Supplementary Materials

In vivo AAV1 transduction with hRheb(S16H) protects hippocampal neurons by BDNF production

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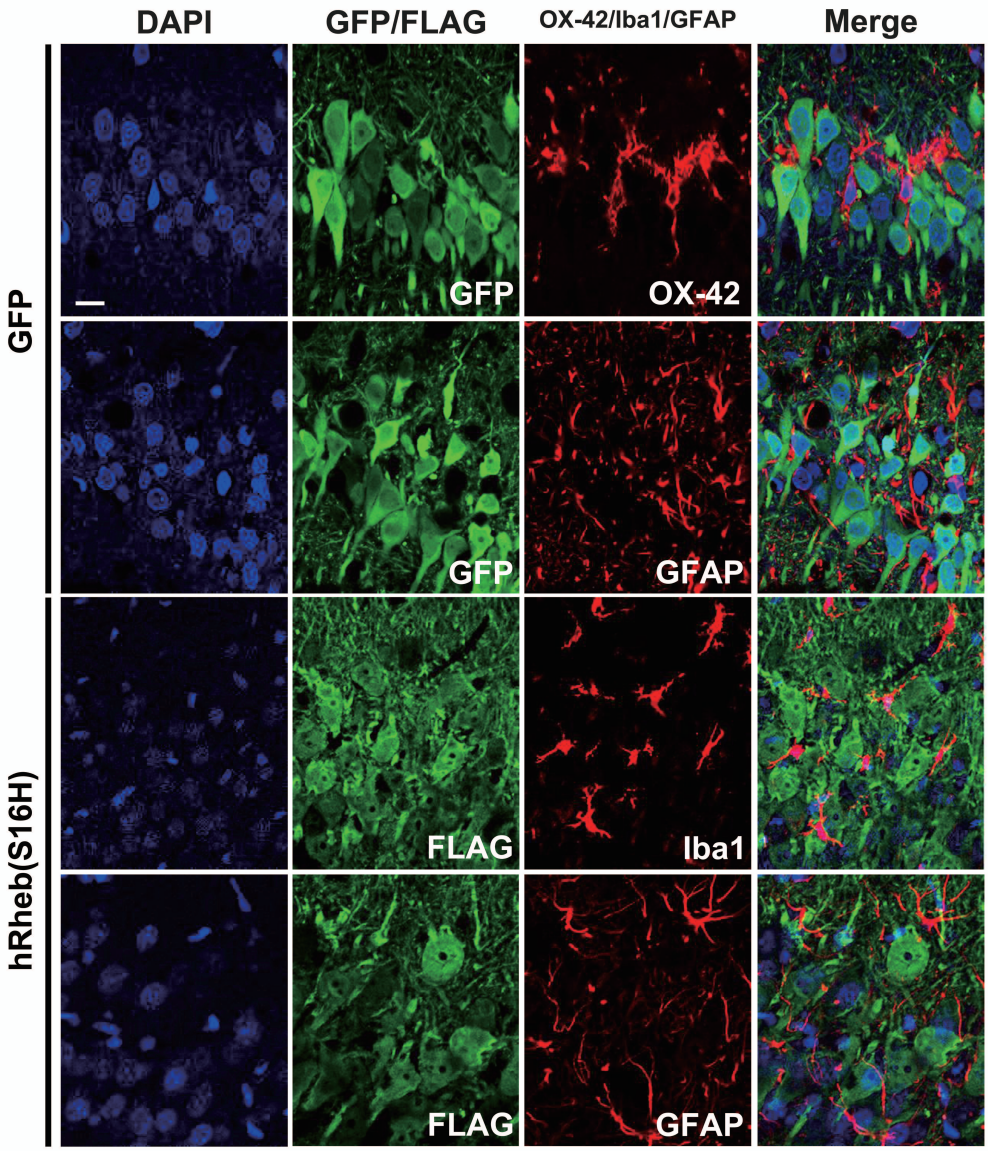
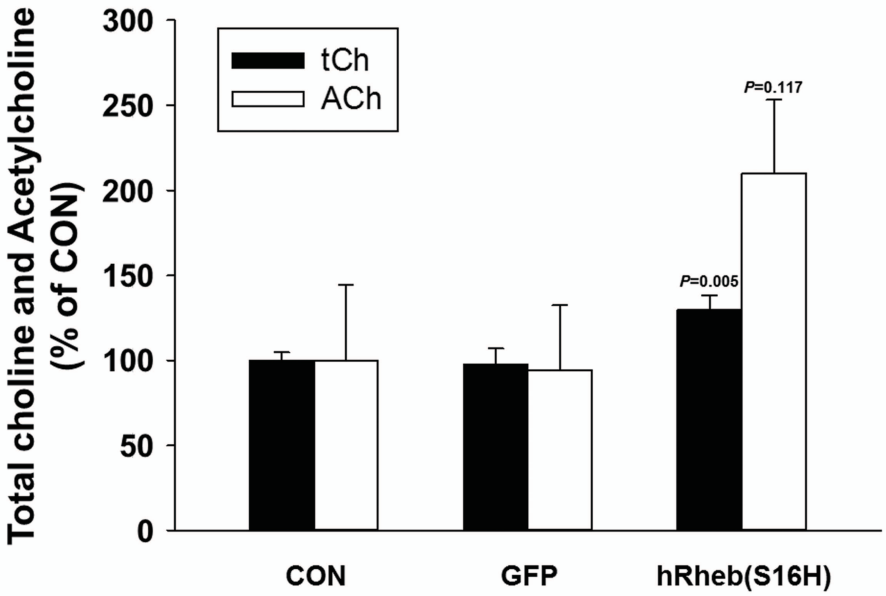
Supplementary informations:


DAPI
GFP/FLAG
OX-42/Iba1/GFAP
Merge
GFP
hRheb(S16H)
GFP
OX-42
GFP
GFAP
FLAG
Iba1
FLAG
GFAP

Supplementary Figure S1: Non-transduction of hippocampal microglia and astrocytes by AAV-GFP or AAV-hRheb(S16H) in normal adult SD rats.

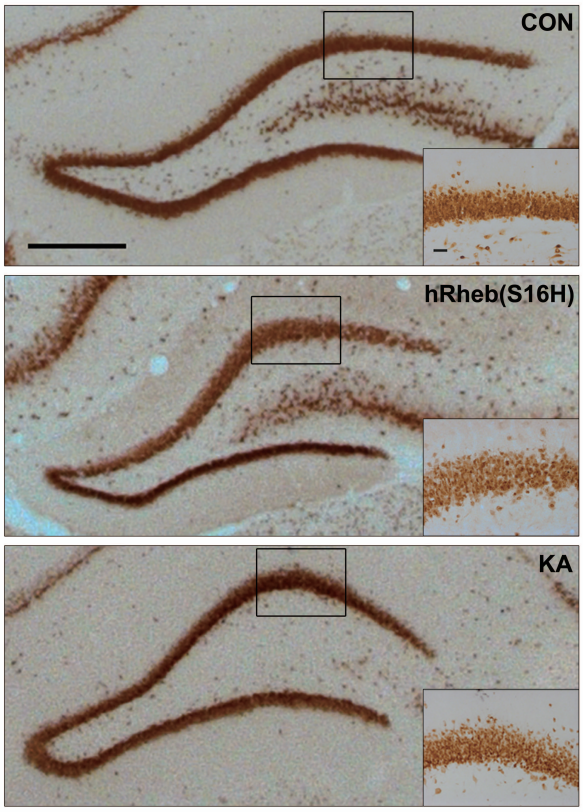
Rats received an unilateral injection of AAV-GFP or AAV-hRheb(S16H) into the hippocampal CA1 region and 4 weeks later they were sacrificed for immunofluorescence labeling. The triple-labeling for DAPI (blue), GFP (green) and OX-42/GFAP (red), or for DAPI, FLAG (green) and Iba1/GFAP (red) shows that transgene expression is not identifiable within microglia and astrocytes of the hippocampus for each vector. Scale bar, 20μm.


Total choline and Acetylcholine (% of CON)
300
250
200
150
100
50
0
tCh
ACh
P=0.117
P=0.005
CON
GFP
hRheb(S16H)

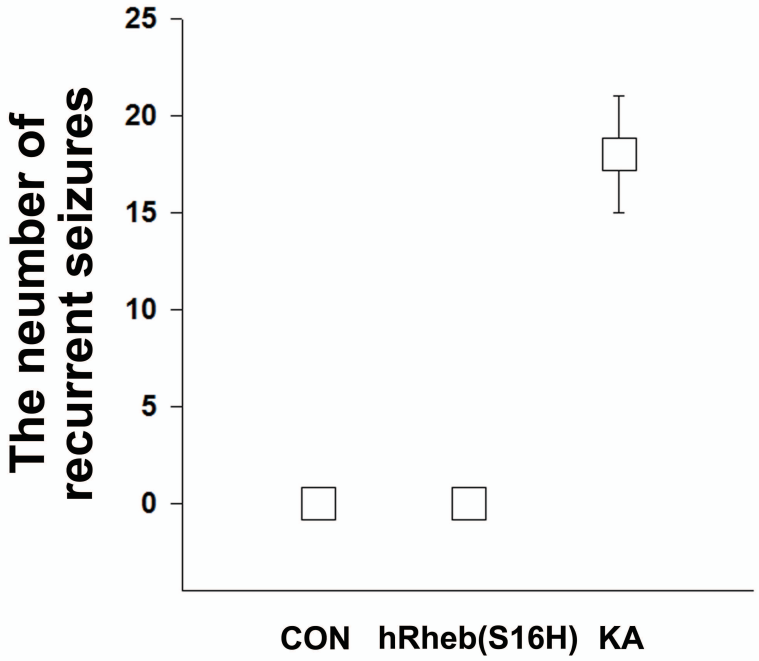
Supplementary Figure S2: The levels of total choline (tCh) and acetylcholine (ACh) in the rat hippocampus.

The amounts of tCh and ACh were measured at 4 weeks after the injection of AAV-GFP or AAV-hRheb(S16H), and the levels in the ipsilateral hippocampus were expressed quantitatively as a percentage compared to the contralateral control. An increase in tCh is observed in the hRheb(S16H)-expressed hippocampus compared to the intact/GFP controls, and the level of ACh shows a modest alteration with no significant change compared to the intact/GFP controls (One-way ANOVA and Student-Newman-Keuls analysis; n = 8, each group).

a

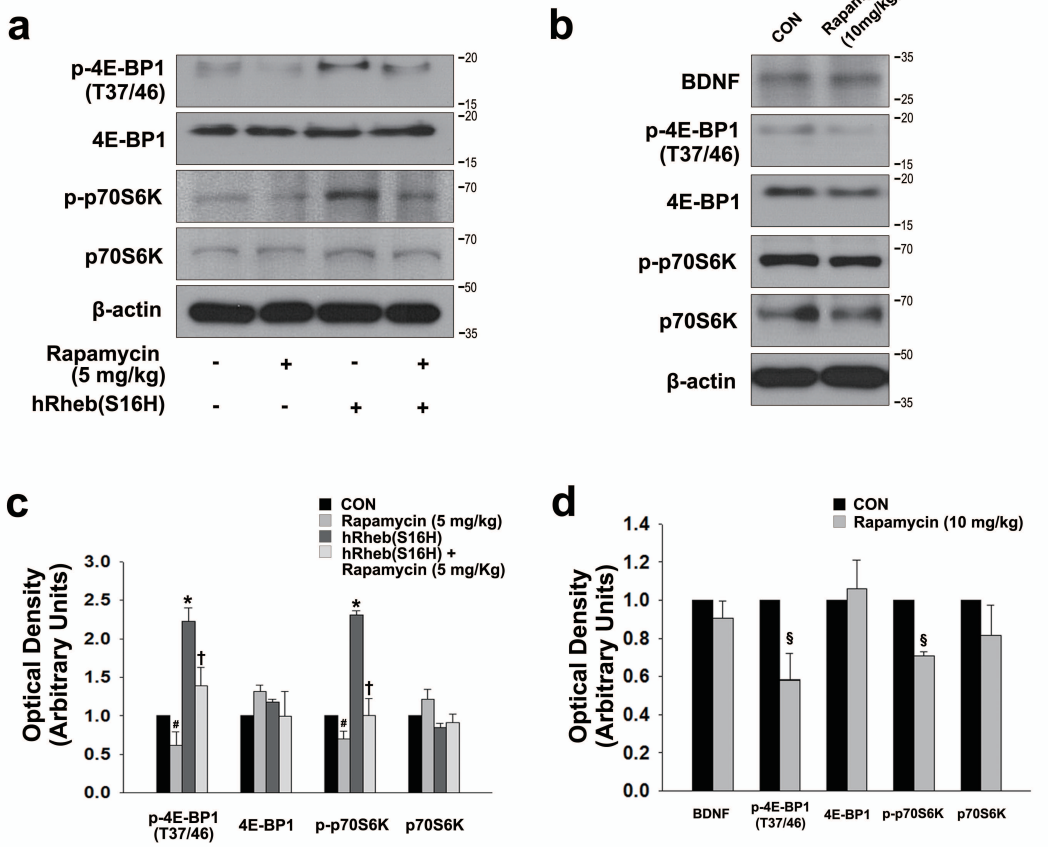

CON
hRheb(S16H)
KA

b


The neumber of recurrent seizures
25
20
15
10
5
0
CON
hRheb(S16H)
KA

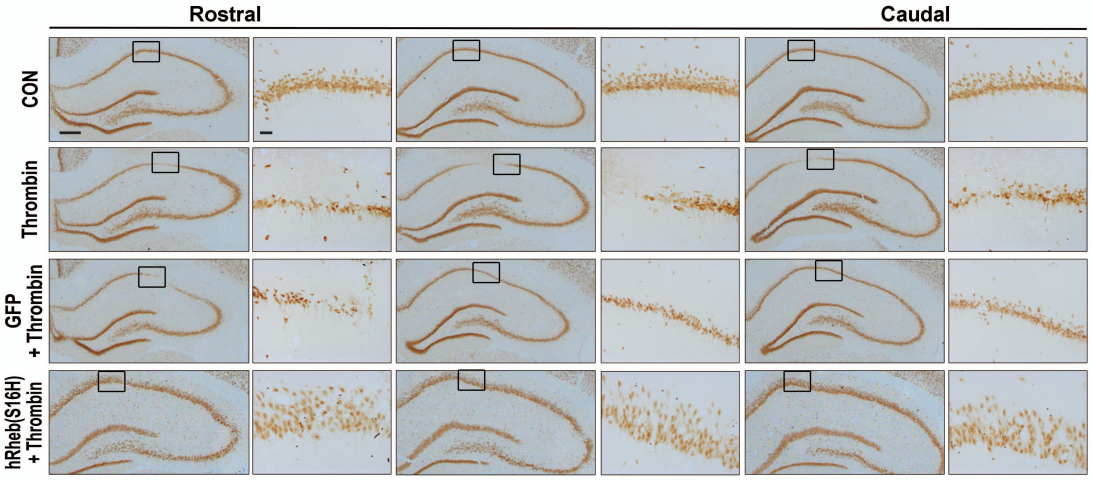
Supplementary Figure S3: Cytoarchitectural changes in the granule cell layers by hRheb(S16H) transduction and behavioral tests for seizures.

Rats received a unilateral intra-hippocampal injection of hRheb(S16H) or kainic acid in the hippocampus, and they were sacrificed at 25 days after hRheb(S16H) or kainic acid treatment for immunohistochemical staining. For the behavioral tests for seizures, video recording was performed for 4 days starting 3 weeks after the post-injection of hRheb(S16H) or kainic acid. **(a)** hRheb(S16H) treatment shows granule cell dispersion with an increase in the area of NeuN-positive neurons in the dentate gyrus compared to the intact controls. Kainic acid also induces granule cell dispersion. However, there is no increase in the neuronal area in the dentate gyrus compared to the intact controls. Scale bars, 500 μm and 40 μm. **(b)** The graph shows the number of recurrent seizures after hRheb(S16H) or kainic acid treatment. Note that AAV-hRheb(S16H)-injected rats do not show seizure-like behaviors compared to the kainic acid-treated rats. n = 5, each group.


a
p-4E-BP1 (T37/46)
4E-BP1
p-p70S6K
p70S6K
β-actin
Rapamycin (5 mg/kg)
hRheb(S16H)
b
CON
Rapamycin (10mg/kg)
BDNF
p-4E-BP1 (T37/46)
4E-BP1
p-p70S6K
p70S6K
β-actin
c
CON
Rapamycin (5 mg/kg)
hRheb(S16H)
hRheb(S16H) + Rapamycin (5 mg/Kg)
Optical Density (Arbitrary Units)
p-4E-BP1 (T37/46)
4E-BP1
p-p70S6K
p70S6K
d
CON
Rapamycin (10 mg/kg)
Optical Density (Arbitrary Units)
BDNF
p-4E-BP1 (T37/46)
4E-BP1
p-p70S6K
p70S6K

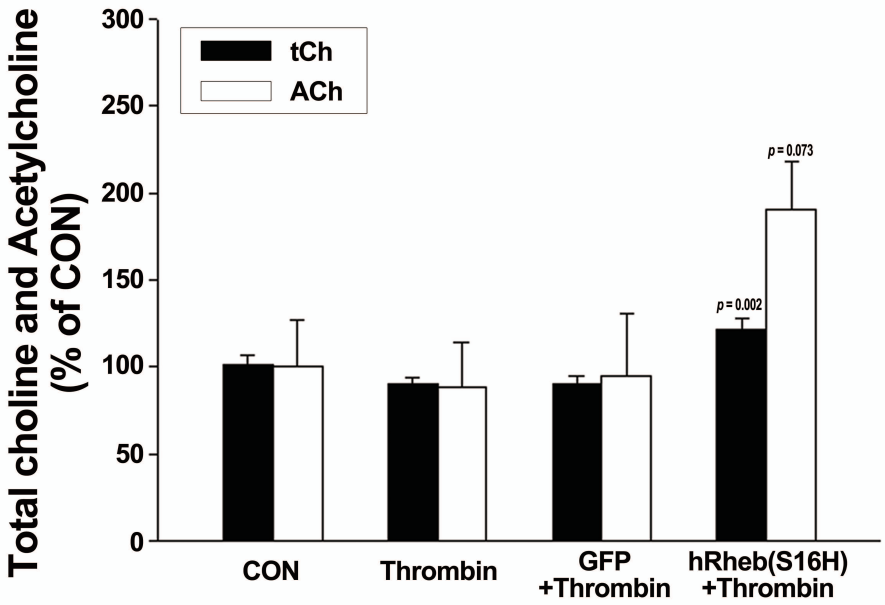
Supplementary Figure S4: Effects of rapamycin on the hRheb(S16H)-increased mTORC1 activity.

(a) Western blot analysis of mTORC1 activity in the hippocampus. Rats were post-treated with rapamycin (5 mg/kg, intraperitoneal injection), starting 3 weeks after hRheb(S16H) injection and continued daily until 4 weeks post-injection. The brain tissues treated with rapamycin or AAV-GFP in the absence of hRheb(S16H) were used as controls. Treatment with 5 mg/kg of rapamycin alone decreases the levels of p-4E-BP1 and p-p70S6K compared to that in intact controls (CON), and the hRheb(S16H)-increased levels of p-4E-BP1 and p-p70S6K are reduced by treatment with rapamycin. **(b)** Western blot analysis of BDNF expression and mTORC1 activity in the hippocampus after treatment with 10 mg/kg rapamycin for 7 days. Similar to the previous results (Figure 4), treatment with 10 mg/kg rapamycin does not alter the basal level of BDNF compared to controls (CON). However, its treatment shows a significant decrease in the levels of p-p-4E-BP1 and p-p70S6K compared with CON, suggesting that rapamycin inhibits mTORC1 activity. **(c and d)** The histogram results show a quantitative analysis based on the density of the p-4E-BP1, 4E-BP1, p-p70S6K, and p70S6K bands normalized to the β-actin band for each sample. (c) and (d) indicate the analyzed data of (a) and (b), respectively. All values represent the mean ± SEM of three pooled samples for each group. $^{*}p < 0.01$ and $^{\dagger}p < 0.05$, significantly different from CON and hRheb(S16H) alone, respectively (One-way ANOVA and Student-Newman-Keuls analysis). $^{\#}p < 0.05$ and $^{\S}p < 0.05$, significantly different form each CON (*t*-test).


Rostral
Caudal
CON
Thrombin
GFP
+ Thrombin
hRheb(S16H)
+ Thrombin

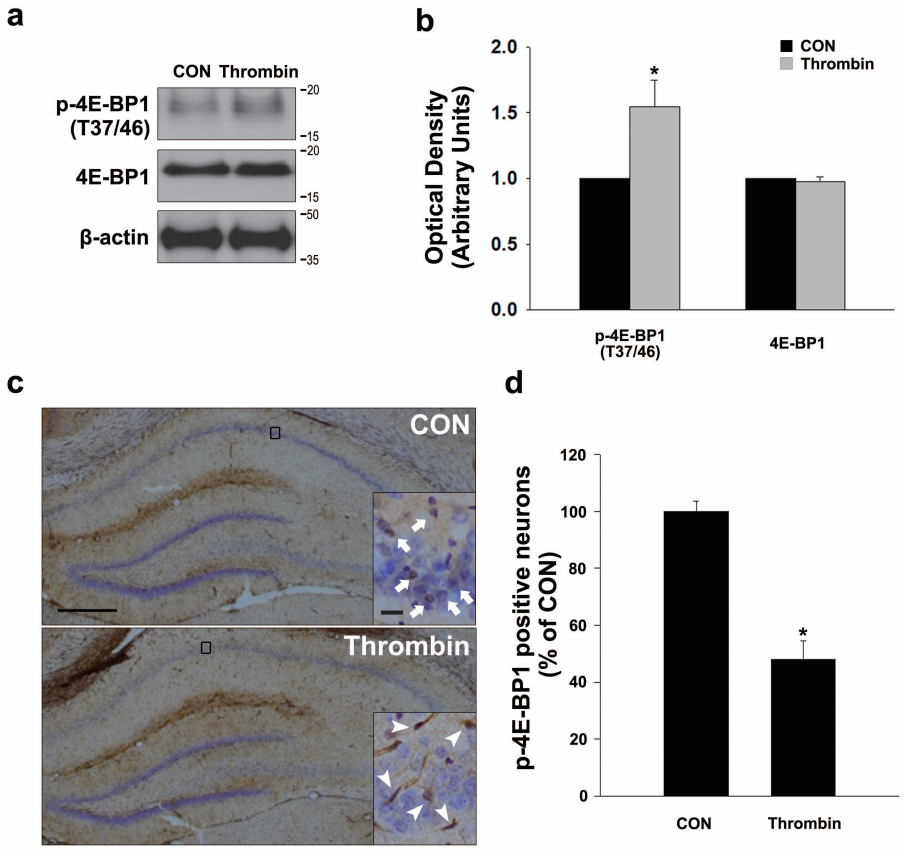
Supplementary Figure S5: Thrombin causes neuronal cell death in the hippocampus.

Rats received a unilateral injection of thrombin into the hippocampal CA1 layer at 3 weeks after the injection of viral vectors, and immunohistochemical staining for NeuN was performed at 1 week following thrombin treatment. The sections, which were used to count neurons in the CA1 region, were obtained at 3.3, 3.6, 4.16, and 4.3 mm posterior to the bregma. The series of sections show neuronal death in the hippocampal CA1 region after thrombin-treatment. However, the transduction of hippocampal neurons with hRheb(S16H) prevents neuronal cell death against thrombin-induced neurotoxicity compared to thrombin alone. GFP has no neuroprotection as described in Figure 5. Scale bars, 500 μm and 20 μm.


Total choline and Acetylcholine (% of CON)
300
250
200
150
100
50
0
tCh
ACh
p = 0.073
p = 0.002
CON
Thrombin
GFP +Thrombin
hRheb(S16H) +Thrombin

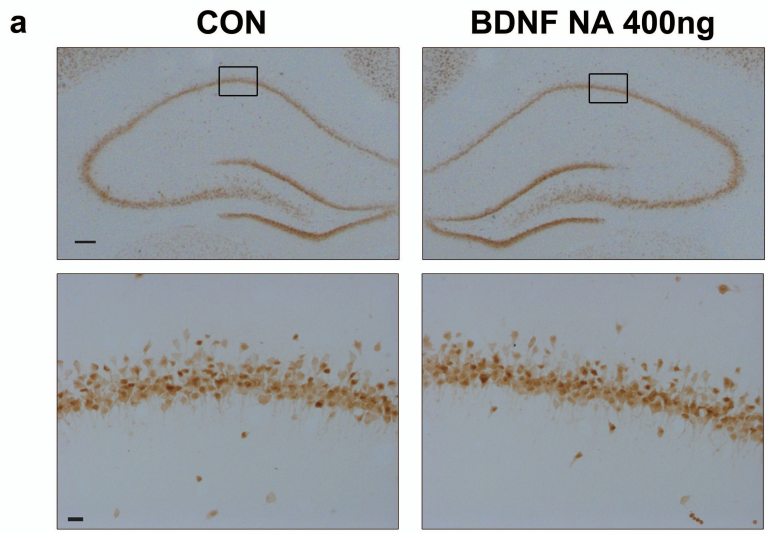
Supplementary Figure S6: hRheb(S16H)-increased tCh and ACh are preserved in the thrombin-treated hippocampus.

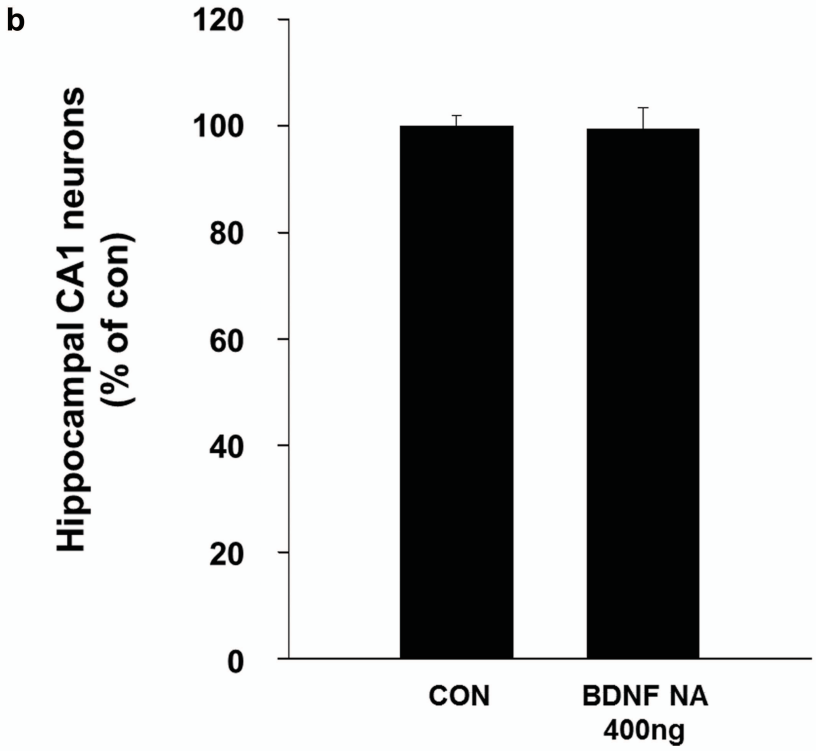
Rats received a unilateral intra-hippocampal injection of thrombin (20 U) at 3 weeks post injection of viral vectors, and the amounts of tCh and ACh were measured at 1 week after thrombin treatment. The levels in the ipsilateral hippocampus were expressed quantitatively as a percentage compared to the contralateral control (CON). The group of GFP+thrombin was used as a control for hRheb(S16H)+thrombin. The amounts of tCh and ACh showed no significant difference between thrombin alone and intact controls. hRheb(S16H)-increased tCh and ACh (Supplementary Figure S2) were preserved in the thrombin-treated hippocampus. $p = 0.002$ and $p = 0.073$, significantly different from CON, respectively (One-way ANOVA and Student-Newman-Keuls analysis; n = 4, each group).


a
CON Thrombin
p-4E-BP1 (T37/46)
4E-BP1
β-actin
-20
-15
-20
-15
-50
-35
b
CON
Thrombin
Optical Density (Arbitrary Units)
2.0
1.5
1.0
0.5
0.0
*
p-4E-BP1 (T37/46)
4E-BP1
c
CON
Thrombin
d
p-4E-BP1 positive neurons (% of CON)
120
100
80
60
40
20
0
*
CON
Thrombin

Supplementary Figure S7: Thrombin reduces mTORC1 activity in the hippocampal neurons.

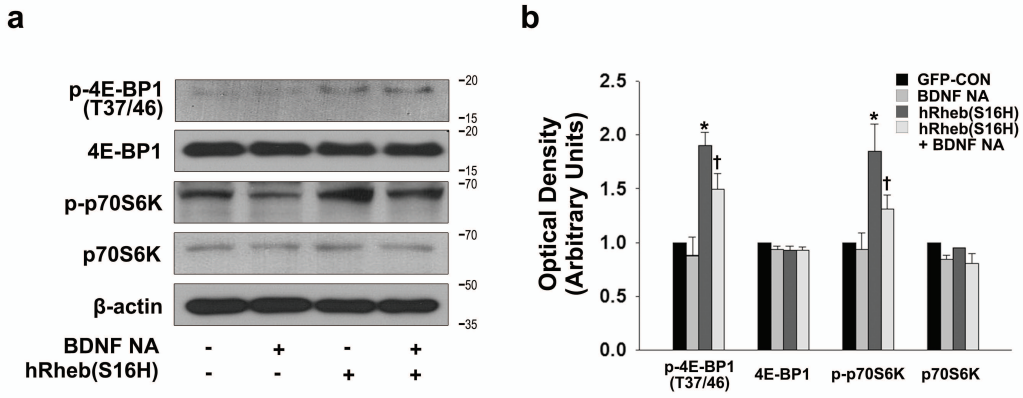
(a) Western blot analysis of mTORC1 activity in the hippocampus. Rats received a unilateral injection of thrombin into the hippocampal CA1 layer. The results show that mTORC1 activity is increased in the thrombin-treated hippocampus. **(b)** The histogram results show a quantitative analysis of p-4E-BP1 and 4E-BP1 bands normalized to the β-actin band for each sample. All values represent the mean ± SEM of three pooled samples for each group. *$p < 0.05$, significantly different from controls (CON) (*t*-test). **(c)** Brain sections were stained with anti-p-4E-BP1 at 1 week post-injection of thrombin. Immunoperoxidase staining for p-4E-BP1 shows that brown reaction products (p-4E-BP1, arrows and arrow-heads) are observed in the Nissl-positive (blue) neurons and non-neuronal cells in the CON and thrombin-treated hippocampus, respectively, suggesting that thrombin decreases mTORC1 activity in the hippocampal neurons, but not in non-neuronal cells. Scale bars, 500 μm and 20 μm. **(d)** The number of p-4E-BP1-positive neurons in the ipsilateral hippocampus, which measured as described in the methods for counting of hippocampal CA1 neurons with some modifications, was quantitatively expressed as a percentage compared with the contralateral controls (CON). *$p < 0.001$ (*t*-test; n = 4, each group).


a
CON
BDNF NA 400ng


b
Hippocampal CA1 neurons
(% of con)
120
100
80
60
40
20
0
CON
BDNF NA
400ng

Supplementary Figure S8: Treatment with BDNF neutralizing antibodies (BDNF NA) has no neurotoxicity.

(a) Rats received a unilateral injection of BDNF NA (400 ng in 4 μl) into the hippocampal CA1 region and NeuN immunostaining was performed at 1 week following BDNF NA treatment. The lower panels show higher magnifications of each CA1 layer. Scale bars, 200 μm and 20 μm. **(b)** The quantitative analysis showed no significant change in the number of hippocampal neurons in the CA1 layer (*t*-test; n = 4, each group).


a
p-4E-BP1 (T37/46)
4E-BP1
p-p70S6K
p70S6K
β-actin
BDNF NA - + - +
hRheb(S16H) - - + +
20
15
20
15
70
70
50
35
b
Optical Density (Arbitrary Units)
2.5
2.0
1.5
1.0
0.5
0.0
GFP-CON
BDNF NA
hRheb(S16H)
hRheb(S16H) + BDNF NA
*
†
*
†
p-4E-BP1 (T37/46)
4E-BP1
p-p70S6K
p70S6K

Supplementary Figure S9: BDNF neutralization attenuates the hRheb(S16H)-increased mTORC1 activity in the hippocampus.

Rats received an intra-hippocampal injection of 300 ng BDNF neutralizing antibodies (BDNF NA) at 3 weeks after AAV-hRheb(S16H) injection, and they were then sacrificed at 2 days post-injection of BDNF NA for Western blot analysis of mTORC1 activity. **(a)** BDNF NA alone has no effect on the levels of p-4E-BP1 and p-p70S6K compared to GFP-controls (GFP-CON). However, in the presence of hRheb(S16H), mTORC1 activity is significantly attenuated by treatment with BDNF NA compared to hRheb(S16H) alone. **(b)** The histogram results show a quantitative analysis of p-4E-BP1, 4E-BP1, p-p70S6K, and p70S6K bands normalized to the β-actin band for each sample. All values represent the mean ± SEM of three pooled samples for each group. $^{*}p < 0.05$ and $^{\dagger}p < 0.05$, significantly different from GFP-CON and hRheb(S16H) alone, respectively (One-way ANOVA and Student-Newman-Keuls analysis).