig. 7). Changes in CRF-IR between virgin and lactating females under colchicine treatment did not parallel the changes in CRF expression that have previously been documented by us and others. Immunoreactive CRF neurones and fibres were more prominent in lactating compared to virgin females (Figs 4 and 6). In the median eminence, CRF-IR was limited to the external zone in both female groups.

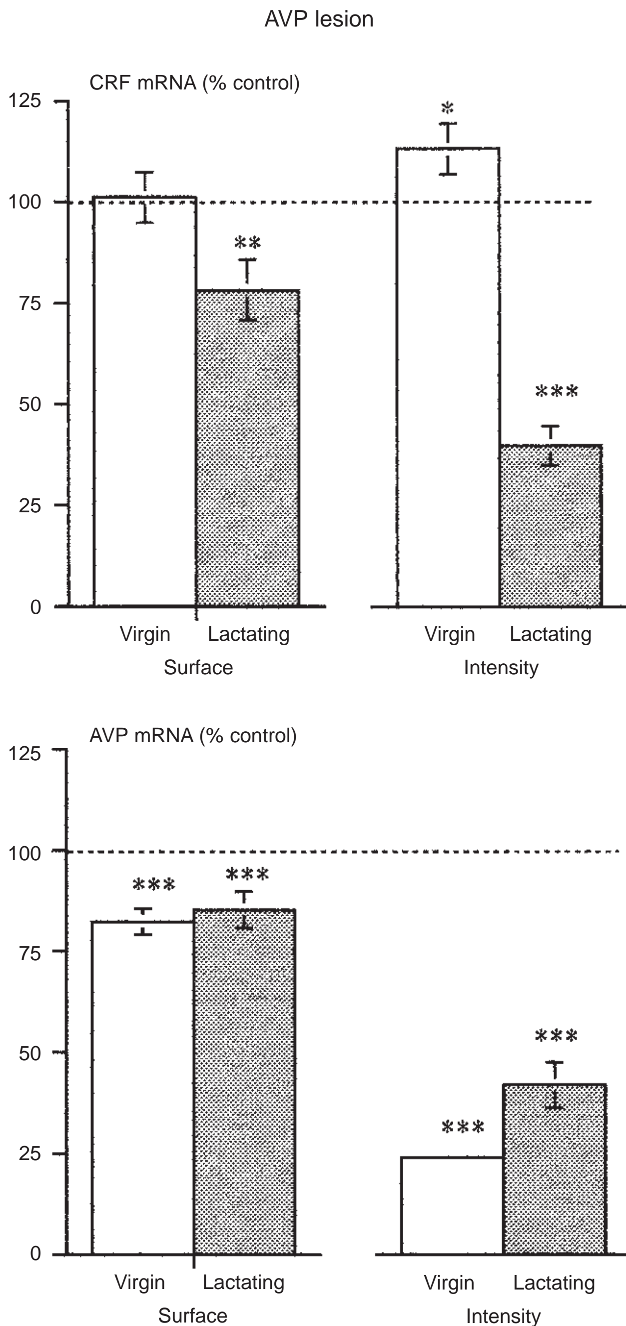

FIG. 3. Effects of arginine vasopressin (AVP) lesions (AVP-Tx) of the paraventricular nucleus (PVN) on the expression of corticotropin-releasing factor (CRF) (top panel) mRNA in the parvocellular portion of the hypothalamic PVN and AVP mRNA (bottom panel) in the magnocellular and parvocellular portions of the hypothalamic PVN (surface). To determine the intensity of AVP mRNA labelling, only the parvocellular portion of the PVN was considered. Females received lesions on day 2 of lactation and were sacrificed on day 14 of lactation (grey bars). A similar time course was followed for virgin females (open bars). Results are expressed as percentage of the control, immunoglobulin (Ig)G-Tx injected animals for the size of the labelled area as well as for the intensity of labelling (grains). Statistical differences compared to IgG-Tx injected females are shown on top of the bars. Values from the IgG-Tx group are shown in Table 1. Data are presented as mean ± SEM and for each female group, 23–48 sections from 5–6 females were used. **$P<0.01$; ***$P<0.001$ compared to the IgG-Tx group for virgin and lactating females, based on the number of sections analysed.

A more refined analysis of cellular colocalization in lactating females (Fig. 5) indicated that both peptides can exist in neurones clearly identified according to their size as parvocellular neurones, but also in neurones whose size was intermediate between parvo and magnocellular neurones. Increased colocalization was also observed along hypothalamic processes confirming that increased colocalization is not restricted to sites where exclusive modifications of either synthesis (cellular bodies) or release (terminals in the median eminence) can occur.

Discussion

In these studies, we have demonstrated that lactating females exhibit a reduction in CRF expression accompanied by an increased synthesis and expression of AVP in parvocellular neurones of the PVN, with a large portion of these coexpressing CRF immunoreactivity.

Using immunotargeted lesions directed to either CRF or AVP-containing neurones, we found that the ricin-A CRF/AVP antibody conjugates had different effects in virgin and lactating rats. In virgins, lesions were specific for each immunotargeted peptide as demonstrated previously for male rats (28). After CRF lesion, the reduction in CRF mRNA expression (Fig. 2) or immunoreactivity (not shown) in parvocellular PVN neurones was small, but significant compared to their control group (IgG-Tx). The efficiency of the AVP lesion in reducing the number of neurones expressing AVP in the PVN was similar to that of the CRF lesion, both resulting in a 18% reduction in hybridized mRNA signal. The synthetic activity of parvocellular AVP neurones was greatly compromised by the AVP lesions since AVP mRNA content of parvocellular PVN neurones, as measured by the intensity of labelling was reduced by 76% compared to controls. In the magnocellular division of the PVN, however, the decline in labelling intensity for AVP mRNA following AVP lesions was much smaller (27%). The difference in sensitivity to the toxin of these two cellular populations within the PVN could not be explained by differences in the site of injection, but rather by a differential cellular access of the antibody associated to the toxin. Indeed, we previously documented that recognition of the target cell by the antibody-toxin mix requires the expression of some portions of the peptide precursor at the cellular surface (34). Because of the large intracellular pool of AVP in magnocellular neurones compared to that of the parvocellular region in virgin females, these magnocellular neurones might be in a more 'static' state of synthesis and therefore express less precursor molecules on their surface. Although we have not documented these changes in precursor availability directly, the increased sensitivity of parvocellular neurones to the lesion compared to magnocellular neurones argues for such a mechanism.

Interestingly, the AVP lesion in virgin females resulted in a small, but significant increase in the expression of CRF in parvocellular neurones compared to control IgG-treated animals. This increased synthetic activity of CRF neurones following damage to neighbouring AVP-containing neurones suggests that some degree of communication between parvocellular neurones expressing CRF and those expressing both CRF and AVP occurs. Whether this communication is

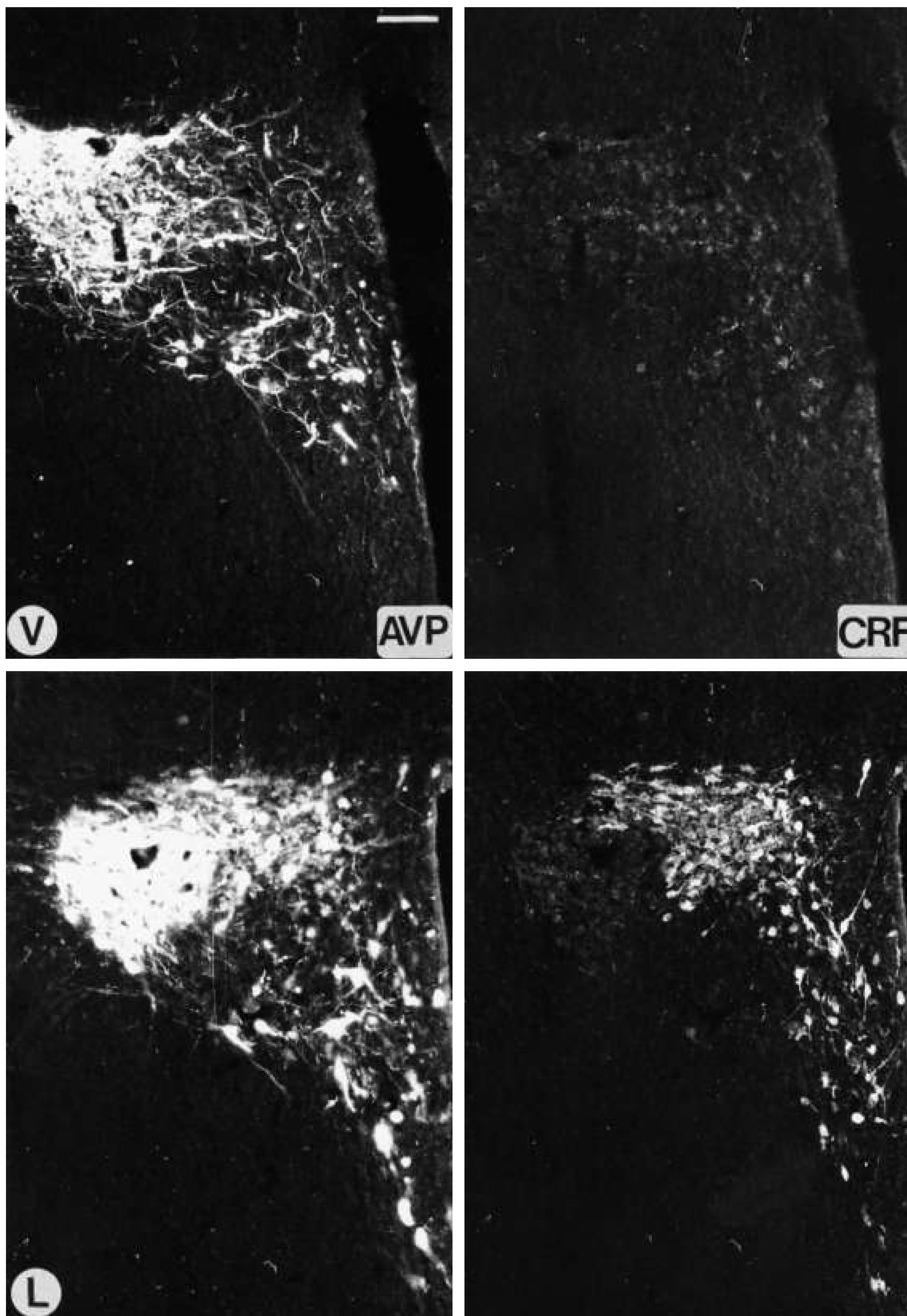

FIG. 4. Immunostaining of arginine vasopressin (AVP) (left) and corticotropin-releasing factor (CRF) (right) in the paraventricular nucleus of virgin (V, top) and lactating (L, bottom) females on day 14 of lactation. Both peptides were detected on the same coronal sections in animals receiving colchicine 24 h earlier. Pups were separated from the mothers at the time of colchicine treatment. Bar = 100 μm.

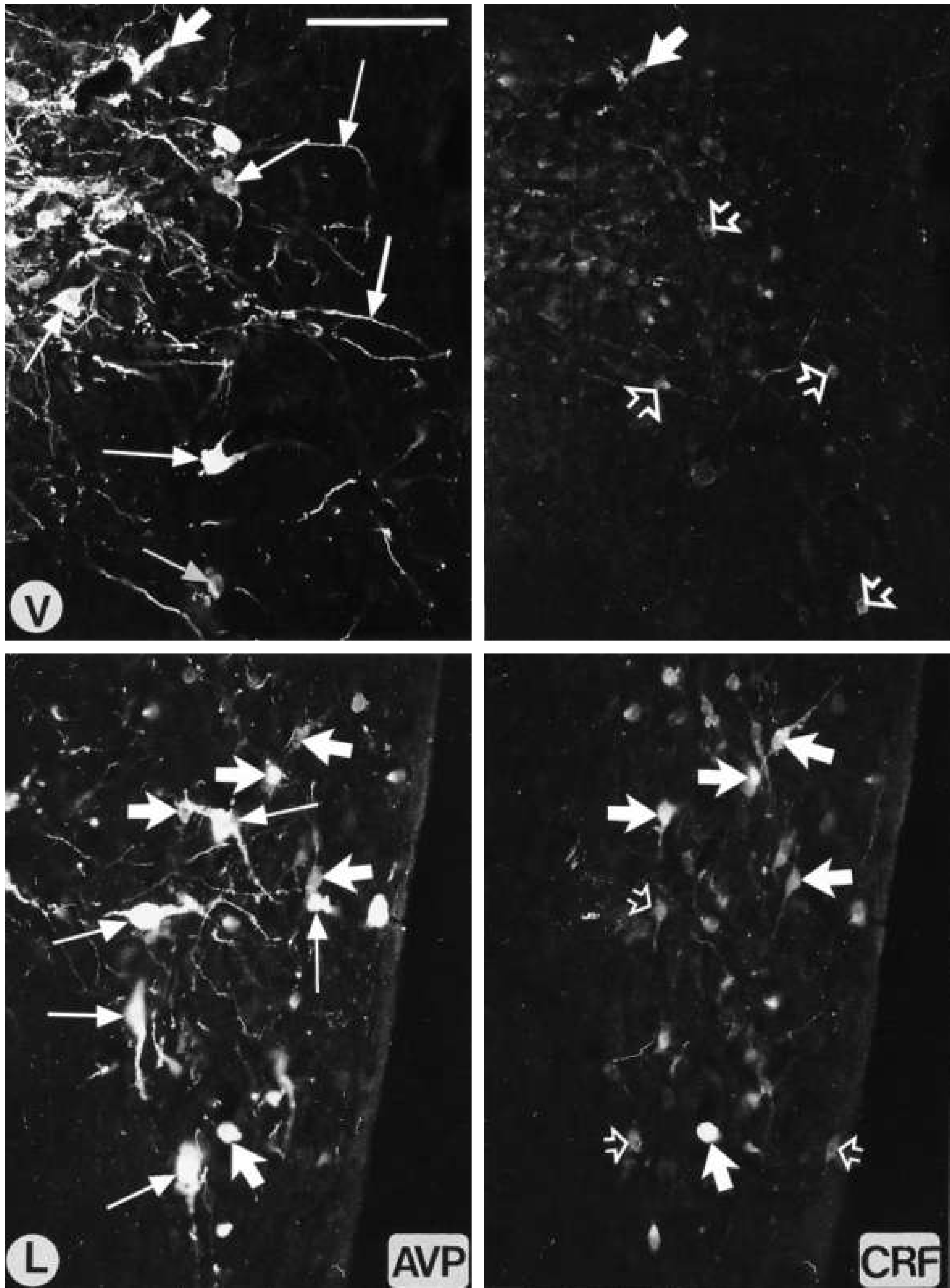

FIG. 5. Immunostaining of arginine vasopressin (AVP) (left) and corticotropin-releasing factor (CRF) (right) in the hypothalamus of virgin (top) and lactating (bottom) females on day 14 of lactation. All animals received colchicine treatment 24 h earlier and pups were separated from the mothers at the time of colchicine treatment. A portion of the parvocellular division of the paraventricular nucleus is shown. Cells containing exclusively CRF or AVP are depicted with open or long thin arrows, respectively. The large filled arrows show double staining. Bar = 100 μm

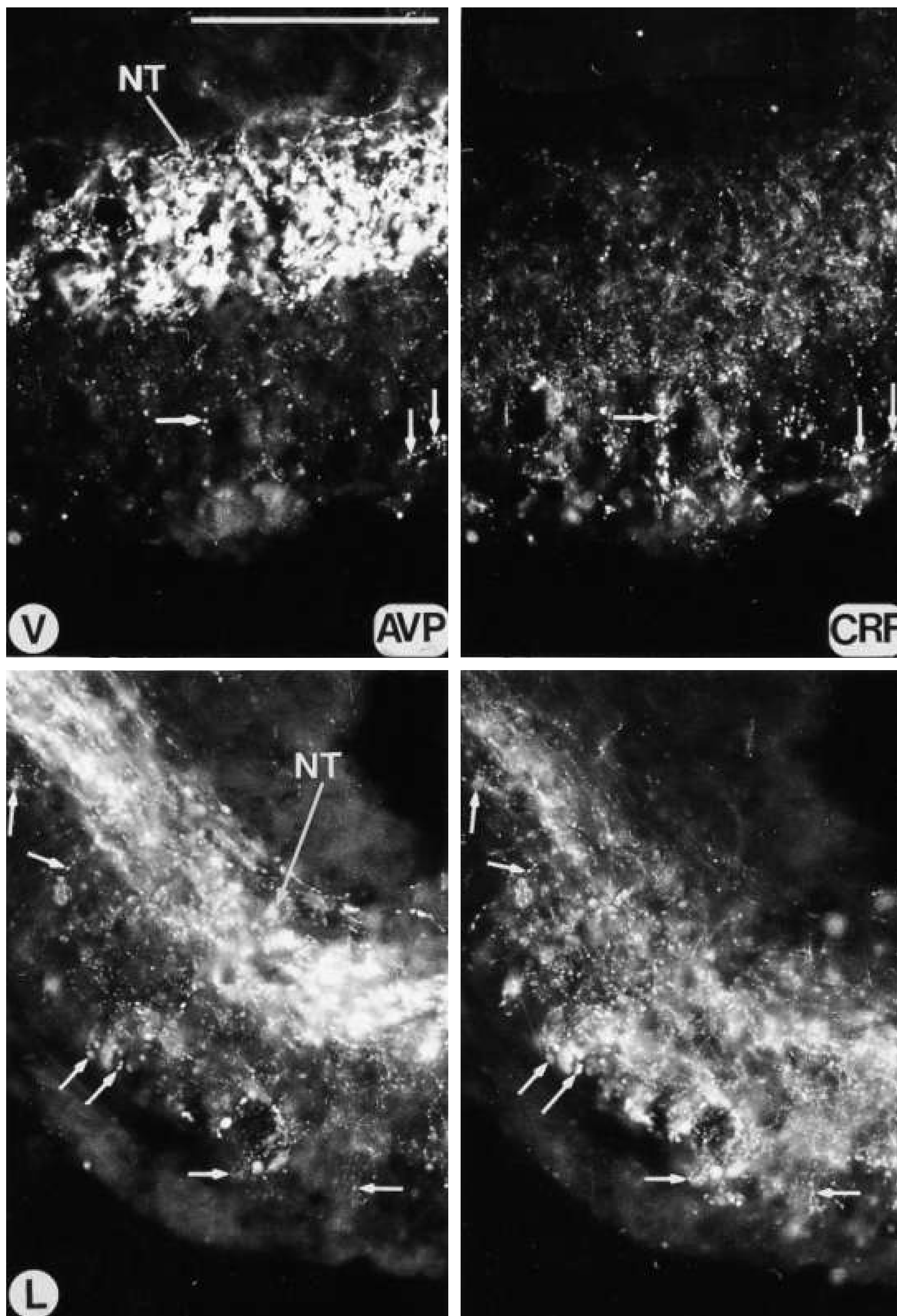

FIG. 6. Immunostaining of arginine vasopressin (AVP) (left) and corticotropin-releasing factor (CRF) (right) in the median eminence of virgin (V, top) and lactating (L, bottom) females on day 14 of lactation. All animals received colchicine treatment 24 h earlier and pups were separated from the mothers at the time of colchicine treatment. Both peptides were detected on the same coronal sections and arrows indicate terminals colocalizing both peptides. Note the dramatic increase in colocalization of both neuropeptides in the external layer of lactating females compared to virgins (arrows). NT, neurohypophysial tract. Bar = 100 μm.

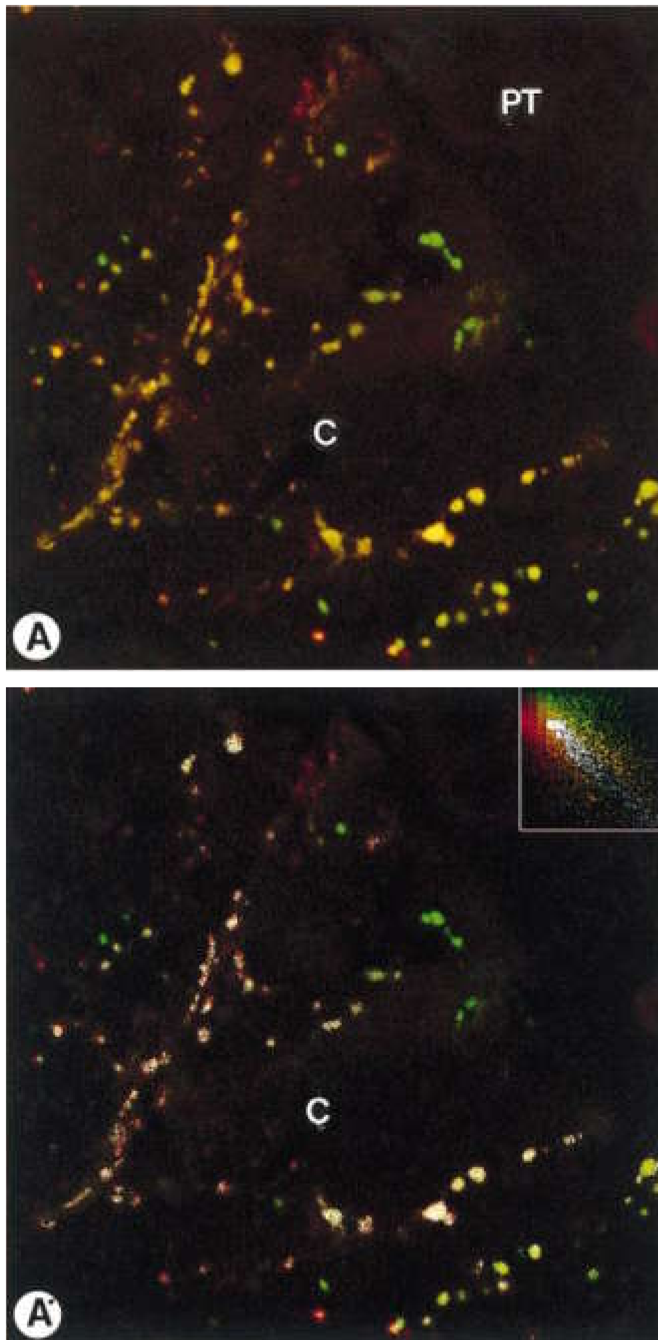

FIG. 7. Double localization of corticotropin-releasing factor (CRF) (green) and arginine vasopressin (AVP) (red) immunoreactivity in the external layer of the median eminence of lactating females on day 14 of lactation. Colocalization of both peptides using confocal microscopy is indicated by yellow (A) or white (A′) staining, and can be analysed semiquantitatively as shown in the insert. C, capillary; PT, pituitary *pars tuberalis*. Magnification ×400.

direct via intranuclear synaptic contacts or mediated by activated microglia is unclear. In our experiments, lesion-induced microglial activation could participate in the control of neuropeptide synthesis in the PVN since such activation has been demonstrated to occur up to 4 weeks after lesions (35).

In marked contrast to the specificity of the lesions in virgin females, in lactating females we found that CRF lesion affected both CRF and AVP mRNA expression in parvocellular neurones and that AVP lesion also reduced CRF expression in parvocellular neurones of the PVN. These results strongly suggest that increased parvocellular colocalization of AVP and CRF in the lactating female favours targeting and functional destruction of CRF neurones by increasing the overall synthetic activity of these neurones (at least with respect to AVP). Since the efficiency of toxin penetration and lesion depends on the state of synthetic activity of neuropeptidergic neurones (36), by expressing increased AVP, parvocellular CRF neurones become more sensitive to the effects of the lesions. Indeed, we observed a greater reduction in size of the labelled area and intensity of labelling for both CRF and AVP lesions in lactating females (CRF: $P < 0.01$ for both surface and grains).

Lactation-induced enhanced CRF/AVP colocalization was confirmed by double immunohistochemical staining for CRF and AVP on control IgG-Tx treated females. Colocalization of CRF and AVP was increased at all levels of analysis in lactating compared to virgin females, including cell bodies of the parvocellular PVN, hypothalamic processes and fibre terminals in the external zone of the median eminence. Thus, increased production of AVP by parvocellular CRF neurones is likely accompanied by increased axonal transport (processes) and release (fibre terminals) of these two neuropeptides by lactation-induced changes in hormonal milieu. Although hypophysial portal concentrations of CRF and AVP have not been compared between virgin and lactating females, we suggest that higher levels of AVP are reaching the anterior pituitary to modify the control of ACTH secretion during lactation. Indeed, we recently demonstrated a shift in the sensitivity of the pituitary in favour of AVP over CRF in lactating compared to virgin females (25). This increased secretion of AVP might be specifically directed towards anterior pituitary function since microdialysis studies conducted from the last 3 days of gestation through day 10 of lactation failed to show any elevation in intranuclear AVP release in either the supraoptic or paraventricular nuclei (37) and plasma concentrations of AVP in lactating females are not different from virgin females or mothers separated from their pups for 48 h (38).

Increased AVP immunoreactivity and mRNA expression were observed in both the magnocellular and parvocellular portions of the PVN during lactation, leading to increased AVP-IR in the internal and external layers of the median eminence. Only a subset of parvocellular CRF neurones expressed AVP in lactating females. Colocalization was seen in neurones clearly identified according to their size as parvocellular neurones, but also in neurones whose size was intermediate between parvo and magnocellular neurones, emphasizing the heterogeneous nature of the parvocellular PVN neurones (39). Interestingly, the increase in AVP-IR was particularly marked in the dorsomedial region of the parvocellular division, a region immediately adjacent to clearly defined magnocellular neurones. Whether local interaction of strongly activated magnocellular neurones with neighbouring parvocellular neurones could favour the

TABLE 1. Comparison of Corticotropin-releasing Factor (CRF) and Arginine Vasopressin (AVP) mRNA Expression in the PVN of Virgin and Lactating Females on Day 14 of Lactation.

	CRF mRNA		AVP mRNA	
Females	Total surface (μm^2)	Intensity (μm^2)	Total surface (μm^2)	Intensity (μm^2)
Virgins	42320 ± 2114 (42)	6120 ± 495 (44)	45692 ± 1804 (46)	mPVN: 6673 ± 344 (46) pPVN: 1461 ± 12 (46)
Lactating	20693 ± 2092 (40)	3427 ± 591 (37)	60128 ± 3277 (46)	mPVN: 11186 ± 1463 (30) pPVN: 4605 ± 24 (30)

All values in lactating females were significantly ($P < 0.01$) different from those of virgins. Values represent the mean ± SEM and the number of determinations is indicated in parenthesis. mPVN, magnocellular PVN; pPVN, parvocellular PVN.

enhancement of AVP production in specific regions of the PVN is currently unknown.

In our study, we observed a parallel increase in AVP mRNA expression and protein in the PVN of lactating compared to virgin females. In contrast, we found a significant reduction in CRF mRNA expression in lactating females compared to virgins that was not mirrored by a reduction in CRF-IR. Quite unexpectedly, we observed higher levels of immunoreactivity in CRF neurones of lactating compared to virgin females under conditions of colchicine treatment. It is generally believed that colchicine treatment increases immunoreactivity of peptides having a rapid turnover and release (40). However, this might not remain true for all physiological conditions or neuropeptides examined and a dissociation between mRNA levels and protein content is often reported in the literature. Alternatively, pup removal following colchicine treatment might have contributed to the increased CRF immunoreactivity seen 24 h later although increased CRF mRNA expression following pup removal is generally not observed before 48 h of separation (19).

Our observation of increased colocalization of AVP in CRF neurones of the PVN, together with the opposite changes in CRF and AVP expression found during lactation in parvocellular neurones provide important evidence for a dissociation in the cellular regulation of these two neuropeptides within the same neurone. A rapid dissociation between CRF and AVP transcription has been documented following acute stress in male rats (41). In these studies, differences in the kinetics of activation of the various transcription factors (fos, jun, CREB, etc.) was suggested to be responsible for the differential rate of neuropeptide transcription (42). Similarly, differences in the nature or rate of production of transcription factors might explain the opposite regulation of CRF and AVP in parvocellular neurones during lactation. In fact, stress-induced activation of c-*fos* in the PVN is considerably reduced in lactating compared to virgin females (43). Alternatively, it is possible that differential sensitivity of the CRF and AVP genes to activated glucocorticoid receptors might explain the dissociation between CRF and AVP transcription given the tonically elevated glucocorticoid levels in lactating females. It is remarkable that in adult male rats, coexpression of AVP in CRF neurones of the PVN coincide with a decreased action of glucocorticoids, either by total removal of endogenous glucocorticoids after adrenalectomy or under chronic stress conditions where elevated levels of glucocorticoids are measured, but appear to be less efficient to elicit negative feedback on the adrenocortical axis (44). In the lactating female, concentrations of glucocorticoids are generally reported to be increased (16), but unlike the situation of chronic stress, the ability of glucocorticoids to inhibit ACTH secretion is not impaired by lactation (22). In fact, glucocorticoid bioavailability might be enhanced since significant reductions in circulating corticosterone binding globulin have been recently documented in lactating females (24). Thus, it appears that tonically elevated glucocorticoid secretion in suckled females does not prevent the increased expression of AVP in parvocellular neurones of the PVN. This suggests that other central inputs activated by the suckling stimulus can supersede glucocorticoid feedback and specifically activate AVP expression in all PVN neurones (parvocellular and magnocellular) while reducing CRF expression.

We conclude from these experiments that the physiological condition of lactation induces large increases in coexpression of CRF and AVP in parvocellular neurones of the PVN controlling adrenocortical activity. Furthermore, we have demonstrated that differential regulation of CRF and AVP expression in these neurones increases their sensitivity to immunotargeted lesions. We propose that the opposite regulation of CRF and AVP gene expression in parvocellular neurones of lactating females might be due to differential production of hypothalamic transcription factors or sensitivity to glucocorticoid negative feedback of both genes.

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