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# Abnormal benzodiazepine receptor function in the depressive-like behavior of diabetic mice

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**Abstract**

We previously reported that streptozotocin (STZ)-induced diabetic mice exhibited depressive-like behavior in the tail suspension test. In this study, we examined the involvement of benzodiazepine receptor functions in this diabetes-induced depressive-like behavior in mice. STZ-induced diabetes significantly increased the duration of immobility without affecting spontaneous locomotor activity. This increase was dose-dependently and significantly suppressed by a benzodiazepine receptor antagonist, flumazenil (0.1–1 mg/kg, i.v.). However, flumazenil (0.1–1 mg/kg, i.v.) did not affect the duration of immobility in non-diabetic mice. Furthermore, flumazenil (1 mg/kg, i.v.) had no significant effect on spontaneous locomotor activity in either non-diabetic or diabetic mice. The benzodiazepine receptor inverse agonist methyl β-carboline-3-carboxylate (β-CCM; 0.03–0.3 mg/kg, i.v.) dose-dependently and significantly increased the duration of immobility in non-diabetic mice, but not in diabetic mice. β-CCM (0.3 mg/kg, i.v.) significantly suppressed spontaneous locomotor activity in non-diabetic mice, but not in diabetic mice. These results indicate that diabetic mice may have enhanced negative allosteric modulation by benzodiazepine receptor ligands, such as diazepam binding inhibitors, under stressful conditions, but not free-moving conditions, and this abnormal function of benzodiazepine receptors may cause, at least in part, the expression of depressive-like behavior in diabetic mice.
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*Keywords:* Diabetes; Flumazenil; β-CCM; Tail suspension test; Depressive-like behavior; Benzodiazepine receptor

## 1. Introduction

It has been recognized that patients with diabetes have a higher prevalence of depression than the general population (Berlin et al., 1997; Peyrot and Rubin, 1997; Anderson et al., 2001; Petrak et al., 2003). Diabetic patients with depression also show poor glycemic control (Lustman, 1988; Lin et al., 2004). In addition, psychological troubles are regarded as risk factors for the future development of diabetes-related complications (Lustman et al., 2000; de Groot et al., 2001). However, little information is available to resolve this problem. In animal studies, streptozotocin (STZ)-treated rodents are often used as an animal model of type 1 diabetes because STZ induces pancreatic β-cell death and hyperglycemia associated with the decreased insulin secretion (Arison et al., 1967; Hohenegger and Rudas, 1971; Tarui et al., 1987). STZ-induced diabetic rodents show changes in the central nervous system as indicated by neurochemical, electrophysiological, morphological and behavioral studies (Hilakivi-Clarke et al., 1990; McCall, 1992; Biessels et al., 1996; Magarinos and McEwen, 2000). We previously reported that STZ-induced diabetic mice exhibited depressive-like behavior in the tail suspension test (Kamei et al., 2003), which is often used to screen putative antidepressants (Steru et al., 1985). However, depressive-like behavior was not observed in mice in the early stage of STZ-induced diabetes or in hyperglycemic mice induced by glucose injection (Kamei et al., 2003). Since STZ does not cross the blood–brain barrier and has an early excretion rate (Schein, 1969; Karunanayake et al., 1974), we have suggested that the depressive-like behavior in STZ-induced diabetic mice is induced by the diabetic state rather than by STZ itself.

We previously reported that psychological stress-induced analgesia was greater in STZ-induced diabetic mice than in non-diabetic mice (Kamei and Ohsawa, 2000). In addition, the anxiety-like state in an unfamiliar environment was enhanced in STZ-induced diabetic mice compared with non-diabetic

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mice (Kamei et al., 2001). Interestingly, these diabetes-induced behavioral changes were transiently normalized by acute injection of flumazenil, a selective benzodiazepine receptor antagonist (Kamei and Ohsawa, 2000; Kamei et al., 2001). Furthermore, benzodiazepine receptor inverse agonist-treated non-diabetic mice showed behavioral changes similar to those observed in STZ-induced diabetic mice (Kamei and Ohsawa, 2000; Kamei et al., 2001). Therefore, we have suggested that the endogenous negative modulation of benzodiazepine receptors may be enhanced in the STZ-induced diabetic state, and this alteration may contribute, at least in part, to the behavioral changes observed in STZ-induced diabetic mice.

It is well established that γ-aminobutyric acid (GABA) and benzodiazepine receptors play important roles in the pathogenesis and therapeutics of depression (Petty et al., 1995; Shiah and Yatham, 1998; Brambilla et al., 2003; Tunnicliff and Malatynska, 2003). Clinical studies have demonstrated that plasma and brain GABA levels in depressive subjects are decreased (Petty et al., 1992; Sanacora et al., 1999). In addition, GABA synthesizing enzyme glutamate decarboxylase activity is significantly decreased in the postmortem brain of patients with depression (Perry et al., 1977). It has also been reported that the levels of diazepam binding inhibitor (DBI), an endogenous substance that shows inverse agonistic properties toward benzodiazepine receptors (Guidotti et al., 1978, 1983), are increased in cerebrospinal fluid in patients with depression (Barbaccia et al., 1986; Roy, 1991). These reports indicate that GABAergic dysfunction may be closely related to the pathogenesis of depression. Since the benzodiazepine anxiolytic alprazolam has therapeutic properties toward depression (Petty et al., 1995), it has also been suggested that normalization of GABAergic dysfunction and/or enhancement of GABAergic neurotransmission may lead to the amelioration of depressive symptoms.

Based on these reports, it is possible that the depressive-like behavior in STZ-induced diabetic mice may be attributed to GABAergic dysfunction associated with the abnormal function of benzodiazepine receptors. To clarify this hypothesis, we examined the involvement of benzodiazepine receptor function in diabetes-induced depressive-like behavior in mice.

## 2. Materials and methods

### 2.1. Animals

Male ICR mice (Tokyo Laboratory Animals Science Co., Ltd., Tokyo), 4 weeks of age and weighing approximately 20 g at the beginning of the experiments, were used. They were housed 10 per cage and had free access to food and water. The animal room was maintained at 24±1 °C and 55±5% humidity with a 12-h light–dark cycle (light on at 08:00 h, light off at 20:00 h). Animals were rendered diabetic by an injection of streptozotocin (200 mg/kg, i.v.) dissolved in citrate buffer at pH 4.5. Age-matched control mice were injected with the vehicle alone. Blood glucose levels were determined using a glucose analyzer (ANTSENSE II, Sankyo Co. Ltd., Tokyo, Japan). Six-week-old mice (i.e. 14 days after the induction of diabetes) with hyperglycemia (plasma glucose levels>400 mg/dl) were defined as diabetic. This study was carried out in accordance with the Guide for the Care and Use of Laboratory Animals as adopted by the Committee on the Care and Use of Laboratory Animals of Hoshi University, which is accredited by the Ministry of Education, Science, Sports and Culture.

### 2.2. Drugs

The drugs used in this study were streptozotocin (Sigma Chemical Co., St. Louis, MO, USA), flumazenil solution (Anexate®; Yamanouchi Pharmaceutical Co., Tokyo, Japan), and methyl β-carboline-3-carboxylate (β-CCM; Sigma). Flumazenil was diluted with saline. β-CCM was dissolved in a small volume of 0.1 M HCl, and then diluted with saline, and the pH was adjusted to 4.0 with NaOH just prior to use. Each drug was administered at a volume of 0.1 ml/10 g of body weight.

### 2.3. Experimental procedures

#### 2.3.1. Tail suspension test

The procedure was according to our previous report (Kamei et al., 2003). The tail suspension apparatus consisted of a white translucent plastic box ($30 \times 30 \times 30$ cm$^3$) with a hook in the middle of the ceiling from which to suspend the mouse. Mice were suspended by the tail using adhesive Scotch tape affixed to the hook which was connected to a strain gauge (TAIL SUSPENSION AMP, Neuroscience Inc., Tokyo, Japan) that picked up all movements of the mouse and transmitted them to a central processing unit which calculated the total duration of immobility and the strength of movements during the 10 min of the test. Each mouse was suspended individually. The movements of the mice were digitized and processed by Super Scope II (GWI; Somerville, MA, USA). The threshold level was set so as to exclude respiration movement. The duration of immobility was defined as the total amount of time that the animal showed no movement. Flumazenil and β-CCM were injected i.v. 5 min before testing. In the combination study, flumazenil was injected i.v. just before i.v. treatment with β-CCM.

#### 2.3.2. Spontaneous locomotor activity

Spontaneous locomotor activity of mice was measured by a digital counter with an infrared sensor (NS-AS01, Neuroscience Inc., Tokyo, Japan). The apparatus detects the movement of animals based on released infrared rays associated with their temperature, and records a digital count. A mouse was placed in a transparent plastic cage ($27 \times 17 \times 13$ cm$^3$), a transparent plastic ceiling was installed, and an infrared sensor was placed at the center of the ceiling. Mice were placed in the measurement cage, and then recording was started. Total activity counts were automatically recorded for 10 min, which was the same as the measurement period in the tail suspension test. Flumazenil and β-CCM were injected i.v. 5 min before testing. In the combination study, flumazenil was injected i.v. just before i.v. treatment with β-CCM.

### 2.4. Statistics

The data were expressed as mean ± S.E.M. Significant differences were determined by one-way and two-way analysis of variance (ANOVA) for factorial comparisons and Dunnett's test for multiple comparisons. Student's *t*-test or Aspin–Welch's *t*-test was used to evaluate differences between two groups. *P*-values less than 0.05 were considered significant.

## 3. Results

### 3.1. Effect of flumazenil on the duration of immobility in the tail suspension test in non-diabetic and diabetic mice

The duration of immobility was significantly longer in diabetic than in non-diabetic mice (Fig. 1). Flumazenil (0.1–1 mg/kg, i.v.) dose-dependently and significantly suppressed the prolonged duration of immobility in diabetic mice to the same levels as observed in non-diabetic mice [$F(3,32)=5.375$, $p<0.01$] (Fig. 1). However, flumazenil (0.1–1 mg/kg, i.v.) had no significant effect on the duration of immobility in non-diabetic mice [$F(3,30)=0.143$, $p=0.9332$] (Fig. 1). Two-way ANOVA revealed that the duration of immobility was significantly affected by diabetes [$F(1,62)=7.080$, $p<0.01$] and diabetes × drug interaction [$F(3,62)=2.958$, $p<0.05$].

### 3.2. Effect of β-CCM on the duration of immobility in the tail suspension test in non-diabetic and diabetic mice

β-CCM (0.03–0.3 mg/kg, i.v.) dose-dependently and significantly increased the duration of immobility in non-diabetic mice to the same levels as observed in diabetic mice [$F(3,34)=3.382$, $p<0.05$] (Fig. 2). However, β-CCM (0.03–0.3 mg/kg, i.v.) had no significant effect on the duration of immobility in diabetic mice [$F(3,32)=0.948$, $p=0.4292$] (Fig. 2). Two-way ANOVA revealed that the duration of immobility was significantly affected by diabetes [$F(1,66)=10.116$, $p<0.01$], but not diabetes × drug interaction [$F(3,66)=1.209$, $p=0.313$].

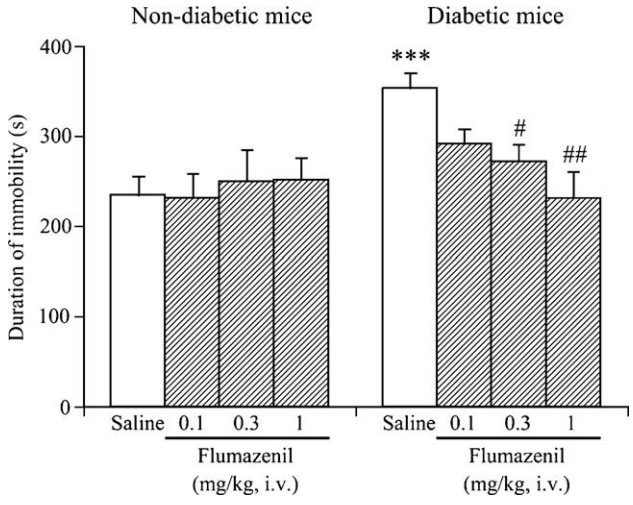


Fig. 1. Effect of flumazenil on the duration of immobility in the tail suspension test in non-diabetic and diabetic mice. Each column represents the mean ± S.E.M. of 8–10 mice. ***$p<0.001$ vs. saline-treated non-diabetic mice (Student's *t*-test). $^{\#}p<0.05$ and $^{\#\#}p<0.01$ vs. respective saline-treated group (Dunnett's test).

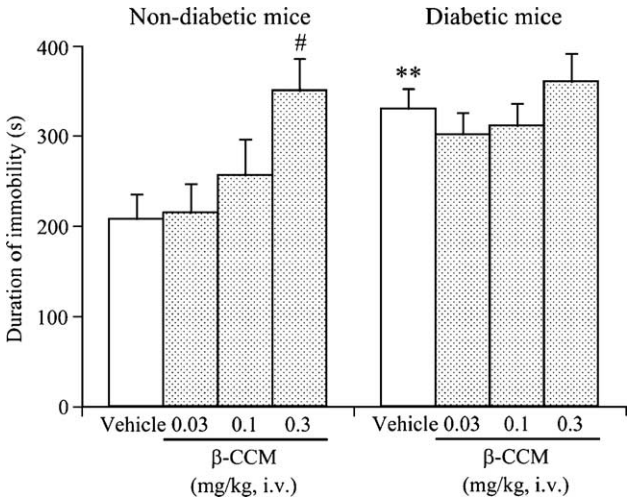


Fig. 2. Effect of β-CCM on the duration of immobility in the tail suspension test in non-diabetic and diabetic mice. **$p<0.01$ vs. vehicle-treated non-diabetic mice (Student's *t*-test). $^{\#}p<0.05$ vs. respective vehicle-treated group (Dunnett's test). Each column represents the mean ± S.E.M. of 8–10 mice.

The β-CCM (0.3 mg/kg, i.v.)-induced marked prolongation of immobility time in non-diabetic mice was dose-dependently and significantly antagonized by the pretreatment with flumazenil (0.03–0.3 mg/kg, i.v.) [$F(3,33)=5.470$, $p<0.01$] (Fig. 3).

### 3.3. Effects of flumazenil and β-CCM on spontaneous locomotor activity in non-diabetic and diabetic mice

There was no significant difference in the spontaneous locomotor activity for 10 min between non-diabetic and

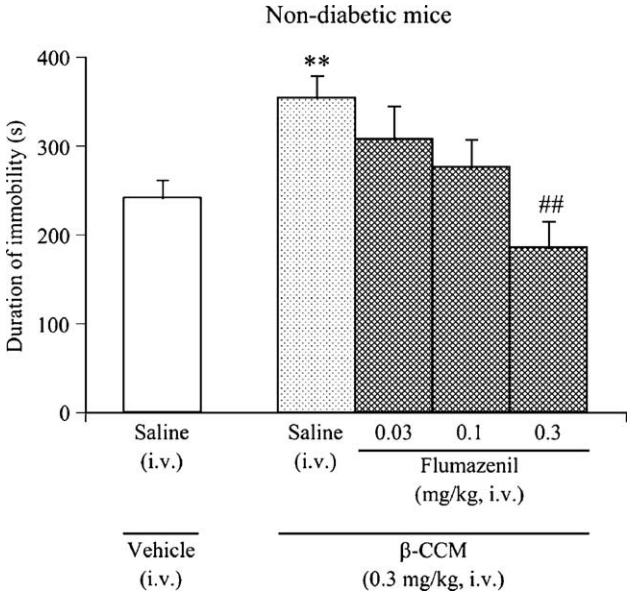


Fig. 3. Effect of flumazenil on the β-CCM-induced prolongation of immobility in non-diabetic mice. **$p<0.01$ vs. saline plus vehicle-treated non-diabetic mice (Student's *t*-test). $^{\#\#}p<0.01$ vs. saline plus β-CCM-treated non-diabetic mice (Dunnett's test). Each column represents the mean ± S.E.M. of 8–10 mice.

Table 1
Effects of flumazenil and β-CCM on spontaneous locomotor activity in non-diabetic and diabetic mice

| | Non-diabetic mice | Diabetic mice |
|---|---|---|
| Saline (i.v.) | 374.4±18.7 | 358.4±24.7 |
| Flumazenil (1 mg/kg, i.v.) | 413.6±12.4 | 373.8±13.8 |
| Vehicle (i.v.) | 373.5±22.7 | 331.3±29.8 |
| β-CCM (0.3 mg/kg, i.v.) | 165.9±52.4** | 265.8±32.7 |
| Flumazenil (0.3 mg/kg, i.v.)+ β-CCM (0.3 mg/kg, i.v.) | 372.5±13.0## | Not determined |

Each value represents the mean±S.E.M. of 10 mice.
** $p<0.01$ vs. respective vehicle-treated mice (Aspin–Welch's $t$-test).
## $p<0.01$ vs. β-CCM-treated non-diabetic mice (Aspin–Welch's $t$-test).

diabetic mice (Table 1). Flumazenil (1 mg/kg, i.v.) had no significant effect on spontaneous locomotor activity in both non-diabetic and diabetic mice (Table 1). β-CCM (0.3 mg/kg, i.v.) significantly reduced spontaneous locomotor activity in non-diabetic mice, but not in diabetic mice (Table 1). Two-way ANOVA revealed that spontaneous locomotor activity was significantly affected by β-CCM [$F(1,36)=14.299$, $p<0.001$], but not by diabetes [$F(1,36)=0.638$, $p=0.4296$] or diabetes × drug interaction [$F(1,36)=3.871$, $p=0.0569$]. Pretreatment with flumazenil (0.3 mg/kg, i.v.) completely antagonized the β-CCM (0.3 mg/kg, i.v.)-induced reduction in locomotor activity in non-diabetic mice (Table 1).

## 4. Discussion

In the present study, diabetic mice showed a prolonged duration of immobility without any difference in spontaneous locomotor activity. This result is consistent with our previous reports (Kamei et al., 2003; Miyata et al., 2004) and this altered behavior was termed depressive-like behavior. We also reported that several antidepressants such as fluoxetine, fluvoxamine and desipramine were less active in diabetic mice in the tail suspension test (Kamei et al., 2003; Miyata et al., 2004). However, the mechanism(s) was still unclear why diabetic mice exhibited the depressive-like behavior. In this study, the depressive-like behavior in diabetic mice was suppressed by treatment with flumazenil. However, this treatment did not affect the duration of immobility in non-diabetic mice. We previously reported that the doses of flumazenil used in this study did not affect the exploratory behavior in non-diabetic mice in the hole-board test (Kamei et al., 2001). Therefore, it is likely that flumazenil at this dose range has the antagonistic property. We also demonstrated that β-CCM produced depressive-like behavior in non-diabetic mice, and this effect was antagonized by flumazenil. Therefore, we can speculate that the endogenous negative modulation of benzodiazepine receptors may be enhanced in diabetic mice and this alteration may elevate the depressive-like state in mice in the tail suspension test. This idea is supported by the previous report indicating that the $GABA_A$ receptor antagonist bicuculline-induced seizure was sensitized by diabetes (Tutka et al., 1998). Therefore, it is likely that flumazenil blocked the enhanced negative allosteric modulation of benzodiazepine receptors and reduced the duration of immobility in diabetic mice. We previously observed that the benzodiazepine receptor agonist diazepam also suppressed depressive-like behavior in diabetic mice (Kamei et al., 2003). Therefore, it can be speculated that diazepam counterbalanced the enhanced negative allosteric modulation of benzodiazepine receptors and reduced the duration of immobility in diabetic mice. Based on these findings, it is possible that the attenuation of negative allosteric modulation of benzodiazepine receptors causes the reduction of immobility in diabetic mice.

Several β-carboline derivatives have anxiogenic-like properties in the Vogel punished drinking test (Corda et al., 1983), the potentiated startle test (Hijzen and Slangen, 1989), the light–dark test (Belzung et al., 1987), the elevated plus-maze test (Pellow and File, 1986) and the hole-board test (File et al., 1985; Kamei et al., 2001) in rodents. In addition, β-carbolines reduce social interactive and aggressive behaviors but increase avoidance behavior in rodents tested in pairs (File et al., 1985; Beck and Cooper, 1986). In this study, we observed that treatment with β-CCM increased the duration of immobility and decreased spontaneous locomotor activity in non-diabetic mice. β-CCM at this dose range does not elicit convulsion in either non-diabetic or diabetic mice (Ohsawa and Kamei, 1999). However, we observed in this study that some non-diabetic mice demonstrated slight freezing behavior after the injection of β-CCM (0.3 mg/kg, i.v.) (data not shown). It has been reported that β-carbolines elicit fear-related freezing in primates at a dose higher than that which elicits the suppression of exploratory behavior (Kalin et al., 1992). Therefore, β-CCM-induced depressive-like behavior in non-diabetic mice is partly associated with hypolocomotion including fear-related freezing behavior. In contrast to the result in non-diabetic mice, β-CCM had less of an effect on the duration of immobility and spontaneous locomotor activity in diabetic mice. We previously reported that a higher dose of β-CCM was needed to induce convulsion in diabetic than in non-diabetic mice (Ohsawa and Kamei, 1999). Furthermore, the anxiogenic-like effect of β-CCM is also less in diabetic than in non-diabetic mice (Kamei et al., 2001). Therefore, the enhanced endogenous negative modulation of benzodiazepine receptors in diabetic mice may be independent of the sensitivity of benzodiazepine receptors. Interestingly, flumazenil had no effect on spontaneous locomotor activity in both non-diabetic and diabetic mice. This result strongly indicates that the enhanced endogenous negative modulation of benzodiazepine receptors is not expressed under free-moving conditions in diabetic mice. In our previous studies, a similar abnormal function of benzodiazepine receptors in diabetic mice was detected after psychological stress treatment and in an unfamiliar environment (Kamei and Ohsawa, 2000; Kamei et al., 2001). Based on these findings, we suggest that the enhanced endogenous negative modulation of benzodiazepine receptors in diabetic mice may be induced by exposure to stress.

Diazepam binding inhibitor (DBI), an 86-amino-acid polypeptide, is an endogenous substance that shows inverse agonistic properties toward benzodiazepine receptors in the human and rat brain (Guidotti et al., 1978, 1983). Proteolytic cleavage of DBI generates several biologically active frag-

ments that also show inverse agonistic properties toward benzodiazepine receptors (Ferrero et al., 1986; De Mateos-Verchere et al., 1998). It has been reported that the release of DBI is stimulated by exposure to stress (Ferrarese et al., 1991). Therefore, it can be speculated that the secretion or cleavage of DBI under stressful conditions may be increased in diabetic mice. This speculation may explain our present and previous findings (Kamei and Ohsawa, 2000; Kamei et al., 2001). It has been reported that octadecaneuropeptide, the active fragment of DBI, displaces β-CCM (Ferrero et al., 1986). Therefore, it is possible that β-CCM did not induce further increase in duration of immobility in diabetic mice since increased endogenous ligands such as DBI and its active fragments under stressful condition may competitively block the effect of β-CCM in diabetic mice.

It is well established that GABAergic dysfunction including elevated DBI levels is an important factor in the pathogenesis of depression (Barbaccia et al., 1986; Roy, 1991; Petty et al., 1995; Shiah and Yatham, 1998; Brambilla et al., 2003; Tunnicliff and Malatynska, 2003). We have demonstrated that the hypnotic activities of ethanol and pentobarbital were markedly attenuated in diabetic mice (Ohsawa and Kamei, 1997; Kamei et al., 2005). Since pentobarbital and ethanol exert their hypnotic actions by interacting with $GABA_A$ receptors (Schulz and Macdonald, 1981; Hunt, 1983), we speculated that GABAergic function may be attenuated by diabetes. Dong et al. (1999) reported that DBI and its active fragment octadecaneuropeptide decreased the hypnotic effect of pentobarbital. Therefore, diabetic mice may have GABAergic dysfunction associated with the abnormal function of benzodiazepine receptors and this alteration may cause, at least in part, the expression of behavioral changes such as depressive-like behavior. Although further studies are required to clarify the relationship between GABAergic dysfunction and the pathophysiology of depression in diabetes, it is possible that the abnormal function of benzodiazepine receptors may be partly involved in the higher incidence of depression in diabetes.

In conclusion, we suggest that the depressive-like behavior in the tail suspension test in STZ-induced diabetic mice may be associated, at least in part, with the abnormal function of benzodiazepine receptors.

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