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Stress-Induced Increase in Brain Neuroactive Steroids: Antagonism by Abecarnil

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BARBACCIA, M. L., G. ROSCETTI, F. BOLACCHI, A. CONCAS, M. C. MOSTALLINO, R. H. PURDY AND G. BIGGIO. *Stress-induced increase in brain neuroactive steroids: Antagonism by abecarnil.* PHARMACOL BIOCHEM BEHAV **54**(1) 205–210, 1996. – Acute foot shock stress elicits a selective and time-dependent increase of neuroactive steroid (pregnenolone, progesterone, allotetrahydrodeoxycorticosterone) concentrations in rat brain cortex, accompanied by a marked increase of plasma corticosterone. The brain cortical neuroactive steroid levels peaked between 10 and 30 min poststress and returned to control values by 2 h. Abecarnil (0.3 mg/kg, IP), a beta-carboline derivative with anxiolytic properties, completely antagonized the effect of foot shock on brain cortical neuroactive steroids. A single administration of the anxiogenic beta-carboline FG 7142 (15 mg/kg, IP), in contrast, mimicked the effect of foot shock. These data support the hypothesis for the existence of a functional relationhip between brain neuroactive steroid concentrations and $GABA_A$ receptor function/emotional state of the animal.

Neuroactive steroids 3,Alpha-21-dihydroxy-5alpha-pregnan-20-one (allotetrahydrodeoxycorticosterone, THDOC) Pregnenolone Progesterone Corticosterone Foot shock $GABA_A$ receptor Beta-Carboline Abecarnil FG 7142

PREGNENOLONE, progesterone, and some of their A-ring reduced metabolites can be produced by neural tissue, both in the central and peripheral nervous systems, and for this are referred to as neurosteroids (1,2,12,22,23,27,28,33,34). Moreover, after in vivo administration or direct in vitro application to cells, neurosteroids have been shown to regulate important neuronal functions via genomic (35) and membrane-mediated actions, among others, via specific interactions with ligand-gated ionotropic receptors such as $GABA_A$ receptors (26,28, 31,37). For this they have also been referred to as neuroactive steroids (28). Among the different neuroactive steroids the 3alpha-hydroxy-5alpha reduced derivatives of progesterone and deoxycorticosterone, namely, 3alpha-OH,5alpha-pregnan-20-one (allopregnanolone, AP) and 3,alpha,21-dihydroxy-5alpha-pregnan-20-one (allo-tetrahydrodeoxy-corticosterone, THDOC) are the most potent and efficacious known positive modulators of the $GABA_A$ receptors (28,31).

These receptors have been suggested to play a pivotal role in the regulation of the animal's emotional state (6). Accordingly, anxiety and/or proconflict promoting conditions, such as acute stress, alter brain GABAergic neurotransmission. Decreased GABA-mediated chloride ion fluxes and increased [^{35}S]-TBPS binding have been reported after several acute stress paradigms (handling, CO_2 inhalation, foot shock, swim stress) (6,14,19,21,36,38). These stress-induced biochemical alterations are accompanied by behavioral changes, i.e., a decreased punished response in the Vogel's test, an index of anxiety in the animal (8,15,16), and an increased sensitivity to both the anxiolytic action of benzodiazepines (10,20) and to the convulsant action of isoniazid, an inhibitor of GABA synthesis (39). These effects of acute stress on the GABAergic neurotransmission are mimicked by an acute administration of anxiogenic beta-carbolines, i.e., negative allosteric modulators of the $GABA_A$ receptor complex, and prevented by prior treatment of the animals with anxiolytics that act as positive allosteric modulators of the $GABA_A$ receptor complex (6,8). AP and THDOC also enhance in vitro $GABA_A$ receptor function (26,28,31,33,37) and, when administered in vivo, show anxiolytic and/or anticonvulsant properties (5,9,24,28). While evidence is accumulating on the pharmacological actions of

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neuroactive steroids at the level of $GABA_A$ receptors (5, 9,24,28), little is known, as yet, on their physiological role in the modulation of neurotransmission in the central nervous system (CNS). The cerebral cortical levels of select neurosteroids are altered by several acute stress paradigms, i.e., forced swimming, handling maneuvers that preceed sacrifice, and CO_2 inhalation (3,4,32).

As we observed differences in the type of neuroactive steroids affected by different stressors, i.e., handling vs. CO_2 inhalation (3,4), we extended our previous studies by analyzing the changes in brain neurosteroid levels elicited by mild foot shock. Moreover, we investigated the effect of anxiolytic and/or anxiogenic drugs, acting either as positive or negative allosteric modulators of the $GABA_A$ receptor, on the neurosteroid brain content in control and foot shock-stressed rats. Foot shock, like CO_2 inhalation and handling (3,4), altered in a time-dependent manner the brain cortical pregnenolone and progesterone levels. However, at variance with CO_2 inhalation, foot shock also induced a marked and time-dependent increase of THDOC brain cortical levels. The effect of foot shock on brain neuroactive steroid levels was prevented by a pretreatment with abecarnil, an anxiolytic beta-carboline derivative, acting as a positive modulator of the $GABA_A$ receptor complex (40). In contrast to abecarnil, but similarly to foot shock, the anxiogenic benzodiazepine receptor ligand FG 7142 (18,29) also increased the brain cortical levels of neuroactive steroids. These results, together with previous evidence showing a preminent involvement of the GABAergic transmission in the neurochemistry of stress, suggest that the brain neuroactive steroid levels are sensitive to anxiogenic/anxiolytic conditions that alter the basal GABAergic tone and therefore may play a physiological role in its modulation.

METHOD

Animals

Male Sprague-Dawley CD rats (Charles River, Como, Italy), with body masses of 200–250 g, were housed under standard laboratory conditions with a 12 L : 12 D cycle, a constant temperature of 22 ± 2°C, and water and food ad lib. The animals were habituated for 4 days to the manipulations relative to the experimental procedures that preceed killing (see below). Animal care and handling throughout the esperimental procedure was performed in accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC). The experimental protocols involving exposure of rats to CO_2 and foot shock were approved by the Animal Ethical Committee of the University of Cagliari.

Foot Shock and CO_2 Exposure

The foot shock consisted of a series of electrical foot shocks delivered in individual boxes with floors made of brass rods, 2 cm apart. Shocks were given for 5 min by a stimulator which delivered, for 500 ms, every 500 ms, a shock of 0.2 mA.

Rats were exposed to a mixture of O_2/CO_2 (65%/35%) in a hermetically closed box with a 35 liter capacity. Control rats were placed in the same box for 1 min but not exposed to the gas. Delivery of CO_2 was monitored with a commercial analyzer. To minimize the stress associated with sacrifice the rats were habituated to the new environment (foot shock and CO_2 boxes) and to the handling manoeuvers that preceed killing for 4 days. They were picked up from their cages, held for 1 min in the foot shock or CO_2 boxes, and either introduced in the microwave holder (for the brain steroids measurement) or placed with their head through the blades of a guillotine for animal sacrifice (for the plasma steroid measurement). After each session, that was repeated four times a day for 4 consecutive days, until approximately 80% of the animals assumed their final position in the microwave holder or under the guillotine without opposing resistance, the rats were returned to their home cages.

The animals were killed at the time periods indicated after CO_2 exposure or foot shock either by guillotine, for the plasma steroid analysis, or by focussed microwave irradiation (4 s) to the head, for brain steroid measurement. This latter procedure results in a virtually instantaneous inactivation of enzymes present in brain tissue, thus minimizing postmortem steroid metabolism. Brains were rapidly (less than 1 min) excised from the skull, the cerebral cortices were dissected, and frozen to −20°C until steroids were measured. Blood was collected from the trunk in heparinized test tubes, centrifuged at 900 × *g* for 20 min at room temperature, and the plasma was frozen until assayed for steroids.

Steroid Extraction and Assay

Steroids were extracted and purified as previously described (3,34). Briefly, steroids present in cerebral cortical homogenates (400 mg tissue in 4 ml of phosphate-buffered saline) were extracted three times with ethylacetate, 1 : 1 (vol : vol). The organic phases were dried under vacuum, the residue was dissolved in 5 ml of *n*-hexane and applied to Seppak-silica cartridges (Waters), and components were eluted with *n*-hexane/2-propanol (7 : 3, vol : vol). Steroids were further purified by high-performance liquid chromatography (HPLC) on a 5 μm Lichrosorb-diol column (250 by 4 mm) (Merck) with a gradient of 2-propanol in *n*-hexane. Because we previously observed that cholesterol, which coelutes from the Lichrosorb-diol column with progesterone, decreases the sensitivity of the radioimmunoassay for progesterone, this latter steroid was separated from cholesterol by washing the corre-

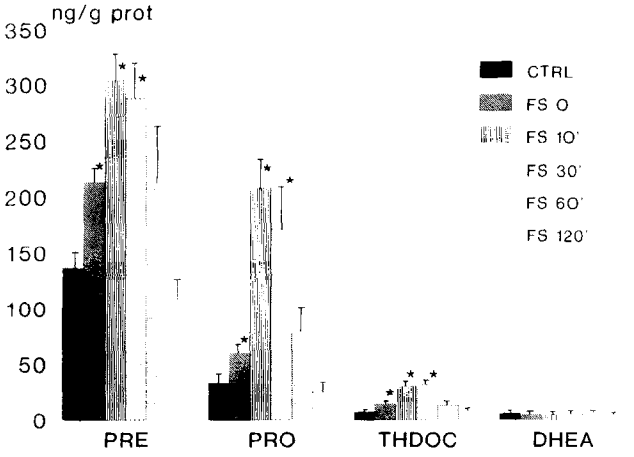

FIG. 1. Foot shock elicits selective and time-dependent changes of neuroactive steroid content in rat brain cortex. Foot shock (FS) (0.2 mA × 500 ms every 500 ms) was delivered for 5 min. The rats were killed by focussed microwave irradiation (4 s) to the head immediately after (time 0), 10, 30, 60, and 120 min after the foot shock session. PRE: pregnenolone; PRO: progesterone; THDOC: allotetrahydrodeoxycorticosterone; DHEA: dehydroepiandrosterone. Each bar represents the mean ± SEM of 10 animals. $*p < 0.05$, when compared to the respective control value.

TABLE 1

CO_2 INHALATION DOES NOT CHANGE THE THDOC CONTENT IN RAT BRAIN CORTEX

	THDOC (ng/g protein)
Control	4.6 ± 1.0
CO_2, 10 min	6.6 ± 1.5
CO_2, 30 min	8.0 ± 3.6
CO_2, 60 min	6.7 ± 2.5

A gas mixture of O_2/CO_2 (65%/35%) was delivered for 1 min in a hermetically closed box (see the Method Section). The rats were killed after CO_2 inhalation at the time points indicated by focussed microwave irradiation (4 s) to the head. Each value represents the mean ± SEM of five animals.

sponding dried HPLC fractions twice with 200 μl of dimethylsulphoxide and water (400 μl). Progesterone was extracted from the aqueous phase twice with 1.5 ml of *n*-hexane. The removal of cholesterol increased the sensitivity of the progesterone radioimmunoassay five- to 10-fold over that previously described (3). The recovery of each steroid through the extraction-purification procedures (70 to 80%) was monitored by adding trace amounts (4000 to 6000 cpm, specific activity 20–60 Ci/mmol) of [^{3}H]-labeled standards to the brain tissue homogenate. THDOC was then quantified by radioimmunoassay as previously described (32), and other steroids measured by radioimmunoassay using specific commercial antibodies (ICN, Costa Mesa, CA).

Protein concentration was measured by the method of Lowry et al. (25), with bovine serum albumin as standard. The steroid plasma concentrations were measured in 1 ml of plasma extracted three times with 1.5 ml of ethylacetate. Pregnenolone, progesterone, corticosterone, dehydroepiandrosterone, allotetrahydrodeoxycorticosterone, and dimethylsulphoxide were obtained from Sigma (Milan, Italy). All other organic solvents (HPLC grade) and reagents were of the best available quality from commercial sources.

Abecarnil and FG 7142 were obtained from Schering, AG, Berlin, Germany.

Statistical Analysis

Statistical analysis was performed by using the two-tailed Student's *t*-test.

RESULTS

Foot shock delivered for 5 min elicited a marked and time-dependent increase of brain cortical neuroactive steroids (Fig. 1). In rats killed by focussed microwave irradiation to the head immediately after stress application the pregnenolone, progesterone, and THDOC cortical content was increased (+69%, +82%, and +105%, respectively). The levels of these steroids continued to increase, reaching a peak between 10 and 30 min, and returned to control values by 120 min. At variance with foot shock, CO_2 inhalation did not affect THDOC brain cortical levels (Table 1). The plasma pregnenolone and progesterone concentrations, that in control rats were approximately 50- and 10-fold lower than the respective brain values, were maximally increased at time 0 and remained elevated up to 1 h after foot shock (Table 2). The plasma corticosterone levels were also dramatically increased in foot shock-stressed rats, indicating a strong activation of the hypothalamus-pituitary-adrenal (HPA) axis. Similar to CO_2 inhalation (3,4), foot shock failed to alter, at any time point examined, either the brain or plasma dehydroepiandrosterone (DHEA) levels. We investigated the effect of a pretreatment with abecarnil, i.e., an anxiolytic beta-carboline derivative acting as positive allosteric modulator at $GABA_A$ receptors (40), on the neurosteroid content in rat brain cortex. Abecarnil (0.3 mg/kg, IP), given 30 min before sacrifice, failed to change the basal pregnenolone and progesterone, while only slightly decreased THDOC levels, but antagonized the brain neuroactive steroid increase induced by foot shock (Table 3).

In contrast to abecarnil but similar to foot shock, FG 7142 (15 mg/kg, IP, 30 min), an anxiogenic and proconvulsant beta-carboline (18,29), significantly enhanced pregnenolone (+108%, +120%), progesterone (+206%, +120%), THDOC (+50%) and corticosterone (+74%) in brain cortex and plasma, respectively (Table 4).

DISCUSSION

The present data show that foot shock, a procedure that causes anxiety and proconflict behavior in the rat (15,39), and the anxiogenic benzodiazepine receptor ligand FG 7142,

TABLE 2

FOOT SHOCK (FS) ELICITS SELECTIVE AND TIME DEPENDENT CHANGES IN RAT PLASMA STEROID LEVELS

	(ng/ml)			
	PRE	PRO	CORT	DHEA
Control	0.21 ± 0.014	0.41 ± 0.08	46 ± 16	0.058 ± 0.005
FS (0 min)	1.90 ± 0.34*	8.70 ± 0.85*	396 ± 30*	0.052 ± 0.006
FS (30 min)	2.20 ± 0.33*	7.40 ± 0.94*	330 ± 17*	0.051 ± 0.005
FS (60 min)	1.00 ± 0.23*	3.60 ± 0.82*	224 ± 42*	0.049 ± 0.008
FS (120 min)	0.35 ± 0.01	0.60 ± 0.10	70 ± 13	0.055 ± 0.007

PRE: pregnenolone; PRO: progesterone; CORT: corticosterone; DHEA: dehydroepiandrosterone. For experimental details see legend to Fig. 1. Each value represents the mean ± SEM of five animals.

*$p < 0.05$ when compared to the respective control value.

TABLE 3

ABECARNIL ANTAGONIZES THE FOOT SHOCK (FS)-INDUCED INCREASE OF NEUROACTIVE STEROID LEVELS IN RAT BRAIN CORTEX

	(ng/g protein)		
	PRE	PRO	THDOC
Control	126 ± 21	22 ± 5.0	14.5 ± 2.5
FS	212 ± 31*	68 ± 18*	26.5 ± 3.2*
Abecarnil	160 ± 20	23 ± 6.4	6.80 ± 2.9
FS + Abecarnil	82 ± 30	27 ± 5.6	9.80 ± 1.2

PRE: pregnenolone; PRO: progesterone; THDOC: allotetrahydrodeoxycorticosterone. The rats received abecarnil (0.3 mg/kg, IP) 30 min before the foot shock session and were killed immediately after the stress application. During the 4 days habituation schedule the rats were also habituated to the IP injection. Each value represents the mean ± SEM of 10 animals.

*$p < 0.05$ when compared to the respective control values.

similar to other acute stress paradigms (3,4,8,32), increase the brain cortical content of neuroactive steroids. Foot shock stress elicits changes in pregnenolone and progesterone brain cortical content similar to other stress paradigms, i.e., CO_2 inhalation and handling, but it also uniquely increases THDOC. Foot shock, in contrast to CO_2 inhalation (3,4), also markedly activates the HPA axis, as demonstrated by the conspicuous increase in corticosterone plasma levels. The HPA axis is under the control of multiple neurotransmitter systems (30). Among others, GABA has been reported to exert an inhibitory control on the release of corticotropin releasing hormone (CRH) (11,30). Therefore, the decrease in GABAergic tone elicited by acute stress (6–8,15,19,21,36,38) may be responsible for the observed elevation in plasma and brain steroid concentrations.

To date, THDOC and AP are the most potent $GABA_A$ receptor-positive modulators (28,33) that can be synthesized in the body. While AP synthesis has been demonstrated to occur in the brain (12,28), THDOC appears to be mostly of adrenal origin (28,32). The data reported here and previous observations showing an increase in allopregnanolone levels in rats exposed to CO_2 inhalation and swim stress (3,4,32) suggest that when the GABAergic tone is low, as it occurs after acute stress, the brain concentration of either or both steroids active at the $GABA_A$ receptors is increased. We do not have, as yet, an explanation for the finding that the various stressors appear to differently affect the brain neurosteroid content. The time frame of each stress paradigm may play a crucial role. In fact, handling-habituated rats are exposed repeatedly, for 4 consecutive days, to the maneuvers that preceed killing as opposed to naive rats, which are only exposed once, while CO_2 and foot shock-stressed animals are challenged only once for 1 and 5 min, respectively. On this basis one could also conceive that the various stressors differently alter neurotransmitter systems that may be relevant in the control of neuroactive steroid production. Indeed, cyclic AMP was previously shown to play a role in the control of neurosteroid levels in brain cortical minces (2,34), and it has been shown that acute stress increases the brain cyclic AMP levels (41). One could speculate that the degree or the duration of the cyclic AMP increase after stress may be one of the factors responsible for the selectivity of the neurosteroid types affected by the various stressors. One should also be reminded that CO_2 inhalation may elicit, at cellular level, metabolic changes, presumably not induced by foot shock, that may, directly or indirectly, affect steroidogenesis. In this respect it seems appropriate to

TABLE 4

THE ANXIOGENIC BETA-CARBOLINE FG 7142 INCREASES BRAIN AND PLASMA STEROID LEVELS

	(ng/g prot or ng/ml)			
	PRE	PRO	THDOC	CORT
Brain Cortex				
Control	130 ± 13	31 ± 7.0	18 ± 2.5	n.d.
FG 7142	270 ± 34*	95 ± 6.5*	27 ± 1.8*	n.d.
Plasma				
Control	0.5 ± 0.06	1.5 ± 0.03	n.d.	109 ± 33
FG 7142	1.1 ± 0.10*	4.1 ± 0.045*	n.d.	190 ± 18*

PRE: pregnenolone; PRO: progesterone; THDOC: allotetrahydrodeoxy-corticosterone; CORT: corticosterone. The rats received FG 7142 (15 mg/kg, IP) 30 min before sacrifice. During the 4-day habituation schedule the rats were also habituated to the IP injection. Each value represents the mean ± SEM of five animals.

*$p < 0.05$, when compared to the respective control value.

emphasize that the HPA axis appears more sensitive to foot shock than to CO_2 inhalation (3,4). Whether the differences in the type and extent of neurosteroid increase elicited by the various acute stressors bear some relevance on the long-term effects of acute and/or repeated stress is an intriguing question that deserves further investigation.

The immediate and persistent enhancement of brain cortical THDOC levels may help in restoring the $GABA_A$ receptor function altered by foot shock and, therefore, control the acute stress-elicited activation of discrete neuronal populations, i.e., dopaminergic, cholinergic, and catecholaminergic (6,13), which has been shown to be antagonized by positive allosteric modulators of the $GABA_A$ receptor (13,17). This hypothesis is consistent with previous observations demonstrating that changes in [^{35}S]-TBPS binding as well as in the behavioral parameters related to GABAergic transmission show a similar time course, i.e., they are altered immediately after a foot shock session and return to basal values by 1-2 h (6,39). The existence of a relationship between brain neurosteroid levels, on one side, and GABAergic tone/emotional state of the animal, on the other, is suggested also by the opposite effects on brain and plasma steroid levels exerted by abecarnil and FG 7142, two beta-carboline derivatives with anxiolytic (40) and anxiogenic (18,29) action, respectively. In fact, abecarnil (0.3 mg/kg), which per se did not alter pregnenolone and progesterone brain and plasma levels but decreased corticosterone in plasma and THDOC in brain cortex, completely antagonized the foot shock-induced increase in brain steroid content. In contrast, FG 7142 mimicked the effect of stress by increasing steroid concentrations both in plasma and brain.

To our knowledge this is the first evidence that the effect of stress on brain neuroactive steroid levels is antagonized by an anxiolytic drug and mimicked by an anxiogenic ligand of the benzodiazepine binding site associated to $GABA_A$ receptors.

Since these compounds modulate in an opposite manner $GABA_A$ receptor function, FG 7142, like foot shock stress, enhances in vitro and in vivo [^{35}S]-TBPS binding while abecarnil reduces it, the present results suggest the existence of a functional relationship between changes in the $GABA_A$ receptor function, the emotional state of the animal, and the brain neuroactive steroid concentrations.

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