Pharmacology Biochemistry and Behavior, Vol. 66, No. 1, pp. 39–45, 2000

0091-3057/00/$–see front matter

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PII S0091-3057(00)00237-9

Caffeine-Induced Increases in the Brain and Plasma Concentrations of Neuroactive Steroids in the Rat

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Received 20 January 2000; Revised 01 February 2000; Accepted 02 February 2000

CONCAS, A., P. PORCU, C. SOGLIANO, M. SERRA, R. H. PURDY AND G. BIGGIO. *Caffeine-induced increases in the brain and plasma concentrations of neuroactive steroids in the rat.* PHARMACOL BIOCHEM BEHAV **66**(1)39–45, 2000.—The effects of caffeine, a naturally occurring stimulant, on the brain and plasma concentrations of neuroactive steroids were examined in the rat. A single intraperitoneal injection of caffeine induced dose- and time-dependent increases in the concentrations of pregnenolone, progesterone, and 3α-hydroxy-5α-pregnan-20-one (allopregnanolone) in the cerebral cortex. The increases were significant at a caffeine dose of 25 mg/kg and greatest (+188, +388, and +71%, respectively) at a dose of 100 mg/kg in rats killed 30 min after caffeine administration. Caffeine also increased the plasma concentrations of pregnenolone and progesterone with a dose-response relation similar to that observed in the brain, whereas the caffeine-induced increase in the plasma concentration of allopregnanolone was maximal at a dose of 50 mg/kg. Caffeine increased the plasma concentration of corticosterone, but it had no effect on the brain or plasma concentrations of 3α,21-dihydroxy-5α-pregnan-20-one and dehydroepiandrosterone. Moreover, the brain and plasma concentrations of pregnenolone, progesterone, and allopregnanolone were not affected by caffeine in adrenalectomized-orchiectomized rats. These results suggest that neuroactive steroids may modulate the stimulant and anxiogenic effects of caffeine. © 2000 Elsevier Science Inc.

Caffeine Allopregnanolone Neuroactive steroids Corticosterone HPA axis Brain Plasma Rat

CAFFEINE, a methylxanthine that exhibits a variety of stimulant effects on the central nervous system, is one of the most widely used psychotropic substances. It is consumed as a component of tea, coffee, and cola drinks, and also enters the body in the form of medications used to treat asthma (38) as well as apnea in newborns (8). Ingestion of moderate doses of caffeine usually reduces drowsiness and fatigue and results in a more rapid and clearer flow of thought (39), effects similar to those elicited by anxiogenic drugs (19,23) and opposite to those induced by drugs usually used in the treatment of anxiety (21). In higher doses, caffeine induces nervousness and insomnia in normal individuals (22), and it increases the level of anxiety, especially in patients prone to anxiety and panic attacks (41). Moreover, in excessive doses, caffeine produces tonic-clonic seizures in both rats (12) and mice (27).

Caffeine alters the function of various neurotransmitter systems that contribute to the regulation of cognitive processes, emotional state, sleep pattern, arousal, and fear (30), effects that are similar to those of stressful stimuli or to changes associated with mood or emotional disorders. Physiologic or pharmacologic conditions, such as stress, the menstrual cycle, and pregnancy (2–4,14,16,36), as well as treatment with anxiogenic or antidepressant drugs (2,4,42,43), that affect mood, emotional state, or cognitive function have recently been shown to induce rapid and marked increases in the concentrations of certain neuroactive steroids in the plasma and brain of animals or humans. Among the neurosteroids so affected, 3α-hydroxy-5α-pregnan-20-one (allopregnanolone) and 3α,21-dihydroxy-5α-pregnan-20-one (allotetrahydrodeoxycorticosterone, or THDOC) are the most

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potent and efficacious positive allosteric modulators of γ-aminobutyric acid type A ($GABA_A$) receptors (24,26). The systemic administration or direct intracerebroventricular injection of these steroid derivatives induces pharmacologic (anxiolytic, sedative, hypnotic, anticonvulsivant) effects similar to those elicited by anxiolytic drugs (5,7,13,28). These observations have suggested that changes in the synthesis of neuroactive steroids might contribute directly not only to normal brain physiology but also to a variety of neurologic and psychiatric disorders. Indeed, a selective decrease in the plasma and cerebrospinal fluid concentrations of allopregnanolone, and the normalization of these concentrations after treatment with antidepressant drugs, has been described in individuals with major depression (40,43). Moreover, the plasma concentration of allopregnanolone appears to correlate with a feeling of well being in women with premenstrual syndrome (44).

We have now investigated whether the administration of caffeine, a drug able to induce behavioral and neurochemical changes similar to those triggered by stressful events, affects the concentrations of allopregnanolone or THDOC, or those of their precursors progesterone and pregnenolone, in the brain or plasma of rats. Moreover, in order to establish the role of the brain in the effect of caffeine on the concentration of neuroactive steroids, we examined the effect of this drug in rats that had been subjected to adrenalectomy-orchiectomy (Adx-Orx).

METHOD

Animals

Male Sprague-Dawley CD rats (Charles River, Como, Italy) with body masses of 200 to 250 g were studied. After arrival at the animal facility, rats were acclimatized to the new housing conditions for at the least 1 week. Animals were housed six per cage under an artificial 12-h-light, 12-h-dark cycle (light on from 0800 to 2000 h) 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.

Adrenalectomy-orchietomy was performed under chloral hydrate anesthesia (400 mg per kilogram of body mass IP), after which the rats received isotonic saline as drinking water. Animal care and handling throughout the experimental procedures were in accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC).

Drug Treatment

Caffeine was dissolved in distilled water at a concentration appropriate for an injection volume of 3 ml/kg. Control rats received an equal volume of distilled water. Rats were injected with a single dose of caffeine (25 to 100 mg/kg IP) 15, 30, 60, or 120 min before killing.

Steroid Extraction and Assay

Animals were killed either by guillotine (for plasma steroid measurements) or by focused microwave irradiation (70 W/cm for 4 s) to the head (for brain steroid measurements). This latter procedure results in a virtually instantaneous inactivation of brain enzymes, thus minimizing postmortem steroid metabolism. Brains were rapidly (<1 min) removed, and the cerebral cortices were dissected and frozen at −20°C until steroid extraction. Blood was collected from the trunk into heparinized tubes and centrifuged at 900 × g for 20 min, after which the plasma was frozen until assayed for steroids.

Steroids were extracted and purified as previously described (3). Briefly, steroids present in cerebral cortical homogenates (400 mg of tissue in 4 ml of phosphate-buffered saline) were extracted three times with an equal volume of ethyl acetate. The combined organic phases were dried under vacuum, the resulting residue was dissolved in 5 ml of *n*-hexane and applied to Seppak-silica cartridges (Waters), and residue components were eluted with a mixture of *n*-hexane and 2-propanol (7:3, v/v). Steroids were further purified by high-performance liquid chromatography on a 5-μm Lichrosorb-diol column (250 × 4 mm) with a gradient of 2-propanol in *n*-hexane. Because we previously observed that cholesterol, which coelutes from the Lichrosorb-diol column with progesterone, reduces the sensitivity of the radioimmunoassay (RIA) for progesterone, this latter steroid was separated from cholesterol by washing the corresponding dried column fractions twice with 200 μl of dimethylsulfoxide and 400 μl of water. 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 RIA 5- to 10-fold relative to 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) of ^{3}H-labeled standards to the brain tissue homogenate. Steroids were then quantified by RIA as previously described (3,36). Protein concentration was measured as described (25) with bovine serum albumin as standard. Plasma steroid concentrations were measured in 1 ml of plasma after extraction three times with 1.5 ml of ethyl acetate.

Chemicals

Caffeine, pregnenolone, progesterone, allopregnanolone, THDOC, DHEA, and corticosterone were from Sigma (Milan, Italy). Rabbit or sheep antiserum to allopregnanolone and THDOC were generated and characterized as previously described (36). Antisera to pregnenolone, progesterone, DHEA and corticosterone were obtained from ICN (Costa Mesa, CA). All other reagents and organic solvents (HPLC grade) were of the best available quality from commercial sources.

Statistical Analysis

Data are presented as means ± SEM. The statistical significance of differences was assessed by analysis of variance followed by Newman-Keuls test. A p value of < 0.05 was considered statistically significant.

RESULTS

Effects of Caffeine on Neuroactive Steroid Concentrations in the Cerebral Cortex

The injection of caffeine (25 to 100 mg/kg IP) resulted in dose-dependent increases in the cerebral cortical concentrations of pregnenolone, progesterone, and allopregnanolone that were apparent after 30 min (Fig. 1). The effects of caffeine on the cortical concentrations of these three steroids were already significant (+72, +90, and +47%, respectively) at the dose of 25 mg/kg, with the greatest observed effects (+188, +388, and +71%, respectively) apparent at the dose of 100 mg/kg. In contrast, caffeine had no significant effects on the brain concentrations of THDOC and dehydroepi-

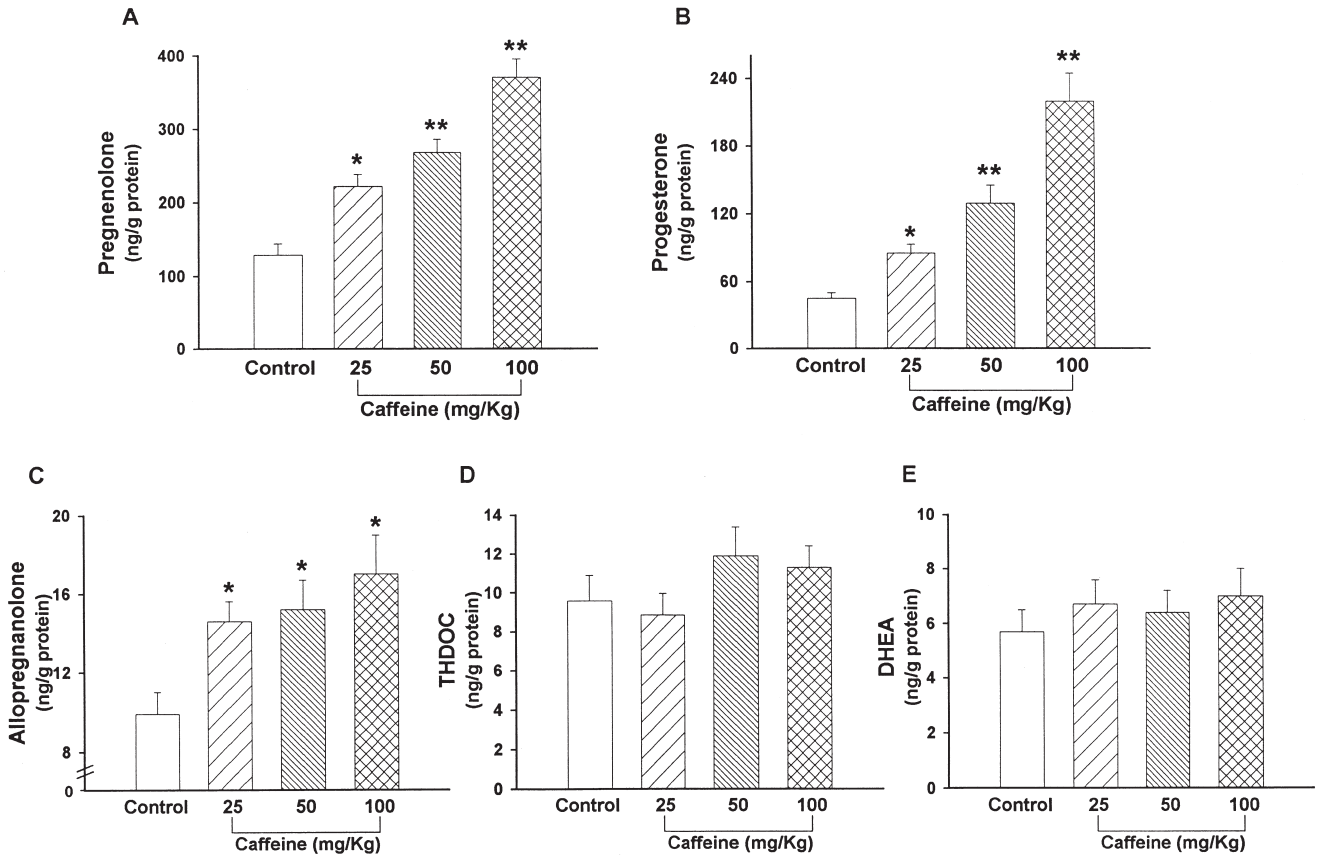

FIG. 1. Effects of caffeine on steroid concentrations in the cerebral cortex. Rats were injected with caffeine (25 to 100 mg/kg IP) 30 min before killing, after which the concentrations of pregnenolone (A), progesterone (B), allopregnanolone (C), THDOC (D), and DHEA (E) were measured in cerebrocortical homogenates. Control animals received the same volume of vehicle. Data are expressed as nanograms of steroid per gram of homogenate protein and are means ± SEM of values from 10 to 15 rats. $*p < 0.05$, $**p < 0.01$ vs. the respective control value.

androsterone (DHEA), a steroid formed metabolically by side chain cleavage of pregnenolone.

The effects of caffeine at the dose of 25 mg/kg on the cerebrocortical concentrations of pregnenolone, progesterone, and allopregnanolone were also time dependent (Fig. 2). The brain concentration of pregnenolone was significantly increased (+67%) 15 min after drug administration and was maximal (+109%) at 60 min. The concentration of progesterone was maximal at 15 min (+183%), remained significantly greater than that in control rats at both 30 and 60 min (∼+90%), and had returned to control values by 120 min after the administration of caffeine. The cortical concentration of allopregnanolone was maximal (+42%) at 30 min and had returned to control values by 60 min. The concentrations of THDOC and DHEA were not affected by caffeine administration at any of the times examined.

Effects of Caffeine on Neuroactive Steroid Concentrations in Plasma

Intraperitoneal administration of caffeine induced dose-dependent increases in the plasma concentrations of pregnenolone and progesterone that were apparent after 30 min (Table 1), similar to its effects on the brain concentrations of these steroids. The effects of caffeine on the plasma concentrations of pregnenolone and progesterone were significant (+46 and +177%, respectively) at a dose of 25 mg/kg and greatest (+108 and +362 %) at the dose of 100 mg/kg. In contrast to its action in the brain, the maximal effect of caffeine on the plasma concentration of allopregnanolone was apparent at the dose of 50 mg/kg. The plasma concentrations of THDOC and DHEA were not significantly affected by caffeine, whereas that of corticosterone, the major adrenal corticosteroid in the rat, was increased by caffeine in a dose-dependent manner. The effect of caffeine on the plasma concentration of corticosterone was significant (+96%) at the dose of 25 mg/kg and greatest (+304%) at the dose of 100 mg/kg.

Effects of Adx-Orx on the Caffeine-induced Changes in the Concentrations of Neuroactive Steroids in the Cerebral Cortex and Plasma of Rats

To determine whether the increases in the cerebrocortical concentrations of neuroactive steroids induced by caffeine were attributable to increased synthesis of these steroids in the brain or to an increased supply from peripheral steroidogenic tissues, we examined the effects of caffeine in rats that had been subjected to Adx-Orx 1 week previously. As expected (11), the brain and plasma concentrations of pregnenolone, progesterone, allopregnanolone, and THDOC were markedly decreased in Adx-Orx rats (Table 2). Furthermore, caffeine (25 mg/kg IP) had no significant effects on the

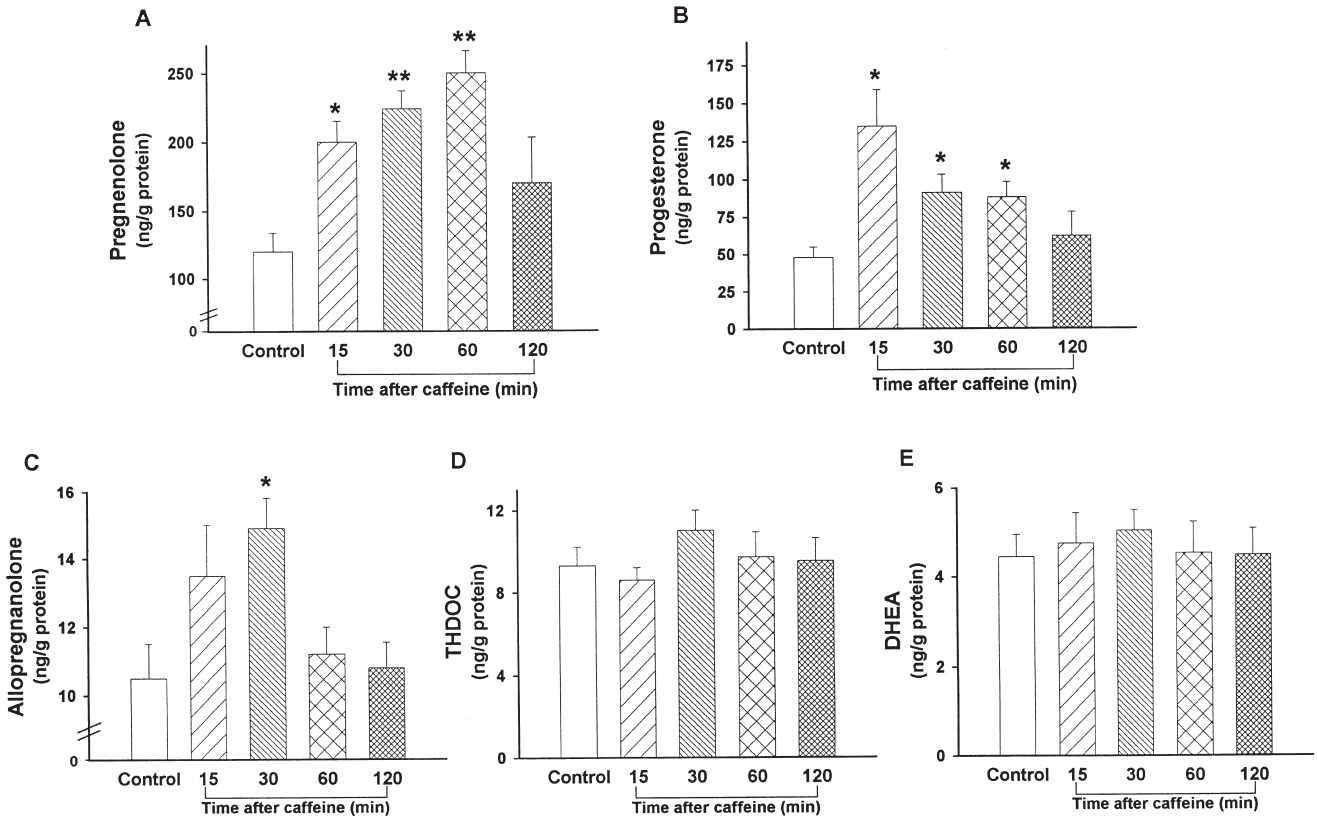

FIG. 2. Time courses of the effects of caffeine on cerebrocortical steroid concentrations. Rats were injected with caffeine (25 mg/kg IP) at the indicated times before killing, after which the concentrations of pregnenolone (A), progesterone (B), allopregnanolone (C), THDOC (D), and DHEA (E) were determined in cerebrocortical homogenates. Control animals received the same volume of vehicle. Data are expressed as nanograms of steroid per gram of homogenate protein and are means ± SEM of values from 8 to 10 rats. *$p < 0.05$, **$p < 0.01$ vs. the respective control group.

concentrations of these steroids in either the cerebral cortex or plasma of Adx-Orx rats measured 30 min after treatment.

DISCUSSION

We have shown that the IP administration of caffeine, a naturally occurring stimulant of the central nervous system, resulted in dose-dependent increases in the plasma and brain concentrations of allopregnanolone as well as in those of its precursors pregnenolone and progesterone. The doses of caffeine required for these effects are similar to those that induce anxiety-like behavior in rats. Caffeine induces experimental anxiety in the open-field and elevated plus maze tests (6) as well as reduces levels of social interaction among rats (20).

The effects of caffeine on the plasma and brain concentrations of neuroactive steroids are similar to those of anxiogenic drugs (2,4), including those of direct and indirect inhibitors of the $GABA_A$ receptor complex that induce proconflict effects

TABLE 1

EFFECTS OF CAFFEINE ON PLASMA STERIOD CONCENTRATIONS

	Steroid concentration (ng/ml)					
Treatment	Pregnenolone	Progesterone	Allopregnanolone	THDOC	DHEA	Corticosterone
Control	3.7 ± 0.4	3.9 ± 0.84	2.7 ± 0.3	3.51 ± 0.4	0.32 ± 0.04	137 ± 33
Caffeine (25 mg/kg)	5.4 ± 0.2*	10.8 ± 0.9**	3.7 ± 0.4	5.4 ± 0.5	0.29 ± 0.03	269 ± 20*
Caffeine (50 mg/kg)	7.2 ± 0.8*	12.4 ± 1.7**	4.2 ± 0.3*	5.2 ± 0.5	0.22 ± 0.01	392 ± 45**
Caffeine (100 mg/kg)	7.7 ± 1.0*	18.0 ± 1.9**	3.9 ± 0.2*	5.0 ± 0.6	0.41 ± 0.06	554 ± 58**

Caffeine was administered intraperitoneally 30 min before killing. Control animals received the same volume of vehicle.

Data are expressed as nanograms of steroid per milliliter of serum and are means ± SEM of values from 15 to 20 rats. *$p < 0.05$, **$p < 0.01$ vs. the respective control value.

TABLE 2

EFFECTS OF CAFFEINE ON CEREBROCORTICAL AND PLASMA CONCENTRATIONS OF NEUROSTEROIDS IN Adx-Orx RATS

	Cerebral cortex (ng/g protein)			Plasma (ng/ml)		
Steroid	Sham surgery	Adx-Orx	Adx-Orx + caffeine	Sham surgery	Adx-Orx	Adx-Orx + caffeine
Pregnenolone	109.2 ± 7.3	68.6 ± 2.9*	72.0 ± 3.2	5.4 ± 0.4	2.1 ± 0.2*	2.5 ± 0.3
Progesterone	47.2 ± 3.2	22.2 ± 2.1*	25.4 ± 2.4	5.4 ± 0.3	0.9 ± 0.06*	1.0 ± 0.07
Allopregnanolone	8.7 ± 0.6	4.7 ± 0.3*	4.9 ± 0.3	3.6 ± 0.4	1.4 ± 0.1*	1.6 ± 0.39
THDOC	7.8 ± 0.6	4.4 ± 0.5*	4.0 ± 0.3	3.8 ± 0.35	1.2 ± 0.09	1.5 ± 0.1

Caffeine (25 mg/kg, IP) or vehicle was administered 30 min before killing.

Data are expressed as nanograms of steroid either per nanogram of cerebrocortical homogenate protein or per milliliter of serum, and are means ± SEM of values from 8 to 12 rats. *$p < 0.05$ vs. corresponding value for sham-operated rats.

in rats and experimental anxiety in humans and other primates (15,19,32). Moreover, the effects of caffeine are also similar to those induced by various acute stress paradigms. Thus exposure of rats to mild foot shock, CO_2 inhalation, forced swimming, or handling maneuvers that precede killing induces rapid and marked increases in both brain and plasma concentrations of neuroactive steroids (2–4,36), and these effects are antagonized by systemic administration of anxiolytic drugs (2,4). Classical anxiolytic agents also antagonize the anxiogenic effects of caffeine both in humans and rodents (37). Our present data together with the results of previous studies (2–4,36) thus show that both pharmacologic treatments and experimental conditions that induce anxiety-like or conflict behavior also induce increases in the plasma and brain concentrations of neuroactive steroids.

The caffeine-induced increases in the cerebrocortical concentrations of neuroactive steroids showed distinct time courses. Whereas the concentration of progesterone was maximal at 15 min after caffeine administration and remained significantly increased at 60 min, the concentrations of pregnenolone and allopregnanolone increased more slowly, reaching a peak at 60 and 30 min, respectively. The rapid increase in the brain concentration of progesterone might result from an increased conversion of pregnenolone to progesterone and might underlie a subsequent increase in the synthesis of allopregnanolone. The delayed increase in the brain concentration of allopregnanolone may thus be a consequence of the rapid and greater increase in that of progesterone and may be of functional relevance to the stimulant effect of caffeine. Given that allopregnanolone is one of the most potent positive modulators of $GABA_A$ receptors (24,26), the delayed increase in the brain concentration of this neurosteroid may represent a homeostatic mechanism to reduce or counteract the neuronal excitability and anxiogenic-like action elicited by caffeine. This conclusion is consistent with our previous data showing acute stress is associated with a rapid (<10 min) and marked increase in the brain concentration of progesterone but with a slower and longer-lasting increase (maximal at 30 min) in that of allopregnanolone (2–4). It is worth nothing that while the maximum increase in plasma allopregnanolone is achieved at a lower dose of caffeine (50 mg/kg) the increase in either plasma pregnenolone and progesterone or the allopregnanolone response in the brain is dose-related being greatest at 100 mg/kg. These data might suggest that in the rat brain the synthesis/accumulation of allopregnanolone seems to be strictly related to the plasma and brain progesterone concentration and not to the plasma level of allopregnanolone and invite to speculate that, under the effect of caffeine, the synthesis of allopregnanolone in the brain cortex might be greater than in plasma: for instance, a high level of progesterone in plasma may be more economically maintained with an inhibition of progesterone metabolism.

Caffeine induces a stresslike response of the hypothalamic-pituitary-adrenal (HPA) axis in rats. Thus through a stimulatory action on the release of corticotropin-releasing factor (CRF), caffeine elicits marked increases in the plasma concentrations of corticosterone and adrenocorticotropic hormone (ACTH) (31). Moreover, caffeine induces behavioral and neurochemical effects in rats that are similar to those triggered by CRF or stressful stimuli (4,9,36). The HPA axis plays an important role in determining the peripheral and central concentrations of neuroactive steroids either under stressful conditions or after administration of anxiogenic drugs (4). Thus the observations that caffeine induces both neurotransmitter release (10,33) and anxiety-like behavior associated with increases in the plasma and brain concentrations of neuroactive steroids suggest that the HPA axis might mediate such actions of caffeine. This conclusion is also consistent with our present data showing that caffeine failed to increase the concentrations of neuroactive steroids in either the brain or plasma of Adx-Orx rats, and that this drug elicited a marked increase in the plasma concentration of corticosterone in control rats. However, the possibility that the adrenalectomy-induced removal of catecholamines as well as at other agents produced by the adrenal cortex might influence the synthesis of neurosteroids in the brain cannot be ruled out.

A single dose of allopregnanolone or progesterone is able to inhibit the stress-induced release of CRF, ACTH, and corticosterone as well as to reduce the anxiogenic effect of CRF (34). Moreover, allopregnanolone, like benzodiazepines, prevents the stress-induced increases in dopamine and acetylcholine release in the rat cerebral cortex and hippocampus, respectively (18,29). Thus the transient increase in the brain concentration of allopregnanolone triggered by caffeine may reflect a physiologic mechanism for reducing the activation of the neuroendocrine and neurochemical pathways associated with the state of arousal and for limiting the extent of neuronal excitability. This conclusion is consistent with the notion (4,36) that neuroactive steroids function to counteract overexcitation of the central nervous system.

It is not clear why caffeine did not affect the brain and plasma concentrations of THDOC, whose synthesis appears to be restricted largely to the adrenal glands (35). However,

this observation is consistent with our previous data showing that the brain concentration of this neuroactive steroid in rats is differentially affected by various stressors. It was thus not affected by CO_2 inhalation and handling (2,3) but was markedly increased by foot shock and forced swimming (2,4,36). Differences in the neurotransmitter systems or molecular endocrinologic mechanisms that underlie the actions of caffeine and various stressors may contribute to the observed differences in the effects of these stimuli on the concentrations of neuroactive steroids.

A competitive antagonistic action at both A_1 and A_2 adenosine receptors is thought to be primarily responsible for the physiologic, biochemical, and behavioral effects of caffeine (17). Caffeine-sensitive adenosine receptor subtypes thus likely mediate the regulation of the HPA axis by this drug. Indeed, deamination of endogenous adenosine by treatment with adenosine deaminase was previously shown to result in an increase in ACTH release from anterior pituitary cells, suggesting that endogenous adenosine may exert an inhibitory tone on the release of this hormone (1). Blockade of adenosine receptors by caffeine might thus underlie the caffeine-induced increases in the abundance of neuroactive steroids.

In conclusion, our data provide the first biochemical evidence for an effect of caffeine on brain neurosteroid concentrations. Because both caffeine and neurosteroids are implicated in the regulation of emotional behavior, our data may shed new light on the neurochemical mechanisms that underlie the neurobiology and pharmacology of anxiety.

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