Supplemental Information

Supplemental Methods

Brain tissue and clinical pathological assessment

Clinical and neuropsychological evaluation criteria for the Religious Orders Study cohort have been published previously (1-3). Upon entry into the Religious Orders Study cohort, subjects were deemed to not have any comorbid conditions contributing to cognitive impairment. Antemortem cognitive assessments performed within a year before death included the Mini Mental State Exam (MMSE) and a battery of 19 neuropsychological tests (3, 4). A global cognitive *z*-score (GCS) was also compiled for each subject based on the neuropsychological battery (3, 4). A board-certified neurologist made a clinical diagnosis for each Religious Orders Study participant. Subjects were clinically categorized as no cognitive impairment (NCI), mild cognitive impairment (MCI) insufficient to meet criteria for dementia, or Alzheimer's disease (AD) (Table 1). The majority of AD subjects from the Religious Orders Study cohort used in this study were classified as mild to moderate AD based upon cognitive and neuropathological criteria (Table 1). An additional cohort of end-stage AD subjects {n = 5; 81.5 years ± 9.5 standard deviation (SD)} was also used for the microarray studies. Although there is no consensus criteria for the clinical classification of MCI (5), the present MCI population was defined as subjects with impaired cognitive testing who were not found to have frank dementia by an examining neurologist (1, 6), similar to criteria used by other independent experts in the field (7, 8).

At autopsy, tissue blocks containing the hippocampal complex were immersion-fixed in 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.2 for 24-72 hours at 4˚C, paraffin embedded, and cut on a rotary microtome at 6 μm thickness. Adjacent tissue slabs were also

snap-frozen in liquid nitrogen for subsequent real-time quantitative polymerase chain reaction (qPCR) and immunoblotting. A series of tissue sections were prepared with neuropathological evaluation including visualization and quantitation of senile plaques and neurofibrillary tangles (NFTs) using thioflavine-S, modified Bielschowsky silver stain, and antibodies directed against amyloid-β peptide (Aβ; 4G8, monoclonal, Covance, Princeton, NJ) and tau (PHF1, monoclonal, a gift of Dr. Peter Davies) (1-3). Additional sections were stained for Lewy bodies using antibodies directed against ubiquitin (13-1600, monoclonal, Invitrogen, Carlsbad, CA) and α-synuclein (18-0215, monoclonal, Invitrogen). Exclusion criteria included Lewy body disease, mixed dementias, Parkinson's disease, frontotemporal dementia, argyrophilic grain disease, and stroke. A board certified neuropathologist blinded to the clinical diagnosis performed the neuropathological evaluation. Neuropathological designations were based on National Institute on Aging (NIA) Reagan, Consortium to Establish a Registry for Alzheimer's Disease (CERAD), and Braak staging criteria (9-11). Amyloid burden and apolipoprotein E (ApoE) genotype were determined for each case as described previously (1, 4, 11-13).

Tissue preparation for microarray analysis

Acridine orange histofluorescence (2, 14, 15) and bioanalysis (2100, Agilent Biotechnologies, Palo Alto, CA) (16, 17) were performed on each brain prior to performing downstream genetic analyses to ensure that high quality RNA was present in hippocampal tissue sections. RNase-free precautions were used throughout the experimental procedures, and solutions were made with 18.2 mega Ohm RNase-free water (Nanopure Diamond, Barnstead, Dubuque, IA).

Briefly, deparaffinized tissue sections were blocked in a 0.1 M Tris (pH 7.6) solution containing 2% donor horse serum (DHS; Sigma, St. Louis, MO) and 0.01% Triton X-100 for 1 hour and then incubated with a primary antibody directed against nonphosphorylated neurofilament proteins (RMdO20) (18) in a 0.1 M Tris/2% DHS solution overnight at 4°C in a humidified chamber. Sections were processed with the ABC kit (Vector Labs, Burlingame, CA) and developed with 0.05% diaminobenzidine (Sigma), 0.03% hydrogen peroxide, and 0.01 M imidazole in Tris buffer for 10 minutes (17, 19). Tissue sections were not coverslipped or counterstained and maintained in RNase-free 0.1 M Tris prior to laser capture microdissection (LCM).

Single cell microaspiration and Terminal Continuation (TC) RNA amplification

Linearity and reproducibility of the TC RNA amplification procedure has been published previously, including the use of CA1 neurons as input sources of RNA (19-21). The TC RNA amplification protocol is available at http://cdr.rfmh.org/pages/ginsberglabpage.html. Briefly, CA1 neurons were homogenized in 500 μl of Trizol reagent (Invitrogen), extracted with chloroform, and precipitated utilizing isopropanol (22). RNAs were reverse transcribed in the presence of poly d(T) primer (100 ng/ml) and TC primer (100 ng/ml) in 1X first strand buffer (Invitrogen), 2 μl of linear acrylamide (Applied Biosystems, Foster City, CA), 10 mM dNTPs, 100 mM DTT, 20 U of RNase inhibitor (Superase-in; Applied Biosystems) and 200 U of reverse transcriptase (Superscript III, Invitrogen). Single stranded cDNAs were digested and then placed in a thermal cycler using a solution consisting of 10 mM Tris (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, and 10 U RNase H (Invitrogen) in a final volume of 100 μl. The thermal cycler program ran as follows: RNase H digestion step at 37ºC, 30 minutes; denaturation step 95ºC, 3 minutes;

primer re-annealing step 60ºC, 5 minutes. Samples were purified by column filtration (Vivaspin 500; Sartorius Stedim, Goettingen, Germany). Hybridization probes were synthesized by *in vitro* transcription using ^{33}P incorporation in 40 mM Tris (pH 7.5), 6 mM $MgCl_2$, 10 mM NaCl, 2 mM spermidine, 10 mM DTT, 2.5 mM ATP, GTP and CTP, 100 mM of cold UTP, 20 U of RNase inhibitor, 2 KU of T7 RNA polymerase (Epicentre, Madison, WI), and 120 mCi of ^{33}P -UTP (10 mCi/ml; MP Biomedicals, Solon, OH) (22, 23). The reaction was performed at 37°C for 4 hours. Radiolabeled TC RNA probes were hybridized to custom-designed cDNA arrays without further purification.

Custom-designed cDNA array platforms and array hybridization

Arrays were prehybridized (2 hours) and hybridized (12 hours) in a solution consisting of 6X saline–sodium phosphate–ethylenediaminetetraacetic acid (SSPE), 5X Denhardt's solution, 50% formamide, 0.1% sodium dodecyl sulfate (SDS), and denatured salmon sperm DNA (200 mg/ml) at 42°C in a rotisserie oven (20, 23). Following the hybridization protocol, arrays were washed sequentially in 2X SSC/0.1% SDS, 1X SSC/0.1% SDS and 0.5X SSC/0.1% SDS for 15 min each at 37°C. Arrays were placed in a phosphor screen for 24 hours and developed on a phosphor imager (GE Healthcare). All array phosphor images were adjusted to the same brightness and contrast levels for data acquisition and analysis.

Data collection and statistical analysis for custom-designed microarrays

Hybridization signal intensity was determined utilizing ImageQuant software (GE Healthcare). Briefly, each array was compared to negative control arrays utilizing the respective protocols without any input RNA. Expression of TC amplified RNA bound to each linearized

cDNA (approximately 576 cDNAs/ESTs on the array) minus background was then expressed as a ratio of the total hybridization signal intensity of the array (a global normalization approach). Global normalization effectively minimizes variation due to differences in the specific activity of the synthesized probe and the absolute quantity of probe (23, 24). These data do not allow the absolute quantitation of mRNA levels. However, an expression profile of relative changes in mRNA levels was generated. Boxplots and scatterplots were graphed using GraphPad Prism (version 5; GraphPad Software, La Jolla, CA) and S-PLUS 7.0 (Tibco, Somerville, MA), respectively.

qPCR

qPCR was performed on frozen micropunches from the hippocampal CA1 region NCI (n = 11), MCI (n = 6), and mild/moderate AD (n = 6) cases from the Religious Orders Study. Among them, 2 NCI and 2 AD cases were also part of the microarray study. Taqman qPCR primers (Applied Biosystems) were utilized for the following genes: *rab3* (Hs00326824_m1), *rab4* (Hs01106488_m1), *rab5* (Hs00991293_g1), *rab7* (Hs01115139_m1), *rab24* (Hs01585713_g1), *rab27* (Hs00608302_m1), *TrkA* (Hs01021011_m1), *TrkB* (Hs01093096_m1), *TrkC* (Hs00983880_m1), $p75^{NTR}$ (Hs00182120_m1), and the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*; Hs02758991_g1). Standard curves and cycle threshold (Ct) were measured using standards obtained from total human brain RNA. Samples were run in triplicate for the qPCR assessments. Negative controls consisted of the reaction mixture without input RNA.

Immunoblot analysis

Frozen microdissected hippocampal tissue samples were homogenized in a 20 mM Tris-HCl (pH 7.4) buffer containing 10% (w/v) sucrose, 1 mM ethylenediaminetetraacetic acid (EDTA), 5 mM ethylene glycol-bis (ß-aminoethylether)-N,N,N',N'-tetra-acetic acid (EGTA), 2 mg/ml of the following: (aprotinin, leupeptin, and chymostatin), 1 mg/ml of the following: {pepstatin A, antipain, benzamidine, and phenylmethylsulfonyl fluoride (PMSF)}, 100 μg/ml of the following: {soybean trypsin inhibitor, Na-p-tosyl-L-lysine chloromethyl ketone (TLCK), and N-tosyl-L-phenylalanine chloromethyl ketone (TPCK)}, 1 mM of the following: (sodium fluoride and sodium orthovanadate). All protease inhibitors were purchased from Sigma. Identical amounts of homogenates (10 μg) were loaded into a gel electrophoresis apparatus, subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE; 4-15% gradient acrylamide gels; Bio-Rad, Hercules, CA), and transferred to nitrocellulose by electroblotting (Mini Transblot, Bio-Rad). Membranes were developed affinity–purified secondary antibodies conjugated to IRDye 800 (Rockland, Gilbertsville, PA) and visualized using an infrared detection system (Odyssey, LiCor). Immunoblots were quantified by densitometric software supplied with the instrument.

rab5 overexpression by infection of human skin fibroblasts

Human forearm skin fibroblasts from normal control subjects were grown in minimum essential medium (Invitrogen), supplemented with 15% fetal bovine serum (Hyclone Perbio South, Logan, UT), 2 mmol/L glutamine (BioWhittaker, Walkersville, MD), and penicillin-streptomycin (Invitrogen). Cells were maintained at 37°C and 5% carbon dioxide and passaged using standard protocols as described by the supplier. Cell confluency was consistently 85%-

90%. Transfection of fibroblasts with rab5 viral vector constructs was performed as described previously (25, 26). Forty-eight hours after initially seeding at 3.5 x 10^5 cells per 60-mm dish, cells were washed with warm phosphate-buffered saline (PBS) and infected for 5 hours with the recombinant viral constructs (rab5wt, rabQ79L, rabS34N, LacZ, or mock transfected with sucrose) in complete media, using 20 μl of virus per 60-mm dish with a multiplicity of infection of 2.0 (25). For lysate preparation, cell monolayers were scraped into cold PBS and centrifuged (1600 x *g*) for 10 minutes at 4°C and the pellet resuspended in lysis buffer containing a protease inhibitor cocktail consisting of 10 mmol/L sodium fluoride, 5 mmol/L sodium pyrophosphate, 1 mmol/L of the following: (zinc chloride and sodium orthovanadate), 0.5 mmol/L of the following: (dithiothreitol, and PMSF), and 1 μg/ml of the following: (antipain, aprotinin chymostatin, leupeptin, and pepstatin) (26). Identical amounts of homogenates (10 μg) were subjected to SDS-PAGE identical to the brain tissue homogenates as above. qPCR assessment was performed on total RNA extracted from fibroblasts 48 hours after infection using Trizol reagent (Invitrogen). RNA quality and quantity were evaluated using the RNA 6000 Pico kit (Agilent Technologies, Santa Clara, CA).

Knockdown of *TrkB* using siRNA

Predesigned *TrkB* siRNA constructs were purchased from Applied Biosystems (Silencer *TrkB* 753 and *TrkB* 754) (27) along with positive and negative control siRNAs to assess nonspecific gene knockdown effects. Human forearm skin fibroblasts from normal controls were prepared as described above. Approximately 1 million cells were seeded onto 100 mm dishes, and cultured with antibiotic-free media 24 hours before transfection (28). siPortAmine (Applied Biosystems) was used as transfection agent for the Silencer siRNA constructs. For immunoblots,

lysates were prepared in the same manner as the fibroblasts infected with *rab5* constructs above (26). Identical amounts of homogenates (10 μg) were subjected to SDS-PAGE and immunoblot analysis was performed and quantified as above. The Silencer 753 *TrkB* siRNA construct produced specific *TrkB* knockdown, whereas the Silencer 754 construct knocked down all of the genes and proteins sampled nonspecifically, and was not employed as part of the quantitative analysis.

Table S1. qPCR analysis: clinical, demographic, and neuropathological characteristics by diagnosis category.

		Clinical Diagnosis			Comparison by diagnosis group
		NCI (n = 11)	MCI (n = 6)	AD (n = 6)	
Age at death (years)	Mean ± SD (Range)	85.5 ± 4.9 (76-92)	84.0 ± 4.4 (79-91)	85.7 ± 6.2 (80-95)	$p = 0.8$[a]
Number (%) of males		5 (45%)	3 (50%)	3 (50%)	$p = 1.0$[b]
Educational level	Mean ± SD (Range)	17.8 ± 4.5 (8-25)	18.8 ± 2.8 (15-23)	18.7 ± 1.8 (16-21)	$p = 0.8$[a]
MMSE	Mean ± SD (Range)	27.6 ± 1.1 (26-29)	27.2 ± 3.1 (22-30)	19.3 ± 3.3 (15-24)	$p = 0.002$[a]*
ApoE ε4 allele (%)		0	1 (17%)	4 (67%)	$p = 0.02$[b]
PMI (hours)	Mean ± SD (Range)	5.1 ± 2.9 (2.4-12.4)	4.7 ± 2.0 (2.3-7.6)	4.3 ± 2.0 (2.2-7.3)	$p = 0.9$[a]
Distribution of Braak scores	0 I/II III/IV V/VI	0 2 8 1	0 2 3 1	0 0 1 5	$p = 0.01$[c]
Distribution of NIA Reagan diagnosis (likelihood of AD)	No AD Low Intermediate High	0 3 7 1	0 3 2 1	0 0 2 4	$p = 0.03$[c]
CERAD diagnosis	No AD Possible Probable Definite	3 0 6 2	2 0 3 1	0 0 1 5	$p = 0.03$[c]

[a]One-way ANOVA
[b]Fisher's exact test
[c]Kruskal-Wallis test
*(NCI & MCI) > AD

Abbreviations: AD, Alzheimer's disease; CERAD, Consortium to Establish a Registry for Alzheimer's Disease; MCI, mild cognitive impairment; MMSE, Mini-Mental State Exam; NCI, no cognitive impairment; NIA, National Institute on Aging; PMI, postmortem interval; qPCR, real-time quantitative polymerase chain reaction.

Table S2. Immunoblot analysis[‡]: clinical, demographic, and neuropathological characteristics by diagnosis category.

		Clinical Diagnosis			Comparison by diagnosis group
		NCI (n = 15)	MCI (n = 8)	AD (n = 17)	
Age at death (years)	Mean ± SD (Range)	76.7 ± 12.3 (43-92)	86.6 ± 6.3 (79-95)	84.2 ± 7.8 (62-97)	$p = 0.04$[a]
Number (%) of males		10 (67%)	3 (38%)	5 (29%)	$p = 0.1$[b]
Educational level [§]	Mean ± SD (Range)	19.0 ± 3.3 (12-25)	18.8 ± 2.8 (15-23)	15.3 ± 3.8 (11-21)	$p = 0.07$[a]
MMSE[§]	Mean ± SD (Range)	28.2 ± 1.1 (26-30)	27.2 ± 3.1 (22-30)	12.0 ± 7.8 (0-22)	$p < 0.0001$[a]*
PMI (hours)	Mean ± SD (Range)	10.6 ± 9.0 (2.3-30.5)	6.6 ± 3.9 (2.3-12.4)	9.0 ± 6.4 (2.7-23.0)	$p = 0.5$[a]
Distribution of Braak scores[§]	0 I/II III/IV V/VI	2 6 6 0	0 1 5 0	0 0 0 14	$p < 0.0001$[a]*

Abbreviations as in Table S1.

[‡]Tissue samples for the immunoblot analysis were obtained from the Religious Orders Study (8 NCI, 6 MCI, 6 AD), and supplemented by tissue samples obtained from UPENN (6 NCI, 8 AD) and the Harvard Brain Bank (1 NCI, 2 MCI, 3 AD).

[§]Educational level was not available for 4 NCI, 2 MCI, and 4 AD cases. MMSE was not available for 5 NCI, 2 MCI, and 4 AD cases. Braak scores were not available for 1 NCI, 2 MCI, and 3 AD cases.

[a]Kruskal-Wallis test

[b]Fisher's exact test

*(NCI & MCI) > AD

Table S3. Microarray-derived gene expression levels of 5 severe AD cases: mean ± standard error of the mean (SEM). This supplemental table is associated with Figure 2.

rab4	*rab5*	*rab7*	*rab10*	*rab24*	*rab27*	*rab1*	*rab3*
4.54 ± 0.30	5.60 ± 0.63	5.83 ± 0.39	3.78 ± 0.65	4.46 ± 0.57	3.43 ± 0.23	3.86 ± 0.45	2.57 ± 0.22

p75^{NTR}	*TrkA*	*TrkB*	*TrkC*
2.61 ± 0.44	1.07 ± 0.14	1.40 ± 0.28	0.80 ± 0.13

Analysis was performed by mixed models analysis for repeated measures, with random intercept, Kenward-Roger denominator degrees of freedom, and unstructured covariance structure.

Microarray assessment in these end-stage cases demonstrates similar expression levels for the following genes evaluated in 7 mild/moderate AD cases from the Religious Orders Study cohort in bold. Note discrepant expression levels especially for *TrkA*, *TrkB*, and *TrkC* (significantly higher in end-stage cases), underscoring the importance of separating mild/moderate AD cases from end-stage disease, particularly in terms of high-affinity neurotrophin receptor expression.

rab4	*rab5*	*rab7*	*rab10*	*rab24*	*rab27*	*rab1*	*rab3*
4.86 ± 0.40	**5.87 ± 0.44**	**5.32 ± 0.30**	2.60 ± 0.35	**4.98 ± 0.47**	**3.35 ± 0.40**	2.64 ± 0.30	**2.34 ± 0.21**

p75^{NTR}	*TrkA*	*TrkB*	*TrkC*
2.93 ± 0.44	0.78 ± 0.10	0.83 ± 0.15	0.55 ± 0.10

Table S4. Association between select rab GTPase and neurotrophin receptor gene expression levels and clinical, demographic, and neuropathological variables. This supplemental table is associated with Figure 3.

	*P-values**											
	rab1	*rab3*	*rab4*	*rab5*	*rab7*	*rab10*	*rab24*	*rab27*	*TrkA*	*TrkB*	*TrkC*	*p75^{NTR}*
Braak score	-	0.01	-	-	**0.004**	-	0.03	-	-	**0.004**	-	-
Reagan diagnosis	-	0.01	0.02	0.02	**0.0007**	-	**0.004**	-	-	**0.003**	-	-
CERAD diagnosis	-	0.03	-	0.03	**0.007**	-	-	-	-	**0.002**	-	-

*Analyses were performed by mixed models analysis. Only p-values < 0.05 are shown. Statistically significant p-values (< 0.01) are bolded for emphasis.

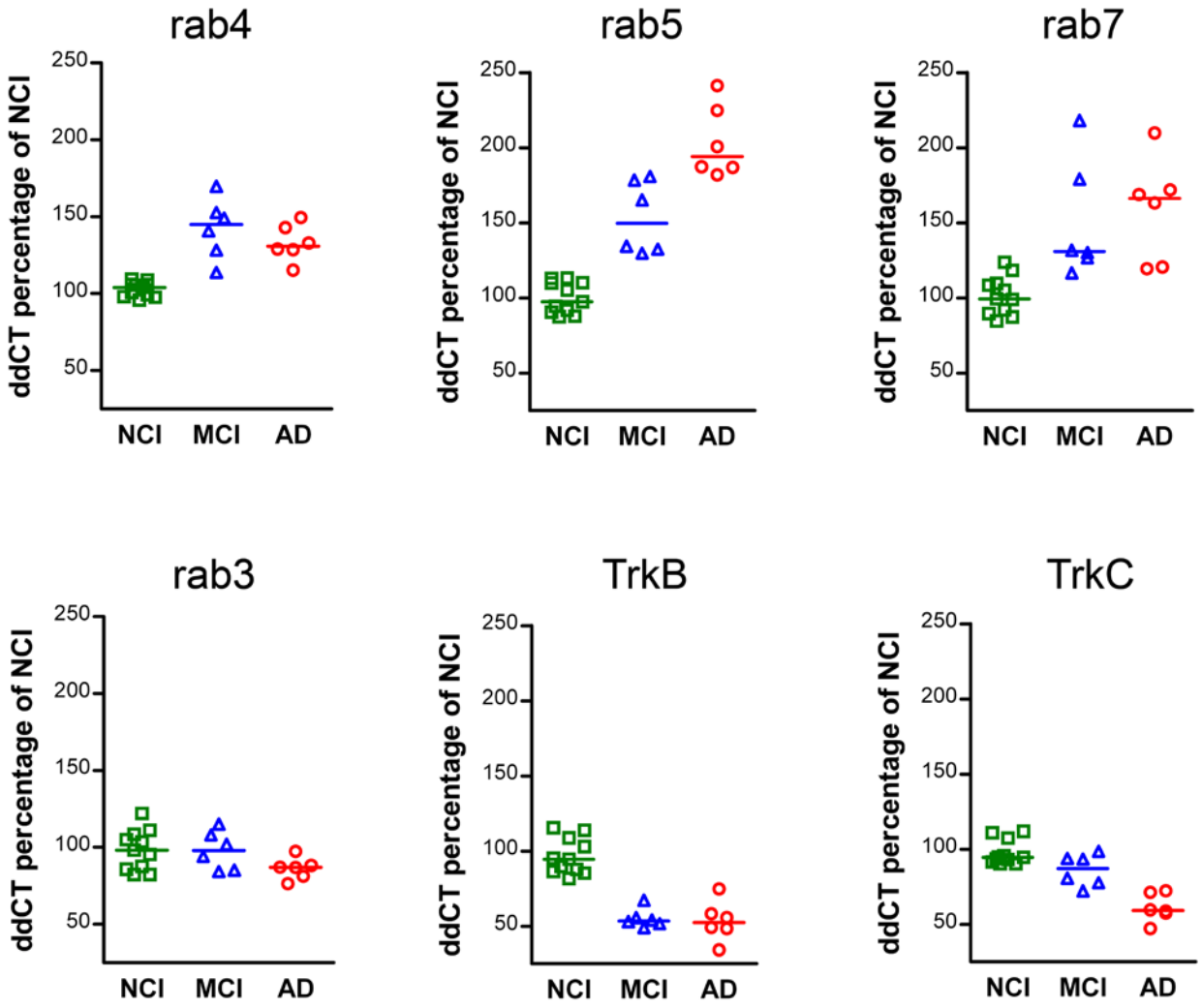

Figure S1. qPCR validation of CA1 neuron microarray findings. Scatterplots (NCI, green squares; MCI, blue triangles; AD, red circles) depict statistically significant up regulation of *rab4* and *rab7* in AD and MCI, indicative of an 'early' alteration ($p < 0.001$). Significant up regulation of *rab5* was also observed ($p < 0.001$) with AD>MCI>NCI, consistent with an 'early', increasing alteration with MCI significantly higher than AD and AD significantly higher than MCI. No differences were observed across the clinical pathological cohort for *rab3* expression. In contrast, statistically significant down regulation of *TrkB* was observed ($p < 0.001$), with AD & MCI<NCI, indicative of an 'early' alteration. Significant down regulation of *TrkC* was also observed ($p < 0.001$) with AD<MCI<NCI, suggesting a 'step down' alteration through the progression of AD.

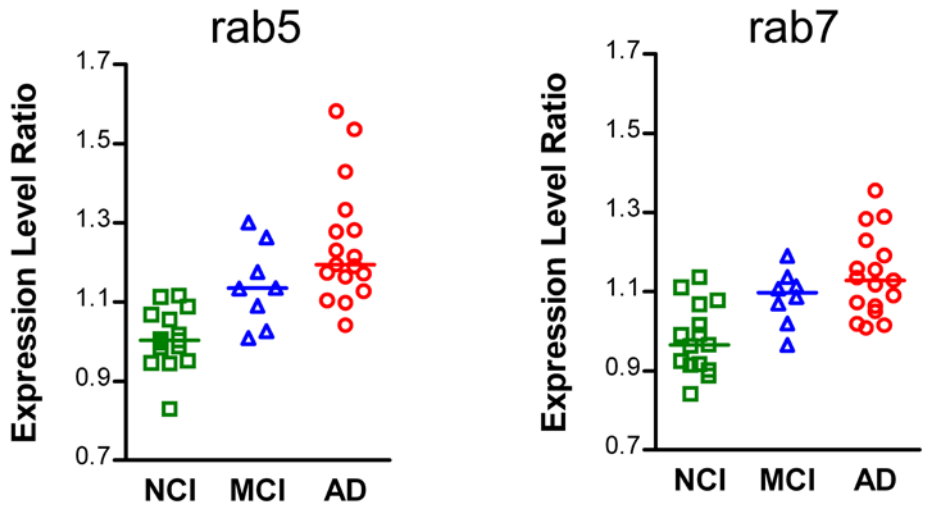

Figure S2. Scatterplots (NCI, green squares; MCI, blue triangles; AD, red circles) showing highly significant up regulation of rab5 protein levels within the hippocampus in AD and MCI ($p < 0.0001$) compared to NCI, consistent with microarray and qPCR observations of an 'early' alteration in rab5 expression. Significant up regulation of rab7 expression ($p < 0.01$; AD>MCI>NCI was also found, indicative of an 'early' alteration that was a 'step-down' between MCI and AD. Data is presented as rab5 and rab7 levels normalized to β-tubulin (TUBB).

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