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# CORTICOTROPHIN-RELEASING FACTOR, VASOPRESSIN AND PRO-OPIOMELANOCORTIN mRNA RESPONSES TO STRESS AND OPIATES IN THE RAT

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SUMMARY

1. Rats underwent either: (1) acute or chronic morphine or naloxone administration; (2) simple morphine withdrawal or naloxone-precipitated withdrawal in morphine-dependent animals; or (3) stress from I.P. administration of hypertonic saline.

2. Quantitative *in situ* hybridization histochemistry was performed using synthetic oligonucleotide probes for corticotrophin-releasing factor (CRF), vasopressin, pro-opiomelanocortin (POMC), dynorphin, enkephalin and oxytocin mRNAs. The paraventricular and supraoptic nuclei were examined in all studies and the arcuate nucleus and pituitary gland in the acute withdrawal study.

3. Neither acute nor chronic morphine administration altered either (a) hypothalamic parvocellular or magnocellular CRF mRNA, or (b) anterior pituitary or pars intermedia POMC mRNA.

4. Naloxone-precipitated morphine withdrawal resulted in a marked increase in parvocellular (but not magnocellular) CRF mRNA within 4 h and levels remained elevated through 24 h. There was no change in arcuate nucleus or pars intermedia POMC mRNA, but in the anterior pituitary there was a delayed increase, significant at 24 h.

5. Simple morphine withdrawal without the use of naloxone did not result in any change in CRF mRNA but there were increases in magnocellular vasopressin and dynorphin mRNA, presumably related to decreased water intake.

6. Intraperitoneal hypertonic saline stress also resulted in a marked accumulation of both parvocellular CRF and vasopressin mRNA without any concomitant change in magnocellular vasopressin mRNA. Increased translation of CRF mRNA was also evidenced by increased immunoreactive CRF detected by immunocytochemistry.

## INTRODUCTION

Corticotrophin-releasing factor (CRF) is produced in the parvocellular cells of the paraventricular nucleus (PVN) of the hypothalamus (Antoni, Palkovits, Makara,

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Linton, Lowry & Kiss, 1983; Swanson, Sawchenko, Rivier & Vale, 1983) and is now well established as having a major role in the control of the release of adrenocorticotrophin (ACTH) and other pro-opiomelanocortin (POMC)-related peptides by anterior pituitary corticotrophs. Adrenalectomy results both in increased CRF immunostaining and CRF mRNA levels (Jingami, Matsukura, Numa & Imura, 1985; Young, Mezey & Siegel, 1986) in the PVN and POMC mRNA in the pituitary gland (Jingami *et al.* 1985), which can both be reversed by replacement with glucocorticoids. The response of this system to stress-induced activation of the hypothalamo-pituitary–adrenal axis has not been defined. Chronic intermittent stress results in elevated POMC mRNA in the anterior pituitary (Hollt, Przewlocki, Haarmann, Almeida, Kley, Millan & Herz, 1986), but evidence for stress-induced changes in CRF activity is less clear. Portal plasma CRF concentrations have been reported to be elevated after haemorrhage (Plotsky & Vale, 1984) but do not change following the powerful stimulus to ACTH secretion of insulin-induced hypoglycaemia (Plotsky, Bruhn & Vale, 1985) or during hypothermia-induced inhibition of ACTH (Gibbs, 1985*b*).

Arginine vasopressin also has corticotrophin-releasing activity (Yates, Russell, Dallman, Hedge, McCann & Dhariwal, 1971; Gillies & Lowry, 1979; Gillies, Linton & Lowry, 1982) and is released into hypothalamo-hypophyseal portal blood in high concentrations (Gibbs, 1985*a*). Adrenalectomy results in an increased number of vasopressin-expressing cells in the parvocellular paraventricular nucleus (Tramu, Croix & Pillez, 1983; Kiss, Mezey & Skirboll, 1984; Sawchenko, Swanson & Vale, 1984; Wolfson, Manning, Davis, Arentzen & Baldino, 1985), an increase in the proportion of CRF neurosecretory vesicles in the median eminence which also contain vasopressin (Whitnall, Mezey & Gainer, 1985), down-regulation of both vasopressin receptors (Antoni, Holmes & Kiss, 1985) and vasopressin-induced phosphatidyl-inositol turnover (Todd & Lightman, 1987) in the anterior pituitary and an increase in vasopressin concentration of portal blood (Koenig, Meltzer, Devane & Gudelsky, 1986; Dow, Fink, Robinson & Tannahill, 1987; Eckland, Todd & Lightman, 1988). As with corticotrophin-releasing factor, however, the response of the parvocellular vasopressin system to stress has not been described.

There is evidence that opioids may be important in the control of the hypothalamo-pituitary–adrenal axis. Acute administration of morphine either directly into the hypothalamus (Lotti, Kokka & George, 1969) or intraperitoneally (Buckingham & Cooper, 1984) results in increased ACTH secretion, whilst animals rendered tolerant to morphine failed to release ACTH or corticosterone in response to further injections of morphine or to the stress of laparotomy under ether anaesthesia (Buckingham & Cooper, 1984). The recent report of intragranular colocalization of methionine-enkephalin octapeptide with both CRF and vasopressin provides a morphological basis for endogenous opioid effects (Hisano, Tsuruo, Katoh, Daikoku, Yanaihara & Shibasaki, 1987).

In the present studies we investigate the response of parvocellular and magnocellular CRF and vasopressin mRNA to the stressful stimulus of I.P. hypertonic saline, and the CRF and POMC mRNA responses to acute and chronic administration of opiates. Opiate withdrawal was also studied using two separate paradigms of naloxone-precipitated opiate withdrawal and the more gradual (and presumably less stressful) cessation of chronic morphine administration.

## METHODS

Male Sprague–Dawley rats weighing 200–230 g were housed four to six to a cage in a 12 h light:dark temperature- and humidity-controlled environment. They were allowed free access to food and either tap water or (experiment 2) 0·03% increasing to 0·05% morphine in water. All studies were commenced at 09·00 h.

*Experiment 1.* Rats were denied access to drinking water at 09·00 h at which time a single injection of 1·5 mol saline (1·8 ml/100 g body weight) was injected I.P. and the animals killed at 13·00 h. This experiment has previously been reported in part in the context of the vasopressin response to osmotic stimuli (Lightman & Young, 1987*a*).

*Experiment 2.* Rats were divided into three groups: The control group received a subcutaneous injection of saline twice on day 1; once on day 2 following which spent osmotic minipumps (Alzet-2ML1; Alza, Palo Alto, U.S.A.) were implanted subcutaneously under ether anaesthesia. The morphine-treatment group received subcutaneous morphine 8 mg/kg twice a day on day 1, 16 mg/kg at 09·00 h on day 2, then at 18·00 h on day 2 osmotic minipumps were inserted which delivered 60 mg/kg per day. The third group was given naloxone 5 mg/kg on day 1 and on day 2 were implanted with osmotic minipumps delivering 20 mg/kg per day. After 12 days the subcutaneous minipumps were removed under ether anaesthesia and 2 h later (time 0) and at 3, 7 and 23 h, naloxone (3 mg/kg) was injected subcutaneously to the control and morphine-treated animals.

*Experiment 3.* Rats were divided into two groups. One group was given free access to drinking water whilst in the other group this was replaced with 0·03% morphine, increasing to 0·05% after 2 weeks. These drug solutions were accepted by the rats without any marked changes in their volume of fluid intake. After a total of 6 weeks morphine was replaced with regular drinking water and rats were killed after 18, 26, 50 and 75 h.

At predetermined times in each study the animals were removed from their cages, decapitated and their brains (and in experiment 2, their pituitary glands also) were rapidly removed and frozen on dry ice. Twelve micrometre sections were cut through the hypothalamus at the level of the PVN and of the arcuate nucleus and through the pituitary glands. The sections were warmed to room temperature and processed by the method of Young, Bonner & Brann (1986). The probes were made on an Applied Biosystems DNA Synthesizer (courtesy Dr M. Brownstein, NIMH, MD, U.S.A.) and have been described previously (Lightman & Young, 1987*a*). The probes were labelled using $^{35}$S-deoxyATP (1000 Ci/mmol, New England Nuclear) and terminal deoxynucleotidyl transferase (Bethesda Research Laboratories) to specific activities of $2–7·5 \times 10^3$ Ci/mmol. Hybridization, washes, exposure of film and nuclear emulsion, image analysis and calculation of copies of probe hybridized have also been described previously in Lightman & Young (1987*a*).

The *in situ* hybridization histochemistry results represent a minimum of five animals and are expressed as total copies of probe hybridized nucleus$^{-1}$ section$^{-1}$ in the brain or as copies per $\mu m^3$ tissue in the pituitary, and are presented as mean ± S.D. Statistical analysis was performed using Dunnetts multiple-comparison one-way analysis of variance. A $P$ value of $< 0·05$ was considered significant.

*Immunocytochemistry study.* Three rats (male, Sprague–Dawley, 200 g), one a control uninjected and one 4 h and another 6 h after intraperitoneal injection of 1·5 mol NaCl (see experiment 1), were perfused with 170 ml of ice-cold 4% paraformaldehyde in 0·15 M-sodium phosphate buffer, PH 7·4. The brains were removed and 3 mm slices containing the PVN were soaked for 2 h in the ice-cold perfusate and then ovenight at 4 °C in 20% sucrose in phosphate-buffered saline (9 g NaCl, 0·122 g $KH_2PO_4$, 0·815 g $NA_2PO_4$). Frozen 32 $\mu$m sections were cut and placed in phosphate-buffered saline at room temperature for four 1 h changes. The free-floating sections were then transferred to phosphate-buffered saline containing 0·1% hydrogen peroxide for 15 min at room temperature. They were then rinsed in phosphate-buffered saline and placed in 0·4% Triton X-100 for 2 h. The sections were then rinsed in phosphate-buffered saline, placed in 3% normal goat serum and 0·3% Triton X-100 in phosphate-buffered saline for 30 min and transferred to the same solution containing the rabbit anti-CRF antiserum rC70 at 1:2000; courtesy Dr W. Vale (Vale, 1987). This incubation was performed at 4 °C for 48 h. The sections were then rinsed in phosphate-buffered saline, placed in normal goat serum again, and then incubated with goat biotinylated anti-rabbit immunoglobulin antiserum (1:200, Vector Laboratories, Burlingame, CA, U.S.A.) for 45 min at room temperature. After rinsing in phosphate-buffered saline, the sections were processed with the avidin–biotin kit (Vectastain ABC kit, Vector Laboratories) according to the manufacturer's instructions. The sections were mounted onto glass slides, dried and cover-slipped.

## RESULTS

Vasopressin, oxytocin and dynorphin mRNA were clearly localized in the supraoptic nucleus (SON) and magnocellular PVN, while CRF was found not only in the parvocellular division of the PVN, but also in the magnocellular PVN and SON as previously described (Lightman & Young, 1987*a*). It was not possible to

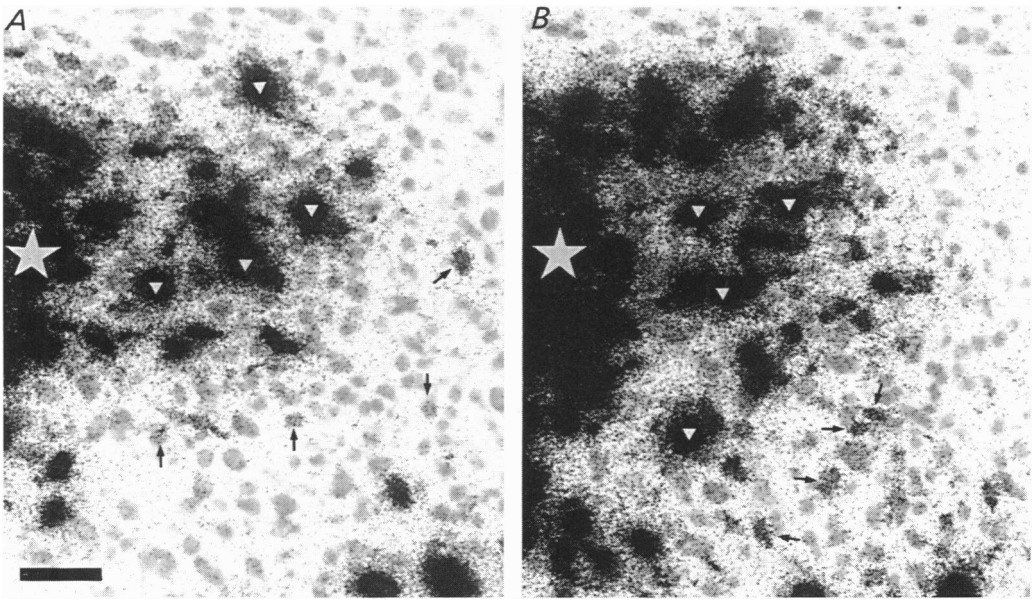


Fig. 1. Vasopressin transcripts in the paraventricular nucleus from normal (*A*) and hypertonic saline-treated (*B*) females after 3 months exposure. The magnocellular neurones in a portion of the posterior magnocellular core (☆) and individually (▽) are greatly over-exposed (usual exposure is less than 1 day). However, some of the labelled parvocellular neurones are indicated by arrows. They are more numerous and heavily labelled after the hypertonic saline treatment. Bar equals 50 $\mu$m.

differentiate magnocellular and parvocellular vasopressin mRNA with the level of resolution afforded by X-ray film due to the overlap of the medial magnocellular cells with the parvocellular cells and the considerably greater amount of vasopressin mRNA in these magnocellular cells. When sections were exposed directly to nuclear emulsion and exposed for 3 months, however, the hybridization of vasopressin probe to the parvocellular cells could be clearly distinguished (Fig. 1).

The hypothalamic CRF and vasopressin mRNA responses to hypertonic saline and the CRF mRNA response to the acute opiate study are shown in Figs 1–3. There was clearly a marked CRF and vasopressin response to hypertonic saline stress in

Fig. 2. Dark-field photomicrograph of the distribution of autoradiographic grains representing CRF probe hybridized in cells of the paraventricular nucleus of the hypothalamus. The parvocellular neurones are found between the magnocellular core of the nucleus (☆) and the third ventricle (V). *A*, control animals; *B*, 24 h after naloxone-precipitated withdrawal; and *C*, 4 h after I.P. hypertonic saline. Exposure was for 3 weeks. Bar equals 100 $\mu$m.

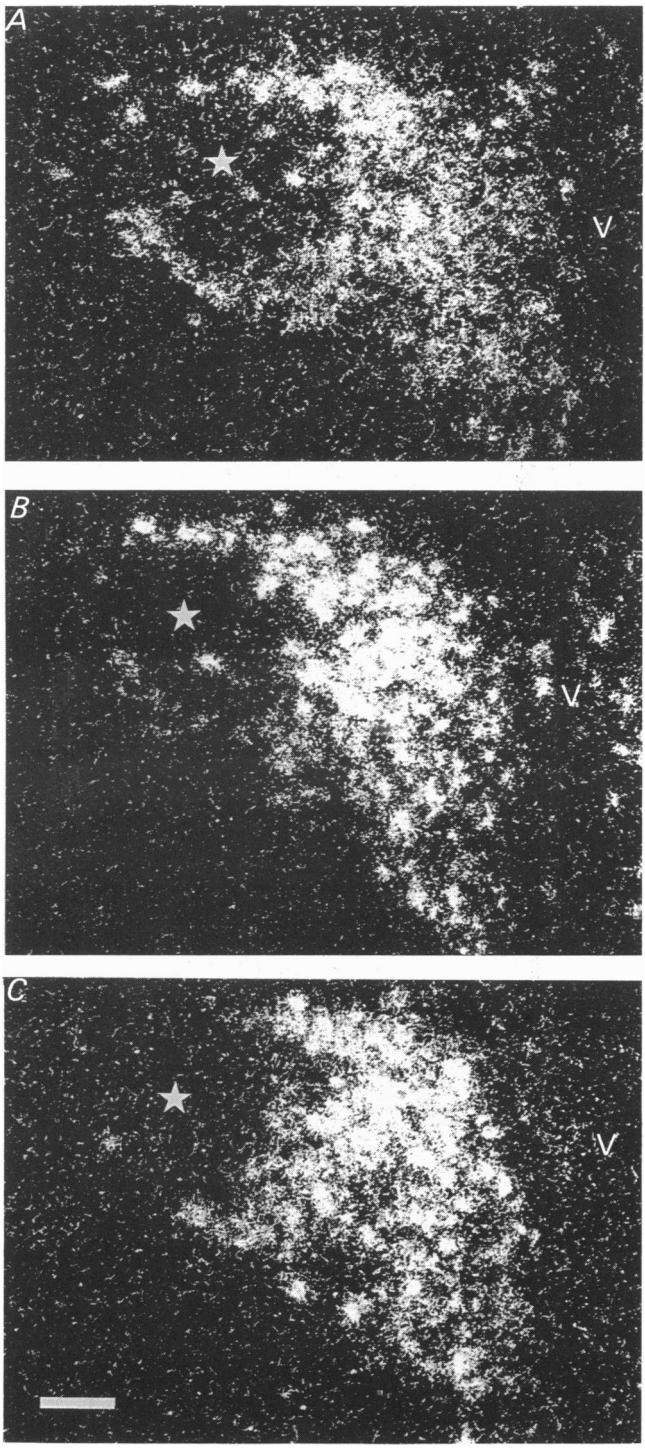


Fig. 2. For legend see opposite.

parvocellular cells of the PVN but no significant response in magnocellular vasopressin mRNA or of CRF mRNA in cells of the purely magnocellular SON. Acute opiate administration on day 1 (M1) and day 2 (M2) had no effect on CRF or vasopressin mRNA levels although there was an increase in magnocellular dynorphin mRNA. Three or 12 days of morphine administration had no effect on the mRNA

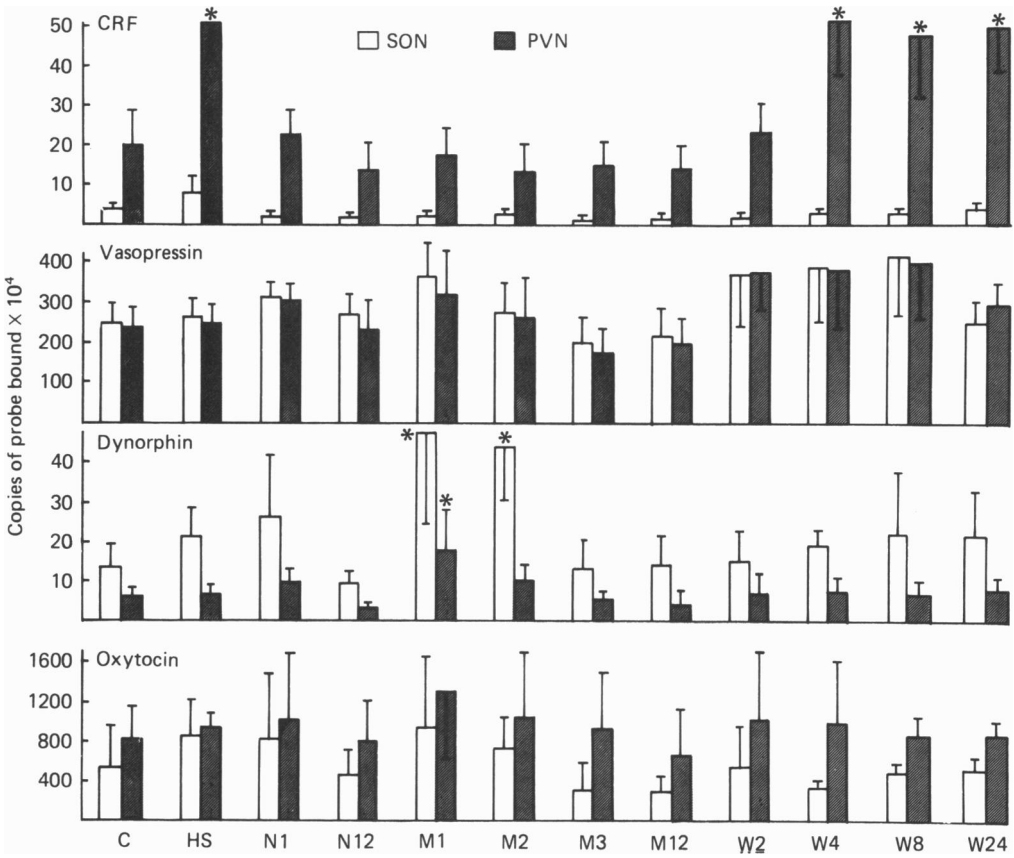


Fig. 3. Quantitative *in situ* hybridization histochemical estimation of paraventricular nucleus (PVN) corticotrophin-releasing factor (CRF), vasopressin, dynorphin and oxytocin mRNA levels. Results expressed as copies of probe hybridized per PVN per section. C, control; HS, 4 h after I.P. injection of hypertonic saline; N1, 4 h after injection of naloxone; N12, after 12 days naloxone infusion; M1, 4 h after first injection morphine; M2, 4 h after morphine on day 2; M3, after 16 h morphine infusion on day 3; M12, after 12 days morphine infusion; W2–W24, 2–24 h after naloxone-precipitated opiate withdrawal. $*P < 0{\cdot}05$.

levels of any of the hypothalamic peptides studied. Following naloxone-induced opiate withdrawal there was a rapid accumulation of paraventricular parvocellular CRF mRNA which rose to significantly increased levels within 4 h and remained elevated at 24 h. Morphine withdrawal was not associated with any change in SON magnocellular CRF mRNA nor in total vasopressin, oxytocin or dynorphin mRNA. Sections hybridized with vasopressin probe were not dipped in nuclear emulsion and differentiation of magnocellular and parvocellular vasopressin mRNA could not therefore be performed.

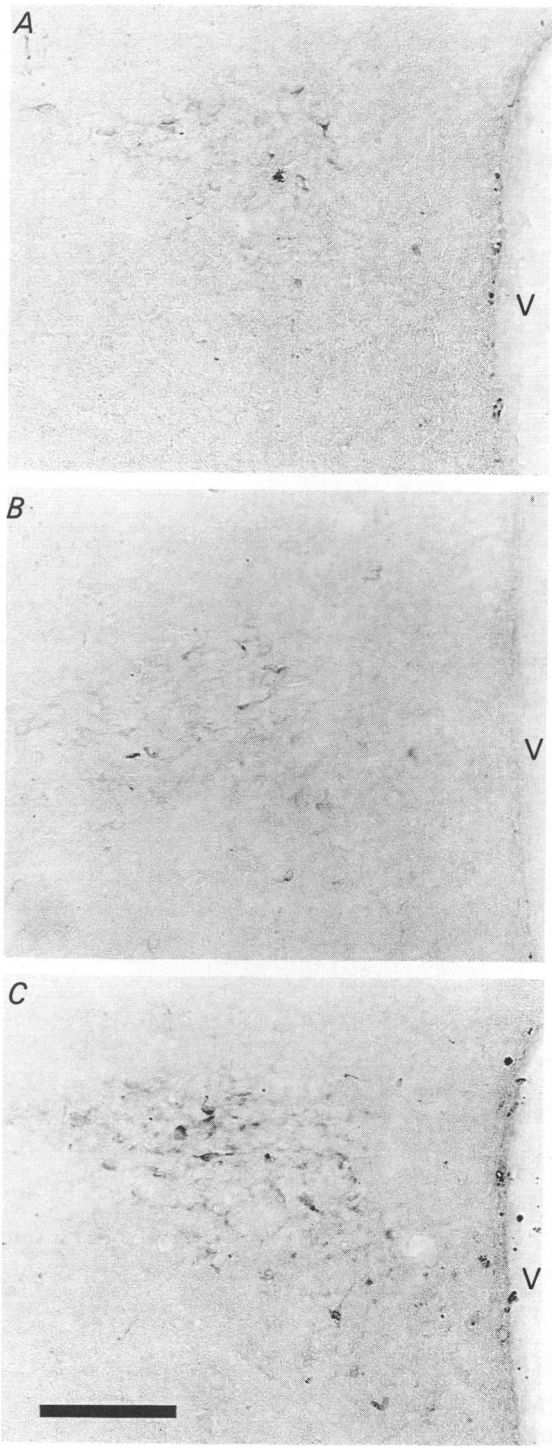


Fig. 4. Immunocytochemical analysis of paraventricular nucleus corticotrophin-releasing factor: *A*, control animal; *B*, 4 h after I.P. hypertonic saline; and *C*, 6 h after I.P. hypertonic saline. V, third ventricle. Bar equals 100 $\mu$m.

Immunocytochemical studies on the parvocellular paraventricular content of CRF confirmed that in addition to the increase in CRF mRNA there is also an increase in the amount of paraventricular immunoreactive CRF peptide itself. This study, which was performed in the absence of colchicine, is presented in Fig. 4.

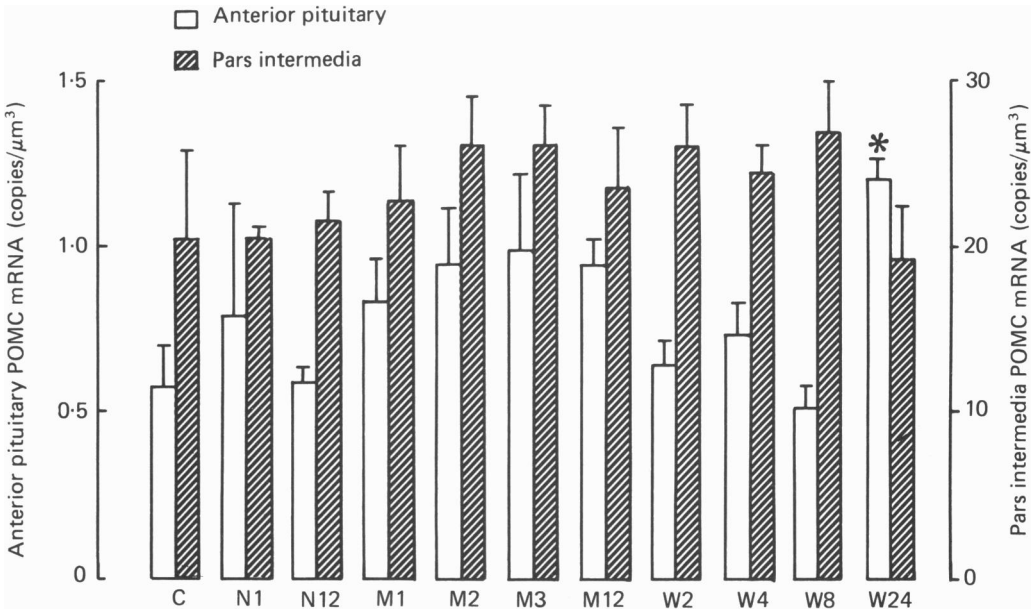


Fig. 5. Quantitative *in situ* hybridization histochemical estimation of pro-opiomelanocortin (POMC) mRNA in the pars intermedia and anterior lobes of the rat pituitary gland. Results expressed as copies of probe hybridized per $\mu m^3$ tissue. C, control; N1, 4 h after injection of naloxone; N12, after 12 days naloxone infusion; M1, 4 h after first injection of morphine; M2, 4 h after morphine on day 2; M3 after 16 h morphine infusion on day 3; M12 after 12 days morphine infusion; W2–W24, 2–24 h after naloxone-precipitated opiate withdrawal. $*P < 0{\cdot}05$

The pituitary POMC mRNA levels in response to morphine and naloxone-precipitated morphine withdrawal are seen in Fig. 5. Administration of morphine had no significant effect on POMC mRNA in either the anterior pituitary or pars intermedia. Following naloxone-precipitated opiate withdrawal an increase in POMC mRNA was delayed and only apparent at 24 h, whilst in contrast there was no change in POMC mRNA in the pars intermedia.

The response of arcuate nucleus POMC mRNA was only measured (a) in control animals; (b) after 12 days of morphine treatment; and (c) at 8 h after naloxone-precipitated opiate withdrawal. At these time points there was no significant change in arcuate nucleus POMC mRNA although the scattered distribution of POMC-containing cells in the arcuate nucleus made this study rather less amenable to quantitative *in situ* hybridization histochemistry. Expressing the results as a percentage of the mRNA found in control nuclei we found the amount of probe bound to be: controls, $100 \pm 20\%$; 12 days morphine treated, $97 \pm 20\%$; 8 h after naloxone-precipitated opiate withdrawal, $111 \pm 61\%$ (mean $\pm$ S.D.).

The response to simple non-naloxone-precipitated opiate withdrawal following 6

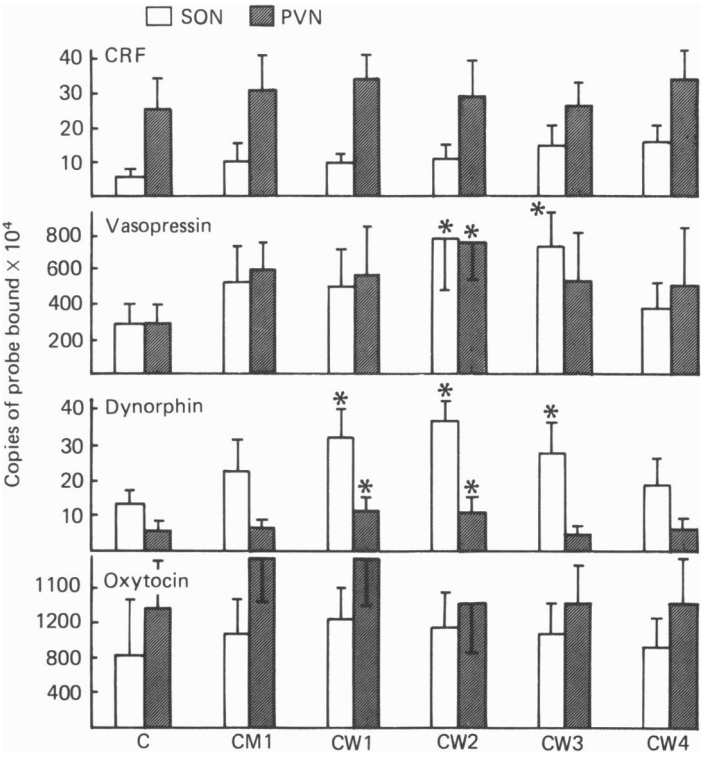


Fig. 6. Quantitative *in situ* hybridization histochemical estimation of paraventricular nucleus corticotrophin-releasing factor (CRF), vasopressin, dynorphin and oxytocin mRNA levels following simple morphine withdrawal. C, control; CM1, after 6 weeks chronic morphine treatment; CW1, 18 h after morphine withdrawal; CW2, 26 h after morphine withdrawal; CW3, 50 h after morphine withdrawal; CW4, 74 h after morphine withdrawal. $*P < 0{\cdot}05$.

weeks of chronic morphine administration was quite different (Fig. 6). These rats did show opiate withdrawal weight loss amounting to 2·3% of their body weight at 18 h, 3% at 24 h, 4·7% at 50 h and 3·3% at 74 h. In spite of this there was no change in CRF mRNA and no appearance of enkephalin mRNA which we have previously shown in naloxone-precipitated opiate withdrawal (Lightman & Young, 1987*b*). There was, however, a significant increase in magnocellular vasopressin and dynorphin mRNA.

## DISCUSSION

The increased secretion of ACTH following adrenalectomy clearly involves paraventricular parvocellular neurones which not only show increased levels of immunoreactive CRF and vasopressin but also show increases in CRF and vasopressin mRNA (see Gillies & Lowry, 1986 and Antoni, 1986 for reviews). It is, however, unknown whether a similar process occurs during stress. In the present study we have been able to confirm that following two stressful stimuli CRF mRNA rapidly accumulated within 4 h, and this mRNA must be active in the translation of CRF itself since we also have the immunocytochemical evidence for an accumulation

of CRF immunoreactivity without colchicine pre-treatment. In one of the studies (hypertonic saline) we also looked at changes in parvocellular vasopressin mRNA and confirmed that this also increases within 4 h of the stress stimulus. This is the first time that stress has been demonstrated to increase levels of both CRF and vasopressin parvocellular paraventricular mRNA and suggests that the potentiation of the corticotrophin-releasing activity of CRF by vasopressin which is seen *in vitro* (Gillies *et al.* 1982) also occurs *in vivo* in the conscious animal.

The parvocellular and magnocellular mRNA responses to stimuli appear to have very different time courses. Intraperitoneal hypertonic saline is not only a potent stressor, it is also a potent osmotic stimulus and results in a 70-fold increase in plasma oxytocin and a 10-fold increase in plasma vasopressin within 30 min (authors, unpublished data). In spite of this massive stimulation of magnocellular cells to release vasopressin and oxytocin there is no change in their vasopressin or oxytocin mRNA at 4 h, although by 24 h after a chronic osmotic stimulus the levels have begun to rise (Lightman & Young, 1987*a*). It is clear therefore that parvocellular vasopressin (and CRF and enkephalin) mRNA responds much more rapidly than magnocellular vasopressin (or oxytocin) to this combined stress and osmotic stimulus. We would presume that this is related to the much greater stores of vasopressin in the terminals of the neural lobe than in those in the median eminence, such that any release of vasopressin, CRF or enkephalin at the median eminence results in a much more rapid need for replenishment and synthesis of new peptide. The biochemical stimulus to this accumulation of mRNA is not at present understood.

The elevation of pituitary POMC mRNA also lagged behind that of the PVN CRF mRNA. Although CRF mRNA was increased at 4 h following opiate withdrawal, pituitary POMC mRNA only increased at 24 h. The relationship of the time course of the CRF mRNA increase to that of changes in portal plasma CRF are unknown, so we cannot be sure whether this delay represents a lag in the POMC mRNA response to CRF, the time necessary for transport of CRF to the median eminence, or a combination of the two. It is interesting that this increase was limited to the anterior lobe of the pituitary which suggests that the pars intermedia has little if any role in the ACTH response to stress.

These results are consistent with the studies of Hollt *et al.* (1986) who gave chronic intermittent electric foot shock and found that maximally elevated POMC levels and mRNA levels were not obtained until 5 days, and were not associated with any change in combined intermediate–posterior pituitary POMC mRNA. Hollt & Haarmann (1984) have also shown that chronic infusion of CRF over 4–8 days results in selective anterior lobe POMC mRNA increases of a similar magnitude.

Morphine has been known to stimulate the pituitary adrenal axis since 1955 (George & Way) and it also potentiates the ACTH response to a subsequent stress (Buckingham & Cooper, 1984). Morphine-tolerant animals, however, failed to release ACTH or corticosterone in response to further injections of the opiate or to stress. Hypothalami from these tolerant animals also failed to release CRF in response to various stimuli (Buckingham & Cooper, 1984). These results suggest that opiates play a major role in hypothalamo-pituitary control of ACTH secretion. We were unable to find any effect of morphine or naloxone administration on hypothalamic CRF or pituitary POMC mRNA. This suggests that any effects of opiates on CRF are

short lived and do not have a significant effect on CRF turnover. Opiates might also act via effects on other biologically active corticotrophin-releasing factors (e.g. vasopressin, oxytocin or cholecystokinin).

The difference between the responses to simple opiate withdrawal and naloxone-precipitated opiate withdrawal are of great interest. Only naloxone-precipitated withdrawal results in increased parvocellular CRF mRNA (and also enkephalin mRNA – Lightman & Young, 1987*b*) suggesting a stress situation comparable to that found after I.P. hypertonic saline.

The oral ingestion of morphine in drinking water is a simple method to achieve long-term physical dependence (Stolerman & Kumar, 1979) as confirmed by the animals' loss of weight following morphine deprivation. Although this chronic stress following morphine deprivation did not result in any changes in enkephalin or CRF mRNA, there was a marked increase in magnocellular vasopressin and dynorphin mRNA which correlates well with the dramatic decrease in water consumption which occurs at this time (McMillan, Leander, Wilson, Wallace, Fix, Redding & Turk, 1976). Vasopressin and dynorphin are co-localized in the same magnocellular cells, and these changes therefore appear to represent the result of activation of one particular cell type. This increase in vasopressin and dynorphin mRNA is similar to that which has been demonstrated by ourselves and other workers following a prolonged hypertonic osmotic stimulus (reviewed in Lightman & Young, 1987*a*).

In the urethane-anaesthetized, morphine-dependent, lactating rat, naloxone-precipitated withdrawal results in a rapid increase in oxytocin cell firing rate, plasma oxytocin concentration and intramammary pressure (Bicknell, Leng, Lincoln & Russell, 1988). Naloxone-precipitated opiate withdrawal in our conscious rats did not result in any change in hypothalamic oxytocin mRNA. This suggests that either the intracerebroventricular infusion of morphine utilized by Russell may effect oxytocin neurones in a different way to our subcutaneous infusions, or the anaesthetized rat may behave differently from the conscious rat or most likely the large stores of oxytocin in the neural lobe do not need rapid replenishment after a single acute stimulus.

In conclusion we have demonstrated for the first time that a stress stimulus in the conscious rat results in a rapid accumulation of both CRF and vasopressin mRNA, suggesting a physiological role for the potentiating effect of vasopressin on the corticotrophin-releasing properties of CRF. Naloxone-precipitated opiate withdrawal also results in significant increases in CRF mRNA within 4 h, and after a delay of 24 h a selective increase in anterior pituitary but not pars intermedia POMC mRNA. Acute or chronic opiate or opiate antagonist administration or simple opiate withdrawal in tolerant animals do not effect CRF mRNA or pituitary POMC mRNA levels. These results provide further information on the role of opioids in the hypothalamo-pituitary–adrenal axis and suggests that naloxone-precipitated opiate withdrawal activates this stress axis in a similar fashion to I.P. hypertonic saline.

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