

## Modification of the Effects of Benzodiazepines on the Exploratory Behaviors of Mice on a Hole-Board by Diabetes

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**ABSTRACT**—The effect of diabetes on the emotional behavior of mice was examined using an automatic hole-board apparatus. Changes in the emotional state of mice were evaluated in terms of changes in exploratory activity; i.e., total locomotor activity, numbers and duration of rearing and head-dipping, and latency to the first head-dipping. The number and duration of head-dipping in diabetic mice were less than those in non-diabetic mice. Diazepam (0.1–0.56 mg/kg, i.p.) dose-dependently increased the number and duration of head-dipping at doses that did not produce sedation in both diabetic and non-diabetic mice. In contrast, methyl- $\beta$ -carboline-3-carboxylate (1 and 2 mg/kg, i.p.) decreased the number and duration of head-dipping in non-diabetic mice, but not in diabetic mice. The number and duration of head-dipping in diabetic mice were increased by treatment with flumazenil (0.1 and 0.3 mg/kg, i.v.). These doses of flumazenil did not affect the number or duration of head-dipping in non-diabetic mice. The present data indicate that diabetic mice exhibited anxiety in the hole-board test and that a benzodiazepine receptor antagonist affected the attenuated number and duration of head-dipping in diabetic mice. The heightened anxiety in diabetic mice may be due to the dysfunction of the benzodiazepine receptor and/or of central inhibitory systems.

**Keywords:** Exploratory behavior, Anxiety, Benzodiazepine, Hole-board test, Diabetes

Diabetes has been reported to be associated with behavioral changes in animals (1, 2). Enhanced retention of passive avoidance training in mice (3, 4), increased grooming activity in a novel environment in rats (5) and poor retention of a previously learned avoidance response in a T-maze in mice (6) have been reported. Furthermore, diabetic rats showed significantly more anxiogenic activity than non-diabetic rats in open-field, elevated plus maze, zero maze and social interaction tests (7). Humans subjects with type I diabetes mellitus performed poorly on tasks requiring visual and motor efficacy and somatosensory discrimination, compared with age-matched, non-diabetic subjects (8). Furthermore, it is well established that anxiety disorders are common among patients with diabetes (9, 10).

The hole-board test, which was first introduced by Boissier and Simon (11, 12), provides a simple method for measuring the response of an animal to an unfamiliar environment. Previously, the hole-board test has been used to assess emotionality, anxiety and/or responses to stress in

animals (13). Some advantages of this test are that several behaviors can be readily observed and quantified, which makes possible a comprehensive description of the animal's behavior. However, these advantages are also a defect in that the behaviors affected by anxiety- and/or anxiogenic-relevant manipulations often vary between animals. Therefore, to overcome this problem, it is important to identify behavior(s) of animals that are affected by anxiety and/or an anxiolytic state. We recently modified this apparatus and developed an automatic hole-board apparatus (14). In our recent research, this system has been a useful tool for objectively estimating various emotional states of animals (14).

Several investigators have previously examined the effect of benzodiazepine anxiolytics such as diazepam on behavior in the hole-board test. However, the results from previous investigations are controversial; i.e., exploratory behaviors were either increased (15, 16) or decreased (17). Recently, we demonstrated using the automatic hole-board apparatus that diazepam and chlordiazepoxide increase head-dipping behavior, while benzodiazepine-receptor inverse agonists, such as methyl- $\beta$ -carboline-3-carboxylate

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( $\beta$ -CCM) and *N*-methyl- $\beta$ -carboline-3-carboxamide (FG7142), decrease head-dipping behavior (14). Under our conditions, an anxiolytic state in animals may be reflected by an increase in head-dipping behavior, while an anxiogenic state may be reflected by a decrease in head-dipping behavior.

The primary aim of this study was to characterize the exploratory behavior and anxiety in diabetic mice using the hole-board test. We also investigated the changes in the pharmacological actions of benzodiazepine receptor modulators, such as diazepam and  $\beta$ -CCM, in the mice in the hole-board test with diabetes.

## MATERIALS AND METHODS

### *Animals*

Male ICR mice (Tokyo Laboratory Animals Science, Tokyo), weighing about 20 g at the beginning of the experiments, were used. They had free access to food and water in an animal room, which was maintained at  $24 \pm 1^\circ\text{C}$  with a 12-h light-dark cycle. Animals were rendered diabetic by an injection of streptozotocin (200 mg/kg, i.v.) prepared in 0.1 N citrate buffer at pH 4.5. Age-matched control mice were injected with vehicle alone. The experiments were conducted 2 weeks after injection of vehicle or streptozotocin. Mice with serum glucose levels above 4000 mg/l were considered diabetic. The body weights of diabetic and non-diabetic mice were  $25.6 \pm 0.3$  g ( $n = 226$ ) and  $33.6 \pm 0.4$  g ( $n = 226$ ), respectively. The serum glucose levels of diabetic and non-diabetic mice were  $5420.8 \pm 235.0$  mg/l ( $n = 226$ ) and  $1896.6 \pm 690.0$  mg/l ( $n = 226$ ), respectively.

### *Behavioral test*

The results in the hole-board test were determined automatically as described by Takeda et al. (14). The hole-board apparatus was made of a gray wooden box (50  $\times$  50  $\times$  50 cm) with four holes 3 cm in diameter equally spaced in the floor. An infrared beam sensor was installed on the wall to detect the numbers and duration of rearing and head-dipping behaviors and the latency to the first head-dipping. Other behavioral performance, such as locus and the distance of movement (total locomotor activity (cm)) of mice, was recorded by an overhead color CCD camera. The heads of the mice were painted yellow and the color CCD camera followed the center of gravity. Data from the CCD camera were collected through a custom-designed interface (CAT-10; Muromachi Kikai, Tokyo) as a reflection signal. Head-dipping behaviors were double-checked via an infrared beam sensor and an overhead color CCD camera. All of the data were analyzed and stored in a personal computer using analytical software (Comp ACT HBS, Muromachi Kikai).

For the hole-board experiments, each animal was placed in the center of the hole-board and allowed to freely explore the apparatus for 5 min. Total locomotor activity, numbers and duration of rearing and head-dipping, and latency to the first head-dipping were recorded automatically. Each mouse was used only once.

### *Drugs*

The drugs used in the present study were streptozotocin (Sigma, St. Louis, MO, USA), diazepam (Wako, Tokyo),  $\beta$ -carboline-3-carboxylate ( $\beta$ -CCM; Research Biochemicals Inc., Natick, MA, USA) and flumazenil (Anecate®; Yamanouchi Pharmaceutical, Tokyo). Diazepam was suspended in vehicle consisting of 9% Tween 80 in saline.  $\beta$ -CCM was dissolved in a small volume of 0.1 N HCl, then diluted with saline, and the pH was adjusted to 4.0 with NaOH just prior to use.  $\beta$ -CCM was injected 30 min before testing. Diazepam was injected i.p. 30 min before testing. Flumazenil was injected i.v. 5 min before testing.

### *Statistical analyses*

The data are expressed as means with S.E.M. The statistical significance of differences between groups was assessed with an analysis of variance (ANOVA) followed by the Bonferroni/Dunn test.

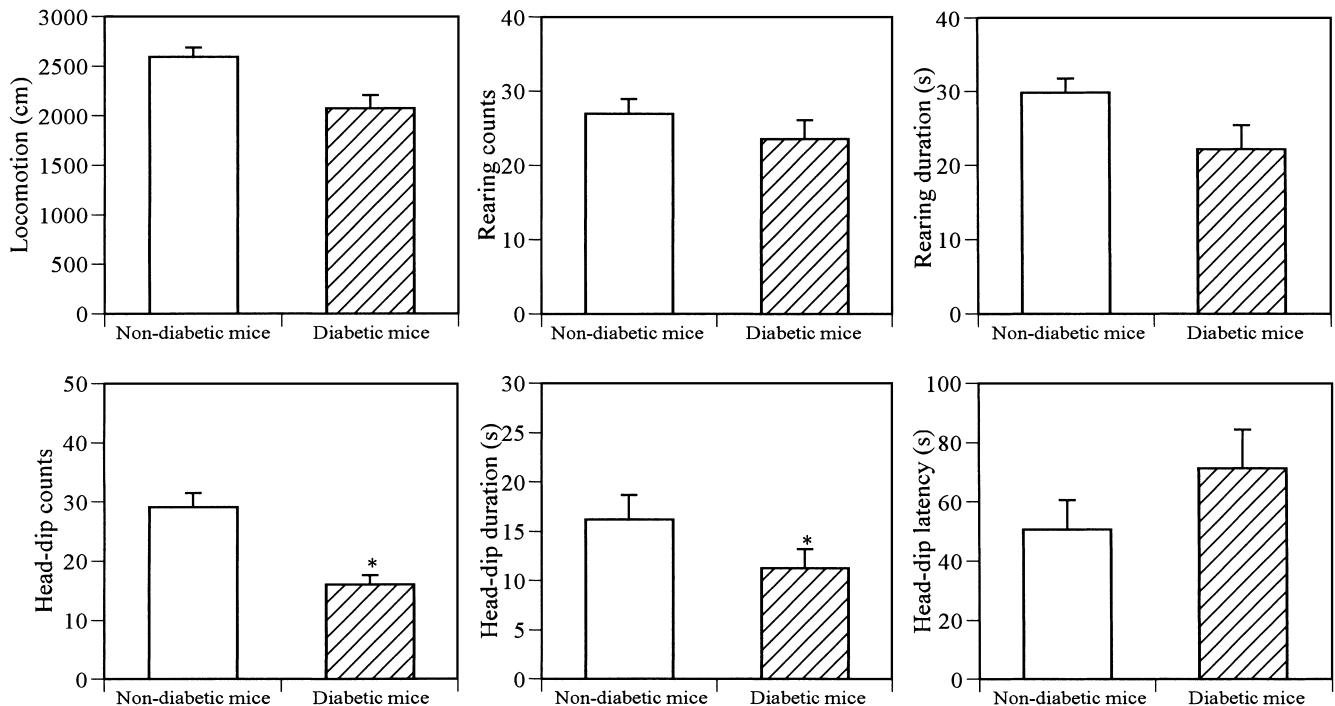
## RESULTS

### *Effect of diabetes on exploratory behavior in mice*

The effect of diabetes on exploratory behavior in mice is shown in Fig. 1. The number and duration of head-dipping behaviors in diabetic mice were significantly less than those in non-diabetic mice. However, there were no significant differences in locomotor activity, the number or duration of rearing behaviors, or the latency to head-dipping between non-diabetic and diabetic mice.

### *Effect of diazepam on exploratory behavior in non-diabetic and diabetic mice*

The effects of diazepam on exploratory behavior in non-diabetic and diabetic mice are shown in Fig. 2. Diazepam did not significantly modify locomotor activity, the number or duration of rearing behaviors or the latency to head-dipping in non-diabetic or diabetic mice. In contrast, the number and duration of head-dipping behaviors were dose-dependently and significantly increased by treatment with diazepam, at doses of 0.1 to 0.56 mg/kg, i.p., in both non-diabetic and diabetic mice. The dose-response effects of diazepam on the number and duration of head-dipping behaviors in non-diabetic and diabetic mice are summarized in Fig. 3. Diazepam had less of an effect in diabetic mice than in non-diabetic mice. Indeed, diazepam only had a significant effect on the number and duration of head-



**Fig. 1.** Effect of diabetes on exploratory behavior in mice tested on the hole-board. Each column represents the mean with S.E.M. of 10 (non-diabetic) to 16 (diabetic) mice. \* $P < 0.05$  vs non-diabetic mice.

dipping behaviors when the highest dose (0.56 mg/kg, i.p.) was used.

#### *Effect of the benzodiazepine inverse agonist $\beta$ -CCM on exploratory behavior in non-diabetic and diabetic mice*

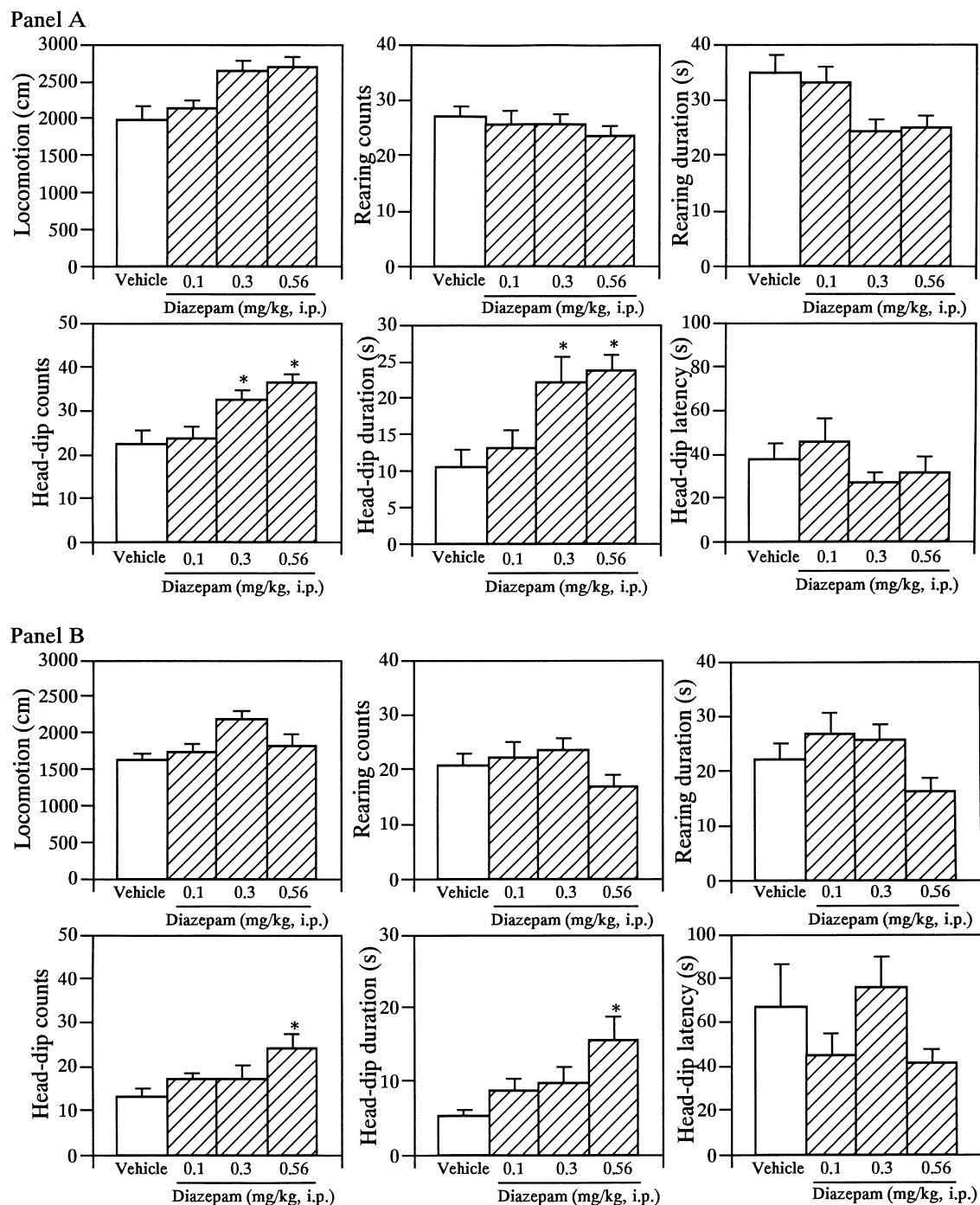
The number and duration of head-dipping behaviors were dose-dependently decreased following treatment with  $\beta$ -CCM (1 and 2 mg/kg, i.p.) in non-diabetic mice, and this decrease was statistically significant at 2 mg/kg (Fig. 4, panel A). However,  $\beta$ -CCM did not modify locomotor activity, the number or duration of rearing behaviors or the latency to head-dipping in non-diabetic mice. Furthermore,  $\beta$ -CCM also did not significantly modify any exploratory behaviors in diabetic mice (Fig. 4, panel B).

#### *Effect of the benzodiazepine receptor antagonist flumazenil on exploratory behavior in non-diabetic and diabetic mice*

Flumazenil did not significantly modify any exploratory behaviors in non-diabetic mice (Fig. 5, panel A). On the other hand, as shown in Fig. 5 (panel B), flumazenil, at doses of 0.1 and 0.3 mg/kg, i.v., dose-dependently increased the number and duration of head-dipping behaviors in diabetic mice, and this increase was statistically significant at 0.3 mg/kg. However, locomotor activity, the number and duration of rearing behaviors, and the latency to head-dipping in diabetic mice were not affected by flumazenil (Fig. 5, panel B).

## DISCUSSION

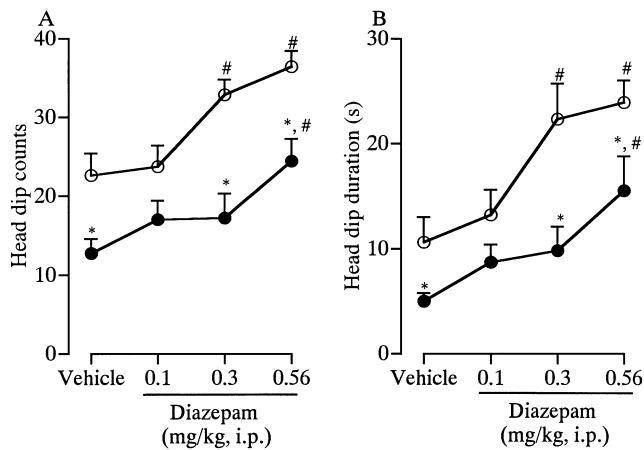
In the present study, the typical benzodiazepine anxiolytic diazepam increased the number and duration of head-dipping without sedation in both non-diabetic and diabetic mice. Furthermore, anxiogenics such as  $\beta$ -CCM, a  $\beta$ -carboline derivative, produced effects on head-dipping behavior that were opposite those of diazepam in non-diabetic mice. These observations are consistent with recent reports of an increase in the number and duration of head-dipping in the hole-board test following injection with a non-sedative dose of diazepam and a decrease in the number and duration of head-dipping in the hole-board test following injection with  $\beta$ -CCM and FG7142 (*N*-methyl- $\beta$ -carboline-3-carboxamide) (14). We previously suggested that changes in head-dipping behavior may reflect the anxiogenic and/or anxiolytic state of animals (14). The present data strongly support this suggestion because typical anxiolytics increase, while anxiogenics decrease, the number and duration of head-dipping in non-diabetic mice. In the present study, the number and duration of head-dipping in diabetic mice is less than those in non-diabetic mice. The sensorimotor deficient-induced behavioral changes and perhaps even locomotor impairment may affect the number and the duration of head-dipping. In the present study, although there was not a significant change, locomotor activity in diabetic mice was reduced



**Fig. 2.** Effect of diazepam on exploratory behavior in non-diabetic (panel A) and diabetic mice (panel B) tested on the hole-board. Diazepam (0.1 – 0.56 mg/kg, i.p.) or vehicle was injected 30 min prior to the measurement of exploratory behavior. Each column represents the mean with S.E.M. of 10 (vehicle-treated) to 16 (diazepam-treated) mice. \* $P < 0.05$  vs vehicle-treated group (open column).

by 20% as compared with non-diabetic mice. However, we previously indicated that locomotor impairment did not occur in diabetic mice, since the long-term (3 h) spontaneous locomotor activity in diabetic mice was significantly greater than that in non-diabetic mice (18, 19). Further-

more, although there was not a significant change, locomotor activity in non-diabetic mice was increased by about 25% when mice were pretreated with higher doses of diazepam. Based on these results, it seems likely that the reduction in spontaneous locomotor activity in diabetic



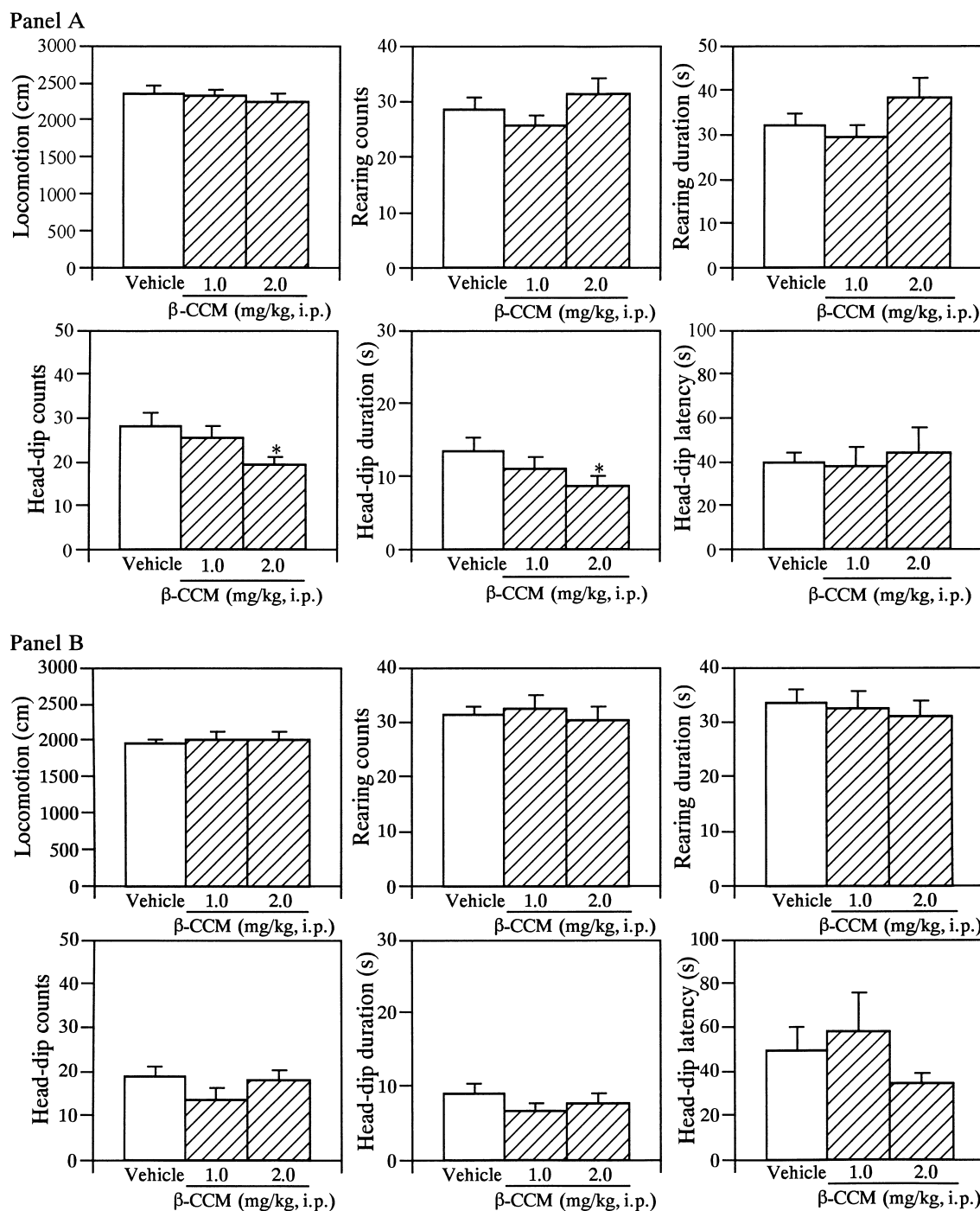
**Fig. 3.** Dose-response curves of diazepam for the number (A) and duration of head-dipping (B) in non-diabetic (open circle) and diabetic mice (closed circle). Diazepam (0.1–0.56 mg/kg, i.p.) or vehicle was injected 30 min prior to the measurement of exploratory behavior. Each column represents the mean with S.E.M. of 10 (vehicle-treated) to 16 (diazepam-treated) mice. \* $P < 0.05$  vs respective non-diabetic mice. # $P < 0.05$  vs respective vehicle-treated group.

mice during the task of the present study may reflect the anxiety response of an animal to an unfamiliar environment, rather than the locomotor impairment. Furthermore, the possibility that the locomotor impairment may affect the number and the duration of head-dipping was also negated by our previous finding that more than 50% reduction of the locomotor activity by sedative dose of diazepam had no significant effect on the head-dipping counts and duration (14). Some earlier investigations also reported increased anxiety in diabetic rats in the plus maze (20) and resident intruder tests (21). Recently, it has been reported that diabetic rats exhibit increased anxiety, as assessed by various paradigms such as the open-field, plus-maze, social interaction and zero-maze tests (7). These previous studies, together with the present findings, indicate that diabetic mice exhibit a heightened anxiogenic state.

The benzodiazepine-GABA hypothesis of anxiety is well accepted (22). In the present study, although diazepam produced a dose-dependent anxiolytic effect in diabetic mice, this effect was lower in diabetic mice than in non-diabetic mice. These results are consistent with our previous observations that the duration of the diazepam-induced loss of the righting reflex was shorter in diabetic mice than in non-diabetic mice (23), and the seizure threshold of a benzodiazepine receptor inverse agonist,  $\beta$ -CCM, was markedly increased in diabetic mice compared to that in non-diabetic mice (23). In the present study, we also observed that treatment with flumazenil, a benzodiazepine receptor antagonist, significantly increased the frequency and duration of head-dipping behavior in diabetic mice, but not in non-diabetic mice. On the other hand,  $\beta$ -CCM, a

benzodiazepine receptor inverse agonist, significantly and dose-dependently decreased the frequency and duration of head-dipping behavior in non-diabetic, but not in diabetic mice. It has also been reported that the anxiolytic effect of diazepam is less marked in diabetic rats than in non-diabetic rats (7). On the other hand, Ramanathan et al. (7) reported that hyperglycemic rats exhibited augmented anxiety in open-field exploratory behavior, elevated plus maze and elevated zero maze behavior, and social interaction tests. The above characteristics indicate that benzodiazepine receptors in diabetic mice are dysfunctional.

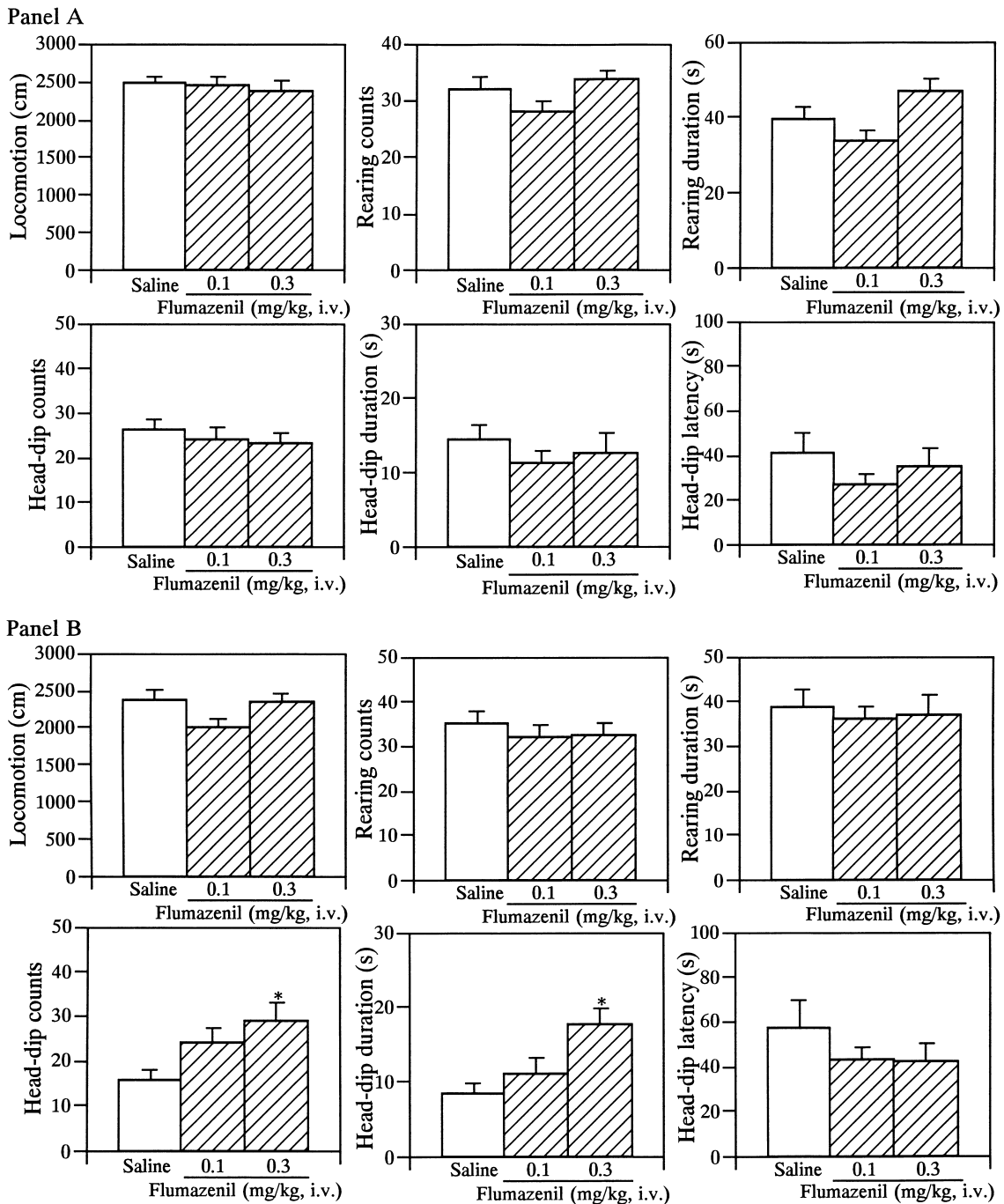
Similar to the effects of treatment with anxiogenics, exposure of mice to acute restraint stress also produced a decrease in head-dipping behavior (14). Furthermore, this decrease in head-dipping behavior produced by acute restraint stress was reversed by treatment with diazepam (14). These results suggested that acute restraint stress may produce anxiety, and that the decrease in head-dipping behavior reflects this emotional state. We recently demonstrated that socio-psychological stress produced marked antinociception in diabetic mice, but not in non-diabetic mice (24). Furthermore, socio-psychological stress-induced antinociception in diabetic mice was significantly reduced by treatment with diazepam (24). We also demonstrated that treatment with flumazenil significantly antagonized the socio-psychological stress-induced antinociception in diabetic mice (24). On the other hand,  $\beta$ -CCM significantly and dose-dependently enhanced the socio-psychological stress-induced antinociception in non-diabetic, but not in diabetic mice (24). Based on these results, we suggested that some endogenous substance(s) that act(s) as an inverse benzodiazepine receptor agonist may also be involved in the enhancement of socio-psychological stress-induced antinociception in diabetic mice (24). A polypeptide with high affinity for benzodiazepine receptors was originally isolated from brain tissue of rodents and humans and called diazepam binding inhibitor (25). In depressive, anxiogenic and schizophrenic patients, significantly higher concentrations of diazepam binding inhibitor and a down-regulated  $\gamma$ -aminobutylic acid (GABA)<sub>A</sub> receptor level in the cerebrospinal fluid have been reported (26). Diazepam binding inhibitor acts on benzodiazepine binding sites and negatively modulates GABAergic transmission (27). Diazepam binding inhibitor has been reported to exert benzodiazepine receptor inverse agonist activity, since it elicits a proconflict response in behavioral paradigms when injected i.c.v. in the rat (25). Furthermore, elevated levels of a diazepam binding inhibitor-like immunoreactive compound have been reported in patients diagnosed with depression with concomitant anxiety (26). Diazepam binding inhibitor has been previously shown to inhibit glucose-stimulated insulin release from pancreatic islet  $\beta$  cells (28, 29). Suk et al. (30) demonstrated that cDNA related to diazepam binding inhi-



**Fig. 4.** Effect of  $\beta$ -CCM on exploratory behavior in non-diabetic (panel A) and diabetic mice (panel B) tested on the hole-board.  $\beta$ -CCM (1.0 and 2.0 mg/kg, i.p.) or its vehicle was injected 30 min prior to the measurement of exploratory behavior. Each column represents the mean with S.E.M. of 10 (vehicle-treated) to 16 ( $\beta$ -CCM-treated) mice. \* $P < 0.05$  vs vehicle-treated group (open column).

bitor was expressed in most tissues including liver, lung, tonsil and thymus, in addition to pancreatic islet and sera, in diabetic patients. Based on these results, it is tempting to speculate that the diazepam binding inhibitor level is capable of increasing rapidly in the diabetic condition. Thus, it

is possible that the increase in diazepam binding inhibitor release may be due, at least in part, to the enhanced anxiogenic state in diabetic mice. This possibility is further supported by the present observation that the treatment with flumazenil, a benzodiazepine receptor antagonist, signifi-



**Fig. 5.** Effect of flumazenil on exploratory behavior in non-diabetic (panel A) and diabetic mice (panel B) tested on the hole-board. Flumazenil (0.1 and 0.3 mg/kg, i.v.) or saline was injected 5 min prior to the measurement of exploratory behavior. Each column represents the mean with S.E.M. of 8 (vehicle-treated) to 16 (flumazenil-treated) mice. \* $P < 0.05$  vs vehicle-treated group (open column).

cantly increased the frequency and duration of head-dipping behavior in diabetic mice. However, further studies are necessary before this possibility can be established with greater certainty.

In conclusion, the present results indicate that diabetic mice show enhanced anxiety in an unfamiliar environment.

The enhanced anxiogenic state in diabetic mice may be due to an increase in an endogenous negative allosteric modulator of benzodiazepine receptors, such as diazepam binding inhibitor or  $\beta$ -carboline, and/or dysfunction of central GABA<sub>A</sub>/benzodiazepine receptors.

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