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

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Effects of repeated swim stress on the binding of 125I-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 125I-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 125I-iomazenil binding. These results suggest that we can investigate the pathophysiology of stress and anxiety with 125I-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 123I-iomazenil has been used to visualize this receptor in humans.4-7 Patients with panic disorder have shown changes in the amount of 123I-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 123I-iomazenil binding.

Earlier studies that examined changes in the benzodiazepine receptor induced by experimental anxiety or environmental stress yielded conflicting results, reporting either a decrease11-14 or increase15,16,17 in the binding. More recently, specific binding of 1H-flumazenil was measured in vivo and in vitro in laboratory animals subjected to repeated swim stress18 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 123I-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
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/kg 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.
In vitro binding experiment
To determine $^{125}\text{I}$-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 $-40^\circ\text{C}$ 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 $^{125}\text{I}$-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 ($^{125}\text{I}$-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$^{30}$ 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 $p < 0.05$.

RESULTS

Distribution of $^{125}\text{I}$-iomazenil binding sites in vivo and in vitro
Autoradiograms demonstrating regional distribution of $^{125}\text{I}$-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 $^{125}\text{I}$-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 125I-lomazenil as dependent variable

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

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

Table 2 Two-way ANOVA with stress and drug as independent factors and the in vitro binding of 125I-lomazenil as dependent variable

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

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 125I-lomazenil 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.

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**DISCUSSION**

*Effects of repeated swim stress on \(^{125}\text{I}-\text{iomazenil binding}*

*In vivo* binding of \(^{125}\text{I}-\text{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}\text{I}-\text{iomazenil* to those of} \(^{3}\text{H}-\text{flumazenil: both of these ligands show a stress-induced decrease in the binding *in vivo* but not *in vitro*.}^{14,19}

Our results validate the research on anxiety disorders with \(^{125}\text{I}-\text{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\(^{20,21}\) and competitively inhibits the binding of \(^{3}\text{H}-\text{diazepam,}^{3}\text{H}-\text{flunitrazepam and}^{3}\text{H}-\text{flumazenil.}^{24}\) These reports suggest that endogenous ligands, increased by repeated swim stress, inhibit the binding of \(^{125}\text{I}-\text{iomazenil in vivo.}

*Effects of diazepam on \(^{125}\text{I}-\text{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}\text{I}-\text{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 \(^{3}\text{H}-\text{flumazenil in vivo,}^{32}\) 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}\text{I}-\text{iomazenil.}

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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 $^{125}$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 $^{125}$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. 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. So 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 ($B_{\text{max}}$) and affinity ($K_c$) 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 $^{125}$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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