GABA-induced facilitation of the periodic bursting activity of oxytocin neurones in suckled rats

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1. GABAergic innervation of oxytocin neurones is particularly abundant during lactation, but little is known about its functional role. In this study, the role of $GABA_A$ receptors in the suckling-induced bursting activity of oxytocin neurones was investigated in lactating rats. $GABA_A$ agonists or antagonists were applied by pressure injection into the immediate neighbourhood of recorded neurones while simultaneous recordings were made from oxytocin neurones in the contralateral supraoptic nucleus.

2. GABA and the GABA agonist isoguvacine decreased the basal electrical activity while application of $GABA_A$ antagonists (picrotoxin and gabazine) increased the basal electrical activity. However, in marked and unexpected contrast, application of GABA and isoguvacine facilitated or triggered milk-ejection reflex bursting activity whereas $GABA_A$ antagonists interrupted this reflex activity.

3. Systemic injection of hypertonic saline is known to increase the firing rate of neurones in the supraoptic nucleus and temporarily to interrupt suckling-induced bursting activity. Application of GABA into one supraoptic nucleus counteracted this inhibitory effect on milk ejection.

4. These observations can be explained if the role of the important GABAergic innervation of oxytocin neurones during lactation is to favour the expression of the stereotyped suckling-induced bursting activity. It might do this by attenuating inputs unrelated to suckling which are incompatible with bursts.

Dense GABAergic inputs have been shown to innervate the magnocellular neurones in the paraventricular (PVN) and supraoptic (SON) nuclei of the hypothalamus (Theodosis, 1988; Decavel & Van den Pol, 1990). This GABAergic innervation markedly increases at the end of gestation and during lactation, and exclusively concerns the oxytocin neurones (Gies & Theodosis, 1994). The qualitative importance of GABA in the PVN and SON during parturition and lactation raises the problem of its functional role. Indeed, it is only during these two particular physiological states that oxytocin neurones develop stereotyped synchronous bursting activity leading to pulsatile oxytocin release (Wakerley & Lincoln, 1973; Belin, Moos & Richard, 1984). In contrast to this response to suckling, other stimuli (including haemorrhage and hypovolaemia) induce tonic activation of oxytocin neurones. These observations raise the possibility that GABA could play a particular role in regulating bursting activity. Extensive literature clearly shows that GABA exerts a potent depressant action on the basal discharge of magnocellular neurones (see review by Renaud & Bourque, 1991). This action could be due to membrane hyperpolarization and to marked reduction in membrane resistance (Mason, Poulain & Cobbett, 1987; Randle & Renaud, 1987) and probably involves the $GABA_A$ receptors expressed by magnocellular neurones (Herbison, 1994). Indeed, the great majority of the spontaneous and evoked inhibitory postsynaptic potentials (Randle & Renaud, 1987) and currents (Wuarin & Dudek, 1993) on these neurones are mediated by GABA acting via $GABA_A$ receptors. However, less is known about the function of GABA in the suckling-induced bursting activity. A recent study in suckled rats showed that bilateral intra-SON injections of both $GABA_A$ agonists or antagonists, interrupted the periodic pattern of milk ejections (Voisin, Herbison & Poulain, 1995). The hypothesis proposed to explain these results was that GABAergic inputs play an important role in the maintenance of the mechanisms underlying the milk-ejection reflex. However, the electrical activity of oxytocin neurones was not recorded. In the present study, the role of $GABA_A$ receptors in the suckling-induced bursting activity of oxytocin neurones was investigated in lactating rats. $GABA_A$ agonists or antagonists were applied by pressure to the immediate neighbourhood of recorded neurones via double-barrelled micropipettes, while simultaneous recordings were made

from oxytocin neurones in the contralateral supraoptic nucleus. Data obtained using this alternative experimental approach suggest a different conclusion regarding the role of GABA.

METHODS

Extracellular electrophysiological recordings were performed on lactating Wistar rats (Depré, France; 250–350 g body weight) between days 8 and 12 postpartum. Dams were anaesthetized with urethane (ethyl carbamate, 1·2 g kg^{-1} given in a single I.P. injection). A cannula was inserted in the jugular vein for I.V. injection of Brietal (9 mg kg^{-1}; Lilly, Strasbourg, France) to supplement anaesthesia when necessary, or for injection of known doses of oxytocin (Sandoz, Basel, Switzerland) to test sensitivity of the mammary gland. A cannula was also inserted in the thoracic mammary gland and connected to an electromagnetic pressure transducer (Viggo-spectramed, Oxnard, CA, USA) for measurement of intramammary pressure peaks as an index of oxytocin release. The rats were then placed in a stereotaxic frame. Xylocaine (1% solution; Rhône-Poulenc, Rorer, France) was injected S.C. to all surgical sites and at the points of contact with the stereotaxic frame. After trephining, a microsyringe (10 μl Hamilton) was inserted into the third ventricle (A = 8, L = 0, H = 3, stereotaxic atlas of Albe-Fessard, Libouban & Stutinsky, 1966) for I.C.V. injection of oxytocin (1 μl of 1 μM solution in 0·9% NaCl) to facilitate the milk-ejection reflex when necessary (Freund-Mercier & Richard, 1984). A bipolar stimulating electrode was inserted into the pituitary stalk at the limit of the neurohypophysis (A = 4·7, L = 0, H = 0·5) for antidromic identification of magnocellular neurones in the SON. The exact location of the electrode was confirmed by a peak of intramammary pressure evoked by repetitive electrical stimulation (40 Hz, 8–12 V, 0·5 ms for 4 s).

Electrophysiological recordings from magnocellular neurones were performed with glass micropipettes filled with 0·5 M sodium acetate solution (9–15 MΩ impedance) and connected to conventional electrophysiological apparatus. The extracellular electrical activities were displayed on an Astromed chart recorder (Astromed SNC, Trappes, France) and simultaneously stored on computer by means of a CED 1401 interface card (Cambridge Electronic Design, Cambridge, UK). Test drugs were injected into one SON while recording the electrical activity of a nearby oxytocin neurone using a double micropipette, the pressure pipette being connected to a pneumatic picopump (WPI Inc., Sarasota, FL, USA). Simultaneously, an oxytocin neurone was recorded in the contralateral SON nucleus. Home-made double-barrelled micropipettes were constructed by gluing the tip of the pressure micropipette 20–40 μm above that of the recording microelectrode. Nitrogen pressure was adjusted to between 40 and 100 Pa according to the tip diameter of the pressure micropipette (10–20 μm). Drugs were delivered by applying pressure pulses with a square waveform and lasting 20 ms every 15–30 s until a change in firing rate and/or bursting activity occurred. These micropulses could be repeated over a period of 10 min in order to maintain the induced changes in electrical activity. Calibration of ejection pulses performed under microscopic observation revealed that the total volume ejected for each application varied between 10 and 15 nl. Such a volume has been shown to spread through the nucleus and to affect a critical pool of neurones which, by mutual driving will induce parallel changes in the bursting activity of the oxytocin neurones located in the other magnocellular nuclei (Lambert, Moos & Richard, 1993). However, because the pipette tip was partly occluded by the tissue while it was lowered through the brain to the SON, the volume injected was difficult to control and was probably never exactly equivalent to that quoted. Neurones which showed no response (no change in basal activity) to pressure ejection of GABA agonists or antagonists were excluded from analysis because lack of responsiveness was probably due to blockage of the pressure micropipette. Indeed, in the majority of tests (45 out of 54), drug injections induced clear and rapid changes in basal activity.

All the substances injected into the SON were dissolved in a cerebrospinal fluid-like medium (NaCl, 126·5 mM; $NaHCO_3$, 27·5 mM; KCl, 2·4 mM; KH_2PO_4, 0·5 mM; $CaCl_2$, 1·1 mM; $MgCl_2$, 0·83 mM; Na_2SO_4, 0·5 mM; glucose, 5·9 g l^{-1}; pH adjusted to 7·4). The drugs tested were GABA (10^{-4} M; Research Biochemicals International), the $GABA_A$ agonist isoguvacine HCl (10^{-4} M; Research Biochemicals International), the $GABA_A$ antagonists picrotoxin (10^{-6} M; Sigma) and gabazine (10^{-5} or 10^{-4} M; SR 95531, a gift from Sanofi, Montpellier, France).

Suckling stimulus with ten pups was applied 3 h after induction of anaesthesia in order to trigger the milk-ejection reflex. Intranuclear injections of the different pharmacological substances were performed either during an on-going milk-ejection reflex, after the occurrence of three to four regular bursts or about one hour after the beginning of suckling in the cases when suckling failed to trigger the milk-ejection reflex. In some cases the effect of the GABAergic drugs was compared with that of oxytocin injected I.C.V. (1 μl of 1 μM solution, i.e. 1 ng; Freund-Mercier & Richard, 1984). In another series of experiments, a hyperosmotic stimulus (single I.P. injection of 2 ml of 1·5 M NaCl solution) was applied during an on-going series of reflex milk ejection either alone or in combination with intra-SON injection of GABA.

Data analyses

Oxytocin neurones were identified by their antidromic response to electrical stimulation of the neurohypophysis and by their bursting activity (high frequency bursts of spikes) occurring periodically 10–15 s before each milk ejection. Vasopressin neurones displayed phasic activity (periods of activity separated by clear phases of silence) which was never correlated with reflex milk ejections during suckling.

The parameters considered in the electrophysiological studies of oxytocin neurones during an on-going milk-ejection reflex were: (i) the basal activity (in spikes s^{-1}) excluding any burst-related activity and calculated every 10 or 100 s; (ii) the burst periodicity quantified by the time intervals (in min) between two successive bursts (or milk ejections when no oxytocin neurone was recorded); (iii) the burst amplitude (total number of spikes per burst) and (iv) the burst characteristics, i.e. the mean and maximal instantaneous firing frequency during the burst. When suckling failed to trigger the milk-ejection reflex, the main parameters considered were: (i) the basal activity (in spikes s^{-1}) and the time lag for the occurrence of the first burst after intranuclear drug injections and (ii) the burst amplitude and characteristics. In every case, the effects of drugs on basal activity were considered (i) as a function of time (kinetics of action) and (ii) during the 100 s preceding the burst.

For statistical analysis all values were expressed as means ± S.E.M. and Student's paired t test was used to compare respective values before and after drug injections when more than five data values were available.

RESULTS

Drugs were injected into one SON while performing paired recordings of SON–SON oxytocin neurones. However, due to the pressure injection procedure, neurones within the injected nucleus were often lost during or a few minutes after injection. Although, in most cases other oxytocin neurones could be rapidly recorded, they have been taken into account only to attest for the synchronization of bursts.

Effect of GABA or isoguvacine during suckling

Intra-SON injections of both GABA and isoguvacine decreased the basal electrical activity of oxytocin neurones but also, unexpectedly, facilitated the occurrence of milk-ejection bursts, both during an on-going series of reflex milk ejections and in rats failing to eject milk (Fig. 1*A* and *B*).

During an on-going milk-ejection reflex, facilitation of bursting activity was the only effect observed in seven out of ten tests. Drug-induced changes in basal and bursting activity could be followed in three neurones within the injected SON and in six neurones within the contralateral SON (two paired SON–SON recordings). Both drugs rapidly depressed the basal activity of oxytocin neurones within the injection site (including neurones lost while injecting drugs) but not the contralateral SON neurones (Figs 1*A* and 3). This decrease persisted during drug application, then wore off progressively with time. Facilitation of burst occurrence was observed during or just after applying the drugs (i.e. while the basal activity was depressed) and concerned either the first burst following injection (observed in five tests with GABA or isoguvacine) or several successive bursts (up to five bursts, observed in two tests with GABA). The facilitatory effect occurred simultaneously in oxytocin neurones of the two SON as attested by paired recordings and/or the constant correlation between intramammary pressure peaks and bursts. When considering the interburst interval for the first burst facilitated by intra-SON injection, the mean value for all seven tests (8·3 ± 1 min) was significantly smaller than the three preceding interburst intervals (respectively, 13·8 ± 1·8 min, $P < 0·01$; 13·4 ± 2·4 min,

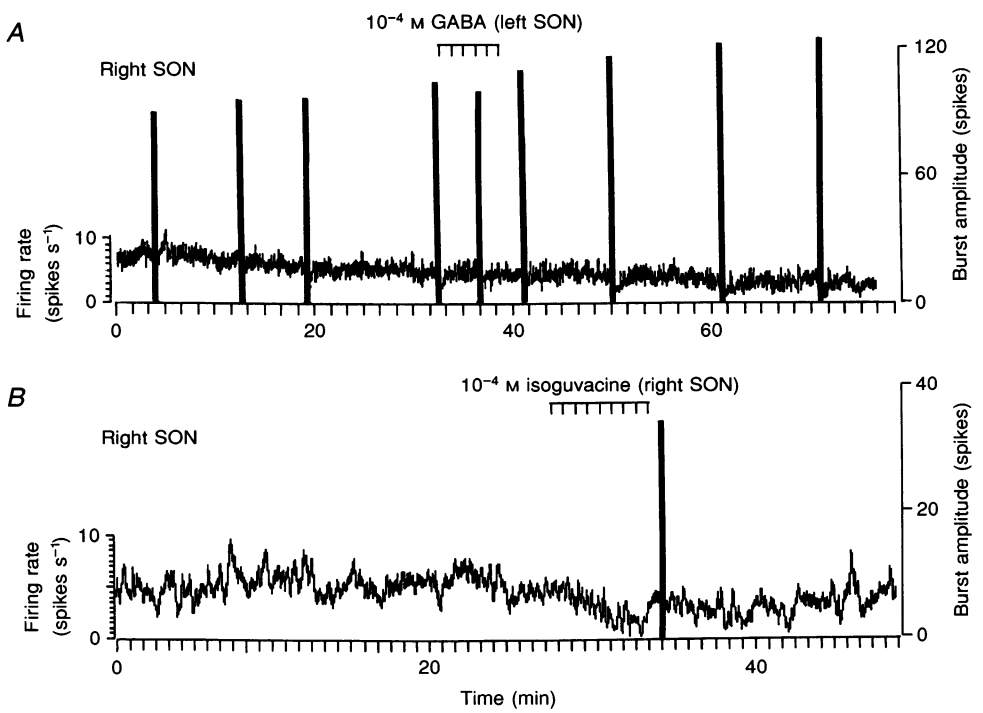

Figure 1. Facilitatory effects of intra-SON injections of GABA or isoguvacine during suckling

Graphs represent the bursting activity of oxytocin neurones. The continuous trace shows the evolution of the basal activity (in spikes s^{-1} every 10 s) and the vertical bars represent the bursts, the height of which is proportional to their amplitude (total number of spikes per burst). *A*, during an on-going milk-ejection reflex, GABA was injected into the left SON while recording the bursting pattern of an oxytocin neurone in the right SON. GABA facilitated the occurrence of the two following bursts, the interburst intervals being reduced as compared with control bursts before injection. The basal activity of the neurone was not affected but a slight increase in the amplitude of bursts following injection occurred. *B*, in the absence of milk-ejection reflex, isoguvacine was injected into the right SON 1 h after the beginning of suckling while recording an oxytocin neurone within the injected nucleus. Isoguvacine decreased the basal activity and triggered the occurrence of one burst during decreased activity.

$P < 0{\cdot}05$; and $17{\cdot}1 \pm 1{\cdot}8$ min, $P < 0{\cdot}001$). Thereafter, bursts continued at regular intervals as they had done during the control period (Fig. 3).

With respect to burst amplitude, a slight increase was observed for neurones within the injected SON despite the fact that their basal activity was still lower than before injection (firing rate during 100 s preceding the respective bursts: 2·6 *vs.* 3·9 spikes s^{-1}). An increase in burst amplitude was not consistently observed for the neurones in the contralateral SON (Fig. 3). The profile of instantaneous frequency of the facilitated bursts was identical to that of control bursts, although their maximal and mean instantaneous frequency were greater (see example in Fig. 2*A*).

In three out of ten tests, facilitation of burst occurrence was preceded by a period during which burst amplitude was decreased. As for the previous cases, GABA and isoguvacine decreased the basal activity, but in the present cases, the amplitude of the one to three subsequent burst(s) also decreased, though there was no apparent change in their periodicity. As this inhibitory effect on burst amplitude wore off, the facilitation of burst occurrence developed. Changes in electrical activity could be followed for three neurones in the injected SON and two contralateral ones. Since neurones in both nuclei were affected similarly, results were pooled. Their mean basal activity measured during the 100 s preceding the first inhibited burst was $1{\cdot}1 \pm 0{\cdot}2$ spikes s^{-1} compared with $1{\cdot}7 \pm 0{\cdot}3$ spikes s^{-1} for the control bursts. The mean amplitude of these reduced bursts was 34 ± 11 spikes compared with 57 ± 14 spikes for the control bursts. Facilitation of burst occurrence affected one to three successive bursts. The values of the first reduced interburst

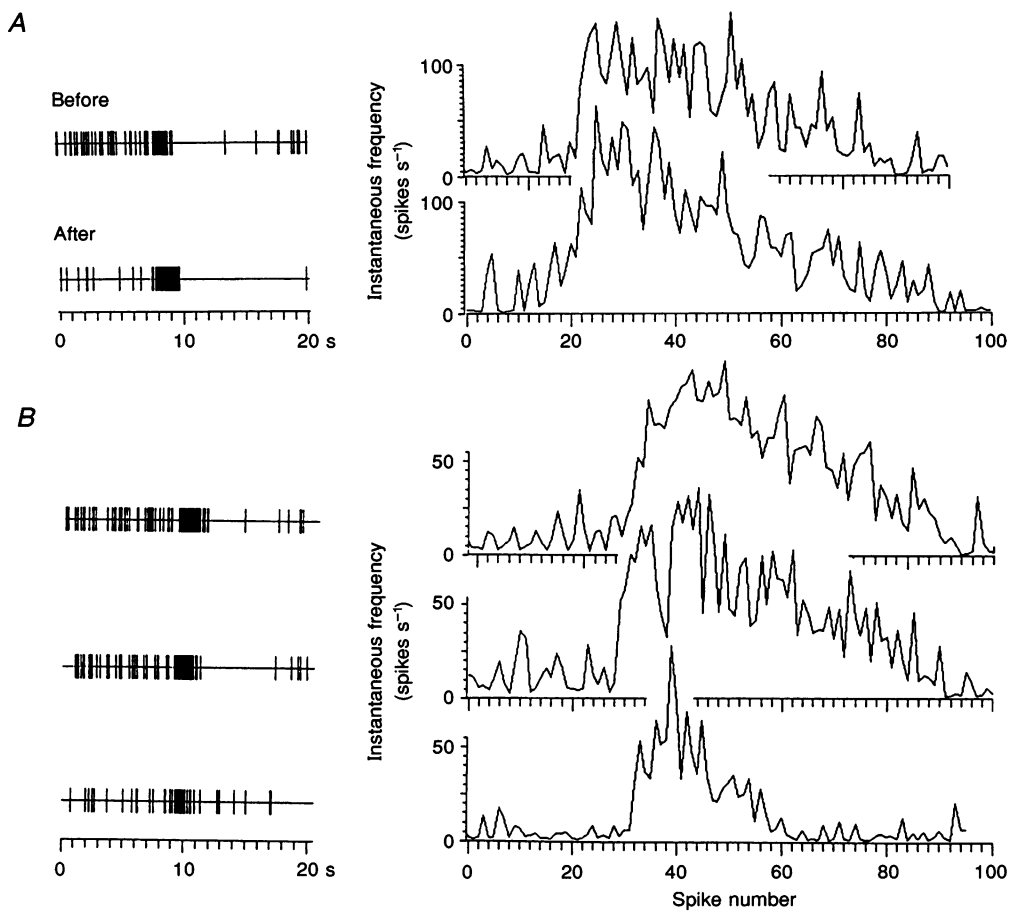

Figure 2. Characteristics of bursts facilitated or triggered by GABA

Left traces show the pattern of action potentials while right traces show the instantaneous frequency for each of the intervals in the left section. Instantaneous frequency is plotted against spike number. *A*, profile of instantaneous frequency of bursts recorded before and after GABA application to an oxytocin neurone in the immediate neighbourhood of injection site. GABA increased maximal instantaneous frequency and mean instantaneous frequency within the burst, but the profile of activity was similar to that of the control burst. *B*, profile of instantaneous frequency of bursts triggered by GABA in three rats failing to milk eject. Patterns of triggered bursts are similar to those of previously recorded suckling-induced bursts (see example in *A*), i.e. with the highest frequencies within a few spikes after the onset of each burst then gradually declining.

intervals were, respectively, 4·5, 9 and 3·2 min instead of 8·7, 20·5 and 9·2 min before GABA application. For the first bursts occurring at reduced intervals, the mean basal activity 100 s before was still decreased (1·2 ± 0·1 spikes s^{-1}) and the mean burst amplitude was 45 ± 11 spikes.

In three rats failing to display a milk-ejection reflex more than 1 h after suckling, GABA (4 times) or isoguvacine (3 times) was injected into one SON. In all seven tests, drug application triggered the occurrence of either one burst in all oxytocin neurones recorded (Fig. 1*B*) and/or one milk ejection.

Due to the pressure injection procedure, only two oxytocin neurones within the injected SON could be followed after injection until bursts occurred. Nevertheless, recordings of four oxytocin neurones in the contralateral SON (one paired recording) and measurements of intramammary pressure provided good evidence for the triggering effect of the drugs and the synchronization of oxytocin neurones in the different nuclei. Repetitive pulses of drugs were given every 30 s over a mean period of 8 ± 2 min. Such injections decreased the basal activity of the neurones within the injected nucleus but not of the contralateral neurones. The single burst occurred 1–7 min after the end of the injection

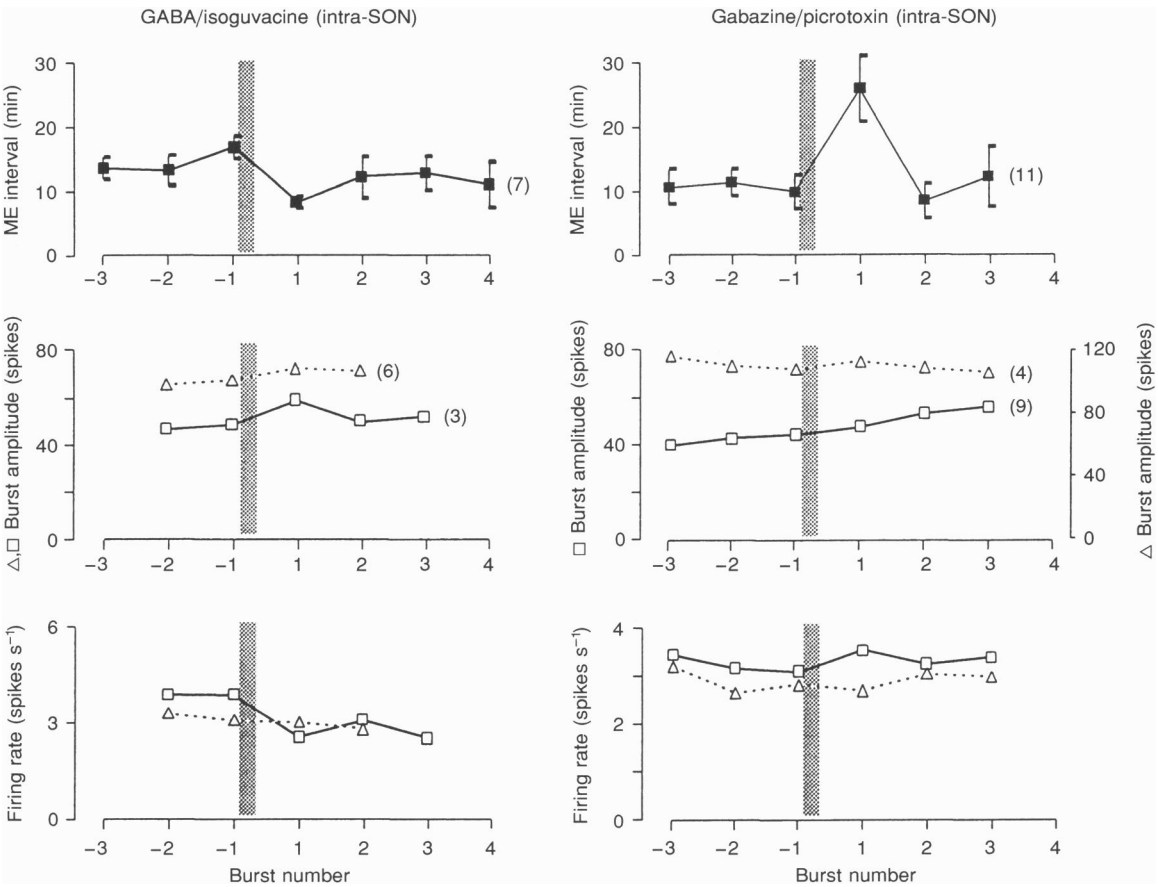

Figure 3. Mean effect of intra-SON injections of $GABA_A$ agonist or antagonist on parameters characterizing the firing pattern of oxytocin neurones

Upper graphs, mean intervals (in min) for the successive bursts or milk ejections (ME intervals) before (burst numbers −3 to −1) and after (burst numbers 1 to 4) injection. The number in parentheses indicates the number of tests. Middle graphs, mean burst amplitude (in spikes) of the successive bursts recorded on neurones within the injected nucleus (□) and on contralateral neurones (△). In each case the number of neurones is indicated in parentheses. Lower graphs, mean firing rate (in spikes s^{-1}) recorded during the 100 s period preceding each successive burst for the same neurones within the injected nucleus or in the contralateral SON. GABA and isoguvacine facilitated the bursting pattern as attested by the decrease in burst interval and slight increase in burst amplitude (particularly for neurones within the injected SON). This facilitatory effect was accompanied by a decrease in firing rate of neurones within the injected SON. The $GABA_A$ antagonists gabazine and picrotoxin strongly delayed the occurrence of the next burst or milk ejection following injection. For both neurones within the injected SON or in the contralateral SON, the delayed bursts occurred with normal amplitude. The firing rate before the delayed burst did not differ from the control rate.

(mean delay, 4·1 ± 1 min) while the basal activity of the neurones within the injected nucleus returned to control values but was still decreased (the values recorded 100 s before the bursts were, respectively, 1·6 and 1·4 spikes s^{-1} instead of 2·1 and 2·1 spikes s^{-1} before drug injection, for the two neurones recorded). The mean burst amplitude of the six neurones recorded was 39 ± 8 spikes. The characteristics of the triggered bursts were similar to that of bursts normally recorded during suckling (see examples in Fig. 2*B*).

In the same rats, the effectiveness of I.C.V. oxytocin to trigger the reflex was also tested either alone ($n = 5$) or in combination with GABA ($n = 3$). Injections were given 15–20 min after a drug-induced burst. Oxytocin alone had little effect since in only two out of the five tests did it trigger bursts or milk ejections (one burst in one of the tests and two in the other; triggering delay, 4·7 and 7 min). In the three tests when GABA was simultaneously injected into one SON, I.C.V. oxytocin injections were effective, being followed by one, two and three bursts in each of the tests, respectively (triggering delays, 1·7, 7·5 and 11 min).

Effect of $GABA_A$ antagonists during suckling

During the regular occurrence of bursts or milk ejections, the intra-SON injections of picrotoxin ($n = 4$) or gabazine ($n = 7$) transiently increased the basal activity of the oxytocin neurones but significantly delayed the occurrence of the next bursts (Fig. 4) and milk ejections. Indeed, the mean burst interval after drug injection (25·9 ± 5 min) was significantly higher than the three intervals recorded before or during injection (respectively, 10·7 ± 2·7, 11·4 ± 2 and 9·9 ± 2·6 min; $P < 0.05$; Fig. 3). Thereafter, bursts recurred at intervals similar to those before injection.

Changes in basal and bursting activity were examined in nine neurones within the injected SON and four neurones in the contralateral SON (two paired recordings). The increase in basal activity was moderate and observed for neurones within the injected SON only. The effect was rapid in onset and maximum during drug application (4·0 ± 0·6 *vs.* 2·9 ± 0·6 spikes s^{-1} before injection; $P < 0.01$) and then progressively returned to control values within 8–18 min for injections of short duration (1–3 min). The increase in basal activity could be sustained for 30 min in cases of repetitive injections every 5 min. Nevertheless, the basal activity had returned to control level when the delayed burst occurred, as shown by the values of firing rate recorded 100 s before bursts in Fig. 3. Examining more precisely the occurrence of bursts revealed that the antagonist injections delayed either the first burst (7/11 tests) or the second burst (4/11 tests) following the beginning of the injection. Indeed, as shown in Fig. 4, a burst could occur during the injection of the antagonist just

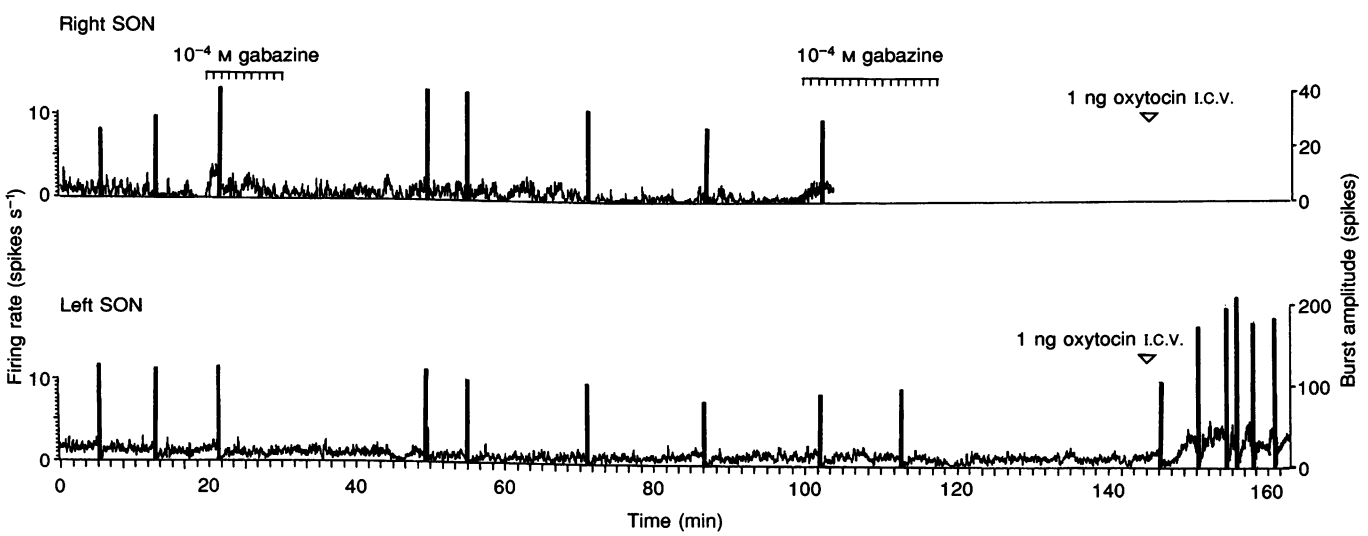

Figure 4. Effect of gabazine ($GABA_A$ antagonist) on the bursting pattern of pair-recorded oxytocin neurones, one being within the injected SON (right SON, upper trace) the other within the contralateral SON (left SON, lower trace)

In this example, gabazine was tested twice, with a 70 min interval between tests, after a period during which bursts (vertical bars) occurred at regular intervals with constant amplitude (total number of spikes quantified by the height of the bar). Minipulses of gabazine were delivered every 30–60 s over 10–20 min. In both cases, gabazine induced a slight increase in basal activity (continuous trace) of the neurone within the injected SON and delayed the occurrence of the following burst for about 30 min (compared with a 7–10 min delay before gabazine). In each case, a burst occurred at the beginning of the injection. The neurone within the injected SON was lost during the second injection of gabazine. Since the second interruption of the bursting pattern by gabazine lasted more than 30 min, oxytocin was injected I.C.V. in order to restart the milk-ejection reflex.

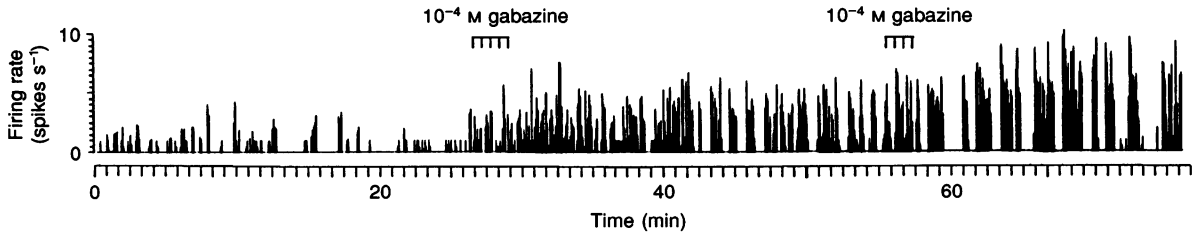

Figure 5. Effect of intra-SON injection of gabazine on the firing rate of a vasopressin neurone
The first injection of gabazine tonically increased the firing rate for about 10 min. Thereafter, activity became clearly phasic. The second injection reinforced this phasic activity by increasing both firing rate and duration of active phases.

as the basal activity of the neighbouring oxytocin neurone increased. This delayed effect on burst periodicity could be due to the fact that the small volume first injected only affected few neighbouring neurones without triggering the known 'cross-talk' between nuclei. Further injection recruited the minimal pool of oxytocin neurones within the SON necessary to affect the periodicity of oxytocin neurones in the four nuclei (see discussion in Lambert *et al.* 1993). There was no significant change either in the characteristics or in the amplitude of the successive bursts following antagonist injection although a slight increase could be observed for some neurones within the injected SON (see example in Fig. 3).

In rats without milk ejections during suckling, intra-SON injections of gabazine ($n = 7$) induced a sustained and tonic increase in the basal activity of all the neurones within the injected SON, but in no case did a burst or milk ejection occur. The increase in basal activity of the seven putative oxytocin neurones (continuous basal activity) was rapid in onset, the maximum being recorded during the first 100 s ($5{\cdot}6 \pm 1{\cdot}5$ *vs.* $3{\cdot}1 \pm 0{\cdot}6$ spikes s^{-1} before injection; $P < 0{\cdot}05$), and lasted 15–20 min after a single injection, but could be sustained for a longer time (35–70 min) with repetitive injections every 5–10 min. Injecting oxytocin I.C.V. just after the excitatory effect of gabazine triggered the milk-ejection reflex in only one out of six tests.

On five occasions, vasopressin neurones displaying more or less regular phasic activity were recorded during gabazine injection. Four of the neurones were located within the injected SON, one being in the contralateral SON. In all cases, application of the drug over a period of 1–2 min led to sustained change in the activity which increased often tonically during the first 5–10 min and then became clearly more phasic (Fig. 5). The fact that the contralateral neurone was similarly affected to those within the injected nucleus, suggested a 'cross-talk' between vasopressin neurones of the different nuclei, as already described for the oxytocin neurones (Moos & Richard, 1989; Lambert *et al.* 1993).

Effect of GABA during hyperosmotic stimulation

Though the effect of hypertonic saline during a milk-ejection reflex has been already described (Negoro, Honda, Uchide & Higuchi, 1987), this stimulus was tested using our experimental conditions in order to study the effect of GABA on the evoked response.

Hyperosmotic stimulus was applied during an on-going milk-ejection reflex in eight suckled rats while recording the bursting activity of ten SON neurones (two SON–SON paired recordings). Injecting I.P. 2 ml of hypertonic saline progressively and dramatically increased the basal activity of all the oxytocin neurones recorded but had complex effects on the bursting pattern (Fig. 6). There was first a

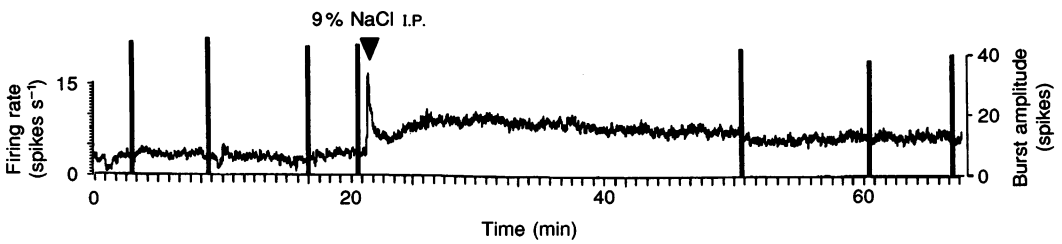

Figure 6. Effect of hyperosmotic stimulus on the bursting pattern of oxytocin neurones during suckling
Injection of 2 ml of hypertonic saline I.P. (9% NaCl) induced a sustained increase in basal activity (continuous trace) of the oxytocin neurone and delayed the occurrence of the next burst (vertical bar) for about 30 min. Thereafter, bursts recurred at regular intervals while the basal activity slightly decreased.

dramatic interruption in burst occurrence; then, when bursts recurred, there was an enhancement of their amplitude but not of their periodicity. The effect on firing rate was at its maximum 10 min after injection, the mean frequency (6·4 ± 0·6 spikes s^{-1}) being significantly greater than the control value recorded for 5 min before injection (2·5 ± 0·3 spikes s^{-1}, $n = 10$; $P < 0·001$; Fig. 7). The mean interburst interval following hypertonic saline injection was 23·7 ± 4·8 min, a value significantly longer than that recorded just before applying the stimulus (6·6 ± 0·6, 8 ± 0·7 and 8·2 ± 0·7 min, $n = 8$; $P < 0·01$; Fig. 7). After this interruption of the reflex, the subsequent bursts recurred at regular intervals as during the control period, but their amplitude was greater (Fig. 7). High values (difference significant at $P < 0·01$) were recorded for the five bursts following hypertonic saline while the basal activity remained at a high level. Thus, when considering the firing rate during the 100 s preceding each burst, values were still significantly higher ($P < 0·001$) than those during the control period (Fig. 7) and this excitatory effect could be observed for at least one hour (in two tests, up to the 8th burst recorded after stimulation).

In five tests, intra-SON application of GABA was performed 1–2 min after I.P. injection of hypertonic saline. Applying GABA during several minutes counteracted the interrupting effect of the hyperosmotic stimulus on burst occurrence (Fig. 8). Indeed, the mean delay for the occurrence of the next burst following 'hypertonic saline plus GABA' was reduced to 12·3 ± 2·6 min (compared with 23·7 ± 4·8 min without GABA; see previous paragraph) and this value did not significantly differ from control values (three respective values before injection: 8·8 ± 2·3, 6 ± 2 and 8·1 ± 1·4 min; Fig. 7).

Basal and bursting activity could be analysed during these tests for six neurones, three being within the injected SON,

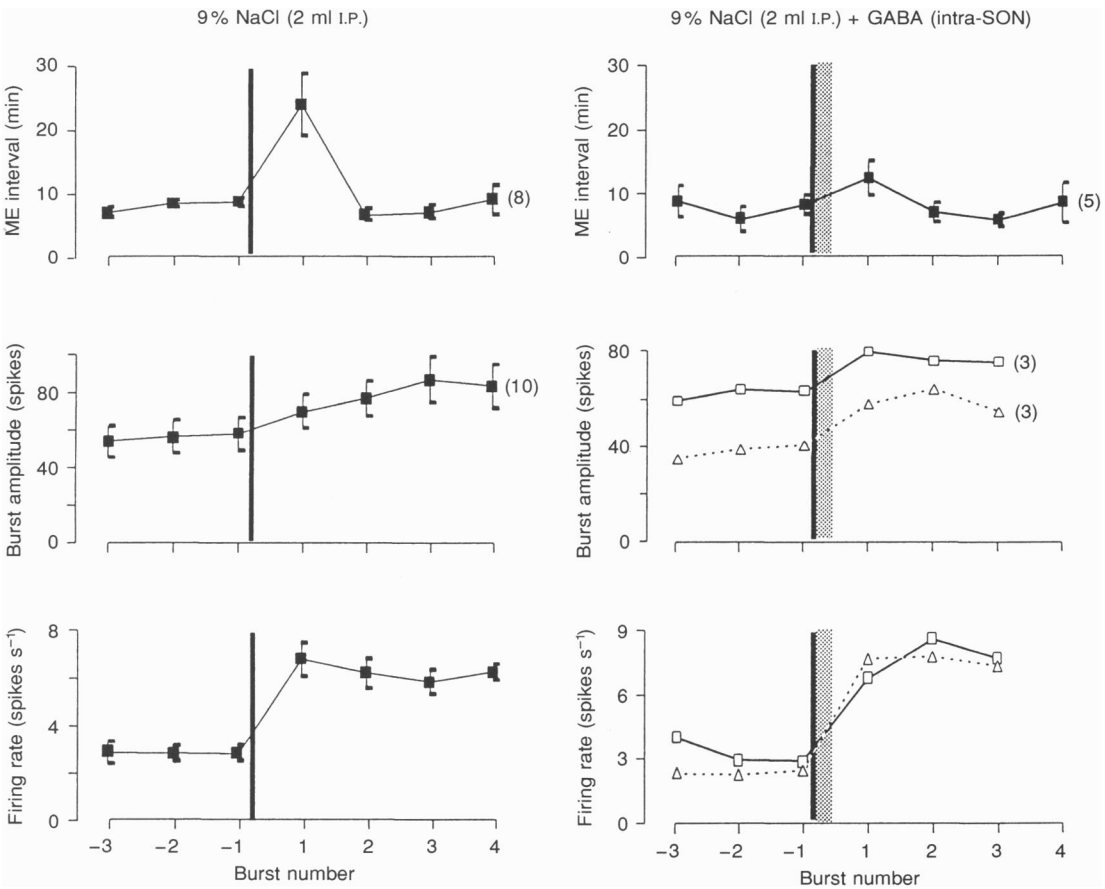

Figure 7. Characteristics of the bursting pattern of oxytocin neurones after applying a hyperosmotic stimulus alone or in combination with intra-SON injection of GABA

Injection of hypertonic saline alone strongly delayed the occurrence of the next burst, as attested by the significant increase in interburst or milk-ejection (ME) interval. After this blocking period, bursts recurred at regular intervals with increased amplitude (number of spikes in the burst) while the basal firing rate was still enhanced. Intra-SON injection of GABA counteracted the inhibitory effect of the hyperosmotic stimulus on burst periodicity since the next burst occurred at an interval not significantly different from the pre-injection ones. Increases in burst amplitude and in firing rate before the successive bursts were comparable for both neurones within the injected SON or in the contralateral SON.

three in the contralateral SON (one paired recording). As shown in Fig. 8, GABA partly counteracted the excitatory effect of the hyperosmotic stimulus on the basal activity for the neurones within the injected SON but not for the contralateral neurones. However, when considering the firing rate 100 s before bursts, there were no apparent differences between neurones within the injected SON and in the contralateral SON (Fig. 7). As in the cases when hyperosmotic stimulus was tested alone, bursts thereafter recurred at regular intervals with greater amplitude than during the control period (Fig. 7).

DISCUSSION

In the present study, intra-SON blockage of $GABA_A$ receptors by $GABA_A$ antagonists increased the basal activity of oxytocin neurones but unexpectedly interrupted the suckling-induced bursting activity, and in rats failing to eject milk, $GABA_A$ antagonists never triggered milk-ejection bursts. Conversely, occurrence of bursts during suckling was facilitated or even triggered by $GABA_A$ agonists, although the basal activity was decreased. These facilitated bursts were not chance occurrences, because the interval prior to the triggered burst was significantly shorter than the preceding inter-burst intervals. Furthermore, they were not injury-induced discharges because (i) simultaneous bursts were recorded in the contralateral SON, (ii) bursts occurred during a period of reduced basal activity and (iii) burst characteristics (profile of instantaneous firing frequency) were identical to those of suckling-induced bursts. Finally, changes in burst occurrence were not due to pressure injection procedure, since, according to the drug injected, the bursts were either delayed or facilitated, and since control injections with cerebrospinal fluid-like medium had no effects (Moos & Richard, 1989).

Tonic *versus* bursting activation

The fact that GABA-induced burst facilitation developed on top of an inhibition of basal activity is surprising, especially when compared with the facilitatory effect induced by oxytocin where both basal and bursting activities simultaneously increased (Freund-Mercier & Richard, 1984). On the other hand, any increase in basal activity of oxytocin neurones does not systematically lead to bursting facilitation. For example, systemic hypertonic saline (Negoro *et al.* 1987 and present data) or I.C.V. injection of acetylcholine (Richard, Moos & Freund-Mercier, 1988) produced a strong and sustained increase in basal activity, but an interruption of bursting, at least in the short term. Similar effects were observed with $GABA_A$ antagonists. Thus, whatever the stimulus used to tonically

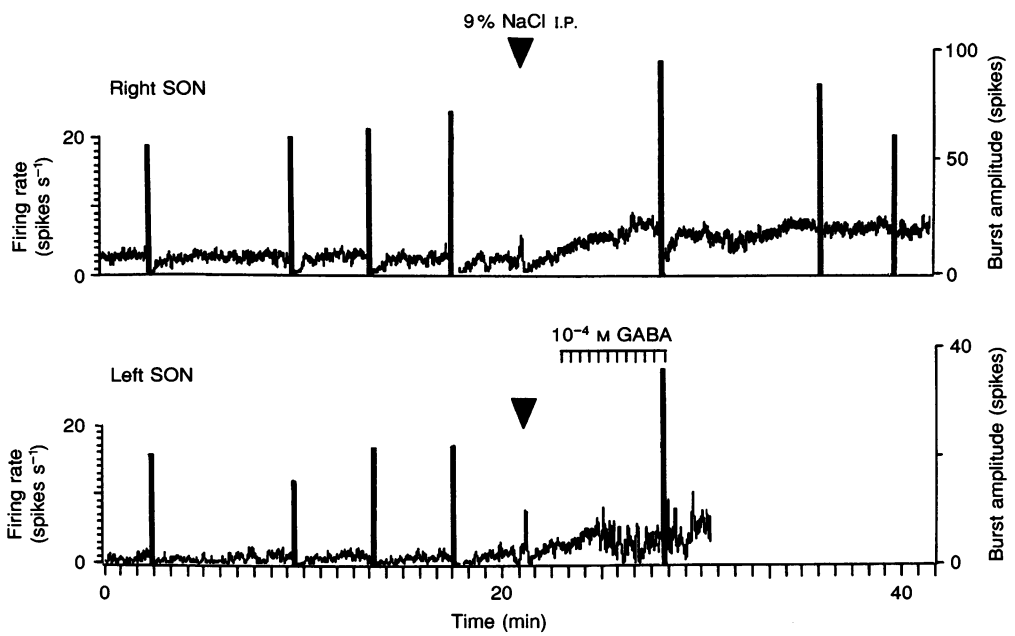

Figure 8. Effect of intra-SON GABA injection on the response induced by hypertonic saline during suckling-induced bursting pattern

Two SON neurones were pair-recorded and GABA was injected into the left SON about 2 min after I.P. injection of hypertonic saline (9% NaCl). Minipulses of GABA were given during about 5 min. Such injections depressed the basal activity (continuous trace) of the neurone within the injection site thus partly counteracting both the increase in firing rate and the interrupting effect on bursting pattern normally induced by hypertonic saline. The next burst (vertical bar) was, however, slightly delayed and occurred within 10 min, a value significantly lower than that recorded when applying the hyperosmotic stimulus alone (see example in Fig. 6 and mean values in Fig. 7).

activate the oxytocin neurones, the resulting activation can compromise the ability of oxytocin neurones to exhibit bursts. Blockage of the milk-ejection reflex by hypertonic saline or bicuculline cannot be explained by EEG desynchronization which lasted a few minutes only (Negoro *et al.* 1987; Voisin, Chapman, Poulain & Herbison, 1994). In the cases of acetylcholine or $GABA_A$ antagonists, the tonic activation lasted 10–20 min and while the excitatory effect wore off, bursts recurred at regular intervals with unchanged amplitude. In the case of the hyperosmotic stimulus, bursts periodically recurred although the neurones still displayed enhanced basal activity, and their amplitude was then increased. Such simultaneous and parallel increase of burst amplitude and basal activity has been previously discussed (Richard *et al.* 1988) and could reflect enhanced excitability as observed after facilitation by oxytocin (Freund-Mercier & Richard, 1984). However, the burst periodicity did not increase after hyperosmotic stimulus whereas it did during oxytocin facilitation. This suggests that the amplitude and periodicity of bursts are not simply related to the level of firing and cannot be explained merely in terms of increased or decreased excitability.

Possible mechanisms of action of GABA

Although not fully justified on the basis of the present recordings, the results suggest a range of membrane potentials within which bursts can develop in oxytocin neurones. Similar speculation was also made by Voisin and co-workers (1994). Once the threshold is reached bursts can be triggered and then facilitated but, above an 'upper limit', the ability to burst is impaired. The first step could depend on local endogenous oxytocin as suggested by (i) the local increase in intra-SON oxytocin release that occurs before the first milk-ejection burst at the beginning of suckling and (ii) the recruitment of bursting oxytocin neurones by centrally injected oxytocin (see review by Richard *et al.* 1988). Maintaining the membrane potential below the 'upper limit' could depend on GABA inputs. Such effect may have developed after injecting small amounts of GABA into the SON since bursts were facilitated or triggered. GABA might act either presynaptically or postsynaptically by selective conductance shunts, thus modifying the membrane properties of oxytocin neurones in order to favour bursting. The suckling stimulus probably involves the GABA afferents in a subtle manner so that the evoked IPSPs (Randle & Renaud, 1987; Tasker & Dudek, 1993; Wuarin & Dudek, 1993) limit or modulate the effects of excitatory inputs, in particular the non-selective ones which could impair the generation of high frequency bursts in oxytocin neurones. The fact that intra-SON GABA counteracted the interrupting effect of hyperosmotic stimulus on the bursting pattern favours this hypothesis. On the other hand, activation of $GABA_A$ receptors beyond a critical level inhibited the ability of oxytocin neurones to burst. Indeed, further amounts of GABA injected in the vicinity of these neurones decreased the burst amplitude. Similarly, Voisin *et al.* (1994) reported a blockage of the milk-ejection reflex after perfusing both SON with 400 nl of muscimol (10^{-5} to 5×10^{-4} M). High levels of $GABA_A$ agonist may act on oxytocin neurones by shifting their membrane potential below the voltage range compatible with burst generation, or by shunting conductances involved in bursting activity. Alternatively, high amounts of GABA could diffuse farther and implicate other indirect mechanisms for regulating oxytocin neurones.

The GABAergic regulation of the phasic activity of vasopressin neurones appears more straight forward. These neurones were clearly activated and became phasic after $GABA_A$ receptor blockage (present data). The evolution and change in phasic activity were quite similar to those occurring after hypertonic saline (Brimble & Dyball, 1977) or haemorrhage (Poulain, Wakerley & Dyball, 1977). In these cells, the phasic activity is an intrinsic property contingent upon synaptic inputs comprising random occurrence of EPSPs and IPSPs. Lowering the level of excitatory synaptic inputs by GABA will tend to stop firing, as previously demonstrated by several authors (see review by Renaud & Bourque, 1991), whereas increasing this level by GABA receptor antagonists will have the reverse effect, as presently shown.

Functional implications

The effect of $GABA_A$ antagonists in the SON, namely enhanced firing and interruption of the milk-ejection reflex, constitutes strong evidence for the physiological involvement of GABA afferents in this reflex. The efficiency with which the $GABA_A$ agonist triggers or facilitates burst occurrence during reduced basal activity supports this hypothesis. Accordingly, we have also noticed a progressive decrease in the firing rate in most oxytocin neurones at the beginning of suckling until the bursts were triggered (F. C. Moos, personal observations). Such a decrease could reflect the progressive involvement of GABA afferents in the mechanisms triggering the milk-ejection reflex. Thus, the bursting pattern could be triggered and maintained by a prolonged activation of GABA afferents, i.e. a long-lasting mild inhibitory GABAergic influence. As discussed above and as also suggested by Voisin and co-workers (1995), GABA could act by selectively attenuating non-suckling excitatory inputs and their related evoked oxytocin release. Indeed, GABA and muscimol ($GABA_A$ agonist) have been previously shown to block the release of oxytocin induced by haemorrhage (Roberts & Robinson, 1991) or hypertonic saline (Bisset, Chowdrey, Fairhall & Gunn, 1990; Roberts & Robinson, 1991). This could explain why lactating rats displayed reduced responses to hyperosmotic stimulus (Hartman, Rosella-Dampman & Summy-Long, 1987; Higuchi, Honda, Takano & Negoro, 1988; Koehler, Mc Lemore, Martel & Summy-Long, 1993), stress (Higuchi *et al.* 1988) or hypovolaemia (Koehler, Mc Lemore, Martel and Summy-Long, 1994) when compared with virgin rats. These stimuli have been demonstrated to tonically activate

oxytocin neurones (Brimble & Dyball, 1977; Poulain *et al.* 1977), and would counteract their bursting pattern. This supports and further develops the hypothesis proposed by Voisin *et al.* (1995) that GABAergic inputs play an important role in the maintenance of the mechanisms underlying milk-ejection reflex.

Thus, the expression of bursting activity during suckling would imply that oxytocin neurones are subjected to increased GABA release. Surprisingly, despite the enhanced GABAergic innervation of SON during lactation (Gies & Theodosis, 1994), the basal release of GABA has been reported not to differ between virgin and lactating rats (Voisin *et al.* 1994). Furthermore, GABA release within the SON did not apparently change during the milk-ejection reflex, whereas it increased during a large elevation of blood pressure (Voisin *et al.* 1994). However, considering the well-known desensitization of $GABA_A$ receptors (Weiss, Barnes & Hablitz, 1988) and the necessity for oxytocin neurones to be submitted to mild but prolonged GABAergic inhibition to express bursting activity, it is probable that GABA release during suckling was pulsatile, localized to the synaptic environment and rapidly taken up so that frequent minipulses may not have been detected by the dialysis procedure, as was suggested by the authors (Voisin *et al.* 1994).

Finally, our present results again raise the problem of the functional interconnections between the four nuclei. It remains unclear how a modification of the electrical activity (membrane properties) of a critical pool of oxytocin neurones within one nucleus changed the bursting pattern of the whole population. This problem has been already debated and both direct and indirect connections between nuclei have been suggested for oxytocin neurones (Moos & Richard, 1989; Lambert *et al.* 1993). Cross-talk has been recently shown between both SON during osmotic challenge (Summy-Long, Neumann, Terrel, Koehler, Gestl, Landgraf and Kadekaro, 1994). A recent study using anterograde tracers has suggested that a pool of parvocellular neurones in the 'dorsochiasmatic area' could ensure cross-talk between the four magnocellular nuclei (Thellier, Moos, Richard & Stoekel, 1994*a*). Indeed, this area is reciprocally connected not only to both PVN and SON (Thellier *et al.* 1994*a*) but also to some related extrahypothalamic areas (Thellier, Moos, Richard & Stoekel, 1994*b*) known to be involved in osmoregulation (e.g. median preoptic nucleus, subfornical organ, etc.) or in the milk-ejection reflex (e.g. bed nucleus of the stria terminalis, ventrolateral septum, etc.). This connecting area, which seems to comprise GABAergic neurones (see discussion in Thellier *et al.* 1994*b*), could be one of the sources of GABAergic inputs and may play a major role in the regulation of burst periodicity and burst synchronization of the oxytocin neurones in the four magnocellular nuclei.

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