Resilience of Precuneus Neurotrophic Signaling Pathways Despite Amyloid Pathology in Prodromal Alzheimer's Disease

Supplement 1

Supplemental Subject Data and Methods

Clinical and Neuropathological Evaluations

Memory tests consisted of the East Boston Memory immediate and delayed recall, Logical Memory immediate and delayed story recall, Consortium to Establish a Registry for Alzheimer's Disease (CERAD) Word List Memory immediate and delayed recall, and CERAD Word List Recognition were computed. At consensus conferences, neurologists and neuropsychologists reviewed clinical and medical records and family interviews before assigning a final clinical diagnosis. Neuropathological evaluation was applied as we reported previously (1, 2, 3, 4). Parkinson's disease and dementia with Lewy body cases were excluded. No cases were treated with anticholinesterase inhibitors.

Tissue

For [^{3}H]PiB binding, samples were added to a homogenization buffer (250 mM sucrose, 20 mM Tris base) containing protease inhibitors (Sigma 100X). Another aliquot was for Aβ ELISA (see main text) and another was diluted to 10 mg tissue/mL with potassium PBS (pH 7.4) for western blotting.

Antibodies

All antibodies used in western blot are commercially available and their specificity has been characterized (5, 6, 7) (see Table S1). The antibodies include: proNGF polyclonal

antiserum (1:50, H-20, Santa Cruz, CA); purified TrkA rabbit polyclonal affinity-purified (1:100, Fitzgerald, Acton, MA); sortilin (1:1000) and $p75^{NTR}$ rabbit polyclonal antibody (1:500) obtained from Abcam (Cambridge, MA); p44/p42 MAPK rabbit antibody (Erk1/2) (1:1000), phospho-p44/p42 MAPK (Erk1/2) (Thr202/Tyr204) (E10) mouse antibody (1:2000), SAPK/JNK (56G8) rabbit antibody (1:1000), and phospho-SAPK/JNK (Thr183/Tyr185) (81E11) rabbit antibody (1:2000) (Cell Signaling Technology, Danvers, MA). Loading control was b-tubulin monoclonal antibody (1:4000) (Millipore, Bedford, MA). The following IgG monoclonal antibodies were used for histology: APP/Ab (6E10; 1:1000, Covance, Princeton, NJ), tau AT8 (pSer202/Thr295) (1:1000, ThermoFisher, Pittsburgh, PA), tau TOC-1 (8, 9) (oligomeric tau) (1:400) and Tau C3 (10) (truncated tau at aspartic acid 421) (1:4000) (gifts from L. Binder, Northwestern University Medical Center, Chicago, IL).

Quantitative Immunoblotting

Membranes were blocked in Tris-buffered saline (TBS)/0.05%Tween-20/5% milk for 60 minutes (20^{o}C) with the exception of proNGF, which was blocked in 0.5X TBS/0.5% milk for 20 min, and phospho-JNK and phospho-Erk, which were blocked in TBS/0.05%Tween-20/3% BSA for 60 minutes. After 60-minute primary antibody incubation, membranes were incubated overnight (4^{o}C). After washes (TBS/0.05% Tween-20), membranes were incubated for 1 hour (20^{o}C) with horseradish peroxidase-conjugate goat anti-mouse IgG secondary antibody (1:4000, Pierce, Rockford, IL), horseradish peroxidase-conjugated goat-anti rabbit IgG secondary antibody (1:4000, Bio-Rad, Hercules, CA), goat anti-rabbit IRDye 800CW secondary antibody (1:8000, LI-COR, Lincoln, NE) or goat anti-mouse IRDye 800 CW secondary antibody (1:8000, LI-COR) depending on the first antibody (Table S1). Immunoreactivity was visualized by

enhanced chemiluminescence (Pierce) on a Kodak Image Station 440CF (Perkin-Elmer, Wellesley, MA) or by an Odyssey Infrared Imager (LI-COR). Bands were quantified using Kodak 1D or Odyssey Infrared Imager software. Protein signals were normalized to b-tubulin and samples analyzed in three independent experiments.

Histofluorescence and Immunohistochemistry

Sections were washed in TBS to remove cryoprotectant and incubated in 0.1 M sodium metaperiodate (Sigma, St. Louis, MO) in TBS to inactivate endogenous peroxidase activity. Tissue was permeabilized in TBS containing 0.25% Triton-X (Thermofisher, Waltham, MA) and blocked in TBS containing 3% goat serum for 1 hr. Sections were incubated with appropriate antibody dilutions overnight (20^{o}C) in 0.25% Triton X-100, 1% goat serum solution, then washed in TBS containing 1% goat serum prior to incubation with the secondary antibody biotinylated goat anti-mouse (1:200 dilution for 1 hr) (Vector Laboratories, Burlingame, CA). Following TBS washes, sections were incubated using the Vectastain ABC kit (Vector Laboratories) for 1 hr, rinsed (0.2 M sodium acetate, 1.0 M imidazole buffer, pH 7.4) and developed in acetate-imidazole buffer (0.05% 3,3'-diaminobenzidine tetrahydrochochloride, DAB, Sigma) with or without 1% nickel sulfate. Reaction was terminated in acetate-imidazole buffer, tissue mounted on glass slides, dehydrated through graded alcohols, cleared in xylene and cover slipped using DPX (Biochemica Fluka, Switzerland). TOC-1 antibody staining was performed on sections mounted on charged slides that were boiled in 0.01 M citric acid (pH 8.5) for 20 min for antigen-retrieval.

Plaque Load Quantification

Three randomly selected microscopic fields in three sections (nine images total per marker per case); positive signal was segregated from background by applying a color threshold (pixel intensity cutoff) that was kept constant for each marker. Image quantification was performed using Image J freeware (NIH, Bethesda, Maryland). Morphological characteristics differentiated diffuse and compact/cored plaques. X-34 positive dystrophic neurites confirmed that all compact/cored plaques were neuritic.

Statistical Analysis

Analyses were performed using Sigma Stat 3.5 (Systat Software, Inc., San Jose, CA) or SAS 9.2 (SAS Institute Inc., Cary, NC) and graphed using box and scatter plots (Sigma Plot 10.0; Systat Software, Inc.).

Table S1. Summary of antibodies

	Antibodies	Epitope or Immunogen	Dilution	Company and Catalog #
ProNGF	rabbit polyclonal to NGF (H-20)	N-terminus of NGF	1:50	Santa Cruz Biotechnology; # sc-548
TrkA receptor	rabbit polyclonal to TrkA	External domain of TrkA	1:100	Fitzgeral Industries International; # 20R-TR013
$p75^{NTR}$ receptor	rabbit polyclonal to p75 NGF receptor	aa' 250-350 of $p75^{NTR}$	1:500	Abcam; # ab38335
Sortilin	rabbit polyclonal to Sortilin	C-terminus of sortilin	1:1000	Abcam; # ab16640
JNK	rabbit polyclonal to SAPK/JNK (56G8)	Human JUNK2/MBP	1:1000	Cell Signaling Technology; # 9258
Phospho-JNK	rabbit polyclonal to phospho SAPK/JNK (81E11)	phospho-Thr 183, phospho-Tyr 185	1:2000	Cell Signaling Technology; # 4668
Erk	rabbit polyclonal to p44/42 MAPK (Erk1/2)	Erk1 (p44), Erk2 (p42)	1:1000	Cell Signaling Technology; # 9102
Phospho-Erk	mouse monoclonal to phospho p44/42 MAPK (Erk1/2) (E10)	phospho-Thr202, phospho-Tyr 204	1:2000	Cell Signaling Technology; # 9106
β-tubulin	mouse monoclonal to tubulin (KMX-1)	tubulin from polycephalum myxamoebae	1:4000	Millipore; # MAB3408
APP/Aβ (6E10)	mouse monoclonal to human Aβ	residues1-16 of Aβ	1:2000	Covance; # SIG-39320
Tau (AT8)	mouse monoclonal to phosphorylated tau	phosphor-Ser202, phospho-Thr 205	1:1000	ThermoFisher; # MN1020
Tau TOC-1	mouse monoclonal to oligomeric tau	cross-linked tau dimers	1:400	Gift from L. Binder
Tau C3	mouse monoclonal to truncated tau	C-terminus of tau truncated at aspartic acid 421	1:4000	Gift from L. Binder

Table S2. Summary of precuneus soluble $A\beta_{1-42}$ and [^{3}H]PiB levels in RROS cases, by clinical diagnosis category

	NCI (*n* = 14)	MCI (*n* = 14)*	AD (*n* = 14)	*p*-value[a]	Pair-wise comparison
Soluble $A\beta_{1-42}$	5.5 ± 6.9 (0.06 - 21.6)[b]	5.6 ± 5.3 (0.06 - 18.1)	13.7 ± 7.0 (0.68 - 27.6)	0.004	(NCI, MCI) < AD
[^{3}H]PiB	126.8 ± 92.0 (37 - 325)	165.8 ± 138.7 (28 - 423)	305.6 ± 148.7 (83 - 662)	0.003	(NCI, MCI) < AD

AD, Alzheimer's disease; MCI, mild cognitively impaired; NCI, no cognitive impairment; RROS, Rush Religious Order Study.

*Amyloid data not available for one MCI case.

[a]Kruskal-Wallis test, with Dunn's correction for multiple comparisons.

[b]Mean ± standard deviation (range).

Table S3. Summary APP/Aβ, 6-CN-PiB and X-34 plaque density/load by clinical diagnosis

	NCI (*n* = 6)	**MCI (*n* = 6)**	**AD (*n* = 6)**	***p*-value[a]**	**Pair-wise comparison**
APP/Aβ plaque density (6E10)	0.74 (0-5.39)[b]	4.80 (0.69-6.11)	4.67 (4.21-5.57)	0.071	--
Diffuse 6-CN-PiB	0 (0-0.86)	0.22 (0-2.67)	0.38 (0.12-0.90)	0.059	--
Compact 6-CN-PiB	0 (0-0.67)	0.77 (0-1.23)	0.92 (0.43-1.58)	0.030	NCI < AD
Total 6-CN-PiB load	0 (0-1.20)	1.05 (0-2.68)	1.06 (0.54-1.84)	0.060	--
Diffuse X-34	0 (0-1.39)	0.67 (0-2.50)	0.53 (0.08-1.04)	0.099	--
Neuritic X-34	0 (0-1.78)	0.73 (0-1.68)	1.06 (0.61-1.18)	0.10	--
Total X-34 load	0 (0-2.19)	1.13 (0-2.51)	1.27 (0.66-1.62)	0.11	--

AD, Alzheimer's disease; MCI, mild cognitively impaired; NCI, no cognitive impairment.
[a]Kruskal-Wallis test, with Dunn's correction for multiple comparisons.
[b]Median (range) of log-transformed plaque density (number/mm^2) or load values.

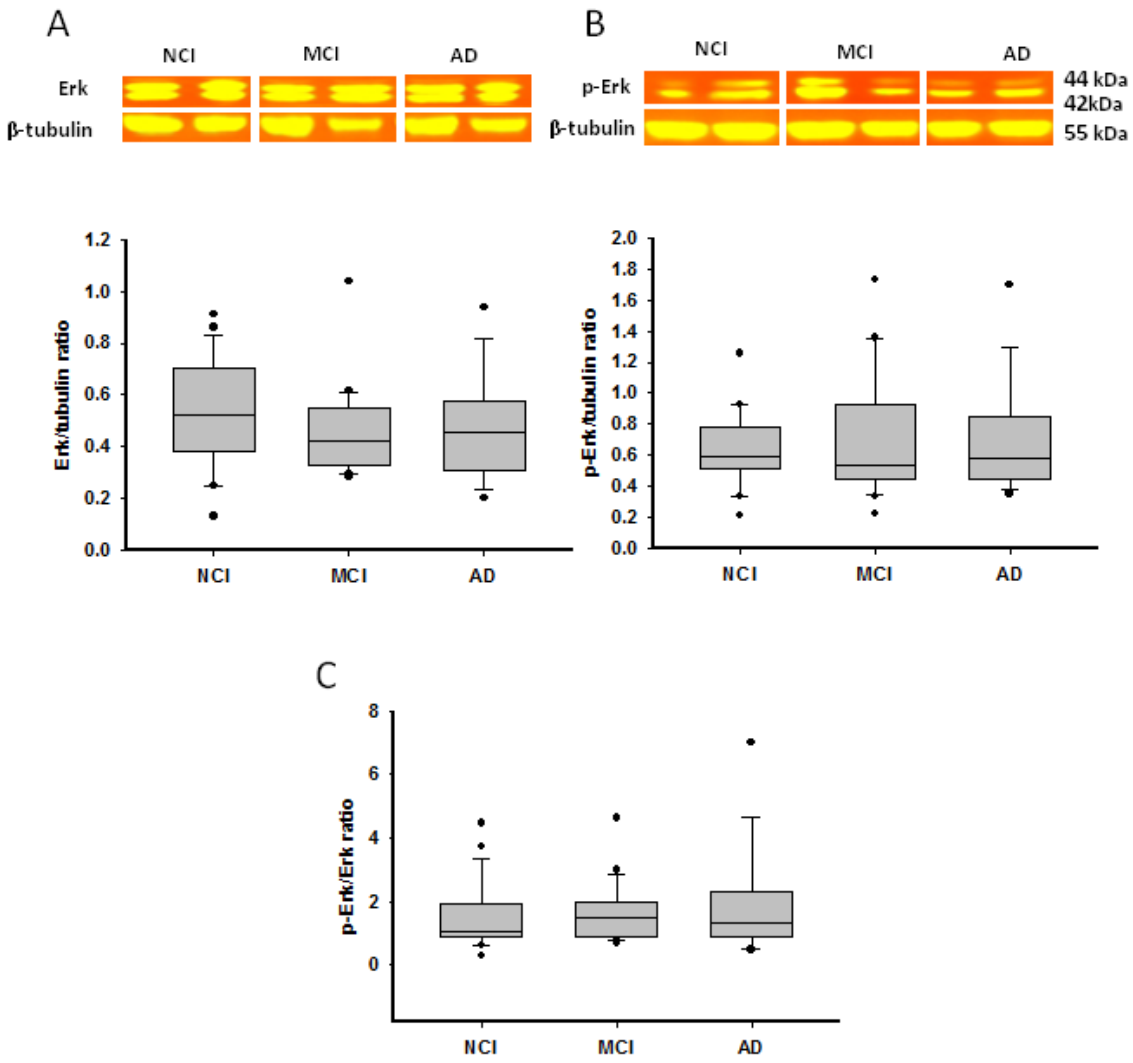

Figure S1. Representative immunoblots and box plots of precuneus levels of total Erk, phospho-Erk (p-Erk) in cases clinically diagnosed as NCI, MCI and AD. Box-plot of the phospho-Erk/Erk ratio is also presented. β-tubulin probe was used to normalize the immunoreactive signals obtained by densitometry in the blots. Levels of Erk (A), phospho-Erk (B) and phospho-Erk/Erk ratio (C) revealed no statistical differences across the clinical groups. Black dots in box-plots indicate outliers. AD, Alzheimer's disease; MCI, mild cognitively impaired; NCI, no cognitive impairment.

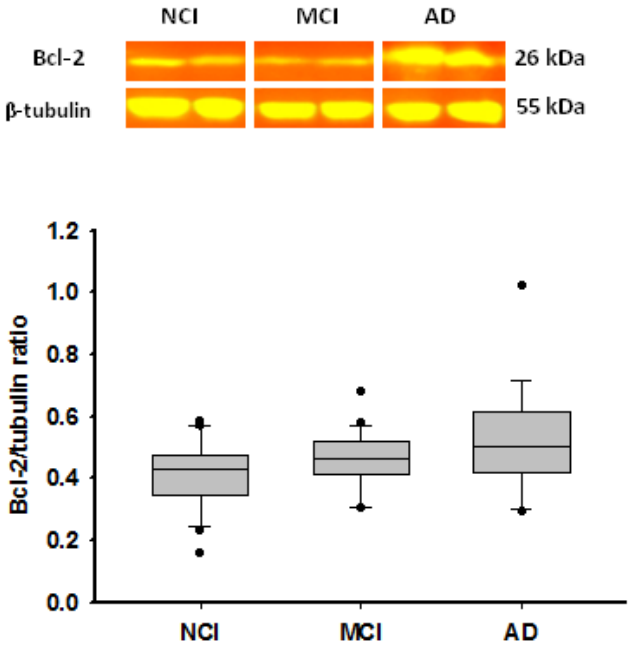

Figure S2. Representative immunoblot and box plot of precuneus levels of Bcl-2 in NCI, MCI and AD cases. β-tubulin probe was used to normalize the immunoreactive signals obtained by densitometry. Levels of Bcl-2 were significantly higher in AD compared to NCI (**p* = 0.043). Black dots in box-plots indicate outliers. AD, Alzheimer's disease; MCI, mild cognitively impaired; NCI, no cognitive impairment.

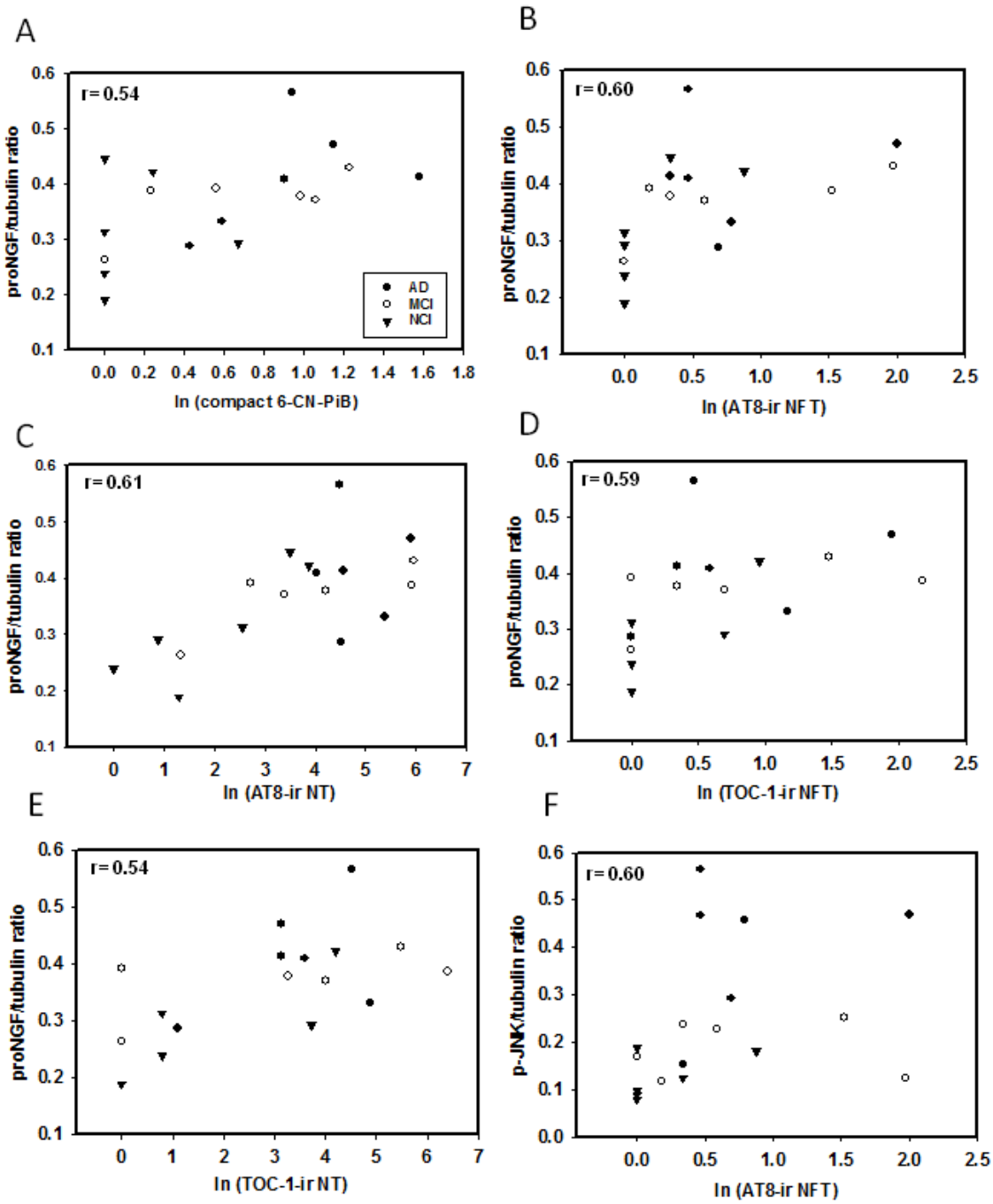

Figure S3. Precuneus proNGF levels correlated positively of compact (neuritic) 6-CN-PiB load (A) as well as AT8 positive NFT (B) and NT (C) and TOC-1 positive NFT (D) and NT (E) density using natural logarithm transformation (ln) of the data. Phospho-JNK correlated positively with the ln transformed data of AT8 positive NFT (F) density. NCI, filled triangles; MCI, open circles; AD, filled circles. AD, Alzheimer's disease; MCI, mild cognitively impaired; NCI, no cognitive impairment; NFT, neurofibrillary tangle; NT, neuropil thread.

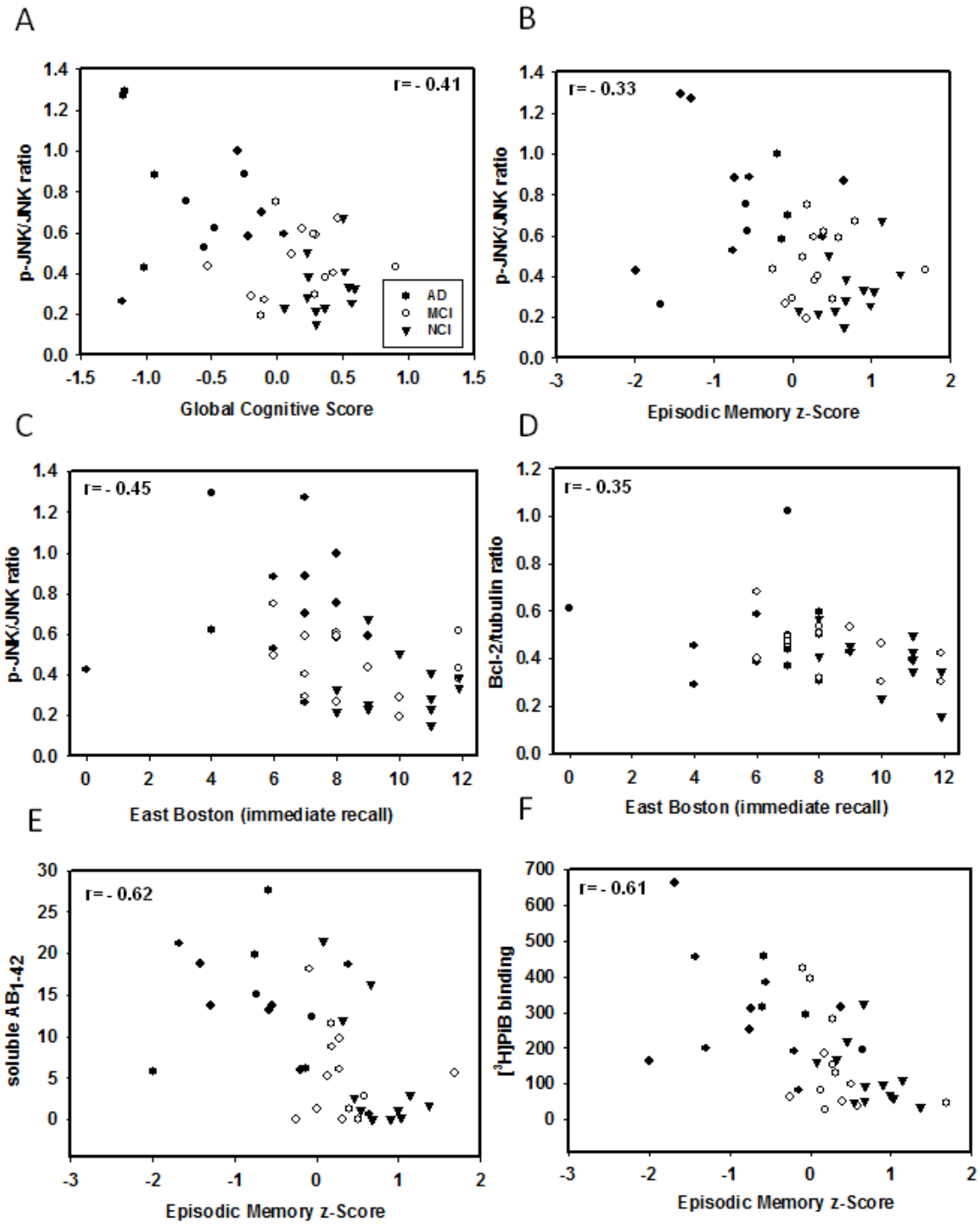

Figure S4. Precuneus phospho-JNK/JNK (p-JNK/JNK) ratio levels correlated negatively with several of the cognitive tests: global cognitive score (A), episodic memory z-score (B) and East Boston memory immediate recall (C), while Bcl-2 levels only correlated with East Boston memory immediate recall (D). Precuneus soluble $Ab_{1\text{-}42}$ (E) and [^{3}H]PiB binding (F) levels correlated also negatively with episodic memory z-score. NCI, filled triangles; MCI, open circles; AD, filled circles. AD, Alzheimer's disease; MCI, mild cognitively impaired; NCI, no cognitive impairment.

Supplemental References

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