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Swim Stress Selectively Alters the Specific Binding of a Benzodiazepine Antagonist in Mice

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PARK, C. H., A. HITRI, L. G. LUKACS AND S. I. DEUTSCH. *Swim stress selectively alters the specific binding of a benzodiazepine antagonist in mice.* PHARMACOL BIOCHEM BEHAV 45(2) 299–304, 1993.—The ability of flurazepam to antagonize the electrical precipitation of tonic hindlimb extension is reduced 24 h after mice are forced to swim for 10 min in cold water (6°C). Presumably, this reduction in flurazepam's antiseizure efficacy reflects an environmental stress-induced modification of the $GABA_A$ receptor complex. The current study employed a variety of complementary in vitro approaches to characterize the delayed effects of cold-water swim stress on binding parameters of the $GABA_A$ receptor complex that may be associated with flurazepam's reduced antiseizure efficacy. The specific binding of [^{3}H]flunitrazepam and the potentiation of this binding by chloride ions did not change after stress in the cerebral cortex, hippocampus, and cerebellum. Moreover, swim stress did not alter the ability of GABA to inhibit the binding of [^{35}S]*t*-butylbicyclophosphorothionate (TBPS), a ligand that is a specific biochemical marker of the GABA-associated chloride ionophore, to crude membranes prepared from the cerebral cortex and cerebellum. Swim stress was associated with alterations of the specific binding of [^{3}H]Ro 15-1788, a benzodiazepine receptor antagonist, to crude hippocampal and cerebellar membranes. The results are considered in the context of new insights derived from molecular cloning studies of the $GABA_A$ receptor complex.

Stress $GABA_A$ receptor complex Ro 15-1788

A variety of behavioral and pharmacologic data support a role for the $GABA_A$ receptor complex in mediating an organism's response to a stressful experience (5). Interestingly, reports of environmental stress-induced changes in the specific binding of selective ligands to this complex have been inconsistent and often subtle (2,6). Recent molecular cloning studies of the $GABA_A$ receptor complex may provide insight into some of the seemingly inconsistent observations reported in the literature. For example, in a social stress paradigm, cortical levels of mRNA for the α_1- and γ_2-subunits were increased 4 h after stress in intruder mice (10). These elevated levels of mRNA persisted for 72 h after the stressor; levels of mRNA for these subunits were unchanged in resident mice. Presumably, these social stress-induced alterations in levels of mRNA correspond to the stress-associated biochemical changes of the $GABA_A$ receptor complex (10).

The variable effects of stress on the specific binding of selective ligands to the $GABA_A$ receptor complex could be due to a variety of factors, including differences in the stress paradigms themselves, strain and species of animal, regional sensitivity to the effects of stress, and nature of ligands used to characterize components of the $GABA_A$ receptor complex. Moreover, the emergence of stress-induced changes and their direction may follow a temporal sequence that differs among brain regions. The observation has also been made that some stress-induced changes in receptor binding may be more easily appreciated with an in vivo approach to their measurement than with conventional in vitro filtration binding assays (4, 21). The nature of the ligands used to characterize the $GABA_A$ receptor complex may present a contributing variable and necessitate the complementary assessment of radiolabeled benzodiazepine agonist and antagonist ligand binding. The effects of "obtunding" concentrations of CNS depressants were found to selectively alter the "antagonist-favored" conformation of the benzodiazepine binding site on the $GABA_A$ receptor complex, while sparing agonist-preferring sites (17). Conse-

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quently, the effect of stress on the $GABA_A$ receptor complex and its temporal sequence may exhibit a "ligand-dependent" action and differentially affect the agonist and antagonist states of the benzodiazepine binding site.

In this investigation, several complementary in vitro approaches to the pharmacological characterization of the $GABA_A$ receptor complex were applied to examine the delayed effects of a single session of cold-water swim stress in three different regions of the mouse brain (i.e., the cerebral cortex, hippocampus, and cerebellum). Specifically, 24 h after a single 10-min session of cold-water swim stress, we studied the specific binding of [^{3}H]flunitrazepam and [^{3}H]Ro 15-1788, the potentiation of [^{3}H]flunitrazepam binding by chloride ions, and the inhibition of [^{35}S]*t*-butylbicyclophosphorothionate ([^{35}S]TBPS) binding by GABA. Our laboratory observed that the ability of flurazepam to antagonize the electrical precipitation of tonic hindlimb extension in mice is reduced 24 h after a single 10-min session of cold-water swim stress (5,12). This study was designed to identify the biochemical changes in the $GABA_A$ receptor complex that accompany this stress-induced reduction in flurazepam's antiseizure efficacy. Thus, we elected to use a variety of complementary in vitro approaches to examine cold-water swim stress-induced changes in the $GABA_A$ receptor complex to detect subtle modifications of the complex that might result from the selective transcription of specific molecular subunit forms in different brain regions.

METHOD

Subjects

Experimentally naive, male NIH Swiss mice weighing approximately 30 g and housed in hanging wire cages in groups of five animals/cage were used throughout the experiments. Animals were maintained in a temperature-controlled vivarium on a 12 L : 12 D cycle and had free access to food and water.

Stress Procedure

Mice were forced to swim in cold water (6°C) for up to 10 min. Twenty-four hours after the session of cold-water swim stress, animals were killed by decapitation in parallel with a group of unstressed control mice. This procedure was approved by the Animal Studies Subcommittee and Research Committee of this Department of Veterans Affairs Medical Center. Brains were immediately removed and dissected on ice. Cerebral cortices, hippocampi, and cerebella were stored frozen at −70°C prior to preparation of tissue homogenates.

Tissue Preparation

Crude membranes were prepared from the cerebral cortex, hippocampus, and cerebellum according to the procedure described by Havoundjian et al. (8) with only slight modification. Briefly, tissue was homogenized in 50 vol 50 mM Tris-HCl buffer (pH 7.4) with a Brinkmann Polytron (5 s at maximal speed). The homogenate was then centrifuged at 20,000 × *g* for 20 min (4°C). The resulting pellets were washed four times in 50 vol 50 mM Tris-citrate buffer (pH 7.4) containing 100 mM NaCl. The final pellet was resuspended in 50 vol 50 mM Tris-citrate buffer (pH 7.4). The concentration of protein in the final tissue suspension was determined by the method of Lowry et al. (11).

[^{3}H]Flunitrazepam and [^{3}H]Ro 15-1788 Binding

The specific binding of [^{3}H]flunitrazepam (specific activity 93 Ci/mmol; New England Nuclear, Boston, MA) and [^{3}H]Ro 15-1788 (83.2 Ci/mmol; New England Nuclear) was studied in an incubation volume of 1 ml using 100 μl of either a [^{3}H]flunitrazepam or [^{3}H]Ro 15-1788 solution, 100 μl of tissue homogenate (approximately 0.1 mg protein), and Tris-citrate buffer to the final volume. Nonspecific binding was determined with the addition of 100 μl midazolam (final concentration 10 μM) and represented less than 10% of the total binding. The assay was initiated with the addition of tissue homogenate and proceeded for 60 min at 4°C. In the experiments examining the chloride ion dependence of [^{3}H]flunitrazepam binding, tissue homogenate was incubated in the presence of varying concentrations of NaCl (25–400 mM). The reaction was terminated by addition of 5 ml ice-cold Tris-citrate buffer and immediate vacuum filtration through Whatman GF/B glass fiber filters (Whatman, Clifton, NJ). The filters were rinsed three times with 5 ml ice-cold buffer and counted using liquid scintillation spectrometry.

[^{35}S]TBPS Binding

The binding of 2 nM [^{35}S]TBPS (approximately 140–160 Ci/mmol; New England Nuclear) was performed according to the method described by Havoundjian et al. (8) with only slight modification. Binding was studied in an incubation volume of 1.0 ml consisting of 100 μl [^{35}S]TBPS solution, 500 μl tissue homogenate (approximately 0.4–0.5 mg protein), 100 μl 2 M NaCl, 100 μl of various concentrations of GABA solution, and buffer to final volume. Nonspecific binding was determined using 10 μM picrotoxinin and represented less than 20% of the total binding. The reaction was initiated with the

TABLE 1

COMPARISON OF THE EFFECT OF STRESS ON [^{3}H]FLUNITRAZEPAM BINDING IN MOUSE BRAIN REGIONS

	Cortex		Hippocampus		Cerebellum	
	Control	Stress	Control	Stress	Control	Stress
K_d (nM)	1.89(0.75)	1.95(0.57)	2.74(1.99)	2.51(1.07)	3.7 (3.40)	3.10(2.4)
B_{max} (pmol/mg protein)	0.81(0.10)	0.83(0.10)	0.84(0.30)	0.81(0.30)	0.24(0.3)*	0.21(0.4)*

Regional differences for B_{max} were significant (Kruskal-Wallis ANOVA, χ^2 (5) = 12.3, p = 0.03); they were not significant for K_d (χ^2 (5) = 4.2, p = 0.51). B_{max} in the cerebellum was four-fold lower than the B_{max} in the cerebral cortex (*Wilcoxon rank-sum with Mann-Whitney *U*-test, z = 2.4, p = 0.016) of both control and stressed mice. Hippocampi and cerebella were pooled from six mice, while the cerebral cortices were stored individually at −70°C until the time of assay. Aliquots of frozen pulverized brain regions were homogenized and assayed in duplicate for [^{3}H]flunitrazepam binding. Values are the median (range) for the binding constants obtained from three to four experiments. The equilibrium binding constants were determined by Scatchard analysis using computerized equilibrium binding data analysis (13).

addition of [^{35}S]TBPS and proceeded at 25°C for 100 min. The reaction was terminated by filtration through Whatman GF/B glass fiber filters. The filters were washed twice with 5 ml ice-cold Tris-citrate buffer (pH 7.4) and counted using liquid scintillation spectrometry.

Data Analysis and Statistics

The equilibrium binding constants were determined by Scatchard analysis. The raw data, expressed as disintegrations per minute (DPM), were subjected to equilibrium binding data analysis (EBDA), using the Collection of Radioligand Binding Analysis Programs (13). The program provided a mean and SEM for replicate determinations at each point. For each point, the program calculated the amount of bound (total, specific) and unbound (free) ligand concentration. The initial estimates of the dissociation constant (K_d), and maximum number of binding sites (B_{max}) were obtained from Scatchard plots using EBDA, while for the final estimate of the constants the LIGAND program was used. All statistical analyses were carried out on an IBM PC AT computer using the Number Cruncher Statistical System version 5.01 (9) and by the GB-STAT Professional Statistics and Graphics version 3.0 (7).

RESULTS

Experiment 1

Using [^{3}H]flunitrazepam (Table 1), regional differences were observed in the maximal density of central benzodiazepine binding sites [Kruskal-Wallis ANOVA, $\chi^2(5) = 12.3$, $p = 0.03$]. B_{max} in the hippocampus did not differ from that in the cerebral cortex, but the B_{max} in the cerebellum was four-fold lower than the B_{max} in the cerebral cortex of both control and stressed mice (Wilcoxon rank-sum with Mann-Whitney U-test, $z = 2.4$, $p = 0.016$). Irrespective of treatment condition, there was no apparent difference in the affinity constant among the three regions, $\chi^2(5) = 4.2$, $p = 0.51$. Twenty-four hours after a single session of cold-water swim stress, K_d and B_{max} values did not differ from control values in the three brain regions.

Experiment 2

The effect of stress was further investigated with respect to the ability of chloride ions to potentiate the binding of [^{3}H]flunitrazepam to crude membrane preparations prepared from the three brain regions. Figure 1 demonstrates the effect of increasing concentrations of chloride ions on [^{3}H]-flunitrazepam binding in the cerebral cortex. The chloride ion effect was significant in both stressed, $\chi^2(5) = 26.2$, $p = 0.0001$, and unstressed mice, $\chi^2(5) = 23.4$, $p = 0.0003$. There was no effect of stress on the ability of chloride ions to potentiate [^{3}H]flunitrazepam binding in the cerebral cortex. This failure of stress to affect the ability of chloride ions to potentiate [^{3}H]flunitrazepam binding was also observed in the hippocampus and cerebellum (data not shown).

The effect of stress on the potentiation of [^{3}H]flunitrazepam binding by chloride ions was also evaluated in saturation studies in the presence of various concentrations of NaCl. Figure 2 illustrates the comparison of Scatchard analyses of [^{3}H]flunitrazepam binding in the presence and absence of 200 mM NaCl in cerebral cortex from stressed and control mice. The addition of chloride ions decreased the K_d and increased the B_{max} to the same extent in the two treatment conditions [K_d, $\chi^2(3) = 0.87$, $p = 0.85$; B_{max}, $\chi^2(3) = 5.2$, $p = 0.15$]

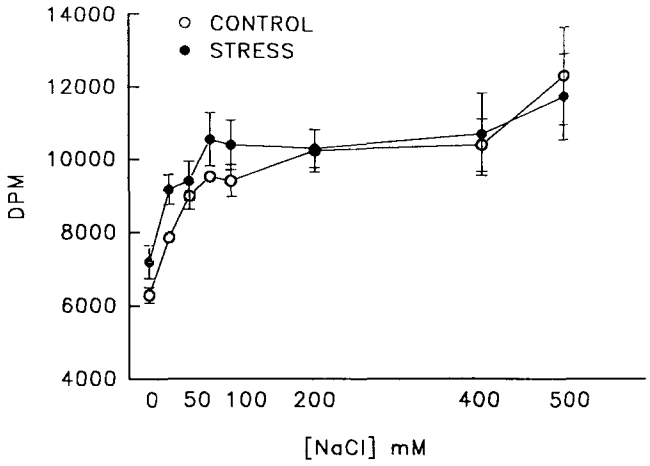

FIG. 1. Potentiation of [^{3}H]flunitrazepam binding by chloride ions in stressed and unstressed mouse cerebral cortex. [^{3}H]Flunitrazepam binding was assayed as described in the text. Each point represents the mean (SEM) disintegrations per minute (DPM) obtained in duplicate assays on individual cortices from five to six animals with the exception of the point at 75 mM NaCl, which is a duplicate determination performed on a single animal. A Kruskal-Wallis analysis of variance indicated significant enhancement of [^{3}H]flunitrazepam binding by chloride ions in both control ($p = 0.0001$) and stressed mice ($p = 0.002$).

Similar results were obtained in the presence of 50, 100, and 500 mM NaCl.

Experiment 3

GABA showed dose-dependent inhibition of the binding of [^{35}S]TBPS in the cerebral cortex and cerebellum [C_{50} values of 3.3 (0.1) and 2.25 (0.2) μM, respectively, with Hill coefficients greater than unity; see Table 2]. There was no stress-induced modification of GABA's ability to inhibit the binding of [^{35}S]TBPS in either brain region, $\chi^2(3) = 7.32$, $p = 0.06$.

Experiment 4

Using [^{3}H]Ro 15-1788 (Table 3) to measure benzodiazepine receptor binding, regional differences were observed in both the maximal density, $\chi^2(5) = 18.6$, $p = 0.002$, and the affinity constant, $\chi^2(5) = 13.6$, $p = 0.02$, irrespective of the treatment. There was a differential effect of stress on both B_{max} and K_d values across the three brain regions. In the cerebral cortex, there was a small (9%) but significant stress-induced reduction in B_{max} ($z = 1.9$, $p = 0.05$) without a significant change in K_d ($z = 1.0$, $p = 0.27$). In the hippocampus, there was no change in B_{max} ($z = 0.87$, $p = 0.38$), while there was a 30% stress-induced reduction in K_d ($z = 2.6$, $p = 0.009$). Finally, in the cerebellum,there was a 27% stress-induced increase in B_{max} ($z = 2.2$, $p = 0.028$) accompanied by a 25% decrease in K_d ($z = 2.4$, $p = 0.016$).

DISCUSSION

In previous work, our laboratory has shown that the ability of flurazepam, a prototypic benzodiazepine agonist, to antagonize the electrical precipitation of tonic hindlimb extension in the mouse is reduced 24 h after a single session of cold-water swim stress. This stress-induced reduction of flurazepam's antiseizure efficacy persisted for up to 72 h after the

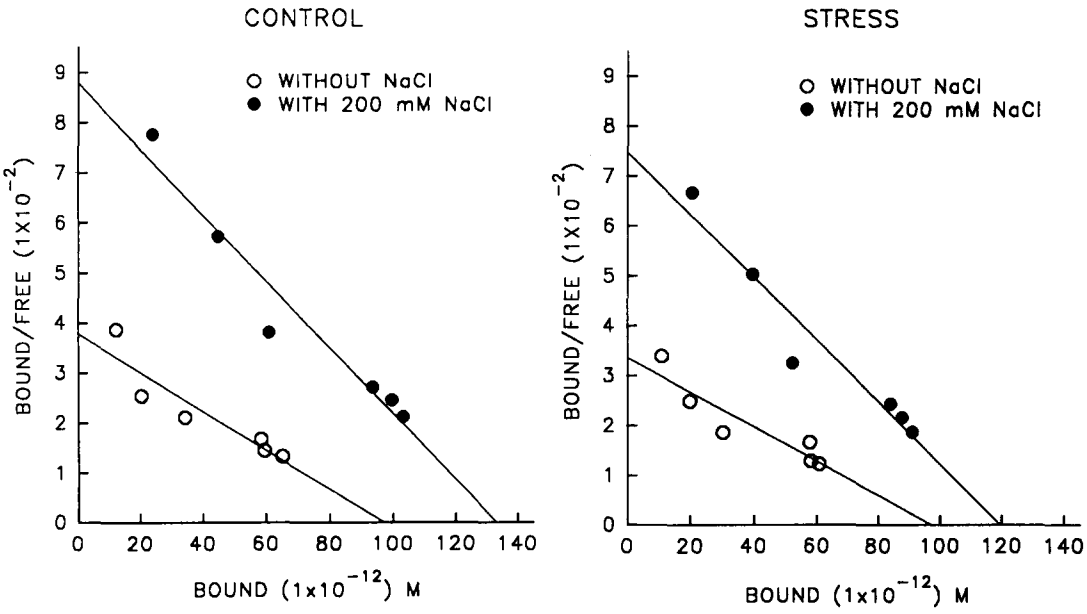

FIG. 2. Typical Scatchard plots of [^{3}H]flunitrazepam binding in the cerebral cortex of a stressed and unstressed mouse. [^{3}H]Flunitrazepam saturation curves were generated in the presence and absence of 200 mM NaCl in duplicate binding assays. The experiment was repeated three times. The following representative equilibrium binding constants were determined by Scatchard analysis using the computerized equilibrium binding data analysis (13): unstressed control, K_d = 2.56 nM, B_{max} = 0.99 pmol/mg protein (without NaCl); K_d = 1.55 nM, B_{max} = 1.33 pmol/mg protein (with 200 mM NaCl); stressed, K_d = 2.8 nM, B_{max} = 0.95 pmol/mg protein (without NaCl); K_d = 1.59 nM, B_{max} = 1.34 pmol/mg protein (with 200 mM NaCl).

single session of stress (5,12). The reduced efficacy of flurazepam was not a function of a lowering of the threshold voltage for seizure production in general, but was specific to the pharmacological action of flurazepam. Presumably, this altered antiseizure efficacy of flurazepam reflects a stress-induced modification of the $GABA_A$ receptor complex. We used a variety of complementary in vitro approaches to explore potential stress-induced alterations in the $GABA_A$ receptor complex.

The efficacy and potency of chloride ions to potentiate the binding of [^{3}H]flunitrazepam to rat cerebral cortical membranes was increased immediately after a 10-min session of ambient temperature swim stress (8,19). In the current study, a stress-induced alteration in the ability of chloride ions to potentiate [^{3}H]flunitrazepam binding was not observed 24 h after a single 10-min session of cold-water swim stress in mice. The conflicting observations could be accounted for by differences in species (i.e., rat vs. mouse) and temperatures (i.e., ambient vs. cold water) between the two studies and potential temporal differences in the expression of stress-induced changes. An immediate increase in chloride ion potentiation of [^{3}H]flunitrazepam binding could reflect rapid posttranslational modifications of existing $GABA_A$ receptor complexes that respond to moment-to-moment fluctuations in the "stressful" level of the environment. In fact, the intracellular domains of many of the polypeptide subunits contain consensus sequences for enzymatic phosphorylation by protein kinases A and C and protein tyrosine kinases (15). The relevance of these phosphorylation sites to modulation of GABAergic transmission was recently shown. The cAMP-dependent phos-

TABLE 2

INHIBITION OF [^{35}S]TBPS BINDING BY GABA IN STRESSED AND UNSTRESSED MOUSE BRAINS

	Control		Stress	
	IC_{50}	n_H	IC_{50}	n_H
Cortex	3.15(1.0)	1.34(0.3)	3.30(1.3)	1.12(0.9)
Cerebellum	2.31(1.3)	1.14(0.4)	2.65(0.5)	1.26(0.0)

IC_{50}, inhibition constant [1 × 10^{-6}] M: χ^2 (3) = 7, p = 0.07. n_H, Hill coefficient: χ^2 (3) = 1.58, p = 0.66. GABA inhibition curves were generated by incubating 2 nM [^{35}S]TBPS with seven concentrations of GABA (1–50 μM). The inhibition constants and Hill coefficients were determined from computer-generated curves using the EBDA and LIGAND programs (see text).

TABLE 3

SUMMARY OF [^{3}H]RO 5-1788 BINDING CONSTANTS IN THE STRESSED AND UNSTRESSED TREATMENT CONDITIONS

	Cortex		Hippocampus		Cerebellum	
	Control	Stress	Control	Stress	Control	Stress
K_d (nM)	0.61(0.56)	0.74(0.47)	0.96(0.16)	0.67(0.16)*	1.00(0.48)	0.75(0.39)*
B_{max} (pmol/mg protein)	1.12(0.24)	1.02(0.17)*	1.25(0.37)	1.12(0.22)	0.44(0.38)	0.56(0.08)*

Regional differences were indicated by both B_{max} (χ^2 (5) = 18.6, p = 0.002, and K_d χ^2 (5) = 13.6, p = 0.02). The effect of treatment was regionally selective. In the cortex, there was a 9% stress-induced reduction in B_{max} (*z = 1.9, p = 0.05); in the hippocampus, there was a 30% stress-induced reduction in K_d (*z = 2.6, p = 0.009); in the cerebellum, there was a 27% stress-induced increase in B_{max} (z = 2.2, p = 0.028) and a 25% decrease in K_d (z = 2.4, p = 0.016). Hippocampi and cerebella were pooled from six mice while the cerebral cortices were stored individually at −70°C until the time of assay. Aliquots of frozen pulverized brain regions were homogenized and assayed in duplicate for [^{3}H]Ro 15-1788 binding. Values are the median (range) for the binding constants obtained from three to four experiments. The equilibrium binding constants were determined by Scatchard analysis using computerized equilibrium binding data analysis (13).

phorylation of the β_1-subunit at a specific serine residue resulted in $GABA_A$ receptor complexes that were less sensitive to GABA and altered the desensitization kinetics of the α_1-β_1 complex to GABA (15).

Consistent with the observation regarding no delayed stress-induced change in the chloride ion potentiation of [^{3}H]flunitrazepam binding, the ability of GABA to inhibit the specific binding of [^{35}S]TBPS was not altered 24 h after swim stress in mice. The ability of both chloride ions to potentiate [^{3}H]flunitrazepam binding and GABA to inhibit the binding of [^{35}S]TBPS are biochemical indices of the allosteric interactions between the GABA agonist recognition site, benzodiazepine modulatory site, and the channel. The specific binding of [^{35}S]TBPS is used as a marker of the GABA-associated chloride ionophore.

Interestingly, of all the complementary in vitro measures examined in the current study, the only one that reflected a potential stress-induced change in the $GABA_A$ receptor complex was the specific binding of [^{3}H]Ro 15-1788. Thus, the data suggest that stress stimulates the selective transcription of molecular subunit forms that result in relatively stable antagonist-preferring benzodiazepine binding sites, as opposed to the agonist-preferring conformation. In the study of Quinlan and Firestone (17), the complementary assessment of radiolabeled benzodiazepine agonist and antagonist binding has been applied to the study of the effects of "obtunding" concentrations of ethanol and diethylether on the $GABA_A$ receptor complex (17). In this study, acute incubation of rat forebrain membranes with ethanol (250 mM to 1 M) resulted in a reduction in the specific binding of [^{3}H]Ro 15-1788, without a significant change in the specific binding of [^{3}H]flunitrazepam. The reduction in radiolabeled antagonist binding by acute exposure of the membrane preparation to ethanol was due to an increase in the apparent K_d. Acute exposure of the membrane preparation to diethylether also resulted in a reduction of specific [^{3}H]Ro 15-1788 binding due to a decreased affinity of the tissue for ligand rather than to a reduction in the maximal density of [^{3}H]Ro 15-1788 binding sites. The data suggest that "obtunding" concentrations of these CNS depressants may selectively alter the "antagonist-favored" conformation of the benzodiazepine binding site on the $GABA_A$ receptor complex, while sparing agonist-preferring sites. The authors referred to their observations as examples of "ligand-dependent" effects of ethanol and diethylether (17).

It is now apparent that the $GABA_A$ receptor complex is a heterooligomeric protein composed of as many as five distinct, although homologous, classes of polypeptide subunits (15,18). Each of the polypeptide subunits has four transmembranous hydrophobic domains. The hydrophobic domains of the pentameric protein complex enclose the GABA-associated chloride ionophore. Moreover, there exists microheterogeneity among the individual subunit forms, that is, there are multiple molecular forms of the individual subunits and the alleles encoding these multiple molecular forms do not necessarily exist on the same chromosome (3). For example, alleles encoding the α-subunit have been identified on chromosomes 4, 5, and X. The sensitivity of the benzodiazepine binding site to agonist (e.g., flurazepam), inverse receptor agonist (e.g., FG-7142), and antagonist (e.g., Ro 15-1788 or flumazenil) is determined by the unique combination of the individual polypeptide subunits (20). For example, the insertion of the γ_2-subunit confers sensitivity to benzodiazepine agonists, whereas the expression and insertion of the α_5-subunit confers a unique sensitivity to inverse benzodiazepine receptor agonists. Moreover, the extent to which a benzodiazepine agonist can facilitate the GABA-gated chloride ion conductance of a single receptor complex could be a stable function of its subunit composition. There are also regional differences noted in the expression of specific subunit forms. Thus, different stress paradigms may be associated with the selective transcription of specific molecular subunits, leading to the expression and insertion of functional GABA-gated channels with stable pharmacological properties. Conceivably, the ability of stress to induce the selective transcription of different molecular forms is regionally specific in the brain. If one considers all the possible combinations of the known molecular forms of each subunit, in excess of 2,000 unique $GABA_A$ receptor complexes may exist. Thus, the recent insights gained from molecular biology suggest an enormous potential for plasticity and could account for the seemingly inconsistent reports in the literature (1,3). Further, the data suggest regional differences in the sensitivity to this stress-induced selective transcription of the antagonist-preferring benzodiazepine binding site. The effect of swim stress on the selective transcription of subunit forms may not be a nonspecific one as increased binding of [^{3}H]Ro 15-1788 was observed in the hippocampus and cerebellum, but not in the cerebral cortex.

The release of an endogenous inverse benzodiazepine receptor agonist (i.e., a β-carboline) has been reported to occur in response to stress in the rat (14,16). It may be that the

selective synthesis of $GABA_A$ receptor complexes with the antagonist-preferring benzodiazepine binding site represents a compensatory response to the release of endogenous inverse agonist in the brain. Finally, the stress-induced reduction in the antiseizure efficacy of flurazepam may be due to the selective synthesis of new benzodiazepine antagonist-preferring $GABA_A$ receptor complexes. Conceivably, flurazepam is less efficacious in the presence of a stress-induced increase in the population of antagonist-preferring $GABA_A$ receptor complexes.

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