Left-right functional lateralization in the rat dorsal and ventral hippocampus

DOCTORAL DISSERTATION

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Abstract

The human brain has two hemispheres, and the left-right functional lateralization is widely known. However, this feature is not specific to humans, and many behavioral studies have suggested the existence of left-right hemispheric differences in various animal species. In addition, several previous studies have suggested anatomical and functional hemispheric differences in the rodent hippocampus. In the present study, I researched the existence of functional left-right differences in the rat dorsal and ventral hippocampus. The experimental results showed that the right, but not the left, ventral hippocampus contributes predominantly to coping with anxiety-like behaviors; however, the left and right ventral hippocampi may work together in some cases in a complementary manner. This is the first study to reveal lateralization in the ventral hippocampus. In addition, the right, but not the left, dorsal hippocampus contributes predominantly to short-term memory, while the left, but not the right, dorsal hippocampus contributed to long-term memory. The experimental results also suggest the effect of interhemispheric interaction on memory formation. This is the first study to report the possibility of hemispheric interactions in DH. These results indicate the existence of a left-right functional difference in the rat hippocampus, suggesting that rats use both the left and right hemispheres appropriately in response to their surrounding environment. These evidences may contribute to the understanding of the significance of the left-right functional lateralization.

Acknowledgements

I would like to thank my supervisor, Dr. Yoshio Sakurai for his continuous guidance, scientific advice, valuable suggestions, and mental care for continuing research during last 5 years. He always respected my opinions and my research interests and allowed me to pursue my research in any way I wanted. His student-centered approach to teaching allowed me to carry out my "original" research. Without his consistent and helpful instruction, I believe I could not have achieved this research. Second, I would like to express my heartfelt gratitude to Dr. Hiroyuki Manabe and Dr. Junya Hirokawa for their helpful support. I got important suggestions and experimental techniques from them to carry out my research. Finally, I would like to express my greatest thanks to my family, friends and others who have always encouraged me.

Table of contents

List of Figures and Tables

Chapter 1. General introduction

1.1. Hemispheric lateralization in humans

The human brain has two hemispheres, and it is widely known that there are many functional differences between these two hemispheres ("Hemisphere Function in the Human Brain By S. J. Dimond and J. G. Beaumont (Pp. 398; £7.50.) Elek Science: London. 1974.," 1975). For example, the most well-known functional lateralization would be language. Paul Broca identified a specific brain region in the left hemisphere related to language production (Broca, 1861) and Carl Wernicke identified another region in the left hemisphere related to language comprehension (Wernicke, 1874). Other brain functions with left-right differences are known to include right hemisphere-dominated spatial processing (Heilman et al., 2000; Roth & Hellige, 1998) and right hemisphere-dominated face recognition (Rhodes, 1985), and emotional processing that differs between the left and right hemispheres (Alves et al., 2008; Demaree et al., 2005).

As these reports show, there is a great deal of evidence for functional differences in the human brain, but the significance and origins of this phenomenon remain unclear. Why are our brains divided into two parts and why do the two brains have separated functions? To approach this understanding, more micro-level neuroscientific studies in the animal brain are needed.

1.2. Behavioral lateralization in animals

As explained in the previous section, there are many functional differences in the human brain. However, this feature is not specific to humans, and many behavioral studies have suggested the existence of left-right hemispheric differences in various animal species (Frasnelli, 2013; Frasnelli et al., 2012; Güntürkün et al., 2020; Güntürkün & Ocklenburg, 2017; Halpern et al., 2005; Hamada, 2020; Ocklenburg et al., 2016) (Fig. 1).

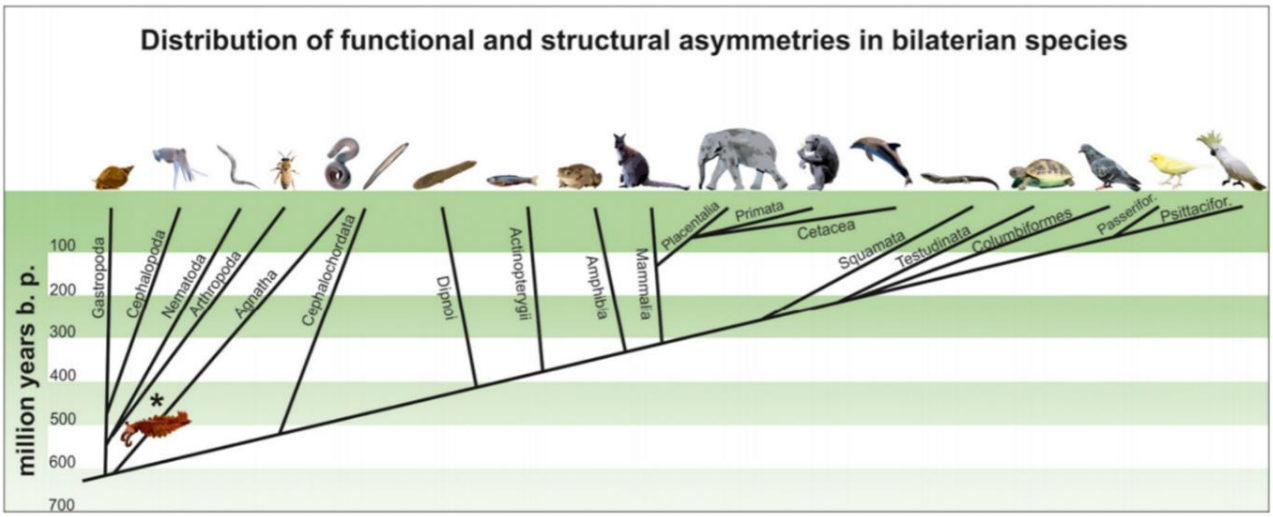

Figure 1. The phylogeny of lateralization.
Lateralized species that represent major taxa of the animal phylum. A wide range of animal species, including invertebrates, as well as fish, amphibians, birds, and mammals, have been found to exhibit behaviors that involve left-right differences. The image was modified from (Güntürkün et al., 2020).

For example, lateralized behaviors during aversive situations have been repeatedly reported, toads exhibit faster avoidance responses at the presentation of a snake model in the left visual field than in the right visual field (Lippolis et al., 2002) (Fig. 2).

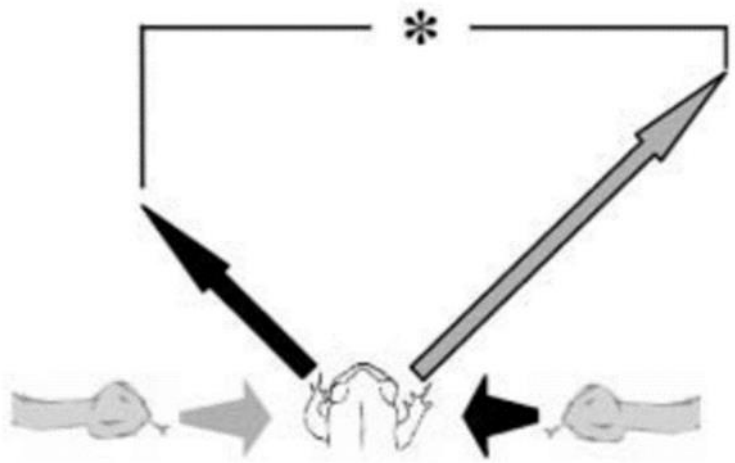

Figure 2. Lateralized predator-escape response in toads.
Occurrence of leaping behavior in response to a predator (a snake head) introduced from the left or right lateral visual fields. Lippolis et al. demonstrated that the left hemifield was more reactive than the right one to the predator stimulus. (Lippolis et al., 2002). The image was modified from (Robins, 2005).

Because information from the left hemifield is sent to the right brain hemisphere through the optic chiasm, these results suggest dominance of the right hemisphere in controlling the fight-or-flight (anti-predator) responses. This left eye/right hemisphere preference during aversive situations has been reported in many animals, such as lizards (Deckel, 1995), chicks (Rogers et al., 2004), teleost fishes (Sovrano et al., 1999), dunnarts (Lippolis et al., 2005), dogs (Siniscalchi et al., 2010), cattle (Robins & Phillips, 2010), horses (Austin & Rogers, 2007), and baboons (Casperd & Dunbar, 1996). Such evidence in many animal studies indicates the possibility that the existence of right hemispheric dominance in emotional responses is common to almost all animals that have brain hemispheres.

These evidences strongly suggest that animals also have left-right differences, at least in behavioral levels. However, little is known about the brain regions and the functions in which the

left-right differences between the hemispheres of the animal brain are found. Therefore, neuroscientific experiments with the animal brain are needed to approach this issue.

1.3. Hemispheric lateralization in rodents

There is little evidence of left-right hemispheric differences reported in rodents. Among the several reported studies, the most notable are the findings on the functional left-right differences related to fear, anxiety, and stress and on the anatomical and functional left-right differences in the hippocampus.

As an example of the former; several studies have reported that some brain regions related to fear/anxiety and stress responses have functional lateralization. Amygdala (AMG) and medial prefrontal cortex (mPFC) are well-known structures associated with fear/anxiety and stress responses. Coleman-Mesches and Mcgaugh have reported that the inactivation of the right AMG with muscimol decreases inhibitory avoidance learning (Coleman-Mesches & McGaugh, 1995a). Ji and Neugebauer have revealed that the right AMG (CeLC) is more preferentially involved in the process of the pain sensation (Ji & Neugebauer, 2009). Sullivan and Gratton have shown that the ibotenic lesions of the right, but not the left, mPFC lead to lower plasma corticosterone levels and smaller ulcers after chronic restraint stress in rats (Sullivan & Gratton, 1999). Hamani et al. have reported that high-frequency deep brain stimulation to not the right but the left mPFC decreased immobility time in the forced swim test, a behavioral test to assess depression-like behavior (Hamani et al., 2010). These findings strongly indicate the existence of left–right functional lateralization in some brain structures,

which lead to lateralized behaviors during aversive stimuli.

As an example of the latter; the left-right anatomical and functional differences have been reported in the rodent hippocampus. In particular, Shinohara et al. have revealed that mice hippocampal CA1 pyramidal cell synapses differ in size, shape, and glutamate receptor expression depending on the laterality of presynaptic origin (Shinohara et al., 2008) (Fig. 3).

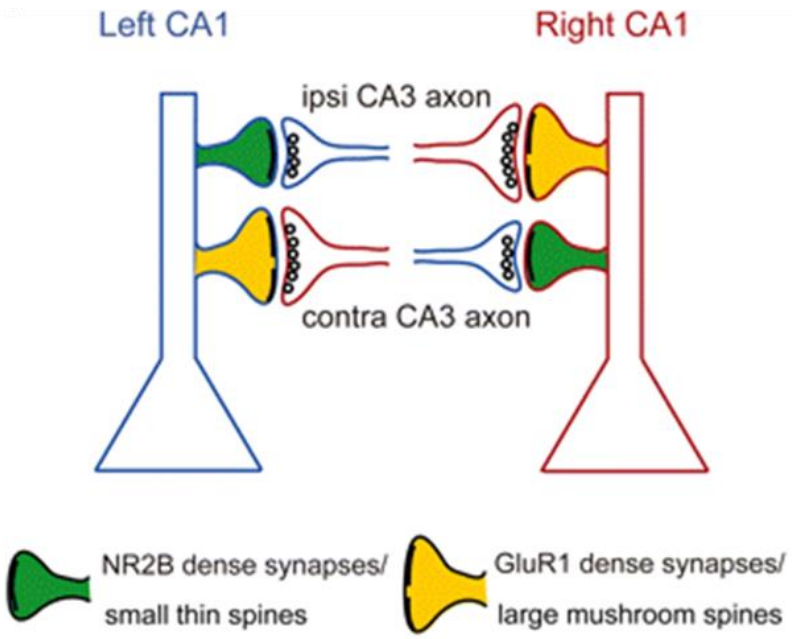

Figure 3. Left-right difference of hippocampal synapses.
Left-right difference of mice CA1 pyramidal cell synapses. Spines that connect to small synapses with axon terminals from the left CA3 have NR2B dense. On the other hand, spines that connect to large synapses with axon terminals from the right CA3 have GluR1 dense and large synapses. The image was modified from (Shinohara et al., 2008).

Kohl et al. have found that left CA3 input produced more long-term potentiation at CA1 synapses than right CA3 input as a result of differential expression of GluN2B subunit–containing NMDA receptors (Kohl et al., 2011). The findings of the study by Shipton et al. were highly suggestive,

demonstrating that the optogenetic silencing of the left CA3 alone impaired long-term memory (LTM) performance in the reward exploration task, whereas the unilateral silencing of either the left or right CA3 caused short-term memory (STM) deficits in the spontaneous alternation task and the spatial novelty preference task (Shipton et al., 2014).

These evidences strongly suggest that rodents have left-right hemispheric differences. However, it is still unclear what functional differences there are in the anxiety and memory functions controlled by the hippocampus. In particular, the left-right differences reported in brain regions other than the hippocampus are all reports about right-dominant functions, and examining the hippocampus, which has been suggested to have different advantages in both left and right hemispheres, is very useful for clarifying the significance of the functional dissociation between the left and right hemispheres and interhemispheric interactions. Therefore, I designed some experiments based on my own ideas to approach these unanswered questions and obtained two results presented in the following chapters.

Chapter 2. Hemispheric lateralization in the rat ventral hippocampus

2.1. Introduction

Functional lateralization between left and right human brain hemisphere is well-known. However, brain lateralization is not human-specific. There are many species that perform some actions asymmetrically (Halpern et al., 2005; Frasnelli et al., 2012; Frasnelli, 2013; Ocklenburg et al., 2016; Güntürkün and Ocklenburg, 2017; Güntürkün et al., 2020; Hamada, 2020), and one of the most well-studied phenomenon is lateralized behaviors during aversive situations. As I introduced in chapter 1, the left eye/right hemisphere preference during aversive situations has been reported in many animal (Austin & Rogers, 2007; Deckel, 1995; Lippolis et al., 2005; McKenzie et al., 1998; Robins & Phillips, 2010; Siniscalchi et al., 2010; Sovrano et al., 1999). Such evidence in many animal studies indicates the possibility that the existence of the right hemispheric dominance in emotional responses is common to most animals that have two hemispheres. However, which brain structure in each hemisphere underlies functional lateralization remains unclear.

Looking at the findings from behavioral neuroscience, several studies have reported that some brain structures related to fear/anxiety and stress responses have functional lateralization. As I described in Chapter 1, functional left-right differences have been identified in several brain regions that control fear/anxiety and stress, for example, AMY and mPFC.

On a different note, it is widely known that the hippocampus in rodents can be divided anatomically and functionally into two regions, dorsal hippocampus (DH) and ventral hippocampus (VH) (David M. Bannerman et al., 2014; Fanselow & Dong, 2010; Harland et al., 2018; Lee et al., 2019; Trompoukis & Papatheodoropoulos, 2020) (Fig. 4).

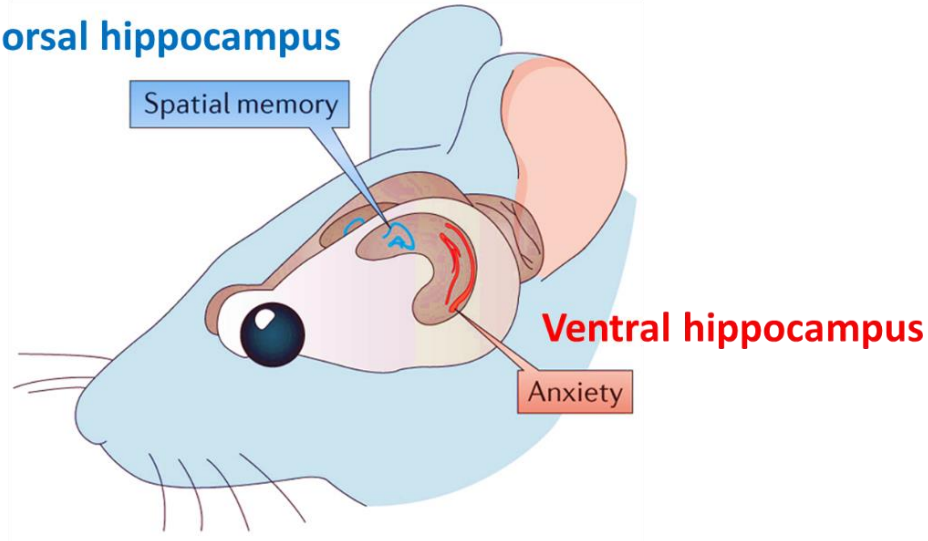

Figure 4. Distinct contributions of the dorsal and ventral hippocampus to behavior. Rodent hippocampus is dissociated into two regions. The dorsal hippocampus (posterior hippocampus in primates) controls the spatial memory functions, whereas the ventral hippocampus (anterior hippocampus in primates) controls the anxiolytic functions. The image was modified from (David M. Bannerman et al., 2014).

Many previous researches have demonstrated that VH is involved in the same functions as AMG and mPFC, particularly anxiety-like behavior (Kjelstrup et al., 2002; Roohbakhsh et al., 2009), fear conditioning (D.M Bannerman et al., 2003; Pentkowski et al., 2006), and autonomic responses (Scopinho et al., 2013). In fact, these three regions (AMY, mPFC, and VH) share anatomical and functional connectivity (Hoover & Vertes, 2007; Lesting et al., 2011, 2013; Padilla-Coreano et al.,

2016; Pikkarainen et al., 1999). These findings may suggest that there is a common left-right difference in these three regions. On the other hand, DH is involved in learning and memory (Maras et al., 2014; Reichel et al., 2017) and has been shown to have left–right hemispheric differences in memory processing (Kohl et al., 2011; Shipton et al., 2014) and spatial learning (Klur et al., 2009; Shinohara et al., 2012). The left and right DH have different numbers of cells (Lister et al., 2006), types of genes (Klur et al., 2009; Moskal et al., 2006), proteins (Samara et al., 2011), types and densities of synaptic receptors (Kawahara et al., 2013; Shinohara, 2009), and they also produce different gamma power after stress exposure in isolation rearing (Benito et al., 2016; Shinohara et al., 2013).

These findings clearly imply the functional lateralization in rodent DH. However, there has been no study regarding the functional lateralization in rodent VH. Thus, I investigated whether VH exhibits functional lateralization during aversive situations. In several previous studies, the elevated plus maze (EPM) (Kjelstrup et al., 2002), successive alleys test (SAT) (McHugh et al., 2004), and light–dark box test (D.M Bannerman et al., 2003) were used to measure anxiety-like behaviors of the VH-injured rodents. In the present study, I used the SAT, a modified version of the EPM, that was developed by Deacon (Deacon, 2013). In this test, the width of successive alleys is gradually narrowed, and the anxiety levels of the animal gradually change from the first wide alley to the last narrow one. I used structural lesion to investigate the functional lateralization of VH during different anxiety levels in rats. Risk assessment behavior has been related to anxiety and VH function in laboratory animals, and the detailed neural mechanisms of VH for such behavior remain to be

revealed. The present study could be one of the first steps to substantiate the occurrence of VH functional lateralization in anxiety-like behavior in animals. This study is written based on (Sakaguchi & Sakurai, 2017).

2.2. Material and methods

2.2.1. Animals

Experimental subjects were male Wistar albino rats (n = 48, Shimizu Laboratory Supplies, Kyoto, Japan) that were aged 8-9 weeks old and weighed 210–250 g at the time of the surgery. The rats were individually housed in cages with free access to food and water under a light–dark cycle, with the light period between 08:00 and 21:00 h. Behaviors were tested between 10:00 and 12:00 h. All experiments were performed in accordance with the Guidelines for Animal Experiments at Doshisha University and with the approval of the Animal Research Committee of Doshisha University.

2.2.2. Surgery

One week before the experiment, the rats were anesthetized with sodium pentobarbital (40 mg/kg, i.p.). Lesions were made by passing anodal direct current (2 mA, 30s) using the Lesion Making Device (53500, UGO BASILE SRL, Gemonio, VA, Italy) and a stainless bipolar electrode (150 μm, UB-9007, UNIQUE MEDICAL Co., LTD., Tokyo, Japan). The electrode was inserted into

bilateral, right, or left VH ((1) AP, −4.5 mm from bregma; ML, ±5.0 mm from bregma; DV, −6.0 mm from dura; (2) AP, −5.5 mm; ML, ±5.2 mm; and DV, −6.5 mm). For sham lesions, the electrode was lowered to the same coordinates, but no current was passed. All groups consisted of 12 rats. All rats were allowed to recover for 7 days and were handled for 5 min each day.

2.2.3. Apparatus

The experimental apparatus (Fig. 5) was the successive alleys, as devised by Deacon (Deacon, 2013), and I followed its experimental procedure. In brief, the apparatus was composed of four 30-cm-long alleys. The widths and side walls of the alleys got gradually narrower and lower as the number of alleys increases (Alley 1, 9-cm width/30-cm height; Alley 2, 9-cm width/2.5-cm height; Alley 3, 6.7-cm width/0.5-cm height; and Alley 4, 3.5-cm width/0.3-cm height). Alleys 1–4 were painted black, gray, white, and white, respectively. The alley surface was placed 50 cm above the floor under 200 lx illumination. Behaviors were recorded using a camera (BSW32KM03SV, BUFFALO INC., Aichi, Japan) that was mounted directly above the apparatus.

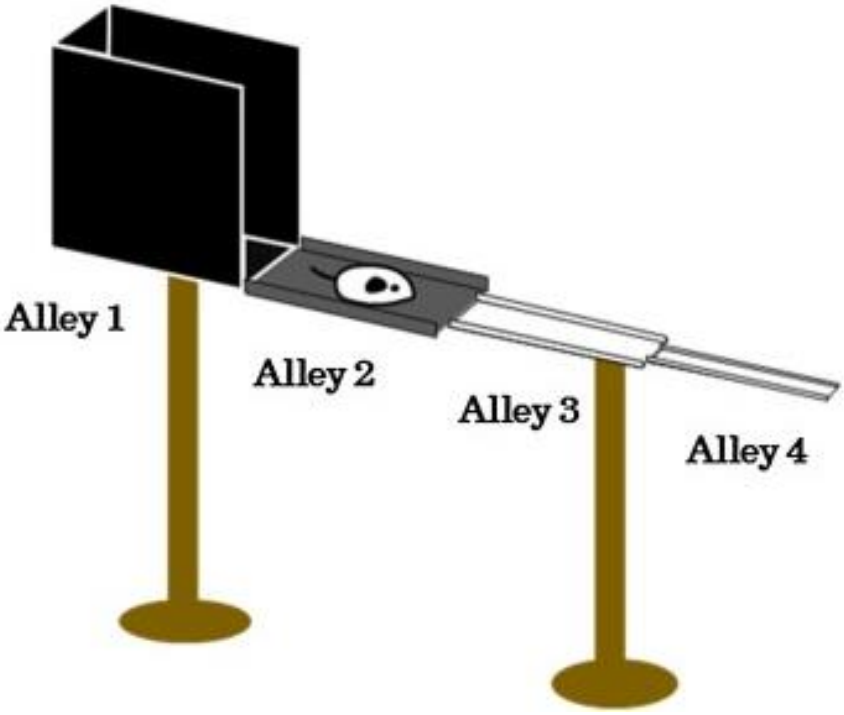

Figure 5. Schematic image of the Successive alleys test.
Each alley is labeled Alley 1, 2, 3, and 4. The animals were allowed to explore the apparatus for 10 min per day.

2.2.4. Successive alleys test

First, the rats were placed in Alley 2 with faced the direction of Alleys 3 and 4. The animals were then allowed to explore the apparatus for 10 min. A trial consisting of this procedure was performed once a day for 7 days (Days 1–7) continuously. After each trial, the surfaces of all alleys were cleaned with a towel containing 70% ethanol. From the recorded videos of the animals, the time spent in each alley and the number of entries into each alley were calculated by using a system for automated analysis (ANY-maze software, Stoelting Co., IL, USA). An entry was scored if the animals moved into the next alley with 80% or more of their bodies (this criterion was considered to be comparable to the invasion of all four of the animal's paws in this software). The ratio of Alley

4/Alley 3 entries (number of entries into Alley 4 compared with those into Alley 3) was an index of how often the rats entered Alley 4 after entering Alley 3. A value of 0 would mean that the rats never entered Alley 4, and a value of 0.5 would mean that the rats always entered Alley 4 (if the rats always entered Alley 4 through Alley 3, the ratio of Alley 3/Alley 4 entries would be 2:1). Theoretically, a value of 1 could be expected (no movement anywhere after entering Alley 4 for the first time during the test), but in this experiment, there were no individuals that never moved from Alley 4, so the value was always less than 0.5.

2.2.5. Histology

On Day 8, the rats in all groups were deeply anesthetized with an overdose of sodium pentobarbital (220 mg/kg) and were perfused with 0.01 M PBS and 4% paraformaldehyde (PFA). The brains were then removed and stored in PFA overnight, before transferring them to 30% sucrose PBS. I obtained coronal brain sections (50 μm) using a cryostat and mounted them on slides. Cresyl violet solution was used to detect the lesion area. Brain regions were identified according to the Rat Brain Atlas (Paxinos & Watson, 2007). The lesion sizes were calculated using a software program (ImageJ software, National Institutes of Health, MD, USA).

2.2.6. Data analysis

Experimental data are shown as the means ± SEM. Two-way analysis of variance (ANOVA) with Condition (Sham lesion, Bilateral lesion, Right lesion, Left lesion) as the between-subject factor

and Day as the within-subject factor, followed by post-hoc Tukey–Kramer method was used to analyze these results; the time spent in each alley and the number of entries into each alley on successive 6 days (Days 2–7) and the results of the sum of entries and the ratio of Alley 4/Alley 3 entries during all 7 days (Days 1–7) among the Sham, Bilateral, Right, and Left lesion groups. One-way ANOVA followed by post-hoc Dunnett method was used to analyze the time spent in each alley and the number of entries into each alley on Day 1. The Student's t-test was used to analyze the sum of entries on Day 1 vs. on Day 7. The regression based TOST equivalence test (Package 'equivalence' of the R software (https://cran.r-project.org/)) was used to analyze the equivalence of the lesion extents.

2.3. Results

2.3.1. Histology

I observed that the stereotaxic passing of an anodal direct current destroyed most VH structures. Fig. 6A shows a raw sample of the electrical lesion, and Fig. 6B indicates the lesion areas of the Bilateral, Right, and Left lesion groups (n = 12 in each group). The extent of the lesion is shown with reference to the horizontal sections found in the Rat Brain Atlas (Paxinos & Watson, 2007). Minimum lesion areas (gray color) were observed in the ventral dentate gyrus, CA1, and CA3, but maximum lesion areas (black color) were not observed in the structures outside of the hippocampus. The lesions in the right and left hemispheres were highly symmetrical. Fig. 6C represents the lesion

sizes of individual rats (n = 12) in each group (Bilateral lesion, Right lesion, and Left lesion groups). Fig. 6D shows the same data with means ± SEM. There was no significant difference among these four sites in all three sections (AP = −4.56, T = 0.68, P = 0.44; AP = −5.52, T = 0.41, P = 0.51, and AP = −6.32 mm, T = 0.32, P = 0.67). The Sham lesion group had little-to-no damage in these areas.

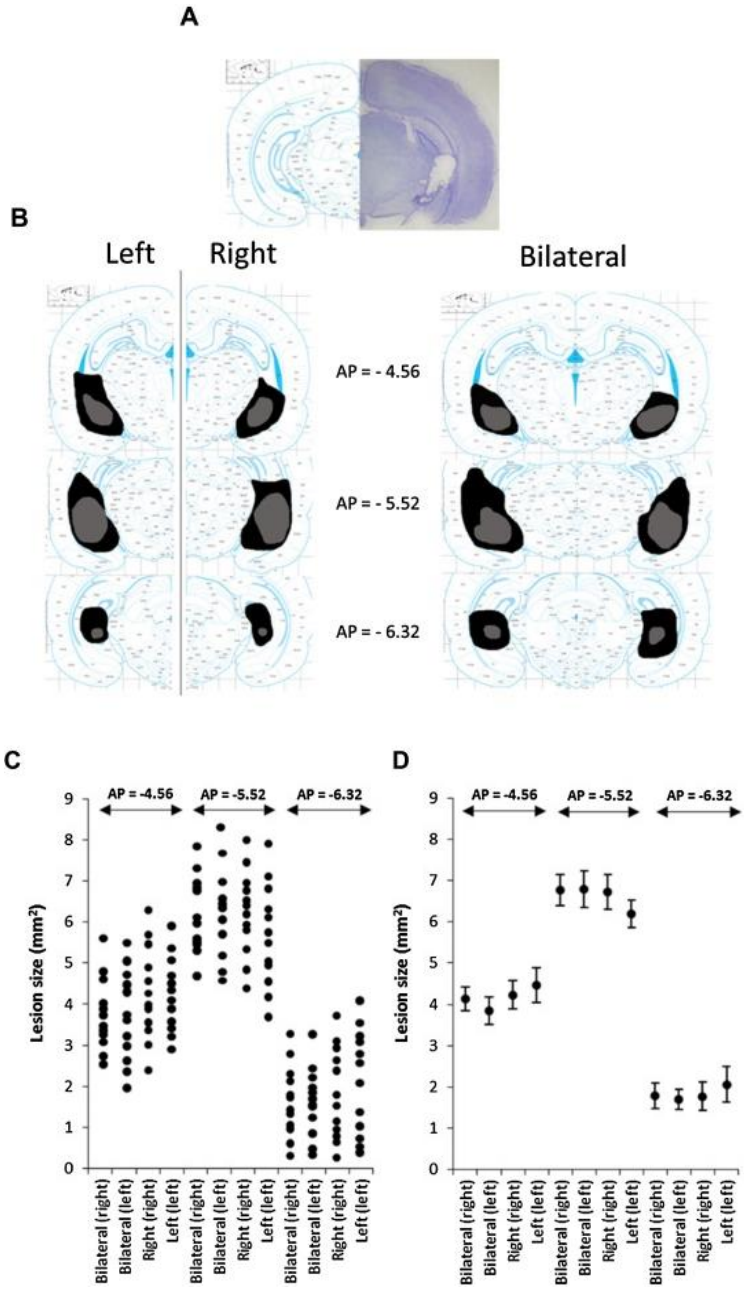

Figure 6. Locations of the lesioned areas.

(A) A raw sample of an electrical lesion of the ventral hippocampus. This section was stained with cresyl violet to identify brain regions more clearly. The brain maps (AP = −4.56, −5.52, and −6.32 mm) derived from Paxinos and Watson (Paxinos & Watson, 2007) represent (B) left, right, and bilateral lesion areas. The gray area indicates the minimum extent of tissue damage and the black area indicates the maximum. (C) Distribution of the lesioned areas of all rats for Bilateral lesion (right hemisphere), Bilateral lesion (left hemisphere), Right lesion (right hemisphere), and Left lesion (left hemisphere) groups in AP = −4.56, −5.52 and −6.32 mm. (D) Means ± SEM of the lesioned areas of each group. No significant differences were detected among the groups.

2.3.2. Behavioral test

SAT was used to measure anxiety-like behavior that depend on the anxiety levels. The apparatus is shown in Fig. 5. No rats fell from the apparatus during the experiments. The results of the two-way ANOVA are summarized in Table 1 and 2. On Day 1, the one-way ANOVA showed a significant effect for the time spent in Alleys 1, 2, and 4 ($F(3, 44) = 9.00$, $P < 0.001$; $F(3, 44) = 6.89$, $P < 0.001$; $F(3, 44) = 3.87$, $P = 0.015$, respectively) (Fig. 7A). The post-hoc comparisons revealed that the Bilateral lesion group spent significantly less time in Alley 1 ($P < 0.001$) and long times in Alleys 2 ($P = 0.0012$) and 4 ($P = 0.022$) compared with the Sham lesion group. On Days 2-7, the two-way ANOVA showed a significant effect of the factor "Condition" for the time spent in Alleys 1, 2, 3, and 4 ($F(3, 264) = 143.13$, $P < 0.001$; $F(3, 264) = 41.15$, $P < 0.001$; $F(3, 264) = 25.01$, $P < 0.001$; $F(3, 264) = 101.69$, $P < 0.001$, respectively). The effect of factor "Day" and the interaction between factors were not observed (see Table 1). The post-hoc comparisons revealed the following differences on the factor "Condition", which are listed in increasing amount of the time spent: Bilateral group (B) < Right lesion group (R) < Left lesion group (L) < Sham lesion group (S) in Alley 1 (all, $P < 0.001$); S < L < R = B in Alley 2 (S-L, $P = 0.013$; S-R, $P < 0.001$; S-B, $P < 0.001$; L-R, $P < 0.001$; L-B, $P < 0.001$; R-B, $P = 0.52$); S < L < R = B in Alley 3 (S-L, $P = 0.0015$; S-R, $P < 0.001$; S-B, $P < 0.001$; L-R, $P < 0.001$; L-B, $P < 0.001$; R-B, $P = 0.59$) ; and S = L < R < B in Alley 4 (S-L, $P = 0.13$; S-R, $P < 0.001$; S-B, $P < 0.001$; L-R, $P < 0.001$; L-B, $P < 0.001$; R-B, $P < 0.001$) ("<" indicates a significance and " = " indicates no significance) (Fig. 7B).

Table 1. Statistical results (P-value, * indicates $P < 0.05$; post-hoc test) of the time spent in each alley on Days 2–7.

	Factor	Alley 1	Alley 2	Alley 3	Alley 4
Two-way ANOVA	Condition	$P < 0.001$*	$P < 0.001$*	$P < 0.001$*	$P < 0.001$*
	Day	$P = 0.16$	$P = 0.35$	$P = 0.16$	$P = 0.29$
	Condition × Day	$P = 0.90$	$P = 0.96$	$P = 0.42$	$P = 0.42$
Post-hoc test (Condition)	Sham-Left	$P < 0.001$*	$P = 0.013$*	$P = 0.0015$*	$P = 0.13$
	Sham-Right	$P < 0.001$*	$P < 0.001$*	$P < 0.001$*	$P < 0.001$*
	Sham-Bilateral	$P < 0.001$*	$P < 0.001$*	$P < 0.001$*	$P < 0.001$*
	Left-Right	$P < 0.001$*	$P < 0.001$*	$P = 0.016$*	$P < 0.001$*
	Left-Bilateral	$P < 0.001$*	$P < 0.001$*	$P < 0.001$*	$P < 0.001$*
	Right-Bilateral	$P < 0.001$*	$P = 0.52$	$P = 0.59$	$P < 0.001$*

Table 2. Statistical results (P-value, * indicates $P < 0.05$; post-hoc test) of the number of entries in each alley on Days 2–7 and the ratio of Alley4/Alley 3 entries.

	Factor	Alley 1	Alley 2	Alley 3	Alley 4	Sum (Day1 vs Day7)	Alley 4/Alley 3
Two-way ANOVA	Condition	$P = 0.46$	$P < 0.001$*	$P < 0.001$*	$P < 0.001$*	$P < 0.001$*	$P < 0.001$*
	Day	$P = 0.015$*	$P = 0.0046$*	$P = 0.0077$*	$P = 0.015$*	$P = 0.0028$*	$P < 0.001$*
	Condition × Day	$P = 0.64$	$P = 0.92$	$P = 0.94$	$P = 0.84$	$P = 0.77$	$P < 0.001$*
Post-hoc test (Condition)	Sham-Left	$P = 0.87$	$P = 0.53$	$P = 0.87$	$P = 0.99$	—	$P = 0.77$
	Sham-Right	$P = 0.38$	$P < 0.001$*	$P < 0.001$*	$P < 0.001$*	—	$P < 0.001$*
	Sham-Bilateral	$P = 0.81$	$P < 0.001$*	$P < 0.001$*	$P < 0.001$*	—	$P = 0.015$*
	Left-Right	$P = 0.83$	$P = 0.013$	$P < 0.001$*	$P < 0.001$*	—	$P < 0.001$*
	Left-Bilateral	$P = 0.99$	$P < 0.001$*	$P < 0.001$*	$P < 0.001$*	—	$P < 0.001$*
	Right-Bilateral	$P = 0.90$	$P = 0.35$	$P < 0.001$*	$P < 0.001$*	—	$P = 0.19$

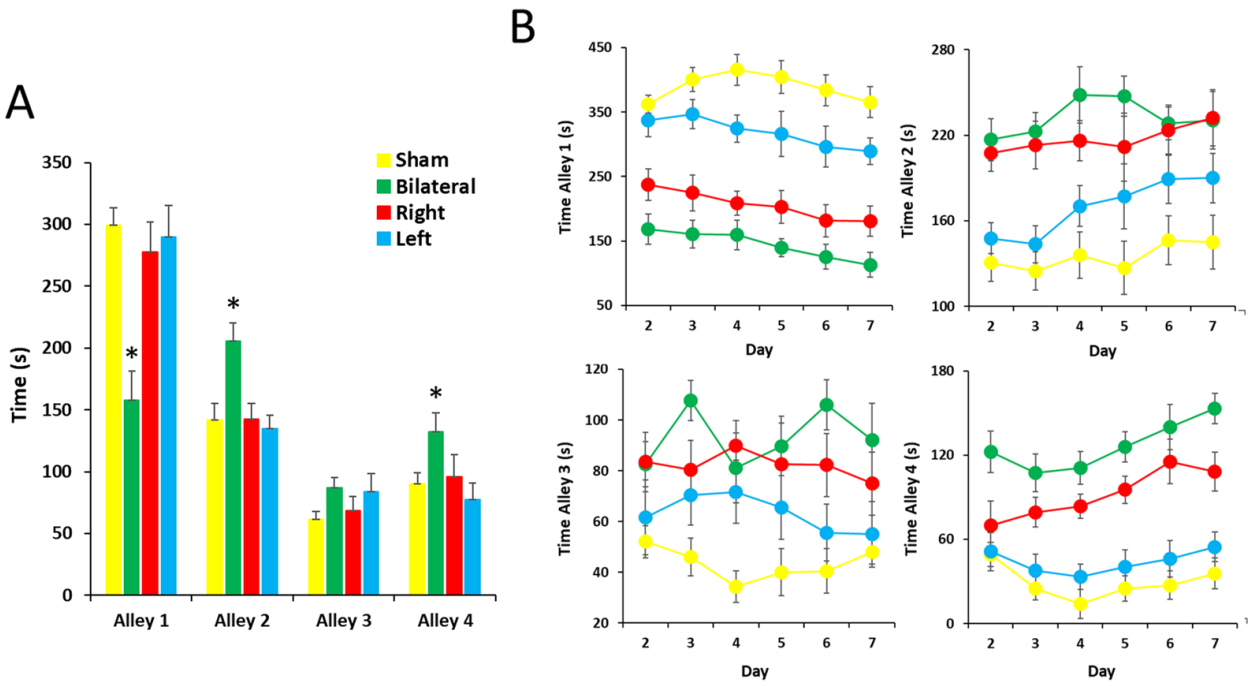

Figure 7. SAT results of the time spent in each alley.
All the rats in the Sham (Yellow), Bilateral (Green), Right (Red), and Left (Blue) lesion groups (n = 12 in each group) explored the alleys for 10 min. Time spent in each alley are shown for Day 1 (A) and for Days 2–7 (B). All data are shown as means ± SEM. *$P < 0.05$ compared with the Sham lesion group. "n.s." means non-significant.

Analysis of the number of entries on Day 1, the one-way ANOVA showed a significant effect in Alleys 3 and 4 ($F(3, 44) = 3.57$, $P = 0.021$; $F(3, 44) = 3.33$, $P = 0.28$, respectively) (Fig. 8A). The post-hoc comparisons revealed that the Bilateral lesion group had more entries into Alleys 3 ($P = 0.020$) and 4 ($P = 0.018$) compared with the Sham lesion group. On Days 2-7, the two-way ANOVA showed a significant effect of the factor "Condition" for the number of entries in Alleys 2, 3, and 4 ($F(3, 264) = 15.39$, $P < 0.001$; $F(3, 264) = 59.41$, $P < 0.001$; $F(3, 264) = 90.39$, $P < 0.001$, respectively). The effect of factor "Day" was also observed in Alleys 1, 2, 3, and 4 ($F(3, 264) = 2.89$, $P = 0.015$; $F(3, 264) = 3.48$, $P = 0.0046$; $F(3, 264) = 3.22$, $P = 0.0077$; $F(3, 264) = 2.87$, $P = 0.015$,

respectively). The effect of the interaction between factors was not observed (see Table 2). The post-hoc comparisons revealed the following differences on the factor “Condition”, which are listed in increasing amount of entries: S = L = R = B in Alley 1 (S-L, $P = 0.87$; S-R, $P = 0.38$; S-B, $P = 0.81$; L-R, $P = 0.83$; L-B, $P = 0.99$; R-B, $P = 0.90$); S = L < R = B in Alley 2 (S-L, $P = 0.53$; S-R, $P < 0.001$; S-B, $P < 0.001$; L-R, $P = 0.013$; L-B, $P < 0.001$; R-B, $P = 0.35$), S = L < R < B in Alley 3 (S-L, $P = 0.87$; S-R, $P < 0.001$; S-B, $P < 0.001$; L-R, $P < 0.001$; L-B, $P < 0.001$; R-B, $P < 0.001$), and S = L < R < B in Alley 4 (S-L, $P = 0.99$; S-R, $P < 0.001$; S-B, $P < 0.001$; L-R, $P < 0.001$; L-B, $P < 0.001$; R-B, $P < 0.0068$) (Fig. 8B). The two-way ANOVA showed a significant effect of the factors “Condition” and “Day” for the sum of entries to all four alleys (Condition, $F(3, 264) = 18.08$, $P < 0.001$; Day, $F(3, 264) = 5.07$, $P < 0.0028$) (Fig. 8C). The effect of the interaction between factors was not observed (see Table 2). The two-way ANOVA showed a significant effect of the factors “Condition”, “Day”, and “the interaction between factors” for the ratio of Alley 4/Alley 3 entries (Condition, $F(3, 264) = 29.84$, $P < 0.001$; Day, $F(3, 264) = 12.12$, $P < 0.001$; the interaction, $F(3, 264) = 4.13$, $P < 0.001$) (Fig. 8D). The post-hoc comparisons revealed the following differences on the factor “Condition”, which are listed in increasing amount of the ratio: S = L < R = B (S-L, $P = 0.77$; S-R, $P < 0.001$; S-B, $P = 0.015$; L-R, $P < 0.001$; L-B, $P < 0.001$; R-B, $P = 0.19$). The values of the Bilateral and Right lesion groups were approximately 0.5 on all 7 days, whereas those of the Sham and Left lesion groups decreased from approximately 0.45–0.3 in the first 3 days and then increased to approximately 0.4 in the last 4 days.

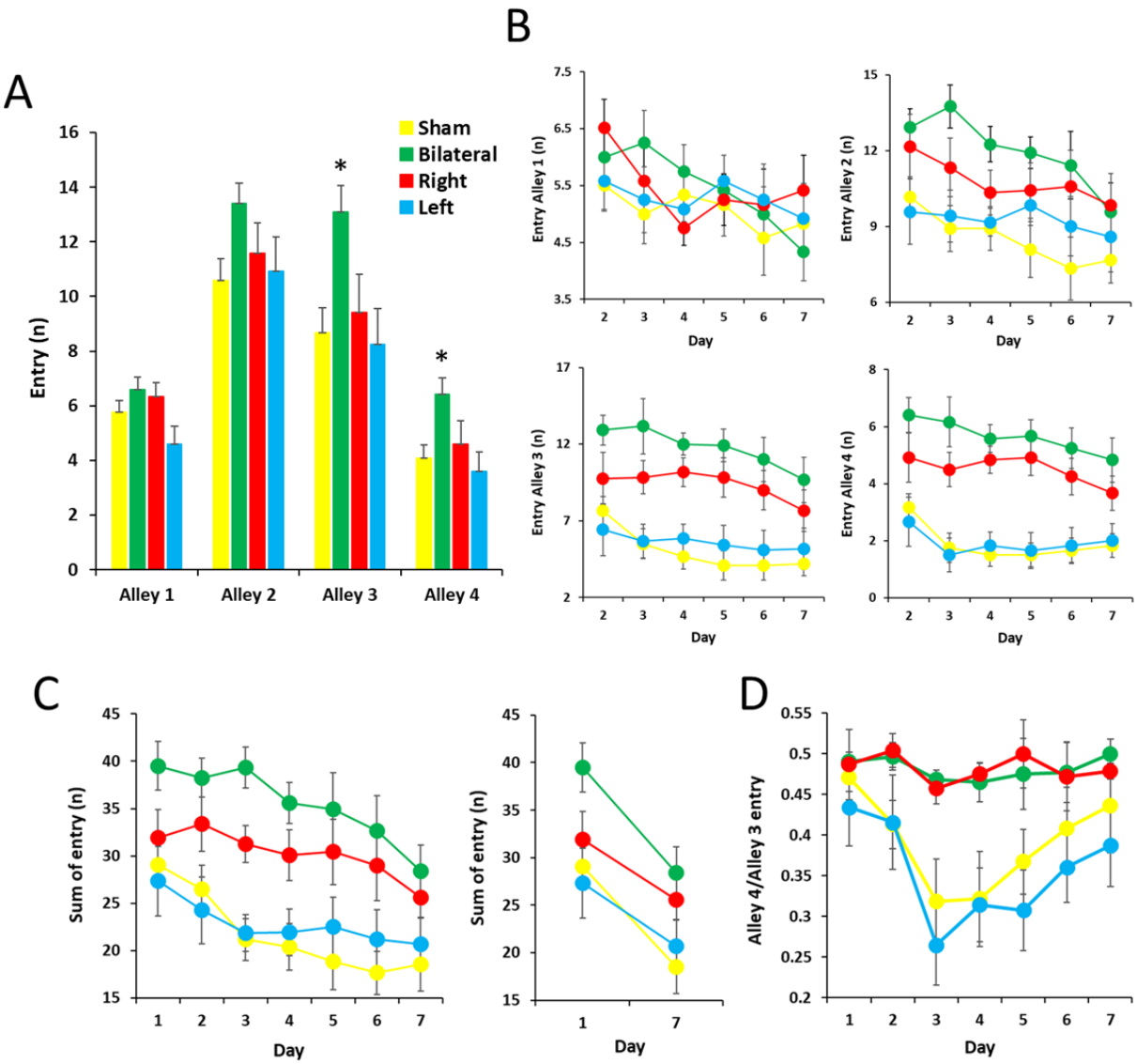

Figure 8. Results of the number of entries into each alley.

All the rats in the Sham (Yellow), Bilateral (Green), Right (Red), and Left (Blue) lesion groups (n = 12 in each group) explored the alleys for 10 min. The number of entries into each alley are shown for Day 1 (A), and Days 2–7 (B). **P* < 0.05 compared with the Sham lesion group. The sum of entries on all 7 days (C-left), the sum of entries on the first and last days (C-right) (**P* < 0.05 compared with Day 1), and ratio of Alley4/Alley 3 entries (D) are shown. All data are shown as means ± SEM. "n.s." means non-significant.

2.4. Discussion

This study aimed to assess whether the rat VH possesses functional lateralization in response to an aversive situation and to reveal, if any, what external factors make the lateralization exhibited.

From behavioral experiments, I found functional lateralization associated with the anxiety in the VH. Moreover, I revealed that the emergence of lateralization depended on the anxiety level. Therefore, I concluded that the rat VH has some lateralized functions that enable adaptive behavior according to different aversive situations.

2.4.1. Behavioral test

Functional dissociation along the hippocampal long-axis has already been described, and the rodent VH mainly regulates fear/anxiety and stress responses (Maras et al., 2014). It has been revealed that this region is functionally associated with AMG, mPFC, and hypothalamus (Fanselow & Dong, 2010). Therefore, I investigated how the left and right VH performed during anxiogenic situations by measuring the time spent in and the number of entries into each alley of the SAT for 7 days. On Day 1, the Bilateral lesion group spent less time in the Alley 1 and more time in the Alleys 2 and 4 than the Sham lesion group (Fig. 7A). The number of entries into the Alleys 3 and 4 were also more in the Bilateral lesion group than in the Sham lesion group (Fig. 8A). These results suggest that bilateral VH lesions lead to decreased anxiety-like behavior, in agreement with Mchugh et al. (McHugh et al., 2004). The results of that study (McHugh et al., 2004) differed slightly from our results, in that they observed a longer time spent only in the Alley 2. This might be explained by the differences in experimental conditions, such as the brightness of the light or the time of each trial. In contrast to the Bilateral lesion group, lesions in the left and right VH did not affect the anxiety-like behaviors, showing that there was no lateralization in these two groups. Other brain areas related to anxiety or

fear, such as AMG and mPFC, might work complementarily instead of the unilateral VH. In contrast, analysis of the time spent in each anxiogenic alley (Alleys 2, 3, and 4) from Days 2–7 resulted in a pattern (listed in increasing amount of time spent) of S < L < R = B in Alley 2, S < L < R = B in Alley 3, and S = L < R < B in Alley 4 (Fig. 7B). These results suggested that either the right VH was consistently dominated to the left one in mediating anxiety-like behavior or the left and right VH worked together against relatively weak anxiety (such as Alleys 2 and 3, with wider widths); the right VH exclusively worked against strong anxiety (such as Alley 4, with a narrower width). The fact that there was a significant difference between the Sham and Right lesion groups and no significant difference between the Sham and Left lesion groups may have confirmed these possibilities.

The number of entries into each anxiogenic alley resulted in a pattern (listed in increasing number of entries) of S = L < R = B in Alley 2 and S = L < R < B in Alleys 3 and 4 (Fig. 8B). This suggests that the right VH is more active on entry into anxiogenic areas. Moreover, the ratio of Alley 4/Alley 3 entries was also significantly different between the Right and Bilateral lesion groups and the Sham and Left lesion groups (Fig. 8D). The Right and Bilateral lesion groups had a consistent value of approximately 0.5 on all 7 trial days. In contrast, the Sham and Left lesion groups showed more widely variable values. On Days 1–3, the values gradually decreased from approximately 0.5–0.3, indicating that the animals in the Sham and Left lesion groups experienced stronger anxiety in Alley 4 during this period. In contrast, on Days 5–7, the values gradually increased to nearly 0.5, indicating that the animals in these two groups were habituated to Alley 4 during the latter periods of the test. These results suggested that the right VH worked more strongly to exhibit behaviors associated with

anxiety than the left VH, in agreement with other studies of lateralized behaviors (A Robins, G Lippolis, A Bisazza, G Vallortigara, 1998; Austin & Rogers, 2007; Deckel, 1995; Lippolis et al., 2002, 2005; McKenzie et al., 1998; Robins & Phillips, 2010; Siniscalchi et al., 2010; Sovrano et al., 1999) and brain functions (Coleman-Mesches & McGaugh, 1995a, 1995b; Ishikawa et al., 2015; Ji & Neugebauer, 2009; Kiyokawa et al., 2012; Mizoguchi et al., 2000; Sullivan & Gratton, 1999; Young & Williams, 2013). Although the interaction between factors was detected in this statistical treatment, it is considered to be the result of the influence of both of the factors, since significant differences were found in both the Condition and the Day. In addition, the total number of entries into the alleys decreased in all groups from Day 1–7 (Fig. 8C), indicating that both unilateral and bilateral VH lesions, gradually formed over this time period, had no influence on the spatial memory.

The above results suggest the existence of functional differences in the left and right VH, which affect anxiety-like behavior. Although the equivalence of the extent of damage in the left and right hemispheres is one of the most crucial factors in revealing brain functional lateralization, there was no significant difference between the left and right VH in the degree of the damage between all groups, as shown in Fig. 6C and 6D. Therefore, it could not be considered that the interhemispheric differences in the present study could be attributed to different degrees of damage between the hemispheres. However, as the lesion method in this study was electrical damage, the present research cannot exclude the possibility of functional compensation by other brain regions. Thus, further verification of the functional lateralization with reversible functional inhibition methods will be

necessary. Moreover, in vivo electrophysiological methods should also be used in future work to investigate left/right asymmetrical activation in greater detail.

2.4.2. General discussion

The left eye/right hemisphere preference in avoidance behaviors has been repeatedly reported in many animals. Thus, the dominance of the right brain is no doubt involved in adaptive behaviors to cope with aversive situations. Additional research has also suggested that AMG and mPFC also display functional lateralization associated with fear/anxiety, pain processing, and stress responses (Coleman-Mesches & McGaugh, 1995a; Ji & Neugebauer, 2009; Kiyokawa et al., 2012, 2016; Neveu & Moya, 1997; Sullivan & Gratton, 1999). Furthermore, in recent years, many studies have pointed out that VH plays an important role in these same functions (D.M Bannerman et al., 2003; Kjelstrup et al., 2002; Pentkowski et al., 2006; Roohbakhsh et al., 2009; Scopinho et al., 2013). In this study, I confirmed the relationship between the VH and anxiety-related functional lateralization. The VH projects to AMG (Pikkarainen et al., 1999), mPFC (Jin & Maren, 2015), and hypothalamus (Kishi et al., 2000), all of which are part of neural circuits that control emotion, indicating that all these regions may exhibit functional lateralization. In fact, the hippocampus, AMG, and mPFC have left/right differences in the amounts of neurotransmitters secreted in those areas after exposure to stressors and release of corticosterone (Neveu & Moya, 1997; Sullivan & Gratton, 1999), noradrenaline (Spasojevic et al., 2013), dopamine (Thiel & Schwarting, 2001), serotonin (Andersen & Teicher, 1999; Belcheva et al., 2007), and angiotensin (Tashev & Stefanova, 2015). A considerable advantage

of this functional lateralization in emotional circuits might be that the activation of a unilateral brain region allows the animal to more quickly perform some urgent behaviors (e.g., fight or flight response). In contrast, animals might be able to perform higher behaviors to cope with complex situations or solve difficult problems by working their two hemispheres interactively.

2.5. Conclusion

In this study, I investigated functional lateralization in the rat VH in response to aversive stimuli that cause different anxiety levels. The results of the present experiments for anxiety-like behavior revealed that (1) VH possessed a noticeable functional lateralization associated with behavior required in the SAT, (2) the right VH more dominantly worked at the Alley 4 (anxiogenic-like situation) than the left VH, and (3) the extent of the functional difference depended on the Alley area (considered as anxiety levels) in the SAT, with Alley 4 (expected for stronger anxiety) enhancing right-hemispheric dominance of VH and Alleys 2 and 3 (expected for weaker anxiety) making it less distinct. This is the first study to reveal functional left–right lateralization of VH and its dependence on the aversiveness of situations. These findings provide new insights on the functional lateralization and its interaction in the brain for adaptive behaviors.

Chapter 3. Hemispheric lateralization in the rat dorsal hippocampus

3.1. Introduction

As described in Chapter 1, some animal species show lateralized behavior (Halpern et al., 2005; Frasnelli et al., 2012; Frasnelli, 2013; Ocklenburg et al., 2016; Güntürkün and Ocklenburg, 2017; Güntürkün et al., 2020; Hamada, 2020). Some of those reports include findings on memory. For example, honeybees could recall STM of odor association when tested using their right antennae, conversely LTM was accessed mainly via the left antenna, suggesting the time-dependent shift from right to left antenna (Rogers & Vallortigara, 2008).

More recently, left-right anatomical (Kawahara et al., 2013; Moskal et al., 2006; Samara et al., 2011; Shinohara, 2009) and functional (Belcheva et al., 2007; Jordan et al., 2019; Klur et al., 2009; Sakaguchi & Sakurai, 2017; Shinohara et al., 2012; Shipton et al., 2014) differences have been reported in the rodent hippocampus. Rodent hippocampus has a so-called trisinaptic circuit consisting of Dentate gyrus (DG), Cornu ammonis 3 (CA3), and CA1, with fibers on each side of CA3 communicating to the ipsilateral CA1 (Schaffer collaterals) and fibers communicating to the opposite CA3 and CA1 via the hippocampal commissure (Associational commisural pathway) (Swanson et al., 1978) (Fig. 9).

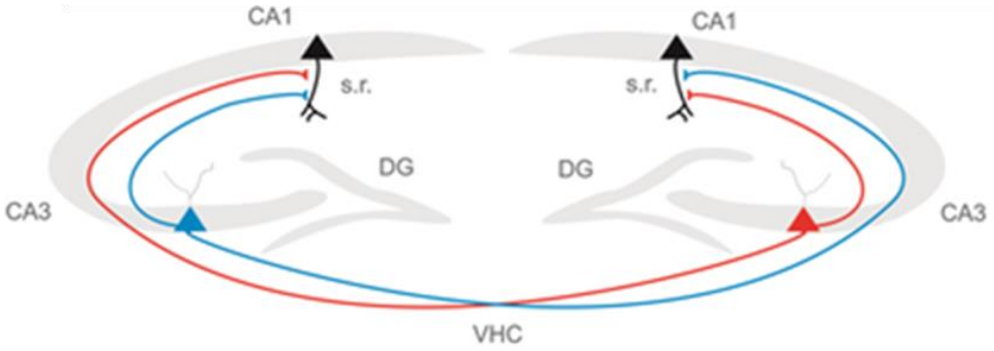

Figure 9. Components and Circuits of the rodent hippocampus.
The hippocampus is mainly composed of Dentate gyrus (DG), Cornu ammonis 3 (CA3) and Cornu ammonis 1 (CA1). Left and right CA3 (blue and red, respectively) project to ipsilateral CA1 via the Schaffer collaterals and to contralateral CA1 via the ventral hippocampal commissure (VHC). The image was modified from (Jordan, 2020).

In particular, the findings of the study by Shipton et al. (Shipton et al., 2014) were highly suggestive, demonstrating that the optogenetic silencing of the left CA3 alone impaired LTM performance in the reward exploration task, whereas the unilateral silencing of either the left or right CA3 caused STM deficits in the spontaneous alternation task and the spatial novelty preference task. However, unlike the results of Shipton et al. (Shipton et al., 2014), previous studies have reported that the right hippocampus contributes predominately to LTM tasks, such as the Barnes maze test (Shinohara et al., 2012) and the active avoidance test (Belcheva et al., 2007), and the unilateral advantage of the left or right hemisphere in LTM is not consistent among the studies. On the other hand, right hippocampal dominance in STM has been suggested in humans (Abrahams et al., 1999). In rodents, however, there is no such study except Shipton et al. (Shipton et al., 2014), and follow-up experiments are needed.

Therefore, it is necessary to investigate this complex left-right hemispherical functional separation for STM and LTM with further experiments. In addition to the functional inhibition of the unilateral hippocampus that has been reported in the previous studies (Jordan et al., 2019; Kawahara et al., 2013; Sakaguchi & Sakurai, 2017; Samara et al., 2011; Shinohara, 2009; Shipton et al., 2014), functional facilitation may contribute to elucidating the functional separation of the left-right hippocampus in rodents.

In the present study, I confirmed the reproducibility and consistency of the left-right hippocampal difference in STM and LTM by the hippocampal lesion experiment. In addition, I investigated how the activation of the unilateral hippocampus by electrical stimulation affects the performance of STM and LTM. From these experiments, I obtained data on the actual role of the left and right hippocampi for STM and LTM formation. This study is written based on (Sakaguchi & Sakurai, 2020).

3.2. Material and methods

3.2.1. Animals

Experimental subjects were male Wistar albino rats (Shimizu Laboratory Supplies, Kyoto, Japan) that were aged 9 weeks old at the time of the surgery. The rats were individually housed in cages with free access to food and water under a light-dark cycle, with the light period between 08:00

and 20:00 h. The rats were randomly assigned to the lesion group and the stimulation group. All experiments were performed in accordance with the Guidelines for Animal Experiments at Doshisha University and with the approval of the Animal Research Committee of Doshisha University.

3.2.2. Surgery

One week before the experiment, the rats were anesthetized with isoflurane (2.5 %, 2.5 L/min) via an anesthetic vaporizer (MK-AT200, MUROMACHI KIKAI Co. LTD., Tokyo, Japan).

In the lesion group (for both STM and LTM experiments), electrical lesions were made by passing anodal direct current (1 mA, 30 s) using a lesion-making device (53500, UGO BASILE SRL, Gemonio, VA, Italy) and a stainless bipolar electrode (150-μm diameter, UB-9007, UNIQUE MEDICAL Co., LTD., Tokyo, Japan). The electrode was inserted into the right or left dorsal hippocampus (DH) ((1) AP, −3.0 mm from bregma; ML, ±2.0 mm from bregma; DV, −3.0 mm from dura; (2) AP, −4.0 mm; ML, ±3.0 mm; and DV, −3.0 mm; (3) AP, −5.0 mm; ML, ±4.0 mm; and DV, −3.0 mm). Brain regions were identified according to the Rat Brain Atlas (Paxinos & Watson, 2007). For sham lesions, the electrode was lowered to the same coordinates, but no current was passed.

In the stimulation group (for both STM and LTM experiments), a head device for electrical stimulation was mounted on the rats' skull. A bipolar electrode was prepared with coated tungsten wire (300-μm diameter, UNIQUE MEDICAL Co. LTD., Tokyo, Japan; the coating is peeled off to

0.5 mm from the tip). The electrodes were inserted into the bilateral, right, or left DH (AP, −3.0 mm from bregma; ML, ±2.0 mm from bregma; DV, −3.0 mm from dura) and were fixed with dental cement and screws. All rats were allowed to recover for 7 days and were handled for 5 min each day.

3.2.3. Stimulation

Electrical stimulation for 10 min was performed in the left and right stimulation groups 10 min before each behavioral test started. The electrodes were connected to the isolated stimulator (Model DS3, Brain Science Idea Co. Ltd., Osaka, Japan) and the train/delay generator (Model DG2A, Brain Science Idea Co. Ltd., Osaka, Japan). The stimulation parameters were 100 μA, 130 Hz, and 90 μs. In the Sham group, the electrodes were connected to the stimulator, but no current was passed.

In addition, to confirm that the stimuli used in this experiment properly activated neurons in the hippocampus, an additional 6 rats (named Stim group, n = 3; Sham group, n = 3) were similarly stimulated for 10 min under free-moving conditions. After 2 hours, these animals were subjected to histology treatment.

3.2.4. Spontaneous alternation test

In both the lesion and the stimulation experiments, there were two meta-groups, STM- and LTM-task. The rats of STM-task groups (Sham, L-lesion, and R-lesion) were tested in two short-term memory tasks, the spontaneous alternation test (SPAT) and the novelty preference test (NPT). All rats were tested using one task each day. The rats of LTM-task groups (Sham, L-lesion, and R-lesion)

were tested in a long-term memory task, the object location test (OLT). On the day of each behavioral test, the home cage was moved to the experimental room 2 h before the start of the test for habituation.

For the SPAT, a T-shaped maze was used (Fig. 10). It was made of transparent acrylic plates. It was comprised of three arms which were each 75 cm long, 10 cm wide, and 40 cm high. Rats were gently placed at the tip of one of the three arms (Start arm). They were then allowed to explore the maze for 10 min. The Start arm was chosen randomly for each rat. After each test, the apparatus was carefully cleaned with a towel containing 70 % ethanol. This was done to prevent the exploratory behavior of other rats from being influenced by olfactory stimuli produced by the previous rats. Behaviors were recorded using a camera (BSW32KM03SV, BUFFALO INC., Aichi, Japan) mounted directly above the apparatus, and the total number of alternations and entries into each of the three arms were calculated by a software program (ANY-maze software, Stoelting Co., IL, USA). The alternation rate was calculated using the following equation: (number of entries into the arm not entered in the preceding two entries)/((total number of entries into all the arms) − 2). Rats were considered to have entered an arm when all four of the animal's paws were located in that arm.

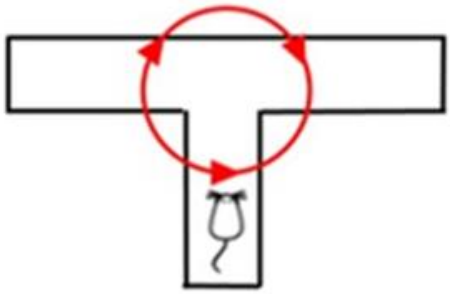

Figure 10. Schematic image of the Spontaneous alternation test.
A T-shaped maze made of transparent acrylic plates was used. The animals were allowed to explore the apparatus for 10 min.

3.2.5. Novel preference test

For the NPT, a Y-maze was used (Figure 11). It was made of transparent acrylic plates and comprised of three 75 cm long, 10 cm wide, and 40 cm high arms. First, one of the arms (named "Novel arm") was blocked with an opaque acrylic plate. Subsequently, rats were gently placed at the Start arm (one of the two unblocked arms) and they were allowed to explore the two unblocked arms (named "Familiar arms") for 5 min. Afterwards, the rats were moved to their home cages for 1 min, then the plate blocking the Novel arm was removed and the rats were placed at the Start arm again and were allowed to explore all three arms for 3 min. The Start arm and Novel arm were chosen randomly for each rat. After each trial, the device was rotated 120 degrees in a randomly selected direction and carefully cleaned with a towel containing 70 % ethanol. Behaviors were recorded using the camera, and the percentage of time spent in the Novel arm and the entries into each of the three arms were calculated.

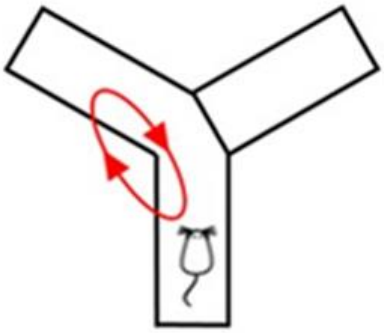

Figure 11. Schematic image of the Novel preference test.
A Y-shaped maze made of transparent acrylic plates was used. In the first session, the animals were allowed to explore the two arms for 5 min. In the second session (1 min after), they were allowed to explore all the three arms for 3 min.

3.2.6. Object location test

For the OLT, a 45 cm × 60 cm × 45 cm box made of white styrofoam boards was used (Fig. 12). First, rats were allowed to explore the empty apparatus for two consecutive days (two hours per day) for habituation. On the third day, the two rectangular parallelepiped blocks (5 cm × 5 cm × 10 cm) made of wood were placed 5 cm away from one plane. Subsequently, rats were gently placed at the center of the apparatus, and they were allowed to explore for 10 min (Note that the rats of the stimulation groups were stimulated for 10 min just prior to this exploration.). After the rats were returned to their home cages, one block (named “Novel block”) was placed in another corner of the device, and one of the previously presented blocks (named “Familiar block”) was placed at the same position as before. After 24 h (the fourth day), the rats were allowed to explore the box again for 3 min (Note that the rats of the stimulation groups were stimulated for 10 min just prior to this exploration). After each test, the apparatus was carefully cleaned with a towel containing 70 % ethanol. Behaviors were recorded using the camera, and the total time during which the rat's nose

touched the Familiar and Novel blocks and the discrimination index (DI, (Novel time − Familiar time)/(Novel time + Familiar time)) were calculated.

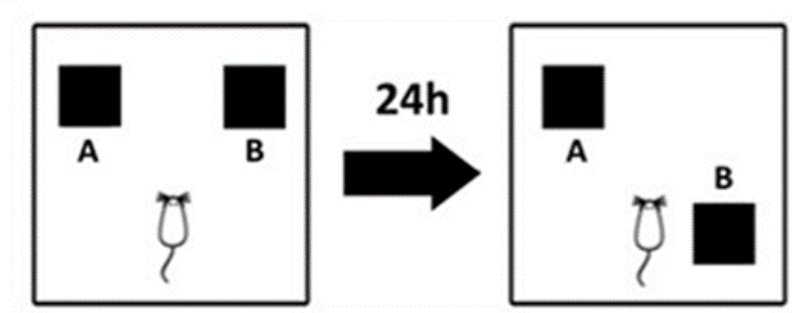

Figure 12. Schematic image of the Object location test.
The apparatus was composed of a styrofoam board box and rectangular parallelepiped blocks. In the first session, the animals were allowed to explore the box and the objects for 10 min. In the second session (24h after), they were allowed to explore the box and the repositioned objects for 3 min.

3.2.7. Histology

In day following the completion of behavioral tests, the rats were deeply anesthetized with an overdose of sodium pentobarbital (220 mg/kg, Kyoritsuseiyaku Corporation, Tokyo, Japan) and were perfused with 0.01 M Phosphate Buffered Saline (PBS, Nacalai Tesque, Kyoto, Japan) and 4 % paraformaldehyde (PFA, Nacalai Tesque, Kyoto, Japan). The brains were then removed and stored in PFA overnight. I obtained coronal brain sections (50 μm) using a microslicer (DTK-3000, Dosaka EM Co. Ltd., Kyoto, Japan) and mounted them on slides, then cresyl violet solution was used as a background stain to detect the lesion area and the inserted site of the electrode with a microscope (Axioplan 2 Imaging, Carl Zeiss Microscopy, LLC, NY, USA) equipped with a camera (DFC300 FX,

Leica Microsystems Inc., IL, USA). Brain regions were identified according to the Rat Brain Atlas (Paxinos & Watson, 2007). The numbers of neurons in the cortex just above the hippocampus (AP, −3.0 mm from bregma; ML, ±2.0 mm from bregma; DV, −1.5 mm from dura) were counted with ImageJ software (National Institutes of Health, Bethesda, MD, USA). First, section images were digitized in gray scale with eight bits and then noise smoothing was performed for all images. Second, the background noise of each image was eliminated, and the obtained images were thresholded to convert them into binary ones. The threshold used for each image was set to 120–180 points, and the circularity values was set to 0.60–1.00 points.

3.2.8. Immunohistochemistry

I used a free-float method for immunohistochemistry. The sections of the Stim and Sham groups (AP =3.50 mm) were blocked with a solution containing 5 % goat serum (G9023, Sigma-Aldrich, MO, USA) for 1 h. After washing in buffer, sections were incubated with rabbit anti-c-fos antibody (1:1000 dilution, sc-52, Santa Cruz Biotechnology, CA, USA), overnight, at 4 ℃. Then sections were washed and incubated with goat anti-rabbit immunoglobulin G (1:1000 dilution, ab150077, Abcam, Cambridge, UK) for 2 h at room temperature. Finally, the stained slices were mounted on slide glasses and coverslipped with the mounting reagent containing DAPI (Fluoro-KEEPER Antifade Reagent, Nacalai Tesque, Kyoto, Japan). Each section was scanned at 20 × magnification using the light microscope equipped with the camera. The numbers of c-fos-positive cells in hippocampal subregions (dentate gyrus (DG), CA3, and CA1) were counted with the ImageJ

software. The image editing method and the cell counting method were applied as described in section 3.2.7. The threshold used for each image was set to 80–100 points, and the circularity values was set to 0.80–1.00 points.

3.2.9. Data analysis

Data analyses were performed with BellCurve for Excel (Social Survey Research Information Co. Ltd., Tokyo, Japan). Experimental data are shown as means ± SEM. One-way ANOVA followed by post-hoc Tukey–Kramer method was used for all statistical comparisons.

3.3. Results

3.3.1. Histology

In the lesion experiment, I observed that the stereotaxic passing of an anodal direct current destroyed most DH structures. Fig. 14a shows a raw sample of an electrical lesion. Fig. 14e (for STM-task) and 15a (for LTM-task) indicate the lesion areas (minimum lesion areas, gray color; maximum lesion areas, black color) of the left and right lesion groups (n = 6 in each group). The extent of the lesion is shown with reference to the horizontal sections found in the Rat Brain Atlas (Paxinos & Watson, 2007). The Sham lesion group had little-to-no damage in these areas. In addition, Figs. 14b and 14c shows enlarged section images of the cortex just above the lesion area of the ipsi- and contra-lateral hemispheres, respectively. In both the left and right lesion groups, there was no significant

difference between the number of neurons on the ipsi-lateral (n = 6) and the contra-lateral (n = 6) hemispheres (left; $F(1, 10) = 0.029$, $P = 0.87$, right; $F(1, 10) = 0.22$, $P = 0.65$) (Fig. 14d).

In the stimulation experiment, I observed that the electrode tip was accurately placed into the intra-hippocampus. Figs. 16a, 16b/17a, and 16c/17b show the raw section sample, and the insertion site of each individual of the left group and right group, respectively (n = 6 in each group). In addition, in order to confirm that stimulation with the parameters used in this experiment can definitely activate hippocampal neurons, the expression of c-fos protein, an activation marker of neurons, was quantified 2 h after performing stimulation for 10 min. Fig. 13a shows the electrode insertion site (n = 3) and Fig. 13b shows raw samples of c-fos expression in the DG, CA3, and CA1 of the Stim and Sham groups. Fig. 13c shows the number of positive cells in each subregion. Compared to the Sham group, the number of positive cells in the Stim group was significantly higher in the DG ($F(1, 4) = 178.21$, $P = 0.0030$), CA3 ($F(1, 4) = 98.83$, $P = 0.0050$), and CA1 ($F(1, 4) = 158.08$, $P = 0.0023$).

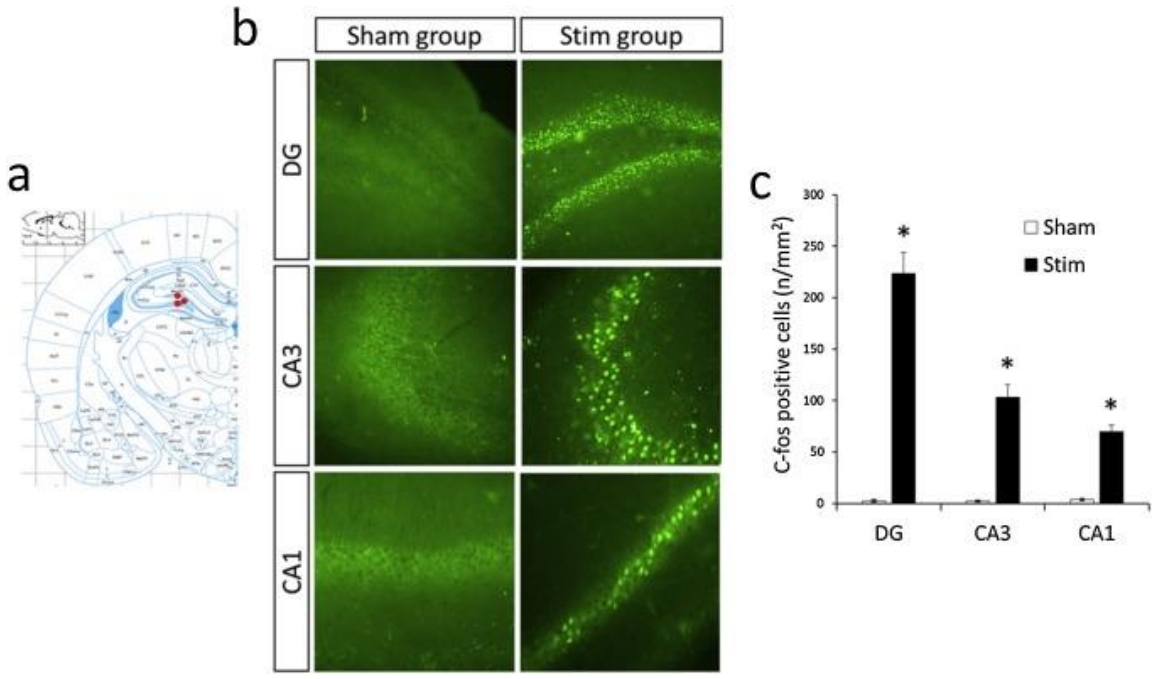

Figure 13. C-fos expression by the electrical stimulation.

(a) Insertion sites of the stimulation electrode (the Stim group, n = 3). The electrodes were inserted into the hippocampus (AP, −3.0 mm from bregma; ML, ±2.0 mm from bregma; DV, −3.0 mm from dura). (b) Raw sections of DG, CA3, and CA1 of the Sham and the Stim groups. (c) Number of the c-fos positive cells. White and black bars represent the Sham (n = 3) and the Stim groups (n = 3), respectively. All measures are shown as means ± SEM and * indicates $P < 0.05$.

3.3.2. STM in the lesion experiment

The SPAT and the NPT were used to measure STM. In the lesion experiment, in the SPAT, the one-way ANOVA showed a significant effect of the factor "lesion effect" for the alternation rate ($F(2, 15) = 11.48$, $P = 0.00094$) (Fig. 14f). The post-hoc comparisons revealed that the right lesion group (n = 6) was significantly lower than that for the Sham group (n = 6) or for the left lesion group (n = 6) (P = 0.0047 and P = 0.0042, respectively). There was no significant difference between the Sham group and the left lesion group ($P = 0.052$). On the other hand, the one-way ANOVA showed no significant effect of the factor "lesion effect" for the total entry number ($F(2, 15) = 0.11$, $P = 0.90$)

(Fig. 14g). In the NPT, the one-way ANOVA showed a significant effect of the factor “lesion effect” for the novel arm rate ($F(2, 15) = 5.60$, $P = 0.015$) (Fig. 14h). The post-hoc comparisons revealed that the right lesion group (n = 6) was significantly lower than that for the Sham group (n = 6) or for the left lesion group (n = 6) ($P = 0.0033$ and $P = 0.0053$, respectively). There was no significant difference between the Sham group and the left lesion group ($P = 0.40$). On the other hand, the one-way ANOVA showed no significant effect of the factor “lesion effect” for the total entry number ($F(2, 15) = 0.096$, $P = 0.91$) (Fig. 14i).

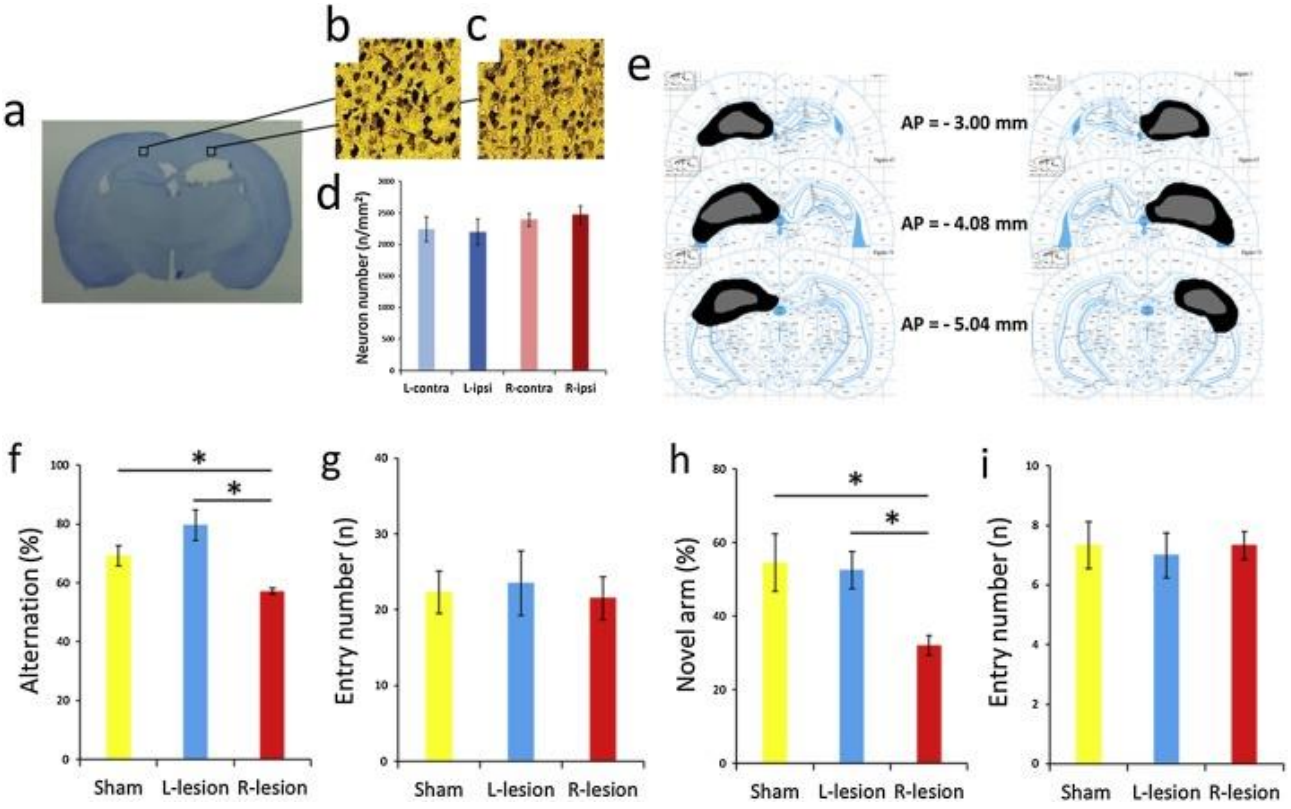

Figure 14. The results of lesion experiment for STM.

Images of sections showing the lesion sites for the (a) raw sample, and (e) maximum (black) and minimum (gray) lesion areas of the left (n = 6, left side three figures) and right lesion (n = 6, right side three figures) groups. A 10×raw sample of the cortical area (AP = 3.00) located just above the hippocampus in (b) ipsi-lateral hemisphere (lesion side) and (c) contra-lateral hemisphere (opposite side). (d) The number of neurons in the ipsi- and contra-lateral hemisphere. (f–i) The results of STM tasks. (f) Alternation rate in the SPAT. (g) Total entry number in the SPAT. (h) Novel arm rate in the NPT. (i) Total entry number in the NPT. Yellow, blue, and red bars represent the Sham (n = 6), the left (L) lesion (n = 6), and the right (R) lesion (n = 6) groups, respectively. All measures are shown as means ± SEM and * indicates $P < 0.05$. STM, short-term memory; SPAT, spontaneous alternation test; NPT, novel preference test.

3.3.3. LTM in the lesion experiment

The OLT was used to measure LTM. The one-way ANOVA showed a significant effect of the factor "lesion effect" for the DI ($F(2, 15) = 3.81$, $P = 0.045$) (Fig. 15b). The post-hoc comparisons revealed that the left lesion group (n = 6) was significantly lower than that for the Sham group (n = 6)

($P = 0.031$). There was no significant difference between the Sham group and the right lesion group ($P = 0.26$), and between the left lesion group and the right lesion group (n = 6) ($P = 0.080$).

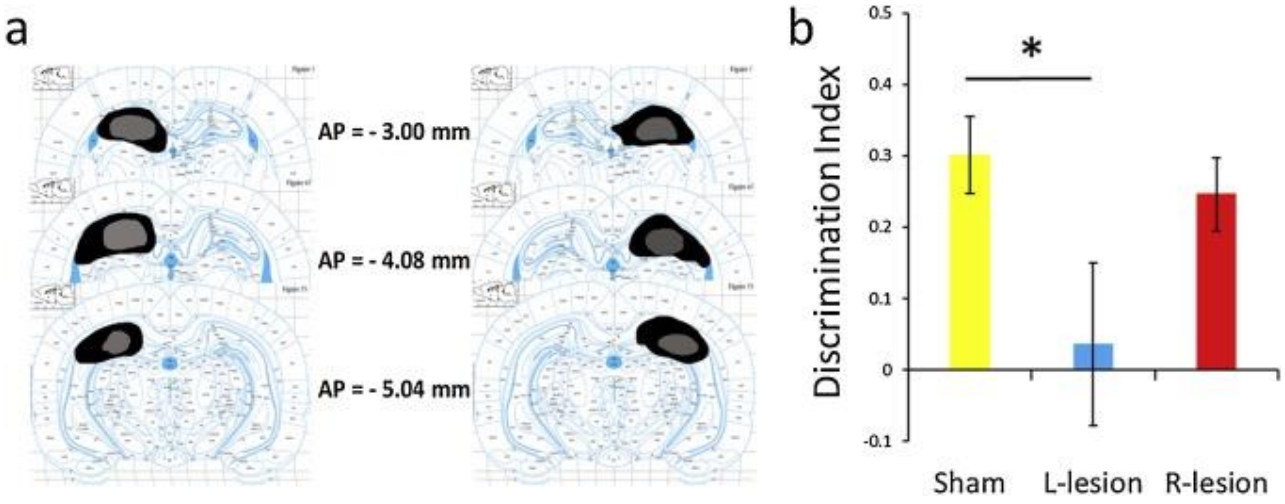

Figure 15. The results of lesion experiment for LTM.
Images of sections showing the lesion sites for the (a) Maximum (black) and minimum (gray) lesion areas of the left (n = 6, left side three figures) and right lesion (n = 6, right side three figures) groups. (b) The results of LTM task. The DI 24 h after the sample presentation in the OLT. Yellow, blue, and red bars represent the Sham (n = 6), the left (L) lesion (n = 6), and the right (R) lesion (n = 6) groups, respectively. All measures are shown as means ± SEM and * indicates $P < 0.05$. LTM, long-term memory; DI, discrimination index; OLT, object location test.

3.3.4. STM in the stimulation experiment

In the stimulation experiment, in the SPAT, the one-way ANOVA showed a significant effect of the factor "lesion effect" for the alternation rate ($F(2, 15) = 8.07$, $P = 0.0042$) (Fig. 16d). The post-hoc comparisons revealed that the left stimulation group (n = 6) was significantly lower than that for the Sham group (n = 6) or for the right stimulation group (n = 6) ($P = 0.0032$ and $P = 0.026$, respectively). There was no significant difference between the Sham group and the right stimulation

group ($P = 0.30$). On the other hand, the one-way ANOVA showed no significant effect of the factor "lesion effect" for the total entry number ($F(2, 15) = 0.25$, $P = 0.79$) (Fig. 16e). In the NPT, the one-way ANOVA showed a significant effect for the novel arm rate ($F(2, 15) = 7.17$, $P = 0.0065$) (Fig. 16f). The post-hoc comparisons revealed that the left stimulation group (n = 6) was significantly lower than that for the Sham group (n = 6) or for the right stimulation group (n = 6) ($P = 0.0053$ and $P = 0.031$, respectively). There was no significant difference between the Sham group and the right stimulation group ($P = 0.40$). On the other hand, the one-way ANOVA showed no significant effect of the factor "lesion effect" for the total entry number ($F(2, 15) = 0.33$, $P = 0.72$) (Fig. 16g).

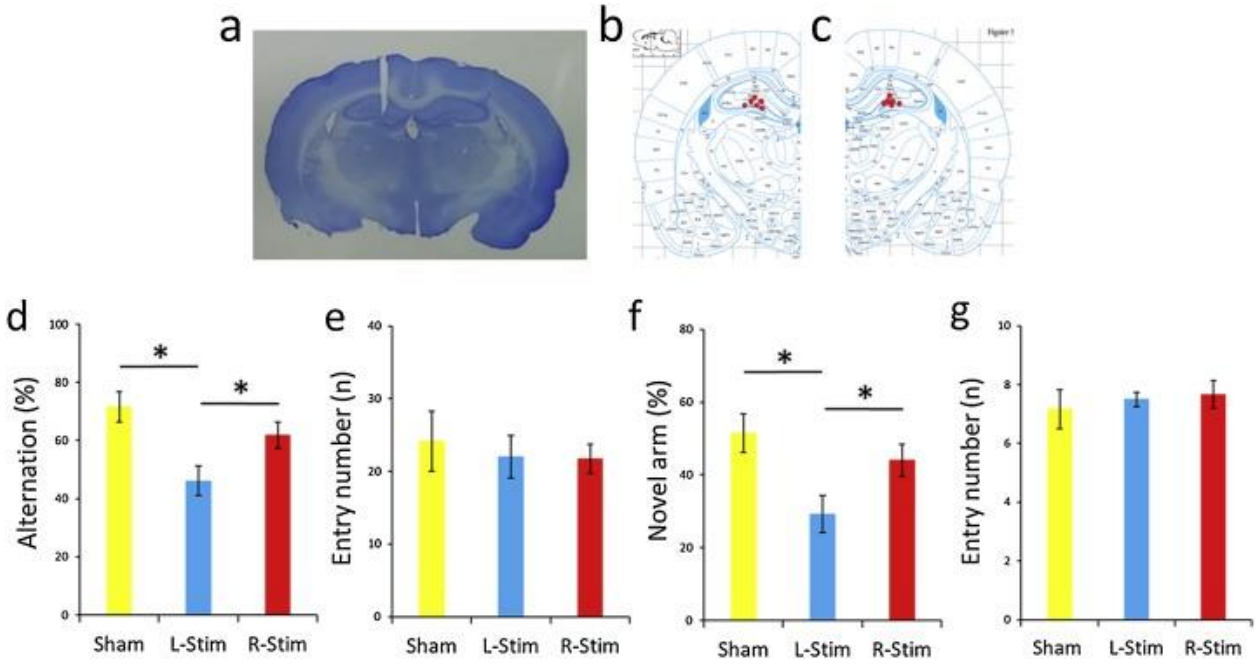

Figure 16. The results of stimulation experiment for STM.

(a–c) Insertion site of the stimulation electrode. Images of sections showing the insertion site for the (a) raw sample, (b) individual insertion sites of the left stimulation group (n = 6), and (c) individual insertion sites of the right stimulation group (n = 6). (d–f) The results of the STM tasks. (d) Alternation rate in the SPAT. (e) Total entry number in the SPAT. (f) Novel arm rate in the NPT. (g) Total entry number in the SPAT. Yellow, blue, and red bars represent the Sham (n = 6), the left (L) stimulation (n = 6), and the right (R) stimulation (n = 6) groups, respectively. All measures are shown as means ± SEM and * indicates $P < 0.05$. STM, short-term memory; SPAT, spontaneous alternation test; NPT, novel preference test.

3.3.5. LTM in the stimulation experiment

In the OLT, the one-way ANOVA showed a significant effect of the factor "lesion effect" for the DI ($F(2, 15) = 6.17$, $P = 0.011$) (Fig. 17c). The post-hoc comparisons revealed that the left stimulation group (n = 6) was significantly lower than that for the Sham group (n = 6) or for the right stimulation group (n = 6) ($P = 0.026$ and $P = 0.0076$, respectively). There was no significant difference between the Sham group and the right stimulation group ($P = 0.38$).

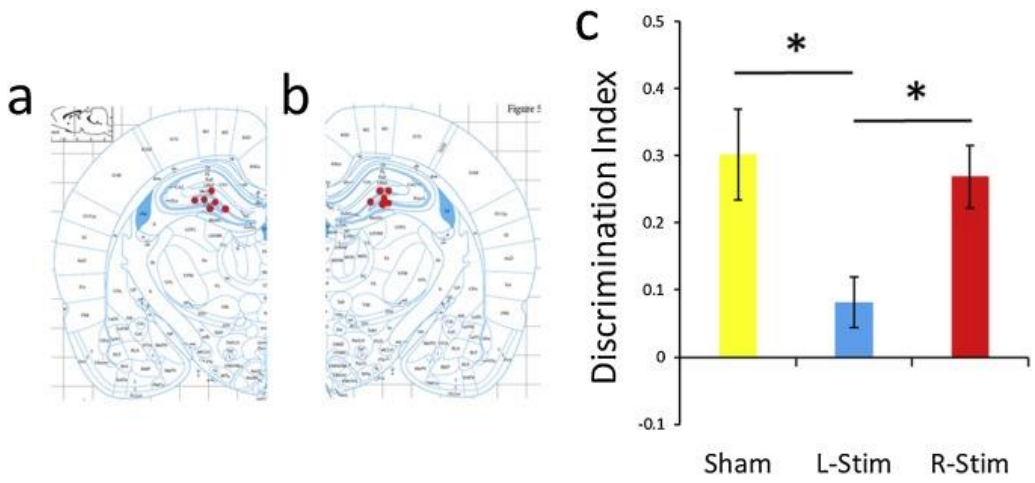

Figure 17. The results of stimulation experiment for LTM.

(a and b) Insertion site of the stimulation electrode. (a) Individual insertion sites of the left stimulation group (n = 6) and (b) those of the right stimulation group (n = 6). (c) The results of the LTM task. The DI in the OLT. Yellow, blue, and red bars represent the Sham (n = 6), the left (L) stimulation (n = 6), and the right (R) stimulation (n = 6) groups, respectively. All measures are shown as means ± SEM and * indicates $P < 0.05$. LTM, long-term memory; DI, discrimination index; OLT, object location test.

3.4. Discussion

The purpose of the present study was to elucidate how the left and right hippocampi contribute to the formation of STM and LTM.

3.4.1. Left-right differential roles for STM

In the lesion experiment, only the right hippocampal lesion impaired both the alternation rate in the SPAT (Fig. 14f) and the novel arm preference in the NPT (Fig. 14h). The lesion areas were not extended beyond the hippocampal structure (Fig. 14e). In addition, there was no significant difference between the numbers of neurons in the corteces just above the ipsi-lateral dorsal hippocampus (lesion side) and the contra-lateral one (Fig. 14d). Considering the cortical involvement in the formation of

both STM and LTM (Lara & Wallis, 2015; Simons & Spiers, 2003), it was necessary to confirm the cortical damage, but the present result shows that the lesion method I used had a specific effect on the hippocampal structure without affecting cortical neurons. Moreover, there was no significant difference in the number of entries in the SPAT and in the NPT between the Sham group and left/right lesion groups (Figs. 14g and 14h), this suggests that rats had no motor impairment by the hippocampal lesion. Therefore, these results indicate that the right hippocampus predominantly contributes to the formation of STM required for the present tasks. Our results differ from those of the results by Shipton et al. (Shipton et al., 2014), which showed that unilateral optogenetic inactivation of both left and right mice CA3 alone during hippocampus-dependent STM tasks impairs the task performance, and suggests that both the left and right hippocampi contribute to STM formation. Such different results might be due to the difference in animal species (rat vs. mouse), target brain region (whole hippocampus vs. CA3), and/or the difficulty of the tasks. On the other hand, in the stimulation experiment, the electrical stimulation of the left hippocampus before testing of the tasks impaired both the alternation rate in the SPAT (Fig. 16d) and the novel arm preference in the NPT (Fig. 16f). The insertion sites of the electrode were properly placed in the intra-hippocampus (Figs. 16b and 16c) and the stimulation induced an increase in the number of c-fos positive cells in all the hippocampal subregions (DG, CA3, and CA1) (Fig. 13c). Moreover, there was no significant difference in the number of entries in the SPAT and in the NPT between the Sham group and left/right lesion groups (Figs. 16e and 16g), this suggests that rats had no motor impairment by the hippocampal stimulation. Therefore, these results indicate that hyper-excitation of neuronal activity in the left hippocampus

inhibited the formation of STM. Taken together, the results of our two experiments suggest that the right hippocampus has a facilitating role for the formation of STM, whereas the left hippocampus has a suppressive role for the formation of it. The left and right hippocampi may utilize the interhemispheric interaction via the hippocampal commissure and interact to excite or inhibit one another during STM formation.

3.4.2. Left-sided specialization for LTM

In the lesion experiment, only the left hippocampal lesion impaired the DI in the OLT (Fig. 15b). The lesion areas were not extended beyond the hippocampal structure (Fig. 15a). These results indicate that the left hippocampus predominantly contributes to the formation of LTM, agreed with the result of Shipton et al. (Shipton et al., 2014). Additionally, in the stimulation experiment, the electrical stimulation of the left hippocampus before testing of the tasks impaired the DI in the OLT (Fig. 17c). The insertion sites of the electrode were properly placed in the intra-hippocampus (Figs. 17a and 17b). These results indicate that hyper-excitation of neuronal activity in the left hippocampus inhibited the formation of LTM. Taken together, the results of our experiments suggest that unlike the results of the STM tasks, only one side (left) of the hippocampus is expected to contribute to LTM. This means that hemispheric interaction might be unnecessary for LTM formation. In addition, it is considered that a refined mechanism that requires an appropriate level of neural activity in LTM formation process. This may be because excessive synchronous firing of cell populations incorporates unnecessary information into the episode, thereby disrupting the accuracy of LTM.

Our results are consistent with Shipton et al. (Shipton et al., 2014), but inconsistent with the previous studies showing that the right hippocampus contributes predominately to the LTM tasks, such as the Burns maze task (Shinohara et al., 2012) and the active avoidance task (Belcheva et al., 2007). This contradiction may be due to the different types of reinforcer, i.e., the negative/avoiding vs. positive/approaching stimuli, such as Belcheva et al. (foot shock) (Belcheva et al., 2007) and Shinohara et al. (light exposure) (Shinohara et al., 2012) vs. Shipton et al. (food reward) (Shipton et al., 2014) and Jordan et al. (novel object exploration) (Jordan et al., 2019). The serotonin involvement in the dorsal hippocampal asymmetry (Belcheva et al., 2007), the asymmetrical contribution of left/right ventral hippocampus to cope with anxiety (Sakaguchi & Sakurai, 2017), and the functional asymmetry in the dorsal hippocampus may depend on emotional types as well as the memory types (STM/LTM) and should be considered in the future research. In addition, the strategies used during the behavioral tests for STM and LTM used in the present research differ from test to test (e.g., allothetic and idiothetic navigation strategies (Whishaw & Brooks, 1999)), more detailed follow-up experiments using tests in which STM and LTM can be quantified in the same parameter using delayed non-matching to sample, etc., are essential in the future. The accumulation of further findings on left-right differences of hippocampal functions and interhemispheric interactions will contribute to clarifying the actual state of functional divisions and coordination between two hemispheres.

3.4.3. Conclusion

In this study, I investigated functional lateralization in the rat DH for STM and LTM. The results of the present experiments for STM and LTM revealed that (1) DH possessed a noticeable functional lateralization associated with memory formation, (2) the right DH has a facilitating role for the formation of STM, whereas the left DH has a suppressive role, and (3) only one side (left) of DH is expected to contribute to LTM. These findings provide new insights on the functional lateralization and its interaction in the brain for adaptive behaviors.

Chapter 4. General discussion

The present research aimed to determine how inhibition of unilateral left or right hippocampal function in the rat brain affects anxiety-like behavior and short- and long-term memory, and to derive the significance of the existence of left-right differences based on these results.

4.1. Functional left-right difference in the ventral hippocampus

In Chapter 2, I conducted unilateral VH lesion surgery to detect functional left-right differences on anxiety-like behavior in the VH. In the SAT anxiety-like behavioral test, the time spent in Alley 2 and 3, which produce weak anxiety to animals, was resulting in Sham < Left < Right < Bilateral lesion or Sham < Left < Right = Bilateral lesion, suggesting that the left VH also makes some contribution to anxiety coping, although right VH is dominant. On the other hand, the time spent in Alley 4, which produce strong anxiety to animals was resulting in Sham = Left < Right < Bilateral lesion, suggesting that right VH is dominant and left VH contributes little to severe anxiety coping. In summary, these results show that the left and right VH contribute complementarily to weak anxiety, while only the right VH contributes to strong anxiety; the present study is the first to find a functional left-right difference in rodent VH (Fig. 18). This suggests that there is a left-right hemispheric difference in rodent brain functions involved in coping with anxiety-like behaviors, and as shown in the SAT results, the left-right difference may allow the rats to flexibly switch between

unilateral and bilateral functions and to express behaviors that are better adapted to the changing environment. In other words, the presence of the hemispheric difference may induce continuous, non-discrete behavioral changes and finer adjustments when adapting to the environment than the absence. This may be one aspect of the significance of the hemispheric lateralization.

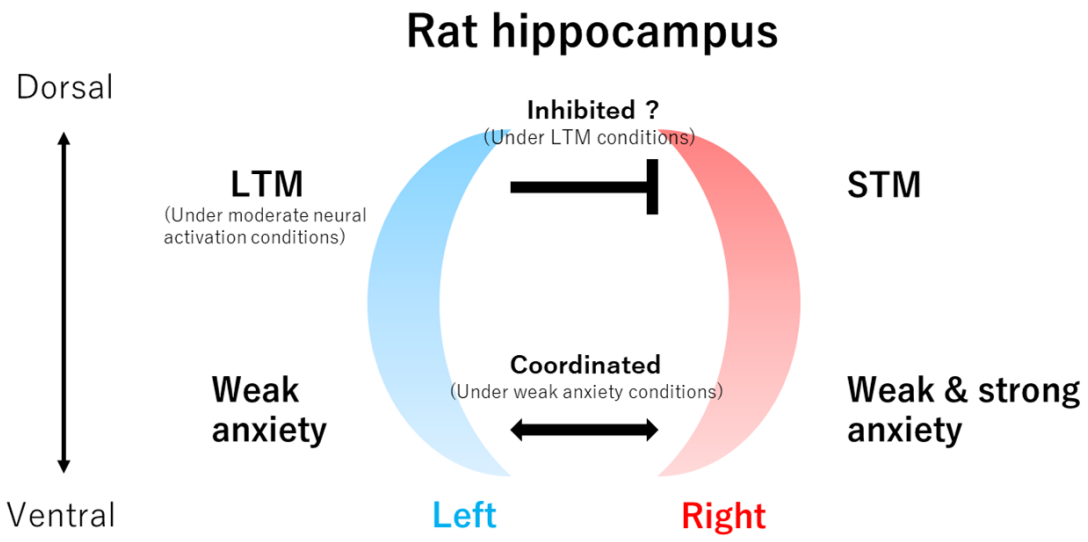

Figure 18. Summary of the lateralized functions in the rat DH and VH.
Illustrated summary of the conclusion of this thesis. The left hippocampus contributes to LTM under moderate neural activation conditions (DH) and to coping with weak anxiety (VH). The right hippocampus contributes to STM (DH) and coping with both weak and strong anxiety (VH). The left DH may act inhibitory to the right hippocampus during LTM formation. The left and right VH may interact in a coordinated manner to cope with weak anxiety.

4.2. Functional left-right difference in the dorsal hippocampus

In Chapter 3, I conducted unilateral DH lesion surgery to detect functional left-right differences on STM and LTM in DH. In the short-term memory test using the SPAT and NPT, lesion of the left DH did not affect the performance, while lesion of the right DH impaired it, suggesting

that the right DH has a dominant function in STM. In the long-term memory test using the OLT, lesion of the right DH did not affect the performance, while lesion of the left DH impaired it, suggesting that the left DH has a dominant function in LTM. One possible explanation for the discrepancy that although lesion of the right DH induces an impairment in STM, but it does not affect LTM is as follows: In STM, the left DH is involved only in acquisition, the right DH in acquisition and retention, and the right lesion impairs retention, resulting in an impairment in STM; In LTM, the left DH is involved in acquisition and consolidation, the right DH is involved only in acquisition, and the right lesion does not impair consolidation, resulting in an impairment in LTM. In addition, stimulation of the right DH did not affect the performance in the SPAT and NPT, whereas stimulation of the left DH impaired it, indicating that excessive activation of the left DH caused a decrease in STM. Stimulation of the right DH did not affect the performance in the OLT, whereas stimulation of the left DH reduced it, indicating that excessive activation of the left DH causes a decrease in LTM. In summary, these results indicate that the right DH is usually involved in facilitating STM and the left DH is involved in inhibiting it (Fig. 18). In addition, only the left DH usually functions facilitatively for LTM, but over-activation of the left DH is interfering with LTM. This suggests that the left DH somehow regulates or inhibits the right-dominant function, STM. Interhemispheric inhibition is known to occur in the brains of patients with mental diseases (Bajwa et al., 2008; Chalah et al., 2018; Munévar et al., 2018) and in the rat somatosensory cortex (Palmer et al., 2012, 2013), where the dominant hemisphere functions to inhibit the other side of the brain when it is functioning. The results obtained in the present study may reflect the effects of this interhemispheric inhibition.

These findings suggest that the left and right DH are functionally separated, and the significance of this laterality is that they can share control of different functions, and that inhibition between the two hemispheres coordinates finer functions and the resulting finer behaviors.

4.3. The significance of functional left-right differences

As described in the previous sections, the present research revealed the existence of functional left-right differences in the dorsal and ventral regions of the rat hippocampus. Furthermore, the findings also suggested the significance of the left-right differences.

In a previous study, Rogers et al. proposed the hypothesis of dispersed attention with chicks, allowing them to be alert for enemy attacks while feeding (Rogers et al., 2004) (Fig. 19). In birds, it is possible to take advantage of the influence of light on the development of visual lateralization. Chicks that had hatched from eggs exposed to light performed well on a dual task in which they performed the feeding task (considered to be left hemisphere dominance) while simultaneously monitoring for a model predator (considered to be right hemisphere dominance), whereas those incubated in the dark performed poorly (Rogers et al., 2004). Birds are known to have no corpus callosum and poor communication between the left and right hemispheres (Suárez et al., 2018; Ünver & Güntürkün, 2014; Zeier & Karten, 1973), and functional dissociation between the left and right hemispheres may be more developed and valued in these animals than the fine regulation of behavior by interhemispheric inhibition, described in the previous section.

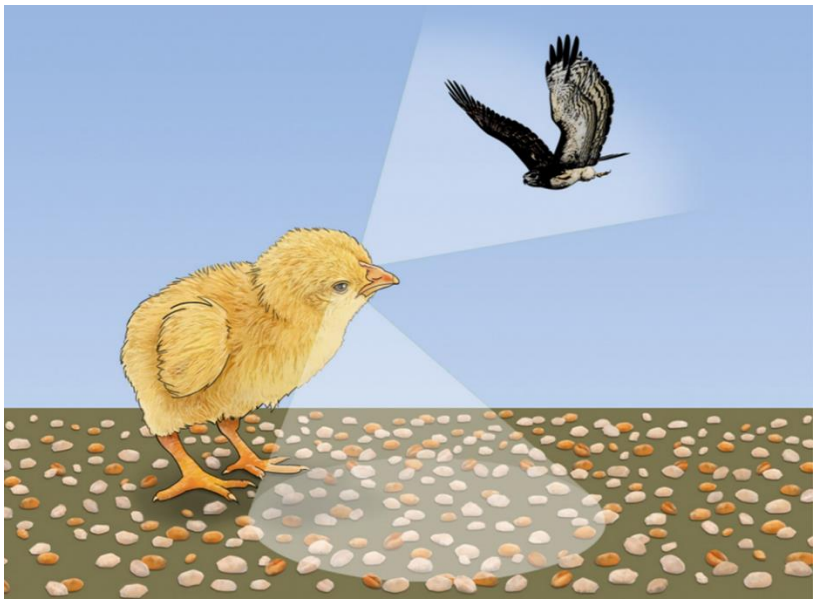

Figure 19. Hypothesis of the significance of functional left-right differences.
Brain lateralization in domestic chicks is associated with an ability to perform two tasks in parallel. Chicks had to find grains among pebbles and were simultaneously forced to be alerted for predator attack. Birds had brain lateralization could success this task, while nonlateralized failed. The image was modified from (Güntürkün et al., 2020).

4.4. Future research

There is still a lack of research on functional left-right differences in the animal brain. In addition, it is not possible to study the significance of the left-right differences that are common to the whole brain by focusing on only a few brain regions. Therefore, it is necessary to examine the anatomical and functional differences between left and right hemispheres in all brain regions. In addition, the experiments conducted in the present study were by the classic experimental methods, such as lesion and electrical stimulation of brain regions, use of latest and specific techniques, such as optogenetics and chemogenetic tools (designer receptors exclusively activated by designer drugs; DREADDs) would provide more accurate results. Such evidences would facilitate a better

understanding of left-right differences. Furthermore, STM and LTM include subdivisions of processes such as acquisition, consolidation, and recall, and it will be necessary to construct more detailed experiments to investigate how the left-right differences are observed in which phases of these processes. In order to clarify the significance of the left-right differences, it is important to study not only the left-right differences but also inter-hemispheric interactions, to investigate what information is exchanged between the two hemispheres and what kind of functions they contribute to. By conducting such experiments in a wide variety of animal species and examining them from a comparative biological perspective, future researches will be able to make a strong contribution to our understanding of the significance and origins of left-right differences, and of essential functions such as the left hemisphere-biased language area in the human brain. These results will also contribute to our understanding of the neural basis and fundamental causes of psychiatric and developmental disorders that show inter-hemispheric (Chang et al., 2019; Zhao et al., 2017) or long-distance inter-regional loss of connectivity (Courchesne & Pierce, 2005) and partial volume loss in the corpus callosum (Haar et al., 2016), and to the development of treatments for these disorders.

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