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Effects of Physical and Psychological Stress on 5-HT2A Receptor-mediated Wet-dog Shake Responses in Streptozotocin-induced Diabetic Rats.

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Abstract

Several epidemiological and clinical studies have indicated that the prevalence of psychiatric disorders is higher in diabetic patients than in the general population. In the present studies, we examined the behavioral changes in streptozotocin-induced diabetic rats, and investigated the effects of physical and psychological stress on the hippocampal BDNF levels and on the serotonin 2A (5-HT2A) receptor-mediated wet-dog shake responses. The streptozotocin (60 mg/kg, i.p.)-induced diabetes had no significant effects on the immobility time in the forced swim test or on locomotor activity in the open-field test. Moreover, there was no significant difference in the wet-dog shake responses induced by DOI, a 5-HT2A receptor agonist, between nondiabetic and diabetic rats. Five-day exposure to physical (electric footshock) and psychological (non-footshock) stress had no signifi cant effect on the hippocampal BDNF level in diabetic or nondiabetic rats. The 2 types of stress had no significant effect on the DOI-induced wet-dog shake responses in nondiabetic rats. In diabetic rats, the repeated exposure to physical stress markedly increased the DOI-induced wet-dog shake responses, but the repeated exposure to psychological stress had no effect. These results suggest that exposure to physical stress augmented the susceptibility to the wet-dog shake responses to 5-HT2A receptor stimulation in streptozotocin-induced diabetic rats.

KEYWORDS: streptozotocin, physical stress, psychological stress, 5-HT2A receptor, wet-dog shake

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Several epidemiological and clinical studies have indicated that the prevalence of psychiatric disorders is higher in diabetic patients than in the general population. In the present studies, we examined the behavioral changes in streptozotocin-induced diabetic rats, and investigated the effects of physical and psychological stress on the hippocampal BDNF levels and on the serotonin 2A (5-HT$_{2A}$) receptor-mediated wet-dog shake responses. The streptozotocin (60 mg/kg, i.p.)-induced diabetes had no significant effects on the immobility time in the forced swim test or on locomotor activity in the open-field test. Moreover, there was no significant difference in the wet-dog shake responses induced by DOI, a 5-HT$_{2A}$ receptor agonist, between nondiabetic and diabetic rats. Five-day exposure to physical (electric footshock) and psychological (non-footshock) stress had no significant effect on the hippocampal BDNF level in diabetic or nondiabetic rats. The 2 types of stress had no significant effect on the DOI-induced wet-dog shake responses in nondiabetic rats. In diabetic rats, the repeated exposure to physical stress markedly increased the DOI-induced wet-dog shake responses, but the repeated exposure to psychological stress had no effect. These results suggest that exposure to physical stress augmented the susceptibility to the wet-dog shake responses to 5-HT$_{2A}$ receptor stimulation in streptozotocin-induced diabetic rats.

Key words: streptozotocin, physical stress, psychological stress, 5-HT$_{2A}$ receptor, wet-dog shake

S tress has been suggested to be an important etiological factor in many psychiatric diseases. In addition, epidemiological and clinical studies have shown that diabetes is related to psychiatric disorders, including depression. Investigators have found that major depression was present in 14.7%, and elevated depression symptoms in 26% of diabetic patients [1], and that the incidence of psychiatric disorders is equally common in those with either type 1 or type 2 diabetes [2]. Although the factors associated with the development of depression remain unclear, it is commonly assumed that the development and exacerbation

of disease probably results from an interaction among psychological, physical and genetic factors [2].

Postsynaptic serotonergic responses to stress are mediated by a number of different serotonin (5-HT) receptor subtypes. Among them, the subtypes, the 5-HT$_{2A}$ receptor plays special roles in the serotonergic responses to stress, and has been suggested to be involved in affective disorders, anxiety disorders and depression [3, 4]. Several studies have found elevated numbers of 5-HT$_{2A}$ receptors in the post-mortem brains of suicide victims and depressed subjects [5, 6]. Recently, neurotrophins and 5-HT have both been implicated in the pathophysiology of depression and in the mechanisms of antidepressant treatments. Protein immunoreactivity of brain-derived neurotrophic factor (BDNF), a member of the neurotrophin superfamily, is reduced in both the prefrontal cortex and hippocampus of suicide subjects [7], and is elevated in the post-mortem tissue from antidepressant-treated patients [8]. Moreover, it has been reported that BDNF promotes 5-HT neuron development [9], and augments 5-HT synthesis and turnover [10, 11]. Interestingly, it has also been reported that immobilization stress decreases the expression of BDNF within the rat hippocampus, and that pretreatment with 5-HT$_{2A}$ receptor antagonists blocks the effects of stress on BDNF expression [12].

The streptozotocin-induced hyperglycemic state has been used as an animal model for diabetes mellitus, and some experiments have been conducted in animal models to study the relationships between diabetes and anxiogenic or depressive behavior [13]. However, the effects of physical and psychological stress on 5-HT$_{2A}$ receptors and BDNF are not clear. In order to study the responses or physiological changes caused by physical and psychological stress, various laboratory techniques, such as forced swim stress, footshock stress and social stress, have been used [13–15]. One of these methods, an apparatus called a communication box, subjects animals to both psychological stress and physical stress at the same time [16]. In the communication box paradigm, one group of animals receives electric footshocks and the resulting distress responses are allowed to reach the unshocked animals in neighboring boxes. These stress exposures have been demonstrated to cause both acute and chronic behavioral and physiological changes in animals [15, 17]. On the other hand, DOI, a 5-HT$_{2A}$ receptor

agonist, produces wet-dog shakes, and this behavior is useful for studying the function of 5-HT$_{2A}$ receptors [18]. Thus, in the present study, we used the communication box paradigm to investigate the effect of physical and psychological stress on DOI-induced wet-dog shake responses and hippocampal BDNF levels in streptozotocin-induced diabetic rats.

Materials and Methods

Animals.   Male Wistar strain rats (at 6–7 weeks of age) were obtained from Charles River (Yokohama, Japan). All animals were housed at 2 rats/cage (42 cm long × 26 cm wide × 15 cm high). The animal room was maintained at $22 \pm 1 °C$ under a 12 h/12 h light/dark cycle with lights on from 7:00 AM. Food and water were available ad libitum. Animals were rendered diabetic by an injection of streptozotocin (60 mg/kg, i.p.). The control rats were injected with the vehicle alone. Blood glucose levels were determined using a glucose analyzer (Arkray Glucocard Diameter-alpha GT-1661; ARKRAY Inc., Kyoto, Japan). Animal experiments were performed in compliance with the Guidelines for Animal Experimentation and with the approval of the Committee of Animal Experimentation, Ehime University School of Medicine.

Drugs.   Streptozotocin (Sigma-Aldrich Co., St. Louis, MO, USA) was dissolved in 0.05 M citrate buffer at pH 4.5 immediately before administration. ($\pm$)-1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane (DOI; Sigma-Aldrich Co.) was dissolved in saline. All drugs were injected at a volume of 0.1 ml per 100 g body weight.

Experimental procedures.   Experiment 1: Behavioral changes in diabetic rats (Fig. 1). Two groups of animals were established to investigate the behavioral changes, and 8–11 animals were used for each group. The forced swim test, elevated plus-maze test and DOI-induced wet-dog shake responses were examined for 14 days after the administration of streptozotocin or vehicle. Locomotor activity was measured for 13 days after the administration using an open-field apparatus.

Experiment 2: Effect of physical and psychological stress on BDNF levels and 5-HT$_2$ agonist-induced behavior. Six groups of animals (n = 6 for each group) were established to investigate the effects of physical

and psychological stress on the BDNF levels in the hippocampus and on the DOI-induced wet-dog shake responses in the streptozotocin-induced diabetic rats. Three groups were administered streptozotocin, and the other 3 groups were administered vehicle. Seven days after the injections, the rats were exposed to 2 types of stress, physical (electric footshock) stress and psychological (non-footshock) stress for 5 days; *i.e.,* 2 of the groups that had received streptozotocin or vehicle were exposed to physical stress, 2 were exposed to psychological stress, and 2 were not exposed to stress (control). Six groups were thus formed: a nondiabetic-control group, nondiabetic-physical stress-exposed group, nondiabetic-psychological stress-exposed group, diabetic-control group, diabetic-physical stress-exposed group, and diabetic-psychological stress-exposed group. Each group was administered DOI 24 h after the last exposure to physical or psychological stress. All behavioral observations were performed between 10:00 AM and 4:00 PM each day.

Physical and psychological stress exposures. Physical and psychological stress was administered using a communication box (Fig. 2) according to the method of Funada and Hara [19]. This box ($53 \times 53 \times 44$ cm) was equipped with a floor grid composed of 0.5 cm-diameter stainless steel rods placed 1.3 cm apart. The box consisted of 9 small compartments (17×17 cm) divided by transparent plastic walls. Plastic plates were placed on the grid floors of 5 compart-
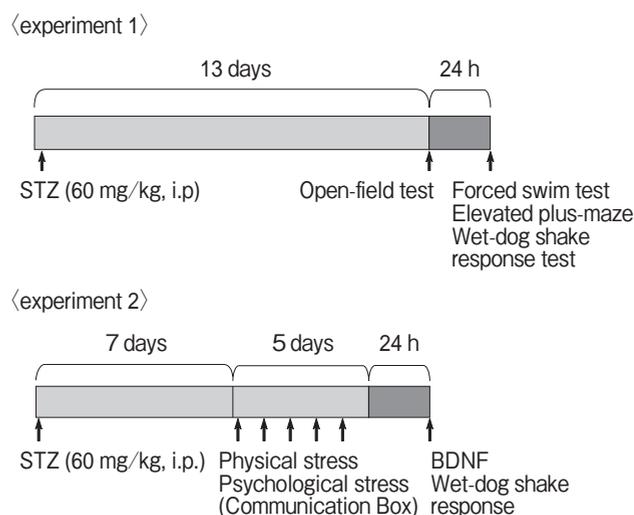
ments to prevent the rats from receiving electric shocks. An electric footshock generator (MSG-001; Toyo Sangyo, Toyama, Japan) was used to produce a scrambled electric footshock (2 mA) through the floor grid lasting for 10 sec at intervals of 90 sec for 30 min.

Forced swim test. The forced swim test was performed 14 days after the administration of streptozotocin. It was carried out in a cylindrical container (15.5 cm diameter, 37 cm height) filled with water to a depth of 20 cm, as described by Porsolt *et al.* [20]. The water was maintained at 25 ± 1 °C. On the day before the forced swim test, each animal was placed in the cylinder for a 13-min preswim. Following swimming, the animals were dried with a towel and kept warm before returning to the home cage. On the forced swim test day, immobility times were recorded during the 6-min swim test using a digital video camera. All data was calculated using Ethovision 3.0 (Noldus Information Technology, Wageningen, the Netherlands).

Open-field test. In order to investigate general changes in activity, rats were assessed for changes in locomotor activity 13 days after the administration of streptozotocin. For the open-field test, rats were individually placed in an acrylic apparatus (69 cm in diameter $\times 28$ cm high wall) with the gray floor divided into 19 squares. Locomotor activity (grid lines crossed with the 4 paws) was measured during a period of 6 min. Illumination was provided by a bulb (100 W) placed above the center of the field, while the rest of the room was in darkness.

Elevated plus-maze test. Anxiety-related behavior was evaluated using the plus-maze test. The plus-maze, consisting of 2 opposite open arms ($50 \times$

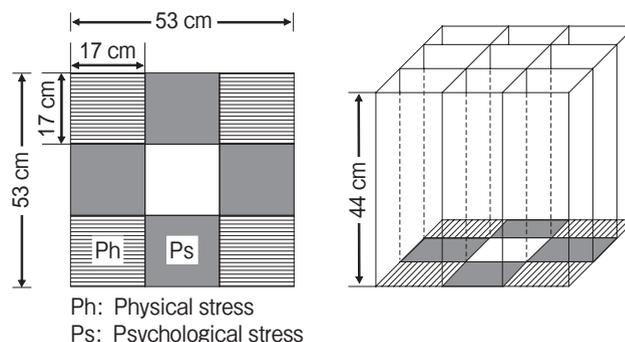Fig. 1   Schematic presentation of the protocol.

Fig. 2   Schematic presentation of a communication box.

10 cm) and 2 enclosed arms (50 × 10 × 40 cm) was elevated 50 cm above the floor. The arms extended from a central platform (10 × 10 cm). Rats were placed individually on the central platform and were allowed to enter freely into both the open and enclosed arms. The behavior of each rat over an 8-min period was monitored by using a video camera. Arm entry was defined as the entry of all 4 paws into 1 arm. The time spent in the open arms and enclosed arms, and the number of open and enclosed arm entries, were recorded. The time spent in open arms (time in open arms) and the number of entries into open arms (open arm entries) were calculated as the percentage of the time spent in all 4 arms, and of the total arm entries, respectively.

DOI-induced wet-dog shakes. 　Rats were placed individually into clean, transparent plastic cages (42 cm long × 26 cm wide × 15 cm high). Immediately after the subcutaneous administration of DOI (1 mg/kg), the number of wet-dog shakes was recorded over a 30-min period.

Measurement of BDNF protein. 　At 24 h after the last exposure to physical and psychological stress, the animals were sacrificed by decapitation. The brains were quickly removed and dissected on ice, separating the hippocampus. Samples were frozen at −80 °C before homogenization. Sections were homogenized in a lysis buffer (137 mM NaCl, 20 mM TRIS, 1% NP40, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride (PMSF), 10 μg/ml aprotinin, 1 μg/ml leupeptin, 0.5 mM sodium vanadate). The homogenates were centrifuged at 10,000 × g for 20 min, and the supernatants were collected and processed for quantification of BDNF with a BDNF Emax Immuno Assay System kit (Promega, Madison, WI, USA). An enzyme-linked immunosorbent assay (ELISA) was performed using the BDNF Emax Immuno Assay System Kit (Promega) according to the manufacturer's instructions [21]. Nunc Maxisorp 96-well immunoplates were coated with 100 μl/well of Anti-BDNF Monoclonal Antibody and incubated overnight at 4 °C. The plates were then incubated in a block and sample buffer at room temperature for 1 h. Next, the samples were added to the coated wells (100 μl) and shaken for 2 h at room temperature. The antigen was incubated with an Anti-Human BDNF polyclonal antibody for 2 h at room temperature with shaking, and then with an anti-IgY antibody conjugated

to horseradish peroxidase (HRP) for 1 h at room temperature. Finally, the plates were incubated with tetramethylbenzidine solution for 15 min, and 1 M hydrochloric acid was added to the wells. The colorimetric reaction product was measured at 450 nm. Standard curves were plotted for each plate. BDNF concentrations were determined from the regression line for the BDNF standard provided by Promega, which ranged from 7.8 to 500 pg/ml. Values of sections were above 16 pg/ml for each plate.

Statistical analysis. 　Values are expressed as the means and S.E.M. of each group. Data were analyzed using Student's *t*-test or one-way analysis of variance (ANOVA) followed by Tukey's test. *P* values less than 0.05 were considered significant.

Results

Experiment 1: Behavioral changes in diabetic rats. 　Fig. 3 shows the results of the forced swim test and open-field test. Student's *t*-test revealed no significant differences in the duration of the immobility time in the forced swim test between diabetic and nondiabetic rats. The locomotor activity in the open-field apparatus was also not different between the groups (Student's *t*-test).

Fig. 4 shows the effect of streptozotocin-induced diabetes on the elevated plus-maze test. There were no significant differences in the percent duration of open arm entries or the number of open arm entries
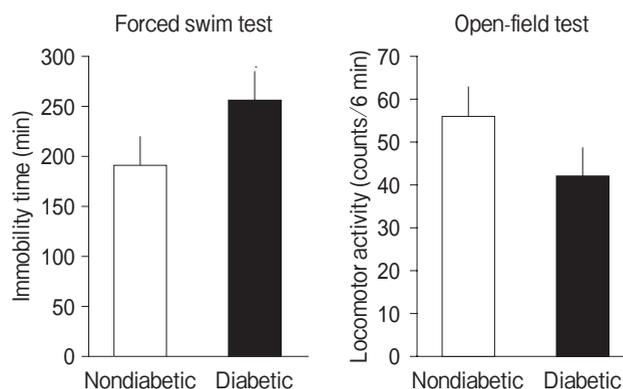

Fig. 3　Effect of streptozotocin-induced diabetes on the immobility time in the forced swim test and locomotor activity in the open-field test in rats. Each column represents the mean ± S.E.M. (n = 8∼11).

between the diabetic and nondiabetic rats.

Experiment 2: Effect of physical and psychological stress. Table 1 shows the effect of physical and psychological stress on the body weight and blood glucose levels in diabetic or nondiabetic rats. The body weight of the nondiabetic-control group was not significantly different from that of the diabetic-control group 14 days after streptozotocin administration. Exposure to physical and psychological stress had no effect on the body weight in the nondiabetic rats, but the body weight of the diabetic-physical stress-exposed group was significantly lower compared with that of the diabetic-control group. However, psychological stress did not have any effect on the body weight of the diabetic rats. Streptozotocin treatment significantly increased blood glucose levels. Both physical and psychological stress had no effect on the blood glucose levels in the nondia-

betic and diabetic rats.

Fig. 5 shows the effect of physical and psychological stress on DOI-induced wet-dog shakes in diabetic or nondiabetic rats. One-way ANOVA revealed a significant effect on DOI-induced wet-dog shake responses $[F_{(5, 30)} = 8.732, \ p < 0.0001]$. The *post hoc* comparisons using Tukey's test showed significantly higher wet-dog shake responses in the diabetic-physical stress-exposed group ($p < 0.001$, respectively) compared with those in the nondiabetic-physical stress-exposed group, diabetic-control group or diabetic-psychological stress-exposed group. However, exposure to physical and psychological stress had no significant effect on the hippocampal BDNF protein level in diabetic or nondiabetic rats (Fig. 6).
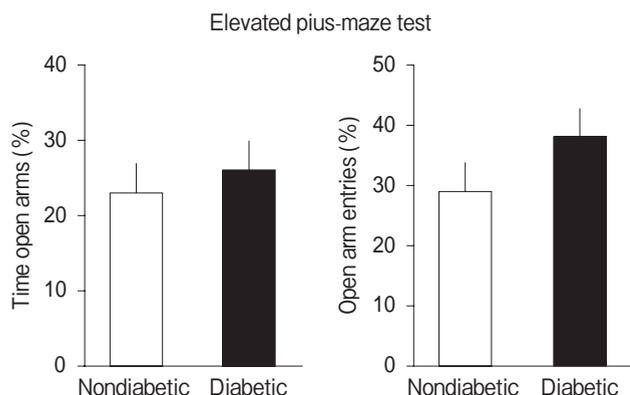

Fig. 4 Effect of streptozotocin-induced diabetes on the duration of open arm entries and the number of open arm entries in the elevated plus-maze test in rats. (n = 11).
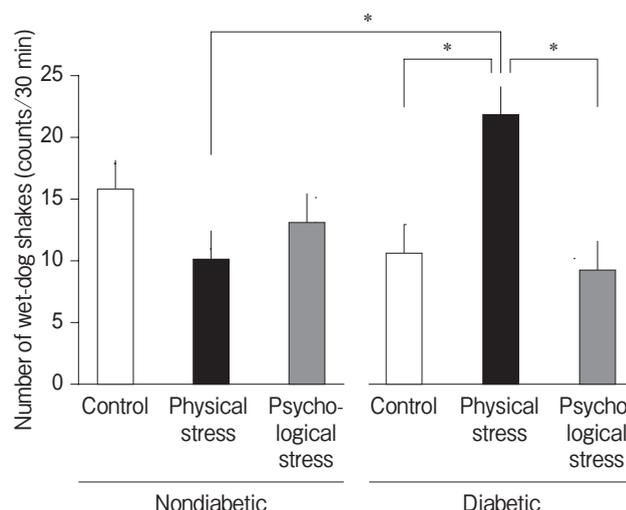

Fig. 5 Effect of physical and psychological stress on DOI-induced wet-dog shakes in streptozotocin- or vehicle-treated rats. Each column represents the mean ± S.E.M. (n = 6), *$p < 0.01$ (one-way ANOVA followed by Tukey's test).

Table 1 The effect of physical and psychological stress on the blood glucose levels and body weight in streptozotocin- or vehicle-treated rats

	Nondiabetic			Diabetic		
	Control	Physical stress	Psychological stress	Control	Physical stress	Psychological tress
Body weight (g)	285.7 ± 3.52	280.0 ± 3.43	297.7 ± 3.32	282.3 ± 7.05	241.0 ± 14.75[#]	271.8 ± 22.44
Blood glucose level (mg/dL)	92.3 ± 4.34	98.7 ± 6.16	111.0 ± 8.56	374.3 ± 76.30[**]	357.3 ± 60.04	326.8 ± 92.80

Each value represents the mean ± S.E.M. (n = 6). **$p < 0.01$ vs nondiabetic control, #$p < 0.05$ vs diabetic control (one-way ANOVA followed by Tukey's test).

Discussion

Diabetes has been reported to induce behavioral changes in animals. Ramanathan *et al.* [22] reported that streptozotocin-induced diabetic rats showed significantly more anxiogenic activity than nondiabetic rats in the elevated plus-maze, zero-maze and social interaction tests, when these experiments were performed 3 days after streptozotocin administration. Hilakivi-Clarke *et al.* [13] reported no significant effect on the plus-maze test of anxiety, but there was a significantly lengthened immobility time in the forced swim test, a putative animal model of depression, in streptozotocin-treated mice. Furthermore, Kamei *et al.* [23] reported that immobility time was significantly longer in diabetic than in nondiabetic mice in the tail suspension test. In the present study, streptozotocin-induced diabetes showed a tendency to increase the duration of open arm entries in the elevated plus-maze test and the immobility time in the forced swim test. However, there were no statistically significant differences compared to nondiabetic control rats, indicating that the diabetic rats do not have markedly higher anxiogenic activity or a markedly higher degree of depression than the nondiabetic rats. In addition, streptozotocin-induced diabetes showed a tendency to reduce locomotor activity in the open-field test. Several studies have shown that basal locomotor activity of streptozotocin-treated rats is lower than that of control rats [13, 23], but it has also been shown that amphetamine-induced hyperlocomotor
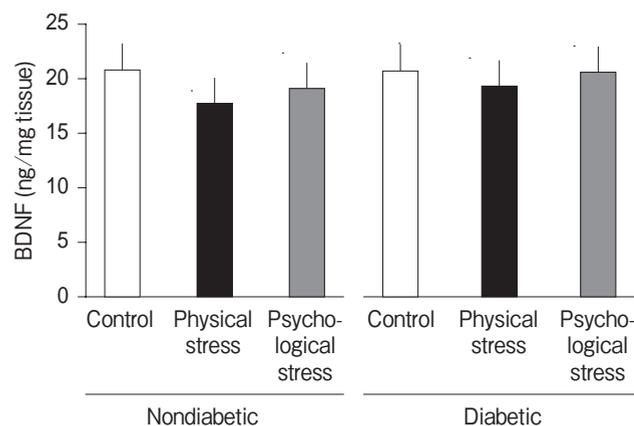

Fig. 6 Effect of physical and psychological stress on the hippocampal BDNF protein level in streptozotocin- or vehicle-treated rats. Each column represents the mean $\pm$ S.E.M. ($n = 6$).

activity increases in streptozotocin-treated rats [24]. Therefore, these differences in the behavioral changes in diabetic animals may be related to differences in the experimental conditions, strain or period after streptozotocin administration.

Both systemic administration and microinjection of DOI into the medial prefrontal cortex elicit dose-dependent wet-dog shake or head- twitch responses [25, 26]. These responses can be blocked by selective 5-HT$_{2A}$ receptor antagonists, indicating that they are mediated by central 5-HT$_{2A}$ receptors [25, 27]. These findings indicate that these responses are mediated by central 5-HT$_{2A}$ receptors. Miyata *et al.* reported that streptozotocin-induced diabetes produces long-lasting increases in 5-HT turnover rates in the mouse midbrain and frontal cortex [28]. In addition, they found that streptozotocin-induced diabetes inhibits the DOI-induced head-twitch response in mice, but that the number and affinity of 5-HT$_{2A}$ receptors in the frontal cortex are not affected by diabetes [29]. Thus, the detailed mechanism of the decreased head-twitch response in diabetic mice is still unknown. In the present study, streptozotocin-induced diabetes had no effect on the wet-dog shake responses induced by DOI in rats.

Repeated exposure to social or physical stressors increases the density of 5-HT$_{2A}$ receptors in the cortex [30, 31], and exposure to 63 days of psychological stress (strobe light and white noise) or 14 days of forced swim stress was reported to increase the DOI-stimulated wet-dog shake responses [30, 32]. In this study, 5 days of exposure to physical and psychological stress using a communication box had no significant effect on the DOI-induced wet-dog shake responses in nondiabetic rats. However, in streptozotocin-induced diabetic rats, the wet-dog shake responses were augmented by exposure to physical stress, but psychological stress had no effect. These results suggest the increased vulnerability of 5-HT receptors to physical stress in diabetics. Thorre *et al.* [33] reported that streptozotocin-induced diabetes reduces 5-HT synthesis and metabolism in rat brains, and a hippocampal microdialysis study has shown that the extracellular 5-HT levels increase throughout restraint stress in vehicle-, but not in streptozotocin-pretreated rats. These findings suggest that the number of 5-HT$_{2A}$ receptors in the brains of the diabetic-physical stress-exposed group may have been increased via a feed-

back-control mechanism. Taken together, these findings and the results of the current study suggest that exposure to physical stress increased the susceptibility to the wet-dog shake responses to 5-HT_{2A} receptor stimulation in streptozotocin-induced diabetic rats. However, further studies are needed to determine whether or not selective 5-HT_{2A} receptor antagonists block the increased wet-dog shake responses in diabetic-physical stress-exposed rats.

Generally, there are several possible explanations for the differences between the effects of physical stress and psychological stress on the wet-dog shake responses in the streptozotocin-induced diabetic rats. First, these differences may be due to differential effects on the neurotransmitter systems. Psychological stress has been reported to cause a mild enhancement of noradrenaline turnover in rat brain, whereas physical stress causes a remarkable increase in the noradrenaline turnover [34]. Moreover, it has reported that exposure to psychological stress, but not physical stress, increases diazepam-binding inhibitor mRNA expression in mouse brains [35]. Another possible explanation is the endocrinological difference. Previous studies have reported that physical stress, but not psychological stress, increases the plasma corticosterone level in rats during psychological stress induced by the communication box [36]. In addition, the chronic administration of adrenocorticotropic hormone (ACTH) increases plasma corticosterone levels and enhances the wet-dog shake response induced by DOI in rats [37]. Therefore, the endocrinological differences between physical and psychological stress may be responsible for the different effects of these stressors regarding the effects of physical and psychological stress on the wet-dog shake responses.

Seki *et al.* [38] and Nitta *et al.* [39] reported reduced protein and mRNA levels of BDNF in the cortex and hippocampus 4 weeks after streptozotocin administration. However, in the present study, there was no significant effect on the hippocampal BDNF protein level 2 weeks after streptozotocin administration. This result indicates that the increased DOI-induced wet-dog response in the diabetic-physical stress-exposed group was unrelated to BDNF expression. However, previous studies have shown that immobilization stress decreases the expression of BDNF within the rat hippocampus, and pretreatment with 5-HT_{2A} receptor antagonists blocks the effects of stress on BDNF expression [12]. Moreover, the downregulation of BDNF mRNA and protein in the rat hippocampus by corticosterone has been reported [40]. Thus, further studies will be necessary to clarify the involvement of BDNF in the vulnerability to physical and psychological stress in diabetics.

In conclusion, the major finding of the present study was that exposure to physical stress augmented the susceptibility to wet-dog shake responses due to 5-HT_{2A} receptor stimulation in streptozotocin-induced diabetic rats. This finding suggests that the increased sensitivity of the 5-HT_{2A} receptor system may be related to some aspect of affective disorder in diabetics.

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