Supplementary Data File 2

Measurements of ChAT, AChE and BChE expression, specific activity and function

Measurement of ChAT was performed as described previously.[1] Briefly, colon muscle extracts were homogenised in K_2PO_4 buffer 50 mM containing EDTA (2 mM), followed by resuspension and homogenisation in the same buffer containing NaCl 500 mM and resuspension and homogenisation in the same buffer containing 0.1% Triton X 100. The samples were diluted to between 0.3 – 0.7 mg/ml. High binding 384-well microtiter plates (Nunc Maxisorb, Denmark) were pre-coated overnight at 4°C with 75 µL/ well of MAB 3447 (1/250 in the coating buffer). After washing, triplicates of the samples or recombinant ChAT (all 10 µL/ well) and choline chloride standards (50 µM, two-fold serial dilution in TBS, 50 µL/ well) were applied to the plate. All samples and the ChAT standards were applied as native and denatured samples (by heating in a thermal cycler 3 times 8 min at 99°C).

Cocktail-A, prepared in TBS-T 0.05% buffer, contained acetyl coenzyme-A (62.5 µM), phosphotransacetylase (1.25 U/ ml), acetyl-phosphate (8.75 mM), choline chloride (15 µM) and eserine hemisulfate (0.25 mM; Sigma-Aldrich). 40 µL of this solution was added to each well, aside from those containing the choline chloride standards. The plate was sealed and incubated in a humid chamber for 1 h at 38°C under constant, gentle linear shaking. Immediately after incubation, 25 µl/ well of Cocktail B (50 mM PBS, pH-7.6, containing Streptavidin-HRP (1/ 5000, Invitrogen), choline oxidase (0.93 U/ ml), 4-aminoantipyrine (3 mM) and phenol (6.3 mM; Sigma-Aldrich) was added to all wells, including the choline standards. Reaction absorbance was monitored using a microplate spectrophotometer reader (Infinite® M1000, Tecan) at 500 nm wavelength. The concentration of ChAT in the samples was determined using an ELISA (abx257416, Abbexa, UK) as per manufacturer's instructions, and expressed per mg protein. Specific activity of the enzyme was determined as activity/ expression.

Specific AChE and BChE activity was measured using a modified Ellman's colorimetric assay in the presence of the selective butyrylcholinesterase inhibitor ethopropazine (100 µM; Sigma-Aldrich) and acetylthiocholine (0.5 mM) as the substrate (for AChE activity), or in presence of the selective AChE inhibitor BW284C51 (1 µM; Sigma-Aldrich) and

butyrylthiocholine (5 mM) as the substrate (for BChE activity).[2,3] Expression was determined using sandwich ELISA as previously described. [4]

1 Vijayaraghavan S, et al. Regulated extracellular choline acetyltransferase activity - The plausible missing link of the distant action of acetylcholine in the cholinergic anti-inflammatory pathway. *PLoS One* 2013;8:e65936.

2 Ellman GL, Courtney KD, Andres Jr, V, et al. A new and rapid colorimetric determination of acetylcholinesterase activity. *Biochem Pharmacol* 1961;7:88–95.

3 Darreh-Shori T, et al. Sustained cholinesterase inhibition in AD patients receiving rivastigmine for 12 months. *Neurology* 2002;59:563–572.

4 Darreh-Shori T, Kadir A, Almkvist O, et al. Inhibition of acetylcholinesterase in CSF versus brain assessed by 11C-PMP PET in AD patients treated with galantamine. Neurobiol Aging 2008;29:168-184.