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Effect of Conditioned Fear Stress on Serotonin Metabolism in the Rat Brain

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INOUE, T., T. KOYAMA AND I. YAMASHITA. *Effect of conditioned fear stress on serotonin metabolism in the rat brain.* PHARMACOL BIOCHEM BEHAV **44**(2) 371–374, 1993.—The effects of electric foot-shock stress (EFS) and conditioned fear stress (CFS) on serotonin [5-hydroxytryptamine (5-HT)] metabolism in seven various brain regions of the rat were studied by measuring tryptophan, 5-HT and 5-hydroxyindoleacetic acid (5-HIAA). EFS for 30 min increased tryptophan levels in almost all brain regions but did not change 5-HT levels in any regions. It increased 5-HIAA levels in the medial prefrontal cortex (mPFC), nucleus accumbens, and amygdala. CFS (exposure to an environment paired previously with foot-shock) increased defecation and induced freezing behavior. It failed to change tryptophan and 5-HT levels in any brain regions but increased 5-HIAA level only in the mPFC. In contrast to EFS, which increased 5-HT metabolism in several other brain regions, increased metabolism of 5-HT was especially marked in the mPFC after CFS, regarded as psychological stress.

Serotonin 5-HIAA Tryptophan Conditioned fear stress Freezing behavior Foot-shock stress Medial prefrontal cortex

STRESS has been reported to increase serotonin [5-hydroxytryptamine (5-HT)] metabolism by several studies (1,2,6,8, 9,17,20,21,23,26). Acute stress (restraint and electric shock) increases brain 5-hydroxyindoleacetic acid (5-HIAA) levels (2,6,8,17,20,21,23). These results suggest that stress activates central serotonergic systems. Because the stressors used by these studies were restraint and foot-shock, these treatments gave physical influence to rats in the experiments. There remains a possibility that increased metabolism of 5-HT is produced by physical stimuli (pain, etc.).

Fanselow reported that rats exhibited freezing behavior when tested in the same location where they had been shocked following a 24-h interval after delivery of electric foot-shock, and this suggested that postshock freezing was produced by conditioned fear elicited by cues associated with shock (10). Recently, two classes of anxiolytics, diazepam (5,11) and buspirone (5), have been reported to reduce freezing response. Thus, conditioned fear stress (CFS) is regarded as a psychological stress without physical stimuli and a simple animal model of anxiety or fear. There have been relatively few studies of the effects of CFS on brain monoamine metabolism (3,15,29). Although CFS has been reported to increase dopamine metabolism (3,15) and noradrenaline metabolism (29), little is know about 5-HT metabolism.

The purpose of this study is to determine whether CFS changes 5-HT metabolism in the rat brain. We examined the behavioral changes and measured the levels of tryptophan, 5-HT, and 5-HIAA in the discrete rat brain regions, comparing with the effect of foot-shock stress.

METHOD

Animals

Male Sprague-Dawley rats obtained from Shizuoka Laboratory Animal Center (Shizuoka, Japan), weighing 250–300 g at the start of the experiment, were housed four per cage and maintained in a 12 L : 12 D, temperature-controlled environment, with free access to food and water. Experiments began after a 14-day period of acclimatization.

Experimental Procedures

In electric foot-shock stress (EFS) sessions, rats were subjected to inescapable EFS for 30 min [2.5-mA scrambled shock, variable-interval schedule with a mean intershock interval of 30 s (5–55 s) and shock duration of 30 s] in a chamber with a grid floor (19 × 22 × 20 cm, Medical Agent Co., Kyoto, Japan). Control rats were placed in the shock chamber but no current was applied to the floor of the chamber. One group of animals was decapitated immediately and another group 24 h after the end of the stress period.

In CFS sessions, rats were subjected to inescapable electric foot-shock (same parameters as above) for 30 min. Control rats were placed in the shock chamber for 30 min but shocks

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were not delivered. Twenty-four hours after the shock, rats of either group were again placed and observed for 30 min in the shock chamber but no current was applied to the floor of the chamber. During the observational period, freezing behavior and defecation score (bolus count/30 min) were recorded. Rats of either group were decapitated immediately after the end of the session.

Biochemical Analysis

After decapitation, brains were immediately removed, frozen, and stored at −70°C. Frozen brains were cut at 300-μm thickness in a cryostat at −10°C. Seven different brain regions were punched out with small stainless steel needles according to the method of Palkovits and Brownstein (24). Tissue obtained was homogenized by ultrasonication in 250 μl ice-cold 0.2 N perchloric acid containing 20 μg/ml *l*-cysteine. After centrifugation at 14,000× *g* for 1 min, tryptophan, 5-HT, and 5-HIAA in supernatants were assayed by high-performance liquid chromatography with electrochemical detection (HPLC-ECD). The HPLC system consisted of an EP-10 liquid chromatograph pump (Eicom, Kyoto, Japan), an ERC-3310 degasser (ERMA, Tokyo, Japan), a reversed-phase ODS column, chemcosorb 5-ODS-H 250 × 4.6mm (Chemco, Tokyo, Japan), an ECD-100 electrochemical detector (Eicom), and a Chromatocorder 12 (SIC, Tokyo, Japan). The mobile phase for 5-HT and 5-HIAA assay was 0.1 M phosphate buffer, pH 3.2, containing 11% methanol, 1.7% tetrahydrofuran, 60 mg/l Na_2EDTA, and 350 mg/l octanesulfonic acid. The mobile phase for tryptophan assay was 0.1 M sodium acetate buffer, pH 4.5, containing 10% methanol and 20 mg/l sodium octyl sulfate. The buffer was filtered before use. Separations were conducted isocratically at 35°C with a flow rate of 1.0 ml/min. The electrochemical detector was set at a potential of 0.7 V for 5-HT and 5-HIAA assay and 0.85 V for tryptophan assay. Protein levels in pellets were measured by the Lowry et al. method (22), using bovine serum albumin as a standard.

Behavioral Observations

Behavior was recorded using a time-sampling procedure (10) during 30 min. Every 10 s, the behavior in which the animal was currently engaged was classified as either freezing or activity. Freezing was defined as the lack of all observable movement of the body and vibrissae except those related to respiration. All other behavior was scored as activity. Defecation score (bolus count/30 min) was recorded.

Data Analysis

All data are presented as the means ± SEM of the individual values of rats from each group. The statistical analysis of the data was performed using the two-tailed Student's *t*-test. The effects of foot-shock stress on freezing behavior were evaluated by Mann-Whitney's *U*-test.

RESULTS

Freezing and Defecation

In the CFS group, freezing behavior was observed markedly during the initial 10 min but not observed in controls (Fig. 1).

CFS significantly increased defecation for 30 min [defecation score (bolus count/30 min): CFS group, 6.8 ± 0.6, n = 8; control group, 1.8 ± 0.6, n = 8].

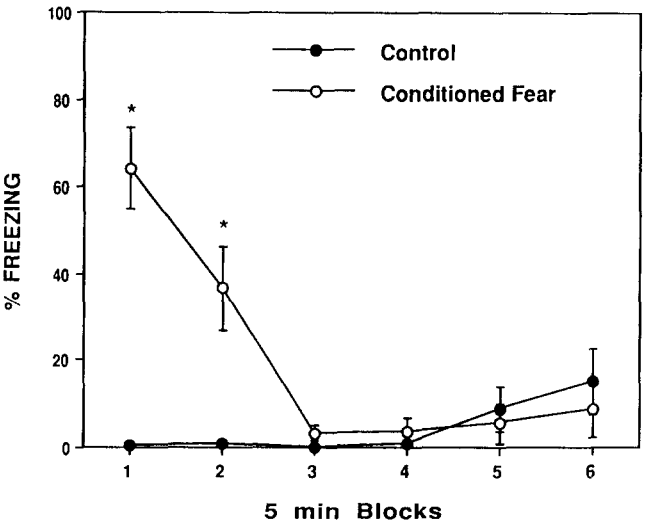

FIG. 1. Mean percent ± SEM of freezing scored for each 5-min block of testing. Behavior was sampled at 10-s intervals. Significantly different from controls (*$p < 0.01$).

5-HT Metabolism

EFS significantly increased tryptophan levels in the medial prefrontal cortex (mPFC), nucleus accumbens, striatum, amygdala, lateral hypothalamus, and hippocampus (Fig. 2). EFS did not change 5-HT levels in any brain regions but significantly increased 5-HIAA levels in the mPFC, nucleus accumbens, and amygdala (Fig. 2). Tryptophan, 5-HT, and 5-HIAA levels were not changed in any brain regions 24 h after EFS (data not shown).

CFS significantly increased 5-HIAA levels only in the mPFC but did not change tryptophan and 5-HT levels in any brain regions (Fig. 3).

DISCUSSION

When rats are placed in a situation that has come to be associated with foot-shock through the process of Pavlovian conditioning, they react with the species-specific defensive response of freezing (11). Postshock freezing is produced by conditioned fear elicited by cues associated with shock and no part of postshock freezing is an unconditional response directly elicited by shock (10). Two classes of anxiolytics, benzodiazepines (5,11) and buspirone (5), reduce the freezing response. Shock-induced freezing is enhanced by ICV administration of corticotropin-releasing factor (CRF) (25), whose systems are suggested to be involved in the stress response and attenuated by CRF antagonists ICV (19). These suggest that shock-induced freezing is modulated by endogenous CRF systems in the CNS. In the present study, CFS not only induced freezing behavior but also increased defecation, regarded as an index of emotionality (14). These results confirmed that CFS was effective as stress.

EFS was demonstrated to increase brain tryptophan levels in almost all brain regions. An increase in brain tryptophan levels during stress has been reported by several authors (6,8,9,20,21). Two studies reported that foot-shock stress increased tryptophan levels in every brain region determined (8,9). These results are consistent with our results. In contrast to EFS, we observed that CFS did not change brain trypto-

phan levels. The potentiation of CFS after repeated foot-shock stress also failed to increase brain tryptophan levels (our unpublished data). Because of the result in CFS, it is assumed that the increased levels of brain tryptophan during EFS are produced by physical stimuli such as pain or more intense emotional reaction caused by EFS.

The present study demonstrated that EFS increased 5-HIAA levels in the mPFC, nucleus accumbens, and amygdala but did not change 5-HT levels in any brain regions. Several studies have also found that acute stress increased brain 5-HIAA levels (2,6,8,20,21). Regional differences in increased 5-HT metabolism under stress were variable and increases of 5-HT metabolism in the prefrontal cortex, olfactory tubercle, hypothalamus, cingulate cortex, striatum, and hippocampus have been reported (2,8,20). In the present study, the effect of CFS on 5-HT metabolism was characteristic as compared with that of EFS. CFS did not change 5-HT levels in any brain regions but increased 5-HIAA levels only in the mPFC. Because shock-induced elevation of 5-HIAA levels diminished 24 h after foot-shock, an increase in 5-HIAA level in the mPFC induced by CFS is not due to prolonged effects of EFS. The finding that CFS increases 5-HT metabolism has not been reported by other authors.

The electrophysiological activity of the dorsal raphe nucleus serotonergic neurons has been reported to be unchanged during stress (16). Commissiong claimed that monoamine metabolite studies alone cannot be used as the sole criterion of a neurophysiological functional correlate (4). However, it has been suggested that stress-induced increases of 5-HT turnover do not merely reflect its intraneuronal metabolism but also increased functional activity of serotonergic neurons (1). In our unpublished data, foot-shock stress increased 5-HIAA level and 5-HT efflux in the mPFC determined using in vivo microdialysis although the effect of CFS on 5-HT release in the mPFC has not been studied. We would like to conclude, at least tentatively, that a CFS-induced increase of 5-HT metabolism reflects an increase in the activity of serotonergic neurons in the mPFC.

What resulted in the regional differences of 5-HT metabolism between EFS and CFS is unclear. One possible reason is that the mPFC is responsible for psychological stress and other brain regions are nonspecifically affected by physical stimuli such as pain, etc. Herman et al. reported that conditioned fear stress caused an increase in dopamine (DA) metabolism in the rat anteromedial frontal cortex but no change in several other brain regions (15). The role of the frontal cortex in emotional behavior has been also suggested by other authors (12,27). Another possible reason for the regional differences is that the serotonergic response in the mPFC is the most responsive to stress. Deutch and Roth claimed that the

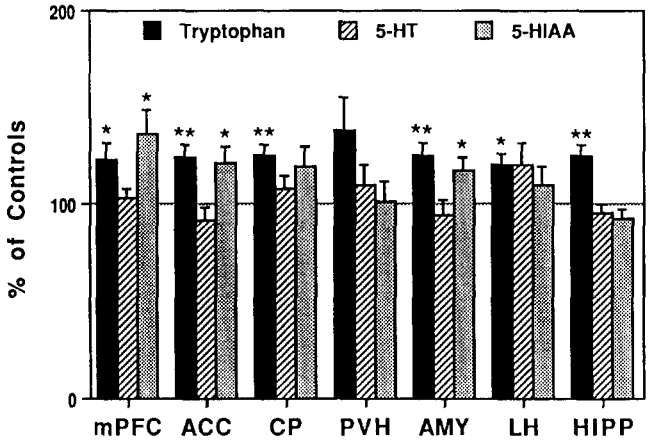

FIG. 2. Effects of electric foot-shock stress for 30 min on tryptophan, 5-hydroxytryptamine (5-HT), and 5-hydroxyindoleacetic acid (5-HIAA) levels of different brain regions. Results are means with SEM of data obtained on seven to eight rats and are expressed as percentage of respective control values. Significantly different from controls ($*p < 0.05$, $**p < 0.01$). mPFC, medial prefrontal cortex; ACC, nucleus accumbens; CP, caudate putamen; PVH, paraventricular nucleus of the hypothalamus; AMY, amygdala; LH, lateral hypothalamus; HIPP, hippocampus.

CONTROL VALUES (pmol/mg PROTEIN)

	Tryptophan	5-HT	5-HIAA
mPFC	356.9 ± 20.2	24.8 ± 1.8	4.9 ± 0.3
ACC	283.7 ± 8.3	27.0 ± 1.9	18.7 ± 1.0
CP	226.6 ± 8.1	7.0 ± 0.3	14.3 ± 1.6
PVH	370.8 ± 33.8	26.2 ± 2.3	15.9 ± 1.0
AMY	398.7 ± 13.0	26.4 ± 1.9	10.2 ± 0.3
LH	363.9 ± 17.3	16.4 ± 1.9	8.1 ± 0.8
HIPP	214.0 ± 6.2	2.6 ± 0.1	2.6 ± 0.1

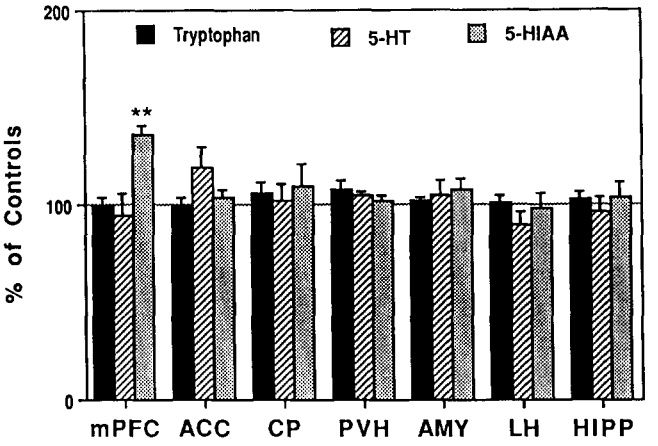

FIG. 3. Effects of conditioned fear stress for 30 min on tryptophan, 5-hydroxytryptamine (5-HT) and 5-hydroxyindoleacetic acid (5-HIAA) levels of different brain regions. Results are means with SEM of data obtained on seven to eight rats and are expressed as percentage of respective control values. Significantly different from controls ($**p < 0.01$). mPFC, medial prefrontal cortex; ACC, nucleus accumbens; CP, caudate putamen; PVH, paraventricular nucleus of the hypothalamus; AMY, amygdala; LH, lateral hypothalamus; HIPP hippocampus.

CONTROL VALUES (pmol/mg PROTEIN)

	Tryptophan	5-HT	5-HIAA
mPFC	344.1 ± 12.9	22.1 ± 1.6	7.6 ± 0.6
ACC	346.3 ± 16.1	27.5 ± 2.4	13.4 ± 1.1
CP	297.2 ± 21.5	7.8 ± 0.8	8.8 ± 1.3
PVH	364.8 ± 12.4	22.2 ± 0.5	12.9 ± 0.5
AMY	345.9 ± 5.1	22.7 ± 1.7	8.6 ± 0.5
LH	309.8 ± 14.3	22.4 ± 1.9	8.1 ± 0.7
HIPP	259.1 ± 9.6	2.9 ± 0.1	2.6 ± 0.2

selective increase in DA metabolism in the PFC observed after exposure to mild stressors is not observed after exposure to stressors of either greater intensity or after exposure of longer duration to relatively mild stressors (7). We also found that in contrast to EFS, which increased DA metabolism in several brain regions including the striatum and nucleus accumbens, increased metabolism of DA was especially marked in the mPFC after CFS in animals used in this experiment (unpublished data). The potentiation of CFS after repeated footshock stress increased DA metabolism in the mPFC and almost all other brain regions determined in the same way in which EFS increased DA metabolism in several regions (our unpublished data). These indicated that the dopaminergic response in the mPFC is the most responsive to stress. However, the serotonergic response to CFS is different from the dopaminergic one. Increased metabolism of 5-HT following CFS after repeated foot-shock is also marked in the mPFC but not observed in other regions except the paraventricular nucleus of the hypothalamus (our unpublished data). These findings suggest the possibility that the mPFC is responsible for psychological stress. Because CFS is regarded as an animal model of anxiety or fear, this biochemical response of the serotonergic system to CFS in the mPFC might be related specifically to the feeling of anxiety or fear.

Recently, 5-HT receptor-related anxiolytics have been developed, which consist of 5-HT_{1A} agonists, 5-HT_2 antagonists, and 5-HT uptake inhibitors (13,18,28). From animal and human anxiety studies, the brain serotonergic system has been implicated in the psychopathology of anxiety states. Our data may support this 5-HT hypothesis of anxiety.

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