Supplemental Experimental Procedures

Chemicals and Antibodies

Staurosporine, cycloheximide, and zVAD were purchased from Calbiochem (La Jolla, California). Recombinant caspase 3 was purchased from Pharmingen (San Diego, CA). The anti-caspase 3 active fragment, anti-caspase 3, anti-EGFR, anti-HA, anti-Myc antibodies were purchased from Cell Signaling (Danvers, MA). The anti-BACE C-terminal antibody was purchased from Affinity Bioreagents (ABR, Golden, CO). The polyclonal antibody A8717, raised against the C-terminus of APP, and the anti-β-tubulin antibody were purchased from Sigma (St Louis, MO). The monoclonal antibody, WO2, raised against 1-17 amino acids of Aβ region was a gift from Dr. Beyreuther. The Asp-1 antibody was purchased from Oncogene (Cambridge MA). The anti-TACE antibody was purchased from Santa Cruz (Santa Cruz, California). The anti-Cu,Zn-SOD antibody was a gift from Dr. Naoyuki Taniguchi. The monoclonal antibodies anti-GGA3 and anti-β-catenin were purchased from Transduction Laboratories (Newington, NH). BACE was detected in human brains by SECB1, which recognizes amino acids 296-310 of BACE amino terminus (Li et al., 2004; Yang et al., 2003). The GAPDH antibody was purchased from Chemicon, Temecula CA.

Cell culture, Western blot analysis, and induction of apoptosis

H4 human neuroglioma cells expressing APP751 (H4-APP751) and APP-SWE (H4-APPSWE) were grown in DMEM containing 10% FBS, 200 μg/mL G418, 250 μg/mL zeocin, 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/mL streptomycin. For the induction of apoptosis, we used staurosporine or etoposide (Calbiochem). For time-

course experiments, cells were seeded at a density of $2x10^6$ cells per 100 mm dish and treated with STS (1µM) or etoposide (100 µg/mL). In order to inhibit caspase activation a sister plate of cells was pre-treated with zVAD (100µM, Calbiochem) for 1 hr before STS treatment. At different time points (0, 3, 6, 9, 12, and 24 hr), the cells were scraped, centrifuged, and then lysed in buffer containing 1% NP40. Western blot analysis was performed as previously described (Tesco et al., 2003). Densitometry analysis was performed on a Macintosh computer using the public domain NIH Image program (developed at the U.S. National Institutes of Health and available on the Internet at http://rsb.info.nih.gov/nih-image/) or using a Versadoc Imager and QuantityOne software (BioRad).

RNA Isolation and Northern Blot Analysis

Total RNA was extracted using TRIZOL (Invitrogen). Northern blot analysis was performed as previously described (Koh et al., 2001). Human cDNA of BACE was labeled with [γ-32P]dCTP (PerkinElmer, Norwalk CT) using random hexanucleotide primers (Prime-a-gene labeling system; Promega Madison WI).

Metabolic Labeling and Pulse-Chase Experiments

H4-APP751 cells were preincubated in methionine/cysteine-free (starve) medium for 30 min, after which they were incubated in starve medium supplemented with 1 mCi of [^{35}S]methionine/cysteine (Amersham, Piscataway NJ) per well for 60 min (pulse). Then, cells were incubated in the presence of excess amounts of cold methionine/cysteine for indicated time (chase). The cells were then washed, lysed in radioimmunoprecipitation

assay (RIPA) buffer (1% sodium deoxycholate, 0.1% SDS, 1% Triton X-100, 5 mM EDTA, 50 mM Tris, pH 8, 150 mM NaCl), and immunoprecipitated with the specific antibodies. Samples were separated by SDS-PAGE using 4-12% gels, fixed, dried, and exposed to film or a phosphorimaging screen (Bio-Rad). Images were analyzed using a Personal Molecular Imager FX and quantified using Quantity One software (Bio-Rad, Hercules CA). For BACE pulse-chase experiments, the anti-BACE antibodies were unable to immunoprecipitate endogenous BACE. Thus, H4-APP751 cells were transfected with 10 μg of pcDNA-BACE-myc cDNA using Superfect (Quiagen, Bothell WA) according to the manufacturer' s protocol. 24 hr following transfection, cells were harvested and pooled together to avoid difference in transfection efficiency and plated again. After 24 hr, cells were metabolically labeled as described above.

Middle cerebral artery occlusion

Female Charles River Sprague-Dawley rats (250g, Wilmington, MA) were acclimatized for three days before surgery. Bilateral ovariectomy was performed 2 weeks before MCAO. The University of North Texas Health Science Center Animal Care and Use Committee approved all animal procedures. Ischemic stroke was induced by occlusion of the middle cerebral artery (MCAO) as described before (Wen et al., 2004). The animals were anesthetized and decapitated at the desired time after the onset of reperfusion (12, 24 and 48 hrs). The brains were harvested, separated into ischemic and non-ischemic hemisphere, dissected in cortex (Ctx) and sub-cortex (Suc-Ctx), and frozen in liquid nitrogen. Then, the tissues were homogenized in RIPA buffer and analyzed by WB.

Real time PCR

Total RNA was extracted using RNeasy Mini Kit (Qiagen). Equal quantities of DNAse treated RNA samples were subjected to cDNA synthesis using Superscript III Reverse Transcriptase (Invitrogen). Subsequently, SYBR Green Master PCR Mix (Applied Biosystems) and target-specific PCR primers for GGA3 (5'-GGGACAGGGTGTGAGAAAG-3' and 5'- AGAGGGGATCAGCGTCCTAT-3') and GAPDH (5'-GGTCTCCTCTGACTTCAACA-3' and 5'-GTGAGGGTCTCTCTCTTCCT-3') were used for amplification of cDNA samples with iCycler real time PCR machine (Bio-Rad). PCR primers were designed to amplify a region flanking two different exons and the target specificity of PCR products were confirmed by sequencing. Standard curve method was used to obtain GAPDH normalized GGA3 values.

Site-directed mutagenesis

Site-directed mutagenesis was perfomed using QuickChange site-directed mutagenesis kit (Stratagene) according to manufacturer's instructions. The primers used to produce the D313A substitution: (forward) CCTTAACCCTGCCTGCCTCGGAAGGAAAC and (reverse) GTTTCCTTCCGAGGCAGGCAGGGTTAAGG. The primers used to produce D328A mutation were: (forward) GGCACGCTCATCGCCCTTGCGGAGCTGG and (reverse) CCAGCTCCGCAAGGGCGATGAGCGTGCC. The primers used to produce D333A mutation were: (forward) GACCTTGCGGAGCTGGCCACGACCAACAG and (reverse) CTGTTGGTCGTGGCCAGCTCCGCAAGGTC. The primers used to produce D428A mutation were: (forward) CAGTCCGACCTGGCCTTCTTCAGCCCC and

(reverse) GGGGCTGAAGAAGGCCAGGTCGGACTG. The GGA3DN was produced with the introduction of a stop codon. The primers used to produce GGA3DN were: (forward) CCCTGCCTGACTAGGAAGGAAACAGTCAGTGC and (reverse) GCACTGACTGTTTCCTTCCTAGTCAGGCAGGG. The resulting cDNA constructs were sequenced for verification.

Lentiviral RNAi

Custom-designed lentiviral vectors (pLKO.1) carrying expression cassettes that express shRNAs that target human and mouse GGA3 gene were purchased from Sigma. The packaging of the virus was performed as previously described (Sena-Esteves et al., 2004)

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Supplemental Figure Legend

Fig. 1 Depletion of GGA3 by siRNA increases EGFR levels

A-B: the graph represents mean ± SEM of 4 measurements of GGA3 or EGFR protein levels, respectively, in H4-APP751cells treated with siGGA3 or siNeg. Levels of EGFR were measured 72 hr after BACE and siGGA3 or siNeg co-transfection. Densitometry was performed using Versadoc Imager and QuantityOne software (Bio-Rad). GGA3 or EGFR densitometry values were normalized against GAPDH values. Mann-Whitney test was used for statistical analysis.

Fig. 2 Depletion of GGA3 by lentiviral RNAi increases both ectopically expressed and endogenous BACE in N2A cells

A: Murine N2A cells were infected with lentivirus expressing either shRNA negative control or shRNA for murine GGA3 gene. Three different murine shRNA lentivirus were tested. After 72 hr, myc-tagged BACE vector was transfected in N2A cells. After additional 72 hr, GGA3 protein levels were determined by WB with anti-GGA3 antibody (Trasduction Laboratories). BACE protein levels were detected by WB using anti-myc polyclonal antibody (Cell signaling). GAPDH was used as loading control. B-C: the graphs represent mean ± SEM of 4 GGA3 or BACE levels measurements, respectively. Densitometry was performed using Versadoc Imager and QuantityOne software (Bio-Rad). GGA3 and BACE densitometry values were normalized against GAPDH values. The levels of GGA3 were 40% and 20% in cells infected with 310 or 306 lentivirus, respectively, compared to levels in cells infected with negative control virus. The levels

of BACE were 150% and 200% in cells infected with 310 or 306 lentivirus, respectively, compared to levels in cells infected with negative control virus. D: N2A cells were infected as described above. After 72 hr GGA3 protein levels were determined by WB with anti-GGA3 antibody (Trasduction Laboratories). Endogenous BACE protein levels were detected by WB using anti-BACE polyclonal antibody (Affinity Bioreagents). GAPDH was used as loading control. E: the graph represents mean ± SEM of 3 BACE levels measurements,. Densitometry was performed using Versadoc Imager and QuantityOne software (Bio-Rad). GGA3 and BACE densitometry values were normalized against GAPDH values. The levels of endogenous BACE were 140% in cells infected with 306 lentivirus, compared to levels in cells infected with negative control virus.

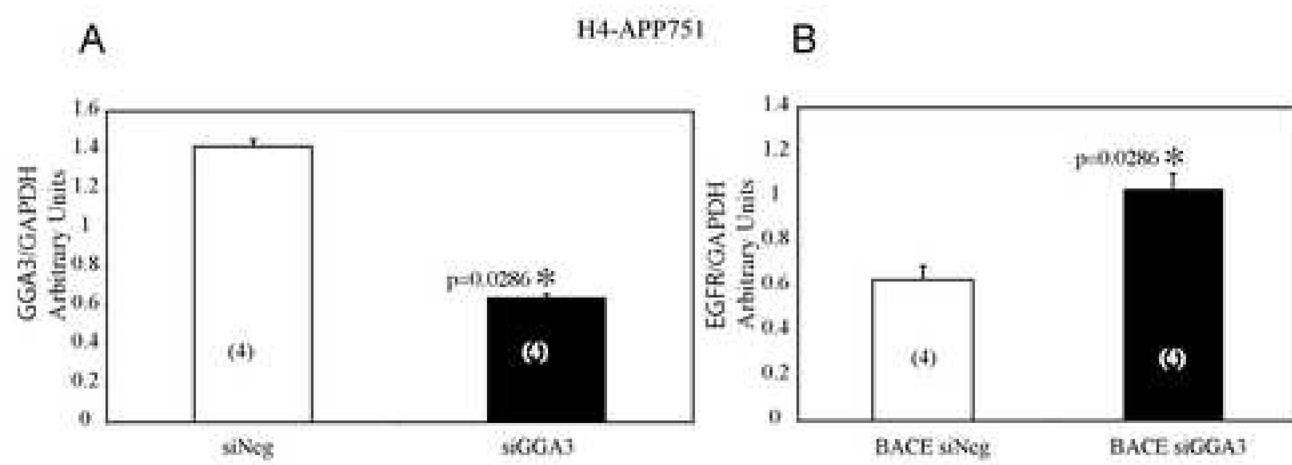
Fig. 3 Depletion of GGA3 affects APP processing independently of γ-secretase activity

A: H4 cells expressing either APP-751 or the APP-CTF (APP105) were infected with lentivirus expressing either shRNA negative control or shRNA for human GGA3. After 72 hr, GGA3 protein levels were determined by WB with anti-GGA3 antibody (Trasduction Laboratories). GAPDH was used as loading control. B-C: the graphs represent mean ± SEM of 6 Aβ40 measurements by ELISA in H4-APP751 or H4-APP105 APPSWE, respectively. Aβ concentration was normalized against the concentration of protein in cell lysates. Unpaired T-test with Welch correction was used for statistical analysis.

Fig. 4 Levels of GGA3 are decreased in AD cerebellum

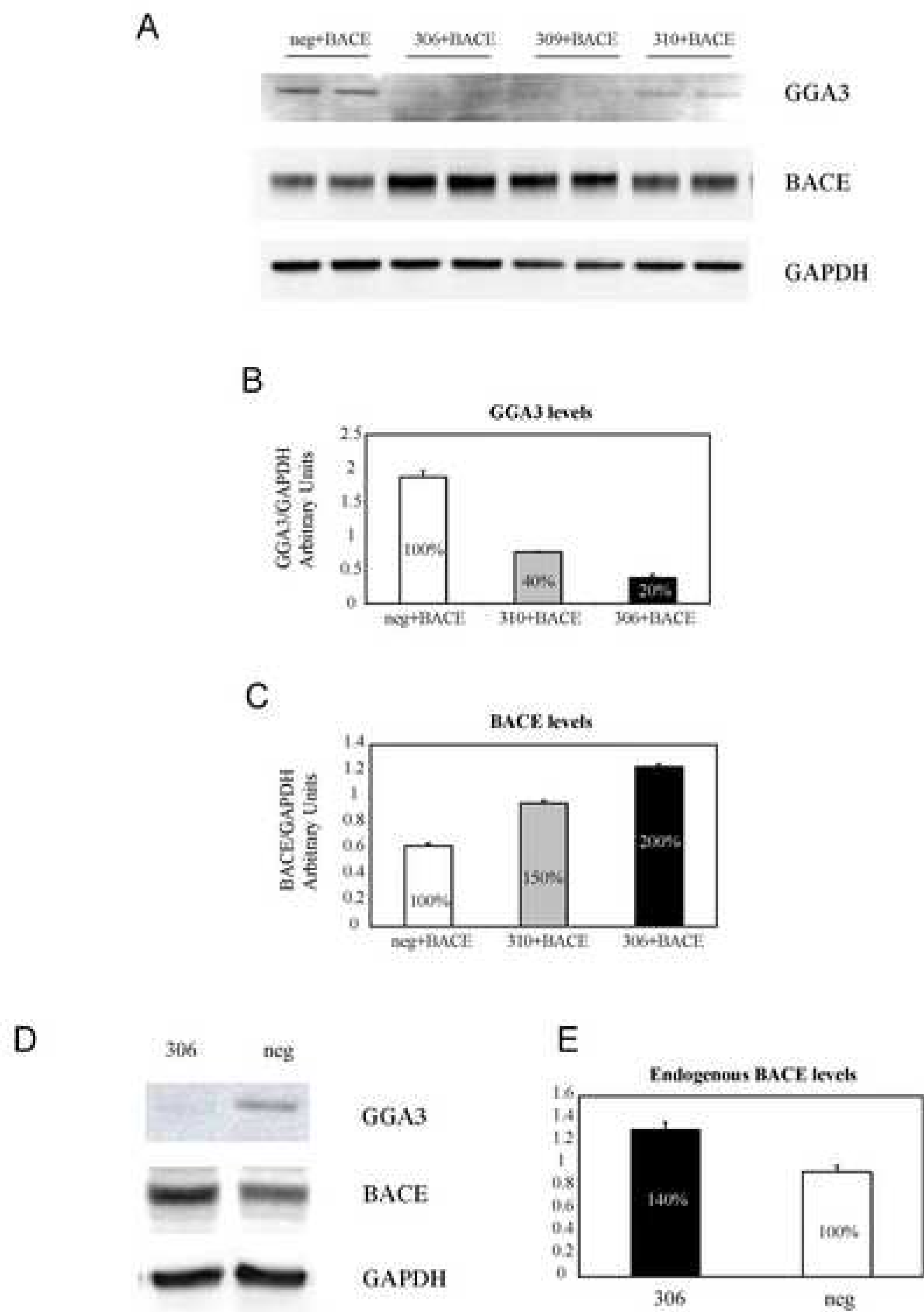
A: Western Blot analysis of cerebellum of human brains. AD = Alzheimer's disease. ND = non-demented control. BACE was detected by SECB1. GGA3 was detected by anti-GGA3 antibody. GAPDH was used as loading control. B-C**.** BACE and GGA3 densitometry values were normalized against GAPDH values. At least triplicate of each samples were analyzed. The graphs represent mean ± SEM of 18 ND and 18 AD. Unpaired t-test and unpaired t-test with Welch correction ware used to perform statistical analysis of BACE and GGA3 levels, respectively. D-E. Linear correlation analysis between GGA3 levels in temporal cortex (TC) and cerebellum in ND and AD, respectively. The dotted line indicates the 95% confidence interval. F: the graph represents the distribution of AD versus ND relative to levels of GGA3 both in TC and cerebellum.

Figure



Suppl Fig 1

Figure



Suppl Fig. 2

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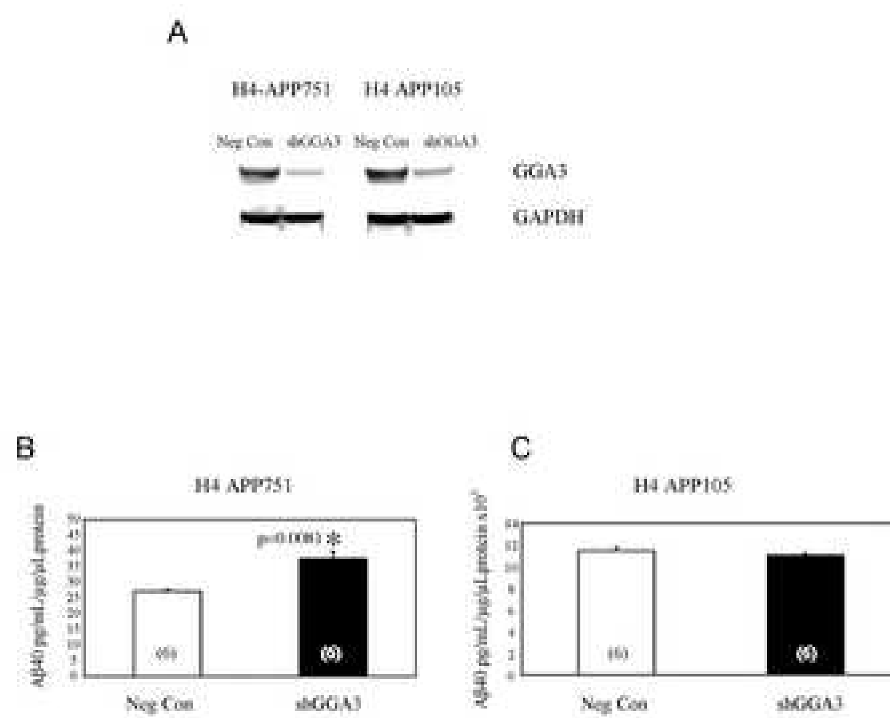

Suppl Fig. 3

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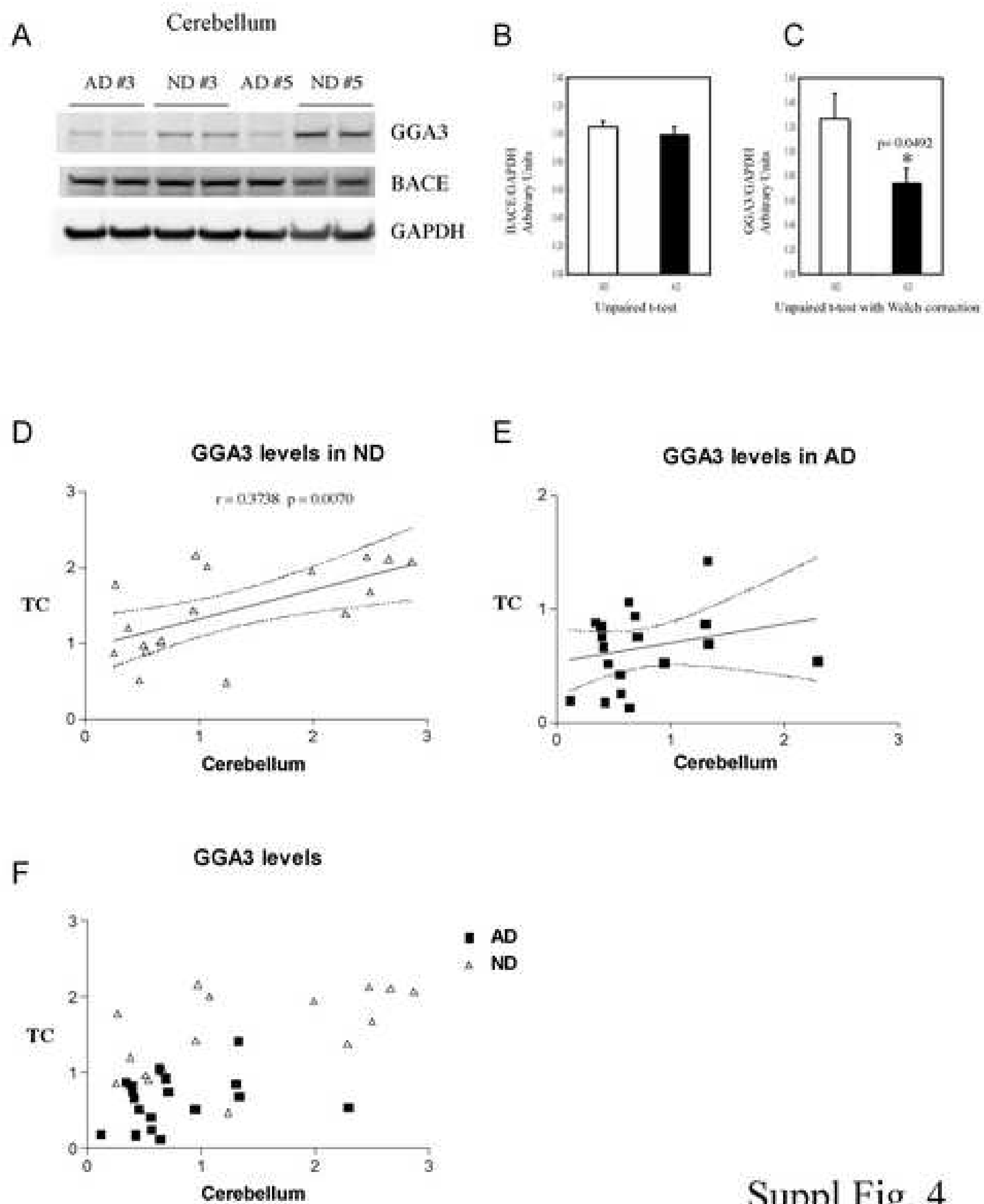

Suppl Fig. 4