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Supplemental Information

HMG-CoA Reductase Inhibitors Bind to PPARα to Upregulate Neurotrophin Expression in the Brain and Improve Memory in Mice

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Supplemental data
Supplemental Figures

Figure S1 (part A)

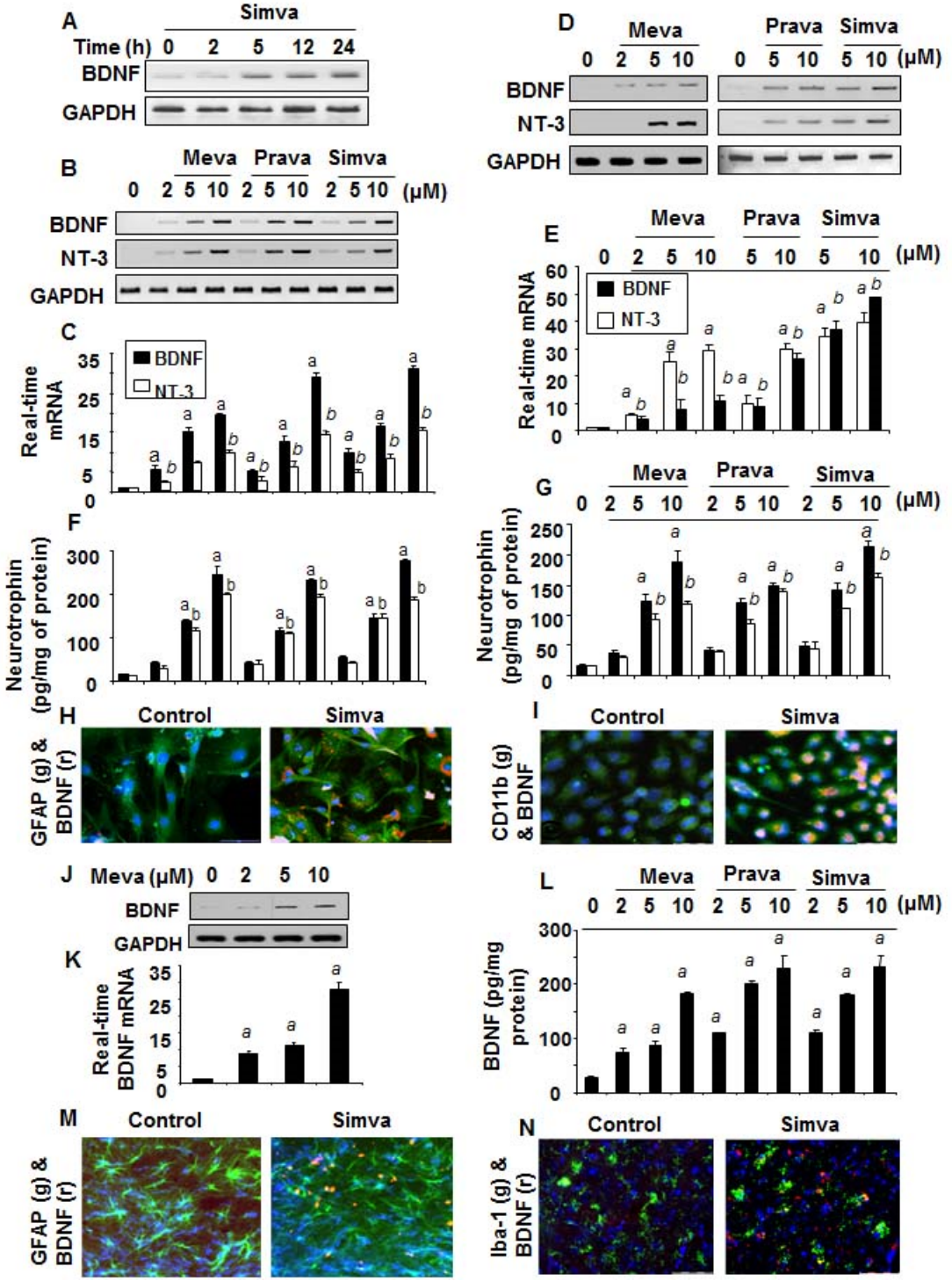

Figure S1 (part B)

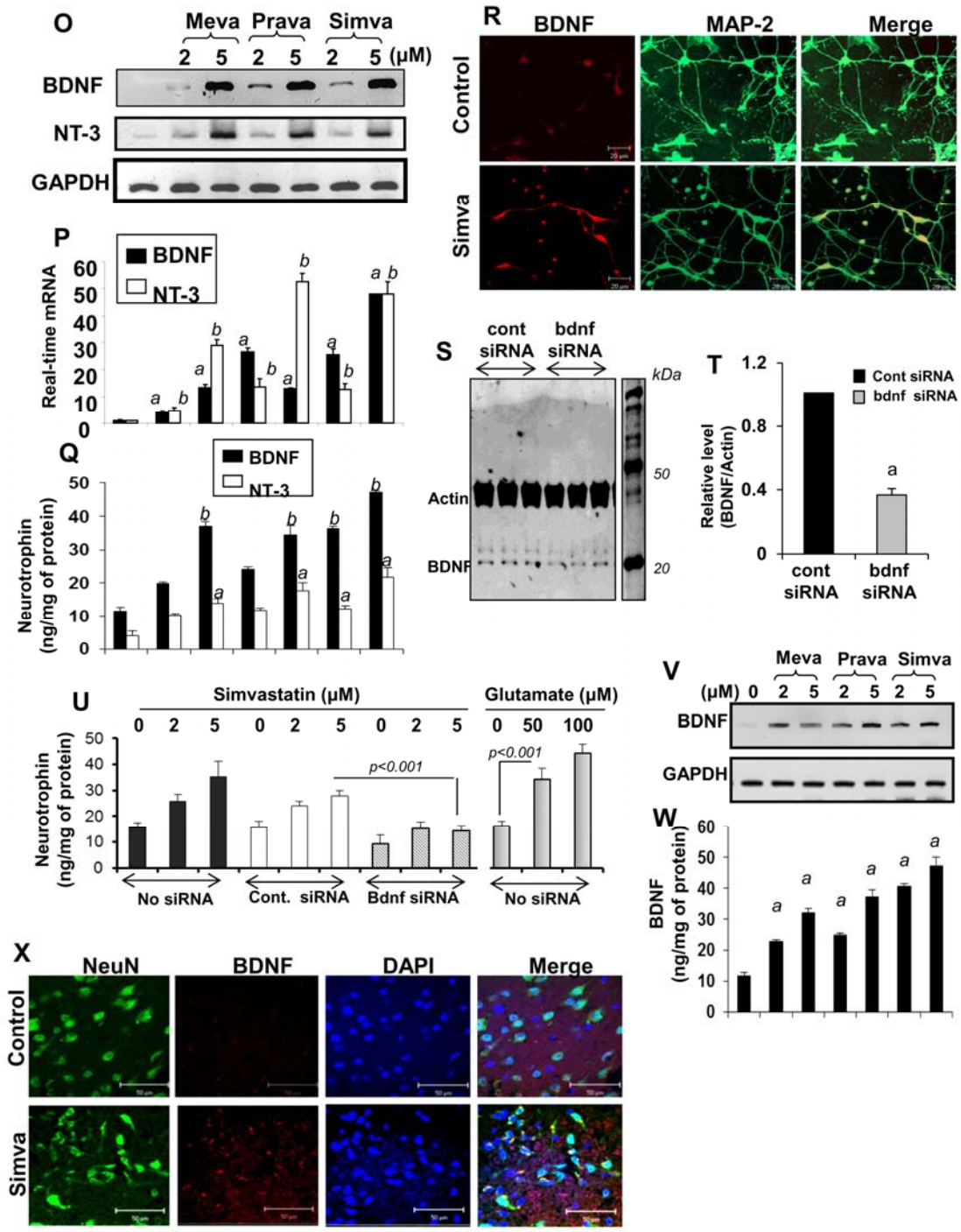

Figure S1, related to Figure 1. Statins upregulate the expression of neurotrophic factors in astrocytes, microglia and neurons. A) Mouse primary astrocytes were stimulated with 5 μM simvastatin for different time periods followed by monitoring the mRNA expression of BDNF by semi-quantitative RT-PCR. Mouse primary astrocytes (B,

C) and microglia (D, E) were stimulated with different doses of mevastatin, pravastatin, and simvastatin under serum-free conditions. After a 5-h incubation, the mRNA expression of BDNF and NT3 was analyzed by semi-quantitaive RT-PCR (B and D) and quantitative real-time PCR (C and E). Data are means ± SEM of three independent experiments. $^{a}p<0.05$, $^{b}p<0.01$, and $^{c}p<0.001$ *vs* control BDNF; $^{a*}p<0.05$, $^{b*}p<0.01$, and $^{c*}p<0.01$ vs control NT3. Similarly, $^{g}p<0.01$, $^{h}p<0.01$, and $^{i}p<0.001$ vs control BDNF; $^{g*}p<0.05$, $^{h*}p<0.05$, and $^{i*}p<0.01$ vs control NT3. After a 24-h incubation, protein levels of BDNF and NT3 were monitored in the supernatents of astrocytes (F) and microglia (G) by ELISA. $^{d,e,f,j,k,l}p<0.05$ vs control BDNF and $^{d*,e*,f*,j*,k*,l*}p<0.05$ vs control NT3. Mouse primary astrocytes (H) and microglia (I) were treated with 10 μM simvastatin under serum-free conditions. After 24 h, BDNF expression was analyzed by immunofluorescence. Human primary astrocytes were treated with different doses of mevastatin for 5 h followed by mRNA analysis of BDNF by semi-quantitaive RT-PCR (J) and quantitative real-time PCR (K). Data are means ± SEM for three independent experiments. $^{m}p<0.01$ vs control. (L) After a 24-h incubation, the protein level of BDNF was monitored in supernatants by ELISA. Data are means ± SEM of three independent experiments with $^{n}p<0.01$, $^{o}p<0.005$, and $^{p}p<0.005$ vs control. Mice (n=*5*) were fed simvastatin (1mg/kg bwt/d) via gavage. After 5 d, animals were perfused and their cortical sections were analyzed for BDNF (red) with either GFAP (M) or Iba-1 (N). Results represent analysis of two sections from each of five mice per group.

Mouse cortical neurons were stimulated with different doses of mevastatin, pravastatin and simvastatin under serum free condition. After 5 h of incubation, the mRNA expression of BDNF and NT-3 was analyzed by semi-quantitaive RT-PCR (O) and quantitative real-time PCR (P). Data are mean ± SD of three independent experiments. $^{a}p<0.001$ *vs control BDNF;* $^{b}p<0.001$ *vs control NT-3,* After 24 h of incubation, levels of BDNF and NT-3 in the supernatents of neurons (Q) were assayed by ELISA. Data are mean ± SD of three independent experiments. $^{a}p<0.001$ *vs control BDNF;* $^{b}p<0.001$ *vs control NT-3,* The protein expression of BDNF was analyzed by immunofluorescence analyses (R). S) Mouse cortical neurons were transfected with control or BDNF siRNA and after 48 h of transfection, the level of BDNF was examined by Western blot. Actin was run as a loading control. T) Bands were scanned and presented as relative to control. $^{a}p<0.001$ *vs control-siRNA*. U) Cells were transfected with control or BDNF siRNA. After 48 h of transfection, cells were stimulated with simvastatin for another 24 h. In a parallel experiment, cells were also stimulated with glutamate for 24 h. Supernatants were analyzed for BDNF by ELISA. Results are mean $\pm$ SD of three separate experiments. Human fetal neurons were incubated with different doses of mevastatin, pravastatin and simvastatin under serum free condition. After 5 h of incubation, the mRNA expression of BDNF was analyzed by RT-PCR (V) and after 24 h of stimulation, protein levels of BDNF and NT-3 were measured in supernatants by ELISA (W). Data are mean ± SD of three independent experiments. $^{a}p<0.001$ *vs control BDNF;* $^{b}p<0.001$ *vs control NT-3*. (X) Male C57/BL6 mice (6-8 wk old) were treated with simvastatin (1 mg/kg bwt/d) via gavage. After 7 d, animals were perfused and their cortical sections were analyzed for the expression of BDNF [Red = BDNF; Green = NeuN; Blue = DAPI]. Results represent analysis of three sections of each of three mice (n=3) per group.

Figure S2 (part A)

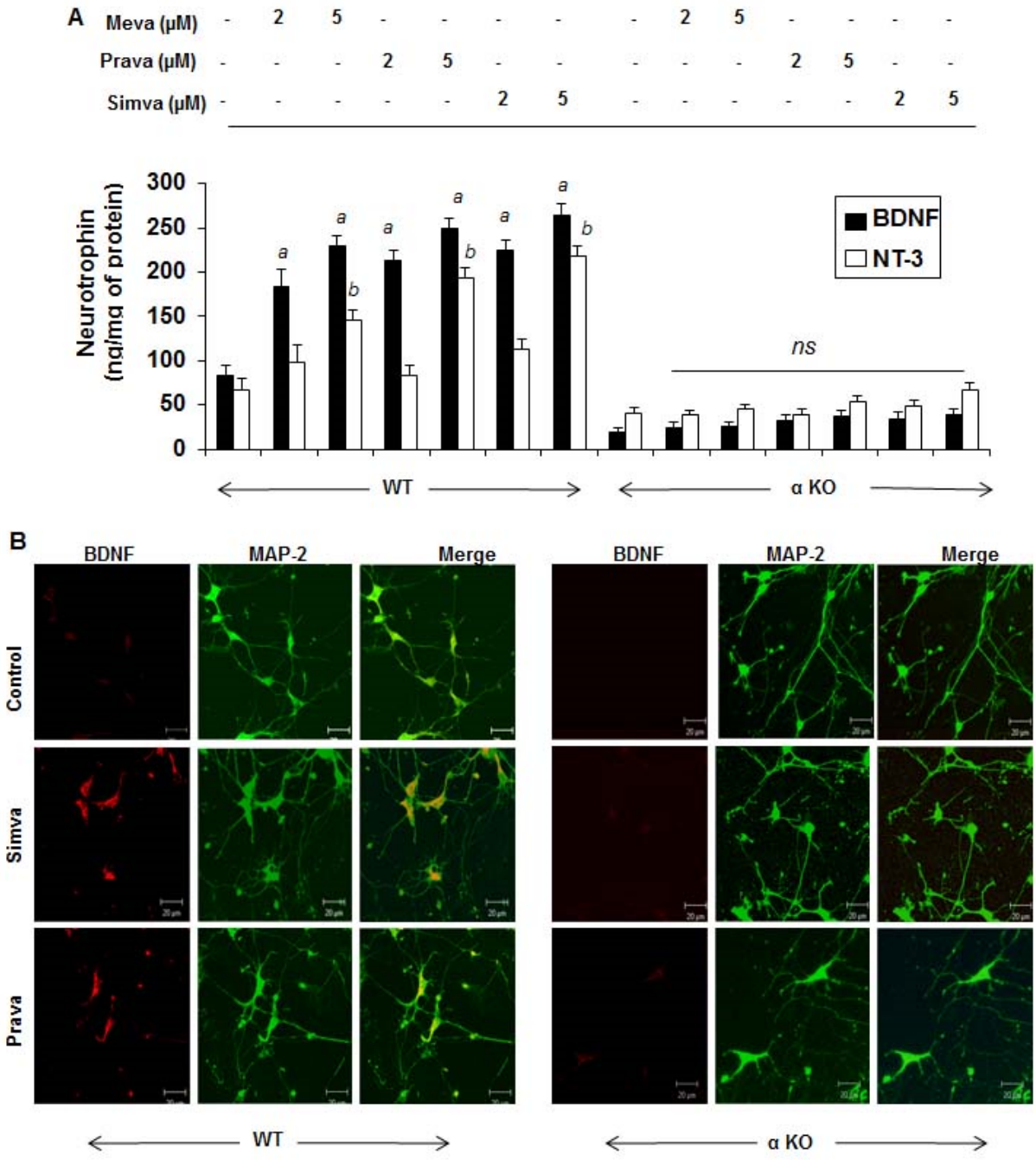

Figure S2 (part B)

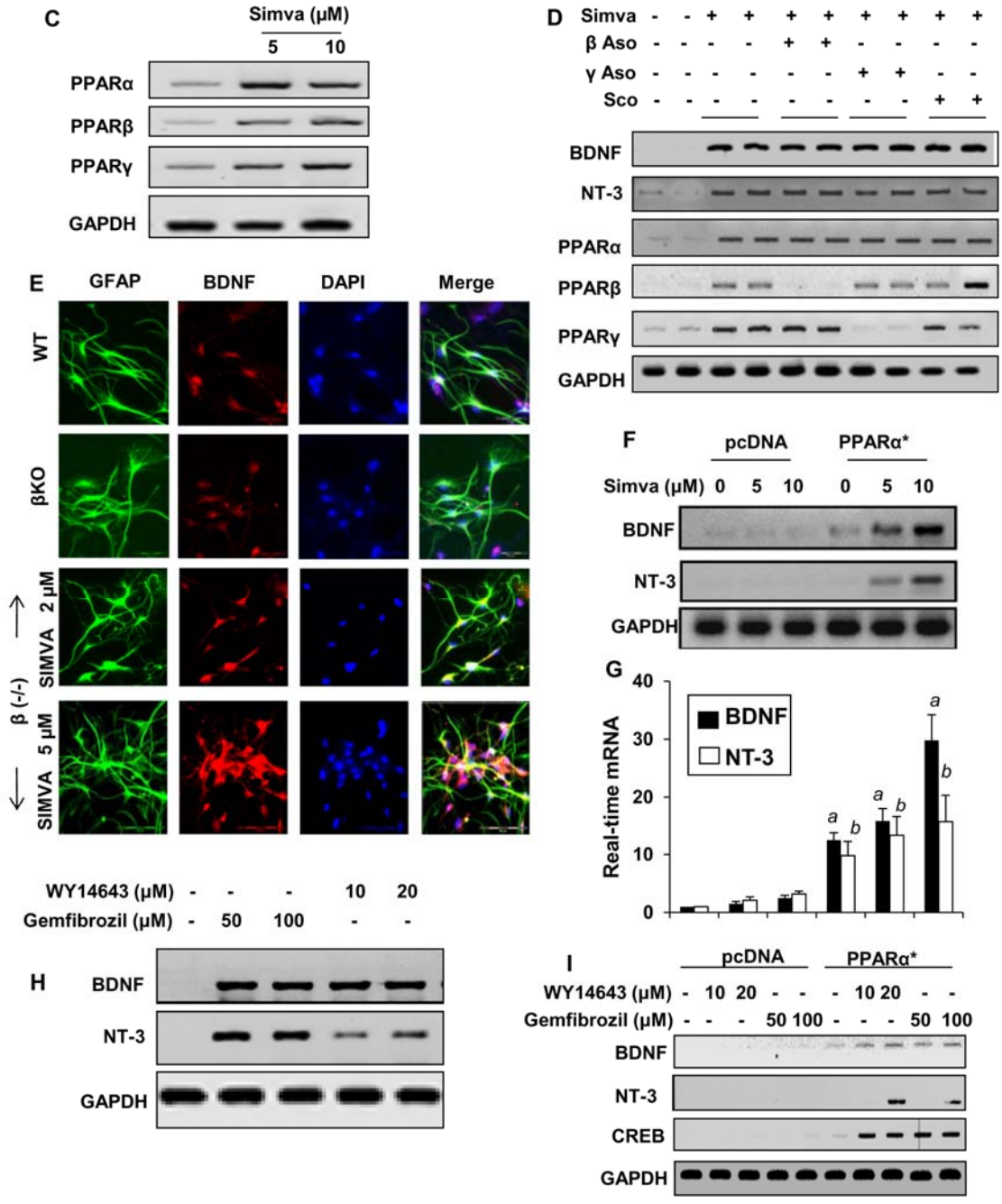

Figure S2, related to Figure 2. **Role of PPARs in statin-mediated increase in neurotrophic factors in primary mouse neurons and astrocytes.** (A) Mouse cortical neurons isolated from WT and *Ppara* null fetus (E18) were stimulated with different doses of mevastatin, pravastatin and simvastatin under serum free condition. After 24 h of stimulation, levels of BDNF and NT-3 proteins were analyzed in supernatants by ELISA. Data are mean ± SD of three independent experiments. *[a]p<0.001 vs control BDNF; [b]p<0.001 vs control NT-3. NS, not significant vs KO control.* (B) WT and *Ppara* null neurons were stimulated with 5 μM simvastatin or pravastatin under serum free condition. After 24 h, cells were double-labeled for BDNF and MAP-2. Results represent three independent experiments. C) Mouse primary astrocytes were treated with different doses of simvastatin for 5 h followed by analysis of PPARα, β and γ mRNAs by RT-PCR. D) Mouse astrocytes were incubated with PPARβ and γ antisense oligoneucleotides (AsO) for 24 h. After that, cells were incubated with 10 μM of simvastatin for 5 h followed by mRNA analysis of BDNF, NT3, PPARα, PPARβ, and PPARγ by semi-quantitative RT-PCR. Scrambled oligoneucleotide (ScO) were used as a negative control in this experiment. E) Astrocytes isolated from *Pparb* null mice were stimulated with simvastatin under serum-free condition for 24 h followed by double-label immunofluorescence for GFAP and BDNF. *Ppara* null astrocytes were transfected with pcDNA3 (an empty vector) or *Ppara* over-expression construct. After 24 h, cells were stimulated with different doses of simvastatin for 5 h followed by mRNA analysis of BDNF and NT-3 by RT-PCR (F) and real-time PCR (G). Data are mean ± SD of three independent experiments. *[a]p<0.001* vs control-pcDNA-BDNF; *[b]p<0.001* vs control-pcDNA-NT-3. H) Cells were treated with different concentrations of WY14643 and gemfibrozil for 5 h followed by mRNA analysis of BDNF and NT3 by semi-quantitative RT-PCR. I) *Ppara* null astrocytes were transfected with pcDNA3 or *Ppara* over-expression construct. After 24 h, cells were stimulated with different doses of WY14643 and gemfibrozil for 5 h followed by mRNA analysis of CREB, BDNF and NT-3 by RT-PCR. Results represent three independent experiments.

Figure S3

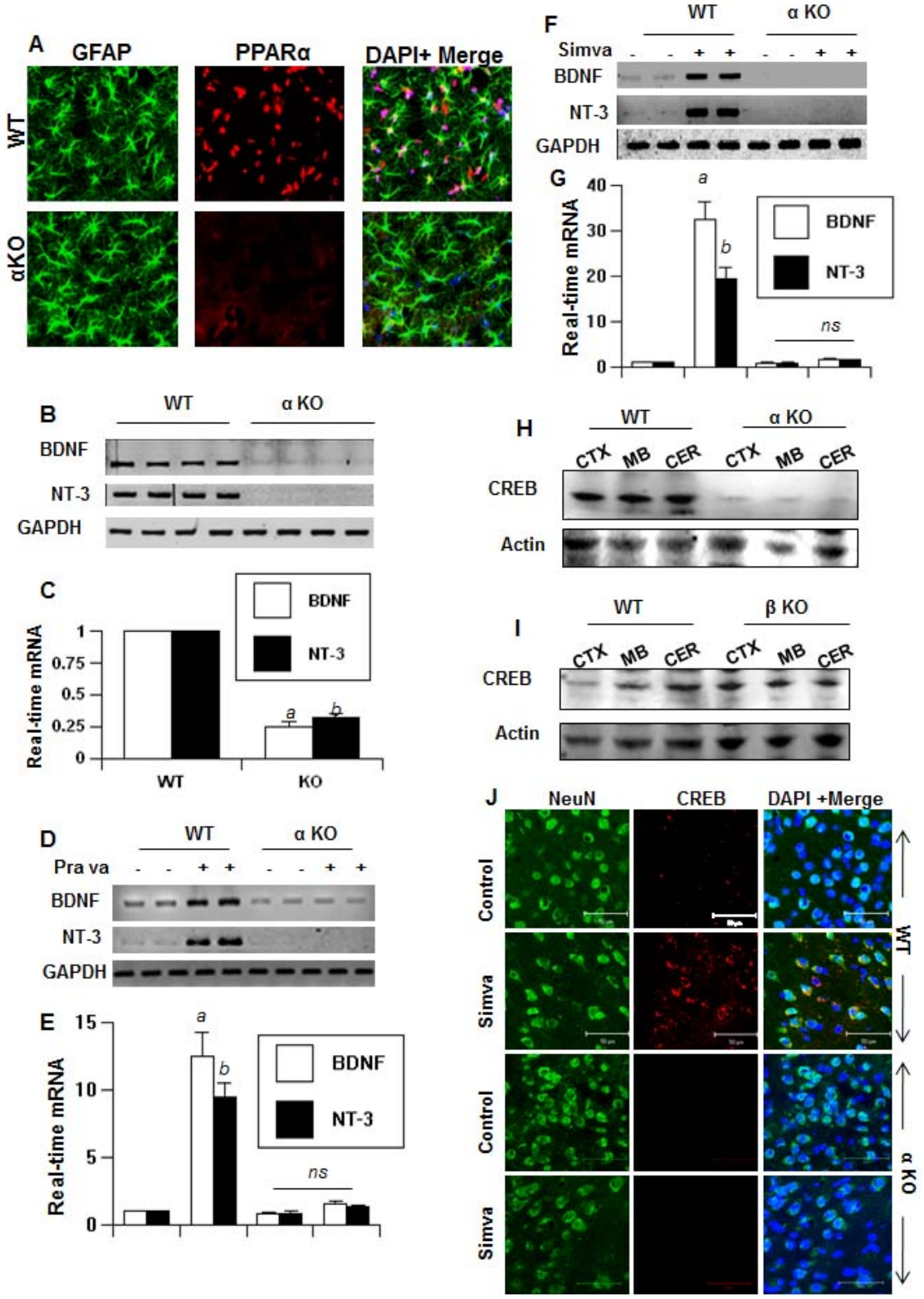
A
GFAP
PPARα
DAPI+ Merge
WT
αKO
B
WT
α KO
BDNF
NT-3
GAPDH
C
BDNF
NT-3
Real-time mRNA
1
0.75
0.5
0.25
0
a
b
WT
KO
D
WT
α KO
Pra va
- - + + - - + +
BDNF
NT-3
GAPDH
E
15
10
5
0
a
b
ns
BDNF
NT-3
Real-time mRNA
F
WT
α KO
Simva
- - + + - - + +
BDNF
NT-3
GAPDH
G
40
30
20
10
0
a
b
ns
BDNF
NT-3
Real-time mRNA
H
WT
α KO
CTX
MB
CER
CTX
MB
CER
CREB
Actin
I
WT
β KO
CTX
MB
CER
CTX
MB
CER
CREB
Actin
J
NeuN
CREB
DAPI +Merge
Control
Simva
Control
Simva
WT
α KO

Figure S3, related to Figure 2 and Figure 5. Statins stimulate the expression of neurotrophins and CREB *in vivo* in the CNS via PPARα. (A) Cortical sections of WT and *Ppara*-null (α KO) mice were double-labeled with GFAP and PPARα. DAPI was used to visualize nucleus. Results represent analysis of two sections of each of 4 mice per group. Cortices of six to eight week old male WT (*n =4*) and α KO mice (*n= 4*) were analyzed for the mRNA expression of BDNF and NT3 by semi-quantitaive RT-PCR (B) and quantitative real-time PCR (C). Data are mean ± SEM of four mice (n=4) per group. $^{a}p<0.001$ *vs control BDNF;* $^{b}p<0.001$ *vs control NT-3.* Hippocampal sections of WT and α KO mice were immunostained with BDNF using DAB-staining protocol. WT and α KO mice (n=4 in each group) were fed pravastatin (1 mg/kg bwt/d) (D, E) and simvastatin (1mg/kg bwt/d) (F, G) for one week followed by analysis of BDNF and NT-3 mRNAs in the cortex by semi-quantitative RT-PCR (D & F) and real-time PCR (E & G). Control mice received vehicle (0.1% methyl cellulose) only. Data are mean ± SEM of four mice per group. $^{a}p<0.001$ vs vehicle-control-BDNF; $^{b}p<0.001$ vs vehicle-control-NT-3. (H) Cortex (CTX), midbrain (MB), and cerebellum (CER) of WT and α KO mice were immunoblotted for CREB. (I) CREB immunoblot analysis in the different parts of the brain of WT and *Pparb*-null (β KO) mice. (J) Wild type and α KO mice (n=4 per group) were treated with simvastatin (1mg/kg bwt/d) via gavage for 7 d. After that, animals were sacrificed and their cortical sections were double-labeled for NeuN (green) and CREB (red). Results represent analysis of two sections of each of 4 mice per group.

Figure S4 (part A)

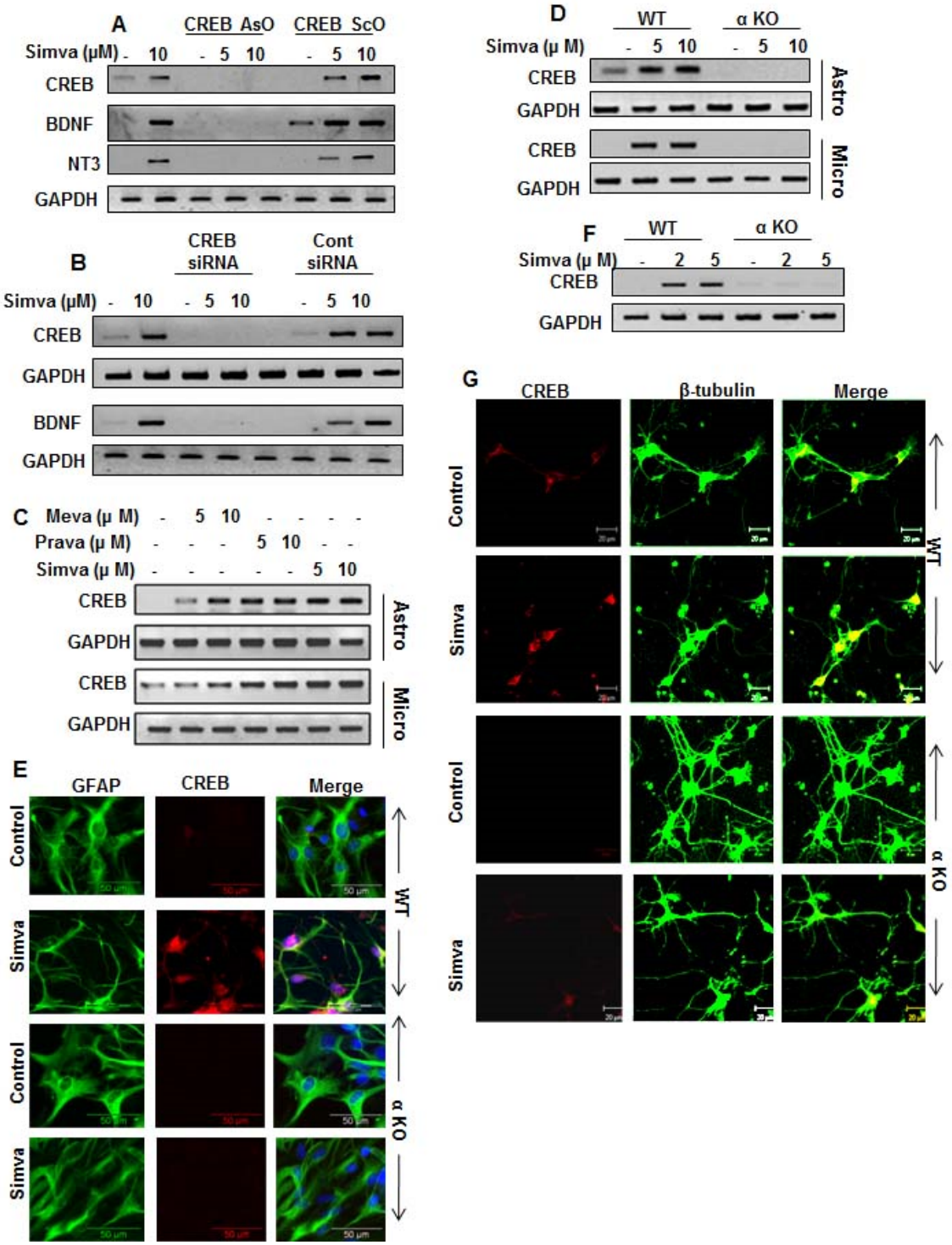

Figure S4 (part B)

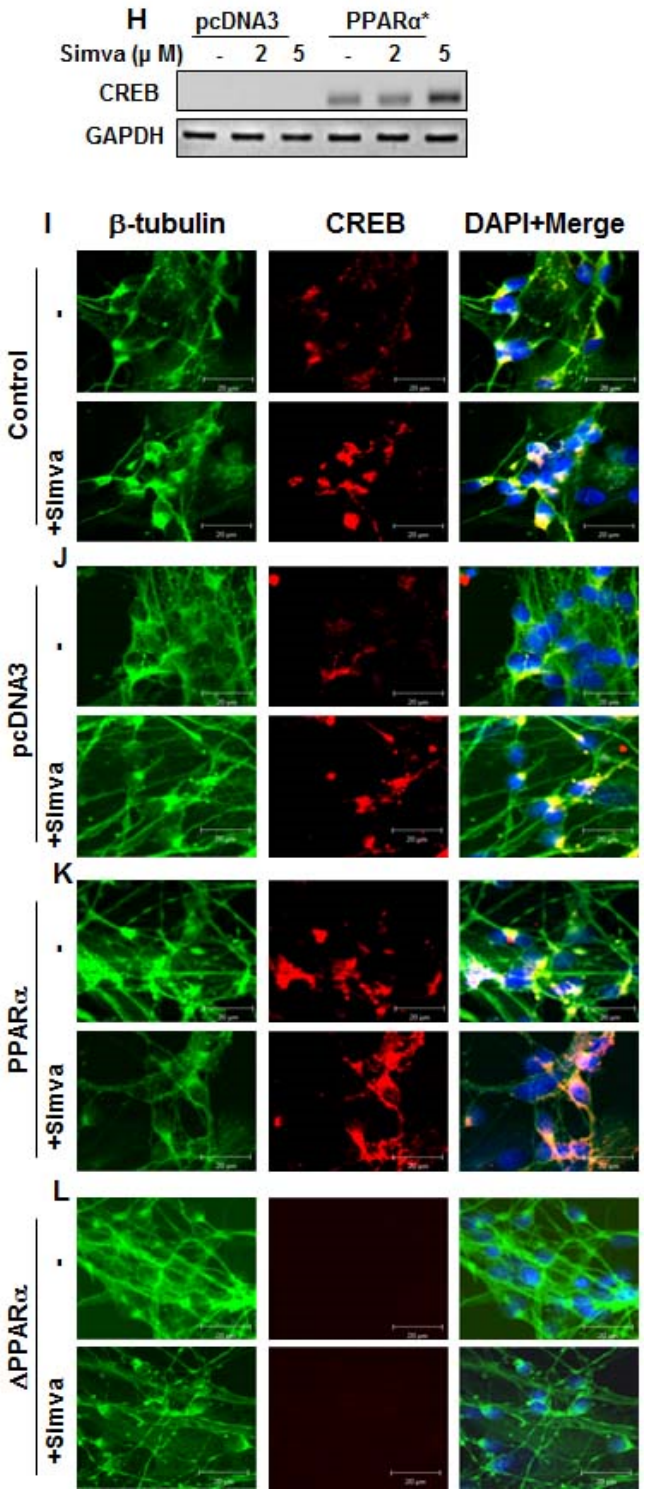

Figure S4, related to Figure 5. Statins stimulate the expression of BDNF and NT-3 in glial cells and neurons via PPARα-dependent transcriptional regulation of CREB.
(A) Primary mouse astrocytes were incubated with CREB antisense (AsO) and scrambled (ScO) oligonucleotides. After 24 h, cells were treated with simvastatin for 6 h followed by monitoring the mRNA expression of CREB, BDNF and NT-3. (B) Mouse cortical neurons were transfected with CREB siRNA and control siRNA. After 24 h of transfection, cells were treated with simvastatin for 6 h followed by analysis of BDNF and CREB mRNAs by RT-PCR. C) Primary mouse astrocytes (*top*) and microglia (*bottom*) were treated with three different statins under serum free condition for 6 h followed by monitoring the mRNA expression of CREB by RT-PCR. (D) Astrocytes (*top*) and microglia (*bottom*) isolated from WT and *Ppara*-null mice were treated with simvastatin for 6 h followed by mRNA analysis of CREB by RT-PCR. (E) Astrocytes isolated from WT and *Ppara*-null mice were treated with simvastatin for 24 h followed by double-labeling of GFAP and CREB. F) Cortical neurons isolated from WT and *Ppara*-null mice were treated with simvastatin for 6 h followed by monitoring the mRNA expression of CREB by RT-PCR. G) After 24 h of stimulation, neurons were double-labeled for CREB and β-tubulin. Results represent three independent analyses. H) *Ppara*-null astrocytes were transfected with *Ppara* over-expression construct. After 24 h of transfection, cells were treated with simvastatin under serum free condition for 6 h followed by monitoring the mRNA analysis of CREB by RT-PCR. (I-L) Human fetal neurons were isolated, cultured and transfected with *Ppara* over-expression construct, dominant negative construct (ΔPPARα) and empty vector (pcDNA3). After 24 h of transfection, cells were treated with simvastatin for 6 h followed by double-labeling of CREB and β-tubulin (I, Control; J, pcDNA3; K, PPARα; L, ΔPPARα). Results represent three independent experiments.

Supplementary Methods

Lentiviral cloning of FL*Ppara* and L331M/Y334D ΔsbdP*para*:
Site directed mutation: Mouse PPARα ORF cloned in pCMV6-AC-GFP vector (cat # MG 227641) was purchased from Origene. MG227641 was mutated at Leu331 with methionine (L331M) and Tyr334 with aspartate (Y334D) by site-directed mutagenesis kit (Stratagene). Two primers in opposite orientation were used to amplify the mutated plasmid in a single PCR reaction. The PCR product was precipitated with ethanol and then phosphorylated by T4 kinase. The phosphorylated fragment was self-ligated by T4 DNA ligase and digested with restriction enzyme DpnI to eliminate the non-mutated template. The mutated plasmid was cloned and amplified in Escherichia coli (DH5α strain) competent cells.
Generating pLenti6.3/V5-TOPO® constructs of FLPpara and ΔsbdPpara
Briefly, each construct was amplified by PCR, using primer pair (sequence) and every product had a single adenosine (A) to the 3' end. Then the TOPO cloning reaction was performed using Invitrogen kit (K5315-20) with pLenti6.3/V5-TOPO vector. For

transformation One-Shot Stbl3 competent cells were used. Sequencing of the clones was performed at ACGT Inc.

Producing Lentivirus in 293FT Cells

293FT cells were cultured with 95% confluency in Opti-MEM media without antibiotics. Next day, ViraPower™ Packaging Mix (9 μg/reaction) and pLenti expression plasmid DNA containing either FL*Ppara* or Δsbd*Ppara* (3 μg/reaction) (12 μg total) were mixed in 1.5 mL of serum-free Opti-MEM® I Medium. In another tube, 36 μL of Lipofectamine® 2000 was added in 1.5 mL of serum-free Opti-MEM® I Medium with gentle mix. After 5 min of incubation at room temperature, both the reactions were combined and incubated for 20 mins. After that, the mixture was applied to HEK-293FT cells and incubated overnight at 37°C in a humidified 5% CO_2 incubator. Next day, the media would be replaced with serum-free Opti-MEM media and further incubated for 48-72 hrs at 37°C in a humidified 5% CO_2 incubator and then sup containing viral particles was collected. Viral particles were concentrated with lenti-concentrator solution and MOI was calculated.

Breeding and development of FAD5X/*Ppara*-null animals. Female 5XFAD mice overexpressing human amyloid precursor protein (APP) with Swedish (K670N, M671L), Florida (I716V), and London (V717I) Familial Alzheimer's Disease (FAD) mutations along with human Presenilin 1 (PS1) harboring two FAD mutations, M146L and L286V, were crossed to male mice null for PPARα (The Jackson Laboratory, #008154). Through a series of four inbred generations, three double-transgenic lines possessing mutated APP and PSEN1 on a PPARα null background were produced. Bigenic male mice maintained transgenic for the 5XFAD mutations and homozygous for the PPARα depletion [FAD5X/*Ppara*-null] were used for experimentation. Animal experiments were conducted in accordance with institutional approval and National Institutes of Health guidelines.

Electrospray ionization (ESI)-MS analysis of PPARα-simvastatin interaction

Primary mouse astrocytes were treated with simvastatin (10 μM) for 2 h under serum free condition followed by washing of cells four times with sterile PBS to remove any unbound simvastatin. Then cells were homogenized in ice-cold nondetergent hypotonic buffer [10 mM HEPES (pH 7.9), 1.5 mM $MgCl_2$, 10 mM KCl, 100 mM DTT, protease and phosphatase inhibitor cocktail]. After 10 min of additional incubation in the hypotonic buffer, the homogenate was centrifuged at 8,000 g at 4°C for 10 min. Next, the pellet was homogenized in ice-cold extraction buffer [10 mM HEPES (pH 7.9), 1.5 mM $MgCl_2$, 0.21 M NaCl, 0.2 mM EDTA, 25% (v/v) glycerol, 100 mM DTT, protease and phosphatase inhibitor cocktail], placed on a rotating shaker at 4°C for 1 h, and then centrifuged at 18,000 g for 10 min. The supernatant (nuclear fraction) was incubated with 1.5 μg of GST PPARα LBD (Protein One) at 4°C for 6 h in a rotating shaker. The reaction mixture was passed through glutathione column (Pierce® GST Spin Purification Kit), washed four times [50 mM Tris HCl (pH 7.4), 100 mM NaCl, protease and phosphatase inhibitor cocktail] and then eluted with free glutathione. The eluate was transferred to methanol:chloroform:water (4:3:1) mixture and then centrifuged at 14,000 rpm for 90 sec. The organic phase was collected, evaporated in the speedvac, reconstituted with 30 μL chloroform, and analyzed by ESI-MS.

In Silico structural analyses of PPARα, β, and γ complexed with simvastatin.

Ligand Preparation

The ligand simvastatin was subjected to LigPrep module implemented in Tripos software[X1], which converted the 2D to 3D structure. Then using the ionization engine, the ligand was prepared at pH 7.0 ± 1. The appropriate stereoisomers were generated along with the low energetic conformers.

Protein Preparation

The crystal structures for PPARα (3VI8.pdb), β (3GWX.pdb), and γ (3U9Q.pdb) were imported from the pdb databank. The protein preparation module of Tripos was utilized to fix up the hydrogen bonding orientation, bond orders, charges, missing side chain atoms, missing loop, protonation at physiological pH, and side chain bumps. Finally, staged minimization was performed for all three protein structures.

Docking of the Ligands

The Surflex docking module[X1] implemented in Tripos was used to carry out the docking of simvastatin in PPARα, β and γ crystal structures. After the docking, three major scoring functions such as Total Score (a function of $-\text{Log}K_d$), Crash Score (penalty score reflecting the inappropriate penetration of the ligand into the active site pocket) and Polar Score (depicting all the favorable polar interactions) were obtained.

We also computed the binding free energy of simvastatin in PPARα, using Molecular Mechanics Generalized Born Surface Area approach[X]. To account for the structural deformation upon binding, we included adaptation expense that accounts for changes in the intramolecular energetics (ΔG^0_{int}). For ligand strain energy, we specified a 5å region of the receptor from the centroid of the ligand to be flexible so that the protein structure was relaxed in the computation of the binding energy of the ligands.