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Effects of Mild Dehydration Stress on Spatial Learning and Brain Transcriptome Profiles in Aged Mice

노인기 마우스에서 가벼운 탈수 스트레스가 공간 기억 능력 및 뇌 전사체 변화에 미치는 영향 연구

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Abstract

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Severe dehydration has detrimental impact on various physiological functions in a whole lifespan. However, the effects of dehydration on brain functions in the elderly have not been fully elucidated yet. Therefore, we aimed to study the effects of mild dehydration on cognitive functions in the elderly using 2-year-old C57BL/6 mice. A limited access to water bottle of 15 min/day for 2 weeks sufficiently established dehydration mice model (DEH) and their physiological parameters and cognitive functions were compared with those of control mice with ad libitum water intake (CON). Dehydrated mice showed significantly higher level of plasma osmolality and vasopressin expression in the brain than control mice. FACS analysis of peripheral blood presented the percent of neutrophils was higher in DEH com-

pared to CON. DEH had significantly higher concentration of serum corticosterone than CON. Depression statuses determined by TST were not different between DEH and CON groups. Next, their spatial learning and memory were measured by the Barnes maze test. Surprisingly, dehydrated mice showed better performance in spatial learning and spent less time to find the target during the test than control mice. To find the underlying mechanism, transcriptome of hippocampus were analyzed by RNA-sequencing. Transcripts related to functions of synaptic plasticity and long-term potentiation were dramatically activated in dehydrated mice compared to those of control. The immunostaining analysis of GFAP+ cells in DG and CA1 regions of hippocampus showed the numbers of astrocyte were greatly higher in DEH than CON. Taken together, although mild dehydration led to increase in the numbers of inflammatory cells in the blood, it promoted spatial learning and memory by modulating ADH-corticosterone axis and thereby enhancing synaptic plasticity. These results might provide new insights in the roles of mild physiological stress of dehydration played in cognitive functions in senescence.

Key words: Cognitive decline, Hippocampus, Spatial memory, Corticosterone, RNA-seq, Synaptic plasticity

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List of Abbreviations

AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid type glutamate
ANOVA	analysis of variances
DEG	differentially expressed gene
GABA	gamma-Aminobutyric acid
GFAP	glial fibrillary acidic protein
HPA axis	hypothalamic-pituitary-adrenal axis
LTP	long term potentiation
NMDA	N-methyl-D-aspartate
PCA	principal component analysis

I. Introduction

Water is the most abundant, but crucial and indispensable nutrient for all known life forms. In human body, water carries out innumerous functions, such as protecting organs, maintaining body temperature, and transporting nutrients (Jéquier and Constant 2010, Kim and Shin 2016). It has been well established that proper hydration status is essential to maintain physiological homeostasis and a healthy life. In healthy adults, water approximately comprises up to 70% of their body weight (Arnaud 1998). However, as senescence progresses, the proportion of water in human body starts to decrease from 70% to 50% of its body weight (Friis-Hansen et al. 1951, Hooper et al. 2014). It is thought to be that this decrement occurs, because the sense of thirst becomes less sensitive as people getting older and this leads to imbalance in volume of water intake and excretion (Ainslie et al. 2002). Furthermore, according to the recent study, the volume of water lost through skin and respiration also increases with age and it can negatively impact water homeostasis in elderlies (Dmitrieva and Burg 2011). Therefore, this reduction of body water in aged people means that the capacity for buffering against water-loss is also reduced, which makes elderlies more vulnerable to dehydration than young ones (Rikkert, Melis, and Claassen 2009).

There are two main types of dehydration, salt loss and water loss de-

hydration. As briefly mentioned above, as aging ensues, the mechanism which regulates thirst and fluid ingestion response become less effective (Morley et al. 1998, Mack et al. 1994). According to US National Health and Nutrition Examination Survey III cohort, about 28% of elderlies over 70 years old are exposed to water-loss dehydration (Stookey 2005). This implies that the water-loss dehydration is becoming the major nutritional and health problem in the post aged society.

The impact of dehydration on human has been researched in various aspects such as physical, physiological, and cognitive function. A majority of previous studies regarding physical and physiological impacts reported that severe dehydration status have association with decreased physical performance and chronic health problems such as kidney stone, infections, and pressure ulcers in elderlies (Hooper et al. 2014, Rikkert, Melis, and Claassen 2009, Rolland et al. 2006). However, in the case of cognitive function, the effects of mild dehydration has been controversial. Elderlies put in acute dehydration showed depressive mood and decreased cognitive performance in short term memory and attention span, whereas other older people put in condition of mild dehydration did not show any impairment in cognitive function or depressive mood (Suhr et al. 2004, El-Sharkawy, Sahota, and Lobo 2015, Secher and Ritz 2012, Ganio et al. 2011). Furthermore, according to the

recent study, the subject exposed to acute water-loss dehydration showed decreased performance in attention, memory, and psychomotor processing compared to those of control group. However, accumulated evidences are not sufficient enough to conclude dehydration was the cause of this results, because rehydration did not make any improvement in impaired cognitive function (Suhr et al. 2004). Thinking of innumerous functions of water in human body, it is plausible to assume water-loss dehydration is likely to have some effects on cognitive function. Still, the exact impact of dehydration on cognitive function remains controversial (Benton and Young 2015).

Cognitive function, especially impairment in spatial learning is an inevitable consequence of natural aging (Baquer et al. 2009, Erickson and Barnes 2003). The process of this cognitive deficit is not yet fully elucidated, but there are psychological factors that prevent or ameliorate the process. Stress is the biological response of an organism to changes in environmental condition. It can activate the hypothalamic-pituitary-adrenal (HPA) axis and make adrenal gland to secrete glucocorticoid (Bizon et al. 2001, Ulrich-Lai and Herman 2009). Glucocorticoids have many physiological roles in human body and they are associated with nutrient metabolism, stress integration and cognitive function (Porter, Herman, and Landfield 2001, Sapolsky, Krey, and McEWEN 1986). Since water-loss dehydration can be a stressor and activate

the HPA axis, it may be able to affect cognitive function negatively or positively. Furthermore, improper hydration state will increase the plasma osmolality, then osmoreceptors at hypothalamus will detect this increase and signal posterior pituitary to secrete arginine vasopressin which also can modulate the secretion of glucocorticoid from adrenal gland (Toufexis et al. 1999, GOLAND et al. 1991, McKinley and Johnson 2004). These rather complex associations between dehydration and stress is may be the cause of controversial and inconsistent results of previous studies regarding cognitive function and dehydration in aged people might be due to the level of stress they received in experiments.

Previous studies about the relationships between dehydration and cognitive function showed that acute severe dehydration was associated with decline in cognitive performance, but little is documented about the effects of mild dehydration and even those results are inconsistent. Thus we aimed to study the effects of mild dehydration on cognitive function in the aged. We hypothesized that mild dehydration may have different impact on cognitive function from acute severe dehydration. To test this hypothesis, mild dehydration model was induced in 2-year-old C57BL/6 mice by restricting their access to a water bottle for 15 minutes per day. Plasma osmolality and brain vasopressin mRNA level was evaluated to check whether mild dehydration

was successfully induced or not. Also, all mice were tested by The Barnes maze and tail suspension test (TST) to evaluate their spatial learning and depression during water restriction period. Then, trancriptome analysis was conducted to elucidate the underlying molecular mechanism of mild dehydration induced changes in spatial learning ability. These results might provide new insight in the impact of dehydration on spatial learning and cognitive function in senescence and it may be applicable to clinical purposes.

II. Materials and Methods

1. Laboratory animals

Male C57Bl/6J mice, 2-year-old, (Jackson Laboratory, ME, USA) were housed at animal facility in the college of veterinary medicine of Seoul National University. Mice were randomly divided into two groups: Control (CON, n=8) and Dehydrated (DEH, n=12). All mice were given ad libitum access to rodent diet (AIN-93G) and kept under 12:12-hour light-dark cycle with 60% relative humidity. After completing all tests, mice were sacrificed via intraperitoneal 20% urethane (U2500, Sigma-Aldrich, MO, USA) injection and collected blood, whole brain and hippocampus. Collected bloods were clotted at room temperature, then centrifuged at 2,000 x g for 10 minutes and collected resulting supernatant. All collected samples, except blood, were stored in liquid nitrogen immediately then moved to -80ºC deep freezer. All experimental procedures were carried out according to guidelines that approved by International Animal Care and Use Committee (IACUC) in Seoul National University (Approval Number: SNU-131203-4, Seoul, South Korea).

2. Water restriction

Mice in dehydrated group had restricted access to water for 14 days. Dehydrated mice only had access to water bottle for only 15 minutes per day during day time, while other animals in control group were given free access to water bottle during whole experiment. To verify mild water dehydration was successfully carried out, body weight, diet and water intake of mice were recorded on daily basis. Also, after sacrificing mice, plasma osmolality was measured using Fiske 210 Micro-Sample Osmometer in all mice (Fiske, Norwood, MA, USA).

3. Barnes maze test

The Barnes maze test is a psychological laboratory tool for assessing spatial learning and cognitive function at laboratory animals. The Barnes maze was constructed and tested as the previous research described (Patil et al. 2009).The test was lasted for 4 days with 2 training trial for each day and after each trial, the entire maze was thoroughly cleaned with 70% ethanol to remove any trace of last trial. On the fourth day, to assess spatial learning ability of mice, only one trial was carried out for each mouse. Each run was video recorded and analyzed by Ethovision XT10 (Nodulus, Wageningen,

Netherlands) to assess parameters such as latency to find the target hole, total moved distance, and mean velocity.

4. Tail suspension test

TST was performed as Can et al. proposed (Can et al. 2012). TST was conducted at two time periods, before water restriction and after 1 week of water restriction. All mice were suspended at the bar of TST apparatus by attaching adhesive paper tape to their tail. The distance between the floor and all mice was adjusted to approximately 20 cm. The test itself was lasted for 6 minutes and it was all video recorded. The immobility time of during the test was analyzed by using Ethovision XT10 (Nodulus, Wageningen, Netherlands).

5. Corticosterone quantification

Deep frozen serum samples were thawed and prepared at room temperature. Serum corticosterone was quantified by triplicate using corticosterone Enzyme-Linked ImmunoSorbent Assay (ELISA) kit (ab108821, Abcam, UK) following the protocol provided by the manufacturer.

6. Immunohistochemistry

Mouse hippocampi were dissected from whole brain samples and were used for immunohistochemistry. Sagittal sections (35μm thick) were cut by microtome (Leica CM1850, Leica Microsystems, Wetzlar, Germany) and floated onto SuperFrost®Biomedicals (Santa Monica, CA, USA). It was applied according to standard protocol and incubated for 24 hours at 4ºC and washed with PBS (3 times * 10 minutes). After incubation with primary antibodies, secondary antibody, Alex 488 (1:500, Thermo Fisher Scientific, MA, USA) was conjugated to glial fibrillary acidic protein (GFAP). After 1 hour incubation with secondary antibody at room temperature, slides were washed with PBS (3 times * 10 minutes). DAPI was stained with DNA-specific fluorescent Hoeschst 33342 (Thermo Fisher Scientific, MA, USA) and then slides were mounted on a slide with Gel/Mount aqueous mounting medium (Sigma-G0918, Sigma Aldrich, MO, USA). Fluorescent imaging of sections were conducted using Leica LSM410 microscope (Leica Microsystems, Wetzlar, Germany). Fluorescent imaging of sections were conducted using Leica LSM410 microscope (Leica Microsystems, Wetzlar, Germany).

7. FACS (fluorescence activated cell sorter) analysis

After sacrificing mice, peripheral blood was immediately collected. Collected bloods were incubated with ammonium-chloride-potassium buffer at room temperature to isolate peripheral blood lymphocytes from whole blood for 5 minutes. Then, it was stained with FACS buffer made of 1 X phosphate-buffered saline, 0.1 % bovine calf serum and 0.05 % sodium azide at 4 ºC for 30 minutes. This buffer also contained fluorescein isothiocyanate (FITC)-conjugated monoclonal antibody to Ly6G (eBioscience, San Diego, CA, USA) and phycoerythrin-conjugated monoclonal antibody to CD11b (eBioscience, San Diego, CA, USA). After sufficient washing with FACS buffer, stained cells were analyzed with FACScalibur (BD bioscience, Franklin Lakes, NJ, USA) and Flowjo software (Tree star, Ashland, OR, USA).

8. RNA isolation

Using DNA-free RNA isolation kit (RNAqueous-4PCR kit; Ambion, TX, USA), total RNA was extracted from hippocampus of mice as protocols provided in manufacturer's manual. Extracted total RNA quality and yield were checked using NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, MA, USA).

9. RNA - Sequencing analysis

Total RNA was isolated from mice hippocampus as described in above. The isolated total RNA was checked its integrity and quantity by using Agilent 2100 Bioanalyzer (Agilent Technologies, CA, USA) and mRNA was isolated from 1.5 μg of total RNA sample with MicroPoly(A)Purist kit (Thermo Fisher Scientific, DE, USA) following manufacturer's protocol. Isolated mRNA samples were fragmentized and hybridized with Ion Total RNA seq kit v2 (Thermo Fisher Scientific, DE, USA). Then, according to the protocol provided by manufacturer, cDNA was synthesized through reverse transcription of prepared mRNA samples. Synthesized cDNA was tagged with barcode at 3' terminus with Ion express™ RNA-Seq Barcode kit (Life technologies, Carlsbad, CA, USA). Only cDNA with more than 150 base pairs were used to construct cDNA library. Template-positive Ion Sphere Particles were generated by using Ion PI™ Hi-Q™ OT2 200 kit and Ion OneTouch™ System kit (Life technologies, Carlsbad, CA, USA). Ion Sphere Particles were enriched with diluted cDNA library and loaded onto Ion Proton PI chip v3 (Life technologies, Carlsbad, CA, USA). RNA-seq analysis of loaded chip was performed using Ion Ptoton™ Semiconductor sequencing system (Thermo Fisher Scientific, DE, USA).

10. Bioinformatic analysis of RNA - Sequencing data

Using Partek Genomics Suite software v6.6 (Partek, Saint Louis, MI, USA), the level of mRNA was normalized and quantified. Normalized sequences were aligned with UCSC mouse reference geoneme mm10 and mapped to the GENCODE Genes-release M8. Transcripts have more than 0.5 reads per kilobase per million mapped reads (RPKM) values with changes over 2 fold were screened. With these transcripts, we conducted ANOVA and excluded transcripts with more than 0.05 p-value. Further analysis like pathway analysis and functional enrichment were conducted with Ingenuity Pathway Analysis software (Qiagen, Hilden, Germany).

11. Quantitative real-time PCR

As described above total RNA was extracted from mouse brain and it was used to synthesize cDNA by using MessageSensor^{TM} RT kit (Ambion, TX, USA). The qPCR was performed using SYBR-GREEN master mix (Applied Biosystem, CA, USA) combined with appropriate dilution of cDNA and relative mRNA level was quantified by $2^{-\Delta\Delta Ct}$ method. Vasopressin and Brain Derived Neurotropin Factor (BDNF) gene expression level was

assessed and normalized by housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in each sample. Below is the primer sequence for each gene. BDNF: Forward 5'-AAAGTCCCGGTATCCAAAGGCCAA–3', Reverse 5'- TAGTTCGGCATTGCGAGTTCCAGT-3'. GAPDH: Forward 5'-TGCACCACCAACTGCTTAG-3'.Reverse 5'-GATGCAGGGATGATGTTC-3'. Vasopressin: Forward 5'-CCAGGATGCTCAACACTACG-3', Reverse 5'-CTCTTGGGCAGTTCTG- 3'.

12. Statistical analysis

All statistical analyses excluding RNA sequencing results were carried out using Prism Graphpad version 5 (GraphPad Software Inc., CA, USA). Unpaired Student's *t*-test was used to check effects of dehydration for parameters like body weight, diet and water intake, TST, FACS analysis and the Barnes maze test results. Data were presented with the mean + SEM.

III. Results

1. Water restriction regime induced mild dehydration in aged mice.

After one week of acclimation to new environment, 2-year-old male C57BL/6J mice were randomly assigned to control group (CON, n=12) and dehydration group (DEH, n=8) (Fig. 1). Through the experimental period, DEH group of mice were provided with water bottle for only 15 minutes a day at same time. DEH mice daily consumed approximately 25% of water compared to CON group that had free access to water bottle during experimental period (Fig. 2A; $p < 0.001$). During first 3 days of water restriction period, the mean weight of diet intake in DEH group was dramatically decreased to about 25% of diet intake of CON (Fig. 2B). However, as water restriction prolonged, mice of DEH group started to consume more diet and it was restored to 77% of CON's (Fig. 2B; $p < 0.005$). Although, body weight of DEH gradually decreased during experimental period due to the reduction in water and diet consumption, there was no significant difference between DEH and CON (Fig. 2C). To verify whether the water restriction regime efficiently induced mild dehydration in those mice, we determined the levels of plasma osmolality and brain vasopressin mRNA expression, Mice in DEH

had significantly higher plasma osmolality and vasopressin mRNA expression level than those in CON (Fig. 3A, B; $p < 0.005$).

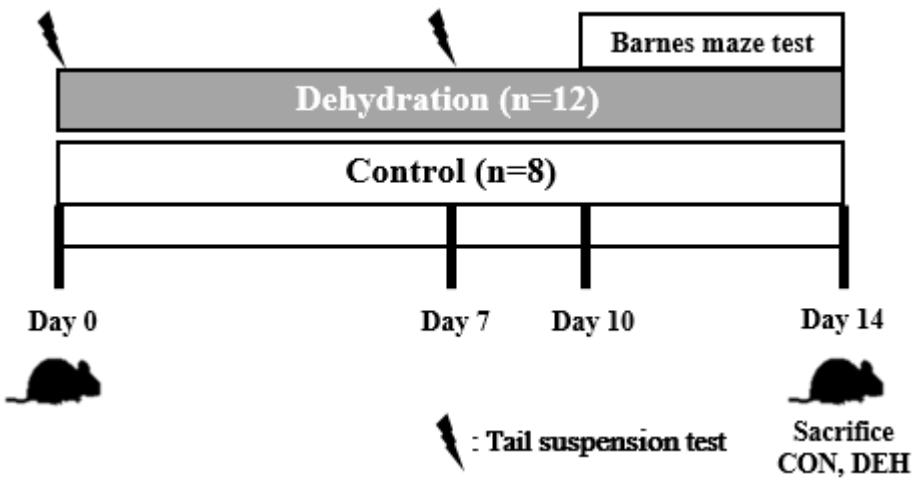

Figure 1. Experimental scheme

Mild dehydration was induced in aged mice during 2 weeks of experimental period. 2-year-old male C57BL/6J mice were randomly assigned to two groups, CON and DEH. During whole period of the experiment, CON mice consumed water *ad libitum*, while DEH mice had limited access to the water bottle. Water bottle was provided to DEH group for only 15 minutes a day. TST was conducted 2 times, at day 0 and 7. After 10 days of water restriction, The Barnes maze test was performed for 4 days and all mice were sacrificed at the end of the Barnes maze test.

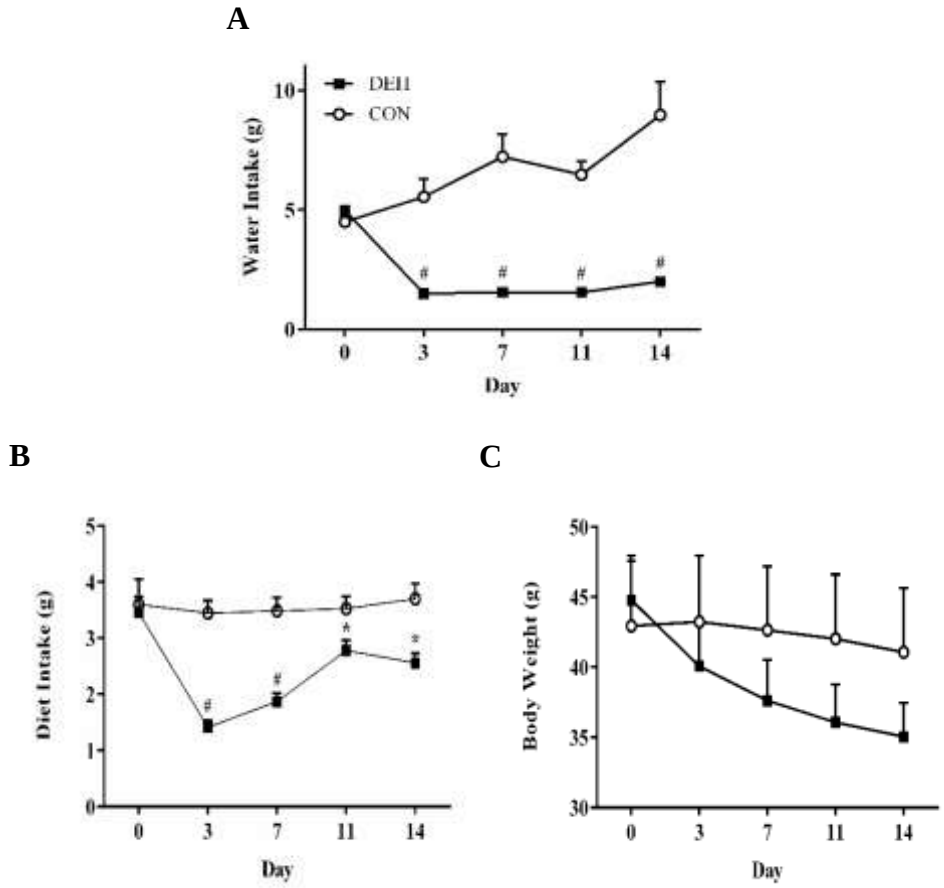

Figure 2. Mild dehydration induced physiological changes

(A) The daily water intake of DEH group was greatly reduced during water restriction period. (B) As water restriction started, daily diet intake of DEH mice was dramatically decreased to almost one third compared to CON. However, it gradually followed up to 80% of normal range. (C) The body weight of DEH mice gradually decreased, but there was no significant difference between DEH and CON. All data are shown as mean + SEM. Student's *t*-test was performed to determine significant difference in DEH compared with CON. * $p < 0.05$, #$p<0.001$

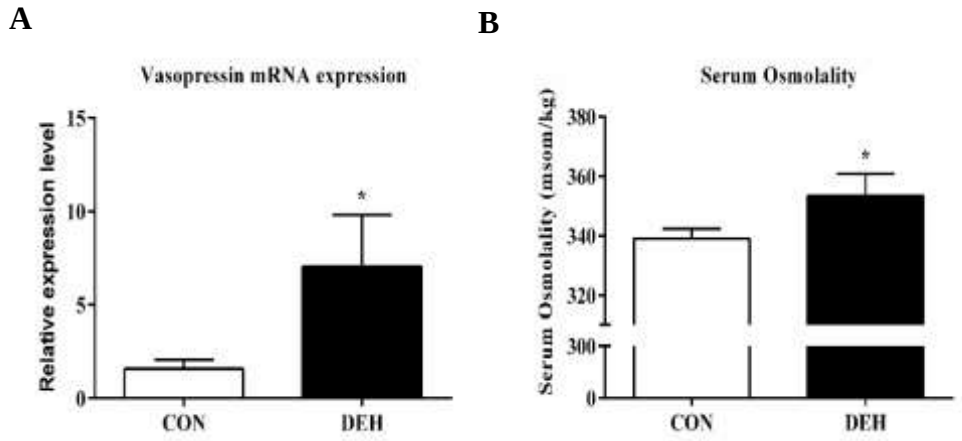

Figure 3. Two weeks of mild dehydration was enough to increase plasma osmolality and brain vasopressin mRNA level

(A) Serum osmolality was assessed by using Osmometer. 2 weeks of mild dehydration was sufficient enough to significantly increase plasma osmolality in DEH group. (B) Brain vasopressin mRNA level was quantified by qPCR and the transcriptome level of vasopressin was normalized with GAPDH. In DEH, the level was almost 7 times increased compared to CON. All data are shown as mean + SEM. Student's *t*-test was performed to determine significant difference in DEH compared with CON. * $p < 0.05$

2. Spatial learning ability of aged mice was improved by mild dehydration

To study the effect of dehydration on cognitive function, we conducted the Barnes maze test in both DEH and CON group. After 10 days of water restriction, both group started to take the Barnes maze training trial for 4 consecutive days and the test trial was run at last day.

First, to validate the efficacy of the Barnes maze protocol we used, we conducted the Barnes maze test using young mice that were 6-week-old C57BL/6J mice (n=4). Those young mice started to show the significant reduction in the latency to find the goal at day 3 compared to the performance at day 1. However, on the contrary to those young mice, old mice in this experiment failed to show the effect of training to find the goal (Fig. 4A; $p < 0.005$). This result confirms that the protocol we applied was valid and efficient in spatial learning. In the case of CON and DEH mice, surprisingly, a decrease in primary latency was observed in day 4 in DEH mice, indicating that they learned the task over the 4-day training period (Fig. 4B; $p < 0.005$). Mice in CON did not show any effect of 4-day training in finding the target hole (Fig. 4B).

The average speed (cm/sec) from the center to enter the target hole

was analyzed. Fig. 5A is the representative heatmap images of training phase and test day. We can observe from heatmap images that CON mice showed no improvement in spatial learning performance after 4 days of training, while DEH mice showed large improvement in finding the escape box (Fig. 5C). Average speed (cm/sec) of both CON and DEH group did not show significant difference between them due to the large individual difference in the group, however, as training progressed, each group showed opposite trend that average velocity of DEH increased, while that of CON decreased (Fig. 5D).

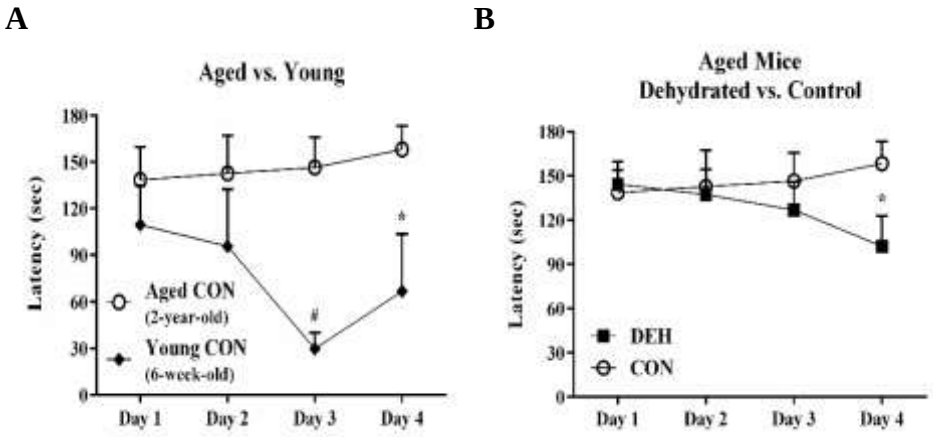

Figure 4. Primary latency of Young, CON, and DEH during the Barnes maze test

(A) Efficacy of the protocol used in this experiment was validated by conducting the Barnes maze test with young and aged mice. Young mice showed better performance in finding the target hole at day 3 and 4. (B) At day 4, the primary latency of dehydrated mice was significantly lower than that of CON. All data are shown as mean + SEM. Student's *t*-test was performed to determine significant difference in DEH compared with CON. * $p < 0.05$

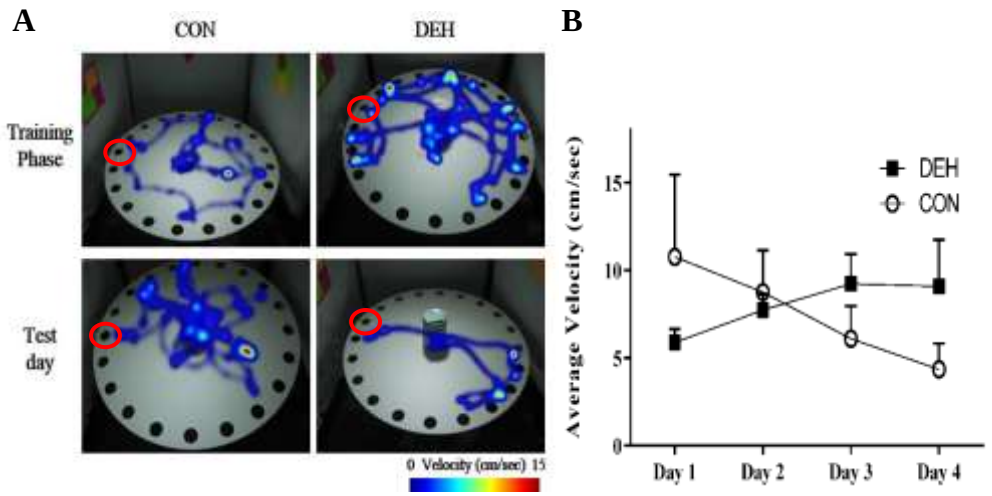

Figure 5. Heatmap images of first and last day of the Barnes maze test

(A) Representative heatmap images of each group at first and last day of the Barnes maze test. DEH mice showed decreased wondering distance compared to CON. Red circle indicates the target hole. (B) Total distance mice moved was divided by their primary latency. All data are shown as mean + SEM. Student's *t*-test was performed to determine significant difference in DEH compared with CON.

3. Gene expression in mice hippocampus was largely altered by mild dehydration

To elucidate the molecular mechanism of mild dehydration in improving spatial memory, we conducted next generation RNA sequencing analysis of mice hippocampus. Total 7 mRNA sample from mice hippocampus, 3 from DEH and 4 from CON, were prepared and loaded on ISP beads. After amplifying loaded samples, ISP beads were stacked to two Ion PI^{TM} chips. Loading density of both chips were 97 % and 96 % and enrichment rate was 100% (Fig. 6A and B).

Before proceed to pathway and functional analysis, we checked the variation of gene expressions in DEH and CON by conducting principle component analysis (PCA) with the RNA sequencing data. The result of PCA clearly demonstrated the difference in transcriptome profile between dehydrated and control mice, which means mild dehydration indeed altered gene expression profile in mice hippocampus (Fig. 7).

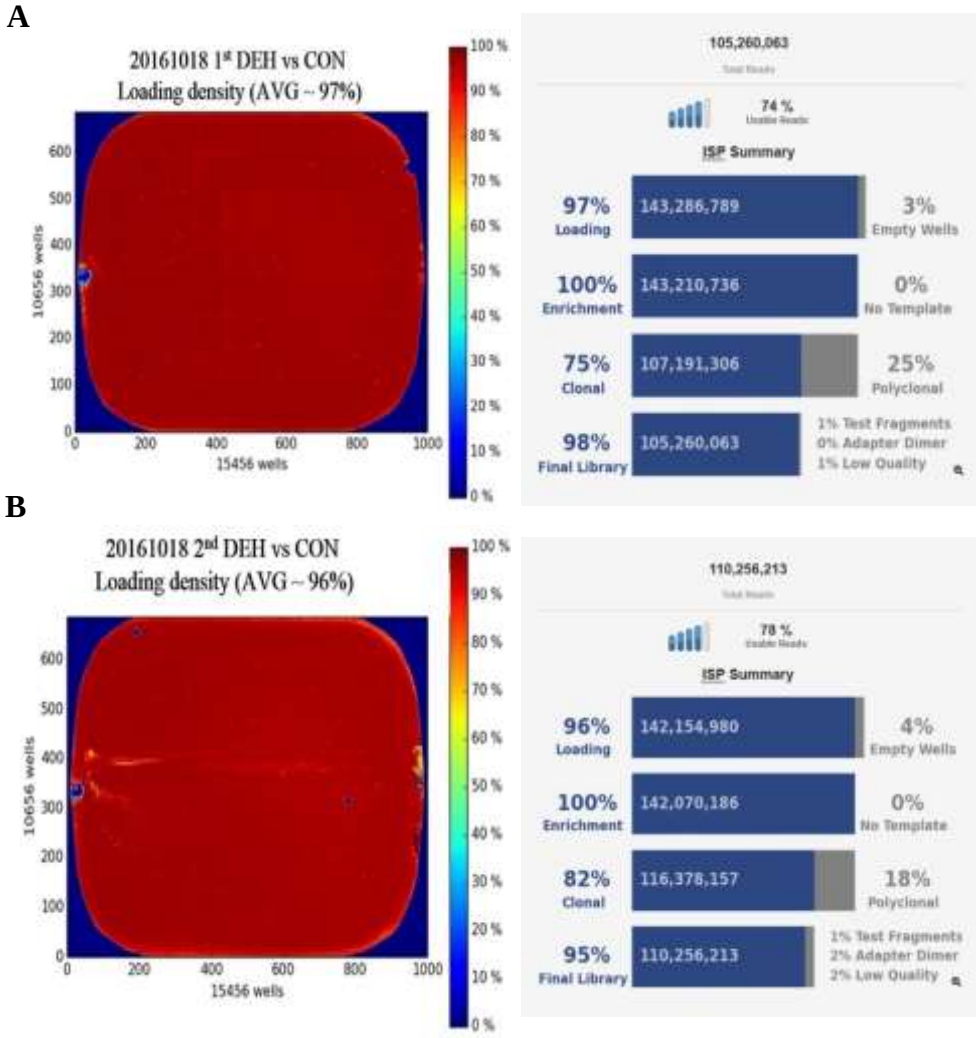

Figure 6. Loading density of IPS beads on Ion™ chips

RNA sequencing analysis was conducted to analyze transcriptome profiles of hippocampus. IPS beads of DEH and CON cDNA libraries were loaded onto two Ion™ chips. The degree of loading density was expressed by colors on chips.

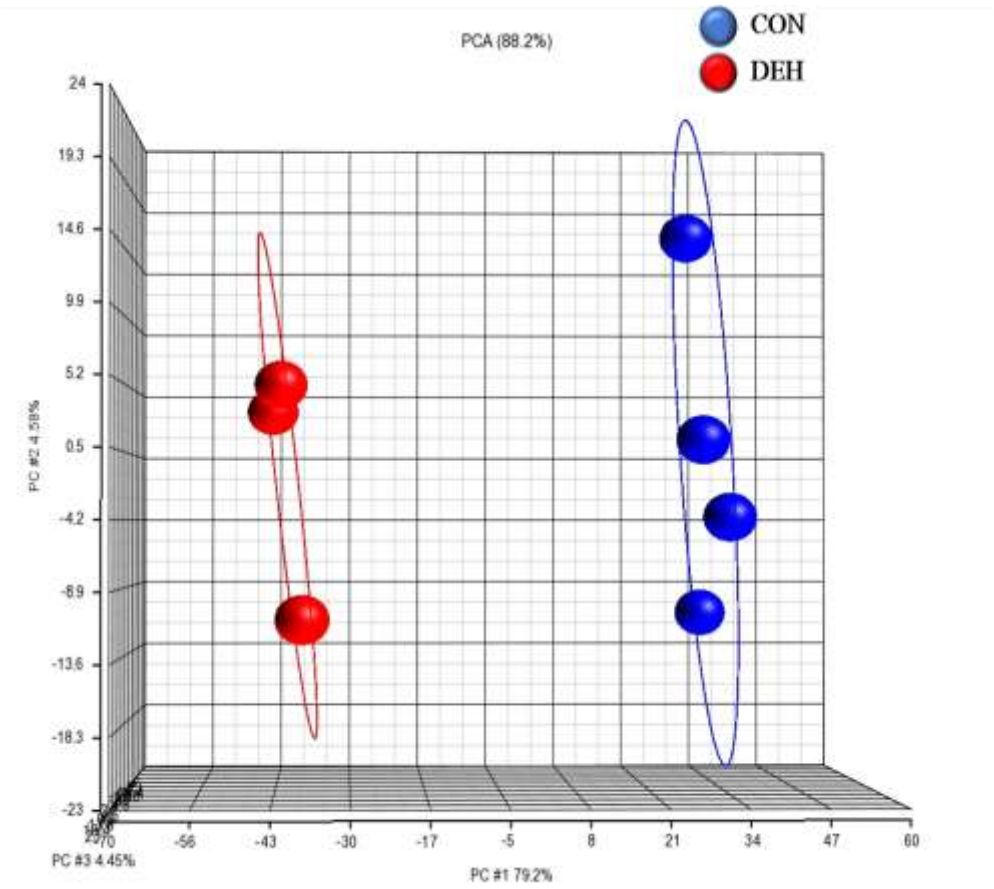

Figure 7. 3-D score plot of principal component analysis

Principal component analysis of RNA-seq data from hippocampus of dehydrated and control mice. Each sphere on the plot represents individual mouse and colors of it indicates which group it belongs. Red and blue spheres indicate DEH and CON.

4. Genes regulating plasticity of synapse were differentially expressed by mild dehydration

From total RNA sequencing data, we first trimmed data using RPKM. Genes with less than 0.5 RPKM were cutoff, then we identified differentially expressed genes by ANOVA analysis ($p<0.05$). Among these differentially expressed genes (DEGs), genes showed at least 2 fold up or down regulation, 1109 genes, were selected and used for further analysis.

With these genes, hierarchical clustering analysis was conducted to investigate trancriptome profiles of both groups. Differentially expressed gene profiles of DEH and CON were very distinctive and readily distinguishable between them (Fig. 8). Each column clustered within same group first and lastly clustered with other group. This finding implies that mild dehydration significantly altered gene expression in mice hippocampus.

To gain further insight about these differentially expressed genes, we conducted functional category analysis with IPA. Top 10 most significantly altered functional category of these genes were such as; cell morphology, cellular development, cellular growth and proliferation, cell cy-

cle, lipid metabolism, small molecule biochemistry, molecular transport, cell death and Survival, cell-to-cell signaling and interaction and cellular assembly and organization (Fig. 9).

We confirmed that mild dehydration induced significant molecular changes in genes regulating cell physiology. This results well coincide with the immunohistochemistry result, significant increment in number of astrocytes in hippocampus region.

Additionally, we carried out pathway analysis to investigate genes associated with hippocampal memory function. Surprisingly, we found a well-known molecular pathway that deeply associated with spatial learning ability and memory formation, the plasticity of synapse. Total 15 genes, regulating plasticity of synapse, were differentially expressed in hippocampus by the impact of mild dehydration (Fig. 10). Molecules like DBN1, EPHB2, ERCC1, GRIA2, GRIK2, KSR1, LEPR, MYO6, PICK1, PIP5K1C, ADD2, and CAMK2B were up-regulated and CPEB1, FMR1 and ATP2B2 were down-regulated in hippocampus of dehydrated mice.

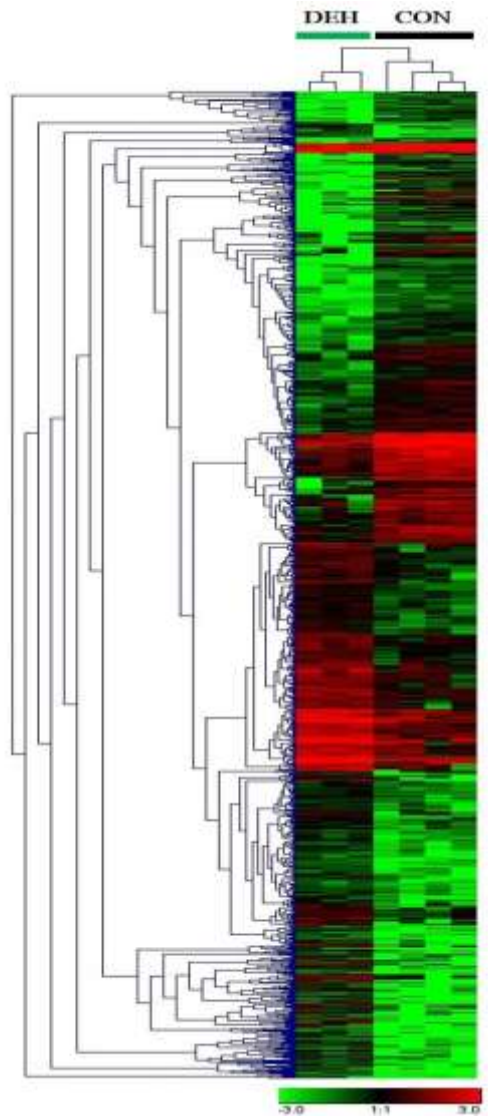

Figure 8. Hierarchical clustering analysis of hippocampal transcriptomes

The heatmap shows differentially expressed genes in hippocampus of mice. Each column and rows represents sample mouse and single gene. Red and green colors indicate up or down regulated genes, respectively.

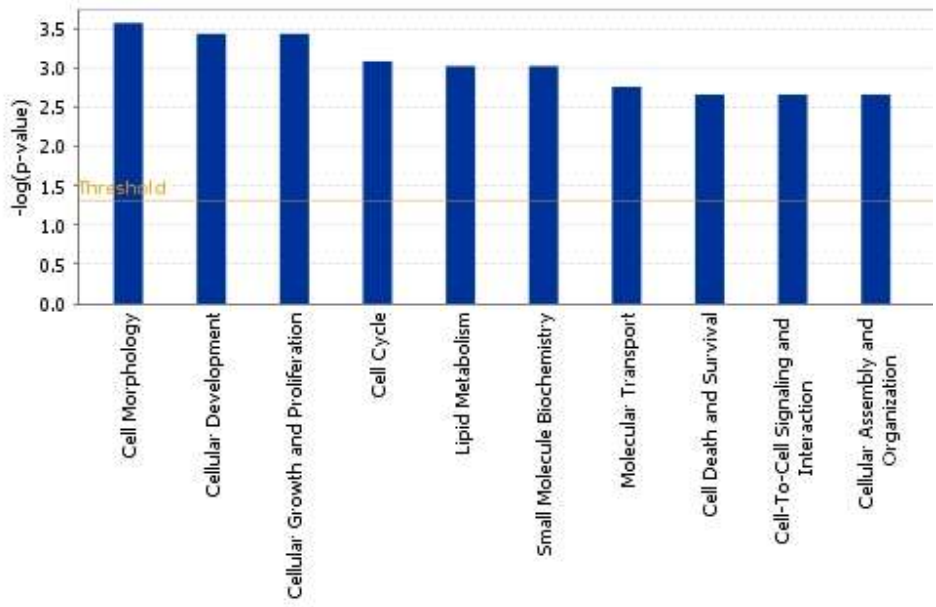

Figure 9. Top 10 biological function categories altered by mild dehydration in hippocampus

Top biological and functional categories of differentially expressed genes were analyzed and categorized using IPA. Biological functions that significantly altered are represented with –log(*p*-value) by Fisher's exact test.

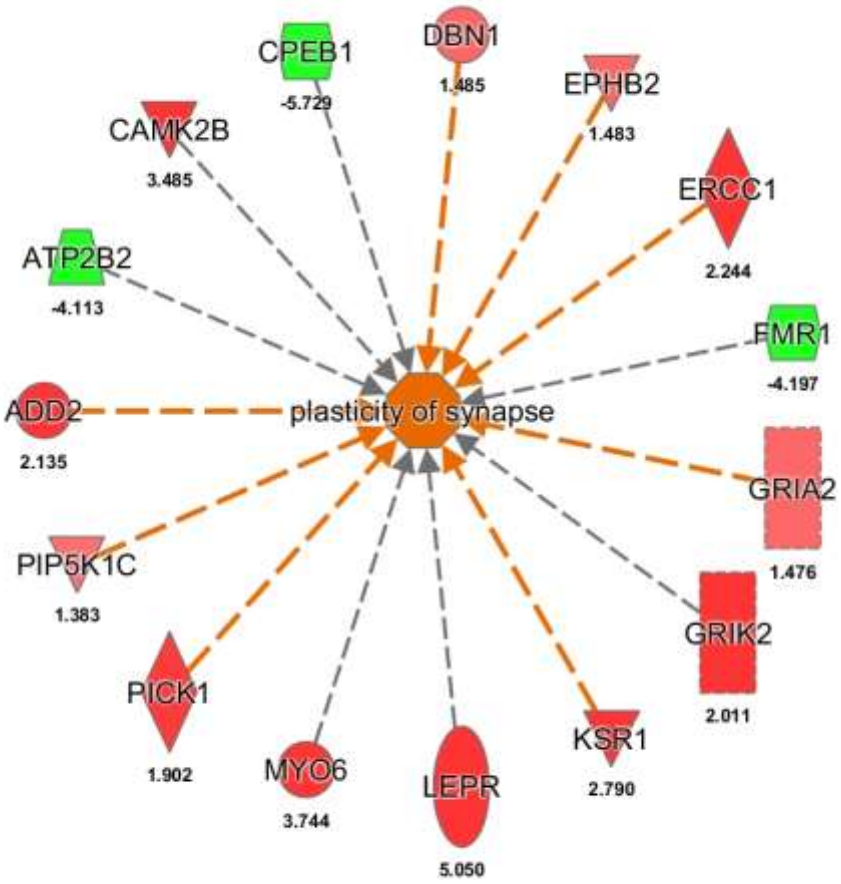

Figure 10. Genes modulating plasticity of synapse were differentially expressed in hippocampus of dehydrated mice

Among differentially expressed genes, genes associated with plasticity of synapse were selected and presented. Red colored genes are up-regulated, while green ones are down-regulated. The numbers under genes are $\log_2$ fold changes of expression level of each gene.

5. Increment of corticosterone level induced changes may have beneficial role at spatial learning

Mild dehydration can stimulate the secretion of corticosterone through two ways, vasopressin and HPA axis. Therefore, we investigated serum corticosterone concentration using ELISA. There was significant increment in serum corticosterone concentration in DEH compared to CON (Fig. 11A; $p < 0.005$). Additionally, we investigated whether mild dehydration increased mRNA expression of BDNF in mice hippocampus. Because, BDNF is well known to have associations with the improvement of hippocampal memory. However, in contrast with the results of the Barnes maze test, Water restriction had not significantly affected expression of BDNF mRNA, but it was slightly higher in DEH than CON (Fig. 11B).

TST was conducted to assess the impact of mild dehydration in mood and anxiety of mice. Before water restriction started, total immobility time between CON and DEH mice were not significantly different. There was also no significant difference in immobility time in both group after the 1 week of mild dehydration (Fig. 12).

It is well established that corticosterone concentration and the density of astrocytes in hippocampus have positive correlation (Carter, Hamilton,

and Thompson 2013, Bridges, Slais, and Syková 2008). Thus, we hypothesized that if BDNF is not responsible for better spatial learning capability of dehydrated mice, astrocytes might be. To verify this hypothesis, we conducted double quantitative immunohistochemistry to assess the number of GFAP-positive cells, Astrocyte, and total number of cells in hippocampus of mice. The result showed that mild water restriction increased the number of astrocytes in DEH mice compared to CON (Fig. 13). We also confirm the number of astrocytes in Young CON to check the validity of our immunohistochemistry protocol, and Young CON had significantly higher number of astrocytes than CON (Fig. 13; $p < 0.005$).

Furthermore, FACS analysis was conducted to investigate the impact of corticosterone and mild dehydration at inflammatory response. As a result of them, the frequency of $CD11b^{+}$ $Ly6G^{high}$ cells, neutrophil related, was higher in dehydrated mice, but with no significance (Fig. 14).

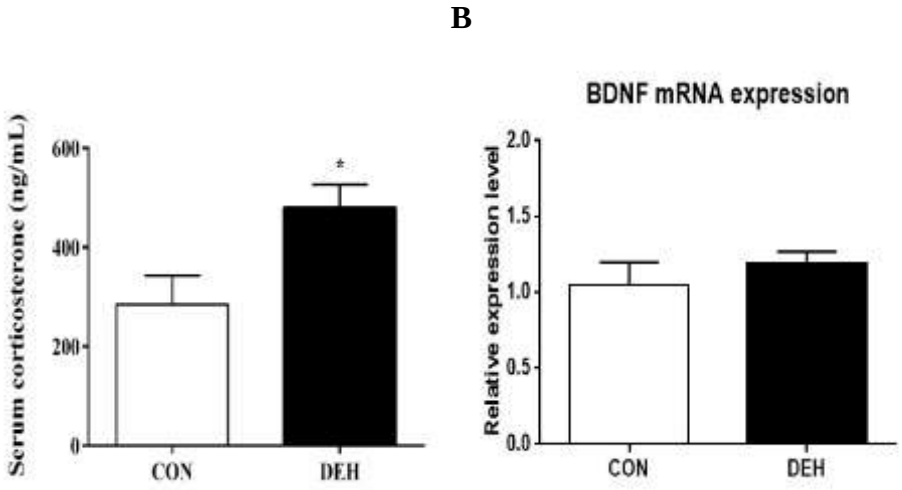

Figure 11. Serum corticosterone was increased by mild dehydration, but not brain BDNF

(A) The level of serum corticosterone in dehydrated mice was significantly higher than control mice. (B) Brain BDNF mRNA level was quantified by qPCR and the transcript level of BDNF was normalized with GAPDH. All data are shown as mean + SEM. Student's *t*-test was performed to determine significant difference in DEH compared with CON. * $p < 0.05$

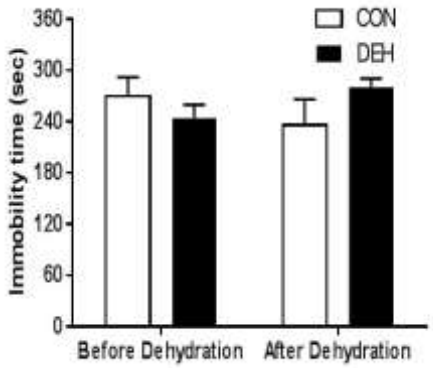

Figure 12. TST result of before and after dehydration

TST was performed for 2 times before and after mild dehydration induced. There was no difference in immobility time between both groups. All data are shown as mean + SEM. Student's *t*-test was performed to determine significant difference in DEH compared with CON.

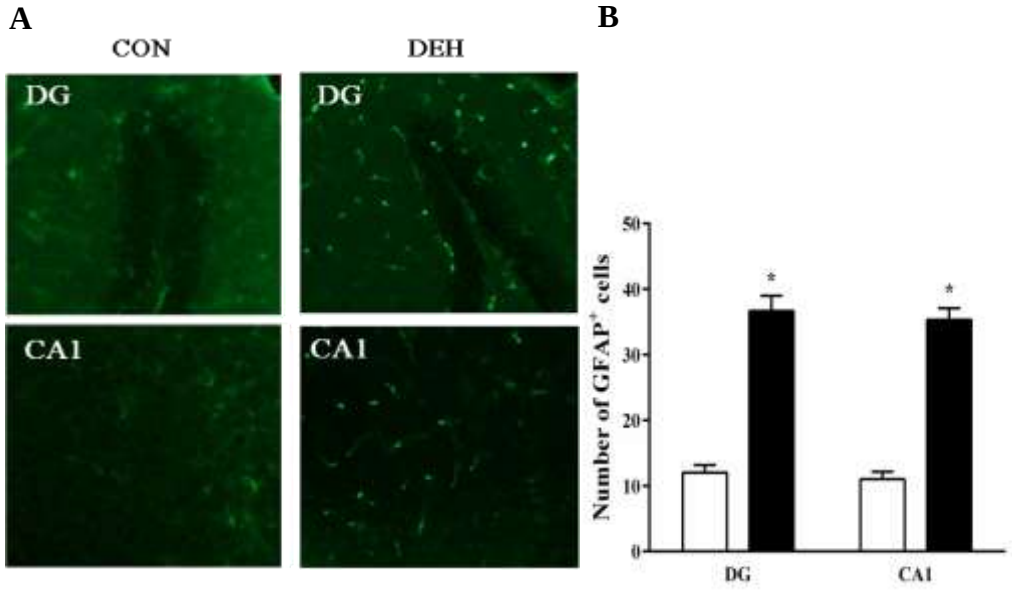

Figure 13. Immunostaining of GFAP$^+$ cells in hippocampus

(A) Mice hippocampus region of each group was stained to analyze the molecular changes induced by mild dehydration. (B) Total mean number of GFAP+ cells in CA1 and DG region of hippocampus were assessed and it was significantly higher in Young and DEH than CON. All data are shown as mean + SEM. One-way ANOVA was performed to determine significant difference in DEH and Young compared with CON. * $p < 0.05$

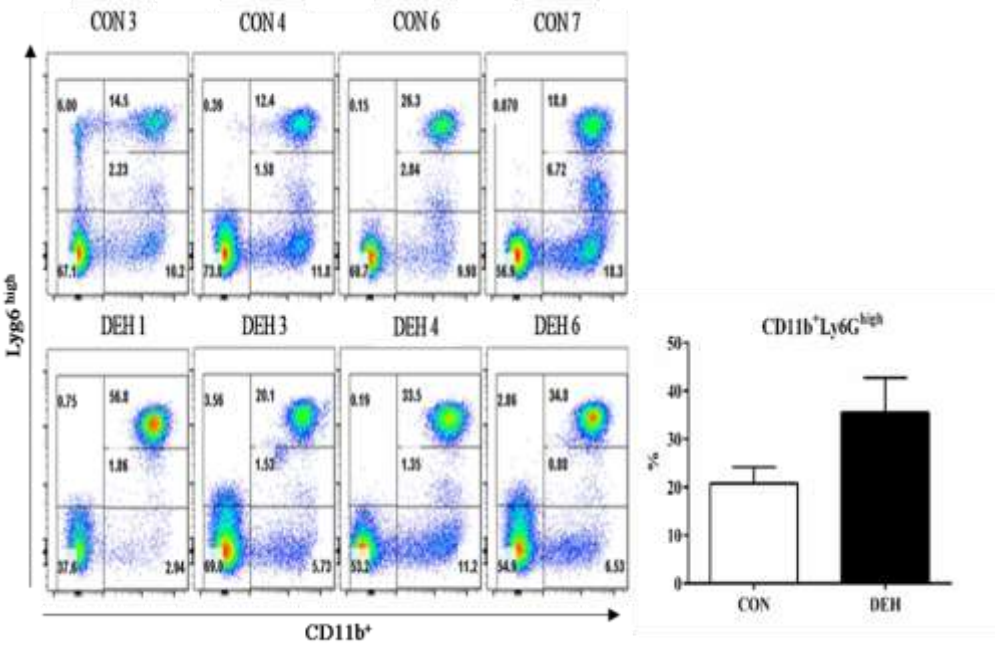

Figure 14. FACS analysis of peripheral blood of aged mice

Peripheral blood of both DEH and CON were analyzed by flow cytometry to evaluate the effect of mild dehydration on the expression of CD11b+Ly6Ghigh cells, neutrophils, in aged mice. All data are shown as mean + SEM.

IV. Discussion

The impact of dehydration on biological functions of life forms have been widely believed and predicted to be solely detrimental from past to present. However, the result of our study showed that 2 weeks of mild dehydration in aged mice improved spatial learning memory, not impairing it. Many researchers previously reported that dehydration has strong association with the impairment of cognitive function (Faraco et al. 2014, El-Sharkawy, Sahota, and Lobo 2015, Suhr et al. 2010). However, the degree of dehydration they induced in these studies was severe, not mild. Dehydrated state was induced by completely depriving water from rodents for 48 hours. In the recent study that deployed 48 hours of water deprivation protocol, there was 18% decrement of body weight compared to base weight after 48 hours. However, in our study, we used water restriction protocol, not deprivation and as a result, there was only 11% of decrement in body weight compared to base weight after 3 days of dehydration. This result implies that we induced mild dehydration in aged mice, not severe dehydration.

During 2 weeks of water restriction period, the mean daily water intake of dehydrated mice was dramatically reduced. Considering the feeding habit of mice, it also stands to reason that the mean daily diet intake was reduced. Since both water and diet intake were decreased, it is a very logical

result that dehydrated mice showed reduction in the body weight. There was, however, no significant difference in body weight of DEH and CON, because we used the mild dehydration regime, not acute and severe one. To prove the induction of mild dehydration in aged mice, we checked plasma osmolality, the golden reference for assessing status of water homeostasis, and it was significantly increased in dehydrated mice (Cheuvront et al. 2010). Furthermore, to be sure about induction of dehydrated state, we also investigated brain vasopressin mRNA level. Vasopressin is a major hormone that modulates cellular water permeability and constriction of blood vessels to maintain water homeostasis in the body (Nielsen et al. 1995). As expected, the mRNA level of vasopressin was significantly higher in DEH than CON. These physiological changes are also observed in other dehydration inducing studies (Begg, Sinclair, and Weisinger 2012, Bekkevold et al. 2013). Therefore, we can safely assume that our water restriction strategy was effective to induce mild dehydration in aged mice.

As briefly mentioned above, there have been not many studies of the association between cognitive function and mild dehydration in the aged. One study induced mild dehydration in older adults by exercise, and there was significant impairments in cognitive function and mood of dehydrated people (Ganio et al. 2011). However, it is impetuous to conclude that mild

dehydration has detrimental impacts on cognitive function as severe dehydration does. Because, in this study, mild dehydrated state was generated by making participants exercise, and exercise itself is known to be negatively associated with cognitive function (Baker et al. 2010, Dietrich and Sparling 2004). Furthermore, many other previous studies also deployed the exercise, heat exposure or water deprivation as a dehydration triggering factor. Indeed, cognitive deficits were observed in these studies (D'anci et al. 2009, Cian et al. 2001, Masento et al. 2014, Armstrong et al. 2012, Szinnai et al. 2005). However, it is known that these factors are also associated with cognitive function itself. Hence, it might be improper to purport mild dehydration and decline in cognitive function have causal relationship. Therefore, to elucidate underlying association between mild dehydration and spatial learning, we induced water-loss mild dehydration in aged mice by limiting access to a water bottle, without applying other external influences.

After inducing mild dehydration in aged mice for 7 days, we conducted the Barnes maze test to assess the impact of long term mild dehydration on spatial learning memory and cognitive function of senescent mice. The Barnes maze test is a widely used psychological laboratory tool that was proposed by Carol Barnes to test spatial learning ability and cognitive function of rodents (Barnes 1979). There are other behavior tests that are used to

evaluate cognitive function such as, Morris water maze and radial arm maze, but these tests are using strong stress to motivate mice to learn spatial tasks and this intense stress can directly affect spatial memory (Hölscher 1999). Furthermore, since we are measuring the impact of dehydration in aged mice, additional water exposure to mice should be avoided. Therefore, we selected and used the Barnes maze to measure learning and memory function of aged mice. During the Barnes maze test, some mice were not willing to enter the escape box, even though they found the right hole right after they start exploring the maze. Similar behaviors, looking or poking the target hole without entering it, were also reported in other study too (Patil et al. 2009). Due to these unwilling situations, a previous study used a different analysis tactics based on assessing primary latency, primary path length, and average speed, and we also adopted this method in analyzing our the Barnes maze test result (Harrison et al. 2006). The validity of our Barnes maze protocol was checked by using 6-week-old mice. They showed significantly reduced latency to find the goal at day 3 and 4. This results were observed in other study that used the Barnes maze test in aged mice (Liu et al. 2011). This proves the validity of the Barnes maze protocol we used in this experiment. Surprisingly, dehydrated mice showed significant reduction in primary latency compared to control mice at day 4. Furthermore, there was clearly visible difference in representative heatmap image of the last Barnes maze trial. This

interesting results was in accordance with our hypothesis that the effect of mild dehydration on cognitive function might be different from that of acute severe dehydration. This implies that there is underlying molecular mechanisms not elucidated yet linking mild dehydration and cognitive function.

To elucidate the underlying molecular mechanism of the improvement in spatial memory by mild dehydration, we conducted transcriptome analysis using next generation RNA sequencing method. Before carrying out bioinformatics analysis, we checked the quality of the RNA-Seq data. The loading density of proton chips and total number of reads were above the minimum requirements. To gain optimal results, it is recommended to score at least 50 % of loading density, and our scores were fulfilled this requirement. Thus, we proceed to bioinformatics analysis of RNA-Seq data. From the result of PCA, we can observe the clearly distinctive gene expression pattern in DEH and CON. From this result, we were able to see the significant changes in gene expression profile of hippocampus by mild dehydration. Then, we screened and aligned transcripts that have more than 0.5 RPKM values with changes over 2 fold from total RNA-seq data compared to that of CON. The number of transcripts met these conditions was 1152 and they were analyzed using hierarchical clustering model. It was clearly visible that profiles of DEGs between dehydrated and control mice were significantly

different. To find out what biological functions were altered by these DEGs, we conducted functional category analysis and aligned 10 most significantly altered categories between DEH and CON. Most of top 10 classifications were associated with cellular physiology. The most significantly influenced functional category was cellular morphology. We conducted further analysis to verify which biological function was responsible for improvement in spatial learning in aged mice.

Various biological functions that belong to the cellular morphology category were thoroughly examined and it was found out that the plasticity of synapse was the most significantly affected biological function in this category. It is well established that long term potentiation (LTP) and synaptic plasticity is directly associated (Larkman and Jack 1995). LTP, the underlying mechanism of learning and memory formation, is a process that strengthens the efficacy of synaptic strength plasticity through repetitive chemical stimulation between synapses (Lüscher and Malenka 2012, Cooke and Bliss 2006, Miyamoto 2006). The initiation of LTP is started with Ca^{2+} influx through N-methyl-D-aspartate (NMDA) receptor in postsynaptic region. Increased level of intracellular Ca^{2+} stimulates and activates calcium/calmodulin-dependent protein kinase II (CaMKII) (Lisman, Yasuda, and Raghavachari 2012). Through multiple interactions with CaMKII and other

proteins, α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) receptors are activated and phosphorylated and this modification increases sensitivity for glutamates (Maren et al. 1993). Furthermore, activated AMPA receptors mediates CaMKII-CREB pathway to stabilize induced LTP and consequently increases synaptic plasticity (Minichiello 2009).

Interestingly, total 15 genes modulating the plasticity of synapse were differentially expressed and 13 out of 15 genes were upregulated in dehydrated mice. The most upregulated gene among them was *Lepr*, the leptin receptor encoding gene. In multiple previous studies, leptin receptor deficient mice showed reduced brain weight, neurons, density of dendritic spines, and abnormal expression of neurological protein (Stranahan et al. 2009, Udagawa et al. 2006, Ahima et al. 1999). Furthermore, according to the recent report, leptin might have a promoting role in promoting GABAergic transmission by increasing the number of synapses and their plasticity (Guimond et al. 2014).

Among other upregulated genes, *ADD2, MYO6, DBN1*, *GRIA2, GRIK2, Pick1 and CaMKIIB* are closely related with inducing LTP in brain. First, *ADD2* encodes β-adducin the membrane skeleton protein. According to a recent study. β-adducin knockout mice showed deterioration in hippocampal LTP and long term memory formation (Rabenstein et al. 2005, Porro et al.

2010). *MYO6* encodes the actin based protein that is highly expressed in neuronal cells (Biemesderfer et al. 2002, Bridgman 2004). *MYO6* knock-out mice showed significant reduction in the number of synapses and disruption in stimulation-induced internalization of AMPA receptors, an essential component of LTP initiation in hippocampus (Osterweil, Wells, and Mooseker 2005). Also, it was reported that MyosinVI motor complex has a possible role in facilitating LTP in hippocampus (Yano et al. 2006). *GRIA2* and *GRIK2* encode a subunit for glutamate receptor, NMDA and kainite receptor. GluR2, subunit of NMDA receptor encoded by *GRIA2*, plays a critical role in initiating LTP (Isaac, Ashby, and McBain 2007). $GluR_6$ is the subunit of kainite receptor and it is encoded by *GRIK2*. Kainite receptor is not directly associated with LTP, however it modulates the amount of released neurotransmitter from presynaptic region and also enhances signal transmission at postsynaptic cell (Contractor et al. 2000, Huettner 2003). Furthermore, according to recent study, children with mutation in *GRIK2* showed impairment in higher cognitive and motor function (Guzmán et al. 2017). *PICK1* encodes a calcium sensing protein that interacts with C-kinase 1. Its close relationship with AMPA receptor has been well studied and documented in multiple reports (Malinow and Malenka 2002, Collingridge, Isaac, and Wang 2004, Bredt and Nicoll 2003). Recently, it was also founded direct association between PICK1 and NMDA receptor in inducing LTP (Terashima et al.

2008). As mentioned above, CaMKII is needed to flux Ca^{2+} into cells to initiate LTP and β isoform of CaMKII is encoded by *CaMKIIB*. CaMkII is highly expressed in hippocampus and its necessity for normal synaptic plasticity and learning was thoroughly studied (Van Woerden et al. 2007). Moreover, recent study also reported that β isoform of CaMKII plays a critical role in modulating synaptic plasticity (Borgesius et al. 2011). These upregulations of genes modulating LTP among DEGs strongly supports the better spatial learning and memory of dehydrated mice.

Previous studies have found that spatial learning and memory are closely associated with stress (Mendl 1999, McEwen and Sapolsky 1995). It is hypothesized that glucocorticoid, the hormone secreted in the body when exposed to stress, is the connecting link between hippocampal memory and stress, because, glucocorticoid receptors are very densely populated in hippocampus (McEwen 2007, Holsboer and Ising 2010, Snyder et al. 2011, Mirescu and Gould 2006). The effect of glucocorticoids on cognitive function, especially on hippocampus-related memory like spatial learning, is in inverted U-shaped relationship which means that it has beneficial effect on cognitive function at optimal level, while too high or low level of glucocorticoids impairs the function (Erickson and Barnes 2003, Mizoguchi et al. 2009, Greendale et al. 2000, Seeman et al. 1997, Wolf et al. 2002). Furthermore,

the secretion of vasopressin and glucocorticoid are closely related, because vasopressin can stimulate adrenal glands to secrete glucocorticoid through HPA-axis (Buckingham 2006). This means that stress induced by mild dehydration and vasopressin can interactively reinforce the corticosterone secretion from adrenal glands. Thus, we measured the level of serum corticosterone, the main glucocorticoid of rodents, to find out if this relationship is responsible for the improvement of spatial learning in dehydrated aged mice. Indeed, the concentration of serum corticosterone was higher in DEH than CON and this means elevated corticosterone level might be the reason of spatial memory improvement in DEH mice.

According to prior studies, high corticosterone concentration is associated with depression and anxiety like behaviors in rodents (Kalynchuk et al. 2004, Johnson, Fournier, and Kalynchuk 2006). It is reported that depression induced by high corticosterone concentration can interfere with the memory formation and spatial learning and deteriorate them (Wong et al. 2007, Song et al. 2006). To rule out this possibility, TST was conducted to check whether mild dehydration induced the depression and anxiety in aged mice or not. There are various behavior tests that are used to measure depression or anxiety in rodents. Among them, forced swim test (FST) and TST is widely used. FST and TST are behavior tests used to measure the stress in-

duced depression (Cryan, Mombereau, and Vassout 2005, Petit-Demouliere, Chenu, and Bourin 2005). TST was selected over FST and deployed it in this experiment due to the same reason why the Barnes maze test was selected over Morris water maze test. There was no significant difference in immobility time between DEH and CON. Thus, we can rule out the negative impact of depression on cognitive function in DEH and CON.

The impact of cortircosterone on hippocampus has been studied by many researchers. Previous works on the effect of corticosterone at hippocampus have found that high corticosterone level is associated with increment in numbers of astrocytes in hippocampus (Bridges, Slais, and Syková 2008, Lambert et al. 2000). Astrocytes are known to be associated with numerous functions in hippocampus, such as propagating intercellular Ca^{2+} signal, providing metabolic substrates to neuron and modulating synaptic plasticity (Achour and Pascual 2010, Fiacco, Agulhon, and McCarthy 2009, Rouach et al. 2008). Hippocampus is composed of multiple sub-regions, and among these sub-regions, Cornu Ammonis1 (CA1) and dentate gyrus (DG) are known to be deeply associated with spatial learning and memory. CA1 is the main site where LTP occurs and LTP is the signaling cascade that strengthens synapses between neurons and the primary mediator of memory formation and synaptic plasticity in brain (Herring and Nicoll 2016,

Abraham 2003, Cooke and Bliss 2006). In DG region of hippocampus, the generation of new neurons is maintained throughout the adulthood (Overstreet-Wadiche and Westbrook 2006, Cameron and Mckay 2001, Aimone, Wiles, and Gage 2006). These newly born neurons are integrated to spatial memory supporting circuitry and improve signal transduction associated with spatial learning (Zhao et al. 2006, Emery et al. 2005, Kee et al. 2007). With these backgrounds, we decided to measure the density of astrocytes in CA1 and DG of hippocampus. Dehydrated mice showed increased numbers of astrocytes in both DG and CA1. Since, neurogenesis declines with aging, maintaining and preserving existing neurons become more important and this preservation of the neuron is the main function of astrocytes. Therefore, mild dehydration induced increment in corticosterone and astrocytes might be responsible for the improvement of synaptic plasticity and long term potentiation and consequently, better spatial learning capability.

The aim of this study is to clarify the underlying mechanism of mild dehydration and cognitive function with using aged animal model. It was demonstrated that mild dehydration induced stress paradoxically promoted spatial learning ability of dehydrated mice through improving synaptic plasticity. Indeed, dehydration has detrimental physiological impacts on human body, however it might be applicable for ameliorating the deficit in cognitive

function as aging ensues.

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국문초록

노인기 마우스에서 가벼운 탈수 스트레스가 공간 기억 능력 및 뇌 전사체 변화에 미치는 영향 연구

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박 인 순

성인에서 심각한 탈수는 인생 전반에 걸쳐 여러 생리학적 기능에 부정적인 영향을 미친다. 하지만, 노인에게 탈수가 뇌 기능에 미치는 영향에 대해서는 아직 완벽히 밝혀지지 않았다. 그러므로, 우리는 2년령의 C57BL/6 마우스를 이용하여 노인에서 가벼운 탈수가 인지 능력에 미치는 영향을 규명하려 한다. 하루 15분간만 수통에 접근을 허용하여 탈수 마우스 모델을 만들어냈으며, 이런 탈수가 유도된 쥐들의 생리학적 지표와 인지 능력을 물이 무제한으로 공급된 대조군의 마우스들과 비교하였다. 탈수가 유도된 마우스들은 대조군의 쥐들에 비해 높은 혈장 삼투압농도와 뇌에서의 항 이뇨호르몬의 유전자 발현 정도를 가지고 있었다. 또한 탈수가 유도된 마우스들에서 부신 피질 호르몬의 하나인 코르티코스테로이드의 혈장에서의 농도가 높았다. 꼬리매달기 실험법을 통하여 실험군과 대조군의 마우스들 모두에서 우울증 상태를

확인하였으나, 두 그룹 사이에 유의미한 차이는 없었다. 다음으로 반즈 미로 실험을 통하여 마우스에서 공간 학습과 기억 능력을 측정하였다. 놀랍게도, 탈수 마우스가 반즈 미로 실험에서 대조군 마우스에 비해서 목표물을 찾는데 더 짧은 시간이 걸렸으며, 더 뛰어난 공간 학습과 기억 능력 보였다. 이런 탈수가 인지 능력에 미치는 영향의 근본적인 메커니즘을 규명하기 위해서, 마우스의 해마를 리보핵산 서열결정 분석을 실행하였다. 그 결과, 탈수 마우스의 해마에서 시냅스의 가소성과 장기 강화 작용에 관련된 전사체들의 발현량이 매우 증가해있었다. 이런 전사체의 변화가 정말로 생물학적 변화로 이어졌는지 확인하기 위해서, 면역염색화학법을 이용하여 마우스의 해마 지역에서 GFAP 양성 세포들의 수를 분석하였고, 해마의 CA1과 DG 지역 모두에서 탈수 쥐의 성상 세포의 수가 더 높았다. 이런 결과들을 종합하여 생각해 볼 때, 분명 가벼운 탈수는 혈장 삼투압을 높이는 등의 부정적인 영향도 있었지만, 항 이뇨호르몬 – 코티코스테론 축 조정을 통하여 시냅스의 가소성을 증진시킴으로써 공간 학습과 기억 능력을 증진하였다. 그러므로, 이런 결과들을 통해서 가벼운 생리학적 스트레스가 노인기의 인지 능력에 미치는 영향에 대한 새로운 영향에 대한 연구에 귀중한 정보를 제공할 수 있을 것이다.

주요어 : 인지능력 저하, 해마, 공간 기억, 코르티코스테론, 리보핵산 분석, 시냅스 가소성

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