Functional changes in GABA_A receptor stimulation during the oestrous cycle of the rat

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1 Slices of rat cuneate nucleus were used to study whether or not gonadal steroids influence the γ -aminobutyric acid (GABA) system *in vivo*. Females in different stages of the oestrous cycle as well as steroid-treated (oestrogen, progesterone or both) ovariectomized animals were used.

2 Functional changes in the GABA_A receptors were assayed using the effects of potentiators (benzodiazepine, barbiturate) and antagonists (picrotoxin) on the muscimol control dose-response curves.

3 The potentiating effect of the benzodiazepine, flurazepam was unchanged during the oestrous cycle, and the hormone treatments did not alter this effect.

4 During oestrus, an increase was seen in the potentiating effect of the barbiturate (pentobarbitone). This suggests a synergistic effect between barbiturates and gonadal steroids. Progesterone treatment also increased the effect of pentobarbitone.

5 The antagonistic action of picrotoxin was unaffected during the oestrous cycle. However, progesterone (or progesterone and oestrogen) treatment reduced the potency of picrotoxin.

6 This study supports the idea that endogenous steroids (presumably progesterone) affect the GABA_A receptors during the oestrous cycle by a mechanism associated with the barbiturate site of the GABA_A receptor complex.

Keywords: GABA_A-receptors; muscimol; flurazepam; pentobarbitone; oestrous cycle of the rat; progesterone; oestrogen

Introduction

The inhibitory neurotransmitter, γ -aminobutyric acid (GABA) is recognised by at least two different receptors (GABA_A and GABA_B receptors). Stimulation of the GABA_A receptor evokes an increase in membrane chloride conductance. This effect generally leads to membrane hyperpolarization. However, presynaptic inhibition is associated with a depolarizing phenomenon, primary afferent depolarization (PAD) (see Levy, 1980 for review).

The GABA_A receptor forms a complex with different binding sites for benzodiazepines, barbiturates and other related compounds (Olsen, 1982). Numerous candidates with different chemical structures have been proposed as endogenous modulators of the GABA_A receptor complex, for instance polypeptides (diazepam binding inhibitor, Guidotti *et al.*, 1983) and β -carbolines (Braestrup *et al.*, 1983). Steroid modulation of the GABA_A receptor complex has also been shown (Simmonds & Turner, 1987; Majewska, 1987; Lambert *et al.*, 1987; Gee *et al.*, 1988).

Progesterone, because of its anticonvulsive (Bäckström, 1976; Landgren *et al.*, 1987) and anaesthetic (Seyle, 1942; Meyerson, 1967; Norberg *et al.*, 1987) properties, is of particular interest in this context. The central nervous system depression brought about by progesterone is thought to be mediated by metabolites, e.g. 3 α -hydroxy pregnane-20-one (Majewska *et al.*, 1986; Simmonds & Turner, 1987; Norberg *et al.*, 1987) acting on GABA_A receptors. It has been shown by *in vitro* studies that the effect of steroids on the GABA_A receptor complex is similar to that of barbiturate stimulation (Harrison & Simmonds, 1984; Majewska *et al.*, 1986; Simmonds & Turner, 1987; Lambert *et al.*, 1987; Turner & Simmonds, 1989). Picrotoxin antagonizes the depolarization by blocking the barbiturate site (Simmonds, 1981). Steroids have the ability to reduce the inhibition induced by picrotoxin (Simmonds *et al.*, 1984). The effect of certain pregnane steroids may also involve the GABA/benzodiazepine Cl⁻ ionophore (Simmonds & Turner, 1987; Majewska, 1987; Lambert *et al.*, 1987). The mechanism by which steroids interact with the

GABA_A receptor complex is not fully understood. However, taken together the *in vitro* studies, the documentation of progesterone-related changes in mood and altered central neuronal excitability, all suggest that central nervous GABAergic transmission is likely to be influenced by steroids under physiological and pathophysiological conditions. To explore this question further under controlled experimental conditions, we used female rats at different stages of the oestrous cycle. Hormonal treatment (oestrogen, progesterone or both) was also given to ovariectomized animals to investigate the role of progesterone alone and after oestrogen priming. Functional changes in the GABA_A receptor complex were investigated by the cuneate-slice method previously described by Simmonds (1978; 1980; 1981). GABA and GABA_A receptor agonists (e.g. muscimol) depolarize afferent nerves (PAD) in the cuneate nucleus slice. This effect is potentiated by benzodiazepines (e.g. flurazepam) and barbiturates (e.g. pentobarbitone).

Methods

Female Sprague-Dawley rats (specified pathogen-free, purchased from A-lab, Sollentuna, Sweden) weighing 336 $\pm$ 6.9 g were kept under a reversed day-night cycle (12/12 h, with the light off between 09 h 00 min and 21 h 00 min). They had free access to water and commercial food pellets.

One group of animals was ovariectomized under pentobarbitone anaesthesia (about 60 mg kg⁻¹, i.p.). The other group consisted of unoperated females. The animals were used in the experiment 1 to 2 months after surgery.

The cuneate slice method

To obtain a functional measurement of the GABA_A receptor complex, the cuneate nucleus slice preparation described by Simmonds (1978; 1980; 1981) was used. The animals were stunned and decapitated at a low cervical level with a guillotine. An incision was made at the level of obex, the medulla oblongata was then placed in ice-cold Krebs medium. A razor

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blade was used to obtain two slices (≈ 0.6 – 0.8 mm thick) containing the dorsal funiculus and cuneate nucleus of each side. The slices were placed in a two-compartment bath so that the cuneate nucleus was in one compartment and the dorsal funiculus projected through a greased slot to the other compartment. The direct current between the two compartments was recorded continuously on a chart recorder with Ag/AgCl electrodes. Both compartments were perfused with Krebs medium, but drugs were added only to the cuneate nucleus compartment.

Two slices were taken from each animal, and the effects of flurazepam and pentobarbitone were tested on one slice (slice a) and the effects of two doses of picrotoxin on the other slice (slice b) (Lindgren & Simmonds, 1987). The drug-induced peaks were measured at their highest amplitude.

Muscimol was used routinely as an agonist (Simmonds, 1980), dissolved in the Krebs medium and the cuneate nucleus was perfused for 2 min. The control doses were $2.5 \mu\text{M}$, $5 \mu\text{M}$ and $10 \mu\text{M}$ muscimol, these doses having been shown to be in the lower part of the dose-response curve in this preparation by Simmonds (1980), thus reducing desensitization of the GABA_A receptor complex. During picrotoxin treatment the doses of muscimol were increased up to $20 \mu\text{M}$ ($3 \mu\text{M}$ picrotoxin) and $80 \mu\text{M}$ ($30 \mu\text{M}$ picrotoxin), to obtain approximately the same response as the control values. Picrotoxin ($3 \mu\text{M}$ and $30 \mu\text{M}$), flurazepam ($1 \mu\text{M}$) and pentobarbitone ($10 \mu\text{M}$) were all dissolved in the Krebs medium and perfused for 30 min before and during the redetermination of the muscimol dose-response curves. During flurazepam or pentobarbitone perfusion the doses of muscimol were decreased to $1.25 \mu\text{M}$, $2.5 \mu\text{M}$ and $5 \mu\text{M}$. All these doses gave responses in the lower part of the dose-response curves. A recovery time of about 20 min was allowed between each dose given. To assess any non specific changes in the responsiveness of the slices, responses to superfusion with an additional 3.9 mM KCl (for 5 min) were monitored regularly. All experiments were performed at room temperature (≈ 18 – 22°C).

The potentiation or inhibition by drug-perfusion was measured as the leftward or rightward shift of the log dose-response curves. Parallel shifts of the log dose-response curves (Figure 2) allowed dose-ratios (log units) to be calculated. Dose-ratios were calculated from the intersections of the dose-response curves with a horizontal line (Simmonds, 1980). Only those experiments where the dose-curves were shifted in an approximately parallel fashion were used.

Steroid treatment

Vaginal smears were taken daily by the lavage technique (between 08 h 00 min and 09 h 00 min) to determine the various stages of the oestrous cycle (dioestrus, prooestrus, oestrus) in intact females.

Ovariectomized rats were either not treated or treated with progesterone (1 mg per animal, s.c. 3–3.5 h before they were killed), oestradiol benzoate ($25 \mu\text{g kg}^{-1}$, s.c. 52 h before they were killed) or oestradiol benzoate followed 48 h later by progesterone (dose as above). The hormonal treatments were chosen with regard to doses which are known to induce a central nervous response, e.g. to activate female copulatory behaviour (lordosis response) (Meyerson, 1967).

Drugs

The following drugs were used: muscimol (Sigma, St. Louis, U.S.A.), pentobarbitone sodium salt (Apoteksbolaget, Sweden), flurazepam (Roche), picrotoxin (EGA-Chemie) isethionic acid, oestradiol benzoate and progesterone (Sigma, St. Louis, U.S.A.). The steroids were dissolved in olive oil. The Krebs medium contained (mM): NaCl 118, KCl 2.1, KH_2PO_4 0.93, CaCl_2 2.5, MgSO_4 2.2, NaHCO_3 25 and glucose 11, and a mixture of 93.5% O_2 and 6.5% CO_2 was bubbled through it.

Statistical methods

All values are means $\pm$ s.e.mean; statistical comparisons were made by analysis of variance (ANOVA) followed by Tukey HSD multiple comparisons test. The levels of significance were set at $P < 0.05$ and $P < 0.01$.

Results

Cl^- dependence

In order to assess the Cl^- dependence of the method in our hands, Cl^- in the Krebs medium was replaced by isethionate. The depolarization induced by $10 \mu\text{M}$ muscimol was determined after 40%, 60% and 80% reduction of the Cl^- concentration. When 80% of the Cl^- was substituted, a significant decrease in depolarization was seen (Figure 1), thus establishing the Cl^- dependence of the cuneate method.

Response to K^+

Regularly, or at least at the beginning and end of an experiment, the response to K^+ was measured (raised to 6 mM in the Krebs medium). This K^+ -induced depolarization was used to test the condition of the slice. Only preparations which gave at least a 0.3 mV response to the first application of KCl were used and subsequent experimental data were accepted only if the response to KCl at the end of the experiment (6–8 h) was not less than 80% of the initial response. The KCl-induced depolarizations were in slice (a); before experiment: $0.42 \text{ mV} \pm 0.028$ ($n = 56$), after experiment: $0.39 \text{ mV} \pm 0.028$ ($n = 56$), and in slice (b); before experiment: $0.37 \text{ mV} \pm 0.033$ ($n = 46$), after experiment: $0.36 \text{ mV} \pm 0.027$ ($n = 46$).

Effect of oestrous cycle on the GABA_A system

Muscimol produced a concentration-related depolarization of the dorsal funiculus fibres in the cuneate slice ($10 \mu\text{M}$ muscimol: $0.47 \text{ mV} \pm 0.06$, $5 \mu\text{M}$ muscimol: $0.32 \text{ mV} \pm 0.04$, $2.5 \mu\text{M}$ muscimol: $0.20 \text{ mV} \pm 0.03$). The recovery time was about 20 min, with a slower recovery for the highest dose (Figure 2).

Flurazepam When flurazepam ($1 \mu\text{M}$) was perfused, a parallel shift to the left in the muscimol dose-response curve was seen (Figure 2).

During the oestrous cycle, no significant change (Table 1) (ANOVA; $F = 2.43$, $P > 0.11$) was seen of the potentiation of muscimol by flurazepam.

Figure 1 Reduction of responses to $10 \mu\text{M}$ muscimol in rat cuneate nucleus slice by replacing 20%, 40% and 80% of the Cl^- concentration with isethionate. The values are expressed as a percentage (of muscimol-induced depolarization) with s.e.mean shown by vertical bars ($n = 3$ – 5). Statistical significance (compared with the 100% value) was calculated by Student's t test (* $P < 0.05$).

Figure 2 Original tracings of responses to 2 min applications of muscimol and potentiation by pentobarbitone 10 μM . (a) Note that, the tracings run from the right to the left. The recovery time was about 20 min after each given dose. (b) Effects of flurazepam 1 μM (○) and pentobarbitone 10 μM (□) on log dose-response curves for muscimol (data from a); control (●). The results represents a single experiment from one animal and each point is a single response. The approximately parallel shift represents a 0.27 log unit shift (flurazepam) and a 0.40 log unit shift (pentobarbitone).

Pentobarbitone Pentobarbitone (10 μM) caused a greater leftward shift of the muscimol dose-response curve than flurazepam (Figure 2).

The pentobarbitone stimulation was significantly increased during the oestrous cycle (ANOVA; $F = 6.23$, $P < 0.01$). When animals were in oestrus, pentobarbitone caused a significantly greater potentiation of muscimol than when they were in dioestrus (Table 1).

Picrotoxin Upon exposure to picrotoxin, an approximately parallel shift to the right in the muscimol dose-response curve was obtained, this antagonism was dose-dependent (Table 1).

Animals in different stages of the oestrous cycle showed no changes in the antagonism of muscimol by picrotoxin (ANOVA; $F = 0.28$, $P > 0.76$ and $F = 0.39$, $P > 0.68$ for 3 μM and 30 μM).

Effects of hormone treatment on the GABA_A system

In order to establish the role of different hormones in the potentiation of muscimol, female rats were ovariectomized and some were given oestrogen, progesterone or both. The hormones were injected 1 to 2 months after surgery in a single dose.

Flurazepam The addition of flurazepam to the medium perfusing slices from ovariectomized, untreated rats caused a leftward shift of the muscimol dose-response curve, similar to that in slices from unoperated rats (Table 2).

The hormone treatments did not significantly alter the effect of flurazepam (ANOVA; $F = 0.85$, $P > 0.48$). Comparison between the oestrous cycle and hormone treatments showed a difference between oestrus and ovariectomized animals (0.25 ± 0.029 log unit vs 0.14 ± 0.026 log unit, respectively, $t = 2.40$, $P < 0.05$).

Table 1 Potentiation of effects of muscimol by flurazepam pentobarbitone and antagonism by picrotoxin on cuneate slices during the oestrous cycle in rats

Oestrous cycle	Fluraz (1 μM)	Pentobarb (10 μM)	Picrot (3 μM)	Picrot (30 μM)
Dioestrous	0.17 (8) ± 0.027	0.26 (8) ± 0.031	0.54 (7) ± 0.089	1.03 (7) ± 0.068
Prooestrous	0.24 (7) ± 0.036	0.30 (7) ± 0.038	0.51 (7) ± 0.028	1.09 (7) ± 0.027
Oestrous	0.26 (8) ± 0.029	0.41 (8)** ± 0.037	0.57 (6) ± 0.080	1.04 (6) ± 0.089

The potentiation or antagonism of muscimol was measured as lateral shifts (log units) of the muscimol dose-response curve (see Methods). The values are means $\pm$ s.e.mean, (n). Statistical significance (compared to dioestrous) was calculated by ANOVA followed by Tukey HSD test (** $P < 0.01$).

Table 2 Potentiation of muscimol by flurazepam, pentobarbitone and antagonism by picrotoxin in cuneate slices from ovariectomized and ovariectomized and hormone-treated rats

Hormone treatment	Fluraz (1 μM)	Pentobarb (10 μM)	Picrot (3 μM)	Picrot (30 μM)
Ovariectomized	0.14 (9) ± 0.026	0.22 (9) ± 0.040	0.62 (6) ± 0.039	1.21 (6) ± 0.036
Ovariectomized + oestrogen	0.17 (7) ± 0.027	0.29 (7) ± 0.033	0.50 (5) ± 0.082	1.11 (5) ± 0.074
Ovariectomized + progesterone	0.20 (9) ± 0.037	0.36 (9)* ± 0.052	0.37 (8)** ± 0.053	0.93 (8)* ± 0.084
Ovariectomized + oest and prog	0.20 (8) ± 0.033	0.34 (8)* ± 0.025	0.46 (7)* ± 0.048	0.98 (7)* ± 0.063

Effects of oestrogen (oest; 25 $\mu\text{g kg}^{-1}$, s.c., 3–3.5 hours before rats were killed), progesterone (prog; 1 mg/animal, s.c., 48 h before rats were killed) or oestrogen and progesterone on the potentiation of muscimol by flurazepam (Fluraz; 1 μM), pentobarbitone (Pentobarb; 10 μM) or antagonism of muscimol by picrotoxin (Picrot; 3 μM , 30 μM) in cuneate nucleus slices from ovariectomized rats. The potentiation or antagonism was measured as lateral shifts (log units) of the muscimol dose-response curve (see Methods). The values are means $\pm$ s.e.mean, (n). Statistical significances (compared to ovariectomized) were calculated by ANOVA followed by Tukey HSD test (* $P < 0.05$, ** $P < 0.01$).

Pentobarbitone In slices from ovariectomized, untreated rats, pentobarbitone shifted the muscimol dose-response curve to the left (Table 2). The potentiation induced by pentobarbitone was greater than that of flurazepam.

In comparing the hormone treatments, there was a tendency towards a difference (ANOVA; $F = 2.81$, $P < 0.057$). Treatment with progesterone or oestrogen combined with progesterone significantly increased the potentiation due to pentobarbitone of the muscimol-induced depolarization of the dorsal funiculus.

Comparison between the oestrous cycle and hormone treatments showed a difference between oestrus and ovariectomized animals (0.41 ± 0.037 log unit vs 0.22 ± 0.040 log unit, respectively, $t = 3.21$, $P < 0.01$).

Picrotoxin Antagonism of the muscimol response by picrotoxin at $3 \mu\text{M}$ and $30 \mu\text{M}$ differed with the different hormone treatments (ANOVA; $F = 3.48$, $P < 0.03$, $F = 3.20$, $P < 0.04$ for $3 \mu\text{M}$ and $30 \mu\text{M}$, respectively).

Progesterone reduced the antagonism of picrotoxin; this was seen with both doses of picrotoxin (Table 2). The combined treatment with oestrogen and progesterone also diminished the antagonism of picrotoxin on muscimol-induced depolarization.

Discussion

The present study addresses the question of whether or not gonadal steroids influence the GABA system *in vivo*. In order to measure functional GABA_A receptors, the cuneate slice method was used (Simmonds, 1978; 1980; 1981). When GABA or GABA agonists are perfused over the cuneate nucleus, a depolarization is produced which is associated with presynaptic inhibition (Schmidt, 1971).

An important aspect of this study was to establish the cuneate slice method in female rats. It was shown that flurazepam, pentobarbitone and picrotoxin had the same modulatory effect in ovariectomized and dioestrus animals as in male rats.

The chloride-dependence of the method was determined by reducing the Cl^- concentration in the Krebs medium. The muscimol-induced depolarization decreased with lower (20%) concentrations of chloride. It has been shown by Simmonds (1978) that replacing 80% of the Cl^- in the medium reduced GABA depolarization. This was confirmed in our pre-experimental study. These results emphasise the role of chloride in the depolarization of fibres of the dorsal funiculus.

GABA is subject to uptake processes. This problem was overcome in the present study by use of the agonist muscimol which is not a good substrate for GABA uptake processes (Simmonds, 1980); also, muscimol is more potent than GABA in the cuneate nucleus slice (Pickles & Simmonds, 1980). In *in vitro* studies both steroids (Simmonds *et al.*, 1984) and barbiturates (Harrison & Simmonds, 1983) reduce the potency of picrotoxin as an antagonist at the GABA_A receptor complex. It was therefore also considered important to study the inhibitory effect of picrotoxin in animals under different hormonal *in vivo* conditions.

When flurazepam was added to the perfusion medium there was a shift to the left of the muscimol dose-response curve (two fold) which corresponded well with the results obtained by Simmonds (1981). The potentiating effect of flurazepam was unchanged during the oestrous cycle, nor did the hormone treatments have any effect. This demonstrates that neither oestrogen nor progesterone plays a role in the potentiation of the GABA_A receptor complex brought about by benzodiazepines.

Pentobarbitone caused a greater modulation than flurazepam, the effect being about a 3 fold shift to the left of the muscimol dose-response curve. During oestrus this effect increased to a 4 fold shift, suggesting a synergistic effect between pentobarbitone and the hormone levels during oestrus. In the female rat, serum levels of progesterone rise to

a single peak (from about 25 ng ml^{-1} to about 150 ng ml^{-1}), whereas oestradiol levels in serum show a more irregular pattern with more numerous peaks (from about 50 pg ml^{-1} to about 250 pg ml^{-1}) during oestrus (Södersten *et al.*, 1981). It has been proposed that steroid hormones potentiate the GABA_A receptor complex in a barbiturate-like manner *in vitro* (Harrison & Simmonds, 1984; Majewska *et al.*, 1986; Simmonds & Turner, 1987; Lambert *et al.*, 1987; Turner & Simmonds, 1989). Majewska and colleagues (1989) have recently shown that pregnancy and the postpartum period alter the binding sensitivity of brain GABA_A receptors to muscimol. The authors suggest that this effect is a result of the action of certain endogenous steroids. By using voltage-clamp recordings from bovine isolated chromaffin cells, Cottrell *et al.* (1987) could show that the steroid anaesthetic, alphaxalone, activated GABA_A receptors in a barbiturate-like manner. Gee *et al.* (1988) studying steroid structure-activity relationships suggest, however, that the steroids and anaesthetic barbiturates do not share a common site of action. Although we do not suggest identical mechanisms of action our results do indicate that the alterations in steroid levels during the oestrous cycle affect the GABA_A receptor by a mechanism associated with the barbiturate site of the GABA_A receptor complex.

The treatment with oestrogen, progesterone or a combination of both given to ovariectomized rats had no effect on the flurazepam-induced potentiation of muscimol. However, the pentobarbitone-induced potentiation of muscimol was significantly increased when progesterone or progesterone and oestrogen were given. This further supports the hypothesis that progesterone acts in a synergistic manner in association with a barbiturate-like mechanism. This investigation cannot answer the question of whether it is progesterone itself or metabolites of the hormone that are acting on the GABA_A receptor complex. However, as the potentiation of the muscimol effect was seen 3 to 3.5 h after the steroid injection (but not after 5 h, Westerling *et al.* unpublished data) and the effect is presumably mediated through membrane mechanisms, it is reasonable to assume that progesterone metabolites are active and not the hormone itself. However, we cannot exclude genomic activation giving rise to products which are responsible for the potentiation of the muscimol stimulation. If progesterone acts directly at the membrane, the effect seems to be a long-lasting modulation of the GABA_A receptor complex.

Pentobarbitone and picrotoxin have been shown to have reciprocal actions on single GABA_A receptor channels (Twyman *et al.*, 1989). In our study picrotoxin inhibition became less pronounced with progesterone or progesterone and oestrogen treatments. This further supports the idea that progesterone acts at or close to the same site as barbiturates on the GABA_A receptor complex. However, during the oestrous cycle no effect on the picrotoxin inhibition could be seen. This might be due to differences in efficiency between the effects of exogenous and endogenous hormones. However, endogenous hormones seem to have a greater effect on pentobarbitone dose-response shifts. This disparity needs further investigation.

In our study, effects of *in vivo* manipulation could be seen in an *in vitro* assay. Majewska and colleagues (1985) showed that adrenalectomy reduced muscimol binding in certain brain areas *in vitro*. The authors suggest that glucocorticoids act directly at the membrane level to alter the affinity of GABA_A receptors.

We conclude that there is a functional change in the GABA_A receptor complex during the oestrous cycle due to the actions of progesterone and/or its metabolites. The results further suggest an association of the steroid action with the barbiturate site of the GABA_A receptor complex.

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