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NALOXONE EXCITES OXYTOCIN NEURONES IN THE SUPRAOPTIC NUCLEUS OF LACTATING RATS AFTER CHRONIC MORPHINE TREATMENT

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SUMMARY

1. Lactating rats were implanted with a cannula in a lateral cerebral ventricle to deliver morphine (up to 50 μg/h) chronically from a subcutaneous osmotically driven mini-pump. After infusion of morphine for 5 days the rats were anaesthetized with urethane and prepared with ventral surgery for recording the electrical activity of single, antidromically identified neurones in the supraoptic nucleus.

2. A single I.V. injection of naloxone (5 mg/kg) in these rats provoked a long-lasting, large increase in intramammary pressure, but in control rats had negligible effects. Concentrations in plasma of oxytocin, measured by radioimmunoassay in samples of femoral arterial blood, rose from $44{\cdot}7 \pm 2{\cdot}5$ to $1072{\cdot}1 \pm 89{\cdot}5$ pg/ml (means $\pm$ S.E.M.) 6 min after naloxone in the morphine-treated rats. In control rats, the concentration of oxytocin in plasma rose only from $42{\cdot}1 \pm 2{\cdot}9$ to $125{\cdot}1 \pm 28{\cdot}2$ pg/ml after naloxone.

3. Naloxone produced a transient increase in arterial blood pressure in morphine-treated but not control rats. Concentrations in plasma of vasopressin, measured by radioimmunoassay in samples of femoral arterial blood, rose in morphine-treated rats from $7{\cdot}4 \pm 2{\cdot}4$ to $29{\cdot}2 \pm 3{\cdot}7$ pg/ml after naloxone, but did not rise significantly in control rats.

4. Naloxone (1–5 mg/kg) produced a prompt and prolonged increase in the discharge rate of each of ten continuously active (putative oxytocin) cells recorded from ten morphine-treated rats. The discharge rate of the six cells tested at the highest dose (5 mg/kg) increased by an average of 6·3 Hz (360 %) within 5 min, and the firing rate remained elevated for at least 30 min; the discharge rate of six continuously active supraoptic neurones recorded in control rats was not affected by naloxone.

5. The firing activity of five phasic (putative vasopressin) supraoptic neurones in morphine-treated rats was increased for at least 30 min by the injection of naloxone; these increases were the result of a raised intraburst firing rate with no change in burst duration or frequency. One phasic neurone was inhibited for 15 min, and one phasic neurone was unaffected.

6. The excitatory effects of naloxone on neurones in the supraoptic nucleus of morphine-treated rats were not explained by changes in blood pressure or osmolarity and did not depend on suckling or a cholinergic pathway.

7. The concentrations of oxytocin in plasma and the operation of the milk-ejection reflex were similar in the controls and morphine-treated rats, prior to naloxone. These findings indicate tolerance to initial inhibitory effects of morphine on oxytocin secretion. Likewise, before naloxone the concentrations of vasopressin in plasma and firing activity of phasic neurones were similar in the morphine-treated and control groups, indicating tolerance to any effects of morphine on vasopressin neurones.

8. The uniform excitation of continuous neurones, accompanied by massive secretion of oxytocin provoked by naloxone in the morphine-treated rats, indicates dependence on morphine in the central mechanisms activating these cells. This phenomenon is inconsistent and less marked for phasic neurones.

INTRODUCTION

Opioids inhibit oxytocin secretion induced by suckling, chemical transmitters, or electrical stimulation of the neurohypophysis. The inhibitory action includes naloxone-reversible effects within the neurohypophysis, either analogous to presynaptic inhibition or exerted via pituicytes (see Bicknell, 1985; Lincoln & Russell, 1986, for reviews). Inhibition of oxytocin secretion in response to suckling, and hence interruption of the milk-ejection reflex, by morphine is due to its action on the neurohypophysis (Clarke, Wood, Merrick & Lincoln, 1979). There are several opioid peptide systems within the neurohypophysis, acting upon different opioid receptor subtypes. It is not known on which of these receptors morphine acts to inhibit oxytocin secretion, though studies on secretion from the isolated neurohypophysis indicate that the κ-opioid receptor subtype may be involved (Bicknell, Chapman & Leng, 1985*a*). Opioids also inhibit the electrical activity of putative oxytocin neurones *in vitro* and *in vivo*, although neither morphine nor naloxone affect the intermittent, bursting pattern of electrical activity of these neurones seen in response to suckling (Clarke *et al.* 1979).

Chronic intracerebroventricular (I.C.V.) infusion of morphine initially blocks milk ejection but this effect is lost within 3 days despite increasing doses of morphine, provided maternal behaviour remains intact (Russell, 1984). The loss of effect of morphine is consistent with the development of tolerance in the oxytocin neurosecretory system. In other systems tolerance is a usual consequence of chronic morphine treatment (Krueger, Eddy & Sumwalt, 1941; Jaffe, 1985); the cellular mechanisms involved have not been elucidated (West & Miller, 1983).

In lactating rats treated chronically with morphine, I.V. injection of the opiate antagonist naloxone provokes a large and prolonged increase in intramammary pressure indicative of a massive release of oxytocin (Russell, 1984). In other systems such hypersensitivity to naloxone is attributed to a compensatory reaction of cells with opiate receptors during opioid exposure, and the excitation of the system when the opioid is withdrawn demonstrates dependence (Collier, 1980; Cuthbert, Francis & Collier, 1983; Redmond & Krystal, 1984).

The present experiments were performed first to confirm and quantify the

oxytocin release in these circumstances by measurement of plasma hormone levels with radioimmunoassay, and second, since opioids can inhibit oxytocin neurones both centrally and in the neurohypophysis, to determine whether oxytocin release after naloxone was the consequence solely of effects in the neurohypophysis or whether naloxone activated the cell bodies of oxytocin neurones in the morphine-dependent rats. Accordingly, we injected naloxone I.V. while recording the electrical activity of single neurones from the supraoptic nucleus of morphine-treated and control lactating rats, under urethane anaesthesia. Blood samples were taken before and after naloxone for measurement of oxytocin or vasopressin. Some of the data in this paper have been communicated to the Physiological Society (Bicknell, Leng, Lincoln & Russell, 1984*b*; Bicknell, Chapman, Leng & Russell, 1985*c*).

METHODS

Animals

Lactating, primiparous Sprague–Dawley rats were used, each nursing litters adjusted to ten pups within 48 h of birth. Rats were housed at 21–23 °C on a 12 h light–12 h dark cycle, and fed standard (breeder) diet.

Morphine infusion

On days 2–4 post-partum (day of parturition = day 1) rats were anaesthetized with ether, and a stainless-steel cannula was inserted stereotaxically through a hole drilled in the skull so that its tip was in the right lateral cerebral ventricle (2 mm lateral, 3 mm posterior to bregma, 4·0 mm below skull surface). The cannula was fashioned from 21 gauge stainless-steel tubing, 1 cm long, bent at 90 deg 4·5 mm from its tip, and was held in place by dental acrylic bonded to two stainless-steel screws (3·2 mm, 10 BA) in the skull. The cannula was connected via an 18 cm coil of Polythene tubing (1·22 mm o.d., 0·76 mm i.d.) to an osmotic mini-pump implanted subcutaneously (Alzet 2001, rated 1 μl/h for 168 h). Morphine sulphate (BP) was dissolved in sterile, pyrogen-free distilled water and finally sterilized by filtration (Millex-GS, 0·22 μm). The cannula and tubing were filled before insertion with 40 μl of this solution at a concentration of 10 μg/μl, followed by 40 μl at 20 μg/μl, separated by 1·0 μl air, and connected, after insertion, to a mini-pump containing morphine sulphate solution at 50 μg/μl. The increase in concentration of morphine each 40 h was intended to delay the loss of effects due to tolerance. In control rats the infusion system contained vehicle only.

Monitoring

Effects of the morphine infusion were monitored daily. Litter weight change and the presence of milk in the pups' stomachs were used to assess milk transfer, and indirectly the release of oxytocin. Size of the nest, care of young and nursing behaviour were assessed to monitor maternal behaviour which can be disrupted by morphine (Russell & Spears, 1984) and the consequent failure to suckle removes an input to oxytocin neurones. Rectal temperature was measured daily as an indicator of the effectiveness of the infusion system; morphine raises rectal temperature throughout its administration by this method (Rayner, Robinson & Russell, 1988).

Preparation for pressure recordings and injections

Four days after the infusion system was implanted each rat was separated from nine of its ten pups overnight, and the following morning was anaesthetized with urethane (ethyl carbamate, 1·25 g/kg I.P.). A cannula was inserted in the trachea; Polythene cannulae filled with heparinized saline were placed in the left jugular and right femoral veins for injection of naloxone and oxytocin solutions, respectively, and in the right femoral artery for withdrawal of blood and to monitor blood pressure (via Endevco 8503-15A transducers and Kontron Elektronik BS314, Lectromed MX216 or Grass polygraph recorders). Cannulae filled with 0·9% NaCl were inserted into at least two nipple ducts of the left abdominal and thoracic mammary glands to record intramammary pressure (via Gould Statham P231d pressure transducers and a Kontron 314 chart recorder or

Grass 7C polygraph). Rectal temperature was maintained close to 37 °C by a thermostatically controlled electric blanket (BioScience, U.K., CFP 8185). The rats were not suckled during these experiments. Six vehicle-infused and seven morphine-infused rats were prepared to this stage only for injection of naloxone and withdrawal of blood samples for vasopressin measurement 10 min before and 5 min after naloxone.

In a further eighteen vehicle-infused and fourteen morphine-infused rats cannulae were inserted into two nipple ducts and into the left jugular vein; no other surgery was performed. One hour after completion of surgery the sensitivity of the mammary glands to I.V. oxytocin was measured. After a further 90 min either naloxone (5 mg/kg) or saline was injected I.V.; 20 min later the rats were decapitated for collection of trunk blood into chilled, heparinized centrifuge tubes. Plasma was separated by centrifugation at 4 °C, acidified, and stored at −20 °C until assay for both oxytocin and vasopressin.

Preparation for electrophysiological recording

Rats prepared as above were placed in a stereotaxic frame, and the left supraoptic nucleus and the neural stalk were exposed by ventral surgery (Leng, 1981). A bipolar stainless-steel stimulating electrode (Type SNEX 200, Rhodes Medical Instruments) was placed on the neural stalk, and a glass micropipette filled with 0·9% NaCl (resistance 15–30 MΩ) was introduced into the region of the supraoptic nucleus to record extracellularly the electrical activity of single neurones.

Stimuli were applied to the neural stalk at 0·3 Hz (matched biphasic pulses, 1 mA peak-to-peak, 1 ms pulse width) during the search for neurones of the supraoptic nucleus. Such neurones satisfied the constant latency and collision tests for antidromic identification following stimulation of the neural stalk. Spike trains were recorded on a polygraph and on magnetic tape; spike waveforms (unfiltered DC recordings using a Grass P16 preamplifier) were analysed during the course of an experiment using a Nicolet 4094 averaging digital oscilloscope. Spike trains were analysed on-line by constructing interspike-interval histograms of 8 min segments of recording, and by a sequential log of firing rate using a Commodore 8032 microcomputer and software supplied by Biodata U.K. Ltd. Subsequent data analysis included the construction of sequential interspike-interval histograms from each block of 500 action potentials. The activity of a single neurone from each rat was monitored for up to 3 h. This included at least 15 min prior to injection of naloxone. Neurones classified as 'phasic' fired in bursts of > 5 s duration separated by silent periods of > 5 s duration; the intraburst firing rate exceeded 4 Hz. Neurones classified as 'continuous' fired uninterruptedly at a mean rate exceeding 2·5 Hz.

Drugs

Naloxone hydrochloride (Endo Laboratories) was given at doses of 1–5 mg/kg body wt I.V. (solution: 10 mg/ml in 0·9% saline) over 1 min. Atropine sulphate B.P. (British Drug Houses) was given to some rats (dose; 2·5 mg/kg body wt I.V.; solution; 5 mg/kg 0·9% saline) 15–30 min after naloxone. Hexamethonium bromide (Koch–Light Laboratories) was given as an I.V. injection of 0·05 ml of a 100 mM solution in 0·9% saline. Oxytocin was given I.V. in doses of 0·1, 0·25, 0·5 or 1 milliunits (Syntocinon, Sandoz Products; 10 milliunits/ml in 0·9% saline) to assess mammary gland sensitivity.

Blood sampling

The first 1 ml arterial blood sample was drawn into a heparinized syringe 10 min before the naloxone injection, and at least 1 h after completion of surgery. This blood was immediately replaced by 1 ml 0·9% saline at 37 °C. The second sample was taken 5–6 min after the naloxone injection, and immediately replaced with 0·5 ml 0·9% warmed saline containing the resuspended erythrocytes from the first blood sample. The blood sampling procedure affected neither blood pressure nor the discharge of any of the neurones recorded.

Radioimmunoassay

Plasma from centrifuged blood samples was acidified by addition of 10 μl of 10 M-acetic acid per millilitre plasma and stored at −70 °C until assay. Plasma samples were extracted on Sep-Pak C18 cartridges (Waters Associates, Harrow, Middx) following a modification of the method of La Rochelle, North & Stern (1980). Samples were reconstituted in assay buffer and oxytocin and vasopressin measured using specific antisera (Moore, Lutterodt, Burford & Lederis, 1977; Sheldrick

& Flint, 1981) as previously described (Bicknell, Brown, Chapman, Hancock & Leng, 1984*a*). Recoveries of iodinated or synthetic oxytocin and vasopressin were greater than 90% and plasma levels are uncorrected for recovery. For each series of experiments, oxytocin and vasopressin were measured in single assays to eliminate inter-assay variability. The intra-assay coefficients of variation were 8·7% for oxytocin and 9·0% for vasopressin. Assay sensitivities were 3·0 pg for oxytocin and 0·4 pg for vasopressin.

RESULTS

As described in the accompanying paper (Rayner *et al.* 1988), in the morphine-treated rats rectal temperature was raised compared with the controls during morphine treatment. Morphine infusion disrupted maternal behaviour irreversibly in four out of twenty-five rats in this study. The affected rats failed to maintain nests, to retrieve, group or nurse their young, which were cold and without milk in their stomachs when weighed each day (Rayner *et al.* 1988). Only those rats still exhibiting maternal behaviour, and still receiving a suckling stimulus on the day of separation from the young were used in the subsequent experiments.

Oxytocin release in response to naloxone

In the rats prepared for electrophysiological recording naloxone did not increase intramammary pressure in any of the controls, but in the morphine-treated rats intramammary pressure increased by 5–20 mmHg starting within 1 min of naloxone injection, and remained elevated for over 15 min. In the rats prepared with minimal surgery, for collection of trunk blood, similar increases in intramammary pressure were seen in all seven morphine-infused rats given naloxone. These responses began at $0{\cdot}6 \pm 0{\cdot}4$ min after the naloxone injection and peaked at $4{\cdot}1 \pm 1{\cdot}5$ min (peak increase in pressure, $9{\cdot}1 \pm 1{\cdot}7$ mmHg). No intramammary pressure changes followed saline injection in either morphine-infused or vehicle-infused rats. Smaller, late increases in intramammary pressure followed naloxone injection in four out of nine vehicle-infused rats. These increases began at $8{\cdot}2 \pm 0{\cdot}6$ min after the injection and peaked at $12{\cdot}6 \pm 2{\cdot}8$ min (peak increase, $3{\cdot}2 \pm 1{\cdot}7$ mmHg; $n = 4$).

Prior to naloxone injection in rats sampled serially, the concentrations of oxytocin in plasma were similar in eight control and five morphine-treated rats (means ± S.E.M., $42{\cdot}08 \pm 2{\cdot}9$ and $44{\cdot}7 \pm 2{\cdot}5$ pg/ml, respectively). After I.V. injection of naloxone (5 mg/kg), the plasma concentration of oxytocin in the control rats rose about 3-fold within 5 min, to a mean of $125{\cdot}1 \pm 28{\cdot}2$ pg/ml ($P < 0{\cdot}01$, paired t test). In morphine-treated rats plasma oxytocin rose 25-fold within 5 min, to a mean of $1072{\cdot}1 \pm 89{\cdot}5$ pg/ml ($P < 0{\cdot}01$, paired t test). This proportionate increase is significantly greater than that seen in the control group (Fig. 1; $P < 0{\cdot}01$, Mann–Whitney U test). Plasma oxytocin levels in trunk blood samples from rats prepared with minimal surgery were again similar in nine vehicle-infused and seven morphine-infused rats following control (saline) injection ($23{\cdot}4 \pm 1{\cdot}4$ and $24 \pm 1{\cdot}8$ pg/ml respectively). Oxytocin levels were elevated in nine vehicle-infused rats injected with naloxone 20 min previously ($41{\cdot}3 \pm 5{\cdot}9$ pg/ml; $P < 0{\cdot}01$), but significantly more elevated in seven morphine-infused rats injected with naloxone (177 ± 33 pg/ml; $P = 9{\cdot}3 \times 10^{-5}$, Kruskal–Wallis test).

Vasopressin secretion

The plasma concentration of vasopressin prior to naloxone injection was similar in the control and morphine-treated rats (means ± S.E.M., 10·2 ± 5·6 pg/ml, $n = 6$ and 7·4 ± 2·4 pg/ml, $n = 5$, respectively, no significant difference). After naloxone (5 mg/kg I.V.), the concentration of vasopressin increased to 29·2 ± 3·7 pg/ml in the

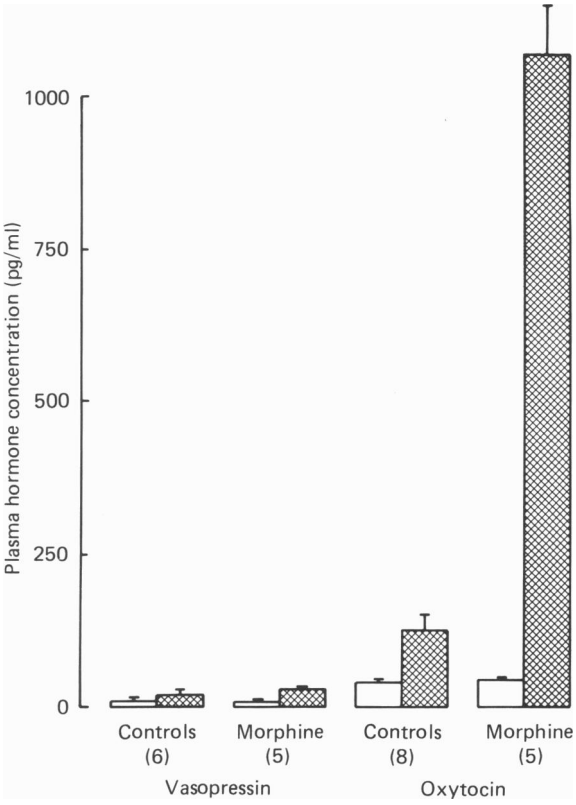

Fig. 1. Oxytocin and vasopressin measured in plasma of control and morphine-treated rats 5 min before (open bars) and 5 min after (shaded bars) injection of naloxone (5 mg/kg I.V.). Oxytocin levels were significantly raised following naloxone in control rats ($P < 0·01$, paired t test; $n = 8$) but the increase was significantly greater in the morphine-treated rats (Mann–Whitney U test, $P < 0·01$). Vasopressin levels were not significantly influenced by naloxone in the control rats; in morphine-treated rats there was a significant increase ($P < 0·01$) but the size of this increase was small in comparison with the effects on oxytocin. Number of experiments given in parentheses.

morphine-treated rats ($P < 0·01$, paired t test), and to 19·3 ± 9·3 pg/ml in the control group (rise not significant). The difference between the post-naloxone values in the control and morphine-treated groups is not significant. Vasopressin levels in trunk blood from rats prepared with minimal surgery were not significantly different between any groups (vehicle-infused rats: 22·2 ± 7·8 pg/ml, $n = 9$ control-injected rats; 8·5 ± 3·0 pg/ml, $n = 9$ naloxone-injected rats. Morphine-infused rats: 32·6 ± 15·7 pg/ml, $n = 8$ control-injected rats; 31·3 ± 13·5 pg/ml, $n = 7$ naloxone-injected rats; $P = 0·54$, Kruskal–Wallis.)

Blood pressure responses to naloxone

In the rats prepared for electrophysiology, mean arterial blood pressure (calculated as diastolic + $\frac{1}{3}$ pulse pressure) in the 5 min prior to removal of the first blood sample in the control group was 70 ± 6 mmHg and in the morphine-treated group 66 ± 8 mmHg (not significant). After naloxone, mean arterial blood pressure did not change in the controls, but rose within 1 min by a maximum of 10 mmHg and remained elevated for up to 2 min in the morphine-treated rats.

In the rats prepared for vasopressin measurement, mean arterial blood pressure was similar in the control and morphine-treated groups in the 5 min prior to naloxone (78 ± 10 mmHg, $n = 8$ and 81 ± 6 mmHg, $n = 6$, respectively) but higher than in the rats prepared for electrophysiology. After naloxone, mean arterial blood pressure did not change in the controls (mean change 0 ± 3 mmHg after 2 min), but rose by a mean of 7 ± 2 mmHg in the morphine-treated rats, and as in the rats prepared for electrophysiology, returned within 3 min to the pre-naloxone value.

Naloxone responses of supraoptic neurones in vehicle-treated rats

The responses of twelve supraoptic neurones to naloxone (5 mg/kg) were studied. Six of these were phasically active and were thus probably vasopressin cells (Poulain & Wakerley, 1982). The other six were continuously active (putative oxytocin) neurones.

The firing rate of no cell altered by more than 15% over the 10 min following naloxone injection, and overall there was no significant change in firing rate. In these control rats, the mean firing rates of each of the six continuous cells before the injection of naloxone ranged between 2·6 and 7·9 Hz over the 10 min prior to naloxone injection; the mean change in firing rate from these values, measured from the sixth to the seventh minute after the start of the naloxone injection, was $-0{\cdot}1 \pm 0{\cdot}2$ Hz, a decrease of 3%, and not statistically significant (Fig. 2). None of the phasic cells changed their firing rate or pattern after the injection of naloxone.

Naloxone responses of supraoptic neurones in morphine-treated rats

The responses of seventeen supraoptic neurones, each from a different rat, to naloxone were studied. Seven of these neurones were phasically active and ten were either continuously active (six cells) or were firing too slowly to be classified initially as either phasic or continuous, although the firing rates of these four neurones were within the normal range for supraoptic neurones (Poulain & Wakerley, 1982).

The initial mean firing rate of each of the ten non-phasic neurones, before naloxone was injected, ranged from 0 to 5·0 Hz; their firing rates and patterns were similar to those of non-phasic cells in the control rats. Each of the ten neurones responded within 30 s of the start of the naloxone injection with a clear increase in firing rate (Fig. 3). The firing rate reached a maximum within 5–10 min of the injection and then declined gradually, but the firing rate of each cell remained above the pre-naloxone rate for as long as the cell was recorded – in every case at least 30 min and in two cells for more than 2 h. Six of these cells were tested with a dose of 5 mg naloxone/kg, one with a dose of 2·5 mg/kg, and three at a dose of 1 mg/kg. For the six cells tested at the high dose, the mean increase in firing rate at 6 min after the injection was

6·3 ± 1·3 Hz (360%; Fig. 2). All ten neurones fired continuously after naloxone, throughout the duration of recording, with interspike-interval histograms similar to those of continuously firing neurones following activation by I.P. injection of hypertonic saline (Brimble & Dyball, 1977), i.e. with modes in the range 35–60 ms

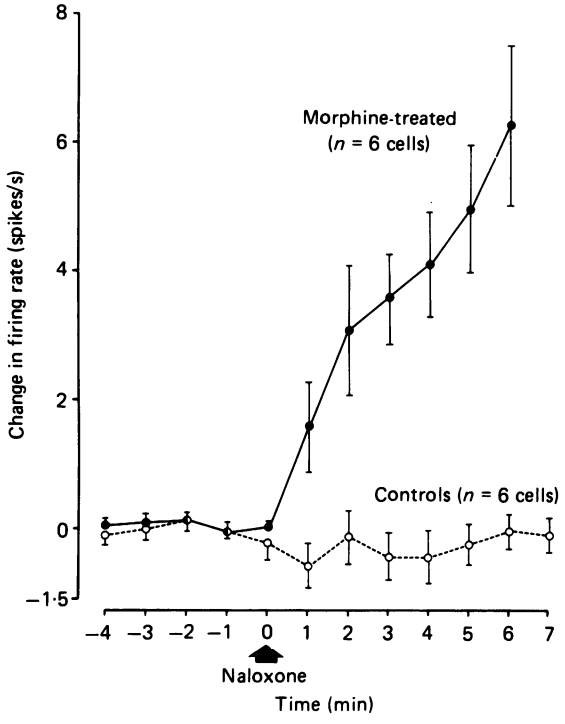

Fig. 2. Effects of naloxone (5 mg/kg I.V.) on the firing rate of six putative oxytocin cells from control rats and six from morphine-treated rats. There was no significant effect on the activity of the cells from control rats, but each of the cells in morphine-treated rats was strongly excited. Data are means (±S.E.M.) of the firing rates in each minute, expressed as differences from a mean spontaneous firing rate measured over the 10 min preceding the naloxone injection.

and with fewer than 20% of intervals in the range 0–30 ms (data not shown). Three of these cells were tested with a second, identical injection of naloxone at least 30 min after the first, and each again showed a prolonged, but weaker excitatory response.

Phasic neurones

The initial firing pattern and mean firing rates of the seven neurones tested with naloxone, and those of seven other phasic cells recorded before naloxone, were similar to those of phasic cells in the vehicle-treated controls and in normal animals: intraburst firing rates were all in the range 4–8 Hz, mean burst durations in the range 10–40 s, mean interburst intervals in the range 15–30 s, and modal interspike intervals in the range 35–55 ms.

Following I.V. injection of 5 mg naloxone/kg, one of the phasic neurones was

inhibited for 15 min, one was unaffected, and the remaining five neurones each increased their firing rate for at least 30 min. The mean increase in overall firing rate of the five excited cells was 2·0 Hz. In these cells, the effects of naloxone began within 30 s. Although they continued to fire phasically after the injection of naloxone, with burst lengths and silence lengths similar to the pre-naloxone pattern of activity,

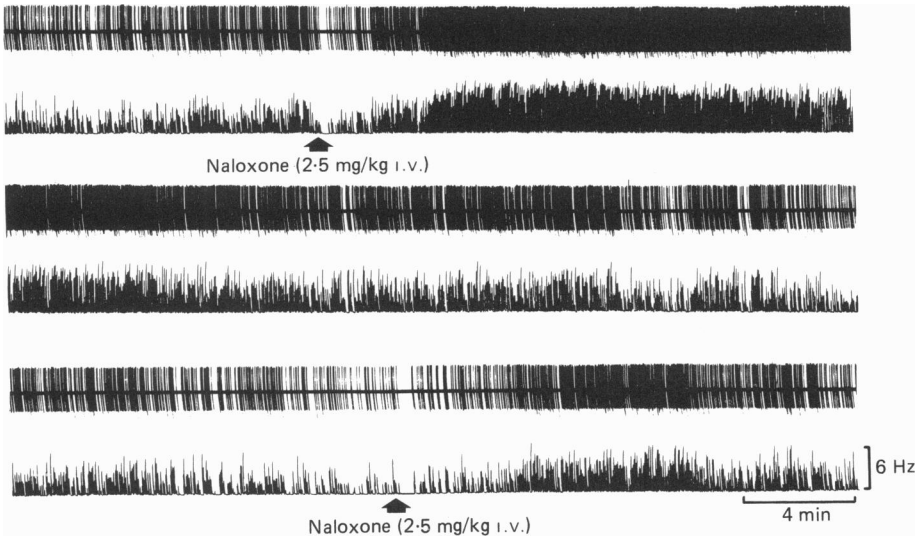

Fig. 3. Effects of naloxone on a continuously active (putative oxytocin) supraoptic neurone from a morphine-treated rat: continuous polygraph record showing (upper trace) single spikes and (lower trace) integrated rate-meter record. The arrows mark the beginning of each injection. Naloxone (2·5 mg/kg I.V.) provoked a prolonged rise in firing rate of this neurone; a second injection given more than 1 h later produced a second, smaller excitation.

firing within bursts was characterized by unusual irregularity. Short, high-frequency discharges (6–12 spikes per train at up to 50 Hz) occurred frequently within the bursts (Fig. 4). This behaviour, which was clearly evident when monitoring the firing audio-visually, was analysed in a comparison of interspike-interval histograms before and after naloxone. In each of four excited cells, there was a greatly increased incidence of short (< 40 ms) intervals after naloxone (Fig. 5). This response is in marked contrast to the response of normal phasic neurones to haemorrhage or osmotic stimulation, characterized by prolongation of bursts rather than changes in intraburst firing rate or pattern (Fig. 6).

Action potential characteristics

Antidromically identified neurones in the supraoptic nucleus were also characterized by the waveforms of their action potentials, which were prolonged and had a distinct second peak on the descending slope (Mason & Leng, 1984). These characteristics were present in both the control and the morphine-treated rats, and were not affected by naloxone (data not shown).

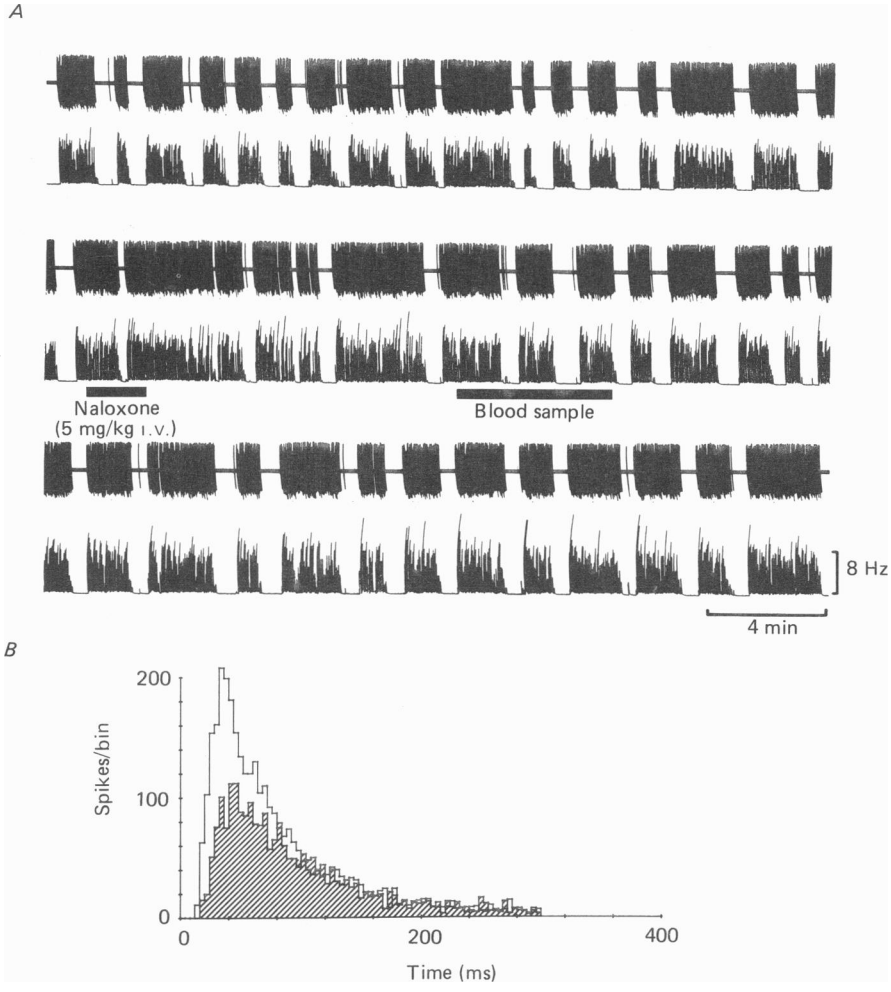

Fig. 4. Effects of naloxone on a phasic (putative vasopressin) neurone from the supraoptic nucleus of a morphine-treated rat. *A*, continuous polygraph record showing (upper trace) single spikes and (lower trace) rate-meter record. Naloxone (5 mg/kg I.V.) did not produce a marked change in the phasic pattern of discharge: burst lengths and silent lengths following injection were similar to those observed before. However, the intraburst firing rate was elevated, and within bursts there was an increased incidence of brief high-frequency trains. This change is apparent in a comparison of interspike-interval histograms. *B*, shaded histogram, before naloxone; open histogram, after naloxone. These histograms were constructed from 8 min segments of discharge, and the comparison shows that following naloxone there was an increased incidence in the number of short (< 50 ms) interspike intervals but a very similar incidence of relatively long (> 100 ms) intervals.

Cholinergic blocking agents

As I.C.V. carbachol can elicit comparable intramammary pressure responses to those seen in the present experiments with naloxone (Clarke, Fall, Lincoln & Merrick, 1978; Rayner *et al.* 1988), we attempted to determine whether the same pathway is involved by administering I.V. atropine (2·5 mg/kg), which blocks the carbachol-

induced response. Neither atropine (five cells tested), nor the nicotinic cholinergic antagonist hexamethonium (four cells tested) affected the elevated neuronal firing rate (Fig. 7). Both atropine and hexamethonium were effective in producing a sustained fall in blood pressure of at least 20 mmHg.

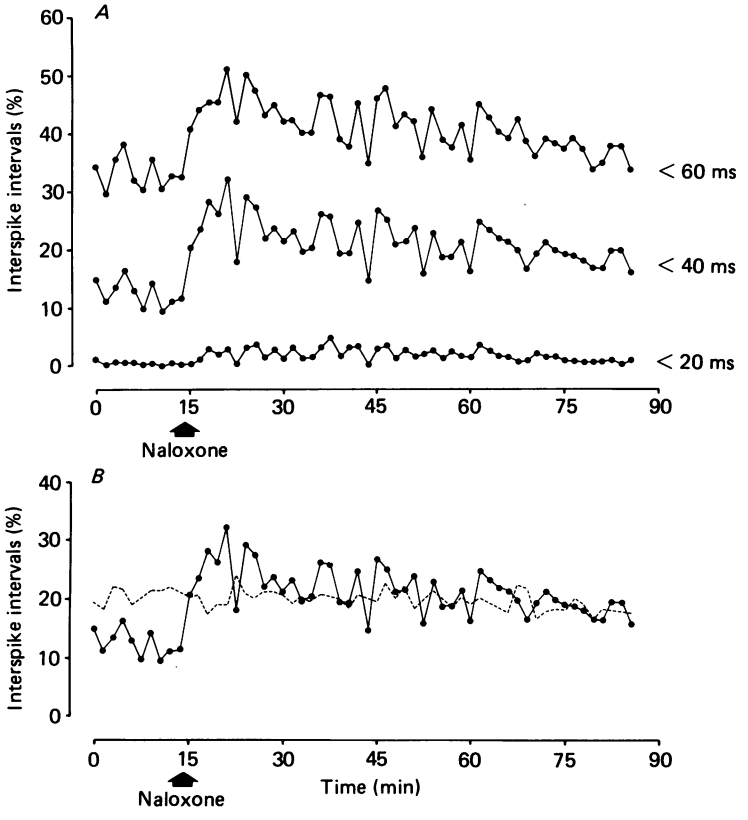

Fig. 5. Analysis of the change in firing pattern of the phasic cell shown in Fig. 4 following naloxone. Interspike intervals were constructed from consecutive blocks of 500 spikes. *A*, the percentage of intervals < 20, < 40 and < 60 ms in duration in each histogram are plotted. Following naloxone there is a sustained increase in the proportion of short intervals. *B*, the continuous line gives the percentage of intervals < 40 ms in each histogram, as given above; the dashed line shows the percentage of intervals in the range 40–60 ms. Following naloxone, there is a change specifically in the proportion of intervals < 40 ms in duration.

Oxytocin-releasing capacity of continuous neurone firing pattern

Naloxone increased the firing rate of continuous neurones in the morphine-treated rats to 5–15 Hz within 5 min. Electrical stimulation of the neurohypophysis in normal rats at 5 Hz rarely produces a change in intramammary pressure, and stimulation at 10 Hz produces a smaller rise than was seen in the morphine-treated rats (data not shown). However, the pattern of stimulation is an important factor in determining hormone release (Dutton & Dyball, 1979; Bicknell *et al.* 1984*b*). To determine whether the increase in firing rate of continuous neurones by naloxone in

morphine-treated rats could evoke release of sufficient oxytocin to cause the observed increase in intramammary pressure we attempted to mimic this cell-body excitation in experiments on three normal lactating rats. A 9 min segment of a recording from a probable oxytocin neurone in a morphine-treated rat, made during a naloxone injection, was used to stimulate the neural stalk. Each recorded action

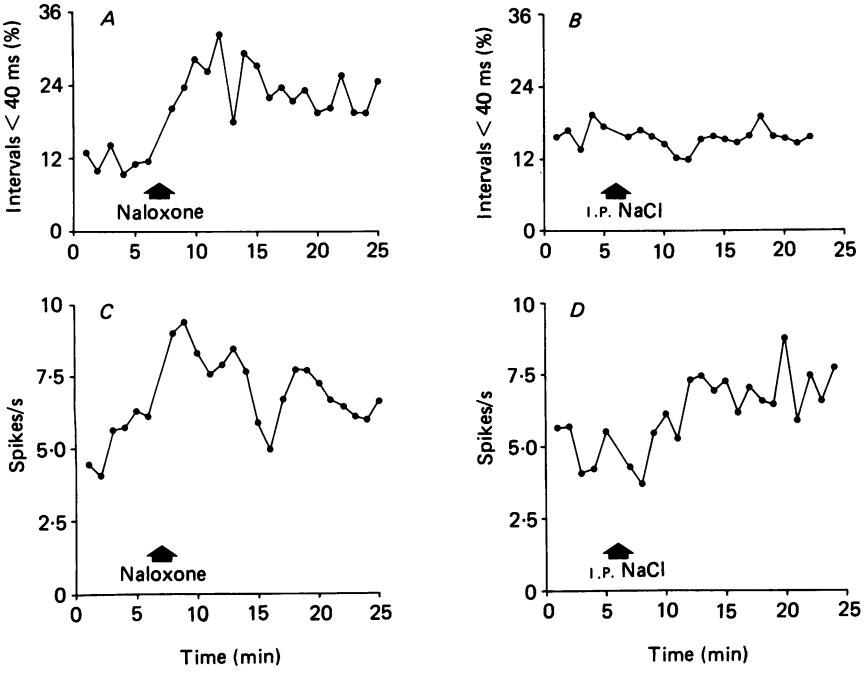

Fig. 6. Data from the phasic cell shown in Figs 4 and 5. Change in incidence of short interspike intervals following naloxone (*A*) compared with change in mean firing rate (*C*). Osmotic stimulation (I.P. injection of 1 ml of 1·5 M-NaCl) did not produce a similar change in firing pattern (*B*) although it did produce a sustained increase in firing rate (*D*). The cell was tested with the osmotic stimulus 90 min after injection of naloxone.

potential triggered a stimulus pulse (1 mA; 1 ms) to the neural stalk, and the mammary gland response to the stimulation was monitored. Stimulation with the discharge activity of this neurone (mean rate 3·0 Hz) in the 2 min before the morphine-treated rat was given naloxone produced no change in intramammary pressure; but the rate (steady increase to 12·5 Hz) and pattern of activity recorded in the 6 min following naloxone stimulated a large, fluctuating rise in intramammary pressure in each normal rat. These responses followed a time course similar to that seen in morphine-treated rats after naloxone (Fig. 8). Naloxone (5 mg/kg I.V.) increased the response to such stimulation in each of the rats studied.

To investigate whether the observed increases in firing rate of oxytocin neurones could account for the very large rises in plasma oxytocin seen following naloxone injection in morphine-infused rats, we measured the oxytocin released in normal rats by electrical stimulation of the neural stalk with a discharge frequency and pattern recorded in a morphine-infused rat following naloxone. In six rats, the neural stalk

was stimulated electrically using the 9 min segment of recording used in the above experiments and shown in Fig. 8. Naloxone was injected at the appropriate time during the stimulation, and 1 ml arterial blood samples were taken 6 min before and 6 min following the injection. Again, the stimulation resulted promptly in large and prolonged fluctuations in intramammary pressure. Plasma oxytocin levels measured

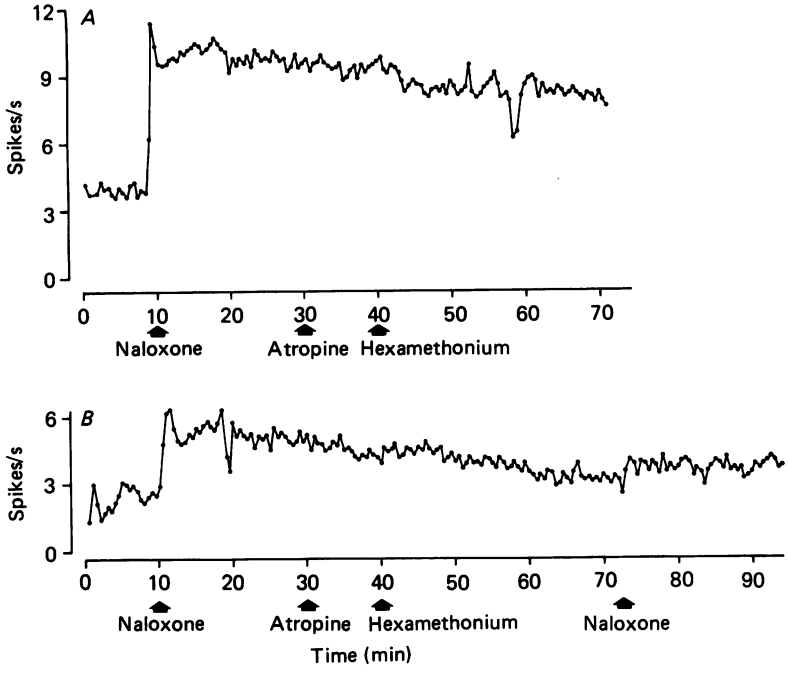

Fig. 7. Responses of two continuously active (putative oxytocin) cells (*A* and *B*) from the supraoptic nucleus of morphine-treated rats. Both cells showed a dramatic and sustained increase in firing rate following injection of naloxone (5 mg/kg I.V.). The response of neither cell was affected by I.V. injection of either atropine or hexamethonium. In the cell in *B*, a second injection of naloxone, given about 1 h after the first, produced a smaller response.

before stimulation were $27{\cdot}4 \pm 4{\cdot}6$ pg/ml, and after stimulation and naloxone were 1725 ± 520 pg/ml (range 418·8–3907 pg/ml). In five control rats, prepared identically but not stimulated, plasma levels before and after naloxone were $24{\cdot}5 \pm 2{\cdot}7$ and $75{\cdot}5 \pm 26{\cdot}2$ pg/ml respectively (range 18·2–175·9 pg/ml; $P < 0{\cdot}01$ *vs.* stimulated).

Neurones outside the supraoptic nucleus

To investigate whether naloxone produced similar excitatory effects to those seen in supraoptic neurones indiscriminately in the anterior hypothalamus, five neurones just outside the supraoptic nucleus were studied in morphine-treated rats during naloxone injection (5 mg/kg). These cells were not activated by stimulation of the neural stalk, and fired with action potentials which lacked the second depolarizing peak typical of action potentials from supraoptic neurones. Three of the neurones were promptly silenced by naloxone injection. No recovery was observed in two cells within the following 20 min, and the third cell returned to its control firing rate

within 40 min. One neurone showed a marked, but transient, excitation lasting for 2 min; for the next hour this cell fired with an altered pattern of discharge, but with a mean discharge rate similar to the pre-naloxone rate. One cell was unaffected by naloxone.

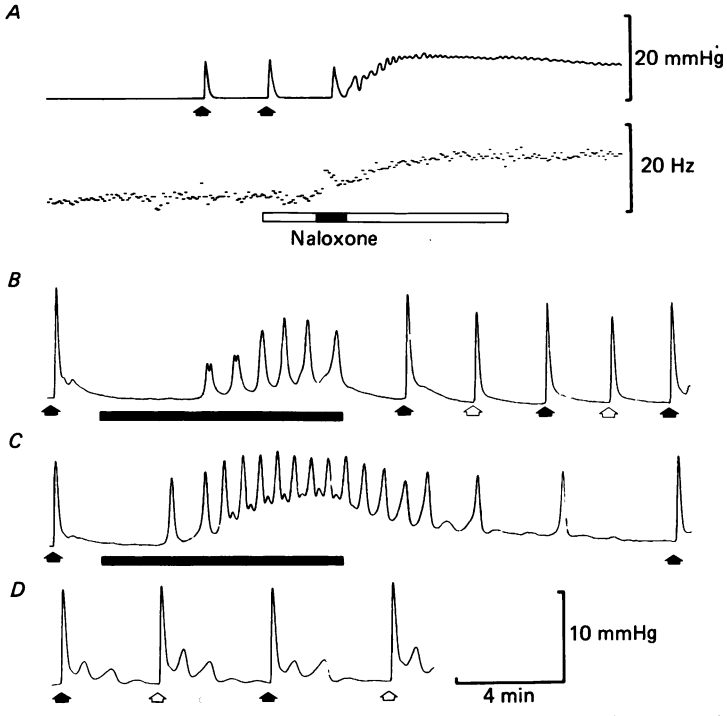

Fig. 8. *A*, intramammary pressure response (top) to 1 milliunit oxytocin (arrows) and to naloxone (filled bar; 5 mg/kg I.V.) in a morphine-treated rat, shown above the simultaneously recorded activity of a continuously active supraoptic neurone. *B–D*, intramammary pressure recording from a morphine-naive rat in response to 1 milliunit oxytocin (filled arrows), to high-frequency neural stalk stimulation (open arrows; 90 pulses at 50 Hz; 1 mA), and to neural stalk stimulation where the pulses were given according to the pattern of discharge recorded from the neurone illustrated in *A* (filled bars). The portion of recording used is indicated by the open bar in *A*. *B* shows the initial response to this stimulation, *C* shows the response following I.V. injection of 5 mg naloxone/kg. Even in morphine-naive rats naloxone potentiates the effects of neural stalk stimulation. Note that the response to high-frequency stimulation exceeds the response to 1 milliunit oxytocin I.V. after naloxone (*D*), whereas before naloxone 1 milliunit oxytocin I.V. evoked the greater response. Following naloxone there has been a change in gland sensitivity to exogenous oxytocin. Such a change was often observed and may reflect the altered basal level of plasma oxytocin.

DISCUSSION

The present data confirm that naloxone precipitates a massive release of oxytocin in lactating rats treated chronically with I.C.V. morphine. By contrast, naloxone causes relatively little vasopressin release in such rats. The extent of the increase in oxytocin secretion is similar to the increase in luteinizing hormone secretion in male rats given naloxone after morphine treatment for 48 h (Cicero, Owens, Schmoeker & Meyer, 1983); this response may be preceded by increased secretion of luteinizing-

hormone-releasing hormone. The marked release of oxytocin after naloxone-induced withdrawal from morphine is interpreted as showing, in the mechanisms leading to oxytocin release, the presence of opioid receptors that bind morphine in a manner reversible by naloxone.

In morphine-infused rats, plasma oxytocin levels increased by over 20-fold, whereas the firing rate of oxytocin neurones increased by 3- to 4-fold (by a mean of 6·3 Hz). Is this increase in firing rate sufficient to account for the observed change in oxytocin release? It is well established that the release of oxytocin is strongly facilitated with increasing frequencies of stimulation. *In vitro*, the amount of oxytocin released per stimulus pulse from the isolated neurohypophysis is approximately doubled with every doubling of stimulation frequency between 6 and 30 Hz (Leng & Bicknell, 1984). *In vivo*, oxytocin release following systemic osmotic stimulation rises 5-fold in response to a mean increase of 2·8 Hz in the firing rate of oxytocin neurones (Brimble, Dyball & Forsling, 1978). Thus the observed 6·3 Hz (360%) increase in firing rate is consistent with a 7- to 10-fold increase in oxytocin release. In addition however, oxytocin release evoked from the neurohypophysis of normal rats is approximately doubled in the presence of naloxone (Bicknell & Leng, 1982). Thus the combination of the increased firing rate and the expected facilitatory effects of naloxone at the neurohypophysis may be expected to produce at least a 14- to 20-fold increase in oxytocin release – close to what was observed in the morphine-infused rats. This was tested directly: electrical stimulation of the neural stalk in normal rats with a rate and pattern mimicking the activation of oxytocin neurones in morphine-dependent rats, together with I.V. naloxone, resulted in plasma oxytocin levels at least as high as those measured in morphine-dependent rats following naloxone.

Central or neurohypophysial site of action?

An important question is whether the opioid receptors at which naloxone acts in morphine-dependent rats are on the perikarya of oxytocin neurones, on some afferent input to them, or in the neurohypophysis. In control rats there was a significant rise in oxytocin release after naloxone, demonstrating active inhibition by endogenous opioids. Similar effects of naloxone or naltrexone have been reported in conscious, morphine-naive rats during parturition, water deprivation or after haemorrhage (Hartman, Miller, Rosella-Dampman, Emmert & Summy-Long, 1984; Summy-Long, Miller, Rosella-Dampman, Hartman & Emmert, 1984). In our control rats, the electrical activity of oxytocin cell bodies did not change after naloxone, thus the site of action of naloxone must be on the axons of oxytocin neurones or in the neurohypophysis (cf. Clarke *et al.* 1979). However, the present results demonstrate that there is also a central site of action of opioids on oxytocin neurones. In morphine-treated rats the receptors occupied by morphine cannot be only in the neurohypophysis because in these rats naloxone increased the electrical activity of oxytocin cell bodies. The simplest hypothesis, that opiate receptors are located on the oxytocin cell perikarya, is consistent with the effects of opiates *in vitro* on oxytocin neurones in morphine-naive hypothalamic slices (e.g. Wakerley, Noble & Clarke, 1983) and with the reported presence of fibres and terminals of opioid-peptide neurones close to oxytocin neurones in the paraventricular and supraoptic nuclei.

A wide range of opioid peptides are associated with oxytocin neurones. Leu-enkephalin, met-enkephalin and dynorphin are present in the neurohypophysis in, respectively, separate fibres, oxytocin terminals and vasopressin terminals (Martin, Geis, Holl, Schafer & Voigt, 1983; van Leeuwen, Pool & Sluiter, 1983; Whitnall, Gainer, Cox & Molineaux, 1983), while β-endorphin could reach the oxytocin terminals from the circulation. Terminals containing adrenocorticotrophin 1–39 are found near to oxytocin neurones in the supraoptic and paraventricular nuclei, and these arise from presumptive pro-opio-melanocortin neurones in the arcuate nucleus (Sawchenko, Swanson & Joseph, 1982). Both δ- and μ-receptors may be involved centrally in the control of oxytocin neurones (Muehlethaler, Gaehwiler & Dreifuss, 1980; Wakerley *et al.* 1983); the rat hypothalamus is rich in opioid μ-receptors (Goodman, Snyder, Kuhar & Young, 1980), and there are opioid binding sites in the supraoptic and paraventricular nuclei (Atweh & Kuhar, 1983; Clark, Hall, Brown, Daum, Sharif, Day & Hughes, 1986).

Naloxone may have excited oxytocin neurones in the morphine-treated rats via actions on afferent pathways. Neither morphine nor naloxone consistently affect the activation of oxytocin neurones by suckling (Clarke *et al.* 1979), but the sensitivity of other pathways to opioids is not established. The subfornical organ and organum vasculosum of the lamina terminalis, structures essential for osmotic activation of oxytocin neurones (Russell, Hatton & Robinson, 1984), contain opioid-peptide receptors and nerve terminals (Finley, Maderdrut & Petrusz, 1981; Atweh & Kuhar, 1983). This anterior pathway may have a cholinergic link (Hatton, Ho & Mason, 1983), but in the present experiments cholinergic antagonists failed to influence the responses of oxytocin cells to naloxone. Oxytocin neurones are relatively insensitive to changes in blood pressure (Harris, 1979) and the fall in blood pressure after cholinergic antagonists were given had no effect on their firing rate. The failure of atropine and hexamethonium to affect the excitation of oxytocin neurones by naloxone in morphine-treated rats excludes a cholinergic pathway from involvement, although stimulation of oxytocin release by acetylcholine is blocked by opioids (Haldar, Hoffman & Zimmerman, 1982).

The physiological role of the central endogenous opioid system is not established. As naloxone had no effect on the electrical activity of oxytocin neurones in our anaesthetized control rats, the system is not tonically active in these conditions, unlike that in the neurohypophysis.

Tolerance to morphine develops centrally

Chronic I.C.V. morphine initially blocks the suckling-induced milk ejection in lactating rats, but this reflex returns to normal, despite the continuing presence of morphine, within 3 days of the start of morphine infusion (Russell, 1984). At this time, prior to naloxone injection, the plasma levels of oxytocin were similar in control and morphine-treated rats. These findings indicate that tolerance to morphine develops after exposure for 3 days. As the neurohypophysis itself is one site of inhibition of suckling-induced oxytocin secretion (Clarke *et al.* 1979), morphine tolerance must develop both centrally and in the neurohypophysis.

In control rats, naloxone increased oxytocin secretion by an action on the neurohypophysis. Since the concentrations of oxytocin in plasma before naloxone

were similar in the control and morphine-treated rats, it is possible that there was also endogenous opioid-peptide inhibition in the neurohypophysis in the latter, and lack of cross-tolerance between these opioids and morphine as has been suggested from *in vitro* studies (Bicknell, Chapman, Leng & Russell, 1985*b*). The mechanism of tolerance in other systems is not certain, but a decreased number of opioid receptors has not been demonstrated (discussed by Redmond & Krystal, 1984) and loss of effects on ionized calcium influx with increased ability of the affected cells to produce cyclic AMP may account for the phenomenon (Collier, 1980; West & Miller, 1983).

Dependence in the mechanisms leading to activation of oxytocin cells

During opiate exposure, the reaction of an opioid-sensitive cell to offset the inhibitory effect of the opiate restores normal excitability. When the opiate is displaced by naloxone the cell is left hyperexcitable, a cellular expression of dependence (Cuthbert *et al.* 1983). The effects of withdrawal are thus a magnified, mirror-image of the initial effect of the opiate. The dramatic excitation of oxytocin neurones by naloxone, which occurred only in morphine-treated rats, suggests that dependence develops in the mechanisms leading to the activation of oxytocin cells.

Similar neuronal excitation following naloxone administration to rats treated chronically with morphine has been observed in the locus coeruleus, frontal cortex, and the ventromedial nucleus of the hypothalamus (Aghajanian, 1978; Fry, Herz & Zieglgansberger, 1980; Dafny, 1982). Opiate dependence is also expressed by the final cholinergic neurones in the gut myenteric plexus (Cuthbert *et al.* 1983). Hippocampal CA1 cells are inhibited during naloxone-induced withdrawal, possibly due to dependence in inhibitory neurones that synapse on the CA1 cells (French & Zieglgansberger, 1982). The few neurones outside the supraoptic nucleus that we studied in morphine-treated rats did not show the long-lasting, large excitatory response to naloxone seen in oxytocin neurones, and indeed three of five cells were inhibited.

The lack of change in action potential waveform in any of the oxytocin neurones after naloxone indicates no gross change in the calcium-dependent depolarization that is reflected in the second component of the action potential (Mason & Leng, 1984). Acutely, morphine prolongs the calcium-dependent after-hyperpolarization in enteric neurones (Tokimasa, Morita & North, 1981), but the effects of opiates on the similar hyperpolarization in supraoptic neurones (Andrew & Dudek, 1984) are not known.

Vasopressin neurones

In comparison to the effects on oxytocin release, the increase in vasopressin release in morphine-treated rats after naloxone was small, and not significantly different from that in controls. In normal conscious rats naloxone has no effect on unstimulated vasopressin secretion (Kamoi, White & Robertson, 1979; Knepel, Nutto, Anhut & Hertting, 1980; Hartman *et al.* 1984). Opioids given peripherally or by I.C.V. injection can inhibit vasopressin secretion and responses to osmotic stimulation or excitatory transmitters (Aziz, Forsling & Woolf, 1981; Summy-Long,

Rosella & Keil, 1981; Summy-Long, Keil, Sells, Kirby, Chee & Severs, 1983), although there are also stimulatory effects with large doses, and antidiuretic actions of opiates that probably do not involve vasopressin (Huidobro & Huidobro-Toro, 1979; Aziz *et al.* 1981). According to some, though not all, reports, electrically stimulated vasopressin secretion from the neurohypophysis *in vitro* is inhibited by β-endorphin, D-Ala-D-Leu-enkephalin and morphine (Iversen, Iversen & Bloom, 1980; Clarke & Patrick, 1983; Wright & Clarke, 1983). However, inhibition by morphine is not reversed by naloxone (Wright & Clarke, 1983), and antagonism with naloxone of endogenous opioids released from the neurohypophysis during electrical stimulation selectively enhances oxytocin rather than vasopressin secretion (Bicknell & Leng, 1982; Knepel & Meyer, 1983). Opioids inhibit vasopressin neurones *in vivo* in a naloxone-reversible manner (Arnauld, Cirino, Layton & Renaud, 1983; Wright & Clarke, 1983), but *in vitro* in hypothalamic slices, few vasopressin cells are inhibited by morphine or other opioids (Muehlethaler *et al.* 1980; Pittman, Hatton & Bloom, 1980; Wakerley *et al.* 1983), suggesting that opioids act on an afferent input rather than directly on the neurones.

The present results in morphine-treated rats confirm that the neurohypophysis is not the only site of action of morphine with respect to vasopressin neurones. The five phasic cells stimulated by naloxone showed an abnormal pattern of excitation in that activity was increased only within bursts which were not lengthened or more frequent. Stimulation of vasopressin neurones by osmotic pressure or haemorrhage increases burst length and has little effect on frequency within bursts (Poulain & Wakerley, 1982). The effects of naloxone in morphine-treated rats were thus not those that would be expected if the osmotic or cardiovascular inputs to the neurones were stimulated. The small change in vasopressin secretion in the morphine-treated rats could reflect mixed inhibitory and excitatory effects on vasopressin neurones, but the pattern of excitation may also be important. Increased proportion of short interspike intervals without an increase in burst length is not expected to increase vasopressin release *pari passu* (Bicknell *et al.* 1984*b*).

Conclusion

The most striking withdrawal responses induced by naloxone were seen in putative oxytocin neurones, and these led to a massive increase in oxytocin secretion. This appears to be the first description of opiate tolerance and dependence in an identified peptidergic neuronal system. There is some evidence that an endogenous opioid system modulates oxytocin release during parturition in the rat (Leng, Mansfield, Bicknell, Dean, Ingram, Marsh, Yates & Dyer, 1985; Cutting, Fitzsimons, Gosden, Humphreys, Russell, Scott & Stirland, 1986). The question thus arises as to whether tolerance and dependence ever develop to the endogenous opioids, and whether in particular a withdrawal response occurs under physiological circumstances as a mechanism to evoke hypersecretion of oxytocin. Parturition in the rat is certainly associated with a large and sustained release of oxytocin, and the mechanisms responsible for this have yet to be established.

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