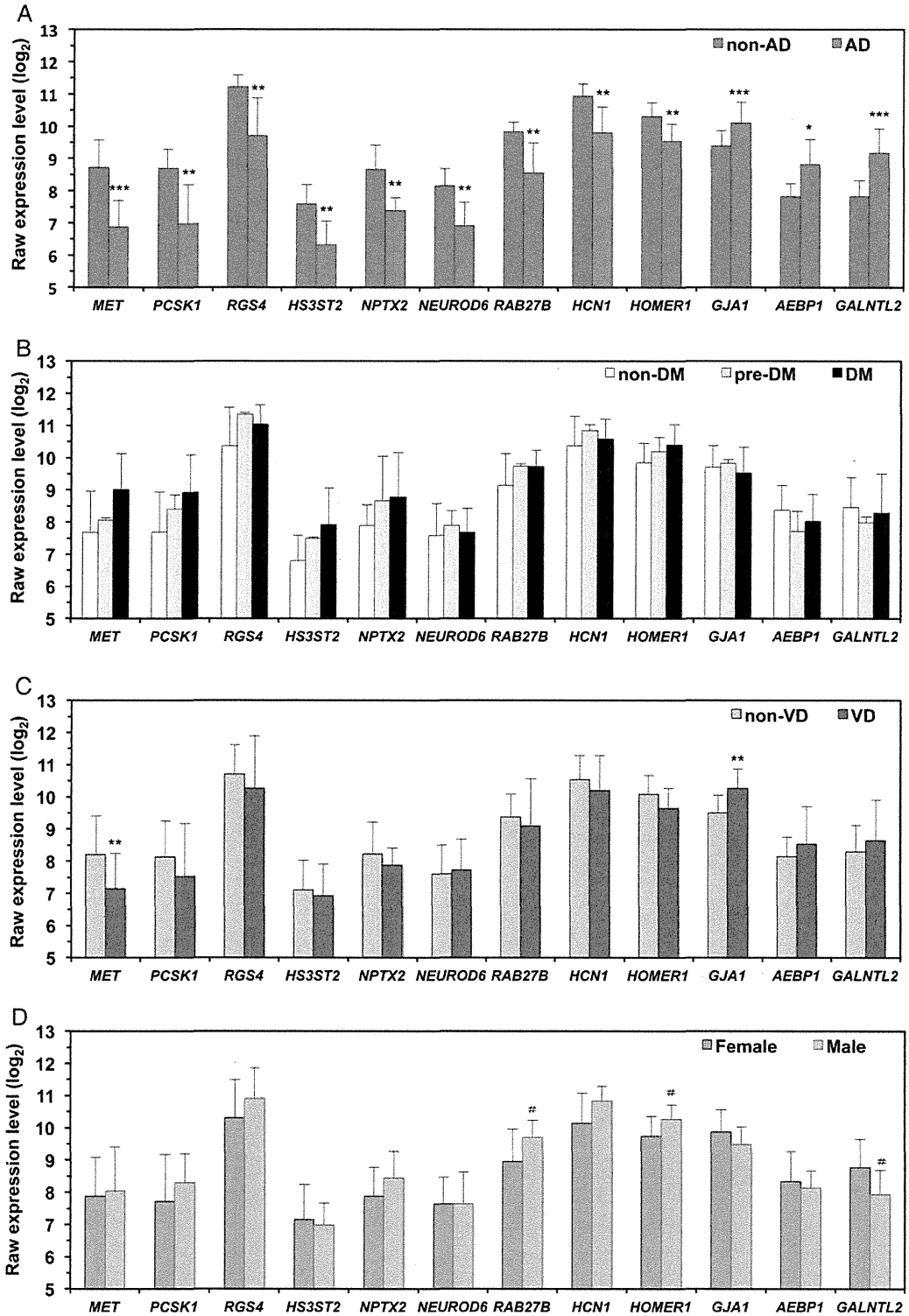


**Figure 2.** Comparison of the raw expression levels of 12 genes whose expression was significantly altered in the AD hippocampus. (*A*) Comparison between non-AD and AD cases. Dark blue box, non-AD ($N = 10$); red box, AD ($N = 7$). (*B*) Comparison between non-DM and pre-DM/DM cases. Open box, non-DM ($N = 12$); gray box, prediabetes (pre-DM, $N = 2$); black box, DM ($N = 3$). (*C*) Comparison between non-VD and VD cases. Green box, non-VD ($N = 13$); purple box, VD ($N = 4$). (*D*) Comparison between female and male cases. Orange box, female ($N = 9$); light blue box, male ($N = 8$). Four-way ANOVA was performed with the list of 1387 transcript clusters altered in the hippocampus, and the *P*-value for each comparison was determined by Fisher's Least Significant Difference method. $Log_2$ transformed mean values with SD for the raw expression levels of 12 genes are shown in each bar graph. $^{\#}P < 0.05$, $^{*}P < 0.01$, $^{**}P < 0.005$, $^{***}P < 0.001$. In *B*, non-DM ($N = 12$) and pre-DM + DM ($N = 5$) were compared; none of the 12 altered genes showed a significant difference.

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. 2*B*). 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. 2*C*,*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. 3*A*; 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. 3*B*; 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. 3*C*; 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; $N=3$) and homozygous (3xTg-AD-H; $N=3$) male mice for $APP_{Swe}$ and $MAPT_{P301L}$ transgenes with a homozygous $PS1_{M146V}$ 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. 4*A*).

**Table 3**
Genes significantly enriched in genetic disorders among those whose expression was significantly altered in AD hippocampus

| Diseases and disorders | *P*-value* | Genes** |
|---|---|---|
| Schizophrenia | 6.77E-14 | *APBA2, ATP2B2, CHRNB2, CPLX1, EGF, EGR3, ELAVL4, GABRA1, GABRA4, GABRA5, GABRG2, GLRB, HOMER1, HPRT1, LARGE, NEFL, PCSK1, PPP1R16B, PRKCB, RGS4, RGS7, RIT2, SLC17A7, SLC7A11, SNAP25, STMN2, SYT4, SYT7, TXNIP* |
| Bipolar disorder | 6.08E-06 | *ARL15, ATP2B2, CA5B, CHRNB2, CNGA3, DUSP6, FABP3, FAM19A1, GABBR2, GABRA1, GABRA4, GABRA5, GABRG2, GALNTL2, KCNJ6, LDB2, MAN1A1, NDFIP2, NEFL, PHACTR1, PRKCB, PTPRN2, RGS4, RIT2, SYN1, WDR49* |
| Coronary artery disease | 1.04E-04 | *ARL15, ATRNL1, FAM19A1, GABRA1, GABRA4, GABRA5, GABRG2, GFRA2, HS3ST2, HS6ST3, IFLTD1, KCNK9, LAMA4, LARGE, LDB2, MAPK9, NEDD4L, NPTXR, PHACTR1, PTPRN2, RIT2, SEL1L3, SNTB1, VEGFA, WBSCR17* |
| Crohn's disease | 2.46E-04 | *APBA2, ATP2B2, ATRNL1, DLGAP1, FAM19A1, GABRA4, GLRB, HCN1, HOPX, HPRT1, HS6ST3, IL12RB2, LDB2, MAN1A1, NEDD4L, PTPRN2, QPCT, RGS7, RIMBP2, RIT2, SEL1L3, SNAP25, SYT7* |
| Noninsulin-dependent diabetes mellitus | 4.15E-03 | *AACS, ANO3, ARL15, CA5B, CHRNB2, COL21A1, GDAP1L1, HS3ST2, HS6ST3, KCNJ6, LARGE, LDB2, NPTXR, PCSK1, PRKCB, RAB27B, RGS7, RIT2, SNTB1, TSPAN5, VEGFA, WBSCR17, YWHAG* |
| Amyotrophic lateral sclerosis | 2.30E-08 | *ATP2B2, ATP2B3, FAM19A1, FRMPD4, GABBR2, GABRA1, GABRA4, GABRA5, GABRG2, GALNTL2, INA, LARGE, NEFH, NEFL, NPTXR, PFKP, PPP1R16B, PTPRN2, RIMBP2, SYN1, VCAN, WBSCR17* |
| Huntington's disease | 2.79E-07 | *AEBP1, ATP2B2, CCKBR, GABRA1, GABRA4, GABRA5, GABRG2, GJA1, GLRB, GNG3, HOMER1, HPCA, MAN1A1, NEFL, OXR1, PRKCB, PTPN3, PTPN5, RGS4, SLC17A7, SNAP25, VCAN* |
| Alzheimer's disease | 1.77E-04 | *ANO3, ATP6V1G2, ATRNL1, CHRNB2, EGF, FAM19A1, FRMPD4, GABBR2, GABRA1, GABRA4, GABRA5, GABRG2, HOMER1, LARGE, MAPK9, NEFH, NEFL, PREP, PRKCB, SLC6A7, WBSCR17* |
| Parkinson's disease | 2.21E-03 | *ATRNL1, CA5B, CHRNB2, FRMPD4, GABBR2, GABRA1, GABRA4, GABRA5, GABRG2, HOMER1, KCNJ6, SH3RF1, SNAP25, SYN1* |
| Obesity | 1.54E-04 | *AEBP1, CA5B, CCKBR, CHRNB2, CPT1C, GABRA1, GABRA4, GABRA5, GABRG2, PCSK1, SYT4, VEGFA* |

Note: Diseases and disorders in which more than 10 genes are enriched are listed.
*P-value by Fisher's exact test. **Upregulated genes are shown with underline.

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 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. 4*B*). The raw expression level of *Pcsk1* 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. 4*C*).

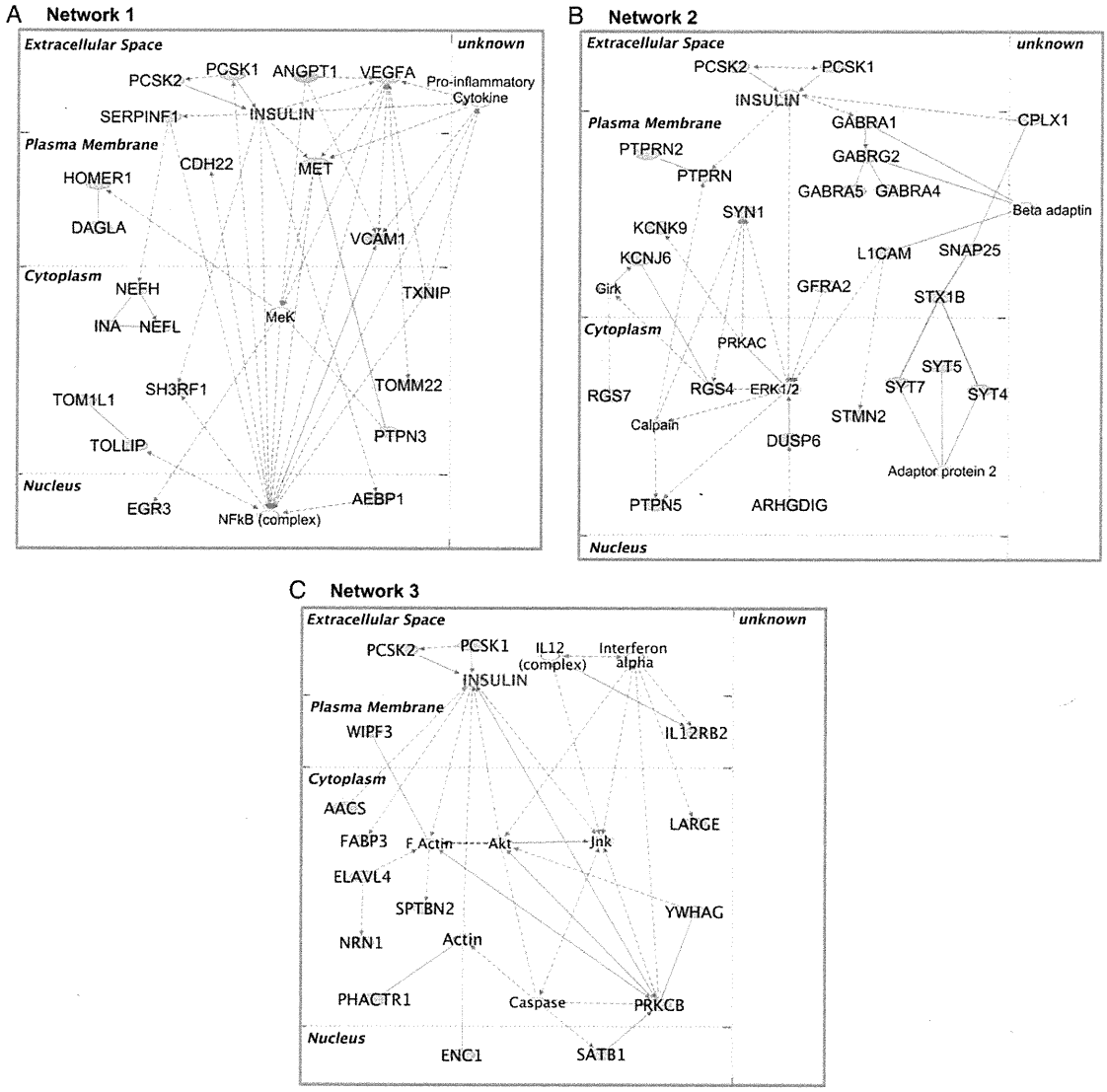


**Figure 3.** Top 3 networks of genes whose expression was significantly altered in the AD hippocampus. Among the top 200 transcription clusters shown in Supplementary Table S6, 145 genes were eligible for generating networks excluding microRNA–mRNA interactions by IPA. (*A*) Network 1 includes 16 downregulated genes (*MET, PCSK1, PTPN3, SERPINF1, VEGFA, NEFH, EGR3, HOMER1, INA, DAGLA, CDH22, NEFL, TOM1L1, TOLLIP, SH3RF1, TOMM22*), and 4 upregulated genes (*AEBP1, TXNIP, VCAM1, ANGPT1*). (*B*) Network 2 consists of 23 downregulated genes (*RGS4, GABRA1, GFRA2, CPLX1, KCNK9, RGS7, ARHGDIG, GABRG2, STMN2, L1CAM, SYT7, SYT5, GABRA4, KCNJ6, STX1B, GABRA5, SNAP25, PTPRN, SYT4, DUSP6, SYN1, PTPN5, PTPRN2*). (*C*) Network 3 consists of 13 downregulated genes (*IL12RB2, PRKCB, WIPF3, 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 PCKS2 are known to be localized in secretary granules in the cytoplasm, but some amount of these proteins may be secreted into the extracellular space.

### *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 noninsulin-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 4*B*). *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. 5*A,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. 5*C*). We found that expression level of PCSK1 was significantly diminished in 3xTg-AD-H brains, including in the cerebral cortex (Fig. 5*A*) and hippocampus (Fig. 5*B,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

**Table 4**
Altered expression of marker genes for various brain cell types in 3xTg-AD hippocampus

| Cell type | Gene symbol | Relative expression (% non-Tg) | |
|---|---|---|---|
| | | 3xTg-AD-h | 3xTg-AD-H |
| Astrocytes | *Gfap* | 110.99 | 118.48 |
| | *S100b* | 96.74 | 98.40 |
| | *Aqp4* | 90.51 | 95.28 |
| | Mean | 99.41 | 104.05 |
| | SD | 10.50 | 12.59 |
| Oligodendrocytes | *Mbp* | 100.87 | 99.36 |
| | *Sox10* | 105.74 | 91.73 |
| | *Mog* | 93.31 | 99.61 |
| | *Mag* | 104.80 | 111.46 |
| | mean | 101.18 | 100.54 |
| | SD | 5.66 | 8.15 |
| Microglia | *Cd68* | 107.96 | 105.79 |
| | *Aif1* | 96.85 | 95.73 |
| | *Lgals3* | 82.67 | 92.88 |
| | *Emr1* | 114.35 | 101.75 |
| | mean | 100.46 | 99.04 |
| | SD | 13.89 | 5.82 |
| Neurons | *Rbfox3* | 104.23 | 100.62 |
| | *Eno2* | 104.26 | 102.52 |
| | *Chga* | 100.56 | 107.48 |
| | *Syp* | 100.57 | 99.01 |
| | *Nefh* | 106.52 | 97.41 |
| | *Nefl* | 102.08 | 100.09 |
| | *Nefm* | 99.64 | 98.33 |
| | *Snap25* | 99.61 | 98.72 |
| | *Tubb2b* | 104.12 | 104.93 |
| | *Tubb2a* | 103.14 | 106.69 |
| | *Tubb2b* | 100.77 | 105.11 |
| | *Tubb5* | 107.12 | 104.99 |
| | *Tubb4* | 100.68 | 107.81 |
| | *Tubb6* | 99.67 | 99.55 |
| | *Tubb1* | 101.90 | 100.76 |
| | *Tubb2c* | 93.66 | 92.52 |
| | *Tubb3* | 96.19 | 84.98 |
| | mean | 101.45 | 100.68 |
| | SD | 3.4 | 5.76 |

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 3*A*,*C* and 4*B*). 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 (*PSEN2*, *RER1*, *ZNT3*, *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

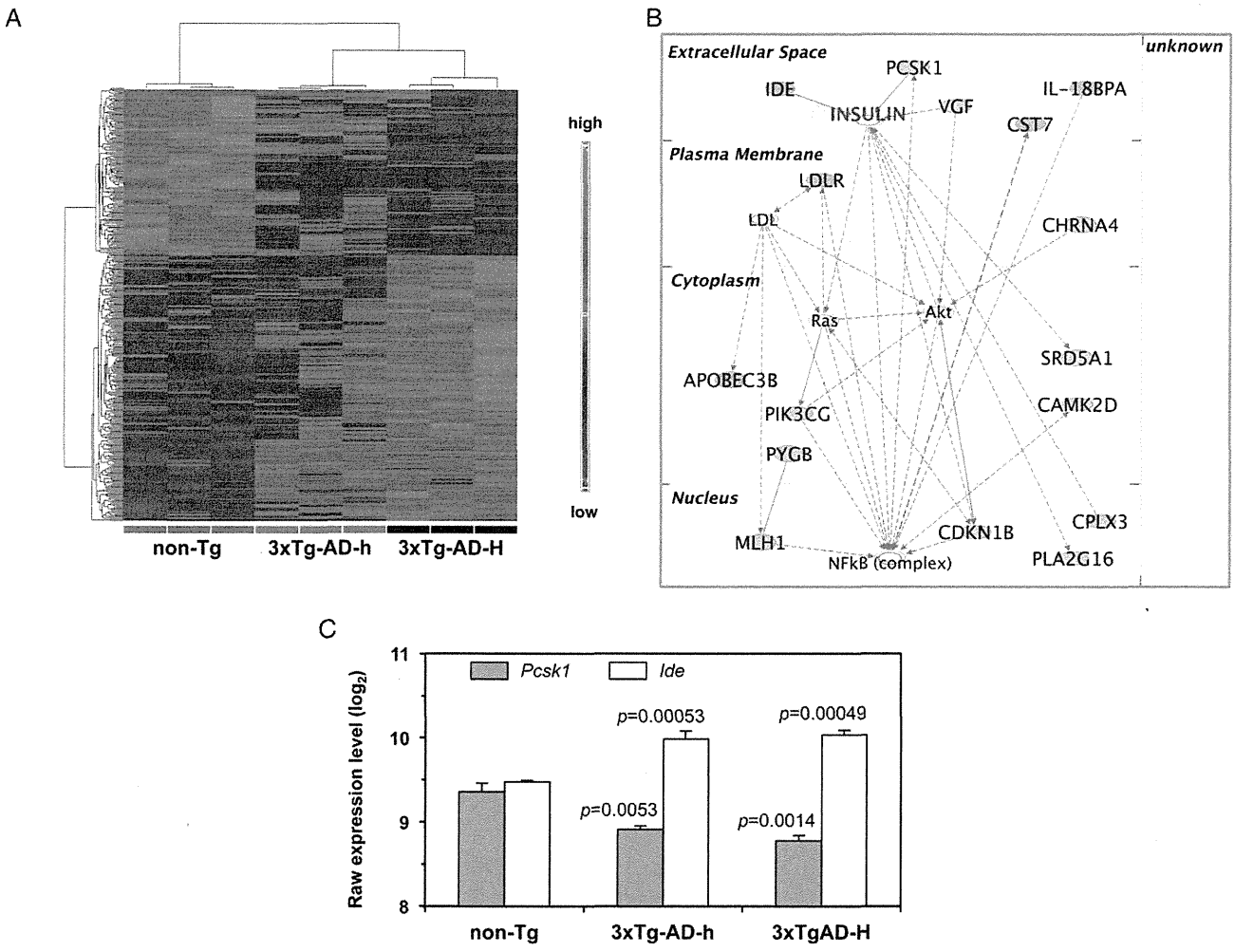


**Figure 4.** Results of microarray analysis of the 3xTg-AD mice. (*A*) Cluster heat map of the 406 transcript clusters based on individual expression data in the hippocampi of non-Tg (green), 3x-Tg-AD-h (magenta), and 3x-Tg-AD-H (black) mice ($N = 3$ for each group). Hierarchical and partitioning clustering of the 406 transcript clusters was performed among the 3 groups. In the heat map, blue represents a lower expression level and red indicates a higher expression level. (*B*) The top network of genes whose expression was significantly altered in the hippocampus of 3xTg-AD-H mice. Among the top 406 transcription clusters shown in Supplementary Table S7, only 120 genes were eligible for generating networks excluding microRNA–mRNA interactions; the most relevant network includes 11 downregulated genes (*Srd5a1, Mlh1, Cdkn1b, Pcsk1, Camk2d, Cplx3, Vgf, Chrna4, Pygb, Pik3cg, Pla2g16*), and 5 upregulated genes (*Cst7, Ide, Apobec3b, Ldlr, Il18bp*). Solid lines indicate direct interactions and dashed lines indicate indirect interactions. Downregulated molecules are shown in green and upregulated ones are shown in red. (*C*) Comparison of the raw expression levels for *Pcsk1* and *Ide* genes whose expression was significantly altered in the 3xTg-AD hippocampus. One-way ANOVA was performed with the list of 406 transcript clusters in hippocampus, and a *P*-value for the comparison with non-TG was determined using Fisher's Least Significant Difference method. $Log_2$ transformed mean values with SEMs of the raw expression levels for each gene are shown in the bar graph.

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. 4*B*), 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-κB. For example, IFNG protein and

**Table 5**
List of genes significantly enriched in genetic disorders among those whose expression was significantly altered in the hippocampi of homozygous 3xTg-AD mice

| Diseases and disorders | *P*-value* | Genes** |
|---|---|---|
| Bipolar disorder | 4.64E-05 | *Abca6, Adamts3, Akr1e2, Ca7, Camk2d, Chrna4, Cit, Cntnap5, Dpp10, Ide, Kcns1, Nme6, Oprd1, Osbpl10, Plxnd1, Ptprt, Rcn1, Slc1a1, Uqcc, Vgf* |
| Noninsulin-dependent diabetes mellitus | 1.41E-02 | *Adamts3, Akr1e2, Bfsp2, Ca7, Chrna4, Cntnap5, Glra3, Hpcal1, Ide, Ldlr, Pcsk1, Ptprt, Rai14, St3gal1, Stac, Tdp1, Unc13c* |
| Coronary artery disease | 8.39E-03 | *Adamts2, Adamts3, C9orf68, Camk2d, Cntnap5, Hpcal1, Itga8, Kiaa1467, Ldlr, Mamdc2, Pamr1, Ptprt, Pygb, Slc1a1, St3gal1, Tdp1* |
| Alzheimer's disease | 1.41E-02 | *Camk2d, Chrna4, Cit, Cntnap5, Fis1, Glt8d2, Ide, Ldlr, Osbpl10, Pamr1, Ptprt, Slc6a7, St3gal1* |
| Parkinson's disease | 3.13E-02 | *Ca7, Camk2d, Chrna4, Cntnap5, Cxorf40a/ Cxorf40b, Osbpl10, Ptprt, Rai14, Unc13c* |
| Obesity | 1.36E-02 | *Ca7, Chrna4, Ldlr, Npbwr1, Pcsk1, Sstr1, Vgf* |
| Immediate hypersensitivity | 1.58E-02 | *Fcer1g, Ide, Klk10, Pik3cg, Stac, Syne2* |

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.

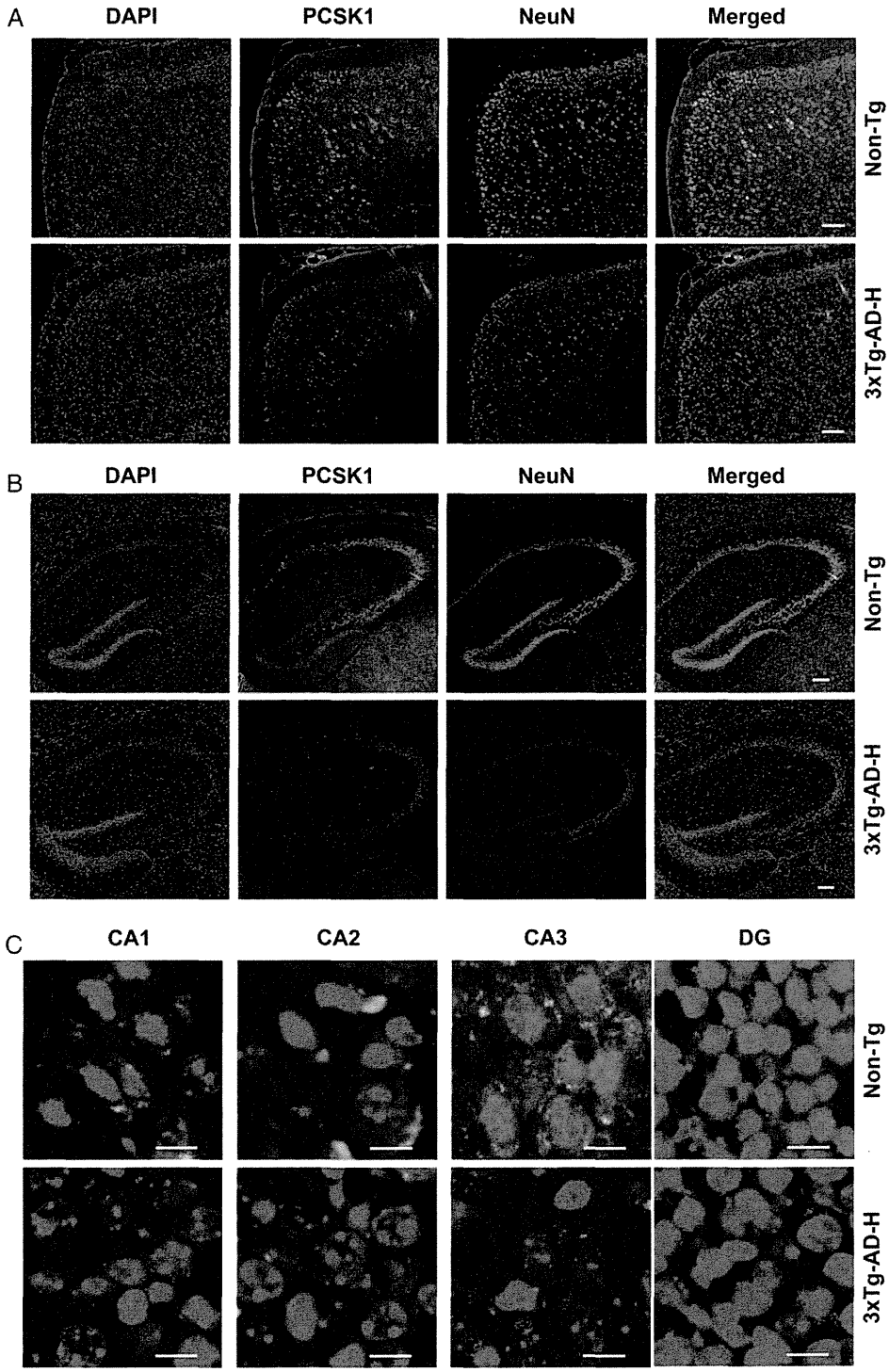


**Figure 5.** Evaluation of PCSK1 protein levels in mouse brain by laser scanning immunofluorescence confocal microscopy. (*A*) PCSK1 expression in the cerebral cortex. (*B*) PCSK1 expression in the hippocampus. (*C*) Magnified images of the hippocampal subregions CA1, CA2, CA3, and DG. Brain sections were prepared from 15-month-old non-Tg and 3x-Tg-AD-H male mice. Sections were reacted with anti-PCSK1 antibody (green) and an anti-NeuN antibody (red), and nuclear DNA was counterstained with DAPI (blue). Scale bars: *A*, *B*, 100 μm; *C*, 20 μm.

IL1B 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,

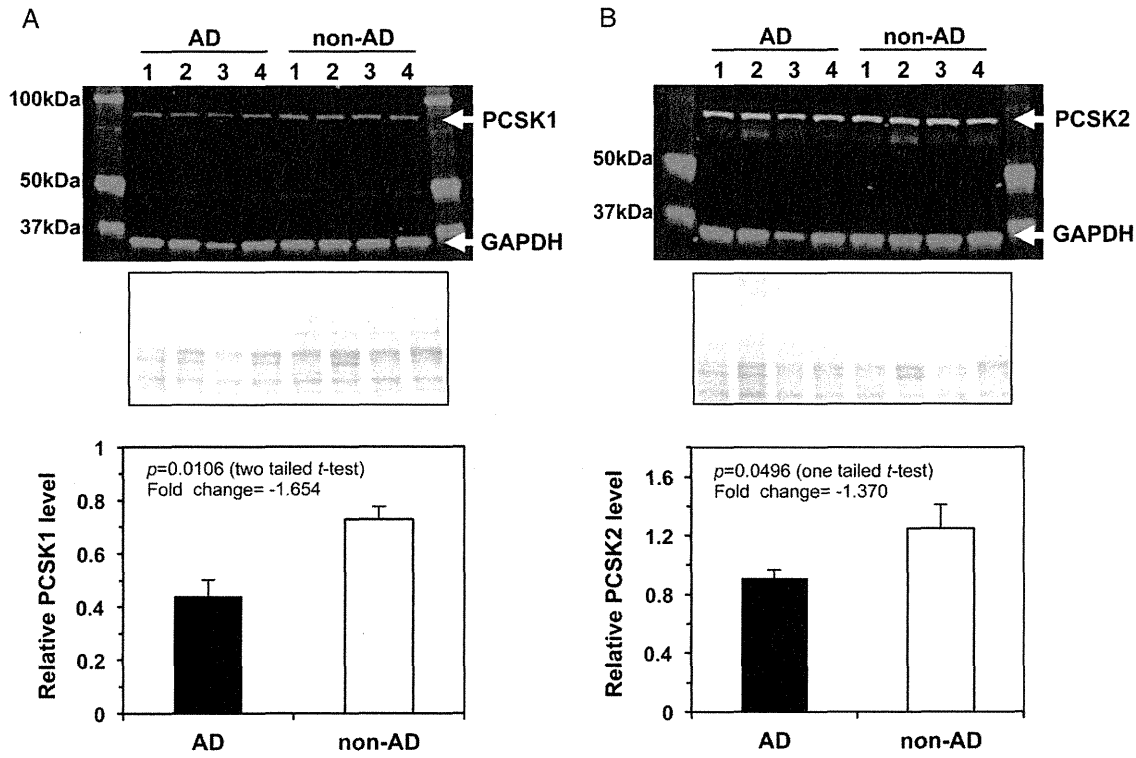


**Figure 6.** Evaluation of PCSK1 and PCSK2 protein levels in the hippocampal samples by western blot analysis. Hippocampal lysates (12 $\mu$g protein/lane) prepared from AD (No. 3, 4, 7, and 13 listed in see Supplementary Table S2) and non-AD brains (No. 19, 20, 21, and 28 listed in see Supplementary Table S2) were run on 10% SDS-PAGE gels and subjected to western blot analysis for PCSK1 (*A*), PCSK2 (*B*), and GAPDH proteins (top panels). Ponceau S staining (middle panels) was conducted to confirm the equal loading of samples and normalization. The relative intensities of bands were quantified using an Odyssey infrared imaging system, normalized to the intensity of Ponceau S staining, and are shown in bar graphs (bottom panels). *P*-values from an unpaired *t*-test are shown.

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 3*B* 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. 3*B*), 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.

### *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. 2*A*, 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 Jośko 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 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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## Notes

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