British Journal of Pharmacology (1996) 118, 839-846 © 1996 Stockton Press All rights reserved 0007-1188/96 $12.00

Functional correlation between allopregnanolone and [^{35}S]-TBPS binding in the brain of rats exposed to isoniazid, pentylenetetrazol or stress

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1 The relation between changes in the cerebral cortical concentration of allopregnanolone and γ-aminobutyric acid (GABA) type A receptor function after intracerebroventricular injection of this neurosteroid was investigated in male rats.

2 Intracerebroventricular administration of allopregnanolone (1.25 to 15 μg) produced a maximal increase (100 fold at the highest dose) in cortical allopregnanolone concentration within 5 min; the concentration remained significantly increased at 15 and 30 min, before returning to control values by 60 min.

3 The same treatment induced a rapid and dose-dependent decrease in the binding of *t*-[^{35}S]-butylbicyclophosphorothionate ([^{35}S]-TBPS) to cerebral cortical membranes measured *ex vivo*, an effect mimicked by the benzodiazepine midazolam but not by the 3β-hydroxyepimer of allopregnanolone. The time course of changes in [^{35}S]-TBPS binding paralleled that of brain allopregnanolone concentration.

4 In a dose-dependent manner, allopregnanolone both delayed the onset of convulsions and inhibited the increase in [^{35}S]-TBPS binding to cortical membranes induced by isoniazid. The potency of allopregnanolone in inhibiting [^{35}S]-TBPS binding in isoniazid-treated rats was approximately four times that in control animals.

5 The ability of allopregnanolone to decrease [^{35}S]-TBPS binding in isoniazid-treated rats also correlated with its anticonvulsant activity against pentylenetetrazol-induced seizures as well as its inhibitory effect on the increase in [^{35}S]-TBPS binding induced by foot shock.

6 The results indicate that the *in vivo* administration of allopregnanolone enhances the function of $GABA_A$ receptors in rat cerebral cortex and antagonizes the inhibitory action of stress and drugs that reduce GABAergic transmission.

Keywords: $GABA_A$ receptor; neurosteroid; allopregnanolone; [^{35}S]-TBPS binding; isoniazid; pentylenetetrazol; stress; seizures; brain cortex

Introduction

Our understanding of the mechanism of action of endogenous neuroactive steroid derivatives was greatly increased by the observation that 3α-hydroxy-5α-pregnane-20-one (allopregnanolone) and 3α,21-dihydroxy-5α-pregnane-20-one (tetrahydrodeoxycorticosterone, or 5α-THDOC) allosterically enhance the function of γ-aminobutyric acid type A ($GABA_A$) receptors *in vitro* with a potency and efficacy similar to or greater than those of classical anxiolytic, hypnotic, and anticonvulsant drugs (Majewska *et al.*, 1986; Harrison *et al.*, 1987; Majewska, 1992; Lambert *et al.*, 1995).

These neurosteroids increase the permeability of the $GABA_A$ receptor-associated Cl^- channel, as revealed by an increase in GABA-induced uptake of $^{36}Cl^-$ into rat isolated brain vesicles or cultured neurones (Majewska *et al.*, 1986; Turner *et al.*, 1989; Im *et al.*, 1990) as well as by a decrease in t-[^{35}S]-butylbicyclophosphorothionate ([^{35}S]-TBPS) binding in brain homogenates (Majewska *et al*, 1986; Gee *et al.*, 1988; Turner *et al.*, 1989), effects mimicked by positive modulators of GABAergic transmission and opposite to those elicited by GABA antagonists (Gee *et al.*, 1986; Obata & Yamamura, 1986; Biggio *et al.*, 1990). The application of patch- and voltage-clamp techniques to cultured cells further demonstrated that neurosteroid modulation of $GABA_A$ receptor function results from a direct activation of the $GABA_A$ receptor complex in addition to an allosteric enhancement of GABA-evoked currents (Barker *et al.*, 1986; Harrison *et al.*, 1987; Lambert *et al.*, 1990; Puia *et al.*, 1990; Hill-Venning *et al.*, 1994). These *in vitro* observations are supported by *in vivo* data showing that the predominant pharmacological effects produced by these neurosteroids are similar to those of drugs (benzodiazepines, barbiturates, general anaesthetics) that enhance the activity of the GABA-gated Cl^- channel (Harrison & Simmonds, 1984; Holzbauerr *et al.*, 1985; Crawley *et al.*, 1986; Belelli *et al.*, 1990; Bitran *et al.*, 1991a; 1993; Wieland *et al.*, 1991; Kokate *et al.*, 1994; Lambert *et al.*, 1995).

We have now investigated whether there is a functional correlation between changes in the activity of the GABA-dependent Cl^- channel and the brain concentration of allopregnanolone after *in vivo* administration of this neurosteroid to rats. $GABA_A$ receptor function was assessed by measuring *ex vivo* the binding of [^{35}S]-TBPS to cerebral cortical membranes of rats administered allopregnanolone by intraventricular injection. We have previously shown that such binding of [^{35}S]-TBPS to specific recognition sites associated with the GABA-dependent Cl^- channel is the best available biochemical parameter for revealing changes in the activity of GABAergic synapses induced *in vivo* either by the adminstra-

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tion of drug or by exposure of the animals to specific environmental conditions (Biggio *et al.*, 1990; Concas *et al.*, 1990). Thus, we have shown that stressful stimuli such as handling, mild foot-shock or CO_2 inhalation, or drugs such as benzodiazepine receptor inverse agonists, isoniazid, and other negative modulators of $GABA_A$ receptor function, increase [^{35}S]-TBPS binding in rat cerebral cortex and other brain areas in a dose-dependent manner, an effect opposite to that induced by administration of benzodiazepines and GABA agonists (Concas *et al.*, 1988; 1990; 1993; Serra *et al.*, 1989b; 1992; 1994; Biggio *et al.*, 1990; Sanna *et al.*, 1992).

With this biochemical model, we have now shown that allopregnanolone enhances $GABA_A$-receptor-coupled Cl^- channel function in a dose- and time-dependent manner and, like benzodiazepines, antagonizes the inhibitory effects of isoniazid, pentylenetetrazol and stress on central GABAergic transmission. Moreover, by measuring the time-dependent changes in allopregnanolone concentration in the brain, we established the range of *in vivo* concentrations over which the neurosteroid is active at $GABA_A$ receptors. Indeed, the time courses of the *ex vivo* binding of [^{35}S]-TBPS and the concentration of allopregnanolone in the cerebral cortex after intracerebroventricular administration of the neurosteroid are consistent with a functional relation between allopregnanolone concentration and $GABA_A$ receptor function.

Methods

Animals

Male Sprague-Dawley rats (Charles River, Como, Italy) with body masses of 175 to 200 g were studied. After arrival at the animal facility, rats were acclimatized to the new housing conditions for at least 1 week. Animals were housed six per cage under an artificial 12-h-light, 12-h-dark cycle (light from 08 h 00 min to 20 h 00 min) at a constant temperature of 22 ± 2°C and a relative humidity of 65%. They had free access to water and standard laboratory food throughout the entire experimental period.

Intracerebroventricular drug administration

Two days before the experiment, rats were anaesthetized with chloral hydrate (400 mg per kilogram of body mass i.p.), placed in a stereotaxic apparatus, and implanted with a stainless steel guide cannula that was fixed to the skull (AP −8.0, ML −1.2, DV −2.5, according to the Paxinos atlas) and kept open with a stainless steel stylet. On the day of the experiment, the stylet was gently removed and replaced with an injector (outer diameter, 0.35 mm), which produced 2.0 mm from the guide cannula in order to reach the lateral ventricle. Placement of the cannula in the lateral ventricle was verified in several of the animals by dye injection.

Allopregnanolone, $3\beta,5\alpha$-pregnanolone, and midazolam were dissolved in 45% 2-hydroxypropyl-β-cyclodestrin by sonication for 4 h and injected into the lateral ventricle through the injector, which was connected by polyethylene tubing to a 5-μl Hamilton microsyringe mounted on a perfusion pump that was set at an injection rate of 5 μl min^{-1}. Control rats received an equal volume of vehicle.

Measurement of brain allopregnanolone

Rats were killed at various times (5 to 60 min) after intracerebroventricular injection of allopregnanolone either by guillotine, for plasma analysis, or by focused microwave irradiation (4 s) of the head, for brain measurement. This procedure results in a virtually instantaneous inactivation of enzymes in the brain tissue, thus minimizing postmortem steroid metabolism. Brain was rapidly (in <1 min) excised from the skull and the cerebral cortices were dissected and frozen at −20°C until steroid extraction. Blood was collected from the trunk into heparinized test tubes and centrifuged at 900 *g* for 20 min at room temperature and the plasma was frozen until assayed for steroids.

Allopregnanolone was extracted and purified as previously described (Barbaccia *et al.*, 1994). Briefly, allopregnanolone present in cerebral cortical homogenates (400 mg of tissue in 4 ml of phosphate-buffered saline) was extracted three times with equal volumes of ethyl acetate. The pooled organic phase was dried under vacuum, the residue was dissolved in 5 ml of *n*-hexane and applied to Seppak silica cartridges (Millipore), and components were eluted with *n*-hexane and 2-propanol (7 : 3, v/v). Allopregnanolone was further purified by high-performance liquid chromatography on a 5-μm Lichrosorb-diol column (250 by 4 mm) (Merck) with a 0 to 30% (v/v) gradient of 2-propanol in *n*-hexane. The recovery of allopregnanolone throughout the extraction-purification procedures (70 to 80%) was monitored by adding trace amounts (4000 to 6000 c.p.m.) of [^{3}H]-allopregnanolone standard to the brain homogenate. The allopregnanolone plasma concentration was measured in 1 ml of plasma extracted three times with 3 ml of ethylacetate. Allopregnanolone was then quantified by radioimmunossay as described (Purdy *et al.*, 1991).

Proteins were assayed by the method of Lowry *et al.* (1951), with bovine serum albumin as standard.

[^{35}S]-TBPS binding assay

Rats were killed by guillotine and the cerebral cortex was rapidly dissected from the brains and homogenized with a Polytron PT 10 (setting 5, 20 s) in 75 volumes of ice-cold 50 mM Tris-citrate buffer (pH 7.4 at 25°C) containing 100 mM NaCl. The homogenate was centrifuged at 20,000 *g* for 20 min, and the resulting pellet was resuspended in 75 volumes of 50 mM Tris-citrate buffer without salt and used for the assay. [^{35}S]-TBPS binding was determined in a final volume of 500 μl, consisting of 200 μl of cerebral cortical membranes (0.15 to 0.20 mg of protein), 50 μl of [^{35}S]-TBPS (70 to 100 Ci mmol^{-1}; final assay concentration, 1 nM), 50 μl of 2 M NaCl, and 200 μl of 50 mM Tris-citrate buffer. Incubations (25°C) were initiated by addition of membranes and terminated after 90 min by rapid filtration through glass-fibre strips (Whatman GF/B). The filters were rinsed with two 4-ml portions of ice-cold Tris-citrate buffer in a Cell Harvester filtration manifold (model M-24, Brandel), and filter-bound radioactivity was quantitated by liquid scintillation spectrometry. Nonspecific binding was defined as binding in the presence of 100 μM picrotoxin.

Saturation analysis of [^{35}S]-TBPS binding was performed with seven different concentrations of ligand (2.5 to 500 nM). The concentration of ^{35}S-labelled ligand was kept constant by dilution of 2.5 nM [^{35}S]-TBPS with unlabelled TBPS. Scatchard analysis of the binding data was performed with an iterative computer programme (LIGAND) written for the IBM-PC.

Isoniazid treatment

Isoniazid (isonicotinic acid hydralize) was dissolved in physiological saline and administered (375 mg kg^{-1}, s.c.) 30 min before intracerebroventricular injection of allopregnanolone or midazolam. Animals were observed for the appearance of convulsions for at least 3 h or killed 45 min after injection of isoniazid for measurement of [^{35}S]-TBPS binding.

Pentylenetetrazol treatment

Pentylenetetrazol (PTZ) was dissolved in physiological saline and administered (75 mg kg^{-1}, i.p.) at various times after intracerebroventricular injection of allopregnanolone. Rats were observed for 60 min after PTZ injection to determine the incidence of convulsions.

Foot-shock stress

Rats were habituated to the new environment (foot-shock box) and to the handling manoeuvres that precede killing in order to reduce stress. Thus, they were picked up from their cages, held for 1 min in the foot-shock box, had their heads forcibly introduced under the blades of a guillotine, and then put back in the original cages. This procedure was repeated four times a day for 6 to 8 days until ~80% of the animals assumed the final position with their neck under the guillotine blades without offering any resistance. The animals were divided into control and foot-shock groups.

The foot-shock apparatus (Lafayette Instruments, Lafayette, IN, U.S.A.), consisted of a Plexiglas box with two opaque sides, measuring 28 by 22 by 27 cm. A stimulator delivered a shock of 0.2 mA of 500-ms duration every second. The rats were shocked continuously for 5 min and then killed by guillotine. Control rats were kept in the same box for 5 min but not exposed to the electric shocks.

Statistical analysis

Data are presented as means ± s.e.mean. Biochemical data were analysed by analysis of variance (ANOVA) followed by Scheffe's test. Behavioural data were analysed by Fisher's exact probability test or by ANOVA followed by Scheffe's test. A P value of <0.05 was considered statistically significant.

Chemicals

Allopregnanolone, 3β,5α-pregnanolone, and 2-hydroxypropyl-β-cyclodestrin were obtained from Research Biochemicals (Milan, Italy). Midazolam was kindly provided by Hoffmann La Roche (Basel, Switzerland). Isoniazid and PTZ were from Sigma (Milan, Italy). [^{35}S]-TBPS (70 to 100 Ci $mmol^{-1}$) and [^{3}H]-allopregnanolone (45 to 75 Ci $mmol^{-1}$) were from New England Nuclear. Other drugs and materials were obtained from commerical sources.

Results

Effects of intracerebroventricular administration of allopregnanolone on cortical and plasma allopregnanolone concentrations

Allopregnanolone was detected in both the cerebral cortex (4.8 ± 0.6 ng g^{-1} of tissue) and plasma (1.38 ± 0.34 ng ml^{-1}) of rats intracerebroventricularly injected with vehicle alone (Table 1). Injection of allopregnanolone (1.25 to 15 μg 5 μl^{-1}) resulted in a rapid, marked, and dose-dependent increase in the cortical concentration of this neurosteroid. At a dose of 15 μg, allopregnanolone injection produced an ~100 fold increase in cortical allopregnanolone concentration within 5 min; the concentration remained significantly increased (~60 and 30 fold, respectively) at 15 and 30 min, before returning to control values by 60 min. The cortical concentration of allopregnanolone showed maximal increase of ~30 and 60 fold 5 min after injection of 1.25 and 2.5 μg of allopregnanolone, and thereafter rapidly decreased, achieving control values at 60 min.

The increases in plasma allopregnanolone concentration after intracerebroventricular injection of this neurosteroid were less pronounced and of shorter duration than those in the cerebral cortex. The lowest dose (1.25 μg) produced a maximal increase in plasma allopregnanolone of approximately 3 fold within 5 min, with values having returned to control levels by 15 min. With doses of 2.5 and 15 μg, plasma allopregnanolone concentration also peaked at 5 min (approximately 5 and 25 fold increases, respectively), remained significantly increased after 15 min, and returned to control values by 30 min.

Effects of allopregnanolone and midazolam on [^{35}S]-TBPS binding

[^{35}S]-TBPS binding was measured *ex vivo* to unwashed membrane preparations from cerebral cortices of rat injected intracerebroventricularly with allopregnanolone. Although the concentration of GABA in this binding assay might differ between the various brain preparations from different treatment of rats the conditions of [^{35}S]-TBPS binding did not show great variability because the fmol mg^{-1} protein in the different experimental groups were changed not more than 15%.

Intracerebroventricular injection of allopregnanolone (1.25 to 15 μg 5 μl^{-1}) resulted in a dose-dependent decrease in [^{35}S]-TBPS binding to unwashed membranes prepared from the cerebral cortex of rats killed 5 min after injection (Figure 1a). At doses of 1.25 and 2.5 μg, which produced ~30- and 60 fold increases in cortical allopregnanolone concentration, this neurosteroid had no effect on [^{35}S]-TBPS binding, whereas the threshold dose (5 μg) induced a 20% decrease. The maximal effect ($-59 \pm 4\%$) was apparent at a dose of 15 μg, which increased the allopregnanolone concentration in the cerebral cortex ~100 fold. In contrast, the 3β-hydroxyepimer of allopregnanolone had no significant effect on [^{35}S]-TBPS binding.

As expected, midazolam also inhibited [^{35}S]-TBPS binding measured *ex vivo*. Intracerebroventricular injection of this benzodiazepine decreased [^{35}S]-TBPS binding with a potency and efficacy (maximal effect of $-62 \pm 5\%$ at a dose of 15 μg) similar to those of allopregnanolone (Figure 1a).

At a dose of 15 μg, the effect of allopregnanolone on [^{35}S]-TBPS binding was maximal ($-73 \pm 5\%$) within 2.5 min after injection, was still significant at 15 min ($-36 \pm 6.6\%$), and returned to control values by 30 min (Figure 1b).

Computer-assisted nonlinear regression analysis of binding data from saturation studies revealed that the effect of allopregnanolone (15 μg) on [^{35}S]-TBPS binding was attributable to a decrease (−35%) in the total number of recognition sites (B_{max}) and an increase (+219%) in the apparent dissociation constant (K_d) (Figure 2).

Table 1 Time course of changes in allopregnanolone concentration in the cerebral cortex and plasma after intracerebroventricular injection of various doses of allopregnanolone

	Cerebral cortex (ng g^{-1}) *Allopregnanolone dose* (μg)			*Plasma* (ng ml^{-1}) *Allopregnanolone dose* (μg)		
Time (min)	*1.25*	*2.5*	*15*	*1.25*	*2.5*	*15*
0	4.5 ± 1.8	6.0 ± 2.2	4.0 ± 1.8	1.61 ± 0.27	1.82 ± 0.26	0.71 ± 0.27
5	150 ± 38.1*	278 ± 61.6*	480 ± 100*	3.93 ± 0.96*	6.1 ± 1.05*	32.6 ± 9.5*
15	19 ± 4.5*	37 ± 6.6*	301 ± 42*	2.74 ± 0.71	3.8 ± 0.56*	14 ± 4.6*
30	15 ± 3.8*	26 ± 4.2*	146 ± 59*	1.91 ± 1.3	1.94 ± 0.28	5.5 ± 2.5
60	3.0 ± 1.4	5.0 ± 2.3	6.2 ± 1.6	1.12 ± 0.84	1.63 ± 0.31	2.3 ± 0.81

Rats were killed at the indicated times after the administration of allopregnanolone. Time 0 values refer to administration of vehicle. Data are means ± s.e.mean of five rats (each sample assayed in duplicate). *$P<0.01$ vs. vehicle-treated rats (ANOVA followed by Scheffe's test).

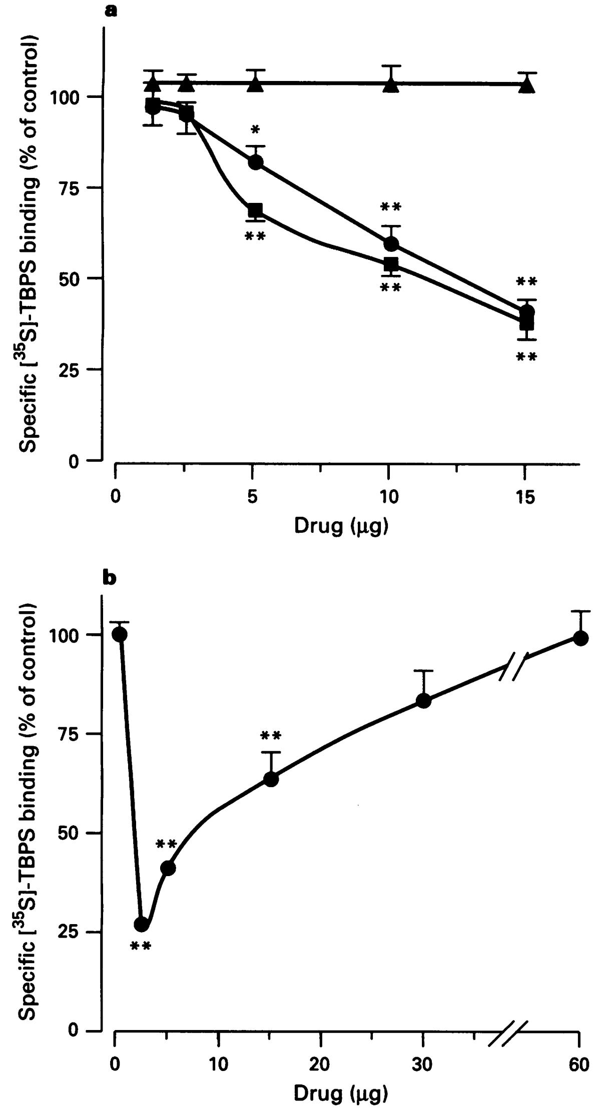

Figure 1 (a) Effects of intracerebroventricular injection of allopregnanolone (●), midazolam (■), and 5α, 3β-pregnanolone (▲) on [^{35}S]-TBPS binding to rat cerebral cortical membranes. Animals were killed 5 min after drug injection. (b) Time course of the allopregnanolone-induced decrease in [^{35}S]-TBPS binding. Animals were killed at the indicated times after injection of 15 µg of allopregnanolone. In both (a) and (b), control rats received an equal volume of vehicle. Unwashed cortical membranes were incubated with 1 nM [^{35}S]-TBPS for 90 min at 25°C. Data are expressed as a percentage of control (vehicle-treated rats) and represent means ± s.e.mean of four separate experiments, each performed with three rats per group. Specific binding of [^{35}S]-TBPS in the control group was 35 ± 5 fmol mg^{-1} protein ($n=16$). *$P<0.05$, **$P<0.01$ vs. vehicle-treated rats (ANOVA followed by Scheffe's test).

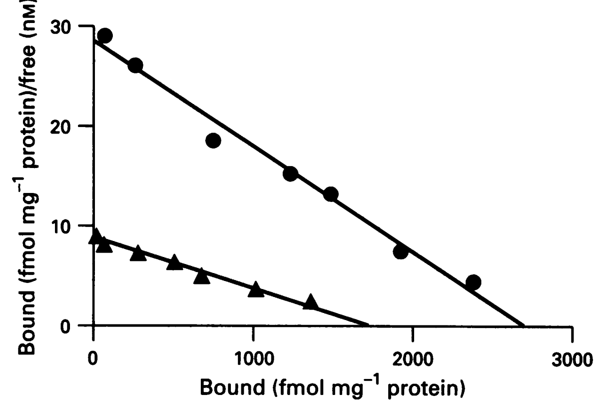

Figure 2 Effects of intracerebroventricular injection of allopregnanolone on the B_{max} and K_d of [^{35}S]-TBPS binding sites in rat cerebral cortical membranes. Rats were killed 5 min after the administration of vehicle (●) or 15 µg of allopregnanolone (▲). Unwashed cortical membranes were incubated in the presence of seven different concentrations (2.5 to 500 nM) of [^{35}S]-TBPS. Data are from a typical experiment that was repeated three times with similar results. Control: $B_{max}=2699$ fmol mg^{-1} protein; $K_d=95$ nM. Allopregnanolone: $B_{max}=1754$ fmol mg^{-1} protein, $K_d=208$ nM.

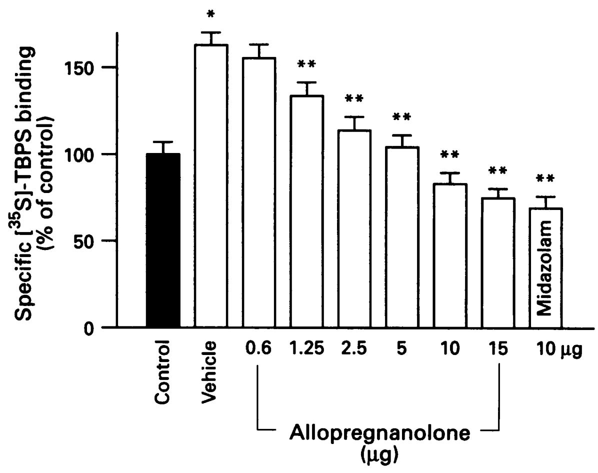

Figure 3 Effects of intracerebroventricular administration of allopregnanolone or midazolam on the isoniazid-induced increase in [^{35}S]-TBPS binding to rat cerebral cortical membranes. Allopregnanolone (0.6 to 15 µg), midazolam (10 µg), or vehicle was injected 30 min after the administration of isoniazid (375 mg kg^{-1}, s.c.). Rats were killed 45 min after injection of isoniazid (open columns) or saline (solid columns). Data are expressed as a percentage of control (saline-treated rats) and are means ± s.e.mean of four separate experiments, each performed with three animals per group. *$P<0.05$ vs. control (ANOVA followed by Scheffe's test). **$P<0.05$ vs. rats treated with isoniazid and vehicle (ANOVA followed by Scheffe's test).

Effects of allopregnanolone on the increase in [^{35}S]-TBPS binding and convulsions induced by isoniazid

To investigate further the action of allopregnanolone on GABA$_A$ receptor function, we evaluated the effect of this neurosteroid in an experimental model in which GABAergic transmission is reduced. Thus, we assessed the ability of allopregnanolone to antagonize the inhibitory action of isoniazid on GABAergic transmission. Subcutaneous administration of isoniazid induces both seizures (Horton, 1980) and a dose and time-dependent increase in [^{35}S]-TBPS binding to unwashed cortical membranes prepared from rat brain (Serra *et al.*, 1989b; Biggio *et al.*, 1990). Thus, with isoniazid-treated rats, it is possible to evaluate the effects of drugs acting on GABAergic transmission at both the pharmacological (seizures) and biochemical ([^{35}S]-TBPS binding) levels in the same animal.

Intracerebroventricular injection of allopregnanolone (0.6 to 15 µg) 30 min after subcutaneous administration of isoniazid (375 mg kg^{-1}) resulted in a dose-dependent inhibition of the isoniazid-induced increase in [^{35}S]-TBPS binding to unwashed cortical membranes prepared from rats killed 15 min later (Figure 3), an effect mimicked by the intracerebroventricular injection of midazolam. The potency of allopregnanolone with regard to its effect on [^{35}S]-TBPS binding was greater in isoniazid-treated rats than in control rats. Thus, doses of 1.25 and 2.5 µg of allopregnanolone, which resulted in only four and eight fold increases in cortical

Table 2 Effect of intracerebroventricular injection of allopregnanolone on seizures and death induced by isoniazid

Allopregnanolone (μg)	*Latency of convulsions* (min)	*No. of animals showing convulsions*	*Mortality*
0	48 ± 4	10/12	6/12
5	64 ± 5^a	8/12	4/12
10	72 ± 7^a	8/12	$1/12^b$
15	85 ± 13^a	6/12	$0/12^b$

Allopregnanolone or vehicle (zero dose) was injected 30 min after the administration of isoniazid (375 mg kg^{-1}, s.c.). Animals were observed for 3 h after isoniazid administration. $^aP<0.05$ vs. isoniazid plus vehicle (ANOVA followed by Scheffe's test). $^bP<0.05$ vs. isoniazid plus vehicle (Fisher's exact probability test).

Table 3 Effect of intracerebroventricular injection of allopregnanolone on seizures elicited by pentylenetetrazol (PTZ)

Allopregnanolone (μg)	*Latency of convulsions* (min)	*No. of animals showing convulsions*	*Mortality*
0	2.10 ± 1.2	12/12	2/12
2.5	5.29 ± 0.6^a	10/12	0/12
5.0	11.26 ± 2.8^b	9/12	0/12
10	32.35 ± 12.8^b	$7/12^c$	0/12
15	>60	$0/12^d$	0/12

Allopregnanolone or vehicle (zero dose) was injected 5 min before the administration of PTZ (75 mg/kg, i.p.). Animals were observed for 1 h after PTZ administration. $^aP<0.05$, $^bP<0.001$ vs. vehicle plus PTZ (ANOVA followed by Scheffe's test). $^cP<0.05$, $^dP<0.001$ vs. vehicle plus PTZ (Fisher's exact probability test).

allopregnanolone concentration 15 min after injection, respectively, and had no effect on [^{35}S]-TBPS binding in cortical membranes of control rats, inhibited the isoniazid-induced increase in [^{35}H]-TBPS binding by 45 and 76% respectively.

Isoniazid induced tonic-clonic seizures in ~80% of control animals within 50 min (Table 2). Administration of 5, 10, or 15 μg of allopreganolone delayed the onset of isoniazid -induced convulsions by 16, 24, and 37 min, respectively, and increased in a dose-dependent manner the percentage of animals that survived.

Effect of allopregnanolone on PTZ-induced seizures

We also investigated the effect of intracerebroventricular administration of allopregnanolone on PTZ-induced seizures. Injection of allopregnanolone 5 min before administration of PTZ (75 mg kg^{-1} i.p.) delayed the onset of convulsions and reduced the percentage of animals showing seizures in a dose-dependent manner (Table 3). At the lowest dose tested (2.5 μg), which resulted in a ~60 fold increase in the cortical allopregnanolone concentration, this neurosteroid produced a significant increase (two fold) in the latency of seizures but failed to affect significantly the number of animals showing convulsions. In contrast, at a dose of 15 μg, allopregnanolone prevented the onset of convulsions for at least 60 min. The protective activity of allopregnanolone peaked 5 min after administration of the maximally effective dose (15 μg), persisted for 45 min, and was no longer apparent after 60 min (Figure 4).

Effect of allopregnanolone on the stress-induced increase in [^{35}S]-TBPS binding

The activity of the GABA$_A$ receptor-associated Cl$^-$ channel is rapidly reduced by stressful environmental stimuli (Drugan *et al.*, 1989; Serra *et al.*, 1989a; Biggio *et al.*, 1990). Thus, foot-shock stress increases [^{35}S]-TBPS binding in rat cerebral cortex, an effect prevented by anxiolytic drugs and mimicked by anxiogenic β-carboline derivatives (Concas *et al.*, 1988; 1990; 1993; Biggio *et al.*, 1990; Serra *et al.*, 1992; 1994). Consistent with the results obtained with isoniazid-treated rats, allopregnanolone (0.6 to 5 μg) antagonized the increase in [^{35}S]-TBPS binding induced by foot-shock stress in a dose-dependent manner (Figure 5). Low doses (1.25 and 2.5 μg) of this neurosteroid, which had no effect on [^{35}S]-TBPS binding in control rats, antagonized the stress-induced increase in [^{35}S]-TBPS binding when injected 10 min before foot shock. The same doses of allopregnanolone injected 60 min before stress failed to prevent the increase in [^{35}S]-TBPS binding (data not shown).

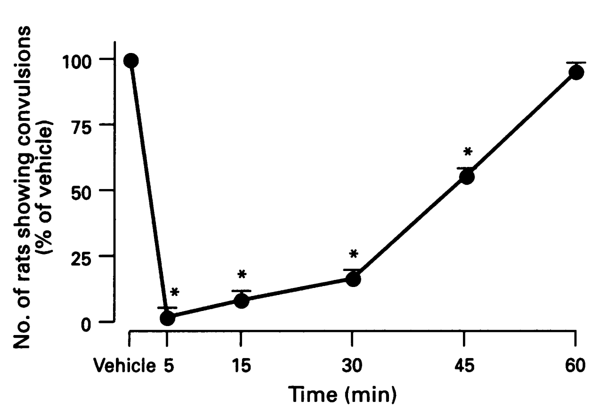

Figure 4 Time course of the protective effect of allopregnanolone against PTZ-induced seizures. PTZ (75 mg kg^{-1}, i.p.) was administered at the indicated times after injection of allopregnanolone (15 μg). Data are means ± s.e.mean of 8 to 12 rats and are expressed as a percentage of control (rats treated with vehicle 5 min before PTZ). *$P<0.01$ vs. vehicle plus PTZ (Fisher's exact probability test).

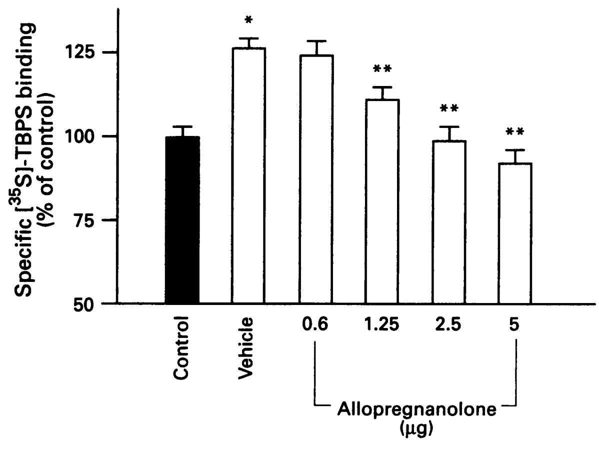

Figure 5 Effects of intracerebroventricular injection of allopregnanolone on the increase in [^{35}S]-TBPS binding to rat cerebral cortical membranes induced by foot-shock stress. Allopregnanol.·ne (0.6 to 2.5 μg) or vehicle was injected 10 min before a 5-min session of foot-shock stress after which rats were killed immediately (open columns). Control rats were not exposed to foot shock (solid columns). Data are expressed as a percentage of control and are means ± s.e.mean of four separate experiments, each performed with four animals per group. *$P<0.05$ vs. control rats (ANOVA followed by Scheffe's test). **$P<0.05$ vs. vehicle-treated rats (ANOVA followed by Scheffe's test).

Discussion

$GABA_A$ receptors participate in the regulation of a variety of psychophysiological phenomena, including anxiety, stress, sleep, depression, seizures and sexual functions (Berretini & Post, 1984; Majewska *et al.*, 1989; Biggio *et al.*, 1995). Thus, characterization of the biochemical, behavioural, and physiological consequences of $GABA_A$ receptor activation may contribute to our understanding of the molecular events that underlie neurological and psychiatric disorders.

In addition to the well-characterized synthetic positive and negative modulators of the $GABA_A$ receptor complex, such as benzodiazepines, barbiturates, and β-carbolines, several endogenous neurosteroids – steroids synthesized from cholesterol in the central nervous system, where they are present independently of peripheral sources (Baulieu & Robel, 1990) – directly modulate $GABA_A$ receptor function (Majewska *et al.*, 1986; Harrison *et al.*, 1987; Gee *et al.*, 1988; Turner *et al.*, 1989; Puia *et al.*, 1990; Lambert *et al.*, 1990; 1995). One of these neurosteroids, allopregnanolone, is the most potent and efficacious endogenous compound shown to enhance $GABA_A$ receptor function *in vitro*.

We have now shown that administration of allopregnanolone directly into the lateral ventricle results in a rapid and marked increase in $GABA_A$ receptor function, as assessed by *ex vivo* measurement of [^{35}S]-TBPS binding to rat cerebral cortical membranes. The observed changes in [^{35}S]-TBPS binding showed a similar time course to that of the changes in allopregnanolone concentration in the same brain area. The *in vivo* efficacy of allopregnanolone in inhibiting [^{35}S]-TBPS binding resembled that of midazolam, an anxiolytic and anticonvulsant benzodiazepine. In contrast, intracerebroventricular administration of the 3β-hydroxyepimer of allopregnanolone had no effect, even at high doses (10 to 15 μg), on [^{35}S]-TBPS binding subsequently measured in cortical membranes. This latter observation is consistent with previous data showing that a 3α-hydroxyl group is required for the pharmacological activity of steroids at the $GABA_A$ receptor complex *in vitro* (Harrison *et al.*, 1987). Together with previous studies showing that allopregnanolone enhances GABAergic transmission *in vitro* (Majewska *et al.*, 1986; Harrison *et al.*, 1987; Gee *et al.*, 1988; Turner *et al.*, 1989), our *in vivo* data support a relation between this neurosteroid and $GABA_A$ receptor function.

Our present study has established the concentration-range in the brain over which allopregnanolone is able to counteract the impairment in GABAergic transmission induced either by stress or pharmacologically.

The pharmacologically effective doses of allopregnanolone in rats exposed to stress, isoniazid, or PTZ ranged from 1.25 to 15 μg. The time course of changes in brain allopregnanolone concentration after intracerebroventricular injection paralleled that of anticonvulsant activity as well as that of changes in cortical [^{35}S]-TBPS binding. Thus, the cortical allopregnanolone concentration after injection of low or high doses peaked within 5 min, which coincided with the time of the maximal effects on [^{35}S]-TBPS binding and on PTZ-induced convulsions. Furthermore, 60 min after injection, when the brain concentration of allopregnanolone had returned to baseline values, these effects were no longer apparent.

The potency of allopregnanolone was increased (approximately four fold) in animals in which GABAergic transmission was inhibited. By inhibiting GABA synthesis, isoniazid decreases central GABAergic transmission, increases [^{35}S]-TBPS binding, and, after a delay of 50 to 60 min, elicits convulsions (Horton *et al.*, 1979; Horton, 1980; Serra *et al*, 1989b). Thus, we have proposed that isoniazid-treated animals constitute a model for investigating, at the same time and in the same animal, the biochemical and pharmacological efficacy *in vivo* of drugs that enhance GABAergic transmission (Serra *et al.*, 1992; 1994).

Intracerebroventricular injection of allopregnanolone in isoniazid-treated rats inhibited the isoniazid-induced increase in [^{35}S]-TBPS binding in the cerebral cortex and delayed the onset of convulsions in a dose-dependent manner. A similar effect of [^{35}S]-TBPS binding was also obtained with midazolam.

Allopregnanolone inhibited the isoniazide-induced increase in [^{35}S]-TBPS binding at doses as low as 1.25 and 2.5 μg, which failed to affect [^{35}S]-TBPS binding in control animals. At a dose of 2.5 μg, allopregnanolone also produced a significant delay in the onset of seizures induced by PTZ, a Cl^- channel blocker. Consistent with these results, Bitran *et al.* (1991a), showed that intracerebroventricular injection of allopregnanolone elicits anxiolytic effects with a potency similar to that observed for antagonism of PTZ-induced convulsions.

The fact that the potency of allopregnanolone in enhancing the function of $GABA_A$ receptors appears markedly increased under pharmacological conditions (PTZ- or isoniazid-treated rats) in which GABAergic transmission is reduced may be relevant to the observation that the brain concentrations of this and other neurosteroids are altered in various physiological conditions, such as stress, the oestrous cycle, and pregnancy, in which $GABA_A$ receptor function is reduced (Majewska *et al.*, 1989; Purdy *et al.*, 1991; Corpechot *et al.*, 1993; Barbaccia *et al.*, 1994; 1996).

In agreement with these results, we have now shown that doses of allopregnanolone as low as 1.25 and 2.5 μg also inhibited the increase in [^{35}S]-TBPS binding induced by foot-shock, a stressful condition known to reduce $GABA_A$ receptor function (Biggio *et al.*, 1990) and to alter the emotional state of animals (Corda & Biggio, 1986). Accordingly, rats exposed to foot-shock stress show an increase in [^{35}S]-TBPS binding to cortical membrane preparations and exhibit conflict behaviour, effects mimicked by anxiogenic drugs and antagonized by anxiolytics (Corda & Biggio, 1986; Concas *et al.*, 1988; 1990; 1993; Serra *et al.*, 1992; 1994).

The greater potency of allopregnanolone under conditions of reduced GABAergic transmission may represent a physiological compensatory mechanism through which this compound and other endogenous neuroactive steroids are able to restore the impairments in $GABA_A$ receptor function associated with various pathological and physiological states. Although neurosteroids are normally present in neuronal tissue at low concentrations, their abundance increases markedly during the oestrous cycle ($\sim$4 to 12 ng g^{-1}) and pregnancy ($\geqslant$30 ng g^{-1}) (Paul & Purdy, 1992; Corpechot *et al.*, 1993), as well as in response to various stressful stimuli ($\sim$5 to 12 ng g^{-1}) (Purdy *et al.*, 1991; Barbaccia *et al.*, 1994; 1996). These brain allopregnanolone concentrations, ranging from 4 to 30 ng per gram of tissue, appear to be associated with changes in $GABA_A$ receptor function, as assessed by various behavioural and biochemical studies (Majewska *et al.*, 1989; Biggio *et al.*, 1990; Bitran *et al.*, 1991b; 1993; Finn & Gee, 1993).

The mean cortical concentrations of allopregnanolone measured 15 min after intracerebroventricular injection of 1.25 to 2.5 μg of this neurosteroid, doses that restored GABAergic transmission in isoniazid-treated animals and foot-shocked rats and delayed the onset of PTZ-induced convulsions, ranged from 19 to 37 ng g^{-1}. Although this way of administration may not lead to a homogeneous, even distribution of allopregnanolone throughout the brain, these levels are similar to those achieved in various physiological conditions. Changes in brain allopregnanolone concentration may therefore play an important role in modulation of $GABA_A$ receptor function in response to various environmental and pathophysiological conditions. Majewska *et al.* (1989) showed that the marked decrease in brain neurosteroid concentrations that occurs after parturition in rats is paralleled by a decrease in the density of $GABA_A$ receptors and an increase of their affinity. Hence, a functional relation between allopregnanolone concentration and $GABA_A$ receptor function appears to exist in rat brain; increases in allopregnanolone concentration appear to enhance $GABA_A$ receptor function, whereas a reduction in the synthesis or release of this neurosteroid may inhibit GABAergic function.

In conclusion, the present study on the pharmacology of allopregnanolone on GABAergic transmission in the rat demonstrated that the *in vivo* administration of this neurosteroid enhances the function of the GABA-coupled chloride channel and antagonizes or reduces the inhibitory action of stress and drugs that decrease GABAergic transmission.

This work was supported by Grant 94.00519.PF41 from the National Research Council (CNR) (target project "Prevention and Control Diseases Factor") Subproject "Stress".

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(Received September 28, 1995
Revised January 18, 1996
Accepted February 16, 1996)