Genomics Data 10 (2016) 38–50

Contents lists available at ScienceDirect

Genomics Data

journal homepage: www.elsevier.com/locate/gdata

Behavioral and omics analyses study on potential involvement of dipeptide balenine through supplementation in diet of senescence-accelerated mouse prone 8

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ARTICLE INFO

Article history:
Received 22 July 2016
Received in revised form 2 September 2016
Accepted 7 September 2016
Available online 9 September 2016

Keywords:
Balenine
Mouse
SAMP8
Alzheimer's disease
Behavior
Whole genome DNA microarray

ABSTRACT

This study investigates effects of dipeptide balenine, as a major component of whale meat extract (hereafter, WME), supplementation on senescence-accelerated mouse prone 8 (SAMP8), an Alzheimer's disease (AD) model at level of learning and memory formation and brain expression profiles genome-wide in brain. Mice fed experimental balenine (+WME) supplemented diet for 26 weeks were subjected to four behavioral tests – open field, Y-maze, novel object recognition, and water-filled multiple T-maze – to examine effects on learning and memory. Brain transcriptome of SAMP8 mice-fed the WME diet over control low-safflower oil (LSO) diet-fed mice was delineated on a 4 × 44 K mouse whole genome DNA microarray chip. Results revealed the WME diet not only induced improvements in the learning and memory formation but also positively modulated changes in the brain of the SAMP8 mouse; the gene inventories are publically available for analysis by the scientific community. Interestingly, the SAMP8 mouse model presented many genetic characteristics of AD, and numerous novel molecules (*Slc2a5*, *Treh*, *Fbp1*, *Aldob*, *Ppp1r1a*, *DNase1*, *Agxt2l1*, *Cyp2e1*, *Acsm1*, *Acsm2*, and *Pah*) were revealed over the SAMR1 (senescence-accelerated mouse resistant 1) mouse, to be oppositely regulated/recovered under the balenine (+WME) supplemented diet regime by DNA microarray and bioinformatics analyses. Our present study demonstrates an experimental strategy to understand the effects of dipeptide balenine, prominetly contained in meat diet, on SAMP8, providing new insight into whole brain transcriptome changes genome-wide. The gene expression data has been deposited into the Gene Expression Omnibus (GEO): GSE76459. The data will be a valuable resource in examining the effects of natural products, and which could also serve as a human model for further functional analysis and investigation.

1. Introduction

The dipeptide, balenine (beta-alanyl-N tau-methyl histidine), has been identified long back in the extracts of muscles from 9 species of mammal, including man and chicken by comparative chromatography in combination with amino acid sequencing techniques [1]. As mentioned by Harris and Milne, 1987, "the dipeptide contained equimolar amounts of beta-alanine and N tau-methyl histidine with beta-alanine as the N-terminus [1]. Further, it was proposed that balenine, like carnosine and anserine is to be regarded as a normal constituent of muscle" [1].

To briefly give a background to the current study, our interest in looking at novel molecules for their potential beneficial effects on the human body was developed while investigating the nucleoprotein (NP) from salmon soft roe [2], especially how NP diet affects the brain and neurodegenerative disorders/diseases therein [3]. Among the

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http://dx.doi.org/10.1016/j.gdata.2016.09.004

major human diseases, and of great concern, are the neurodegenerative disorders, such as Parkinson's disease and Alzheimer's disease (AD), and which seriously limit the health and well-being of the world's aging population. Thus, the brain (and especially neurodegenerative disorders/diseases) becomes a hot topic for current researchers in this field, including our own group. With this backdrop, our present research examines the imidazole dipeptide, balenine – a major component of the meat, especially whale meat that is known to contain balenine in high concentration [4–6], in context of it being an important anti-fatigue food. This assumption is based on previous research, which shows the anti-fatigue effects with a ddY [7] swimming mouse model [8], and in humans under physical load and by daily activities [9]. These two studies [8,9] suggested that whale meat through diet, in mice and as a supplement for human subjects, was most probably involved in "high efficiency for muscle endurance in mice" [8] and "attenuating high workload induced fatigue in humans" [9], indicating possible utilization of the whale meat as an anti-fatigue food, and maybe more. As balenine is the major constituent, and the objective of our research, we used the whale meat extract (WME) as a test sample to conduct the present research. The availability of the whale meat at Kyodo Senpaku Co. Ltd. (Tokyo, Japan) also helped to utilize it for our research as a candidate meat sample. It should be re-emphasized that we were primarily interested in the imidazole dipeptide, balenine, contained in the meat as an essential molecule/compound [4–6] behind these observed benefits for human health [8,9], and not about whaling per se. To note, research into human wellness includes studies into the major lifestyle diseases and the benefits of not only searching for a suitable and healthy lifestyle but also and more importantly utilizing the potential of naturally occurring and protective compounds in our environment (plant or animal).

In the present study, we report the results of our investigation into the effects of balenine through WME-supplemented diet in a mouse model of AD, the senescence-accelerated mouse prone 8 (SAMP8), using behavior and molecular genetic approaches to better understand the central nervous system dysfunction [10]. The reason for using SAMP8 is that it is a widely used model for AD and numerous studies have been conducted in relation to cognitive impairment (learning and memory deficits) and pathology, including oxidative stress and gene and protein expression analyses (for review see [11,12]). Our present study builds up on two sets of experiments investigating the experimental balenine (+WME)-supplemented diet over the low-safflower oil (hereafter, LSO) diet as a control diet, on SAMP8 and SAMR1 (senescence-accelerated mouse resistant 1) learning and memory deficits (experiment 1) and whole genome DNA microarray-based transcriptomics profiling in conjunction with Ingenuity Pathway Analysis (IPA; experiment 2). Results revealed that balenine (+WME) supplementation in the diet of SAMP8 mouse resulted in an increase in the level of learning and memory formation and positive changes in the transcriptome of the brain in regards to cellular energy metabolism, suggested through the observation of recovery of gene expressions in the SAMP8 model over the not supplemented (control diet) mouse, and the potential involvement of the dipeptide balenine, as a major source of the beta-amino acid, beta-alanine, and other peptides to the cells.

2. Results

2.1. The experimental design and strategy for investigating effects of balenine (+WME)-supplemented diet on SAMP8 mice

With an aim to investigate the potential beneficial effects of balenine (+WME) we have here utilized a mouse model of SAMP8, and which also serves as an AD model. In this study, we linked behavioral analysis with omics approach via whole genome DNA microarray chip experiment and IPA analysis to reveal changes in the SAMP8 mice brain with or without WME supplemented diet and over the SAMR1 mice brain. As schematically presented in Fig. 1, the SAMP8 and SAMR1 mice were fed WME or control LSO diets for 26 weeks, followed by tests to determine improvements in learning and memory and thereafter extraction of total RNA for unraveling the brain transcriptome to first identify changes genome-wide and then attempt to delineate potential molecular factors behind the suggested improvements in learning and memory. Previous studies have also reported the WME-induced anti-fatigue effect in mouse and human [8,9], and that was one reason for carrying out the present research. The other reason for why the meat component was used on the SAMP8 mice model, we provide a preliminary data on the radical (superoxide anion, O^{2-}) scavenging activity between different edible parts of the whale oil and meat (see Online Resource 1 [13]). These data revealed that meat strongly removed O^{2-} compared to other groups in vitro, suggesting its potential as an anti-oxidant or containing anti-oxidative properties, which was useful in context of the SAMP8 mice model. Next, we present below the results of each of the four behavioral tests and the transcriptome profiles of the SAMP8 mouse brains under balenine (+WME) supplemented diet.

2.2. Behavioral tests on the SAMP8 and SAMR1 mice fed balenine (+WME)-supplemented diet

To investigate potential balenine (+WME) diet-induced mechanisms underlying effects for learning and memory in SAMP8 as AD model mouse, we performed four tests. Firstly, mice were evaluated by open field test as measurement of locomotor activity. SAMR1 mice had significantly reduced locomotor activity (SAMR1 LSO; 852.8 ± 60.5). No differences between SAMP8 LSO and SAMP8 WME groups were observed (SAMP8 LSO; 1520.5 ± 93.9, SAMP8 WME; 1405.7 ± 76.1) (Fig. 2a). Secondly, the Y-maze test results showed that in the SAMP8 WME group degradation of spatial memory formation significantly improved compared to SAMP8 LSO group (SAMR1 LSO; 49.6 ± 4.2, SAMP8 LSO; 41.5 ± 4.0, SAMP8 WME; 56.6 ± 1.8%; $p < 0.05$) (Fig. 2b). Thirdly, for the NOR test, there were no differences among any of the groups (SAMR1 LSO; 65.0 ± 4.9, SAMP8 LSO; 44.9 ± 2.3, SAMP8 WME; 54.4 ± 4.5%) (Fig. 2c). Fourthly, the WMTM test revealed that the SAMP8 WME diet group had reduced escape time from the water in a time-dependent manner, and the observed escape time was significantly different at the 3rd day compared to SAMP8 LSO group (SAMP8 LSO; 135.9 ± 31.3, SAMP8 WME; 52.5 ± 5.8 2nd, $p < 0.02$), but the number of inserted blind array were no different among any of the groups (Fig. 2d).

2.3. Global gene expression (transcriptomics) analysis of SAMP8 mice fed with balenine (+WME)-supplemented diet over SAMP8 LSO (−WME) fed diet

DNA microarray analysis revealed 274 and 224 up (≧1.5-fold)- and down (≦0.75-fold)-regulated genes in the balenine (+WME)-supplemented diet SAMP8 mouse whole brain, interestingly over the much higher number of genome-wide changes in gene expressions in the −WME (i.e. LSO diet only) SAMP8 mouse, where 904 and 613 up (≧1.5-fold)- and down (≦0.75-fold)-regulated genes were being altered (Fig. 3). It can also be said that the gene abnormalities (i.e. changed gene expressions) seen in the SAMP8 model mice brains are being strongly affected by the balenine (+WME) supplementation. As to what is the nature of these gene expressions was the next question, we answer below. These genes are listed in Online Resource 2 (+WME, up- and down-regulated gene lists) and Online Resource 3 (−WME (LSO), up- and down-regulated gene lists). These results provide not only the first inventory of WME supplemented diet-influenced genes (see Online Resource 2) in the animal (mouse) brain but also a comprehensive list of gene expressions in a mouse model of AD, i.e. SAMP8 (see Online Resource 3). To note, log transformed fold (log2 fold) change is used to represent the expression levels [14]. The reason for using ≧/≦1.5/0.75-fold as the cut-off value in the present experiment was to consider

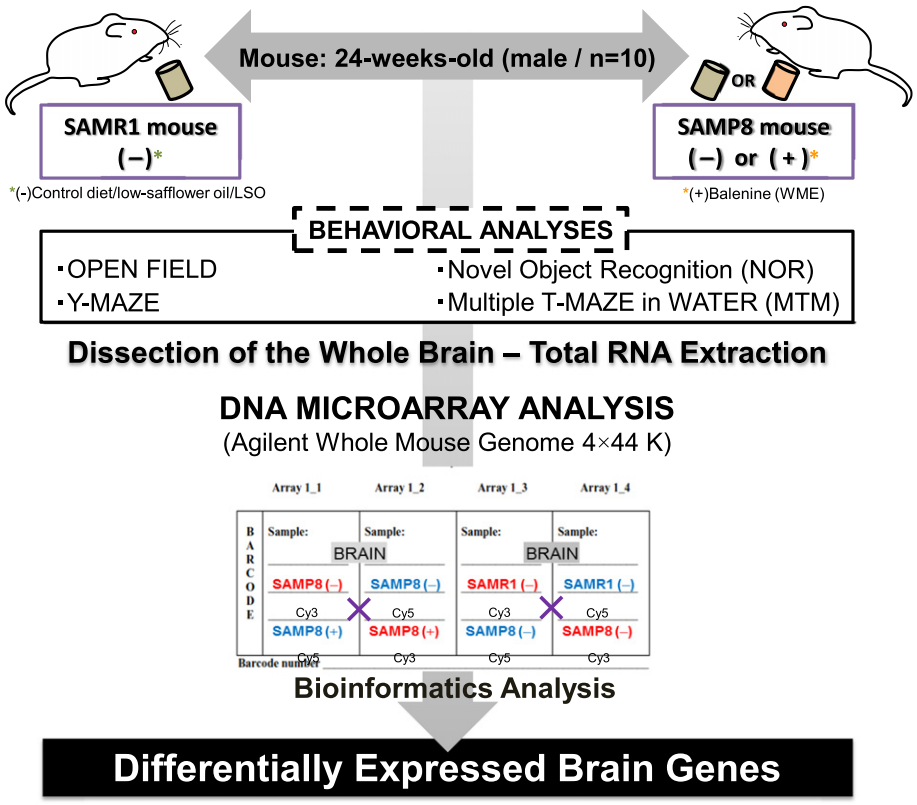

Fig. 1. The experimental strategy for investigating the effects of balenine (+WME) supplementation diet in the senescence-accelerated mouse prone 8 (SAMP8) mouse. 24-weeks-old SAMP8 and senescence-accelerated mouse resistant 1 (SAMR1) mice were housed in individual cages till 50-weeks-old. Following the chow (CE-2) for 24 weeks, mice were fed low-safflower oil (LSO) diet as control diet or balenine through whale meat extract (WME) diet powders until sacrificed for 26 weeks. Two analyses were performed, four behavior tests for learning and memory followed by whole-genome DNA microarray experiment using whole brain in conjunction with bioinformatics analysis. Details are in the text.

even smaller fold-change value genes in the experiment for downstream bioinformatics analyses. Moreover, as our experiment utilizes the two-color dye-swap approach (see below, Section 4.5) a highly confident (severe selection) differentially expressed gene list was generated in the analysis.

2.4. Comparative analysis of the genes oppositely regulated in the SAMP8 mice fed the balenine (+WME)-supplemented diet and their role in cellular energy metabolism

The above DNA microarray analysis data also revealed 92 genes expressions which were recovered in the SAMP8 (+WME) mouse brain over the same genes expression status in the SAMP8 LSO (−WME) mouse brain. The color codes (red, up-regulated; green, down-regulated) mark these genes in Table 1. Each gene recovery, either up-expressed or down-expressed, is indicated by their ratios. Looking at the genes expressions oppositely regulated by the diets (+WME or LSO), and their functions in major metabolic pathways in the cell, some major trends were noticed in possible modulations of the energy metabolism via the tricarboxylic acid (TCA) cycle. Some of these genes (*Slc2a5*, *Treh*, *Fbp1*, *Aldob*, *Ppp1r1a*, *DNase1*, *Agxt2l1*, *Cyp2e1*, *Acsm1* and *Acsm2*, and *Pah*) expressions are mentioned in a schematic pathway revolving around the said metabolic pathways depicting their up- or down-regulations status, in Fig. 4.

2.5. Functional categorization and IPA analyses of differentially expressed brain genes of SAMP8 mice fed with balenine (+WME)-supplemented diet and SAMP8 LSO (−WME) fed diet

The functionally categorized genes post-analysis using the SABiosciences PCR array list (Qiagen) are presented as color coded up-regulated (red) and down-regulated (green) genes numbers, for each category in both the SAMP8 (+WME) and SAMP8 LSO (−WME) mouse brain (see Online Resource 4). At a glance the number of genes and gene categories highly expressing in the SAMP8 mouse brain under control (LSO) diet were reversed in the balenine (+WME)-fed SAMP8 mouse brain (see also, Table 1). For a wider bioinformatics analysis, a biological function and network analysis was performed using the IPA tool generating numerous networks and pathways revealing the balenine (+WME)-supplemented diet affecting the SAMP8 mouse brain in complex ways (see Online Resources 5 and 6). Each sample condition IPA analysis also provided us with a list of top molecules that are gene candidates for the potential mechanism/s underlying the balenine (+WME)-supplemented diet effects in the SAMP8 model mouse (Fig. 5).

3. Discussion

Our study indicates that the balenine (+WME)-supplemented diet has first, brought about a change in the behavior of the SAMP8 mice, namely, the improvement in the learning and memory formation, and secondly, caused numerous positive genome-wide changes in the brain of SAMP8 mouse over the LSO diet (control)-fed mouse. Inadvertently, these genomic data also revealed that the SAMP8 (AD model) mouse-fed LSO (−WME) had more numbers of changed gene expressions (1517 genes) than the SAMP8 mouse-fed (+WME) diet (498 genes) (Fig. 3). This can be expected as the SAMP8 is an AD model mice [11,12]. Interestingly many genes known to be affected in AD were also found to be present among these differentially expressed genes and when comparing the SAMP8 brains to the SAMR1 brains (seealso Table 1 and Fig. 5). We discuss some of these genes below in context of the recovery of gene expressions in the brain following the

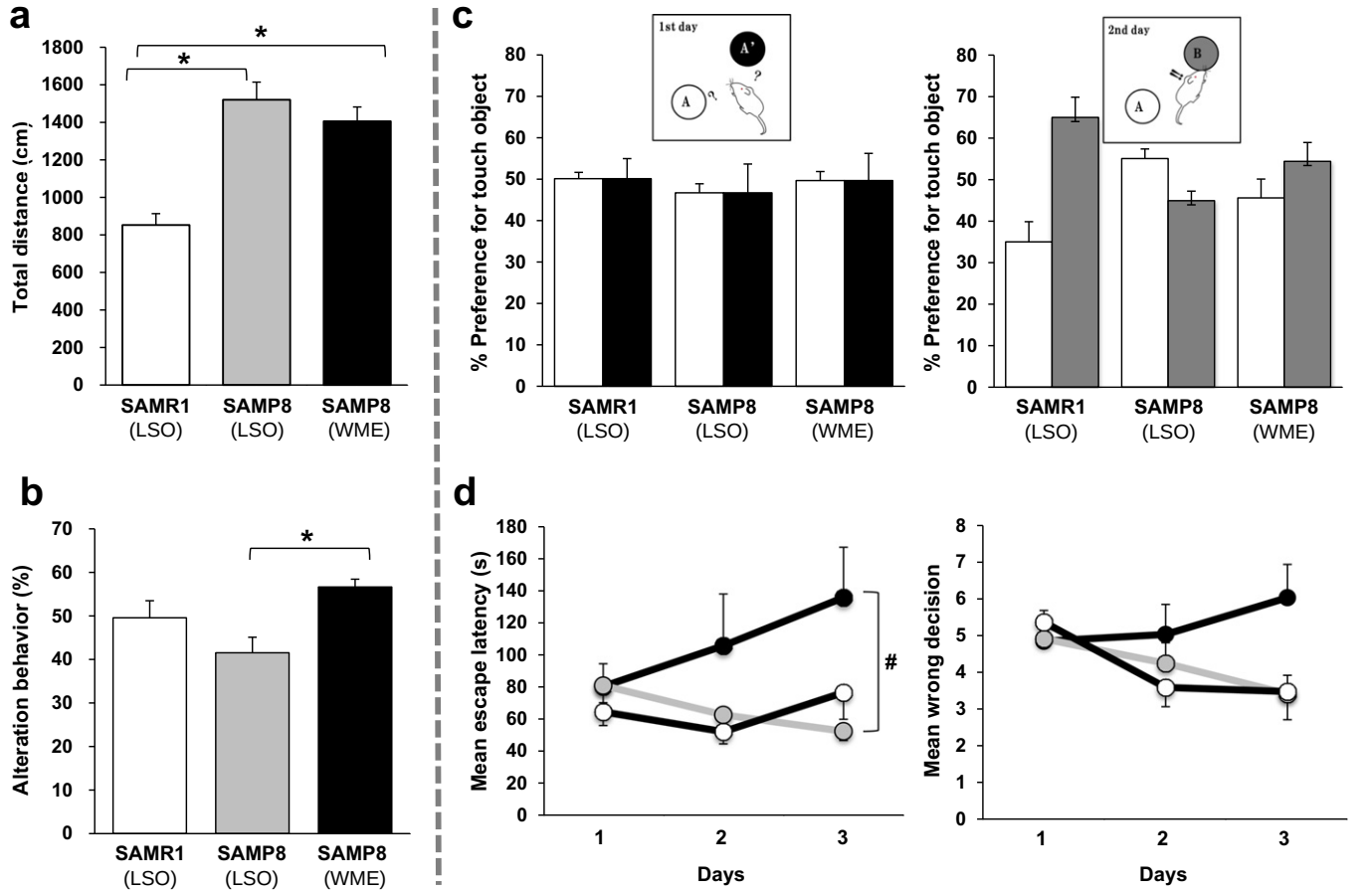

Fig. 2. The four behavioral tests for examining the balenine (+WME)-supplemented diet or not (LSO) effects in the SAMP8 mouse. We performed open field test (a), Y-maze test (b), novel object recognition test (NOR; c), and water-filled multiple T-maze test (WMTM; d). (a) Locomotor activity of SAMR1 mice were significantly decreased compared to SAMP8 LSO and WME group mice. However, no significant differences were observed between SAMP8 LSO group and balenine (+WME) group. (b) In the evaluation of learning impairment using Y-maze test, the SAMP8 WME group showed a significant alteration in behavior compared to the SAMP8 LSO group. No differences were seen between SAMP8 (+WME) and SAMR1 LSO groups (one-way comparison *post-hoc test*; $^{*}p < 0.05$). (c) In the training for novel object recognition, first during the training period two objects (A and A′) were presented to a mouse. Second, during the testing phase, one of the conditioned objects was replaced by a differently colored novel object (A and B). During training all mice groups were found to equally touch the objects (A and A′). During testing, SAMR1 LSO and SAMP8 (+WME) groups increased the percentage of touching novel objects, however there were no differences among groups. (d) In the escape latency from multiple T-maze in water maze, the SAMP8 (+WME) group showed a significantly faster escape latency on day 3 compared to SAMP8 LSO group (repeated measures one-way ANOVA, Bonferroni's multiple comparison tests; $^{\#}p < 0.02$). LSO: low-safflower oil.

feeding of mouse with the balenine (+WME)-experimental diet over the control diet (LSO, −WME). The present study thus provides genes and their potential roles/functions in context of the utilization of the balenine as a possible supplement for human wellness, especially in regards to the AD model used in this study.

In the behavioral part of this study, SAMP8 balenine (+WME)-diet group showed significantly differences compared to SAMP8 LSO diet (control) group in Y-maze and WMTM tests. Two previous reports have shown that WME-induced the anti-fatigue effect in mouse and human [8,9], concluding that balenine as imidazole dipeptide [4–6] in the WME might affect the muscle endurance. Searching the literature we found that another imidazole dipeptide, carnosine, has been reported to have protective effects from ischemic vascular dementia [15], and that dietary supplementation of carnosine suppressed the accumulation amyloid-β in hippocampal region in 3 × Tg-AD mice as an AD model [16]. The chicken breast meat is known to abundantly contain carnosine [17]. That balenine is abundantly and specifically present in whale meat has also been reported [18], but there is no study to our knowledge on its (balenine) phygiological role. However, based on previous researches and our preliminary data (see Online Resource 1), we have considered the present study whereby, the results suggest an improvement in learning and memory function (Fig. 2) in SAMP8 balenine (+WME)-fed supplemented diet mice. Our results also lead us to speculate that balenine may exert a similar function as the imidazole dipeptide carnosine [19]. This may include possible roles as activators of the enzyme adenosine triphosphate, formation of energy-rich phosphorus compounds, buffers (protective action on the mitochondrial structures), as have been discussed for anserine and carnosine, mostly in the muscle [19]. This however needs to thoroughly examined in the mouse brain and proved in the future studies. As will be discussed below, the balenine (+WME)-supplemented diet is seen to cause, in the brain of the SAMP8 mouse, a recovery in the energy requirements or balance of the brain power via providing energy substrates and critical amino acids, including the non-essential amono acid beta-alanine (Fig. 4), that could be directly derived from balenine, in the WME degradation.

Table 1 lists the genes (92) oppositely regulated/recovered by the balenine (+WME)-supplemented diet. These genes present insight into the positive effects of the balenine-rich (WME) diet on the SAMP8 mouse, which we find to be impaired in metabolic functions as exemplified by the plotting of these genes on the energy metabolism pathway/s shown in Fig. 4. Though speculative at this stage, this DNA microarray-based gene expression data and analysis points towards the fact that the balenine (WME) diet might exert a positive effect on the glucose and gluconeogenesis, ethanol fermenation, fatty acid

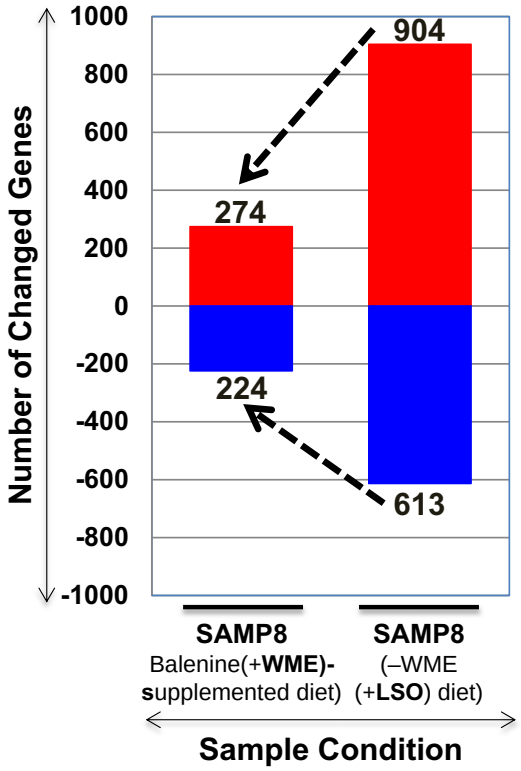

Fig. 3. Differentially expressed genes in the whole brain of SAMP8 mouse with or without balenine (WME) supplementation. The histograms show the up (red)- and down (blue)-regulated gene (≧/≦ 1.5/0.75-fold; log transformed fold (log2 fold) change) numbers in each sample condition. Balenine (+WME) indicates SAMP8 mice fed with the experimental diet containing balenine as described in the Materials and methods section. −WME refers to the SAMP8 mice fed the control diet (LSO, low-safflower oil).

degradation (a major route is the mitochondrial beta-oxidation pathway), aromatic amino acids, and finally the TCA cycle. In other words, it implies that balenine (+WME)-supplemented diet might provide the necessary energy resources impaired in the SAMP8 mouse to provide the brain with necessary metabolites and amino acids for its proper functioning. Here, in relation to the above mentioned balenine (+WME) positively affecting learning and memory functions in the SAMP8 mice and the glucose metabolism pathway genes being affected genome-wide, we would like to mention some previous literature pointing to a link between learning memory and glucose metabolism in aging brain and in causes of disorders causing dementia [20–25]. For example, a study by Mayer and co-workers (1990) inducing irreversible hyperglycemia and depression of cerebral glucose metabolism via the intracerebroventricular (i.c.v.) or intraperitoneal (i.p.) administration of streptozotocin (STZ), which is known to damage insulin-producing cells, examined the behavioral functions (locomotor activity, learning and memory) in rat [24]. Results revealed that the STZ-i.c.v. injection rat was impaired in learning tasks compared with control groups and the STZ-i.p. injection group, suggesting impairment of passive avoidance learning might reflect a poorer acquisition and/or retention of memory in these rats [24]. This lead those authors to further suggest that poor cerebral regulation of glucose would be a primary metabolic event involved in impairments of learning and memory [24]. In 2003, Hoyer summarized studies specifically focused on the role of cerebral glucose metabolism and the function of insulin in the brain regarding memory capacity under normal and pathological conditions, including AD [25,26]. An interesting result to mention from that paper is the finding that *Ginkgo biloba* extract (EGb 761®) exerts a beneficial effect on STZ-damaged rats returning them to completely normal values of cerebral energy metabolism, including partial compensation of the deficits in learning, memory and cognitive ability [25]. Indirectly, these data provide support to the beneficial effects of balenine (+WME) diet on abnormalities in behavior and possible link to the recovery of the energy metabolism in the SAMP8 mouse model.

In the following paragraphs, we discuss some of these genes presented in Table 1, first taking an example of the glucose metabolism/transport-related *Slc2a5* gene. *Slc2a5*, which is a solute carrier family 2 (facilitated glucose transporter) member 5 (*Glut5*), is down-expressed in balenine(+WME) over the LSO (−WME) diet. Although several of the GLUTs have been identified in the brain, it is GLUT1, which is the key transporter of glucose through the blood brain barrier [27]. The GLUT 5 (*Glut5*/*Slc2a5*), known as a fructose transporter having very low affinity for glucose, has been shown to be expressed in human and rat microglia cells but its functional significance remains unknown [27,28]. Although glucose metabolism alterations have been suggested to play a role in AD, the mechanism remains unclear as also the link to AD [27]. Nevertheless, altered brain glucose levels and metabolism are suggested to be impaired in neurodegenerative diseases, and this might be the case with the SAMP8 model. Therein, we come across the second gene, namely *Treh* [29], which conversely to *Slc2a5* is highly expressed in the balenine(+WME) condition. This implies the TREHALASE, catalyzing the hydrolysis of trehalose into glucose providing an alternative source of energy [30,31], is available in the balenine (+WME) SAMP8 mouse and might be utilized for the production of glucose, based on the *Treh* gene expression level. It is also interesting to note that this implies an additional availability of trehalose, and which if might be proven in later studies, would be important factor in the improved learning and memory we find in the SAMP8 (+WME) mice. The reason may lie in the fact that this disaccharide is also known as an antioxidant protecting cells from injury, suppressing protein aggregation including having cellular and behavioral beneficial effects in animal models of neurodegenerative disorders [32].

These results lead us to speculate on the availability of glucose in the SAMP8 mice fed the balenine (+WME)-supplemented diet, which might help in recovering the said glucose deficiency in these mice. Similarly, we see the increased up-expression of the *Fbp1* (fructose-1,6-bisphosphate 1) and *Aldob* (aldolase B, fructose-bisphosphate) genes in the (+WME) SAMP8 mouse, again pointing towards activated glycolysis pathway, and leading to the TCA cycle. The other significant genes positively regulated by the balenine (+WME) supplemented diet in SAMP8 mouse were, alanine-glyoxylate aminotransferase 2-like 1 (*Agxt2l1*; glutamate catabolism, promoting the conversion of L-alanine + glyoxylate to pyruvate + glycine, metabolism of L-glutamate), cytochrome P450, family 2, subfamily e, polypeptide 1 (*Cyp2e1*; conversion of ethanol to acetaldehyde), acyl-CoA synthetase medium-chain family member 1 and 2 (*Acsm1*, *Acsm2*; priming or activation of the fatty acids by forming fatty acyl-CoA thioesters), phenylalanine hydroxylase (*Pah*; tetrahydrobiopterin (BH_4)-dependent enzyme phenylalanine 4-hydroxylase converts phenylalanine to tyrosine), and deoxyribonuclease 1 (*DNase1*; nucleates to ribose-5-P) (Fig. 4). Further, than their generally known functions, these genes are suggested here, in this study, to act also for the production of substrates to be utilized for the energy metabolism in the mitochondria as shown in Fig. 4.

Other than the possibility of the above genes presented in Fig. 4, towards improved energy metabolism in the (+WME) SAMP8 mouse brain, we would also like to discuss some unique characteristics of these gene products in relation to disease, specifically neurodegenerative disorders. The case of the novel gene up-expressed, *Agxt2l1*, a class II aminotransferase [33] is presented. First, the AGXT2L1 is a brain enzyme which might be involved in the pathogenesis of severe neuropsychiatric disturbances, and second, despite it being a transaminase was recently shown to have a stronger lyase activity (phospholipid metabolism) than an aminotransferase in human [34]. Taking the case of the human study, AGXT2L1 acts on O-phosphoethanolamine (PEA) irreversibly degrading it to acetaldehyde, phosphate and ammonia

Table 1
Genes oppositely regulated in the SAMP8 mouse fed the balenine (+WME)-supplemented diet. Color codes (red, up-expressed; green, down-expressed) mark the genes expressions recovering in the SAMP8 balenine (+WME) mouse brain over the same genes expression state in the SAMP8 LSO (−WME) mouse brain. The genes differentially expressed in the SAMP8 mice brain (LSO diet) were determined using SAMR1 mice brain(LSO diet) gene expression data as a reference. LSO, Low-safflower oil.

Gene Count(s): 92 genes		SAMP8 LSO/ SAMR1 LSO	SAMP8 +WME/ SAMP8 LSO
Symbol	Description	Ratio (average)	Ratio (average)
Igk–V19–14	gb|*Mus musculus* anti–DNA light chain (Vk19) mRNA, partial cds. [U59155]	3.91	−3.84
Igkv6–17	ens|Ig kappa chain V–V region MPC11 Precursor [Source:UniProtKB/Swiss–Prot;Acc:P01633] [ENSMUST00000103391]	2.32	−0.84
ENSMUST00000008011	ens|predicted gene 9946 Gene [Source:MGI Symbol;Acc:MGI:3643134] [ENSMUST00000008011]	2.04	−0.90
Cyp2g1	ref|*Mus musculus* cytochrome P450, family 2, subfamily g, polypeptide 1 (Cyp2g1), mRNA [NM_013809]	1.95	−1.64
Cyp2a5	ref|*Mus musculus* cytochrome P450, family 2, subfamily a, polypeptide 5 (Cyp2a5), mRNA [NM_007812]	1.80	−0.87
Cyp2a4	ref|*Mus musculus* cytochrome P450, family 2, subfamily a, polypeptide 4 (Cyp2a4), mRNA [NM_009997]	1.78	−0.81
Rya3	ref|*Mus musculus* antimicrobial peptide RYA3 (Rya3), mRNA [NM_194357]	1.78	−1.89
Cyp2f2	ref|*Mus musculus* cytochrome P450, family 2, subfamily f, polypeptide 2 (Cyp2f2), mRNA [NM_007817]	1.74	−0.70
ENSMUST00000108834	ens|RIKEN cDNA 4930438A08 gene Gene [Source:MGI Symbol;Acc:MGI:1921238] [ENSMUST00000108834]	1.73	−0.97
ENSMUST00000103746	ens|immunoglobulin lambda chain, variable 1 Gene [Source:MGI Symbol;Acc:MGI:96530] [ENSMUST00000103746]	1.70	−0.63
Igh–VJ558	ens|Ig alpha chain C region [Source:UniProtKB/Swiss–Prot;Acc:P01878] [ENSMUST00000103412]	1.66	−1.00
Gbp1	ref|*Mus musculus* guanylate binding protein 1 (Gbp1), mRNA [NM_010259]	1.56	−0.55
Lcn2	ref|*Mus musculus* lipocalin 2 (Lcn2), mRNA [NM_008491]	1.55	−0.62
S100a8	ref|*Mus musculus* S100 calcium binding protein A8 (calgranulin A) (S100a8), mRNA [NM_013650]	1.54	−1.32
Fgf21	ref|*Mus musculus* fibroblast growth factor 21 (Fgf21), mRNA [NM_020013]	1.53	−1.93
Cyp2g1	ref|*Mus musculus* cytochrome P450, family 2, subfamily g, polypeptide 1 (Cyp2g1), mRNA [NM_013809]	1.51	−1.62
Scgb1c1	ref|*Mus musculus* secretoglobin, family 1C, member 1 (Scgb1c1), mRNA [NM_001099742]	1.45	−1.82
Ugt2a1	ref|*Mus musculus*UDP glucuronosyltransferase 2 family, polypeptide A1 (Ugt2a1), mRNA [NM_053184]	1.45	−1.23
Camp	ref|*Mus musculus*cathelicidin antimicrobial peptide (Camp), mRNA [NM_009921]	1.41	−1.41
S100a9	ref|*Mus musculus* S100 calcium binding protein A9 (calgranulin B) (S100a9), mRNA [NM_009114]	1.40	−1.26
AK087643	gb|*Mus musculus*2 days pregnant adult female oviduct cDNA, RIKEN full–length enriched library, clone:E230031M10 product:novel transcript associated with phospholipase A2, group IB, pancreas, receptor, full insert sequence. [AK087643]	1.34	−0.68
Ltf	ref|*Mus musculus* lactotransferrin (Ltf), mRNA [NM_008522]	1.29	−1.21
Egr2	ref|*Mus musculus* early growth response 2 (Egr2), mRNA [NM_010118]	1.27	−0.72
Egr2	ref|*Mus musculus* early growth response 2 (Egr2), mRNA [NM_010118]	1.26	−0.75
Egr2	ref|*Mus musculus* early growth response 2 (Egr2), mRNA [NM_010118]	1.24	−0.74
Egr2	ref|*Mus musculus* early growth response 2 (Egr2), mRNA [NM_010118]	1.24	−0.75
Egr2	ref|*Mus musculus* early growth response 2 (Egr2), mRNA [NM_010118]	1.23	−0.73
Egr2	ref|*Mus musculus* early growth response 2 (Egr2), mRNA [NM_010118]	1.22	−0.74
Egr2	ref|*Mus musculus* early growth response 2 (Egr2), mRNA [NM_010118]	1.22	−0.73
Egr2	ref|*Mus musculus* early growth response 2 (Egr2), mRNA [NM_010118]	1.20	−0.71
Egr2	ref|*Mus musculus* early growth response 2 (Egr2), mRNA [NM_010118]	1.20	−0.73
Egr2	ref|*Mus musculus* early growth response 2 (Egr2), mRNA [NM_010118]	1.20	−0.74
Fos	ref|*Mus musculus* FBJ osteosarcoma oncogene (Fos), mRNA [NM_010234]	1.15	−0.94
Fos	ref|*Mus musculus* FBJ osteosarcoma oncogene (Fos), mRNA [NM_010234]	1.14	−0.97
Fos	ref|*Mus musculus* FBJ osteosarcoma oncogene (Fos), mRNA [NM_010234]	1.14	−0.95
AK143879	gb|*Mus musculus* 6 days neonate spleen cDNA, RIKEN full–length enriched library, clone:F430214N12 product:unclassifiable, full insert sequence. [AK143879]	1.14	−0.74
Fos	ref|*Mus musculus* FBJ osteosarcoma oncogene (Fos), mRNA [NM_010234]	1.14	−0.95
Fos	ref|*Mus musculus* FBJ osteosarcoma oncogene (Fos), mRNA [NM_010234]	1.13	−0.95
Fos	ref|*Mus musculus* FBJ osteosarcoma oncogene (Fos), mRNA [NM_010234]	1.13	−0.95
Mmp13	ref|*Mus musculus* matrix metallopeptidase 13 (Mmp13), mRNA [NM_008607]	1.13	−0.80
Fos	ref|*Mus musculus* FBJ osteosarcoma oncogene (Fos), mRNA [NM_010234]	1.12	−0.96
Fos	ref|*Mus musculus* FBJ osteosarcoma oncogene (Fos), mRNA [NM_010234]	1.11	−0.96

Table 1. *(continued)*

Igh–VJ558	ens|Ig alpha chain C region [Source:UniProtKB/Swiss–Prot;Acc:P01878] [ENSMUST00000103412]	1.11	−0.72
Fos	ref|*Mus musculus* FBJ osteosarcoma oncogene (Fos), mRNA [NM_010234]	1.09	−0.92
Fos	ref|*Mus musculus* FBJ osteosarcoma oncogene (Fos), mRNA [NM_010234]	1.09	−0.95
Igh–VJ558	ens|Ig alpha chain C region [Source:UniProtKB/Swiss–Prot;Acc:P01878] [ENSMUST00000103412]	1.08	−0.62
Ngp	ref|*Mus musculus* neutrophilic granule protein (Ngp), mRNA [NM_008694]	1.02	−1.55
Nr4a1	ref| *Mus musculus* nuclear receptor subfamily 4, group A, member 1 (Nr4a1), mRNA [NM_010444]	1.01	−0.67
Ear10	ref|*Mus musculus* eosinophil–associated, ribonuclease A family, member 10 (Ear10), mRNA [NM_053112]	0.95	−1.36
Ppbp (=CXCL7)	ref|*Mus musculus* pro–platelet basic protein (Ppbp), mRNA [NM_023785]	0.90	−0.77
Ddx58	ref|*Mus musculus* DEAD (Asp–Glu–Ala–Asp) box polypeptide 58 (Ddx58), mRNA [NM_172689]	0.90	−0.73
Scn9a (=Nav1.7)	ref|*Mus musculus* sodium channel, voltage–gated, type IX, alpha (Scn9a), mRNA [NM_018852]	0.78	−0.63
Nr4a3	ref|*Mus musculus* nuclear receptor subfamily 4, group A, member 3 (Nr4a3), mRNA [NM_015743]	0.74	−0.72
Ltf	ref|*Mus musculus* lactotransferrin (Ltf), mRNA [NM_008522]	0.74	−0.92
Retnlg	ref|*Mus musculus* resistin like gamma (Retnlg), mRNA [NM_181596]	0.68	−0.81
Hsd17b12	gb|*Mus musculus* 10, 11 days embryo whole body cDNA, RIKEN full–length enriched library, clone:2810004A21 product:hydroxysteroid (17–beta) dehydrogenase 12, full insert sequence. [AK076088]	0.53	−0.59
1600017P15Rik	gb|*Mus musculus* adult female placenta cDNA, RIKEN full–length enriched library, clone:1600017P15 product:unclassifiable, full insert sequence. [AK005488]	−0.54	0.63
Mettl7b	ref|*Mus musculus* methyltransferase like 7B (Mettl7b), mRNA [NM_027853]	−0.69	1.01
ENSMUST00000112652	gb|*Mus musculus* 16 days neonate heart cDNA, RIKEN full–length enriched library, clone:D830032D10 product:unclassifiable, full insert sequence. [AK085942]	−0.75	0.80
Aldob	ref|*Mus musculus* aldolase B, fructose–bisphosphate (Aldob), mRNA [NM_144903]	−0.76	1.76
	gb|*Mus musculus* adult male pituitary gland cDNA, RIKEN full–length enriched library, clone:5330407B06 product:weakly		

Table 1. *(continued)*

Agxt2l1	similar to ALANINE:GLYOXYLATE AMINOTRANSFERASE 2 HOMOLOG 1, SPLICE FORM 1 [Homo sapiens], full insert sequence. [AK030395]	−0.76	0.60
Treh	ref|*Mus musculus*trehalase (brush–border membrane glycoprotein) (Treh), mRNA [NM_021481]	−0.78	1.30
Clec2h	ref|*Mus musculus* C–type lectin domain family 2, member h (Clec2h), mRNA [NM_053165]	−0.78	0.96
Fbp1	ref|*Mus musculus* fructose bisphosphatase 1 (Fbp1), mRNA [NM_019395]	−0.78	1.93
Aldob	ref|*Mus musculus* aldolase B, fructose–bisphosphate (Aldob), mRNA [NM_144903]	−0.80	1.77
Defa–rs12	ref|*Mus musculus* defensin, alpha, related sequence 12 (Defa–rs12), mRNA [NM_007846]	−0.84	1.18
Tmem27	ref|*Mus musculus* transmembrane protein 27 (Tmem27), mRNA [NM_020626]	−0.86	2.09
AU018778	ref|*Mus musculus* expressed sequence AU018778 (AU018778), mRNA [NM_144930]	−0.87	2.87
ENSMUST00000060246	ens|RIKEN cDNA 1110008E08 gene Gene [Source:MGI Symbol;Acc:MGI:1915750] [ENSMUST00000060246]	−0.93	0.89
2810405F15Rik	ref|*Mus musculus* RIKEN cDNA 2810405F15 gene (2810405F15Rik), non–coding RNA [NR_033447]	−0.95	0.65
Aldob	ref|*Mus musculus* aldolase B, fructose–bisphosphate (Aldob), mRNA [NM_144903]	−0.96	2.09
Fxyd2	ref|*Mus musculus* FXYD domain–containing ion transport regulator 2 (Fxyd2), transcript variant b, mRNA [NM_052823]	−0.97	1.91
Gtpbp8	gb|*Mus musculus* adult retina cDNA, RIKEN full–length enriched library, clone:A930006C13 product:unclassifiable, full insert sequence. [AK044298]	−0.97	0.98
TC1676807	tc|Q6NS85_MOUSE (Q6NS85) Sgk protein, partial (45%) [TC1676807]	−1.05	0.96
Pah	ref|*Mus musculus* phenylalanine hydroxylase (Pah), mRNA [NM_008777]	−1.05	2.10
Smchd1	gb|*Mus musculus* 0 day neonate eyeball cDNA, RIKEN full–length enriched library, clone:E130019A11 product:unclassifiable, full insert sequence. [AK053453]	−1.06	0.64
Acsm2	ref|*Mus musculus*acyl–CoA synthetase medium–chain family member 2 (Acsm2), nuclear gene encoding mitochondrial protein, transcript variant 3, mRNA [NM_146197]	−1.07	3.81
S100g	ref|*Mus musculus* S100 calcium binding	−1.10	3.57

(continued on next page)

Table 1. *(continued)*

	protein G (S100g), mRNA [NM_009789]		
Cyp4b1	ref|*Mus musculus* cytochrome P450, family 4, subfamily b, polypeptide 1 (Cyp4b1), mRNA [NM_007823]	−1.14	2.46
Fam83d	ref|*Mus musculus* family with sequence similarity 83, member D (Fam83d), mRNA [NM_027975]	−1.19	0.61
Slc17a3	ref|*Mus musculus* solute carrier family 17 (sodium phosphate), member 3 (Slc17a3), transcript variant 1, mRNA [NM_134069]	−1.32	2.54
Keg1	ref|*Mus musculus* kidney expressed gene 1 (Keg1), mRNA [NM_029550]	−1.53	3.52
Pdzd2	gb|*Mus musculus* 0 day neonate eyeball cDNA, RIKEN full–length enriched library, clone:E130016M22 product:unclassifiable, full insert sequence. [AK087419]	−1.61	0.59
Slc34a1	ref|*Mus musculus*solute carrier family 34 (sodium phosphate), member 1 (Slc34a1), mRNA [NM_011392]	−1.63	3.22
B430316J06Rik	gb|*Mus musculus* 4 days neonate male adipose cDNA, RIKEN full–length enriched library, clone:B430316J06 product:unclassifiable, full insert sequence. [AK046698]	−1.76	1.58
Cyp2j5	ref|*Mus musculus* cytochrome P450, family 2, subfamily j, polypeptide 5 (Cyp2j5), mRNA [NM_010007]	−1.84	4.17
Acsm1	ref|*Mus musculus*acyl–CoA synthetase medium–chain family member 1 (Acsm1), mRNA [NM_054094]	−1.93	4.34
Cyp2e1	ref|*Mus musculus* cytochrome P450, family 2, subfamily e, polypeptide 1 (Cyp2e1), mRNA [NM_021282]	−2.05	3.30
Cyp2j5	ref|*Mus musculus*cytochrome P450, family 2, subfamily j, polypeptide 5 (Cyp2j5), mRNA [NM_010007]	−2.48	4.70
Dnase1	ref|*Mus musculus*deoxyribonuclease I (Dnase1), mRNA [NM_010061]	−2.50	4.40
Acsm2(ACSM2B)	ref|*Mus musculus*acyl–CoA synthetase medium–chain family member 2 (Acsm2), nuclear gene encoding mitochondrial protein, transcript variant 3, mRNA [NM_146197]	−3.13	4.15
Kap	ref|*Mus musculus* kidney androgen regulated protein (Kap), mRNA [NM_010594]	−4.06	5.94

[34]. As described by Schiroli and co-workers (2013) [34], and we quote "PEA is both a byproduct of sphingolipid degradation and a precursor of glycerophospholipid biosynthesis and it can accumulate when membrane remodeling or degradation increase"; such an accumulation can lead to mitochondrial dysfunction [35]. Thus, AGXT2L1 might act as a protector of the toxic metabolite increase in cells or in the case of disease might serve as a compensatory mechanism to alleviate the effects of excessive membrane degradation [34]; is this the case in the SAMP8 mouse brain, though we do not know at present. The *Agxt2l1* gene was also recently shown to be one of the three strongly up-regulated brain genes in the mouse model of psychiatric disorders/neurological diseases, such as compound C and lithium therapy, which have been used to study the effects on chromatin-modifying enzymes such as the histone deacetylase [36]. These data suggest an involvement of the *Agxt2l1* gene in AD, though it should be noted that this gene function needs further investigation and validation as a novel target of lithium and neuroleptics, thus providing greater relevance to its decreases in depression and suicide and increases in bipolar disorder and schizophrenia [37].

Another exciting finding of an up-expressed gene is *Pah*, which, interestingly, causes a reduction in the amino acid phenylalanine turnover in chronic inflammation conditions that is known to affect dopamine, adrenaline and noradrenaline synthesis, as tyrosine is the precursor of these neurotransmitters [38]. Moreover, a cross-regulation between the immune response and severe metabolic changes is known and a connection therein with depression and cognitive impairment, which is in turn linked with changes in amino acid metabolism and neurotransmitter (serotonin, L-DOPA, dopamine, noradrenaline and adrenaline) synthesis [38–41]. As recently reported by Gostner and co-workers (2015), the low-inflammatory stress-associated dysregulation of amino acid metabolism may be part of the pathophysiology of common HIV-associated neuropsychiatric conditions [38]. These reports led us to speculate that SAMP8 mice model of AD brains might have an amino acid imbalance and that is being possibly recovered in part by the up-expression of genes, such as *Pah*, with the balenine (+WME)-supplemented diet. Herein, again the role of balenine (in the WME) may be called to question and further investigation.

From the functional gene categories, the gene numbers in antifungal response, NFκB signaling targets, cancer inflammation and immunity crosstalk, inflammatory responses and autoimmunity related categories were highly dominant in the SAMP8 mouse (LSO diet) brain. Contrastingly, these functional categeories were considerably reduced in gene numbers in the SAMP8 mouse-fed balenine (+WME) diet, brain (see Online Resource 3). Looking carefully at the literature it was found that brain infections have recently become an important topic in relation to AD [42–44]. A most recent publication showed that different brain regions (central nervous system) of AD patients are infected with fungal pathogens [45]. However, as those authors mention, it is not clear as to whether the fungal agents were the cause of AD or the patients, who were elderly, had a poor adaptive immune response resulting in them having fungal infections [45]. As also recently reported, researchers have considered the idea that AD might be an infectious disease or that infectious agents, include bacteria are risk factors for AD [46,47]. The gene category for antibacterial response was also dominant in the SAMP8 (LSO diet) mouse brain (see Online Resource 3). Neuroinflammation or the inflammatory processes also are thought to play an important part in the pathogenesis of AD [48–50], and these gene categories are also well represented in the SAMP8 (LSO diet) mouse brain (see Online Resource 3).

Combining the powerful approach of DNA microarray chip technology with the IPA bioinformatic tool, the possible brain gene interactions and pathways/networks could be delineated. These networks, one through six, for both SAMP8 mouse-fed balenine (WME) and LSO diets are shown in Online Resources 5 and 6. The top molecules presented in Fig. 5, of which some can also be seen in the Table 1 (oppositely regulated expressions in the balenine + WME mouse-fed diet), suggest their importance as key genes in the said positive effects of balenine (+WME) supplementation. It would be interesting to analyze these genes (*Kap*, *Cyp2j5*, *S100G*, *Keg1*, *UGT2B28*, *Igkv6-14*, *HLA-A*, *CCL19*, etc.) individually and further bioinformatics analysis would shed light on their possible role/function and modulation by balenine (+WME)-supplemented diet in the SAMP8 mouse. For example, the most highly up-regulated and top molecule (Fig. 5), namely, *Kap* gene that was also the most down-regulated gene in SAMP8 mouse-fed LSO diet showed a dramatic recovery in its expression with the balenine (+WME) diet (Table 1). *Kap* encodes for the kidney androgen-

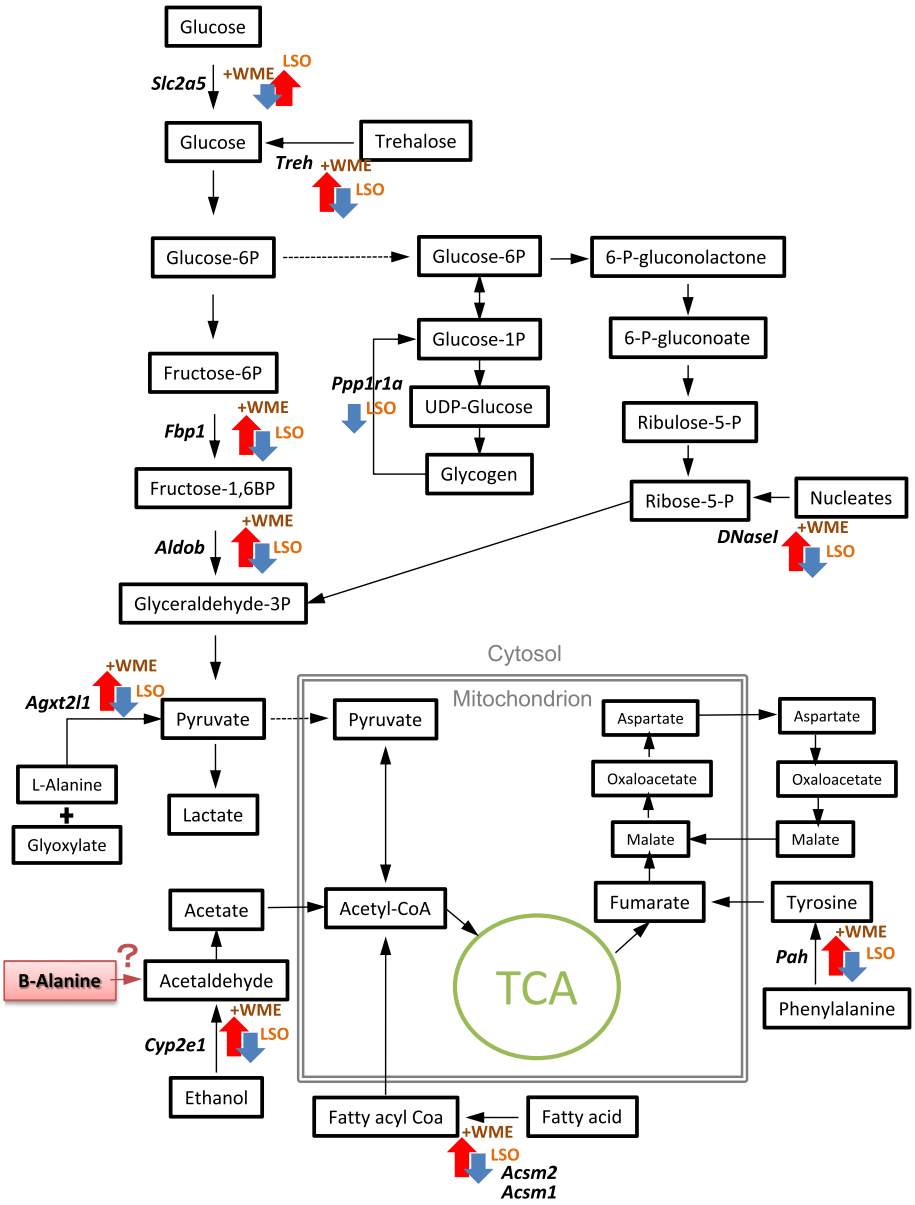

Fig. 4. The genes of the major cellular metabolic pathways influenced in the whole brain of SAMP8 mouse with or without balenine (WME) supplementation. The red and blue bold arrows indicate up-regulated and down-regulated expression of genes (*italicized*) located intermediate to the respective substrates and products. Balenine (+WME) indicates SAMP8 mice fed with the experimental diet containing balenine as described in the Materials and methods section. −WME refers to the SAMP8 mice fed the control diet (LSO, low-safflower oil).

regulated protein precursor [51], and its functions in the kidney are being widely investigated but nothing is known about it in the brain. *Kap* expression and presence raises new questions about its possible role in the brain of the SAMP8 mouse.

In summary, this research is a first such study describing the effects of balenine (in WME) as a dietry supplement in a mice model (SAMP8) using both behavioral tests and high-throught DNA microarray-based omics approach, which provides new insight into the effects of the meat supplemented diet on the AD model mouse brain. To note, a previous study has also investigated the aging-related gene expression profiles in the frontal cortext of SAMP8 [52]. In particular, the energy metabolism pathways discussed above and amino acids provision to the brain point towards the beneficial positive effects of balenine (+WME) diet on the SAMP8 mouse, which are interesting but require further experimental evidence. Our study helps set up a model system to investigate further the effects of specific whale meat compoments, such as the imidazole dipeptide balenine, and most probably other natural meats/products for possible beneficial effects on the human body. In addition, the obtained gene information resource in this study might serve well in investigating and designing new therapies that target these genes/proteins/enzymes of the said metabolic pathways for AD. The upcoming studies in our group will not only look directly at the effects of balenine in animal models of AD (SAMP8)/neurological diseases, but also utlize other omics-based approaches, proteomics [53] and metabolomics to try link the genes from this microarray analysis to their products for a better understanding of how the balenine (+WME) supplemented diet affects the observed positive effects on brain recovery and function in the AD model mouse. We would argue at the end that this study was conducted only as a test to try get an insight into the positive effects of the dipeptide balenine (though dietry supplementation) a major component of the whale meat that formed/forms the diet of not only ancient, and to some extent current, Japanese people, but also in other whale eating traditions in the world.

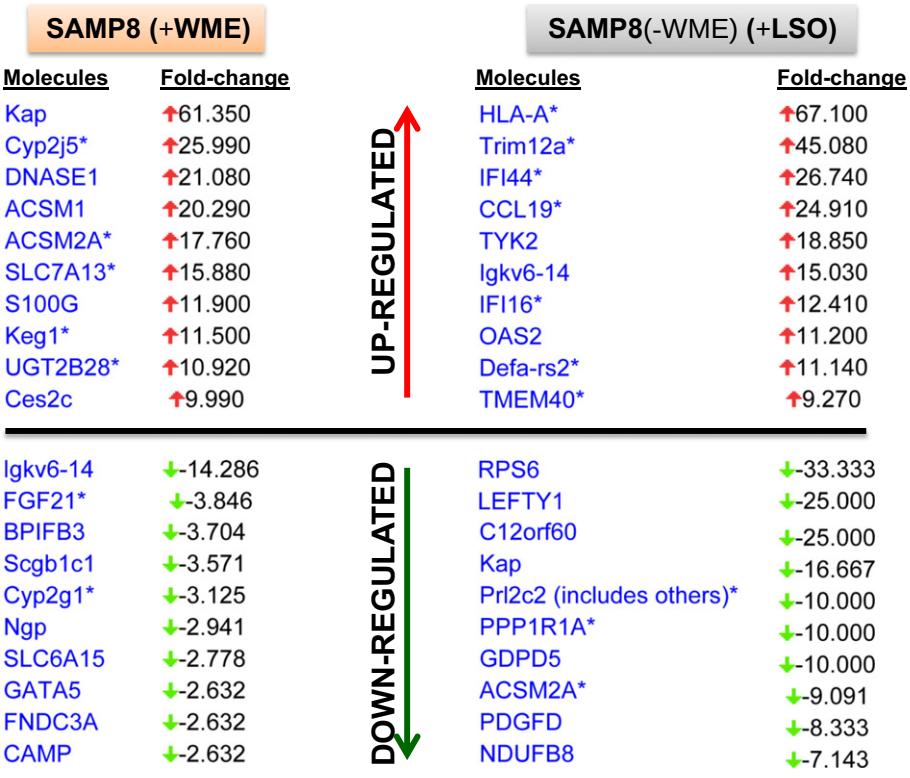

Fig. 5. The top molecules identified by IPA analysis in the whole brain of SAMP8 mouse with or without balenine (WME) supplementation. Gene names are in blue, and the up- and down-regulated genes along with the fold changes are indicated by red and green bold arrows for each sample condition. Balenine (+WME) indicates SAMP8 mice fed with the experimental diet containing balenine as described in the Materials and methods section. −WME refers to the SAMP8 mice fed the control diet (LSO, low-safflower oil).

4. Materials and methods

4.1. Animals and husbandary, supplemented diet, and sampling of whole brain

Six-weeks-old SAMP8 and SAMR1 mice (CLEA, Tokyo, Japan) were housed at the Animal Institution Facility in Showa University, and maintained in individual cages in a ventilated animal room with controlled temperature and relative humidity under a 12 h light: 12 h dark regime (8:00 AM, lights turned on). Mice were fed chow (CE-2, CLEA Japan) and tap water ad libitum until 24-weeks-old. Then, 24-weeks-old SAMs mice were given experimental diets, balenine (+WME)-supplemented diet and LSO as a control diet, and both in a powder form. The WME was prepared from red meat of Antarctic minke whale *Balaenoptera bonaerensis* taken from the Japanese Whale Research Program under Special Permit in the Antarctic-Phase II in 2009/2010 by heat, enzyme and drying treatments, and as provided by Kyodo Senpaku Co. Ltd., Tokyo (Japan). The quality standard of whale meat was measured by Marugei Co. Ltd., (Hyogo, Japan), and results of the analysis are shown in Online Resource 7. The LSO diet components are shown in Online Resource 8. SAMP8 mice were randomly given balenine (in WME) or LSO diet, respectively. SAMR1 mice were given only the LSO diet, until 50-weeks-old. The behavioral tests, as detailed below, were performed at the timing of 49-weeks-old for eight days. At the end of the experiment (50-weeks of age) following the behavioral analysis and the last day of the feeding, the mice were removed their cages, decapitated and their brains carefully removed on ice. The whole brains were quickly frozen in liquid nitrogen in sterile freeze tubes, and stored at −80 °C, till extraction of total RNA (Online Resource 9) for downstream DNA microarray analysis (see also Sections 4.4. and 4.5.). All animal studies were conducted in accordance with the "Standards Relating to the Care Management of Experimental Animals" (Notice No. 6 of the Office of Prime Minister dated March 27, 1980) and with approval from the Animal Use Committee of Showa University (Approval Number: #04093).

4.2. Behavioral tests

To evaluate the effect of WME on the mouse, behavioral tests were carried out as follows: open field test, Y-maze test, novel object recognition test (NOR) and water-filled multiple T-maze (WMTM) [54]. The number of animals in each test was as follows: SAMP8 LSO, n = 8; SAMP8 WME, n = 10; SAMR1 LSO, n = 10. Behavioral testing was performed between 10:00 AM and 6:00 PM. To habituate the animals to the testing room conditions, mice were transferred to the behavior testing room 1 h (9:00 AM to 10:00 AM) prior to beginning the first trial. The test room was not illuminated by the overhead lights but by using indirect lighting that pointed upwards to the roof. All apparatus were purchased from Bio-Research Co. Ltd. (Tokyo, Japan), and analysis of behavior test were analyzed by the SMART software 2.0 (Bio-Research Co. Ltd.). Each test was performed consequently for 8 days (open field test, 1 day; Y-maze test, 1 day; NOR, 2 days; WMTM, 4 days). We describe below, in detail, each test, and the images of each of the four apparatus set-up are schematically depicted in Online Resource 10.

4.2.1. Open field test

Mice were moved to the test room in their home cages and were handled by the base of their tails at all times. Each mouse was placed into the one of the four corners of the open field (45 cm × 45 cm × 40 cm) and allowed to explore the apparatus for 5 min (see Online Resource 10a). After 5 min, the mouse were returned to its home cage, and the open field was cleaned with oxidative water and permitted to dry between tests, followed by the second mouse.

4.2.2. Y-maze test

The Y-maze apparatus consisted of three arms made of gray plastic joined in the middle to form a "Y" shape (see Online Resource 10b). The walls of the arms were combinated, and the one arm component spec is 300 × 60 × 150 mm. The mouse was placed into one of the arms of the maze (start arm) and allowed to explore freely all three arms of the maze for 8 min. The number and the sequence of arms entered were recorded. The dependent variables were activity, defined as the number of arms entered, and percent alternation, calculated as the number of alternations (entries into three different arms consecutively) divided by the total possible alternations.

4.2.3. Novel object recognition test

The NOR test was performed using the open field box (see, open field test above) (see Online Resource 10c). Mice were individually habituated to the apparatus for 10 min at the 1st day (training day). The mouse can freely explore the open field area and touch the novel objects (A and A′) for 10 min. The animal behavior was recorded and we did a touch count, i.e., the number of times the mouse touched the objects was counted. On the next day, mice were individually let into the open field area again. We observed the mouse noticing change in the color of the object (A and B). If the mouse remembered the color of the object from the previous day, the tendency of the mouse moving towards the novel object is greater. This behavior alteration was recorded and the number of touches to the novel object was counted. Only 5 min were given to each mouse on the 2nd day.

4.2.4. Water-filled multiple T-maze test

The WMTM test is known as the multiple learning and memory test [54]. WMTM consists of seven T-maze combinations (20 cm wall height × 9 cm width), which are filled with water (22 ± 1 °C) to a height of 16 cm (see Online Resource 10d). During training (day 1), mice were habituated to the water by swimming three times a day. Mice remembered the goal position in order to escape from the water. During testing (days 2, 3 and 4), mice were placed at the start position in the water and allowed to swim freely for a maximum of 180 s. We measured when the mouse entered into the blind alley or not, and the time taken to arrive at the goal position. The interval time of this test is 2 h, and we performed this test three times in a day for three consecutive days.

4.3. Statistical analysis

All statistical analyses were performed with the Sigma Plot 12 (Hulinks, Tokyo, Japan) software package. Differences between three groups were assessed using the one-way analysis of variance (ANOVA) followed by Bonferroni's post-hoc test. Values were reported as the mean ± SE. Statistical significance was defined as $p < 0.05$.

4.4. Total RNA extraction

The deep-frozen whole mouse brain in a freeze tube was transferred from the deep freezer to a container with liquid nitrogen. The tissue was then carefully transferred to a pre-chilled (in liquid nitrogen) mortar and ground with a pestle to a very fine powder with liquid nitrogen [55–57]. Sample powders were immediately used for the next step of total RNA extraction by the QIAGEN RNeasy Mini Kit (QIAGEN, Maryland, USA) or stored at −80 °C. Prior to DNA microarray analysis, the total RNA, quantity and quality measured spectrophotometrically with NanoDrop (Thermo Scientific, Wilmington, DE, USA) and re-confirmed using formaldehyde-agarose gel electrophoresis [55–57]. The obtained total RNA quality measurements revealed good quality as demonstrated by the $A_{260/280}$ values > 1.8, $A_{260/230}$ > 1.8; in our experiments, aiming for a higher value of >2.0 to 2.3 [55–57]. Further, we obtained total RNA in optimum quantity of >300 ng/μl. The total RNA was visualized for confirming pre-experimental integrity of the total RNA subunits by gel electrophoresis as shown in Online Resource 9.

4.5. DNA microarray chip-based whole genome brain transcriptome profiling

The total RNA that was extracted from the brain tissues for each animal was pooled in each group (SAMP8 LSO; SAMP8 WME; SAMR1 LSO) prior to DNA microarray analysis. As mentioned in Online Resource 9, for total RNA extraction individual biological replicates for SAMP8 LSO (n = 5), SAMP8 +WME (balenine) (n = 4), and SAMR1 LSO ()−WME) (n = 5) were used. These RNAs were pooled for application to the DNA microarray chip for one experiment. In addition, two technical replicates were performed by using two DNA microarray chips following the two-color dye-swap approach (detailed in Fig. 1). The reason for pooling the total RNA and not individual tissues is first, due to the usually larger variability at the tissue level than at the RNA level for estimating the gene expression, and second, in the use of lesser number of chips that was an economical/cost-effective option for the highly expensive microarray analysis [55,58,59]. All the steps followed in sequence provided confidence that we had the best quality total RNA for use in the DNA microarray chip. As a last step, the Agilent mouse whole genome 4 × 44 K (G4122F) DNA slide (composed of four chips on one slide) was used for the microarray analysis, which was performed according to the Agilent instructions (Agilent Technologies, Santa Clara, CA, USA) and detailed in our publications [55–57,60]. Our established DNA microarray analysis experiment is also based on the two-color dye-swap approach providing a more-stringent selection condition for changed gene expression profiling than a simple single/two-color approach [55]. Briefly, total RNA (200 ng) was labeled with either Cy3 or Cy5 dye using an Agilent Low-RNA-Input Fluorescent Linear Amplification Kit (Agilent Technologies). Fluorescently labeled targets of control (SAMP8 −/SAMR1 −) as well as treatment (SAMP8 +/SAMP8 −) indicated in Fig. 1 were hybridized to the same microarray slide with 60-mer probes. A flip-labeling (dye-swap or reverse labeling with Cy3 and Cy5 dyes) procedure was followed to nullify dye bias associated with unequal incorporation of the two Cy dyes into cDNA. Differentially expressed genes were considered genes that were up-regulated in chip-1 but down-regulated in chip-2 for each group. Hybridization and wash processes were performed according to the manufacturer's instructions, and the hybridized microarrays were scanned using an Agilent Microarray scanner G2505C. To detect significantly differentially expressed genes between the control and treated samples, each slide image was processed using Agilent Feature Extraction software (version 11.0.1.1). This program measures the Cy3 and Cy5 signal intensities of whole probes. Dye bias tends to be signal-intensity-dependent; therefore, the software selected probes using a set-by-rank consistency filter for dye normalization. Said normalization was performed by LOWESS (locally weighted linear regression), which calculates the log ratio of dye-normalized Cy3- and Cy5 signals and the final error of the log ratio. Statistical significance of the log ratio values obtained for individual microarray probes was calculated using the "most conservative error model" default function of the Feature Extraction software, by which both a propagated and a universal error model are evaluated, and the higher (more conservative estimate of error) p-value of two error models was reported (Agilent Technologies [55]). For the analyses, threshold of significant differentially expressed genes is <0.01 (for the confidence that the feature was not differentially expressed). Additionally, erroneous data generated due to artifacts were eliminated before data analysis by the software.

4.6. Functional categorization of differentially expressed genes

Pathway- and disease state-focused gene classification of the differentially expressed genes in the brain were classified based on the available categories of >100 biological pathways or specific disease states in the SABiosciences PCR array list (Qiagen; www.sabiosciences.com) for *Mus musculus*.

4.7. IPA analysis

The biological function and network analyses were performed using IPA (Ingenuity® Systems, www.ingenuity.com). The dataset from the microarray [balenine diet (WME) supplementation in SAMP8 (+) or in SAMP8 (−)], which includes the differentially expressed (≧/≦ 1.5/0.75-fold compared with control) genes and their corresponding fold change values, was uploaded as an Excel spread sheet into the IPA tool. To create gene networks, the genes were overlaid onto a global molecular network that was developed from information that was contained in the ingenuity knowledge base. The functional analysis identified the biological functions that were most significant to the dataset (p-value < 0.05) according to a right-tailed Fisher's exact test.

4.8. Access to gene array data

The outputs of microarray analysis used in this study are available under the series number GSE 76459 (whole genome - http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE76459; at the NCBI Gene Expression Omnibus (GEO) public functional genomics data repository (http://www.ncbi.nlm.nih.gov/geo/info/linking.html).

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.gdata.2016.09.004.

Conflict of interest

The authors declare no conflict of interests.

Acknowledgments

SS greatly appreciates the financial support from Kyodo Senpaku Co. Ltd., Tokyo, Japan for funding the current research. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript. Authors appreciate the help of Mr. Gaku Tamura (computer programmer) and Dr. Tetsuo Ogawa (Showa University) for their help with development of an Excel program to sort the list of gene expression changes into the pathway- and specific disease states-focused gene classifications. Authors also thank Dr. Kazue Satoh and Ms. Michiru Ootsuka (Showa University) for the free-radical scavenging activity test.

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