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^{125}I-iomazenil binding shows stress- and/or diazepam-induced reductions in mouse brain: Supporting data for ^{123}I-iomazenil SPECT study of anxiety disorders

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Effects of repeated swim stress on the binding of ^{125}I-iomazenil were examined in the brains of diazepam-treated and non-treated mice. The mice were orally administered diazepam or vehicle (0.5% ethylene glycol) and subjected to daily swim stress (at 20°C for 10 min) for seven consecutive days. The distribution and the amount of ^{125}I-iomazenil binding were analyzed autoradiographically after *in vivo* and *in vitro* binding experiments. Repeated swim stress decreased the *in vivo* binding in the hippocampus ($p < 0.05$) and cerebral cortex ($p < 0.05$) of vehicle-treated mice but caused no significant changes in diazepam-treated mice. Subchronic treatment with diazepam decreased the *in vivo* binding approximately 50% in all brain regions examined ($p < 0.01$). The *in vitro* experiment, however, revealed no significant changes except in the hippocampus, where a small but significant decrease in the binding was observed after subchronic treatment with diazepam ($p < 0.01$). The stress- or diazepam-induced reductions seem to represent alterations in the *in vivo* environment related to ^{125}I-iomazenil binding. These results suggest that we can investigate the pathophysiology of stress and anxiety with ^{123}I-iomazenil SPECT. Care must be taken concerning the effects of benzodiazepines.

Key words: iodine-125-iomazenil; benzodiazepine receptor; repeated swim stress; diazepam; autoradiography

INTRODUCTION

CENTRAL-TYPE benzodiazepine receptor plays a major role in regulating anxiety.[1-3] Single-photon emission computed tomography (SPECT) with ^{123}I-iomazenil has been used to visualize this receptor in humans.[4-7] Patients with panic disorder have shown changes in the amount of ^{123}I-iomazenil binding when they are drug-free.[8-10] To further evaluate the significance of these findings over the course of the illness, the effects of benzodiazepines need to be clarified, since most patients with anxiety disorders are treated with these drugs. Unfortunately there is still a dearth of animal data demonstrating stress-induced and/or benzodiazepines-induced changes in the amount of ^{125}I-iomazenil binding.

Earlier studies that examined changes in the benzodiazepine receptor induced by experimental anxiety or environmental stress yielded conflicting results, reporting either a decrease[11-14] or increase[12,15-17] in the binding. More recently, specific binding of ^{3}H-flumazenil was measured *in vivo* and *in vitro* in laboratory animals subjected to repeated swim stress[18] or social isolation.[19] The results of these studies have demonstrated reductions in the binding *in vivo* but have failed to show any changes *in vitro*. Therefore *in vivo* experiments seem to be more sensitive to the stress-induced changes than *in vitro* experiments.

In the present study we measured ^{125}I-iomazenil binding autoradiographically by using *in vivo* and *in vitro* techniques, and evaluated the effects of repeated swim stress and/or diazepam on the binding. The purposes of

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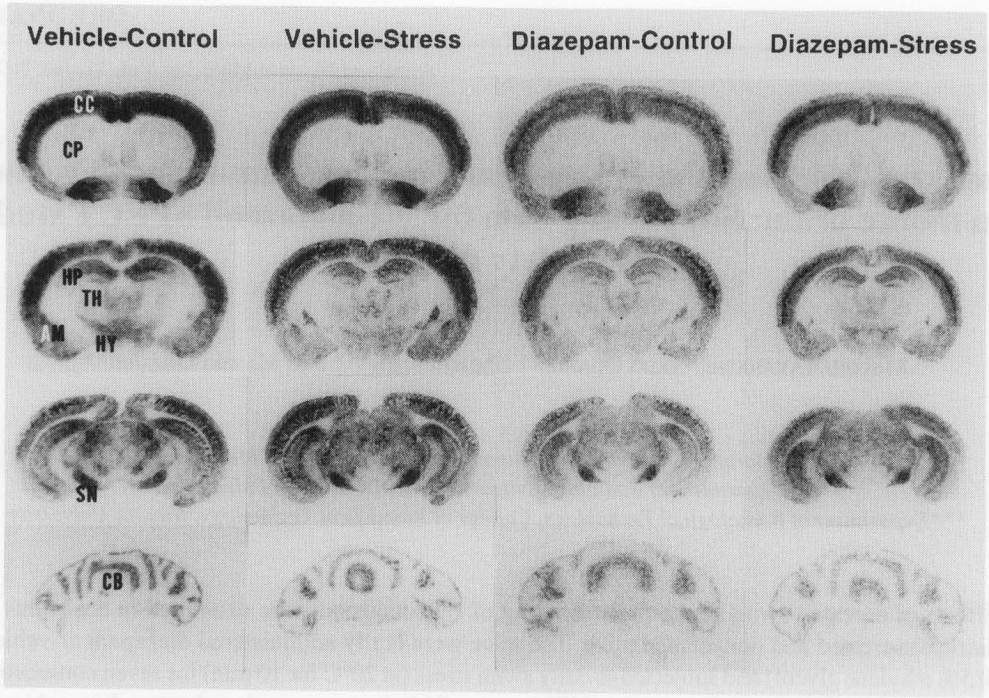

Fig. 1 Autoradiographic images of coronal mouse brain sections obtained 2 h after ^{125}I-iomazenil injection. The binding was measured on cerebral cortex (CC), caudate putamen (CP), globus pallidus (GP, images not shown), amygdala (AM), hippocampus (HP), thalamus (TH), hypothalamus (HY), substantia nigra (SN), and cerebellum (CB). Note that the Vehicle-Stress group demonstrates reduced binding in the hippocampus and cerebral cortex compared to the Vehicle-Control group. Subchronic treatment with diazepam causes diffuse reductions in the binding.

the study were: (1) to examine whether diazepam affects stress-induced changes in the binding of ^{125}I-iomazenil injected into mice; and (2) to determine whether those changes correlate to the alterations in receptor binding measured *in vitro*.

MATERIALS AND METHODS

Animals

Male ICR mice (6–8 W) were used in all experiments. Prior to use they were housed in groups of three or four on a 12 h light-dark cycle with free access to food and water. The mice were randomly assigned to 4 groups: Vehicle-Control group (n = 8 and 7 for the *in vivo* and *in vitro* experiments, respectively); Vehicle-Stress group (n = 9 and 6); Diazepam-Control group (n = 9 and 6); and Diazepam-Stress group (n = 8 and 6). All animal-use procedures were approved by the Niigata University Animal Experimentation Committee.

Repeated swim stress and drug administration

Mice were subjected to swim stress in a tank of water (30 cm diameter, 30 cm depth, 19–21°C) for 10 min. This stress procedure was repeated for seven consecutive days at random times between 8 : 00 a.m. and 5 : 00 p.m. Non-stressed mice remained as controls.

To examine the effects of diazepam, each group of mice received diazepam (25 mg/*l* in 0.5% ethylene glycol) or vehicle (0.5% ethylene glycol) in drinking water during the period of repeated swim stress. The average intake of diazepam was 4.72 ± 0.22 and 4.41 ± 0.15 (mean ± SEM) mg/kg per day in stressed and control groups, respectively, and the difference was not significant as determined by Student's t-test.

In vivo *binding experiment*

The *in vivo* binding experiment was performed twenty-four hours after the last swim stress. To estimate total and nonspecific binding, each group was subdivided into two, and administered either clonazepam (5 mg/kg in 10% dimethyl sulfoxide, i.p., Hoffman-LaRoche, Japan) or vehicle (10% dimethyl sulfoxide, i.p.), respectively. Immediately thereafter, 740 kBq of ^{125}I-iomazenil (specific activity 81.4 TBq/mmol, Medi-Physics, Japan) was injected into a lateral tail vein. The mice were sacrificed by decapitation 120 min after the injection, and the brains were removed rapidly and immersed into isopentane on dry ice for about 15 sec. Coronal sections of the brains were cut in a cryostat at −15°C. The 20 μm thick sections were collected on poly-L-lysine-coated glass slides and dried at room temperature for at least 120 min.

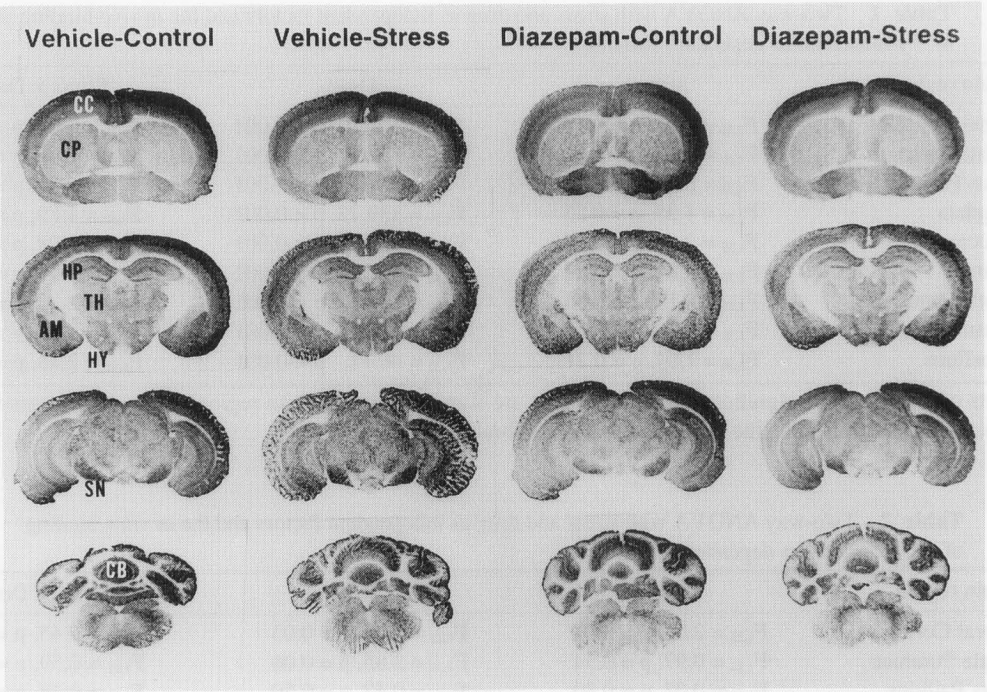

Fig. 2 Autoradiographic images of coronal mouse brain sections obtained 60 min after incubation with ^{125}I-iomazenil (16.3 pM) in 25 mM KH-PO_4 buffer, pH 7.4, containing 150 mM NaCl at room temperature. Clonazepam displaced greater than 90% of total binding. Note that the Diazepam-Control group demonstrates slightly reduced binding in the hippocampus compared to the Vehicle-Control group.

In vitro *binding experiment*

To determine ^{125}I-iomazenil binding *in vitro*, mice were decapitated twenty-four hours after the last swim stress. The brain sections were prepared as described above and stored at −40°C until use. For *in vitro* labeling, slide-mounted sections were preincubated for 30 min in 25 mM KH-PO_4 buffer, pH 7.4, containing 150 mM NaCl at room temperature. The sections were then incubated with ^{125}I-iomazenil (16.3 pM) for 60 min at room temperature in the same buffer as used in the preincubation. Nonspecific binding was defined by adding clonazepam (1 μM) to the incubate. The sections were rinsed four times for 1 min in ice-cold KH-PO_4 buffer, dipped in ice-cold distilled water, and dried under a stream of cool air.

Autoradiography

The sections were apposed to imaging films (X-OMAT AR, Kodak) with calibrated standards (^{125}I-microscales, Amersham) for 2–7 days at 4°C; the films were then removed and developed. Autoradiograms obtained were analyzed by means of a Macintosh computer-based image analysis system (Image, NIH). The atlas of Paxinos and Watson[20] was used to assist in the verification of structures. According to the density of the calibrated standards, the density of the binding was expressed as percent injected dose per gram of polymer for the *in vivo* measurements, or as femtomoles per milligram of polymer for the *in vitro* measurements.

Statistics

All results were analyzed by two-way analysis of variance (ANOVA) for differences between group factors of stress (Stress or Control) and drug (Diazepam or Vehicle). ANOVA was followed by post-hoc comparison (Tukey's test). In all cases, the criterion for significance was $p \leq 0.05$.

RESULTS

Distribution of ^{125}I-iomazenil binding sites in vivo *and* in vitro

Autoradiograms demonstrating regional distribution of ^{125}I-iomazenil binding sites were obtained by *in vivo* (Fig. 1) and *in vitro* (Fig. 2) techniques. Non-specific binding *in vivo* was at the level of the film background. We therefore considered the binding 120 min after the injection as highly specific. In the *in vitro* experiment, clonazepam displaced more than 90% of total binding. Values for specific binding were obtained by subtracting the amount of binding with clonazepam from the total value.

Alterations in the binding of ^{125}I-iomazenil measured in vivo

Figure 3 demonstrates the *in vivo* binding after repeated

Table 1 Two-way ANOVA with stress and drug as independent factors and the *in vivo* binding of ^{125}I-iomazenil as dependent variable

Brain region	Stress	Drug	Stress × Drug
Cerebral Cortex	$F_{1,30} = 1.84$, $p = 0.19$	$F_{1,30} = 120.04$, $p < 0.001$	$F_{1,30} = 6.06$, $p = 0.02$
Caudate Putamen	$F_{1,30} = 0.24$, $p = 0.63$	$F_{1,30} = 67.88$, $p < 0.001$	$F_{1,30} = 2.95$, $p = 0.10$
Globus Pallidus	$F_{1,30} = 0.40$, $p = 0.53$	$F_{1,30} = 51.07$, $p < 0.001$	$F_{1,30} = 0.91$, $p = 0.35$
Amygdala	$F_{1,30} = 1.38$, $p = 0.25$	$F_{1,30} = 159.73$, $p < 0.001$	$F_{1,30} = 1.89$, $p = 0.18$
Hippocampus	$F_{1,30} = 2.52$, $p = 0.12$	$F_{1,30} = 84.57$, $p < 0.001$	$F_{1,30} = 7.28$, $p = 0.01$
Thalamus	$F_{1,30} = 0.01$, $p = 0.92$	$F_{1,30} = 58.78$, $p < 0.001$	$F_{1,30} = 0.99$, $p = 0.33$
Hypothalamus	$F_{1,30} = 0.19$, $p = 0.66$	$F_{1,30} = 84.83$, $p < 0.001$	$F_{1,30} = 2.33$, $p = 0.14$
Substantia Nigra	$F_{1,30} = 0.20$, $p = 0.66$	$F_{1,30} = 53.53$, $p < 0.001$	$F_{1,30} = 0.51$, $p = 0.48$
Cerebellum	$F_{1,30} = 1.64$, $p = 0.21$	$F_{1,30} = 46.12$, $p < 0.001$	$F_{1,30} = 2.45$, $p = 0.13$

Effects of stress are not significant. Effects of drug are significant in all brain regions examined. Stress × drug interactions are significantin the hippocampus and cerebral cortex.

Table 2 Two-way ANOVA with stress and drug as independent factors and the *in vitro* binding of ^{125}I-iomazenil as dependent variable

Brain region	Stress	Drug	Stress × Drug
Cerebral Cortex	$F_{1,21} = 2.01$, $p = 0.17$	$F_{1,21} = 5.64$, $p = 0.03$	$F_{1,21} = 0.45$, $p = 0.51$
Caudate Putamen	$F_{1,21} = 0.97$, $p = 0.34$	$F_{1,21} = 3.85$, $p = 0.06$	$F_{1,21} = 0.59$, $p = 0.45$
Globus Pallidus	$F_{1,21} = 0.07$, $p = 0.80$	$F_{1,21} = 0.47$, $p = 0.50$	$F_{1,21} = 0.28$, $p = 0.60$
Amygdala	$F_{1,21} = 0.06$, $p = 0.81$	$F_{1,21} = 0.01$, $p = 0.94$	$F_{1,21} = 0.14$, $p = 0.71$
Hippocampus	$F_{1,21} = 1.09$, $p = 0.31$	$F_{1,21} = 7.94$, $p = 0.01$	$F_{1,21} = 5.59$, $p = 0.03$
Thalamus	$F_{1,21} = 0.91$, $p = 0.35$	$F_{1,21} = 2.06$, $p = 0.17$	$F_{1,21} = 0.05$, $p = 0.83$
Hypothalamus	$F_{1,21} = 0.88$, $p = 0.36$	$F_{1,21} = 0.23$, $p = 0.64$	$F_{1,21} = 0.48$, $p = 0.50$
Substantia Nigra	$F_{1,21} = 0.01$, $p = 0.93$	$F_{1,21} = 2.58$, $p = 0.12$	$F_{1,21} = 0.21$, $p = 0.65$
Cerebellum	$F_{1,21} = 0.90$, $p = 0.36$	$F_{1,21} = 1.06$, $p = 0.31$	$F_{1,21} = 0.04$, $p = 0.85$

Effects of stress are not significant. Effects of drug are significant in the hippocampus and cerebral cortex. Stress × drug interaction is significant in the hippocampus.

swim stress for the vehicle-treated and diazepam-treated mice. Results of two-way ANOVA are summarized in Table 1. Two-way ANOVA revealed significant effects of drug ($p < 0.001$) and no significant effects of stress in all brain regions examined. There were also significant stress × drug interactions in the hippocampus ($p < 0.05$) and cerebral cortex ($p < 0.05$), indicating different effects of stress in the vehicle-treated and diazepam-treated mice.

In the hippocampus, post-hoc comparisons demonstrated significantly decreased binding after repeated swim stress for the vehicle-treated mice (18.8% reduction, $p < 0.05$) but no significant difference for the diazepam-treated mice. Post-hoc comparisons also revealed significantly decreased binding after subchronic treatment with diazepam both in the control (52.2% reduction, $p < 0.01$) and in the stressed mice (35.2% reduction, $p < 0.01$).

Similarly, in the cerebral cortex, post-hoc comparisons demonstrated significantly decreased binding after repeated swim stress in the vehicle-treated mice (14.3% reduction, $p < 0.05$) but no significant difference in the diazepam-treated mice. Post-hoc comparisons also revealed significantly decreased binding after subchronic treatment with diazepam both in the control (50.1% reduction, $p < 0.01$) and in the stressed mice (37.0% reduction, $p < 0.01$).

Alterations in the binding of ^{125}I-iomazenil measured in vitro

Figure 4 demonstrates the *in vitro* binding after repeated swim stress in the vehicle-treated and diazepam-treated mice. Results from two-way ANOVA are summarized in Table 2. Two-way ANOVA revealed significant effects of drug in the hippocampus ($p < 0.01$) and cerebral cortex ($p < 0.05$), and no significant effects of stress. There was a significant stress × drug interaction in the hippocampus ($p < 0.05$), indicating different effects of drug in the stressed and control mice.

In the hippocampus, post-hoc comparisons demonstrated significantly decreased binding after subchronic treatment with diazepam in the control mice (7.5% reduction, $p < 0.01$) but no significant difference in the stressed mice. Alterations in the binding after repeated swim stress were not significant in any brain regions, including the hippocampus.

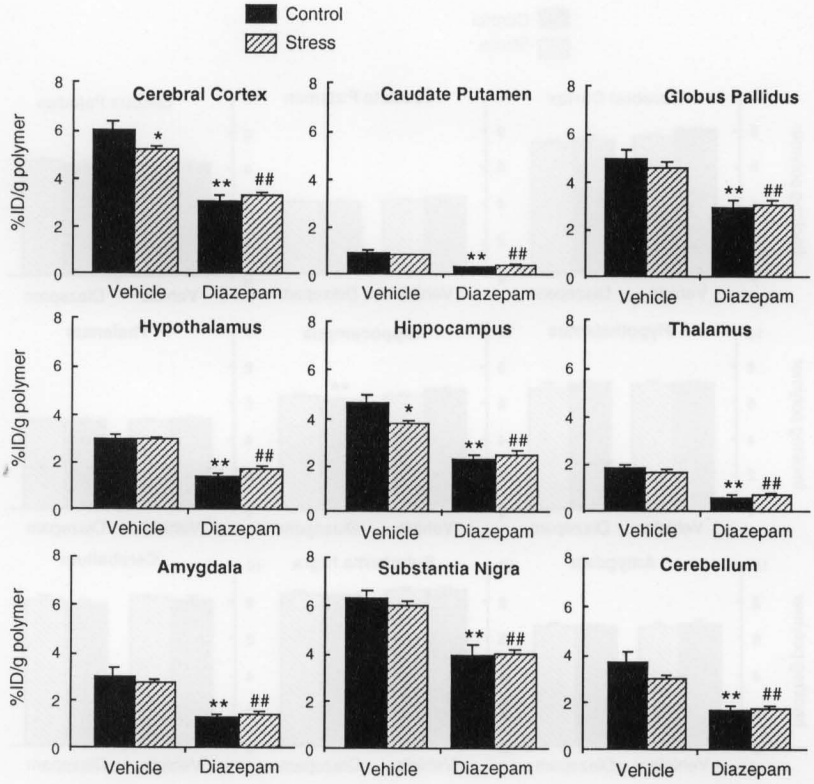

Fig. 3 Alterations in the *in vivo* binding of ^{125}I-iomazenil after repeated swim stress and/or subchronic treatment with diazepam in different brain regions. Results are means with SEM of data on eight to nine mice and are expressed as %ID/g polymer. * Significant difference from Vehicle-Control group, $p < 0.05$; **Significant difference from Vehicle-Control group, $p < 0.01$; ##Significant difference from Vehicle-Stress group, $p < 0.01$.

DISCUSSION

Effects of repeated swim stress on ^{125}I-iomazenil binding

In vivo binding of ^{125}I-iomazenil was significantly decreased after repeated swim stress in the hippocampus and cerebral cortex (Fig. 3). Stress-induced reductions *in vivo* were not accompanied by reductions *in vitro* (Fig. 4). These results indicate similar binding characteristics of ^{125}I-iomazenil to those of ^{3}H-flumazenil: both of these ligands show a stress-induced decrease in the binding *in vivo* but not *in vitro*.[18,19]

Our results validate the research on anxiety disorders with ^{123}I-iomazenil SPECT. Reductions in amount of binding are predicted for the hippocampus as well as for the cerebral cortex, but earlier studies have shown decreased binding only in the cerebral cortex and not yet in the hippocampus, probably due to the limited spatial resolution of the SPECT image.[8,9] We need to identify the hippocampus precisely and measure the binding reliably to elucidate the changes in this region.

The discrepancy between the binding *in vivo* and *in vitro* suggests that the stress-induced reductions do not represent changes in the number and/or affinity of the receptor, but represent changes in the *in vivo* environment involved in the binding of receptor ligands. Recently Ferrarese et al.[21] have shown that acute noise stress increases diazepam binding inhibitor (DBI) levels in the hippocampus. DBI is an endogenous ligand specific to the benzodiazepine receptor[22,23] and competitively inhibits the binding of ^{3}H-diazepam, ^{3}H-flunitrazepam and ^{3}H-flumazenil.[24] These reports suggest that endogenous ligands, increased by repeated swim stress, inhibit the binding of ^{125}I-iomazenil *in vivo*.

Effects of diazepam on ^{125}I-iomazenil binding

Benzodiazepines either induce a decrease[25–29] or cause no change[30,31] in the *in vitro* binding of the receptor, depending on the differences in dose, duration or binding affinity of the drugs employed.[28,29] Subchronic treatment with diazepam (p.o.) did not cause significant changes in the *in vitro* binding of ^{125}I-iomazenil except in the hippocampus, where a small but significant decrease was observed (Fig. 4), but the *in vivo* experiment showed more evident and more diffuse reductions in the binding than the *in vitro* experiment did (Fig. 3). Because benzodiazepines have been reported to occupy the receptor and inhibit the binding of ^{3}H-flumazenil *in vivo*,[32] diazepam-induced reductions *in vivo* do not seem to result from the decrease in the number of receptors. Diazepam may occupy the benzodiazepine receptor and inhibit the binding of ^{125}I-

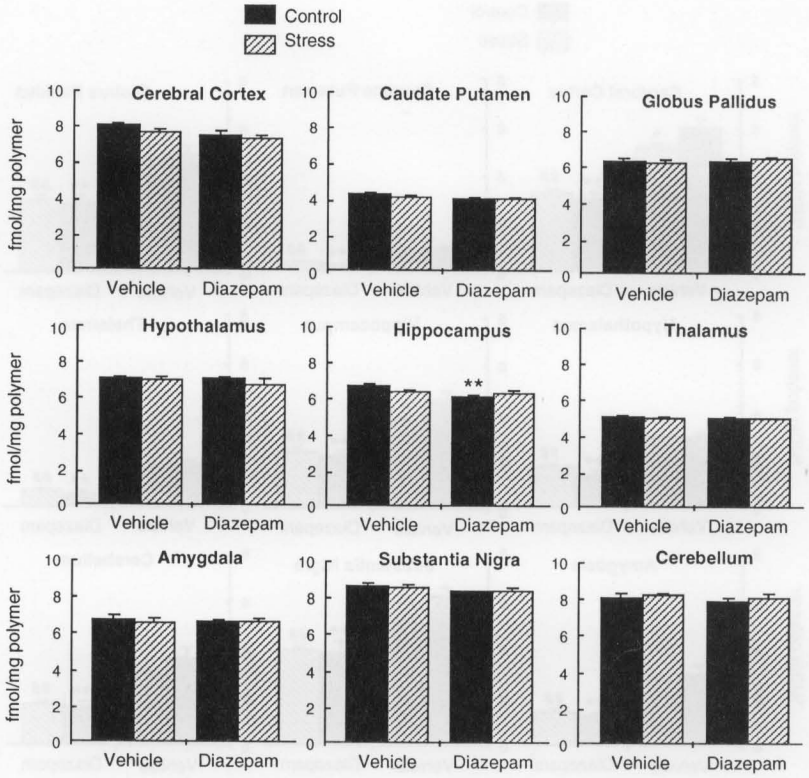

Fig. 4 Alterations in the *in vitro* binding of ^{125}I-iomazenil after repeated swim stress and/or subchronic treatment with diazepam in different brain regions. Results are means with SEM of data on six to seven mice and are expressed as fmol/mg polymer. **Significant difference from Vehicle-Control group, $p < 0.01$.

iomazenil in the mouse brain, although it seems to be washed out *in vitro*.

The stress-induced reductions observed *in vivo* are small compared to the reductions induced by diazepam. When we examine patients with ^{123}I-iomazenil SPECT, we should carefully evaluate the use of benzodiazepines because these drugs may change the binding more clearly than stress or anxiety does.

Effects of diazepam on stress-induced reductions in ^{125}I-iomazenil binding

The diazepam-treated mice did not demonstrate the stress-induced reductions *in vivo* (Fig. 3). Conversely, ^{125}I-iomazenil injected into the diazepam-treated mice occupied a number of receptors irrespective of stress. These results further suggest the difficulty of using ^{123}I-iomazenil SPECT to investigate the patients treated with benzodiazepines. Decreased binding in these patients may merely represent the receptor occupied with these drugs.

The molecular mechanisms underlying these findings are not clear. In mice diazepam may reduce the sensitivity to stress or it may modify stress-induced alterations in the brain. Failure of diazepam to decrease the *in vitro* binding after repeated swim stress, especially in the hippocampus (Fig. 4), indicates that stress also modifies the effects of diazepam. If repeated swim stress increases DBI levels in the brain, then DBI, diazepam, and ^{125}I-iomazenil competitively inhibit each other from binding to the benzodiazepine receptor.

Limitations

The following limitations need to be considered in interpreting the differences between *in vivo* and *in vitro* results. First, several methodological factors may account for the decreased binding *in vivo*. These factors include stress-induced alterations in cerebral blood flow or in ^{125}I-iomazenil metabolism or biodistribution. But Weizman et al.[18] demonstrated that repeated swim stress caused no significant difference in ^{14}C-iodoantipyrine distribution or in whole brain concentrations of clonazepam. Second, stress and/or diazepam may affect the time course of ^{125}I-iomazenil binding *in vivo*. Because *in vivo* binding of receptors does not reach a state of equilibrium, apparent changes in receptor binding depend on the time of measurement after the tracer injection,[33,34] so that kinetic parameters need to be determined by compartment model analysis. Third, we used a single concentration of ^{125}I-iomazenil in the *in vitro* binding experiment. To determine the maximal binding capacity (B_{max}) and affinity (K_d) of the receptor, saturation experiments should be

performed. Finally, concentrations of diazepam and DBI should be measured in the brain sections before and after preincubation to evaluate the involvement of exogenous and endogenous ligands in the *in vitro* binding.

CONCLUSION

(1) Binding of ^{125}I-iomazenil injected into mice decreased significantly in the hippocampus and cerebral cortex after repeated swim stress, but no stress-induced reductions were observed in diazepam-treated mice.

(2) The stress- or diazepam-induced reductions *in vivo* represented alterations in receptor function that were not detectable *in vitro*.

Our animal data suggest that we can investigate the pathophysiology of stress and anxiety with ^{123}I-iomazenil SPECT. We should carefully evaluate the effects of benzodiazepines in interpreting the results obtained in patients with anxiety disorders.

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