

Supporting Information

Hébert *et al.* 10.1073/pnas.0710263105

SI Methods

Patient Information. The non-dementia ($n = 21$), dementia ($n = 34$) and non-AD dementia ($n = 9$) patients were from the geriatric department of E. Roux Hospital at Limeil-Brevannes and the Lille CH&U Hospital, France (ADERMA network). They represent all patients who were hospitalized for various disorders and died at this hospital, excluding those whose family opposed autopsy, or for whom postmortem delay was more than 24 h. Cognitive status was evaluated using the MiniMental State Examination (MMSE) and the Clinical Dementing Rating (CDR) score. Clinical diagnosis was summarized as AD, AD (possible, probable), vascular dementia, mixed dementia (AD with a strong vascular involvement revealed by investigations), or dementia (for patients with an uncertain clinical diagnosis). Similarly, neuropathologic, biochemical and genetic data for each patient are summarized in [supporting information \(SI\) Dataset S1](#). Blocks from the anterior temporal cortex or cerebellum were dissected from each case and snap frozen in liquid nitrogen.

Transfections, DNA Cloning, and Luciferase Assays. 200,000 HeLa or 500,000 SK-N-SH cells were plated in 6 well plates. The next day, cells were transfected with 25–75 nM of premiRs (Ambion), a scrambled sequence (Ambion), or wild-type human BACE1 cDNA (cloned in pSG5 vector) using lipofectamine 2000 following manufacturer's instructions. 48 h posttransfection, cells were processed for immunoblot analysis. The 3'UTR of BACE1 and the TK promoter were amplified from human chromosomal DNA and cloned into the pGL3-luciferase basic vector (Promega). Sequences of primers and cloning strategy are available on request. The BACE1 luciferase mutant construct was generated using the QuikChange II XL Site-Directed Mutagenesis kit (Stratagene). For the luciferase assays, 75 nM of premiRs (Ambion) were co-transfected with the sensor vector and the Renilla control vector (Promega). 26–28 h posttransfection, the measurements were performed using the Dual luciferase reporter assay kit (Promega).

Quantitative RT-PCR (mRNA). Total RNA was extracted using the mirVana PARIS kit (Ambion) following the manufacturer's instructions. RT-PCR as well as quantitative PCR was performed as described (48) using an ABI 7000 Sequence Detector. Human BACE1: 5'CACCACCAACCTTCGTTTGC 3' and 5'AGTTCCTGATGGTTTCTGGC 3'. Human β -Actin: 5'CACCTGAAGTACCCCATGG 3' and 5'TGCCAGATTTTCTCCATGTCG 3'.

Northern Blotting (microRNA). Total RNA was extracted using the mirVana PARIS kit (Ambion) following the manufacturer's instructions. The microRNA probes were generated using the mirVana miRNA Probe Construction kit (Ambion) following manufacturers instructions (see list below). 5 μ g of total RNA was resolved on 15% TBE-urea/polyacrylamide precast gels (Invitrogen) and transferred to Hybond XL membrane (Amersham). RNA was immobilized by baking for 15 min at 80°C followed by UV cross-linking. Membranes were prehybridized in prehybridization buffer (5xSSPE, 0.5x deionized formamide, 50 μ g/ml heparin, 0.5% SDS, 1xDenhardt's, 1%ss DNA) for at least 5h at 42°C. Small RNAs were detected using P³² end-labeled antisense RNA oligonucleotides in hybridization buffer (prehybridization buffer + 10% dextranulphate) overnight. Membranes were rinsed 3x in wash solution (0.3x SSPE, 0.5% SDS)

followed by one wash in wash solution (0.3x SSPE, 0.5% SDS) for 1h at 60°C. Sealed blots were exposed to phosphorimager plates.

Northern Blotting (mRNA). A premade "mouse brain aging blot" (See-gene) was used which contained ~20 μ g total RNA per lane isolated from mouse brain at different developmental stages. The blot was subsequently hybridized with probes specific for mouse GAPDH and BACE1 mRNAs. The probe for BACE1 (a fragment of 947 bp) was PCR amplified from BACE1 cDNA. The GAPDH probe (543 bp) was provided by the company.

Quantitative RT-PCR (microRNA). The Taqman microRNA reverse transcription kit (Applied Biosystems) and the Taqman Universal PCR master mix (Applied Biosystems) were used. The quantitative PCR procedures were carried out following manufacturer's instructions provided with the Taqman microRNA assays (Applied Biosystems). Relative expression was calculated by using the comparative CT method. Out of three candidate reference genes (RNU19, RNU48 and hsa-miR-16), miR-16 (for human patient data) or RNU19 (for mouse data) was selected as normalizing control using the GeNorm software (<http://medgen.ugent.be/~jvdesomp/genorm>).

MicroRNA Microarray. Total RNA was extracted from frozen human brain material using the mirVana PARIS kit (Ambion) following the manufacturer's instructions. Samples for microRNA profiling studies were processed by Asuragen Services (formerly Ambion Services, Austin, TX), according to the company's standard operating procedures. The microRNA enriched fraction was obtained by passing 10 μ g of total RNA (from human brain) through a flashPAGE Fractionator apparatus (Ambion Inc.) and cleaned and concentrated using the flashPAGE Reaction Clean-Up Kit (Ambion Inc). The 3' ends of the RNA molecules were tailed and labeled using the mirVana miRNA Labeling Kit (Ambion Inc.) according to the manufacturer's instructions. Amine-modified nucleotides were incorporated during the poly (A) polymerase mediated tailing reaction, and Cy5 succinimide esters [Amersham Biosciences (GE Healthcare)] were conjugated to the amine moieties on the microRNAs. Hybridization to the mirVana miRNA Bioarrays V2 (Ambion Inc.) was performed using the mirVana miRNA Bioarray Essentials Kit (Ambion Inc., cat#1567). The Cy3 fluorescence on the arrays was scanned at an excitation wavelength of 532 nm using a GenePix 4200AL scanner (Molecular Devices). The fluorescent signal associated with the probes and local background was extracted using GenePix Pro (version 6.0, Molecular Devices).

Thresholding and signal scaling were generated using algorithms selected by Asuragen, as implemented as part of the miRNA Standard Service Premium Analysis (miSSP package). The background adjusted fluorescent values generated by GenePix Pro, were normalized for each microRNA using a variation stabilization transformation method described by Huber *et al.*, 2002 (4). Hypothesis testing with one-way ANOVA or t-Test depending on the number of groupings in the experimental design.

For Multiple Group comparisons, we use the One-way ANOVA (Analysis Of Variance) model to test the null hypothesis, which states there is no difference between groups. The goal is to filter out genes that have the same expression level across all groups.

Pair-wise comparisons are carried out on differentially expressed genes identified by ANOVA to see how they differ from each other. For each pair of treatments, a two-sample t test is carried out for every gene and multiplicity correction is followed to control the false discovery rate (FDR) using a step-up

approach as described by Benjamini and Hochberg (3) using an FDR of 5%. This method is referred to as “protected Least Significant Difference (LSD)”. The detailed miRNA lists and associated information such as fold-change and P values are reported in [Dataset S2](#) and [Dataset S3](#).

1. Hebert SS, et al. (2004) Coordinated and widespread expression of gamma-secretase in vivo: evidence for size and molecular heterogeneity. *Neurobiol Dis* 17:260–272.
2. Hebert SS, et al. (2006) Regulated intramembrane proteolysis of amyloid precursor protein and regulation of expression of putative target genes. *EMBO Rep* 7:739–745.
3. Benjamini Y, Hochberg Y (1995) Controlling the false discovery rate: a practical and powerful approach to multiple testing. *J R Stat Soc B* 57:289–300.
4. Huber W, von Heydebreck A, Sultmann H, Poustka A, Vingron M (2002) Variance stabilization applied to microarray data calibration and to the quantitation of differential expression, *Bioinformatics* 18:596–5104.

Hébert et al. www.pnas.org/cgi/content/short/0710263105

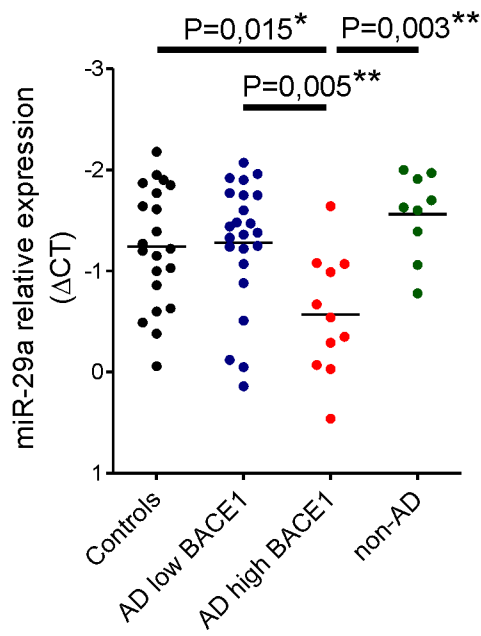
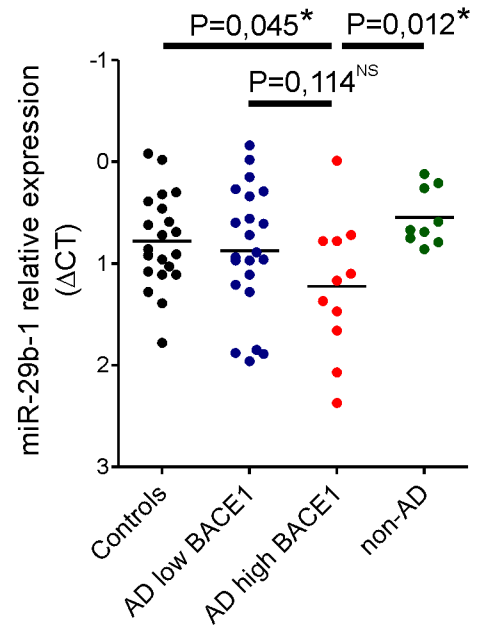
A**B**

Fig. S3. (A and B) Crude ΔC_t values obtained from qRT-PCR used to generate Fig. 4 A and B. Relative expression is shown using the mean as the controls group as reference. Note that a lower ΔC_t value indicates a higher miR-29 expression level. Ubiquitously expressed miR-16 was used as normalization control. Every data point reflects the mean of three independent RT reactions. The graph mean is shown. Statistical significance was determined by a Mann-Whitney test, where $*$ = $P < 0.05$, $**$ = $P < 0.01$.

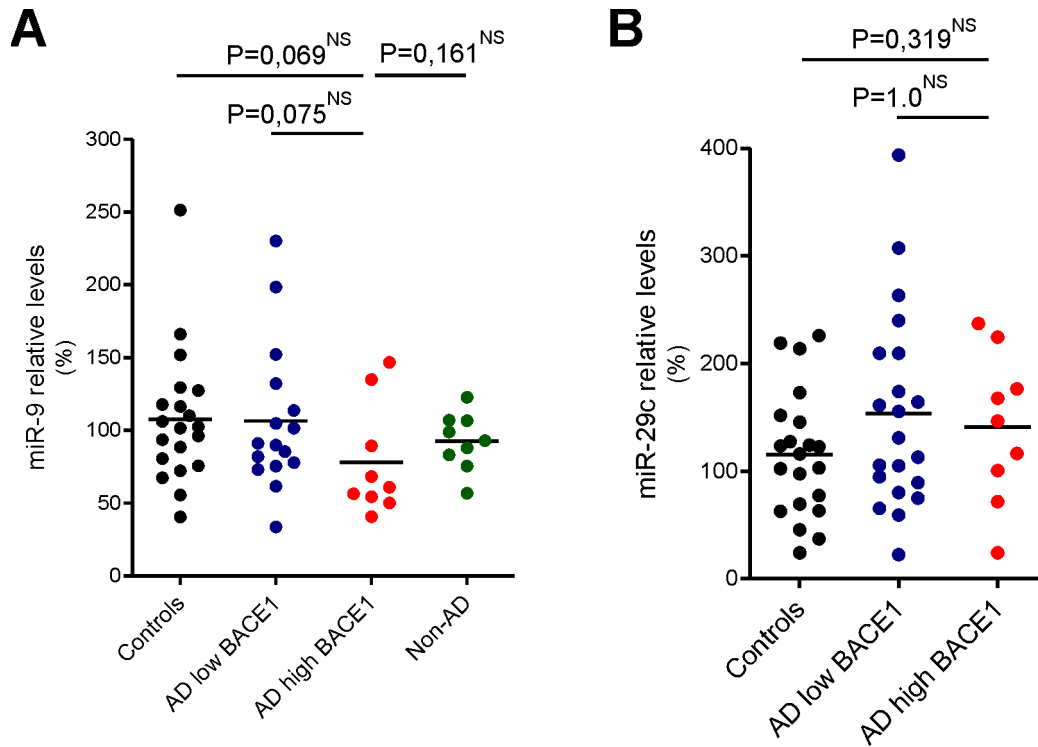


Fig. S4. (A) Quantitative RT-PCR of miR-9 in controls ($n = 20$), AD patients with low BACE1 ($n = 16$), AD patients with high BACE1 ($n = 9$) and non-AD dementia patients ($n = 9$). Relative expression is shown as percentage using the mean of the controls group as reference. (B) Quantitative RT-PCR of miR-29c in controls ($n = 19$), AD patients with low BACE1 ($n = 20$) and AD patients in high BACE1 ($n = 8$). Relative expression is shown as percentage using the mean of the controls group as reference. The graph mean is shown. In all experiments, ubiquitously expressed miR-16 was used as normalization control. Every data point reflects the mean of three independent RT reactions. Statistical significance was determined by a Mann-Whitney test.

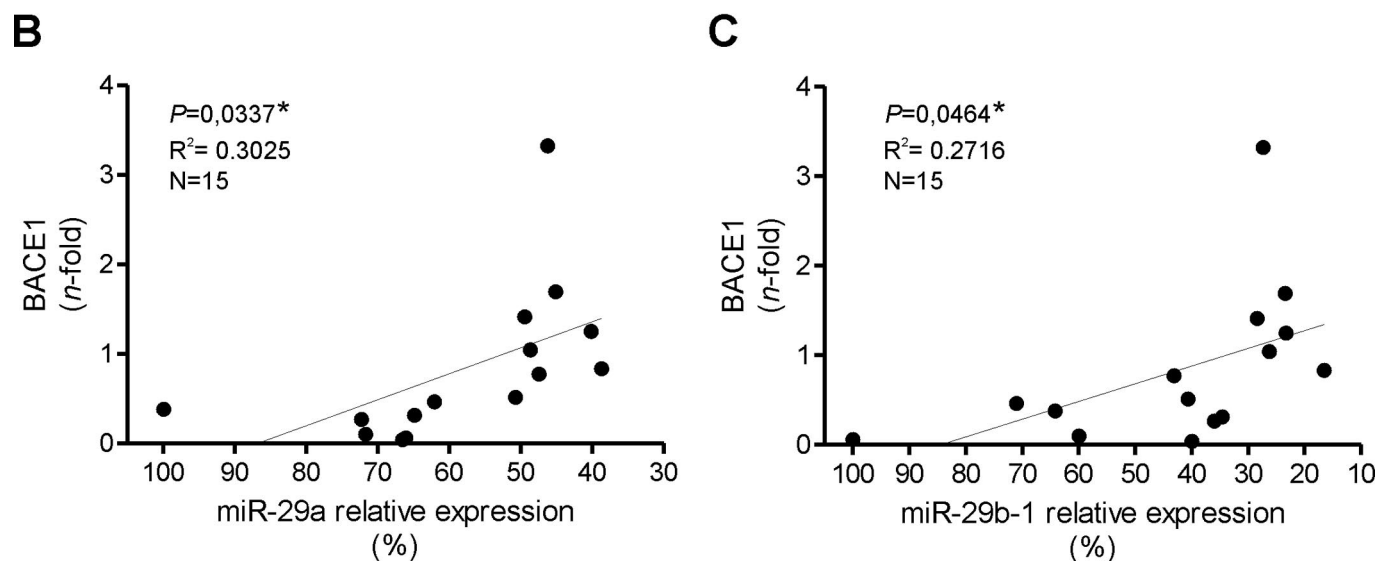
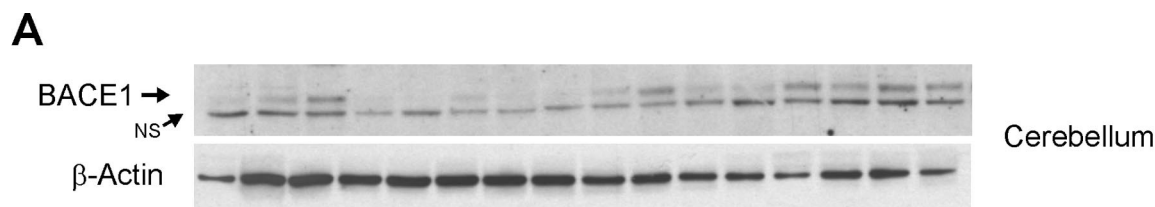


Fig. S5. (A) Western blot analysis of BACE1 (probed with C-terminal antibody) in sporadic AD cerebellum ($n = 15$) is shown. β -Actin was used as normalization control. NS, non-specific band. (B) Significant correlation between BACE1 protein, miR-29a ($P = 0.0337$, *Pearson's* correlation test) or miR-29b-1 ($P = 0.0464$) in AD cerebellum ($n = 15$) as assessed by quantitative RT-PCR. Relative expression is shown as percentage using the lowest Δ Ct value (here, highest miR levels) of miR-29a/b-1 as 100%. Ubiquitously expressed miR-16 was used as normalization control. Every data point reflects the mean of three independent RT reactions. Densitometric quantifications of BACE1 protein levels in sporadic AD cerebellum ($n = 15$) was performed using the average of all samples as reference (i.e., 1 fold). Statistical significance was calculated using a Mann-Whitney test, where $*$ = $P < 0.05$.

Other Supporting Information Files

[Dataset S1](#)

[Dataset S2](#)

[Dataset S3](#)