Supplementary Material

Modulation of Cytosolic Phospholipase A2 as a Potential Therapeutic Strategy for Alzheimer's Disease

SUPPLEMENTARY METHODS

1. Panning and identification of phage clones

The complete protocols about biopanning and clones isolation and amplification are fully described in our previous paper [45].

The biopanning was performed against the C2-domain of the cPLA$_2$-IVA (C2-cPLA$_2$-IVA, Recombinant cPLA$_2$ [1-178], MyBiosource, San Diego, USA) diluted at 100 µg/mL for the first round and 50 µg/mL for the second and the third rounds of selection.

Wells were blocked with PFBB (Protein-free [TBS] Blocking Buffer, Pierce, Fisher Scientific, Brussels, Belgium) for 1 h at room temperature.

Specific buffer HCN (HEPES 160 mM, NaCl 300 mM, $CaCl_2$ 20 mM, pH 7.4) supplemented with Tween-20 (C2-HCN-T; 0.1%, 0.3% and 0.5% for the rounds 1, 2, and 3 respectively) was used as dilution buffer for the phages and the washings. The incubation times were increased for the PFBB-coated well during the rounds allowing to remove non-specific phages (60, 90, and 120 min respectively) whereas it was decreased for the C2-cPLA$_2$-IVA-coated well (120, 90, and 60 min respectively).

2. Evaluation of the apparent dissociation constant (K^*_d) of C2-cPLA$_2$-targeted clones

The C2-cPLA$_2$-IVA was immobilized overnight at 4°C in $NaHCO_3$ 0.1M pH 7.4 at a concentration of 10 µg/mL (1 well per dilution of one phage clone, 1 well as blank).

Immobilized wells and an equivalent number of non-immobilized wells (for a specific affinity of phage clones) were blocked with PFBB for 1 h at room temperature. A range of 10 dilutions (1:1) of the tested phage clone was prepared in HCN supplemented with Tween-20 0.5% (C2-HCN-T) and added in the wells (100 µL/well) for 2 h at room temperature under stirring after rinsing the plate 6 times with 200 µL of C2-HCN-T. The plate was rinsed again, and the bound phage clones were detected using the monoclonal anti-M13 antibody coupled to the Horse Radish Peroxidase (HRP; Amersham Pharmacia Biotech Benelux, Roosendaal, The Netherlands) diluted

1:5000 in HCN supplemented with BSA 0.5%. After a final rinsing step, the revelation was performed by adding a volume of 200 µL of ABTS (22 mg of 2,2'-azino-bis(3-éthylbenzothiazoline-6-sulfonic acid in 100 mL of $C_6H_5Na_3O_7$ • $2H_2O$ 50 mM, pH 4.0) supplemented with H_2O_2 (1.714 µL H_2O_2 30% per mL of ABTS). The optic density was measured at 405 nm (differential filter 630 nm) using the SpectraMax M2 plate reader (Molecular Devices, Workingham, Berks, United-Kingdom).

3. Evaluation of the inhibitory concentrations 50% (IC_{50}) of C2-cPLA$_2$-targeted clones

The IC_{50} of C2-cPLA$_2$-targeted clones was determined using a constant concentration of the target and a range of dilutions of phages.

The first step was the determination of the K^*_d of the C2-domain of the cPLA$_2$-IVA used during the biopanning. For this purpose, the 2-Arachidonoyl-1-stearoyl-sn-glycero-3-phosphocholine (Sigma-Aldrich) was immobilized at a concentration of 100 µg/ml. The target was first solubilized in chloroform at 1 mg/mL and transferred into a glass tube to evaporate. Then the target was resolubilized in ethanol 95% (magnetic stirring and heating at 80°C) at 100 µg/mL. Finally, 100 µL of this solution were transferred in 96-well plate pre-warmed at 37°C and dried overnight. After blocking with PFBB for 1 h, a range of 10 dilutions of the C2-domain of the cPLA$_2$-IVA (from 900 nM to 50 nM, prepared in C2-HCN-T) were incubated for 2 h at 37°C. The C2-domain bound was detected by successive incubations with 1) polyclonal anti-human cPLA$_2$ made in goat (Antibodies-Online GmbH, Aachen, Germany) at 5 µg/mL in C2-HCN-T (0.1% Tween), 2) biotin-conjugated anti-goat IgG antibody made in horse (Vector Labconsult, Brussels, Belgium) at 5 µg/mL in phosphate buffer (Na_2HPO_4 • 12 H_2O 10 mM, NaH_2PO_4 • H_2O 10 mM, NaCL 150 mM, pH 7.8) supplemented with 0.1% Tween-20 and 0.2% BSA, and 3) Vectastain ABC kit (Vector Labconsult) diluted 1:100 in TBS. Finally, the revelation was performed by addition of ABTS/H_2O_2 as previously described.

The IC_{50} of C2-cPLA$_2$-targeted clones was determined using a constant concentration of the C2-domain of the cPLA$_2$-IVA (corresponding to the K^*_d, prepared at 2xK^*_d in C2-HCN-T 0.1%) and a range of 10 dilutions (1:2) of C2-cPLA$_2$-targeted clones (range of their K^*_d) in C2-HCN-T supplemented with 0.1% BSA. Clones and C2-domain of the cPLA$_2$-IVA were pre-incubated (1:1) in Eppendorf for 30 min before their transfer into immobilized wells (1.5 h, 37°C). Blanks were incubated with C2-HCN-T. The C2-domain bound was detected as described above.

Supplementary material_Results

TABLES

Table S1 – Statistic analysis (One Way ANOVA) of the « Number of errors » for each Barnes test (T)

	APP/PS1 : MAP-(PLP25)$_3$-(LRPep2)$_1$	**C57BL/6J x C3H/HeJ**	**APP/PS1 : NSP2**	**APP/PS1 : 10 months**
APP/PS1 : MAP-(PLP25)$_3$-(LRPep2)$_1$	/	T1 : p<0.001 T4 : p<0.05	T5 : p<0.05	T1 : p<0.05 T2 : p<0.05 T3 : p<0.05 T4 : p<0.05 T5 : p<0.05
C57BL/6J x C3H/HeJ	/	/	T1 : p<0.05	
APP/PS1 : NSP2	/	/	/	T2 : p<0.05
APP/PS1 : 10 months	/	/	/	/

Table S2 – Statistic analysis (One Way ANOVA) of the « Percentage of time spent in the quadrant of interest » for each Barnes test (T)

	APP/PS1 : MAP-(PLP25)$_3$-(LRPep2)$_1$	**C57BL/6J x C3H/HeJ**	**APP/PS1 : NSP2**	**APP/PS1 : 10 months**
APP/PS1 : MAP-(PLP25)$_3$-(LRPep2)$_1$	/		T4 : p<0.05 T6 : p<0.05 T8 : p=0.055	T4 : p<0.05 T5 : p<0.05
C57BL/6J x C3H/HeJ	/	/		T5 : p<0.05
APP/PS1 : NSP2	/	/	/	
APP/PS1 : 10 months	/	/	/	/

Table S3 – Statistic analysis (Student) of the « Total number of plaques »

	APP/PS1 : 5 months	**APP/PS1 : NSP2**	**APP/PS1 : MAP-$(PLP25)_3$-$(LRPep2)_1$**	**APP/PS1 : 10 months**
APP/PS1 : 5 months	/	p<0.001	p<0.05	p<0.001
APP/PS1 : NSP2	/	/	p<0.001	p<0.001
APP/PS1 : MAP-$(PLP25)_3$-$(LRPep2)_1$	/	/	/	p<0.05
APP/PS1 : 10 months	/	/	/	/

Table S4 – Statistic analysis (Student) of the «Number of plaques in the hippocampus»

	APP/PS1 : 5 months	**APP/PS1 : NSP2**	**APP/PS1 : MAP-$(PLP25)_3$-$(LRPep2)_1$**	**APP/PS1 : 10 months**
APP/PS1 : 5 months	/	p<0.05	p<0.05	p<0.05
APP/PS1 : NSP2	/	/	p<0.05	p<0.001
APP/PS1 : MAP-$(PLP25)_3$-$(LRPep2)_1$	/	/	/	p<0.05
APP/PS1 : 10 months	/	/	/	/

Tablea S5 – Statistic analysis (Mann-Whitney) of the « Total area cortex » labeled by the $cPLA_2$-IVA

	NMRI	**C57BL/6J x C3H/HeJ**	**APP/PS1: 5 months**	**APP/PS1: 10 months**	**APP/PS1: MAP-$(PLP25)_3$-$(LRPep2)_1$**	**APP/PS1: NSP2**
NMRI	/	p<0.05	p<0.001	NS	p<0.05	p<0.001
C57BL/6J x C3H/HeJ	/	/	p<0.001	NS	p<0.05	p<0.001
APP/PS1: 5 months	/	/	/	p<0.001	p<0.001	NS
APP/PS1: 10 months	/	/	/	/	p<0.05	p<0.05
APP/PS1: MAP-$(PLP25)_3$-$(LRPep2)_1$	/	/	/	/	/	p<0.001
APP/PS1: NSP2	/	/	/	/	/	/

Table S6 – Statistic analysis (Mann-Whitney) of the « Total area hippocampus » labeled by the cPLA$_2$-IVA

	NMRI	**C57BL/6J x C3H/HeJ**	**APP/PS1: 5 months**	**APP/PS1: 10 months**	**APP/PS1: MAP-(PLP25)$_3$-(LRPep2)$_1$**	**APP/PS1: NSP2**
NMRI	/	NS	p<0.05	NS	p<0.05	p<0.001
C57BL/6J x C3H/HeJ	/	/	p<0.05	NS	NS	p<0.05
APP/PS1: 5 months	/	/	/	NS	p<0.05	NS
APP/PS1: 10 months	/	/	/	/	p<0.05	NS
APP/PS1: MAP-(PLP25)$_3$-(LRPep2)$_1$	/	/	/	/	/	p<0.001
APP/PS1: NSP2	/	/	/	/	/	/

Table S7 – Statistic analysis (Mann-Whitney) of the « Total area cortex » labeled by the NMDAR

	NMRI	**C57BL/6J x C3H/HeJ**	**APP/PS1: 5 months**	**APP/PS1: 10 months**	**APP/PS1: MAP-(PLP25)$_3$-(LRPep2)$_1$**	**APP/PS1: NSP2**
NMRI	/	NS	p<0.001	p<0.001	p<0.05	p<0.001
C57BL/6J x C3H/HeJ	/	/	p<0.05	p<0.05	NS	p<0.001
APP/PS1: 5 months	/	/	/	NS	p<0.05	NS
APP/PS1: 10 months	/	/	/	/	NS	p<0.05
APP/PS1: MAP-(PLP25)$_3$-(LRPep2)$_1$	/	/	/	/	/	p<0.05
APP/PS1: NSP2	/	/	/	/	/	/

Table S8 – Statistic analysis (Mann-Whitney) of the « Total area hippocampus » labeled by the NMDAR

	NMRI	**C57BL/6J x C3H/HeJ**	**APP/PS1: 5 months**	**APP/PS1: 10 months**	**APP/PS1: MAP-$(PLP25)_3$-$(LRPep2)_1$**	**APP/PS1: NSP2**
NMRI	/	p<0.05	p<0.05	NS	NS	NS
C57BL/6J x C3H/HeJ	/	/	NS	NS	NS	NS
APP/PS1: 5 months	/	/	/	NS	NS	NS
APP/PS1: 10 months	/	/	/	/	NS	NS
APP/PS1: MAP-$(PLP25)_3$-$(LRPep2)_1$	/	/	/	/	/	NS
APP/PS1: NSP2	/	/	/	/	/	/

Table S9 – Statistic analysis (Mann-Whitney) of the « Total area cortex » labeled by the p199-Tau

	C57BL/6J x C3H/HeJ	**APP/PS1: 5 months**	**APP/PS1: 10 months**	**APP/PS1: 2 years**	**APP/PS1: MAP-$(PLP25)_3$-$(LRPep2)_1$**	**APP/PS1: NSP2**
C57BL/6J x C3H/HeJ	/	p<0.05	p<0.05	p<0.001	p<0.05	p<0.001
APP/PS1: 5 months	/	/	NS	NS	p<0.001	NS
APP/PS1: 10 months	/	/	/	NS	p<0.001	NS
APP/PS1: 2 years	/	/	/	/	p<0.001	NS
APP/PS1: MAP-$(PLP25)_3$-$(LRPep2)_1$	/	/	/	/	/	p<0.001
APP/PS1: NSP2	/	/	/	/	/	/

FIGURES

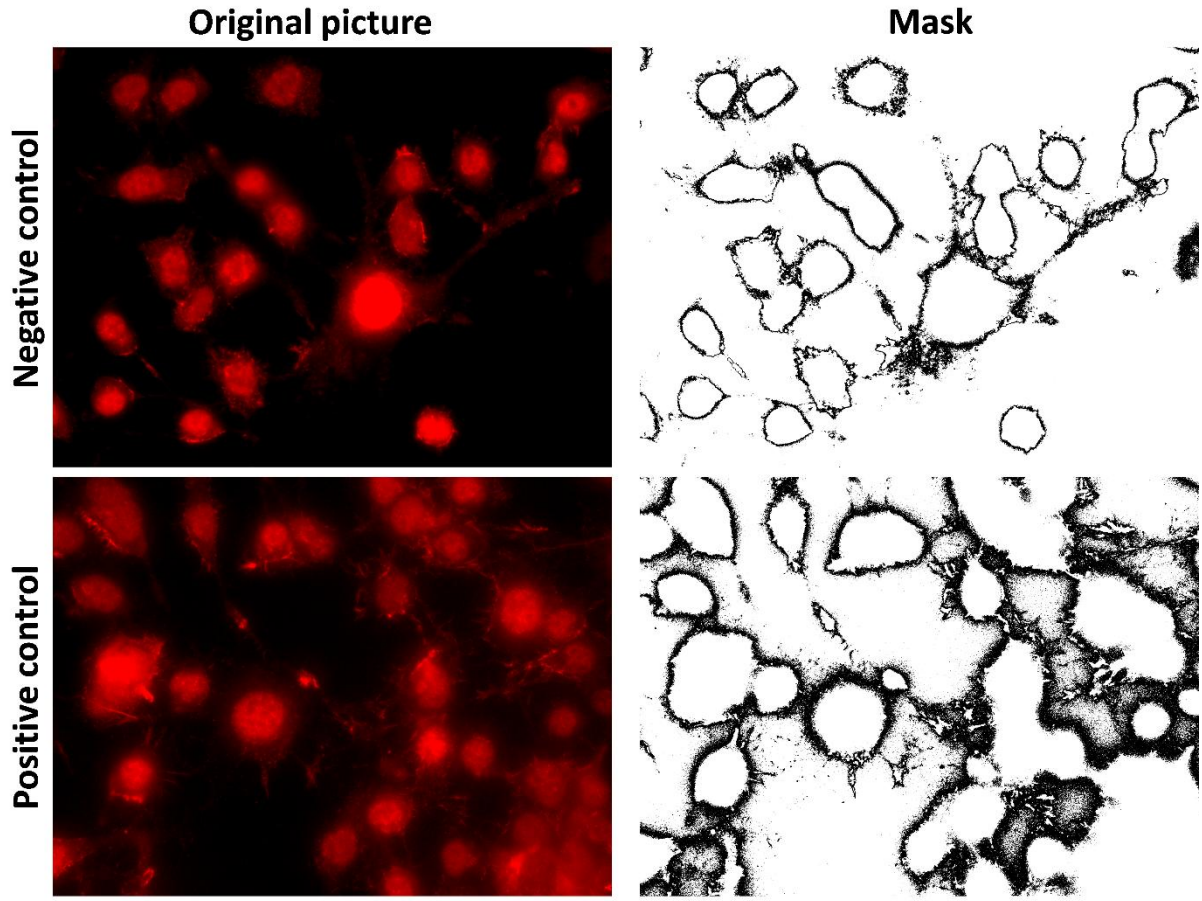


Fig. S1. Isolation of the cell processes stained for the cPLA$_2$-IVA by the establishment of a threshold with ImageJ. A particle analysis of the generated mask was then performed to quantify to surface occupied by the processes.

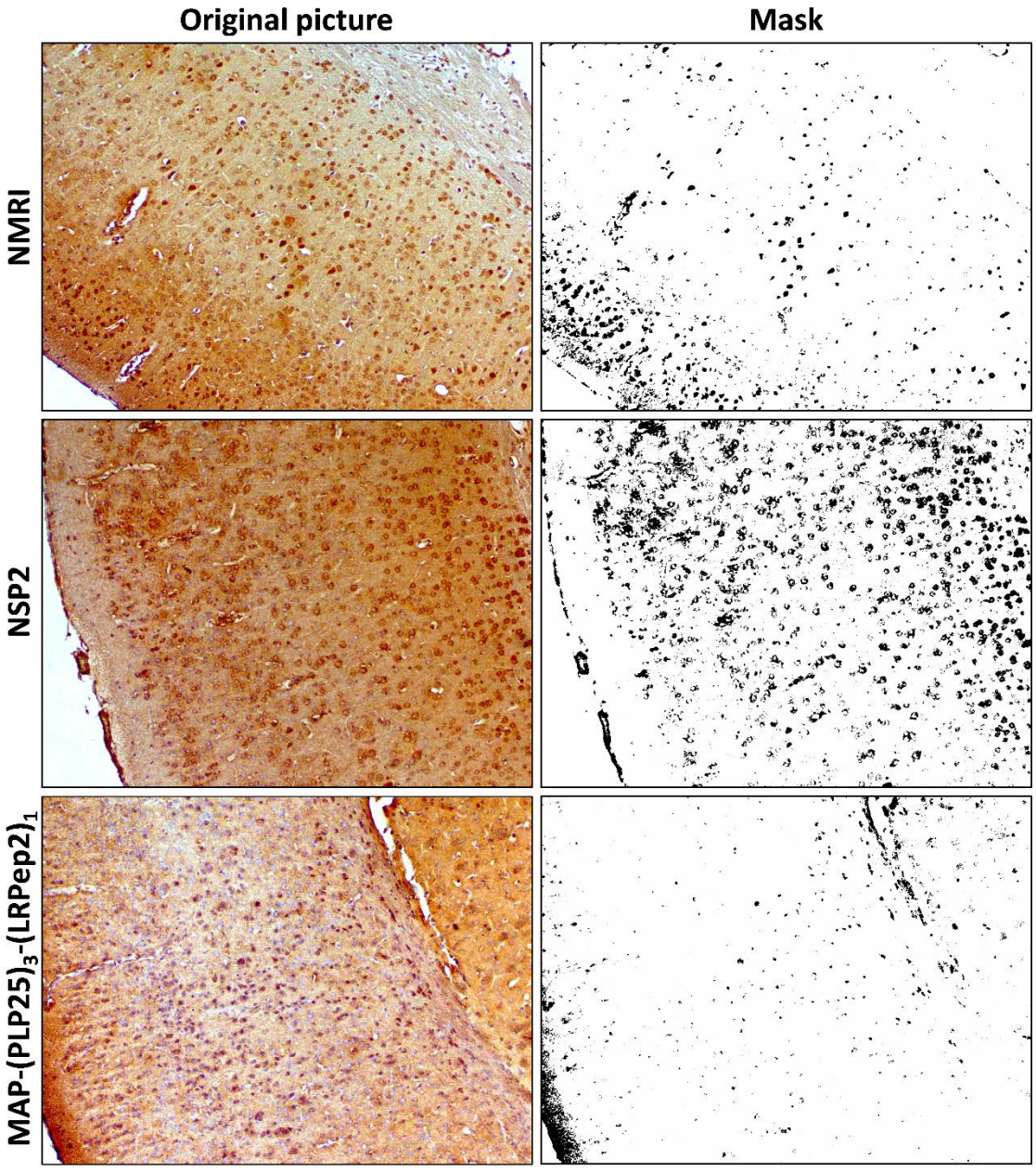


Fig. S2. Example of the DAB staining isolation in cell bodies on brain slices using the ImageJ software. A threshold was determined on NMRI brain slices (different in the cortex and the hippocampus) and was then applied to the other conditions. The mask generated is used to perform a particle analysis, giving the total area stained by DAB.

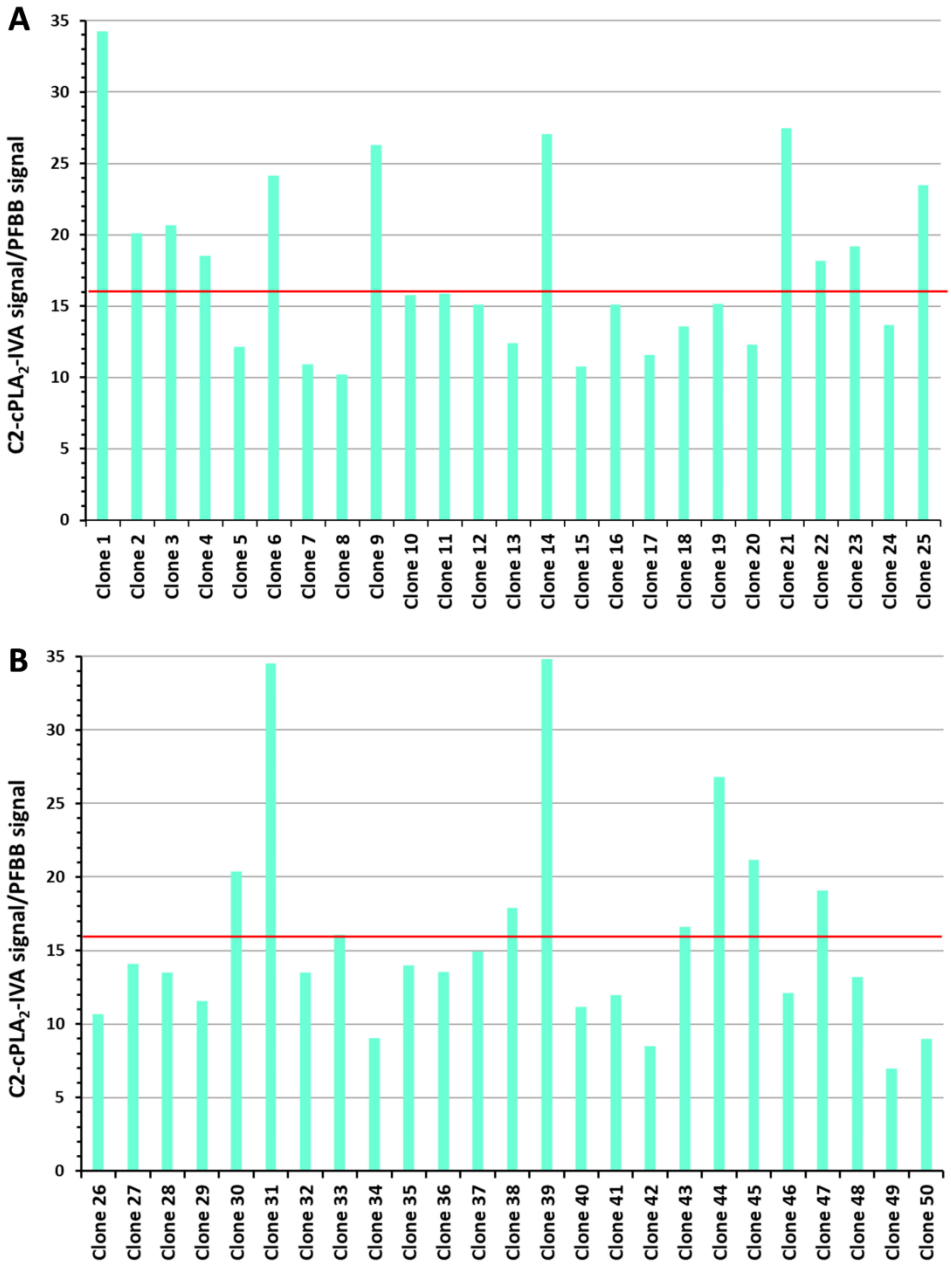
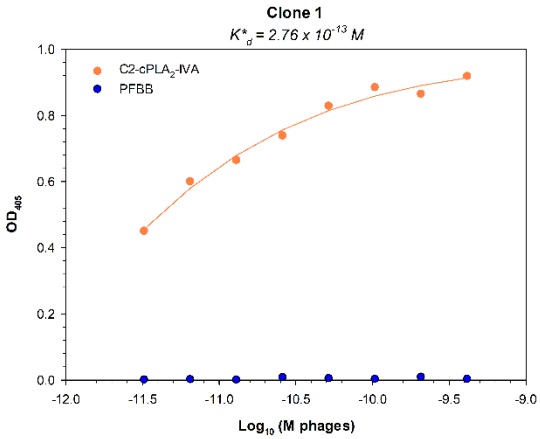
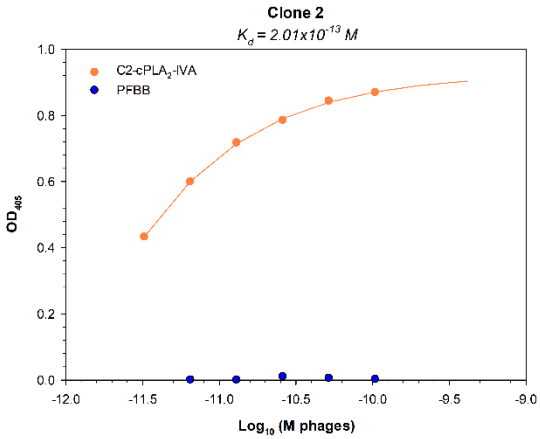


Fig. S3. (A-B) Specific affinity (C2-cPLA$_2$-IVA/PFBB) of the 50 clones isolated from the pool of the 3rd round of panning. (A) Clones 1 to 24. (B) Clones 25 to 50. Red line: mean = 16.79.


Clone 1
K^*_d = 2.76 x 10^{-13} M
C2-cPLA$_2$-IVA
PFBB
OD$_{405}$
Log$_{10}$ (M phages)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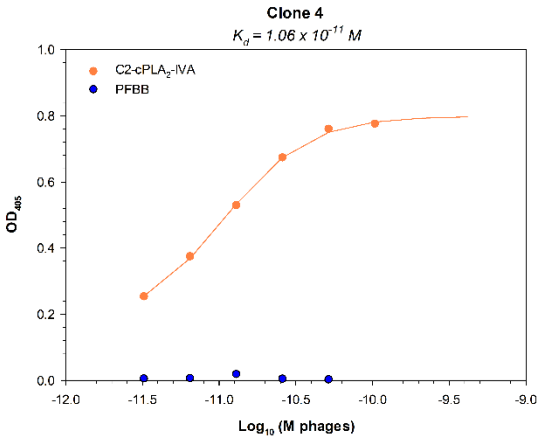
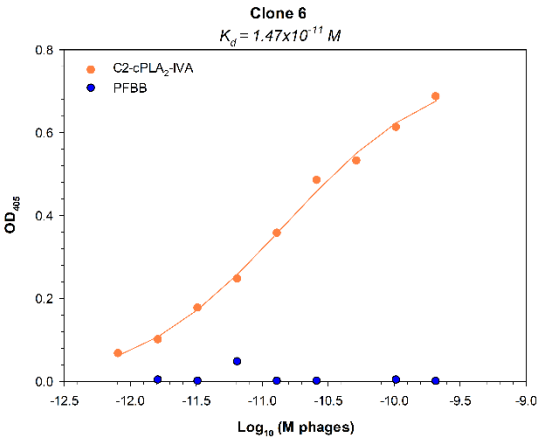
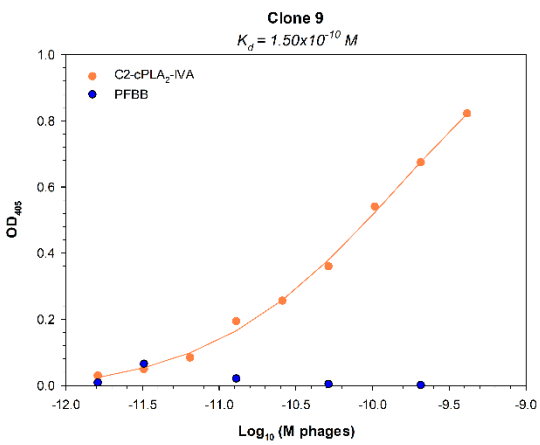
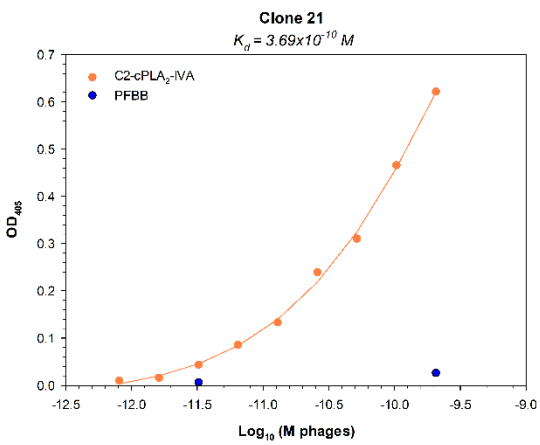
K_d = 2.01x10^{-13} M
C2-cPLA$_2$-IVA
PFBB
OD$_{405}$
Log$_{10}$ (M phages)


Clone 4
K_d = 1.06 x 10^{-11} M
C2-cPLA$_2$-IVA
PFBB
OD$_{405}$
Log$_{10}$ (M phages)


Clone 6
K_d = 1.47x10^{-11} M
C2-cPLA$_2$-IVA
PFBB
OD$_{405}$
Log$_{10}$ (M phages)


Clone 9
K_d = 1.50x10^{-10} M
C2-cPLA$_2$-IVA
PFBB
OD$_{405}$
Log$_{10}$ (M phages)


Clone 21
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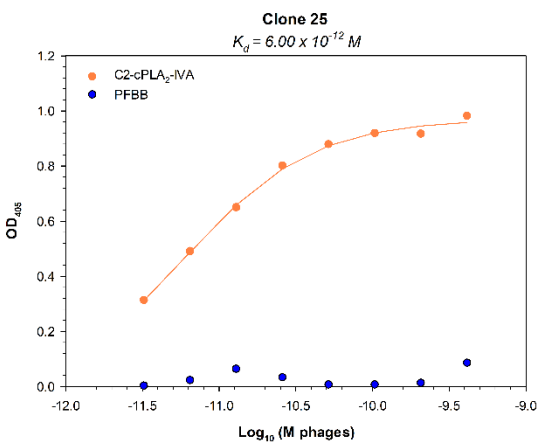
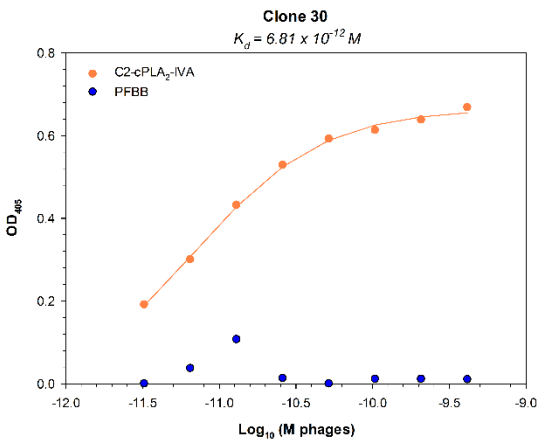
C2-cPLA$_2$-IVA
PFBB
OD$_{405}$
Log$_{10}$ (M phages)


Clone 25
K_d = 6.00 x 10^{-12} M
C2-cPLA$_2$-IVA
PFBB
OD$_{405}$
Log$_{10}$ (M phages)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K_d = 6.81 x 10^{-12} M
C2-cPLA$_2$-IVA
PFBB
OD$_{405}$
Log$_{10}$ (M phages)

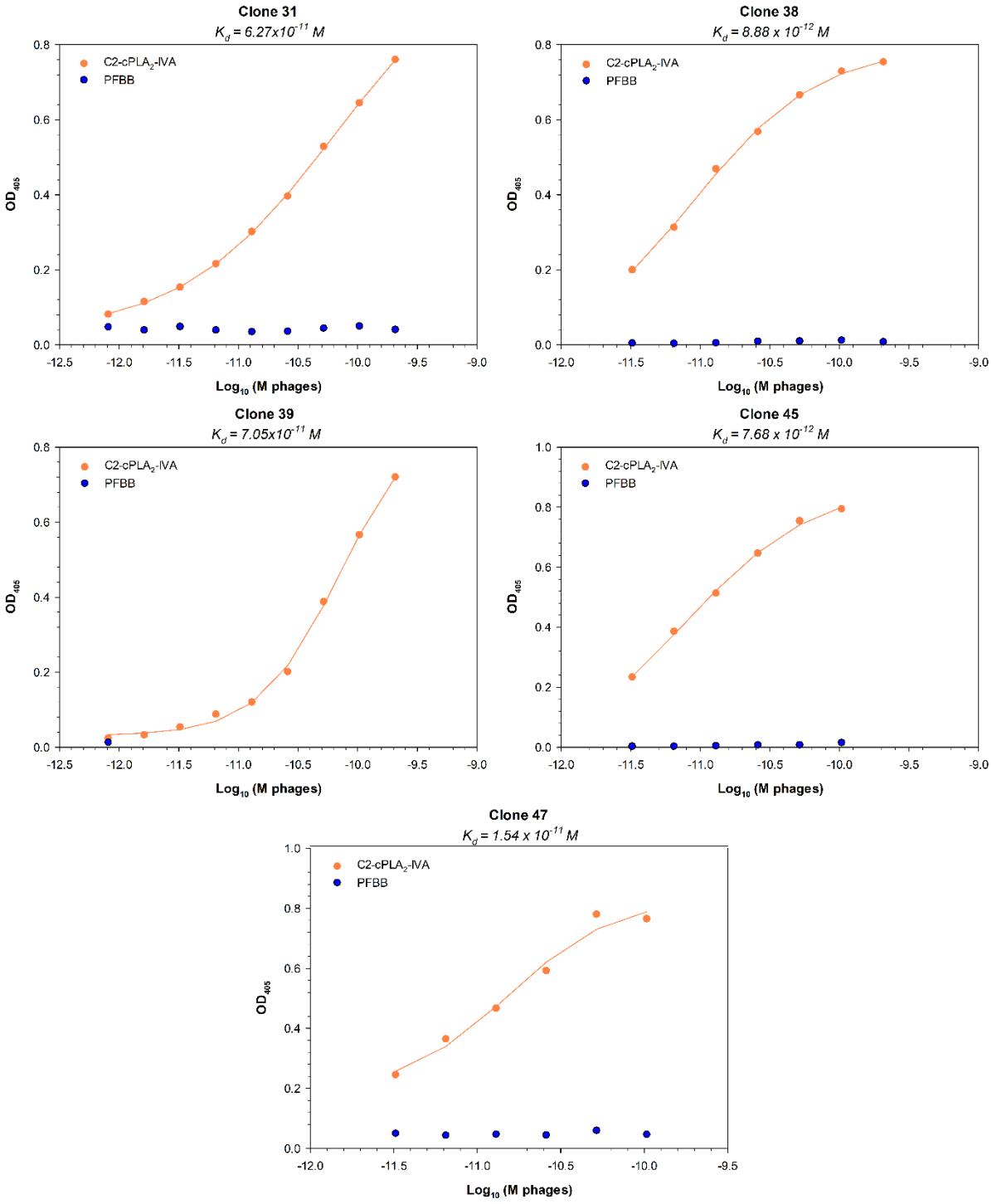

Fig. S4. The titration curves used to determine the apparent dissociation constants (K^*_d) of the 13 representative phage clones against the C2-cPLA$_2$-IVA (in orange) and the PFBB (in blue). They reflect the dose-dependent binding of peptides to C2-cPLA$_2$-IVA.

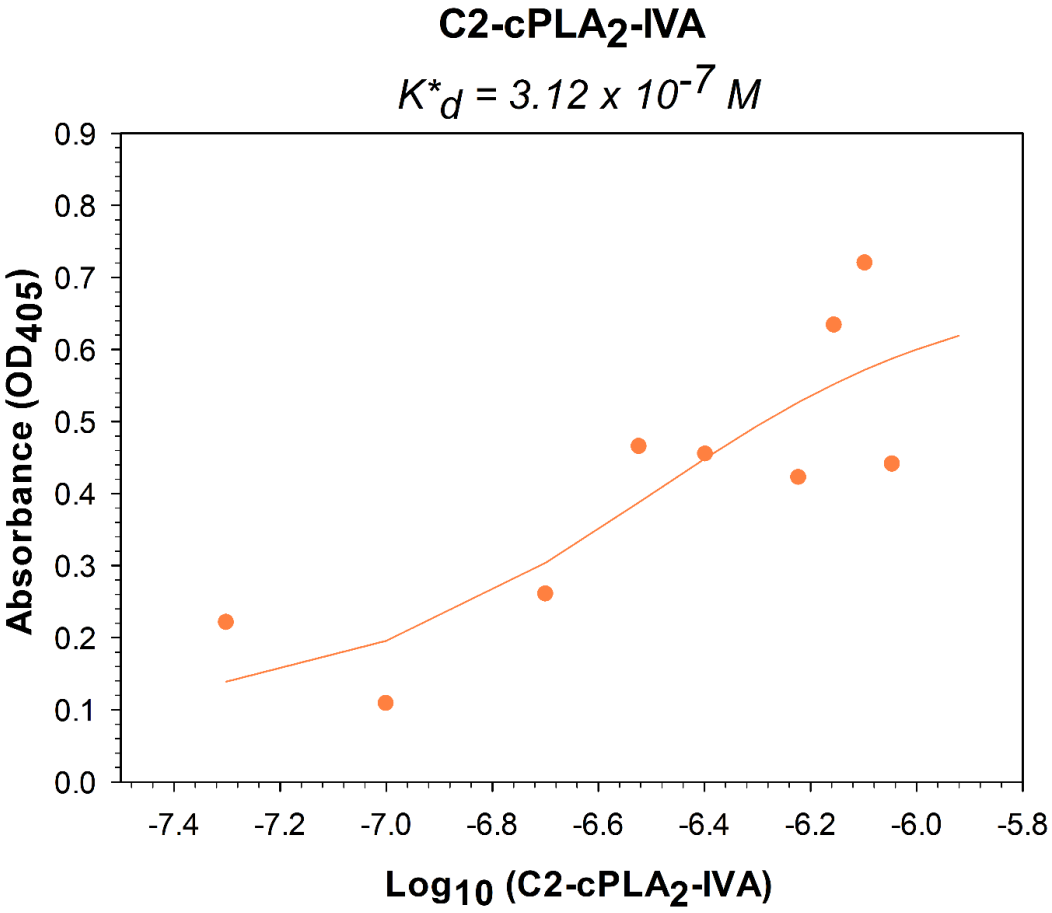

Fig. S5. Apparent dissociation constant (K^*_d) of the C2-cPLA$_2$-IVA against the PC.

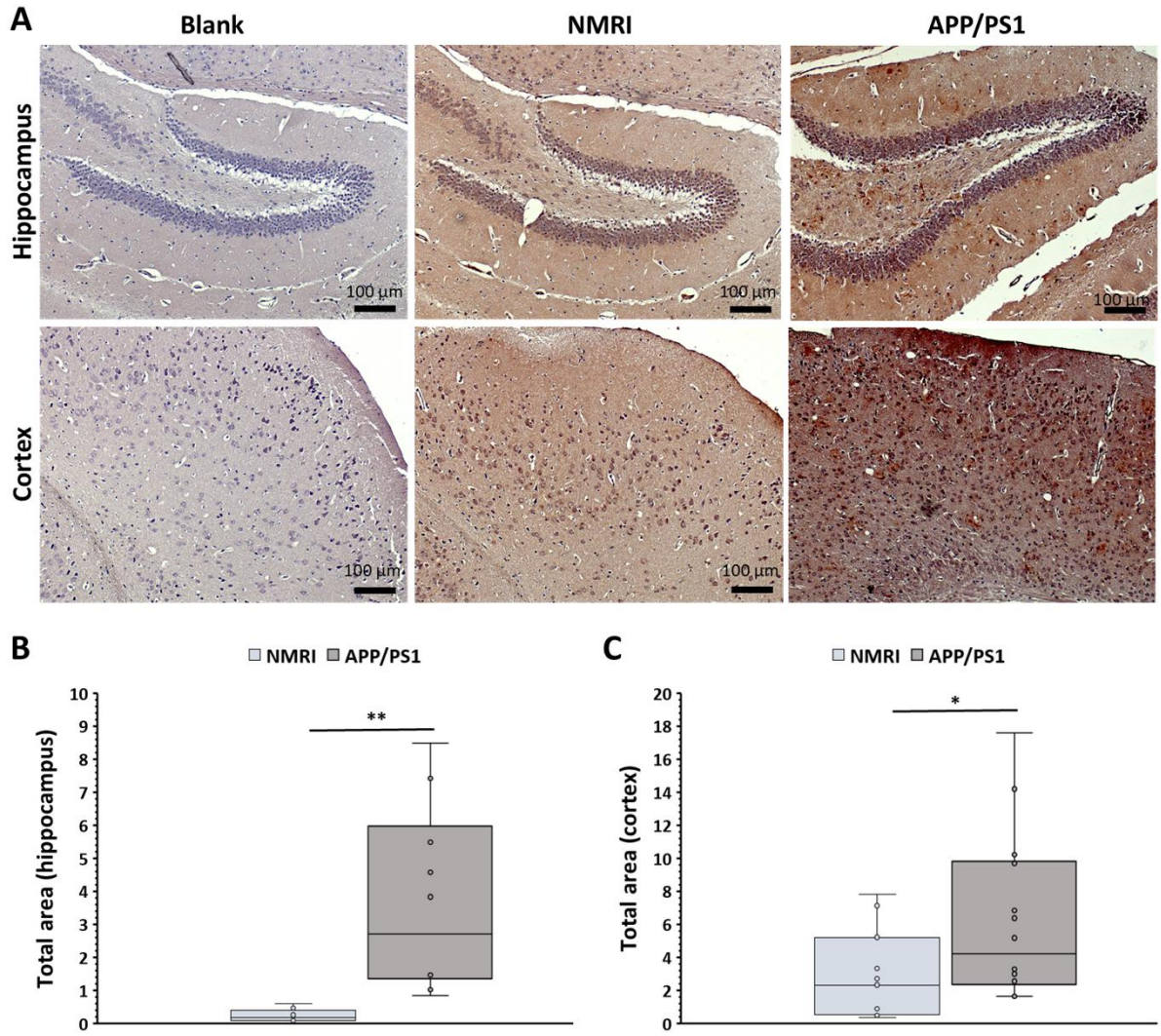

Fig. S6. (A) cPLA$_2$-IVA detected by IHC on healthy NMRI and APP/PS1 (2 years) brain slices. cPLA$_2$-IVA is highlighted by the DAB and appears in brown. (B-C) Total area labeled by the cPLA$_2$-IVA in the cell bodies in the hippocampus (B) and the cortex (C). * $p<0.05$, ** $p<0.001$ (Mann-Whitney).

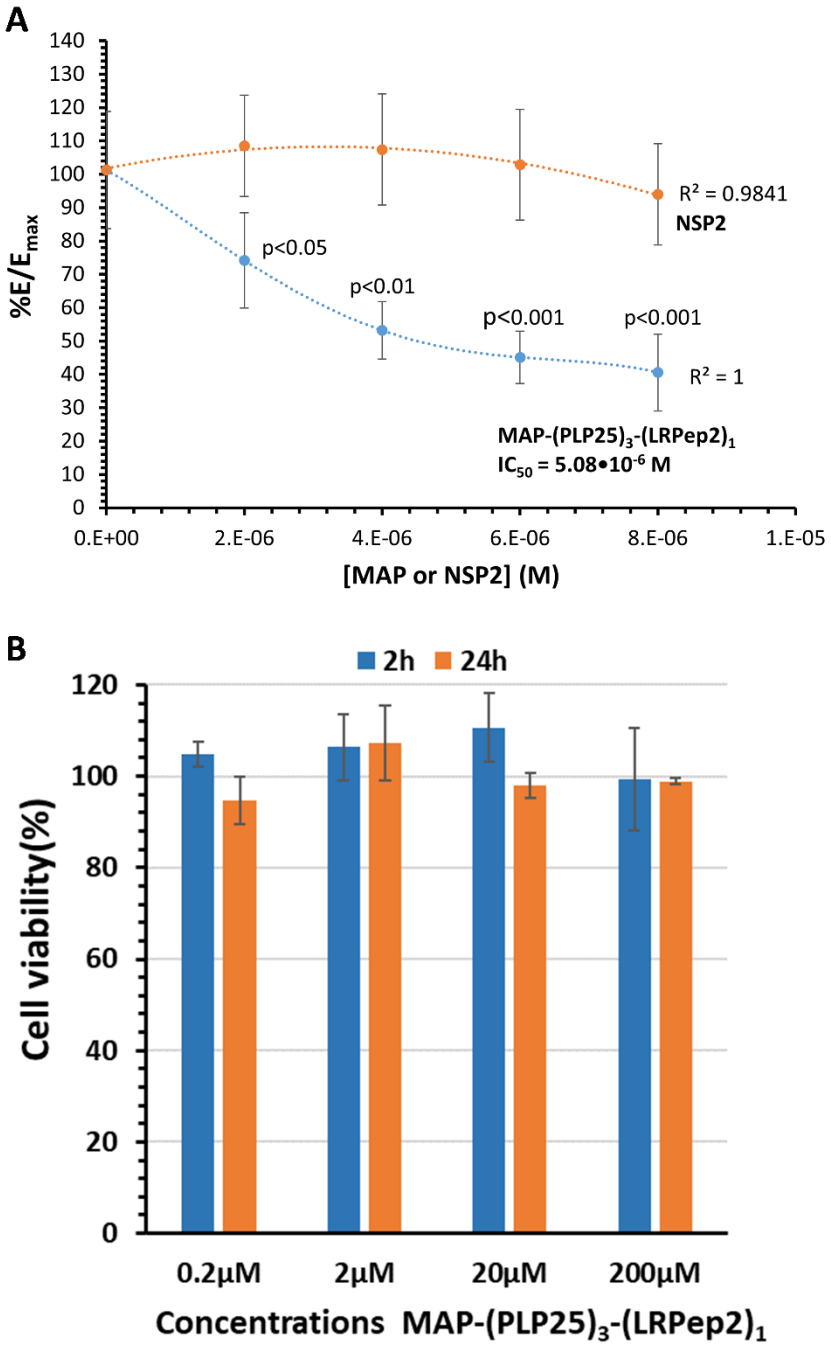

Fig. S7. (A) IC_{50} of MAP-$(PLP25)_3$-$(LRPep2)_1$ determined by immunofluorescence on NT2/$D1_n$ cells; NSP2 is used as non-specific control peptide. The statistical significance of the inhibitory effect of MAP-$(PLP25)_3$-$(LRPep2)_1$ compared to NSP2 on cPLA_2-IVA translocation to membrane of cell processes was determined by Student *t* test. (B) HepaRG cell viability following the incubation for 2 or 24 hours with MAP-$(PLP25)_3$-$(LRPep2)_1$ (0.2, 2, 20 and 200µM). Control cells correspond to 100% of viability. No statistical significance was identified between the different experimental groups.

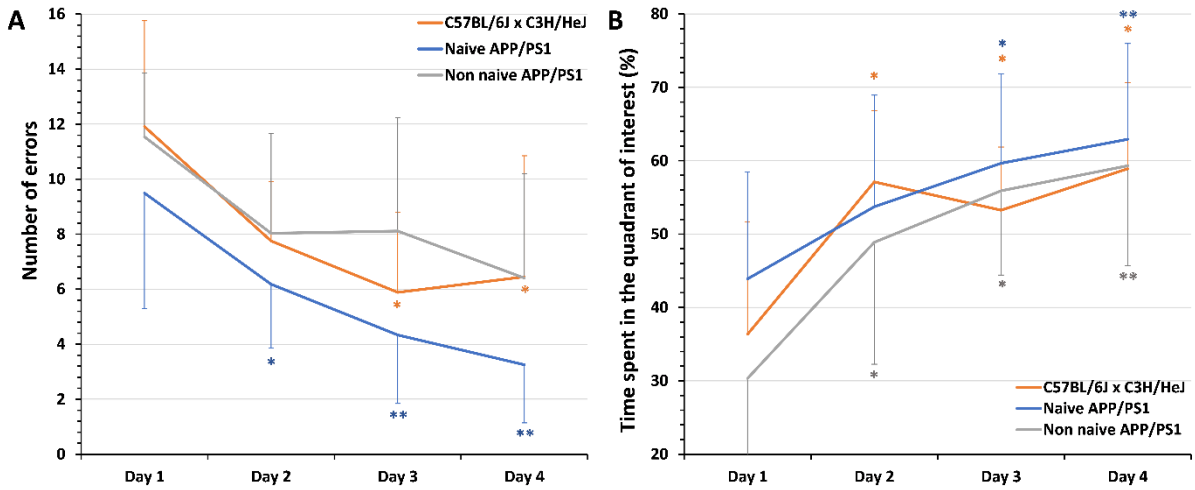

Fig. S8. Analysis of the behavior of healthy (C57BL/6J x Hej, n=8) and APP/PS1 mice (naive mice: n=10; non naive mice: n=8) during the learning of the Barnes maze. (A) Total number of errors; (B) Percentage of time spent in the quadrant of interest. One WAY Anova vs. day 1, * $p<0.05$, ** $p<0.01$.

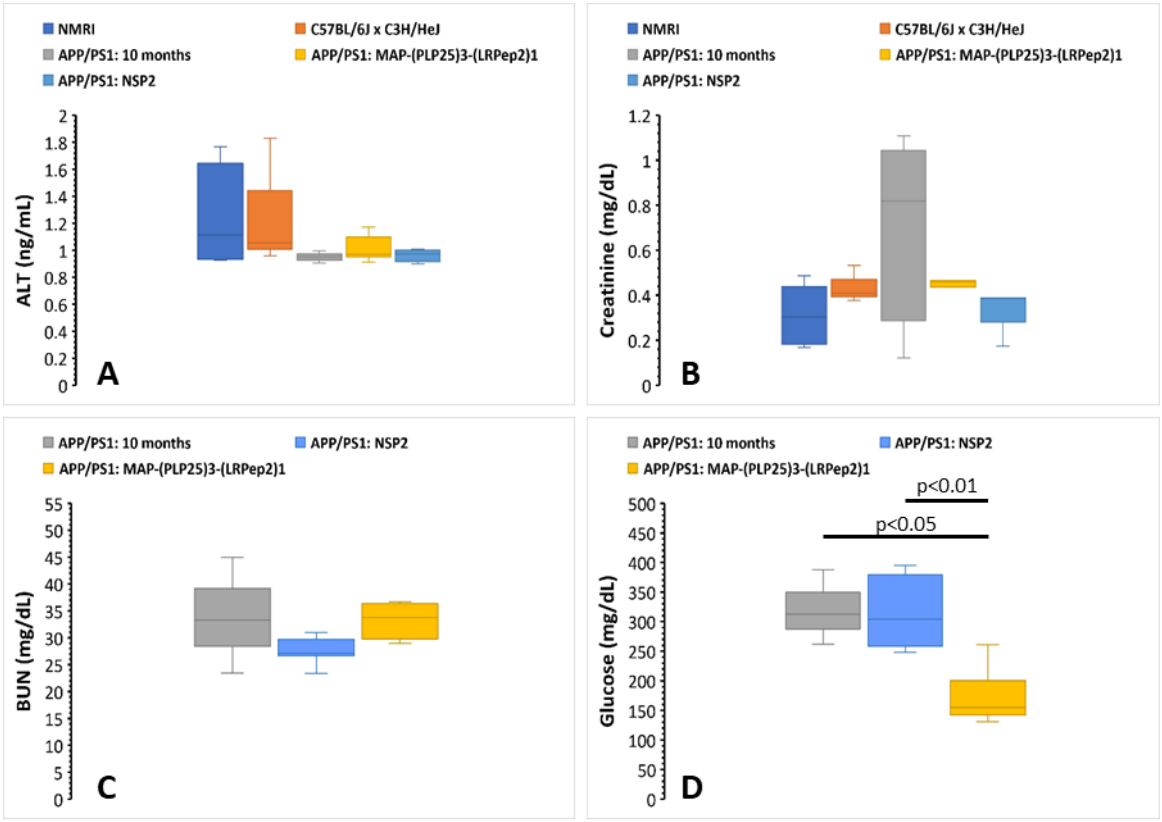

Fig. S9. Measurement of blood plasma ALT (A), creatinine (B), BUN (C) and glucose (D) in healthy NMRI and C57BL/6JxC3H/HeJ as well as APP/PS1 mice, treated with MAP-$(PLP25)_3$-$(LRPep2)_1$ or NSP2, or non-treated (APP/PS1 10 months). No statistical significance was identified between the different experimental groups for ALT, creatinine, and BUN.