UNIVERSITY OF CALIFORNIA, SAN DIEGO

Local dentate circuits support spatial working memory regardless of position along the longitudinal hippocampal axis

A thesis submitted in partial satisfaction of the requirements
for the degree Master of Science

in

Biology

by

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2014

The thesis of Ernie Hwaun is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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Chair

University of California, San Diego

2014

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ACKNOWKLEDGEMENTS

I would like to thank Dr. Jill K. Leutgeb for giving me the opportunity to join her lab. I learned important skills needed for basic research from her lab. In addition, the research experience in her lab reinforced my passion in the field of neuroscience. Dr. Leutgeb has always offered support and guidance whenever needed.

I would also like to thank Dr. Veronica C. Piatti and Dr. Takuya Sasaki for teaching me the techniques necessary for the experiments. Specifically, Dr. Piatti taught me how to train animals, prepare brain tissues, and quantify volume of brain area using Cavalieri methods. Dr. Piatti performed all the surgeries and perfusion in this study. Dr. Sasaki taught me how to use Matlab to analyze data. Their guidance is crucial for my intellectual development.

Next, I would like to thank all of the members of the Leutgeb lab for their support. I would especially like to thank Maria Josic for training the animals and quantifying the volume of the hippocampus.

Lastly, I would like to thank all of my friends and family who have encouraged and supported me while I worked towards this graduate degree.

ABSTRACT OF THE THESIS

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Master of Science in Biology

University of California, San Diego, 2014

Professor Jill K. Leutgeb, Chair

The dentate gyrus (DG) is the initial site of information processing in the hippocampus. Previous studies have shown that the rostral-dorsal DG (rdDG) is essential in the rat's ability to discriminate similar spatial locations. In addition, the DG has been shown to support spatial working memory(WM). However, the question of how the DG supports spatial WM remains unclear. We demonstrated that spatial WM performance in rats is not selectively supported by a specific region along the longitudinal axis of the

DG. Rats with 20 –40% volume reduction specific to the rdDG or the caudal-ventral DG (cvDG) did not impair spatial WM, suggesting a mechanism independent of the anatomical location of the DG could support WM. To further elucidate the mechanism responsible for WM in the DG, we investigated whether a compensatory activity of neurons existed in the remaining DG of the rdDG or cvDG lesion rats. Using immediate early gene *c-fos* to label active neurons in the DG granule cell layer, we quantified the number of active neurons for each group (control, rdDG lesion, and cvDG lesion) through stereological methods. The fraction of *c-fos* labeled granule neurons in the lesion groups did not change compared to control. Thus, WM is supported in rats with cvDG or rdDGlesions without compensatory activity of granule neurons.The result implies that local dentate circuits with a network in which sparsity is retained can support dentate-dependent memory.

I:

Introduction

Overview of the Dentate Gyrus

The hippocampus is crucial for memory of specific events in humans and animals (Squire LR, 1992). Depending on their unique anatomical organization, different subregions within the hippocampus contribute to various aspects of memory. The dentate gyrus (DG) is the initial site of information processing in the hippocampus. Dentate granule cells (DGCs) receive major inputs from layer II of the entorhinal cortex through perforant pathway and send outputs to CA3 through mossy fibers (Amaral DG and Witter MP, 1989). The large number of DGCs relative to entorhinal cells makes perforant pathway highly divergent. In addition, new neurons are incorporated into the DG throughout an animal's lifetime to expand the number of DGCs (Gage FH, 2000).Thus, the DG is hypothesized to disperse the entorhinal inputs onto CA3 neurons to form distinct patterns (Treves and Rolls, 1992, 1994).

Functional disparity along the longitudinal axis of the DG

In support of the DG's hypothesized role in differentiating similar pattern, a study shows that animals with selective rostral-dorsal DG (rdDG) lesion cannot distinguish similar places (Gilbert et al.,2001). Furthermore, electrophysiological recordings in rdDG demonstrate that the firing pattern of DGCs is more sensitive to small changes in space than in CA3 (Leutgeb et al., 2007). Thus, rdDG plays an important role in cognitive aspect of memory.

On the other hand, animals with selective caudal-ventral DG (cvDG) lesion are unable to distinguish similar odors after a delay (Weeden et al., 2014). Furthermore, optogenetic activation of cvDG has been shown to suppress innate anxiety (Kheirbek et al., 2012), suggesting cvDG is involved in emotional aspect of memory. Additionally,

one computational model has postulated the importance of the CA3-DG backprojection in sequence coding (Lisman et al., 2005). Since there is more extensive CA3-DG backprojection in cvDG than in rdDG (Myers and Sharfman, 2011), cvDG can be an ideal site to support sequence coding relevant for working memory.

The DG supports working memory

The DG has also been shown to specifically support spatial working memory (WM) instead of spatial reference memory (Niewoehner et al., 2007). WM refers to the ability to hold relevant information temporarily that can be used to guide behavior later while spatial reference memory is the ability to recognize a place. Animals without the DG cannot store temporary information of space. However, the mechanism of how the DG supports spatial WM remains unclear. Given the functional differences along the longitudinal axis of the DG, WM might rely more on one subregion of the DG than the other.

Investigate the mechanism of how the DG supports WM

To elucidate how the DG supports WM, this study first aimed to determine the DG subregion necessary for WM by inducing lesion in either rdDG or cvDG with colchicine. A non-match-to-sample task on eight-arm radial maze was used to evaluate WM performance. Locating the DG subregion can inform us whether the ability to discriminate similar spatial input, which is dependent on rdDG, or the hypothesized role of DG-CA3 backprojection in WM, which is more extensive in cvDG, is more relevant for WM. However, it is also possible that neither rdDG lesion nor cvDG lesion alone impairs WM, suggesting a mechanism independent of the DG position along the longitudinal axis.

The second part of this study sought to examine the neuronal activity in the remaining DG after partial DG lesion. Since the majority of the DGCs are silent in behaving animals, they become potential source of compensation for WM after partial DG lesion, assuming the total number of active DGCs is critical for WM. Recruiting silent DGCs is possible through the mossy cells, which are the excitatory interneurons that connect the DGCs along the longitudinal axis of the DG. Another possibility is that the level of neuronal activity in the remaining DG is unaffected by partial DG lesion, suggesting a lack of interaction along the longitudinal axis of the DG during a WM task. Instead,the interaction between local dentate circuits and their downstream target, CA3, might play an important role to support WM. Determining the presence of compensatory activity in the remaining DG can inform us whether the interaction within the DG or the interaction between the DG and CA3 is more relevant for WM. We used the rats with either rdDG lesion or cvDG lesion from the first part of the study and labeled the DGCs that expressed c-Fos, which is an immediate early gene. The expression of c-Fos has been shown to increase in response to high frequency of neuronal activity (Douglas R.M. et al, 1988). Next, we used the stereological methods to quantify the number of DGCs that expressed c-Fos and its density in the dentate granule cell layer (DGCL). Levels of c-Fos expression indicated the level of activity of the DG along the longitudinal axis while the rats did the spatial WM task.

II:

Results

Behavioral performance

The eight-arm radial maze task included sample phase and choice phase (Figure 1A). During the sample phase, the rats visited pseudorandomly selected four arms to retrieve reward. Next, the rats obtained access to all of the eight arms. Only the remaining arms that the rats had not been to would have reward. Any re-entry to an arm was considered a WM error. A trial without any WM error was considered correct. The WM performance was quantified by the percentage of correct trials on each day. The behavioral results showed that only the entire DG lesion group had significantly impaired WM performance while the cvDG and rdDGlesion groups had comparable WM performance to the control group (Figure 1B).

Quantification of the DG lesion

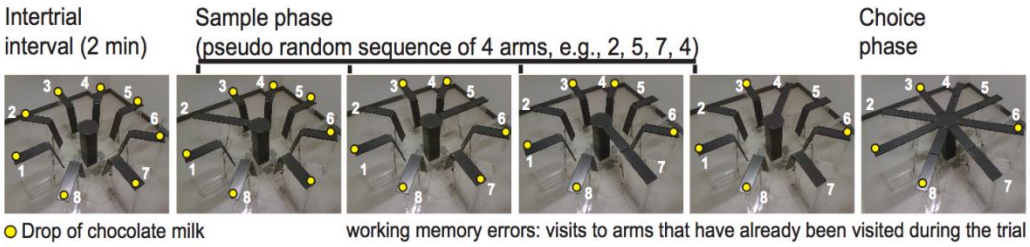
To induce selective lesion in the DG, we injected colchicine to various positions along the longitudinal axis of the DG (see materials and methods for details). The volume of DGCL reduced substantially while the rest of the hippocampus, including CA1 and CA3, was intact after colchicine injection (Figure 2A& B). Both the cvDG and rdDG lesion groups had 20% - 30% overall volume reduction of DGCL compared to the control group. However, the volume reduction of DGCL for the two lesion groups was limited to their corresponding areas, as shown in Figure 2B. On the other hand, the overall volume reduction in the entire DG lesion group was 60% - 80% compared to the control. In the entire lesion group,both caudal-ventral and rostral-dorsal DG region had substantial decrease in the volume of DGCL.

Number of active neurons in the DG

The neurons that expressed c-Fos were sparsely distributed in the DGCL, as shown in Figure 3A. Stereological methods were used to estimate the number of c-Fos labeled DG granule cells in different groups. The control group had a significant increase in the number of c-Fos labeled cells in both cvDGregion and rdDGregion compared to the home cage group(Figure 3B). On the other hand, there was no significant difference in the number of c-Fos labeled cells between the control group and the cvDGlesion group in the rdDG region and between the control group and the rdDGlesion group in the cvDG region. But a significant decrease in the number of c-Fos labeled cells was seen at the lesion sites in the DG for both lesion groups.

In Figure 3C, the density of c-Fos labeled cells in the DGCL was calculated by dividing the number of c-Fos labeled cells to the volume of the DGCL for each rat. There was no significant difference between the control group and the two lesion groups in both the cvDGregion and rdDG region.

A.



B.

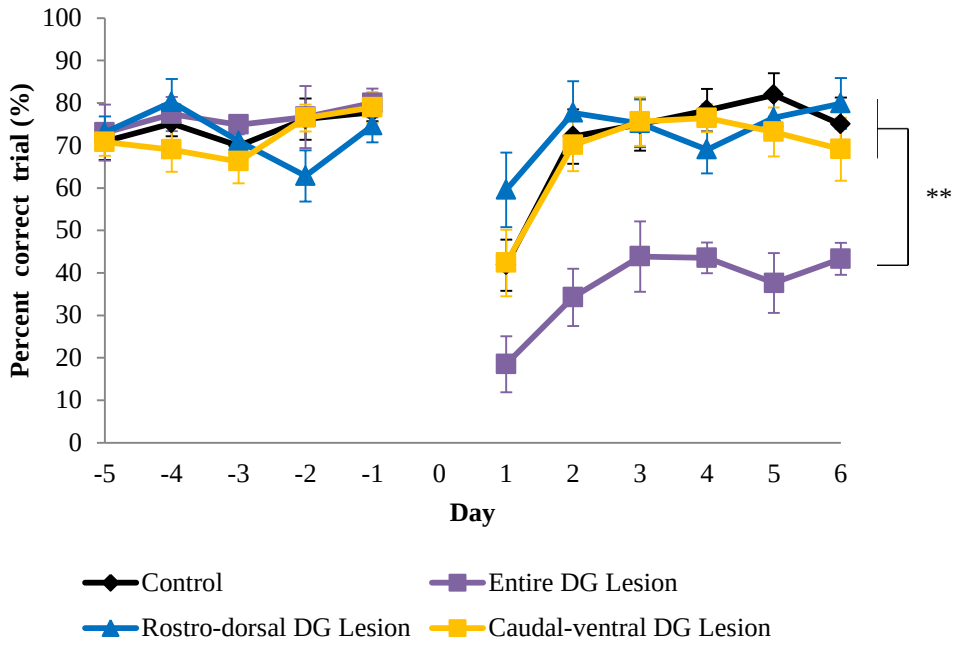
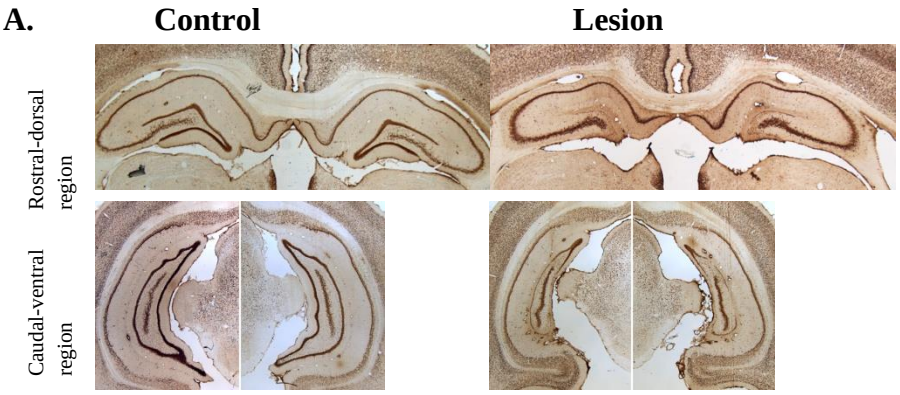


Figure 1. Working memory deficit after complete lesions of the dentate gyrus, but not after restricted lesions to either the rostral-dorsal or cadual-ventral subregions.

(A) An example of the eight-arm radial maze task is shown. The rats were forced to visit four pseudorandom arms to retrieve food reward. Next, all of the eight arms were raised during the choice phase. To complete the task correctly, the rats had to retrieve all the reward without revisiting any previous arm.

(B) Working memory performance is quantified by the percentage of correct trials on each training day. Day 0 was the surgery day while Day 1 was the first testing day after surgery. The control group (n=10), the rostral-dorsal DG lesion group (n=9), and the caudal-ventral DG lesion group (n=9) performed significantly better than the entire DG lesion group (n=11, $p<0.01$, Tukey test). Error bar was SEM.



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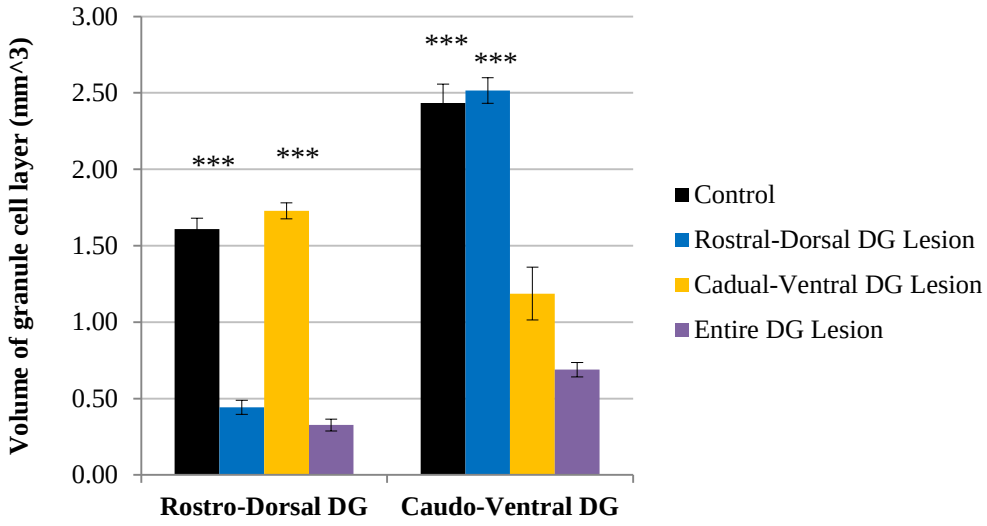


Figure 2.Volume reduction of dentate granule cell layer (DGCL).

(A) NeuNimmunostaining of the rats' brain slices from the control group and the lesion group included caudal-ventral DG and rostral dorsal DG.The size of DGCL decreased substantially as evidence by the comparison between the lesion slices and the control slices.

(B) Average volume of DGCL in rostral dorsal DG and caudal-ventral DG was quantified by the Cavalieri method for each group. Significant volume reduction of DGCL was seen in the corresponding lesion sites ($P<0.0001$, Tukey test). There were 8, 6, 8,7 rats in the control group, rostral dorsal DG lesion group, caudal-ventral DG lesion group, entire DG lesion group, respectively. Error bar was SEM.

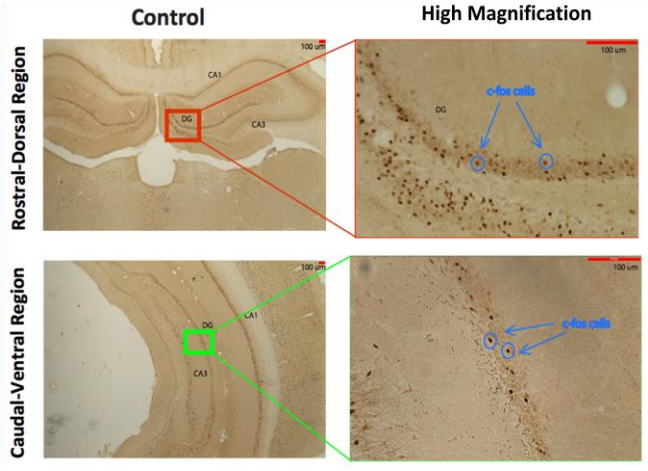
Figure 3. No compensation in the number of c-Fos labeled DG neurons in partial DG lesion.

(A) c-Fos immunostaining of the rats' brain slices from the control group included caudal-ventral DG and rostral-dorsal DG. The red boxes in the pictures of low magnification (left) showed the location of the pictures of high magnification (right). The red bar on the top left of each picture was a 100-μm scale bar.

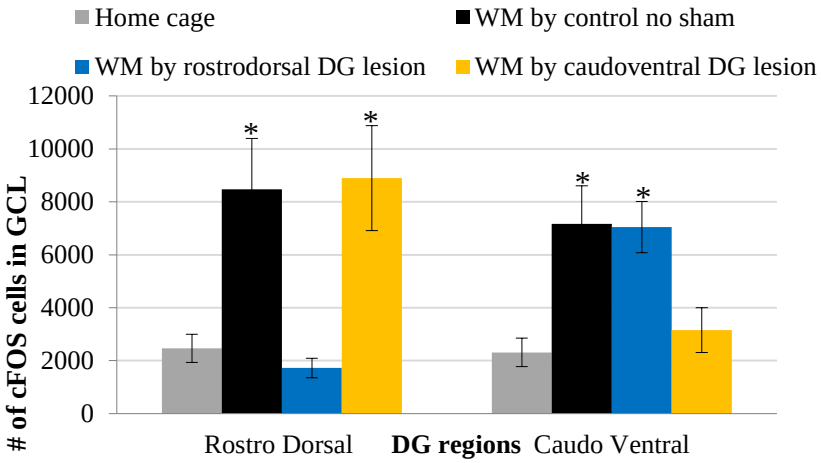
(B) Number of c-Fos labeled cells in each group was quantified by stereological methods. A significant increase in the number of c-Fos labeled cells in both rostral-dorsal DG and caudal-ventral DG was seen in the control group (n=8) compared to the home cage group (n=7,$p<0.05$, Wilcoxon Test). However, there was no significant difference between the control group and the caudal-ventral lesion group (n=8) in rostral-dorsal DG and between the control group and the rostral-dorsal DG lesion group (n=6) in caudal-ventral DG.Error bar was SEM.

(C) The density of c-Fos cells remained unchanged after partial DG lesion. The number of c-Fos cells was divided by the volume of the corresponding DG subregions. The bar graph showed the average density of each group (control, n=7;rostral-dorsal DG lesion, n=6; caudal-ventral DG, n=8). Error bar was SEM.

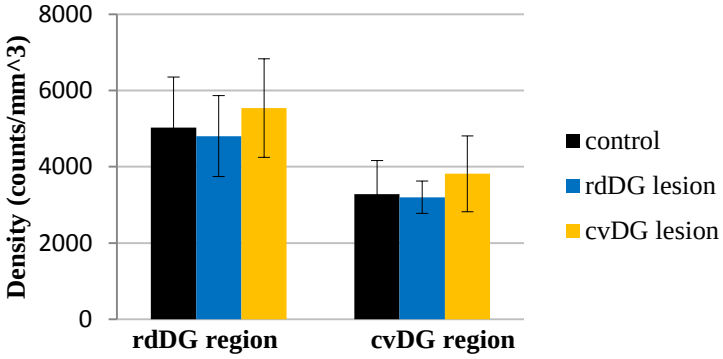
A.


Control
High Magnification
Rostral-Dorsal Region
Caudal-Ventral Region
c-fos cells
DG
CA1
CA3

B.


Home cage
WM by control no sham
WM by rostrodorsal DG lesion
WM by caudoventral DG lesion
# of cFOS cells in GCL
12000
10000
8000
6000
4000
2000
0
Rostro Dorsal
DG regions
Caudo Ventral

C.


Density (counts/mm^3)
8000
6000
4000
2000
0
rdDG region
cvDG region
control
rdDG lesion
cvDG lesion

III:

Discussion

Spatial WM is not localized to a subregion of the DG

NeuN immunostaining of the brain slices shows that colchicine selectively lesions the DGCL. The result confirms colchicine as a selective drug that kills the DGCs. Furthermore, the selective lesion to cvDG and rdDG is successful as evidenced by the volume reduction of the DGCL limited to the corresponding DG regions. The rats with lesion in both DG regions display spatial WM deficit, which confirms the DG is required for spatial WM. Since both the cvDG lesion group and the rdDG lesion group show no impairment on spatial WM from our previous study, the mechanism used by the DG to support spatial WM is not localized to a specific region along the longitudinal axis of the DG. Therefore, neither the ability to discriminate similar spatial inputs nor the DG-CA3 backprojection in caudal-ventral hippocampus is required for WM. The result suggests a novel DG mechanism that is independent of the position along the longitudinal axis for spatial WM. The finding is in contrast with the studies that have shown functional differences along the longitudinal axis of the hippocampus (Bannerman et al. 2004, Fanselow and Dong 2010, Kesner et al. 2011).

The level of neuronal activity stays the same in the remaining region of the DG

The active DGCs during the WM task are shown by c-Fosimmunostaining of the brain slices. The dispersed c-Fos labeled cells indicates the sparse network in the DG. The majority of the DGCs remain silent when they receive inputs from entorhinal cortex. However, the rats that perform the WM task have significantly higher number of active DGCs compared to the rats that stay in home cage. Therefore, the high level of c-Fos expression in the DG correlates with the WM task. In the DG lesion groups, the level of c-Fos expression at the remaining region of the DG is similar to that of control. The

results imply that the remaining DG alone can support WM without recruiting more DGCs from the same region. In other words, no compensatory activity is seen in the remaining DG of the DG lesion groups. Consequently, the total number of active DGCs is not critical to support WM. The result implies a lack of interaction between rdDG and cvDG during a spatial WM task. Instead, the interaction between the DG and CA3 through mossy fiber might be relevant for WM.

Looking Forward

The present study shows that it is necessary to remove both rdDG and cvDG to impair spatial WM. Additionally, removing part of the DG does not affect the remaining DG in terms of the number of active neurons. Thus, it is reasonable that the remaining DG supports WM through its direct downstream target, CA3, in the hippocampus.

Interestingly, a recent study demonstrates electrophysiological evidence for spatial WM in CA1, which is the direct downstream target of CA3 (Jadhav et al., 2012). In this study, a brief but intense high-frequency oscillation (100-250 Hz) known as sharp-wave ripple was disrupted during behaviors. Animals with disrupted awake sharp-wave ripples had impaired performance in a spatial alternation task. Because sharp-wave ripples originate from CA3, the DG might help generate sharp-wave ripples (Csicsvari et al., 2000). To test this hypothesis, it is necessary to do extracellular recordings in CA3 after complete DG lesion. Since the structure of CA3 is intact after the DG lesion, extracellular recording of this area is possible, though the tissue shrinkage can make targeting CA3 difficult. If the DG contributes to generating sharp-wave ripples in CA3, then the animals with complete DG lesion will have less sharp-wave ripples during a WM

task. The results from the proposed study can potentially reveal the electrophysiological mechanism used by the DG to support spatial WM.

IV:

Materials and Methods

Animals

4 months oldLong Evan male rats were used for this study. All the rats were maintained on a 12/12-h light/dark inverted cycle throughout the course of the experiment, so we could perform the experiments in the dark phase of the animals. Each rat was housed in one individual box. Food restriction on the rats started two days before the training and two weeks after the surgery. During food restriction, the percent weight of the rats was kept at around eighty five. Once the rats were at learning plateau, which was defined as over sixty percent of correct trials, for five days, food restriction was removed and the rats received either colchicine or sham injection two days after.

Behavior on the Eight-Arm Radial Maze

Initially, the rat was allowed to explore all the arms of the eight-arm radial maze in a ten-minute habituating session. At the end of each arm, there was a drop of chocolate milk that attracted the food-deprived rats. The rat received one habituating session per day. Once the rat became comfortable moving around on the radial maze and started to eat chocolate milk, the sound of opening and closing the arms was introduced and a thirty-minute training session started on the following day. Each training session could include up to ten training trials. One training trial was consisted of forced phase and choice phase. During the forced phase, four baited arms were randomly opened one at a time for the rat to retrieve chocolate milk at the end of the arm. In other words, the rat was forced to visit the pseudo randomly-selected four baited arms sequentially. The four arms couldn't be all adjacent to each other because the rats could solve the task in a stereotypical strategy. For example, the rats could always turn right to solve the task. This kind of stereotypical strategy didn't involve hippocampus. After the rat visited the fourth

arm, all the arms opened at the same time. The rat could choose whichever arm to visit at this point, but only the four arms that he hadn't visited during the forced phase had the chocolate milk (correct arms). A training trial ended when the rat retrieved all the chocolate milk and started to move back towards the center of the radial maze. There was a two-minute wait between trials to ensure that the information from the previous trial didn't interfere with the current trial. During the wait, a cylinder was placed on top of the rat and the eight arms were re-baited with chocolate milk. The rat completed a correct trial only when he visited all the correct arms once. During the choice phase, if the rat re-entered one or more arms that he already visited (errors), the trial was considered incorrect. The rat's performance was evaluated on the percent correctness of trials and the average of errors in one training session. When the rats reached a criterion of an average over sixty percent of correct trials in five consecutive days with an average error no more than one, they were schedule for one random chosen surgery. After two weeks of recovery from the surgeries, the rats receive the training on the same task again until their performances were stable for five consecutive days.

Surgeries

The rats were scheduled for one of the following surgeries: control no sham, lesion or shams control in the dorsal DG, the ventral DG, or the entire DG. The DG lesions were performed by multiple and bilateral stereotaxic injections of colchicine in the entire or different parts of the DG. In the entire DG lesion, there were six sites of colchicine injection on one hemisphere while there were only two in the dorsal and ventral lesion. Colchicine was shown to be a selective drug to kill DGCs (Goldschmidt et al., 1982). The concentration of colchicine was 2.5μg/μL in 0.1M PB. 0.04μL was

injected at each site at a rate of 0.2nL/second. Shams control surgeries were performed injecting buffer phosphate 0,1M, which was where the colchicine was dissolved. For control no sham surgeries the craniotomy was done without any injection.

Histology and Stereology

The rats were perfused one hour after they completed the last training session with paraformaldehyde 4 %. The rats' brains were then placed in 30% sucrose solution until the brains sank to the bottom of the test tubes. Serial sections (40μm) were cut through the whole brain coronally on a Leica microtome. The brain slices were stored in PBS with sodium azide.

One in every six brain slices were used to do NeuN and c-Fos immunohistochemistry. On the first day, the brain slices were pre-treated with 0.6% hydrogen peroxide in 1x PBS solution for 15 minutes. Next, the brain slices were placed in blocking solution for 60 minutes. The brain slices were then placed to primary antibody solution in PBS overnight. The dilution factor was 1 to 10000 for NeuN and 1 to 1400 for c-Fos. On the second day, the brain slices were placed in secondary antibody solution in PBS overnight after primary antibody solution was washed off. The dilution factor for both NeuN and c-Fos was 1 to 1000. On the third day, the brain slices were placed in ABC solution for 1 hour after secondary primary antibody was washed off. Then, the brain slices were transferred to the DAB solution, which was consisted of 1 DAB tablet, 30 μL $NiCl_2 \cdot 6H_2O$, and 5 μL of 30% H_2O2 (was added just before use) in 10 mL of 1x PBS solution. The DAB reaction time was 1 minute for NeuN and 20 minutes for c-Fos. After the DAB reaction was completed, the stained brain slices were mounted on microscope slides.

The stained brain slices were examined by using a Leica microscope and a CCD camera. Cavalieri method was used to quantify the volume of the granule cell layer of the DG while Stereology method was used to quantify the number of c-Fos labeled DGCs with the help of Stereo Investigator software. The whole DG was separated into two parts: rostral-dorsal and caudal-ventral. The rdDG was considered as the upper and anterior half of the DG while the cvDG was considered as the lower and posterior half.

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