Diabetes mellitus (DM) is considered to be a risk factor for dementia including Alzheimer’s disease (AD). However, the molecular mechanism underlying this risk is not well understood. We examined gene expression profiles in postmortem human brains donated for the Hisayama study. Three-way analysis of variance of microarray data from frontal cortex, temporal cortex, and hippocampus was performed with the presence/absence of AD and vascular dementia, and sex, as factors. Comparative analyses of expression changes in the brains of AD patients and a mouse model of AD were also performed. Relevant changes in gene expression identified by microarray analysis were validated by quantitative real-time reverse-transcription polymerase chain reaction and western blotting. The hippocampi of AD brains showed the most significant alteration in gene expression profile. Genes involved in noninsulin-dependent DM and obesity were significantly altered in both AD brains and the AD mouse model, as were genes related to psychiatric disorders and AD. The alterations in the expression profiles of DM-related genes in AD brains were independent of peripheral DM-related abnormalities. These results indicate that altered expression of genes related to DM in AD brains is a result of AD pathology, which may thereby be exacerbated by peripheral insulin resistance or DM.

Keywords: animal model, hippocampus, insulin, microarray, postmortem brains

Introduction

More than 20 million people worldwide suffer from dementia, and this number is expected to exceed 80 million by 2040 because of the rapid increase in the numbers of elderly (Ferri et al. 2005). The prevalences of all-cause dementia and Alzheimer’s disease (AD) in the general population of Japanese elderly have increased significantly over the past 20 years, especially among subjects aged ≥75 years (Sekita et al. 2010). Thus, it is important to establish effective prevention strategies for dementia, and particularly for AD. To reach this goal, it is essential to understand the risk factors for developing dementia, including AD, in the elderly population.

Several recent studies have indicated effects of insulin and glucose metabolism on the risk of developing dementia, especially AD (Kuusisto et al. 1997; de la Monte and Wands 2008; Schrijvers et al. 2010). The results of the Hisayama study suggested that hyperinsulinemia and hyperglycemia caused by insulin resistance accelerate the formation of neuritic plaques (NPs) in combination with the effect of the APOE ε4 allele, a major risk factor for AD (Matsuzaki et al. 2010).

To identify molecular pathological alterations in AD brains, we performed interspecies comparative microarray analyses using RNA prepared from postmortem human brain tissues donated for the Hisayama study (Katsuki 1966; Matsuzaki et al. 2010; Sekita et al. 2010), and hippocampal RNAs from the triple-transgenic mouse model of AD (3xTg-AD) (Oddo et al. 2003). We found altered expression profiles of diabetes mellitus (DM)-related genes in AD brains, which were independent of peripheral DM-related abnormalities.

Materials and Methods

Postmortem Brain Tissues

We examined 88 autopsy samples from Hisayama residents obtained between 15 December 2008 and 24 February 2011. Clinical data related to DM or prediabetes were collected as described (Ohara et al. 2011). The study was approved by the Ethics Committee of the Faculty of Medicine, Kyushu University. Written informed consent for all subjects was obtained from their families. Neuropathologic changes were examined as described previously (Matsuzaki et al. 2010). Sections were routinely stained using hematoxylin–eosin, Klüver-Barrera stain, and a modified Bielschowsky method. Specimens from each subject were immunostained using antibodies against phosphorylated microtubule-associated protein tau (MAPT) (AT8, mouse monoclonal, 1:500; Innogenetics, Belgium) and the assessment of AD pathology was conducted according to the Consortium to Establish a Registry for Alzheimer’s Disease (CERAD) guidelines (Mirra et al. 1991) and the Braak stage (Braak and Braak 1991). During autopsy dissection, parts of the frontal cortex, temporal cortex, and hippocampus were cut out from each brain and preserved at −80 °C until RNA preparation.

Animals

3xTg-AD-H mice harboring a homozygous Psen1M146V mutation and homoygous mutant transgenes for APPsw and MAPT9NL transgenes, and nontransgenic control mice (non-Tg) (Oddo et al. 2003) were used in this study. At age 14 months, brains were removed (N=3 male mice of each type) under pentobarbital anesthesia (i.p.), with perfusion of 40 mL of saline via the left ventricle. Hippocampi were isolated and preserved at −80 °C until RNA preparation. The handling and killing of all animals was performed in accordance with the national prescribed guidelines, and ethical approval for the study was granted by the Animal Experiment Committee of Kyushu University.

Gene Expression Profiling with Microarray Analyses

Total RNA was isolated using a combination of Isogen (Nippon Gene, Tokyo, Japan) and the RNeasy Mini Kit (Qiagen, Tokyo, Japan), and analyzed using the Affymetrix Human Genome U133 Plus 2.0 Array (Affymetrix, Santa Clara, CA). The microarray data have been deposited in the Gene Expression Omnibus (GEO) database of the National Center for Biotechnology Information (NCBI) under the accession number GSE69228.
Reverse-Transcription and Quantitative Polymerase Chain Reaction

To validate the microarray data, we performed quantitative real-time reverse-transcription polymerase chain reaction (RT-PCR) on individual genes. We selected 17 genes of interest as potential targets for real-time RT-PCR: the corresponding gene-specific primer pairs are listed in Supplementary Table S1. Three genes, RX1851, ACTB, and GAPDH, were used as internal controls. Each sample was reverse-transcribed to first-strand cDNA using 1 μg of total RNA, random primers and the High-Capacity cDNA Reverse-Transcription Kit (Life Technologies Japan). For each quantitative PCR reaction, 0.5% of the total complementary DNA yield was used. Transcript quantifications were carried out on a Thermal Cycler Dice® Real-Time System Single (Takara, Kyoto, Japan). Each reaction was performed using the appropriate amount of complementary DNA, optimized amounts of forward and reverse primers, and 12.5 mL of 2 × SYBR Green Ready Reaction Mix with Rox (Life Technologies Japan) in a total volume of 25 mL. Dissociation curves were generated for all wells. No primer dimers were observed.

Tissue Processing and Immunofluorescence Microscopy

Animals deeply anesthetized with pentobarbital were perfused intracardially with saline followed by cold 4% paraformaldehyde (PFA) in PBS. The brains were removed, immersed for 12 h in the same 4% PFA fixative, then in 20% followed by 30% sucrose in PBS for 24 h at 4 °C. The brains were stored as paraaffin-embedded blocks. Tissue sections (4-μm thick) were cut from the blocks on a microtome and mounted from warm water (42 °C) onto slides. Sections were allowed to dry overnight at room temperature, deparaffinized in xylene, and rehydrated through a graded ethanol series. Antigen retrieval was performed by boiling sections in plastic Coplin jars containing sodium citrate buffer (10 mM citric acid, 0.05% Tween 20, pH 6.0) using a water bath (100 °C) for 10 min followed by cooling for 30 min to room temperature. Sections were blocked with a solution containing 1 × Block Ace (Dainippon Pharmaceutical, Osaka, Japan) for 30 min at room temperature, incubated with anti-PCSK1 (sc-100579), anti-GAPDH (sc-2768), anti-Peripherin (sc-1722), anti-Syntaxin-1A (sc-14717), and antineuron-specific nuclear protein (NeuN) (AB978, 1:1000, Merck, Tokyo, Japan) antibodies in 10% Block Ace at 4 °C overnight, and then incubated with an Alexa Fluor-labeled second antibody (Invitrogen Japan, Tokyo, Japan) for 45 min at room temperature. Confocal images were acquired using an LSM510 META Confocal Microscope System (Carl Zeiss Microimaging, Tokyo, Japan).

Western Blot Analysis

Frozen hippocampus samples were homogenized in buffer containing 150 mM NaCl, 1.0% NP-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 50 mM Tris-HCl, pH 8.0 with 2% protease inhibitor, and a 5% phosphatase inhibitor cocktail (Nacalai Tesque, Kyoto, Japan) using a tissue homogenizer at 1500 rpm for 30 s. The homogenates were mixed with 2 × SDS sample buffer and subjected to 10% SDS polyacrylamide gel electrophoresis followed by western blotting using anti-PCSK1 (sc-100579), anti-PCSK2 (MAB0018, R&D Systems, Minneapolis, MN, USA), and anti-GAPDH (14C10, Cell Signaling Technology, Beverly, MA, USA) antibodies with appropriate fluorophore-conjugated secondary antibodies (LI-COR, Lincoln, NE, USA). Quantitative detection of fluorescent bands was performed using the Odyssey infrared imaging system (LI-COR). The blots were stained with Ponceau S (Sigma-Aldrich Japan, Tokyo, Japan), and digital images generated by a document scanner were used to quantify total proteins on the blots in Image Gauge software (Fujifilm, Tokyo, Japan).

Statistical Analysis

Gene-level estimates from human microarray data were subjected to 3-way analysis of variance (ANOVA); then, the results of a specific comparison (AD vs. non-AD) were obtained using false discovery rate (FDR, q < 0.05) controlling procedures (Benjamini and Hochberg 1995). Gene-level estimates from mouse microarray data were subjected to ANOVA, and the obtained list of transcript clusters with P < 0.05 was subjected to a specific comparison (3x-TG-AD-H vs. non-Tg) with FDR (q < 0.05) control and a fold-change > 1.3 as a threshold for the comparison. Statistical analysis of western blot data was performed by unpaired t-test using JMP 8.0 software (SAS Institute, Raleigh, NC, USA). A P-value < 0.05 was considered statistically significant.

Results

Clinical and Pathological Features of Subjects

High-quality RNA samples with an RIN ≥6.9 from the gray matter of frontal and temporal cortices and hippocampi of 88 postmortem brains were subjected to microarray analysis. Among these, 26 subjects were pathologically diagnosed as having AD or an AD-like disorder. Only those results that

Table 1

| Comparison | Mean F ratios in 3-way ANOVAs of the microarray data | Mean F ratios |
|------------|--------------------------------------------------|--------------|
|            | frontal cortex A, B, C, Temporal cortex A, B, C, Hippocampus A, B, C |              |
| AD vs. non-AD | 1.77, 3.97, 5.28, 2.15, 5.85, 10.24, 3.50, 22.14, 23.49 |              |
| VD vs. non-VD | 0.83, 0.64, 0.66, 1.06, 1.01, 0.98, 1.37, 2.37, 2.49 |              |
| Sex         | 2.30, 1.91, 2.91, 2.57, 1.33, 1.27, 1.27, 2.73, 3.33, 2.71 |              |

Note: A: mean F ratios for all transcript clusters (33 297) are shown. B: mean F ratios for transcript clusters (1387) exhibiting significantly altered expression between AD and non-AD hippocampal RNAs. C: mean F ratios for the top 200 transcription clusters in B. Statistical power analysis revealed that the smallest sample size that could be set for the 3-way ANOVAs was 5 with a significance (P-value) level of 0.01 and power of 0.8.
Figure 1. Hierarchical and partitioning clustering of the 1387 transcript clusters in the 3 regions of brain. (A) Cluster heat map of the 1387 transcript clusters based on expression data in the hippocampus. (B) Cluster heat map of the 1387 transcript clusters based on expression data in the temporal cortex. (C) Cluster heat map of the 1387 transcript clusters based on expression data in the frontal cortex. AD (red), non-AD (nAD, dark blue); vascular dementia (VD, dark green), non-VD (nVD, purple); diabetes mellitus (DM, black), prediabetes (pre-DM, gray), non-DM (light green); female (F, orange), male (M, light blue). In the heat map, blue represents a lower expression level and red indicates a higher expression level.

passed examinations for quality assurance and quality control of the GeneChip Human Gene 1.0 ST arrays were retrieved. In total, we obtained gene expression profiles from the following:

- 33 frontal cortex samples, among which 15 were from AD patients;
- 29 temporal cortex samples, among which 10 were from AD patients;
- 17 hippocampus samples, among which...
which 7 were from AD patients (see Supplementary Tables S2 and S3). NPs, assessed according to the CERAD guidelines (Mirra et al. 1991), were frequent (score of 3) in all 16 AD brains that provided RNA samples of suitable quality. The distribution patterns of neurofibrillary tangles (NFTs), assessed according to Braak stage (Braak and Braak 1991), were all stage V–VI. Two subjects given a pathological diagnosis of AD had been clinically diagnosed as having vascular dementia (VD), while another 3 subjects with a pathological diagnosis of AD had been clinically diagnosed with DM or prediabetes (defined as a blood glucose level of 140–199 mg/dL 2 h after a 75-g oral glucose tolerance test, or a blood glucose level of 110–125 mg/dL in the fasting condition) (see Supplementary Tables S2 and S3).

**Altered Gene Expression Profiles in the Hippocampus, Temporal Cortex, and Frontal Cortex with AD Pathology**

Three-way ANOVA of the microarray data with AD versus non-AD, VD versus non-VD, and female versus male as factors revealed that the comparison of AD versus non-AD exhibited the highest mean F ratio (3.50) based on expression data for all 33 297 transcript clusters obtained from hippocampal RNAs (Table 1). In total, 348 transcript clusters in the temporal cortex (98 up and 250 down) and 1387 transcript clusters in the hippocampus (569 up and 818 down), but none in the frontal cortex, showed significantly altered expression levels in AD versus non-AD brains (see Supplementary Tables S4 and S5). Of the 348 transcript clusters in the temporal cortex, 125 were also among the 1387 transcript clusters in the hippocampus. The mean F ratios for the 1387 transcript clusters identified in the hippocampus (Table 1) confirmed that the gene expression profile in the hippocampus is the most significantly altered in AD brain. No genes in any cluster showed a significant difference in expression levels between patients with DM or prediabetes (data not shown).

Hierarchical and partitioning clustering of the 1387 hippocampal transcript clusters (Fig. 1A) based on data from hippocampal samples revealed clustering of the 7 AD cases separately from the 10 non-AD cases, with statistical significance. Using data from temporal cortex samples, 9 of 10 AD cases were clustered together (Fig. 1B). Using data from frontal cortex samples, 6 and 8 AD cases were separately clustered out of 15 AD cases, and 8 and 9 non-AD cases were separately clustered out of 18 non-AD cases (Fig. 1C). Thus, the expression profiles of the 1387 transcript clusters identified as being altered in the hippocampus are similarly changed in the temporal and frontal cortices, but to lesser extents.

**Genes Whose Expression Levels are Significantly Altered in AD Hippocampus**

To retrieve genes whose expression levels were significantly altered in AD brains in comparison with non-AD brains, it is essential to consider the changes in the population of brain cells in AD brains. Therefore, we compared the expression levels of genes encoding specific markers for 4 major types of brain cells, namely, neurons, astrocytes, oligodendrocytes, and microglia (Table 2). The expression levels of 10 neuronal markers, including *RBFOX3* encoding NeuN (Dredge and Jensen 2011), which is expressed in about 68% of cells in the gray matter of the adult cerebral cortex (Azevedo et al. 2009), were consistently decreased in AD brains relative to the levels in non-AD brains, most significantly in the hippocampus. Conversely, the expression levels of *GFAP, S100B*, and *AQP4* transcripts, representing the astrocyte population, and to a lesser extent those for *AIF1, LGALS3, CD68*, and *EMR1* representing the microglial population, were increased, especially in the temporal cortex and hippocampus. The expression levels of *MBP, SOX10, MOG*, and *MAG*, representing the oligodendrocyte population, were largely unchanged. These data are likely to reflect neuronal loss and gliosis in AD brains; neuronal loss is most evident in the hippocampus, and gliosis is most evident in the temporal cortex and hippocampus.

Taking the mean relative expression levels of these markers in different brain regions (Table 2) into account, we selected the top 200 transcript clusters that exhibited a fold-change >1.563 from among the 1387 transcript clusters identified as being altered in the hippocampus (see Supplementary Table S6). In AD brains, 143 of the 200 transcript clusters were markedly downregulated in the hippocampus beyond the level expected based on the cell population change. Because of the population change in AD brains, the number of upregulated genes in AD brains was likely to have been underestimated (57 transcript clusters in see Supplementary Table S6).

We next individually analyzed the raw expression levels of 12 genes showing significant alterations (downregulated: *MET, PCSK1, RGS4, H3S3T2, NPTX2, NEUROD6, RAB27B, HCN1, HOMER1; upregulated: GJA1, AEBP1, GALNTL2*) in 3 brain regions from each subject (see Supplementary Fig. 1, left panels), confirming that AD hippocampi exhibited the most significant alterations of gene expression (Fig. 2A). The expression levels of individual exons within the 12 genes were also most significantly altered in the hippocampi of AD patients (see Supplementary Tables S2 and S3).

**Table 2**

| Cell type | Relative expression (% non-AD) |
|-----------|-------------------------------|
| Marker gene | Frontal cortex | Temporal cortex | Hippocampus |
| Astrocytes | GFAP | 126.95 | 162.93 | 136.25 |
| | S100B | 101.32 | 128.95 | 125.96 |
| | AQP4 | 107.40 | 132.57 | 146.39 |
| | Mean | 111.89 | 141.98 | 136.20 |
| | SD | 13.39 | 18.66 | 10.22 |
| Oligodendrocytes | MBP | 101.57 | 102.15 | 96.83 |
| | SOX10 | 96.41 | 100.62 | 103.07 |
| | MOG | 104.55 | 130.64 | 116.20 |
| | MAG | 108.60 | 116.36 | 102.50 |
| | Mean | 103.28 | 112.44 | 104.65 |
| | SD | 4.34 | 14.05 | 8.20 |
| Microglia | CD68 | 103.86 | 134.19 | 110.55 |
| | AIF1 | 96.81 | 114.67 | 107.54 |
| | LGALS3 | 103.20 | 114.04 | 112.30 |
| | EMR1 | 101.13 | 115.21 | 102.74 |
| | Mean | 101.20 | 119.53 | 108.28 |
| | SD | 3.12 | 5.78 | 4.19 |
| Neurons | RBFOX3 | 84.26 | 79.43 | 63.82 |
| | ENO2 | 98.75 | 94.25 | 78.10 |
| | CHGA | 94.11 | 89.88 | 53.31 |
| | Tubb | 96.40 | 96.03 | 86.51 |
| | SYN | 92.00 | 86.73 | 65.49 |
| | NEFH | 90.60 | 96.27 | 54.13 |
| | NF2M | 97.17 | 90.80 | 64.43 |
| | NEFL | 89.76 | 83.80 | 58.93 |
| | SNAP25 | 86.35 | 79.55 | 60.83 |
| | SYT1 | 87.70 | 82.85 | 63.15 |
| | Mean | 91.71 | 87.96 | 64.87 |
| | SD | 4.86 | 6.45 | 10.27 |
Brains, being altered to a lesser extent in the temporal cortex and much less so in the frontal cortex, in accordance with the pathological severity (see Supplementary Fig. 1, right panels).

None of the 12 genes examined exhibited a significant alteration between non-DM and prediabetes/DM cases (Fig. 2B). The genes that were downregulated in AD hippocampus
exhibited slightly increased expression in subjects with prediabetes or DM, but this increase was not statistically significant. Among the 12 genes with altered expression, a few genes (MET and GJA1 for VD; RAB27B, HOMER1 and GALNTL2 for sex) exhibited moderate but statistically significant alterations between non-VD and VD or between sexes (Fig. 2C,D).

Among the top 200 transcription clusters, 147 genes were Functions/Pathways eligible genes in the computational gene network prediction tool IPA. These were categorized as genes significantly relevant to genetic disorders [105], neurological diseases [85], gastrointestinal diseases [74], and others. Genes categorized into genetic disorders were subcategorized as genes significantly relevant to schizophrenia [29], bipolar disorder [26], coronary artery disease [25], Crohn’s disease [23], noninsulin-dependent DM [23], amyotrophic lateral sclerosis [22], Huntington’s disease [22], AD [21], Parkinson’s disease [14], obesity [12], and others (Table 3).

Among the top 200 transcription clusters, 145 genes were eligible for generating IPA networks. The most relevant network included downregulated genes such as MET, PCSK1, PTPN3, SERPINF1, and VEGFA, and upregulated genes such as AEBP1 and TXNIP (Fig. 3A; Network 1). The second-most relevant network consisted of the genes encoding GABA receptors (GABRA1, GABRA4, GABRA5, GABRG2), synaptotagmin members, syntaxin, potassium channels, and regulators of G protein signaling. Expression of all of these genes was markedly decreased in the AD hippocampus (Fig. 3B; Network 2), reflecting the neuronal dysfunction in AD brain. The third-most relevant network consisted of genes regulated by insulin signaling pathways, as discussed below (Fig. 3C; Network 3). The alterations in the expression levels of the genes constituting these 3 networks were well preserved in the temporal cortex and to a lesser extent in the frontal cortex of AD brains (see Supplementary Table S6).

**Altered Gene Expression Profiles in Mouse AD Hippocampus**

We next performed microarray analysis of hippocampal RNA prepared from 14-month-old 3xTg-AD hemizygous (3x-Tg-AD-h; =3) and homozygous (3xTg-AD-H; N=3) male mice for APPswe and MAPTp301, transgenes with a homozygous PstI-M146V mutation and non-Tg mice (N=3). The transgenic mice exhibited severe learning and memory deficits with progressive development of amyloid plaques and NFTs as previously described (Oddo et al. 2003). We compared the expression levels of genes encoding specific markers for the 4 major types of brain cells, and found no differences among the 3 groups (Table 4), supporting a previous observation that there is no obvious neuronal loss in 3xTg-AD mice (Oddo et al. 2003). Then, 2713 transcript clusters showing a significant difference among the 3 groups (ANOVA, P<0.05) were further compared between samples from non-Tg mice and each line of 3xTg-AD mice with FDR control (q<0.05). As a result, 406 clusters from 3xTg-AD-H samples and 243 clusters from 3xTg-AD-h samples were found to have a fold-change >1.3 compared with non-Tg samples (see Supplementary Table S7). Ninety-three transcript clusters were shared between these groups. Hierarchical clustering of the 406 transcript clusters identified as having changed in 3xTg-AD-H samples was performed among the 3 groups, revealing that the expression profiles in 3xTg-AD-H samples were significantly different from those in non-Tg samples, and that the differences were partly shared by 3xTg-AD-h samples (Fig. 4A).

Among the 406 mouse transcription clusters, 109 genes were Functions/Pathways eligible genes in IPA. These were categorized as genes significantly relevant to genetic disorders [62], neurological disease [43], gastrointestinal disorders [35], and others. Genes categorized into genetic disorders were subcategorized as genes significantly relevant to bipolar disorder [20], noninsulin-dependent DM [17], coronary artery disease [16], AD [13], Parkinson’s disease [9], obesity [7], and others (Table 5). These categories and subcategories were essentially the same as the those detected as relevant in the AD hippocampus. Among the 406 transcription clusters, only 120 genes were eligible for generating IPA networks, and the most relevant network included 11 genes that were downregulated and 5 that were upregulated in the hippocampi of 3xTg-AD-H mice (Fig. 4B). The raw expression level of Pcsle1 was most significantly decreased in the hippocampi of 3xTg-AD-H mice and to a lesser extent in those of 3xTg-AD-h mice in comparison with non-Tg mice, while that of Ide was significantly increased in the hippocampi of both 3xTg-AD-H and 3xTg-AD-h mice (Fig. 4C).
Altered Expression of DM-Related Genes in Human and Mouse AD Hippocampus

A comparison of the altered gene expression profiles in human and mouse AD brains revealed that expression of genes relevant to non-insulin-dependent diabetes and obesity was significantly altered in the presence of AD pathology, as was that of genes relevant to neuronal function or brain dysfunction (Tables 3 and 5). IPA revealed that some of the genes dysregulated in both humans and mice are regulated by insulin signaling (Figs 3 and 4B). Pcsk1, encoding proprotein convertase subtilisin/kexin type 1, which is essential for proinsulin processing together with PCSK2 (Seidah et al. 1999), was placed upstream of insulin in the mouse network along with Ide, encoding insulin-degrading enzyme, the expression of which was significantly increased in the hippocampus.

We then examined expression of PCSK1 protein in mouse brain by laser scanning immunofluorescence microscopy (Fig. 5). In 15-month-old male non-Tg brains, we detected PCSK1 expression in most neurons in the cerebral cortex and hippocampus (Fig. 5A,B). In non-Tg hippocampus, PCSK1 expression is prominent in CA3 and CA2 subregions and to a lesser extent in CA1 and the dentate gyrus (DG) (Fig. 5C). We found that expression level of PCSK1 was significantly diminished in 3xTg-AD-H brains, including in the cerebral cortex (Fig. 5A) and hippocampus (Fig. 5B,C), as confirmed by microarray data.

Because PCSK1 was the second-most significantly decreased gene in human AD brains, we reconsidered the relationships among the genes in the 3 human networks and found that PCSK2, expression of which was also decreased in

![Figure 3](https://example.com/image.png)

**Figure 3.** Top 3 networks of genes whose expression was significantly altered in the AD hippocampus. Among the top 200 transcription clusters shown in Supplementary Table S6, 145 genes were eligible for generating networks excluding microRNA–mRNA interactions by IPA. (A) Network 1 includes 16 downregulated genes (MET, PCSK1, PTEN, SERPINF1, VEGFA, NEFH, EGR3, HOMER1, INA, DAGLA, CDH22, NEFL, TOM1L1, TOLLIP, SH2B1, ANGPT1), and 4 upregulated genes (AEBP1, TXNIP, VCAM1, ANGPT1). (B) Network 2 consists of 23 downregulated genes (IGF5, GABRA1, GFRα2, CCL5, KCN7, ARHGDIG, GABRG2, STmn2, L1CAM, SYT7, SYT5, GABRA4, KCNJ6, STX1B, GABRA5, SNAP25, PTPrN, SYT4, DUSP6, SYN1, PTPrN, PTPrN2). (C) Network 3 consists of 13 downregulated genes (IL12RB2, PRC8B, WIF3, NRN1, ENC1, SATB1, PHACTR1, ELAVL4, FABP3, AACS, LARGE, SPTBN2, YWHAG). Solid lines indicate direct interactions and dashed lines indicate indirect interactions. Downregulated molecules are shown in green and upregulated ones are shown in red. Encoded molecules were placed in an appropriate subcellular compartment based on IPA, if known. We added PCSK2 into Network 1, insulin, PCSK1, and PCSK2 into Network 2, and PCSK1 and PCSK2 into Network 3. PCSK1 and PCSK2 are known to be localized in secretory granules in the cytoplasm, but some amount of these proteins may be secreted into the extracellular space.
AD hippocampus (~1.502, P = 0.0288, 2-tailed t-test), could be placed upstream of those networks together with PCSK1 and insulin (Fig. 3). Human Networks 1 and 3 and Mouse Network 1 are likely to represent the major insulin signaling network, in which PCSK1 and PCSK2 are essential for insulin production (Figs 3A,C and 3B). We then verified the human microarray data by real-time quantitative RT-PCR analyses (primers shown in see Supplementary Table S1) of 10 genes showing significant alterations as well as PCSK2 and PCSK5–7 in the hippocampus (see Supplementary Table S8). The relative expression level of each gene was highly correlated with the data obtained by microarray analyses (see Supplementary Fig. 2). Among the 5 PCSK members identified, only the expression levels of PCSK1 and PCSK2 were significantly decreased in AD hippocampus (see Supplementary Table S8).

To obtain data supporting the biological relevance of these changes, we examined the levels of PCSK1 and PCSK2 proteins in the hippocampus by western blot analysis. Protein levels of PCSK1 and PCSK2 were significantly decreased in AD cases compared with non-AD subjects (Fig. 6). Thus, we confirmed that the decreases in PCSK1 and PCSK2 mRNA levels in AD hippocampus are indeed reflected in the levels of their translation products.

### Discussion

Microarray analyses of postmortem AD brains have revealed altered expression of neurological and immunological genes, genes encoding inflammatory molecules and genes encoding metabolic enzymes (Colangelo et al. 2002; Brooks et al. 2007; Parachikova et al. 2007; Bossers et al. 2010; Tan et al. 2010). Bossers et al. (2010) reported the results of a systematic search for global gene expression changes in the prefrontal cortex during the course of AD using Braak staging. They identified a number of genes involved in the processing of amyloid precursor protein and amyloid beta (PN2, RER1, NZT3, PCSK1, SST, PACAP, and EGR1) that were initially upregulated in Braak stages I–II, but were significantly downregulated in the late Braak stages V–VI. Moreover, Tan et al. (2010) reported a significantly altered AD transcriptome in the temporal cortices of AD patients, indicative of synaptic dysfunction, perturbed neurotransmission and activation of neuroinflammation. Their lists of significantly altered AD genes contained most of the genes constituting the 3 networks shown in Figure 3 (14 of 20 genes in Network 1; 14 of 23 genes in Network 2; 4 of 13 genes in Network 3), confirming that there are common alterations of gene expression in AD brains from 2 independent cohorts (the Oxford Project to Investigate Memory and Ageing and the Hisayama study). Our study and the studies of Bossers et al. (2010) and Tan et al. (2010) all showed that expression of the PCSK1 gene is reproducibly and most significantly downregulated in the late stages of disease in AD brains. Moreover, our data showed that the extent of PCSK1 downregulation was most significant in the hippocampi of AD brains, with downregulation occurring to a lesser extent in the temporal cortex and to a much lesser extent in the frontal cortex, in accordance with the pathological severity.

### AD Pathology May Alter Insulin Signaling

Several epidemiologic cohort studies, including the Hisayama study, have shown that individuals with DM or insulin resistance exhibit an increased risk of developing AD compared with nondiabetic individuals (Kuusisto et al. 1997; Matsuzaki et al. 2010; Schrijvers et al. 2010). Supporting these epidemiological data, induction of type 1 or type 2 DM in mouse models of AD has been reported to accelerate AD neuropathology and memory dysfunction (Jolivalt et al. 2010; Takeda et al. 2010). Conversely, mouse models of AD are likely to be more susceptible to obesity or insulin resistance (Kohjima et al. 2010). Moreover, it has been shown that insulin is produced in neuronal cells derived from the hippocampus and olfactory bulb in adult rat brain and in isolated neuronal stem cells (Kuwabara et al. 2011), suggesting that insulin produced in neurons may play important roles in the brain. The expression levels of insulin and insulin-like growth factors I and II are known to be markedly reduced in AD brains together with decreased expression of their receptors, suggesting that AD may be a neuroendocrine disorder, namely, type 3 diabetes (Steen et al. 2005). It has also been shown that insulin prevents the loss of surface insulin receptors, oxidative stress, and synaptic spine loss in cultured mature hippocampal neurons caused by Aβ-derived diffusible ligands (De Felice et al. 2009). Moreover, administration of intranasal insulin has been reported to stabilize or improve cognition, function, and cerebral glucose metabolism in adults with mild cognitive impairment or AD (Craft et al. 2012). Taken together, our results strongly suggest that AD pathology alters insulin signaling in the brain.

In 3xTg-AD mice, insulin signaling in the hippocampus is likely to be significantly diminished based on the decreased
expression of downstream genes such as Srd5a1 (Lubik et al. 2011), Cdkn1b (Bhatt et al. 2005) and Pla2g16 (Duncan et al. 2008). This downregulation may be caused by a reduction in the insulin level owing to decreased expression of Pcsk1, and may also be due to increased expression of Ide, which degrades insulin and/or Aβ peptides in a competitive fashion (Farris et al. 2003). Moreover, genes involved in insulin secretion, such as Vgf (Watson et al. 2005) and Cplx3 (Reim et al. 2005), were also found to be downregulated in 3xTg-AD mice in the present study (Fig. 4B), suggesting that AD pathology diminishes the production and secretion of insulin in brain.

In the present study, we observed significantly decreased expression of both PCSK1 and PCSK2 in human AD brains, which may result in a severe reduction in insulin level in AD brains. It has been shown that proinflammatory cytokines alter the expression of genes involved in insulin signaling through activation of NF-kB. For example, IFNG protein and expression of downstream genes such as Srd5a1 (Lubik et al. 2011), Cdkn1b (Bhatt et al. 2005) and Pla2g16 (Duncan et al. 2008). This downregulation may be caused by a reduction in the insulin level owing to decreased expression of Pcsk1, and may also be due to increased expression of Ide, which degrades insulin and/or Aβ peptides in a competitive fashion (Farris et al. 2003). Moreover, genes involved in insulin secretion, such as Vgf (Watson et al. 2005) and Cplx3 (Reim et al. 2005), were also found to be downregulated in 3xTg-AD mice in the present study (Fig. 4B), suggesting that AD pathology diminishes the production and secretion of insulin in brain.

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Table 5

| Diseases and disorders | P-value* | Genes** |
|------------------------|---------|--------|
| Bipolar disorder       | 4.64E-05|        |
| Noninsulin-dependent   | 1.41E-02|        |
| diabetes mellitus      |         |        |
| Coronary artery disease| 8.39E-03|        |
| Alzheimer’s disease     | 1.41E-02|        |
| Parkinson’s disease     | 3.13E-02|        |
| Obesity                | 1.36E-02|        |
| Immediate hypersensitivity| 1.58E-02|        |

Note: Diseases and disorders in which more than 5 genes are enriched are listed.
P-value by Fisher’s exact test. **Upregulated genes are shown with underline.
IL-1β protein are known to decrease expression of PCSK1 in a process that is dependent on NF-κB in rat primary β islet cells (Cardozo et al. 2001). It has been shown that increases in the expression levels of amyloid precursor protein, presenilin-1, presenilin-2, and glycogen synthase kinase 3 (GSK3)-β in peripheral blood mononuclear cells derived from type 2 DM patients were efficiently suppressed by insulin infusion. This suppression was accompanied by significant parallel reductions in NF-κB binding activity (Dandona et al. 2011), thus suggesting that insulin may also counteract NF-κB signaling in the brain.

We also found that the gene expression profile in the brain was not significantly altered by DM or prediabetes (data not shown). Together with the observations in 3xTg-AD mice,
this finding strongly suggests that the primary AD pathology itself diminishes insulin signaling in the brain, and as such, that AD brains are more vulnerable to various pathological insults caused by metabolic impairment or inflammatory responses. Peripheral insulin resistance or DM further exacerbates AD pathology, and is thus a strong risk factor for the progression of AD. It has been reported that gastric bypass surgery for morbidly obese patients with type 2 DM significantly suppresses the increase in expression levels of AD-related genes such as amyloid precursor protein, presenilin-2, and GSK3-β in mononuclear cells, in parallel with marked weight loss and improved insulin resistance (Ghanim et al. 2012). Therefore, it is relevant that cognitive function has been shown to improve with weight loss following bariatric surgery (Gunstad et al. 2011).

Recently, it was shown that insulin-induced hypoglycemic and streptozotocin-induced diabetic rats exhibit significantly decreased expression of GABRA1 with reduced cortical GABA binding (Antony et al. 2010; Sherin et al. 2010, 2012), indicating that Network 2 shown in Figure 3B also represents the effects of insulin signaling impairment owing to the decreased expression of PCSK1 and PCSK2. Moreover, silencing of the CPLX1 gene, which is also part of Network 2 and which was also downregulated in AD brains (Fig. 3B), has been reported to cause strong impairment of insulin secretion in response to glucose (Abderrahmani et al. 2004). Thus, decreased expression of CPLX1 may contribute to the insulin signaling impairment and neuronal dysfunction in AD brains.

![Figure 6](image-url)

**The HGF–MET Axis May Be Involved in Insulin Signaling in Brain**

Expression of MET, encoding a receptor for hepatocyte growth factor (HGF), was most significantly decreased in AD brains (Fig. 2A, see Supplementary Table S6). Expression of MET has been shown to be upregulated by VEGF and HGF (Gerritsen et al. 2003), and we also found that the expression level of VEGF is significantly decreased in AD brains, suggesting that the downregulation of MET gene in AD brains is likely to reflect reduced expression of VEGF, which is upregulated by insulin (Miele et al. 2000). Recently, Fafalios et al. (2011) reported that MET is essential for an optimal hepatic insulin response by directly engaging the insulin receptor (INSR) to form a MET–INSR hybrid complex culminating in a robust signal output. They also found that the HGF–MET system restores insulin responsiveness in a mouse model of insulin refractoriness. Because it has been established that insulin, HGF (Sharma 2010) and VEGF (Góra-Kupilas and Jo/C19 sko 2005) have neuroprotective functions, the altered gene expression profiles in AD brains strongly suggest that a decline in the neuroprotective pathways regulated by these molecules at least partly underlies the neurodegeneration in AD brains.

**Altered Expression of Transcription Factors in AD Brains**

In the human AD brains, several genes encoding transcription factors were significantly downregulated (see Supplementary Table S5).
Table S6). Among them, NEUROD6 is known to be involved in the regulation of neuronal fate in the mammalian retina (Kay et al. 2011) and SATB1 has been shown to play a role during postnatal brain development (Balamotis et al. 2012) as well as in aging, dietary restriction, and insulin-like signaling (Zhang et al. 2009). Expression of the NEUROD6 gene has been shown to be induced by SATB2 (Kay et al. 2011), suggesting that SATB1 may be involved in regulation of NEUROD6 in adult brain, because SATB1 and SATB2 share some targets and cooperatively regulate their expression (Asanoma et al. 2012). NEUROD6 is a basic helix-loop-helix transcription factor that plays important roles in the mammalian central nervous system including the retina (Kay et al. 2011), and has been shown to confer tolerance to oxidative stress by triggering an antioxidant response and sustaining mitochondrial biomass (Uittenbogaard et al. 2010). Thus, downregulation of NEUROD6 in AD brain may also accelerate neurodegeneration.

**Conclusion**

The findings of the present study clearly show that expression of genes involved in insulin signaling related to DM is significantly diminished, likely as a result of AD pathology, even in the absence of peripheral DM-related abnormalities. These findings provide new insights into the molecular mechanisms underlying AD pathology and will help us to develop new strategies for the prevention of and therapy for AD.

**Supplementary Material**

Supplementary material can be found at: http://www.cercor.oxfordjournals.org/

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