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SAMP8 mice have altered hippocampal gene expression in LTP, phosphatidylinositol signaling, and endocytosis pathways

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Abstract

The senescence-accelerated mouse (SAMP8) strain exhibits decreased learning and memory and increased amyloid beta peptide (A β) accumulation at 12 months. To detect differences in gene expression in SAMP8 mice, we used a Control mouse that was a 50% cross between SAMP8 and CD-1 mice and which showed no memory deficits (50% SAMs). We then compared gene expression in the hippocampus of 4 month and 12 month old SAMP8 and Control mice using Affymetrix gene arrays. At 12 months, but not at 4 months, pathway analysis revealed significant differences in the Long Term Potentiation (LTP) (6 genes), Phosphatidylinositol Signaling (6 genes), and Endocytosis (10 genes) pathways. The changes in LTP included MAPK signaling (N-ras, CREB binding protein, protein phosphatase inhibitor 1) and Ca-dependent signaling (ITP receptors 1 and 2 and phospholipase C). Changes in phosphatidylinositol signaling genes suggested altered signaling through PI3-kinase, and Western blotting revealed phosphorylation changes in AKT and 70S6K. Changes in the Endocytosis pathway involved genes related to clathrin-mediated endocytosis (dynamin and clathrin). Endocytosis is required for receptor recycling, is involved in A β metabolism, and is regulated by phosphatidylinositol signaling. In summary, these studies demonstrate altered genes expression in three SAMP8 hippocampal pathways associated with memory formation and consolidation. These pathways may provide new therapeutic targets in addition to targeting A β metabolism itself.

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Keywords

SAMP8 mouse; hippocampal gene expression; memory loss; long term potentiation; phosphatidylinositol signaling; clathrin-mediated endocytosis

1. Introduction

We have studied extensively the SAMP8 mouse, an animal model that spontaneously and gradually develops cognitive impairments by 12 months of age (Morley et al., 2002; Morley et al., 2002a). At 12 months, the SAMP8 mice show impaired footshock avoidance, object recognition with a 24-hour delay, lever press operant condition, and 3-way brightness discrimination compared to 4 month animals (Morley et al., 2002). During this age span there is a 100% increase in amyloid beta peptide (A β) (Kumar et al., 2000; Morley et al., 2002a). A β is thought to play a key role in age-related memory loss and Alzheimer's Disease (AD) (Rosenberg, 2000; Viola, 2008).

Since the hippocampus is important in memory formation, we hypothesized that there may be differences in hippocampal gene expression in SAMP8 mice. These differences may affect key pathways involved in memory formation. The purpose of this study was to compare hippocampal gene expression with age and strain and determine whether specific pathways are altered. A β can act through a number of membrane receptors to affect a number of biochemical pathways (summarized by Balleza-Tapia and Pena, 2009). These pathways include phosphatidylinositol-3-kinase (PI3K), Ca signaling, ERK, CREB, MAPK, RAS, p38, JNK, nitric oxide and reactive oxygen species.

Previous studies have characterized gene expression changes in the SAMP8 hippocampus but used the SAMR1 mouse as a control (Carter et al., 2005; Cheung et al., 2007). The SAMR1 has been bred over many generations, and so it is not closely related to the SAMP8 mouse. To minimize strain differences, we developed a 50% cross between SAMP8 and CD-1 mice, which showed no memory deficits (50% SAMP8). We then compared gene expression in the hippocampus at 4 month and 12 months in the 50% SAM (Control) and SAMP8 mice. Pathway analysis revealed differences in three inter-dependent pathways, some of which have been previously associated with cognitive deficits.

2. Methods

2.1 Animals and Experimental Design

SAMP8 mice were from our in-house animal colony, and CD-1 mice were originally obtained from Charles River Laboratories (Wilmington, MA). The 50% SAMP8 mice were re-derived from the SAMP8 and CD-1 mice as previously described (Flood et al., 1995) (see Results). Mice were housed four to a cage with a 12 hour light-dark cycle and access to food and water ad libitum. All studies were approved by the Institutional Animal Care and Use Committee of the St. Louis VA Medical Center. The general experimental design consisted of four age/ strain groups of 8 mice each: (1) 4 month Control (50% SAMP8); (2) 4 month SAMP8; (3) 12 month Control (50% SAMP8); and (4) 12 month SAMP8 (see Fig. 2A).

2.2 Behavioral Measures

T-maze foot shock avoidance and novel object recognition were measured as previously described (Farr et al., 2012). T-maze is a declarative memory task that requires an intact hippocampus (Farr et al., 2000). Briefly, the maze consists of a black plastic start alley with a start box at one end and two goal boxes at the other (T-maze). The goal box that the mouse first enters on the initial trial is designated as "correct". The mouse is trained until it makes

one avoidance (acquisition). The mouse is then re-tested one week later on the same task until it makes 5 avoidances in 6 consecutive trials (retention). Object-place recognition is a declarative memory task that involves the hippocampus when, as performed here, the retention interval is 24 hours (Hammond et al., 2004). On the first day of training, two similar objects are placed in the maze. Mice are placed in the maze and allowed to explore the objects for 5 minutes. In the 24-hour retention test, one of the same objects is placed in the maze, as well as a new object in a new location. The percent time spent exploring the new versus the old object is recorded. The higher the percent of time the mouse spends exploring the new object, the greater its memory of the old object.

2.3 RNA Processing

Total RNA was extracted from the hippocampus of eight individual mice per group using the RNeasy Lipid Extraction Mini Kit (Qiagen Inc, Valencia, CA) according to the manufacturer's guidelines. Total RNA quality was assessed by running micro-capillary gels on RNA LabChips using the Bioanalyzer 2100 (Agilent Technologies; Palo Alto, CA), confirming the integrity of 18S and 28S ribosomal RNA bands. Total RNA was quantified using the NanoDrop® ND-1000 Spectrophotometer (NanoDrop, Wilmington, DE).

For microarray analysis, four biological replicate RNA samples were generated by combining equal amounts of total RNA (2 µg) from two animals each. This generated samples for a total of 16 arrays (4 independent biological replicates × 4 experimental age/strain groups).

2.4 Microarray Processing

In general, microarray processing of RNA samples was performed as previously described (Haren et al., 2011). Global profiling of hippocampal gene expression was performed using GeneChip® Mouse Genome 430 2.0 Arrays (Affymetrix, Inc.; Santa Clara, CA). These contain 45,000 probe sets that interrogate the expression level of over 39,000 transcripts and variants from over 34,000 well-characterized mouse genes.

The mRNA amplification/labeling process began with a total of 4 µg of total RNA for each of the 16 samples. mRNA amplification/labeling and chip washing/hybridization were conducted according to Affymetrix guidelines. The arrays were then scanned using the Affymetrix GeneChip® Scanner 3000. The resulting raw images (*.DAT files) were converted to computer-readable probe cell intensities (*.CEL files). Quality metrics for each array were monitored by analyzing the accuracy of varying known concentrations of spike-in labeling controls and hybridization/staining controls using Microarray Suite 5.0 (MAS 5.0) algorithms from Affymetrix. GeneChip® Operating Software 1.2, GCOS. MAS 5.0 was used only to assess the quality metrics for each array. Raw microarray data were deposited with GEO Accession Number xxxxxxxxxx.

2.5 Determination of Differential Gene Expression

Differential gene expression was determined as previously described (Haren et al., 2011). Probe cell intensities (*.CEL files) for each array were normalized using accuracy/precision gc-RMA algorithms. These consist of background adjustment, quantile normalization, and a robust probe-set summary of the log-transformed probe-level data by median polishing, while also accounting for probe GC content (Wu and Irizarry, 2005). The balanced design of this study enabled the use of a balanced 2-way ANOVA to assess gene expression differences with respect to strain (Control vs SAMP8) and age (4 vs 12 months) (FDR 0.01) (Pavlidis, 2003; Reiner et al., 2003).

The 902 probes with a $P < 0.01$ in at least one of the pair-wise comparisons (see Fig. 2a) were grouped into 6 distinct expression profile categories: (1) changes in SAMP8 vs Control at 4 months (up or down), (2) changes in SAMP8 vs Control at 12 months (up or down), and (3) changes in 12 month old vs 4 month old mice (up or down) regardless of strain. Grouping of the 902 differentially expressed probes was conducted using $P < 0.05$ for the appropriate pair-wise comparison. For a list of all 902 probes, see the Supplemental Spreadsheet – Characterization of Differential Gene Expression.

As previously described (Haren et al., 2011), each group of differentially expressed genes was statistically analyzed for enrichment/over-representation in functional categories (i.e. Gene Ontologies and Pathways). This was accomplished using the 2x2 Fisher's Exact Test implemented in the web-based DAVID platform (<http://david.abcc.ncifcrf.gov/home.jsp>) (Huang et al., 2009). The default criteria of $P < 0.10$ was used due to the small number of genes. Categories queried included the Biological Function, Cellular Component, and Molecular Function Gene Ontologies; and and KEGG canonical pathways.

2.5 Western Blot Analysis

Total and phosphorylated AKT and 70S6K were determined by Western blot. Whole hippocampus was homogenized in PBS buffer containing 20 mM iodoacetamide, 3 mM EDTA, 1 μ g/ml Pepstatin A, 2 mM PMSF, 0.1% SDS, 0.5% sodium deoxycholate, 1% NP40, and Roche PhosSTOP (one tablet/10 ml). After homogenization, samples sat on ice for 30 min, were centrifuged in a microfuge, and supernatants were frozen. Supernatants were run on a 4–12% Bis-Tris gel and transferred to an immuno-Blot PVDF membrane (BioRad, Hercules, CA). First antibodies were from Cell Signaling (Danvers, MA): total 70S6K (#9202), phosphor-70S6K (Thr 389) (#9205), total AKT (#9272) and phospho-AKT (Ser 473) (#9271). Bands were visualized using a HRP-linked second antibody with ECL detection, and bands were quantitated using an Amersham ImageScanner and software.

3. Results

To detect small differences in the hippocampus related to learning and memory, we originally developed a genetically related control mouse for the SAMP8 through a series of crosses with the CD-1 mouse (Flood et al., 1995). The CD-1 mouse was chosen because it had similar learning and emotional characteristics to the SAMP8. It is also resistant to disease and the mice have large litters. We found that mice with a 50% SAM and 50% CD-1 genetic background exhibited no memory deficits compared to the SAMP8. For the present studies, 50% SAMP8 mice were re-derived as previously described (Flood et al., 1995). The hybrid pups were propagated by inbreeding. The behavioral characteristics were stable over subsequent generations (Fig. 1). Fig. 1 compares behavior in the SAMP8 to behavior in the 50% SAMP8 mice from generations 3–4 and generation 8. T-maze learning and memory and object recognition memory declined significantly in the SAMP8 mouse between 4 and 12 months in all experiments. However, these cognitive measures did not change with age in 50% SAMP8 mice but remained at the 4 month level. The results from generations 3–4 (Fig. 1 – top row) were essentially the same as the results from generation 8 (Fig. 1 – bottom row). This is evidence that our colony of 50% SAMP8 mice retain their behavioral characteristics through multiple breeding cycles. Therefore, for the rest of these studies, we used the 50% SAMP8 mice as Control mice for comparison to the SAMP8 mice themselves.

Hippocampal gene expression was measured by Affymetrix gene chips in Control and SAMP8 mice at 4 and 12 months of age (Fig. 2). The Control (50% SAMP8 mice) were from generations 3–4. The overall analysis of age and strain showed significant differences in 902 (536+172+194) hippocampal genes (Fig. 2A). For a list of all 902 genes, see the Supplemental Spreadsheet – Characterization of Differential Gene Expression. Of these,

there were differences in 194 genes at 4 months (87 increased and 107 decreased) and 536 genes at 12 months (386 increased and 150 decreased). This suggests a divergence in gene expression between 4 and 12 months of age, the same period during which learning and memory deficits arise. Pathway analysis using DAVID (see Methods) revealed changes in several pathways (Fig. 2B). Pathway analysis of all 902 genes showed significant changes in the Phosphatidylinositol Signaling System (10 genes), Endocytosis (15 genes), and Phosphatidylinositol Metabolism (7 genes) pathways. At 4 months, there were differences in the Small Cell Lung Cancer (6 genes), Focal Adhesion (7 genes), and ECM-Receptor Interactions (4) pathways. These pathways are not generally associated with learning and memory. However, at 12 months, there were significant differences in the Long Term Potentiation (LTP) (6 genes), Phosphatidylinositol Signaling (6 genes) and Endocytosis (10 genes) pathways (Fig. 2B). These pathways are inter-related and have all been linked to learning and memory.

3.1 Long Term Potentiation

DAVID flagged changes in the LTP pathway at 12 months but not at 4 months (Fig. 2B). LTP is closely linked to memory in the intact animal (Lynch et al., 2011). The expression of six genes was altered (Table 1 and Appendix Fig. 1). There were three genes from the MAPK signaling pathway – N-Ras (Nras), an inhibitory subunit of protein phosphatase 1 (Ppp1r1a), and Creb-binding protein (Crebbp). The MAPK pathway itself is involved in LTP (Bozon et al., 2003), and the Ras family is involved in memory formation (Ye and Carew, 2010). Expression of all three of these genes was decreased at 12 months in SAMP8 mice (Fig. 3A). Three genes from the Ca signaling component of the LTP pathway were altered at 12 months - the two ITP receptors 1 and 2 and phospholipase c, beta 1 (PLC-B1). The major ITP1 receptor was increased, while the minor ITP2 receptor was decreased (Fig. 3B). The ITP receptors are involved in Ca release and Ca signaling. PLC-B1, part of the complex that generates the key signaling lipids diacylglycerol and I(1,4,5)P3, was increased at 12 months in the SAMs.

3.2 Phosphatidylinositol Signaling

There were significant changes in Phosphatidylinositol Signaling at 12 months - 3 genes each in the PI3K pathway and phosphatidylinositol catabolism genes (Table 1 and Appendix Fig. 2). LTP is impacted by phosphatidylinositol signaling (van der Heide et al., 2006). Genes involved in the synthesis of signaling lipids (phosphatidylinositol synthase and PI3K(III)) were increased in the SAMP8s (Fig. 3C). Genes involved in the degradation of signaling lipids were decreased (ITP-5/6 kinase, IP-5-Patase) (Fig. 3D). Expression of the p85 beta subunit of PI3K was decreased at 4 months in SAMP8 mice. The p85 beta subunit regulates the activity of PI3K. Since this subunit may be inhibitory, the overall effect may increase PI3K activity.

PI3K is a key enzyme in downstream signaling through the AKT/mTOR pathway, which has been implicated in memory (van der Heide et al., 2006). Therefore, we looked for changes in downstream targets of this pathway by Western blotting (Fig. 4). AKT is the direct target of phosphatidylinositol signaling while 70S6K is the target of mTOR, which is a target of AKT. At 12 months, there was a decrease in the phosphorylation of AKT and 70S6K in the SAMs compared to the Control. There was no change in the phosphorylation of ERK and CREB, members of the MAPK signaling pathway (data not shown).

3.3 Endocytosis

The final major pathway showing significant differences was the Endocytosis pathway (Fig. 2B and Fig. 5). There are a number of endocytosis pathways (Doherty and McMahon, 2009). Of the 15 genes that showed significant changes, 13 were associated with the

Clathrin-Mediated Endocytosis (CME) pathway (Table 2). Endocytosis in general plays a role in the formation of hippocampal synapses (Granseth and Lagnado, 2008), and CME has been implicated in A β overproduction and AD (Wu and Yao, 2009). Changes were seen in all the major classes of proteins involved in the endocytosis process (Table 2). The light polypeptide chain of clathrin itself showed changes, as did dynamin 3. In particular, dynamin was markedly reduced at both 4 and 12 months. Dynamin proteins are found around the neck of invaginated CME vesicles and are involved in the fission of the endocytosis membranes to form isolated vesicles (Doherty and McMahon, 2009). Protein turnover via ubiquitination plays an important role in endocytosis, and there were changes in three ligases. Finally, three heat shock proteins (HSP) were increased at 12 months. HSPs play a role in vesicle uncoating (Eisenberg and Greene, 2007), and changes in HSPs have been implicated in neurodegenerative diseases (Chen and Brown, 2007). The changes in these key CME genes are shown in Fig. 5.

3.4 Genes Related to AD

Recently, a number of genes have been implicated in human genome-wide association studies of AD. Therefore we determined whether expression of these or closely related genes was significantly different between Control and SAMP8 mice in our study (Table 3). A recent study reported an association between the human genes PICALM, CLU, and EPHA1 and late-onset AD (Naj et al., 2011). These genes have been associated with AD in other studies. Expression of the corresponding mouse gene Picalm was increased at 4 months, and Clu, EphA7, and Ephb3 were increased at 12 months in SAMP8 mice. Three other genes in our study (Adrb2, Grm3, Tfr) have also been linked to AD. Polymorphisms in the beta 2-adrenergic receptor (Adrb2) have been associated with AD susceptibility (Yu et al., 2008). The mouse gene Grm3, a Group II metabotropic glutamate receptor (mGlu3), was increased at 12 months. A β peptides are thought to disrupt glutamate receptors (Parameshwaran et al., 2008), and a recent study suggested that the mGlu3 receptor is protective against neurotoxicity by A β (Caraci et al., 2011). Finally, the gene for transferrin receptor (Tfrc), an important component of iron metabolism, was decreased at 12 months. Iron overload is thought to contribute to AD and the gene for transferrin interacts with the gene for hemochromatosis to increase the risk for AD (Lehmann et al., 2012).

4. Discussion

These studies identify for the first time gene expression changes in specific hippocampal pathways in 12 month old SAMP8 mice that are associated with learning and memory. LTP is a rapid event that is thought to be the precursor to memory formation. Phosphatidylinositol signaling is involved in both short term and long term memory processes. Through its role in Ca signaling, it plays a role in LTP. Through PI3K signaling, it activates downstream pathways such as the AKT, mTOR, 70S6K pathways. These pathways are involved in memory consolidation. Endocytosis plays a role in memory formation and consolidation through its regulation of neurotransmitter receptor recycling (Granseth and Lagnado, 2008). There were no significant differences in these pathways at 4 months of age, suggesting that changes in these pathways may be related to the decrease in learning and memory seen between 4 and 12 months in the SAMP8 mouse.

There have been four previous studies of hippocampal gene expression in the SAMP8 mouse, all using the SAMR1 strain as a control, that have possible relevance to the present study. One study (Cheng et al., 2007) used subtracted cDNA libraries followed by microarray analysis, and it identified 91 differentially expressed genes in the hippocampus of 12 month old animals. The 53 genes of known function were categorized by the authors as mitochondrial, structural component and regulating function genes, but there was no overlap with the genes in the present study. Another study (Carter et al., 2005) used

Affymetrix gene arrays to study hippocampal gene expression in 3 and 16 month old SAMP8 mice, and they also found changes in genes with a wide range of functions. There was no overlap with the present study except that expression of the protein phosphatase 1-regulatory subunit 1A (Ppp1r1a) gene was markedly decreased in both studies. A third study (Alvarez-Lopez et al., 2013) looked at the effect of exercise in 10 month old SAMP8 and SAMR1 mice using Agilent microarrays to measure gene expression. The authors identified 34 genes which showed strain differences and were modulated by exercise (aging genes responsive to physical exercise), and these genes related mainly to extracellular matrix maintenance. None of these genes were differentially expressed in the present study, which is not surprising as they were screened for the effect of exercise as well as age. Finally, a recent study (Casadesus et al., 2012) compared expression of hippocampal cell cycle re-entry markers with age in SAMP8 and SAMR1 mice. They found increased protein levels of cell cycle markers but not mRNA levels in the SAMP8 mice. We also found no changes in the mRNA levels of cell cycle markers, supporting their conclusion that these changes are post-translational. The fact that there was little overlap between our studies and previous studies may reflect the use of the SAMR1 strain as a control, the use of different technologies, and in some cases the use of female rather than male mice.

4.1 Long Term Potentiation

There were major changes in the LTP pathway involving both MAPK and Ca signaling (Table 1). The most dramatic change in expression was a decrease in the inhibitory subunit of protein phosphatase 1, and, as noted above, this was confirmed in another study (Carter et al., 2005). As part of the LTP pathway, protein phosphatase 1 inactivates CaMKII by dephosphorylation. Therefore, a decrease in the inhibitory subunit could lead to increased phosphatase activity and decreased CAMKII and CREB activity. CAMKII plays a large role in LTP (Lucchesi et al., 2011), and changes in protein phosphatase 1 activity has been shown to modulate LTP via CAMKII (Blitzer et al., 1998). Another important regulator of CAMKII, Ca-calmodulin-dependent protein kinase II inhibitor 1 (Camk2n1), was markedly decreased in both 4 and 12 month old SAMP8 mice. CAMK II inhibitors may play a role in memory formation (Lucchesi et al., 2011). Two other important members of the MAPK pathway, Nras and CREB binding protein were also decreased in 12 month SAMP8 mice. Overall, these changes are consistent with decreased MAPK pathway activity.

With regard to the Ca-dependent pathway of LTP, there were major changes in the expression of PLC-B1 and ITP receptors 1 and 2 (Table 1). The beta-1 subunit of PLC, which generates the key signaling lipids diacylglycerol and I(1,4,5)P₃, was increased at 12 months in the SAMP8s. PLC-B1 has been localized in the hippocampus in association with Gq-coupled receptors such as mGluR5 and with diacylglycerol lipase (Fukaya et al., 2008). This emphasizes the role of PLC-B1 in PI3K mediated signaling. PLC-B1 knockout mice have impaired memory function (McOmish et al., 2008). Old rats with impaired memory have decreased expression of PLC-B1 and decreased phosphatidylinositol turnover (Nicolle et al., 1999).

With regard to the ITP receptors, expression of ITP receptor Type 1 was increased while Type 2 was decreased (Fig. 3B). Type 1 receptors are the predominant receptor subtype in the hippocampal neurons. Type 1 receptors are found mainly in the endoplasmic reticulum of neuronal cells while Type 2 receptors are found in the nuclear region of glial cells (Sharp et al., 1999). The ITP receptors have been linked to altered Ca homeostasis and neurotoxicity. Increased A expression, as seen in SAM mice, may directly act on cells to alter Ca release through ITP receptors (Ferreiro et al., 2004). Glutamate receptors (also increased in SAMs – Table 3) increase cytosolic Ca in part through Ca release from the endoplasmic reticulum (ER), the major site of the type 1 receptors (Ruiz et al., 2009).

Enhanced Ca release through the ITP receptor Type 1 is part of the hypothesis linking AD to altered cell Ca homeostasis and cell death (Camandola and Mattson, 2011).

4.2 Phosphatidylinositol Signaling

Phosphatidylinositol metabolism affects Ca signaling through the signaling lipid I(1,4,5)P₃ and PI3K signaling through PI(3,4,5)P₃. The gene changes seen in the phosphatidylinositol metabolism pathway would seem to favor increased signaling in the SAMP8 hippocampus (Table 1). The increase in phosphatidylinositol synthase and PI3K(III) would be expected to increase lipid synthesis while the decrease in the 5/6 kinase and the IP-5 and IP₄ phosphatases would be expected to inhibit the degradation of the signaling lipids. With regard to the PI3K signaling, there is a decrease in the p85 beta subunit of PI3K and a decrease in Pten in the SAMP8 mice. The class I PI3Ks consist of a p110 catalytic subunit and two regulatory subunits – p85 alpha and beta. In these studies expression of p85 beta was significantly reduced in 4 month old SAMs (Fig. 3C). Regulation of PI3K by the p85 subunits is complex. However, free p85 can inhibit PI3K signaling (Ueki et al., 2003). Thus, a partial reduction in p85 beta could result in an increase in PI3K signaling (Engelman et al., 2006).

Altered PI3K-dependent signaling would have multiple downstream effects, including activation of the AKT-mTOR pathway by PI3K (Wullschleger et al., 2006; Engelman, et al., 2006). Interestingly, we found that phosphorylation of AKT and 70S6K was decreased in the SAMP8 mice (Fig. 4) despite evidence for increased phosphatidylinositol signaling. One possibility is that intracellular A β is interfering with the AKT signaling pathway distal to phosphatidylinositol signaling (Lee et al, 2009). PI3K produces the signaling lipid PI(3,4,5)P₃, which brings together the phosphoinositide-dependent kinase (PKD) and AKT, resulting in the phosphorylation of AKT. Recent studies have provided evidence that soluble A β prevents the phosphorylation of AKT by blocking the association between AKT and PKD (Lee et al, 2009). It may be that phosphatidylinositol signaling is increased in the SAMP8 mice to compensate for the downstream inhibition of the AKT pathway by A β . Recent studies have implicated PI3K (Tohda et al., 2009; Mizuno et al., 2003) and mTOR (Jaworski and Sheng, 2006; Sui et al., 2010) in learning and memory.

4.3 Endocytosis

Since dynamin and Arf6 are markedly decreased at 12 months, it may be that endocytosis is decreased. Arf and its phosphorylation partner ArfGAP are important regulators of both CME and a separate pathway, Arf6-dependent endocytosis (Doherty and McMahon, 2009). Arf6 was decreased at 12 months and ArfGAP2 increased at 4 and 12 months (Fig. 5D). The ArfGAPs function to regulate vesicle formation by dephosphorylating ARF-GTP to form ARF-GDP (Spang et al., 2010). Arf6 may play a role in neuronal development and synaptic plasticity (Jaworski, 2007).

Adaptor proteins and accessory proteins interact with clathrin to recognize and organize the “cargos” that will be transported by the endocytosis vesicles (Doherty and McMahon, 2009). The adapter protein disabled homolog 2 (dab2) was decreased at 12 months and has been shown to be involved in the recycling of many membrane receptors, such as the receptor for transforming growth factor-beta (Penheiter et al, 2010). The protein casitas B-lineage lymphoma (cbl) was increased at 12 months. Cbl proteins function as both ubiquitin ligases and as multifunctional adapter proteins (Schmidt and Dikie, 2005). They play a major role in regulating receptor tyrosine kinase signaling via endocytosis recycling.

Finally, heat shock proteins are involved in vesicle uncoating, and several of these were increased in this study (Fig. 5C). The heat shock proteins auxilin (Dnajc6) and hsc70

(Hsp8a) play a major role (Eisenberg and Greene, 2007), and these were both increased at 12 months. The major 70kDa heat shock protein Hsp1a was also increased.

4.4 Genes Related to AD

The fact that there is a correspondence between genes whose expression is different in the SAMP8 mouse and genes implicated in the incidence of AD (Table 3) provides further support for the importance of these genes in aging and memory loss. First, the genome-wide association studies measure only variations in gene sequences and not gene expression. Second, in many of genes identified in these association studies, the associated variations take place near the gene but not within the gene. Thus, it is inferred, but not proven, that the nearest gene is the one affected by the variation. These studies of the SAMP8 mouse demonstrate changes in corresponding mouse genes that are correlated with late onset AD. This suggests that the SAMP8 may be a useful model for late onset memory loss and AD and its treatment.

The changes seen in both mouse and human Picalm and EphA7 and EphB3 expression may have special relevance to the pathways identified in this present study. Picalm (phosphatidylinositol binding – clathrin assembly protein) binds specific phosphatidylinositols and plays a role in CME (Miller et al., 2011). These dual functions make it a possible link between the Phosphatidylinositol Signaling and Endocytosis pathways identified in these studies. The mouse genes EphA7 and EphB3 are part of the Eph/ephrin cell-to-cell signaling system that regulates synapse formation and neuronal plasticity in the adult (Klein, 2009). In terms of the results of this study, the Eph/ephrin system is capable of stimulating the PI3K, and the system may be regulated by endocytosis (Pitulescu and Adams, 2010).

In summary, these studies demonstrate differences in the LTP, Phosphatidylinositol Signaling, and Endocytosis pathways in the hippocampus of 12 month old SAMP8 mice. These pathways are interrelated (Fig. 6), and differences could contribute to the cognitive differences seen in SAMP8 mice of this age (Fig. 1). The changes in phosphatidylinositol signaling were the most significant in the overall analysis of age and strain differences (10 genes, $P = 0.0022$) (Fig. 2B). Changes in phosphatidylinositol signaling can produce changes in Ca signaling. These, along with changes in MAPK signaling, can regulate LTP and ultimately memory formation (Fig. 6). Changes in phosphatidylinositol signaling could also result in downstream changes in AKT/mTOR/70S6K signaling via PI3K. We observed changes in the phosphorylation of AKT and 70S6K in the present study (Fig. 4). Changes in the AKT/mTOR/70S6K could lead to alterations in the protein synthesis and RNA processing involved in memory consolidation. Finally, phosphatidylinositol signaling can regulate CME. The phosphatidylinositol signaling pathway and the CME pathway are known to interact with each other in multiple ways (Hauke, 2005; Claugue et al., 2009). For example, phosphatidylinositol-(4,5)-bisphosphate is a major regulator of clathrin-coated pit formation (Antonescu et al., 2011). As a result, changes in CME can lead to changes in receptor recycling, such as the AMPA receptors involved in LTP. In addition, endocytosis is involved in APP processing itself (Thinakaran and Koo, 2008), and changes could potentially contribute to the high levels of A β seen in the SAMP8 mouse itself (Erickson et al., 2012). Specific genes in these affected pathways may provide new therapeutic targets for modulating age-related cognitive decline.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Cognitive Deficits in SAMP8 vs. 50% SAMP8 Mice

T-maze acquisition (learning) and T-maze retention (memory) and object recognition were measured as previously described (see Farr et al., 2012 and Methods). The top row of graphs is from generations 3–4 and the bottom row is from generation 8 of the 50% SAMP8 mice. Bars are the mean $\pm$ SD of 8–10 animals from two separate experiments. Asterisks indicate significantly different from 4 months in post-hoc testing ($P < 0.05$). There was a significant strain $\times$ age effect in all measures.

Figure 2. Pathway Analysis of Age and Strain Differences

(A) Differential gene expression. There was differential expression of 194 genes in 4 month mice (87 up,107 down), 536 genes in 12 month mice (386 up,150 down), and 172 genes between 4 and 12 month old mice (81 up,91 down). (B) Pathway analysis of the genes in (A) using DAVID.

A. MAPK – Dependent Signaling

B. Ca – Dependent Signaling

C. PI3K – Dependent Signaling

D. Phosphatidylinositol Catabolism

Figure 3. Relative Levels of Gene Expression in Signaling Pathways

From the array data, relative expression levels (Z-scores) were calculated for genes that showed significant changes in expression. Z-scores were calculated as previously described (Haren et al., 2011) and are a measure of relative gene expression. A positive Z-score indicates increased expression and a negative Z-score indicates decreased expression relative to the average gene expression in the hippocampus (zero line). Bars are the average of 4 independent biological replicates.

Figure 4. Phosphorylation of AKT and 70S6K in 12 Month Old Mice

Total protein and phosphorylated protein were measured using specific antibodies as previously described (Haren et al., 2011). Bars are the mean and standard error of 8 mice. Asterisk indicates that Control (50% SAMP8) mice are significantly different from SAMP8 mice ($P < 0.05$). Western blots of hippocampal samples from four individual animals are shown below each graph.

A. Coated Pit and Adapter Proteins

B. Protein Ligases

C. Heat Shock Proteins/Vesicle Uncoating

D. Stimulators

Figure 5. Relative Levels of Gene Expression in Clathrin-Mediated Endocytosis Pathway
From the array data, Z-scores (see Fig. 3) were calculated for specific categories of genes in the clathrin-mediated endocytosis pathway. Bars are the average of 4 independent biological replicates.

Figure 6. Inter-relationship of Hippocampal Signaling Pathways Altered in SAMP8 Mice
 Observed changes in phosphatidylinositol signaling and related Ca signaling, along with the key changes observed in MAPK signaling, may affect initial long term potentiation and ultimately memory formation. Altered phosphatidylinositol signaling through PI3K and AKT/mTOR/70S6K may alter the protein synthesis and RNA processing necessary for memory consolidation. Finally, phosphatidylinositol signaling is a regulator of clathrin-related endocytosis, which is important in the receptor recycling necessary in memory formation and in APP processing itself. Dotted lines indicate proposed relationships.

Table 1Signaling Pathways Identified by Pathway Analysis – Changes in Individual Gene Expression[†]

Gene Name	Gene Symbol	Change (relative to Control)	P Value
<u>MAPK-Dependent Signaling</u>			
N-ras *	Nras	Down at 12 mo	0.0097
PPase 1-Inhibitor 1a *	Ppp1r1a	Down at 4,12 mo	0.0529, 0.0081
Creb binding protein *	Crebbp	Down at 12 mo	0.0109
<u>Ca-Dependent Signaling</u>			
ITP receptor1 *	Itpr1	Up at 12 mo	0.0030
ITP receptor2 *	Itpr2	Down at 12 mo	0.0078
Phospholipase c, beta1 *	Plcb1	Up at 12 mo	0.0069
<u>PI3K-Dependent Signaling</u>			
PI synthase	Cdipt	Up at 12 mo	0.0055
PI3K (III)	Pik3c3	Up at 12 mo	0.0103
PI3K-subunit 2 (p85 beta)	Pik3r2	Down at 4 mo	0.0060
<u>Phosphatidylinositol Catabolism</u>			
ITP 5/6 kinase	Itpk1	Down at 12 mo	0.0092
IP-5-phosphatase B	Inpp5b	Down at 4,12 mo	0.0083, 0.0014
IP-4-phosphatase type 1	Inpp4a	Down at 4,12 mo	0.0001, 0.0001

[†]The locations of these genes in their respective pathways are shown in Supplemental Appendix Figure 1 (LTP) and Figure 2 (Phosphatidylinositol Signaling). The P values were generated by DAVID.

* Genes in the Long Term Potentiation Pathway

Table 2**Clathrin-Mediated Endocytosis – Changes in Individual Gene Expression**

Gene Name	Gene Symbol	Change (relative to Control)	P Value [†]
<u>Coated Pit and Adapter Proteins</u>			
Clathrin, light polypeptide	Cltb	Up at 4 mo	0.005
Dynamin 3	Dnm3	Down at 4,12 mo	0.0001, 0.0001
Disabled homolog 2	Dab2	Down 12 mo	0.001
<u>Protein Ligases</u>			
Casitas lymphoma	Cbl	Up at 12 mo	0.005
Ring finger protein 41*	Rnf41	Down at 4,12 mo	0.008, 0.044
EH –domain containing 1*	Ehd1	Up at 12 mo	0.002
<u>Vesicle Uncoating/Heat Shock Proteins</u>			
HSP70 cognate 70	Hspa8	Up at 12 mo	0.002
DnaJ(Hsp40) homolog	Dnajc6	Up at 4,12 mo	0.044, 0.005
HSP 70kD1A	Hspa1a	Up at 12 mo	0.001
<u>Stimulators</u>			
ADP-ribosylation factor 6	Arf6	Down at 12 mo	0.005
ArfGAP2	Acap2	Up at 4,12 mo	0.001, 0.001

[†]The P values were generated by DAVID.

Table 3Changes in Genes Implicated in AD[†]

<u>Mouse Gene</u>	<u>Possible Importance</u>	<u>Change</u>	<u>Reference</u>
Pi-binding/clathrin assembly(Picalm)	AD candidate(PICALM)	Up at 4 mo	Naj AC, 2011
Clusterin(Clu)	AD candidate(CLU)	Up at 12 mo	Naj AC, 2011
Eph receptors(Epha7 and Ephb3)	AD candidate(EPHA1)	Up at 12 mo	Naj AC, 2011
Adrenergic receptor beta 2(Adrb2)	AD candidate(ADRB2)	Up at 4 mo	Yu JT, 2008
Glutamate receptor-mGluR3(Grm3)	Glutamate signaling in AD	Up at 12 mo	Parameshwaran K, 2008
Transferrin receptor(Tfrc)	Iron metabolism in AD	Dn at 12 mo	Lehmann DJ 2012

[†]SAMP8 mouse genes (small letters) related to human genes (capital letters) implicated in AD.