Ingestion of dried-bonito broth (*dashi*) facilitates parvalbumin-immunoreactive neurons in the brain, and affects emotional behaviors in mice

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Abstract

[Objectives] Emerging evidence suggests that traditional diets and nutrition have a significant impact on brain development, and could contribute to the promotion of mental health and prevention of psychiatric disorders in children and adolescents. Moreover, deficits in parvalbumin (PV)-immunoreactive and/or GABAergic neurons are closely associated with various psychiatric disorders in children and adolescents. To investigate the possible neural mechanisms of diet involvement in mental health, we analyzed the effects of dried-bonito *dashi* (Japanese fish broth) (DBD) on PV-immunoreactive neurons and emotional behaviors in young mice.

[Methods] Male mice after weaning were fed DBD for more than 4 weeks, and tested with a resident-intruder test for aggressiveness and a forced swimming test for depression-like symptoms. After the behavioral testing, PV-immunoreactive neurons in the brain were immunohistochemically analyzed.

[Results] The results indicated that DBD intake decreased aggressiveness and depression-like symptoms, and increased the densities of PV-immunoreactive neurons in the medial prefrontal cortex (mPFC), amygdala, hippocampus, and superior colliculus. These behavioral changes were correlated with the densities of PV-immunoreactive neurons in the mPFC, amygdala, and hippocampus. However, subdiaphragmatic vagotomy did not affect the effects of DBD on emotional behaviors, although it nonspecifically decreased the densities of PV-immunoreactive neurons.

[Discussion] The results suggest that DBD might modulate emotional behaviors by promoting PV-immunoreactive and/or GABAergic neuronal activity through parallel routes. The present results highlight a new mechanism for diet involvement in brain functions, and suggest that DBD might have therapeutic potential for the promotion of mental health.

Keywords: gut-brain axis; parvalbumin-immunoreactive neuron; amygdala; hippocampus; prefrontal cortex; emotion

Introduction

The brain and gastrointestinal system communicate bi-directionally by parallel routes including the autonomic nervous system and humoral factors (gut-brain axis), which affects emotion, behaviours, and brain development[1,2]. Diet and nutrition are one of the important factors in the gut-brain axis, and could be applied to the prevention of mental health problems in children and adolescents and their health promotion[3,4]. The average age of onset for anxiety and mood disorders is 6 years and 13 years, respectively[5], and recent emerging evidence suggests that diet patterns have a significant impact on emotion and mental disorders in children and adolescents. It has been reported that higher healthy diet quality, which includes fish, vegetables, beef, and legumes, is associated with better quality of mental health, and that improvement of diet quality improved mental health while deterioration of diet quality worsened mental health in adolescents[6]. Consumption of fish, which is one of the healthy foods, is negatively correlated with incidence of depression across the countries[7]. This effect might be attributed to the omega-3 polyunsaturated fatty acids included in fish[8].

Differential formation of excitatory and inhibitory synapses is critical for the functional development of the central nervous system. A subclass of GABAergic inhibitory interneurons is characterized by the co-expression of calcium-binding protein, parvalbumin (PV). Fast-spiking PV-immunoreactive neurons control the outputs of pyramidal neurons and are necessary for gamma oscillation, which facilitates sensory and cognitive information processing[9-12]. PV-immunoreactive neurons slowly mature, and oxidative stress might induce the loss of PV-immunoreactive neurons and/or impairment of their maturation[13-15]. Pathology of PV-immunoreactive neurons has been suggested to be involved in the deficits in various higher brain functions including emotion, anxiety, and learning and memory[11,12,16], as well as neurodevelopmental disorders[17-20]. Effects of high quality diets on children's mental health (see above) could be attributed to the protection of PV-immunoreactive neurons from various biochemical stresses in the brain by those diets.

Dried-bonito dashi (fish broth) (DBD) is a hot-water extract of dried bonito (skipjack tuna, Katsuwonus pelamis), and a traditional Japanese broth widely used in Japanese cuisine to enhance flavor[21]. It is consumed to promote recovery from fatigue in Japan. Experimental studies also reported that DBD intake ameliorated physical and mental fatigue and improved mood states in humans[22,23] and animals2[4,25]. It is noted that DBD includes only amino acids, peptides, proteins, organic acids, nucleotides, and minerals, but not omega-3 fatty acids[26]. Although the neural mechanisms underlying the DBD's effects on the brain were unknown, previous available data suggest that DBD could be used for mental health promotion in children. We hypothesized that DBD's effects on emotion might be attributed to its effects on PV-immunoreactive neurons. In the present study, the effects of DBD intake during development on emotional behaviours and PV-immunoreactive neurons were investigated in mice. The effects of DBD could be mediated through the vagus nerve, which has various receptors for nutrients and humoral factors in the gut, and projects to the brain[1]. Therefore, we further analysed effects of vagotomy on emotional behaviours and PV-immunoreactive neurons during DBD intake.

Materials and methods

Subjects

Four-week-old (at the start of the experiment) C57BL/6J male mice (n = 84, Japan SLC, Hamamatsu, Japan) were used in a preference test for DBD and/or behavioral and immunohistochemical analyses, and 4-week-old C3H/HeJ male mice (n

= 19, Japan SLC, Hamamatsu, Japan) were used as intruders in a resident-intruder test for aggressive behaviors (see below). All C57BL/6J mice were housed in individual cages in a temperature-controlled environment (22 ± 1ºC) with a 12/12-h light/dark cycle (lights were turned on at 07:00, and turned off at 19:00), while the C3H/HeJ mice were housed five per cage. Food (#D12450B; Research Diets, NJ) and tap water were supplied ad libitum for 1 week after their arrival. The mice were handled for three consecutive days before the start of the experiments. All experimental protocols were performed in accordance with the guidelines for care and use of laboratory animals approved by the University of Toyama and the National Institutes of Health's Guide for the Care and Use of Laboratory Animals, and approved by the Ethics Committee for Animal Experiments at the University of Toyama (Permit No. A2011MED-27). All surgeries were performed under anesthesia, and all efforts were made to minimize suffering.

After acclimation in cages for 1 week, the mice were subjected to the following two experiments. In Experiment I, C57BL/J6 male mice (n = 46) were divided into three groups, and used in the preference test for 10% (n = 15) and 100% (n = 15) DBD solution, and control solution (tap water, n = 16) for 7 days (see below). After the initial preference test, these three groups of mice had access to the same solutions for 53 days (10%DBD, n = 10; 100%DBD, n = 10; tap water, n = 11) in the same environment, and were subsequently subjected to the behavioural (aggression test on the 61st day and forced swimming test on the 67th day) and immunohistochemical analyses (see below). In Experiment II, effects of vagotomy were analyzed. The C57BL/6J male mice (n = 38) initially received a surgery (vagotomy, n = 20; sham operation, n = 18), and three mice with sham operation died. After recovery, they were divided into four groups for the preference test for 10% DBD solution (vagotomized, n = 10; sham, n = 7), and control solution (tap water) (vagotomized, n = 10; sham, n = 8) for 60 days. These mice were subsequently subjected to the same behavioural and immunohistochemical analyses. Body weights of all mice in Experiments I and II were measured daily.

Dried-bonito dashi (DBD)

DBD (Hondzukuri Ichiban-Dashi Katsuo; Ajinomoto, Inc., Japan) contained 4% (w/w) dry matter and had a nominal caloric density of 0.14 kcal/g. The macronutrient composition (per 100 g DBD) was 3.44 g protein, 0.8 g ash, and 127 mg sodium; contents of lipid and carbohydrate were negligible (less than 0.1 g)[27]. Taste components per 100 g DBD included substantial sour (640 mg lactate), bitter (651 mg bitter amino acids and peptides), umami (108 mg inosinate and glutamic acid), and sweet amino acids (26 mg glycine and alanine). In the present study, concentrations of DBD were expressed as percentages of this stock (DBD).

Preference test

The two-bottle preference tests were conducted in the animal's home cage, and the fluid intake was measured daily throughout the experiment. Fluid was available through two sipper spouts attached to two 15-mL plastic drinking tubes. One of the tubes contained tap water and the other a test solution of DBD (10 or 100%). The sipper spouts were inserted through holes positioned 2 cm apart in an aluminum plate with an angle of approximately 30° to the horizontal level. The drinking tubes were fixed in place with rubber clips. Throughout the experiment, the left-right positions of the DBD and water bottles were pseudo-randomly alternated to control for side preferences. The sipper spouts were cleaned daily and used randomly to avoid any association between spout cues and DBD cues. All bottles were cleaned and refilled daily.

Surgery

Twenty mice received a subdiaphragmatic total vagotomy according to a previous study[28]. Under tribromoethanol anesthesia (240 mg/kg, i.p.), a midline incision of the abdominal wall was made, and then, the right and left trunks of the vagus nerve were cut at a level just under the diaphragm. Then, the muscles and skin were sutured. After the surgery, the mice were allowed to recover for 1 week, and then subjected to the preference test. Another 18 mice received a sham vagotomy, in which the surgery was performed in the same way as the total vagotomy, except that the vagal nerves were kept intact.

Aggressive behaviors

Aggressive behaviours of C57BL/6J male mice were elicited in a resident-intruder test[29-32]. Before the resident-intruder test, animal bedding of the home cages of the C57BL/6J male mice (resident) was not changed for 10 days. On the testing day, the home cages of the C57BL/6J male mice were bought to a testing room under temperature control (22 ± 1°C) in the dark phase. After 30 min from the transfer of the home cage, an adult C3H/HeJ male mouse (intruder) was introduced into the home cage of the C57BL/6J male mouse. Each intruder was used no more than four times. Duration of the test was limited to 10 min. The behaviors of both mice were recorded by a CCD camera positioned above the cage. By offline analysis of the video, the latency to the first attack, and number and duration of all attacks were registered by an observer blind to the groups. When the resident did not attack the intruder during 10 min, the latency of aggressive behaviour was counted as 10 min.

Forced Swimming test

The procedures used in this study were based on the original version used for mice by Porsolt et al.[33,34] with modifications[11]. Mice were placed in a cylinder (5 cm × 22.5 cm; diameter × height) filled with water (12 cm high). The mice were not able to touch the bottom. The water temperature was set at 25 ± 1°C. The cylinder was placed in a box with infrared cell sensors on the walls to detect swimming activity (SCANET, Melquest Inc., Toyama, Japan). The software set up a rectangle that circumscribed the body of an animal every 0.3 sec. If the animal went out of the rectangle (i.e., a part of the animal body was detected outside the rectangle) 0.3 sec after setting up the rectangle, the software counted it as "movement (swimming)" in that period. If the animal stayed within the same rectangle 0.3 sec after setting up the rectangle, it was defined as "Immobility".

On the first day of acclimation, the mice were placed in water and forced to swim in a single trial of 15 min. On the second day (test day), the mice were placed in water and forced to swim in a single trial of 5 min. Swimming time was continuously recorded every 1 min of each trial in each animal. After the test, the animals were dried with towels and returned to their cages. The data from the second day were analyzed[34].

Immunohistochemistry

Under deep anesthesia with sodium pentobarbital (50 mg/kg body weight, i.p.), the mice were transcardially perfused with heparinized saline (0.9% w/v NaCl), followed by 4% paraformaldehyde dissolved in 0.1 M phosphate buffer (PB) (pH 7.4). After perfusion, the brains were removed from the skull, coronally cut into small blocks, and postfixed in 4% paraformaldehyde overnight. Fixed brain blocks were then cryoprotected in 30% sucrose dissolved in 0.1 M PB and frozen in dry ice. Next, 40-

μm–thick sections were cut, placed in 0.01 M phosphate buffer saline (PBS), transferred into an antifreeze solution (25% ethylene glycol, 25% glycerin, and 50% 0.1 M PB), and then stored at -20°C until immunohistochemical staining was performed.

Five serial sections were collected for every 200 μm, with one used for PV immunocytochemistry, one for Nissl staining with cresyl violet, and three preserved for further use. Sections were stained with mouse monoclonal anti-PV antibodies (1:10,000 dilution in 1% horse serum PBS, Sigma, St. Louis, MO, USA) using the labeling protocol described in our previous study[11,12]. Negative control sections were produced by omitting the primary antibody, and no reaction product was observed in any of the control sections.

Stereological analysis of PV-immunoreactive neurons

Images of the sections were obtained with an all-in-one fluorescence microscope system (BZ-9000, Keyence Corporation, Osaka, Japan). Using the brain atlas by Hof et al.[35] as reference, we counted the immunoreactive cells in each of the three sections anatomically matched to the adjacent Nissl-stained sections located at +1.10, +1.26, and +1.42 mm to the anterior-to-posterior (AP) level from the bregma in the medial prefrontal cortex (mPFC), including the medial orbital cortex, cingulate cortex area, prelimbic cortex, infralimbic cortex, and dorsal peduncular cortex. In the amygdala, we counted the stained cells in the basolateral amygdala (BLA), including the lateral, basal, and accessory basal nuclei, in four sections at -1.44, -1.60, -1.76, and -1.92 mm AP. In the hippocampus and superior colliculus (SC), stained cells were counted in five (-1.60, -1.76, -1.92, -2.08, -2.24 mm AP) and seven (-3.80, -3.96, -4.12, -4.28, -4.44, -4.60, -4.76 mm AP) sections, respectively. These four brain areas have been implicated in pathology of PV-immunoreactive neurons[12,16,18].

The PV-immunoreactive neurons were counted using an optical dissector, an unbiased stereological technique[12,36]. Systematic sampling of the brain was performed by randomly translating a grid with 280.87 × 765.50-μm (mPFC), 259.00 × 372.40-μm (BLA), 682.81 × 323.55-μm (hippocampus), and 422.71 × 270.45-μm (SC) rectangles onto the section of interest using a stereology software (Stereo Investigator v.7.53.1, MicroBrightField, Williston, VT). Each intersection represented a sample site where 200 × 200-μm (mPFC, BLA, hippocampus) and 100 × 100-μm (SC) counting frames with exclusion lines[37,38] were then applied. All randomly distributed, computer-generated sample sites were then examined using a 20× objective. Only PV-immunoreactive cell bodies falling within the counting frame without contact with the exclusion lines were enumerated. Objects seen in the counting frame were counted only if they came into focus within a predetermined 5-μm thick optical dissector positioned 2 μm below the surface of the mounted section as indicated by the Z-axis microcator. The PV-immunoreactive cell density was estimated in each brain area of each animal using the stereology software.

Statistical data analysis

Quantitative data are expressed as means ± SEM. Preference to DBD (preference ratio) in the two-bottle test was expressed as percent intake (DBD intake/total fluid intake x 100). Data were compared by t-test, or two-way or three-way ANOVAs followed by the Bonferroni and Tukey tests. The statistical significance level was set at $p < 0.05$. Correlations between behavioral parameters and PV-immunoreactive neuronal density were analysed by Pearson's correlation coefficient. All statistical data processing was performed using the Statistical Package for the Social Sciences (SPSS) version 19.0 (SPSS Inc., Chicago, IL).

Results

Experiment I (intact mice)

Solution intake and body weight

Figure 1A shows the mean fluid intake during the initial 7 days in the two-bottle test. The results indicated that mice drank more 10% and 100% DBD compared with tap water (t-test, $p < 0.001$). Figure 1B shows the comparison of preference to the solutions. A statistical analysis by one-way ANOVA indicated that there was a significant main effect of group [$F(2, 43) = 3.72$, $p < 0.05$], consistent with a previous study indicating preference for DBD in mice[39]. Post-hoc tests indicated that the mean preference ratio of 10% DBD was higher than that of tap water (Tukey test, $p < 0.05$). Figure 1C shows the changes in body weights in the three groups of the mice during the two-bottle test. A statistical analysis by repeated measures two-way ANOVA revealed that there was no significant main effect of group [$F(2, 28) = 1.42$, $p > 0.05$], indicating that there was no significant difference in body weights among the three groups.

Resident-intruder test

Figure 2 shows the comparison of the parameters of aggressive behaviours among the three groups in the resident-intruder test. In the mean latencies of aggressive behaviors, there was a significant effect of groups [one-way ANOVA: $F(2, 28) = 3.314$, $p < 0.05$] (A). The post-hoc test indicated that the mean latencies of aggressive behaviors were increased in the C57BL/6J mice subjected to the preference test for 10% DBD (10%DBD group) than those subjected to the preference test for tap water only (TW group) (Tukey test, $p < 0.05$). As for the number of aggressive behaviours, there was a trend of main effect of group [one-way ANOVA; $F(2, 28) = 2.584$, $p = 0.093$] (B), and the 10%DBD group tended to show lesser aggressive behaviours.

Furthermore, there was a significant effect of groups in the mean duration of aggressive behaviours [one-way ANOVA; $F(2, 28) = 3.74$, $p < 0.05$] (C). The post-hoc test indicated that the mean durations of aggressive behaviours were decreased in the 10%DBD group than the TW group (Tukey test, $p < 0.05$). These results indicated that DBD decreased aggressive behaviours.

The above results could be ascribed to differences in body weights among the three groups of the resident mice. Therefore, we further analysed the body weights of the mice. The mean body weights of the resident mice from 61st to 67th days was 27.3 ± 0.5 g, while the mean body weights of the intruder mice from the 61st to 67th days was 18.3 ± 0.7 g. There was no significant difference in body weight among the three groups of the resident mice [one-way ANOVA; $F(2, 28) = 2.51$, $p > 0.05$]. Furthermore, the relationship between body weights of the resident mice and each parameter (latency, number, and duration) of aggressive behaviours was analysed. Statistical analyses by Pearson's correlation indicated that there was no significant correlation between body weight and the parameters of aggressive behaviours ($p > 0.05$). These results indicated that the above results were not ascribed to the differences in body weights of the resident mice.

Forced swimming test

The forced swimming test has been previously used to assess depression-related behaviors in animal models that are considered negative symptoms of schizophrenia and autism[40,41]. In the 5-min test on the second day (Fig. 3), the statistical analysis by two-way ANOVA indicated that there were significant main effects of group [$F(2, 140) = 17.92$, $p < 0.001$] and time [$F(4, 140) = 5.55$, $p < 0.001$]. Subsequent post-hoc tests

indicated that the mean swimming time was significantly increased in the 10%DBD and 100%DBD groups than in the TW group (Tukey test, $p < 0.05$). These results indicate that the 10%DBD and 100%DBD groups became less immobile, suggesting that DBD made the mice less depressed.

The above results could be ascribed to differences in body weights of the mice. Therefore, we further analysed the relationship between body weights and swimming time. A statistical analysis by Pearson's correlation indicated that there was no significant correlation between body weight and mean swimming time ($p > 0.05$). These results indicated that the above results were not ascribed to the differences in body weights of the mice.

Immunohistochemical analysis

The above results indicated that 10% DBD showed significant effects in both behavior tests. We further analyzed the effects of 10% DBD on the brain. Figure 4 shows examples of PV-immunoreactive neurons in the mPFC (Aa), basolateral amygdala (BLA) (Ba), hippocampus (Ca), and SC (Da) for the TW group. An increment in PV-immunoreactive neurons was observed in these areas in the 10%DBD group (Ab, Bb, Cb, Db) in comparison to the TW group. Figure 5 shows the mean density (cells/mm3) of PV-immunoreactive neurons in the mPFC (A), BLA (B), hippocampus (C), and SC (D). Statistical comparisons by t-tests in each area indicated that the cell density was significantly higher in the 10%DBD group than in the TW group (mPFC, BLA, hippocampus, and SC, $p < 0.05$).

Experiment II (operated mice)

Preference to DBD solution

Figure 6A shows the comparison of mean fluid intake during 7 days before the behavioural experiments between the sham-operated and vagotomized mice. Statistical analysis by three-way ANOVA indicated that a main effect of group (TW vs. 10%DBD) tended to be significant [$F(1, 62) = 3.56$, $p = 0.064$], and that there were significant main effects of operation (sham vs. vagotomy) [$F(1, 62) = 16.20$, $p < 0.001$], and test solution (TW vs. test solution) [$F(1, 62) = 21.171$, $p < 0.001$]. The results indicated that the vagotomized mice drank more fluid than the sham-operated mice, and the 10%DBD group drank more fluid than the TW group. The increases in the fluid intake in the vagotomized mice are consistent with previous studies suggesting a role of vagus nerve in the negative feedback control of meal size[42,43]. Furthermore, there were also significant interactions between group and test solution [$F(1, 62) = 20.17$, $p < 0.001$]. The results indicated that the mice drank more 10% DBD compared with tap water regardless of vagotomy.

Figure 6B shows the comparison of preference to the solutions. A statistical analysis by two-way ANOVA indicated that there was a significant main effect of test solution [$F(1, 31) = 18.46$, $p < 0.001$].

Resident-intruder test

Figure 7 shows the latencies (A), number (B), and duration (C) of aggressive behaviours of the sham-operated and vagotomized mice in the resident-intruder test. A statistical analysis of the mean latencies by two-way ANOVA indicated that there was a significant main effect of group [$F(1, 31) = 4.35$, $p < 0.05$], while there was no significant main effect of operation [$F(1, 31) = 1.41$, $p > 0.05$], nor significant

interaction between group and operation [$F(1, 31) = 0.08$, $p > 0.05$] (A). These results indicated that the mean latencies were increased in the 10%DBD group regardless of vagotomy. Furthermore, a statistical analysis of the number of aggressive behaviours by two-way ANOVA indicated that there was a significant main effect of group [$F(1, 31) = 4.83$, $p < 0.05$], while there was neither a significant main effect of operation [$F(1, 31) = 1.93$, $p > 0.05$], nor a significant interaction between group and operation [$F(1, 31) = 0.10$, $p > 0.05$] (B). These results indicated that the number of aggressive behaviours was decreased in the 10%DBD group regardless of vagotomy. On the other hand, a statistical analysis of duration of aggressive behaviours by two-way ANOVA indicated that there was a significant main effect of operation [$F(1, 31) = 5.75$, $p < 0.05$], while there was neither a significant main effect of group [$F(1, 31) = 2.31$, $p > 0.05$], nor a significant interaction between group and operation [$F(1, 31) = 0.15$, $p > 0.05$] (C). These results indicated that the duration of aggressive behaviour was increased in the vagotomized mice regardless of the solutions.

The above results could be ascribed to differences in body weights among the four groups of the resident mice. Therefore, we further analysed the body weights of the mice. The mean body weights of the resident mice from 61st to 67th days was 25.7 ± 0.3 g, while the mean body weight of the intruder mice from the 61st to 67th days was 18.5 ± 0.7 g. A statistical analysis by two-way ANOVA indicated a significant difference in body weight among the four groups of the resident mice; there was a significant main effect of operation (vagotomy) [$F(1, 31) = 7.18$, $p < 0.05$], while there was neither a significant main effect of group (DBD) [$F(1, 31) = 0.79$, $p > 0.05$], nor a significant interaction between group and operation [$F(1, 31) = 0.01$, $p > 0.05$]. These results indicated that the mean body weight was slightly, but significantly greater in the vagotomized resident mice than the sham operated resident mice. Furthermore, the relationship between body weights of the resident mice and each parameter (latency, number, and duration) of aggressive behaviours was analysed. Although the mean body weight was slightly, but significantly greater in the vagotomized resident mice than the sham operated resident mice (see above), statistical analyses by Pearson's correlation indicated that there was no significant correlation between body weight and the parameters of aggressive behaviours ($p > 0.05$). These results indicated that the above results were not ascribed to the differences in body weights of the resident mice.

Forced swimming test

Figure 8 shows the mean swimming time in the 5-min test on the second day. A statistical analysis by three-way ANOVA indicated that there were significant main effects of group (tap water vs. 10%DBD) [$F(1, 155) = 8.69$, $p < 0.01$] and time [$F(4, 155) = 7.03$, $p < 0.001$], while there was no significant main effect of operation (sham vs. vagotomy) [$F(1, 155) = 0.77$, $p > 0.05$] and no significant interaction among group, time, and operation [$F(4, 155) = 0.64$, $p > 0.05$]. These results indicate that the 10%DBD group showed more mobility regardless of vagotomy, suggesting that DBD made the mice less depressed.

The above results could be ascribed to differences in body weights of the mice. Therefore, we further analysed the relationship between body weights and swimming time. Although the mean body weight was slightly, but significantly greater in the vagotomized resident mice than the sham operated resident mice (see above), a statistical analysis by Pearson's correlation indicated that there was no significant correlation between body weight and mean swimming time ($p > 0.05$). These results indicated that the above results were not ascribed to the differences in body weights of the mice.

Immunohistochemical analysis

Figure 4 shows examples of PV-immunoreactive neurons in the mPFC (Ac-f), basolateral amygdala (BLA) (Bc-f), hippocampus (Cc-f), and SC (Dc-f) for the TW and 10%DBD groups with sham operation or vagotomy. An increment in PV-immunoreactive neurons was observed in these areas in the 10%DBD group with sham operation (Ad, Bd, Cd, Dd) compared to the TW group with sham operation (Ac, Bc, Cc, Dc). A similar increment in PV-immunoreactive neurons was also observed in these areas in the 10%DBD group with vagotomy (Af, Bf, Cf, Df) compared to the TW group with vagotomy (Ae, Be, Ce, De).

Figure 9 shows the mean density (cells/mm3) of PV-immunoreactive neurons in the mPFC (A), BLA (B), hippocampus (C), and SC (D) in the TW and 10%DBD groups with sham operation or vagotomy. In the mPFC (A), a statistical comparison by two-way ANOVA indicated that there were significant main effects of group (TW vs. 10%DBD) [$F(1, 24) = 19.64$, $p < 0.001$] and operation (sham vs. vagotomy) [$F(1, 24) = 8.330$, $p < 0.05$], while there was no significant interaction between group and operation [$F(1, 24) = 0.01$, $p > 0.05$]. The results indicated that the mean cell densities were higher in the 10%DBD group than in the TW group, and that the mean cell densities were higher in the mice with sham operation than in the mice with vagotomy.

In the BLA (B), a statistical comparison by two-way ANOVA indicated that there were significant main effects of group [$F(1, 24) = 19.23$, $p < 0.001$] and operation [$F(1, 24) = 21.14$, $p < 0.001$], while there was no significant interaction between group and operation [$F(1, 24) = 0.004$, $p > 0.05$]. The data in the BLA replicated that in the mPFC; the mean cell densities were higher in the 10%DBD group than in the TW group, and the mean cell densities were higher in the mice with sham operation than in the mice with vagotomy.

In the hippocampus (C), a statistical comparison by two-way ANOVA indicated that there were significant main effects of operation [$F(1, 24) = 24.97$, $p < 0.001$] and group [$F(1, 24) = 11.97$, $p < 0.05$], while there was no significant interaction between group and operation [$F(1, 24) = 1.56$, $p > 0.05$]. The data in the hippocampus replicated that in the mPFC; the mean cell densities were higher in the mice with sham operation than in the mice with vagotomy, and the mean cell densities were higher in the 10%DBD group than in the TW group.

In the SC (D), a statistical comparison by two-way ANOVA indicated that there were significant main effects of group [$F(1, 24) = 15.41$, $p < 0.005$] and operation [$F(1, 24) = 25.14$, $p < 0.001$], while there was no significant interaction between group and operation [$F(1, 24) = 0.83$, $p > 0.05$]. The data in the SC also replicated that in the mPFC; the mean cell densities were higher in the 10%DBD group than in the TW group, and the mean cell densities were higher in the mice with sham operation than in the mice with vagotomy.

Correlation analysis

The relationship between the cell density of PV-immunoreactive neurons in each brain area and three parameters of aggressive behaviors were analyzed. In the mPFC (Fig. 10A), the results indicated that the immunoreactive cell density was significantly and positively correlated with the latency of aggressive behavior (Pearson's correlation, $r = 0.419$, $p < 0.01$), tended to be negatively correlated with the number of aggressive behaviour (Pearson's correlation, $r = 0.294$, $p = 0.059$), and was significantly and negatively correlated with the duration of aggressive behavior ($r = 0.345$, $p < 0.05$). In the BLA (Fig. 10B), the results indicated that the immunoreactive cell density was significantly and positively correlated with the latency of aggressive behavior (Pearson's correlation, $r = 0.355$, $p < 0.05$), and negatively correlated with the duration

of aggressive behaviour (Pearson's correlation, $r = 0.308$, $p < 0.05$), while there was no significant correlation between the cell density and the number of aggressive behavior (Pearson's correlation, $r = 0.230$, $p > 0.05$). In the hippocampus (Fig. 11A), the results indicated that the immunoreactive cell density was significantly and positively correlated with the latency of aggressive behaviour (Pearson's correlation, $r = 0.417$, $p < 0.01$), while there was no significant correlation between the cell density and the number of aggressive behaviour (Pearson's correlation, $r = 0.206$, $p > 0.05$), and duration of aggressive behaviour (Pearson's correlation, $r = 0.192$, $p > 0.05$). In the SC (Fig. 11B), there was no significant correlation between the cell density and latency of aggressive behaviour (Pearson's correlation, $r = 0.291$, $p = 0.061$), between the cell density and number of aggressive behaviour (Pearson's correlation, $r = 0.162$, $p > 0.05$), and between the cell density and duration of aggressive behaviour (Pearson's correlation, $r = 0.218$, $p > 0.05$). These results indicated that the PV-immunoreactive neurons in the mPFC, BLA, and hippocampus exert suppressive effects on the aggressive behaviours of the resident mice.

In the forced swimming task, the relationship between the cell density of PV-immunoreactive neurons in each brain area and the mean swimming time during the task was analyzed. In the BLA (Fig. 12), the results indicated that the immunoreactive cell density was significantly and positively correlated with the mean swimming time (Pearson's correlation, $r = 0.378$, $p < 0.05$). In the other brain areas (mPFC, hippocampus, SC), there were no significant correlations (data not shown).

Discussion

Effects of DBD intake on aggressive behaviors

Aggression is not a unitary phenomenon, and there are at least two types of aggressive behaviours - offensive and defensive aggressive behaviors[44]. The resident-intruder test induces offensive aggressive of a resident by invasion of an intruder[29]. In the present study, the latencies of aggressive behaviours increased and the duration of aggressive behaviours decreased in the 10%DBD group compared with the TW group, suggesting that the intake of 10% DBD inhibits aggressive behaviours. Furthermore, the onset latencies of aggressive behaviours were positively correlated with the densities of PV-immunoreactive neurons while the number and duration of aggressive behaviours were negatively correlated with the densities of PV-immunoreactive neurons in the mPFC, amygdala, and hippocampus. These findings suggest that the decrease in aggressive behaviours by DBD intake was attributed to the increase in PV-immunoreactive neurons. These findings parallel the results of a previous study in which affiliative behaviours were positively correlated with PV-immunoreactive neurons in the mPFC and hippocampus[12]. It has been reported that chronic administration of antidepressants induces side effects including aggression and violence[45] as well as dematuration of PV-immunoreactive neurons in the mPFC, amygdala, and hippocampus[46,47]. The side effects might be ascribed to dematuration of PV-immunoreactive neurons, which might decrease the number of PV-immunoreactive neurons and inhibitory neurotransmission[47,48].

There is some evidence of GABA involvement in the suppression of aggressive behaviors. Aggressive mice had lower brain GABA concentrations compared to non-aggressive mice[49], and injection of GABA into specific brain regions inhibited aggression[50]. Second, administration of agents to inhibit GABA transaminase increased brain GABA content and decreased aggressive behaviors[51], while valproic acid, which increases brain GABA levels, suppressed impulsive aggression in human patients[52,53]. Third, plasma GABA levels, some of which comes from the brain GABA, were negatively correlated with aggressiveness in human patients[54]. Collectively, these

results suggest that DBD inhibited aggressive behaviors through alteration in the brain GABAergic activity.

Effects of DBD intake on forced swimming

The 10%DBD group showed increased swimming time in the forced swimming test (considered an anti-depression effect). The impairment in the forced swimming test (decreased swimming time) has been proposed as an animal model of depression[33,34]. In the 10%DBD group, the densities of PV-immunoreactive neurons were increased in the mPFC, amygdala, hippocampus, and SC. Human clinical studies reported that the mPFC, amygdala, and hippocampus are related to pathogenesis of mood disorders (see review by Davidson et al.[55]). Furthermore, the mean swimming time was positively correlated with the densities of PV-immunoreactive neurons in the amygdala. These results suggest that the increases in the swimming time by DBD intake were attributed to the increases in PV-immunoreactive neurons. These findings suggest that the increases in PV-immunoreactive neurons induce decreased depressive-like symptoms, while the decreases in PV-immunoreactive neurons induce depressive symptoms. Consistently, D2 receptor knockout mice with increased PV-immunoreactive neurons showed decreased depression-like behaviors[56]. In contrast, mice with truncation of Disrupted-in-Schizophrenia 1 (DISC1), which is associated with psychiatric disorders including depression, showed decreases in PV-immunoreactive neurons and deficits in the forced swimming test[57]. Similar deficits in the same task have been reported as a negative symptom in various animal models of autism and schizophrenia[11,40,41], in which deficits of PV-immunoreactive neurons were reported[58-60]. A recent study also reported that the excitatory synaptic current in PV-immunoreactive neurons was reduced in animals with depressive symptoms, and that selective suppression of PV-immunoreactive neurons promoted depressive symptoms[61].

The above changes in depression-like behaviours might be attributed to changes in the GABAergic activity due to changes in PV-immunoreactive neurons. Impairments in GABAergic neurotransmission or reduced number of GABAergic neurons have been reported to be associated with depression-like symptoms in animals and humans[62-64]. Furthermore, cortical GABA content was increased after treatment of depression in human patients[65,66]. These findings suggest that the behavioral improvement observed in the 10%DBD group might be related to the promotion of GABAergic neurotransmission.

Plausible mechanisms of DBD

The present results indicated that DBD intake increased the number of PV-immunoreactive neurons and improved emotional deficits. Emerging evidence suggests that diet has a significant impact on the brain, which might be mediated through afferent autonomic nerves (vagal and sympathetic nerves), humoral factors released from the gut (peptides, hormones, cytokines, etc.), specific nutritional and biochemical substances included in the food (vitamins, zinc, folate, omega-3 polyunsaturated fatty acids, antioxidants, etc.), gut microbiome, and immune system in the gut[1,2,8,67,68]. DBD includes various amino acids and peptides (histidine, taurine, alanine, anserine, etc.), nucleotides, and over 400 odorants (phenolic compounds, etc.)[26,69]. First, histidine, anserine, and phenolic compounds in DBD have been reported to show antioxidative actions in vitro[70-72]. Furthermore, DBD intake decreased a urinary biomarker of oxidative stress in humans[22]. On the other hand, PV-immunoreactive neurons slowly mature, and oxidative stress might induce the loss of PV-immunoreactive neurons and/or impairment of their maturation (see Introduction). Furthermore, some vulnerability genes (PRODH, DISC-1, G72, dysbindin) for schizophrenia and/or bipolar disease might lead to mitochondrial or antioxidant dysfunction[15]. Thus, lowered antioxidant defenses such as diets low in antioxidants might induce psychiatric

disorders such as depression and schizophrenia in patients with the vulnerability genes, and antioxidant medication might be useful for prevention of these psychiatric disorders[13,73,74]. Consistently, knockout mice with impaired synthesis of glutathione (antioxidant) showed deficits of PV-immunoreactive neurons, and the deficits could be ameliorated by the administration of antioxidant N-acetylcysteine[15]. These findings suggest that the effects of DBD intake might be mediated partly through its antioxidant activity.

Second, vagotomy decreased the densities of PV-immunoreactive neurons in the mPFC, amygdala, hippocampus, and SC, although it did not affect the emotional behaviours. The vagus nerve has various mechano- and chemo-receptors for nutrients and humoral factors, and projects to the nucleus of solitary tract, which in turn projects to the forebrain important for emotional behaviors[75]. Furthermore, vagus nerve stimulation increased the expression of brain derived neurotrophic factors (BDNF) in the rat hippocampus and cortex[76], and BDNF promotes expression of PV and GABAergic neural transmission[77-79]. Consistently, vagus nerve stimulation ameliorated depressive symptoms in humans[80]. These findings suggest that the vagus nerve might partially mediate DBD effects.

Third, DBD contains abundant histidine (68% of amino acids in DBD)[69]. Histidine is a precursor of histamine, which is an important modulator that acts as a trophic and/or neurogenic factor in the developing brain[81]. Perinatal exposure to dioxin induced reduction of PV-immunoreactive neurons in rats and autistic traits in humans[18,19], and lower urinary histidine levels were associated with the neurodevelopmental delay in children in the dioxin-polluted area[82]. It is reported that the peripheral administration of histidine as well as intraventricular administration of H1-receptor agonist decreased depression-like symptoms[83]. Furthermore, histamine directly opens $GABA_A$ receptor and/or potentiates the current mediated by $GABA_A$ receptor in vitro[84], and excites GABAergic neurons in the septohippocampal system[85]. These findings suggest that peripheral histidine in DBD might be converted to histamine in the brain, which in turn might modulate GABAergic neurotransmission. Further studies are required to prove or disprove the above three possibilities.

Conclusion

Clinical studies suggest that deficits in PV-immunoreactive and/or GABAergic neurons are closely associated with various psychiatric disorders[86,87]. Emerging evidence suggests that diets and nutrition have a significant impact on the brain, and could contribute to the promotion of mental health and prevention of psychiatric disorders[67,88]. The present study indicated that DBD intake decreased aggressiveness and depression-like symptoms. These behavioural changes were correlated with densities of PV-immunoreactive neurons in the mPFC, amygdala, and hippocampus. Along with previous studies, the results suggest that DBD might modulate emotional behaviours by promoting PV-immunoreactive and/or GABAergic neurotransmission through antioxidants and histidine included in DBD, and the vagal nerve. The present results highlight a new mechanism for diet involvement in brain functions, and DBD might have therapeutic potential for the promotion of mental health. However, other types of GABAergic neurons such as calbindin-immunoreactive neurons are also implicated in psychiatric disorders such as major depression[89]. Since major depression is associated with metabolic syndrome[90], changes in calbindin-immunoreactive neurons could be ascribed to deteriorated diet patterns in depressive patients with metabolic syndrome. Further studies are required to determine whether DBD is effective to ameliorate behavioural deficits using various animal models of psychiatric diseases with changes in other types of GABAergic neurons including calbindin-immunoreactive neurons.

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Figure legends

Fig. 1. Liquid intake (A), preference of DBD solutions (B) and body weight changes (C) in the three groups in Experiment I.
A: Mean liquid intakes during 7 days in the two-bottle test. In the 10%DBD and 100%DBD groups, the mice have significantly drunk more DBD solutions compared with TW. ***, $P < 0.001$; TW, tap water; DBD, dried bonito broth.
B: Comparison of preference of the solutions shown in A. *, $p < 0.05$.
C: Daily changes in mean body weight in the three groups.

Fig. 2. Comparison of the mean latencies (A), numbers (B), and durations (C) of aggressive behaviors in the resident-intruder test in Experiment I. *, $p < 0.05$.

Fig. 3. Comparison of the mean swimming time in the forced swimming test in Experiment I. Ordinate indicates swimming time in 1 min in the second day. *, $p < 0.05$.

Fig. 4. Photomicrographs of the medial prefrontal cortex (mPFC, A), basolateral amygdala (BLA, B), CA3 subfield of the hippocampus (C), and superior colliculus (SC, D) of the six groups of mice. Number of PV-immunoreactive neurons was decreased in the DBD groups. Intact TW, TW group in Experiment I; Intact DBD, 10%DBD group in Experiment I; Sham TW, sham-operated TW group in Experiment II; Sham DBD, sham-operated 10%DBD group in Experiment II; Vagotomy TW, vagotomized TW group in Experiment II; Vagotomy DBD, vagotomized 10%DBD group in Experiment II; Scale bar = 100 μm.

Fig. 5. Intake of 10% DBD increases the density of parvalbumin-immunoreactive neurons in the mPFC (A), basolateral amygdala (BLA) (B), hippocampus (C), and superior colliculus (SC) (D). *, $p < 0.05$.

Fig. 6. Mean liquid intake of sham-operated and vagotomized mice in Experiment II.
A: Mean liquid intakes during 7 days in the two-bottle test. In the sham-operated and vagotomized 10%DBD groups, the mice have significantly drunk more DBD solutions compared with TW. #, significant difference from TW at $p < 0.05$; *, significant difference from the sham-operated mice at $p < 0.05$; TW, tap water; DBD, dried bonito broth.
B: Comparison of preference of the solutions shown in A. *, $p < 0.05$.

Fig. 7. Comparison of the mean latencies (A), numbers (B), and durations (C) of aggressive behaviors in the resident-intruder test in Experiment II. *, significant difference from TW at $p < 0.05$; #, significant difference from the sham-operated mice at $p < 0.05$.

Fig. 8. Comparison of the mean swimming time in the forced swimming test in Experiment II. Ordinate indicates swimming time in 1 min in the second day. *, significant difference from TW at $p < 0.05$.

Fig. 9. Intake of 10% DBD increases and vagotomy decreases the density of PV-immunoreactive neurons in the mPFC (A), basolateral amygdala (BLA) (B), hippocampus (C), and superior colliculus (SC) (D). *, $p < 0.05$. *, significant difference from TW at $p < 0.05$; #, significant difference from the sham-operated mice at $p < 0.05$.

Fig. 10. Relationships between the parameters [latency (a), number (b), and duration (c)] of aggressive behaviors in the resident-intruder test and PV-immunoreactive neuron density in the mPFC (A) and BLA (B). Significant correlations between

all or some of three parameters of aggressive behaviors and PV-immunoreactive neuron density are observed in the mPFC (A) and BLA (B) ($p < 0.05$).

Fig. 11. Relationships between the parameters [latency (a), number (b), and duration (c)] of aggressive behaviors in the resident-intruder test and PV-immunoreactive neuron density in the hippocampus (A) and SC (B). A significant correlation between latency (a) of aggressive behaviors and PV-immunoreactive neuron density is observed in the hippocampus (A) ($p < 0.05$).

Fig. 12. Significant correlation between the mean swimming time and PV-immunoreactive neuron density in the BLA ($p < 0.05$).

ACKNOWLEDGEMENTS

I would like to express my deepest thanks to Prof. Nishijo who took me into his laboratory. He has shown great kindness and patience in helping me over the years.

Special thanks to Prof. Hori who gave me the opportunity to learn his progressive experimental skills and encouraged me continuously.

I want to thank Dr. Nishimaru for scientific advice, and for providing valuable training and suggestions to improve my thesis.

I wish to express my warm thanks to Dr. Takamura, Dr. Kondoh, Dr. Nakamura and Dr. Matsumoto for helping with the experiments and discussions.

I also thank the laboratory members of System Emotional Science who have helped me. Without their patience and excellent collaborative work, I would not have been able to finish my study.

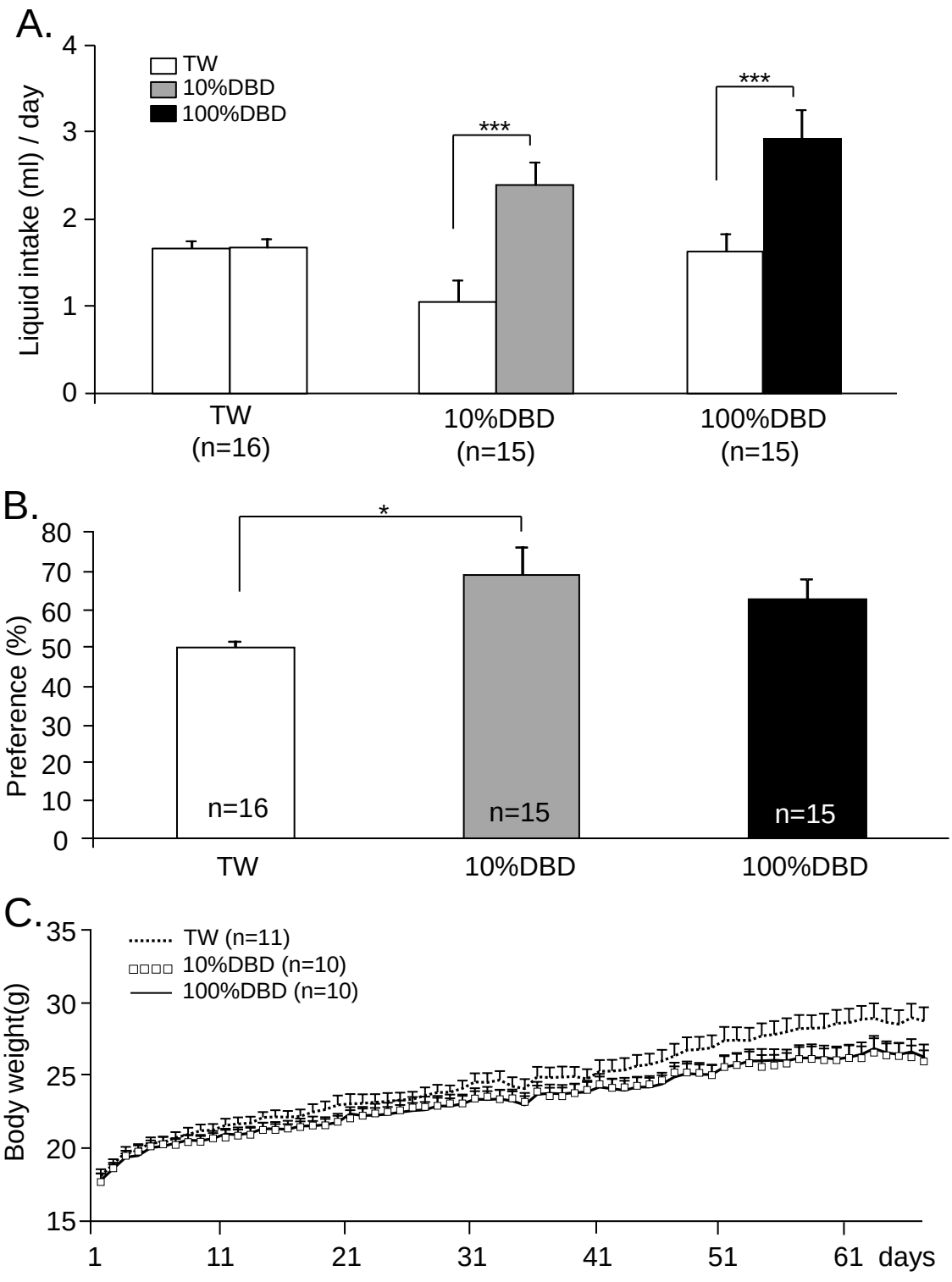
A.
Liquid intake (ml) / day
TW
10%DBD
100%DBD
***
***
TW
(n=16)
10%DBD
(n=15)
100%DBD
(n=15)
B.
Preference (%)
*
n=16
n=15
n=15
TW
10%DBD
100%DBD
C.
Body weight(g)
TW (n=11)
10%DBD (n=10)
100%DBD (n=10)
days

Fig. 1

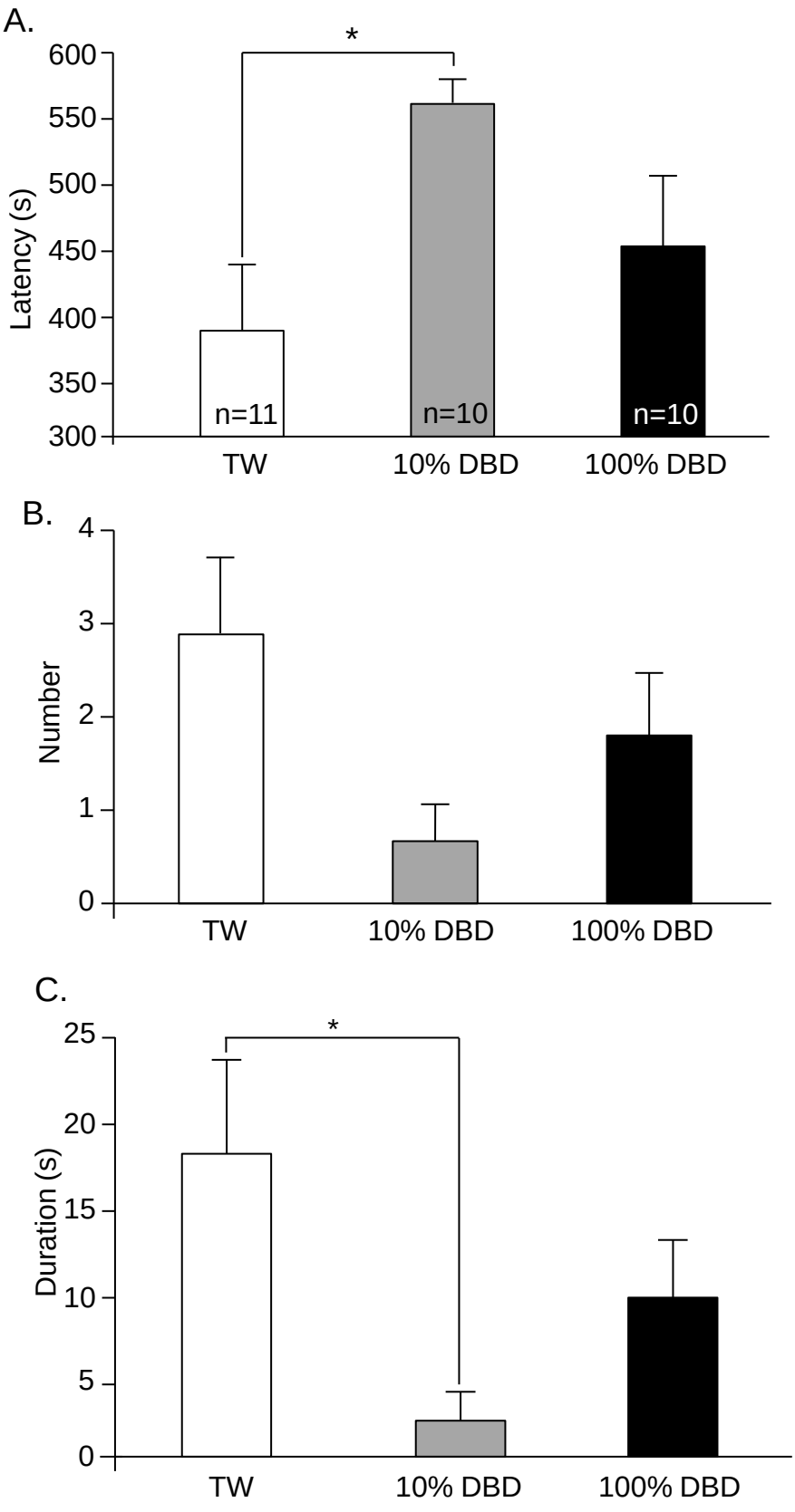
A.
*
600
550
500
450
400
350
300
Latency (s)
n=11
n=10
n=10
TW
10% DBD
100% DBD
B.
4
3
2
1
0
Number
TW
10% DBD
100% DBD
C.
*
25
20
15
10
5
0
Duration (s)
TW
10% DBD
100% DBD

Fig.2

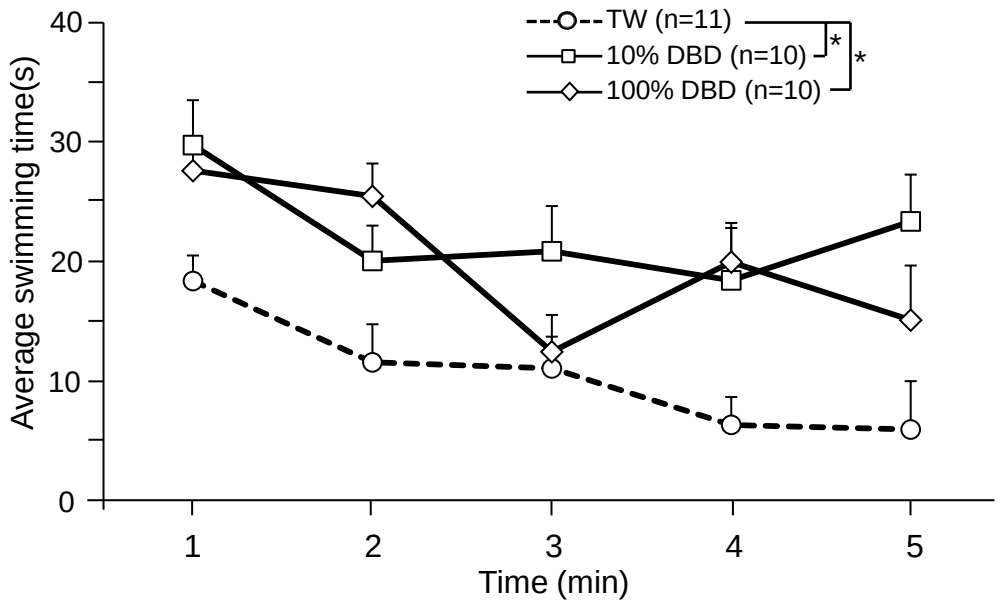
TW (n=11)
10% DBD (n=10)
100% DBD (n=10)
*
*
Average swimming time(s)
40
30
20
10
0
1
2
3
4
5
Time (min)

Fig. 3

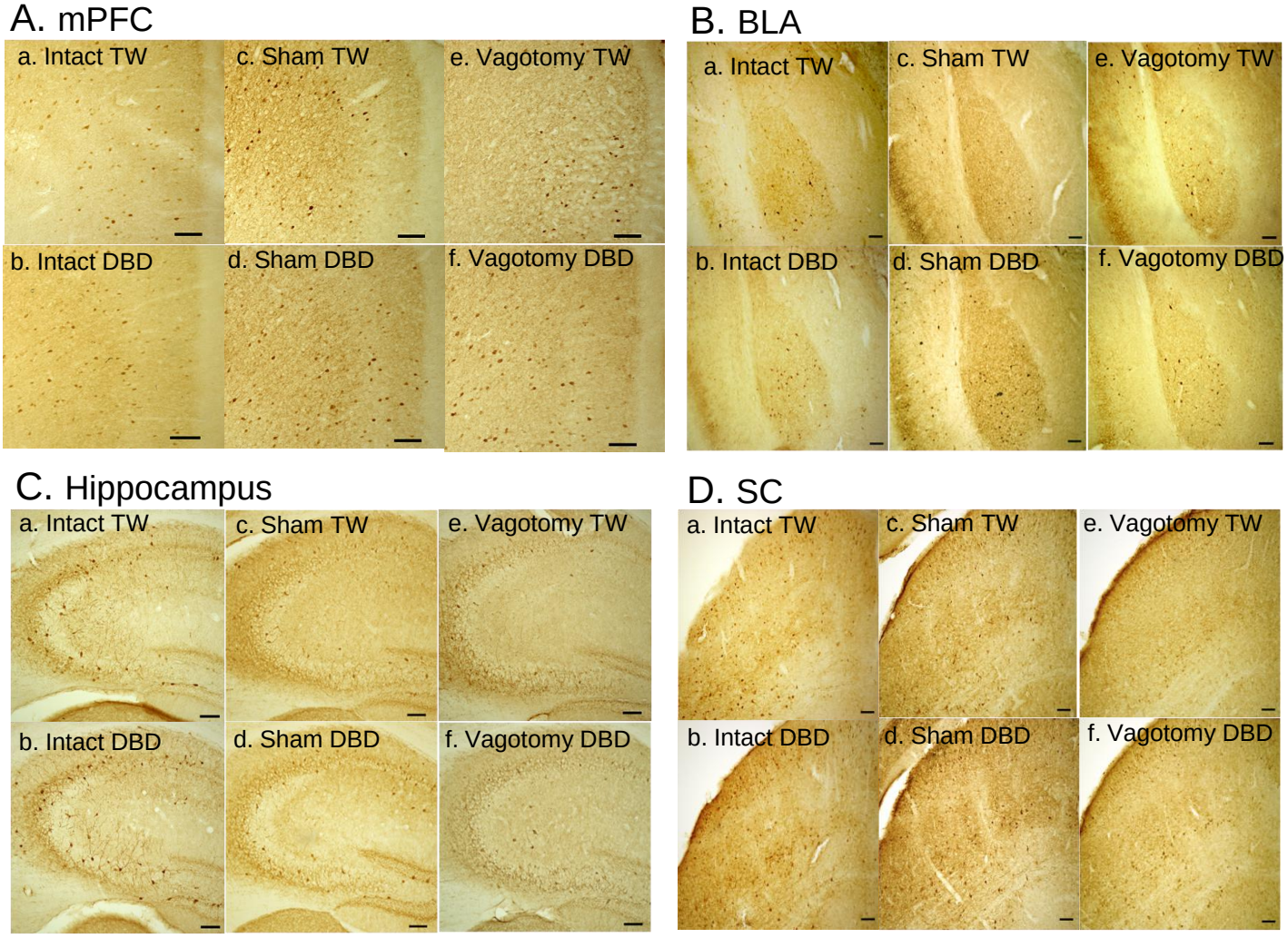
A. mPFC
a. Intact TW
c. Sham TW
e. Vagotomy TW
b. Intact DBD
d. Sham DBD
f. Vagotomy DBD
B. BLA
a. Intact TW
c. Sham TW
e. Vagotomy TW
b. Intact DBD
d. Sham DBD
f. Vagotomy DBD
C. Hippocampus
a. Intact TW
c. Sham TW
e. Vagotomy TW
b. Intact DBD
d. Sham DBD
f. Vagotomy DBD
D. SC
a. Intact TW
c. Sham TW
e. Vagotomy TW
b. Intact DBD
d. Sham DBD
f. Vagotomy DBD

Fig. 4

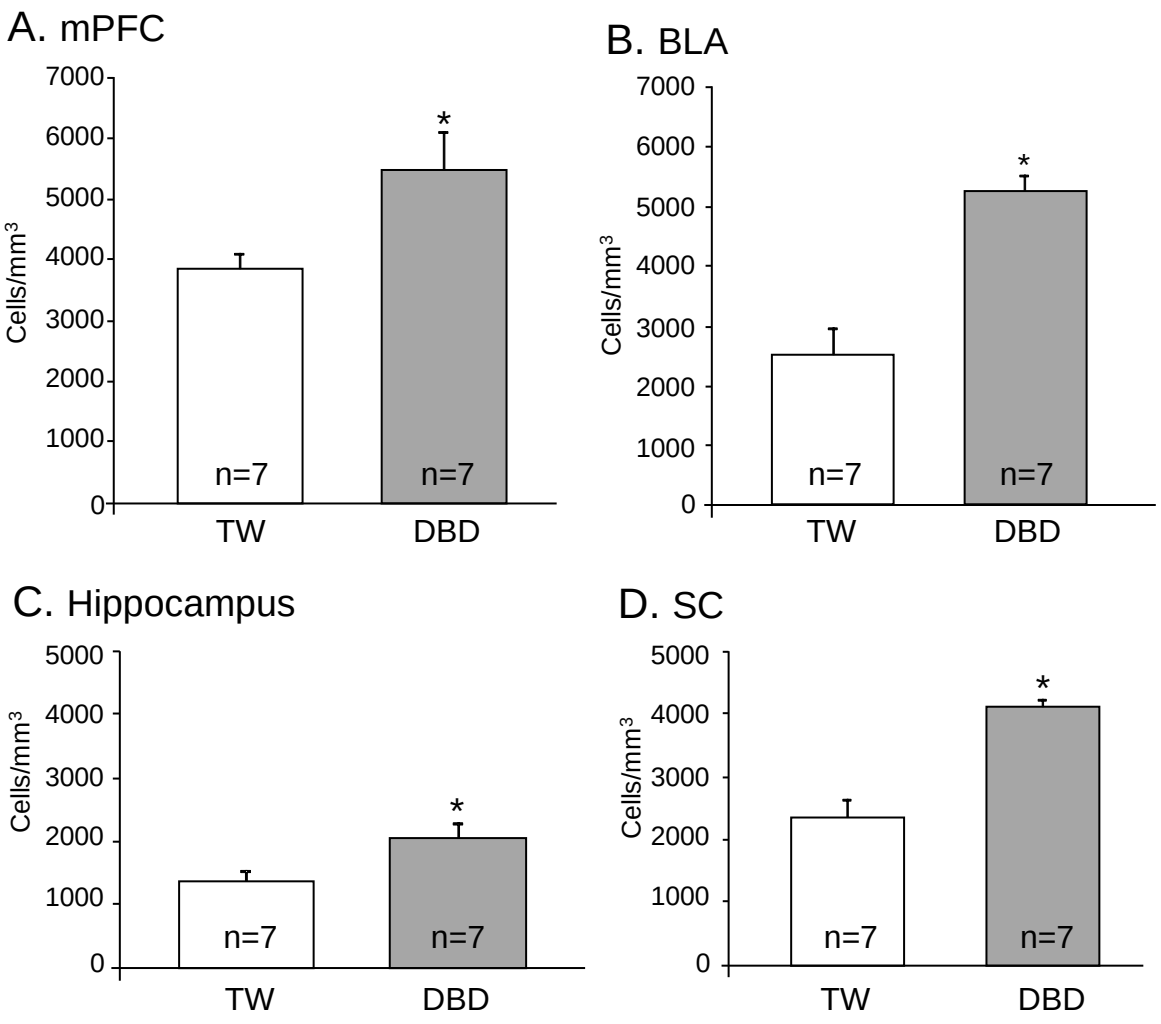
A. mPFC
B. BLA
C. Hippocampus
D. SC
Cells/mm3
7000
6000
5000
4000
3000
2000
1000
0
n=7
TW
DBD
*

Fig. 5

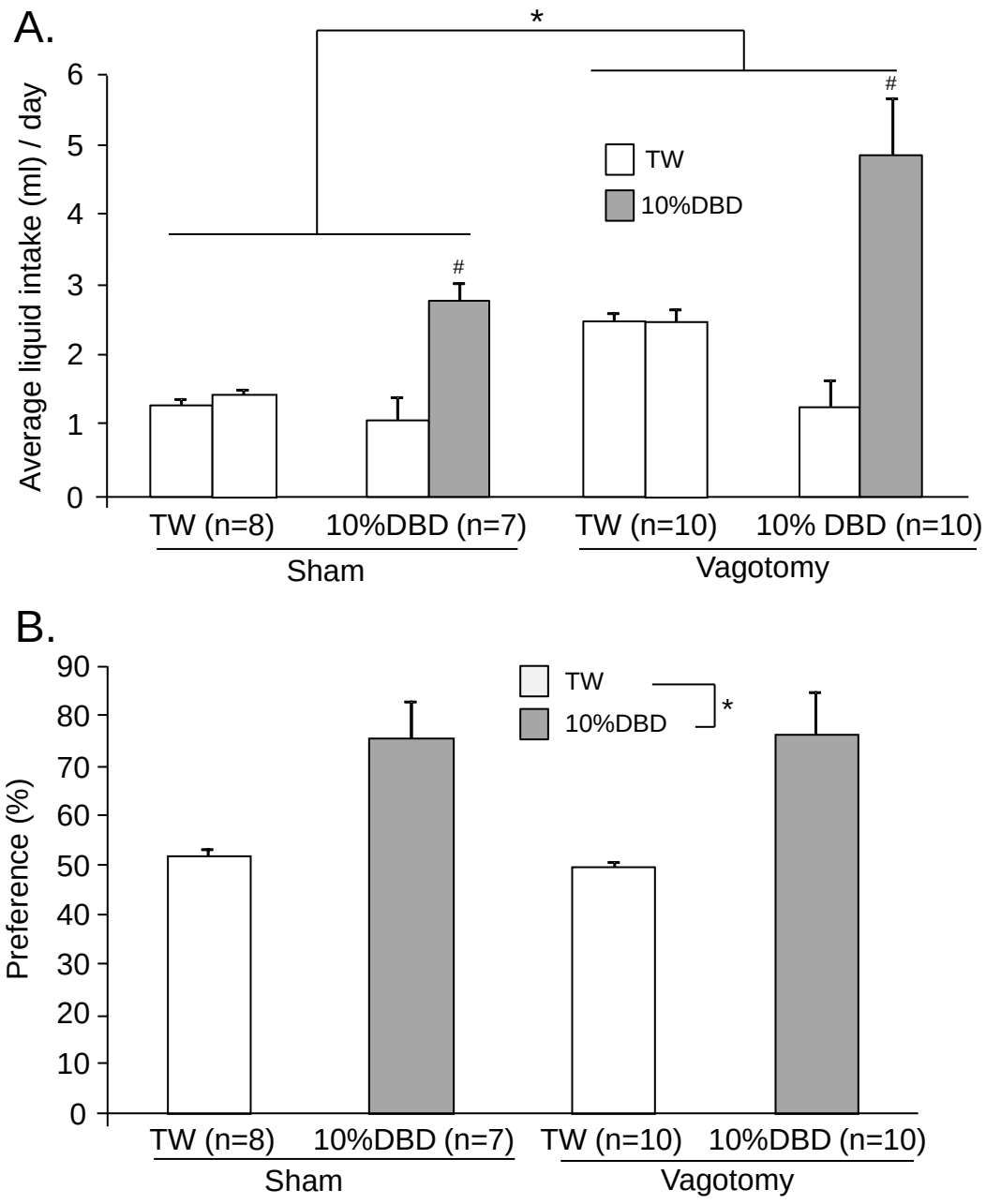
A.
Average liquid intake (ml) / day
0
1
2
3
4
5
6
*
#
#
TW
10%DBD
TW (n=8)
10%DBD (n=7)
TW (n=10)
10% DBD (n=10)
Sham
Vagotomy
B.
Preference (%)
0
10
20
30
40
50
60
70
80
90
TW
10%DBD
*
TW (n=8)
10%DBD (n=7)
TW (n=10)
10%DBD (n=10)
Sham
Vagotomy

Fig. 6

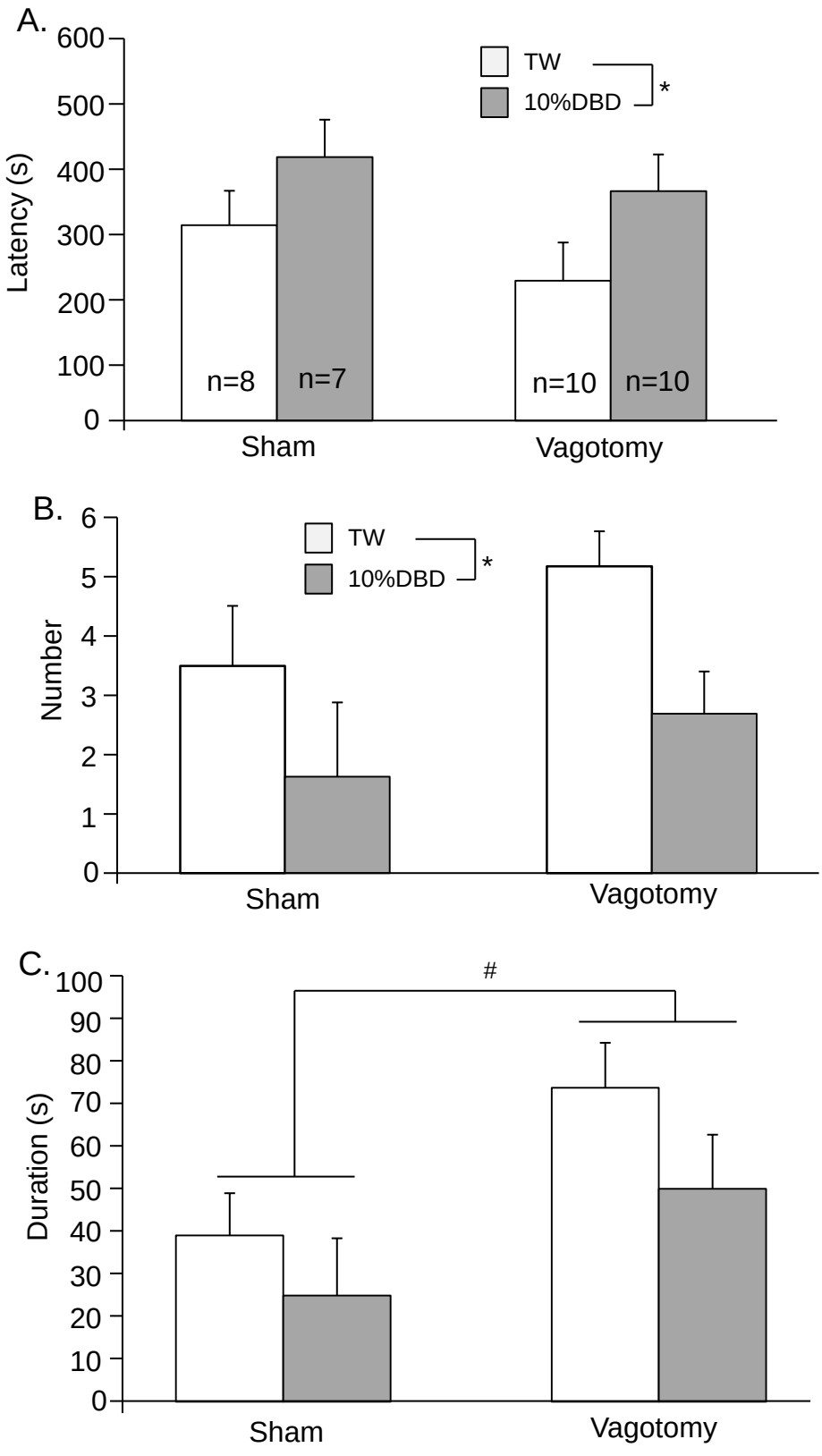
A.
600
500
400
300
200
100
0
Latency (s)
TW
10%DBD
*
n=8
n=7
n=10
n=10
Sham
Vagotomy
B.
6
5
4
3
2
1
0
Number
TW
10%DBD
*
Sham
Vagotomy
C.
100
90
80
70
60
50
40
30
20
10
0
Duration (s)
#
Sham
Vagotomy

Fig. 7

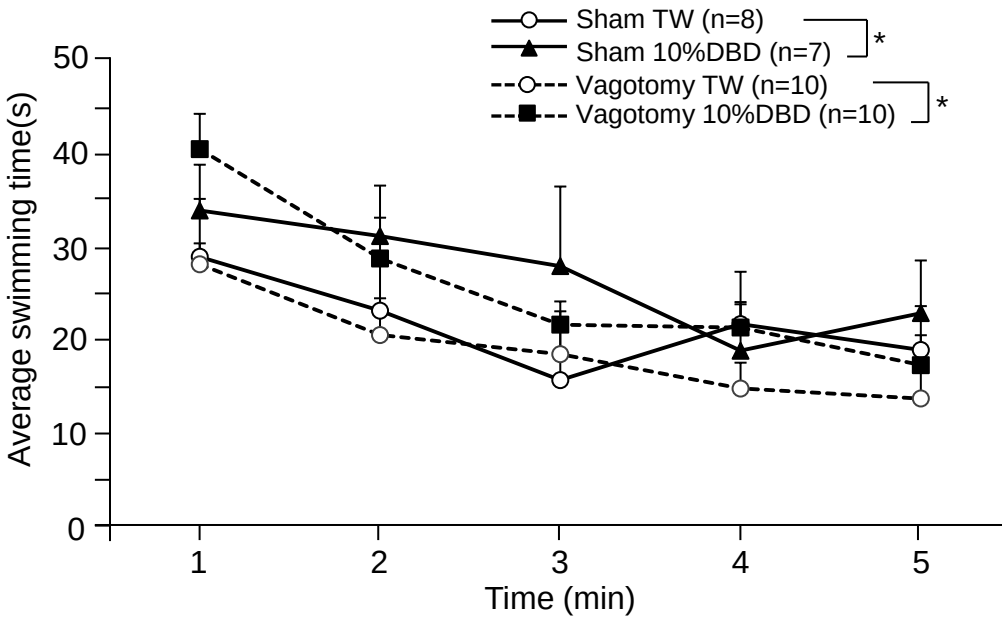
Sham TW (n=8)
Sham 10%DBD (n=7)
Vagotomy TW (n=10)
Vagotomy 10%DBD (n=10)
*
*
Average swimming time(s)
0
10
20
30
40
50
1
2
3
4
5
Time (min)

Fig. 8

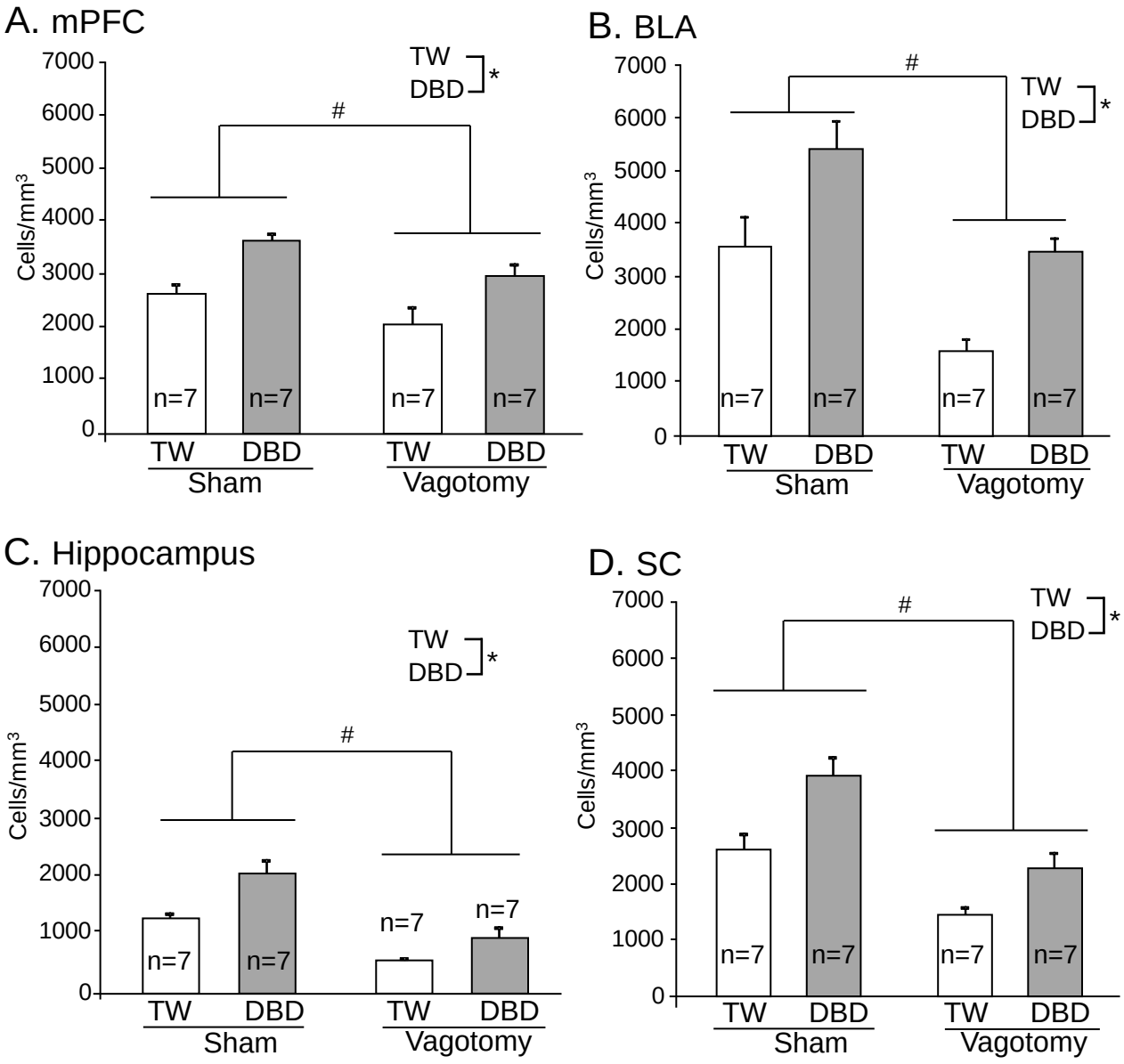
A. mPFC
B. BLA
C. Hippocampus
D. SC
Cells/mm3
7000
6000
5000
4000
3000
2000
1000
0
TW
DBD
*
#
n=7
TW
DBD
Sham
Vagotomy

Fig. 9

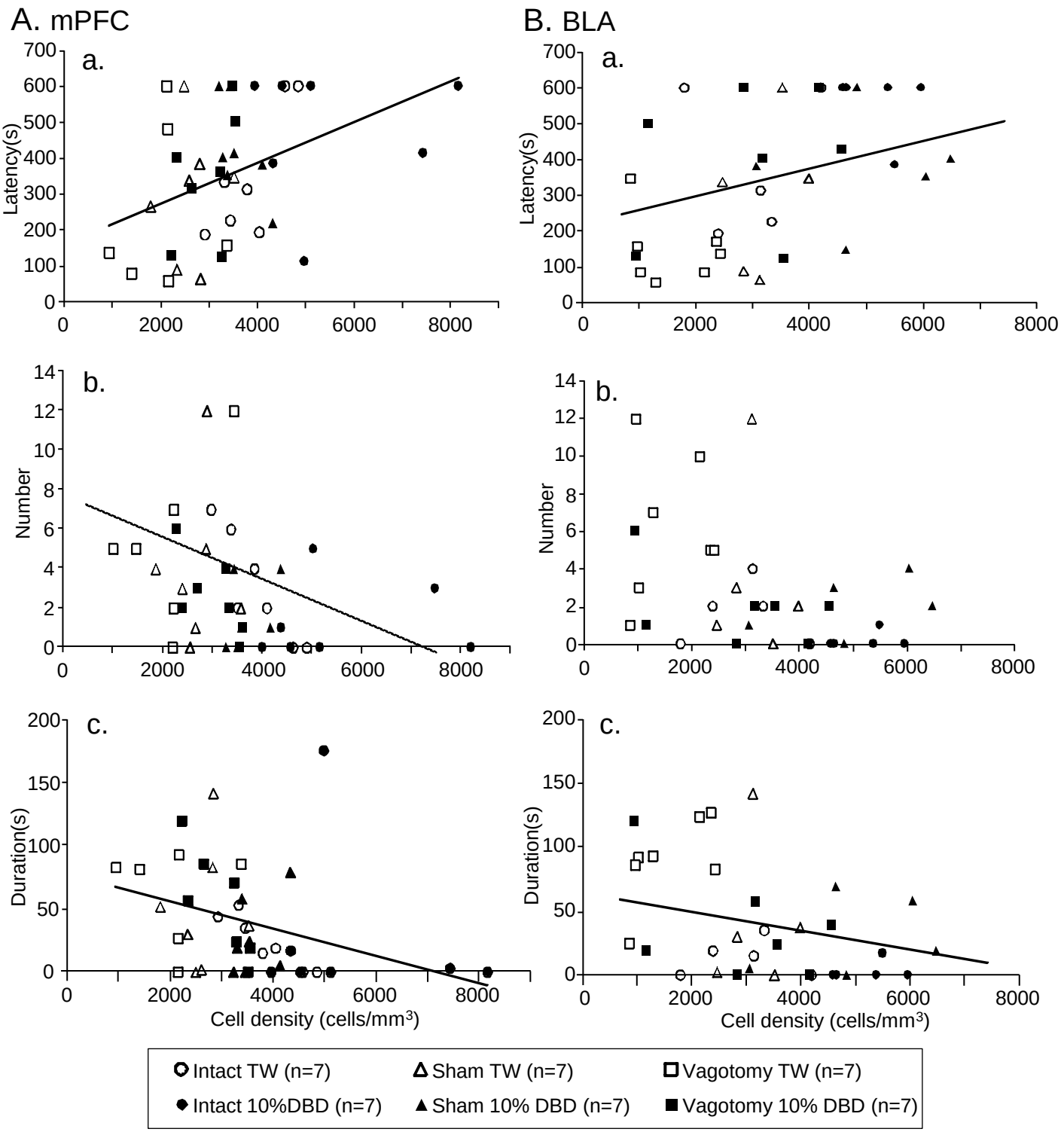
A. mPFC
B. BLA
a.
b.
c.
Latency(s)
Number
Duration(s)
Cell density (cells/mm3)
Intact TW (n=7)
Sham TW (n=7)
Vagotomy TW (n=7)
Intact 10%DBD (n=7)
Sham 10% DBD (n=7)
Vagotomy 10% DBD (n=7)

Fig. 10

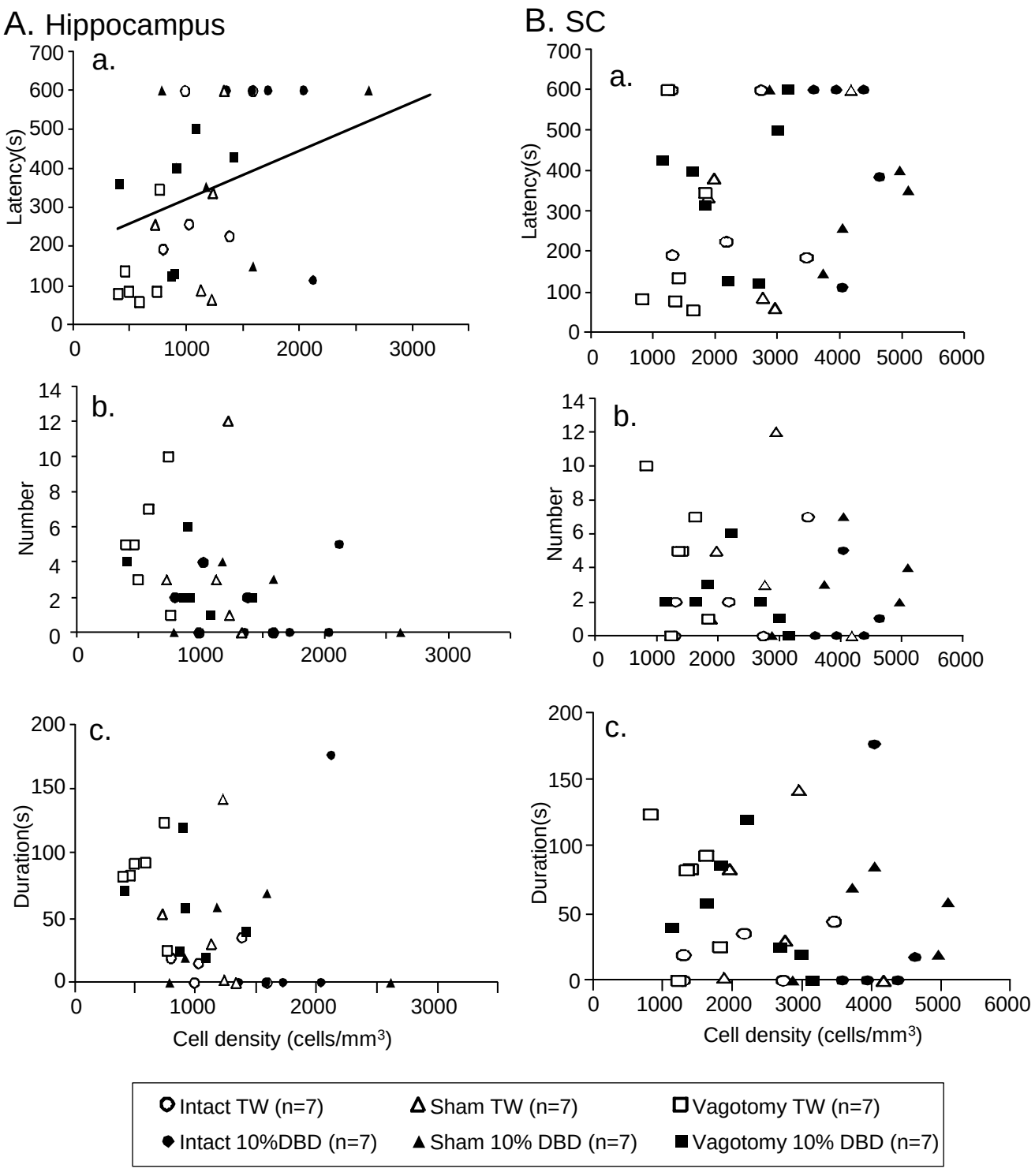
A. Hippocampus
B. SC
a.
b.
c.
Latency(s)
Number
Duration(s)
Cell density (cells/mm3)
Intact TW (n=7)
Sham TW (n=7)
Vagotomy TW (n=7)
Intact 10%DBD (n=7)
Sham 10% DBD (n=7)
Vagotomy 10% DBD (n=7)

Fig. 11

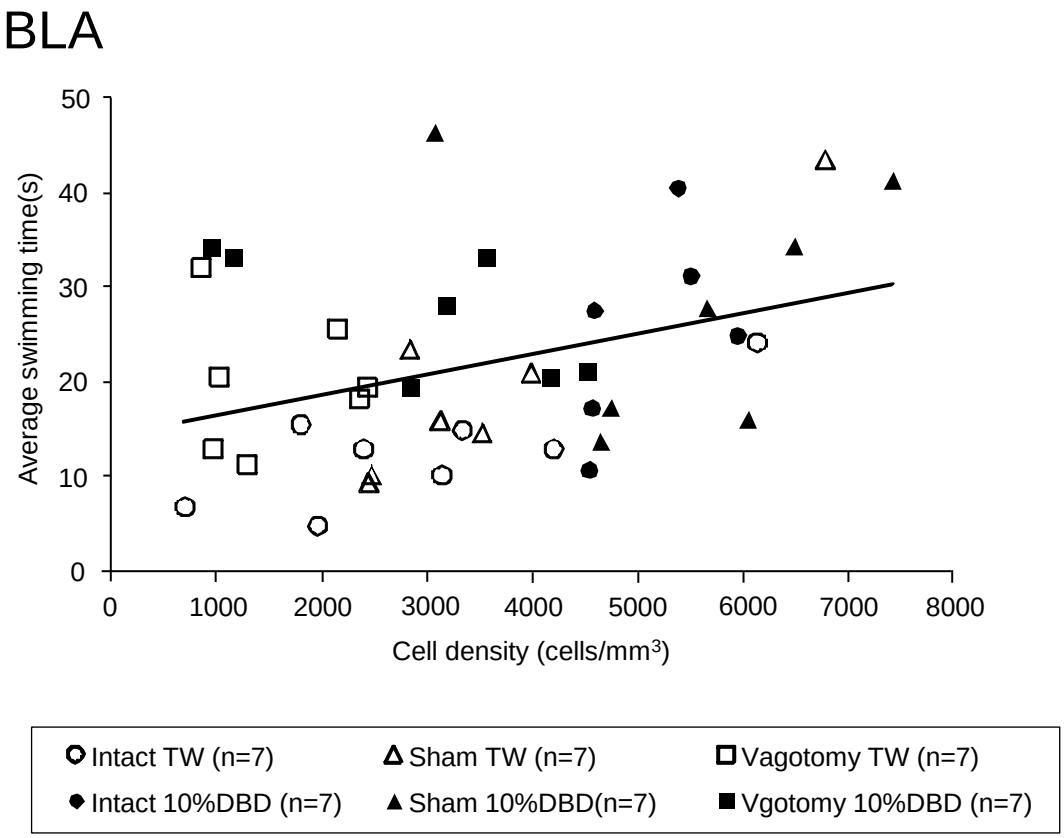
BLA
Average swimming time(s)
50
40
30
20
10
0
0
1000
2000
3000
4000
5000
6000
7000
8000
Cell density (cells/mm3)
Intact TW (n=7)
Sham TW (n=7)
Vagotomy TW (n=7)
Intact 10%DBD (n=7)
Sham 10%DBD(n=7)
Vgotomy 10%DBD (n=7)

Fig.12