*Br. J. Pharmacol.* (1988), **94**, 647–652

# Neonatal capsaicin treatment of rats reduces ACTH secretion in response to peripheral neuronal stimuli but not to centrally acting stressors

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**1** Plasma adrenocorticotrophic hormone (ACTH) concentrations were measured in rats following exposure to anaesthetic agents, after stimulation of peripheral sensory nerves, and during psychological stress.

**2** In rats, kept in their home cages, the i.p. injection of sodium pentobarbitone did not cause an increase in plasma ACTH, whereas injection of urethane increased plasma ACTH several times. In rats transferred to a glass dessicator and inhaling oxygen, plasma ACTH was more than 3 fold higher than in rats in their home cage. Exposure to nitrous oxide, halothane or ether in a glass dessicator produced significantly higher plasma ACTH concentrations when compared to exposure in the home cage.

**3** In rats anaesthetized with pentobarbitone, the electrical stimulation of large myelinated afferents in the sciatic nerve did not trigger a measurable increase in ACTH secretion, whereas stimulation of afferent A$\delta$- and C-fibres significantly elevated plasma ACTH concentrations. Rats treated as neonates with capsaicin showed an attenuated ACTH response to A and C-fibre stimulation.

**4** Similarly, capsaicin pretreatment reduced the increase in ACTH secretion during morphine withdrawal; a similar effect was produced by clonidine.

**5** ACTH secretion following open field exposure, ether stress or hypoglycaemia was not changed by capsaicin pretreatment.

**6** It was concluded that capsaicin-sensitive afferents are involved in the secretion of ACTH elicited by somatosensory forms of stress. Centrally evoked ACTH release is not affected by capsaicin pretreatment.

## Introduction

The plasma concentration of ACTH (adrenocorticotrophic hormone) secreted by the corticotroph cells of the anterior pituitary gland, is elevated in response to stress in animals and man. The afferent pathways to the anterior pituitary gland within the central nervous system, which are involved in the stress-response, have been studied in detail (for review see Feldman, 1985). Less attention has been paid to the type of nerve fibres which transmit peripherally-evoked ACTH release stimuli to the CNS. Recently it has been shown that capsaicin-sensitive primary afferents can be involved in the activation of pituitary ACTH secretion in response to somatosensory stimuli such as cold stress, administration of formalin (i.p.) or surgery (Lembeck & Amann, 1986; Amann & Lembeck, 1987). This class of sensory neurones, which can be selectively destroyed by treatment of neonatal rats with capsaicin, belongs to the C- and A$\delta$-fibre group and contains peptides as neurotransmitters (for review see Pernow, 1983; Buck & Burks, 1986).

The present experiments were carried out (1) to determine ACTH secretion following the injection or inhalation of anaesthetic agents; (2) to study whether, in addition to the stimulation of unmyelinated and thin myelinated afferents, the stimulation of large myelinated afferents can also induce ACTH secretion; and (3) to investigate whether the ACTH response to forms of stress which act peripherally as well as centrally, is modified by neonatal capsaicin

pretreatment. The examples used were morphine withdrawal, insulin-induced hypoglycaemia, open field exposure and ether exposure in a glass dessicator.

## Methods

### *Animals*

Sprague-Dawley rats of either sex were used; they were weaned when 4 weeks old and kept in groups of 4 in cages 42 × 22 × 15 cm with a 12 h light and dark cycle. A standard laboratory diet and water were available *ad libitum*. For the capsaicin experiments, 2 day old rats were anaesthetized with ether and injected subcutaneously with either capsaicin ($50\,mg\,kg^{-1}$) or with an appropriate volume of the vehicle (0.9% w/v NaCl solution containing 10% ethanol and 10% Tween 80). The experiments were carried out when the rats were 8–10 weeks old after the efficacy of the capsaicin pretreatment had been assessed by the wiping test (Gamse, 1982): wiping movements with the forepaws are counted following the instillation of a dilute solution ($10^{-4}$ M) of capsaicin into the eye. Rats with adequate pretreatment did not respond, whereas vehicle-treated rats showed $25 \pm 2$ wipings plus blepharospasm ($n = 10$). On the three days preceding the experiment the rats were weighed and handled by the experimenter. The experiments were performed between 09 h 00 min and 12 h 00 min. Any additional isolation or environmental stresses were avoided. Experiments on conscious rats were done in the room in which the rats were housed or the animals were anaesthetized in this room and then carried to the laboratory. Unless indicated otherwise, the rats were put back into their home cages following exposure to stress.

The duration of the stimuli and the time lag between stimulation and the blood collection were selected according to published findings and our own screening experiments. ACTH levels were measured at a time following the onset of the stimuli when a marked rise in plasma ACTH was known to occur in control rats, but at a time when the feedback inhibition by the stress-induced corticosterone secretion would not yet be effective (Nakane *et al.*, 1985).

The animals were decapitated and blood was collected from the trunk into EDTA-coated tubes. After centrifugation, the plasma was pipetted off and stored at −60°C until the ACTH-assay. For the radioimmunoassay of ACTH a commercially available kit from Immuno Nuclear, Stillwater, MN, U.S.A., was used.

### *Anaesthetic agents*

*Injections* Rats were injected with 0.9% w/v NaCl solution (saline), sodium pentobarbitone ($50\,mg\,kg^{-1}$ i.p.) or urethane ($1.25\,g\,kg^{-1}$ i.p.) and put back into their cages.

*Exposure to volatile anaesthetics* Rats were either placed into a glass dessicator or left in their home cages. An ether-soaked piece of gauze was placed at the bottom of the container, or oxygen, nitrous oxide (80% and 20% oxygen) or halothane (4% in oxygen) was blown into the containers at a flow of $10\,l\,min^{-1}$. Three min after the injections or two min after the end of the gas inhalation the rats were decapitated and blood from the trunk was collected for ACTH determination.

### *Nerve stimulation*

Rats were anaesthetized with sodium pentobarbitone ($50\,mg\,kg^{-1}$ i.p.); one sciatic nerve was exposed along the femur and placed on a bipolar silver electrode. In one group of rats the nerve was not stimulated in order to assess the effect of surgery alone. In two other groups the nerve was stimulated immediately at the end of surgery, either for 2 min at 1 mA, 0.5 ms and 10 Hz (excitation of A- and C-fibres) or at 75 μA, 0.05 ms and 10 Hz (excitation of large myelinated A-fibres only, Cook *et al.*, 1987). The animals were killed 1 min after the end of the stimulation.

### *Morphine withdrawal*

Rats were injected subcutaneously at intervals of 8 h for 3 days with morphine or its solvent (saline) (first two doses: $5\,mg\,kg^{-1}$; next two doses: $10\,mg\,kg^{-1}$; finally doses of $15\,mg\,kg^{-1}$). Four hours after the last injection of morphine the rats received naloxone ($1\,mg\,kg^{-1}$ s.c.) or the solvent only. In the experiments with clonidine this drug was given i.p. ($30\,\mu g\,kg^{-1}$) 10 min before naloxone; 20 min after naloxone the rats were decapitated and trunk blood collected.

### *Open field exposure*

Rats were placed singly for 5 min in a square white box (80 × 80 × 40 cm) which had a grid of black lines, 10 cm apart, painted on the floor. The box was illuminated with a 60 W bulb positioned 80 cm above its floor. Following this stress exposure the rats were put back into their home cages and decapitated 2 min later.

*Hypoglycaemia*

Rats, which had been fasted overnight, received a s.c. injection of 4 u $kg^{-1}$ insulin; they were decapitated 30 min later and trunk blood collected to determine blood glucose and plasma ACTH concentrations. Blood glucose was estimated by the hexokinase 1G6P-DH method (Gluco quant, Boehringer, Mannheim, F.R.G.).

*Statistical analysis*

Data were analysed by use of ANOVA (SPSS) and differences between treatments were determined by Scheffe's multiple comparisons test.

*Substances*

Sodium pentobarbitone (Sanofi Sante Animali, Paris, France), urethane (Fluka, Buchs, Switzerland), halothane (Fluothane, ICI, Macclesfield, U.K.), ether (Merck, Darmstadt, F.R.G.), morphine hydrochloride (Diosynth, Apeldoorn, Holland) and naloxone hydrochloride (DuPont, Geneva, Switzerland) were used. Clonidine was a gift from Boehringer (Ingelheim, F.R.G.), and soluble insulin (Insulin CS) was a gift from Hoechst (Frankfurt, F.R.G.).

## Results

*Anaesthesia*

The results obtained in rats not pretreated with capsaicin are presented in Table 1. Plasma ACTH concentrations were not elevated in rats injected with pentobarbitone when compared with those of rats injected with saline (Table 1). Urethane caused a significant increase in plasma ACTH. Exposure of rats to volatile anaesthetic agents in their home cage had little effect on ACTH secretion. Only halothane caused a significant increase in plasma ACTH.

In contrast, all rats transferred to glass dessicators showed largely elevated plasma ACTH concentrations, whether inhaling oxygen or anaesthetic gases. Although the mean values were higher after inhalation of the anaesthetic gases, the increases were not statistically significant because of large individual variations. Rats pretreated as neonates with capsaicin and exposed to ether in the dessicator showed the same increase in plasma ACTH as rats pretreated with the capsaicin vehicle (see Figure 1c).

*Open field exposure*

Capsaicin pretreated rats and vehicle pretreated rats showed the same significant increase in plasma ACTH when exposed to the open field situation (see Methods and Figure 1b).

**Table 1** Effect of injection or inhalation of anaesthetic agents on plasma ACTH concentrations in normal rats

| *Treatment* | *Plasma ACTH* (pg $ml^{-1}$) *Home cage* | *Glass dessicator* |
|---|---|---|
| Saline i.p. | 90 ± 13 | ND |
| Sodium pentobarbitone i.p. | 100 ± 16 | ND |
| Urethane i.p. | 329 ± 79[a] | ND |
| Oxygen | 70 ± 24 | 244 ± 55[b] |
| Nitrous oxide (20% oxygen) | 130 ± 17 | 318 ± 87[b] |
| Halothane (4% in oxygen) | 159 ± 30[c] | 296 ± 46[b] |
| Ether vapour | 140 ± 13 | 441 ± 92[b] |

The rats were decapitated 3 min after the i.p. injection of the first 3 agents or 2 min after a 60 s exposure to the gases. Mean values ± s.e. mean are shown ($n$ = 6–8), ND: not determined. Significance of difference from appropriate controls: [a] $P < 0.05$ compared to saline or sodium pentobarbitone i.p.; [b] $P < 0.05$ compared to home cage-group; [c] $P < 0.05$ compared to oxygen exposure.

*Hypoglycaemia*

The s.c. injection of insulin (4 u $kg^{-1}$ body weight) in fasted rats lowered the blood sugar concentration

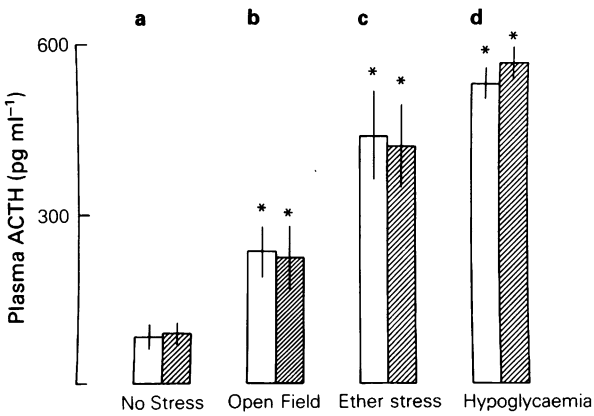


**Figure 1** Plasma ACTH concentrations in vehicle- (open columns) or capsaicin- (hatched columns) pretreated rats in various stressful situations: (b) Open field stress for 5 min, see methods; (c) exposure to ether vapour in a glass box for 1 min; (d) hypoglycaemia was induced in fasted rats by s.c. administration of insulin (for details see methods). Each column represents the mean and vertical lines indicate s.e. mean; $n$ = 6–8. Significance of difference when compared to the unstressed group (a): * $P < 0.05$ (Scheffe's multiple comparison test).

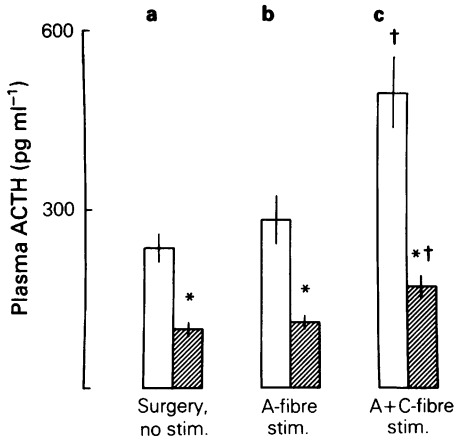


**Figure 2** Effect of neonatal pretreatment with capsaicin on ACTH release evoked by electrical stimulation of sciatic nerve fibres in the rat. Stimulation parameters: (b) Large myelinated A-fibres: 75 $\mu$A, 0.05 ms for 2 min; (c) A + C fibres: 1 mA, 0.5 ms for 2 min. One min after the end of nerve stimulation the animals were decapitated and the trunk blood collected. Open columns: rats pretreated neonatally with vehicle; hatched columns: rats pretreated neonatally with capsaicin (for pretreatment see methods). Each column represents the mean and vertical lines indicate s.e. mean; $n = 6$–8. Significance of differences: * $P < 0.05$ compared to vehicle pretreated rats, † $P < 0.05$ compared to nerve exposure without stimulation (a) or A-fibre stimulation. (Scheffe's multiple comparison test).

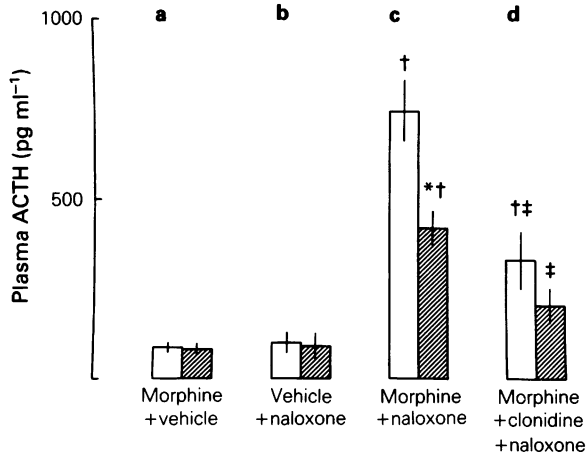


**Figure 3** Effect of neonatal pretreatment with capsaicin on ACTH release due to opiate withdrawal. Rats were injected for 3 days with increasing doses of morphine (from 5 mg kg$^{-1}$ to 15 mg kg$^{-1}$ s.c.) at 8 hourly intervals. Four hours after the last dose of morphine they received naloxone 1 mg kg$^{-1}$ s.c. to induce an opiate withdrawal syndrome. Some rats received an i.p. injection of clonidine (30 $\mu$g kg$^{-1}$) 10 min before naloxone. All rats were decapitated 20 min after naloxone and blood collected from the trunk for ACTH determinations. Open columns: rats pretreated with vehicle as neonates; hatched columns: rats pretreated with capsaicin as neonates. Each column represents the mean and vertical lines indicate s.e. mean; $n = 6$–8. Significance of differences: * $P < 0.05$ compared to vehicle pretreated rats; † $P < 0.05$ compared to morphine or saline plus naloxone treatment. ‡ $P < 0.05$ compared to morphine plus naloxone (Scheffe's multiple comparison test).

from $4.8 \pm 0.3$ to $2.6 \pm 0.3$ mmol l$^{-1}$ (vehicle pretreated rats) and from $4.7 \pm 0.3$ to $2.5 \pm 0.2$ mmol l$^{-1}$ (capsaicin pretreated rats). The fall in blood glucose was accompanied by a more than 5 fold rise in plasma ACTH in both groups of rats (Figure 1d).

*Nerve stimulation*

In Figure 2 plasma concentrations of ACTH following dissection (a) and stimulation (b and c) of the sciatic nerve in capsaicin pretreated and vehicle pretreated rats are presented. In each of the three experimental conditions plasma ACTH was much lower in the capsaicin pretreated rats. In control as well as in capsaicin pretreated rats the increase in plasma ACTH concentration caused by stimulation of large myelinated A-fibres did not differ from that which occurred in animals in which the nerve was placed on the electrode without stimulation. Additional A$\delta$- and C-fibre stimulation caused a two fold increase in ACTH release in control rats but only a moderate response in capsaicin-treated rats.

*Morphine withdrawal*

No differences in plasma ACTH concentrations could be observed between vehicle pretreated or capsaicin pretreated rats when the animals were injected several times with morphine or once with naloxone (see Figure 3a and b). The plasma ACTH concentrations were within the normal range (compare with Figure 1a). Morphine withdrawal, induced by injecting naloxone after repeated doses of morphine, caused a seven fold increase of plasma ACTH concentrations in the control rats within 20 min. In the capsaicin pretreated group the increase was only 4 fold (see Figure 3c). In both groups of rats clonidine attenuated the ACTH response to the morphine withdrawal.

## Discussion

An ACTH releasing effect has been described for many anaesthetic agents, e.g. for urethane (Walker *et al.*, 1986). Under the present experimental conditions sodium pentobarbitone (i.p.) did not increase plasma ACTH concentrations and did not interfere with the ACTH release induced by surgery or nerve stimulation, as has been observed previously (Amann & Lembeck, 1987). The ACTH release observed after ether inhalation (e.g. Nakane *et al.*, 1985; Feldman *et al.*, 1986) may have mainly been due to the change in environment during anaesthesia (compare Table 1, results in home cage and in glass dessicator).

In previous experiments it has been observed that the ACTH release induced by surgery, a classical somatosensory form of stress, is almost abolished by pretreatment of the rats as neonates with capsaicin (Amann & Lembeck, 1987). This was confirmed in the present work. In addition, evidence was obtained to strengthen the assumption that the transmission of peripheral stressful stimuli to the central mechanisms involved in ACTH release, proceeds mainly via C-fibres. In control rats the combined electrical stimulation of C plus A fibres of the sciatic nerve resulted in an increase in plasma ACTH concentrations by more than 100%, when compared with that in rats in which the sciatic nerve was only exposed and not stimulated, or in which only large myelinated A-fibres were stimulated. In contrast, in capsaicin-pretreated rats the increase in plasma ACTH after stimulation of C plus A fibres was much smaller. This small increase may be attributed to the remaining Aδ- and C-fibres after capsaicin pretreatment (5–28%: see Nagy *et al.* 1983; Arvidsson & Ygge, 1986).

The opiate withdrawal symptom may be classified as a partial somatosensory form of stress. From observations in man and primates it is known that morphine withdrawal not only leads to frustration but that signs of severe pain are apparent. Multiple mechanisms are involved (Redmond & Krystal, 1984), among them activation of capsaicin-sensitive afferent fibres (Sharpe & Jaffe, 1986). Repeated administration of morphine might cause chronic inhibition of the release of excitatory neurotransmitters (Yaksh *et al.*, 1980). The injection of a dose of naloxone may then lead to a sudden overshoot in the release of excitatory transmitter(s). The results from the present experiments suggest that neurotransmitters released from primary afferent C-fibres are involved in inducing ACTH release during morphine withdrawal. Clonidine, an $\alpha_2$-adrenoceptor agonist, is known to depress several populations of neurones, especially those in the brain stem (Aghajanian, 1978). It is likely that this is the level at which its depressive action on ACTH secretion during morphine withdrawal is exerted. An effect of clonidine on the first synapse in the dorsal horn of the spinal cord cannot be excluded, but, this is probably not the predominant site involved in the present findings, since clonidine was equally effective in both control and capsaicin-pretreated rats. Clonidine has been shown in several investigations to be a helpful substitute for opiates during the withdrawal phase (Gold & Roehrich, 1987). This could be explained by the fact that opiate receptors and $\alpha_2$-adrenoceptors activate independently the same second messenger system: inhibition of adenylate cyclase via a guanine nucleotide regulatory protein (Aghajanian & Wang, 1986).

As open field stress and exposure to ether in a glass dessicator caused an equal increase in plasma ACTH in control and capsaicin-pretreated rats, we may conclude that these forms of stress activate central neural pathways independent of capsaicin-sensitive afferents or act directly on cells which produce the corticotrophin releasing factor (CRF). Open field exposure has been classified as an emotional form of stress in rats (Goma & Tobena, 1978). Ether exposure of rats in a glass dessicator is widely used as experimental stress; a part of this stress is due to the exposure to a brightly illuminated container.

Although the secretion of adrenaline from the adrenal medulla during hypoglycaemia is, at least in part, regulated by capsaicin-sensitive fibres (Khalil *et al.*, 1984; Amann & Lembeck, 1986), in the present work we did not find an effect of these fibres on ACTH secretion. It is likely that the excitation of central neuronal pathways, or direct effects on CRF or vasopressin cells, to mediate ACTH secretion, predominate during hypoglycaemia (Plotzky *et al.*, 1985).

The central pathways involved in ACTH release include noradrenergic input to the hypothalamus (Feldman *et al.*, 1986) and excitation of CRF-cells in the paraventricular nuclei of the hypothalamus (Rivier *et al.*, 1982; Nakane *et al.*, 1985). Other factors like vasopressin, oxytocin and angiotensin might also interact with CRF (Rivier & Plotzky, 1986).

To summarize, capsaicin-treated rats are a valuable model for differentiating between somatosensory evoked and centrally evoked ACTH release in response to stress. The present results have shown that excitation of afferent Aδ- and C-fibres, but not of large myelinated A-fibres, induces ACTH release via hypothalamic neurosecretory neurones.

This work was supported by the Austrian Scientific Research Funds, grant No. P5616, the Austrian National Bank (grant 2811) and the Pain Research Commission of the Austrian Academy of Sciences.

## References


AGHAJANIAN, G.K. (1978). Tolerance of locus coeruleus neurones to morphine and suppression of withdrawal response by clonidine. *Nature*, **276**, 186–188.

AGHAJANIAN, G.K. & WANG, Y.Y. (1986). Pertussis toxin blocks the outward currents evoked by opiate and α2-agonists in locus coeruleus neurons. *Brain Res.*, **371**, 390–394.

AMANN, R. & LEMBECK, F. (1986). Capsaicin sensitive afferent neurons from peripheral glucose receptors mediate the insulin-induced increase in adrenaline secretion. *Naunyn-Schmiedebergs Arch. Pharmacol.*, **334**, 71–76.

AMANN, R. & LEMBECK, F. (1987). Stress induced ACTH release in capsaicin treated rats. *Br. J. Pharmacol.*, **90**, 727–731.

ARVIDSSON, J. & YGGE, J. (1986). A quantitative study of the effects of neonatal capsaicin treatment and of subsequent peripheral nerve transection in the adult rat. *Brain Res.*, **397**, 130–136.

BUCK, S.H. & BURKS, T.F. (1986). The neuropharmacology of capsaicin: review of some recent observations. *Pharmacol. Rev.*, **38**, 179–226.

COOK, A.J., WOOLF, C.J., WALL, P.D. & McMAHON, S.B. (1987). Dynamic receptive field plasticity in rat spinal cord dorsal horn following C-primary afferent input. *Nature*, **325**, 151–153.

FELDMAN, S. (1985). Neural pathways mediating adrenocortical responses. *Fed. Proc.*, **44**, 169–175.

FELDMAN, S., CONFORTI, N. & MELAMED, E. (1986). Norepinephrine depletion in the paraventricular nucleus inhibits the adrenocortical responses to neural stimuli. *Neurosci. Lett.*, **64**, 191–195.

GAMSE, R. (1982). Capsaicin and nociception in the rat and mouse. Possible role of substance P. *Naunyn- Schmiedebergs Arch. Pharmacol.*, **329**, 205–216.

GOLD, M.S. & ROEHRICH, H. (1987). Treatment of opiate withdrawal with clonidine. *ISI Atlas of Science: Pharmacology*, **1**, 29–32.

GOMA, M. & TOBENA, A. (1978). Reliability of various measures obtained in the open field test. *Psychol. Rep.*, **43**, 1123–1128.

KHALIL, Z., MARLEY, P.D. & LIVETT, B.G. (1984). Neonatal capsaicin treatment prevents insulin-stress induced adrenal catecholamine secretion *in vivo*: possible influence of sensory nerves containing substance P. *Neurosci. Lett.*, **45**, 65–70.

LEMBECK, F. & AMANN, R. (1986). The influence of capsaicin sensitive neurons on stress-induced release of ACTH. *Brain Res. Bull.*, **16**, 541–543.

NAGY, J.I., HUNT, S.P., IVERSEN, L.L. & EMSON, P.C. (1983). Effect of capsaicin on dorsal root afferents. In *Advances in Pain Research and Therapy*. ed. Bonica, J.J. Vol. 5, pp. 77–82. New York: Raven Press.

NAKANE, T., AUDHYA, T., KANIE, N. & HOLLANDER, C.S. (1985). Evidence for a role of endogenous corticotropin-releasing factor in cold, ether, immobilization, and traumatic stress. *Proc. Natl. Acad. Sci. U.S.A.*, **82**, 1247–1251.

PERNOW, B. (1983). Substance P. *Pharmacol. Rev.*, **35**, 85–141.

PLOTSKY, P.M., BRUHN, T.O. & VALE, W. (1985). Hypophysiotropic regulation of adrenocorticotropin secretion in response to insulin-induced hypoglycemia. *Endocrinology*, **117**, 323–329.

REDMOND, D.E. & KRYSTAL, J.H. (1984). Multiple mechanisms of withdrawal from opioid drugs. *Ann. Rev. Neurosci.*, **7**, 443–478.

RIVIER, C.L. & PLOTSKY, P.M. (1986). Mediation by corticotropin releasing factor (CRF) of adenohypophysial hormone secretion. *Ann. Rev. Physiol.*, **48**, 475–494.

RIVIER, C., RIVIER, J. & VALE, W. (1982). Inhibition of adrenocorticotropic hormone secretion in the rat by immunoneutralization of corticotropin-releasing factor (CRF). *Science*, **218**, 377–379.

SHARPE, L.G. & JAFFE, J.H. (1986). Neonatal capsaicin modifies morphine withdrawal signs in the rat. *Neurosci. Lett.*, **71**, 213–218.

WALKER, C.D., PERRIN, M., VALE, W. & RIVIER, C. (1986). Ontogeny of the stress response in the rat: role of the pituitary and the hypothalamus. *Endocrinology*, **118**, 1445–1451.

YAKSH, T.L., JESSELL, T.M., GAMSE, R., MUDGE, A.W. & LEEMAN, S.E. (1980). Intrathecal morphine inhibits substance P release from mammalian spinal cord *in vivo*. *Nature*, **286**, 155–157.


*(Received July 16, 1987*
*Revised January 16, 1988*
*Accepted January 22, 1988)*