Evidence for a Hypothalamic Oxytocin-Sensitive Pattern-Generating Network Governing Oxytocin Neurons *In Vitro*

Pascal Jourdain, Jean-Marc Israel, Bernard Dupouy, Stéphane H. R. Olié, Michèle Allard, Sergio Vitiello, Dionysia T. Theodosios, and Dominique A. Poulain

Institut National de la Santé et de la Recherche Médicale U. 378, Institut François Magendie, F33077 Bordeaux Cedex, France

During lactation and parturition, magnocellular oxytocin (OT) neurons display a characteristic bursting electrical activity responsible for pulsatile OT release. We investigated this activity using hypothalamic organotypic slice cultures enriched in magnocellular OT neurons. As shown here, the neurons are functional and actively secrete amidated OT into the cultures.

Intracellular recordings were made from 23 spontaneously bursting and 28 slow irregular neurons, all identified as oxytocinergic with biocytin and immunocytochemistry. The bursting electrical activity was similar to that described *in vivo* and was characterized by bursts of action potentials (20.1 ± 4.3 Hz) lasting ~ 6 sec, over an irregular background activity. OT ($0.1\text{--}1 \mu\text{M}$), added to the medium, increased burst frequency, reducing interburst intervals by 70%. The peptide also triggered bursting

in 27% of nonbursting neurons. These effects were mimicked by the oxytocin receptor (OTR) agonist [Thr⁴, Gly⁷]-OT and inhibited by the OTR antagonist desGly-NH₂d(CH₂)₅[D-Tyr², Thr⁴]OVT. Burst rhythmicity was independent of membrane potential. Hyperpolarization of the cells unmasked volleys of afferent EPSPs underlying the bursts, which were blocked by CNQX, an AMPA/kainate receptor antagonist.

Our results reveal that OT neurons are part of a hypothalamic rhythmic network in which a glutamatergic input governs burst generation. OT neurons, in turn, exert a positive feedback on their afferent drive through the release of OT.

Key words: organotypic slice cultures; supraoptic nucleus; glutamate; rhythmic network; milk ejection; oxytocin

The nonapeptide oxytocin (OT) is synthesized by magnocellular neurons of the supraoptic, paraventricular, and accessory magnocellular nuclei of the hypothalamus. The axons of these cells terminate in the neurohypophysis from which OT is released into the general circulation to act as a neurohormone in neuroendocrine regulations of parturition, lactation, and body fluid homeostasis.

The model most widely used to study the action of OT has been the lactating rat. Earlier studies (Lincoln and Wakerley, 1974; Poulain and Wakerley, 1982) using extracellular recordings established that in response to the continuous stimulus of suckling, OT neurons display a periodic electrical activation of brief, high-frequency discharges of action potentials recurring every 3–15 min over a more or less regular background activity. Such a bursting activity results in a bolus release of OT into the bloodstream that then acts on the mammary gland to induce milk ejection.

Although many subsequent investigations have tried to determine how the prolonged stimulus of suckling evokes such a periodic neuronal activation, the afferent processing from mammary sensory receptors to the hypothalamus involved in the milk ejection reflex remain elusive, as do the mechanisms underlying the bursting activity itself (Wakerley et al., 1994). For instance,

the periodic bursting activity of OT neurons may result from a gating of afferent impulses (Lincoln and Wakerley, 1975), or, alternatively, it may arise from activation of a pulse generator, existing anywhere along the pathway, up to and including the OT neurons themselves. In addition, we know that centrally released OT regulates the bursting activity and the modalities of the reflex (for review, see Richard et al., 1991), but the means by which it does so remain unclear.

As noted earlier, most data concerning the bursting activity of OT neurons derives from *in vivo* studies, which preclude intracellular recordings necessary to resolve the fine cellular mechanisms underlying such activity. We do not know, therefore, whether OT neurons express specific endogenous membrane properties that could evoke, or at least contribute to, the high frequency discharge. To address these questions, we have been using organotypic slice cultures from postnatal rat hypothalamus. Magnocellular OT neurons develop well in these cultures and retain their specific electrical properties, including a spontaneous bursting behavior driven by synaptic activity (Jourdain et al., 1996).

We here pursue these analyses further. We now provide evidence that bursting electrical activity in OT neurons in culture is similar to that observed *in vivo*, and it results from activation of a rhythmic hypothalamic network, which in turn is influenced by locally released OT.

MATERIALS AND METHODS

Preparation of the cultures. The cultures were prepared using the roller tube method, as described previously (Jourdain et al., 1996). Briefly, brains from 4- to 6-d-old Wistar rat pups were removed, and tissue blocks that included the hypothalamus were quickly dissected and sectioned ($400 \mu\text{m}$) with a McIlwain tissue slicer. Frontal slices containing the supraoptic nucleus (SON) were cut into two parts along the third ven-

Received March 11, 1998; revised May 7, 1998; accepted June 9, 1998.

We are grateful to Drs. H. Gainer, T. Higuchi, M. Morris, and A. Robinson for their generous gifts of antibodies and to Dr. M. Manning for his generosity and help with oxytocin receptor agonists and antagonists.

Correspondence should be addressed to D. A. Poulain, Institut National de la Santé et de la Recherche Médicale U. 378, Institut François Magendie, 1 Rue Camille Saint-Saëns, F33077 Bordeaux Cedex, France. E-mail: dominique.poulain@bordeaux.inserm.fr

Copyright © 1998 Society for Neuroscience 0270-6474/98/186641-09\$05.00/0

tricle, and each part was placed on a glass coverslip coated with heparinized chicken plasma. Thrombin was then added to the coverslip to coagulate the plasma and permit adhesion of the slice to the coverslip. The coverslip was inserted into a plastic flat-bottomed tube (Nunc, Roskilde, Denmark) containing 750 μ l of medium, pH 7.4 (290–295 mOsm), composed of 50% Eagle's basal medium (Life Technologies, Gaithersburg, MD), 25% heat-inactivated horse serum (Life Technologies), and 25% HBSS (Life Technologies) enriched with glucose (7.5 mg/ml); L-glutamate (Seromed, Berlin, Germany) was added at a concentration of 2 mM. No antibiotics were used. The tubes were tightly capped and inserted in a roller drum; the tubes were rotated approximately 15 turns/hr. The medium was replaced once a week.

RIA and HPLC analyses. Extracellular levels of OT were determined in 21- to 30-d-old cultures maintained in normal medium ($n = 16$) and in cultures exposed to elevated levels of extracellular K^+ ($n = 16$). For the latter, media that had been in contact with cells for 7 d were collected and replaced with Yamamoto's solution containing (in mM): NaCl 125, KCl 5, KH_2PO_4 1.25, $MgSO_4$ 1, $NaHCO_3$ 5, $CaCl_2$ 2, HEPES 10, glucose 5, pH 7.4 (290–300 mOsm). This solution was collected after 20 min and replaced by one containing 56 mM KCl and 85 mM NaCl to keep the ionic strength constant. After 10 min, this solution was collected and replaced by normal culture medium, which in turn was collected after 24 hr. All samples were acidified (0.1 M HCl) and passed through a reversed-phase C-18 cartridge (Waters Associates, Milford, MA). After the cartridge was washed with 0.1% trifluoroacetic acid, pH 2.4, it was eluted with 0.1% trifluoroacetic acid (pH 2.4)/MeOH (40:60). Eluates were dried in a vacuum concentrator (Unijet II, Uniequip) and stored at $-20^\circ C$. The dried extracts were dissolved again in 150 μ l of RIA buffer consisting of 0.1 M sodium phosphate buffer, pH 7.4, 0.1% BSA, and 0.01% sodium azide.

For intracellular determinations, attached cells were lysed with 0.1 M HCl and transferred to vials. Each culture dish was rinsed with 500 μ l of 0.1 M HCl, which was added to the lysed cells. The samples were then centrifuged ($12,000 \times g$ for 10 min at $4^\circ C$), and supernatants were extracted as for the culture media. Extracts from hypothalamic slices from 4- to 6-d-old rats, which included the SON, and from neurohypophyses and the SON of adult rats served as controls.

OT was radiolabeled with Na [^{125}I] using the chloramine-T method (Hunter and Greenwood, 1962) and purified by HPLC (μ Bondapak C18 column) using a 0.1% TFA (pH 2.4)/MeOH (59:41) elution. Aliquots of radioiodinated peptide were stored at $-30^\circ C$ in 41% methanol until use. The specific activity was in the range of 1000–2000 Ci/mmol ($37\text{--}74 \times 10^6$ MBq/mmol). Two different rabbit sera that recognize amidated OT (Higuchi et al., 1985; Morris et al., 1992) were used. All dilutions of samples, serum, and tracer were made in RIA buffer.

For RIA, 50 μ l of OT standards (0.25–125 pg/tube) or samples were incubated with 50 μ l of anti-OT serum (at a final dilution of 1:36 000 for 24 hr at $4^\circ C$). Subsequently, 50 μ l of [^{125}I]OT (8,000–10,000 cpm/assay) were added and incubated for 48 hr ($4^\circ C$). Free [^{125}I]OT was separated from antibody-bound [^{125}I]OT by adding 50 μ l of horse serum and 1 ml of polyethyleneglycol (8000). The samples were centrifuged, supernatants were discarded, and the radioactivity of the pellets was counted in an LKB Wallac counter. The concentrations of OT-like materials were determined using OT standard curves.

For HPLC, media and tissue extracts were applied to a Kromasil silica C18 column (250×4.6 mm), which was eluted with a linear gradient of 0.1% TFA (pH 2.4)/MeOH (0–60%, 1 ml/min, 60 min). One-minute fractions were collected, dried in a vacuum concentrator (Unijet II, Uniequip), dissolved again in 50 μ l of RIA buffer, and radioimmunoassayed as described.

To evaluate cell death, we measured lactate dehydrogenase activity using a commercial kit from Boehringer (Bagnole, France). We never detected any significant lactate dehydrogenase activity in our cultures.

Electrophysiological recordings. Conventional intracellular recordings were performed in 2- to 7-week-old cultures. Coverslips were transferred to a temperature-controlled chamber ($37^\circ C$) fixed to the stage of an inverted microscope (Axiovert, Zeiss). They were perfused continuously (2 ml/min) with Yamamoto's solution. The following drugs were added to the medium when required: synthetic OT (Peninsula, Belmont, CA; 0.1–1 μ M), the OTR agonist [Thr⁴, Gly⁷]-OT, the OTR antagonist desGly-NH₂d(CH₂)₅[D-Tyr², Thr⁴]OVT, 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX; RBI, Natick, MA). The potent oxytocic and antioxytocic effects of the OTR agonist and antagonist have been described earlier (Lowbridge et al., 1977; Manning et al., 1995).

Recording microelectrodes were pulled from borosilicated glass capil-

laries [outer diameter, 1 mm; inner diameter, 0.5 mm (Sutter Instruments)] with a horizontal puller (Flaming/Brown-P87) and filled with 1 M potassium acetate with 0.5–1% biocytin (Sigma, St. Louis, MO). Electrode resistance varied from 150 to 250 M Ω . Intracellular potentials were recorded through single electrodes using an Axoclamp 2A (Axon Instruments, Foster City, CA), which also permitted injection of currents. Electrical signals were visualized on an oscilloscope (Tektronics, Beaverton, OR), recorded directly on a pen recorder (Gould 2400, Gould, Glen Burnie, MD), digitized (Neurodata), and recorded on videotape. Unless stated otherwise, values are expressed as mean $\pm$ SD. Each parameter (burst and interburst duration, burst and interburst action potential frequency) was determined before and during application of drugs. Data were analyzed using Student's *t* test.

At the end of the recording session, neurons were filled with biocytin using hyperpolarizing current pulses (1 nA, 200 msec, 1 Hz, 10 min). The cultures were then fixed (2 hr, room temperature) in a freshly prepared solution of 4% paraformaldehyde and 0.15% picric acid in 0.1 M sodium phosphate buffer, pH 7.4.

Identification of recorded neurons. This was performed according to the protocol described in detail in Jourdain et al. (1996). Briefly, after fixation, the cultures were incubated in a mixture of primary antibodies: a monoclonal mouse Ig raised against OT-related neurophysin (OT-Np) (Ben-Barak et al., 1985) and a polyclonal rabbit serum raised against vasopressin-associated neurophysin (VP-Np) (Roberts et al., 1991). They were then treated with a mixture of anti-mouse Igs conjugated to Texas Red, anti-rabbit Igs conjugated to fluorescein (FITC) (Bioss), and streptavidin conjugated to 7-amino-4-methyl-coumarin-3-acetic acid (AMCA) (Bioss). After the cultures were mounted with fluoromount (Vectashield, Vector Laboratories, Burlingame, CA), they were examined with epifluorescence (Leica, Leitz DMR) with appropriate filters. When required, cell counts were obtained by counting all labeled neurons within the slice directly under the microscope.

RESULTS

Release of OT in hypothalamic organotypic cultures

To analyze the role of OT in the electrical activity of our cultured OT neurons, we first determined whether they secrete OT and the form of the peptide released into the cultures. OT immunoreactivity was assayed, therefore, in extracts of cells and culture media derived from 21- to 30-d-old cultures. It coeluted as one peak with the same retention time as amidated OT, the final, secreted form of the peptide in the adult (Gainer and Wray, 1994). As seen in Figure 1, all profiles, whether obtained from cells or media, were similar to those from extracts of the adult SON or neurohypophysis.

The amount of OT immunoreactivity detected in individual cultures varied considerably, both in the media (0.7–19 pg/culture) and in the cells (0.8–26.1 pg/culture). Nevertheless, the level in each culture medium was closely correlated to the number of OT cells present in that culture (Fig. 2A). Moreover, OT levels in the media depended on the number of days of incubation in the same medium (Fig. 3). After raising the levels of extracellular K^+ , a well known procedure to depolarize the neurons and evoke OT release (Douglas, 1974), we detected a clear correlation between the amount of OT in the medium before and after stimulation (Fig. 2B). Last, we studied OT levels in the medium of 60 cultures from which we had obtained recordings from one OT neuron (but that had not been tested with OT or its analog). As seen in Figure 3, cultures in which OT neurons displayed a bursting electrical activity had higher OT levels than cultures with no bursting cells.

Passive membrane properties of cultured OT neurons

The data reported here derive from 51 neurons, each from a different culture, which we were able subsequently to identify as OT neurons by simultaneous immunolabelings for OT-Np and VP-Np of the biocytin-injected cells. None of the OT-Np-positive cells were VP-Np positive.

Figure 1. HPLC of OT immunoreactivity (OT-IR) in extracts of cultured cells, culture medium, adult SON, and neurohypophysis (NH). Note that OT in cultures coeluted in the same fraction as that in adult tissues.

As reported previously (Jourdain et al., 1996), the membrane properties displayed by OT neurons in our cultures were similar to those obtained in other *in vitro* preparations. The OT neurons recorded here had a mean resting membrane potential of -60 ± 5 mV and a mean input resistance of 109 ± 31 M Ω , with a mean membrane time constant of 10.1 ± 1.5 msec. As expected, they displayed spontaneous action potentials (71 ± 7 mV; 1.3 ± 0.2 msec).

Bursting electrical activity of cultured OT neurons

The bursting pattern of electrical activity was analyzed in detail in 23 neurons displaying spontaneously such activity for at least 30 min. Bursts of action potentials had a mean frequency of 20.1 ± 4.3 Hz (range, 10–30 Hz) and a mean duration of 6.2 ± 2.5 sec (range, 3–14 sec). They alternated with periods of irregular activity (mean frequency, 2.7 ± 1.7 Hz; range, 0.5–6 Hz; mean duration, 92 ± 59 sec; range, 20–290 sec) (Fig. 4A). Analysis of the sequential histogram of firing during the bursts revealed two types of profiles. In 55% of the cells, the profile had a skewed bell shape, with the frequency of discharge increasing rapidly to reach a peak (range, 20–45 Hz) and then decreasing more or less exponentially to control values (Fig. 4B₁). In the other cells, the profile was rectangular, with a frequency of discharge stable within the burst (Fig. 4B₂), reaching a maximum of 30 Hz.

Effect of OT on spontaneously bursting OT neurons

The effect of bath application of OT was studied on 12 spontaneously bursting OT neurons (Table 1, Fig. 5A). Apart from one neuron, which remained completely unaffected, OT induced an

Figure 2. A, Spontaneous release of OT in organotypic cultures. Correlation between the number of OT cells and OT immunoreactivity (OT-IR) in medium was $r^2 = 0.83$, $p < 0.001$. Media were collected after 5 d of incubation (see Materials and Methods). B, K⁺-evoked release of OT in organotypic cultures. The total amount of OT released during 56 mM [K⁺] stimulation was closely correlated to OT concentration in the culture medium before the medium was changed ($r^2 = 0.93$, $p < 0.001$).

acceleration of bursting activity in the recorded neurons, an acceleration that was characterized by a pronounced decrease of the mean interburst duration (-70%) and a 10% decrease of the mean basal frequency discharge. Burst duration also showed $\sim 10\%$ reduction, but the mean frequency of discharge during the bursts increased slightly ($+5\%$). Although the effects of OT were significantly reduced after washing, in four of nine cells they were not fully reversed by the end of the recording period. The peptide had no effect on resting membrane potential or input resistance.

Effect of OT on nonbursting OT neurons

Twenty-eight OT neurons that showed an irregular or continuous firing pattern, with a mean frequency of 1.9 ± 0.8 Hz (range, 0.05–5 Hz), were included in this study to test the effect of OT on their electrical activity. In six of these neurons, OT evoked a bursting activity similar to that observed in spontaneously bursting OT neurons after OT application (Table 1, Fig. 5B). After washing, bursting activity persisted but decreased in frequency.

Figure 3. OT-IR in culture medium in relation to the number of days of incubation in the medium and to the number of cultures in which one bursting OT neuron was recorded or not. Note that the probability of recording a bursting neuron was higher in culture with high OT-IR. $p < 0.05$, Mann-Whitney U test. Numbers in brackets indicate numbers of cultures per group.

Figure 4. Bursting activity in OT neurons. *A*, Sequential histogram of action potential discharge (number of spikes per second) over a 10 min period. Note the recurrence of bursts over an irregular background activity. *B*, Examples of action potential discharges during bursts. Action potential frequencies have been calculated every 0.5 sec to visualize peak frequencies. In *B*₁, the profile is very similar to that of milk ejection bursts *in vivo*. In *B*₂, firing is more sustained throughout the burst.

The remaining cells were not affected by OT. None of the recorded cells showed significant changes in their basal firing rate in response to OT.

Effects of OT receptor agonist and antagonist

Bath application of the specific OTR agonist [Thr⁴,Gly⁷]-OT (0.1 μ M) caused an increase in burst incidence in five of six

Table 1. Characteristics of action potential discharges obtained at resting membrane potential, before and after application of OT

	Spontaneous bursting cells ($n = 11$)		OT-induced bursting activity ($n = 6$)
	Before OT	During OT	
Burst duration (sec)	7.0 ± 2.8	$6.5 \pm 2.8^*$	6.1 ± 1.7
Interburst duration (sec)	115 ± 64	$34 \pm 15^*$	35 ± 9
Intraburst frequency (Hz)	19.3 ± 3.9	$20.4 \pm 4.1^{**}$	15.0 ± 1.3
Interburst frequency (Hz)	2.4 ± 1.9	$2.1 \pm 1.7^{**}$	2.8 ± 1.2

Data are expressed as mean $\pm$ SD. Significantly different from control values at $*p < 0.005$, $**p < 0.05$; Student's paired t test.

Figure 5. Effect of OT on the bursting activity of OT neurons. *A*, Example of a spontaneously bursting OT neuron in which bath application of OT (0.1 μ M; 10 min) increased dramatically the frequency of bursts (*), with no effect on their duration or the intraburst frequency of discharge. *B*, Example of a nonbursting OT neuron in which bath application of OT (0.1 μ M; 15 min) switched the pattern of activity from a continuous (*top panel*) to a bursting mode (*bottom panel*).

spontaneously bursting OT neurons tested (Table 2, Fig. 6*A*) and triggered a bursting activity in two of six nonspontaneously bursting cells (Fig. 6*B*). In the former, the agonist significantly decreased the mean interburst duration (-75%) without significantly modifying the mean duration of the bursts. The frequency of discharge was increased during the bursts ($+8\%$) but decreased during the interburst period (-18%). These effects were reversible in all of the cells tested. In the two nonspontaneously

Table 2. Characteristics of action potential discharges measured at resting membrane potential before and after application of the OTR agonist [Thr⁴, Gly⁷]-OT

	Spontaneous bursting cells (<i>n</i> = 5)		OTR agonist-induced bursting activity (<i>n</i> = 2)
	Before OTR agonist	During OTR agonist	
Burst duration (sec)	5.5 ± 1.7	5.4 ± 1.5	4.0–9.5
Interburst duration (sec)	104 ± 42	25 ± 6*	30–77
Intraburst frequency (Hz)	19.6 ± 3.3	21.2 ± 3.0*	13.0–21.0
Interburst frequency (Hz)	2.6 ± 1.1	2.2 ± 1.0*	0.4–3.0

For the spontaneously bursting cells, data are expressed as mean ± SD. Significantly different from control values at **p* < 0.01; Student's paired *t* test. For the two cells in which the OTR agonist induced bursting, the two values are given for each parameter.

Figure 6. Effect of an OTR agonist on the bursting activity of OT neurons. The specific OTR agonist [Thr⁴, Gly⁷]-OT (0.1 μM; 10 min) mimicked the effect of OT, facilitating bursting in a spontaneously bursting neuron (*A*) and evoking bursting in a nonbursting OT-cell.

bursting cells, the OTR agonist triggered a bursting activity similar to that of the previous group (Table 2, Fig. 6*B*). After washing, the bursting activity persisted but the interburst period increased.

The effect of bath application of a specific OTR antagonist, desGly-NH₂d(CH₂)₅[D-Tyr², Thr⁴]OVT (50 μM), was tested on a group of 10 OT neurons. In contrast to the agonist, it

Figure 7. Effect of an OTR antagonist on the bursting activity of OT neurons. *A*, Example of a spontaneously bursting (*) OT neuron whose activity was inhibited by bath application of a specific OTR antagonist desGly-NH₂d(CH₂)₅[D-Tyr², Thr⁴]OVT (50 μM; 10 min). *B*, Example of a spontaneously bursting OT neuron (*top panel*) in which addition of OT (0.1 μM; 10 min) resulted in an increased burst frequency (*middle panel*). Simultaneous application of the OTR antagonist (50 μM; 15 min) inhibited the facilitatory effect of OT (*bottom panel*).

dramatically inhibited spontaneous (Fig. 7*A*) and OT-sensitive (Fig. 7*B*) bursting activity. In six cells displaying spontaneous (*n* = 3) or OT-induced bursts (*n* = 3), the antagonist greatly increased the interburst duration (+240%) and, to a lesser extent, the basal firing rate (+9%). The burst duration, as well as the frequency of discharge during the bursts, was not significantly affected (Table 3). These effects were fully reversible in three of six cases. In three other neurons, the OTR antagonist irreversibly stopped spontaneous (*n* = 2) or OT-induced (*n* = 1) bursting activity. Finally, in one case, the antagonist had no effect on the OT-induced bursting behavior of one neuron.

The agonist and the antagonist induced no change in resting membrane potential and input resistance in all the neurons tested.

OT-sensitive bursts are not caused by intrinsic mechanisms

The influence of intrinsic cell properties on burst generation and duration was assessed by varying the resting membrane potential with sustained current injections. In neurons in which OT induced or increased bursting activity, burst duration and interburst intervals were unaffected by membrane depolarization or hyperpolarization ($n = 6$) (Fig. 8*A₁*, *B*). The intraburst firing rate, however, was highly correlated to membrane potential (Fig. 8*C*). Furthermore, in neurons that were maintained hyperpolarized below spike threshold, recurring volleys of EPSPs (Fig. 8*A₂*)

Table III. Characteristics of action potential discharges obtained at resting membrane potential before and after application of the OTR antagonist

	Before OTR antagonist	During OTR antagonist
Burst duration (sec)	5.0 ± 1.7	5.6 ± 2.0
Interburst duration (sec)	30 ± 10	100 ± 62*
Intraburst frequency (Hz)	20.8 ± 5.1	19.8 ± 4.1
Interburst frequency (Hz)	3.5 ± 1.5	3.8 ± 2.1

Data are expressed as mean ± SD ($n = 6$ cells). Statistically different from control values at * $p < 0.001$; Student's paired t test.

Figure 8. *A*, Effect of membrane potential on bursting activity. Example of an OT cell in which bursting activity was induced by bath-application of OT. The burst persisted when the cell was held at three different membrane potentials. Neither interburst intervals (*A₁*) nor burst duration (*A₂*) was affected by this manipulation. Note that hyperpolarizing membrane potential unmasked an abundant excitatory synaptic activity underlying the bursts (*A₂*, bottom record). In *A₂*, action potentials have been clipped to adjust to the scale. *B*, Summary of the effect of membrane potential on burst and interburst durations. These two parameters were unaffected in six of six cells tested. Data were normalized to the value obtained at -60 mV and are expressed as percentage changes of the mean ± SD. *C*, Summary graph of the effect of membrane potential on action potential discharge observed within the burst. Action potential frequency within the burst increased at depolarized potentials and decreased at hyperpolarized potentials.

underlying the OT-sensitive bursts persisted, as was the case for spontaneous bursts (Jourdain et al., 1996).

In a second set of experiments, we tested whether OT-sensitive bursting activity could be induced or interrupted by applying brief depolarizing or hyperpolarizing current pulses, respectively ($n = 5$). Brief suprathreshold depolarizing current pulses were unable to elicit bursts in an OT neuron that was already bursting or to affect burst duration. Conversely, OT-sensitive bursting activity was unaffected by hyperpolarizing pulses of current applied within the bursts. Current pulses never triggered any endogenous regenerative potentials.

Synaptic influences on OT-sensitive bursting activity

In our earlier studies we found that the spontaneous bursting activity displayed by cultured OT neurons was caused by recurrent volleys of synaptic impulses. Because the excitatory afferent input to these neurons is glutamatergic via AMPA/kainate receptors (Jourdain et al., 1996), we examined the effect of CNQX, an AMPA/kainate receptor antagonist, on the OT-sensitive bursting activity.

CNQX reversibly blocked the fast EPSPs as well as the OT-sensitive bursting activity on all tested cells ($n = 5$) (Fig. 9). This effect was sometimes accompanied by a membrane hyperpolarization. Furthermore, such a blockade persisted after the membrane potential was depolarized to a level at which the firing rate

Figure 9. Effect of CNQX on bursting activity. Example of a cell in which the bursting activity was facilitated by exogenous application of OT (top panel). Addition of 15 μ M CNQX to the perfusion solution (middle panel) fully inhibited the bursting behavior. This effect was reversible on washout of CNQX (bottom panel). Note that during CNQX application, depolarizing membrane potential above spike threshold (-50 mV) enhanced action potential discharge but caused no burst.

was similar to that observed during the interburst periods under control conditions (Fig. 9), indicating that the blockade was not caused by a change in membrane potential of the recorded cell after the removal of a tonic AMPA/kainate excitation.

DISCUSSION

OT neurons develop a characteristic bursting electrical activity before each reflex milk ejection during lactation. We here investigated mechanisms involved in the genesis and modulation of this activity, using OT neurons in organotypic slice cultures from postnatal hypothalamus. As shown previously (Jourdain et al., 1996), the cultured OT neurons possess morphological and basic electrophysiological properties of adult OT neurons, including a capacity for bursting. We show here that such bursting activity shares many characteristics of that recorded *in vivo* during suckling and that it results from the activity of a local circuitry, in which, as *in vivo*, OT secreted by the neurons exerts a positive feedback action.

Bursting activity in cultured OT neurons

In vivo, hypothalamic magnocellular neurons display two types of bursting activity. One is the phasic activity characteristic of vasopressin neurons, typically consisting of periodic bursts with a discharge rate of 5–15 Hz, lasting 5–25 sec, and separated by intervals of similar duration, during which there is no spike activity (Poulain and Wakerley, 1982; Poulain et al., 1988). Endogenous plateau potentials (Andrew and Dudek, 1983) underlie the bursts in phasic activity, and the bursts are not influenced by OT (Freund-Mercier and Richard, 1984). Clearly, the bursting activity recorded in cultured OT neurons was distinct from this phasic activity. In contrast, it offers striking analogies to that recorded from OT neurons *in vivo*. In both cases, bursts have a short duration, lasting a few seconds, with a high frequency of discharge. In half the cases, the temporal profile of burst discharge was similar to that observed *in vivo*. Both *in vivo* and *in vitro*, bursts occur on a background of continuous slow spike discharge, and interburst intervals are very long compared with the bursts.

Nevertheless, we also noted some differences between the activities of OT neurons *in vivo* and *in vitro*. Burst duration was more variable in cultures (3–14 sec) than *in vivo* (2–4 sec), and peak amplitude was smaller (20–45 vs 30–80 Hz) (Poulain and Wakerley, 1982). The average interburst interval was much shorter *in vitro* (97 sec) than *in vivo* (3–5 min), although the longest intervals recorded here (2–5 min) clearly overlap with the shorter intervals recorded *in situ*, especially when the milk ejection reflex is facilitated by OT (Freund-Mercier and Richard, 1984). Such differences may be attributable in part to the anatomic remodeling that occurs in cultures, where OT neurons are much less numerous and less densely packed than in the intact hypothalamus. This may then alter synaptic drive and extracellular fluid homeostasis and, consequently, the characteristics of the bursts.

The closest analogy between the bursting activity recorded here and that described in lactating rats is that they are both modulated by OT. *In vivo*, suckling-induced bursting activity of OT neurons is modulated by endogenous OT (Moos et al., 1989; Neumann et al., 1993a,b), which allows the onset of the reflex (Freund-Mercier and Richard, 1984) and facilitates the occurrence of bursts (Freund-Mercier and Richard, 1981, 1984) and the recruitment of OT cells into bursting (Belin and Moos, 1986). In our cultures, OT also facilitated or induced bursting, and as *in vivo* (Freund-Mercier and Richard, 1984), spontaneous bursting was blocked by an OT receptor antagonist.

Origin of bursting activity in cultured OT neurons

Bursting neurons may be either pacemaker cells, themselves generating the bursts, or followers in a synaptic network. In pacemaker neurons displaying a bursting activity underlain by specific voltage-dependent ionic conductances, burst duration and rhythmicity are highly voltage-dependent [for example, see McCormick and Huguenard (1992) and Destexhe et al. (1996)]. However, the rhythmicity and duration of spontaneous (Jourdain et al., 1996) and OT-induced bursts remained unaffected by depolarizing and hyperpolarizing current pulses given before, within, or after the bursts. Likewise, burst duration was unchanged at various membrane potentials. Furthermore, hyperpolarizing membrane potential below spike threshold unmasked volleys of EPSPs underlying the bursts, volleys that were blocked by CNQX. Finally, we found no evidence for slow regenerative mechanisms such as plateau potentials.

Taken together, then, these observations show that OT-induced bursting activity is similar to spontaneous bursting activity and results from a patterned afferent drive, not from an endogenous mechanism. This pattern is imposed by glutamate neurons, which either could be endogenous pacemaker cells or be driven by a more complex input.

Modulation of bursting activity in cultured OT neurons

Application of OT had very striking effects in inducing or modulating bursting activity. This was not merely a pharmacological effect. The cultured OT neurons secreted OT, and the higher the level of OT released, the greater were the chances of detecting a bursting neuron. Furthermore, spontaneous bursting was blocked by an OTR antagonist. The question arises, then, regarding the site of action of OT. OT neurons possess OT receptors *in situ* (Freund-Mercier et al., 1994). Furthermore, it was shown that OT increased intracellular Ca^{2+} levels (Lambert et al., 1994) and depressed inhibitory GABAergic responses in these cells (Brussaard et al., 1996). Nevertheless, in our experiments, the effect of

OT appeared indirect, via modulation of a periodic presynaptic activity. As already pointed out, bursting had a presynaptic origin, and OT affected the pattern of afferent volleys of EPSPs, with no effect on the membrane potential of OT cells. It appears unlikely, therefore, that the OT receptors located on OT neurons play a critical role in the genesis of bursting activity. This, of course, does not exclude postsynaptic modulation whereby OT would facilitate OT release from OT neurons (Moos et al., 1984), which in turn would act on presynaptic neurons. These autoreceptors may also intervene in other physiological regulations of OT release.

In our previous work, we reported that OT neurons possess NMDA and non-NMDA glutamate receptors. Glutamate appears to be essential in the genesis of the bursts because the afferent volleys underlying bursting were blocked by CNQX. The importance of this input is not surprising because ~25% of synapses on OT neurons are glutamatergic (El Majdoubi et al., 1996, 1997), providing the major excitatory drive to the neurons, essentially via non-NMDA receptors (Wuarin and Dudek, 1993; Boudaba et al., 1997). In our cultures, we believe that AMPA/kainate receptors are most important for conveying glutamatergic input because EPSPs had rapid kinetics and were blocked by CNQX. NMDA receptors exist on cultured OT cells (Jourdain et al., 1996) as they do *in vivo* (Parker and Crowley, 1993). However, it is unlikely that they intervene in burst genesis because they are highly voltage dependent (Hu and Bourque, 1991), but the bursts recorded here were not.

Inhibitory GABA synapses are the other main afferents to OT neurons (Gies and Theodosis, 1994). Although GABA may contribute to hyperpolarize the cells (Jourdain et al., 1996), it cannot be directly responsible for their bursting activity, because once again the bursts were underlain by volleys of excitatory EPSPs, and we found no patterned inhibitory activity.

A model for bursting in OT neurons

In vivo, the pulsatile release of OT attributable to the bursting electrical activity of OT neurons occurs only under two physiological conditions: parturition and suckling. All other stimuli evoke a tonic release linked to a tonic activation of these cells. The bursting activity, therefore, is highly dependent on a particular anatomical and functional organization of the afferent pathways. Moreover, the bursting activity typical of OT neurons has not been detected in acute *in vitro* preparations, where long synaptic afferents are severed (Armstrong et al., 1994). The possibility, then, was that long afferents shape the continuous train of afferent stimuli into periodic drive to OT neurons. In our postnatal cultures, all afferents that were cut degenerated after a few weeks, and there was no neurogenesis (Altman, 1963; Gähwiler et al., 1997). All synaptic input to OT neurons, therefore, can arise only from neurons already present in the hypothalamic slice before it is cultured. Therefore, this strongly suggests that the pulse generator for OT neurons is localized entirely within the hypothalamus. Why such a pulse generator is not active in acute slices remains to be determined. One possibility is that remodeling in slice cultures leads to the formation of a new pattern-generating network peculiar to the cultures, as was reported in the hippocampus [McBain et al. (1989), but see Gähwiler et al. (1997)]. However, this is difficult to reconcile with the fact that the pattern of bursting is so similar to that seen *in vivo*, both in its characteristics and in its sensitivity to oxytocin. More likely, the pattern-generating network does exist *in vivo* but is inhibited in acute *in vitro* preparations. One such example has

been described recently in the stomatogastric ganglion of the lobster, whose intrinsic bursting activity, dependent on afferent input, becomes silent in acute preparations but recovers its rhythmic potential in cultures (Thoby-Brisson and Simmers, 1998).

In conclusion, our results suggest that OT neurons are part of a hypothalamic rhythmic network in which they receive inputs from local glutamate neurons that govern their burst generation. *In vivo*, bursting is highly synchronized between OT cells. Whether this is the case *in vitro* and the pacemaker neurons are responsible for such synchronization remains to be investigated. In any case, OT neurons, in turn, through the release of OT, exert a positive feedback action on their own afferent drive, at a level that is yet unknown. Within this framework, the frequency and duration of action potential discharge within the burst may be greatly modulated by any postsynaptic mechanism that induces a change in membrane potential in the OT cell, as for example, a GABA-induced hyperpolarization. This would then affect the amount of OT released in the periphery, thereby modulating milk ejections, and centrally, thereby modulating the rhythm of the network. *In vivo*, therefore, one can imagine that certain afferent pathways arising from the mammary gland elicit pacemaker properties in the hypothalamic pulse generator, whereas other afferents, such as those involved in osmoregulation (Bourque et al., 1994), do not act on the same component of the network. Others, such as limbic structures (Lebrun et al., 1983), would inhibit the pulse generator. The organotypic cultures of OT neurons that we used here now offer a possibility to unravel the organization of this intrahypothalamic pulse generator.

REFERENCES

- Altman J (1963) Autoradiographic investigation of cell proliferation in the brains of rats and cats. *Anat Rec* 145:573–591.
- Andrew RD, Dudek FE (1983) Burst discharge in mammalian neuroendocrine cells involves an intrinsic regenerative mechanism. *Science* 221:1050–1052.
- Armstrong WE, Smith BN, Tian M (1994) Electrophysiological characteristics of immunochemically identified rat oxytocin and vasopressin neurones *in vitro*. *J Physiol (Lond)* 475:115–128.
- Belin V, Moos F (1986) Paired recordings from supraoptic and paraventricular oxytocin cells in suckled rats: recruitment and synchronization. *J Physiol (Lond)* 377:369–390.
- Ben-Barak Y, Russell JT, Whitnall MH, Ozato K, Gainer H (1985) Neurophysin in the hypothalamo-neurohypophyseal system: I. production and characterization of monoclonal antibodies. *J Neurosci* 5:81–97.
- Boudaba C, Schrader LA, Tasker JG (1997) Physiological evidence for local excitatory synaptic circuits in the rat hypothalamus. *J Neurophysiol* 77:3396–3400.
- Bourque CW, Oliet SHR, Richard D (1994) Osmoreceptors, osmoreception, and osmoregulation. *Front Neuroendocrinol* 15:231–274.
- Brussaard AB, Kits KS, de Vlieger TA (1996) Postsynaptic mechanism of depression of GABAergic synapses by oxytocin in the supraoptic nucleus of immature rat. *J Physiol (Lond)* 497:495–507.
- Destexhe A, Bal T, McCormick DA, Sejnowski TJ (1996) Ionic mechanisms underlying synchronized oscillations and propagating waves in a model of ferret thalamic slices. *J Neurophysiol* 76:2049–2070.
- Douglas WW (1974) Mechanism of release of neurohypophyseal hormones: stimulus-secretion coupling. In: *Endocrinology (handbook of physiology, Vol 4: part 1)* (Knobil E, Sawyer WH, eds), pp 191–224. Washington, DC: American Physiological Society.
- El Majdoubi M, Poulain DA, Theodosis DT (1996) The glutamatergic innervation of oxytocin- and vasopressin-secreting neurons in the rat supraoptic nucleus and its contribution to lactation-induced synaptic plasticity. *Eur J Neurosci* 8:1377–1389.
- El Majdoubi M, Poulain DA, Theodosis DT (1997) Lactation-induced plasticity in the supraoptic nucleus augments axodendritic and axosomatic gabaergic and glutamatergic synapses: an ultrastructural analysis using the disector method. *Neuroscience* 80:1137–1147.
- Freund-Mercier MJ, Richard P (1981) Excitatory effects of intraventric-

- ular injections of oxytocin on the milk ejection reflex in the rat. *Neurosci Lett* 23:193–198.
- Freund-Mercier MJ, Richard P (1984) Electrophysiological evidence for facilitatory control of oxytocin neurones by oxytocin during suckling in the rat. *J Physiol (Lond)* 352:447–466.
- Freund-Mercier MJ, Stoeckel ME, Klein M-J (1994) Oxytocin receptors on oxytocin neurones: histoautoradiographic detection in the lactating rat. *J Physiol (Lond)* 480:155–161.
- Gähwiler BH, Capogna M, Debanne D, McKinney RA, Thompson SM (1997) Organotypic slice cultures: a technique has come of age. *Trends Neurosci* 20:471–477.
- Gainer H, Wray S (1994) Cellular and molecular biology of oxytocin and vasopressin. In: *The physiology of reproduction*, Ed 2 (Knobil E, Neill JD, eds), pp 1099–1129. New York: Raven.
- Gies U, Theodosis DT (1994) Synaptic plasticity in the rat supraoptic nucleus during lactation involves GABA innervation and oxytocin neurons: a quantitative immunocytochemical analysis. *J Neurosci* 14:2861–2869.
- Higuchi T, Honda K, Fukuoka T, Negoro H (1985) Release of oxytocin during suckling and parturition in the rat. *J Endocrinol* 105:339–346.
- Hu B, Bourque CW (1991) Functional *N*-methyl-D-Aspartate and non-*N*-methyl-D-aspartate receptors are expressed by rat supraoptic neurosecretory cells in vitro. *J Neuroendocrinol* 3:509–514.
- Hunter WM, Greenwood FC (1962) Preparation of iodine-131 labelled human growth hormone of high specific activity. *Nature* 194:495–496.
- Jourdain P, Poulain DA, Theodosis DT, Israel JM (1996) Electrical properties of oxytocin neurons in organotypic cultures from postnatal rat hypothalamus. *J Neurophysiol* 76:2772–2785.
- Lambert RC, Dayanithi G, Moos FC, Richard P (1994) A rise in the intracellular Ca^{2+} concentration of isolated rat supraoptic cells in response to oxytocin. *J Physiol (Lond)* 478:275–287.
- Lebrun CJ, Poulain DA, Theodosis DT (1983) The role of the septum in the control of the milk ejection reflex in the rat: effects of lesions and electrical stimulation. *J Physiol (Lond)* 339:17–31.
- Lincoln DW, Wakerley JB (1974) Electrophysiological evidence for the activation of supraoptic neurones during the release of oxytocin. *J Physiol (Lond)* 242:533–554.
- Lincoln DW, Wakerley JB (1975) Factors governing the periodic activation of supraoptic and paraventricular neurosecretory cells during suckling in the rat. *J Physiol (Lond)* 250:443–461.
- Lowbridge J, Manning M, Halder J, Sawyer WH (1977) Synthesis and some pharmacological properties of (4-threonine,7-glycine)oxytocin, 1-(L-2-hydroxy-3-mercaptopropanoic acid), (4-threonine-7-glycine) oxytocin, (hydroxy-4-threonine-7-glycine)oxytocin and (7-glycine)oxytocin: peptides with high oxytocin/antidiuretic selectivity. *J Med Chem* 20:120–123.
- Manning M, Miteva K, Pancheva S, Stoev S, Wo NC, Chan WY (1995) Design and synthesis of highly selective in vitro and in vivo uterine receptor antagonists of oxytocin: comparisons with atosiban. *Int J Pept Protein Res* 46:244–252.
- McBain CJ, Boden P, Hill RG (1989) Rat hippocampal slices “in vitro” display spontaneous epileptiform activity following long-term organotypic culture. *J Neurosci Methods* 27:35–49.
- Mc Cormick DA, Huguenard JR (1992) A model of the electrophysiological properties of thalamocortical relay neurons. *J Neurophysiol* 68:1384–1400.
- Moos F, Freund-Mercier MJ, Guerne Y, Guerne JM, Stoeckel ME, Richard P (1984) Release of oxytocin and vasopressin by magnocellular nuclei in vitro: specific facilitatory effect of oxytocin on its own release. *J Endocrinol* 102:63–72.
- Moos F, Poulain DA, Rodriguez F, Guerné Y, Vincent JD, Richard P (1989) Release of oxytocin within the supraoptic nucleus during the milk ejection reflex in rats. *Exp Brain Res* 76:593–602.
- Morris M, Castro M, Rose JC (1992) Alterations in oxytocin prohormone processing during early development in the fetal sheep. *Am J Physiol* 263:R738–R740.
- Neumann I, Ludwig M, Engelmann M, Pittman QJ, Landgraf R (1993a) Simultaneous microdialysis in blood and brain: oxytocin and vasopressin release in response to central and peripheral osmotic stimulation and suckling in the rat. *Neuroendocrinology* 58:637–645.
- Neumann I, Russell JA, Landgraf R (1993b) Oxytocin and vasopressin release within the supraoptic and paraventricular nuclei of pregnant, parturient and lactating rats: a microdialysis study. *Neuroscience* 53:65–75.
- Parker SL, Crowley WR (1993) Stimulation of oxytocin release in the lactating rat by central excitatory amino acid mechanisms: evidence for specific involvement of *R,S*-alpha-amino-3-hydroxy-5-methylisoxazole-4-propionic acid-sensitive glutamate receptors. *Endocrinology* 133:2847–2854.
- Poulain DA, Wakerley JB (1982) Electrophysiology of hypothalamic magnocellular neurones secreting oxytocin and vasopressin. *Neuroscience* 7:773–808.
- Poulain DA, Brown D, Wakerley JB (1988) Statistical analysis of patterns of electrical activity in vasopressin- and oxytocin-secreting neurones. In: *Pulsatility in neuroendocrine systems* (Leng G, ed), pp 119–154. New York: CRC.
- Richard P, Moos F, Freund-Mercier MJ (1991) Central effects of oxytocin. *Physiol Rev* 71:331–370.
- Roberts MM, Robinson AG, Hoffman GE, Fitzsimmons MD (1991) Vasopressin transport regulation is coupled to synthesis rate. *Neuroendocrinology* 53:416–422.
- Thoby-Brisson M, Simmers J (1998) Neuromodulatory inputs maintain expression of a lobster motor pattern-generating network in a modulation-dependent state: evidence from long-term decentralization *in vitro*. *J Neurosci* 18:2212–2225.
- Wakerley JB, Clarke G, Summerlee AJS (1994) Milk ejection and its control. In: *The physiology of reproduction*, Ed 2 (Knobil E, Neill JD, eds), pp 2283–2322. New York: Raven.
- Wuarin JP, Dudek FE (1993) Patch-clamp analysis of spontaneous synaptic currents in supraoptic neuroendocrine cells of the rat hypothalamus. *J Neurosci* 13:2323–2331.