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Effects of diazepam on ^{125}I-iomazenil-benzodiazepine receptor binding and epileptic seizures in the El mouse

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Objective: To investigate changes in free benzodiazepine receptor density in response to repeated, long-term administration of diazepam in epilepsy, we assessed ^{125}I-iomazenil (^{125}I-IMZ) binding in a mouse model. ***Methods:*** El mice were divided into two groups of 12 mice each which received either no diazepam (El(D[−]) group) or 2 mg/kg of diazepam per week (El(D[+]) group). Nine ddY mice were used as a control. Once each week from the age of 5 to 19 weeks, the El mice received stimulation to produce epileptic seizures 20 minutes after receiving intraperitoneal injections. At 20 weeks of age, a total dose of 0.37 MBq of ^{125}I-IMZ was injected in all mice and their brains were rapidly removed 3 hours later. The incidence of epileptic seizures at the age of 19 weeks and the autoradiograms of the brain were compared. ***Results:*** The incidence of epileptic seizures in response to weekly stimulation was significantly lower in the El(D[+]) group than in the El(D[−]) group ($p < 0.001$). The percent injected doses of ^{125}I-IMZ per gram of tissue in the cortex, hippocampus and amygdala were significantly lower in the El(D[+]) group than in the El(D[−]) group ($p < 0.05$). ***Conclusion:*** The results suggest that diazepam binds competitively to ^{125}I-IMZ as an agonist to free benzodiazepine receptor sites in the cortex, hippocampus and amygdala and shows anticonvulsant effect in El mice.

Key words: ^{125}I-iomazenil, benzodiazepine receptor, epileptic seizure, diazepam, El mouse

INTRDUCTION

NUMEROUS ANIMAL STUDIES have investigated the mechanisms of epileptic seizures.[1–9] The major inhibitory neurotransmitter in the brain is γ-aminobutyric acid (GABA). Epilepsy is a disease whose mechanism is affected by the $GABA_A$-benzodiazepine receptor (BZR) complex. The loss of GABA-mediated inhibitory functions is responsible for the development of epileptic seizures in several animal models.[1,3,9–12]

The radioligand ^{123}I-iomazenil (^{123}I-IMZ), which was developed to diagnose and study cerebrovascular diseases and neurological as well as psychological disorders, is a partial inverse agonist for central BZRs.[13,14] Central BZRs are located on the alpha subunit of $GABA_A$ receptors, which form chloride ion channels distributed throughout the brain. The $GABA_A$-BZR chloride ion channel complex is believed to serve as a major inhibitory neurotransmitter receptor in the mammalian brain.[15,16]

The El mouse was first described in 1959, registered internationally in 1964,[17] and confirmed through electroencephalographic studies to be an authentic epilepsy model in 1976.[18] The El gene, located on chromosome 9, is inherited in an autosomal recessive manner.[19] The El mouse has seizures in response to sensory stimulation with rapid acceleration, such as vertical tossing and seesaw movements.[20–24] The El mouse is a model for genetic sensory-precipitated epilepsy with generalized tonic-clonic convulsions.[25,26]

Diazepam is a benzodiazepine commonly used as an anxiolytic, anticonvulsant, sedative-hypnotic, and muscle relaxant.[27–30] The pharmacologic actions of diazepam are due to enhancement of GABAergic inhibition via the $GABA_A$/BZR chloride-channel complex. Although the pharmacologic actions and mechanisms of diazepam have been extensively studied, how diazepam affects the

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distribution of BZR-specific radioligands in the brain with epilepsy remains unclear.

In the present study, we assessed changes in ^{125}I-IMZ accumulation and the development of epileptic seizures with repeated, long-term administration of diazepam in El mice.

MATERIALS AND METHODS

All experiments were performed according to the Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources, 1996).

Twenty-four El mice weighing 35.5 to 48.8 g, and 9 ddY mice which are mother strain of El mice weighing 38.4 to 46.4 g were used. Mice were housed in plastic cages in groups of 2 or 3 and given food and water ad libitum. The mice were maintained on a 12-hour light-dark cycle (lights on from 08:00 to 20:00 hours) at a room temperature of 22°C to 25°C and a relative humidity of 45%.

We used diazepam as an anticonvulsant because it is a benzodiazepine and is suitable for assessing the state of BZRs. The El mice were then divided into two groups of

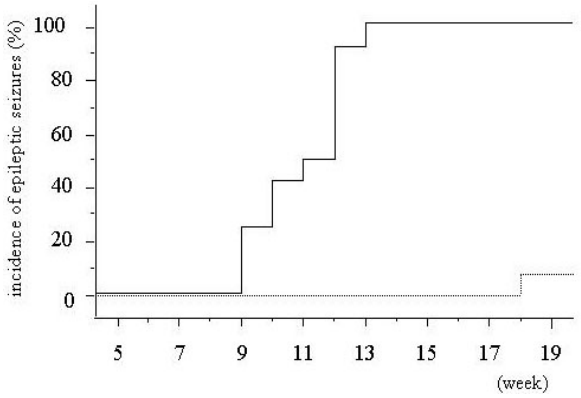

Fig. 1 The incidence of epileptic seizures. The solid line shows the El(D[−]) group. The dotted line shows the El(D[+]) group. Epileptic seizures were first observed at 9 weeks of age and at 13 weeks of age in all mice of the El(D[−]) group. An epileptic seizure was observed at 18 weeks of age in one mouse of the El(D[+]) group. The incidence of epileptic seizures was 100% (12 of 12 mice) in the El(D[−]) group and 8.3% (1 of 12 mice) in the El(D[+]) group at 19 weeks of age. The incidence of epileptic seizures in the El(D[−]) group at 19 weeks of age was significantly higher than that in the El(D[+]) group ($p < 0.0001$).

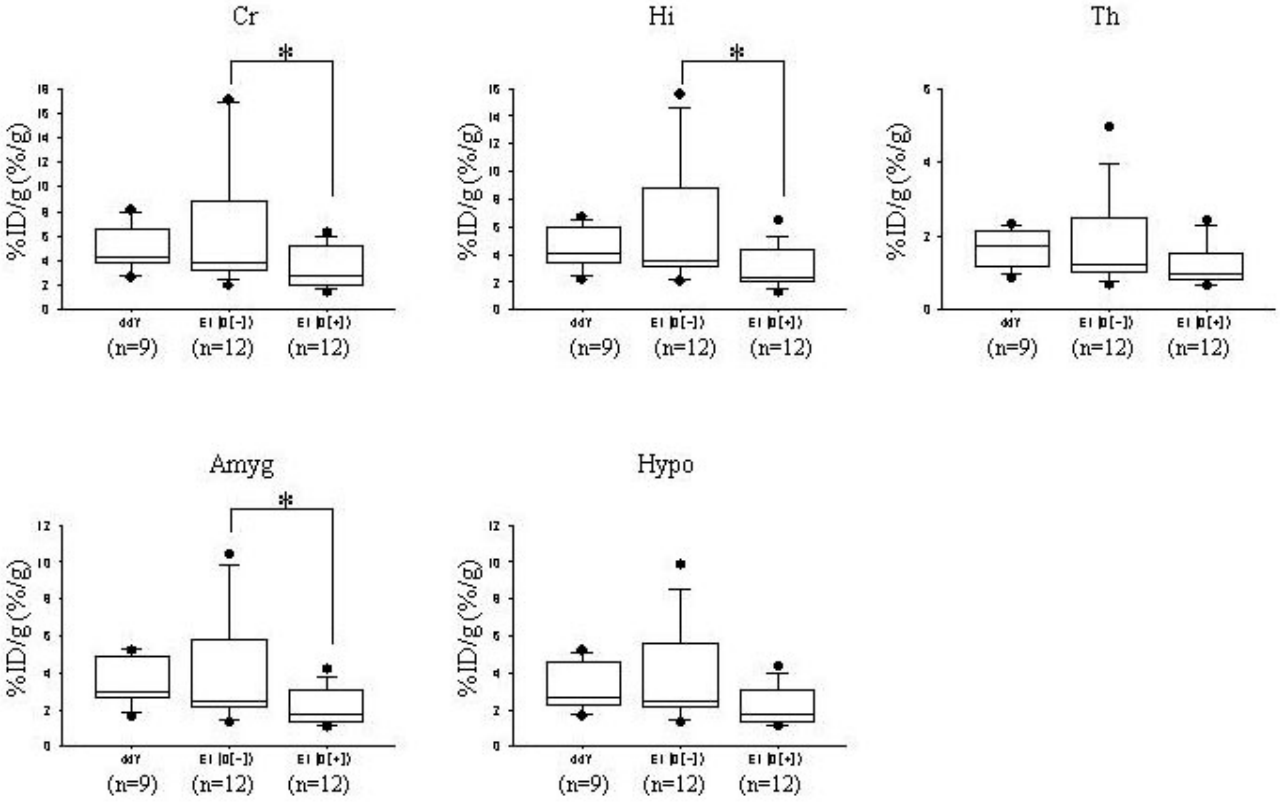

Fig. 2 The %ID/g of the each region. The %ID/g in each region is presented by Box Plots. The %ID/g in the cortex, hippocampus and amygdala of the El(D[+]) group was significantly lower than that of the El(D[−]) group ($p < 0.05$). (Cr: cortex; Hi: hippocampus; Th: thalamus; Amyg: amygdala; Hypo: hypothalamus, *: $p < 0.05$)

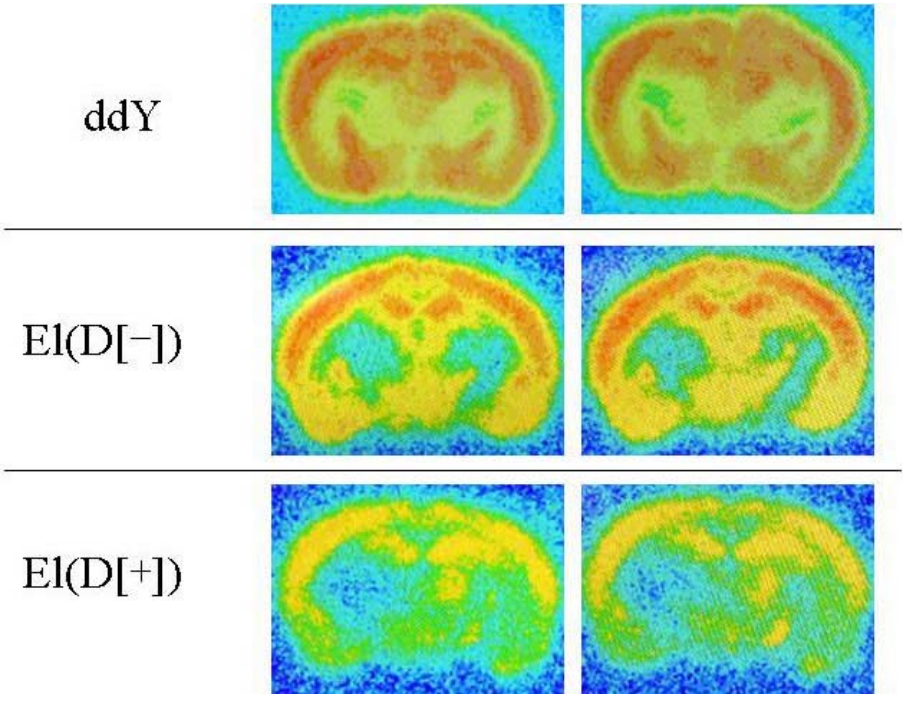

Fig. 3 The autoradiogram of the brain of the ddY, El(D[−]) and El(D[+]) mice. (*Upper:* ddY mouse, *middle:* El(D[−]) mouse, *lower:* El(D[+]) mouse.) The autoradiogram of the El(D[−]) mouse showed mild decreased accumulation of ^{125}I-IMZ in each region compared to that of ddY mouse. The autoradiogram of the El(D[+]) mouse showed severely decreased accumulation of ^{125}I-IMZ in cortex, hippocampus and amygdala and moderately decreased accumulation in hypothalamus compared to that of El(D[−]) mouse. The %ID/g of cortex, hippocampus and amygdala in El(D[+]) mouse was less than 70% compared to that of El(D[−]) mouse.

12 mice each which received weekly intraperitoneal injections containing no diazepam (El(D[−]) group) or 2 mg/kg diazepam (El(D[+]) group). The El mice were placed in the supine position, and diazepam was injected into the intraperitoneal cavity using a 1-m*l* syringe with straight needles.

Once each week from the age of 5 to 19 weeks, the El mice received stimulation to produce epileptic seizures 20 minutes after receiving intraperitoneal injections. All El mice were tossed vertically 30 cm from the bottom of the cage 10 times. When the mouse had an epileptic seizure, the stimulation procedure was stopped immediately.

At 20 weeks of age, all mice were immobilized with ether, and a total dose of 0.37 MBq of ^{125}I-IMZ (1.85 MBq/500 μl; specific activity, 2200 Ci/mmol, saline solution, pH 5.0; Nihon Medi-Physics, Chiba, Japan) was injected into the lateral tail vein of each mouse. The mice were immobilized with ether and decapitated 3 hours after the injection; their brains were rapidly removed and frozen at −70°C in hexane and dry ice. Coronal sections of the brains were cut with a cryostat at −20°C.

Twenty-micrometer-thick sections were collected with adhesive tape and dried for autoradiography at −20°C. The coronal sections were placed on imaging plates (BAS-MP, Fuji Photo Film Co., Ltd., Tokyo, Japan), which were exposed for 5 to 8 days. The imaging plates were then removed and developed. The resulting autoradiograms were analyzed with a fluorescent/radioisotope imaging system (FLA-2000, Fuji Photo Film Co., Ltd.).

Five regions of interest (ROI) representing the cortex, hippocampus, thalamus, amygdala and hypothalamus were determined on both sides of the brain in two coronal sections of autoradiograms (modified from the atlas of Paxinos and Watson[31]). Regional tracer binding was measured according to calibrated standards and expressed as the percent injected dose of ^{125}I-IMZ per gram of tissue (%ID/g).

The incidence of epileptic seizures at the age of 19 weeks was compared between the El(D[−]) and El(D[+]) groups and the %ID/g of the ROIs was compared among ddY, El(D[−]) and El(D[+]) groups. The incidence of epileptic seizures was calculated with the Kaplan-Meier method and analyzed with the log-rank p test. The %ID/g was analyzed with the Fisher's PLSD test. A p value <0.05 was considered to indicate statistical significance.

RESULTS

Epileptic seizures were observed in all 12 mice of the El(D[−]) group and in 1 mouse of the El(D[+]) group.

Epileptic seizures began at 9 to 13 weeks of age in the El(D[−]) group and at 18 weeks of age in the El(D[+]) group and continued until the age of 19 weeks, when the final stimulation was performed. The incidence of epileptic seizures at 19 weeks of age was significantly higher in the El(D[−]) group than in the El(D[+]) group ($p < 0.001$, Fig. 1).

The %ID/g of the cortex, hippocampus and amygdala was significantly lower in the El(D[+]) group than in the El(D[−]) group ($p < 0.05$, Fig. 2). We present autoradiograms of the brain of mice of the ddY, El(D[−]) and El(D[+]) groups in Figure 3.

DISCUSSION

^{123}I-IMZ is extremely effective for detecting epileptogenic foci.[32,33] Although the usefulness of ^{123}I-IMZ for diagnosing epilepsy and for identifying epileptogenic foci has been evaluated, the changes in ^{123}I-IMZ distribution with treatment, such as that with anticonvulsants, have not been fully examined. If ^{123}I-IMZ would reveal specific changes due to treatment with anticonvulsants and thus be used to evaluate the effects of treatment as well as to diagnose epilepsy, the clinical usefulness of ^{123}I-IMZ would be significantly increased. We thought that as a next step we should assess changes in ^{123}I-IMZ distribution for evaluating the effects of treatment in patients with epilepsy. However, because clinical studies can be affected by various factors, such as the type, frequency, and severity of epilepsy, location of epileptogenic focus, time course, and treatment method, we decided to first evaluate the changes in ^{125}I-IMZ distribution in an animal model.

Diazepam has been often used as an anticonvulsant in animal studies. The dose of diazepam producing anticonvulsant effects has ranged from 0.1 and 10 mg/kg and anticonvulsant effects against the stimulation were observed 15–30 minutes after injection in previous mouse studies.[34–39] De Sarro et al. reported that the TD_{50}, which is the dose at which 50% of mice fall from a rotarod, is 3.25 mg/kg.[40] The ideal dose of diazepam would block epileptic seizures as much as possible without toxicity. After referring to past reports, we decided to administer diazepam at a dose of 2 mg/kg via intraperitoneal injection 20 minutes before stimulation to produce epileptic seizures.

Although regional binding of radiolabeled diazepam has been investigated in animal and human studies,[41–43] no consensus has been reached. It is reported that the cerebral cortex and hippocampus show high binding of ^{3}H-diazepam in the brain of rats and humans.[42,43] It is easily understood that cortex and hippocampus have high potential to bind diazepam and diazepam competitively blocks the binding of ^{125}I-IMZ to BZR in the cortex and hippocampus. We think that amygdala has high potential to bind diazepam as well as cortex and hippocampus from our results. However we have no data supporting this theory. Other mechanisms of decreased binding of ^{125}I-IMZ may be present and function together in the amygdala.

Histological studies of El mice have revealed that the CA1/2 pyramidal cell layer and the dentate granule cell layer of the hippocampus are loosely packed and disorganized.[44] Ishida et al. presented electrocorticographic data suggesting a focus complex and epileptogenicity circuit consisting of the parietal cortex and hippocampus, and proposed that the hippocampus is related to the generalization of seizure discharges, serving to maintain and terminate them in El mice.[45] Epileptic seizures start in the hippocampus and propagate to other brain regions. Suzuki et al. reported that [^{14}C]2-deoxyglucose accumulation is decreased in hippocampus, pyriform cortex, entorhinal cortex and amygdala.[46] Our results seem to be consistent with abnormalities reported in previous histological, electrocorticographic and radiologic studies.[44–46] We think that the reason for the decreased binding of ^{125}I-IMZ in the cortex, hippocampus and amygdala is not only the binding affinity, but also the changes of the $GABA_A$/BZR complex occurring in the process of acquisition and regulation of epileptic seizures in El mouse, although further studies are necessary to prove this contention.

Few animal studies have examined changes in BZR-specific radioligand distribution with administration of anticonvulsants. Zanotti et al. found, using diazepam and imidazenil, a new partial BZR agonist, that the maximum numbers of ^{3}H-flumazenil binding sites are reduced in the cerebral cortex (36% decrease) and cerebellum (42% decrease) of rats made tolerant to diazepam. In contrast, no changes in ^{3}H-flumazenil binding occurred in rats after long-term treatment with imidazenil. Specific ^{3}H-flumazenil binding *in vivo* is decreased in the forebrain after long-term treatment with diazepam, but not with imidazenil.[47] Although we used a different animal model and radioligand in our study, the decreased binding of BZR-specific radioligand in the brain with administration of diazepam is consistent with our result.

Our study has clearly shown that repeated, long-term administration of diazepam to El mice prevents epileptic seizures and reduces the binding of ^{125}I-IMZ-BZR in the cortex, hippocampus and amygdala. The El mouse we used is just one of many animal models of epilepsy. We administered diazepam and stimulated El mice with vertical tossing from 5 to 19 weeks of age as a model of epilepsy treated with long-term administration of diazepam. We did not administer diazepam for the final week to exclude as much as possible the acute action of diazepam as an agonist to BZR. The reason why the %ID/g in each region of the El(D[−]) group was not significantly decreased as compared to those of ddY group may be due to this protocol. We believe that further preclinical studies like ours should provide much more detailed and important information.

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