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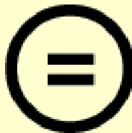

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A THESIS FOR THE DEGREE OF
MASTER OF SCIENCE IN FOOD AND NUTRITION

Effects of Sucrose Solution Intake during Growth Period on Social Aggression and Inflammatory Response in Adult Mice

성장기 동안의 설탕용액 섭취가 마우스 공격성에 미치는 영향 및 관련 기전 연구

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Department of Food and Nutrition
Graduate School
Seoul National University
Jung-Yun Choi

Effects of Sucrose Solution Intake during Growth Period on Social Aggression and Inflammatory Response in Adult Mice

지도교수 신 동 미

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식품영양학과

최 정 윤

최 정 윤의 생활과학 석사학위 논문을 인준함

2016 년 7 월

위 원 장____________________(인)

부위원장____________________(인)

위 원____________________(인)

Abstract

Effects of Sucrose Solution Intake during Growth Period on Social Aggression and Inflammatory Response in Adult Mice

Jung-Yun Choi
Department of Food and Nutrition
The Graduate School
Seoul National University

Aggressive behavior has been traditionally defined as an overt behavior with the intention of inflicting physical damage on other individual. Although aggression has advantages in competitive situations for obtaining food or defending territories and mates from competitors in wild animal, it has been considered as one of the major social problems in the human society. Among extrinsic factors which are considered to affect aggressive behaviors, the association between specific food component in the diet such as sugar and aggressive behaviors has been suggested. Sugars are found in diet either as a natural component

of the food or as an added sugar of foods and beverages. Although added sugar enhances food desirability by sweetening foods and beverages, it provides only empty calories with no nutrient value. Although overconsumption of sugar-sweetened beverages (SSBs) is widely known to be a key contributor to epidemic of overweight and obesity, its effects on behavioral changes have not been fully studied yet. In present study, we examined the long-term effects of SSB on social aggression in mice. 3 week old weaned mice started to drink either 30 w/v% of sucrose solution (S30), plain water (CT), or aspartame solution with equivalent sweetness of the sucrose solution (A30) until they grew to 11 week old adult. Resident-intruder test revealed the total duration spent on any of six different aggressive behaviors- biting, sexual mounting, clinch, lateral threat and upright was significantly longer in S30 than any other groups. The transcriptome analysis of brain tissues presented the gene expression profiles of S30 were readily distinguished from those of CT or A30 in principal component analysis (PCA). Differentially expressed genes in S30 compared to CT or A30 were categorized on their biological functions and top 6 significant categories were all related to immunological disorders. Bioinformatic analysis of upstream regulator molecules that control target gene expressions identified *Tlr4*, *Stat4*, *Chuk* and *Il1b* that lead to inflammatory responses in mice of S30. Heightened levels of corticosterone, an analogue of cortisol for rodents, was observed in S30 and the dramatic increase in the number of CD11b^{+}Gr-1^{+} cells was observed in the same group by FACS analysis. Furthermore, corticosterone resistance was found in mice of S30. Interestingly, the artificial sweetener failed

to mimic the roles of sugar in elevating corticosterone and promoting social aggression. Taken together, these results demonstrate long-term SSB consumption promotes aggressive behaviors, and also inflammatory response in brain and corticosterone resistance mediate the roles played by sugar in promoting social aggression. Our studies provide information useful for development of intervening strategies to control social aggression for public health.

Keywords : Sugar-sweetened beverage, Social aggression, Transcriptome, Inflammatory response, Glucocorticoid resistance

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List of Abbreviations

FACS	Fluorescence activated cell sorter
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
IPA	Ingenuity pathway analysis
PBL	Peripheral blood
PCA	Principal component analysis
PCR	Polymerase chain reaction
RNA	Ribonucleotide acid
S.E.M.	Standard error of the mean
SSB	Sugar-sweetened beverage

I. Introduction

Aggressive behavior has been traditionally defined as an overt behavior with the intention of inflicting physical damage on other individual. The potential for aggressive behavior exists whenever the interests of two or more individuals conflict with broad similarities across species from murine to human (Moyer, 1971). Although aggression has advantages in competitive situations for obtaining food or defending territories and mates from competitors in wild animal kingdom (Nelson, 2005), it has been considered as one of the major social problems in the human society. The World Health Assembly officially declared violence as a major public health issue in 1996 and World Health Organization (WHO) released the first World Report on Violence and Health in 2002 (Krug, Mercy, Dahlberg, & Zwi, 2002) and various forms of violence have incurred huge costs in treating victims and repairing infrastructure. Therefore, it might be important to identify relevant factors to promote or suppress aggressive behavior for effective interventions and, therefore contribution to public health ultimately.

Aggression-related molecules may include neuropeptides and neurotransmitter, which are small protein-like molecules or chemical messengers regulating neuronal signaling. Vasopressin (ADH; antidiuretic hormone) and oxytocin are frequently reported as neuropeptides that are related to aggressive behaviors. Several studies in animals have demonstrated that oxytocin can reduce aggression (Caldwell & Young III, 2006; Consiglio, Borsoi, Pereira, &

Lucion, 2005) while vasopressin is likely to increase male-male aggression in birds and mammals (Veenema, Beiderbeck, Lukas, & Neumann, 2010; Wersinger, Ginns, O'carroll, Lolait, & Iii, 2002). In addition, low level of serotonin, one of the neurotransmitter related to mood, was reported to induce impulsivity and aggression ("Exaggerated aggression and decreased anxiety in mice deficient in brain serotonin," 2012). Recently, transcript levels of genes such as *Mecp2*, *Adrbk2 and Maoa* and a few transcriptional networks including NF-kB and MAPKs signaling have been studied in relation to aggressive behaviors (Malki et al., 2014; Popova, 2006; Tantra et al., 2014). Furthermore, among extrinsic factors, diet, one of the daily necessities of life, has attracted attention due to its effect on behaviors. Diet was believed already to determine not only health and disease, but also spirituality, mental health, intelligence, and temperament including antisocial or aggressive behavior with prevailing concept of health reform movement (Kanarek, 1994).

In this regard, specific food component in the diet could be considered as one of the aggression-modulating factors, which may regulate aggressive behavior. Sugars are found in diet either as a natural component of the food or as an added sugar of foods and beverages. Although added sugar enhances food desirability by sweetening foods and beverages, it provides only empty calories with no nutrient value (Popkin, 2014). Considering potential negative effects of excessive sugar intake, WHO already suggested a guideline to provide recommendations on the intake of free sugars in adults and children, with a particular

focus on the prevention and control of unhealthy weight gain and dental caries (Organization, 2015). Increase in sugar intake could be possibly attributed to rising consumption of sugar-sweetened beverages (SSB) (Slining & Popkin, 2013; Wang, Bleich, & Gortmaker, 2008). Numerous studies have been reported that overconsumption of SSB might be related to metabolic diseases such as obesity and type 2 diabetes and even cancers (Grimes, Riddell, Campbell, & Nowson, 2013; Montonen, Järvinen, Knekt, Heliövaara, & Reunanen, 2007; Schulze et al., 2004; Takahiro et al., 2005). Overconsumption of SSB is extensively studied for the associations with physical health problems, however, its effects on behavioral changes have not been fully studied yet.

The association between aggressive behaviors and sugar consumption remains controversial and inconsistent. One study reported high level of sugar consumption was positively related with destructive-aggressive behaviors in hyperactive children (Prinz, Roberts, & Hantman, 1980). Others also found that reduced sugar intake might be linked to lower incidence of formal disciplinary actions in juvenile prison inmates (Schoenthaler, 1982, 1983a, 1983b). Furthermore, in adolescents, a relationship between levels of consumption of sugar-containing soft drinks and behavior problems was found in a cross-sectional population-based survey (Lien, Lien, Heyerdahl, Thoresen, & Bjertness, 2006). In contrast, no such a correlation was found in the study (Wolraich et al., 1994), in which oppositional or aggressive behaviors were assessed in the school-aged

children that were provided with sugar-sweetened diet. Lack of systemic studies using animal models that enable to examine the causal effect of sugar consumption on behavior problem may contribute to increase in the ambiguousness.

In present study, we aimed to investigate the effects of sucrose solution on social aggression in animal model, therefore, we assessed aggressive behaviors in mice consumed sucrose solution from infancy to adulthood, which were compared to those of artificial sweetener solution or plain water, respectively. Artificial sweetener was established to investigate the effects of sweet taste on aggressive behavior, which was used as a control for sweetness while plain water was used as a control for to study the roles of sucrose itself. In addition, to elucidate underlying mechanism of aggressive behaviors induced by sucrose consumption, transcriptome analysis of brain tissues were performed. Findings from the transcriptome analysis prompted us to assess the changes in numbers of inflammatory cell in peripheral blood using flow cytometry. Further investigation on the relationship between intrinsic glucocorticoid and inflammation was also carried out as to validate glucocorticoid resistance in mice following consumption of sucrose solution. These results provide evidences that continuous consumption of sucrose solution from infancy to adulthood lead to increase in social aggression and insights into understanding the physiological and molecular mechanisms by which sugar play roles in promoting aggressive behaviors.

II. Materials and Methods

1. Experimental animals

3 week-old male C57BL/6J mice after weaning were obtained from Jackson Laboratory (Bar Harbor, Maine, USA) and were acclimated for 4 days. Experimental mice were assigned to three groups depending on the type of drinks; provided with plain water (CT, n = 9), aspartame solution with equivalent sweetness of 30 w/v% sucrose solution (A30, n = 8) (Aspartame 100%, The Nutrasweet Company, USA), or 30 w/v% sucrose solution (S30, n = 8) (Saccharose, Daejung, South Korea) *ad libitum*. Each mouse was kept in individual plastic cages under conditions of animal facility at the college of veterinary medicine of Seoul National University (Seoul, South Korea). All group was provided normal diet composed of 20 kcal% protein, 70 kcal% carbohydrate and 10 kcal% fat (3.85 kcal/g, D12450J, Research diets). Single housing was necessary since aggressive behavior test requires to maintain isolated state from other mice.

On day 54, mice were anesthetized and sacrificed through intraperitoneal injection of 20 % urethane (U2500, Sigma-Aldrich, USA) after fasting. All animal experiments were performed after receiving approval of the Institutional Animal Care and Use Committee of the Institute of Laboratory Animal Re-

sources, Seoul National University (Institutional Animal Care and Use Committee permit number: SNU-140129-6). All experiments were carried out in accordance with the guidelines and regulations.

2. Behavioral assessment

The resident-intruder test was performed to measure and evaluate inter-male aggression in each resident mouse against intruder mouse. Resident mice were housed individually to establish their own territory in home cage. The intruder mice (n=15) were group-housed males, 4 weeks younger than resident mice. They were used once or two times as an intruder, therefore each intruder mouse could confront one or two resident mice. On day 50, each intruder mouse was introduced to a resident mouse in resident's home cage temporarily divided by an acrylic board with several holes. At first, the resident and intruder mouse could experience only by their scent through the holes in acrylic board for 5 minutes in order to inform the resident that there is a stranger in his home cage. Thereafter, acrylic board was removed and they could explore each other for 10 minutes. All process of resident-intruder test were recorded. Latency to first attack, time and frequency of following aggressive behaviors were measured; biting, clinch, sexual mounting, lateral threat, keep down, upright. Duration to show total time of all 6 different aggressive behaviors was also calculated.

3. Serum biochemical analysis

Blood samples of mice were collected by heart puncture under 20 % urethane anesthesia. Serum was taken after blood samples centrifuged at 2,000 rpm for 15 min. in 4 ℃ and stored at -80 ℃. Serum corticosterone levels were measured using Corticosterone ELISA kit (Abcam LLC, Cambridge, UK) in accordance with manufacturer's instructions.

4. RNA isolation

Total RNA was isolated by homogenizing tissue samples of hypothalamus using TissueLyser II (QIAGEN, Crawley, UK) and purified using a DNA-free RNA isolation kit (RNAqueous-4PCR kit; Ambion, Austin, TX, USA) according to the manufacturer's instructions. The integrity and quantity of total RNA were assessed with a NanoDrop 2000 Spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA) before the microarray experiments and its quality was assessed by agarose gel electrophoresis using 1 % gels.

5. Microarray hybridization

For microarray hybridization, RNA samples were first amplified for array analysis using the Illumina TotalPrep RNA Amplification Kit (Ambion, Austin, TX,

USA) in accordance with the manufacturer's instructions. Briefly, 500 ng of total RNA was used to prepare labelled cRNA with overnight incubation according to the manufacturer's protocol. The quality and quantity of the labelled cRNA were monitored using a NanoDrop 2000 Spectrophotometer (Thermo Fisher Scientific, Wilmington, DE). Amplified cRNA was hybridized on MouseWG-6 v2 Expression BeadChip arrays, containing more than 45,200 well-annotated Ref transcripts in mice, according to the manufacturer's standard protocol. The array were scanned on a BeadArray Reader (BeadStation 500G Instrument, Illumina Inc.). Spot images were identified and quantified by the Genome Studio software v1.0.2. (Illumina Inc.).

6. Bioinformatic analysis of microarray data

The raw data from microarray were pre-processed through three steps: background correction was performed, the data were then log-transformed to log2 scale, and normalized by quantile normalization method implemented in the Genome Studio software (Illumina Inc.). Significant difference among three groups was identified using ANOVA test ($p < 0.05$) on log2-transformed normalized intensities by Partek® Genomics Suite software v6.3 (Partek, St Louis, MI) (http://www.partek.com/partekgs). Transcripts with more than 2-fold differential were selected for each specific comparison analyzed.

Principal component analysis (PCA) was carried out by Partek® Genomics Suite software v6.3 (Partek, St Louis, MI) (http://www.partek.com/partekgs). Hierarchical clustering analysis was performed with Genesis software v1.7.5 (Sturn, Quackenbush, & Trajanoski, 2002) using the Pearson correlation distance matrix with average linkage algorithm. Functional identification was categorized by the use of QIAGEN's Ingenuity® Pathway Analysis (IPA® , QIAGEN Redwood City, www.qiagen.com/ingenuity). Upstream regulator analysis was performed to predict upstream regulators with calculated z-score with indirect or direct relationships in dataset through IPA.

7. FACS (Fluorescence activated cell sorter) analysis

Fresh peripheral blood lymphocytes (PBLs) were prepared by incubating blood with ACK (ammonium-chloride-potassium) buffer to lyse red blood cells at room temperature for 3-5 minutes, and stained using FACS buffer (1X phosphate-buffered saline [PBS] with 0.1 % bovine calf serum and 0.05 % sodium azide) containing fluorescein isothiocyanate (FITC)-conjugated monoclonal antibody (mAb) to Gr-1 (RB6-8C5, eBioscience, San Diego, CA, USA) and phycoerythrin (PE)-conjugated mAb to CD11b (M1/70, eBioscience) at 4 °C for 30 minutes. After washing with FACS buffer, the cells were analyzed by a

FACSCalibur (BD Bioscience, Franklin Lakes, NJ, USA) and Flowjo software (Tree star, Ashland, OR, USA) (Shon, Lee, Shin, Choi, & Shin, 2015)

8. Quantitative Real-Time PCR

Synthesis of cDNA was done by MessegeSensor™ RT kit (Ambion, Austin, TX, USA) and mRNA levels were quantified by using Applied Biosystems® SYBR® Green PCR Master Mix. Relative mRNA expression level was calculated by $\Delta\Delta C_T$ method. Detection of vasopressin (NCBI Reference Sequence: NM_009732.1) and oxytocin (NCBI Reference Sequence: NM_011025.4) was performed using each specific primer pairs amplifying vasopressin (forward primer: 5’-CCAGGATGCTCAACACTACG-3’, reverse primer: 5’- CTCTTGGGCAGTTCTGGAAG-3’) and oxytocin (forward primer: 5’-CCTACAGCGGATCTCAGACTGA-3’, reverse primer: 5’-TCAGAGCCAGTAAGCCAAGCA-3’) respectively. Each gene expression levels were normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (forward primer: 5’- TGCACCACCAACTGCTTAG-3’, reverse primer: 5’-GATGCAGGGATGATGTTC-3’). Quantitative PCR results were assessed by melt curve to verify if the correct amplicon was produced.

9. Statistical analysis

All data were expressed as the mean ± S.E.M. Statistical significance (p-value < 0.05) was evaluated by Mann-Whitney U test. Statistical analyses were performed using Graph Pad Prism 5 software (GraphPad Software Inc., La Jolla, CA, USA) and SAS Enterprise Guide 6.1 (SAS Institute Inc., Cary, NC, USA). Significances for functional enrichment of specific genes were determined by a right-tailed Fisher's exact test as the negative log of the probability that the number of focus genes is not due to random chance.

III. Results

1. Mice provided with sucrose solution for long-term period displayed distinct patterns of drink and diet consumption

Right after weaning, 3 week-old male C57BL/6J mice were randomly assigned into three groups and provided with plain water (CT), aspartame solution with equivalent sweetness of 30 % sucrose solution (A30) or 30 % sucrose solution (S30) from infancy to adulthood (Figure 1). While both CT and A30 group were used as control groups for S30 group, especially mice in A30 group were utilized as a comparison group for sweetness of sucrose solution. After providing each solution, we conducted a series of analyses to identify the effects of consuming sucrose solution for long-term period on aggressive behaviors.

Firstly, as shown in Figure 2, the pattern of diet or drink consumption in S30 group was distinguished from those of CT or A30 group. The diet intake in S30 was less than other two groups, about one third of CT or A30 diet consumption (Figure 2A). However, mice in S30 group consumed sucrose solution about two times more than drink consumption in CT or A30 consistently (Figure 2B). Consequently, daily caloric intake calculated by diet and drink consumption was significantly higher in S30 than CT ($p < 0.001$) or A30 ($p < 0.001$). Notably, calories consumed in drink contributed to increased daily caloric intake in S30 whereas calories consumed in diet were one and half of those

in other two control groups. As a result, mice in S30 group tend to intake calories from drink rather than diet.

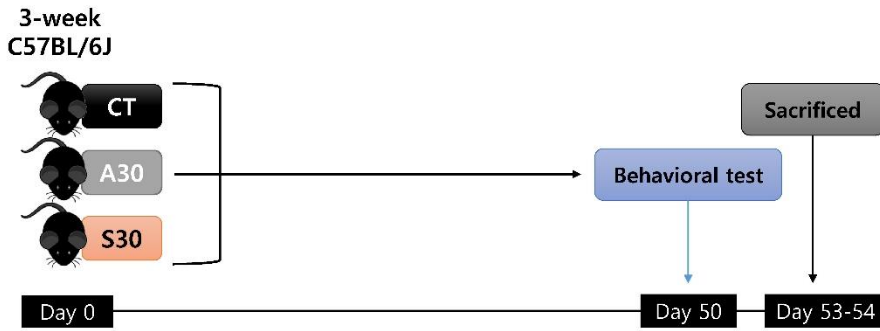

Figure 1. Experimental scheme.

Right after weaning, 3-week old male mice were obtained and randomly assigned into three groups by matching their body weight. Three different kinds of solutions were supplied from infancy to adulthood. 30 % sucrose solution was offered to S30 group. Plain water or aspartame solution with equivalent sweetness of 30 % sucrose solution was provided to CT or A30 as controls respectively. After providing each solutions for 8 weeks, behavioral test were conducted to assess aggressive behaviors in each group. Then, all mice were sacrificed to obtain blood and tissues for further analyses.

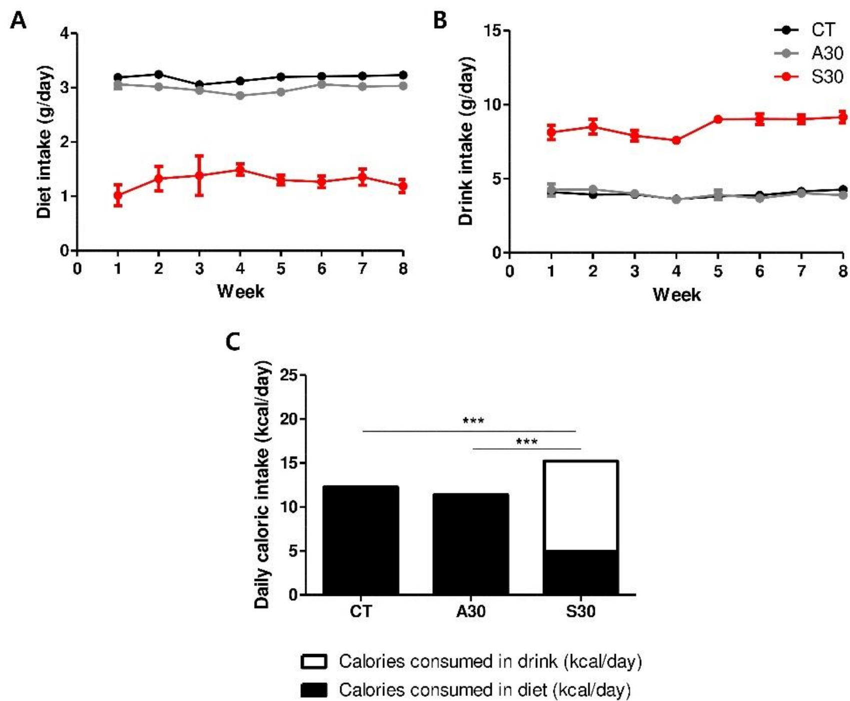

Figure 2. Diet, drink and daily caloric intake.

(A) Diet intake was significantly declined consistently in S30 (n = 8) compared with CT (n = 9) or A30 (n = 8) group. (B) Mice in S30 consumed sucrose solution as a dietary drink about twice amount of drink consumption in CT or A30 group. (C) Daily caloric intake in S30 was significantly higher than other two control groups. Bar graph in white imply calories consumed in drink; bar graph in black imply calories consumed in diet. Daily caloric intake was calculated based on diet and drink intake. All data were presented as the mean ± S.E.M. Mann-Whitney U test was performed to determine significant difference in S30 compared with CT or A30. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

2. Consumption of sucrose solution reduced body weight gain without inhibiting growth curve

Regardless of types of solution, body weight of mice in each group normally increased as they grow up until become adulthood (Figure 3A). However, the growth curve for body weight in S30 was more gradual than those of CT or A30. In particular, mean body weight at 2-week of experimental period was significantly decreased compared with those of CT. This difference in body weight between S30 and CT group was continued throughout whole experimental period. At the end of experiment, the final body weight of S30 was significantly lower than that of CT (Figure 3B). Also, the percent of body weight gain, which means the percent of final body weight compared with initial body weight was significantly lower in S30 than CT or A30 (Figure 3C).

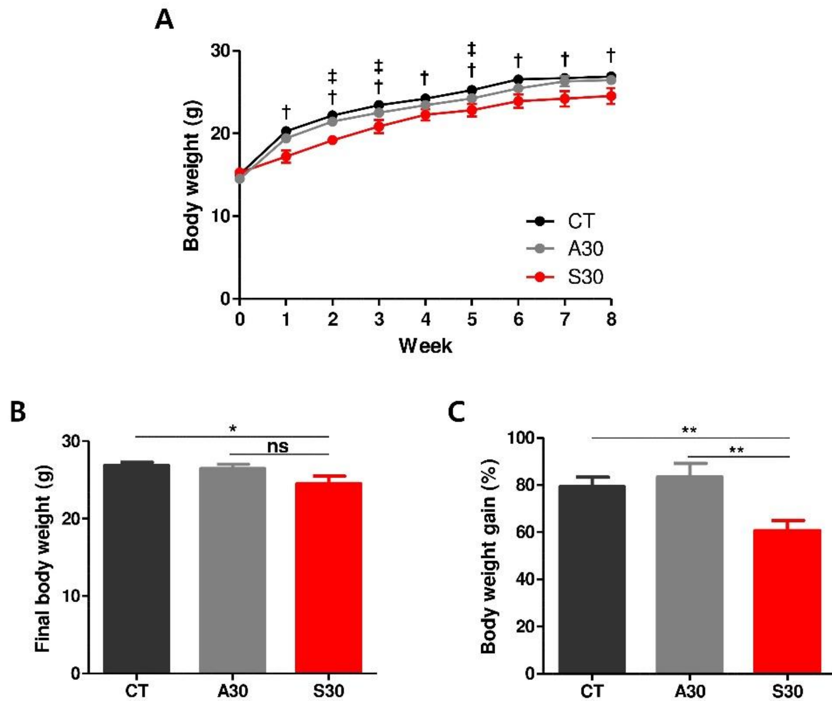

Figure 3. Body weight changes.

(A) Body weight of mice in all group (n = 8-9 per each group) gradually increased until the end of experimental period (8-week) regardless of kinds of solution consumed in each group. Statistical significance was presented; CT versus. S30 (†$p < 0.05$); A30 versus. S30 (‡$p < 0.05$). (B) Final body weight of S30 was significantly declined compared with CT. (C) The percent of body weight gain in S30 group was significantly lower than CT or A30. All data were presented as the mean ± S.E.M. Mann-Whitney U test was performed to determine significant difference in S30 compared with CT or A30. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

3. Long-term consumption of sucrose solution had influence on aggression-related hormones

To identify the effects of consumption of sucrose solution for long-term period on aggression, aggression-related hormones was measured. Since corticosterone secretion was previously reported to be related to aggressive behavior in rodents (Haller, Halasz, Mikics, Kruk, & Makara, 2000), we first examined the serum corticosterone levels in each group. Transcription levels of vasopressin, which is reported as one of the aggression-related hormones showed no significant difference among groups (Figure 4A). Furthermore, increased oxytocin transcription levels were observed in S30 mice compared to mice in aspartame group with significance (Figure 4B). Surprisingly, concentration of serum corticosterone in S30 mice was higher significantly than other groups as shown in Figure 4C. These findings imply that long-term sucrose consumption from infancy to adulthood may lead to changes in aggression-related hormones. Also, aspartame solution failed to mimic the effects of sucrose solution according to different results of S30 from A30, which means that it is more likely that these changes were induced by sucrose itself rather than a sweet taste.

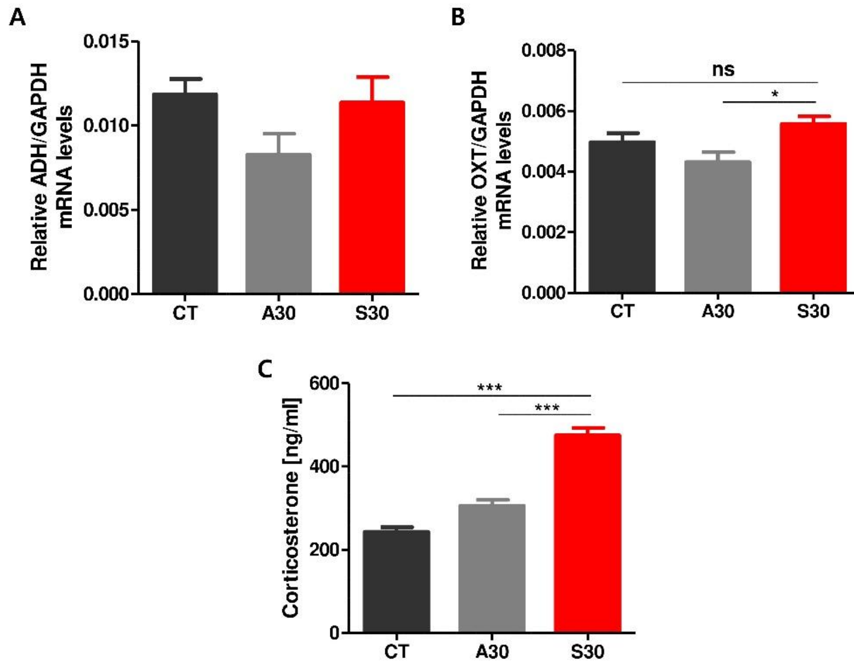

Figure 4. Aggression-related hormones in mice following consumption of plain water, sucrose solution or aspartame solution from infancy to adulthood.

(A, B) Relative mRNA expression level of ADH (A) and oxytocin (B) of hypothalamus in mice following consumption of plain water (n = 9), sucrose solution (n = 8) or aspartame solution (n = 8) for 8-week. Each gene was normalized to GAPDH. (C) Concentration of serum corticosterone following consumption of sucrose solution, aspartame solution, or plain water for 8-week. All data are presented as mean ± S.E.M. Mann-Whitney U test was performed to determine significant difference in S30 compared with CT or A30. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

4. Increased social aggression was induced by long-term consumption of sucrose solution

In order to identify the associations between aggression-related hormones and aggressive behaviors in mice following long-term consumption of sucrose solution, resident-intruder test was conducted to examine male-male territorial aggression. We measured latency to show the time of first attack by resident mice against intruder mice. Also, total time and numbers of different kinds of aggressive behaviors were measured that include biting, sexual mounting, clinch, lateral threat, keep down and upright.

Dramatically, mice in S30 group showed a significant increase in duration, which includes the total time of all six different aggressive behaviors (Figure 5B). No significant difference among groups was observed in latency as shown in Figure 5A. Although each aggressive behavior was not significantly different among groups, there was a tendency of increase in time and number of biting and lateral threat in S30 compared with CT or A30 (Figure 5C). These results suggest that aggressive behaviors could be affected by continuous consumption of sucrose solution for long-term period with various changes in aggression-related hormones.

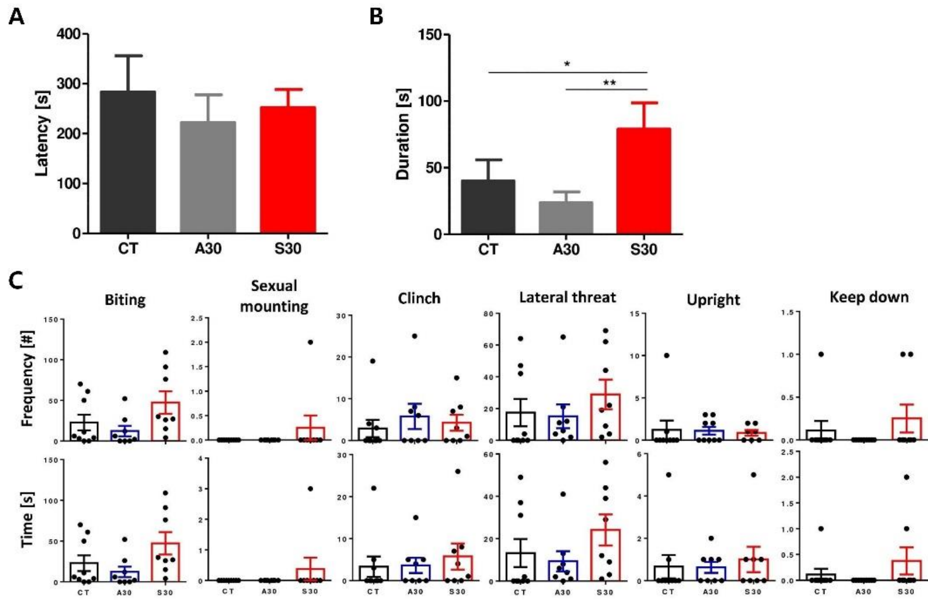

Figure 5. Aggressive behaviors in adult mice consumed plain water, sucrose solution or aspartame solution for long-term period.

(A) Latency to show the time of first attack performed by resident mice following consumption of sucrose solution (n = 8), aspartame solution (n = 8), or plain water (n = 9) for 8-week. (B) Duration to show the total time of all aggressive behaviors. (C) Frequency (upper) and time (lower) of six different kinds of aggressive behaviors: biting, sexual mounting, clinch, lateral threat, upright, and keep down. Each dot linearly displayed by each group means a value of individual mouse. All data are presented as mean ± S.E.M. p-values were generated by Mann-Whitney U test to determine significant difference in S30 compared with CT or A30. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

5. Transcriptome analysis revealed consumption of sucrose solution induced inflammatory response in brain

To identify underlying mechanisms by which consumption of sucrose solution induced aggressive behaviors, transcriptome analysis in brain was performed. Total RNA from hypothalamus of randomly-selected 4 different mice in each group were applied to Illumina whole genome mouse arrays. 3,750 differentially expressed genes were identified among groups by one-way ANOVA test with a cutoff of $p\text{-value} < 0.05$.

Principal component analyses (PCA) confirmed that S30 group was readily distinguished from CT or A30 group (Figure 6A). Since aggressive behaviors seemed to be enhanced by sucrose itself rather than sweet taste, we conducted further analyses using data from S30 group compared with CT. Differentially expressed genes in S30 were categorized based on their biological functions. Also, functional relevance of significant genes modulated by consumption of sucrose solution were examined by right-tailed fisher's exact test for their significance. A large number of differentially expressed genes in S30 were involved in categories such as Cellular Movement, Hematological System Development and Function, Hypersensitivity Response, Immune Cell Trafficking, Hematological Disease and Infectious Disease as top 6 significant biological categories (Figure 6B).

Hierarchical clustering analysis was carried out to show that each

group was well segregated as shown in PCA (Figure 7). Then, we focused on two gene clusters, which showed clearly distinguished gene expression patterns in S30 from to other groups. In cluster A, normalized gene expression value was increased only in S30 whereas in cluster B, gene expression value was decreased only in S30. Furthermore, a total of 68 genes in cluster A were classified to explore biological implications. Functional identification and Fisher's exact test confirmed Inflammatory Response (p = 2.95E-03), Cancer (p = 3.00E-03), and Developmental Disorder (p = 3.00E-03) as the top 3 significant Diseases and Disorders. According to Inflammatory Response, seven molecules were included such as *Mgst1*, *Ces3*, *Spn*, *Abcb4*, *Cxcl1*, *P2rx7*, and *Sh2b3*. In particular, *Cxcl1* plays a role in inflammation and as a chemoattractant for neutrophils. *Sh2b3* encodes a member of the SH2B adaptor family of proteins that are involved in a range of signaling activities by growth factor and cytokine receptors. Likewise, in cluster B, the top three significant Diseases and Disorders detected by IPA were Immunological Disease (p = 1.73E-03), Hematological Disease (p = 2.52E-03), and Cancer (p = 3.12E-03) with associated molecules of *Serpinb3a*, *Tbxa2r*, *Tlr7*, *Slit2*, *Dusp10*, and *Pik3r1* in Immunological Disease.

On the basis of gene expression dataset, we carried out further bioinformatic analysis to explore upstream regulators controlling multiple genes in our dataset. As shown in Figure 8, a number of genes were up- or down-regulated in S30 compared to CT with upstream regulators detected by calculated z-score via IPA such as IL1B (z-score = 2.404), STAT4 (z-score = 2.400),

CHUK (z-score = 2.190), and TLR4 (z-score = 1.946). Interestingly, these up-stream regulators which were predicted as activation state in S30 are known to be mediators of inflammation. Together, augmented inflammation state including activated upstream regulators and genes, which are both associated with pro-inflammatory reaction and inflammatory response was induced by consuming sucrose solution for long-term period in mice.

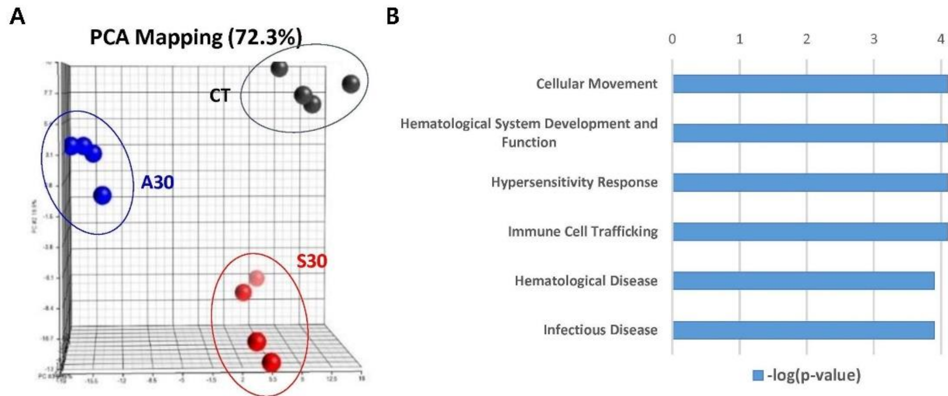

Figure 6. Principal component analysis and functional identification of significantly expressed genes in S30.

(A) 3-D view of principal component analysis scores plot of brain transcriptome. Each spots indicates individual mouse in the group classified by different colors. Black spots, mice consumed plain water (CT, n = 4); Red spots, mice consumed 30 w/v% sucrose solution (S30, n = 4); Blue spots, mice consumed aspartame solution with equivalent sweetness of 30 w/v% sucrose solution (A30, n = 4). (B) Functional identification categorized by IPA; Top six categories of diseases and bio functions was detected in S30 group compared to control group. Statistical significance was calculated by a right-tailed Fisher's exact test in IPA and –log (p-value) is noted on y axis.

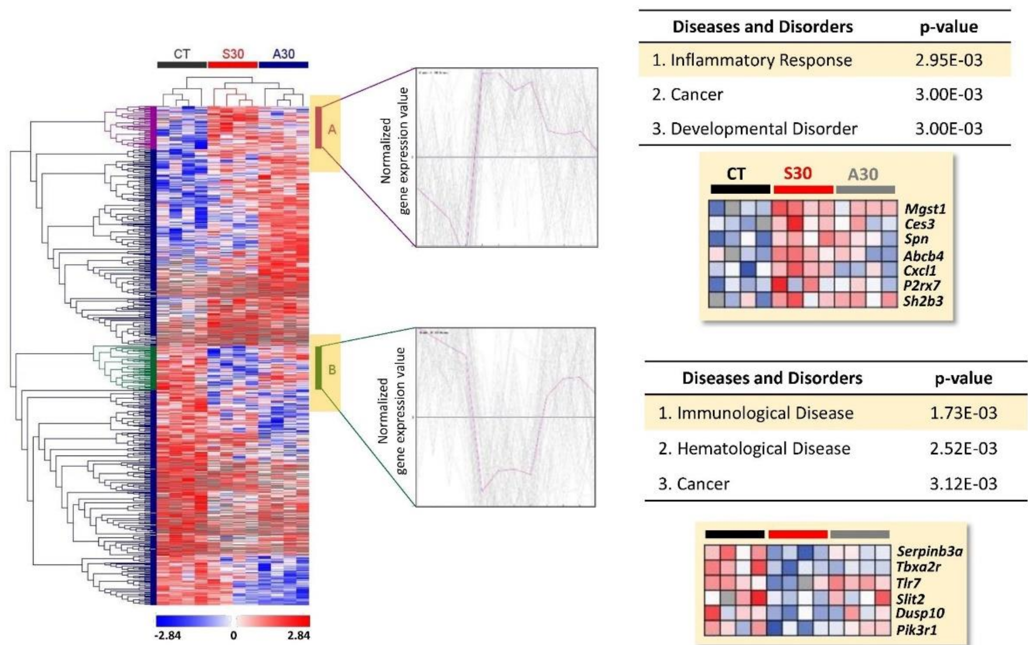

Figure 7. Hierarchical clustering analysis with two clusters and their functional identification.

Hierarchical clustering and heat map of differentially expressed genes (> 2-fold) in either S30 (n = 4) or A30 group (n = 4) compared with CT group (n = 4). Red represents high relative expression and blue represents low relative expression of genes as shown in colored scale bar below the heat map. Clusters were established by gene expression patterns as shown in gene expression view. Cluster A consists of genes that are only up-regulated (n=68) in S30 compared to CT or A30; Cluster B is composed of down-regulated genes (n=73) only in S30 compared to CT or A30.

Table 1. List of top 20 genes in cluster A.

Symbol	Gene name	Fold-change	Molecular function
Setdb2	SET Domain Bifurcated 2	6.817	Histone-lysine N-methyltransferase
Anxa4	Annexin A4	4.139	Calcium-dependent protein binding
Slc27a5	Solute carrier family 27 member 5	4.105	Fatty acid transporter, long-chain-fatty-acid-CoA ligase
Mgst1	Microsomal glutathione S-transferase 1	3.528	Glutathione peroxidase
Creb3l3	cAMP responsive element binding protein 3-like 3	3.431	Transcription regulator
Asph	Aspartate beta-hydroxylase	3.404	Calcium ion binding, transmembrane domain
Dnajb8	DnaJ heat shock protein family (Hsp40) member B8	3.345	Chaperone binding
Ces3	Carboxylesterase 3	3.328	Carboxylic ester hydrolase activity
Spn	Sialophorin	3.252	Protein binding; transmembrane signaling receptor activity
Siah1b	Seven in absentia 1B	3.239	Coenzyme F420-0 gamma-glutamyl ligase activity
Zfp52	Zinc finger protein 52	3.185	Metal ion binding
Gpsm2	G-protein signaling modulator 2	3.052	GDP-dissociation inhibitor activity
Sdcbp2	Syndecan binding protein 2	2.979	Protein binding
Abcb4	ATP binding cassette subfamily B member 4	2.850	ATPase activity
Dag1	Dystroglycan 1	2.708	Actin binding
Tmem32	Membrane magnesium transporter 1	2.676	Cobalt ion transmembrane transporter activity
Nr1d2	Nuclear receptor subfamily 1 group D member 2	2.674	Core promoter sequence-specific DNA binding
5830457O10Rik	Chromosome transmission fidelity factor 8	2.614	DNA binding
Spag7	Sperm associated antigen 7	2.577	Nucleic acid binding

Table 2. List of top 20 genes in cluster B.

Symbol	Gene name	Fold-change	Molecular function
Ltf	Lactotransferrin	-4.031	DNA binding
5730507C01Rik	RIKEN cDNA 5730507C01 gene	-3.424	Nucleic acid binding
Galns	Galactosamine (N-acetyl)-6-sulfatase	-3.343	Catalytic activity
Serpinb3a	Serpin family B member 4	-3.329	Enzyme binding
C030032F19Rik	RIKEN cDNA C030032F19 gene	-3.284	Unknown
Slc4a8	Solute carrier family 4 member 8	-3.198	Anion:anion antiporter activity
Klk7	Kallikrein 1	-3.162	Endopeptidase activity; metal ion binding
2510042H12Rik	RIKEN cDNA 2510042H12 gene	-3.103	Unknown
Gdf3	Growth differentiation factor 3	-2.938	Cytokine activity; growth factor activity
Laptm4b	Lysosomal protein transmembrane 4 beta	-2.839	Protein binding
Nphp1	Nephronophthisis 1 (Juvenile)	-2.814	Protein binding; structural molecule activity
5730593N15Rik	NOTUM, palmitoleoyl-protein carboxylesterase	-2.801	Carboxylic ester hydrolase activity
LOC100048434	Predicted gene 11627	-2.666	Unknown
Olfr1419	Olfactory receptor 1419	-2.656	Odorant binding; olfactory receptor activity
2010005H15Rik	Cystatin A	-2.623	Cysteine-type endopeptidase inhibitor activity
Brd8	Bromodomain containing 8	-2.593	Protein binding; sequence-specific DNA binding transcription factor activity
AI449175	Zinc finger protein 868	-2.555	Metal ion binding; nucleic acid binding
Mcpr	Anaphase promoting complex subunit 1	-2.541	Protein binding
2900027M19Rik	RIKEN cDNA 2900022M12 gene	-2.532	Unknown
Zbtb38	Zinc finger and BTB domain containing 38	-2.519	DNA binding; metal ion binding

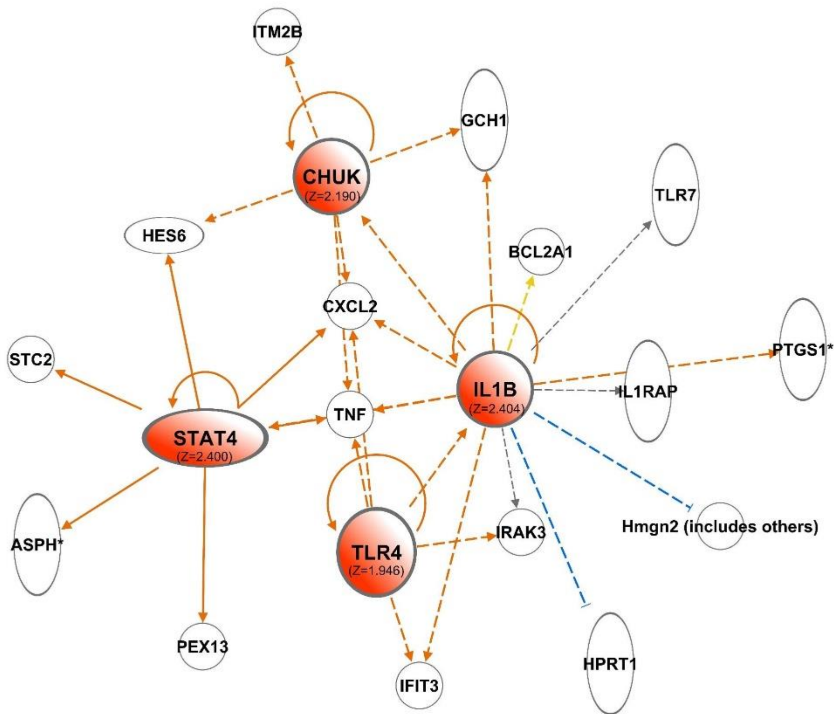

Figure 8. Upstream regulator analysis based on significantly expressed genes in S30.

Top ranked upstream regulators related to inflammation with gene interaction network containing up- or down-regulated genes in S30 compared with CT. Predicted activation state of upstream regulators is determined by z-score calculated by IPA and is displayed by different color; Z-score > 1.5, predicted as activated state marked by orange color. Solid lines imply direct and dotted lines imply indirect transcriptional regulation. Several genes and molecules as detected upstream regulators are involved in pro-inflammatory response (p = 4.40E-09) and inflammatory response (p = 1.60E-08) identified by IPA.

Table 3. List of significant genes for prediction of up-regulators.

Symbol	Gene name	Fold-change	Molecular function
TNF	tumor necrosis factor	3.381	Cytokine activity
HPRT1	hypoxanthine phosphoribosyltransferase 1	-2.447	Guanine phosphoribosyltransferase activity
HMGN2	high mobility group nucleosomal binding domain 2	-3.215	Nucleosomal DNA binding
IFIT3	interferon induced protein with tetratricopeptide repeats 3	3.218	Protein binding
IL1RAP	interleukin 1 receptor accessory protein	2.208	Interleukin-1 binding
IRAK3	interleukin 1 receptor associated kinase 3	2.571	ATP binding; kinase activity
PTGS1	prostaglandin-endoperoxide synthase 1	3.669	Prostaglandin-endoperoxide synthase activity
TLR7	toll-like receptor 7	-2.278	Transmembrane signaling receptor activity
ASPH	aspartate beta-hydroxylase	3.404	Peptide-aspartate beta-dioxygenase activity
PEX13	peroxisomal biogenesis factor 13	2.423	Peroxisome targeting sequence binding; protein binding
STC2	stanniocalcin 2	2.255	Enzyme binding; heme binding;
CXCL2	chemokine (C-X-C motif) ligand 2	2.387	CXCR chemokine receptor binding
GCH1	GTP cyclohydrolase 1	2.214	GTP-dependent protein binding
HES6	hes family bHLH transcription factor 6	2.038	Transcription factor binding
ITM2B	integral membrane protein 2B	2.202	ATP binding; beta-amyloid binding; protein binding

6. The consumption of sucrose solution resulted in increased $CD11b^+Gr\text{-}1^+$ cells in peripheral blood with development of glucocorticoid resistance.

To study if activated inflammatory response in brain could affect distribution of inflammatory cells in peripheral blood (PBL), fluorescence-activated cell sorting (FACS) analysis was conducted. The frequencies of $CD11b^+Gr\text{-}1^+$ cells, known to be increased in condition of inflammation (Shon et al., 2015), were significantly higher in PBL of S30 mice than in CT mice (Figure 9). However, there was no significant difference between S30 and A30.

Since glucocorticoids are frequently used as drugs for their anti-inflammatory function and transcriptome analysis identified that glucocorticoid receptor signaling in brain was down-regulated (Figure 10), we investigate the associations between the percent of $CD11b^+Gr\text{-}1^+$ cells and corticosterone level by linear regression model. As shown in Figure 11, the concentration of serum corticosterone was positively correlated with the percent of $CD11b^+Gr\text{-}1^+$ cells significantly in CT mice ($r^2 = 0.4539$, $p = 0.0466$), implying that secretion of corticosterone known to modulate immune response via anti-inflammatory effect was promoted by increased inflammatory cells in normal condition. In contrast, this correlation shown in CT disappeared in S30 completely ($r^2 = 0.02394$, $p = 0.7145$). In addition, serum corticosterone level and the percent of $CD11b^+Gr\text{-}1^+$ cells were not significantly correlated but still showed a positive

correlation in A30 mice ($r^2 = 0.3214$, $p = 0.1428$). This indicates that serum corticosterone levels were elevated as inflammation provoked by sucrose consumption but did fail to suppress inflammatory response though its anti-inflammatory action. These results suggest that sucrose consumption may affect the development of glucocorticoid resistance despite elevated concentration of corticosterone and result in continuous inflammation status ultimately.

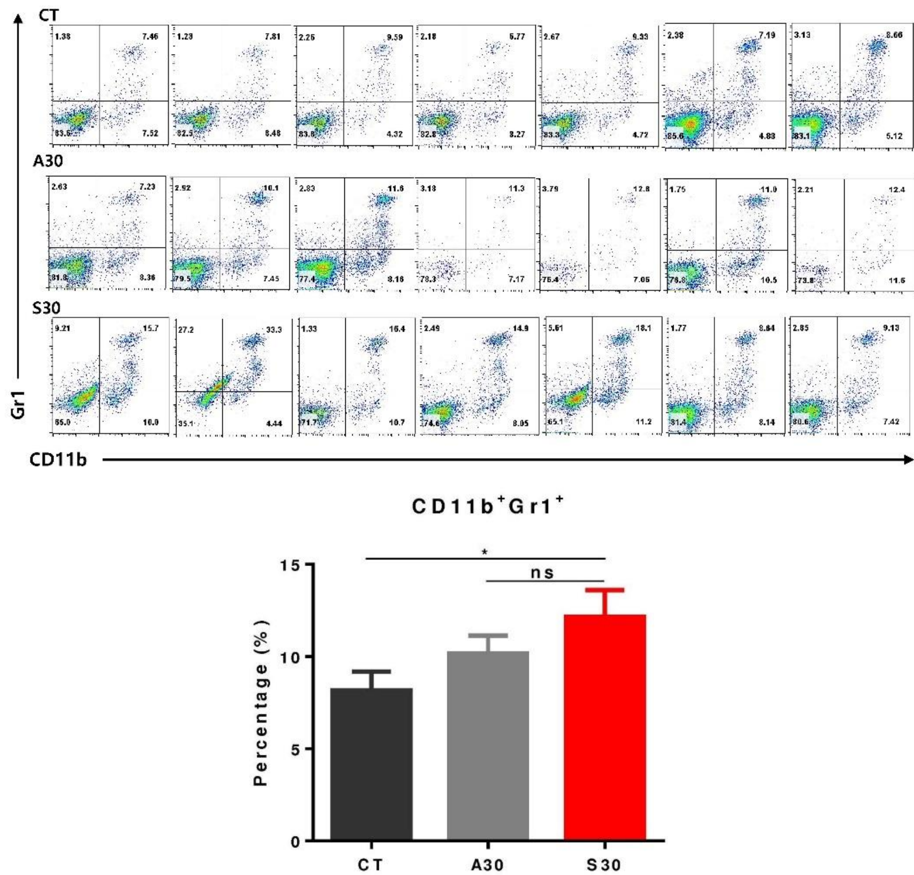

Figure 9. FACS analysis after consuming plain water, aspartame solution or sucrose solution.

The effect of consuming either sucrose solution or aspartame solution from on the expression of $CD11b^{+}Gr\text{-}1^{+}$ cell in peripheral blood (PBL) analyzed by flow cytometry. Representative flow cytometric data are displayed for each group.

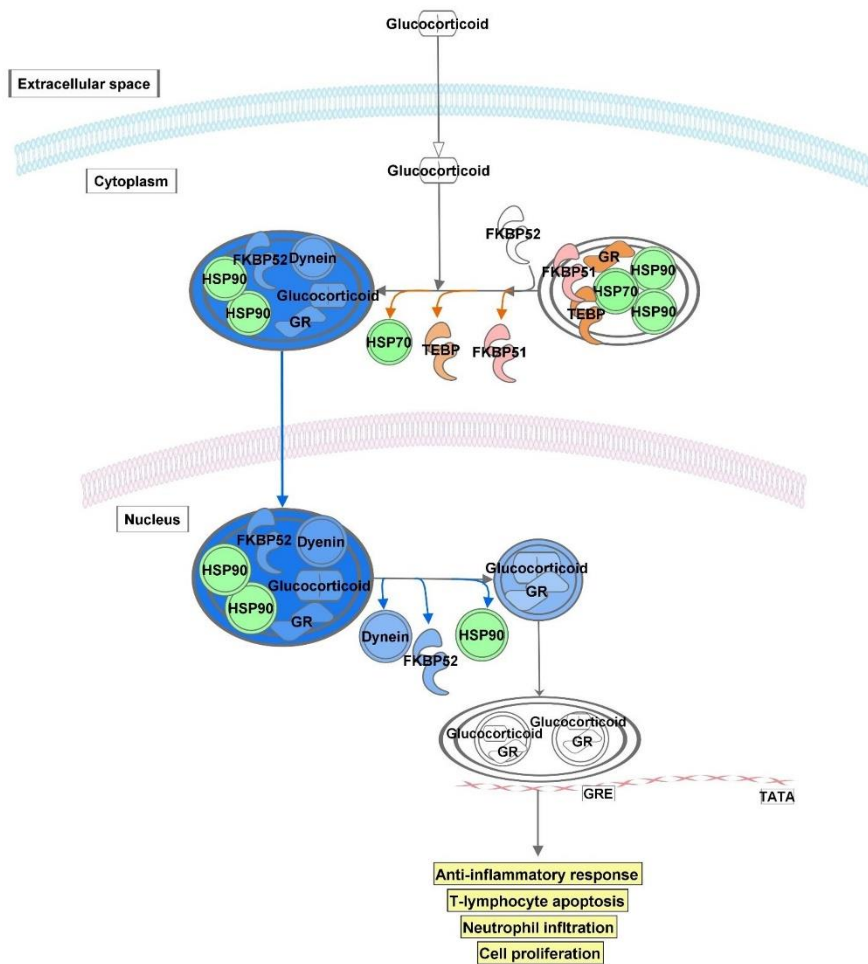

Figure 10. Down-regulated glucocorticoid-receptor signaling by consumption of sucrose solution.

Glucocorticoid complex, which could enter nucleus from cytoplasm predicted as down-regulated with following process. Predicted activation state of genes is determined by IPA and is displayed by different color; predicted as activated state marked by orange color; predicted as inhibited state marked by blue color.

Genes in red or blue color represent up- or down-regulation respectively in S30 compared with CT.

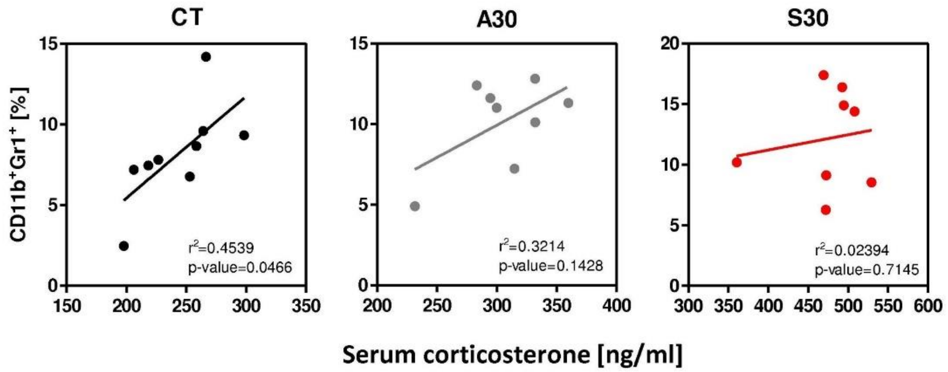

Figure 11. Linear regression model between serum corticosteorone and CD11b$^+$Gr-1$^+$ cell.

Correlations between concentration of serum corticosterone and the percentage of CD11b$^+$Gr-1$^+$ cell in PBL for consuming plain water (CT), aspartame solution (A30), or sucrose solution (S30) in sequence. Each dot indicates each value of individual mouse and each solid line represents equation of correlation elicited from linear regression model. p-value and r^2 are presented in each graph of group respectively.

IV. Discussion

Previously the effects of sucrose consumption on anti-social behaviors remained controversial. The positive correlation between sugar consumption and destructive-aggressive behaviors was firstly found in hyperactive children (Prinz et al., 1980) and it was followed by another study which tested the hypothesis the levels of violent crimes and antisocial behaviors can be reduced by using less sucrose in the diet for juvenile prison inmates (Schoenthaler, 1982, 1983a, 1983b). One year after reducing sugar in their diet, the incidence of serious antisocial behaviors in the subjects declined by 21% (Schoenthaler, 1983b). According to a recent analysis of a national dataset, higher soft drink consumption is associated with a range of aggressive behavior including physical fights in US high school students (Solnick & Hemenway, 2014). Similarly, 5-year-old children with higher soda consumption showed higher total aggression score in a cohort study (Suglia, Solnick, & Hemenway, 2013). On the other hand, others failed to find the associations between sucrose diet and behavioral problems such as conduct problem and oppositional behavior in children. In addition, a meta-analysis of 16 double-blind intervention studies found that sugar does not affect the behavior or cognitive performance of children, but it concluded that the effect of sugar on subsets of children should not be excluded (Wolraich, Wilson, & White, 1995).

Here, to our knowledge, this is the first report to show the promoting effects of long-term sucrose consumption on aggressive behaviors using animal

models. First, we found the dramatic elevation of serum corticosterone level was resulted from short-term high sugar consumption in dose-dependent manner in adult mice. This observation led us to examine the long-term effects that high sugar exposure was started from infancy until to adulthood and we found the heightened level of corticosterone when those mice grew to adult mice compared to control mice. Corticosterone, a more precise marker rather than cortisol for rodents, is a well-established targets in the search for hormonal modulators of social aggression (Haller et al., 2000). In a good accordance with others, we observed the aggressive mice showed higher levels of corticosterone. These results imply that the promoting effect of sugar consumption on the social aggression might be mediated by up-regulating level cortisol hormone. It is also notable that the artificial sweeter did not mimic the role of the sugar playing in elevating corticosterone and aggressive behaviors as well.

Several other hormones have been also reported for their associations with aggressive behaviors. Local release of vasopressin within the lateral septum in adult male rats during resident-intruder test was examined (Veenema et al., 2010). Interestingly, vasopressin release was correlated positively with inter-male aggression and treatment of vasopressin V1a receptor antagonist prevented highly aggressive rats from showing an increase in inter-male aggression. Oxytocin has been reported to mediate intimate behaviors (Consiglio et al., 2005) but conflicting result was reported (DeVries, Young III, & Nelson,

1997). Homozygous oxytocin knock out ($OT^{-/-}$) mice showed a significant decrease in duration of each aggressive attack in resident-intruder test compared with wild type (WT) mice. In addition, $OT^{-/-}$ mice spent significantly less time in aggressive encounters during the 5 min test in a neutral arena than WT mice and the duration of each aggressive attack was significantly shorter in $OT^{-/-}$ than WT mice. Our present study revealed that there was no significant difference in gene expression of vasopressin in S30 mice compared to CT or A30 mice. On the contrary, gene expression of oxytocin in S30 mice was significantly higher than CT or A30 mice only. Dramatically, serum corticosterone level was elevated significantly in S30 mice compared to either CT or A30 mice, which might suggest the relevance between corticosterone and aggressive behaviors rather than the other molecules mentioned above.

The consumption of sucrose solution in S30 mice resulted in not only hormonal changes but also alteration in transcription level compared to CT or A30. We identified augmented inflammatory response with increased expression of inflammation-related genes and their pro-inflammatory upstream regulators in brain of S30 mice through transcriptome analysis. Furthermore, the frequency of $CD11b^{+}Gr\text{-}1^{+}$ cells in PBL was the highest in S30 mice among groups. Surprisingly, serum corticosterone level was positively correlated with the frequency of $CD11b^{+}Gr\text{-}1^{+}$ cells in CT mice unlike mice in S30 group, which failed to maintain positive correlation with sustained high corticosterone

levels regardless of frequency of CD11b$^+$Gr-1$^+$ cells. As greater levels of glucocorticoid such as cortisol are associated with higher numbers of certain monocyte subsets, the correlation between glucocorticoid levels and the frequency of monocyte was utilized as an indirect marker of glucocorticoid resistance (Cohen et al., 2012). Unlike the correlation between corticosterone level and the frequency of CD11b$^+$Gr-1$^+$ cells in CT, disrupted positive correlation shown in S30 mice might imply the development of glucocorticoid resistance resulting in impaired control of inflammatory response. Based on several studies, which reported previously that sucrose consumption may possibly increase inflammatory markers such as C-reactive protein (CRP), interleukin-6 (IL-6), tumor necrosis factor alpha (TNF-α). Furthermore, researches on the association between aggressive behaviors and elevated CRP levels in schizophrenia inpatients (Barzilay et al., 2016), and elevated plasma inflammatory markers in individuals with intermittent explosive disorder (Coccaro, Lee, & Coussons-Read, 2014) are reported. These evidences imply that aggressive behaviors shown in S30 mice are potentially related to augmented inflammation with development of glucocorticoid resistance induced by intake of sucrose solution.

The outcomes shown in A30 mice are variant from the results of S30 mice though sweetness of aspartame solution is equivalent to sucrose solution in present study. In other words, the effects of sucrose solution are distinguished from those of aspartame solution on host metabolism regardless of identical sweetness, suggesting that sweetness is not a key factor. In our research, A30

mice seemed less aggressive than CT mice according to resident-intruder test results. Similarly, previous study found that administration of aspartame acutely in high-dose to rats reduced aggressive attack (Goerss, Wagner, & Hill, 2000). These results could give a false perception that aspartame is beneficial than sucrose. However, a study reported that rats received an aspartame solution would make longer and more numerous aggressive responses by reducing brain tryptophan levels and serotonin synthesis (Kring, 2012). In addition, there are conflicting results regarding toxicity of aspartame (Marinovich, Galli, Bosetti, Gallus, & La Vecchia, 2013) and even the association between intake of aspartame and neuronal or neurobehavioral dysfunction has been reported (Lindseth, Coolahan, Petros, & Lindseth, 2014; Rycerz & Jaworska-Adamu, 2013). These researches suggest that unknown risk concerning intake of aspartame still remains and in-depth studies are required.

High concentration of sucrose solution could provide high carbohydrate diet since a molecule of sucrose is composed of a fructose and a glucose. One study showed that male Göttingen minipigs fed a high fat/cholesterol, low carbohydrate diet for 13 weeks were less aggressive, showed more non-agonistic social contact and had fewer and less severe skin than minipigs fed low fat, high carbohydrate diets (Haagensen et al., 2014). These results confirmed that aggressive behaviors shown in S30 mice was affected by sucrose itself rather than other diet component such as fat. Also, carbohydrate such as starch could possibly have an effect on aggressive behavior.

The present study found the promoting effects of long-term consumption of sucrose solution on aggressive behaviors with controlled animal model. Further comprehensive approaches including transcriptome analysis elucidated underlying mechanism of aggressive behaviors. Aggressive behaviors in adult mice with systemically dys-regulated inflammatory responses and glucocorticoid resistance might be induced by consumption of sucrose solution for long-term period. Next, human studies would be required to validate a direct relationship between sucrose consumption and aggressive behaviors and to investigate other possible adverse effects of sucrose consumption. This study could contribute to public health for effective interventions of social aggression.

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국문초록

성장기 동안의 설탕용액 섭취가 마우스 공격성에 미치는 영향 및 관련 기전 연구

서울대학교 대학원 식품영양학과

최 정 윤

공격적인 행동은 전통적으로 다른 개인에게 신체적인 피해를 주려는 의도를 가진 외현적 행동으로 정의되었으며, 이것은 설치류부터 인간에 이르기까지 그 종을 아울러 유사성을 가진다. 공격적 행동은 야생에서는 식량을 얻거나 경쟁자로부터 영토나 짝을 보호함에 있어 이점이 있었지만, 최근에는 사회의 주요한 문제로 여겨지고 있다. WHO (World health organization) 보고서에 따르면 다양한 형태의 폭력은 희생자를 치료하고 사회 기반 시설을 복구하는데 많은 비용을 필요로 하는데, 공격적 행동이 폭력으로

이어질 수 있다는 점에서 공격적 행동을 촉진 혹은 억제하는 요인들을 규명하고 효과적인 중재방법을 찾는 노력이 필요하다. 공격성과 관련된 여러 요인 중 식이와 관련된 요인인 설탕은 풍미와 보존성 향상을 위해 식품이나 음료에 더해지는 첨가당의 형태로 그 섭취가 증가됨에 따라 주목을 받고 있으며, 특히 가당음료 (sugar-sweetened beverage, SSB)의 섭취 증가는 설탕 섭취 증가에 크게 기여할 수 있다. 가당음료 섭취 증가와 주의력결핍 과잉행동장애 (attention deficit hyperactivity disorder, ADHD)나 반사회적인 행동과의 관련성이 제기되고 있는 가운데, 가당음료의 과다 섭취와 비만, 제 2 형 당뇨병과 같은 대사성질환의 연관성에 대한 연구는 활발히 이루어지고 있지만 행동에 미치는 영향에 대한 연구는 부족한 실정이다. 또한, 이와 관련한 일부 관찰연구 결과도 논란의 여지가 있어 통제된 동물실험을 통한 연구가 필요하다. 따라서 본 연구에서는 영아기부터 성인기까지 장기간의 설탕용액 섭취가 마우스의 공격적인 행동에 미치는 영향을 살펴보고 그 기전을 탐색하고자 하였다. 이유기 후의 3 주령 마우스를 세 그룹으로 나누어 일반 물 (CT), 30 % 의 설탕 용액과 동일한 단맛을 내는 아스파탐 용액 (A30) 그리고 30 % 의 설탕 용액 (S30)을 유아기에서 성인기에 이르는 기간 동안 각각 공급하였다. 이 때, CT 는 설탕 용액 자체에 대한 대조군, A30 은 설탕 용액의 단맛에

대한 대조군으로 각각 사용되었다. 총 8주 동안 각 음용수를 공급한 후 공격성을 측정하는 행동실험을 수행한 결과, S30 그룹에서 6가지 공격적인 행동을 수행한 시간인 duration 이 다른 두 그룹에 비해 유의적으로 증가하였다. 뇌 조직에서의 전사체를 분석한 결과 S30 군에서 유의적으로 변화한 유전자들은 CT 나 A30 에서 변화한 유전자들과는 구분됨이 PCA 분석으로 확인되었다. S30 에서 유의적으로 증가 혹은 감소한 유전자들을 생물정보분석을 통해 기능적으로 분류한 결과 6 개의 유의적인 카테고리가 발견되었는데 이들은 모두 면역 질환과 관련되었다. 또한 Upstream regulator analysis 를 통해 S30 군에서 유의적으로 변화한 유전자들을 바탕으로 4 개의 유의적인 상위 조절자를 발견하였다. 이들은 *Tlr4*, *Stat4*, *Chuk* 그리고 *Il1b* 로, 각각 inflammatory response 또는 pro-inflammatory response 에 관여하는 분자들로 밝혀졌다. 공격성과 관련된 호르몬인 corticosterone 은 S30 군에서 유의적으로 높았고, FACS 분석 결과 $CD11b^{+}Gr\text{-}1^{+}$ 세포 또한 S30 군에서 유의적으로 높은 것으로 나타났다. 더불어 corticosterone 수치와 $CD11b^{+}Gr\text{-}1^{+}$ 세포 간의 상관성을 통해 글루코코르티코이드 저항성의 발생을 간접적으로 측정한 결과 S30 군에서 두 요소 간 양의상관관계가 무너짐을 확인하여 글루코코르티코이드 저항성의 발생을 예측해볼 수 있었다. 흥미롭게도, A30 군에 공급된 아스파탐 용액은 S30 군에 공급된 설탕

용액과 동일한 단맛임에도 불구하고 S30 군에서 장기간 설탕 용액 섭취 후에 보인 공격성 증가, 뇌에서의 inflammatory response 증가, 혈액에서의 corticosterone 수치 증가 혹은 inflammatory cell 의 증가, 글루코코르티코이드 저항성의 발생과 같은 일련의 결과와는 다른 양상을 보였다. 따라서, 이러한 결과들은 유아기부터 성인기에 이르기까지의 장기간의 설탕 용액 섭취가 공격적인 행동을 증가시키며, 뇌에서의 inflammatory response 와 혈액에서의 corticosterone 과 inflammatory cell 이 이를 매개할 수 있음을 시사한다. 이는 염증반응과 공격성과의 연관성을 보고해 온 기존의 연구 결과들을 뒷받침하는 근거가 될 수 있을 것으로 사료된다. 본 연구는 통제된 마우스 실험을 통해 장기간 설탕용액 섭취에 따른 공격성을 표준화된 행동실험으로 측정하고 그 기전을 전사체 분석을 통해 규명했다는 점에서 의의가 있으며, 공격성 완화를 위한 중재에 기여할 수 있을 것이다.

주요어: 설탕 용액, 공격성, 전사체, 염증 반응, 글루코코르티코이드 저항성

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