Central inhibitory effects of muscimol and bicuculline on the milk ejection reflex in the anaesthetized rat

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1. In order to determine whether GABAergic mechanisms are involved in the control of the milk ejection reflex in the rat, we examined the effects of central administration of a $GABA_A$ receptor agonist (muscimol) and antagonist (bicuculline) on the milk ejection reflex in the urethane-anaesthetized rat.

2. Intracerebroventricular (I.C.V.) injection of both muscimol ($n = 17$), at doses of 5, 10 and 20 ng, and bicuculline ($n = 15$), at doses of 0·01, 0·1 and 0·3 μg, inhibited the milk ejection reflex in a dose-dependent manner. The bicuculline-induced inhibition was accompanied by desynchronization of the electroencephalogram and, at the highest dose, by alteration in the sensitivity of the mammary gland to oxytocin. No significant effect on the milk ejection reflex was seen with I.C.V. isotonic saline ($n = 5$).

3. Injection of 20 ($n = 5$) or 40 ng ($n = 2$) muscimol or 0·1 μg bicuculline ($n = 5$) I.C.V. did not significantly alter the rise in intramammary pressure evoked by electrical stimulation of the neurohypophysis.

4. Bilateral 400 nl microinfusions directly into the supraoptic nuclei of either muscimol (20–100 ng ml^{-1}; $n = 10$) or bicuculline (0·15 ng ml^{-1}; $n = 5$) resulted in an inhibition of the milk ejection reflex, which was not accompanied by desynchronization of the electroencephalogram.

5. The effects of I.C.V. injections of muscimol (15 and 20 ng) and bicuculline (0·01, 0·12 and 0·3 μg) on the electrical activity of twenty-seven antidromically identified supraoptic magnocellular neurones were examined. Both compounds resulted in an inhibition of the background firing of oxytocinergic and vasopressinergic cells, and delayed the occurrence of high frequency bursts in oxytocin neurones. In five supraoptic neurones, bicuculline induced a transient activation before inhibition.

6. The powerful inhibitory action on the milk ejection reflex of both muscimol and bicuculline provides evidence for the importance of GABA neurones in maintaining the functional integrity of the mechanisms which allow the intermittent and pulsatile release of oxytocin during suckling.

In the lactating rat, suckling leads to the pulsatile release of oxytocin, which acts on the myoepithelial cells of the mammary gland to induce milk ejection. Reflex milk ejections persist under urethane anaesthesia: they begin 10–20 min after the onset of suckling and then occur every 5–10 min (Lincoln, Hill & Wakerley, 1973). Extracellular recordings of oxytocin neurones during suckling (see Poulain & Wakerley, 1982) show that they maintain a steady background activity ranging from 1 to 10 spikes s^{-1} and that just before each milk ejection the neurones suddenly fire at up to 50 Hz for 1–4 s, with a rise in intramammary pressure inducing milk ejection occurring some 15–20 s later. At the end of each high frequency discharge the neurones display a short (5–20 s) after-inhibition. These high frequency bursts are highly stereotyped. They occur synchronously amongst almost all magnocellular oxytocin cells of the hypothalamus (Belin & Moos, 1986) in response to a continual rather than an intermittent stimulus, and are a specific response to suckling (Poulain, Wakerley & Dyball, 1977). However, we still remain in considerable ignorance about the means by which oxytocin cells fire synchronously, how bursting activity can be elicited specifically as a response to some, but not other excitatory inputs and about how and where the system transduces a continuous stimulus into an intermittent response (see Leng, 1988).

Morphological evidence suggests an important role for the inhibitory neurotransmitter γ-aminobutyric acid (GABA) in regulating the activity of oxytocin neurones (Theodosis, Paut & Tappaz, 1986; Decavel & Van Den Pol, 1990). Supraoptic neurones receive a rich GABAergic innervation: oxytocin and vasopressin cells are contacted in a similar fashion by GABA synapses, which represent nearly half of the synapses within the nucleus (Theodosis *et al.* 1986; Gies & Theodosis, 1994). Moreover, GABAergic terminals often form synapses with two or more postsynaptic oxytocinergic elements in the same plane of section (shared synapses). Such a synaptic configuration is most often encountered in the supraoptic nuclei of lactating animals, which suggests that GABAergic synapses may participate in the structural plasticity that the nucleus undergoes when its neurones are highly stimulated (Theodosis *et al.* 1986; Gies & Theodosis, 1994). The source of GABAergic innervation to the supraoptic nucleus has not been fully investigated but is believed to come at least in part from the perinuclear zone of the supraoptic nucleus (Roland & Sawchenko, 1993). Electrophysiological data also suggest an important role for GABA within the supraoptic nucleus. Extracellular recordings *in vivo* have shown powerful inhibitory actions of GABA on the electrical activity of putative oxytocin neurones (Bioulac, Gaffori, Harris & Vincent, 1978; Arnaud, Cirino, Layton & Renaud, 1983) while *in vitro* experiments have been able to demonstrate a direct hyperpolarizing action of GABA within the supraoptic nucleus (Mason, Poulain & Cobbett, 1987; Randle & Renaud, 1987). Furthermore, evidence of frequent spontaneous GABA-mediated inhibitory postsynaptic potentials in supraoptic neurones (Randle, Bourque & Renaud, 1986; Wuarin & Dudek, 1993) suggests that GABA may be involved in maintaining resting background electrical activity.

In order to determine whether GABAergic mechanisms are involved in the control of the milk ejection reflex in the rat we investigated the effects of intracerebroventricular (I.C.V.) injections of a $GABA_A$ receptor agonist (muscimol) and antagonist (bicuculline) on the pattern of the milk ejection reflex, the amplitude of milk ejections induced by electrical stimulation of the neurohypophysis, and the electrical activity of identified oxytocin neurones in the supraoptic nucleus during suckling. In addition, we examined the effects on the pattern of the milk ejection reflex of bilateral microinfusions of both compounds direcly into the supraoptic nuclei.

METHODS

Animals

All experiments were performed on Wistar rats issued from the INSERM 176 (Bordeaux) breeding unit (280–380 g body weight) on day 9–12 of lactation. Animals were maintained in a 14 h light–10 h dark environment, and given food and water *ad libitum*.

Surgical preparations

On days 8–11 of lactation, lactating rats were separated from all but one of their pups. The following morning, rats were anaesthetized with urethane (1·1 g kg^{-1} I.P.) plus xylazine (4 mg I.M.). This procedure is known to provide a stable level of anaesthesia for several hours (Isherwood & Cross, 1980). A silicone catheter (Silastic, Dow Corning, Midland, MI, USA) was inserted into the right jugular vein. To obtain records of intramammary pressure, a plastic cannula (PP 50, Portex, Hythe, UK) was placed in the teat duct of an inguinal mammary gland and connected to a pressure transducer (P 25, Statham). The animals were then placed in a stereotaxic frame (Narishige Equipment, Tokyo, Japan) where rectal temperature was maintained at 36·5–37·5 °C by a thermostatically controlled electric blanket (Animal Blanket Control Unit, Ealing, UK). A 10 μl Hamilton syringe was positioned stereotaxically with its needle inserted into the third ventricle, and two silver electrodes were positioned in small holes drilled in the frontal cranium to record the electroencephalogram (EEG) on a pen recorder. For electrophysiological recordings, a bipolar stimulating electrode (SNEX 100, Rhodes Medical Instruments, Paris, France) was lowered into the pituitary stalk to enable antidromic activation of magnocellular neurones, and a glass micropipette filled with a 2% Pontamine Sky Blue–0·7 M KCl solution was positioned into the supraoptic nucleus. For microinfusion experiments, instead of positioning a Hamilton syringe with its needle inserted into the third ventricle, bilateral infusion cannulae (o.d. 0·25 mm) were inserted so that their tips were located immediately above or within each supraoptic nucleus. Each infusion cannula was connected to a 10 μl Hamilton syringe by Silastic and polythene tubing. The two syringes were placed in a microlitre infusion pump (Harvard Apparatus, South Natick, MA, USA). For control animals, the connecting tubing, syringes and infusion cannulae were filled with an artificial cerebrospinal fluid (ACSF) of composition (mM): 124 NaCl, 5 KCl, 25 $NaHCO_3$, 5 D-glucose, 2 $CaCl_2$; pH adjusted to 7·4). For experimental rats, the syringes, connecting tubing and infusion cannulae were filled with ACSF containing muscimol or bicuculline.

Milk ejection experiments

Three hours after administering the anaesthetics, nine or ten pups were positioned on the nipples of the mother. Milk ejections were readily characterized by an abrupt and transient rise in intramammary pressure which was followed immediately by the stretch reaction of the pups (Fig. 1). After at least six milk ejections had been observed, 1 ml of either isotonic saline, muscimol (5, 10, 20 ng ml^{-1}; Sigma) or bicuculline methiodide (0·01, 0·1, 0·3 $\mu g\ \mu l^{-1}$; Sigma) was injected into the third ventricle within 1 min following the last milk ejection. For microinfusion experiments, after at least six milk ejections had been observed, 400 nl of ACSF, muscimol (20, 40, 100 ng ml^{-1}) or bicuculline methiodide (0·15 mg ml^{-1}) were simultaneously infused in each supraoptic nucleus over a period of 12 min. A small bubble of air was placed in each connecting tube to monitor the movement of fluid during the infusion. For both I.C.V. infusion and microinfusion experiments changes in the frequency of milk ejections were assessed by determining a confidence interval with a 98% probability estimate of their frequency, according to the data obtained during the pretreatment period, in order to check if any of the values within 30 min of drug treatment were outside the limits as defined above (see Clarke, Fall, Lincoln &

Merrick, 1978) and by calculating the milk ejection ratio (duration of the longest interval between milk ejections following drug injection divided by the mean interval before treatment). The EEG was recorded in lactating rats continuously during the suckling period. The sensitivity of the mammary gland to exogenous oxytocin was checked by giving I.V. synthetic oxytocin (Syntocinon, Sandoz, Basel, Switzerland; 0·5 milliunits (mU)). For microinfusion experiments the positions of the infusion cannulae were ascertained using conventional histological methods. Data were taken only from those animals in which both infusion cannulae were determined to be functioning and found to be located bilaterally just above supraoptic nuclei. In order to estimate the spread of fluid from the infusion cannulae, two rats were implanted with cerebral cannulae as described above and 400 nl of a 2% ammonium ferrous sulphate solution dissolved in ACSF was infused into one supraoptic nucleus over a period of 12 min. Animals were then killed; their brains were removed and placed into a 4% paraformaldehyde fixative containing potassium ferrocyanide and potassium ferricyanide. The following day the brains were sectioned at 50 μm on a vibratome and the spread of the ferrous solution identified (Fig. 7).

In a further set of experiments designed to assess the effects of I.C.V. infusions on the neurohypophysis directly, electrical stimulation of the neurohypophysis (stimulus parameters: 3 s at 50 Hz, biphasic 1 ms square wave; 200–400 μA amplitude peak to peak) was carried out at 5 min intervals to induce a uniform rise in intramammary pressure equivalent to that evoked by a bolus of about 0·5 mU oxytocin I.V. After at least six consistent intramammary pressure responses to electrical stimulation, 1 ml of either muscimol (20, 40 ng μl^{-1}) or bicuculline methiodide (0·1 μg μl^{-1}) was injected into the third ventricle, and the effects on intramammary pressure increases related to electrical stimulation of the neurohypophysis observed for 30 min. Statistical analysis used to assess changes in intramammary pressure increases consisted in determining a confidence interval with a 98% probability estimate of their amplitude, according to the data obtained during the pretreatment period, and to check if any of the values within 30 min of drug treatment were outside the limits as defined above.

Electrophysiological recording experiments

Extracellular neuronal activity was recorded through a preamplifier (MOD-DAM-5A differential amplifier, WPI instruments; pass band, 0·1–30 kHz) connected to an oscilloscope. Signals were stored on a magnetic tape with a Hitachi magnetic tape recorder and simultaneously sent to a signal discriminator (N750 spike analyser, Mentor, Minneapolis, MN, USA) whose output was plotted on a chart recorder. Final analysis of data was carried out using the Spike 2 software for the interface CED 1401 (Cambridge Electronic Design, Cambridge, UK) with a 386 model Twin Set computer. At the end of the experiments, the dye Pontamine Sky Blue was extruded electrophoretically from the recording electrode by passing a current of 5 μA for 10 min and 20 μA for 10 min. Animals were killed by an overdose of urethane, and the brain removed and placed in a 4% paraformaldehyde solution. The position of the tip of the electrode was ascertained using conventional histological methods.

Supraoptic magnocellular neurones were identified by antidromic invasion after electrical stimulation of the pituitary stalk. Oxytocinergic neurones were identified further by either a positive correlation between their bursting pattern of firing and reflex milk ejections or, when milk ejections were not observed, by their response to 20 μg (kg body weight)$^{-1}$ I.V. cholecystokinin (CCK; sulphated CCK-8 from Sigma; Leng, Way & Dyball, 1991). The following parameters were compared before and after I.C.V. injection of either drug (15 or 20 ng muscimol, 0·12 or 0·3 mg bicuculline): (a) background activity, determined as the mean number of spikes s^{-1} over successive 1 min periods (excluding any high frequency burst-related activity); (b) intervals (in min) between successive high frequency bursts; and (c) neurosecretory burst amplitude, determined as the total number of spikes per neurosecretory burst. Statistical analysis used to assess drug-induced changes in background firing consisted in determining for each cell a confidence interval with a 98% probability estimate of values of firing rate, according to data obtained during the pretreatment period. The normality of distribution of these data was ensured previously. Firing was judged to have been significantly inhibited or activated if at least two successive values within 20 min of drug treatment were outside the limits defined above. The duration of the effect upon firing was assessed by counting the number of successive 1 min periods for which the effect was observed. Changes in the frequency of high frequency bursts were assessed following the method of Clarke *et al.* (1978), and by calculating the milk ejection ratio.

All results are given as means ± S.E.M.

RESULTS

Effects of I.C.V. muscimol and bicuculline on the pattern of the milk ejection reflex and EEG activity

Thirty-seven of fifty (74%) anaesthetized rats displayed the milk ejection reflex during suckling. The delay to the first milk ejection varied from 3 to 36 min (median, 10 min). Subsequent reflex milk ejections occurred at intervals from 4·4 ± 1·2 to 7·6 ± 1·3 min (range of the mean of the seven experimental groups in the pre-test period). In all groups spontaneous oscillations in intramammary pressure unrelated to milk ejection were observed during recording (Fig. 1*B*). I.C.V. injection of the vehicle alone did not modify the pattern of the reflex in any animal of the control group ($n = 5$), nor the EEG activity in the three rats in which it was recorded (Fig. 1*A*); the mean milk ejection ratio was equal to 1·1 ± 0·1 (the milk ejection ratio as defined by Clarke *et al.* (1978) is the duration of the longest interval between milk ejections following drug injection divided by the mean interval before treatment).

The $GABA_A$ receptor agonist muscimol at 5, 10 and 20 ng inhibited the milk ejection reflex in 2/5, 4/5 and 7/7 animals, respectively (Fig. 2; $P \leqslant 0·02$). The mean milk ejection ratio in each group was respectively 1·9 ± 0·2, 3·3 ± 0·6 and 6·6 ± 1·1. A significant correlation was found between the dose of muscimol injected and the duration of inhibition, as quantified by this ratio (Fig. 2; $r = 0·81$; $P < 0·001$). During the period of inhibition of the reflex, the mammary gland remained responsive to exogenous oxytocin (Fig. 1*C*). The amplitude of milk ejections occurring after the period of inhibition was the same as

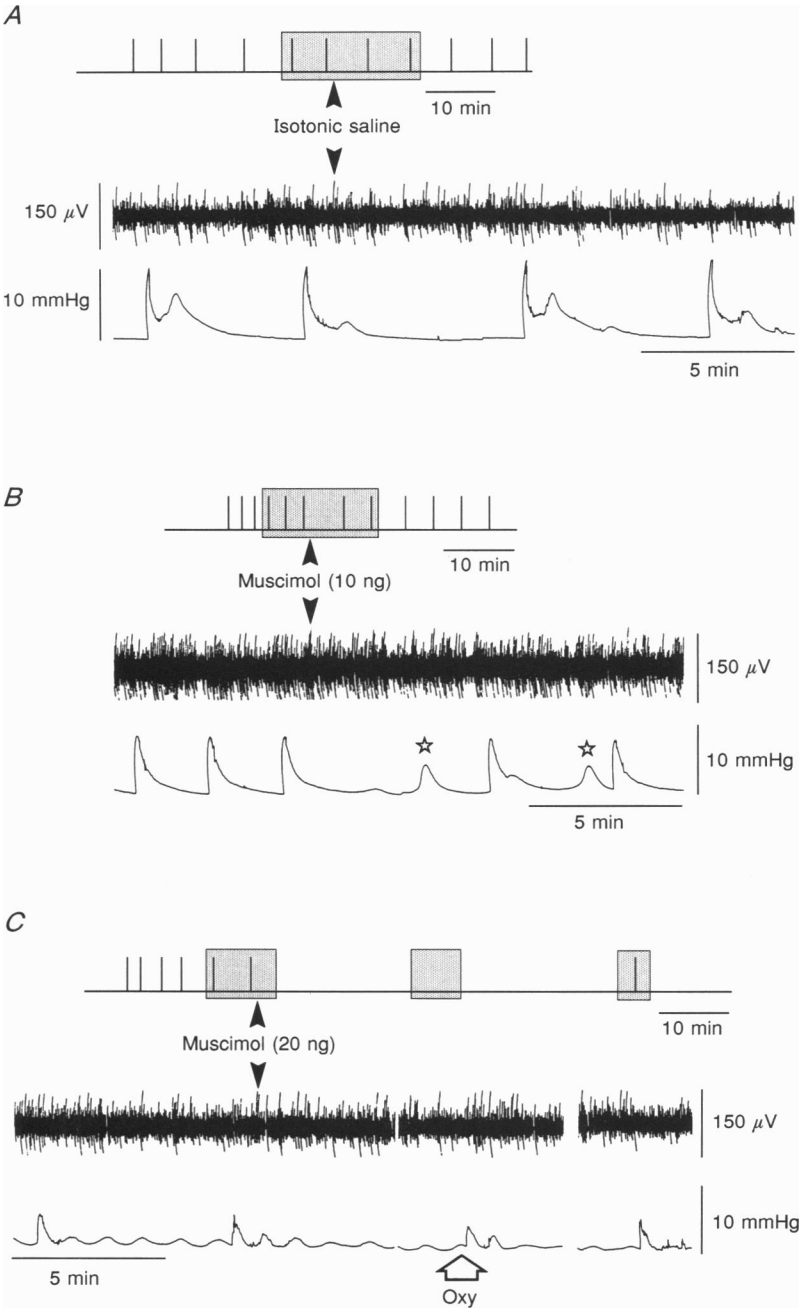

Figure 1. Intracerebroventricular injection of muscimol inhibits the milk ejection reflex in the rat

The effects of I.C.V. injection (arrowheads) of isotonic saline (*A*) or 10 (*B*) and 20 ng (*C*) muscimol on the milk ejection reflex and on the EEG of individual lactating rats. The top line shows a diagramatic representation of each reflex milk ejection (vertical bar) occurring during the time of the experiment. Actual EEG and intramammary pressure recordings (bottom trace) for the periods shaded on the top line are given below at an expanded scale. Note that I.C.V. saline (*A*) had no effect on milk ejection rate and amplitude, while muscimol at both 10 (*B*) and 20 ng (*C*) inhibited the occurrence of milk ejections. In *B* stars mark spontaneous oscillations in intramammary pressure unrelated to high frequency discharges of oxytocin neurones. In *C* note that during the period of muscimol-induced inhibition, the mammary gland sensitivity to oxytocin was not affected, since I.V. injection of 0·5 mU oxytocin (large open arrow, Oxy) elicited an increase in intramammary pressure. In all cases the EEG was not affected by I.C.V. injections.

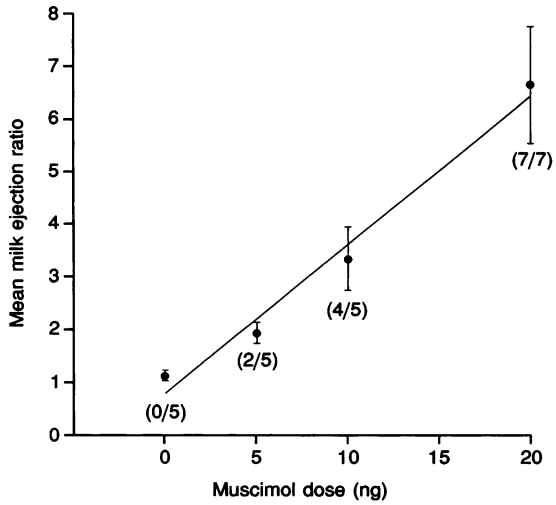

Figure 2. The effect of muscimol upon the milk ejection ratio is dose dependent

The mean milk ejection ratio is plotted against the dose of muscimol used. Each point is the mean value derived from all the rats tested at each dose; the S.E.M. is denoted by the vertical bar. The correlation coefficient is significant at $P < 0{\cdot}001$ ($r = 0{\cdot}81$). The ratio beside each point gives the number of rats in which milk ejection reflex was inhibited *vs.* the number of rats tested at each dose.

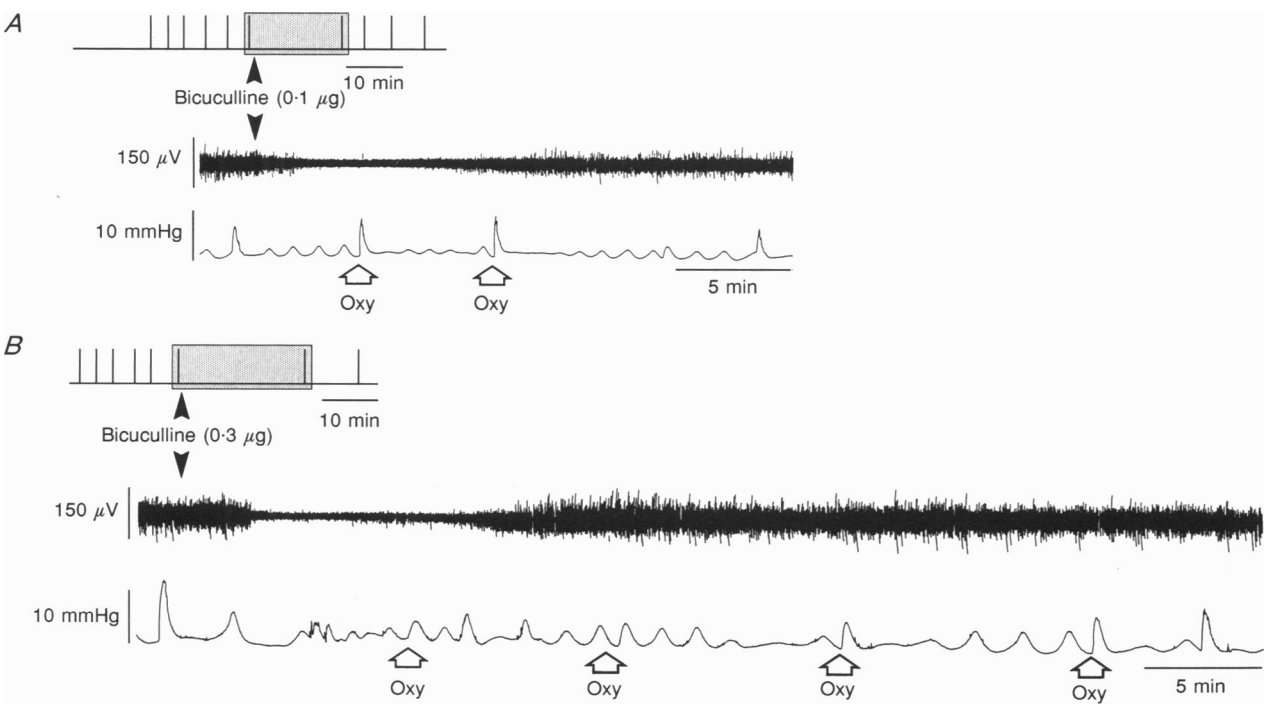

Figure 3. Intracerebroventricular injection of bicuculline inhibits the milk ejection reflex and desynchronizes the EEG

The effects of I.C.V. injection (arrowheads) of bicuculline at 0·1 (*A*) or 0·3 μg (*B*) on the milk ejection reflex and on the EEG of individual lactating rats. The top line shows a diagramatic representation of each reflex milk ejection (vertical bar) occurring during the time of the experiment. Actual EEG and intramammary pressure recordings (bottom trace) for the periods shaded on the top line are given below at an expanded scale. Bicuculline at both 0·1 (*A*) and 0·3 μg (*B*) inhibited the occurrence of milk ejections. In both cases I.C.V. injections provoked an EEG desynchronization. During inhibition of the milk ejection reflex induced by 0·1 μg of bicuculline (*A*), the mammary gland was still responsive to 0·5 mU exogenous oxytocin (open arrow). In *B* note that during the period of bicuculline-induced inhibition, the mammary gland sensitivity to oxytocin was altered, but partially recovered before the end of the period of inhibition.

that observed before I.C.V. administration of muscimol (Fig. 1*B* and *C*). The EEG was recorded in 3/5, 4/5 and 4/7 animals receiving 5, 10 and 20 ng muscimol, respectively (Fig. 1*B* and *C*), and no alteration was observed except for one rat (20 ng), which displayed a slight desynchronization, in terms of amplitude.

The lowest dose of bicuculline (0·01 μg) inhibited the milk ejection reflex in one of five animals tested and the effect was accompanied by a desynchronization of the EEG. At 0·1 μg, bicuculline (Fig. 3*A*) produced desynchronization of the EEG in all animals, in concert with an inhibition of the milk ejection reflex in three out of five rats. The EEG desynchronization lasted for approximately half as long as the inhibition of the milk ejection reflex (Fig. 3*A*). At a dose of 0·3 μg, bicuculline inhibited the milk ejection reflex and desynchronized the EEG in 5/5 animals (Fig. 3*B*); this was accompanied by short-lasting convulsions of the animal. At this dose, transient fluctuations in intramammary pressure were recorded during the period over which the milk ejection reflex was inhibited and mammary sensitivity to exogenous oxytocin was markedly reduced (Fig. 3*B*). The mean milk ejection ratio in each group of bicuculline-treated animals was 2·0 ± 0·6, 2·8 ± 0·4 and 6·4 ± 1·2 in animals receiving 0·01, 0·1 and 0·3 μg bicuculline, respectively. A significant correlation was found between the dose of bicuculline injected and the duration of inhibition as quantified by this ratio ($r = 0·81$; $P < 0·001$).

Effects of I.C.V. muscimol and bicuculline on electrically evoked increases in intramammary pressure

Trains of electrical stimulation of the neurohypophysis (50 Hz, 3 s), at intervals of 5 min, elicited a uniform rise in intramammary pressure equivalent to that evoked by 0·5 mU oxytocin I.V. Injection of 20 ($n = 5$) or 40 ng ($n = 2$) muscimol I.C.V. did not change the rise in intramammary pressure within a 30 min period (Fig. 4*A*). Injection of 0·1 μg bicuculline I.C.V. did not modify the amplitude of electrically evoked increases in intramammary pressure in three animals (Fig. 4*B*), while in two other rats a decrease was observed for just a single stimulation (one at 20 min and one at 30 min post-injection).

Effects of I.C.V. muscimol and bicuculline on the activity of magnocellular neurones in the supraoptic nucleus (SON)

Twenty-seven antidromically identified SON neurones were recorded. These neurones displayed antidromic responses fulfilling the usual criteria of stable latency (range, 7–17 ms), high frequency following, and

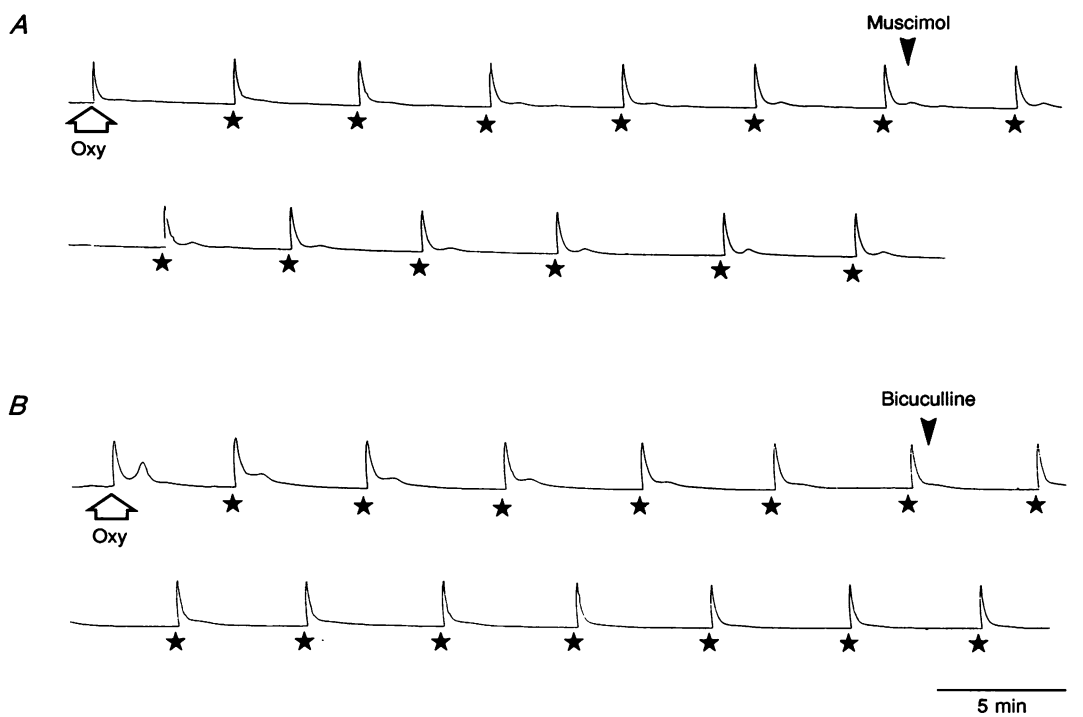

Figure 4. No effects on intramammary pressure rises evoked by electrical stimulation of the neurohypophysis are seen after I.C.V. injection of muscimol or bicuculline

The effects of I.C.V. injection of 20 ng muscimol (*A*) and 0·1 μg bicuculline (B) on milk ejections evoked by electrical stimulation of the neurohypophysis in anaesthetized lactating rats are illustrated. The neural lobe was stimulated (star) every 5 min (50 Hz for 3 s at 200 μA) in order to produce rises in intramammary pressure equivalent to those evoked by I.V. injection of 0·5 mU oxytocin (open arrow). After a control period, muscimol or bicuculline were injected: no effects on intramammary pressure rises were seen after I.C.V. injection of either drug (arrowheads).

cancellation of the antidromic potential by collision with a spontaneous orthodromic potential. The cells were histologically localized within the SON. Fourteen cells were classified as oxytocin neurones: eleven displayed high frequency discharges before reflex milk ejections, and three others were activated by I.V. injections of CCK. Eight cells displayed a phasic pattern of activity, and were considered to be putative vasopressin neurones. Five cells showing a continuous or a slow irregular pattern of firing remained unidentified.

Effect of muscimol on the electrical activity of magnocellular neurones. Muscimol was tested on seven oxytocin neurones (four identified through the milk ejection reflex, and three according to their response to I.V. CCK), two unidentified magnocellular neurones, and three phasic cells. In six out of seven oxytocin neurones, muscimol induced a significant decrease in the basal firing rate. At 15 ng (Fig. 5*A*) the firing of three cells was inhibited from 1·7 ± 0·1 to 0·6 ± 0·2 Hz, for 2–10 min, and at 20 ng (Fig. 5*B*) the firing of three out of four cells decreased from 3·9 ± 0·9 to 0·5 ± 0·1 Hz for 13–21 min. For the neurones identified in rats displaying reflex milk ejections, this effect was accompanied at 15 ng by an increased delay between high frequency discharges (milk ejection ratios of 2·0 and 3·7). High frequency bursts occurring after the administration of the drug were within the same range as those occurring during the pretreatment period. The 20 ng dose disrupted the reflex in two animals until the end of the experiment (at least 45 min). In the two unidentified cells the basal firing rate was inhibited from 3·3 ± 0·1 and 2·4 ± 0·2 to 0·1 ± 0·1 and 0·5 ± 0·1 Hz for 11 and 4 min, respectively. Two phasic neurones were inhibited by 20 ng muscimol for 8 and 15 min (firing rate decreasing from 4·7 ± 2·3 to 0·7 ± 0·6 Hz; Fig. 5*C*), while no effect was noted for the putative vasopressinergic cell which exhibited long silent periods in the pretreatment period. In all cells tested the latency between injection of muscimol and onset of inhibition was less than 1 min.

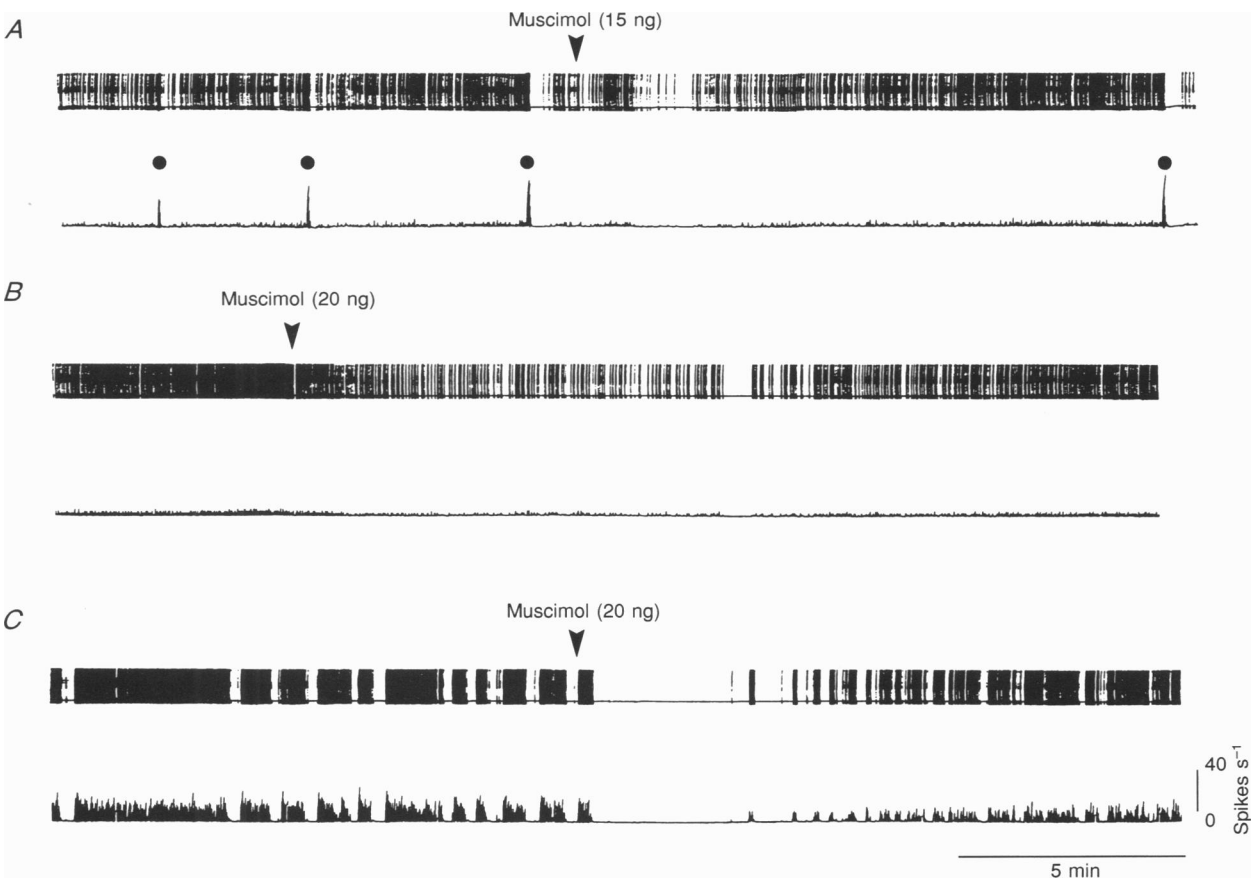

Figure 5. Intracerebroventricular injection of muscimol inhibits the firing of magnocellular neurones

The effects of I.C.V. injections of muscimol on the electrical activity of oxytocin cells (*A* and *B*), and vasopressin cell (*C*) are illustrated. The different traces represent the unit activity and the firing rate in spikes s^{-1}. Arrowheads represent the I.C.V. injection of the drug. In *A*, I.C.V. muscimol (15 ng) transiently inhibited the background firing of oxytocin neurones, and delayed the occurrence of high frequency bursts (marked by the dots). Muscimol (20 ng) also had an inhibitory effect on the electrical activity of continuously firing neurones identified by their response to I.V. CCK (*B*), and on phasic neurones (*C*).

Effect of bicuculline on the electrical activity of magnocellular neurones. Bicuculline was tested on seven oxytocin neurones (all identified through the milk ejection reflex), three unidentified cells, and five phasic neurones. Intracerebroventricular injection of 0·12 μg bicuculline significantly decreased the basal firing of six out of six oxytocin cells for 2–18 min (11·2 ± 3·1 min) with firing rates changing from 5·6 ± 1·9 to 2·0 ± 1·3 Hz. In three cells, bicuculline had a dual effect on the background firing since neurones were activated for a short time, before being inhibited. The activation lasted from 30 s to 3 min and resulted in a mean increase of 1·0 ± 0·4 Hz over the previous basal firing rate. In the three other oxytocin neurones only the inhibitory effect was seen (Fig. 6*A* and *B*). In three animals for which enough high frequency bursts were recorded, the effect on the background firing

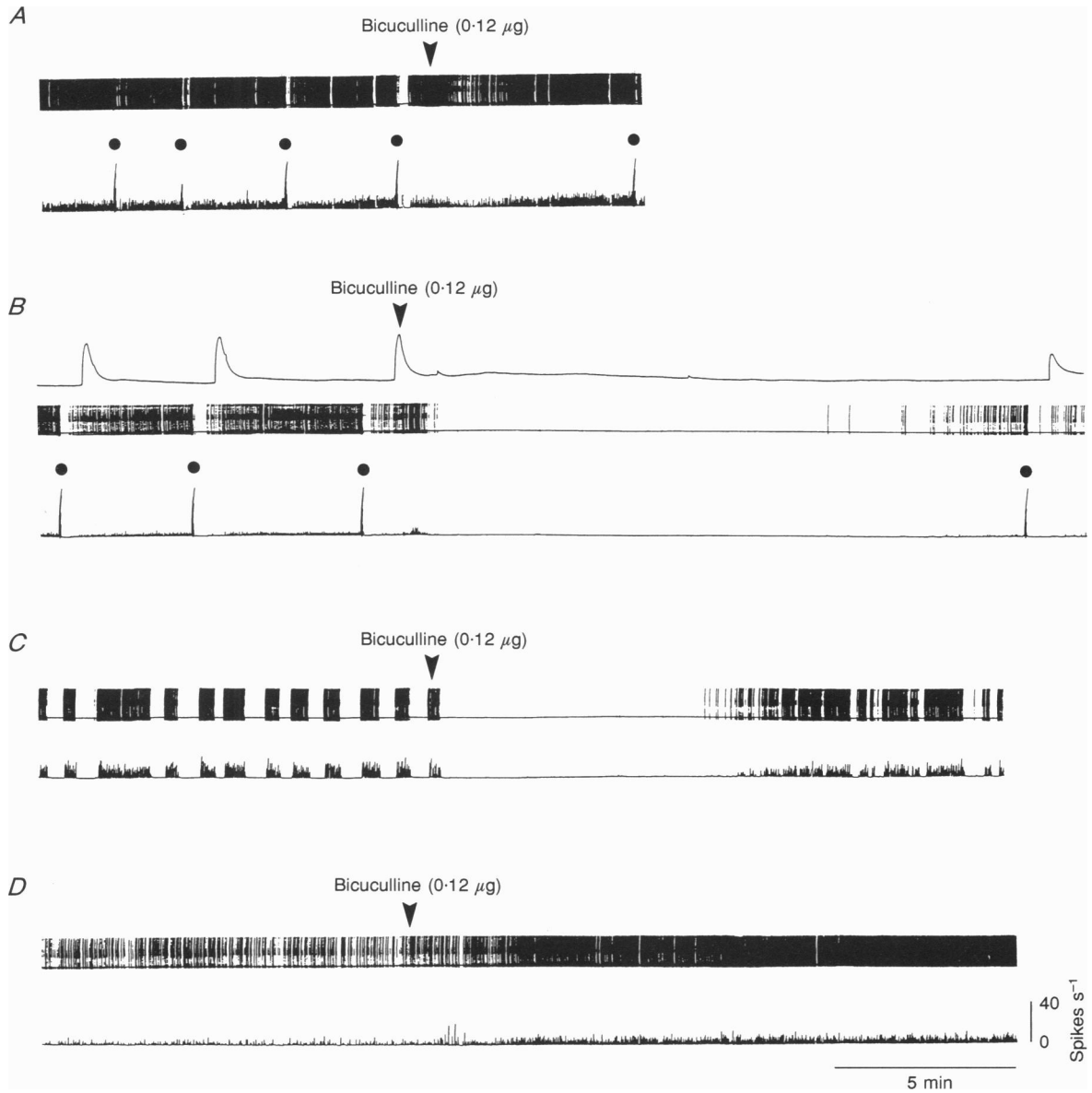

Figure 6. Intracerebroventricular injection of bicuculline inhibits the firing of magnocellular neurones

The effects of I.C.V. injections of bicuculline on the electrical activity of oxytocin cells (*A* and *B*), vasopressin cells (*C*) and control cells (*D*) are illustrated. The different traces represent the intramammary pressure, the unit activity and the firing rate in spikes s^{-1}. Arrowheads represent the I.C.V. injection of the drug. Each circle marks the occurrence of a reflex milk ejection-related burst. Bicuculline (0·12 μg) inhibited the background firing of oxytocin neurones and delayed the occurrence of the coming high frequency burst (*A* and *B*). In some cells a two-step effect on the background firing was observed, since inhibition was preceded by a transient activation (*B*). An inhibitory effect was also observed in phasic neurones (*C*), although in most of the control neurones an activation was seen after I.C.V. bicuculline (*D*).

was accompanied by an increased delay between neurosecretory bursts (milk ejection ratios of 2·7, 4·6 and 5·0). High frequency bursts occurring after the administration of the drug were in the same range of amplitude as those occurring during the pretreatment period. A lower dose of bicuculline (0·01 μg) was also tested in one oxytocin cell and no alteration in the basal firing rate or in the amplitude and frequency of high frequency bursts was observed. The effects of a larger dose of bicuculline, 0·30 μg, were examined in two unidentified neurones with a basal firing rate of 1·2 ± 0·1 and 1·7 ± 0·1 Hz. In both cells, a brief activation (30–60 s) of the electrical activity up to 4 Hz was observed, then a total inhibition (1–3 min), before the cell was lost because of the convulsions of the animal. Another unidentified slow irregularly firing neurone was inhibited (from 0·8 ± 0·1 to 0·02 ± 0·01 Hz) for 12 min by 0·12 μg bicuculline. The firing of four putative vasopressinergic neurones was also inhibited by I.C.V. injection of 0·12 μg bicuculline from 4·2 ± 1·5 to 1·1 ± 1·0 Hz for 5–23 min, but this effect was never preceded by activation (Fig. 6*C*). The lowest dose of bicuculline (0·01 μg) inhibited one phasically bursting cell for a period of 5 min (firing decreasing from 2·2 ± 0·1 to 0·8 ± 0·1 Hz). In all cases, changes in firing began within 1 min of bicuculline injection.

Effect of muscimol and bicuculline on control cells. Recordings were made from twelve cells with spontaneous firing rate ranging from 2·0 ± 0·1 to 25·2 ± 0·1 Hz, which were not antidromically driven, and were found to be located immediately above the supraoptic nucleus. Of these, eight were tested with 0·12 μg bicuculline injected I.C.V.: five were activated (from 2·1 ± 0·9 prior to 4·1 ± 0·9 Hz after I.C.V.; Fig. 6*D*), two inhibited (from 2·4 ± 1·1 to 0·5 ± 0·4 Hz), and one unaffected. The effects of I.C.V. muscimol (15 ng) on the electrical activity was tested on two cells which were inhibited, and two others which were unaffected.

Effects of microinfusions of muscimol and bicuculline directly into the supraoptic nuclei on the pattern of the milk ejection reflex and EEG activity

Seventy per cent of the anaesthetized rats ($n = 30$) displayed the milk ejection reflex during suckling. The delay to the first milk ejection varied from 8 to 44 min (median = 18 min). Subsequent reflex milk ejections occurred at intervals from 5·8 ± 1·4 to 8·0 ± 1·6 min (range of the mean of the experimental groups in the pre-test period). Bilateral microinfusions of ACSF alone did not modify the pattern of the reflex in any animal of the control group ($n = 4$); the mean milk ejection ratio was equal to 1·4 ± 0·1. In the two rats which received a 2% ammonium ferrous sulphate solution, the fluid diffused out of the infusion cannula in a spherical manner to include the supraoptic nucleus and the adjacent dorsal and lateral perinuclear regions (Fig. 7). The average radius of fluid spread from the tip of the infusion cannulae was 400 ± 25 μm.

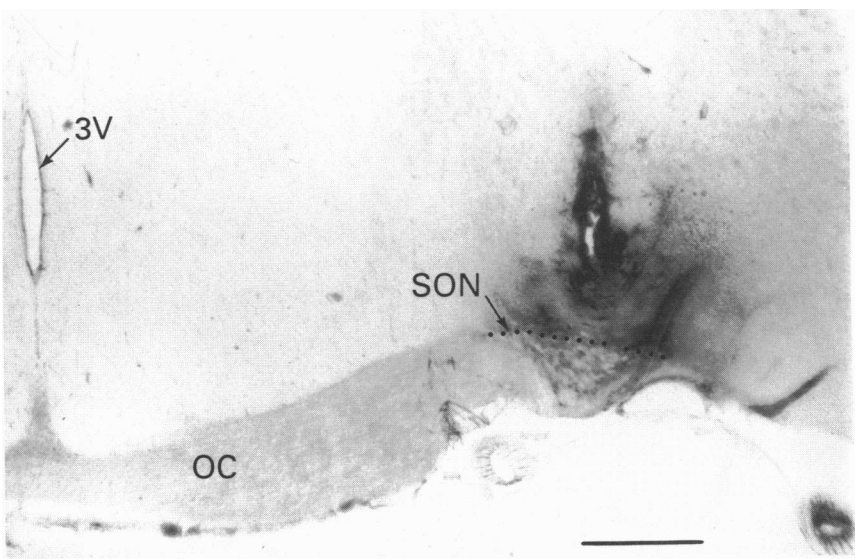

Figure 7. Fluid spread following microinfusion into the supraoptic nucleus

Photomicrograph of coronal section through the supraoptic nucleus (SON) showing the region of fluid spread following a 400 nl infusion of an iron sulphate solution. Note that fluid diffusion included the SON and the adjacent dorsal and lateral perinuclear regions. OC, optic chiasma; 3V, third ventricle. Calibration bar, 0·5 mm.

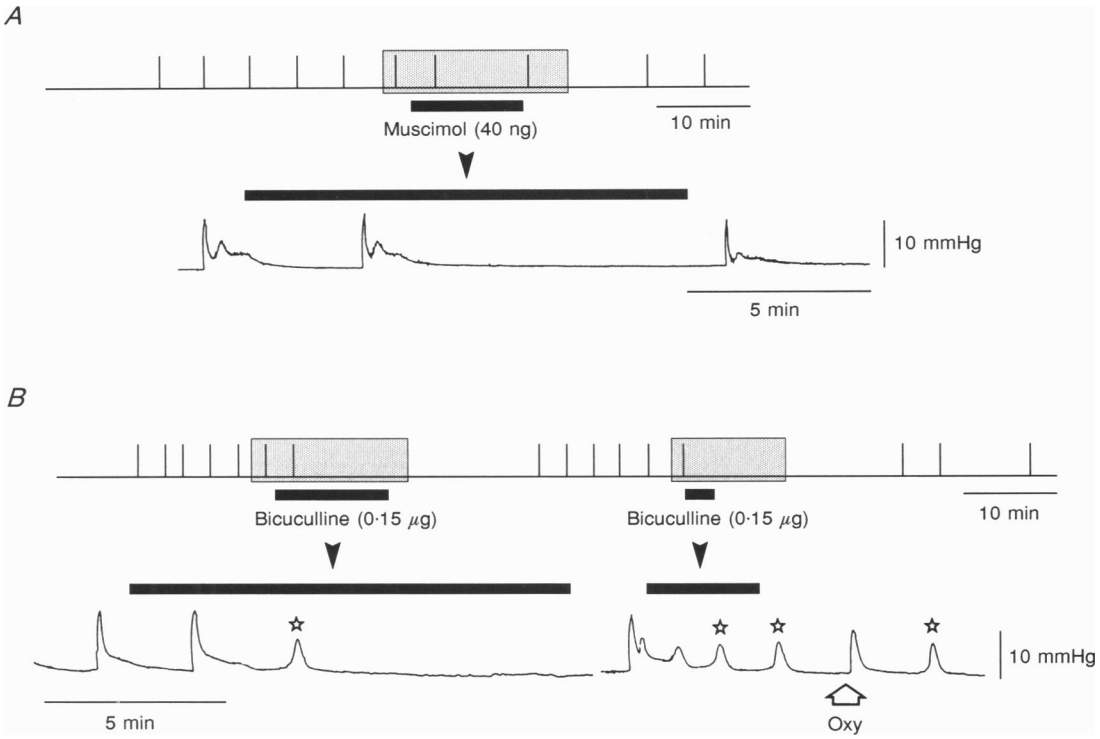

Figure 8. Bilateral microinfusions of both muscimol and bicuculline into the supraoptic nuclei inhibit the milk ejection reflex

The effects on the milk ejection reflex of microinfusions (horizontal bar) of ACSF containing 40 ng ml^{-1} of muscimol (*A*) or 0·15 $\mu g\ \mu l^{-1}$ bicuculline (*B*) are illustrated in individual lactating rats. The top line shows a diagrammatic representation of each reflex milk ejection (vertical bar) occurring during the time of the experiment. Actual intramammary pressure recordings (bottom trace) for the periods shaded on the top line are given below at an expanded scale. In the animal illustrated in *B* a second bilateral microinfusion into the supraoptic nuclei (100 nl of a bicuculline solution at 0·15 $\mu g\ \mu l^{-1}$) performed after the reflex had recovered induced a further inhibition of the milk ejection reflex. Stars mark spontaneous oscillations in intrammmary pressure unrelated to high frequency milk ejections. Such spontaneous oscillations were observed in some animals but no consistent relationship with drug infusion was determined.

Bilateral microinfusions into the supraoptic nuclei of 400 nl of a muscimol solution at 20, 40 and 100 ng ml^{-1} inhibited the milk ejection reflex in 2/3, 2/3 and 4/4 animals, respectively (Fig. 8*A*; $P \leqslant 0{\cdot}02$). The mean milk ejection ratio in each group was respectively $2{\cdot}2 \pm 0{\cdot}4$, $3{\cdot}3 \pm 1{\cdot}4$ and $5{\cdot}4 \pm 2{\cdot}6$. A correlation between the dose of muscimol injected and the duration of inhibition failed to reach significance ($r = 0{\cdot}53$; $P = 0{\cdot}053$). During the period of inhibition of the reflex, the mammary gland remained responsive to exogenous oxytocin, and no alteration of the EEG was observed in the three animals in which it was recorded. In most cases, the inhibitory effect occurred at the end of the period of infusion, and was sometimes delayed. In two other rats in which the infusion cannulae were positioned in such a way that only one supraoptic nucleus was likely to have been influenced by the infusion, muscimol at 20 and 40 ng ml^{-1} failed to inhibit the milk ejection reflex.

Bilateral microinfusions into the supraoptic nuclei of 400 nl of a bicuculline solution at 0·15 mg ml^{-1} inhibited the milk ejection reflex in 5/5 animals (Fig. 8*B*; $P \leqslant 0{\cdot}02$). The mean milk ejection ratio was $5{\cdot}8 \pm 1{\cdot}2$. The EEG was recorded in two cases and was not desynchronized during microinfusions, and no animal showed any sign of convulsions. No alterations in mammary sensitivity to exogenous oxytocin were noted during the period of inhibition of the reflex. The inhibitory effect of bicuculline always appeared before the end of the duration of microinfusion. In one rat a second bilateral microinfusion into the supraoptic nuclei (100 nl of a bicuculline solution at 0·15 $\mu g\ \mu l^{-1}$, for 3 min) performed after the reflex recovered again inhibited the milk ejection reflex (Fig. 8*B*).

DISCUSSION

In conjunction with the wealth of electrophysiological and morphological information indicating a substantial GABAergic innervation of the supraoptic nucleus (Nicoll & Barker, 1971; Bioulac *et al.* 1978; Arnauld *et al.* 1983; Theodosis *et al.* 1986; Mason *et al.* 1987; Randle & Renaud, 1987; Decavel & Van Den Pol, 1990; Wuarin & Dudek,

1993; Gies & Theodosis, 1994), it has not been surprising to observe in our experiments that muscimol inhibited the electrical activity of oxytocin neurones and the milk ejection reflex in suckled rats. Although the concentrations of muscimol required to inhibit the milk ejection reflex were similar to those used by others to show that I.C.V. muscimol inhibits oxytocin release induced by haemorrhage and hypertonic saline infusion (Bisset, Chowdrey, Fairhall & Gunn, 1990; Roberts & Robinson, 1991), they were high compared with those used in experiments elsewhere in the hypothalamus (Osborne, Mataga, Onoe & Watanabe, 1993; Tominaga, Shibata, Hamada & Watanabe, 1994). This is likely to result from the substantial heterogeneity known to exist amongst $GABA_A$ receptors where different subunit combinations show varying affinities for muscimol (McKernan *et al.* 1991; Sivilotti & Nistri, 1991). At present, the precise subunit conformation of the $GABA_A$ receptors expressed by magnocellular neurones is not known but recent work indicates that oxytocin cells located in the supraoptic nucleus express α_1, α_2, β_3 and γ_2 subunits of the $GABA_A$ receptor (Fenelon, Seighart & Herbison, 1994; Herbison, 1994; V. Fenelon & A. E. Herbison, unpublished observations). Irrespective of the precise nature of the $GABA_A$ receptors activated in our study, however, the clear dose–response relationship between I.C.V. muscimol and milk ejection reflex inhibition argues in favour of a physiologically relevant activation of these receptors.

Experimental evidence indicates that $GABA_A$ receptors located in the neurohypophysis may regulate oxytocin release into the bloodstream; GABAergic nerve terminals have been observed on neurosecretory nerve endings in the neurohypophysis (Oertel *et al.* 1982) where low affinity $GABA_A$ receptors are located (Mathison & Dreifuss, 1980) and GABA-activated chloride channels have been identified (Zhang & Jackson, 1993). Further, the activation of these $GABA_A$ receptors inhibits oxytocin release from the isolated neurohypophysis (Dyball & Shaw, 1979). We have been careful in this study to ensure that the effects we observed did not originate from the neural lobe. Principally, milk ejections evoked by electrical stimulation of the pituitary stalk were not altered following muscimol or bicuculline infusions into the third ventricle. Furthermore, during both I.C.V. and microinfusion experiments, a normal milk ejection could always be evoked by the intravenous administration of oxytocin, showing that the sensitivity of the mammary gland was not altered. In some animals recurrent oscillations in intramammary pressure were observed, but they had no consistant relationship to the administration of control saline, muscimol or bicuculline. These oscillations, which are not correlated to the high frequency discharges observed in oxytocin neurones evoked by suckling, are thought to result from a particular mode of mammary gland response to moderate but sustained increases in the basal activity of oxytocin neurones (Poulain & Tasker, 1985).

The precise site at which muscimol acts in the hypothalamus to inhibit the high frequency bursting of oxytocin neurones during suckling is not known. Clearly, the I.C.V. route of administration allows little specificity in determining this site. In an attempt to define better the location of muscimol action, we have carried out a series of bilateral supraoptic nucleus microinfusion experiments where we estimate the diffusion radius to be approximately 0·4 mm. Although we do not know if the spread of iron sulphate through nervous tissue is equivalent to that of GABA-related compounds, the absence of any effects on the EEG following microinfusion experiments compared with I.C.V. administration indicates that these two approaches are not equivalent in the neuronal sites they reach. With present technology, therefore, the microinfusion experiments provide the best possible *in vivo* evidence that activation of $GABA_A$ receptors located within and/or nearby the supraoptic nucleus can effectively inhibit the milk ejection reflex in lactating rats. Certainly, supraoptic oxytocin neurones receive direct GABAergic inputs (Theodosis *et al.* 1986; Decavel & Van Den Pol, 1990; Gies & Theodosis, 1994) and express $GABA_A$ receptors (Fenelon *et al.* 1994; Herbison, 1994), and supraoptic magnocellular neurones are hyperpolarized by direct application of GABA acting through $GABA_A$ receptors (Mason *et al.* 1987; Randle & Renaud, 1987). Hence it is not unreasonable to think that the inhibitory effects of muscimol on both the milk ejection reflex and the activity of identified oxytocin neurones resulted from direct actions on supraoptic magnocellular cells. Since approximately 75% of magnocellular oxytocin neurones activated by suckling are found in the supraoptic nuclei (Poulain & Wakerley, 1982), it is not surprising to find that bilateral supraoptic infusions of muscimol were sufficient on their own to inhibit the reflex, which is known to continue unabated in animals with complete lesions of the paraventricular nuclei (Wakerley, Juss, Farrington & Ingram, 1990). The fact that infusion into one supraoptic nucleus failed to inhibit the reflex, suggests that a critical number of oxytocin neurones must be inhibited to prevent the milk ejection reflex from occurring.

The most intriguing finding of this study has been the observation that bicuculline also inhibited the milk ejection reflex and the high frequency discharges of supraoptic oxytocin neurones in response to suckling. As slow wave sleep is a prerequisite for the occurrence of the milk ejection reflex in the rat (Lincoln, Hentzen, Hin, van der Schoot, Clarke & Summerlee, 1980), it can be argued that the EEG disturbance following I.C.V. injection of bicuculline contributed to the inhibition of the milk ejection reflex. However, if this was the only factor inhibiting the reflex we would have expected to see milk ejections return as the EEG resumed the slow wave pattern. That this was not the case, with the period of milk ejection inhibition greatly exceeding that of the EEG disturbances, indicated a more direct effect of $GABA_A$ receptor blockade on the electrical

activity of oxytocin neurones. Our microinfusion experiments support this contention, as bilateral infusions of bicuculline into the supraoptic nuclei were highly effective in inhibiting the milk ejection reflex without altering the EEG. Such observations suggest that tonic GABA activity within the supraoptic nucleus and/or its immediate vicinity is required for the normal high frequency bursting behaviour of oxytocin neurones in response to suckling.

Whilst the *in vivo* inhibitory actions of muscimol are easily understood and are, in essence, an extension of previous *in vitro* electrophysiological findings relating to GABA in the supraoptic nucleus (Mason *et al.* 1987; Randle & Renaud, 1987), the nature of bicuculline's inhibitory effects are not clear. The site of bicuculline's inhibitory action may be either the same as that of muscimol, for example, directly on the magnocellular neurones, or different from that of muscimol. In the former case, it is worth noting that all spontaneous inhibitory postsynaptic potentials in magnocellular neurones originate from actions of GABA on the $GABA_A$ receptor (Randle & Renaud, 1987; Wuarin & Dudek, 1993). As elsewhere in the brain (Otis, Staley & Mody, 1991), it seems very likely, therefore, that supraoptic oxytocin neurones are subject to a spontaneous and constant barrage of GABA release under normal circumstances (Wuarin & Dudek, 1993). What function this on-going bombardment serves is not known although it has been suggested to play a role in limiting the effect of excitatory input within neural systems (Otis *et al.* 1991). Although we show here that the removal of $GABA_A$ receptor-mediated events by bicuculline does not result in epileptic activity by the oxytocin neurones, we cannot discount the possibillity that the acute withdrawl of GABA actions may have more subtle effects in altering the balance of excitatory and inhibitory influences important for generating high frequency bursting in oxytocin neurones.

Alternatively, muscimol and bicuculline may have different sites of action within the supraoptic nucleus and its immediate vicinity. Recent evidence indicates that ligands of the $GABA_A$ receptor such as muscimol and bicuculline exhibit variability in their affinity for the $GABA_A$ receptor depending on its subtype composition (McKernan *et al.* 1991; Sivilotti & Nistri, 1991; Bureau & Olsen, 1993). Thus, such relative differences between the supraoptic nucleus and neurones in its immediate vicinity may underly the common actions of agonist and antagonist. For example, immunocytochemical studies have shown that cells expressing $GABA_A$ receptors are plentiful in the perinuclear zone of the supraoptic nucleus (Herbison, 1994) and there is evidence to suggest that neurones in this region innervate magnocellular neurones (Tribollet, Armstrong, Dubois-Dauphin & Dreifuss, 1985; Wilkin, Mitchell, Ganten & Johnson, 1989). Hence, the inhibitory effects of bicuculline may arise from the disinhibition of local inhibitory neurones innervating oxytocin neurones while muscimol may inhibit oxytocin neurones directly. Although the neurochemical identity of the putative local inhibitory input is not known, if it is to be GABA itself then the receptors on the oxytocin neurones targeted by these cells must either be relatively insensitive to bicuculline or be $GABA_B$ in nature.

In summary, we report that both muscimol and bicuculline inhibit the milk ejection reflex in lactating rats. The results with muscimol provide the first evidence that the activation of $GABA_A$ receptors to levels above that normally occurring will block the milk ejection reflex. In contrast, the inhibition observed with bicuculline indicates that tonic activity at $GABA_A$ receptors located within the supraoptic nucleus and its immediate vicinity is essential for maintaining the functional integrity of the neural network transducing the suckling stimulus into intermittent bursting activity of oxytocin neurones. Our recent study showing that extracellular GABA concentrations in the supraoptic nucleus do not alter in response to suckling or individual milk ejections (Voisin, Chapman, Poulain & Herbison, 1994) suggests that the role of GABA may be permissive in nature and not involved directly in evoking each high frequency burst of firing. It is of interest to note that GABA has been hypothesized to play a very similar role to help generate pulsatile activity within the gonadotrophin-releasing hormone (GnRH) neurones; extracellular GABA concentrations do not change in relation to individual pulses of luteinizing hormone and both $GABA_A$ receptor agonists and antagonists infused into the immediate vicinity of the GnRH neurones inhibit pulsatile activity (Herbison, Chapman & Dyer, 1991). Together, these observations suggest that an on-going balance of GABA activity may be essential for the normal functioning of several neuroendocrine networks and we provide here such evidence for the magnocellular oxytocin neurones during lactation.

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