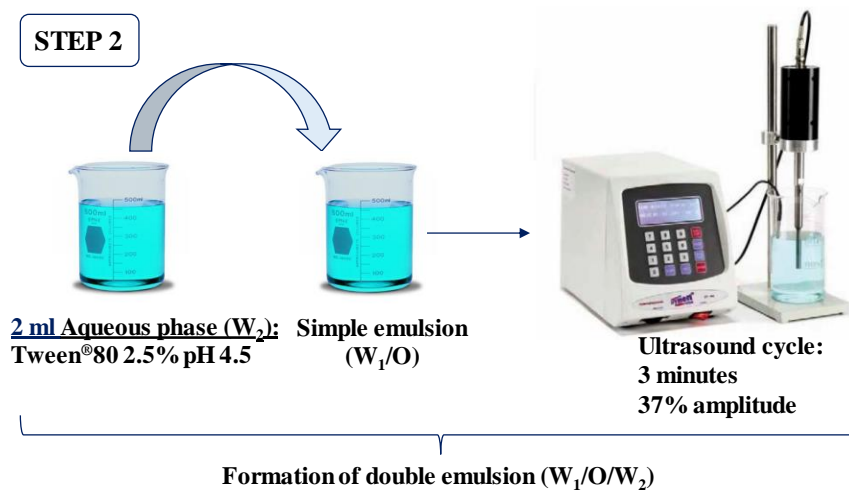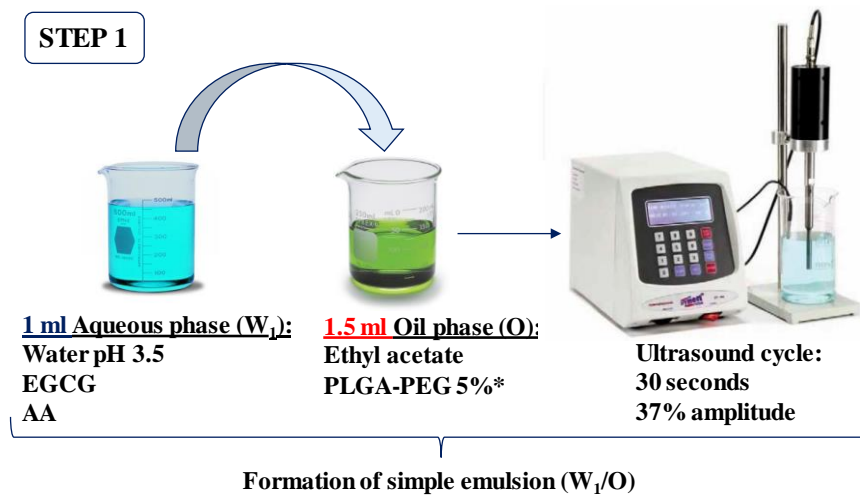# Supplementary material

Dual-drug loaded nanoparticles of Epigallocatechin-3-gallate (EGCG) / Ascorbic acid enhance therapeutic efficacy of EGCG in a APPswe/PS1dE9 Alzheimer's disease mice model.

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Figures

STEP 1

1 ml Aqueous phase (W_1):
Water pH 3.5
EGCG
AA

1.5 ml Oil phase (O):
Ethyl acetate
PLGA-PEG 5%*

Ultrasound cycle:
30 seconds
37% amplitude

Formation of simple emulsion (W_1/O)

STEP 2

2 ml Aqueous phase (W_2):
Tween®80 2.5% pH 4.5

Simple emulsion (W_1/O)

Ultrasound cycle:
3 minutes
37% amplitude

Formation of double emulsion (W_1/O/W_2)

STEP 3

2 ml Outer covering Tween®80 0.04% pH 4.5

Double emulsion (W$_1$/O/W$_2$)

Evaporation of organic solvent under stirring (24h)

Stabilization of double emulsion and evaporation of organic solvent

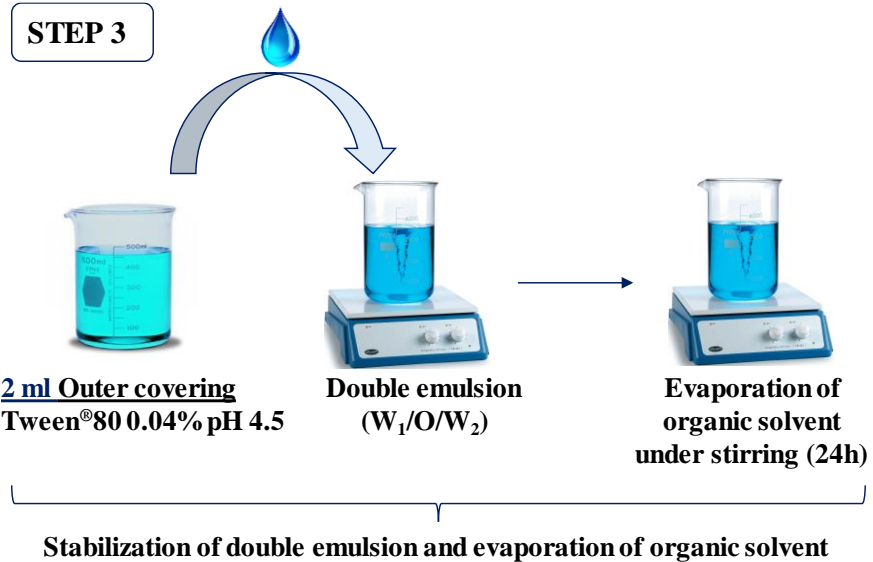**Figure S1.** Fabrication of EGCG/AA NPs by double emulsion method. Final volume of 5 ml. *Percentage of PEG that contains PLGA.

Month 0
Groups (n=10/group):
WT – Non treated
APP/PS1 – Non treated
APP/PS1 – free EGCG
APP/PS1 – EGCG/AA NPs

Month 5
Beginning of behavioral studies:
Morris Water Maze
Novel Object Recognition

GENERATION OF MICE GROUPS

T R E A T M E N T

Month 3
Beginning of treatments
(40 mg/kg/day)

Month 6
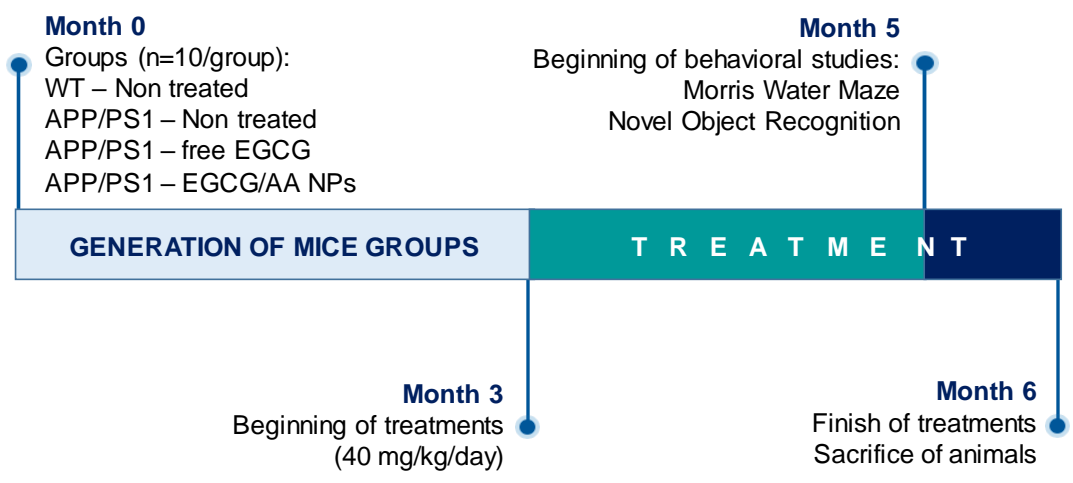Finish of treatments
Sacrifice of animals

Figure S2. Timeline, groups distribution, dosage and behavioural tests of EGCG/AA NPs and free EGCG treatments. Dose of both treatments were recalculated and readjusted every week according to the weight gain of the animals.
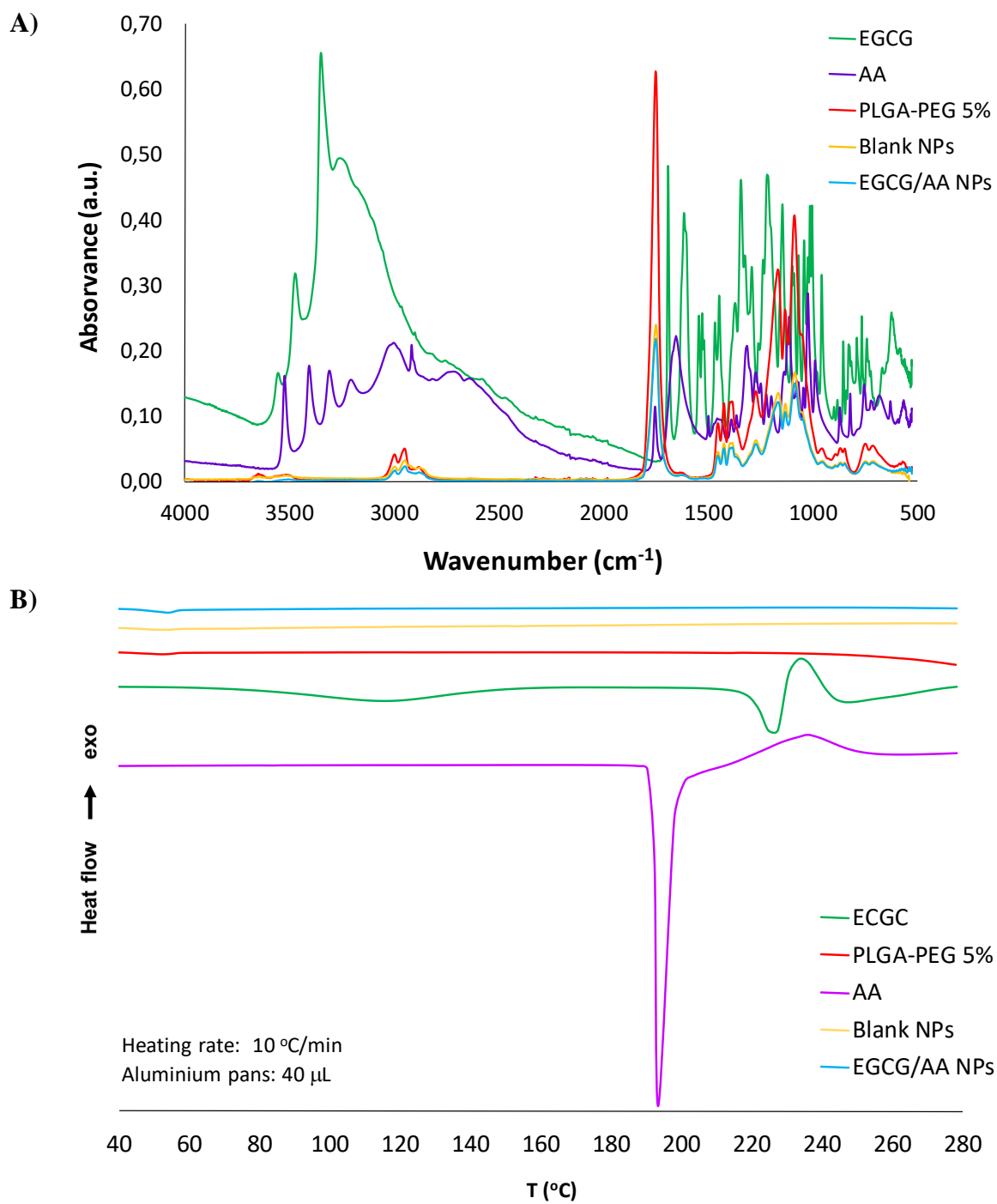
A)

B)

Figure S3. Interaction studies of EGCG/AA NPs compounds. **A)** FTIR spectra. **B)** DSC thermograms.
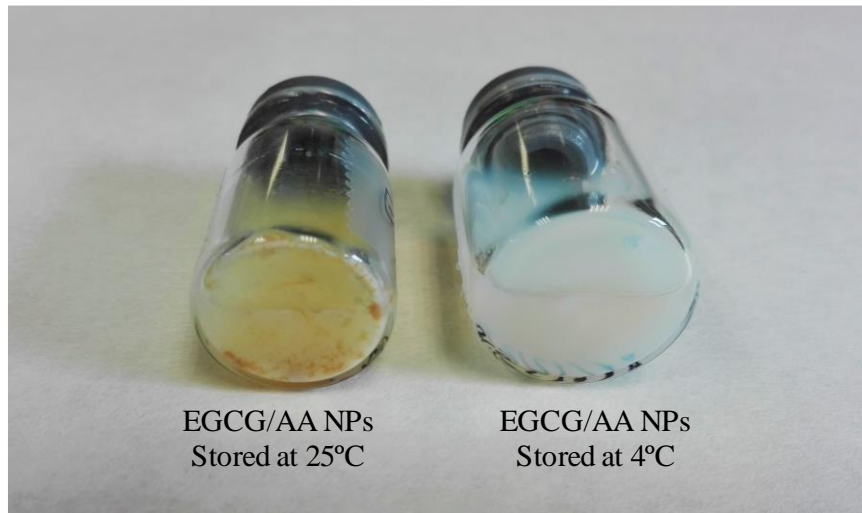
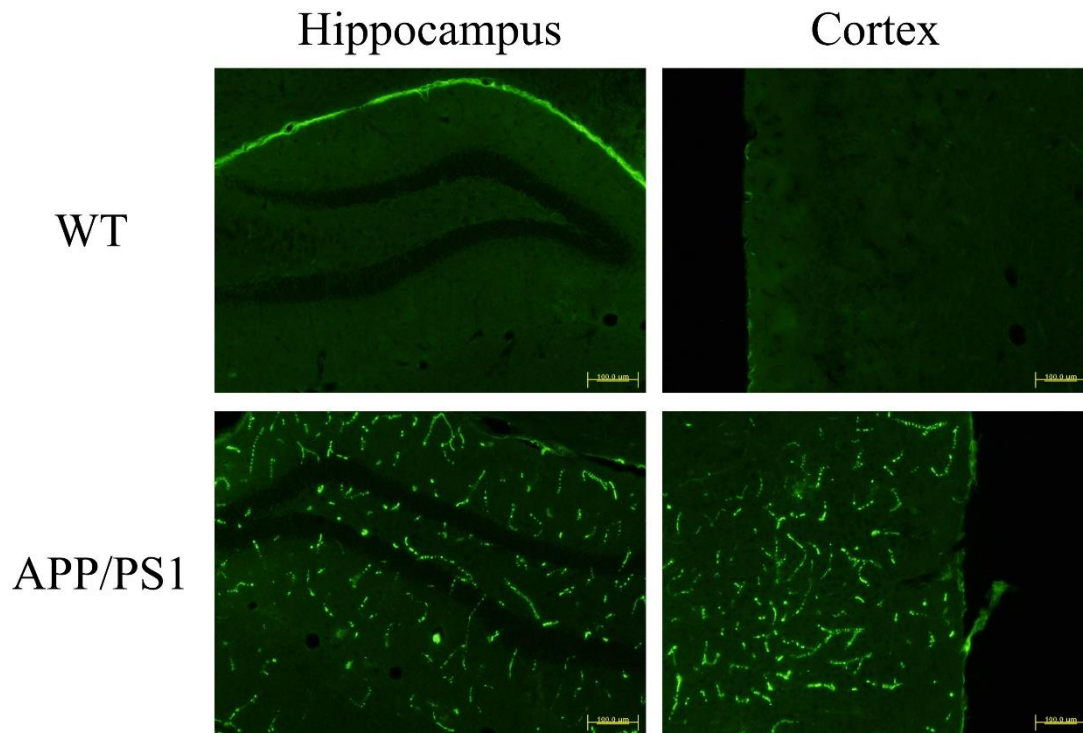**Figure S4**. EGCG/AA NPs appearance at 4th month of storage.

Figure S5. **Endogenous IgG leakage in mouse brains**. C57BL/6 WT and APP/PS1 mice were sacrificed by i.p. injection of ketamine/xylazine (100/10 mg/kg, respectively) and perfused with PFA 4%. Brain coronal sections of 20 µm of thickness were stained with Goat Anti-Mouse IgG (H+L) antibody, AlexaFluor 488 Green (1:1000; Life Technologies, Cambridge, UK). Image acquisition was carried out with an epifluorescence microscope (BX41, Olympus, Germany).

Tables

Table S1. Physicochemical characteristics of EGCG/AA NPs stored at different temperatures

	Stored temperature 25ºC			Stored temperature 4ºC		
Month	**Size (nm)**	**PDI**	**ZP (mV)**	**Size (nm)**	**PDI**	**ZP (mV)**
0	120.3 ± 0.7	0.063 ± 0.012	-12.0 ± 2.1	118.6 ± 1.5	0.072 ± 0.004	-13.5 ± 1.3
1	123.2 ± 1.5	0.059 ± 0.020	-10.0 ± 0.8	119.2 ± 0.9	0.082 ± 0.018	-12.3 ± 0.7
2	120.9 ± 1.2	0.043 ± 0.008	-12.2 ± 2.8	117.9 ± 0.6	0.062 ± 0.017	-13.1 ± 0.5
3	130.5 ± 0.4	0.167 ± 0.018	-12.6 ± 0.8	119.2 ± 1.8	0.093 ± 0.015	-12.1 ± 1.1
4	941.2 ± 9.4	0.700 ± 0.282	-	124.9 ± 0.5	0.107 ± 0.011	-12.6 ± 0.2
5	-	-	-	128.5 ± 2.2	0.095 ± 0.019	-11.5 ± 1.2
7	-	-	-	125.4 ± 2.3	0.121 ± 0.024	-10.3 ± 1.3
9	-	-	-	126.2 ± 0.5	0.150 ± 0.030	-10.4 ± 1.5
11	-	-	-	90.6 ± 2.5	0.250 ± 0.204	-8.6 ± 0.7

Table S2. ANOVA analysis results of the Aβ plaques depositions and Aβ $_{(1-42)}$ peptide levels

Test	Condition	Tukey's multiple comparisons test	Mean Diff.	95% CI of diff.	Significant?	Summary	Adjusted P Value
Aβ plaques	Hippocampal region	APP/PS1 vs. APP/PS1 free EGCG	3.528	0.6070 to 6.449	Yes	*	0.0155
		APP/PS1 vs. APP/PS1EGCG/AA NPs	7.639	4.718 to 10.56	Yes	****	< 0.0001
		APP/PS1 free EGCG vs. APP/PS1 EGCG/AA NPs	4.111	0.9887 to 7.234	Yes	**	0.0081
	Cortical region	APP/PS1 vs. APP/PS1 free EGCG	7.447	4.771 to 10.12	Yes	****	< 0.0001
		APP/PS1 vs. APP/PS1 EGCG/AA NPs	9.114	6.438 to 11.79	Yes	****	< 0.0001
		APP/PS1 free EGCG vs. APP/PS1 EGCG/AA NPs	1.667	-0.9501 to 4.283	No	ns	0.2751
Aβ $_{(1-42)}$ **peptide levels**	Soluble Aβ $_{(1-42)}$ peptide	APP/PS1 vs. APP/PS1 free EGCG	-1.799	-16.08 to 12.48	No	ns	0.9219
		APP/PS1 vs. APP/PS1 EGCG/AA NPs	20.45	6.177 to 34.73	Yes	*	0.0109
		APP/PS1 free EGCG vs. APP/PS1 EGCG/AA NPs	22.25	7.976 to 36.53	Yes	**	0.0073
	Insoluble Aβ $_{(1-42)}$ peptide	APP/PS1 vs. APP/PS1 free EGCG	25.40	-14.96 to 65.76	No	ns	0.2105
		APP/PS1 vs. APP/PS1 EGCG/AA NPs	94.53	54.17 to 134.9	Yes	***	0.0009
		APP/PS1 free EGCG vs. APP/PS1 EGCG/AA NPs	69.13	28.77 to 109.5	Yes	**	0.0046

Table S3. ANOVA analysis results of the behavioural test

Test	Condition	Tukey's multiple comparisons test	Mean Diff.	95% CI of diff.	Significant?	Summary	Adjusted P Value
MWM	Escape latency - Learning process	WT vs. APP/PS1	-33.48	-46.76 to -20.19	Yes	****	< 0.0001
		WT vs. APP/PS1 free EGCG	-18.59	-32.27 to -4.902	Yes	**	0.0054
		WT vs. APP/PS1 EGCG/AA NPs	-9.915	-24.81 to 4.981	No	ns	0.2786
		APP/PS1 vs. APP/PS1 free EGCG	14.89	2.159 to 27.62	Yes	*	0.018
		APP/PS1 vs. APP/PS1 EGCG/AA NPs	23.56	9.540 to 37.59	Yes	***	0.0006
		APP/PS1 free EGCG vs. APP/PS1 EGCG/AA NPs	8.673	-5.731 to 23.08	No	ns	0.3615
	Escape latency - Test day	WT vs. APP/PS1	-43.03	-54.50 to -31.55	Yes	****	< 0.0001
		WT vs. APP/PS1 free EGCG	-15.61	-27.78 to -3.442	Yes	*	0.0103
		WT vs. APP/PS1 EGCG/AA NPs	-2.376	-13.85 to 9.095	No	ns	0.9314
		APP/PS1 vs. APP/PS1 free EGCG	27.42	15.25 to 39.58	Yes	****	< 0.0001
		APP/PS1 vs. APP/PS1 EGCG/AA NPs	40.65	29.18 to 52.12	Yes	****	< 0.0001
		APP/PS1 free EGCG vs. APP/PS1 EGCG/AA NPs	13.23	1.066 to 25.40	Yes	*	0.0309

	Time in target quadrant – Test day	WT vs. APP/PS1	10.36	4.360 to 16.37	Yes	***	0.0003
		WT vs. APP/PS1 free EGCG	3.748	-1.939 to 9.435	No	ns	0.2947
		WT vs. APP/PS1 EGCG/AA NPs	3.000	-2.573 to 8.572	No	ns	0.4685
		APP/PS1 vs. APP/PS1 free EGCG	-6.615	-12.05 to -1.177	Yes	*	0.0126
		APP/PS1 vs. APP/PS1 EGCG/AA NPs	-7.363	-12.68 to -2.046	Yes	**	0.0040
		APP/PS1 free EGCG vs. APP/PS1 EGCG/AA NPs	-0.7487	-5.707 to 4.209	No	ns	0.9759
	Time in border – Test day	WT vs. APP/PS1	-35.14	-49.66 to -20.61	Yes	****	< 0.0001
		WT vs. APP/PS1 free EGCG	-7.718	-22.25 to 6.811	No	ns	0.4634
		WT vs. APP/PS1 EGCG/AA NPs	-6.417	-20.95 to 8.113	No	ns	0.6121
		APP/PS1 vs. APP/PS1 free EGCG	27.42	12.89 to 41.95	Yes	***	0.0002
		APP/PS1 vs. APP/PS1 EGCG/AA NPs	28.72	14.19 to 43.25	Yes	***	0.0001
		APP/PS1 free EGCG vs. APP/PS1 EGCG/AA NPs	1.302	-13.23 to 15.83	No	ns	0.9943
NOR	Exploration time	WT vs. APP/PS1	28.23	18.03 to 38.43	Yes	****	< 0.0001
		WT vs. APP/PS1 free EGCG	16.76	5.354 to 28.16	Yes	**	0.0023
		WT vs. APP/PS1 EGCG/AA NPs	2.544	-8.122 to 13.21	No	ns	0.9131
		APP/PS1 vs. APP/PS1 free EGCG	-11.48	-21.67 to -1.278	Yes	*	0.0231
		APP/PS1 vs. APP/PS1 EGCG/AA NPs	-25.69	-35.06 to -16.32	Yes	****	< 0.0001
		APP/PS1 free EGCG vs. APP/PS1 EGCG/AA NPs	-14.21	-24.88 to -3.547	Yes	**	0.0059