Postsynaptic mechanism of depression of GABAergic synapses by oxytocin in the supraoptic nucleus of immature rat

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1. Oxytocin is known to act on autoreceptors of oxytocin neurones in the supraoptic nucleus (SON). We investigated whether oxytocin modulates putative oxytocin neurones by suppressing the $GABA_A$ receptor-mediated synaptic inputs on these cells.

2. GABAergic inhibitory postsynaptic currents (IPSCs) were recorded from SON neurones in hypothalamic slices from young rats. Oxytocin specifically reduced the amplitude of both spontaneous and evoked IPSCs, without altering their current kinetics.

3. The effect of oxytocin was observed in 70% of the magnocellular neurones recorded from the dorsomedial part of the SON. $d(CH_2)_5OVT$, a specific antagonist of oxytocin receptors, blocked the effect of oxytocin on the IPSCs. Vasopressin had no effect on oxytocin-sensitive SON neurones.

4. The intervals between spontaneous IPSCs were not affected by oxytocin. This suggested that oxytocin had a postsynaptic effect on SON neurones.

5. This postsynaptic origin was further substantiated by application of TTX, which blocked all evoked release but did not prevent the suppressive effect of oxytocin on the amplitude of the spontaneous IPSCs still present in the recording. The selective effect of oxytocin on IPSC amplitude was also maintained in nominally zero extracellular calcium.

6. Intracellular perfusion of SON neurones with GTPγS mimicked the effect of oxytocin on IPSCs, while GDPβS, similarly applied, abolished the effect of oxytocin.

7. Application of calcium mobilizers such as thapsigargin and caffeine also reduced the amplitude of spontaneous IPSCs without significantly altering the frequency at which IPSCs occurred.

8. Thus, oxytocin depresses GABAergic synapses in the SON via modulation of the postsynaptic $GABA_A$ receptors. This would lead to disinhibition of SON neurones sensitive to oxytocin and could, therefore, be a powerful means of controlling the firing of oxytocin neurones.

A well-known function of oxytocin-containing neurones in females is to secrete oxytocin into the blood during the expulsive phase of parturition and during lactation (Poulain & Wakerley, 1982). In males, oxytocin neurone activity has been implicated in penile erection and contraction of the vas deferens (Melis, Stancampiano & Argiolas, 1994). Oxytocin neurones are subject to autoregulation by oxytocin (Freund-Mercier & Richard, 1984; Moos & Richard, 1989) released from their dendrites (Pow & Morris, 1989). Measurements of oxytocin by *in vivo* microdialysis of the supraoptic nucleus (SON) support this view (Neumann, Ludwig, Engelmann, Pittman & Landgraf, 1993*a*; Neumann, Russell & Landgraf, 1993*b*). Injections of specific oxytocin receptor antagonists into the SON during suckling reduce oxytocin release within the SON as well as into the blood (Neumann, Koehler, Landgraf & Summy-Long, 1994). Oxytocin receptors are expressed in the putative oxytocin neurones (Yoshimura *et al.* 1993; but see Freund-Mercier, Stoeckel & Klein, 1994), stimulation of which induces the release of calcium from thapsigargin-sensitive intracellular stores (Lambert, Dayanithi, Moos & Richard, 1994). Moreover, oxytocin directly facilitates firing of oxytocin neurones both *in vivo* and *in vitro* (Yamashita *et al.* 1987; Moos & Richard, 1989). It has also been shown that there are excitatory interconnections between oxytocin-containing, but not vasopressin-containing, neurones (McKenzie, Leng & Dyball, 1995). Thus, oxytocin facilitates firing of oxytocin neurones and a rise in $[Ca^{2+}]_i$ may be the key intracellular messenger that mediates this autoexcitation.

Another important component in the regulation of oxytocin neurones is the inhibitory input from GABAergic interneurones onto the somata and dendrites of these cells (Van

de Pol, 1985). Injections of bicuculline into the SON disrupt patterned and synchronous firing activity of oxytocin neurones during lactation and in consequence, inhibit the milk ejection reflex (Voisin, Herbison & Poulain, 1995). *In situ* patch clamp recordings have shown the occurrence of spontaneous GABAergic IPSCs in magno-cellular neurones in the SON (Wuarin & Dudek, 1993); these cells are known to express $GABA_A$ receptor subunits (Fenelon & Herbison, 1995; Fenelon, Sieghart & Herbison, 1995).

In various cell types, $GABA_A$ receptor activity is suppressed by increases in $[Ca^{2+}]_i$ (Inoue, Oomura, Yakushiji & Akaike, 1986; Mouginot, Feltz & Schlichter, 1991; Chen & Wong, 1995). Since oxytocin induces a rise in $[Ca^{2+}]_i$ in putative oxytocin neurones (Lambert *et al.* 1994), $GABA_A$ receptors in these cells are a possible target for calcium-dependent modulation triggered by oxytocin. Preliminary data have indicated that oxytocin is able to suppress the GABAergic synaptic input in a number of SON neurones recorded under whole-cell voltage clamp (Brussaard, 1995). In the present study we confirm this finding and describe the underlying mechanism of the modulation, showing (i) that oxytocin-induced depression of GABAergic synapses occurs only in a distinct set of SON neurones that are not sensitive to vasopressin, and (ii) that the effect of oxytocin is mediated via postsynaptic modulation of the amplitude of the IPSCs, without alteration in the frequency at which the spontaneous events occur.

METHODS

Hypothalamic slices and patch clamp procedure

Wistar rats aged between 21 and 30 days were used in this study; for slice preparations all were males, except where specified. Rats were killed by decapitation on a guillotine without the use of anaesthetics. Coronal hypothalamic slices (400 μm) were prepared according to the method of Edwards & Konnerth (1992). For this procedure slices were kept on ice-cold artificial cerebrospinal fluid (ACSF; see below). A single slice was selected, incorporating an appropriate part of both SONs. It was submerged in ACSF in a recording chamber perfused continuously at room temperature (20–22 °C) with ACSF gassed with 5% CO_2–95% O_2. Whole-cell recordings from the SON were made using a modification of the *in situ* patch clamp set-up described in Stuart, Dodt & Sakmann (1993). A Zeiss Axioscope top-view microscope with a long working distance water-immersion ×40 objective lens (NA, 0·75) was equipped with Hoffman modulation contrast optics, and used in combination with a high resolution video camera sensitive to infrared light (Philips CCD camera type LDH 702/20, Eindhoven, The Netherlands). Visual light wavelengths (< 700 nm) were filtered to reduce scattering; long wavelengths (>1000 nm) were blocked to reduce heating of the slices. In this set-up the somata and dendrites of the magnocellular neurones could be visualized up to several cell layers deep.

In situ patch clamp recordings

ACSF was prepared using commercially available sterile water (Baxter, Utrecht, The Netherlands) containing (mM): NaCl, 125; $NaHCO_3$, 25; KCl, 3; $NaH_2PO_4.H_2O$, 1·2; $CaCl_2.2H_2O$, 2·4; $MgSO_4.7H_2O$, 1·3; D-glucose, 10 (304 mosmol l^{-1}, gassed with 5% CO_2–95% O_2, pH 7·4). For experiments involving nominally zero extracellular calcium, the bath solution contained a modified, so-called Ca^{2+}-free ACSF containing (mM): NaCl, 125; $NaHCO_3$, 25; KCl, 3; $NaH_2PO_4.H_2O$, 1·2; $MgSO_4.7H_2O$, 1·3; $MgCl_2$, 2·4; D-glucose, 10. Pipette solution was prepared weekly and contained (mM): CsCl, 141; Hepes, 10; MgATP, 2; adjusted to pH 7·2 using CsOH (296 mosmol l^{-1}). Whole-cell recordings were made at room temperature using 2 MΩ patch electrodes. Gigaohm seals were made as described in Stuart *et al.* (1993) with the exception that we used only 10 mbar of positive pressure when approaching and penetrating the slice surface, and 5 mbar when approaching a cell. Seal formation was obtained by instantly switching to 20 mbar of suction using a 10 ml syringe. After seal formation, one or two calibrated suction pulses of 50 ms were used to rupture the membrane.

Criteria for selecting cells were as follows. Whole-cell recordings with an uncompensated series resistance (R_s) > 12 MΩ were rejected. In the accepted recordings, the R_s was usually compensated by about 80% and cell capacitances varied between 12 and 30 pF (measured by whole-cell capacitance compensation of the Axopatch 200A). Recordings in which the R_s changed by more than 20% were also rejected.

Glutamatergic as well as GABAergic synaptic currents were observed in recordings made within 4–5 h after preparation of the slices. For the experiments described here the GABAergic synaptic currents were pharmacologically isolated using 6,7-dinitro-quinoxaline-2,3-dione (DNQX) and D-aminophosphonovalerate (AP5) (non-NMDA and NMDA receptor antagonists, respectively, both at 20 μM; Research Biochemicals International).

Neuropeptides, microperfusion and other manipulations

Oxytocin, vasopressin and [des-glycinamide^9,d$(CH_2)_5$,*O*-Me-Tyr^2, Thr^4,Orn^8]-vasotocin (d$(CH_2)_5$OVT), a specific oxytocin receptor antagonist, were from Saxon Biochem (Bubendorf, Switzerland). Neuropeptides (in ACSF) were microperfused using a Y tube. The Y tube was a flow patch pipette (20 μm tip opening) directed at, and positioned within 300 μm of, the site of recording and connected to gravity-fed reservoirs. The flow patch pipette itself consisted of two compartments, an outer glass compartment to which suction was applied and an inner compartment consisting of a stainless-steel needle with its tip 2 mm above the opening of the glass pipette. An amaranth-tinted peptide solution (0·025%; Merck) was perfused in order to gain visual control of the peptide application to the site of recording. Normally a constant low negative pressure was applied to the outer compartment to draw the neuropeptide solution away from the tip of the needle before it reached the bath. When the negative pressure was released, the amaranth-tinted solution leaving the pipette was seen to reach the recording side in < 2 s. Neither amaranth nor d$(CH_2)_5$OVT (1 μM) affected the amplitude or frequency of IPSCs ($n = 15$; not shown). Cells from which recordings were made were usually located in the second layer of neurones under the slice surface (some 50 μm deep). Some dilution (and therefore delay) of the neuropeptide application at the site of recording may have occurred.

Guanosine-5′-*O*-(3-thiotriphosphate) (GTPγS, tetralithium salt) and guanosine-5′-*O*-(2-thiodiphosphate) (GDPβS, trilithium salt) were from Boehringer Mannheim. Thapsigargin and caffeine were both from Research Biochemicals International.

Analysis

The IPSC recordings were stored on DAT recording tapes (unfiltered and amplified ×5 using the Axopatch 200A amplifier) and analysed off-line, after 1 kHz filtering using a Bessel filter and AD conversion at a sampling rate of 5–10 kHz using a CED 1400

digitizer. For the analysis of evoked as well as spontaneous synaptic currents, the Strathclyde SCAN & CDR software (J. Dempster, University of Strathclyde, Glasgow, UK) was used. Amplitude histograms of spontaneous IPSCs were fitted with a single lognormal distribution (Borst, Lodder & Kits, 1994) using the following likelihood equation:

$$Y = A\exp[-0{\cdot}5\{(\ln X - \mu)/\sigma^2\}]/(\sigma X\sqrt{2\pi}). \quad (1)$$

In this equation, Y is the number of amplitude events, A the relative area under the curve and X the measured amplitude; μ is the mean, and σ the standard deviation, of the underlying normal distribution. The mean (μ') and standard deviation (σ') of the lognormal distribution can be calculated from this by using:

$$\mu' = \exp(\mu + \sigma^2), \quad (2)$$

$$\sigma' = \mu'\sqrt{(\exp(\sigma^2) - 1)}. \quad (3)$$

We then analysed the amplitude of the IPSCs during the recordings (i.e. amplitude *vs.* time plots) by averaging amplitude values after appropriate transformation of consecutive IPSCs (bin size, 20 events). To take into account the non-linearity of the IPSC amplitude distribution (see above), a transformation of amplitude data was necessary. Thus, before analysis of the effect of oxytocin with time, IPSC amplitude values were lognormally transformed,

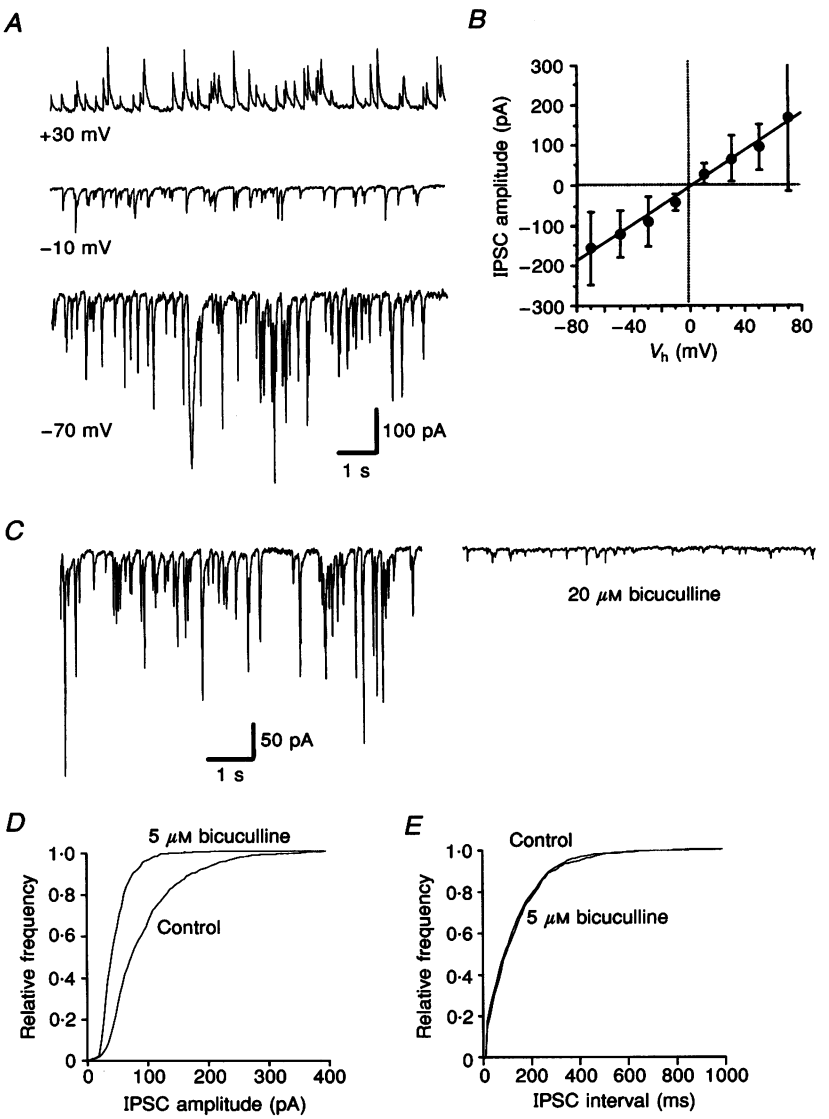

Figure 1. Effect of membrane potential and bicuculline on the amplitude and occurrence of spontaneous IPSCs

A, representative 8 s sweeps at the indicated holding potentials, all obtained from the same magnocellular neurone in the SON. Currents were recorded under approximately equimolar inside and outside chloride conditions, hence the apparent inward instead of outward direction of the synaptic currents at negative holding potentials. *B*, mean spontaneous IPSC amplitude (calculated from fits of single lognormal distributions to cumulative amplitude histograms obtained at each holding potential, V_h) plotted as a function of membrane potential. The IPSCs recorded here reversed at +4 mV; averaged over 4 experiments this occurred at +2·5 ± 3 mV. All recordings were made under whole-cell voltage clamp, in the presence of DNQX and AP5. Recordings shown in *C–E* were made at a V_h of −70 mV.

which yielded normally distributed data. Then regular means (μ) and standard deviations (σ) per twenty IPSCs were calculated. Subsequently, the means (μ') and standard deviations (σ') of the underlying (untransformed) lognormally distributed data were obtained, using eqns (2) and (3). These μ' values per twenty IPSCs and their standard errors are plotted against time in Figs 2, 6, 7, 8, 9 and 10. Throughout the paper amplitude distributions were plotted in a cumulative manner to avoid binning artefacts.

RESULTS

Spontaneous GABAergic IPSCs recorded in isolation

GABAergic IPSCs were recorded from visually identified magnocellular neurones within the SON. Recordings were preferably made in the dorsomedial region of the SON, which has been shown to contain more oxytocin neurones than vasopressin cells (Hou-Yu, Lamme, Zimmerman & Silverman, 1986).

IPSCs were recorded in the presence of DNQX and AP5 which block glutamatergic synaptic inputs. Equimolar internal and external chloride concentrations were used. At negative holding potentials, apparent inward currents were observed (see Fig. 1*A*) and equal and opposite outward currents occurred when the cell was depolarized beyond 0 mV ($n = 4$; Fig. 1*A* and *B*). This suggests that these currents were carried by chloride. Further, in the presence of DNQX and AP5, bicuculline (20 μM) blocked all synaptic activity (Fig. 1*C*), indicating that the synaptic input was via $GABA_A$ receptors.

Amplitude histograms obtained at different holding potentials were not normally distributed, but instead were best fitted with single lognormal distributions (see Methods). To analyse whether IPSC interval measurements were dependent on the signal-to-noise ratio of the recordings, we applied 5 μM bicuculline. This caused a 50% depression in the IPSC amplitude (Fig. 1*D*) without affecting the frequency with which IPSCs occurred (Fig. 1*E*). The minimum IPSC amplitude under these conditions was about 10 pA, while under control conditions the GABAergic IPSCs were well above 20 pA (see below). Thus, even after reduction of IPSC amplitude caused by half-maximal block of postsynaptic receptors, the smallest events were well above our detection threshold.

Activation of oxytocin receptors reduces the amplitude of GABAergic IPSCs

To test whether activation of oxytocin receptors on oxytocin neurones modulates $GABA_A$ receptors, oxytocin was applied in the vicinity of the recorded cell using a microperfusion system. Application of oxytocin reduced the amplitude of spontaneous IPSCs (Figs 2*A* and 3*A*, *B* and *D*). The onset of the effect was slow, taking 15–30 s (Fig. 2*B*). Analysis of the intervals between IPSCs before, during and after application of oxytocin (Fig. 2*C* and 3*D*) showed them

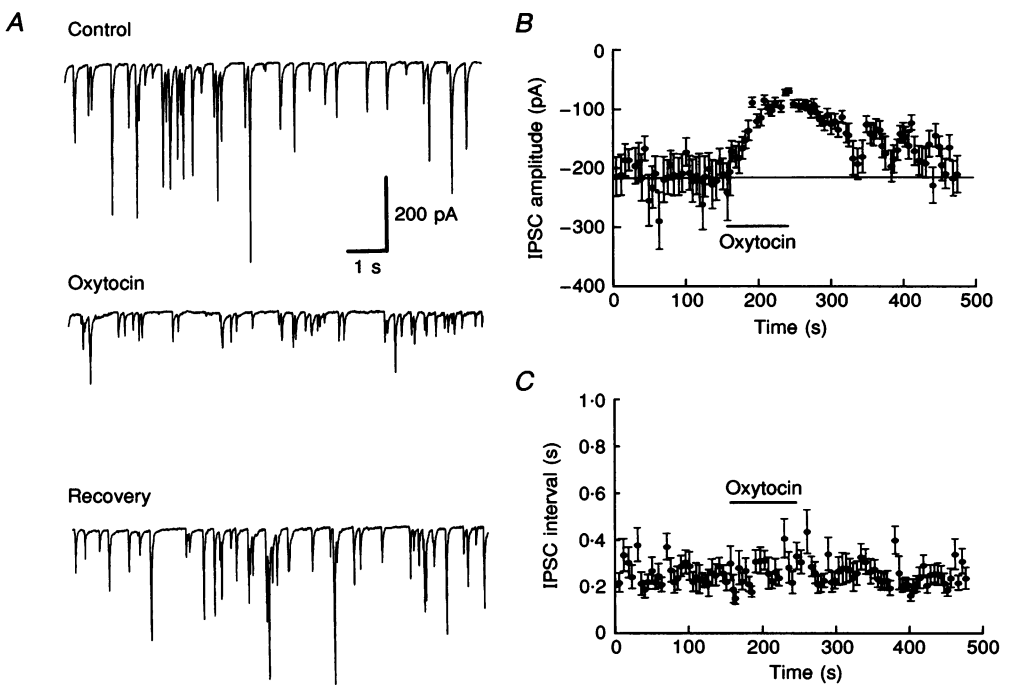

Figure 2. Oxytocin reduces the amplitude of spontaneous GABAergic IPSCs

A, spontaneous IPSCs before, during and after oxytocin application (1 μM). *B*, a series of mean amplitudes of spontaneous IPSCs plotted against recording time in the experiment shown in *A* (the means and standard errors were calculated per 20 IPSCs as described in Methods). Oxytocin was applied after 160 s of recording, as indicated. *C*, a series of interval means from the same period of recording time as shown in *B*. All data are from the same cell, also shown in Fig. 5. V_h, − 70 mV; recordings made in the presence of DNQX and AP5.

to be unaffected by oxytocin. Thus, oxytocin did not alter the chance of GABA release, but did affect the efficiency of its transmission. The effect of oxytocin on the GABAergic IPSCs persisted as long as the application was continued (up to 120 s), but was always reversible by washing. Recovery of the amplitude to original levels took between 3 and 5 min (Fig. 2*B*).

In order to exclude the possibility that changes in the series resistance (R_s) during the recording artificially reduced the amplitude of the synaptic events, regular measurements of R_s were made and rise times of synaptic events were analysed. However, there was no correlation between the amplitude of synaptic events and their rise times (Fig. 3*A–C*). To determine possible effects of oxytocin on the decay kinetics of synaptic currents, we compared the decay of spontaneous IPSCs before and during oxytocin application. To this end, control sweeps and sweeps of synaptic events recorded in the presence of oxytocin were normalized and superimposed on each other. Their exact superimposition showed that oxytocin had no effect on the kinetics of GABAergic IPSCs (Fig. 3*B*).

Oxytocin suppressed spontaneous GABAergic synaptic currents in eighteen out of twenty-three recordings of SON magnocellular neurones in male hypothalami and in five out of eight recordings in female hypothalami. In the eight other recordings, oxytocin had no effect. In the oxytocin-sensitive SON neurones from males, 1 μM oxytocin reduced the mean IPSC amplitude by 46 $\pm$ 10% ($n = 6$) and maximal amplitude by 63 $\pm$ 9% ($n = 6$). In females, 1 μM oxytocin caused a reduction of 58 $\pm$ 23% in mean amplitude and 69 $\pm$ 2% in maximal amplitude ($n = 5$, not significantly different from males).

A dose–response analysis of the effect of oxytocin on the amplitude of spontaneous IPSCs showed that half-maximal reduction in mean or maximal IPSC amplitude occurred at about 200 nM oxytocin; the threshold dose for the effect was 10 nM (Fig. 4).

Oxytocin also appeared to decrease the variation within experiments in the amplitude of spontaneously occurring IPSCs, as shown in Fig. 2*B*. This effect was seen even at low concentrations; 20 nM oxytocin reduced the standard deviation of the IPSC amplitude distribution by 50% (not shown). This probably reflects the not uncommon dependence of σ on μ in non-linearly distributed data. It could be argued that as a result of the reduction in amplitude some IPSCs might fall below the detection

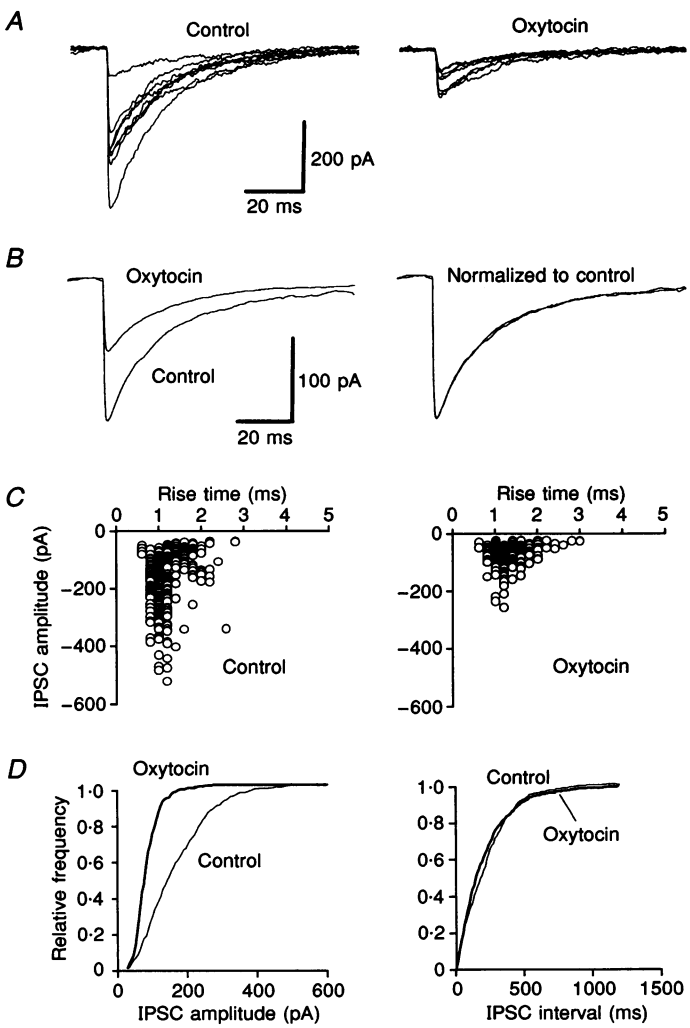

Figure 3. Oxytocin selectively suppresses the amplitude of spontaneous GABAergic IPSCs without altering the kinetics of synaptic currents or their frequency

A, representative (non-consecutive) superimposed sweeps showing IPSCs before and during the application of 1 μM oxytocin. *B*, left, superimposed means of 50 spontaneous IPSCs (lined up according to the mid-point of their rise times) before and during the application of oxytocin. (Events with notches in the rising phase and other obvious doublets were not included.) Right, superimposed means of spontaneous IPSCs before and during application of oxytocin, but normalized to the control, showing no difference in the decay of synaptic events. *C*, scatter plots of rise times *vs.* IPSC amplitude before (left) and during (right) the application of oxytocin. *D*, cumulative amplitude (left) and interval (right) distribution of spontaneous IPSCs recorded before and during the application of oxytocin (> 250 events per condition). All data are from the cell shown in Fig. 4. $V_h = -70$ mV; recorded in the presence of DNQX and AP5.

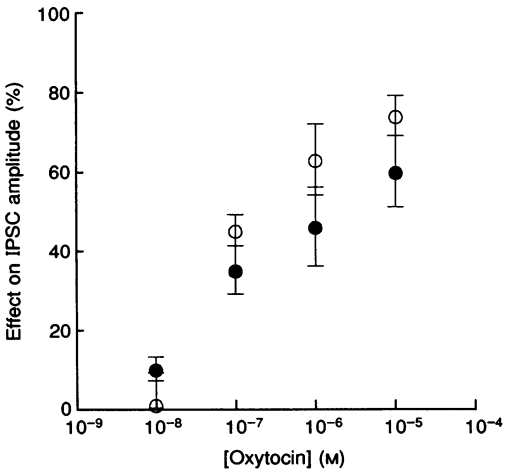

Figure 4. Dose dependency of the oxytocin effect
The effects of oxytocin on the mean (●) and maximal (○) amplitudes of spontaneous IPSCs recorded from oxytocin-sensitive magnocellular neurones in the SON (pooled data from male and female animals presented as means and standard errors, $n \geqslant 4$ per concentration of oxytocin). All data obtained at $V_h = -70$ mV; in the presence of DNQX and AP5.

threshold. This appeared not to be the case since oxytocin had no effect on the frequency at which the spontaneous GABAergic events occurred (see Figs 2*C* and 3*D*).

To study the effect of oxytocin on evoked IPSCs, a concentric bipolar stimulating electrode was placed 200 μm deep in the perinuclear region, 300 μm lateral to the SON (i.e. dorsolateral to the site of recording). IPSCs were elicited and recorded from oxytocin-sensitive SON neurones before, during and after the application of oxytocin. Under control conditions the amplitude of the evoked IPSCs was highly variable within the same recording (Fig. 5*B*, control). On average, oxytocin reduced the amplitude of evoked IPSCs by 48 ± 8% ($n = 5$; Fig. 5*A* and *B*; failures amounted to < 10% of the total number of trials; oxytocin had no effect on the relative number of failures). However, at the median of the IPSC amplitude distribution, the effect of oxytocin seemed larger (65 ± 11%) and the largest IPSCs in the presence of oxytocin were around 200 pA compared with > 800 pA under control conditions.

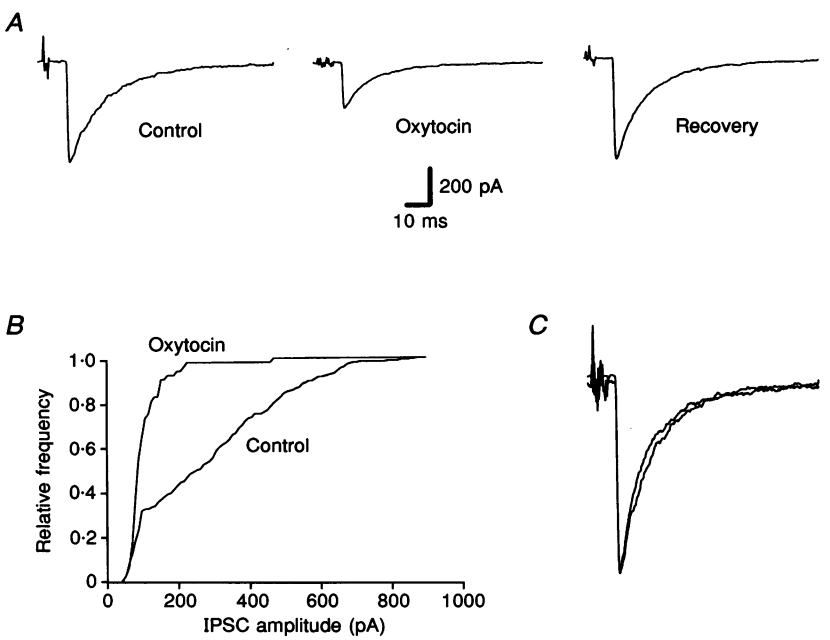

Figure 5. Oxytocin reduces the amplitude of evoked GABAergic IPSCs
A, plots of mean evoked currents before, during and after application of oxytocin at 1 μM (50 consecutive IPSCs following supramaximal stimuli, each at 0·3 μA for 1 ms, given once every 2 s by a bipolar electrode placed in the perinuclear region dorsolateral to the site of recording approximately 300 μm away from the SON). *B*, cumulative amplitude histograms of the evoked IPSCs under control conditions and in the presence of oxytocin. The amplitude of the evoked currents under control conditions was highly variable per event, and ranged from approximately 50 pA up to 800 pA (distribution based on 250 events, excluding failures). *C*, mean of 50 evoked IPSCs recorded in the presence of oxytocin, but here normalized to and superimposed on the mean IPSC recorded under control conditions. Horizontal scaling as in Fig. 5*A*, vertical calibration arbitrary. Note the lack of effect of oxytocin on the decay of evoked synaptic events.

In a further examination of possible effects of oxytocin on decay kinetics of the synaptic currents, we also compared the decay of electrically evoked IPSCs. Control sweeps and sweeps of synaptic events recorded in the presence of oxytocin were normalized and superimposed (Fig. 5*C*). In line with the observations on spontaneous IPSCs (Fig. 3*B*) this showed that there was no effect of oxytocin on the kinetics of the evoked GABAergic IPSCs in these cells.

Brussaard (1996) showed that the effect of oxytocin on evoked potentials was blocked by $d(CH_2)_5OVT$, a specific antagonist of oxytocin receptors. Moreover, the response to oxytocin was reproducible within experiments. In the present study, there was a noticeable dose dependence of the oxytocin effect within experiments; at 10 μM the onset of the reduction in IPSC amplitude was faster and more pronounced than at 1 μM (not shown).

We conclude that oxytocin specifically reduced the impact of the GABAergic input in around 70% of cells in regions where the number of oxytocin neurones was relatively high.

SON neurones are sensitive either to oxytocin or vasopressin

To test the specificity of the oxytocin effect, vasopressin (1 μM) was applied during recordings from magnocellular neurones that were sensitive to oxytocin (1 μM). In no experiment did we observe any effect of vasopressin on either amplitude or frequency of spontaneous IPSCs (results obtained from four different slices, not shown). In the same slices, four cells that were not sensitive to oxytocin did respond to vasopressin, showing a selective reduction in amplitude of spontaneous IPSCs ($50 \pm 8\%$, $n = 4$; Fig. 6), but no change in frequency of synaptic events. This suggests that the inhibitory input to a second population of magnocellular cells within the SON is specifically reduced by vasopressin. Additional experiments using alternate applications of oxytocin and vasopressin during individual recordings (up to three times, not shown) confirmed this conclusion.

Oxytocin effect on GABA synapses in the SON is mediated via a postsynaptic action

TTX, applied at concentrations that block the evoked release of GABA (see Fig. 7*A*), had no effect on the amplitude of spontaneous IPSCs (Fig. 7*B*). Single lognormal equations (see Methods) fitted to amplitude histograms under both conditions gave averages of 212 ± 25 pA (correlation between residuals and normal distribution, $r > 0{\cdot}97$) before TTX and 201 ± 35 pA after TTX ($r > 0{\cdot}97$, not shown, $n = 4$). Moreover, the intervals between spontaneous events both in the absence and presence of TTX showed a stationary pattern. In other words, IPSCs appeared to occur randomly, not in clusters, during the recording.

To further test the nature of spontaneous GABA release in these experiments we compared the amplitudes and frequency of spontaneous IPSCs recorded in normal ACSF with those obtained in Ca^{2+}-free ACSF. From comparisons within experiments and by fitting lognormal functions to amplitude histograms it was shown that the amplitude of spontaneous IPSCs was not significantly altered by Ca^{2+}-free ACSF (reduction of $15 \pm 18\%$, $n = 5$ experiments, $P > 0{\cdot}05$, Student's t test), nor was there any effect on intervals between IPSCs. Nevertheless, in two of these experiments we observed a small reduction in amplitude of spontaneous IPSCs (e.g. Fig. 8*A* shows a 23% reduction in mean amplitude). However, even in these cells, frequency plots obtained in Ca^{2+}-free and normal ACSF completely overlapped (see Fig. 8*B*). On the basis of these data, we estimate that in normal ACSF, either all, or a large majority, of spontaneous IPSCs are derived from monoquantal events.

It is thus likely that the IPSCs in normal ACSF result, to a large extent, from the spontaneous release of single vesicles containing GABA not contingent upon Na^+ or Ca^{2+} channel activity in the presynaptic cell. If this is true, then the observation that oxytocin selectively depresses the amplitude (Fig. 3*A*), but not the frequency, of spontaneous IPSCs (Fig. 3*D*) suggests a postsynaptic rather than a

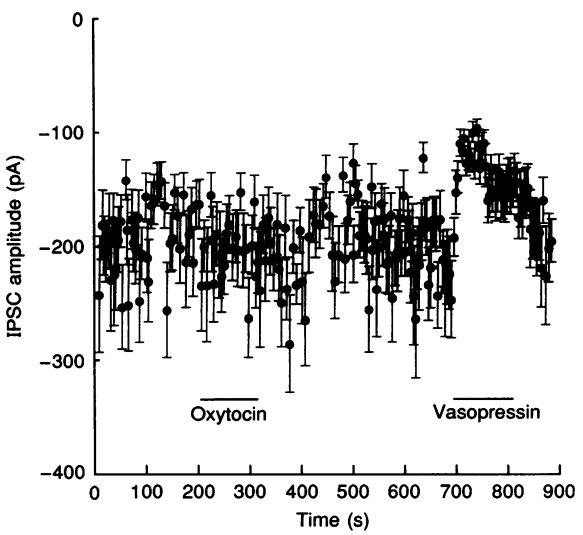

Figure 6. Vasopressin and oxytocin reduce the amplitude of spontaneous GABAergic IPSCs in distinct populations of magnocellular neurones
Shown here are averages of the IPSC amplitudes of a magnocellular neurone not sensitive to 1 μM oxytocin, but sensitive to vasopressin (1 μM) which reduced the amplitude of the spontaneous IPSCs without any effect on the frequency at which the IPSCs occurred (interval plot not shown).

presynaptic origin of the oxytocin effect. This hypothesis was further substantiated in the following four series of experiments.

In the first series, the effect of oxytocin on the spontaneous IPSCs in the continuous presence of TTX was tested, so as to exclude the possibility that oxytocin induces modulation of the action potential-driven release of GABA from the presynaptic cells. In four cells, following a 10 min pre-incubation in TTX which completely abolished the evoked IPSCs (Fig. 7*A*), the effect of oxytocin on the amplitude and frequency of spontaneous IPSCs was recorded. In all four cells oxytocin selectively reduced the mean IPSC amplitude ($54 \pm 4\%$, Fig. 7*C*) without affecting the frequency of IPSCs (not shown).

In the second series, the effect of oxytocin on spontaneous IPSCs recorded in Ca^{2+}-free ACSF was tested. This was to exclude the possibility that oxytocin induced presynaptic modulation of GABA release via a reduction in Ca^{2+} influx into presynaptic terminals. In five cells, following pre-incubation of > 8 min in Ca^{2+}-free ACSF (see Fig. 8*A* and *B*), the effect of oxytocin on spontaneous IPSC amplitude and frequency was recorded. In four out of five cells, oxytocin caused a reduction in the mean IPSC amplitude ($44 \pm 5\%$, Fig. 8*C*) without any effect on the frequency of IPSCs (not shown). The ratio of four out of five SON neurones being sensitive to oxytocin is similar to the ratio in normal ACSF (see above).

In the third series, the sensitivity of postsynaptic $GABA_A$ receptors to intracellular signalling was investigated. G-proteins within the postsynaptic cell were activated by including 200 nM GTPγS, a non-hydrolysable form of GTP, in the recording pipette. Immediately after the start of recording, a progressive run-down of the IPSC amplitude was observed in all five cells examined (Fig. 9*A*). Superimposed synaptic current sweeps (Fig. 9*B*) show that this occurred without a loss in quality of the recording (i.e. the rise time remained around 1 ms).

In the fourth series of experiments, G-proteins within the postsynaptic cell were blocked by inclusion of 200 nM GDPβS (a non-hydrolysable from of GDP) in the pipette medium. The aim was to test whether this prevented the effect(s) of either oxytocin or vasopressin. After pre-incubation for > 8 min, neither the application of oxytocin nor the subsequent application of vasopressin had significant effects on the amplitude (Fig. 9*C*) or frequency (not shown) of the IPSCs ($n = 8$). GDPβS by itself had no effect on the IPSC amplitude.

These experiments strongly suggest that the effect of oxytocin on GABAergic transmission in the SON is mediated through a postsynaptic action.

Caffeine and thapsigargin mimic the effect of oxytocin on spontaneous IPSCs

Since oxytocin induces an increase in $[Ca^{2+}]_i$ via release of calcium from intracellular stores in putative oxytocin neurones (Lambert *et al.* 1994), it is possible that the effect of oxytocin on GABAergic synapses is calcium dependent. To pursue this, we have examined the effects of the calcium-mobilizing agents caffeine, an agonist of the ryanodine receptor, and thapsigargin, which blocks Ca^{2+} re-uptake into calcium stores, including the endoplasmic reticulum. Both

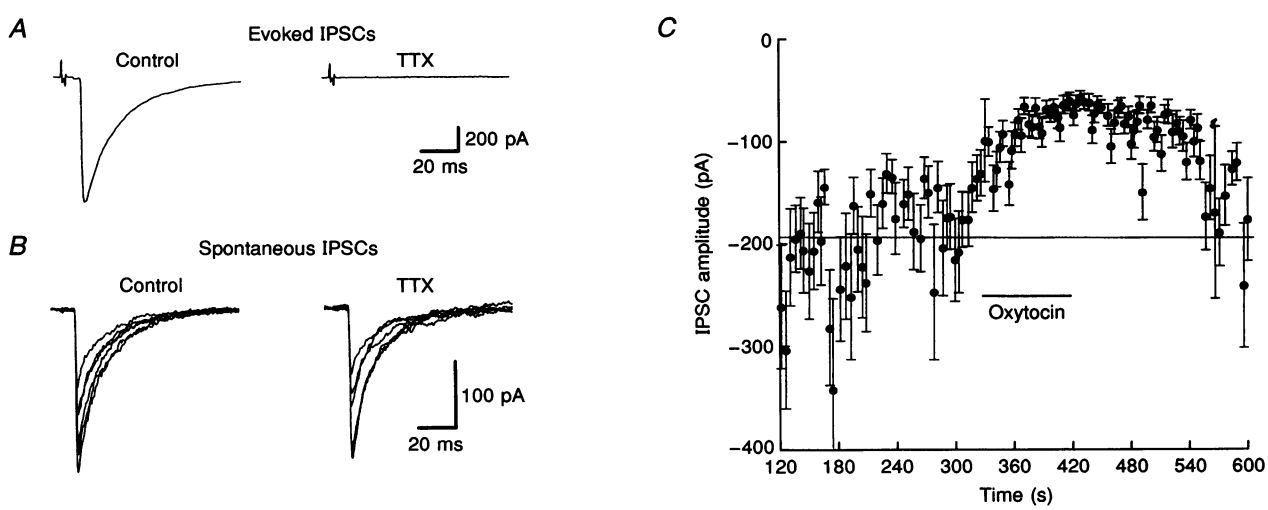

Figure 7. Oxytocin reduces the amplitude of spontaneous IPSCs via a postsynaptic mechanism of action

A, application of TTX (2 μM) completely abolished the evoked GABAergic responses in magnocellular neurones (means of 25 evoked IPSCs under TTX and control conditions). *B*, TTX does not affect the amplitude of spontaneous IPSCs (6 representative spontaneous IPSCs superimposed for each condition). $V_h = -70$ mV; recording in the presence of DNQX and AP5. *C*, after 10 min of preincubation in the presence of 2 μM TTX, oxytocin (5 μM) still caused a large reduction in the amplitude of spontaneous IPSCs. $V_h = -70$ mV; recording in the presence of DNQX and AP5.

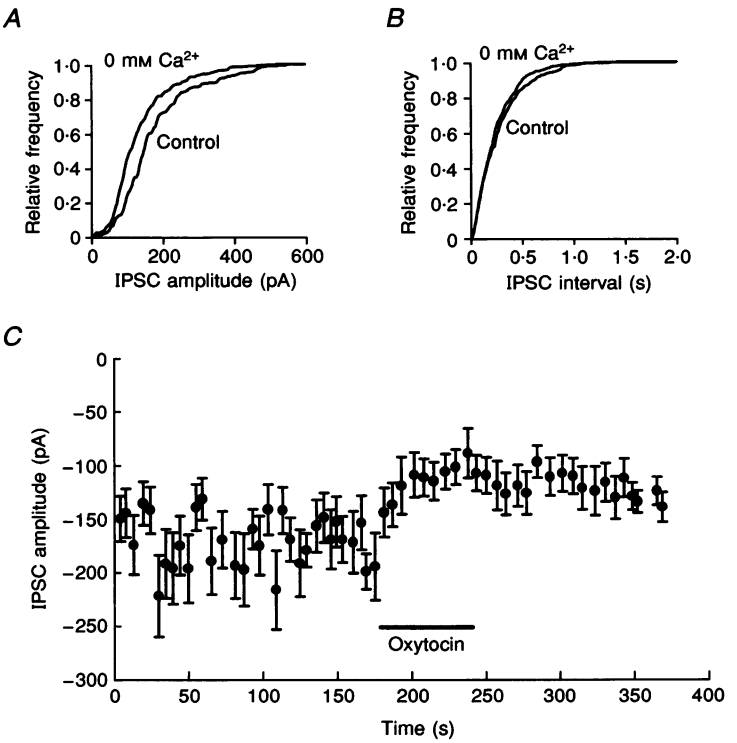

Figure 8. Effects of oxytocin on spontaneous IPSC amplitudes in Ca^{2+}-free ACSF

The effect of changing the bath solution from normal ACSF (2·4 mM $CaCl_2$) to a Ca^{2+}-free ACSF (containing 3·7 mM Mg^{2+} and 0·1 mM EGTA) on the amplitude (*A*) and interval (*B*) of spontaneous IPSCs. Single lognormals fitted to the amplitude distributions before and after 0 Ca^{2+} in this particular experiment showed that the mean IPSC amplitude was reduced from 172 ± 91 pA under normal ACSF to 133 ± 67 pA in Ca^{2+}-free ASCF. Averaged over 5 experiments, the apparent effect on the IPSC amplitude was not significant. *C*, the effect of oxytocin on the IPSC amplitude is still present in Ca^{2+}-free ACSF as can be observed in the amplitude *vs.* time plot (from the same recording as *A* and *B*). $V_h = -70$ mV; recording in presence of DNQX and AP5.

substances induce a marked rise in $[Ca^{2+}]_i$ in putative oxytocin neurones (Lambert *et al.* 1994), possibly by acting on different, distinct calcium stores.

Caffeine (20 mM) caused a slow, 50–70% reduction in the amplitude of spontaneous IPSCs (onset within 20 s lasting > 1 min, $n = 43$; not shown), and also had a transient effect on the frequency of evoked IPSCs (onset within 3 s, duration less than 20 s). Thus, the effect of caffeine was not selective for the postsynaptic cell and was in part mediated through a presynaptic effect on the probability of release of GABA-containing vesicles. Because the effect of caffeine on the frequency of IPSCs occurred before the observed reduction in their amplitude, it is unlikely that the latter is caused by decreased release of GABA. To test this hypothesis we repeated the caffeine experiments in Ca^{2+}-free ACSF. Under this condition caffeine selectively reduced the IPSC amplitude (50–60%) without any effect on the frequency of IPSCs (not shown, $n = 4$), demonstrating that caffeine has postsynaptic effects.

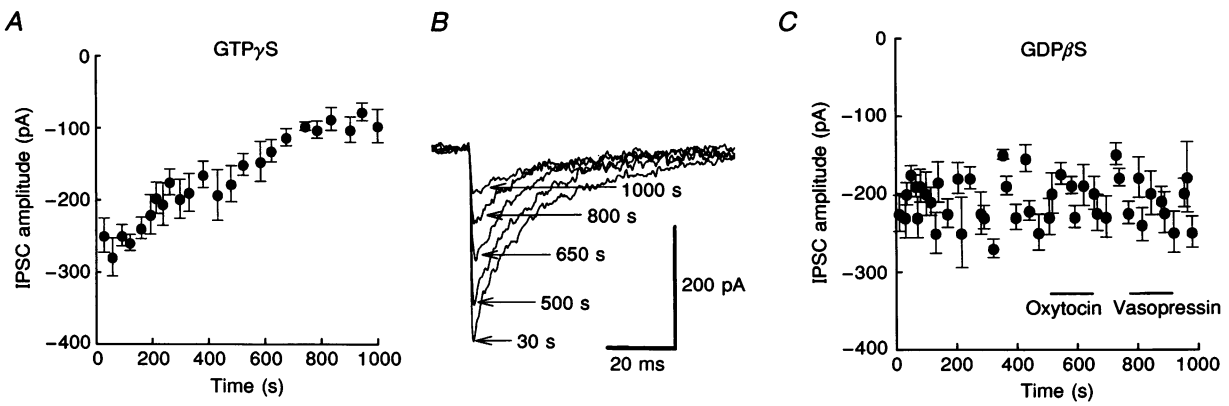

Figure 9. GTPγS induces run-down of the amplitude of spontaneous IPSCs while GDPβS prevents the effect of oxytocin or vasopressin on the IPSC amplitude

A, scatter plot of IPSCs amplitude means (± standard errors per 40 events); 200 nM GTPγS included in the pipette medium resulted in a 50% reduction in the amplitude of IPSCs within 10 min of the start of recording. *B*, 5 representative IPSCs from the recording shown in *A*. Times indicated relative to the beginning of the experiment. *C*, scatter plot (as in *A*) of another experiment in which 200 nM GDPβS was included in the pipette to block G-proteins within the postsynaptic cell. Application of either oxytocin (5 μM) or vasopressin (5 μM) had no effect on any of the 8 magnocellular neurones examined. $V_h = -70$ mV; recording in presence of DNQX and AP5.

Lastly, we tested the effects of thapsigargin. With 200 nM thapsigargin (a concentration normally used on dissociated cells), oscillations occurred in mean IPSC amplitude ($n = 5$; Fig. 10*A*). The significance of this was not clear. At a higher concentration (1 μM), a slow (onset within 60 s) but large reduction in the IPSC amplitude was seen ($n = 4$; Fig. 10*B* and *C*), with only minor effects on the frequency of IPSCs (Fig. 10*D*). Thus the reduction of the IPSC amplitude was significant and mimicked the oxytocin-induced depression observed in other experiments in this study. This observation is in line with the data of Lambert *et al.* (1994) who showed that the oxytocin-induced rise in $[Ca^{2+}]_i$ involves thapsigargin-sensitive intracellular calcium stores.

DISCUSSION

Synaptic depression induced by oxytocin

This study shows that oxytocin causes the depression of GABAergic synaptic inputs in around 70% of cells in the dorsomedial region of the SON. *Post hoc* attempts at immunocytochemical identification of oxytocin and vasopressin in recorded cells were unsuccesful, probably because the low-resistance patch electrodes used in the whole-cell mode readily wash out intracellular neuropeptides. However, it has been shown that the dorsomedial region of the SON is enriched in oxytocin neurones (Hou-Yu *et al.* 1986) and we therefore suggest that oxytocin disinhibits oxytocin neurones via depression of the inhibitory input to these cells.

Oxytocin regulates the GABAergic input in this cell system via a postsynaptic mechanism. This conclusion follows from the observation that oxytocin, either in the presence of TTX or in Ca^{2+}-free ACSF, reduced the amplitude of spontaneous IPSCs, while it did not influence their frequency. The finding that with both Ca^{2+}-free and normal ACSF, single lognormal plots could be fitted to the amplitude histograms suggests that under neither condition do the spontaneous IPSCs display distinguishable multiple components. In line with previous observations in other cell types (Borst, Lodder & Kits, 1994), it is therefore also likely that spontaneous IPSCs recorded from SON neurones at

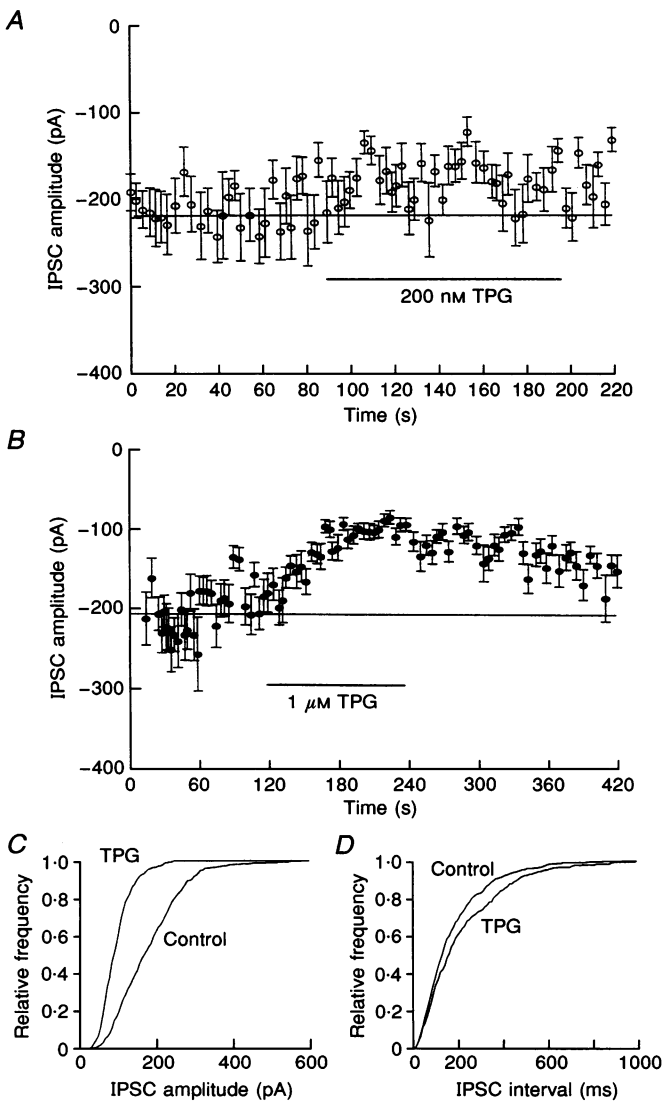

Figure 10. Thapsigargin reduces the amplitude of spontaneous IPSCs

A and *B*, scatter plots of IPSC amplitude means of 2 experiments in which 200 nM (*A*) and 1 μM thapsigargin (TPG) (*B*), were applied during the recordings as indicated. *A*, at 200 nM thapsigargin there were oscillations in the amplitude of IPSCs. *B*, at 1 μM thapsigargin, a slow but significant reduction of the IPSC amplitude occurred; this reversed upon washing the bath with control medium. Note that thapsigargin will diffuse through the membrane, which may explain the slow onset of this effect. *C* and *D*, cumulative amplitude (*C*) and interval (*D*) histograms (of > 250 events) before (control) and during application of 1 μM thapsigargin showed a large effect on the amplitude of the IPSC, but only a minor effect on the interval. $V_h = -70$ mV; recording in presence of DNQX and AP5.

room temperature reflect monoquantal events. This would imply that, while GABA vesicles are still being released at the same frequency by the presynaptic cells, the postsynaptic response to GABA is reduced by oxytocin. In keeping with this idea, the application of GTPγS to the interior of postsynaptic cells mimicked the effect of oxytocin. Thus a constitutive higher activation of postsynaptic G-proteins leads to modulation of $GABA_A$ receptor functioning. With GDPβS in the pipette, the effect of oxytocin was absent. This observation can be explained only by assuming that oxytocin activates postsynaptic G-protein-coupled receptors. Finally, thapsigargin and caffeine, calcium mobilizers known to affect the postsynaptic cells (Lambert *et al.* 1994; Dayanithi, Widmer & Richard, 1996), mimic the effect of oxytocin without substantially altering the frequency of release of GABA by presynaptic cells.

Surprisingly, vasopressin was capable of reducing the amplitude of spontaneous IPSCs in a distinct set of magnocellular neurones that were not sensitive to oxytocin. Further investigation is necessary to confirm this finding, but it would be in line with a recent report that vasopressin may facilitate its own release within the SON (Wotjak, Ludwig & Landgraf, 1995). Indeed, dendritic release of both oxytocin and vasopressin has been observed in the SON (DiScala-Guenot, Strosser & Richard, 1987; Pow & Morris, 1989). Moreover, Lambert *et al.* (1994) reported that around 50% of SON neurones were insensitive to oxytocin but responded to vasopressin by generating a rise in $[Ca^{2+}]_i$; this has been confirmed by Dayanithi *et al.* (1996).

Analysis of the IPSC amplitudes

The reduction in amplitude of the IPSCs by bicuculline did not cause a parallel shift in cumulative IPSC amplitude distribution, but resulted in an altered slope of the distribution (see Fig. 1*D*). Since the IPSC amplitude data are not distributed normally, it appears that bicuculline preferentially reduced the amplitude of the larger IPSCs, while small events remained relatively unaffected. A similar effect on the slope of the cumulative amplitude distribution of spontaneous and evoked IPSCs was observed after the application of oxytocin (Fig. 3*D* and 5*B*). This is due to the fact that in skewed distributions, the mean is positively correlated with the variance (greater means are accompanied by greater variances; Sokal & Rohlf, 1981). Indeed oxytocin affects both the mean and the variance in amplitude of spontaneously occurring IPSCs within experiments (Fig. 2*B*). We cannot exclude the possibility that large monoquantal IPSCs arise from synapses close to or on the somata, whereas smaller events are generated further away from the soma on one of the few apical dendrites. If this is true (which needs further investigation), preferential modulation of $GABA_A$ receptors close to the somata would also induce the observed decrease in variance.

Since spontaneous IPSCs in our recordings are to a large extent monoquantal, the amplitude of the spontaneous IPSCs should be directly related to the number of postsynaptic channels per synaptic bouton. Variation in amplitude of spontaneous IPSCs has been reported to result from substantial variation in the number of postsynaptic receptors per bouton (Borst *et al.* 1994).

Oxytocin reduced only the amplitude of the IPSCs without affecting the decay of the synaptic currents. Therefore, given the monoquantal nature of spontaneous IPSCs in SON neurones, this suggests that oxytocin reduces the number of participating $GABA_A$ receptors per synaptic bouton or affects their single channel slope conductance, or both. Oxytocin does not affect the apparent open time of $GABA_A$ receptors in these cells, since the decay of the IPSCs was not altered. This contrasts with the shortening of the apparent open time of $GABA_A$ receptor channels produced by rises in $[Ca^{2+}]_i$ in frog sensory neurones (Beherends, Maruyama, Tokutomi & Akaike, 1988) and cerebellar granular cells (Martina *et al.* 1994). The original idea (Inoue *et al.* 1986) that intracellular Ca^{2+} reduces the agonist affinity of $GABA_A$ receptors, thereby affecting the burst length of individual $GABA_A$ receptor channels, does not therefore explain the modulatory effect of oxytocin on $GABA_A$ receptors on SON neurones.

Calcium-dependent mechanisms of $GABA_A$ receptor modulation

Our data show that thapsigargin mimics the oxytocin effect on the GABA synapses in the SON. Previous work by others suggests that oxytocin acting via the activation of G-protein-coupled receptors located on putative oxytocin neurones triggers the release of calcium from thapsigargin-sensitive calcium stores (Lambert *et al.* 1994). The time course (onset, 20 s and decay, 3–5 min), as well as the dose dependence (maximum $> 1\ \mu M$), of the oxytocin-induced effects on the GABAergic IPSCs in our experiments is well in line with the oxytocin-induced calcium responses in SON neurones (Lambert *et al.* 1994).

A number of studies show that $GABA_A$ responses may be suppressed by $[Ca^{2+}]_i$ rises (Inoue *et al.* 1986; Taleb *et al.* 1987; Mouginot *et al.* 1991; Martina, Kilic & Cherubini, 1994; Chen & Wong, 1995). Our data on the depression of GABAergic synapses, via a postsynaptic mechanism in putative oxytocin neurones, are in agreement with a study on dissociated porcine intermediate lobe cells (melanotrophs), where thyrotrophin-releasing hormone modulates post-synaptic $GABA_A$ receptor activity via rises in $[Ca^{2+}]_i$ triggered by Ca^{2+} release from intracellular stores (Mouginot *et al.* 1991). The influx of extracellular calcium through calcium channels (Inoue *et al.* 1986), Ca^{2+} ionophores (Martina *et al.* 1994) or NMDA receptor channels (Chen & Wong, 1995) may also affect $GABA_A$ receptor functioning. This may lead to the impression that any rise in intracellular calcium, induced either by the action of another transmitter (e.g. glutamate) or by voltage gating of calcium channels, will reduce the $GABA_A$ receptor function. This is not necessarily true. Firstly, Mouginot *et al.* (1991) found that while calcium mobilization from intracellular stores did

affect the $GABA_A$ receptor functioning, influx of calcium from outside the cell did not. Secondly, earlier work on melanotrophs (Taleb *et al.* 1987) showed that the concentration of intracellular Ca^{2+} may determine whether calcium will enhance or depress $GABA_A$ responses.

Putative calcium-dependent mechanisms that are known to reduce $GABA_A$ receptor functioning include (i) activation of protein kinase C, resulting in the phosphorylation of $GABA_A$ receptors (Krishek, Xie, Blackstone, Huganir, Moss & Smart, 1994), (ii) a direct effect of calcium (> 100 nM) at the intracellular side of the membrane (Taleb *et al.* 1987, Martina *et al.* 1994), and (iii) activation of calcineurin, a calcium-dependent phosphatase (Chen & Wong, 1995).

Control of firing of oxytocin neurones

Most of what is known about oxytocinergic autoregulation comes from studies on female rats (Poulain & Wakerley, 1982). Local release of oxytocin was shown to facilitate the occurrence of the first milk ejection during suckling through recruitment of oxytocin neurones into a burst-firing pattern (Moos, Poulain, Rodriguez, Guerne, Vincent & Richard, 1989; Moos & Richard, 1989). In addition to autoregulation by oxytocin, and the reported regulation by a glutamatergic (Wuarin & Dudek, 1993) and a noradrenergic input (Onaka, Luckman, Guevaraguzman, Ueta, Kendrick & Leng, 1995), the input mediated via $GABA_A$ receptors has also been shown to regulate the firing of oxytocin neurones (Voisin *et al.* 1995). Previously it has been shown that, during lactation, GABA levels within the SON do not substantially alter in response to suckling (Voisin, Chapman, Poulain & Herbison, 1994). If oxytocin neurones autoregulate their own sensitivity to GABA in adults as they do in immature rats, this would lead to a reduction in the impact of the inhibitory input on SON neurones without any change in the release of GABA. Possibly the effect of oxytocin also explains the excitation reported by McKenzie *et al.* (1995) after stimulation of the neural stalk in lactating animals.

Stern & Armstrong (1995) have identified a slow outward current in oxytocin neurones that deactivates during prolonged periods of hyperpolarization. They show that this membrane property of oxytocin neurones gives rise to a rebound depolarization at the end of a prolonged hyperpolarization, as a result of which a burst of action potentials may be induced. It is tempting to speculate that oxytocin, acting via the depression of a sustained GABA input, reinforces this rebound depolarization, which may give rise to a synchronous, suprathreshold excitation generating a burst of action potentials in several oxytocin neurones. Once a burst of spikes is initiated, strong outward rectification in oxytocin neurones (Stern & Armstrong, 1995) may prevent the induction of a repetitive or phasic firing pattern, such as is seen preferentially in vasopressin neurones (but see Armstrong, Smith & Tian, 1994).

Conclusions

The present data support the idea that oxytocin causes depression of GABAergic inputs on oxytocin neurones in the SON. This synaptic depression is induced via a postsynaptic, possibly calcium-dependent mechanism resulting in a reduction of $GABA_A$ receptor function. Release of oxytocin from the dendrites into the extracellular space may thus lead to a synchronous disinhibition of all neighbouring magnocellular neurones expressing the oxytocin receptor. It is conceivable that the GABA input normally maintains a prolonged hyperpolarization. An oxytocin-induced depression of IPSCs may shift the balance between inhibitory and excitatory inputs and result in a sustained depolarization of oxytocin neurones which, as a result of the intrinsic membrane electrical properties (Stern & Armstrong, 1995) could be a powerful means of controlling the firing pattern in these neurones.

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