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# Adrenalectomy-Induced Enhancement of CRF and Vasopressin Immunoreactivity in Parvocellular Neurosecretory Neurons: Anatomic, Peptide, and Steroid Specificity

**P. E. Sawchenko**

The Salk Institute for Biological Studies, and The Clayton Foundation for Research–California Division, La Jolla, California 92037

**Following adrenalectomy (ADX), corticotropin-releasing factor (CRF) and vasopressin immunoreactivity are jointly expressed by a population of parvocellular neurosecretory neurons in the paraventricular nucleus of the hypothalamus (PVH). Because these cells stain positively for CRF, but not for vasopressin, after pretreatment with colchicine, the results suggest the existence of state-dependent alterations in the expression of peptides by neuroendocrine neurons. The present study sought to determine (1) whether other neuropeptides (e.g., neurotensin, met-enkephalin) that have been colocalized with CRF in the parvocellular division of the PVH are influenced similarly by ADX; (2) whether the enhancement of CRF and/or vasopressin immunoreactivity after ADX is limited to neurons of the PVH; and (3) what factors might be involved in the regulation of the expression of these peptides in the PVH. The results confirmed that CRF and vasopressin immunoreactivity are both enhanced, and may be colocalized in a substantial population of parvocellular neurosecretory neurons after ADX; no comparable enhancement of staining for met-enkephalin or neurotensin was observed. The effect of ADX on CRF immunoreactivity was not limited to cells in the PVH, as neurons in the cerebral cortex, amygdala, and the bed nucleus of the stria terminalis also showed heightened CRF immunostaining after ADX; vasopressin immunoreactivity was never colocalized with CRF in these extrahypothalamic sites. Hypophysectomy produced an enhancement of CRF and vasopressin staining in the PVH that was comparable to that seen after ADX, implicating adrenal steroids as primary regulators of peptide expression in this system. Corticosteroid replacement studies in ADX rats indicated that lower doses of dexamethasone attenuated, and higher doses essentially abolished, the expected enhancement of both CRF and vasopressin immunoreactivity after ADX. The relative potency of steroids in mitigating these effects was dexamethasone > corticosterone > deoxycorticosterone > aldosterone. Collectively, these results indicate that the ADX-induced enhancement of CRF and vasopressin immunoreactivity in parvocellular neurosecretory neurons is at least somewhat specific to these peptides and to this cell type. Both peptides would appear to be regulated similarly by adrenal steroids, with glucocorticoids playing a primary role.**

The parvocellular neurosecretory system consists of neurons localized in more or less discrete clusters in the hypothalamus and basal forebrain and their axonal projections, which end on portal vessels in the external lamina of the median eminence. Via these pathways, peptides and amines are delivered to the portal circulation and act to stimulate or inhibit the release of anterior pituitary hormones. With the isolation and characterization of a peptide that has met the criteria postulated for corticotropin-releasing factor (CRF; Vale et al., 1981), and its localization in a sizable population of parvocellular neurosecretory neurons in the paraventicular nucleus of the hypothalamus (PVH; e.g., Bloom et al., 1982; Bugnon et al., 1982; Olschowka et al., 1982; Antoni et al., 1983; Cummings et al., 1983; Merchenthaler et al., 1983a, b; Swanson et al., 1983), an important class of such neurons, which are vital to the initiation of stress responses, has been made accessible to detailed morphological and physiological investigation.

Consistent with the well-established negative feedback control by adrenal steroids and/or ACTH on CRF production and release (Yates and Maran, 1974; Keller-Wood and Dallman, 1984) have been recent reports, from a number of laboratories, that ADX or hypophysectomy enhances immunostaining for CRF in cell bodies in the PVH (Bugnon et al., 1983; Merchenthaler et al., 1983b; Paull and Gibbs, 1983; Swanson et al., 1983). In addition, several groups have recently demonstrated vasopressin immunoreactivity in a large population of CRF-stained parvocellular neurons in the ADX rat (Tramu et al., 1983; Kiss et al., 1984b; Sawchenko et al., 1984b; cf. Piekut and Joseph, 1985), a phenomenon never before seen in intact or colchicine-treated animals. Because vasopressin has the capacity to stimulate ACTH secretion by acting directly on corticotropes in the anterior pituitary, and to interact synergistically with CRF in this regard (e.g., Gillies et al., 1982; Turkelson et al., 1982; Vale et al., 1983a), these results suggest the existence of an adaptive mechanism whereby the expression of a particular neuroactive substance may be unmarked under specific physiological conditions. Although it is not yet clear whether vasopressin is normally produced at low levels by parvocellular

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Correspondence should be addressed to Dr. P. E. Sawchenko, The Salk Institute, P.O. Box 85800, San Diego, CA 92138.

neurosecretory neurons in the PVH, in view of the established interaction between CRF and vasopressin in promoting ACTH secretion, the phenomenon suggests a potential mechanism for enhancing the effectiveness of this neurosecretory pathway under conditions (ADX) that must be interpreted by the brain as demanding an adrenal response.

While these data raise the possibility that neurons in this system, at least, may be capable of displaying a "chemical switching" that is relevant to their function, many questions remain as to the specificity of the effect and its underlying mechanism(s). For example, subsets of CRF-immunoreactive neurons in the PVH have been reported to stain positively for enkephalin, peptide histidine isoleucine (PHI), and neurotensin immunoreactivity (Hökfelt et al., 1983; Sawchenko et al., 1984b) after pretreatment with colchicine, a drug whose capacity to inhibit axonal transport is generally assumed to allow identification of neurons that normally produce an antigen at levels low enough to escape detection using conventional immunohistochemical methodology. The issue of whether these peptides, which are not known to influence the hypothalamo–pituitary–adrenal axis directly, are comparably influenced by ADX speaks directly to the specificity of the effect. Second, it has been reported (Merchenthaler et al., 1983b; Paull and Gibbs, 1983) that neurons outside the PVH may show enhanced CRF immunostaining after ADX or hypophysectomy. We have reported such effects to be limited to the PVH (Swanson et al., 1983). It is therefore important to clarify the generality of the effect and to determine whether any enhanced staining in extrahypothalamic cell groups after ADX may also be associated with the appearance of vasopressin immunoreactivity. Finally, it is of interest to determine what regulatory agents mediate the effect of ADX on peptide expression, and whether CRF and vasopressin immunoreactivities are regulated similarly by each. As noted above, adrenal steroids are well known to exert feedback inhibitory effects on CRF production and release (Keller-Wood and Dallman, 1984), and comprise primary candiates as effector agents in this system. Nevertheless, negative feedback control of CRF by ACTH has also been established (see Yates and Maran, 1974), and a comparison of the effects of ADX and hypophysectomy, which differentially influence plasma ACTH levels while comparably affecting adrenal steroid titers, allows a comparison of the relative importance of these potential regulatory agents.

Brief accounts of some of the results may be found elsewhere (Sawchenko and Swanson, 1984, 1985).

## Materials and Methods

*Animals.* Adult male Sprague-Dawley albino rats, weighing 250–300 gm, were used in all experiments. Animals were either left untreated (normals), injected intracerebroventricularly with colchicine (50 μg in 10 μl saline, 48–72 hr prior to being killed), adrenalectomized (ADX), or hypophysectomized. Adrenalectomies were performed via bilateral incisions in the dorsolateral flanks under ether anesthesia and were checked for completeness and/or the presence of ectopic adrenal cortical tissue by examination of the peritoneum at autopsy. Adrenalectomized rats were given free access to saline (0.9% solution) in addition to normal drinking water. Four hypophysectomized rats, along with littermate controls, were purchased from a commercial supplier (Zivic-Miller, Pittsburgh, PA), and completeness of hypophysectomy surgery was confirmed by examination of the sella turcica at autopsy.

*Perfusion and histology.* All animals were perfused under chloral hydrate anesthesia with ice-cold 4% paraformaldehyde and 0.05% glutaraldehyde in a 2-phase procedure in which the pH of the perfusate was varied (Sawchenko et al., 1982; Swanson et al., 1983). Brains were postfixed overnight at 4°C in the final perfusion solution, to which 10% sucrose was added. Frozen sections 20 μm thick were cut on a sliding microtome, and for indirect immunofluorescence histochemistry, a method described in detail elsewhere was used (Sawchenko and Swanson, 1981). Typically, five 1-in-5 series of sections through the hypothalamus were saved. The third of these was stained with thionin for reference purposes, while the remaining 4 were incubated in the following: (1) a mixture of a C-terminally directed rabbit (polyclonal) antiserum against ovine CRF (C30 of Swanson et al., 1983) and a mouse-derived monoclonal antibody against vasopressin (Hou-Yu et al., 1982); (2) a mixture of an N-terminally directed antiserum against ovine CRF (C24 of Swanson et al., 1983) and the monoclonal antibody against vasopressin; (3) an antiserum against rat CRF (C70); (4) a polyclonal antiserum against vasopressin (Vandesande et al., 1975). Rabbit-derived polyclonal antisera were localized using an affinity-purified, fluorescein-conjugated goat anti-rabbit IgG (Tago Inc., Burlingame, CA), while the monoclonal antibody was localized with a species-specific, rhodamine-conjugated goat anti-mouse IgG (American Qualex, La-Mirada, CA). Control experiments, in which varying concentrations of anti-mouse secondary antiserum were applied to sections previously incubated in rabbit-derived primary antisera (and vice versa), revealed no discernible cross-reactivity of these secondary antisera with primary antisera derived from the heterologous species, thus allowing concurrent localization of vasopressin and CRF immunoreactivity in individual tissue sections (see Discussion in Gerfen and Sawchenko, 1985).

To control for the lower sensitivity characteristic of monoclonal antibodies, material stained with the monoclonal antivasopressin was compared with sections stained with a polyclonal antiserum against vasopressin. Similarly, results from concurrent double-staining experiments (in which the monoclonal antibody was used) were compared with those in which material was stained sequentially with rabbit-derived polyclonal antisera against CRF and vasopressin, using a variation (Sawchenko et al., 1984b) of the sequential double-staining protocol of Tramu et al. (1978).

In some experiments, staining for vasopressin and CRF immunoreactivity was compared with that for other peptides (met-enkephalin, neurotensin) in normal, cholchicine-treated, and ADX rats. Polyclonal antisera against these latter peptides were obtained from Immunonuclear Corp., Stillwater, MN (met-enkephalin) and Dr. M. Brown (neurotensin serum I-II; Brown et al., 1978), respectively. Specific staining in the hypothalamus with each of the antisera used in this study was blocked by preabsorption (overnight at 4°C) with 100 μg/ml of the homologous synthetic peptide, but was not diminished by addition of an equal concentration of any of the other peptides of interest here. Nevertheless, all sequential double-staining experiments employed antisera that were differentially preabsorbed with 100 μg/ml of the heterologous antigen.

*Procedure.* For comparisons of the distribution of neuropeptide immunoreactivity in the PVH and in extrahypothalamic regions, the brains of one each of a total of 6 normal, 6 colchicine-treated, and 6 ADX rats were processed in tandem. Adrenalectomies were performed 7–8 d prior to death. Multiple series of sections through the basal forebrain were prepared as described above to compare, sequentially and concurrently, CRF and vasopressin staining in the PVH, the bed nucleus of the stria terminalis, the central nucleus of the amygdala, and the cerebral cortex.

To compare the effects of ADX and colchicine treatment on CRF, vasopressin, neurotensin, and met-enkephalin immunoreactivity in the PVH, 4 adjoining series of sections from 3 additional untreated, 3 colchicine-treated, and 3 ADX rats were stained for the presence of each peptide. Antiserum C24 (for CRF) and the polyclonal antiserum against vasopressin were used in this experiment.

To examine the effect of hypophysectomy on CRF and vasopressin expression in the PVH, 4 hypophysectomized rats and 4 littermate controls were perfused in pairs at 7, 14, 31, and 59 d after hypophysectomy. Multiple series through the PVH of each rat were prepared for visualization of CRF and/or vasopressin immunoreactivity, as described above.

Steroid replacement studies involved performing adrenalectomies and, under the same anesthetic dose, implanting animals with osmotic minipumps (Alzet; Palo Alto, CA) prepared so as to deliver 1 of 2 doses of the acetate salts of 1 of 4 adrenal steroids. Two groups received the synthetic, long-acting glucocorticoid dexamethasone at dosages (20 or 50 μg/100 gm body wt/d given, respectively, to 4 and 6 rats) somewhat higher than those commonly used to replace glucocorticoid activity


*Figure 1.* Series of line drawings through the rostrocaudal extent of the PVH of an ADX rat showing the distribution of neurons stained for the presence of CRF (*filled circles*), vasopressin (*open circles*), or both peptides (*asterisks*). Doubly stained cells are overwhelmingly concentrated in the medial parvocellular part of the nucleus, while magnocellular vasopressinergic neurons are preferentially localized in the posterior magnocellular part. *Larger* and *smaller open circles* correspond, respectively, to vasopressin-stained neurons with diameters greater than or less than 25 μm. A substantial majority of all CRF-stained cells detectable in the ADX rats express vasopressin immunoreactivity. Most of the vasopressin-stained neurons in the parvocellular division that do not manifest CRF immunostaining are larger, and presumably correspond to magnocellular neurosecretory neurons, some of which are known to be scattered throughout the parvocellular division. Parts of the PVH: *am,* anterior magnocellular; *ap,* anterior parvocellular; *dp,* dorsal parvocellular; *lp,* lateral parvocellular; *mm,* medial magnocellular; *mp,* medial parvocellular; *pm,* posterior magnocellular; *pv,* periventricular. Other structures: *AHA,* anterior hypothalamic area; *BST,* bed nucleus of the stria terminalis; *fx,* fornix; *PT,* parataenial nucleus; *PVT,* paraventricular nucleus of the thalamus; *Re,* nucleus reuniens; *ZI,* zona incerta.

(Singer and Stack-Dunne, 1955) but at which the compound still exhibits little mineralocorticoid activity (Bia et al., 1982). Additional groups received similar doses of the acetate salts of corticosterone (low dose, $n = 4$; high dose, $n = 6$), the principal endogenous glucocorticoid in the rat, deoxycorticosterone (low dose, $n = 3$; high dose, $n = 5$), the major mineralocorticoid in the rat or aldosterone (low dose, $n = 4$; high dose, $n = 5$), a potent and relatively pure mineralocorticoid. A final group ($n = 7$) received infusions of the ethylene glycol (90% by volume)–absolute ethanol (5%)–water (5%) vehicle (Carroll et al., 1981; see also Will et al., 1980). Replacement regimens were in force for 7–8 d, an interval after which we have reliably seen enhancement of both CRF and vasopressin immunoreactivity in the parvocellular division of the PVH. Animals were then perfused and prepared for CRF and vasopressin localization as described above. Cell counts (Abercrombie, 1946) were made in 1 animal judged to be representative of each of the 9 groups, though multiple series of sections from every rat were stained for CRF, vasopressin, and both peptides, as described above.

## Results

The polyclonal antiserum and the monoclonal antibody against vasopressin provided qualitatively similar patterns of staining in each of the experiments described below, although the polyclonal antiserum proved consistently more sensitive. For this reason, counts of the number of vasopressin-stained neurons in the parvocellular division of the PVH were drawn from material stained with the polyclonal antiserum. Similarly, each of the 3 antisera against CRF (2 directed against ovine CRF and one against rat CRF) provided convergent results, with a single exception (see Hypophysectomy, below). Despite the fact that antisera against rat CRF generally provided somewhat more robust staining, the more thoroughly characterized antiserum against ovine CRF (C24; Swanson et al., 1983; Vale et al., 1983b) was used in quantitative studies.

### *Anatomic specificity: the paraventricular nucleus*

A detailed description of the distribution within the PVH of CRF- and vasopressin-immunoreactive neurons in which enhanced staining may be observed after ADX has not yet appeared, and is pertinent to the issue of specificity. The regional distribution of cells staining for ovine CRF, vasopressin, or both antigens (taken from sequentially double-stained material) in an ADX rat is shown in Figure 1. Cell counts for each peptide alone, taken from adjacent series of sections from normal and ADX rats, are given in Table 1. These may be compared to previously published data from colchicine-treated rats (Swanson et al., 1983; Sawchenko et al., 1984a).

The results indicate that the cells exhibiting enhanced CRF

**Table 1. Mean (±SEM) number of CRF- and vasopressin-immunoreactive cells in subdivisions of the PVH in normal and adrenalectomized rats[a]**

| | Subdivision | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Magnocellular | | | Parvocellular | | | | |
| | AM | MM | PM | AP | PV | MP | DP | LP |
| CRF | | | | | | | | |
| Normal | 0 ± 0 | 0 ± 0 | 8 ± 6 | 16 ± 8 | 10 ± 8 | 67 ± 9 | 0 ± 0 | 1 ± 1 |
| ADX | 0 ± 0 | 0 ± 0 | 21 ± 6 | 29 ± 11 | 12 ± 6 | 961 ± 112 | 0 ± 0 | 9 ± 7 |
| Vasopressin | | | | | | | | |
| Normal | 4 ± 3 | 8 ± 6 | 815 ± 115 | 7 ± 7 | 33 ± 4 | 125 ± 13 | 25 ± 6 | 17 ± 9 |
| ADX | 1 ± 2 | 0 ± 0 | 828 ± 70 | 35 ± 10 | 49 ± 7 | 732 ± 42 | 37 ± 12 | 8 ± 1 |

[a] CRF- and vasopressin-immunoreactive neurons were counted in adjacent series of sections from the brains of each of 3 normal and 3 ADX rats.

immunostaining after ADX are overwhelmingly concentrated in the medial parvocellular part of the PVH, as defined by Swanson and Kuypers (1980). At the level indicated in the bottom center panel of Figure 1, a further segregation of CRF-immunoreactive neurons in the dorsal aspect of this subdivision is apparent. The novel locus of vasopressin expression seen after ADX is similarly circumscribed, and a majority of CRF-stained neurons in the medial parvocellular subdivision also shows vasopressin immunoreactivity, though some singly stained cells of each type are reliably seen in the ADX rat.

Comparisons of cell counts in normal and ADX rats support the regional specificity of the effect (Table 1). That is, the increased number of cells expressing each peptide is predominantly attributable to increases in the number of stained neurons in the medial parvocellular subdivision.

*Anatomic specificity: extrahypothalamic cell groups*

Congruent with recent reports (Merchenthaler et al., 1983b; Paull and Gibbs, 1983), the enhancement of immunohistochemical staining for CRF after ADX was not limited to the PVH. Compared to untreated controls, in which only occasional CRF-stained cell bodies were seen, substantial numbers of immunoreactive neurons were apparent after ADX in several forebrain regions. These included the bed nucleus of the stria terminalis, the central nucleus of the amygdala, and the cerebral cortex (Fig. 2). CRF-stained perikarya have been reported previously in each of these regions after pretreatment with colchicine (e.g., Swanson et al., 1983), and these observations were confirmed in the course of the present study. With the parameters employed here, fewer CRF-stained cells typically were apparent at each of these 3 loci after ADX than after colchicine treatment, though their topographies were similar. Other forebrain cell groups in which numerous CRF-stained neurons were visible after colchicine injection displayed no discernibly enhanced staining after ADX. These included the preoptic region of the hypothalamus, the substantia innominata, the lateral hypothalamic area, and, as reported previously (Sawchenko et al., 1984b), oxytocinergic neurons in the *magnocellular* division of the PVH and in the supraoptic nucleus.

Consistent with previous analyses, ADX was again found to reliably enhance staining for vasopressin in a substantial number of neurons in the parvocellular division of the PVH, in precisely those regions in which CRF immunoreactivity was augmented. Never, however, was vasopressin immunoreactivity demonstrated in forebrain regions outside the PVH in which enhanced perikaryal staining for CRF was obvious.

*Peptide specificity*

Comparisons were made of the number and distribution of met-enkephalin- and neurotensin-immunoreactive neurons in the PVH of normal, colchicine-treated, and ADX rats. Met-enkephalin (Hökfelt et al., 1983) and neurotensin (Sawchenko et al., 1984a) have been colocalized with CRF in subsets of neurons in the parvocellular division of the PVH in colchicine-treated animals, and the response of these staining patterns provides a direct indication as to the specificity of the ADX effect on CRF and vasopressin immunoreactivity. The results are shown in Figure 3 and Table 2.

In untreated rats, few, if any, CRF-, neurotensin-, or met-enkephalin-stained neurons were seen in the PVH, while large, vasopressin-immunoreactive neurons centered in, but not restricted to, the magnocellular division of the nucleus were prominent. Pretreatment with colchicine resulted in the appearance of sizable populations of CRF-, neurotensin-, and enkephalin-immunoreactive neurons in the parvocellular division, and sequential double-immunohistochemical staining in selected cases confirmed the presence of neurotensin and met-enkephalin immunoreactivity in respectively smaller (<20%) and larger (>60%) subpopulations of CRF-positive cells in the parvocellular division of the PVH. Vasopressin staining was not discernibly altered after colchicine treatment, and only widely scattered cells (0–20 per brain; Sawchenko et al., 1984a) in the parvocellular division of the PVH were found to stain positively for both CRF and vasopressin immunoreactivity. After ADX, the enhancement of CRF immunoreactivity in cells concentrated in the medial parvocellular part of the PVH was again apparent, as was the appearance of vasopressin immunoreactivity in a great majority (>70%) of these neurons. In adjoining series of sections from the same brains in which these observations were made, no discernible change in the number or distribution of either neurotensin or met-enkephalin immunoreactivity was noted.

*Hypophysectomy*

In agreement with previous findings (Merchenthaler et al., 1983b; Paull and Gibbs, 1983), hypophysectomy resulted in an augmentation of the number and staining intensity of CRF-immunoreactive neurons in the parvocellular division of the PVH, as compared to unoperated littermate controls. This effect was apparent, and at least qualitatively similar, in parts of animals killed at 7, 14, 31, and 59 d after hypophysectomy. It should be noted that 1 antiserum (C24) also revealed CRF staining in a subset of *magnocellular* vasopressinergic neurosecretory neu-

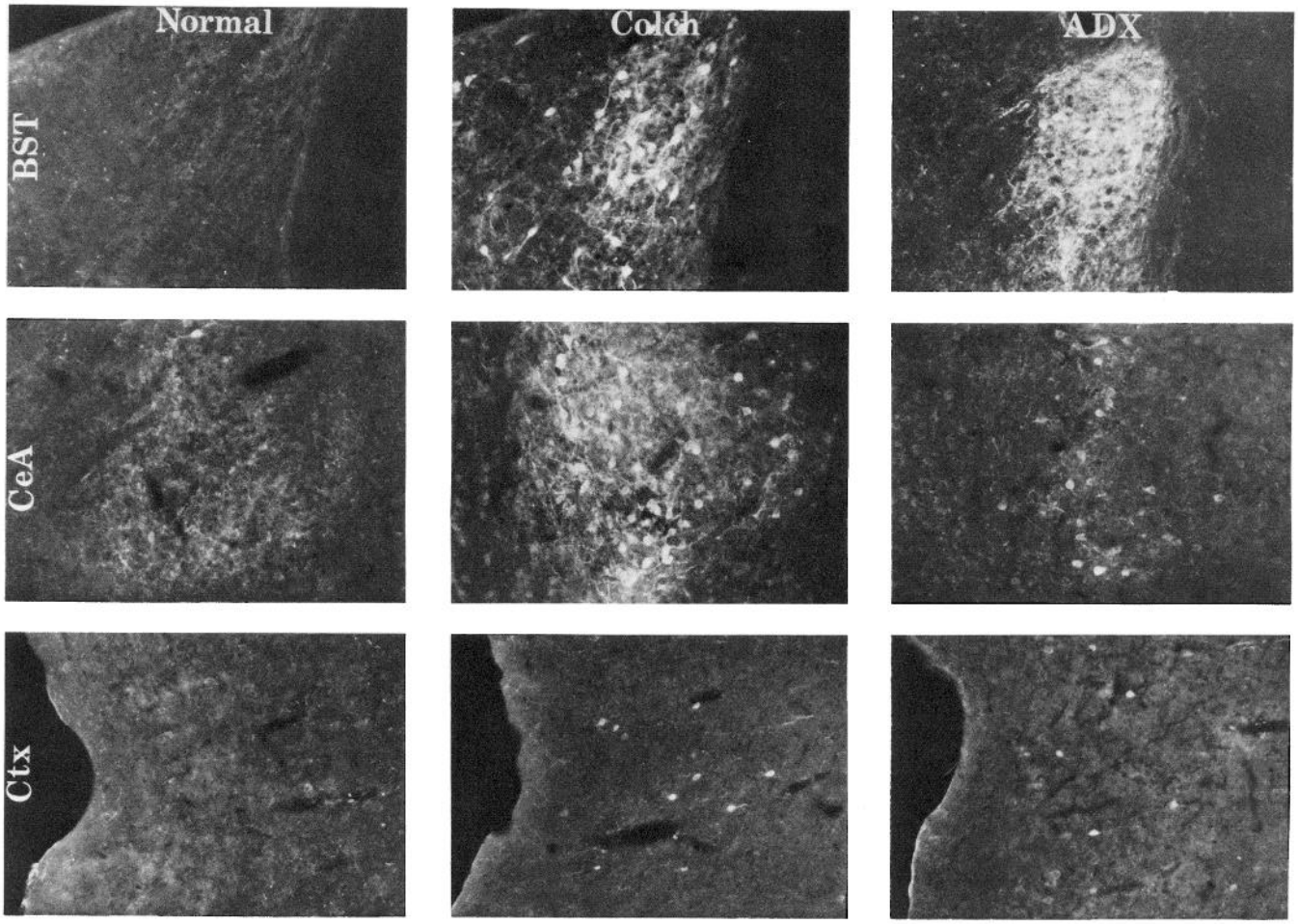


*Figure 2.* Distribution of CRF immunoreactivity in comparable regions of the bed nucleus of the stria terminalis (*BST*), central nucleus of the amygdala (*CeA*), and the perirhinal part of the cerebral cortex (*Ctx*) in *Normal,* colchicine-treated (*Colch*), and *ADX* rats. CRF immunostaining in each of these areas is enhanced after ADX (compared to normal controls), but vasopressin immunoreactivity was never colocalized in these neurons. All micrographs, ×80.

rons of hypophysectomized rats (see Fig. 4), but this was not abolished by preincubation of the antiserum with concentrations of synthetic ovine CRF sufficient to block immunostaining in the parvocellular division, and it was not apparent using antisera C30 or C70.

Like ADX, hypophysectomy also resulted in the appearance of vasopressin immunoreactivity in cells in the parvocellular division of the PVH, where extensive colocalization within CRF-stained neurons was again demonstrated. Although direct comparisons of matched ADX versus hypophysectomized rats have not been undertaken, both CRF and vasopressin staining in the parvocellular divsion were reliably more robust after hypophysectomy than is typically seen after ADX. Cell counts in a hypophysectomized rat killed 7 d after surgery (the same interval we have typically used after ADX; cf. Table 1) revealed 1056 CRF- and 1040 vasopressin-immunoreactive neurons in the parvocellular division, with 84% of the CRF-stained neurons also showing vasopressin immunoreactivity.

*Steroid specificity*

Pronounced differences were observed in the efficacy of various adrenal steroid replacement regimens in antagonizing the effect of ADX on CRF and vasopressin immunoreactivity in the parvocellular neurosecretory system. The paradigm employed here involved concurrent ADX surgery and subcutaneous implantation of steroid- or vehicle-filled osmotic minipumps for 1 week prior to perfusion. Two doses of each of 4 steroids were used, and each group consisted finally of 3–7 rats. Counts of cells in the parvocellular division of the PVH that stained positively for CRF, vasopressin, or for both peptides, obtained from 1 representative animal of each group, are given in Table 3.

Rats administered only the ethanol–ethylene glycol vehicle ($n = 7$) all showed the ADX-induced enhancement of CRF and vasopressin staining that was fully comparable to that described

**Table 2. Mean (±SEM) number of cells in the parvocellular division of the PVH stained for peptide immunoreactivity in untreated, colchicine-treated, or adrenalectomized rats[a]**

| | CRF | Vasopressin | Neurotensin | Met-Enkephalin |
|---|---|---|---|---|
| Untreated | 58 ± 12 | 109 ± 24 | 0 ± 0 | 37 ± 6 |
| Colchicine-treated | 1010 ± 59 | 119 ± 9 | 752 ± 27 | 956 ± 89 |
| ADX | 905 ± 64 | 687 ± 84 | 0 ± 0 | 21 ± 12 |

[a] Counts reflect values obtained from adjacent series of sections from the brains of 3 rats of each type.

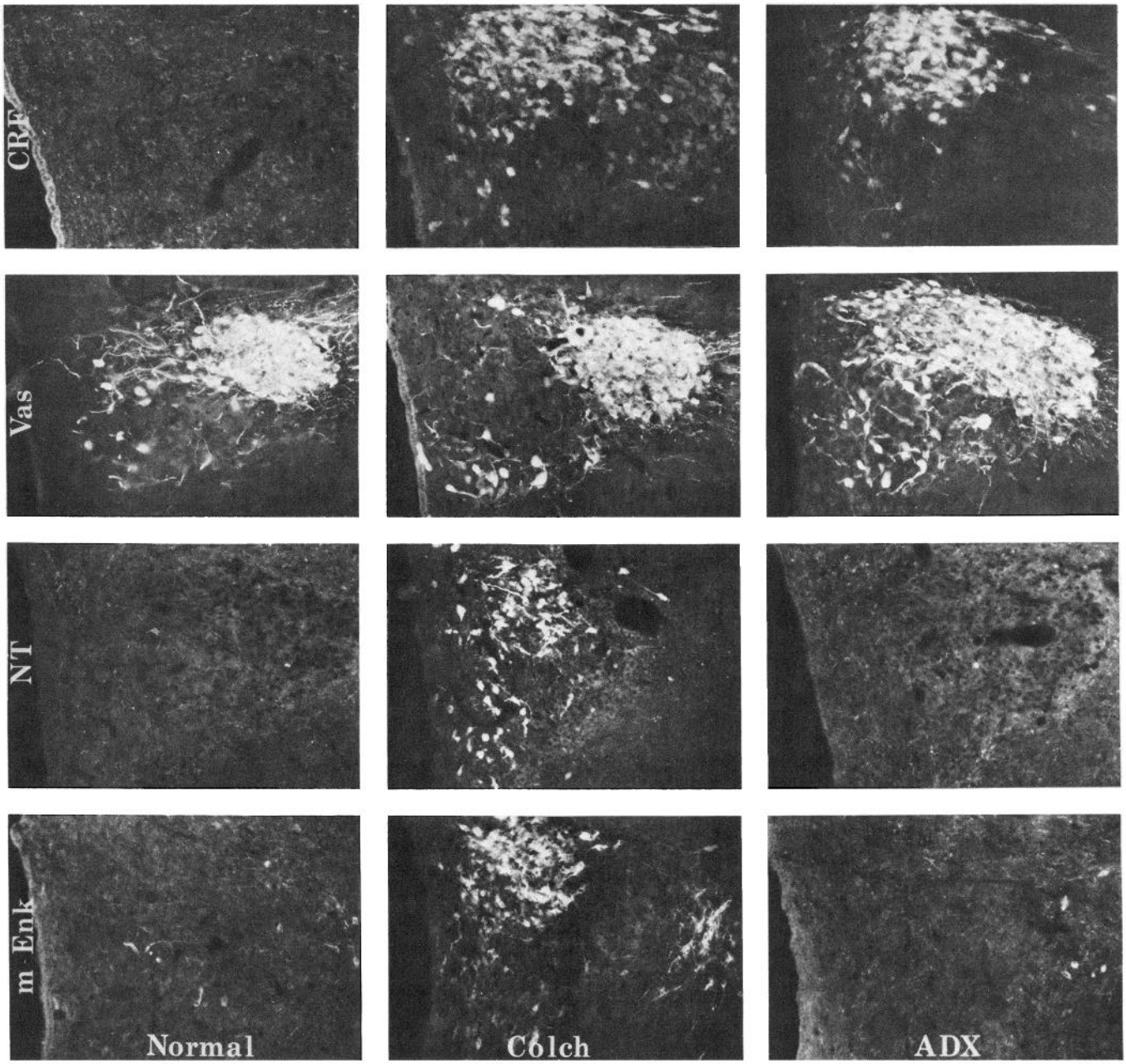


*Figure 3.* Comparison of the distribution of CRF, vasopressin (*Vas*), met-enkephalin (*m-Enk*), and neurotensin (*NT*) immunoreactivity at a similar level of the PVH in *Normal,* colchicine-treated (*Colch*), and *ADX* rats. In the untreated rat, only magnocellular Vas-immunoreactive-ir cells are prominent. Colchicine treatment reveals the presence of CRF, m-Enk, and NT immunoreactivity cells in the parvocellular division of the PVH. Colocalization studies have indicated that m-Enk and NT immunoreactivity coexist with CRF in subsets of parvocellular neurons. ADX enhances CRF and Vas immunoreactivity in parvocellular neurons, while staining for m-Enk and NT is not visibly affected. The effect of ADX appears to be specific to CRF and vasopressin, which alone among the peptides in question are known to stimulate ACTH secretion directly. All micrographs, ×80.

previously (see Fig. 5). In contrast, lower doses ($n = 4$) of dexamethasone attenuated, and higher doses ($n = 6$) virtually abolished the characteristic effects of ADX on both CRF and vasopressin immunoreactivity in the cell group in question. In each experiment, immunoreactivity for both peptides responded similarly to steroid treatment, though staining for vasopressin in the parvocellular division of the PVH, typically less robust than that for CRF, proved more sensitive to dexamethasone treatment. It should be noted that the great majority of the vasopressin-stained neurons that remained in the parvocellular division of the PVH of dexamethasone-treated ADX rats (see Table 3) were large (20–30 μm in diameter) and presumably corresponded to scattered magnocellular neurosecretory neurons interspersed throughout the parvocellular division (cf. Table 1, Rhodes et al., 1981, and Sawchenko and Swanson, 1982).

Consistent with the results regarding the cellular localization of the peptides, dexamethasone treatment also resulted in a corresponding decrement in the ADX-induced enhancement of CRF and vasopressin immunoreactivity in fibers in the external lamina of the median eminence. This was manifested as a re-

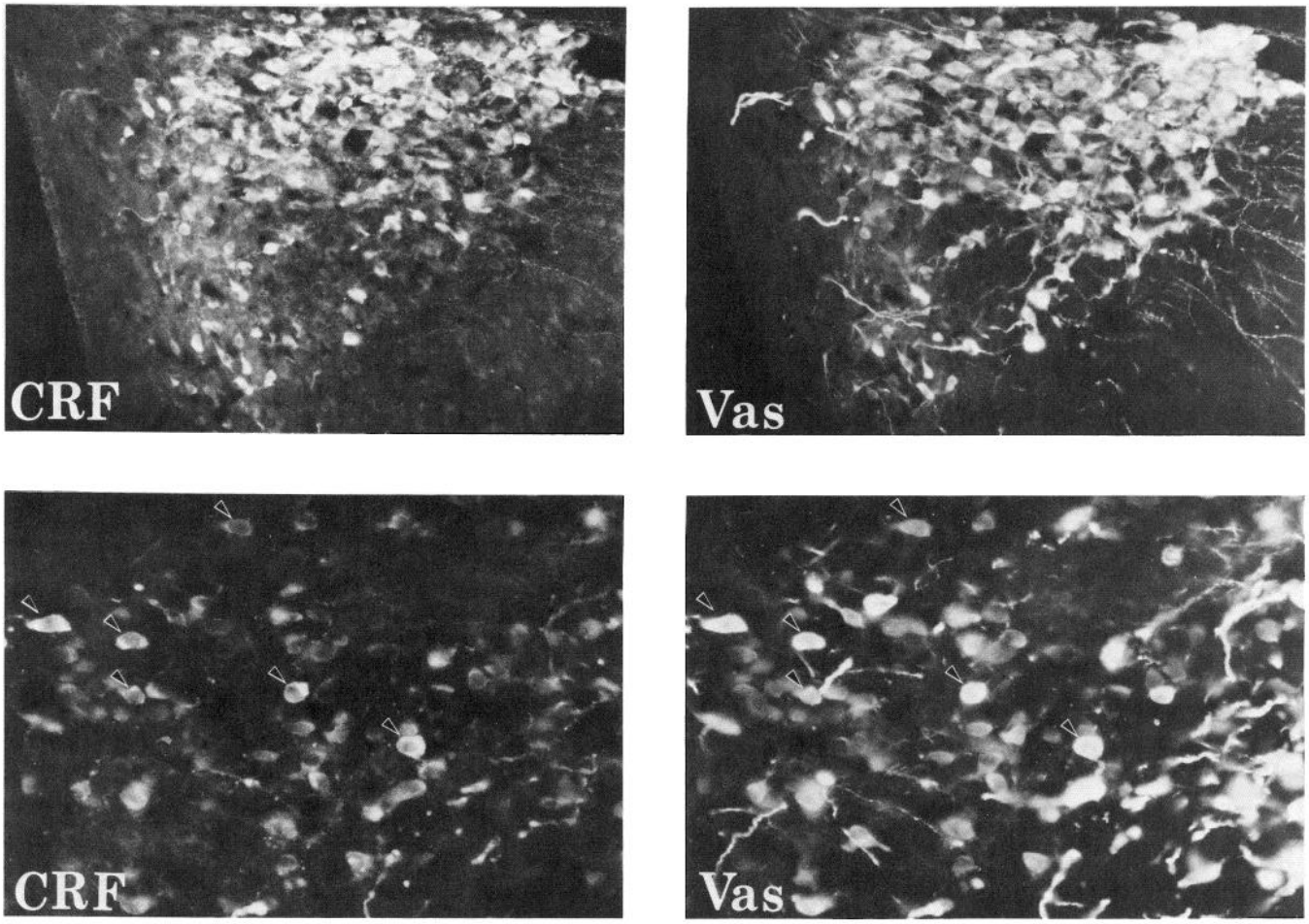


*Figure 4.* *Top,* Distribution of CRF- and vasopressin (*Vas*)-immunoreactive neurons in a doubly stained section through the PVH of an hypophysectomized rat. Like ADX, hypophysectomy results in an enhancement of both CRF and Vas immunostaining in the parvocellular division of the PVH. Apparent CRF immunostaining of some magnocellular neurons was not abolished by preadsorption of anti-CRF with synthetic ovine CRF, and is therefore not specific. Both micrographs, ×125. *Bottom,* Higher-power view of CRF and Vas immunoreactivity in a doubly stained section through the PVH of an hypophysectomized rat. Many doubly stained neurons (*arrows*) are apparent in the parvocellular division of the PVH (*left side* of each photomicrograph), while larger (magnocellular) neurons at the *right* are Vas-immunoreactive only. The enhancement of both CRF and Vas immunoreactivity in parvocellular neurosecretory neurons after hypophysectomy or ADX implicates alterations in adrenal steroid levels as primary mediators of these effects. Both micrographs, ×250.

duction in the intensity of CRF-stained terminals and, especially in those rats receiving higher doses of dexamethasone, a virtual disappearance of vasopressin-stained fibers in the external zone (Fig. 6).

Similar, though less pronounced, effects were observed in animals treated with corticosterone, the principal endogenous glucocorticoid in the rat. Attenuation of the ADX effect on both peptides was apparent after treatment with lower ($n$ = 4) or higher ($n$ = 6) doses of corticosterone, but never was a complete abolition of the responses observed (Fig. 7). Again, CRF and vasopressin immunoreactivity in the parvocellular division of the PVH responded in tandem in these replacement experiments, with vasopressin staining proving consistently more sensitive to the steroids.

Deoxycorticosterone, the steroid thought to be the major endogenous mineralocorticoid in rat, but which possesses signif-

**Table 3. Number of CRF- and vasopressin-immunoreactive neurons in the parvocellular division of the PVH in ADX rats treated with various corticosteroid replacement regimens[a]**

| | | Dexamethasone | | Corticosterone | | Deoxycorti-costerone | | Aldosterone | |
|---|---|---|---|---|---|---|---|---|---|
| | Vehicle | Low dose[b] | High dose[b] | Low dose | High dose | Low dose | High dose | Low dose | High dose |
| CRF | 914 | 148 | 32 | 437 | 324 | 824 | 648 | 864 | 956 |
| Vasopressin | 704 | 164 | 103 | 346 | 146 | 646 | 486 | 732 | 763 |

[a] Counts are from a single animal judged to be representative of each treatment group.

[b] Lower dose was estimated to be 20 μg/100 gm body wt/d; higher dose was 50 μg/100 gm body wt/d.

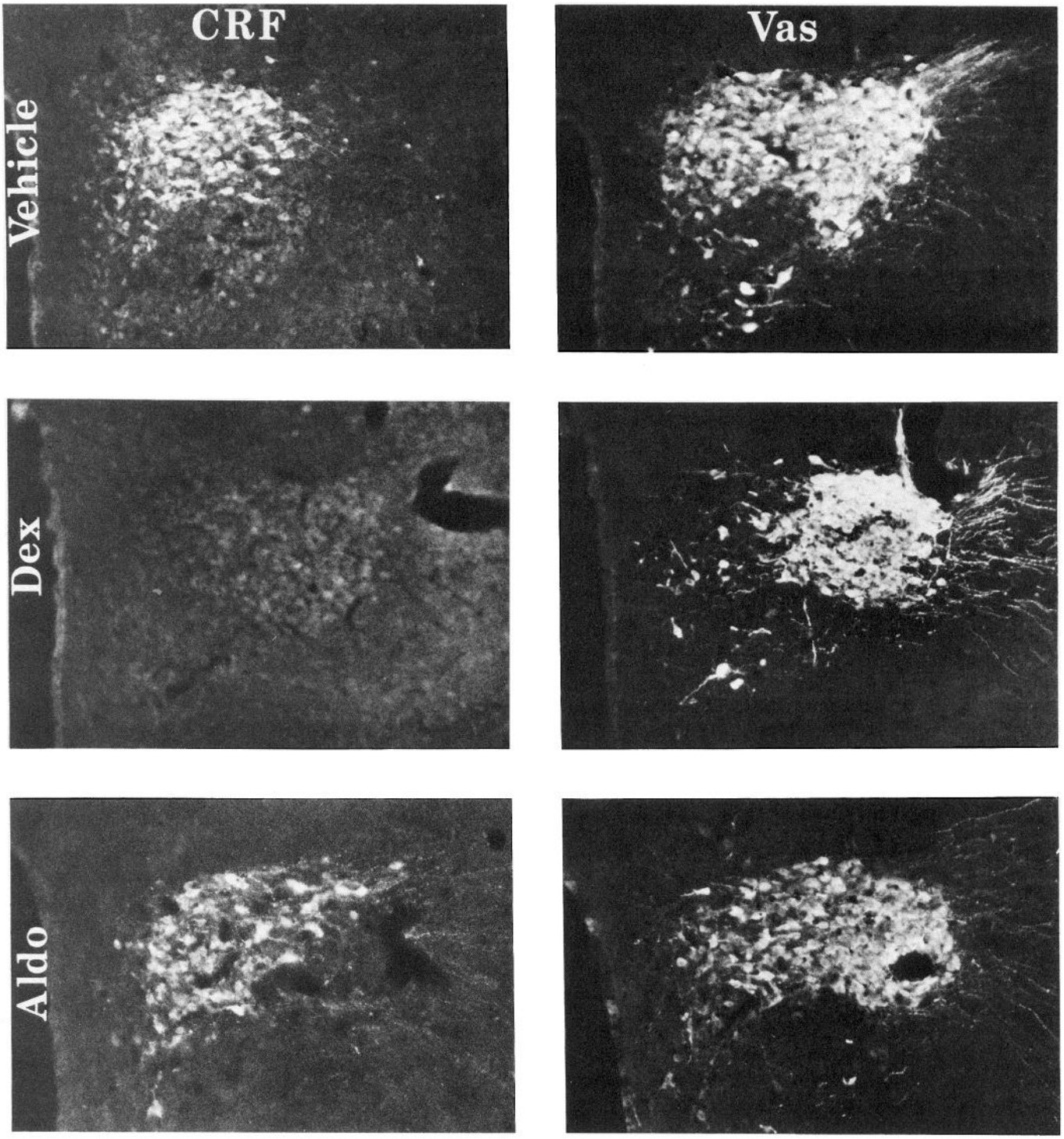


*Figure 5.* Adjacent sections through the PVH stained for CRF and vasopressin (*Vas*) immunoreactivity from ADX rats treated via osmotic minipumps with either a *vehicle* (control), a higher dose of dexamethasone (*Dex,* a synthetic glucocorticoid), or a similar dose of aldosterone (*Aldo*). At these dosage levels, replacement with Dex virtually abolished the ADX enhancement of both CRF and Vas immunoreactivity in the parvocellular division of the PVH (note that Vas-immunoreactive magnocellular neurons are still prominently stained). In contrast, replacement with Aldo had virtually no impact on the response to ADX. The potency of steroids in antagonizing the ADX-dependent enhancement of both CRF and Vas staining was, in descending order, dexamethasone, corticosterone, deoxycorticosterone, aldosterone. All micrographs, ×100.

icant glucocorticoid activity, produced no consistent decrement in CRF or vasopressin staining at lower doses ($n = 3$). Higher doses ($n = 5$) produced, at best, a mildly blunted response (see Fig. 5), in which substantial numbers of cells staining for both peptides remained detectable. Neither lower ($n = 4$) nor higher ($n = 5$) doses of aldosterone, a relatively pure mineralocorticoid, produced any discernible decrement in CRF or vasopressin staining in any aspect of the parvocellular neurosecretory system.

## Discussion

The principal findings of the present study may be summarized as follows: Previous reports of enhanced CRF and vasopressin immunoreactivity in a common population of parvocellular

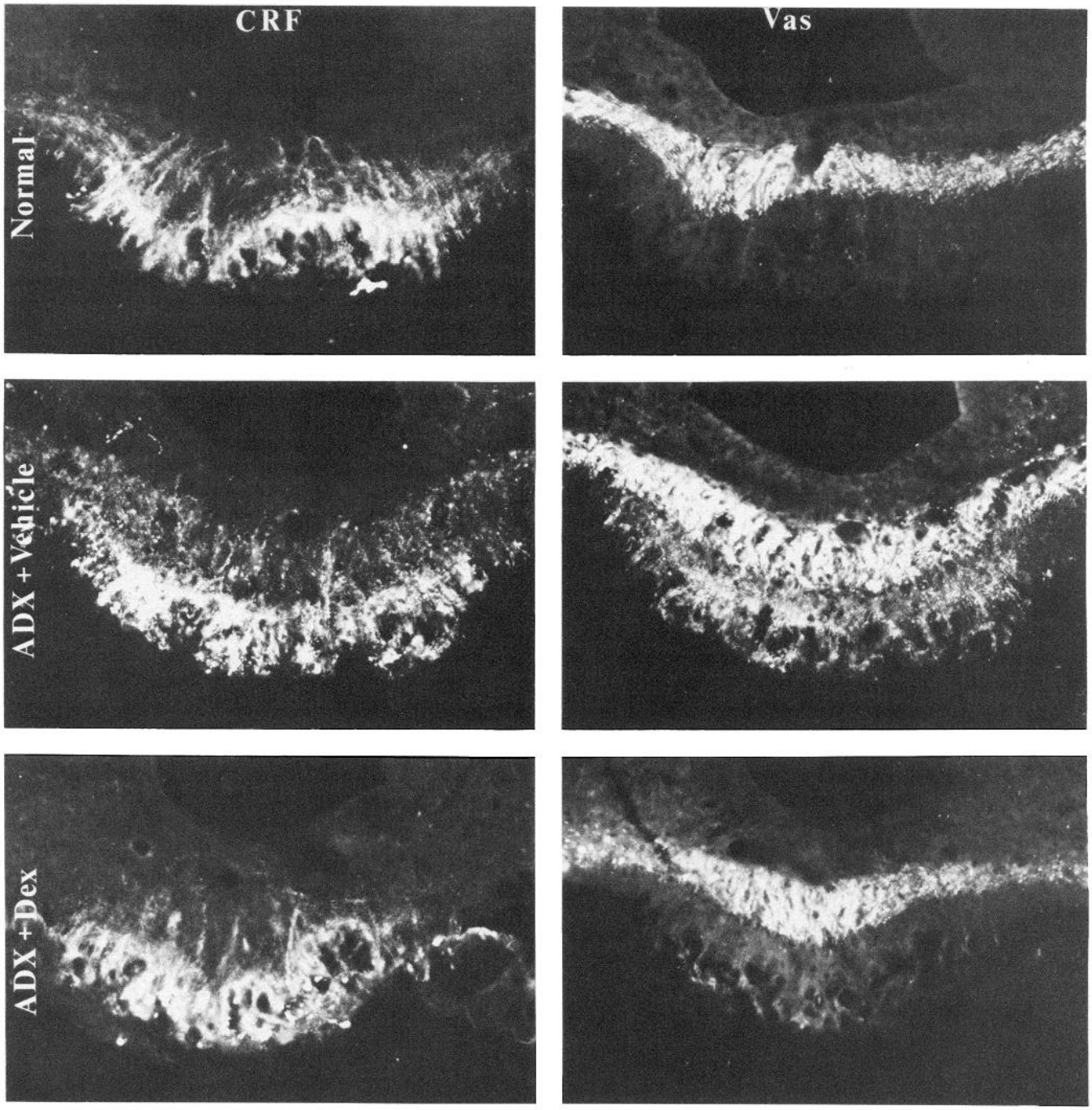


*Figure 6.* Adjacent sections through the median eminence of untreated (*Normal*), *vehicle-treated ADX,* and *dexamethasone-treated ADX* rats, stained for CRF or vasopressin. In the normal rat, CRF-immunoreactive fibers are concentrated in the external lamina of the median eminence, while the preterminal axons of vasopressin-immunoreactive neurons course through the internal lamina; only very few vasopressin-stained fibers in the external lamina are evident. ADX results in an enhancement in the staining intensity of CRF-immunoreactive fibers and in the appearance of a far more widespread and prominent vasopressinergic plexus in the external lamina. Treatment with dexamethasone through the duration of the 7 d period after ADX results in staining patterns that resemble those of the intact rat. All micrographs, ×125.

neurosecretory neurons in the PVH after ADX (Tramu et al., 1983; Kiss et al., 1984b; Sawchenko et al., 1984b) were repeatedly confirmed and localized primarily to one particular (medial parvocellular) subdivision of the nucleus. This response was at least somewhat specific to these peptides, as ADX produced no augmentation of staining for 2 other peptides (met-enkephalin and neurotensin) that have previously been colocalized in subsets of parvocellular CRF-immunoreactive cells of colchicine-treated rats (Hökfelt et al., 1983; Sawchenko et al., 1984a). Similarly, the response also appears to have been specific to a particular cell type. While enhanced CRF immunostaining was apparent in several forebrain regions beyond the PVH after ADX, not all cell groups in which CRF immunoreactivity has been described in colchicine-treated rats responded in this way, and vasopressin immunoreactivity was never evident in those that did. Information on the regulatory agents controlling the expression of CRF and vasopressin in parvocellular neurosecretory neurons was also obtained. Hypophy-

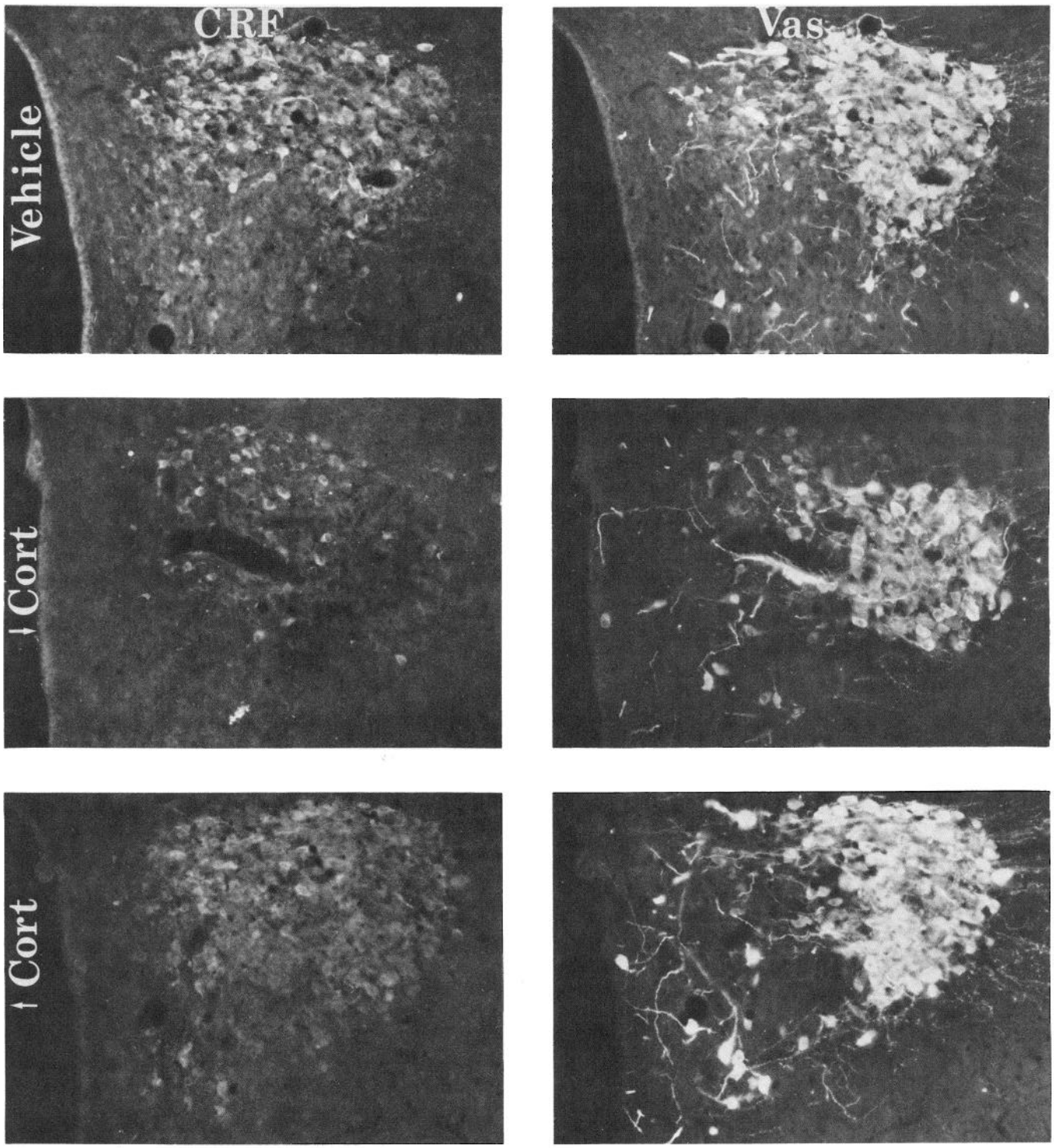


*Figure 7.* Individual sections through the PVH of ADX-steroid treated rats stained concurrently for CRF and vasopressin (*Vas*) immunoreactivity. *Top,* ADX–*vehicle*-treated control. Enhancement and coexpression of CRF and Vas immunoreactivity in parvocellular neurons is again apparent, although the monoclonal anti-Vas tends to stain parvocellular neurons less robustly than the polyclonal antiserum used in other phases of the study. *Middle,* ADX rat treated with lower replacement doses of corticosterone (*Cort*). A clear decrease in the number of parvocellular CRF- and Vas-stained cells is apparent, though some doubly stained cells can still be resolved. *Bottom,* ADX rat treated with high dose of corticosterone. Still fewer, and less brightly stained, CRF- and Vas-stained cells are visible. All micrographs, ×125.

sectomy produced an enhancement of CRF and vasopressin immunoreactivity that was qualitatively similar, and typically even more robust, than that seen after ADX. Such results would suggest a primary role for adrenal steroids as regulators of peptide expression in this system, and a secondary influence of ACTH. Finally, systemic steroid replacement studies identified glucocorticoids, especially dexamethasone, as most potent in mitigating the CRF and vasopressin immunoreactivity to ADX.

### *Methodological considerations*

Immunohistochemical techniques were used to evaluate alterations in peptide expression in the present study. Evidence for

the specificity of the localizations reported here was provided. Results with CRF were obtained using both C- and N-terminally directed antisera against ovine CRF, and localizations of vasopressin immunoreactivity were consistent using either a monoclonal antibody or a polyclonal antiserum. Staining for either peptide was blocked by preincubation of each antiserum with the homologous synthetic peptide, and colocalization results obtained in sequential double-staining experiments were consistent despite adsorption of each antiserum with high concentrations of the heterologous peptide. Further evidence of the authenticity of the vasopressin-immunoreactive peptide expressed by parvocellular neurosecretory neurons may be inferred from our failure to detect vasopressin immunoreactivity in the parvocellular division of the PVH in 3 adrenalectomized, homozygous Brattleboro rats, which nonetheless did manifest enhanced CRF immunostaining, relative to untreated controls of the parent (Long-Evans) strain (P. E. Sawchenko, unpublished observations). In addition, recent *in situ* hybridization experiments, using a synthetic cDNA oligonucleotide probe to a portion of the prohormone for vasopressin, have revealed a broadening of the distribution of vasopressin mRNA in the PVH after ADX and the colocalization of this mRNA and CRF immunoreactivity in individual neurons (Wolfson et al., 1985). Collectively, these observations support the specificity of the basic phenomenon being considered.

Neither immunohistochemistry nor, at its current state of development, *in situ* hybridization provides an absolute assay for the presence of any given peptide or its mRNA in individual neurons. While it might appear that withdrawal of adrenal steroids results in a qualitative "unmasking" of vasopressin expression by parvocellular neurosecretory neurons, the absolute levels of vasopressin gene transcription and translation and of peptide secretion under more normal conditions remain to be determined. On one hand, the failure to detect vasopressin immunostaining in the parvocellular division of the PVH after colchicine treatment, a procedure proven effective in enhancing staining for CRF and other neuropeptides in these neurons, supports, at least, a pronounced quantitative difference in the levels at which vasopressin is normally produced by parvocellular neurons. On the other hand, vasopressin has been detected in relatively high concentrations in the hypophyseal portal circulation of normal and stressed rats (e.g., Gibbs, 1985; Plotsky, 1985).

*Anatomic specificity*

The locus at which CRF and vasopressin immunoreactivity may be jointly expressed is quite restricted. The some 1000 cells on each side of the brain that we typically observe in the PVH of the ADX rat are overwhelmingly concentrated in the medial parvocellular subdivision of the nucleus, as defined by Swanson and Kuypers (1980). Nearly double this number have been reported in colchicine-treated animals (Swanson et al., 1983). While it is not certain whether longer post-ADX survival periods would produce a more robust response, some degree of selectivity seems obvious. Thus, ADX failed to enhance CRF immunostaining in magnocellular neurosecretory neurons (see also Kiss et al., 1984b; Sawchenko et al., 1984b) in which CRF–oxytocin colocalization has been demonstrated in colchicine-treated rats (Burlet et al., 1983; Sawchenko et al., 1984a). Similarly, ADX failed to produce any marked effect on CRF immunostaining in the anterior part of the parvocellular division of the PVH despite the fact that cells in this region also appear to project to the median eminence (Lechan et al., 1980; Wiegand and Price, 1980).

Outside the PVH, ADX also enhanced CRF immunostaining in at least 3 additional forebrain regions: the bed nucleus of the stria terminalis, the central nucleus of the amygdala, and the cerebral cortex (see also Merchenthaler et al., 1983b; Paull and Gibbs, 1983). This was in contrast to our previous analysis (Swanson et al., 1983), which found the effect to be limited to the PVH. Moreover, none of the previous analyses examined possible ADX effects on vasopressin immunoreactivity in extrahypothalamic regions that displayed CRF staining. CRF-immunoreactive cell groups in the brain stem were not examined in the present study, though 1 group has shown an enhancement of CRF immunostaining in the spinal cord following hypophysectomy (Merchenthaler et al., 1983b). A number of forebrain cell groups in which CRF immunostaining was readily demonstrable in colchicine-treated rats (e.g., medial preoptic area, lateral hypothalamic area, substantia innominata) failed to show enhanced staining after ADX, though, again, more subtle responses could have escaped detection. For purposes of the present discussion, the important observations are that the influence of ADX on CRF immunoreactivity was clearly detected in at least some extrahypothalamic cell groups, and that none of these expressed vasopressin immunoreactivity. This is especially pertinent in light of the fact that novel, *gonadal* steroid-dependent sites of vasopressin expression in the bed nucleus and amygdala have recently been reported (e.g., DeVries et al., 1985). Thus, the appearance of vasopressin immunoreactivity does not appear to be inextricably linked to that of the heightened CRF immunoreactivity that follows ADX.

*Peptide specificity*

Six additional neuropeptides have now been reported to coexist with CRF in subsets of parvocellular neurosecretory neurons. Apart from vasopressin, these include enkephalin, PHI (Hökfelt et al., 1983), neurotensin (Sawchenko et al., 1984a), angiotensin II (Lind et al., 1984), and cholecystokinin (Mezey et al., 1985). Evidence presented here and elsewhere suggests that at least some of these are differentially regulated. Comparison of staining patterns for 2 peptides (CRF, vasopressin) that are recognized as secretagogs for ACTH, and of 2 that are not (enkephalin, neurotensin) in normal, colchicine-treated, and ADX rats indicates that while all but vasopressin may be readily demonstrated in the parvocellular division of the PVH after colchicine treatment, only CRF and vasopressin show marked enhancement after ADX. This result argues strongly that the joint response of CRF and vasopressin immunoreactivity in parvocellular neurosecretory neurons is not a reflection of some global metabolic disturbance resulting from ADX, but rather a specific response that is physiologically appropriate and in keeping with the functional association of the 2 peptides.

In line with this view are the recent results of Lind et al. (1984), who demonstrated extensive ADX-dependent colocalization of angiotensin II immunoreactivity with CRF and vasopressin immunoreactivity in parvocellular neurosecretory neurons. Angiotensin II is also generally recognized as an ACTH secretagog (Gaillard et al., 1981; Sobel and Vagnucci, 1982), albeit a relatively weak one. Pretreatment with colchicine *and* ADX was required to demonstrate angiotensin II immunoreactivity in these neurons, but colchicine treatment alone was ineffective in this regard.

One potential challenge to the view that the expression of

corticotropin secretagogs is selectively enhanced by ADX has been posed by recent studies on the role of cholecystokinin (CCK) in the parvocellular neurosecretory system. CCK immunoreactivity has been colocalized with CRF in individual neurons in the parvocellular division of the PVH (Mezey et al., 1985). Evidence for a direct stimulatory action on anterior pituitary corticotropes has been provided (see Mezey et al., 1985), but others have argued for a central site of action (e.g., Itoh et al., 1979; Porter and Sander, 1981). Recent studies in this laboratory have consistently failed to demonstrate any ADX-induced enhancement of CCK immunoreactivity in experiments in which pronounced effects on CRF and vasopressin staining in the parvocellular division of the PVH were obvious (P. E. Sawchenko, unpublished observations). Additional study is clearly required to clarify the role of, and regulatory influences on, CCK in this system.

*Steroid specificity*

The fact that ADX and hypophysectomy produced qualitatively similar effects on CRF and vasopressin immunoreactivity suggests that adrenal steroids are principal regulators of peptide expression in these neurons. It is worthy of note, however, that staining for both peptides, and especially vasopressin, was particularly robust after hypophysectomy, a situation reflected in the fact that cell counts in 1 animal killed 7 d after hypophysectomy (the same interval we have used after ADX) revealed a greater number of vasopressin-stained cells in the parvocellular division of the PVH and a greater percentage of CRF-stained neurons manifesting vasopressin immunoreactivity than we have ever counted after ADX. This would suggest that ACTH may play a regulatory role in this system, since the so-called "short-loop" negative feedback effect of ACTH (see Yates and Maran, 1974) would be eliminated by hypophysectomy but not ADX. It is worthy of mention in this context that the PVH is known to receive an ACTH-immunoreactive input from the intracerebral pro-opiomelanocortin system. Light-microscopic analyses have suggested that the region of the PVH in which CRF-stained neurons are massed is only sparsely laden with ACTH-stained varicosities (Sawchenko et al, 1982; Kiss et al., 1984a). Until this analysis is extended to the ultrastructural level, a potential role for the central ACTH system in influencing peptide dynamics in the PVH will remain open to question.

Systemic steroid replacement experiments revealed that glucocorticoids were far more potent than mineralocorticoids in combating the effect of ADX, and that both CRF and vasopressin immunoreactivity appeared to be regulated similarly by adrenal steroids. The synthetic glucocorticoid dexamethasone proved more potent in this regard than the principal native glucocorticoid in rat, corticosterone. A number of factors may account for this difference, including the generally more pure glucocorticoid properties of dexamethasone, its longer half-life in blood, its lesser propensity to be bound by transcortin (a circulating corticosteroid-binding protein), and its lower rate of dissociation from receptors (see Meyer, 1985, for a review).

In contrast, replacement with aldosterone, a relatively pure and potent mineralocorticoid, had no discernible impact on peptide expression in parvocellular neurosecretory neurons. Deoxycorticosterone, even at higher doses, only minimally inhibited ADX effects on CRF and vasopressin immunoreactivity. While deoxycorticosterone is thought to be the principal endogenous mineralocorticoid in rat (Melby et al., 1972), its potency has been estimated to be 30–70-fold less than that of aldosterone (Gomer-Sanchez et al., 1976), and it does possess significant glucocorticoid activity.

These results are consistent with those of Stillman et al. (1977), who showed the ADX-induced enhancement of vasopressinergic fibers in external lamina to be suppressible by dexamethasone treatment, and of Silverman and colleagues (1981), who showed corticosterone to be more potent than deoxycorticosterone in similar experiments. Recent reports regarding the impact of dexamethasone treatment on CRF immunostaining in the parvocellular neurosecretory system have provided less consistent results. One group has described a dexamethasone-reversible decrement in CRF immunoreactivity in the median eminence 7 d postoperatively (Schipper et al., 1984), a result at odds with those reported here and by others (Bugnon et al., 1983; Merchenthaler et al., 1983a, b; Paull and Gibbs, 1983; Swanson et al., 1983). Another (Paull and Gibbs, 1983) has found that dexamethasone treatment actually enhances CRF immunostaining in the intact rat, and fails to attenuate the enhancement of CRF immunoreactivity in the long-term ADX rat. The results in normal animals are most readily explained by a dexamethasone-induced blockade of CRF release from terminals in the median eminence (Edwardson and Bennett, 1974), resulting in an accumulation of peptide in the cell body. Moreover, considerable time (30 d) elapsed between adrenalectomy and the initiation of replacement therapy, which was in force for only 24 hr. This interval might well be interpreted as allowing time for an accumulation of peptide prior to the onset of steroid treatment, which could then be expected to block peptide release and thereby mask any inhibitory effect of dexamethasone on peptide synthesis. The strategy adopted in the replacement experiments described here was to initiate replacement therapy at the same time as adrenalectomy, thereby pitting the 2 manipulations against one another concurrently, and avoiding the potentially confounding influences of multiple sites and modes of action of corticosteroids in this system.

Thus, aspects of the present results are consistent, or can be reconciled with, those of most previous studies, but none of these studies have compared the effects of manipulation of the steroid environment on both CRF and vasopressin immunoreactivity in cell bodies in the PVH and their terminal fields in the median eminence. Our results suggest that both peptides are regulated similarly by adrenal steroids, though it remains to be determined whether our impression that there is a more quantitative modulation of CRF immunoreactivity, as opposed to a seemingly qualitative masking and unmasking of vasopressin immunoreactivity, holds any validity. No regulatory influence has yet been described that differentially affects these 2 agents in parvocellular neurons of the PVH.

*Conclusions*

The results described here provide further characterization, and help to establish the specificity, of a seemingly novel form of regulation of neuropeptide expression in the rat hypothalamus. Subsets of CRF-containing parvocellular neurosecretory neurons appear capable of expressing at least 6 additional peptides; 3 of these (CRF, vasopressin, angiotensin II) share a functional association as stimulators of corticotropin secretion and appear to be regulated by circulating adrenal steroids. A variety of effects on various aspects of anterior pituitary function, generally exerted at or above the level of the median eminence, have been

attributed to the other peptides (e.g., Meites et al., 1979; Frohman et al., 1982; McCann et al., 1982). The precise roles of enkephalin, neurotensin, PHI, and CCK of paraventricular origin in any aspect of anterior pituitary function remain to be elucidated, as do the identities of regulatory agents that might play upon these peptides. In light of their well-established and readily assayable physiologic role as initiators of the stress response, it seems clear that this population of parvocellular neurosecretory neurons will provide a useful model system for understanding the overall organizational economy of the hypothalamus and for providing at least one framework in which the significance of the apparently widespread, but often puzzling, phenomenon of peptide colocalization may be approached in a meaningful functional context.

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