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Transcriptome Profiling Analysis of Kidneys and Brain in Dehydrated Infant Mice

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Abstract

Transcriptome Profiling Analysis of Kidneys and Brain in Dehydrated Infant Mice

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Water is an essential nutrient as a structural and functional component in the body. To maintain a healthy life optimal hydration status is essential and sufficient water intake is recommended. Compared to adults, infants have higher body water composition ratio, reaching 75%, and immature fluid regulatory system, which leads to higher risk of dehydration. In spite of the importance of water, there is few evidence underlying the impacts of dehydration on physical and cognitive development. We, therefore, investigated the molecular mechanisms by which dehydration draws physiological changes in infancy. 3-week-old infant mice that just finished weaning were provided limited access to a water bottle for fifteen minutes per day for one week (RES 1W) and four weeks (RES 4W) to induce dehydration status while the control group consumed water *ad libitum* with free access to the water bottle for a week (CON 1W)

and 4 weeks (CON 4W). Compared with control mice, mice in the RES group consumed 2.4 times less water. The four-week dehydration resulted in increased plasma osmolality and the transcript level of vasopressin in the brain compared to the control group. Transcriptome analysis was conducted to understand physiological changes in the kidney induced by dehydration. We found that structural and functional kidney development was still ongoing in early life by analyzing the renal transcriptional networks of infant (4 weeks old; CON 1W) and juvenile (7 weeks old; CON 4W) mice. Kidneys in 4 week and 7 week old mice showed significantly distinctive functional gene networks. Gene sets related to cell cycle regulators, fetal kidney patterning molecules and immature glomerular barrier integrity were upregulated in infantile kidneys while heightened expressions of genes associated with ion transport and drug metabolism were observed in juvenile kidneys. Western blot analysis was conducted to validate the protein levels of representative molecules in morphological and functional maturation in the kidney. GPC3 that mediates ureteric bud branching in the metanephros was slightly higher in infantile kidneys at the protein level compared to the juvenile kidneys. CYP4A that is one of the most critical proteins in xenobiotics and lipid metabolism had increased protein levels in juvenile kidneys with normal renal development. Dehydration during infancy suppressed renal growth by interrupting the SHH signaling pathway, which targets cell cycle regulators. Importantly, disruption of the developmental program ultimately led to long-term alterations in renal filtration functions by causing a decline in glomerular filtration barrier integrity. We also investigated whether dehydration affects cognitive function. Dehydration from

infancy to juvenile resulted in impairment of learning ability. In the Barnes maze test, dehydrated mice (RES 4W) showed 4 times longer latency to find a target hole after 4 days of training compared to control mice (CON 4W). Brain weight was determined at the end of experiment and there was no significant difference between control and dehydrated mice. A series of neurotrophic factor was examined for their expression and we found only BDNF expression was significantly lower in RES 4W than CON 4W. The transcript level of BDNF only in hippocampus of RES 4W was significantly different from CON 4W. Next-generation RNA sequencing analysis in the brain revealed that CREB and its downstream target genes related to memory consolidation were significantly dysregulated by dehydration. These results suggest that sufficient water intake is required for normal renal growth and learning development during infancy. This study provides a meaningful perspective of pediatric dehydration with a molecular and physiological explanation of why infants are more vulnerable to dehydration than adults. Furthermore, these findings suggest new insights into the systemic effects of dehydration on development and may provide possible markers for clinical application in pediatric dehydration.

Keywords: Dehydration, Infancy, Transcriptome, Renal development, Cognitive function

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List of Abbreviations

AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid
BDNF	Brain-derived neurotrophic factor
CREB	Cyclic AMP-responsive element-binding protein
CYP	Cytochrome P450
GSEA	Gene set enrichment analysis
LTP	Long-term potentiation
SHH	Sonic hedgehog

I. Introduction

Water is an essential nutrient as a structural and functional component in the human body, being the major constituent of cells and tissues and it functions as a carrier of nutrients and excretions. It also serves as both reactant and product in metabolisms (Jéquier and Constant, 2010). Optimal water intake, therefore, is necessary to maintain a healthy life as water has multiple critical functions (D'Anci et al., 2006; Jéquier and Constant, 2010). It is well documented that improper hydration status negatively impacts on multiple physiological disorders such as difficulties in physical and cognitive performances, and pathological issues (D'Anci et al., 2006; Jéquier and Constant, 2010; Popkin et al., 2010). However, according to reports by the United States Department of Agriculture (USDA) and the National Health and Nutrition Examination Survey (NHANES), most people do not consume sufficient daily water, as a matter of fact, their water intake is far below the recommended amount (Popkin et al., 2010).

Healthy adults regulate fluid balance to maintain homeostasis. Infants, however, have immature fluid regulatory system (Rodriguez-Soriano et al., 1981). They also have a higher percentage of water in their body composition, reaching 75% (D'Anci et al., 2006). Moreover, infants, after weaning, are more prone to drink insufficient water since they begin solid diets and caregivers are likely to be unaware of their thirst (D'Anci et al., 2006). Despite infants seem to be at higher risk for de-

hydration than adults, there have been few studies on which dehydration draws physiological changes in infants. The molecular and physiological background for why infants are more vulnerable to dehydration has not been studied yet as well, and there are few studies on whether dehydration causes physiological changes in infants.

The kidney is a major organ that plays crucial roles in homeostasis in the body. It filters blood to excrete waste products from the body (Jéquier and Constant, 2010) and controls body fluid volume, solute and acid balance within a tight range in cooperation with other endocrine organs (Popkin et al., 2010). The critical functions of the kidney in the fluid regulatory system are implicated in precise regulation of plasma osmolality. When the body senses changes in osmolality, osmoreceptors at the hypothalamus stimulate arginine vasopressin (AVP) release, which promotes fluid reabsorption in the kidney and thirst by which water retention increases (McKinley and Johnson, 2004).

Kidney organogenesis comprises several common developmental steps: proliferation and differentiation of stem cells, epithelial-mesenchymal cell transition, branching and regional segmentation (Dressler, 2009; Humphreys, 2014; Vainio and Lin, 2002). Complex gene regulatory networks mediate cell interactions underlying anatomical and functional modifications in kidney morphogenesis (Kuure et al., 2000; Uhlenhaut and Treier, 2008). Especially, dynamic regulations underlying cell and tis-

sue integrity are essential in kidney function and are highly associated with renal disorders (Welsh and Saleem, 2012). A highly selective permeable glomerular barrier membrane establishes networks with podocyte foot processes and slit diaphragm, a modified form of adherens junction, to gain mature filtration functions (Reiser et al., 2000). Thus, retaining a stable cell and junction architecture is required for normal renal function (Lee et al., 2006; Reiser et al., 2000; Welsh and Saleem, 2012).

Along with progress of microarray and genome sequencing technologies, mechanistic studies underlying fetal kidney development and physiology have been extensively carried out. While recent studies have shown that further growth of the nephrons and elongation of tubular structures occur after birth (Čukuranović and Vlajković, 2005), little is known about how genetic events are regulated in renal growth postnatally (Baxter and Yoffey, 1948; Clark, 1957; Larsson et al., 1980; Wu et al., 2013). Therefore, it still remains to be revealed in terms of manifesting molecular mechanisms for in-depth understanding of the physiological processes in renal maturation after birth.

Cognitive development heavily relies on neuronal plasticity which accounts for learning and memory formation. Emerging evidences describe that long-term potentiation (LTP) is the most contributing mechanism in learning and memory regarding neuronal plasticity. LTP is a signaling cascade that includes diverse gene transcription and protein synthesis mediating synaptic strength (Lamprecht and LeDoux, 2004).

Induction of LTP is initiated from the influx of Ca^{2+} into the cell via NMDA (N-methyl-D-aspartate) receptors at the postsynaptic membrane. The elevation of intracellular Ca^{2+} provokes signaling cascades which in turn regulates AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid) receptor trafficking including its activation and endocytosis. AMPA receptor trafficking is mediated by phosphorylation of GluR1 and it coordinates CaMKII-CREB pathway (Lonze and Ginty, 2002; Minichiello, 2009). After induction of LTP, it is stabilized via activation of CREB (cAMP response element-binding protein) which subsequently increases synaptic strength by regulating the transcription of its downstream target molecules. CREB is a transcription factor which has crucial physiological meaning in various biological processes. Among its regulating factors, genes related to function and plasticity of the nervous system have received enormous attention with respect to learning and memory mechanism (Carlezon et al., 2005). CREB is demonstrated to function for neuronal growth and survival, synaptic plasticity, and neuroprotection by triggering synthesis of proteins such as neurotrophic factors, transcription factors and signaling molecules (Carlezon et al., 2005; Lonze and Ginty, 2002).

Aim of the study

We aimed to study the impacts of insufficient water intake in infant mice, with emphasis on physiological and transcriptional changes in the developing kidney and brain. Here, we report crucial effects of dehydration on the developmental processes in the kidney by profiling global gene expression. We also highlight the changes in learning behavior and transcriptional networks associated with synaptic activity in the brain during sustained dehydration.

II. Materials and Methods

1. Experimental animals

Right after weaning, 3-week-old male C57BL/6 mice were housed for 1 week and 4 weeks and sacrificed at the age of 4 and 7 weeks old, corresponding to infant and juvenile. Animals were randomly assigned into control (CON) and water restriction group (RES). Total 4 groups were used in comparison analysis: CON 1W (4 weeks old, n=5), CON 4W (7 weeks old, n=6), RES 1W (4 weeks old, n=6), RES 4W (7 weeks old, n=8). Animals were maintained in a 12-hour light/dark cycle and fed *ad libitum* an AIN-93G diet. All experimental procedures were approved by the IACUC (Institutional Animal Care and Use Committee) of Seoul National University and conducted according to the IACUC guidelines.

2. Water restriction experiment

Animals in water restriction group were imposed limited access to water. A water bottle was given to animals for 15 minutes a day during the experimental period. Control mice consumed water *ad libitum* with free access to the water bottle.

3. Plasma biochemical analysis

Serum biochemical analysis for blood urea nitrogen (BUN) and serum creatinine was

conducted using a dry-chemistry blood analyzer, Spotchem SP-4410 (Arklay, Kyoto, Japan). Plasma osmolality was determined by Fiske 210 Micro-Sample Osmometer (Fiske, Norwood, MA, USA).

4. Behavioral assessment

Barnes maze test was used to assess the cognitive dysfunction in learning development of infant mice. The maze is a circular platform (92cm diameter) with twenty holes at the edge and elevated 105cm above the floor. One of those holes is connected to a dark tunnel linked to a hidden escape cage. Visual cues with different colors and shapes were provided at the same point during experimental period to give spatial clues to find a target. 30W LED light was placed above the platform, as mice prefer darker environment and avoid bright light, to encourage them to find the escape hole. The whole experiment was conducted in a separate room.

1) Adaptation phase: On the first day, animals had a chance to habituate to the new environment and task. 10 seconds after placing an animal in a cylindrical transparent start chamber in the middle of the maze, the experimenter guided the mouse to an escape hole and gave 3 minutes to freely enter the escape cage through the target hole.

2) Learning phase: After adaptation, 4 days of training followed. During this period, spatial reference learning ability was assessed. The animal was placed in an opaque start chamber for 10 seconds and allowed to explore the maze for 3 minutes. If the mouse failed in finding the target hole within a given time, the experimenter led the

mouse to the hole. Training trials happened twice a day during training period. Interval between trials was kept to 15 minutes. Each trial was video recorded and analyzed by Ethovision XT 10 (Noldus, Wageningen, Netherlands) to estimate latency and cumulative path length.

3) Test trial: On the test day, mice were given only one trial to find the target hole, and estimate latency and cumulative path length were assessed to evaluate spatial learning ability.

5. RNA isolation

Total RNA was extracted from the kidneys and brain using DNA-free RNA isolation kit (RNAqueous-4PCR kit; Ambion, Austin, TX) according to the manufacturer's directions. Total RNA integrity and quantity were assessed with a NanoDrop 2000 Spectrophotometer (Thermo Fisher Scienctific, Wilmington, DE).

6. Microarray hybridization

RNA samples from the kidneys were amplified for microarray analyses using Illimina TotalPrep RNA Amplification Kit (Ambion, Austin, TX). 500ng of total RNA was used to prepare labelled cRNA with overnight incubation. Amplified cRNA was hybridized on Illumina MouseWG-6 Expression BeadChip arrays. The arrays were

scanned with BeadArray Reader (BeadStation 500G Instrument, Illumina Inc.). Identification and quantification of spot images were obtained by Genome Studio software v1.0.2 (Illumina Inc.).

7. Bioinformatic analysis of microarray data

The analysis was performed as described in (Shon et al., 2015). Raw data was log-transformed and normalized by quantile normalization using Genome Studio software (Illumina Inc.). Differentially expressed genes among four groups were identified using an ANOVA ($p<0.05$) by Partek® Genomics Suite software v6.6 (Partek, St Louis, MI). (http://www.partek.com/partekgs). Significant genes with fold change > 1.5 were used for further analysis.

Hierarchical clustering analysis was performed using the Pearson correlation distance matrix with average linkage algorithm in Genesis software v1.7.5 (Sturn et al., 2002). To examine the significance of functional categories which were classified based on Ingenuity Knowledge Base, Gene Set Enrichment Analysis was carried out (http://www.broadinstitute.org/gsea/index.jsp). Mechanistic networks underlying signaling pathways and metabolic pathways were built based on the Ingenuity Knowledge Base. Upstream regulator analysis was carried out to predict upstream regulators in direct or indirect relationships with dataset using Ingenuity Pathway Analysis (IPA) (Krämer et al., 2013). Functional network map of gene sets was constructed by using CytoScape software v3.2.0 (http://cytoscape.org) (Shannon et al.,

2003). The microarray dataset is available at the Gene Expression Omnibus (www.ncbi.nlm.nih.gov/geo/, accession number GSE75604).

8. Next-generation RNA sequencing in the brain

Total RNA from hippocampal region of the brain tissue was prepared following the same processes described above. Total RNA integrity and quantity were assessed with Agilent 2100 bioanalyzer (Agilent Technologies, Palo Alto, CA). Polyadenylated mRNA was isolated from 3µg of total RNA sample using MicroPoly(A)Purist kit (Thermo Fisher Scientific, Wilmington, DE) according to the manufacturer's directions. The mRNA samples were fragmentized using RNase III from Ion Total RNA-Seq Kit v2 (Thermo Fisher Scientific, Wilmington, DE). The fragmented mRNA was then converted to cDNA using Ion Total RNA-Seq Kit v2. Barcoded cDNA libraries were prepared using Ion Xpress™ RNA-Seq Barcode kit (Life Technologies, Carlsbad, CA) and quantified using Agilent 2100 bioanalyzer for sequencing. The cDNA fragments > 150 bp were used and the cDNA library was diluted to 100 pmol for emulsion PCR (em-PCR) on Ion Sphere Particles (ISPs) using Ion PI™ OT2 200 Kit with the Ion OneTouch™ System (Life Technologies, Carlsbad, CA) according the manufacturer's instruction. Enriched template-positive ISPs were loaded on an Ion Proton PI chip v3 (Life Technologies, Carlsbad, CA). Next-generation RNA sequencing of the enriched libraries was performed using Ion Proton semiconductor sequencer (Thermo Fisher Scientific, Wilmington, DE).

9. Bioinformatic analysis of RNA-seq data

The mapped sequencing reads to the mouse reference genome (NCBI mm9) with TopHat and Bowtie algorithm were generated and the raw reads counts were normalized into RPKM (Reads per kilobase of exon model per million mapped read) values to quantify mRNA level by using Partek® Genomics Suite software v6.6 (Partek, St Louis, MI). (http://www.partek.com/partekgs). The normalized counts were mapped to each transcript. Transcripts with RPKM > 1.5 and significant genes with fold change > 1.3 were used for further analysis. Analysis of signaling pathways and metabolic pathways were conducted as described above using Ingenuity Pathway Analysis (IPA).

10. Quantitative RT-PCR

DNase I treated total RNA was converted into cDNA by two step procedure with MessageSensor RT kit (Ambion, Austin, TX) and mRNA levels were quantified by SYBR-GREEN qPCR method (Applied Biosystem, Carlsbad, CA). Relative mRNA expression level was calculated by $\Delta\Delta C_T$ method. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a housekeeping gene for normalization of mRNA expression of each sample. Primer sequences are as in the following. *Avp*: forward 5'-CCAGGATGCTCAACACTACG-3', reverse 5'- CTCTTGGGCAGTTCTGGAAG-3'. *Gapdh*: forward 5'-TGCACCACCAACTGCTTAG-3', reverse 5'-GATGCAGGGATGATGTTC-3'. *Ngf*: forward 5'-AGACTCCACTCACCCCGTG -

3’, reverse 5’-GGCTGTGGTCTTATCTCCAAC-3’. *Nt-3*: forward 5’-GGAGTTT-GCCGGAAGACTCTC-3’, reverse 5’-GGGTGCTCTGGTAATTTTCCTTA-3’. *Bdnf*: forward 5’-AAAGTCCCGGTATCCAAAGGCCAA-3’, reverse 5’-TAG-TTCGGCATTGCGAGTTCCAGT-3’.

11. Western blot analysis

Proteins were extracted from the kidneys. Equal amounts of protein were subjected to SDS-PAGE and transferred to PVDF membranes. The membranes were incubated with anti-GPC3 (1:200, ab66596), anti-CYP4A (1:10000, ab140635) primary anti-bodies from Abcam (Abcam Inc., Cambridge, MA). Anti-α-Tubulin (1:10000, T5168) from Sigma (Sigma Chemical Co., St. Louis, MO) was used as control. Horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (1:5000, #7074S) from Cell Signaling Technology (Cell Signaling Technology Inc., Danvers, MA) or goat anti-mouse secondary anti-body (1:10000, G21040) from Invitrogen (Invitrogen, Carlsbad, CA) were used for detection. Protein bands were visualized using ECL Western blot detection reagents (Amersham Pharmacia Biotech, Piscataway, NJ).

12. Statistical analysis

Data are expressed as mean ± SEM. Statistical significance ($p<0.05$) was evaluated by unpaired Student's t-test between two groups. Statistical analyses were performed using GraphPad Prism 6 software (GraphPad Software Inc., La Jolla, CA).

III. Results

1. Time-limited access to water bottle induces mild dehydrating physiological conditions in infant mice.

Infant mice that just finished weaning were provided limited access to a water bottle for fifteen minutes per day for one week (RES 1W) and four weeks (RES 4W) (Figure 1). Mice in the RES 1W group consumed 2.4 times less water compared to the age-matched control mice in the CON 1W group with *ad libitum* water intake ($p<0.001$). Mice in the RES 4W that grew into juveniles with the same daily water-restriction treatment showed a significant reduction in daily water intake, on average down to a third of that in control mice (CON 4W) ($p<0.001$) (Figure 2A). There was no significant difference in diet intake between two groups during the experimental period while reduced diet intake was observed during the first few days of experiment (Figure 2B and 2C). In order to verify whether the reduction in water intake was sufficient to induce physiological changes in those mice, we determined the levels of plasma osmolality. Mice in RES 1W had significantly higher plasma osmolality than mice in CON 1W ($p<0.001$). The four-week dehydration resulted in increased plasma osmolality as well, although it was not significant ($p=0.083$) (Figure 3A). Next we measured the transcript level of vasopressin in the brain and found dramatic increases in vasopressin production in both RES 1W and RES 4W, compared to CON 1W and CON 4W, respectively ($p<0.001$) (Figure 3B).

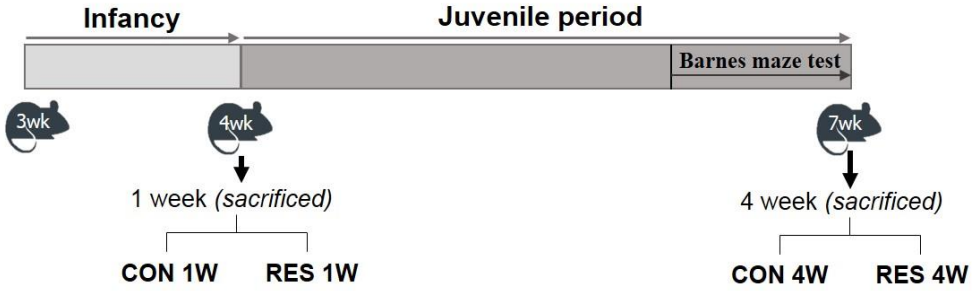

Figure 1. Experimental design

Water restriction experiment was sustained throughout the developing period of mice from infant to juvenile. 3-week-old male mice were assigned into four groups for water restriction experiment. Infant mice were raised for 1 week right after weaning and sacrificed at 4 weeks of age: CON 1W and RES 1W. Juvenile mice were raised for 4 weeks and sacrificed at 7 weeks of age: CON 4W and RES 4W. While the control group (CON 1W and CON 4W) consumed water *ad libitum*, animals in the dehydration group (RES 1W and RES 4W) had access to water bottle limited to 15 minutes a day. Barnes maze test was conducted on the last week of water restriction experiment.

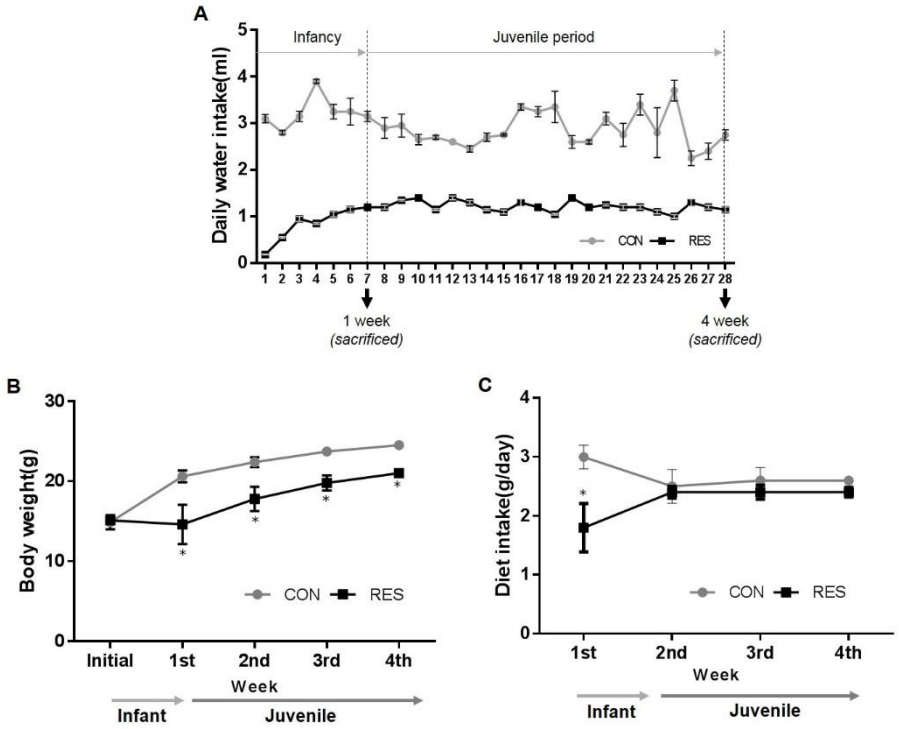

Figure 2. Induction of mild dehydration in infant mice

(A) The dehydration group had reduced water intake by a third compared to the control group. Body weight **(B)** and diet intake **(C)** were also decreased at the onset of dehydration but followed up to the normal range at the end of the experiment. Data are expressed as mean ± SEM. Student's t-test; *$p < 0.05$, ***$p < 0.001$ versus control group.

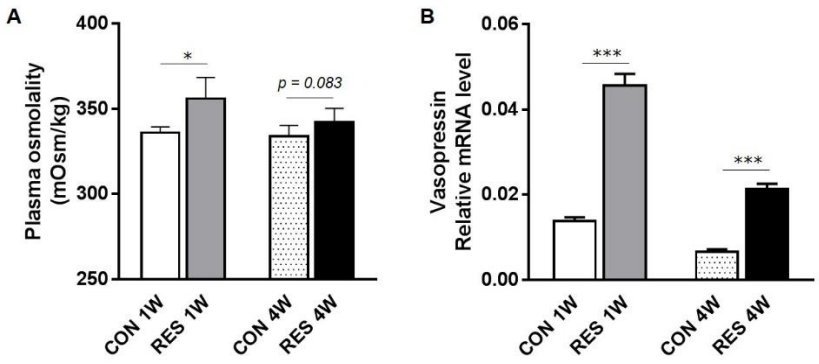

Figure 3. Increased plasma osmolality and brain vasopressin mRNA level

Mild dehydration was generated, resulting in elevated plasma osmolality **(A)** and brain vasopressin mRNA level **(B)** in the dehydrated mice. Data are expressed as mean ± SEM. Student's t-test; *$p < 0.05$, ***$p < 0.001$ versus control group.

2. Gene expression profiling analysis of the kidneys in dehydrated mice and control mice using genome-scale Illumina microarray

To examine the effect of dehydration on kidney growth, the organ weight was measured. The weight of the kidneys increased with age from 0.22g in CON 1W to 0.28g in CON 4W ($p<0.01$), however dehydration caused a decrease of kidney weight in infant mice (RES 1W) by 29% and juvenile mice (RES 4W) by 14% (Figure 4A). To understand the molecular events underlying kidney growth and development from the age of infancy to the juvenile stage during the life cycle and to unveil the mechanism by which dehydration retards kidney growth, we conducted gene expression profiling analysis of kidneys using a genome-scale microarray.

Principal component analysis (PCA) showed that the transcriptome profiles were readily distinguished by either age (CON 1W and CON 4W) or a period of sustained water restriction (RES 1W and RES 4W) (Figure 4B). Differentially expressed genes among the four groups were identified by ANOVA analysis ($p<0.05$). A total of 234 probes were differentially expressed depending on age in the normal control groups (CON 4W vs. CON 1W). Furthermore, 977 probes were detected to distinctively mediate the transcriptional events in the infantile kidneys of the dehydration group (RES 1W vs. CON 1W), while 1,926 differentially expressed probes were in the juvenile kidneys of the dehydration group (RES 4W vs. CON 4W) (Figure 4C).

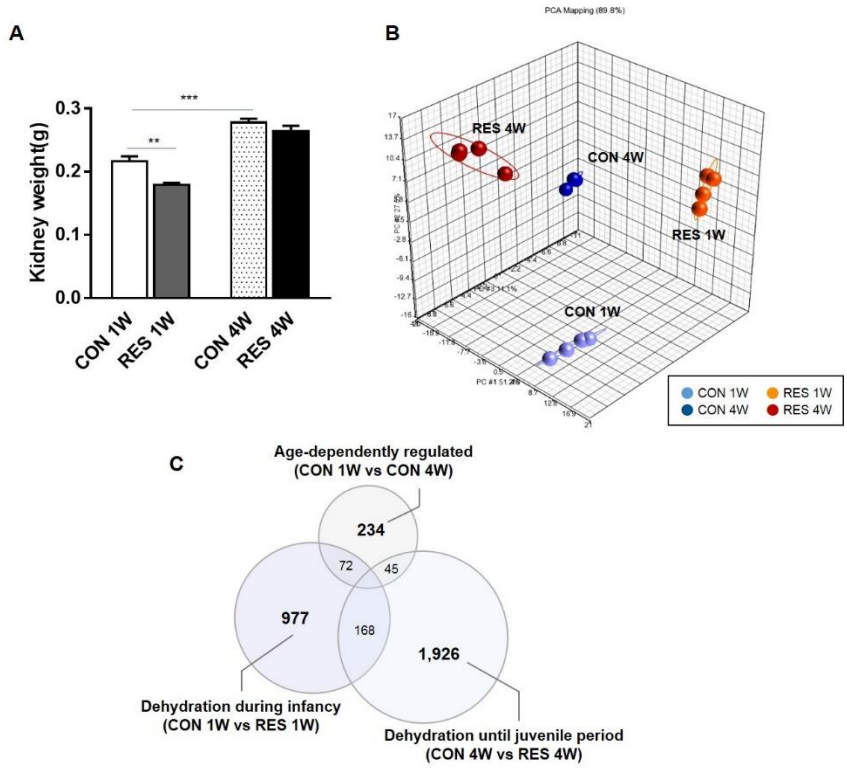

Figure 4. PCA plot and Venn diagram of renal transcriptome

(A) The weight of the kidneys increased with age but dehydration caused decreased weight in the kidneys of infant mice (RES 1W) by 29% and in juvenile mice (RES 4W) by 14%. **(B)** A 3D principal component analysis (PCA) plot of the renal transcriptome data represents significantly distinguishable profiles from each group. Each dot indicates an individual kidney sample. **(C)** The Venn diagram shows the number of probes that were detected with different signal intensity in each dataset. Data are expressed as mean ± SEM. Student's t-test; $^{**}p < 0.01$, $^{***}p < 0.001$ versus control group.

3. Normal infant and juvenile mice present distinct renal transcriptional profiles.

Hierarchical clustering analysis showed that postnatal kidneys at different time points (CON 1W and CON 4W) had distinguishable transcriptome profiles (Figure 5A). 234 genes were differentially expressed in a comparison of CON 1W and CON 4W, with 149 genes upregulated in the kidneys of infant mice (CON 1W) and 85 genes upregulated in juvenile mice (CON 4W). This finding implies that the kidneys of infant and juvenile mice might undergo different biological processes.

To extend our knowledge about the biological process occuring in the infantile kidney, we established a functional network map of differentially expressed genes and assigned gene ontology (GO) terms. Enriched GO categories from gene set enrichment analysis revealed that genes related to the cell cycle, tissue morphology and development were significantly upregulated, whereas they were downregulated in CON 4W (Figure 5B).

Genes including *Aurka, Birc5, Bub1b, Cdc20, Meox1, Pbk, Kif22, Mcm5, Mcm6, Ncaph, Plk1, Ung, Cdca3* were overexpressed (Figure 6A), indicating that developmental cellular processes were still abundant in the kidneys of CON 1W. Among those genes, *Aurkb, Plk1, Mcm5, Kif22* have been found to function in embryonic renal development by mediating cell division (Kim et al., 2011). In addition, *Birc5*, which had about a 6-fold increase in infant mice, has been reported to be expressed only in the tubules and glomeruli of fetal kidney (Schwab et al., 2003). *Meox,*

with a 2-fold increase in CON 1W compared to CON 4W, is one of the important factors in kidney formation, and has a role in epithelial-mesenchymal cell interactions (Quinn et al., 2000). Genes that are essential for DNA synthesis and cell division (*Nasp, Rrm1, Rrm2, Prc1*) were upregulated and then eventually downregulated with age in our dataset. Many of the predictive upstream regulators within gene sets of CON 1W were associated with cell cycle regulation, including *Myc, Tp53, Foxm1, Ccnd1 and Smad3* (Figure 7). *Myc* is highly expressed in the metanephros during renal organogenesis and its disruption is associated with reduced cell proliferation (Bates et al., 2000). These results imply that pathways mediating cell cycle progression were dominantly engaged in the development of infantile kidneys.

We also observed that the cluster of genes participating in basement membrane integrity was highly expressed in infant kidneys compared to juvenile kidneys. Genes encoding extracellular matrix (ECM) components are well known to change their expression levels during kidney tubulogenesis and maturation (Muller and Brandli, 1999). Among them, collagen is a major component that provides structural integrity and its subtypes appear to have different expression levels during glomerular structural development in the kidney (Muller and Brandli, 1999). In our dataset, the members of the Collagen IV family (*Col4a1, Col4a2*) showed two times higher expression in infant than juvenile kidneys (Figure 6A). In concert with the overexpression of glomerular basement membrane (GBM) composing protein, there was elevated expression of *Adamts2, Nid1, Serpinh1 and Wisp1* which participate in ECM

synthesis (Berschneider and Königshoff, 2011; LeBleu et al., 2007) (Figure 6A). Genetic events regulating ECM deposition demonstrate that improvement of GBM integrity occurs in infants, which accounts for structural maturation of the glomerulus after birth. In addition, genes that play roles in ureteric bud formation such as, *Tbx10, Gpc3, Osr2,* and *Nid1* had higher expression in CON 1W than in the CON 4W group. *Snail* is known to regulate mesenchymal to epithelial transformation (Boutet et al., 2006). Diminishing expression of *Snail* along with renal maturation in our dataset was coincident with previous work showing that *Snail* becomes inactive in the mature organ. Taken together, the results indicate that structural development of the kidney is not completed before birth, rather, postnatal kidneys continue to grow during infancy and they undergo the processes necessary for mature structural integrity.

Contrary to the observation that structural morphogenesis was the most significant biological process in the transcriptome analysis of kidneys in CON 1W, upregulated genes in the CON 4W kidneys were associated with molecular transport, renal system development and function, and cellular function and maintenance (Figure 6A). This indicates that functional maturation of the kidney was ongoing followed by morphological development. Genes involved in molecular transport associated with the renal system (*Kcnk5, Slc2a9, Slc6a9, Slc35a3*) were highly expressed with 1.6 to 2 fold changes (Figure 6A). The cytochrome P450 family, involved in bile acid metabolism in extrahepatic tissues, xenobiotics metabolism (*Cyp4b1, Ugt1a10*) and steroids and fatty acid metabolism (*Cyp2e1, Cyp4b1, Cyp7b1, Cyp4a12a*), was also upregulated during the juvenile period (Figure 6A). Furthermore, an immunoblotting

assay was conducted to validate the microarray analysis results for kidney growth in the infants and juveniles. *Gpc3,* which interacts with growth factors to mediate ureteric bud branching in the metanephros (Rivera and Haber, 2005), was slightly higher in infantile kidneys at the protein level compared to the juvenile kidneys. *Cyp4a,* one of the most critical proteins that play a role in xenobiotics and lipid metabolism, had increased protein levels in juvenile kidneys with normal renal development (Figure 6B). These results indicate that the kidneys of juveniles are in the process of developing into functionally matured adult kidneys.

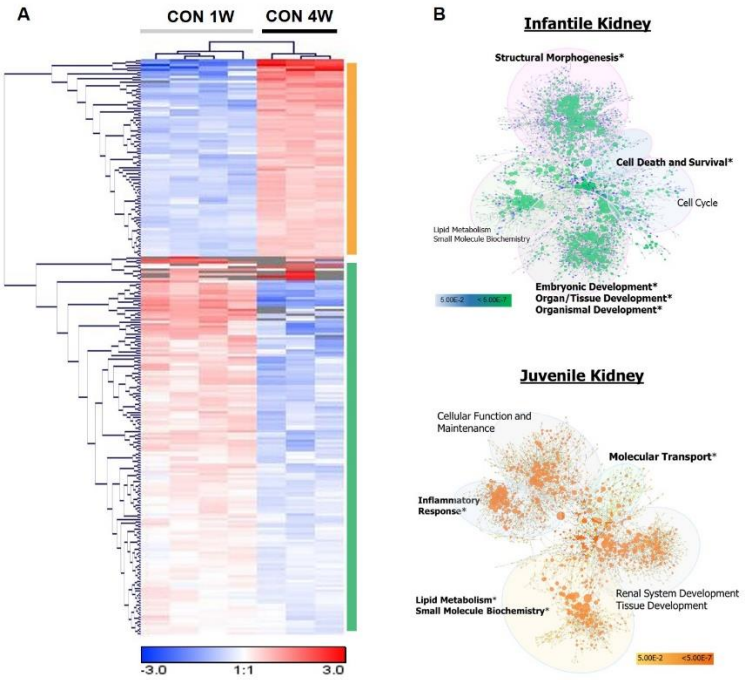

Figure 5. Hierarchical clustering and functional network of renal transcriptome at different time point of age

(A) The heatmap of probesets shows that that postnatal kidneys at different time points (CON 1W and CON 4W) had distinctive transcriptional profiles from hierarchical clustering analysis. Red and blue colors indicate upregulated and downregulated genes, respectively. **(B)** Functional network of gene sets at each time point of age. Gene ontology (GO) was categorized based on Ingenuity pathway analysis (IPA) results. GO terms in bold with asterisk indicate enrichment in GSEA (Gene set enrichment analysis). Each node represents a biological function and its color and size indicate the p-value and number of genes in the cluster, respectively.

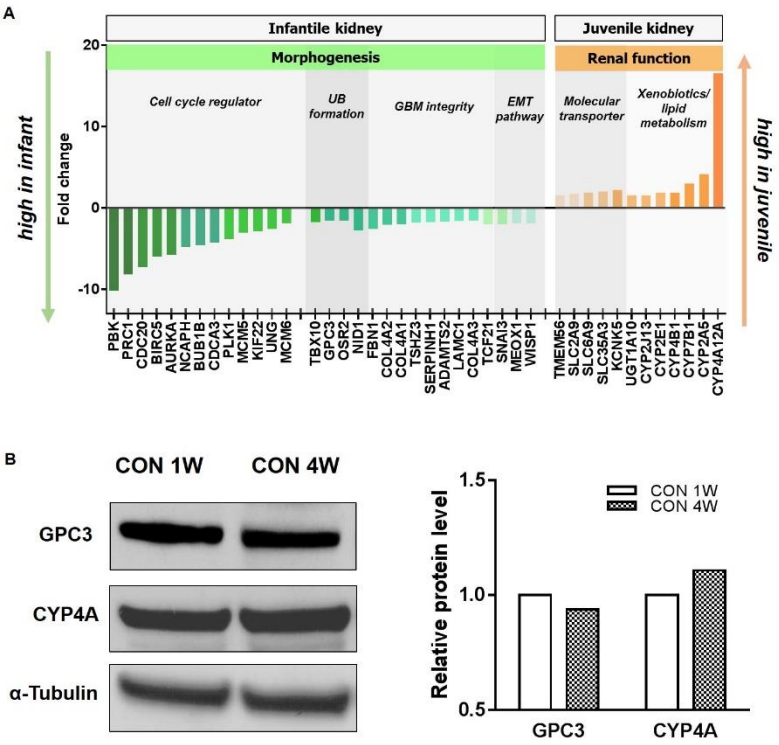

Figure 6. Genes related to postnatal renal development and Western blot assay

(A) Genes related to structural and functional maturation were enriched in the kidneys of infant and juvenile mice, respectively. **(B)** Representative two molecules were assayed by Western blot. Representative samples in each group are shown above. Molecules associated with structural (*Gpc3*) and functional (*Cyp4a*) maturation were abundant in infantile and juvenile kidneys, respectively. UB; ureteric bud, GBM; glomerular basement membrane, EMT; epithelial-mesenchymal transition.

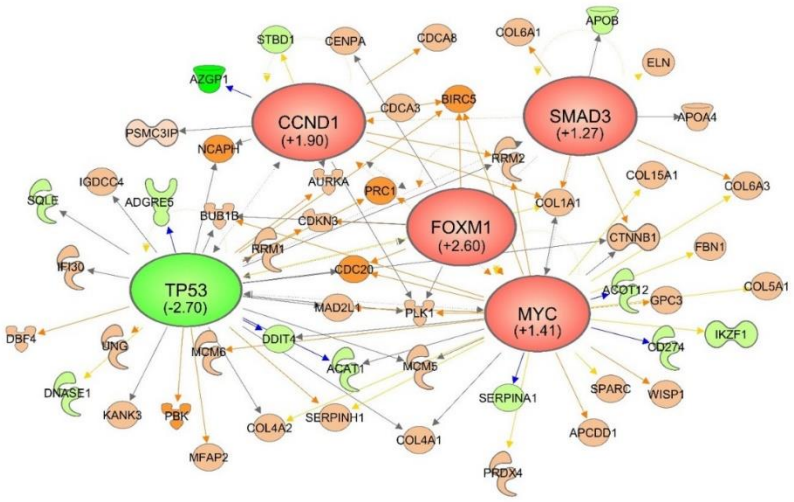

Figure 7. Upstream regulators in infantile renal development

Upstream regulator analysis shows the top predicted regulators that connected to the downstream nodes. Many of predictive upstream regulators associated with cell cycle regulation were found within the gene sets of CON 1W. Activation z-score of each regulator is presented in brackets. Edges indicate predicted relationships, colored with yellow and blue when it led to activation and inhibition, respectively.

4. Dehydration impairs renal growth and development by interrupting Shh signaling in infant mice.

Dehydration resulted in dramatic changes in kidney transcriptome profiles in infant mice. Each functional category of differentially expressed genes in the RES 1W group compared to CON 1W was tested for statistical significance. We found that the cell cycle, organ development and renal system development were in the top seven significant categories (Figure 8A). GSEA (Gene set enrichment analysis) confirmed that the cell cycle was the most enriched biological process regulated by hydration (Figure 8B). These findings suggest that dehydration caused a deterioration in renal growth.

We further performed pathway analysis and found that one of the most crucial molecular pathways that were significantly regulated by dehydration during kidney organogenesis was the Sonic hedgehog (Shh) pathway. It is known to have a role in the expression of GLI transcription factors whose targets are cell cycle regulators (Uhlenhaut and Treier, 2008). Interestingly, a significant reduction in the *Shh* signaling pathway was observed following insufficient water intake (Figure 9A). This subsequently resulted in decreased expression of downstream target genes including cell cycle regulators (*Cdk1, Cdc20, Ccnd1, Plk1, Bub1b, Rrm2, Mcm5, Mcm6*) and cell differentiation related molecules (*Id1, Id2, Rad9, Birc5*) (Figure 9B).

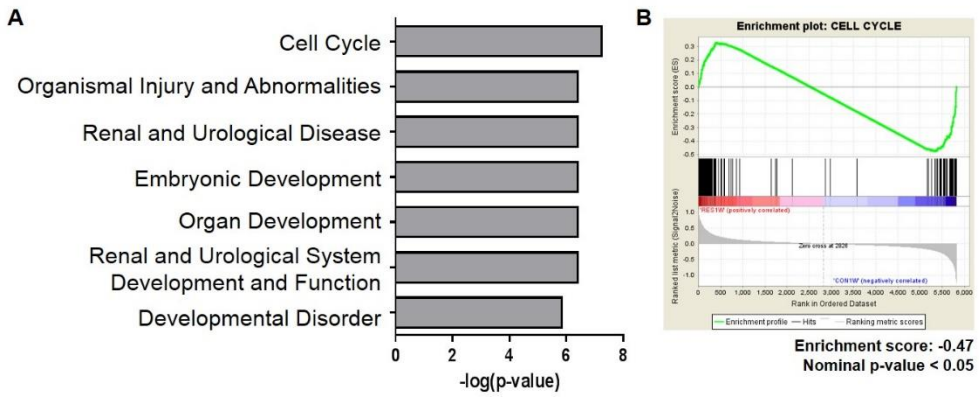

Figure 8. Top biological categories and GSEA of renal transcriptome in dehydrated mice

(A) Top disease and functional categories of differentially expressed genes were classified based on IPA knowledge. Significant biological functions are represented with –log(*p*-value) by Fisher's exact test. **(B)** GSEA (Gene set enrichment analysis) shows enrichment plot of cell cycle related genes in comparison analysis between CON 1W and RES 1W.

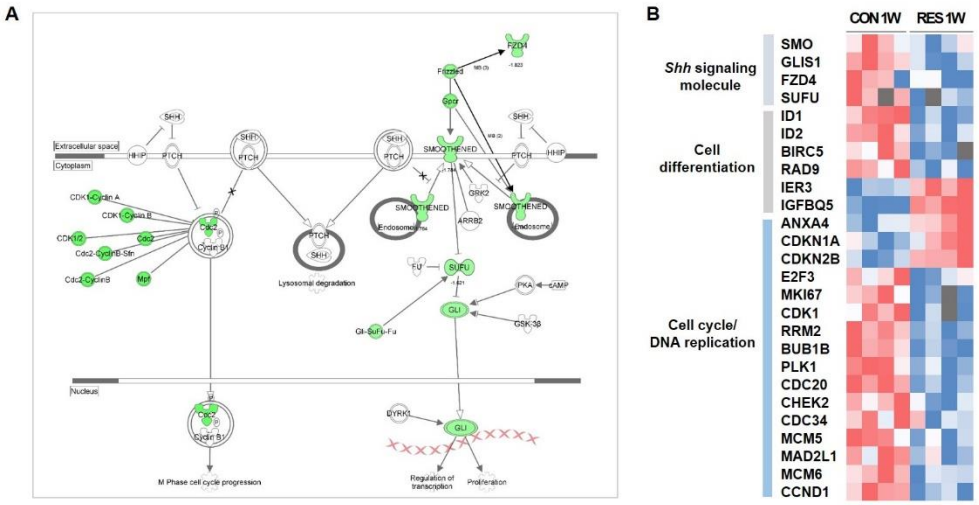

Figure 9. Genes related to Sonic hedgehog signaling pathway

(A) Sonic hedgehog (Shh) signaling pathway was suppressed by dehydration, resulting in impaired renal morphogenesis. Genes in green color were downregulated. **(B)** The heatmap shows downstream target genes and indicates that cell cycle and cell differentiation regulators in the Shh signaling pathway were distinctively regulated by dehydration. Red and blue color code indicates up- and down-regulation.

5. Discordance in glomerular barrier integrity leads to loss of renal function by long term effects of dehydration.

We observed a significant increase in blood urea nitrogen (BUN) ($p<0.05$) but not in serum creatinine in the RES 4W group (Figure 10A and B). To further investigate these results, we conducted comparison analysis of differentially expressed genes in the kidneys of RES 4W compared to CON 4W. There were significant changes in glomerular barrier integrity markers. Glomerular basement membrane component (*Col4a4*; 1.6-fold decrease) and integrins (*Itgb5, Itgb6*; 1.7-fold decreases) were reduced at the transcription level. Additionally, slit diaphragm, which is a modified form of junction structure, showed defects in its organization, with 2-fold reduction in the mRNA level of β–Catennin and ZO-2 (Figure 11). These results indicate that prolonged dehydration ultimately gave rise to breakdown of glomeruli filtration barrier integrity in the kidneys of juvenile mice.

In order to understand the implicit mechanism underlying the deterioration of barrier integrity, we confirmed the top significant pathways by canonical pathway analysis. We found that glomerular structural integrity was intrinsically altered via dysregulation of the epithelial adherens junction and its remodeling pathway (Figure 12). Cadherin (*Cdh1*)-Catennin (*Ctnnb1*) complex and Nectin (*Pvrl1*) complex, which have roles in epithelial cell adhesion integrity and tissue homeostasis, were downregulated by sustained dehydration. Downstream molecules such as *Iqgap1, Actr3, Vav2* were also decreased at the transcription level, indicating that alteration was observed in the network between cell-cell contacts (Figure 13A). *Iqgap1* is a

signaling molecule detected during the establishment of foot processes (Lehtonen et al., 2005). Especially, 3.4-fold increased expression of α-Actinin-4, which is accompanied by foot progress effacement in damaged podocytes (Pavenstädt et al., 2003), was observed in the RES 4W group. In concert with depressed junction structural stability, many of the differentially regulated genes in the dehydration group were related to adherens junction recycling. The downstream effect of junction structure remodeling is implicated in clathrin-mediated endocytosis of the actin cytoskeleton, which appeared to be suppressed in the dehydration group (Figure 13B). Clathrin (*Cltc*), a mediator of vesicle formation during endocytosis of the cadherin complex, was decreased by 1.5-fold at the expression level in RES 4W. A gene encoding clathrin recruiting protein, *Picalm*, also showed decreased expression levels (1.7-fold change). *Rab5c*, with roles in vesicle docking and trafficking, had reduced transcription levels (1.6-fold change) along with depressed fusion of endocytic vesicles (Figure 13B). From these results, we demonstrate that the dynamics of glomerular filtration barrier integrity modulated by adherens junction signaling were dysregulated as a consequence of prolonged dehydration. These results suggest that loss of renal function was derived from glomerular injury accompanied by abnormal renal maturation under the sustained hyperosmolar stress.

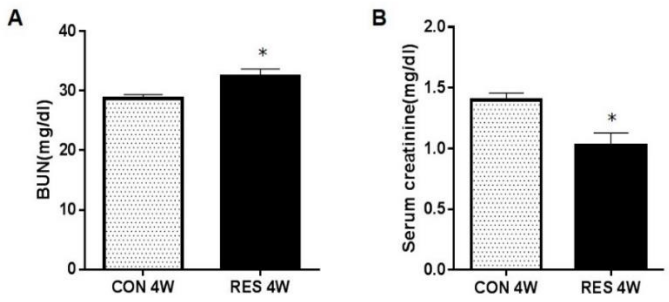

Figure 10. Changes in renal function markers

An increase in blood urea nitrogen (BUN) **(A)** but not in serum creatinine level **(B)** in the dehydration group compared to the control group. Data are expressed as mean ± SEM. Student's t-test; *$p < 0.05$ versus control group.

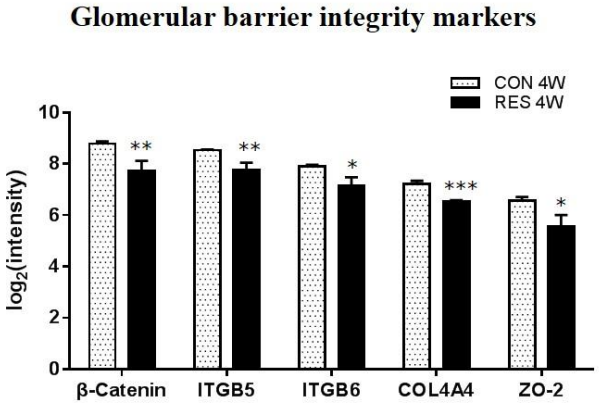

Figure 11. Transcript levels of glomerular barrier components

Perturbed glomerular barrier function was followed by glomerular barrier integrity deterioration by long term effects of dehydration. Log2 normalized intensity of each gene is expressed as mean ± SEM. Student's t-test; *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ versus control group.

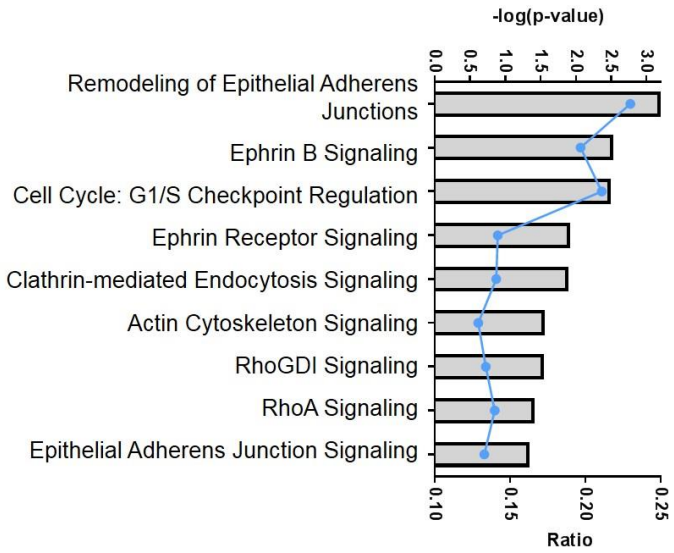

Figure 12. Top canonical pathways related to cell junction dynamics

Top canonical pathways that were differentially regulated following dehydration in juvenile mice were related to cell interaction and contact-dependent signals. Each category was scored with –log(*p*-value) from Fisher's exact test and ratio. Ratio refers to the ratio of the number of molecules that map to the canonical pathway and the number of molecules within the dataset by IPA.

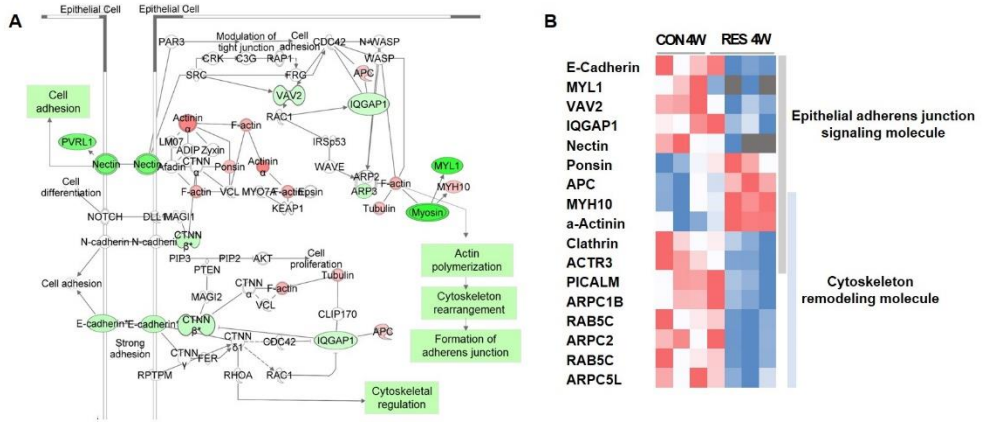

Figure 13. Genes related to adherens junction signaling pathway

(A) Suppression of adherens junction signaling accounts for altered cytoskeleton re-arrangement, which caused alteration of glomerular barrier integrity. Molecules in green and red color indicate down- and up-regulated genes. **(B)** The heatmap shows the signaling molecules related to cell-cell junction and cytoskeleton rearrangement underlying altered membrane integrity.

6. Dehydration has substantial effects on cognitive deficits in learning development.

To confirm the effects of dehydration on cognitive capacity, we used the Barnes maze test, a standardized method for evaluating spatial learning ability in young animals (Attar et al., 2013). Mice were trained for 4 consecutive days after one day of adaptation during the last week of water restriction experiment (Figure 1). We found significant differences in escape latency and total distance moved to reach the target hole between the control group and the dehydration group. Control group with sufficient water intake had a tendency to significantly decreased escape latency ($p<0.001$) and total distance moved ($p<0.05$) as learning the task and the environment during training phase. By contrast, there was relatively small improvement in both values in mice with restricted water intake (Figure 14A and B). Figure 15A shows the heatmap of the tracking that mice moved during the test on the last day. Dehydrated mice showed a fall in performance by wandering around the maze during the test while control mice directly headed to the escape hole on the last day. That is, dehydrated mice spent more time to find the target hole. Relative latency on the test which was compared to that of on the first day in each group is shown in Figure 15B. There was significantly reduced relative latency by approximately 30% in the control group ($p<0.001$). On the contrary, the dehydration group showed an increasing tendency in latency, up to 430% ($p=0.34$) (Figure 15B).

As we confirmed detrimental impacts of dehydration on cognitive function, we aimed to find out the striker molecule which contributed to the changes in the

behavior test. We measured the weight of the brain, and the mRNA level of neurotrophic factors including NGF, NT-3 and BNDF in the brain using quantitative RT-PCR. There was no significant difference in brain weight (Figure 16A). However, it was noteworthy that the mRNA expression of BDNF in the hippocampus was significantly changed with a 1.3-fold decrease in the dehydration group (RES 4W) ($p<0.05$) unlike other neurotrophins which had no differences in expression levels (Figure 16B). Interestingly, it was found that decreased mRNA level of BDNF after dehydration in juvenile mice (RES 4W) corresponded to the level of control infant mice (CON 1W) (Figure 16C). Given that imperative roles of BDNF in synaptogenesis and synaptic plasticity, we hypothesized that dehydration might have gene regulatory effects which caused cognitive deficits in infant mice. We, therefore, further conducted mechanistic analysis by profiling brain transcriptome using next-generation sequencing.

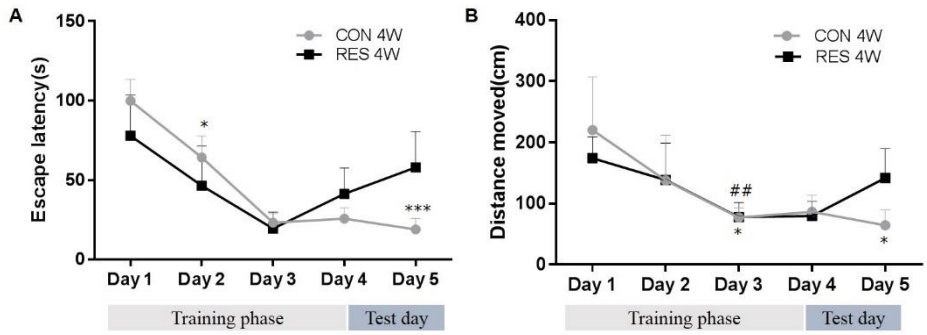

Figure 14. Escape latency and total distance moved during the Barnes maze test

The dehydration group had lower performance of finding the target hole. **(A)** Escape latency was assessed by analyzing the time spent to reach the target hole. Compared to dehydrated mice, control mice showed significantly decreased latency after 4 consecutive day of training. **(B)** Total distance moved in the dehydration group was increased on the test day. Student's t-test; *$p < 0.05$, ***$p < 0.001$ versus Day 1 in control group, ##$p < 0.01$ versus Day 1 in restriction group.

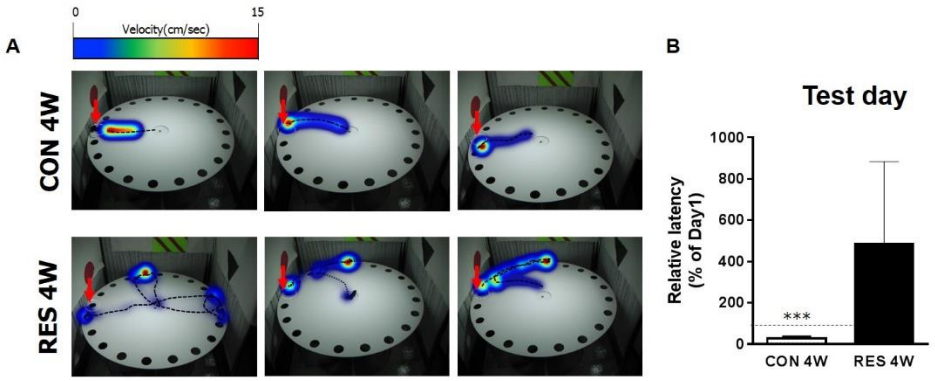

Figure 15. Representative heatmap images of the Barnes maze test

Impaired cognitive function in learning development was observed after dehydration. **(A)** On the last day of the maze test, control group directly headed to the escape hole. Compared to control, dehydrated mice wandered around the platform and took much longer time to get to the target. Arrow indicates the target hole and heatmap presents the time they spent at the point. **(B)** Average of relative latency (%) to the target on the test day was calculated by dividing escape latency (s) on the test day by that of the first day. Dashed line indicates the baseline (100%, latency on the first day of the Barnes maze test). Student's t-test; ***$p < 0.001$ versus Day 1 in each group.

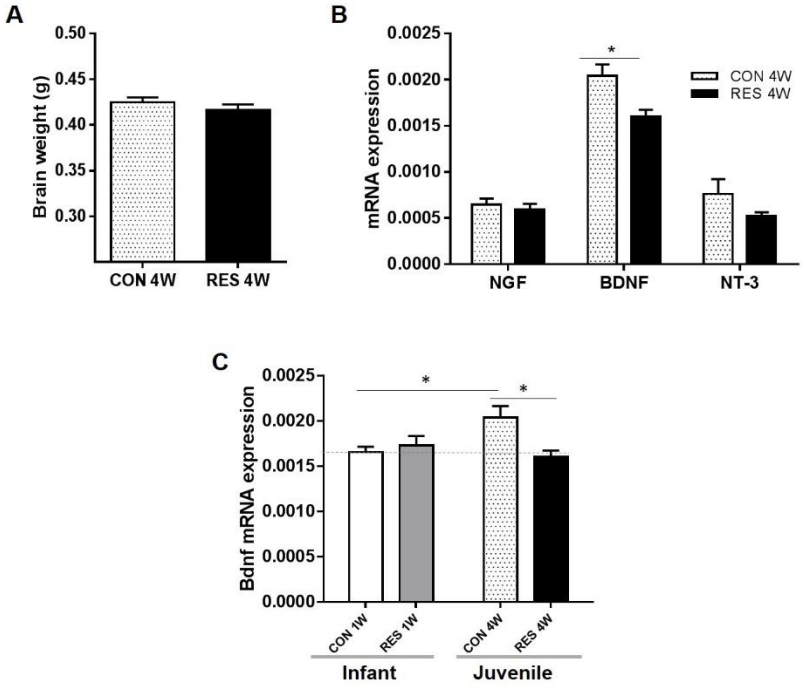

Figure 16. Impacts of dehydration on cognitive function

There were no significant differences in brain weight (A) and the transcript level of other neurotrophic factors such as NGF and NT-3, but significant decrement in BDNF mRNA level (B). Decreased mRNA level of BDNF after dehydration in juvenile mice (RES 4W) corresponded to the level of infant mice (CON 1W) (C). Student's t-test; *$p < 0.05$ versus control group.

7. Dysregulation of LTP signaling is a main contributor of cognitive dysfunction in dehydrated mice.

In order to determine what physiological mechanisms and molecules interfered in cognitive function under hyperosmolar stress, we conducted next-generation RNA sequencing in the hippocampal region. There were 21,879 distinctively regulated transcripts between control and dehydration group. Pathway analysis was performed with transcripts over 1.3 fold change and categorized into biological function and physiological disorders in which distinctively regulated transcripts are involved. Intriguingly, differentially expressed transcripts were predominant over in the categories of behavior, cell to cell signaling and neurological disorders (Figure 17). Figure 18 shows the most significantly regulated gene networks in the brain of the dehydration group. It indicates that gene sets mediating cognitive processes were distinctively regulated by insufficient water intake. Interestingly, many of the genes comprising LTP signaling were significantly dysregulated. Glutamate receptors including AMPA receptors; *Gria2* (1.6-fold), *Gria4* (1.6-fold), and NMDA receptor; *Grin1* (2.2-fold) were differentially regulated at postsynaptic membrane (Table 1). Along with this, it was found that CREB-mediated downstream molecules which play roles in synaptic plasticity; *Egr1* (1.6-fold), *Fos* (2.2-fold), *JunB* (2.2-fold), *Nr4a1* (2.7-fold), *Arc* (2.6-fold), and neuronal cell growth and survival; *Gadd45b* (1.6-fold), *Midn* (1.7-fold), *Btg2* (1.9-fold) were downregulated in the dehydration group by upstream regulator analysis (Figure 19). These results highlight that insufficient water intake led to alter-

ations in neurogenesis and neurotransmission which ultimately converged upon cognitive dysfunction.

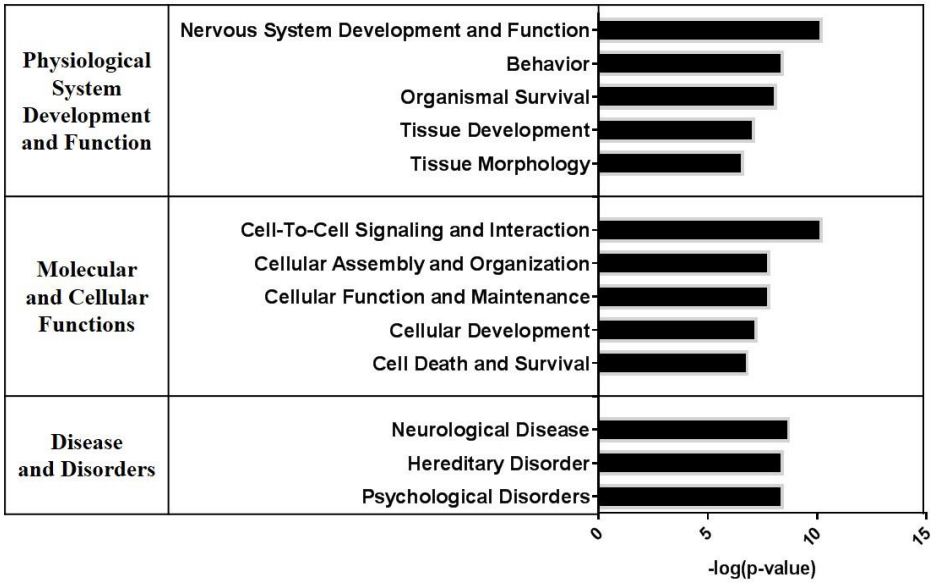

Figure 17. Top biological categories in brain transcriptome

Functional categories were classified based on IPA knowledge. Significant biological functions are represented with –log(*p*-value) by Fisher's exact test.

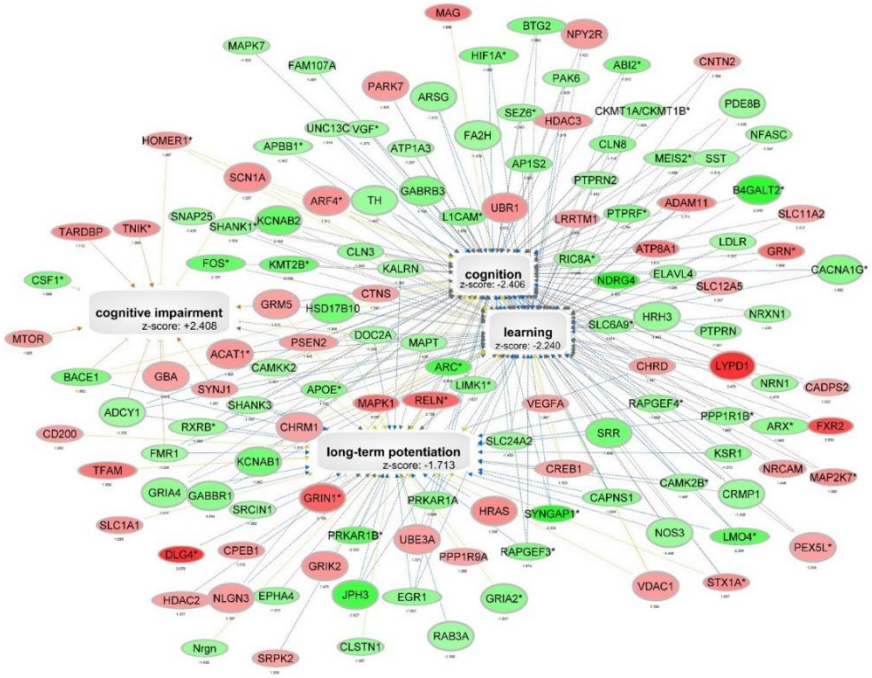

Figure 18. Top significant gene sets related to cognitive dysfunction in dehydrated mice

The most significant gene sets within functional categories were selected based on activation z-score and presented in the box. Each node indicates up- or down-regulated genes with red or green color code.

Table 1. Transcripts related to LTP signaling pathway

Function	Transcript	Gene name	Fold change
Ionotropic glutamate receptor	GRIA2	AMPA 2	-1.627
	GRIA4	AMPA 4	-1.517
	GRIN1	N-methyl D-aspartate 1	2.195
Metabotropic glutamate receptor	GRM2	glutamate receptor, metabotropic 2	-1.338
	GRM5	glutamate receptor, metabotropic 5	1.312
Signaling molecules	DLG4	discs, large homolog 4 (Drosophila)	3.070
	CAMK2B	calcium/calmodulin-dependent protein kinase II beta	-1.497
	CAMK2D	calcium/calmodulin-dependent protein kinase II delta	1.846
	ADCY1	adenylate cyclase 1 (brain)	-1.335
	EPAC1	Rap guanine nucleotide exchange factor (GEF) 3	-1.874
	PLCG1	phospholipase C, gamma 1	-1.490
	PLCL1	phospholipase C-like 1	-1.619
	PPP1CB	protein phosphatase 1, catalytic subunit, beta isozyme	-1.427
	Ppp1cc	protein phosphatase 1, catalytic subunit, gamma isoform	1.497
	c-Raf	Raf-1 proto-oncogene, serine/threonine kinase	1.311
	MAPK1	mitogen-activated protein kinase 1	2.057
	RSK	ribosomal protein S6 kinase, 90kDa, polypeptide 1	-1.807
Transcription factor	CREB1	cAMP responsive element binding protein 1	1.303

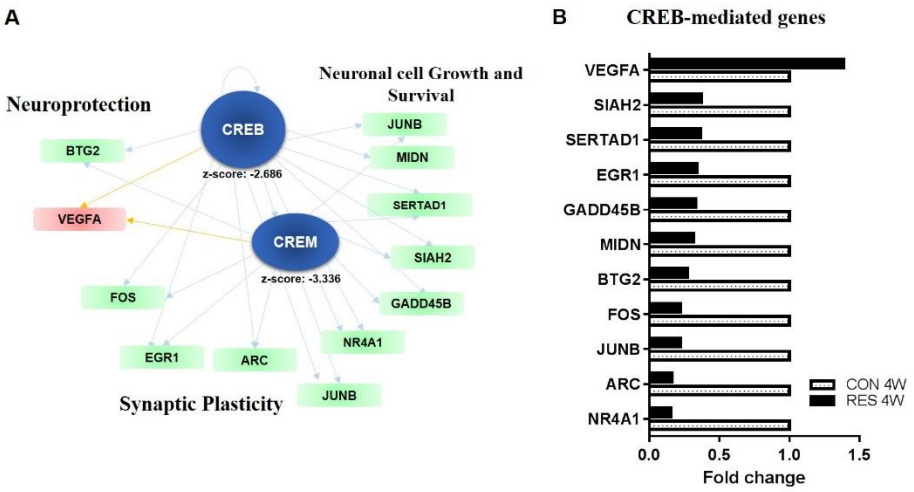

Figure 19. CREB-mediated gene transcription in LTP

(A) The top predicted regulators are in blue and connected to the downstream nodes. Activation z-score of each regulator is presented. Edges indicate predicted relationships, colored with yellow and blue when it is led to activation and inhibition, respectively. **(B)** Molecules in the nodes and their relative mRNA levels are presented in the bar graph.

IV. Discussion

It was widely accepted that nephrogenesis completes during the embryonic period. However, recent studies unearthed new facts showing that later genetic events occur in postnatal renal growth. One study revealed that gene expression from E19.5 to P35 had a postnatal developmental pattern, showing a declining trend in cell differentiation and tissue development with aging in the mouse kidney (Wu et al., 2013). In comparing the kidneys of adults to newborns, molecular transporters were found to be dominant in the transcriptome profiles (Stuart et al., 2001). Concurring with previous results, we identified that kidneys during infancy showed sequential processes indicating structural and functional maturation. Infantile kidneys, at 4 weeks old, were still differentiating and developed into functionally mature kidneys at 7 weeks old. This result provides critical evidence that postnatal care such as environmental and nutritional interventions is required for normal organ development since early renal dysfunction is implicated in various renal disorders in later life (Dressler, 2009). There is, however, a need for further studies to fully elucidate postnatal renal maturation using genetic perturbation animal models to elicit the importance of neonatal intervention for optimal growth.

Gene regulatory networks function in a coordinated fashion in kidney organogenesis to maintain kidney integrity (Burrow, 2000). It is, thus, emphasized that genetic developmental processes during the early postnatal life might be in charge of governing kidney health in later life. Moreover, despite the importance of adequate

hydration in the maintenance of a healthier life, few studies have identified molecular mechanisms underlying the impacts of dehydration (Jéquier and Constant, 2010). More than that, a fundamental methodological issue related to the experimental model of dehydration did not allow us to examine the true effects of dehydration. Acute water deprivation or heat and exercise stimulus were incapable of reflecting practical dehydration conditions due to confounding factors (Lieberman, 2007). Here, we aimed to generate mild dehydration in animals, reproducing the condition of individuals that drink less water daily. With this approach, we were able to verify the fundamental mechanisms underlying sustained moderate dehydration by profiling renal and brain transcriptomes.

We looked into the implications of dehydration throughout the early postnatal stage, from 4 to 7 weeks. Among corresponding mediators in kidney morphogenesis, the *Shh* signaling pathway and downstream target genes involved in the cell cycle and cell differentiation were downregulated as a consequence of dehydration. *Shh* is described as an important signaling molecule in renal patterning and the cell cycle during embryonic renal development (Gill and Rosenblum, 2006). Of its effectors, *Glis1* has a possible regulatory role in retaining normal morphological integrity in the kidney (Kang et al., 2010; Vasyutina and Treier, 2010). Since the transcription level of *Glis1* and its target genes were shown to be reduced in the dehydration group, we identified a morphological disorder with possible implications in cell proliferation, differentiation and death under improper hydration conditions.

As dehydration progressed throughout the developmental period, functional

deficits eventually materialized, with physiological derangements and transcriptional changes in our dataset. Kidney integrity in structure and function is characterized by a series of successive modifications from neonates to aged mice (Čukuranović and Vlajković, 2005; Rodriguez-Soriano et al., 1981). Sequential developmental processes in the kidneys correlate with numerous renal defects such as renal agenesis, hypoplasia and polycystic kidney disease (PKD) (Schedl, 2007). In other words, normal renal organogenesis is necessary for mature functioning (Čukuranović and Vlajković, 2005; Thiagarajan et al., 2011; Welham et al., 2002; Wu et al., 2013) and diverse renal diseases are common consequences of abnormal kidney development (Čukuranović and Vlajković, 2005; Schedl, 2007). That is, it is conceivable that consecutive dehydration along with perturbation of morphogenesis influences sequential developmental steps including system functional maturation. One possible mechanism responsible for functional injury in the hyperosmolar state is demonstrated by the observation that continuous angiotensin II infusion into rats induced injuries in various cell types with worsened renal function (Johnson et al., 1992). In line with this report, kidney specific cellular organization that is composed of networks between podocytes and the glomerular basement membrane was shown to be damaged as the renin-angiotensin system was activated in the dehydration group. Genes associated with glomerular filtration barrier architecture, which is comprised of the glomerular basement membrane, slit diaphragm and junction structure showed different mRNA expression patterns. In addition, many genes related to adherens junction-cytoskeleton dynamics were shown to be downregulated by dehydration. In fact, precise

regulation of junctional adhesion molecules is a decisive element in the assembly of the actin cytoskeleton, ensuring tissue homeostasis and recovery from injuries (Denker and Sabath, 2011). These adhesion molecules have pivotal roles in renal epithelial cell dedifferentiation, proliferation and migration as well. It is likely that the disruption in structural maturation during infancy resulted in defects in morphological integrity in terms of the loss of cell-cell and cell-membrane contacts in juvenile kidneys and failed adaptation to the loss of cells during regeneration (Bonventre, 2003). Meanwhile, once renal cells are injured, loss of intact cells is directly linked to the loss of nephrons (Pavenstädt et al., 2003). Due to degenerative changes in glomerular filtration barrier integrity along with podocyte foot effacement, a limited number of intact nephrons are exposed to hyperfiltration. Therefore, overload of glomerular flow per unit of the kidney might result in glomeruli injuries and subsequent renal functional perturbation, leading to increased BUN (de Barros Sene et al., 2013). It is, therefore, conceivable that reduced membrane integrity impairs glomerular barrier function followed by improper tissue morphogenesis and homeostasis.

Interestingly, prolonged dehydration during infancy resulted in reduced plasma creatinine levels. Based on present knowledge, reduction in the pool size or rate of creatinine metabolism, known as a "creatinine deficit," may account for the reduction in plasma creatinine under pathophysiologic conditions (Doi et al., 2009; Jones and Burnett, 1974). Otherwise, we assume that failure in toxic substance clearance by renal insufficiency resulted in hyper toxic loads, augmenting systemic disorders in animals. While plasma creatinine is one of the most frequently used markers

for kidney disease, it could blunt the early diagnosis of pediatric renal diseases in cases where the clinical marker is arbitrarily estimated. We, hereby, suggest that plasma creatinine might underestimate the significance of renal disorders in pediatric dehydration. Thus, it is noted that different clinical markers are required for pediatric renal disorders.

Since it is hard to assess learning and memory deficits in infancy, there has been scarce mechanistic causality study of cognition and dehydration in infants. One study carried out cerebral circulation assessment with relation to cognitive capacity after 48 hours of complete water deprivation. There was significant alteration in somatosensory cortex blood flow resulted from increased vasopressin and oxidative stress. This indicates acute dehydration induced neural dysfunction which might be related to cognitive deficits (Faraco et al., 2014). In the study of transcriptome analysis, 24 hours of acute dehydration in mouse produced shrinkage in neuronal cell volume which in turn triggered modification in transport of various neurotransmitters including glutamate, taurine, GABA and aspartate (Tang et al., 2011). In intervention study, children who received additional drink showed better performance in cognitive tasks consisting of memory, concentration, attention and executive ability tests. This result demonstrates that supplementary water intake had a beneficial role in short-term memory but it also points out that dehydration might have positive regulatory effects in some other cognitive performances (Fadda et al., 2012). To date, it is controversial whether dehydration causes alteration in cognitive processes. We, therefore,

tried to unravel the underlying implication of sustained dehydration for cognitive development by analyzing the learning behavior and brain transcriptome.

We used the Barnes maze test to measure learning and memory function in infant mice. Barnes maze test is a dry-land format of Morris water maze test for spatial learning and memory (Sunyer, 2007). In terms of water restriction experiment, we utilized Barnes maze in order to avoid exposure to water. Also, it is known that Barnes maze is less stressful to animals, without fear and anxiety (Attar et al., 2013). It was found that dehydrated mice showed abnormal performance in the behavior test. From this result, we identified the negative influence of dehydration on learning ability and measured transcript level of neurotrophins in the brain. Neurotrophin in the brain is a regulatory factor that engages in diverse processes including neuronal development, synaptogenesis and plasticity. Among neurotrophic factors, BDNF is the most notable element that plays a critical role in cognitive development (Park and Poo, 2013). It was, therefore, quite impressive that downregulation of BDNF was found in the dehydration group with no significant perturbations in other neurotrophic factors and the brain weight as well. These results triggered us to investigate the mechanistic bases of dehydration which induced cognitive deficits and large-scale analysis of brain transcriptomes were profiled.

In recent years, numerous neurochemical researches have demonstrated LTP as a primary mediator in learning and memory. LTP is the most widely studied mechanism which induces gene regulatory networks and protein synthesis to consolidate information during learning and memory formation. LTP-induced genes converge

upon CREB which is demonstrated by a transcription regulator in the involvement of induction and maintenance of LTP (Wu et al., 2007). CREB has multiple facets that regulate a wide range of neuropsychological behaviors encompassing learning, addiction and mood disorders (Carlezon et al., 2005). Taking in account its functions for synaptic strength, it regulates neuronal cell growth and differentiation, synaptic plasticity, and neuroprotection. In the dehydration group, AMPA receptors which have roles in neurite outgrowth and stabilization in LTP were significantly reduced. Interestingly, *Arc*, which regulates AMPA receptor trafficking in synaptic plasticity, was reduced as well. It seems that AMPA receptor turnover was disrupted which is strongly linked to dendrite size and growth (Shepherd and Huganir, 2007). Furthermore, although the mRNA level of CREB1 was elevated, its downstream target genes residing in synaptic plasticity and neurogenesis were downregulated, which seem to be ascribed to the maze test results. We assume that reduced transcription of TORC2, one of the coactivators of CREB, might have affected initiation of CREB-dependent gene transcription (Wu et al., 2007) but it needs to be investigated further whether CREB activity was modulated in the protein level to regulate its downstream molecules. It is also noted that, dehydrated mice showed anxiety-like behavior as well in the maze test. Mice in the dehydration group exhibited freezing behavior by halting movement during trials, which is a response to increased anxiety level in mice (Belzung and Griebel, 2001). It was remarkable especially on the fourth day of the training phase, where mice in the dehydration group stopped in movement, showing a reduction in total path length but an increase in time. We assume that it was also

derived by altered CREB activity which regulates transcription of molecules in response to aversive stress during the behavior test.

Meanwhile, at the onset of the experiment, dehydrated mice exhibited reduced diet consumption during the first few days. Despite catching up to normal diet intake after the first week of the dehydration experiment, insufficient energy intake could be a cause of developmental disorders in the situation of impaired renal development and learning ability in pediatric dehydration. Notwithstanding, it is noted that animals have different patterns of eating behavior with reduced appetite under high osmolar stimulus while they are subjected to strict control of osmolality (Greenwood et al., 2015). Given the complexity of the physiological response, therefore, we suggest that changes in eating behavior is a validated consequence of physiological adaptation to water deprivation (Greenwood et al., 2015).

In this research, we demonstrate transcriptional events during renal development in infancy, and show that the improper responses to inadequate water intake in the early postnatal stage heavily rely on the impairment of normal renal maturation. This finding suggests that optimal nutritional intervention would be required for successful renal development. Dehydration also perturbed CREB-dependent gene transcription, a key modulator in synaptic plasticity, during learning and memory consolidation. This finding is meaningful in terms of providing the first mechanistic bases in relation to dehydration and cognitive function. Altogether, this study gives us better insights into the systemic effects of dehydration in functionally immature kidneys of

infants and learning development. It may provide possible markers for clinical applications in pediatric dehydration. In this respect, this study could be a cornerstone providing predictive biomarkers for future examination of known renal diseases and a broadened perspective of pediatric dehydration during development.

V. References

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국문초록

영아기 마우스에서 탈수에 의한 신장 및 뇌에서의 전사체 변화 연구

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물은 인간의 생명과 항상성 유지에 필수적인 영양소로서, 체중의 60%를 차지하는 신체 주요 구성성분이다. 또한 체내 조직과 세포 간에 산소와 영양소 운반하는 매개체이며 혈액량과 혈류를 유지하게 하여 체내 생리적 기능에 필수적인 요소이다. 건강한 성인은 체내 수분 항상성을 유지하기 위해 체내 수분균형이 이루어지지만 영아의 경우 수분조절 시스템이 완전하지 않기 때문에 수분 결핍으로 인한 더 심각한 위험에 노출되어 있다. 더불어 영아는 성인에 비해 체수분이 75%로 높으며 체중 대비 체표면적이 크기 때문에 수분 손실의 위험이 크다. 영아기에서 물이 갖는 영양생리적 기능의 중요성이 더욱 강조되나, 관련된 연구는 매우 미흡하다. 탈수로 인한 체내 생리적 반응 메커니즘에 대한 연구는 실험동물을 이용한 단기간 동안의 수분 결핍 또는 제한

연구들이 수행되어 왔다. 하지만 장기간 동안의 수분섭취 부족의 영향에 대한 연구는 전무하며, 특히 영아기에서 탈수에 의한 생리적 변화에 대한 분자 생물학적 메커니즘 연구와 학습능력과의 상관관계에 대한 연구가 미진하다. 따라서 본 연구는 영유아기에서 청소년기의 생애에 걸친 장기적인 탈수가 신장과 뇌의 전사체에 미치는 영향을 확인함으로써 물이 가지는 영양생리 기능적 중요성을 시스템 생물학적 관점에서 규명하고자 하였다. 이유가 끝난 직후의 3주령 C57BL/6 마우스를 이용하여 1주 또는 4주동안 수분의 제한적인 섭취에 의한 생리적, 생화학적 변화를 관찰하였다. 하루 15분 간 수분을 제한적으로 섭취한 수분제한군은 수분을 자유롭게 섭취한 대조군에 비해 약 30%의 적은 물을 섭취하였다. 체중은 군 간에 큰 차이가 없었으며 탈수 지표인 혈장 삼투압과 뇌하수체에서 항이뇨 호르몬의 유전자 발현이 수분제한군에서 유의적으로 증가하였다. 탈수에 대처하는 영아의 미성숙한 신기능에 대한 이해를 위해 마이크로어레이를 이용하여 신장의 전사체를 분석하였다. 영아기 마우스의 신장은 아직 기관의 구조 및 기능적 성숙 과정에 있었고 수분제한에 의해 기관의 발달 및 성숙에 관여하는 유전자의 발현이 변화하였다. 수분제한군에서 태아기 신장 발생에 중요하며 세포 주기를 조절하는 SHH 시그널이 유의적으로 감소하였다. 지속적인 탈수는 사구체 여과 장벽을 구성하는 세포골격의 리모델링에 관여하는 유전자의 발현을 감소시킴으로써 사구체 여과막의 구조적, 기능적 항상성을

깨트렸다. 이는 결국 신기능의 저하로 이어져 수분제한군에서 혈중 요소 농도가 유의하게 증가하였다. 탈수가 인지능력에 미치는 영향을 확인하기 위해 공간학습능력을 측정하는 Barnes maze 실험과 차세대 염기서열 기법을 이용한 뇌의 전사체 분석을 수행하였다. Barnes maze 실험에서 대조군은 4일간의 학습 뒤 탈출구의 위치를 기억하고 찾아가는 시간이 유의하게 감소한 반면, 수분제한은 증가하여 공간 학습능력이 저해됨을 확인하였다. 뇌의 무게는 군 간에 차이가 없었으나 뇌성장인자 중 BDNF가 해마에서 특이적으로 유전자 발현이 감소한 것으로 보아 탈수가 기억형성 과정에 중요한 영향을 미친 것을 확인하였다. 뇌 전사체 분석 결과, 수분제한군에서 학습과 기억을 관장하는 장기기억강화 시그널에 관여하는 유전자의 발현이 변화하였다. 위 결과를 종합하면, 영유아기에 정상적인 신장 발달 및 학습능력 발달을 위해 충분한 수분 섭취가 필요함을 시사한다. 본 연구는 영아기에서 탈수에 의한 생리적, 학습심리학적 변화를 매개하는 분자 생물학적 메커니즘을 규명한 첫 연구이며, 소아 탈수에 대한 시스템 생물학적 이해와 함께 향후 소아 탈수 및 신질환의 진단 및 치료에 적용할 수 있는 새로운 임상 지표를 제시한다는 점에서 그 의의가 있다.

주요어: 탈수, 영아기, 전사체, 신장 발달, 학습능력

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