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Effect of Psychological Stress on [^{35}S]TBPS Binding in Rat Brain

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FODDI, M. C., M. CINQUANTA AND T. MENNINI. *Effect of psychological stress on [^{35}S]TBPS binding in rat brain.* PHARMACOL BIOCHEM BEHAV **58**(2) 373–377, 1997.—This study was designed to determine whether psychological stress alters the function of the GABAergic synapse, examined as biochemical changes of [^{35}S]*t*-butylbicyclophosphorothionate ([^{35}S]TBPS) binding, in unwashed membranes of rat cerebral cortex. Psychological stress increased the number of [^{35}S]TBPS binding sites by 22%. This enhancement was very similar to that after acute foot shock (24%). Psychological stress was induced very rapidly, because only 1 day after previous foot shock exposure, [^{35}S]TBPS binding was increased by 23%. Diazepam [3 mg/kg intraperitoneally (subcutaneously)] and ipsapirone (5 mg/kg subcutaneously), injected 30 min before psychological stress, antagonized the enhancement of [^{35}S]TBPS binding. This result suggests that psychological stress is a good animal model for investigating the various biochemical changes related to stress, avoiding the physical components associated with most of the normally used stressors and mimicking only emotional state alterations. © 1997 Elsevier Science Inc.

[^{35}S]TBPS binding $GABA_A$ receptors Psychological stress Diazepam Ipsapirone

SEVERAL studies underlining the importance of the benzodiazepine (BDZ)–GABA–chloride ionophore receptor complex in the pharmacology of anxiolytic and anxiogenic drugs have also indicated that recognition sites within the supramolecular $GABA_A$ receptors may be involved in the physiopathology of stress and anxiety situations [see (4) for review]. Stress and anxiety in rats have, in fact, been associated with diminished GABAergic transmission at the GABAergic synapses (5,10). It has also been reported that the function of the GABA-coupled chloride ionophore receptor complex may be rapidly modified by the emotional state of the animal before euthanasia (9). Using an unstressed animal model of handling-habituated rats, it has been found that stress, like anxiogenic drugs, reduces the function of the $GABA_A$ receptor complex (3), an effect mimicked by in vivo administration of different inhibitors of GABAergic transmission and antagonized by anxiolytic drugs. These conclusions were based on the reported decrease in [^{3}H]GABA (6) and [^{3}H]flunitrazepam (21) binding and on an increase in [^{35}S]*t*-butylbicyclophosphorothionate ([^{35}S]TBPS) (10) binding in a brain membrane preparation of rats exposed to different stressful conditions.

Several animal models have been used to study stress, but most of the stressful stimuli differ from anxiety in humans because they are based on strong physical stimuli, such as electric shock, restraint, or noise (20,21,24). Conditioned fear stress (exposure to environmental stimuli previously coupled with inescapable foot shock) is considered a psychological stress without physical stimuli and a simple animal model of anxiety or fear (14,15,25,28). The metabolism of dopamine (16,17), noradrenaline (28), and serotonin (15,18) has been studied extensively after psychological stress, but to our knowledge no studies have been published on the effects of psychological stress on the $GABA_A$ receptor complex. Therefore, this study was designed to determine whether psychological stress alters the function of the GABAergic synapse, examined as biochemical changes of [^{35}S]TBPS binding in unwashed fresh cortical membrane preparations (10), and comparing the effect with that of acute foot shock stress.

MATERIALS AND METHODS

Stress Procedures

Male Sprague–Dawley CD-COBS rats weighing 200–225 g were used. Procedures involving animals and their care were conducted in conformity with institutional guidelines that are in compliance with national (D.L. n. 116, G.U., suppl. 40, 18 February 1992) and international laws and policies (EEC Council Directive 86/609, OJ L 358,1, 12 December 1987; *NIH*

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Guide for the Care and Use of Laboratory Animals, NIH Publ. 85-23, 1985).

Handling-habituated rats were used, housed two per cage. As previously reported (3), these rats were habituated to the manipulations that precede killing. Stress treatments consisted of electric foot shocks delivered in individual boxes with floors made of brass rods 2 cm apart. Shocks were provided by a stimulator that delivered a 0.2-mA shock of 500 ms duration every 500 ms.

The handling-habituated animals were divided into three experimental groups: a control group of rats resting unstressed (see above); an acute foot shock group of rats that received a 10-min foot shock session on the day of the experiment; and a psychological stress group consisting of rats exposed to 4-min foot shock sessions daily for 3 days and, on the 4th day, exposed to a sham foot shock session in which they were put in the same cage where they had experienced the foot shock.

On the day of the experiment, some animals in each group were injected with diazepam [3 mg/kg intraperitoneally (IP)], ipsapirone [5 mg/kg subcutaneously (SC)], or 1-pyrimidinylpiperazine (2 mg/kg IP) 30 min before the stress procedure. Each group consisted of two rats put in the same cage. All rats were killed with a guillotine immediately after the end of the foot shock session or environmental stimuli. Two animals per group were randomly used in each experimental session; experiments were repeated three times.

[^{35}S] TBPS Binding

After killing, the brains were rapidly removed and the cerebral cortices were dissected out on ice. Chilled homogenization buffer was used for the measurement of [^{35}S]TBPS binding. The tissue was homogenized within 5 min of death with an Ultra-Turrax (TP 18-10 setting 3, for 20 s) in 50 vol of ice-cold 50 mM Tris-citrate buffer (pH 7.5 at 25°C) containing 100 mM NaCl, then centrifuged at 20,000 × *g* for 20 min. The resulting pellet was resuspended in 50 vol of 50 mM Tris-citrate buffer, pH 7.5, and used for binding assay.

[^{35}S]TBPS binding (10) was determined in a final volume of 500 μl consisting of 200 μl of tissue homogenate (0.2–0.3 mg protein), 50 μl of [^{35}S]TBPS (specific activity 70–100 Ci/mmol; NEN) at the appropriate concentrations (final assay concentration 2 nM in experiments other than saturation analysis), 50 μl of 2 M NaCl (final concentration 0.2 M), 50 μl of drugs or solvent, and buffer to volume. The concentration of 2 nM was selected in agreement with previous studies (10,23,24) to keep the radioactivity per sample to an acceptable level (150,000–200,000 dpm/sample). On the other hand, this ligand concentration, which is about 1/50 its K_d value (10,11), allows detection of possible variation in either the K_d or B_{max} value (22). After 90 min incubation at 25°C, the samples were rapidly filtered through Whatman GF/B glass-fiber filters that were rinsed with two 4-ml aliquots of ice-cold Tris-citrate buffer using a filtration manifold (Brandel, model M-48). Filter-bound radioactivity was measured by liquid scintillation spectrometry. Nonspecific binding was defined as binding in the presence of 100 μM picrotoxin.

Proteins were assayed by the method of Lowry et al. (19) using bovine serum albumin as standard. Saturation experiments were based on nine different concentrations of ligand (2.5–500 nM). The specific activity of the radioligand was kept constant at 2.5 nM, then diluted with unlabelled TBPS. Kinetic parameters (K_d and B_{max}) were calculated using the LIGAND (22) program running on an IBM AT personal computer.

Chemicals

TBPS (RBI, Natick, MA, USA) was dissolved in dimethyl sulfoxide to an initial concentration of 2 mM, and serial dilutions were made up in 50 mM Tris-citrate buffer, pH 7.4. Ipsapirone (Troponwerke Gmbh, Cologne, Germany) was dissolved in saline and injected SC; diazepam (Roche, Basel, Switzerland) was injected IP as a saline suspension containing two drops of Tween 80.

Statistical Analyses

The data were analysed by one-way and two-way analysis of variance (ANOVA) followed by post hoc comparison (Tukey's *t*-test). The significance of the difference between the two B_{max} values (i.e., controls and stressed rats) was assessed by the extra sum of squares *F*-test criterion on the residual variances, included in the LIGAND program (22).

RESULTS

Acute foot shock stress increased [^{35}S]TBPS binding by 24% (Fig. 1) in unwashed membrane of the rat cerebral cortex, confirming previously reported data (10). Like acute foot shock stress, psychological stress obtained with a sham shock session after three foot shock sessions (4 min, once per day) also significantly enhanced [^{35}S]TBPS binding by 22% (Fig. 1). The similar increases in [^{35}S]TBPS binding found after acute and repeated foot shocks indicated that rats do not become "habituated" to stress after the 3 days of 4-min foot shock sessions.

Saturation experiments in the presence of increasing concentrations of TBPS (Fig. 2) showed that the increase in

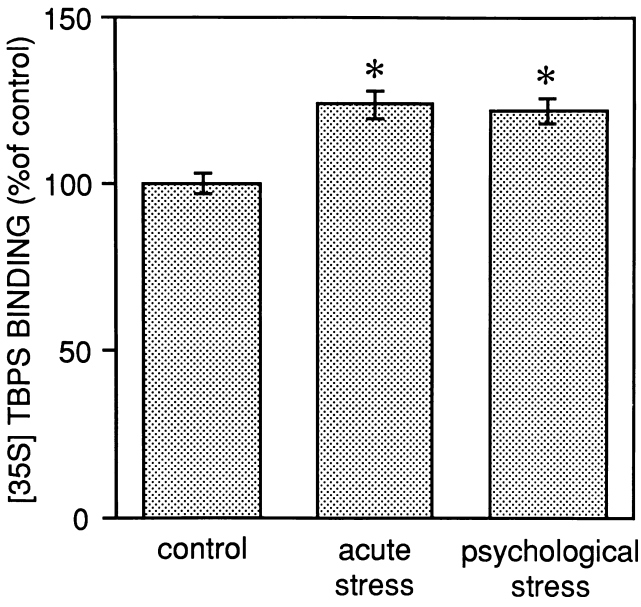

FIG. 1. Psychological stress, like acute foot shock stress, increases [^{35}S]TBPS binding to unwashed rat cortical membranes. *$p < 0.01$ vs. control rats, ANOVA and Tukey's test. Data are means ± SD of six animals per group. Psychologically stressed rats were exposed to foot shock for 4 min, once per day for 3 days, before a sham foot shock session. Acutely stressed rats were exposed to acute foot shock for 10 min the day of the experiment. All rats were killed immediately after the stress session.

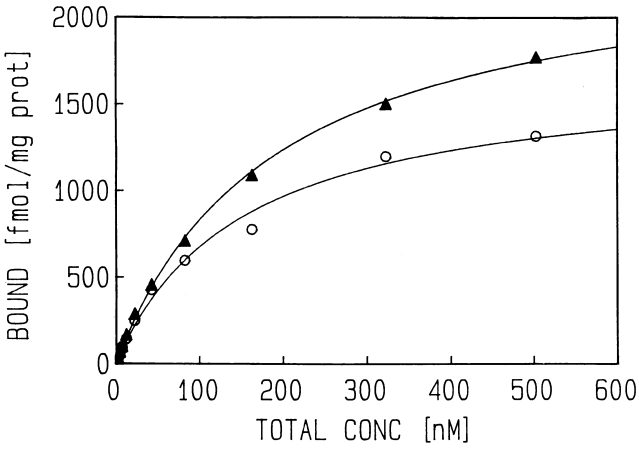

FIG. 2. Psychological stress increases the density of [35S]TBPS binding sites to unwashed rat cortical membranes. Data are from a representative experiment that was replicated three times with very similar results. Nonlinear regression fitting of the saturation curves yielded the following parameters: control rats (○—○): B_{max} = 1469 ± 88, K_d = 105 ± 9; psychologically stressed rats (△—△): B_{max} = 1773 ± 141*, K_d = 122 ± 13. Units: B_{max}, fmol/mg protein ± SD; K_d, nM ± SD. *$p < 0.01$, F-test.

[35S]TBPS binding elicited by psychological stress was exclusively due to a rise in the maximum number of binding sites (B_{max}), with no significant changes in affinity (K_d). The psychological stress could be induced very rapidly in rats, because a similar increase in [35S]TBPS binding (36 ± 2.2%) was found when the sham shock session was done 1 day after a single foot shock exposure.

We also verified the effect of anxiolytic drugs on the increase in [35S]TBPS binding induced by psychological stress. Diazepam was given at a dose of 3 mg/kg IP 30 min before the sham foot shock session, which was carried out after 3 days of 4-min foot shock sessions once per day. Diazepam completely prevented the increase in [35S]TBPS binding associated with the psychological stress (Fig. 3). Figure 4 shows that ipsapirone, 5 mg/kg SC, given 30 min before the sham foot shock session also completely antagonized the effect of psychological stress on [35S]TBPS binding without affecting it in unwashed membranes from handling-habituated rats. 1-Pyrimidinyl-piperazine, a metabolite of ispapirone, injected at a dose of 2 mg/kg IP, did not prevent the increase in [35S]TBPS binding induced by stress (data not shown).

DISCUSSION

The binding of [35S]TBPS to unwashed brain membranes ex vivo is a valid measure of the function of GABAergic synapses in vivo, as it increases when GABAergic transmission is decreased and vice versa (10). This approach has been used to characterize the decrease in GABAergic transmission following foot shock in handling-habituated rats (4,11,24). Although unwashed membrane preparations (containing endogenous substances) are required to show functional modulation of [35S]TBPS binding (10), an increase in [35S]TBPS binding elicited by swim stress has also been reported in well-washed membrane preparations (27). This makes it unlikely that the increase in [35S]TBPS binding in unwashed membranes is due

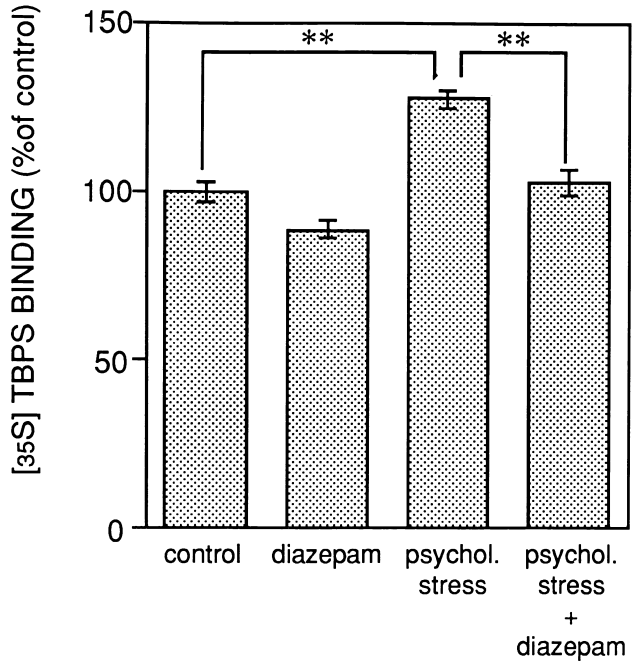

FIG. 3. Diazepam antagonizes the effect of psychological stress on [35S]TBPS binding to unwashed rat cortical membrane. Interaction between diazepam and psychological stress: $p < 0.05$, two-way ANOVA; **$p < 0.01$, ANOVA and Tukey's test. Values are means ± SD of six animals from three different experiments. Diazepam (3 mg/kg IP) was administered 30 min before psychological stress. The animals were killed immediately after the stress session.

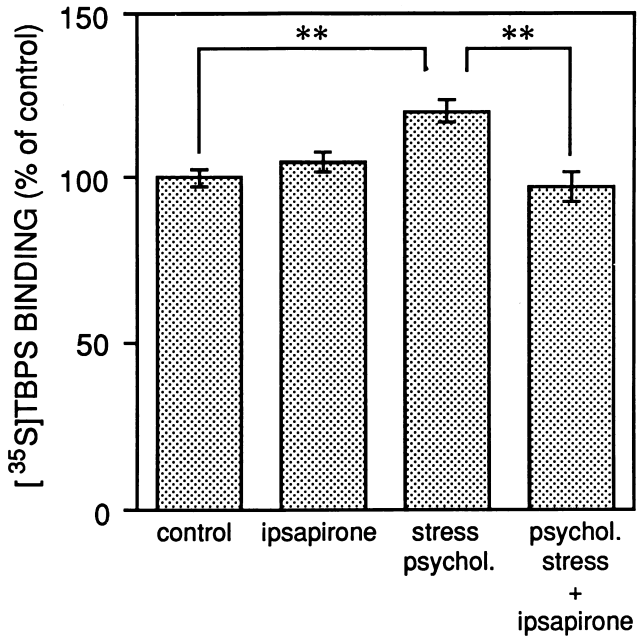

FIG. 4. Ipsapirone antagonizes the effect of psychological stress on [35S]TBPS binding to unwashed rat cortical membrane. **$p < 0.01$, ANOVA and Tukey's test. Data are means ± SD of six animals per group. Ipsapirone (5 mg/kg SC) was administered 30 min before psychological stress. The animals were killed immediately after the stress session.

to a lower GABA concentration in the stressed group, and suggests that the changes are the result of receptor modification in vivo during the stress.

Our present findings indicate that psychological stress causes a drop in GABAergic transmission similar to that described in handling-habituated rats exposed to acute foot shock. The fact that psychological stress is also able to increase [^{35}S]TBPS binding in unwashed cortical membrane of rats suggests that the decrease in GABAergic transmission seen after stress is not related to the physically painful stimuli elicited by stressors used in previous studies.

The increase in [^{35}S]TBPS binding induced by psychological stress, like that induced by acute foot shock (10), was entirely due to an increase in the maximum number of binding sites, without changes in affinity.

We used a standard protocol in which rats were exposed to inescapable foot shock for 3 days before the experiment, although a single foot shock session was enough to elicit conditioned fear associated with the environmental stimulus, as indicated by the similar increase in [^{35}S]TBPS binding found in two experimental conditions (1 day or 3 days of foot shock). This suggests that the experience of a stressor stimulus in handling-habituated rats precipitated a plastic change in the receptor that dropped to a level comparable to that of naive rats.

To further clarify whether the increase in [^{35}S]TBPS binding induced by psychological stress was the consequence of decreased activation of $GABA_A$ receptors, we investigated whether diazepam, a benzodiazepine anxiolytic that facilitates GABAergic transmission (13,24), antagonized it. Pretreatment with diazepam slightly reduced [^{35}S]TBPS binding in unwashed membrane preparation from handling-habituated rats (23,24), although this effect was not significant in our experiment, and completely prevented the increase due to psychological stress. Therefore, it is likely that psychological stress reduced GABAergic transmission either directly, through reduced GABA release, or indirectly, through changes in modulatory neurosteroids (1) or diazepam binding inhibitor (DBI) (12). Diazepam has been reported to reduce freezing behavior (15) and the increase in dopamine turnover (17) induced by psychological stress in rats.

We also tested the effect of ipsapirone, an anxiolytic agent acting as a 5-HT1A partial agonist (26). We used an acute dose of ipsapirone known to have anxiolytic activity in rats (7). Ipsapirone also completely prevented the increase in [^{35}S]TBPS binding induced by psychological stress in rats, an effect not mediated by its metabolite 1-pyrimidinyl-piperazine, which acts as an α_2-adrenoceptor antagonist (2). This finding is consistent with the previously reported antagonism by buspirone of the decrease in ^{3}H-flunitrazepam binding induced by noise stress in rats (21) and with the reduction of freezing behavior induced by psychological stress (15).

The effect of the anxiolytic 5-HT1A agonist and partial agonist on GABAergic transmission merits further comment. Stressor stimuli, including psychological stress (16,18), increase serotonin release in relevant brain regions like the amygdala and prefrontal cortex. It is thus conceivable that drugs like buspirone and ipsapirone, by reducing the firing of 5-HT neurons, counteract the rise in extracellular 5-HT induced by stress. It must also be considered that benzodiazepine anxiolytics such as diazepam, like 5-HT1A agonists, reduce the firing of dorsal raphe 5-HT neurons.

Our results suggest that extracellular 5-HT has a modulatory role in GABAergic transmission, as indicated by the fact that ipsapirone prevented the increase in [^{35}S]TBPS binding induced by psychological stress. One possibility is that released 5-HT, acting on 5-HT3 receptors, inhibits the release of GABA from interneurons (8). We are currently exploring this.

In conclusion, our results indicate that psychological stress, obtained by exposure to environmental stimuli previously paired with inescapable foot shock, is a simple and valid animal model for investigating the various biochemical changes related to stress, avoiding the physical components associated with most of the more commonly used stressors and only mimicking emotional state alterations.

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