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Modulation of $GABA_A$ receptor-mediated IPSCs by neuroactive steroids in a rat hypothalamo-hypophyseal coculture model

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1. We have used the whole-cell configuration of the patch-clamp technique to investigate the effects of neuroactive steroids on $GABA_A$ receptor-mediated synaptic transmission between rat hypothalamic neurones and pituitary intermediate lobe (IL) cells grown in coculture. In order to discriminate between possible pre- and postsynaptic sites of action, the effects of neurosteroids on $GABA_A$ receptor-mediated synaptic currents (IPSCs) were compared with those on $GABA_A$ currents (I_{GABA}) triggered by local application of 50 or 500 μM GABA, which yielded approximately half-maximal and maximal responses, respectively.

2. In primary cultures of rat pituitary IL cells, allopregnanolone (5α-pregnan-3α-ol-20-one) reversibly potentiated I_{GABA} in a dose-dependent manner with a threshold between 0·1 and 1 nM. At a concentration of 10 nM, allopregnanolone increased the response evoked by 50 μM GABA by $+21·4 \pm 5·1$% ($n = 8$), but had no effect on I_{GABA} induced by 500 μM GABA. The β-isomer of allopregnanolone, epipregnanolone (5β-pregnan-3β-ol-20-one, 10 nM), had no effect on I_{GABA} at any concentration of GABA tested.

3. At concentrations lower than 10 μM, pregnenolone sulphate (5-pregnen-3α-ol-20-one sulphate) did not significantly inhibit I_{GABA}. However, at 10 μM, a systematic reduction of I_{GABA} evoked by 50 and 500 μM GABA was observed, with mean values of −80 and −60%, respectively. This blocking effect was reversible and accompanied by a marked acceleration of the decay of $GABA_A$ currents during the application of GABA.

4. In isolated pairs of synaptically connected hypothalamic neurones and IL cells, allopregnanolone (10 nM) augmented the mean amplitude of spontaneous IPSCs (sIPSCs) and electrically evoked IPSCs (eeIPSCs) by about 40% and also increased the mean frequency of sIPSCs. Allopregnanolone (10 nM) also markedly increased the frequency of miniature IPSCs (mIPSCs) recorded in the presence of TTX (0·5 μM), but without modifying their mean amplitude. Epipregnanolone had no effect on the amplitude or frequency of sIPSCs. Neither epipregnanolone nor allopregnanolone modified the time to peak and decay time constants of GABAergic IPSCs.

5. Pentobarbitone (50 μM), a positive allosteric modulator of $GABA_A$ receptors, did not affect the amplitude of sIPSCs or eeIPSCs, but significantly increased the decay time constants of both types of IPSCs. Pentobarbitone had no effect on the frequency of sIPSCs.

6. Pregnenolone sulphate (10 μM) completely and reversibly blocked sIPSCs and eeIPSCs. Progressive block of IPSCs was correlated with a gradual decrease of the mean decay time constant.

7. Our results suggest that, under physiological conditions, allopregnanolone might be a potent modulator of GABAergic synaptic transmission, acting at both pre- and postsynaptic sites. The involvement of pregnenolone sulphate as a modulator of GABAergic IPSCs under physiological conditions is, however, more questionable. The mechanisms of action of both types of neurosteroids are discussed.

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$GABA_A$ receptors are ligand-gated ion channels, formed by oligomeric associations of α, β, γ and δ subunits or homomeric associations of ρ subunits, and possess different allosteric modulatory sites, such as those for barbiturates, benzodiazepines and steroids (MacDonald & Olsen, 1994; Sieghardt, 1995). In particular, neurosteroids that are synthesized by glial cells and neurones in the central nervous system (Majewska, 1992; Paul & Purdy, 1992; Robel & Beaulieu, 1994; Lambert, Belelli, Hill-Venning & Peters, 1995) may play an important role in the fine tuning of GABAergic synaptic transmission. Until now, electrophysiological experiments have been performed almost exclusively on single-channel currents and whole-cell currents induced by activation of recombinant or extrasynaptic $GABA_A$ receptors (Harrison, Majewska, Harrington & Barker, 1987; Puia *et al.* 1990; Twyman & MacDonald, 1992), but relatively little is known about the precise effects of endogenous neurosteroids on GABAergic synaptic transmission.

In order to characterize the actions of such neurosteroids (allopregnanolone, epipregnanolone and pregnenolone sulphate (PS)) at the synaptic level, we have used a coculture system in which hypothalamic neurones form functional GABAergic synapses with endocrine cells of the intermediate lobe of the pituitary (Poisbeau, René, Egles, Félix, Feltz & Schlichter, 1996). In this model the properties of the GABAergic IPSCs are similar to those recorded in acute neurointermediate lobe slices (Schneggenburger & Konnerth, 1992) and the system allows ideal pharmacological access to the synapse as well as the study of the synaptic transmission between pairs of clearly identified cells. In order to discriminate between pre- and postsynaptic actions of the neurosteroids, their effects on IPSCs were compared with those on total $GABA_A$ currents (I_{GABA}) induced by local exogenous application of GABA. A preliminary account of this work has appeared in abstract form (Poisbeau, Jover, Feltz & Schlichter, 1995).

METHODS

The methods for preparing hypothalamo-hypophyseal cocultures and for recording from such cocultures have been described in detail elsewhere (Poisbeau *et al.* 1996) and will therefore only be briefly summarized below.

Tissue culture

Preparation of hypothalamic neurones. Pregnant Wistar rats at day 15 of gestation were anaesthetized with pentobarbitone, the embryos removed and the mothers killed by decapitation. The embryonic diencephalic area corresponding to the basal hypothalamus was dissected out. After enzymatic dissociation in a divalent-free phosphate-buffered solution (PBS) containing Trypsin and EDTA (0·02–0·05%, Gibco, France) for 7 min at 37 °C, mechanical dissociation was performed in 2 ml of Dulbecco's modified Eagle's medium (DMEM, Gibco, France) containing 20% (v/v) fetal calf serum (FCS, Gibco, France) with fire-polished Pasteur pipettes of decreasing tip diameter. The dissociated cells were resuspended in serum-free medium according to the method of Bottenstein & Sato (1979). Hypothalamic neurones were seeded at a density of 400000 cells per dish on 35 mm culture dishes (Costar, Costar Corning Corporation, Cambridge, MA, USA), which had been previously coated with poly-L-lysine.

Preparation of intermediate lobe (IL) cells. Neonatal (1–7 days) Wistar rats were killed by decapitation under diethyl ether anaesthesia. Pituitaries were removed, extensively washed in PBS and the neurointermediate lobes (NILs) were separated from anterior lobes using thin forceps. Melanotrophs were obtained after enzymatic and mechanical dissociation of the NILs following the procedure described above for hypothalamic neurones, and seeded at a density of 80000 cells per dish either on top of the hypothalamic neurones (cocultures) or alone (primary cultures).

Primary cultures and cocultures were maintained in water-saturated atmosphere (95% air, 5% CO_2) at 37 °C. Medium was renewed for the first time after 5 days and subsequently every 3 days.

Preparation and application of substances

Neurosteroids were diluted in the external medium and were bath applied. The rate of perfusion was 10 ml min^{-1} for a total bath volume of 1·5 ml. In experiments on extrasynaptic $GABA_A$ currents, GABA was applied locally with a U-tube (Fenwick, Marty & Neher, 1982). When the effects of steroids on $GABA_A$ responses were tested, these steroids were present at equilibrium in the bath and coapplied (at the same concentration as in the bath) with GABA via the U-tube. With this system the rise time of the solution exchange was of 8–10 ms, as judged from the change in the tip resistance of a patch-pipette during application of a solution with low electrical conductance. Allopregnanolone (5α-pregnan-3α-ol-20-one), epipregnanolone (5β-pregnan-3β-ol-20-one) and pregnenolone sulphate (5-pregnen-3α-ol-20-one sulphate) (all from Sigma) were prepared as 1000 times concentrated stock solutions in ethanol (96%) and stored at 4 °C. The neurosteroids to be tested were dissolved at final concentration in extracellular solution just before the recording session. At a 1/1000 dilution, ethanol alone had no effect on $GABA_A$ currents under the prevailing experimental conditions.

Electrophysiological recordings

All experiments were performed at room temperature (20–22 °C). For experiments concerning the effect of neurosteroids on extrasynaptic $GABA_A$-gated currents, 3- to 7-day-old primary cultures of IL cells without neurones were used. Experiments on GABAergic synaptic currents were performed on 9- to 14-day-old hypothalamo-hypophyseal cocultures. In such cocultures, IL cells were identified as round, phase bright cells devoid of neuritic extensions, whereas neurones had an elongated shape, were relatively phase dark and extended several long neurites (for details see Poisbeau *et al.* 1996). Patch-clamp recordings were made from IL cells in the whole-cell recording configuration (Hamill, Marty, Neher, Sakmann & Sigworth, 1981) using an Axopatch 200A amplifier (Axon Instruments) and low resistance (3–4 MΩ) electrodes. The series resistance during whole-cell recording was between 5 and 8 MΩ. The external medium contained (mM): 135 NaCl, 5 KCl, 5 $CaCl_2$, 1 $MgCl_2$, 5 Hepes and 10 glucose; pH 7·3 (adjusted with NaOH). Pipettes were filled with an intracellular solution containing (mM): 125 KCl or CsCl, 5 $CaCl_2$, 2 $MgCl_2$, 10 Hepes, 10 EGTA and 2 Na_2-ATP; pH 7·3 (adjusted with KOH or CsOH). The estimated intracellular free calcium concentration was 10^{-7} M and the equilibrium potential for chloride ions (E_{Cl}) was −2 mV.

Electrical stimulation

Electrical stimulation was applied to the cell body of the presynaptic neurone with two patch pipettes filled with extracellular solution (resistance, 1 MΩ), glued tip-to-tip under a

stereomicroscope and connected to a pulse generator. The duration of the stimuli was 0·1 ms in all experiments and the stimulation intensity used varied between −10 and −20 V (Poisbeau *et al.* 1996). The stimulation electrodes were lowered onto the neurone after having established the whole-cell recording configuration in the postsynaptic (pituitary) cell and one of them was placed in direct contact with the cell soma of the visually identified presynaptic neurone. In these experiments, we exclusively selected isolated pairs of cells, i.e. an IL cell that was in contact with a single neurite of a single presynaptic neurone.

Data storage and analysis

Acqusition and analysis of $GABA_A$ receptor-gated whole-cell currents and synaptic currents was performed as previously described (Poisbeau *et al.* 1996). Briefly, membrane currents were stored on videotape in digital form (20 kHz) after being filtered at 5 kHz by the internal filter of the Axopatch-200A amplifier. Analysis was performed on current traces filtered at 2 kHz and digitized at 4 kHz using pCLAMP (Fetchex, Axon Instruments) and Axograph II (Axon Instruments) software. Rise times (time to peak) and amplitudes of all IPSCs were measured manually on individual events using the cursor measurement facility of the Axograph software. Miniature IPSCs were recorded in the presence of 0·5 μM tetrodotoxin (TTX, Latoxan, Rosans, France) to block the voltage-dependent Na^+ currents underlying the propagation of the action potential and were analysed as described for the other IPSCs. Rise times are expressed as the total duration of the rising phase from baseline (0%) to the peak (100%) of the synaptic current. Exponential fits of the decaying phases of the synaptic currents were performed with the internal fitting routines of the Axograph software.

All statistical results are expressed as means ± S.D. In order to evaluate statistical differences between sets of data, Student's *t* test was used. The confidence interval was set at 0·05.

RESULTS

Modulation of $GABA_A$ receptor-mediated currents by allopregnanolone, epipregnanolone and pregnenolone sulphate

The effects of allopregnanolone, epipregnanolone and pregnenolone sulphate (PS) were first examined on whole-cell $GABA_A$ currents (I_{GABA}) induced by local application of GABA to IL cells in primary culture. Two different concentrations of GABA, i.e 50 and 500 μM, were used. These concentrations corresponded, respectively, to submaximally effective (approximately half-maximal) and maximally effective (saturating) concentrations of GABA, as inferred from analysis of GABA dose–response curves from IL cells.

Allopregnanolone reversibly potentiated I_{GABA} induced by 50 μM GABA (Fig. 1*A*) but had no effect on I_{GABA} elicited by

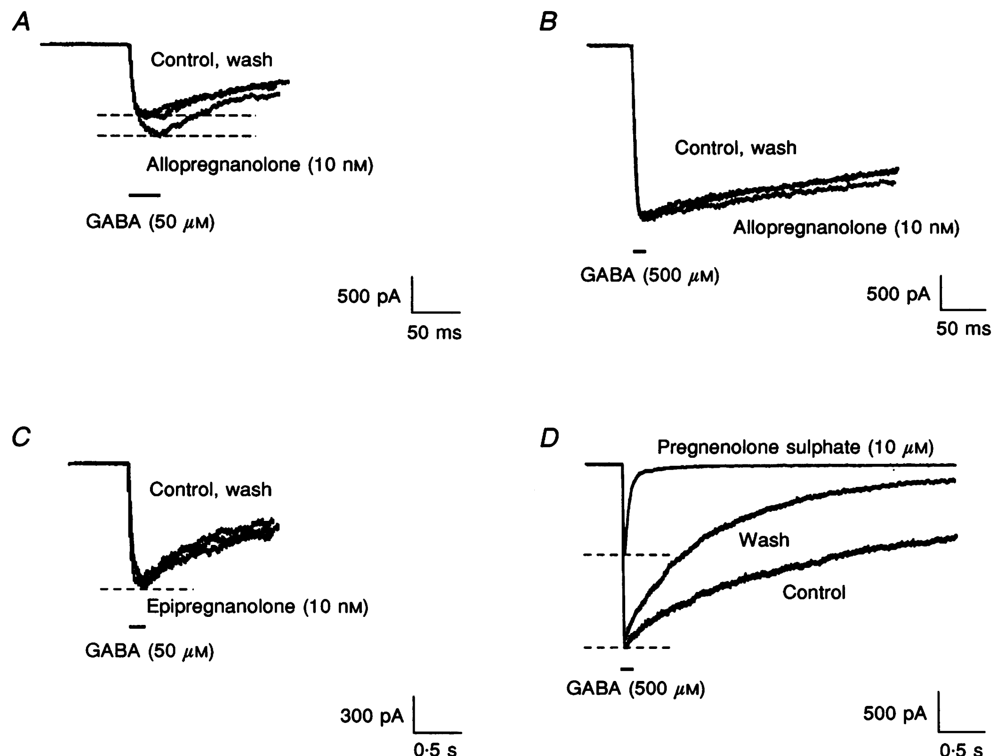

Figure 1. Modulation of total $GABA_A$ currents by allopregnanolone, epipregnanolone and pregnenolone sulphate

A, allopregnanolone (10 nM) reversibly potentiated half-maximal currents evoked by 50 μM GABA. *B*, maximal currents induced by 500 μM GABA were unaffected by allopregnanolone. *C*, epipregnanolone (10 nM) had no effect on submaximal $GABA_A$ currents induced by 50 μM GABA. *D*, effect of pregnenolone sulphate (10 μM) on maximal GABA currents evoked by 500 μM GABA. Note the marked acceleration of the deactivation phase. GABA was applied for the durations indicated by the horizontal bars. Holding potential, −60 mV.

Table 1. Dose dependence of the potentiating effect of allopregnanolone on the $GABA_A$ receptor-mediated whole-cell currents induced by local application of 50 μM GABA

	Concentration of allopregnanolone (nM)			
	0·1	1	10	100
Potentiation of GABA response	Not detectable	12·5 ± 2%	21·4 ± 5·1%	40·2 ± 8%
Number of cells	$n = 3$	$n = 4$	$n = 8$	$n = 5$

The values are given as means ± S.D. All values are statistically different at a confidence interval of 0·05.

500 μM GABA ($n = 13$, Fig. 1*B*). This effect appeared to be dose dependent (Table 1) with a threshold between 0·1 and 1 nM. At a concentration of 10 nM, allopregnanolone potentiated I_{GABA} induced by 50 μM GABA on average by +21·4 ± 5·1% ($n = 8$).

The β-isomer of allopregnanolone, epipregnanolone (10 nM), was without effect on I_{GABA} elicited by either 50 μM GABA (Fig. 1*C*) or 500 μM GABA ($n = 5$, not shown). At a concentration of 10 μM, pregnenolone sulphate inhibited in a reversible manner the currents evoked by 50 μM GABA (−81·4 ± 3·3%; $n = 3$), as well as those induced by 500 μM GABA (−60·1 ± 17%; $n = 6$; Fig. 1*D*). This reduction in amplitude of I_{GABA} was accompanied by a marked decrease of the decay time constant of the current during GABA application. For example, the apparent desensitization time constant, i.e. the decay time constant of the whole-cell current during a 1 s application of 500 μM GABA, was reduced from 1·09 ± 0·65 s in the absence of PS to 0·048 ± 0·009 s ($n = 6$) in the presence of 10 μM PS. The

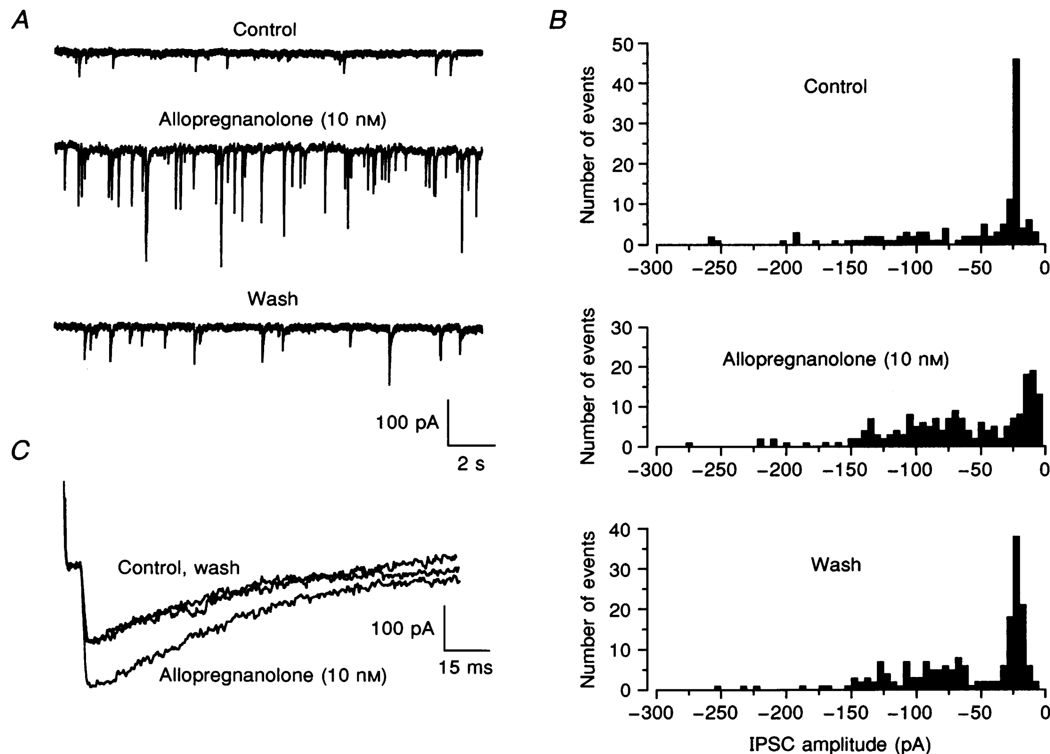

Figure 2. Modulation of sIPSCs and eeIPSCs by allopregnanolone

A, allopregnanolone (10 nM) increased both the frequency and the amplitude of the spontaneous synaptic currents in a reversible manner. *B*, amplitude histograms of the sIPSCs under control conditions (top), during perfusion of 10 nM allopregnanolone (middle) and after washout of allopregnanolone (bottom). Note the increase in the proportion of sIPSCs with amplitudes > 50 pA *versus* sIPSCs with amplitudes < 50 pA during the superfusion of allopergnanolone. Bin width, 5 pA. *C*, allopregnanolone (10 nM) increased the amplitude of eeIPSCs but did not change the time to peak or the kinetics of the decaying phase of the IPSC. Holding potential, −60 mV.

effect of PS on the amplitude of I_{GABA} did not show a clear dose dependence over the range of concentrations tested. At a concentration of 10 nM, PS had no detectable effect on I_{GABA} ($n = 3$) and at concentrations of 100 nM and 1 μM, PS reduced the amplitude of I_{GABA} elicited by 50 μM GABA by $-10{\cdot}7 \pm 6{\cdot}8\%$ ($n = 4$) and $-13{\cdot}3 \pm 9{\cdot}6\%$ ($n = 4$), respectively. These values are not significantly different ($P > 0{\cdot}05$).

Allopregnanolone-induced potentiation of GABAergic IPSCs

It has been previously shown that in our coculture system all synaptic currents recorded in IL cells were mediated by activation of $GABA_A$ receptors and therefore corresponded to GABAergic inhibitory postsynaptic currents (IPSCs) (Poisbeau *et al.* 1996). The majority (> 90%) of the IPSCs had deactivation phases that could be fitted with a monoexponential function.

Allopregnanolone (10 nM) produced a strong and reversible potentiation of spontaneously occurring IPSCs (sIPSCs) recorded in IL cells (Fig. 2*A*), an effect that included an increase in the mean amplitude ($+46{\cdot}9 \pm 3{\cdot}6\%$, $n = 4$) and frequency of sIPSCs ($+86{\cdot}8 \pm 0{\cdot}98\%$, $n = 4$). The kinetic characteristics of sIPSCs were not affected by allopregnanolone. Under control conditions, the mean values of the time to peak and the time constant (τ) of monoexponentially decaying sIPSCs were $1{\cdot}47 \pm 0{\cdot}36$ and $42{\cdot}64 \pm 15{\cdot}07$ ms, respectively ($n = 6$). In the presence of allopregnanolone (10 nM), these values were $1{\cdot}47 \pm 0{\cdot}47$ and $42{\cdot}01 \pm 12{\cdot}06$ ms ($n = 6$). There was no statistically significant difference between the two sets of values ($P > 0{\cdot}05$). Under control conditions the amplitude histogram of sIPSCs showed a majority of events with amplitudes smaller than 50 pA (Fig. 2*B*, top). Although such small events persisted during perfusion of allopregnanolone (Fig. 2*B*, middle) there was an apparent increase in the proportion of sIPSCs with larger amplitudes (> 50 pA). This effect was slowly reversible after washout of allopregnanolone (Fig. 2*B*, bottom, and Fig. 2*A*, bottom trace).

Similar results were obtained for electrically evoked IPSCs (eeIPSCs) recorded from IL cells. As illustrated in Fig. 2*C*, the potentiating effect of allopregnanolone concerned only the amplitude of the eeIPSCs ($+42{\cdot}7 \pm 3{\cdot}4\%$, $n = 4$). The kinetic parameters of the eeIPSCs were unchanged in the presence of allopregnanolone (control: time to peak, $1{\cdot}6 \pm 0{\cdot}3$ ms and τ, $44{\cdot}9 \pm 13{\cdot}8$ ms; 10 nM allopregnanolone: time to peak, $1{\cdot}7 \pm 0{\cdot}4$ ms and τ, $43{\cdot}1 \pm 13{\cdot}9$ ms; $n = 4$; $P > 0{\cdot}05$). The percentage of failures of transmission observed during electrical stimulation experiments was $2{\cdot}8 \pm 3{\cdot}3\%$ under control conditions ($n = 11$). In the

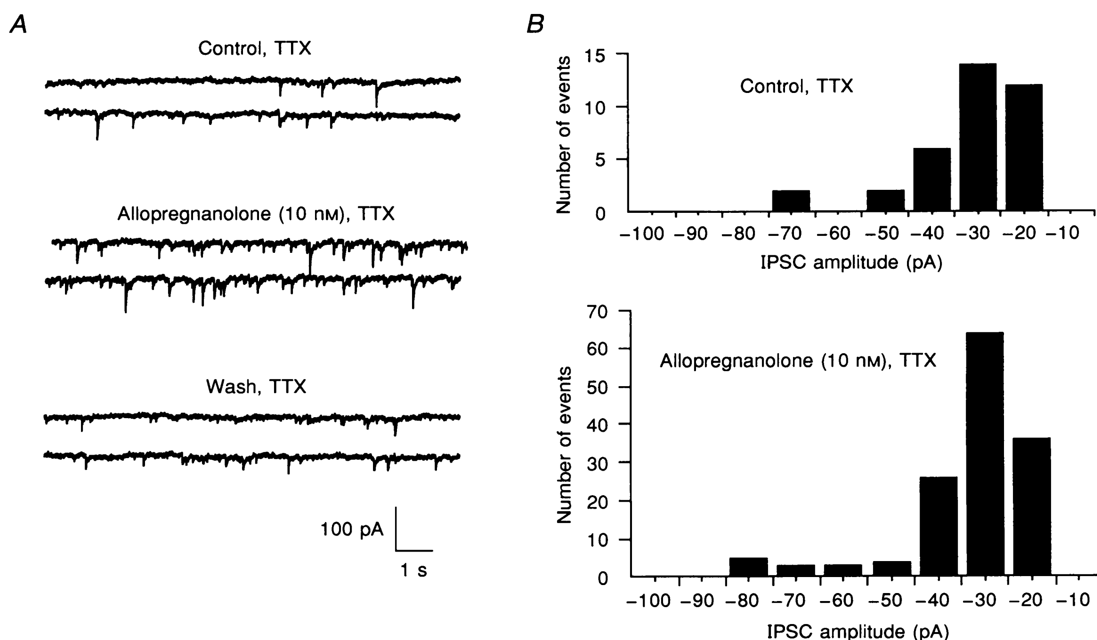

Figure 3. Modulation of miniature IPSCs (mIPSCs) by allopregnanolone (10 nM)

A, allopregnanolone markedly increased the frequency of mIPSCs in a reversible manner. *B*, distribution of amplitudes of mIPSCs under control conditions (top) and in the presence of 10 nM allopregnanolone (bottom). Each histogram was constructed from the events recorded during a period of 1 min. Note the difference in scale of the ordinate axis of the two histograms, which indicates a much larger number of events recorded in the presence of allopregnanolone (total of 141 events) compared with the control (total of 36 events). This phenomenon reflected the increase in frequency of mIPSCs observed in the presence of allpregnanolone. The mean amplitude of mIPSCs was $-26{\cdot}8 \pm 11{\cdot}6$ pA under control conditions and $-28{\cdot}11 \pm 13{\cdot}5$ pA in the presence of allopregnanolone (10 nM). All recordings were made in the presence of 0·5 μM TTX in the extracellular solution in order to isolate mIPSCs. Bin width, 10 pA. Holding potential, −60 mV.

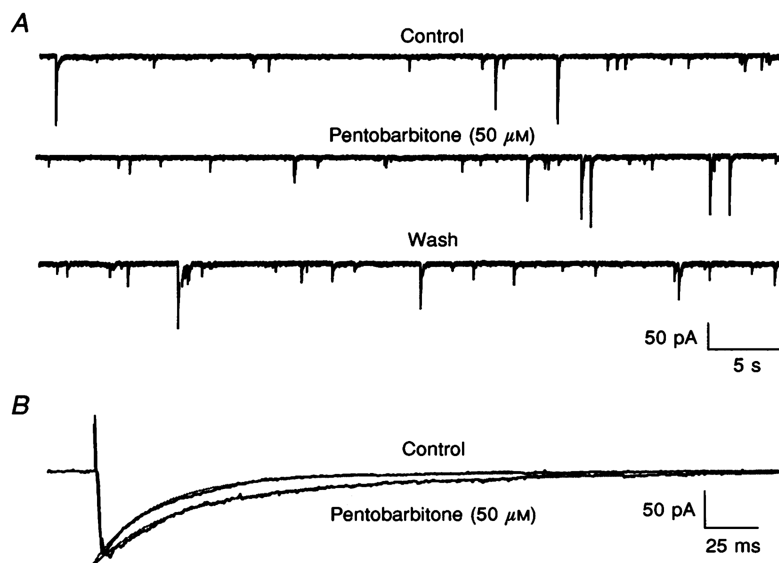

Figure 4. Modulation of IPSCs by pentobarbitone

Pentobarbitone (50 μM) had no effect on the amplitude or frequency of the sIPSCs (*A*) but markedly prolonged the deactivation phase of IPSCs, as illustrated in the case of eeIPSCs (*B*). Note that pentobarbitone did not potentiate the amplitude of the eeIPSCs. The superimposed shadowed lines are monoexponential best fits of the deactivation phases of the eeIPSCs. The time constants were 34·5 ms under control conditions and 65 ms in the presence of 50 μM pentobarbitone. Holding potential, −60 mV.

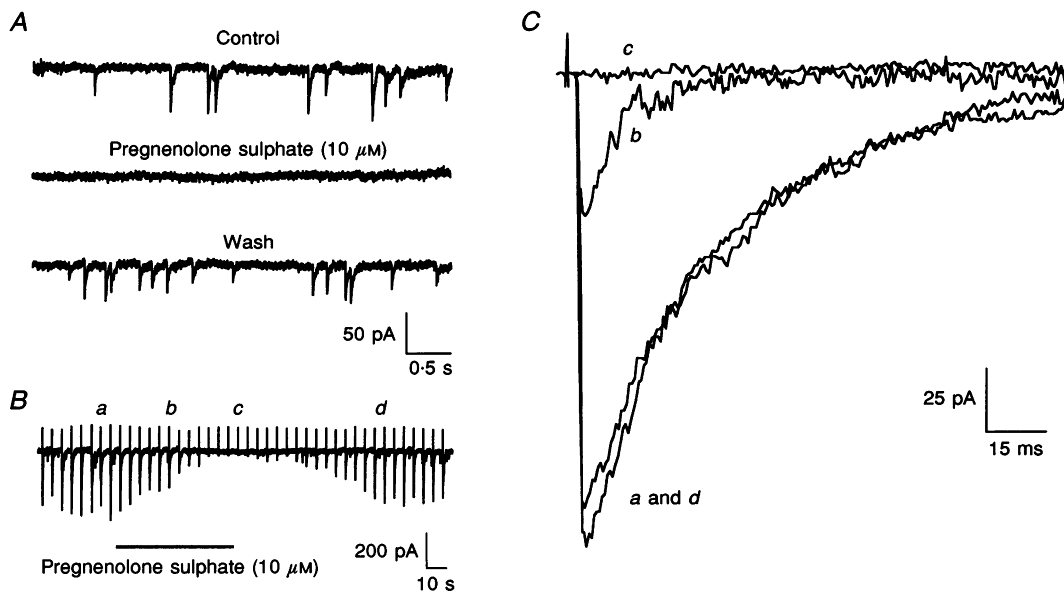

Figure 5. Effects of pregnenolone sulphate on GABAergic synaptic events

A, pregnenolone sulphate (10 μM) completely abolished spontaneous IPSCs in a reversible manner. *B*, eeIPSCs, represented by the vertical deflections of the current trace, were also completely and reversibly blocked by pregnenolone sulphate (10 μM). Note the progressive onset of block during superfusion with pregnenolone sulphate for the period indicated by the horizontal bar. The traces labelled *a*–*d* correspond to representative traces of eeIPSCs under control conditions (*a*), during partial block (*b*) and total block (*c*) of IPSCs by pregnenolone sulphate and after washout of pregnenolone sulphate (*d*). *C*, representation on a faster time scale of traces *a*–*d* from *B*. The traces were superimposed to facilitate comparison. Holding potential, −60 mV.

presence of allopregnanolone, no failures were observed ($n = 4$).

Figure 3 illustrates the effect of allopregnanolone (10 nM) on miniature IPSCs (mIPSCs) recorded in IL cells in the steady-state presence of 0·5 μM TTX. Allopregnanolone clearly increased the frequency of mIPSCs in a reversible manner (Fig. 3*A*), but did not alter the mean amplitude of the synaptic events (control, $-31{\cdot}45 \pm 4{\cdot}6$ pA; allopregnanolone, $-31{\cdot}75 \pm 3{\cdot}6$ pA; $n = 4$; $P > 0{\cdot}05$). The time to peak and the deactivation time constant were not modified by allopregnanolone (control: time to peak, $1{\cdot}6 \pm 0{\cdot}14$ ms and τ, $43{\cdot}3 \pm 8{\cdot}6$ ms; allopregnanolone: time to peak, $1{\cdot}6 \pm 0{\cdot}1$ ms and τ, $46{\cdot}7 \pm 7{\cdot}0$ ms; $n = 4$; $P > 0{\cdot}05$). Figure 3*B* shows an example of the amplitude distribution of mIPSCs under control conditions and in the presence of 10 nM allopregnanolone. Similar observations were made in four different cells.

Pentobarbitone-induced modulation of IPSCs

In order to characterize further the mechanism of action of allopregnanolone, its effect was compared with that of pentobarbitone, a well-established positive allosteric modulator of $GABA_A$ receptors (MacDonald & Olsen, 1994; Sieghardt, 1995). As illustrated in Fig. 4*A*, steady-state perfusion of pentobarbitone (50 μM) did not affect the frequency of sIPSCs recorded in IL cells. The mean amplitude and the time to peak of sIPSCs were also unaffected by pentobarbitone (control: time to peak, $0{\cdot}98 \pm 0{\cdot}11$ ms and mean amplitude, $-52{\cdot}4 \pm 14{\cdot}95$ pA; pentobarbitone (50 μM): time to peak, $1{\cdot}07 \pm 0{\cdot}03$ ms and mean amplitude, $-50{\cdot}19 \pm 16{\cdot}29$ pA; $n = 4$; $P > 0{\cdot}05$). In contrast, the deactivation time constant of sIPSCs was markedly increased (control, $38{\cdot}27 \pm 1{\cdot}95$ ms; pentobarbitone (50 μM), $72{\cdot}1 \pm 5{\cdot}53$ ms). These values are significantly different at a confidence interval of 0·05.

The same observations were made for eeIPSCs. The values of the time to peak and of the mean amplitude of eeIPSCs in IL cells were $1{\cdot}89 \pm 0{\cdot}5$ ms and $-159{\cdot}66 \pm 33{\cdot}07$ pA, respectively, under control conditions, and $1{\cdot}93 \pm 0{\cdot}47$ ms and $-132{\cdot}7 \pm 29{\cdot}9$ pA, respectively, in the presence of 50 μM pentobarbitone ($n = 4$). These sets of values are not significantly different ($P > 0{\cdot}05$). However, as for sIPSCs, the deactivation time constant of eeIPSCs was increased by 50 μM pentobarbitone (control, $39{\cdot}68 \pm 9{\cdot}28$ ms; pentobarbitone, $69{\cdot}71 \pm 18{\cdot}4$ ms). These values are significantly different at a confidence interval of 0·05.

Effect of pregnenolone sulphate on GABAergic IPSCs

PS (10 μM) produced a progressive, complete and reversible block of sIPSCs and eeIPSCs (Fig. 5) in IL cells. The IPSCs recorded just before total block displayed a marked decrease of the monoexponential decay time constant: from $42{\cdot}1 \pm 9{\cdot}6$ to $16{\cdot}2 \pm 4{\cdot}6$ ms ($n = 5$) for eeIPSCs (Fig. 3*B* and *C*) and from $38{\cdot}9 \pm 8{\cdot}7$ to $9{\cdot}8 \pm 2{\cdot}5$ ms ($n = 5$) for sIPSCs (not shown). These values are statistically different at a confidence interval of 0·05. The frequency of the sIPSCs decreased progressively until complete abolition ($n = 5$). PS did not affect the time to peak of IPSCs, which was $1{\cdot}04 \pm 0{\cdot}1$ and $1{\cdot}3 \pm 0{\cdot}26$ ms for sIPSCs ($n = 5$) and eeIPSCs ($n = 5$), respectively.

DISCUSSION

The aim of this study was to characterize the effects of neuroactive steroids on GABAergic synaptic transmission. For that purpose, a coculture model of hypothalamic neurones and intermediate lobe (IL) cells of the rat pituitary was used, which offered a number of advantages for the study of GABAergic synaptic transmission, including optimal space-clamp conditions in the postsynaptic cell due to the absence of membrane processes, clear identification of the postsynaptic (endocrine) cell and presence of a small number ($\leqslant 5$) of synaptic boutons (Poisbeau *et al.* 1996). We have chosen to test the effects of allopregnanolone and pregnenolone sulphate, which are known to respectively potentiate and inhibit $GABA_A$ receptor-gated Cl^- currents, in various preparations (MacDonald & Olsen, 1994; Sieghardt, 1995).

This study shows that allopregnanolone is a powerful positive modulator of $GABA_A$ receptor-gated whole-cell currents and GABAergic IPSCs in IL cells at nanomolar concentrations. These properties confer to allopregnanolone the profile of a possible endogenous modulator of $GABA_A$ receptor function, which might be involved in the fine tuning of GABAergic synaptic transmission *in situ*. Moreover, these results provide evidence that allopregnanolone acts at both pre- and postsynaptic sites. In contrast, pregnenolone sulphate had no clear direct effect on $GABA_A$ receptor currents at concentrations $\leqslant 1$ μM, but strongly inhibited such currents and GABAergic IPSCs at a concentration of 10 μM. Therefore the physiological role of pregnenolone sulphate is more questionable than that of allopregnanolone, although such a role cannot completely be excluded (see below).

Effect of allopregnanolone

Whole-cell $GABA_A$ responses. When tested on isolated IL cells in primary culture, allopregnanolone was found to produce a potentiation of $GABA_A$ receptor currents (I_{GABA}) only when the concentration of GABA used was submaximal (50 μM). In contrast, allopregnanolone failed to potentiate (or inhibit) I_{GABA} elicited by saturating (maximal) concentrations of GABA (500 μM). These results agree well with those obtained with allopregnanolone and 3α-neuroactive steroids in other preparations, such as hippocampal neurones (Harrison *et al.* 1987; Lambert, Peters, Sturgess & Hales, 1990) or recombinant human $GABA_A$ receptors (Puia *et al.* 1990; Puia, Ducic, Vicini & Costa, 1993), and indicated that in our system, allopregnanolone also exerted a positive allosteric modulation of $GABA_A$ receptors expressed by IL cells. The threshold of the effect of allopregnanolone was between 0·1 and 1 nM. For higher concentrations of allopregnanolone (1–100 nM) the potentiation of I_{GABA} was

dose dependent, indicating a receptor-mediated mechanism of action. Moreover, epipregnanolone, which is structurally very close to allopregnanolone, had no effect on I_{GABA} at the concentrations tested (10–50 nM). This observation suggested that the effect on I_{GABA} was not due to a non-specific lipophilic interaction of the neurosteroid with the cell membrane.

GABAergic IPSCs. The effect of allopregnanolone on GABAergic IPSCs was more complex than its effect on I_{GABA}. Indeed, at a concentration of 10 nM, it was found that allopregnanolone not only augmented the mean amplitude of spontaneously occurring IPSCs (sIPSCs) and electrically evoked IPSCs (eeIPSCs), but also markedly increased the frequency of sIPSCs without affecting the time to peak or the deactivation time constant of IPSCs. These observations suggested that allopregnanolone also acted presynaptically to stimulate the release of GABA.

In order to distinguish between the different sites of action of allopregnanolone, its effect was first compared with that of pentobarbitone (50 μM), a well-established positive allosteric modulator of $GABA_A$ receptors (MacDonald & Olsen, 1994; Sieghardt, 1995). Pentobarbitone did not increase (or decrease) the frequency of sIPSCs, indicating the absence of a presynaptic site of action. Moreover, pentobarbitone, in contrast with allopregnanolone, had no effect on the mean amplitude of sIPSCs and eeIPSCs, but markedly increased the deactivation time constant of IPSCs. These results showed that pentobarbitone was able to modulate postsynaptic $GABA_A$ receptor kinetics, as indicated by the increase in the deactivation time constant of the IPSCs, but failed to increase the amplitude of these IPSCs. The latter observation indicated that in the coculture system, the concentration of GABA reached in the synaptic cleft at individual release sites is sufficient to saturate postsynaptic $GABA_A$ receptor clusters, as has been demonstrated for some GABAergic IPSCs in the hippocampus (De Koninck & Mody, 1994; Mody, De Koninck, Otis & Soltesz, 1994). This conclusion is also supported by the fact that allopregnanolone failed to increase the mean amplitude of miniature IPSCs (mIPSCs) recorded in the presence of 0·5 μM TTX. Indeed, if the mIPSCs had been induced by submaximal concentrations of GABA, allopregnanolone would be expected to potentiate the amplitude of these IPSCs. This was not the case.

In our preparation, allopregnanolone (10 nM) did not increase the deactivation time constant of IPSCs. This is in contrast with the results of Harrison *et al.* (1987), who reported a prolongation of IPSC duration in the presence of 1 μM allopregnanolone in hippocampal neurones, a phenomenon that probably reflected an increase in burst open time duration of $GABA_A$ receptor channels (Twyman & Macdonald, 1992). The results of this study are, however, in good agreement with those of Puia *et al.* (1990) on recombinant human $GABA_A$ receptors, showing that allopregnanolone increases the frequency of opening of $GABA_A$ channels without modification of their burst open time duration. It is also important to emphasize that in the coculture system used here, allopregnanolone had the same modulatory action at neuroendocrine synapses (between a hypothalamic neurone and an IL cell) and at neuro-neuronal synapses (between two hypothalamic neurones). Interestingly, the profiles of expression of $GABA_A$ receptor subunits in IL cells (Berman, Roberts & Pritchett, 1994) and in hypothalamic neurones (Wisden, Laurie, Monyer & Seeburg, 1992) are very similar, since both cell types predominantly express the α_2, β_3 and γ_2 subunits of the $GABA_A$ receptor, but differ markedly from hippocampal neurones that in addition express the α_1, α_4 and α_5 subunits. Therefore a subunit-specific effect of allopregnanolone cannot be totally ruled out and could eventually account for the differences in the modulatory action of allopregnanolone between different preparations. Interestingly, it has been shown recently that in cerebellar granule cells, the expression of the δ subunit of the $GABA_A$ receptor selectively suppressed the potentiating effect of 3α,21-dihydroxy-5α-pregnan 20-21 (THDOC) on $GABA_A$ receptor-gated currents without affecting the potency of THDOC to directly activate $GABA_A$ currents at high concentrations (1–10 μM) or the faculty of pregnenolone sulphate to inhibit $GABA_A$ receptor-gated currents (Zhu, Wang, Krueger & Vicini, 1996). Another important point to take into consideration is that the effect of allopregnanolone on the deactivation kinetics of GABAergic IPSCs might be concentration dependent, i.e. would be significant only at concentrations larger than 10 nM. Although attractive, this hypothesis is very difficult to test in our system (especially on miniature IPSCs) because at concentrations $\geqslant$ 10 nM, we frequently observed an important increase in membrane noise associated with a dose-dependent inward current at −60 mV, an effect probably due to direct activation of $GABA_A$ receptors by allopregnanolone (Harrison *et al.* 1987; Majewska, 1992; Lambert *et al.* 1995). Under these conditions, most mIPSCs were masked by the membrane noise and were therefore impossible to analyse.

Allopregnanolone potently and reversibly increased the frequency of sIPSCs, indicating a facilitatory presynaptic effect on the release of GABA. This stimulatory effect was also observed in the presence of TTX (0·5 μM). However, there was a major difference between the results obtained in the two experimental situations. In the absence of TTX, the mean amplitude of sIPSCs was augmented in the presence of allopregnanolone, a phenomenon explained by the occurrence of a higher proportion of larger sized sIPSCs. In the presence of TTX, allopregnanolone still increased the frequency of sIPSCs (mIPSCs), but did not modify the mean amplitude of the synaptic currents. Therefore it was likely that the appearance of larger sized IPSCs depended in part on a TTX-sensitive mechanism. The simplest explanation for this observation would be that allopregnanolone induced the firing of action potentials in the presynaptic neurone in the absence of TTX. Although this possibility cannot be completely excluded, it is important to note that a high proportion of the sIPSCS induced by allopregnanolone in the absence of TTX had amplitudes of between 50 and 100 pA (see Fig. 2*B*), whereas eeIPSCs are usually always

larger than 100 pA in these cells (see Figs 2*C*, 4*B* and 5*C* and Poisbeau *et al.* 1996). This observation might indicate the existence of an additional local mechanism relying on the presence of functional TTX-sensitive Na^+ channels, but which does not depend on the generation of an action potential. One explanation could be that TTX-sensitive Na^+ channels are present close to the site where allopregnanolone depolarizes the synaptic terminal via the activation of presynaptic 'depolarizing' $GABA_A$ receptors. The role of the TTX-sensitive Na^+ channels could be to sustain (or even to amplify) this allopregnanolone-induced depolarization. A $GABA_A$ receptor-mediated membrane depolarization is likely to occur in hypothalamic neurones since it has been shown recently that allopregnanolone induced a dose-dependent increase of the intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) in these neurones by a mechanism involving Ca^{2+} influx (Dayanithi & Tapia-Arancibia, 1996). This effect of allopregnanolone had an EC_{50} value of 10 nM and was blocked by bicuculline and picrotoxin, suggesting that it was due to a $GABA_A$ receptor-mediated membrane depolarization. Moreover, our results are consistent with the existence of such a mechanism at the level of the synaptic terminals of hypothalamic neurones, since allopregnanolone stimulated the frequency of mIPSCs recorded in the presence of TTX, a condition in which propagating action potentials are blocked. Such a phenomenon could account for the increase in frequency of sIPSCs as well as that in the proportion of larger sized sIPSCs observed during the application of allopregnanolone in the absence of TTX. The latter phenomenon could result from an increase in the basal level of $[Ca^{2+}]_i$ induced by allopregnanolone and reflect a facilitation of transmitter release, leading to a higher probability of summation of individual mIPSCs that arise at different release sites. This is also suggested by the observation that allopregnanolone suppressed the failures of transmission during electrical stimulation experiments.

Effect of pregnenolone sulphate

At a concentration of 10 μM, PS blocked GABAergic IPSCs progressively and completely in a reversible manner, but only partially blocked I_{GABA}, even at a non-saturating concentration of GABA. A common feature of the effect of PS on IPSCs and I_{GABA} was a marked decrease in the decay time constants of the currents, a phenomenon that was always observed for IPSCs before complete block (see Fig. 3). Therefore this acceleration of the decaying phase seemed to reflect a purely postsynaptic effect and is consistent with previously described actions of PS on single $GABA_A$ channels, including a decrease in channel opening frequency (Mienville & Vicini, 1989), a reduction in the occurrence of long-lasting openings and a favouring of fast desensitized states (Twyman & MacDonald, 1992; MacDonald & Olsen, 1994). It must, however, be emphasized that PS had no apparent effect on the amplitude of I_{GABA} at concentrations between 10 nM and 1 μM, whereas at a concentration of 10 μM, PS blocked 80% of the GABA response.

Nevertheless, the fact that PS blocked IPSCs completely but only partially blocked I_{GABA} induced by saturating concentrations of GABA suggested the existence of a presynaptic action of PS. Indeed, PS has been shown to inhibit voltage-dependent N- and L-type Ca^{2+} channels in CA1 hippocampal neurones at low micromolar concentrations by a mechanism involving a pertussis toxin-sensitive pathway (ffrench-Mullen, Danks & Spence, 1994). It is not known if such an inhibitory effect of PS on voltage-dependent Ca^{2+} currents also exists in hypothalamic neurones projecting onto IL cells. However, if this was the case, it could account for the additional reduction (i.e. total blockade) of IPSCs compared with its partial blocking effect on I_{GABA} in our coculture system.

Physiological significance

The intermediate lobe of the pituitary is composed of a relatively homogeneous population of endocrine cells (melanotrophs) that secrete α-melanocyte-stimulating hormone (α-MSH). IL cells are innervated by hypothalamic GABAergic neurones (Oertel *et al.* 1982; Vincent, Hökfelt & Wu, 1982) and the secretion of α-MSH is inhibited by GABA (Tomiko, Taraskevitch & Douglas, 1983; Taraskevitch & Douglas, 1985). Therefore factors modulating the efficiency of GABAergic synaptic input to IL cells will also influence the tonic inhibitory control exerted by GABA on the electrical and secretory activities of these endocrine cells. Our results indicate that neurosteroids can regulate negatively (PS) or positively (allopregnanolone) GABAergic synaptic transmission between hypothalamic neurones and IL cells. Of course, an important issue is to know if such modulations are likely to occur *in vivo*. In the hypothalamo-hypophyseal system, synthesis and secretion of allopregnanolone has been demonstrated in the neuro-intermediate lobe of the pituitary (Berman, 1994). It has been estimated that in this structure, the synthetic activity is such that allopregnanolone can reach a local concentration of about 100 nM within a few minutes (Berman, 1994). According to our data, such concentrations would be sufficient to induce a marked potentiation of the GABAergic transmission. Moreover, our results suggest that at the synaptic level, allopregnanolone had a prominent presynaptic effect in facilitating the release of GABA and the summation of unitary IPSCs. At low concentrations, the overall effect of allopregnanolone is probably dominated by this presynaptic component, since it appears that in our system the concentrations of GABA reached in the synaptic cleft are saturating and that under these conditions allopregnanolone cannot further increase the amplitude of individual unitary IPSCs by potentiating postsynaptic $GABA_A$ receptors. However, at concentrations $\geqslant$ 10 nM, we noticed the appearance of a non-inactivating membrane current due to the direct activation of $GABA_A$ receptors by allopregnanolone. This postsynaptic current could contribute significantly to tonic inhibitory control of IL cells mediated by the activation of $GABA_A$ receptors. Consistent with such a possibility, it has been shown that pregnanolone

significantly potentiates the GABAergic inhibition of action potential discharge in spontaneously firing frog IL cells (Le Foll, Louiset, Vaudry & Cazin, 1997).

Unfortunately it is not known if pregnenolone sulphate is also produced in the neurointermediate lobe of the pituitary and in sufficient amounts to represent a significant negative regulator of this GABAergic transmission. Although the effect of PS appears to be relatively dose independent, if the concentration of PS in the vincinity of a GABAergic synapse was to reach 1 μM, one could expect an acceleration of the decaying phase of the IPSCs, and at concentrations close to 10 μM an additional reduction in amplitude of the IPSCs.

Finally, since GABA is a major inhibitory neurotransmitter in the CNS, and since neurosteroids are synthesized in the brain (Paul & Purdy, 1992; Robel & Beaulieu, 1994; Lambert *et al.* 1995), it is likely that similar modulations of GABAergic synaptic transmission by steroids occur in various areas of the central nervous system and our hypothalamo-hypophyseal coculture system could therefore represent a useful model for the detailed study of pre- and postsynaptic actions of neuroactive steroids at central synapses.

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