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Inescapable Shock Reduces [^{3}H]Ro 5-4864 Binding to "Peripheral-Type" Benzodiazepine Receptors in the Rat

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DRUGAN, R. C., A. S. BASILE, J. N. CRAWLEY, S. M. PAUL AND P. SKOLNICK. *Inescapable shock reduces [^{3}H]Ro 5-4864 binding to "peripheral-type" benzodiazepine receptors in the rat.* PHARMACOL BIOCHEM BEHAV **24**(6) 1673–1677, 1986.—[^{3}H]Ro 5-4864 binding to "peripheral-type" benzodiazepine receptors was examined in brain and peripheral tissues of rats subjected to inescapable tailshocks. Two hours after a session of 80 (five-second) inescapable tailshocks, a significant reduction in [^{3}H]Ro 5-4864 (10 mM) binding was observed in membranes from kidney (31%), cerebral cortex (29%), heart (19%) and pituitary (17%) compared to tissues from naive animals. In contrast, inescapable shock did not effect [^{3}H]Ro 5-4864 binding to hippocampal, lung, or adrenal membranes. Scatchard analyses of [^{3}H]Ro 5-4864 binding to renal membranes demonstrated that this session of tailshock reduced the density (B_{max}) of "peripheral-type" benzodiazepine receptors without effecting the apparent affinity (K_d) of the radioligand for these sites. The effects of graded stress on [^{3}H]Ro 5-4864 binding to cerebral cortex and kidney were investigated using 5, 20, or 80 (five-second) inescapable shocks. In cerebral cortical membranes, sessions of either 5 or 20 shocks did not affect, while 80 shocks reduced (29%) [^{3}H]Ro 5-4864 (10 mM) binding. In renal membranes, 5 shocks significantly increased (35%), 80 shocks significantly decreased [^{3}H]Ro 5-4864 (10mM) binding (31%). These findings demonstrate that the density of "peripheral-type" benzodiazepine receptors in both peripheral tissues and the central nervous system can be rapidly modulated by stress.

"Peripheral-type" benzodiazepine receptors Ro 5-4864 PK 11195 Inescapable shock Stress

BENZODIAZEPINE receptors in the central nervous system (CNS) that are coupled to both $GABA_A$ receptors and an associated chloride ionophore have been shown to mediate the principal pharmacologic actions of the benzodiazepines [22, 28, 33]. Recent studies suggest that these receptors may also be important in the response to stress and the physiologic control of anxiety [10, 13, 29]. A physically and pharmacologically distinct class of binding sites for benzodiazepines which is present in both peripheral tissues and the CNS has recently been shown to fulfill many of the criteria of a pharmacologic receptor [25, 30–31, 36].

Despite the unusual distribution of these "peripheral-type" benzodiazepine receptors (PBR) in the rat CNS, with the highest densities in the olfactory bulb and choroid plexus [2, 6, 24], Ro 5-4864 (4'-chlorodiazepam, the prototype ligand for these sites), like the "anxiogenic" beta-carbolines, potentiates both shock-induced suppression of drinking and behavior in the social interaction test [12,27]. The "anxiogenic" actions of Ro 5-4864 can be antagonized by PK 11195 (a high affinity ligand of these "peripheral-type" benzodiazepine receptors), but not Ro 15-1788, suggesting a specific interaction at PBR [27].Higher doses of PK 11195 were also shown to counteract shock-induced suppression of drinking, an effect that was not reversed by Ro 15-1788 [27]. These observations suggest that PBR may also be involved in the physiological control of anxiety or stress independent of the benzodiazepine receptors linking to $GABA_A$ receptors and an associated chloride ionophore.

Since exposure to stressful situations involves activation of peripheral tissues (e.g., endocrine, cardiac and renal organs: [4, 8, 15, 17–18, 35]) that contain relatively high densities of PBR relative to the CNS, we examined the effects of exposure to a stressor (inescapable tailshock) on the equilibrium parameters of ligand binding to PBR in both peripheral tissues and the central nervous system.

We now report a significant reduction in radioligand binding to PBR in both peripheral tussues and the central nervous system two hours after exposure to a session of 80 (five-second) inescapable tailshocks. A detailed examination of this effect in renal membranes demonstrated a reduction in the maximum number of PBR, with no change in the apparent affinity of [^{3}H]Ro 5-4864 for these sites. The effects of inescapable tailshock on PBR were not manifest in every tissue examined, and appeared related to the intensity and

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TABLE 1

PBR DENSITY IN ORGANS FROM RATS RECEIVING 80 INESCAPABLE SHOCKS

Tissue	[^{3}H]Ro 5-4864 Binding (pmole/mg protein) 80 Shocks	Naive	n
Cerebral Cortex	0.18 ± 0.02*	0.26 ± 0.02	4
Hippocampus	0.17 ± 0.04	0.18 ± 0.03	4
Pituitary Gland	3.09 ± 0.08*	3.72 ± 0.14	4
Adrenal Gland	65.71 ± 2.80	58.52 ± 2.85	4
Lung	9.23 ± 0.53	8.25 ± 1.20	4
Heart	4.71 ± 0.27*	5.81 ± 0.36	4
Kidney	6.50 ± 0.86*	9.43 ± 0.89	8
Kidney ([^{3}H]PK 11195)	5.49 ± 0.27*	8.21 ± 0.59	4

The density of [^{3}H]Ro 5-4864 and [^{3}H]PK 11195 binding was determined using a single concentration of ligand (10 nM). *=Significantly different from control (naive rat) values at $p<0.05$ as determined using Student's t-test. Note significant decrease in PBR density in heart, kidney, pituitary and cerebral cortex, but not hippocampus, adrenals, or lung.

duration of the stressor (i.e., the number of shocks in a session) in those tissues that were affected. These results demonstrate specific, stress-induced alterations in the density of PBR in both peripheral tissues and the CNS.

METHOD

Behavioral Procedures

Male Sprague-Dawley rats (Taconic Farms, Germantown, NY) weighing 250–300 g received no treatment (naive) or restrained in Plexiglas escape-yoke wheelturn boxes (15.5×12×17 cm) modeled after those used by Weiss *et al.* [40]. A grooved Plexiglas wheel extended 1.7 cm into the front of the chamber through a hole 8.0 cm from the floor of the box. The rat's tail was extended through a slot in the rear wall of the chamber and was taped to a Plexiglas rod parallel to the floor of the chamber. Shock generators (Lafayette Instruments Model No. 82400) were used to apply 5 (1 mA), 20 (1mA) or 80 (incremented from 1–2 mA) inescapable shocks lasting five seconds through electrodes attached to the rat's tail.

Two hours after the last shock all subjects were killed by decapitation. Tissues were immediately placed in an isotonic sucrose solution, fast frozen and stored at −80°C in order to ensure optimal binding conditions. All tissues were assayed within one week after sacrifice.

Radioligand Binding Assays

Tissues were thawed in a water bath at 50°C. The thawed tissue was homogenized using a Brinkman Polytron (Setting 6–7, 15 seconds) in 50 volumes of 50 mM Tris-HCl buffer (pH 7.4) and centrifuged at 20,000 × g for 20 minutes. The pellets derived from peripheral organs or the CNS were resuspended in 400 and 100 volumes of buffer, respectively. The binding of [^{3}H]Ro 5-4864 or [^{3}H]PK 11195 to PBR was determined according to the method of Weissman *et al.* [37]. Briefly, 0.1 or 0.6 ml of peripheral or brain tissues, respectively (containing 0.02–0.1 mg protein, respectively) was added to each assay tube containing 0.1 ml of radioligand (final concentration, 0.25–10 nM), 0.1 ml of unlabelled drug or buffer, and buffer to a final volume of 1 ml. Assays were performed in triplicate. The reaction was initiated by the addition of tissue and terminated after incubating (0.4°C) for 60 min by rapid filtration over Whatman GF/B filter strips using a Brandel M-24R filtering manifold. Samples were washed with two 5 ml aliquots of ice-cold buffer. The specific binding of [^{3}H]Ro 5-4864 and [^{3}H]PK 11195 was defined as the difference in binding obtained in the presence and absence of unlabelled Ro 5-4864 or PK 11195, respectively (final concentrations=5 μM). The radioactivity retained by the filters was measured in a Bechman LS 5801 liquid scintillation spectrometer, using 8 ml of Ready-Solve MP (Beckman Instruments, Fullerton, CA) as a fluor. [^{3}H]Ro 5-4864 (Sp. Act. 76.5 Ci/mmol) and [^{3}H]PK 11195 (Sp. Act. 75 Ci/mmol) were purchased from New England Nuclear, Boston, MA. Ro 5-4864 was donated by Hoffmann-LaRoche, Nutley, NJ, and PK 11195 was a gift from G. LeFur, Pharmuka Laboratories, Gennevilliers, France. Protein was determined using the Miller modification [26] of the method of Lowry *et al.* [21].

RESULTS

Effects of 80 Inescapable Tailshocks on Radioligand Binding to PBR

In initial experiments, the effect of 80 inescapable tailshocks on [^{3}H]Ro 5-4864 binding was surveyed in several brain areas and peripheral tissues. Eighty inescapable shocks (Table 1) significantly reduced [^{3}H]Ro 5-4864 binding in cerebral cortex (~29%, $p<0.05$), heart (~19%, $p<0.05$), kidney (~31%, $p<0.05$) and pituitary (~17%, $p<0.05$) when compared to naive rats. In contrast, this paradigm did not significantly change [^{3}H]Ro 5-4864 binding to membranes from hippocampus, lung, or adrenal gland (Table 1). The decrease in [^{3}H]Ro 5-4864 binding after 80 shocks was paralleled by a similar decrease in [^{3}H]PK 11195 binding to renal membranes (~33%, $p<0.05$, Table 1). Scatchard analysis of [^{3}H]Ro 5-4864 binding to kidney membranes from animals receiving 80 shocks revealed a 31% decrease in density (B_{max}, 6.50±0.86 vs 9.43±0.89 pmole/mg protein, treatment vs. control), with no change in the apparent affinity (K_d, 1.104±0.14 vs. 0.97±0.06) compared to naive animals (Fig. 1). Differences between shock and naive controls in the first experiment were analysed for statistical significance using the Student's t-test.

In order to determine whether there is a relationship between the number or intensity of shocks applied and changes in the number of PBR, groups of rats received either 5, 20 or 80 tailshocks and [^{3}H]Ro 5-4864 binding was assessed in cortical and renal membranes.

As can be seen in Fig. 2 (right panel), 5 and 20 shocks had no effect on cerebral cortical PBR density while 80 shocks resulted in a significant (29%) decrease. These observations were confirmed by a one-way analysis of variance. The ANOVA revealed a significant treatment (shock) effect: $F(3.16)=3.51$, $p<0.05$. Subsequent Newman-Keuls comparisons ($\alpha=0.05$) indicated that the 80 shock group differed significantly from the naive controls, while the remaining groups did not differ from one another. In contrast, a significant increase (35%) in the density of PBR was found in renal

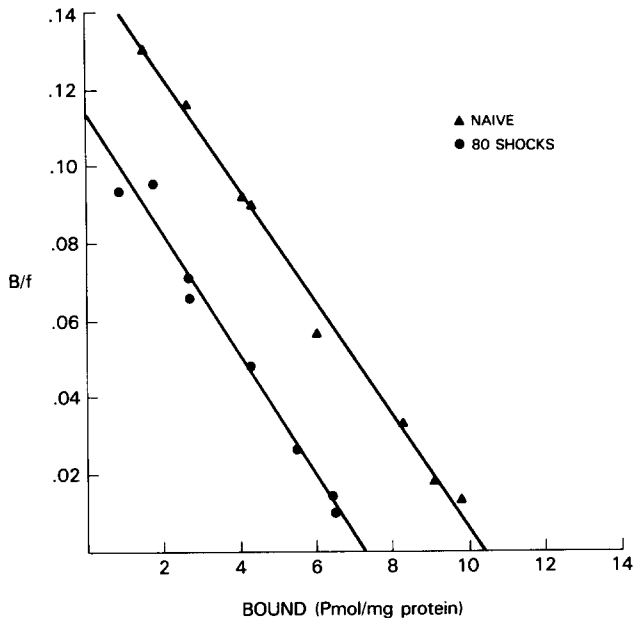

FIG. 1. A representative Scatchard analysis of [^{3}H]Ro 5-4864 binding to renal membranes from 80 shock treated rats (circles) and naive controls (triangles). For the experimental and naive control kidneys, the B_{max} and K_d for [^{3}H]Ro 5-4864 binding were 7.21 pmol/mg protein, 1.10±0.14 mM, and 10.38 pmol/mg protein, 0.97±0.06 nM, respectively. The 35% decrease in [^{3}H]Ro 5-4864 binding to renal membranes from the 80 shock treated rat is significantly different at $p<0.05$. There is no significant difference in the apparent affinity of the PBS for [^{3}H]Ro 5-4864 binding to renal membranes from either group.

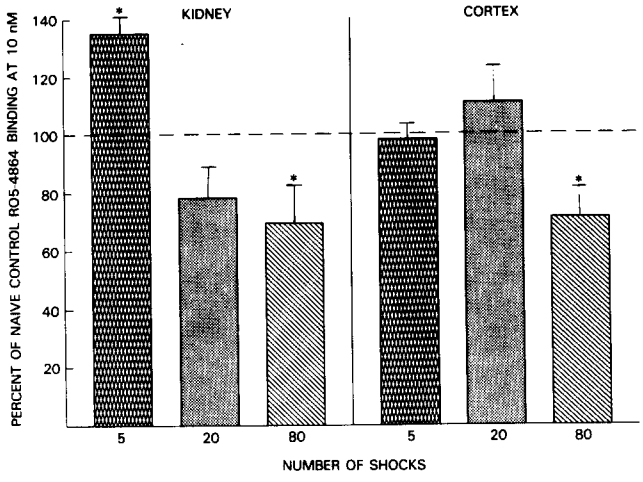

FIG. 2. Effects of different stress durations on [^{3}H]Ro 5-4864 binding to membranes from kidney and cerebral cortex. Rats received either 5, 20 or 80 5-second shocks. A significant increase (35%) in [^{3}H]Ro 5-4864 binding to renal membranes was seen after 5 shocks. However, after 20 and 80 shocks, a 22% and 31% decrease, respectively, in [^{3}H]Ro 5-4864 binding was observed. In contrast, a significant, 29% decrease in [^{3}H]Ro 5-4864 binding was observed in cerebral cortex (right panel) only after 80 shocks. The density of [^{3}H]Ro 5-4864 binding to renal and cortical membranes was determined at a single concentration of ligand (10 nM). Each bar value represents the average percentage difference in [^{3}H]Ro 5-4864 binding to tissue membranes from shock treated animals when compared to their naive controls. *=Significantly different at $p<0.05$, as determined by Newman-Keuls Post-Hoc Test on raw data scores, not percentage differences.

membranes after 5 inescapable shocks, while 20 and 80 inescapable shocks produced a 22 and 31% decrease in PBR density, respectively (Fig. 2, left panel). These observations were confirmed by a one-way analysis of variance. The ANOVA revealed a significant treatment (shock) effect, $F(3,36)=5.46$, $p<0.01$. Subsequent Newman-Keuls comparisons ($\alpha=0.05$) indicated that the 5 and 80 shock groups were significantly different from naive controls, while the 20 shock group was not.

DISCUSSION

This study demonstrates that exposure to 80 (5-sec) inescapable tailshocks elicits a rapid reduction in the density of PBR which is restricted to some peripheral tissues and areas of the CNS. For example, a significant reduction in [^{3}H]Ro 5-4864 binding to PBR was observed in both heart (19%) and kidney (31%), but not in lung or adrenal gland, while in the central nervous system, a significant reduction in binding was observed in cortical (29%) but not hippocampal membranes. The demonstration of an apparent tissue-specific reduction in PBR (unrelated to the density of PBR) mitigates against a nonspecific effect of stress. Tissues were initially surveyed using a concentration of [^{3}H]Ro 5-4864 that was approximately 10 times greater than the apparent K_d. Thus, the reductions in PBR that were observed in these experiments probably reflect alterations in the density of PBR rather than changes in the apparent affinity. Scatchard analysis of kidney membranes prepared from naive and stressed animals confirmed this hypothesis, since a 35% reduction in the B_{max} was observed, using 10 nM [^{3}H]PK 11195 or [^{3}H]Ro 5-4864 (Table 1). The initial tissue survey was performed using a schedule of 80 inescapable tailshocks, which has previously been demonstrated to elicit a number of effects including analgesia [11, 14, 22] and behavioral escape deficits in animals examined 24 hours later [23]. In order to determine whether the density of PBR could be altered in a graded fashion, the effects of 5 and 20 shocks was compared with the standard 80 tailshock regimen in cerebral cortical, and kidney membranes. No changes were observed in cortical tissue from animals that received 5 or 20 tailshocks, while a significant reduction was observed in the 80 tailshock group (Table 1, Fig. 2). In contrast, a significant increase (35%) in [^{3}H]Ro 5-4864 binding was observed in animals receiving 5 shocks, with a graded reduction in [^{3}H]Ro 5-4864 binding in the 20 and 80 tailshock group. This type of rapid "up and down" modulation of PBR density has not been previously reported. However, both increases and decreases in the density of benzodiazepine receptors in the CNS have been reported after exposure to stressful situations [7, 18–20, 32].

The present findings that PBR density may be altered in both peripheral tissues and the CNS as a result of exposure to a stressful stimulus raises the possibility that PBR ligands could produce behavioral effects [12,27] via an effect on peripheral tissues (e.g., cardiovascular, endocrine) rather than a direct effect on PBR in the CNS. However, our findings are consistent with previous studies [12,27] suggesting an asso-

ciation between Ro 5-4864, PBR, and stress/anxiety in rats and argues for a direct effect of Ro 5-4864 on the PBR rather than on the $GABA_A$ receptor gated chloride channels [38,40]. The observation that the proconflict effect of Ro 5-4864 could be blocked by PK 11195 (which does not affect $GABA_A$ receptor gated chloride channels as assessed by [^{35}S]t-butylbicyclophosphorothionate binding; [38,40]) and that higher doses of PK 11195 produce an anticonflict action [27] suggest that occupation of PBR could be involved in the "anxiogenic" actions of Ro 5-4864.

The mechanisms by which PBR density is altered in response to stressful stimuli is unknown. However, since stress can affect cardiovascular, endocrine and renal as well as CNS function, the changes in PBR density that were observed could be due to activation of these systems to different degrees. The graded or biphasic effect of inescapable shock observed in renal and cerebral cortical membranes supports this contention. Several studies have demonstrated that the density of PBR are under hormonal control [1, 3, 9] and that PBR in some tissues (e.g., pineal; [37]) are associated with presynaptic catecholamine nerve terminals, which would further support a role or association of PBR in stress-related phenomena. Further investigation using both pharmacologic and behavioral manipulations will be necessary to clarify the role and regulation of PBR and its relationship to stress and anxiety.

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