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Immunoreactivity of hypothalamo-neurohypophysial neurons which secrete corticotropin-releasing hormone (CRH) and vasopressin (Vp): immunocytochemical evidence for a correlation with their functional state in colchicine-treated rats

G. Alonso, A. Szafarczyk, and I. Assenmacher

Neuroendocrinology Laboratory, UA–639 CNRS, Department of Physiology, University of Montpellier II, F–34060 Montpellier, France

Summary. The specific immunoreactivity of neurons containing corticotropin-releasing hormone (CRH) or vasopressin (Vp) was studied both centrally, in the parvocellular division of the paraventricular nucleus, and distally, in the external median eminence. Control rats were compared with adrenalectomized rats and with animals supplemented with corticosterone or dexamethasone, either without additional treatment, or 24, and 48 h after an intraventricular injection of colchicine. In all groups of animals, colchicine induced a progressive and parallel decrease in both CRH and Vp immunoreactivity within the axons of the external median eminence. A semi-quantitative estimation of this axonal immunostaining showed that the decrease was clearly correlated with the axons' releasing activity according to the different functional states of the adrenocorticotropic system. Increased rates of hormonal release induced by adrenalectomy could be seen in the accelerated depletion of axonal immunoreactivity whereas corticosteroid supplementation had the opposite effect. Correspondingly, the progressive intensification of the CRH and Vp immunoreactivity within the perikarya following colchicine treatment was further markedly enhanced in adrenalectomized rats and diminished after corticosteroid supplementation. Taken together, these data suggest that in these neurons, perikaryal hormone synthesis may be closely related to the releasing activity of the axon terminals. They further point to appropriate colchicine treatment as useful tool for evaluating the functional state of CRH and Vp neurons of the parvocellular paraventricular nucleus under various experimental conditions.

Key words: Corticotropic system – Corticotropin releasing hormone – Vasopressin – Colchicine – Neuronal functional state – Rat

Offprint requests to: G. Alonso (address see above)

Introduction

Measurements of hormonal contents in hypothalamic regions by bioassays or radioimmunoassays (RIA) of tissue extracts have been frequently used to determine the functional state of neurosecretory systems (Hillhouse und Jones 1976; Rougon-Rapuzzi et al. 1978; Patel 1979; Berelowitz et al. 1981; Moldow and Fishman 1982). Since under standardized conditions immunoreactivity of endocrine neurons is assumed to parallel their hormone content (Kraehenbuhl et al. 1980; Benno et al. 1982; Schipper and Tilders 1982) immunocytochemical methods have also been utilized for quantitative estimations of neuronal hormone contents (Parish et al. 1981; Seybold et al. 1981; Bugnon et al. 1983; Schipper et al. 1984). Despite its high sensitivity, immunocytochemical approaches based on visual, microfluorometric or microdensitometric measurements of immunostaining, are certainly less accurate than bioassays or RIAs for quantitative estimations of hormone content variations. Nevertheless, they are unique in allowing comparative estimation of hormone levels within individual neurons, especially in CNS areas with highly intermingled neurosecretory systems. For instance, in the median eminence (ME), which contains two functionally differentiated vasopressin (Vp) systems ending respectively onto the hypophysial portal vessels and in the posterior pituitary (Dierickx et al. 1976; Bock et al. 1980) immunocytochemistry alone may clearly discriminate between the hormonal loads of either system under specific experimental conditions (Stillman et al. 1977; Zimmerman et al. 1977; Silverman et al. 1981; Seybold et al. 1982; Bugnon et al. 1983).

Regarding the CNS control of the corticotropic system, which relates to our current research field, a series of recent studies based on semi-quantitative immunocytochemistry involving a visual estimation

of immunostaining intensities showed marked modifications in the immunoreactivity of CRH and Vp axons in the external ME when the pituitary-adrenal axis was affected (Silverman et al. 1981; Bugnon et al. 1983; Merchenthaler et al. 1983; Paul and Gibbs 1983). Until now, however, no decisive functional interpretations could be drawn from overall modifications in axonal immunostaining. Clearly enough, 24 h after bilateral adrenalectomy the dramatic rise in plasma ACTH levels (Dallman et al. 1972; Van Loon et al. 1982) significantly correlated with a marked decrease in the immunoreactivity of both CRH and Vp axons in the external ME (Bugnon et al. 1983), which appears to be consistent with an increased release of hypothalamic CRF. However, 5 days later, even though persistently high ACTH levels still accounted for an intense hypothalamic stimulation, the CRH axons of the external ME had returned to almost normal immunoreactivity, and a conspicuous increase in immunoreactivity was even noted in the Vp axons (Silverman et al. 1981; Bugnon et al. 1983; Merchenthaler et al. 1983; Paull and Gibbs 1983).

Considering that at the neurosecretory axon terminal level, hormone content actually results from a balance between the hormone's release in the extraneuronal space and the amount of newly synthetized hormone arriving in the nerve ending by rapid axonal transport, we took advantage of the blocking effect of colchicine on axonal transport to gain a closer insight into the functional meaning of immunoreactivity modifications in CRH and Vp neurons after removal of or supplementation of circulating corticosteroids.

Material and methods

Animals

Thirty-six female Sprague-Dawley rats (200–250 g) were housed in temperature-controlled rooms (21 ± 1° C), under a lighting regime of 12 h light (50 lux at 7.00 a.m.) and 12 h darkness. Four groups of 9 rats were examined: (1) intact controls, (2) bilaterally adrenalectomized rats, 7 to 9 days after surgery; (3) intact animals receiving for 4 days corticosterone (15 mg/d; s.c.) and (4) intact animals receiving for 4 days dexamethasone (500 μg/d; s.c.).

Adrenalectomized rats were given a 10‰ NaCl drinking solution, whereas the other animals had free access to tap water. A standard dry food (U.A.R., Paris) was available ad libitum. In each of the 4 groups, 3 series of 3 rats were treated for immunocytochemistry: one series without pharmacological treatment, and the other two series, 24 h or 48 h after an intraventricular injection of colchicine.

Colchicine injection

Under deep pentobarbital anesthesia (60 mg/kg), animals were fixed in a stereotaxic device, and colchicine (75 μg in 10 μl saline) was injected into the lateral ventricle. Injections were given between 10 and 12 a.m. Adrenalectomized rats were injected on day 7 after surgery, while those supplemented with corticosterone or dexamethasone received the toxicant on the 2nd day of corticoid treatment.

Immunocytochemical procedure

Again under deep pentobarbital anesthesia, all animals were sacrificed between 10 and 12 a.m. They were perfused intracardially with phosphate-buffered saline (PBS) followed by a freshly prepared solution of 4% paraformaldehyde in 0.1 M phosphate buffer (pH 6.9) containing 0.2% picric acid. After dissection, the brains were immersed in the same fixative at 4 °C for an additional 24–48 h. They were then rinsed overnight in PBS supplemented with 10% sucrose, frozen in liquid nitrogen, and cut frontally into 10 μm thick sections in a cryostat (American Optical). Sections were mounted on gelatin-coated glass slides and kept for 5 to 7 h at −20 °C before being treated according to the indirect immunofluorescence technique. Sections were incubated overnight at 4 °C in a humid atmosphere with rabbit anti-oCRH$_{41}$ (diluted 1/200) or anti-Vp (diluted 1/200) antisera. The oCRH$_{41}$ antisera were kindly provided by Prof. Charles Oliver from the University of Marseille, and the Vp antisera were prepared in our Laboratory. Sections were then rinsed in PBS and incubated with fluorescein isothiocyanate conjugated with a goat anti-rabbit antiserum (diluted 1/50) for 3 h at room temperature. After rinsing in PBS, they were coverslipped with a mixture of PBS and glycerol (1/3) and examined in a ZEISS fluorescence microscope.

In order to reduce non specific binding of γ-globulins to the brain tissues, sections were preincubated for 1 h at room tempera-

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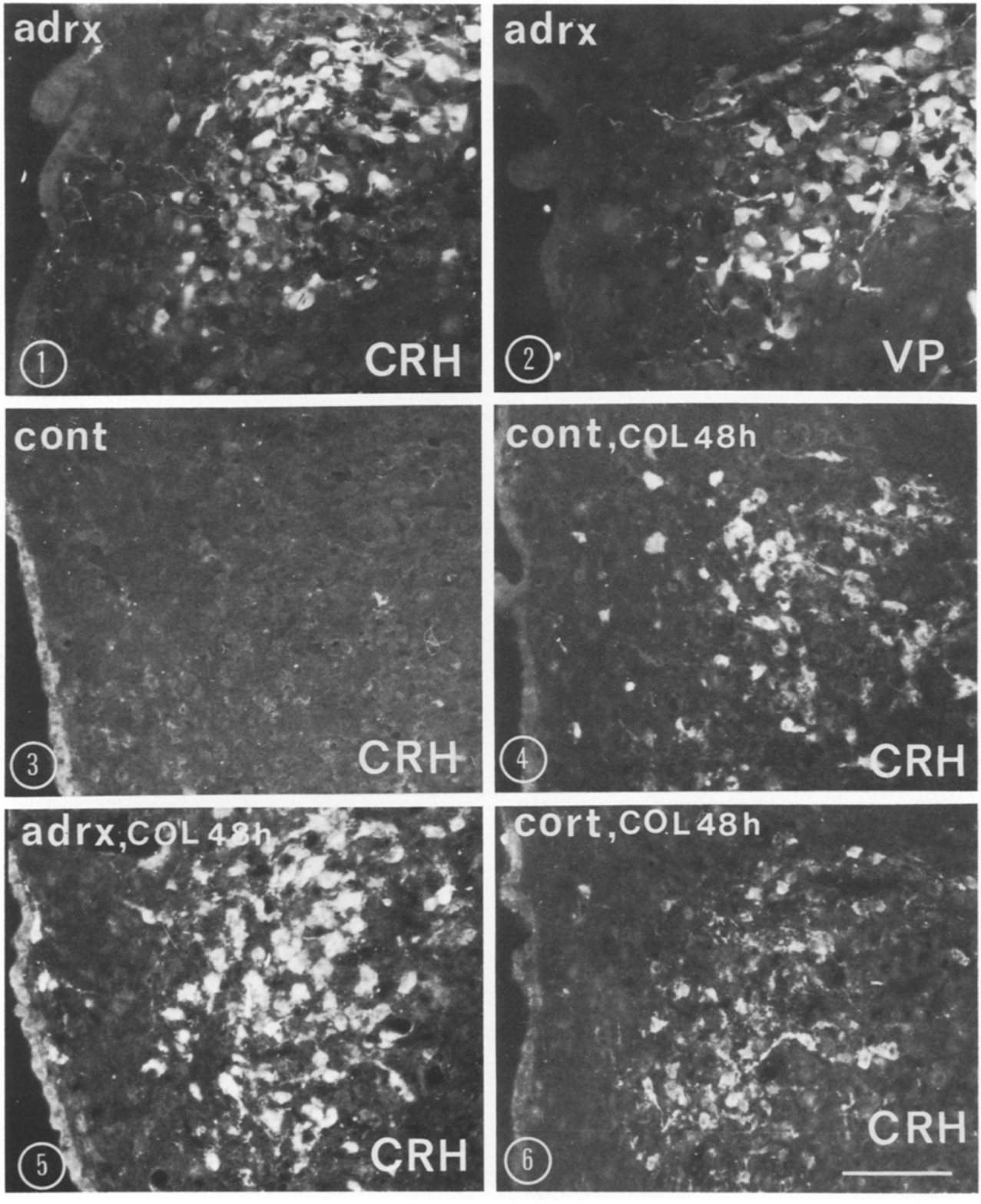
Figs. 1–6. Coronal sections through the midcaudal parvocellular PVN of rats without colchicine treatment (Figs. 1–3) or rats intraventricularly injected with colchicine (Figs. 4 and 5), Figs. 1, 3, 4, 5, 6: CRH antibodies; Fig. 2: Vp antibodies. Calibration bar: 100 μm

Figs. 1 and 2. 8 days adrenalectomized rats without colchicine treatment. CRH (Fig. 1) and Vp (Fig. 2) immunolabeling are observed within perikarya of parvocellular PVN

Figs. 3 and 4. Control rats. No labeled perikarya are observed in the absence of colchicine (Fig. 3) moderate to intense CRH immunostaining is observed within numerous perikarya 48 h after colchicine administration. (Fig. 4)

Fig. 5. 8 days adrenalectomized rats, 48 h after intraventricular injection of colchicine. Amount of labeled perikarya and intensity of labeling are increased, compared to adrenalectomized rats without colchicine (Fig. 1) or to intact rats 48 h after colchicine administration (Fig. 4)

Fig. 6. Corticosterone supplementation, 48 h after intraventricular injection of colchicine. Weak CRH immunolabeling is observed within a few scattered perikarya


adrx
CRH
1
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VP
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cont, COL 48h
CRH
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CRH
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CRH
6

Fig. 1–6

ture with 1/50 diluted goat serum and all antisera were diluted in PBS containing 0.2% gelatin. The specificity of immunostaining was ascertained by incubating control sections with the primary antiserum previously immunoadsorbed with its specific antigen, i.e. 0.1–0.5 mg of synthetic Vp or $oCRH_{41}$/ml of diluted antiserum, for 4 h at room temperature.

Results

Although labeled Vp and CRH-containing neurons could be observed in various brain and hypothalamic areas, the present study focussed on the Vp- and CRH-immunoreactive neurons located in the parvocellular division of the paraventricular nucleus (PVN) which are known to project into the external median eminence.

Animals without colchicine

1) CRH neurons. Except in adrenalectomized rats, who displayed several immunoreactive CRH perikarya in the PVN area, no immunostained perikarya were detected in any region of the hypothalamus in control rats or in animals treated with one of the corticosteroids (Figs. 1 and 3). On the other hand, in all animals, heavily labeled axons or axon terminals were visible all along the external ME, and a more intense labeling generally occurred in a narrow strip located near the portal vessels. Due to the consistent individual variations in immunostaining, no clear differences were apparent in the axonal immunoreactivity of the different rat groups, except for a slight increase generally noted in adrenalectomized rats (Fig. 11), and in animals supplemented with corticosterone (Fig. 15).

2) Vp neurons. Whereas highly labeled Vp perikarya were observed in the magnocellular section of the PVN in all animals examined, the parvocellular PVN displayed labeled perikarya exclusively in adrenalectomized rats (Fig. 2). Nevertheless, as for the CRH neurons, labeled Vp axons occurred in the external ME of all rat groups. In controls and in rats treated with dexamethasone, labeled Vp axons appeared sparsely scattered all along the external layer of the ME, and their density appeared increased in rats receiving corticosterone and in adrenalectomized animals (Figs. 9, 13, 17). By contrast, no treatment significantly affected the dense tract of Vp axons through the internal ME.

Animals injected with colchicine

1) CRH neurons. Contrasting markedly with animals lacking colchicine treatment, labeled CRH perikarya were observed in the parvocellular PVN sections of all treated animals, and in all 4 experimental groups the density of labeled perikarya and their staining intensity appeared maximal 48 h after colchicine injection. However, both parameters of CRH-labeling clearly appeared higher in the PVN of adrenalectomized colchicine-treated rats. (Fig. 5), whereas they were depressed when compared with the intact controls, and even more so compared with adrenalectomized rats, in intact animals supplemented with corticosterone or dexamethasone (Fig. 6).

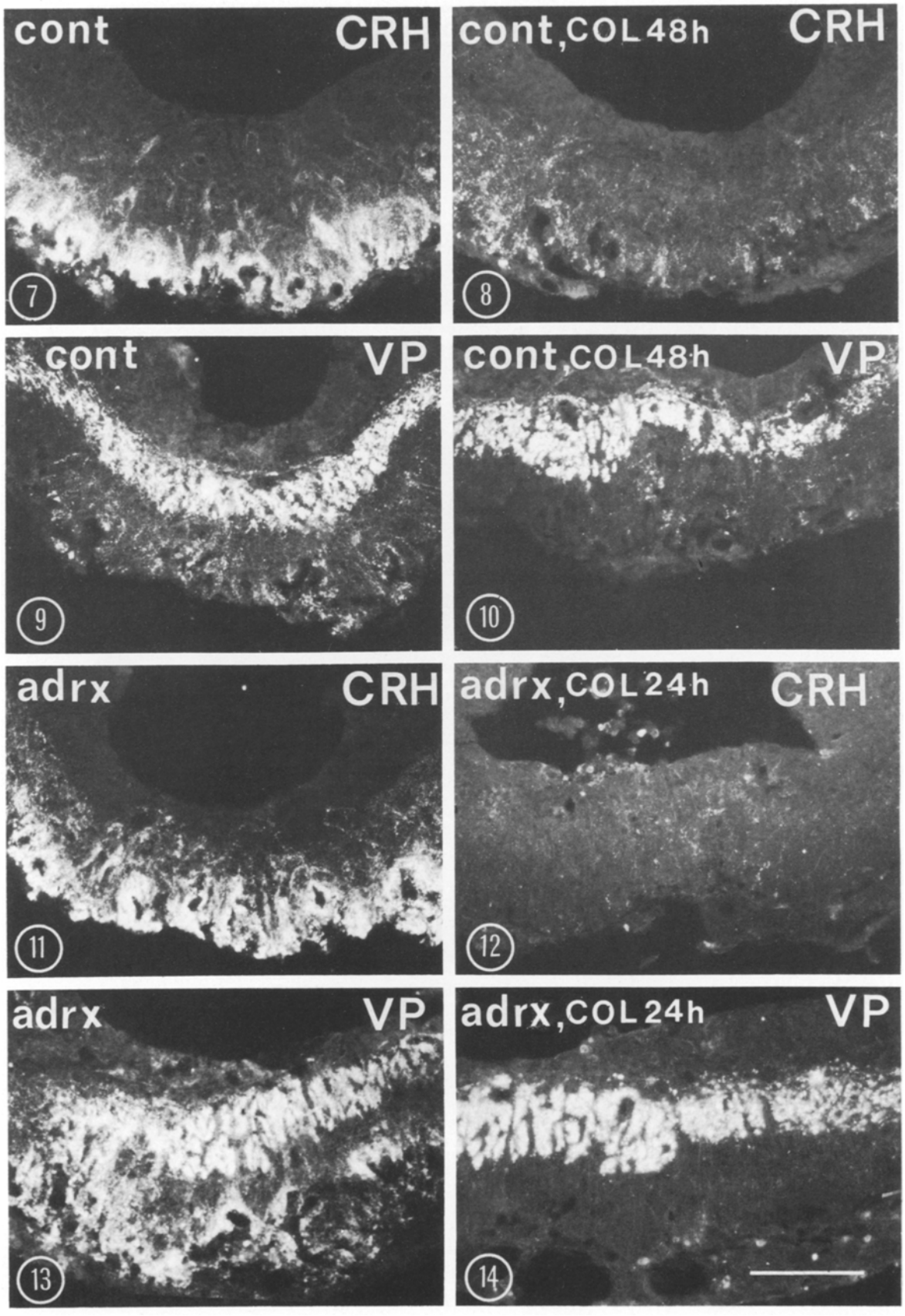
In the external ME, colchicine treatment was always followed by a decline in axonal CRH labeling. In intact controls CRH staining in the external ME had already markedly dropped 24 h after colchicine, and were poorly detectable after 48 h (Figs. 7 and 8). In adrenalectomized rats, the CRH immunostaining had vanished from the ME as early as 24 h after colchicine injection (Figs. 11 and 12), whereas in corticosterone-treated (Figs. 15 and 16) and dexamethasone-treated rats (Figs. 19–20), the immunoreactivity of CRH axons in the external ME was fairly strong even 48 h after colchicine injection (Figs. 15 and 16).

2) Vp neurons. In the magnocellular sections of the PVN colchicine treatment increased immunoreactivity of Vp in the four experimental groups, whereas in parvocellular perikarya Vp immunoreactivity could only be detected in adrenalectomized rats. In the latter group, 48 h after colchicine, Vp-immunoreac-

Figs. 7–20. Coronal sections of rat median eminence without colchicine (Figs. 7, 9, 11, 13, 15, 17, 19) or after intraventricular injection of colchicine (Figs. 8, 10, 12, 14, 16, 18, 20). Figs. 7, 8, 11, 12, 15, 16, 19, 20: CRH antibodies; Figs. 9, 10, 13, 14, 17, 18: Vp antibodies. Calibration bar: 100 μm

Figs. 7–10. Intact rats. 48 h after colchicine administration, immunolabeling of CRH (Fig. 8) or Vp axons (Fig. 10) projecting to the EME is highly decreased, compared to CRH (Fig. 7) and Vp (Fig. 9) axonal immunostaining in untreated animals

Figs. 11–14. 8 days adrenalectomized rats. Without colchicine treatment, immunolabeling of EME axons is slightly or markedly increased, respectively for CRH (Fig. 11) and Vp (Fig. 13) compared to untreated controls (Figs. 7 and 9). 24 h after colchicine, very scarce CRH (Fig. 12) or Vp (Fig. 14) labeled axons are detected in the EME


cont
CRH
7
cont, COL 48h
CRH
8
cont
VP
9
cont, COL 48h
VP
10
adrx
CRH
11
adrx, COL 24h
CRH
12
adrx
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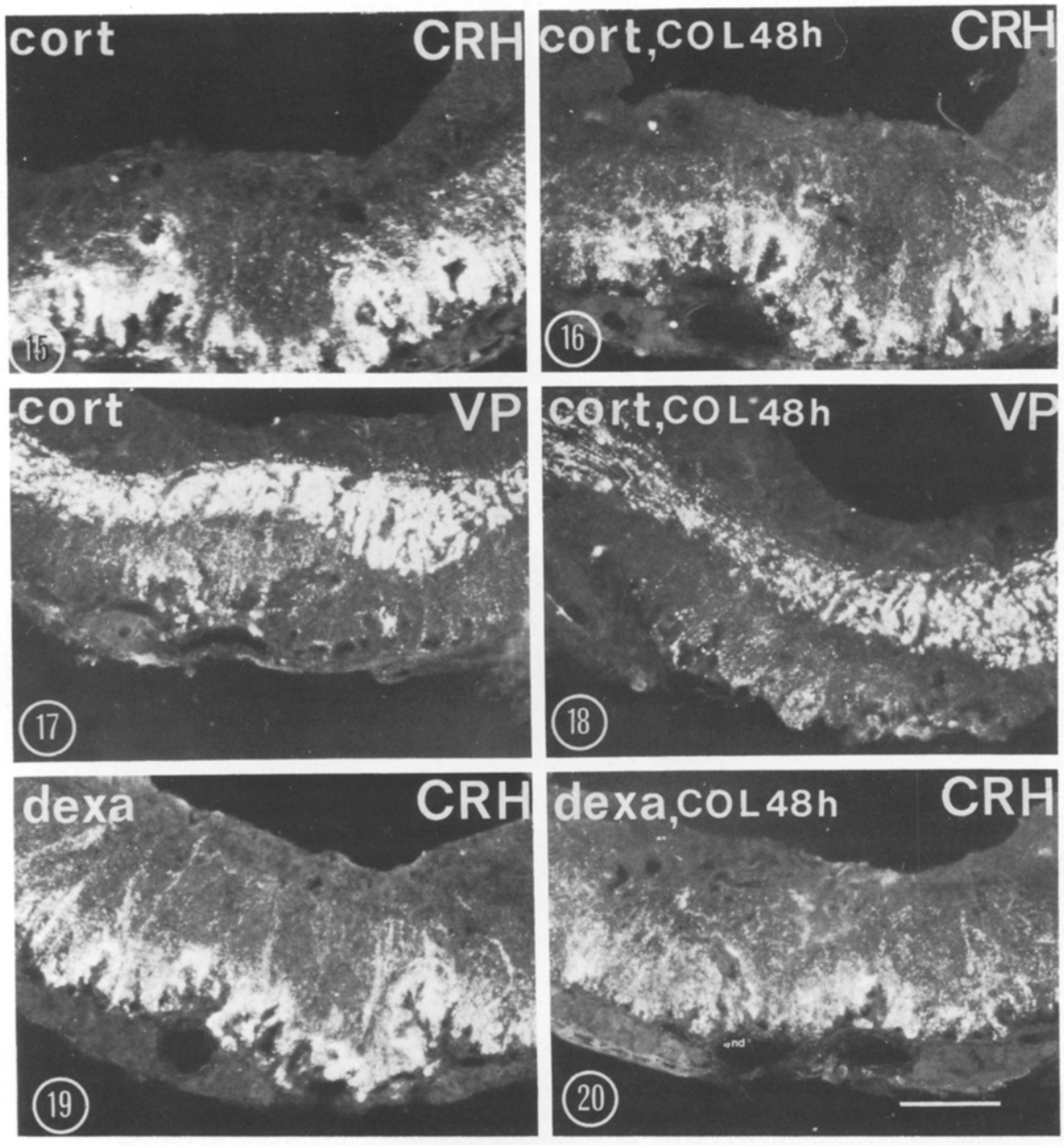
13
adrx, COL 24h
VP
14

Fig. 7–14



Figs. 15–18. Rats supplemented with corticosterone. Without colchicine, immunolabeling of CRH (Fig. 15) or Vp axons (Fig. 17) is slightly increased in the EME, compared to untreated controls (Figs. 7 and 9). 48 h after colchicine, intensity of CRH (Fig. 16) and Vp (Fig. 18) axonal immunolabeling is only slightly decreased and clearly superior to that of controls without corticosterone but with similar colchicine treatment for 48 h (Figs. 8 and 10)

Figs. 19–20. Rats supplemented with dexamethasone. Without colchicine treatment, immunolabeling of CRH axons in the EME (Fig. 19) is similar to that observed in untreated controls (Fig. 7). 48 h after colchicine, CRH immunolabeling (Fig. 20) is markedly decreased, compared to colchicine-treated rats supplemented with corticosterone instead of dexamethasone (Fig. 16), but substantially higher than that of colchicine injected control rats (Fig. 8)

tivity generally exceeded that of adrenalectomized rats without colchicine.

In the external ME, the effect of colchicine on Vp axons generally paralleled its effects on CRH axons. In adrenalectomized rats, Vp staining had already disappeared 24 h after the toxic treatment (Figs. 13 and 14), while some labeled axons were still observed after 48 h in intact controls (Fig. 10). In both corticosteroid-supplemented groups, Vp axons appeared only slightly modified 48 h after colchicine injection (Figs. 17 and 18).

In the ME of all groups, the immunoreactivity of

the Vp axons running down to the posterior pituitary within the tract of the internal layer of the organ generally appeared little affected by colchicine in their rostral sections, but decreased in the most caudal sections of the axons 48 h after colchicine injection.

Discussion

The blocking effect of colchicine on rapid axoplasmic transport is well documented. In radioautographic studies of neurosecretory neurons, the toxicant has been shown to strongly impair the transport of newly synthetized neurosecretory material (NSM) toward the axon terminals, leading to the accumulation of labeled NSM within the proximal neuronal portions (Flament-Durand and Dustin 1972; Dustin et al. 1975; Hindelang-Gertner et al. 1976; Parish et al. 1981) and to a complete lack of newly formed NSM in the axons' terminals (Norström et al. 1971b; Parish et al. 1981). A maximal blockade of rapid axoplasmic transport was shown to occur as early as 5 h after colchicine administration (Karlsson and Sjöstrand 1969) and high doses of colchicine as used in our study, causes irreversible effects (Dustin 1978). Since the hypothalamic regions studied here are known to be easily accessible to substances injected into the cerebral ventricles, it can therefore be assumed that axoplasmic transport in all neurons of the periventricular area was, indeed, completely blocked shortly after colchicine injection and that, from a few hours to 48 h post-injection, no neurosecretory material of central origin reached the neurosecretory nerve terminals at the external median eminence (EME).

Additionally, since it is generally accepted that colchicine has little effect on the releasing mechanisms of neurosecretory material (Douglas and Sorimachi 1972; Norström and Hansson 1973) it was postulated that the immunoreactivity detected at the axons terminals 24 to 48 h after colchicine injections corresponded essentially to the distal neurohormonal stores available before the blockade of axoplasmic transport and to its subsequent extraaxonal release. In the present study, the decrease in specific neurohormone immunoreactivity in the axons' terminals of the EME after intraventricular colchicine injection therefore served as an index for the hormonal release into the perivascular space of the hypophysial portal system.

This special experimental approach was used here to study the neurosecretory release of CRH and Vp, both hormones involved in ACTH release (Gillies and Lowry 1979; Lutz-Bucher et al. 1980; Rivier and Vale 1983) and originating from the parvocellular region of the PVN, where they have even been shown to be occasionally colocalized within the same neurosecretory neurons (Roth et al. 1982; Tramu et al. 1983; Kiss et al. 1984; Sawchenko et al. 1984). Even though, under the present experimental conditions, varying amounts of positive immunostaining in Vp and CRH neurons could be finely discriminated (Table 1) semi-quantitative data based on visual estimation of neuronal immunostaining may be considered with caution and our conclusions were concentrated on striking differences that were obvious on all sections examined. Thus, the results summarized in Table 1 clearly show that two experimental conditions known to have opposite effects on the adrenocorticotropic system i.e. adrenalectomy and corticosteroid administration, led to opposite trends in the speed of the decrease in IR-CRH and IR-Vp at the ME axons terminals after colchicine injection. Compared to the basal state of colchicine-treated, but otherwise intact controls, adrenalectomy led to a rapid and complete depletion of both CRH and Vp from the external ME, whereas corticosteroid supplementation markedly inhibited their axonal release. Consequently, the data first provide a clear morphological illustration for well known physiological observations on the negative feed back effect of circulating corticosteroid levels on related neurohormones' release (Dallman et al. 1972; Hillhouse and Jones 1976; Buckingham 1979). They further demon-

Table 1. Semiquantitative estimation of CRH and Vp immunoreactivity in neurosecretory neurons of rats without colchicine treatment (Col-0) or 24 h (Col-24) and 48 h (Col-48) after an intraventricular injection of colchicine. Intensity of immunostaining is estimated as intense (++++), strong (+++), moderate (++), weak (+) or undetectable (−). pPVN: parvocellular paraventricular nucleus; EME : external median eminence

	CRH		Vp	
Controls	pPVN	EME	pPVN	EME
Col-0	−	+++	−	++
Col-24	+	++	−	+
Col-48	++	+	−	+
8 d. Adrenalectomized				
Col-0	++	++++	++	+++
Col-24	++	−	++	−
Col-48	+++	−	+++	−
Corticosterone inj.				
Col-0	−	++++	−	+++
Col-24	+	+++	−	++
Col-48	+	+++	−	++
Dexamethasone inj.				
Col-0	−	+++	−	++
Col-24	+	++	−	++
Col-48	+	++	−	+

strate that in vivo administred colchicine does not markedly alter the releasing activity of CRH and Vp neurons, which indicates that the neuronal inputs that control these neurons are little affected by the toxicant.

Since it is also generally assumed that colchicine has little if any effect on the synthesis of neurosecretory material (Norström et al. 1971b), it may be postulated that whenever an increase in immunoreactivity was observed within the CRH and Vp perikarya of the parvocellular PVN it was closely correlated with the level of neurohormone synthesis. Taken together, the results obtained here at both perikaryal and axonal levels, clearly favor the concept that in parvocellular CRH and Vp neurons, perikaryal neurohormone synthesis is closely related to the neurons' releasing activity as previously observed in other neurosecretory systems (Norström et al. 1971a; Gainer 1981) and that circulating corticosteroids may affect the functional state of CRH and Vp neurons at both levels. Thus, in the actively releasing neurons of adrenalectomized rats high synthesis levels resulted 24 and 48 h after colchicine injection in a large neurohormonal accumulation within the perikarya, whereas in the neurons of corticosteroid treated animals, the weak releasing activity of the axons was correlated with low levels of synthesis, leading to reduced perikaryal accumulation of neurohormones following colchicine treatment.

Our data further provide significant information related to a few specific questions. First, the parallel variations in CRH and Vp release from the EME of adrenalectomized or corticosteroid-treated rats conforms to the concept that both neurohormones are involved in the stimulatory control of the corticotropic function whether or not colocalized within the same neurons (vide supra). Secondly, the data also provide an alternative explanation for the secondary increase in axonal CRH and Vp immunoreactivity noted from 5 days after adrenalectomy. This axonal load in neurohormone has been interpreted as an index for a decreased CRH and Vp release, resulting from a short feedback of increased ACTH secretion (Motta et al. 1965; Bugnon et al. 1983). At variance with that theory, the colchicine technique clearly showed here that 8 days after adrenalectomy CRH and Vp releases, indeed, were substantially increased. Consequently, the progressive loading of neurosecretory axon terminals in adrenalectomized rats without colchicine may rather result from a progressive stimulation of neurohormone synthesis leading to increased neurohormone supply to the axon terminals, which may compensate and even surpass the losses due to stimulated release.

In conclusion, the estimation of the modifications in neurohormonal immunoreactivity at both the perikaryal and the terminal levels of neurosecretory neurons after colchicine blockade of axoplasmic transport yields a significant improvement for quantitative studies of the regulatory processes controlling endocrine neurons. Clearly enough, the conclusions drawn from visual estimations of morphological parameters used in the present study may only apply for major modifications in immunoreactivity. In this line, the present data indeed, afford a novel insight on the modulation by corticosteroids of the neurohormone synthesis-release balance of CRH and Vp neurons.

Further studies are now in progress using this experimental approach to explore the central regulations of these neurosecretory adrenocorticotropic system by various afferent neurotransmitter systems.

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