Supplemental online methods

Drugs. Morphine sulfate was purchased from Baxter Healthcare Corp. Rapamycin, ascomycin, and Akt inhibitor IV were ordered from CalBiochem. Naltrexone hydrochloride, nor-Binaltorphimine dihydrochloride (ηBNI), naltrindole hydrochloride, CTOP, DAMGO, and LY294002 were purchased from Sigma-Aldrich. Rapamycin, ascomycin, Akt inhibitor IV, and LY294002 were dissolved in 20% DMSO in normal saline solution.

Western blotting. Western blotting was carried out as we have described previously (1). Briefly, bilateral $L_{4/5}$ dorsal root ganglions and $L_{4/5}$ spinal cord were collected and homogenized with ice-cold lysis buffer (10 mM Tris, 5 mM EGTA, 0.5% Triton X-100, 2 mM benzamidine, 0.1 mM phenylmethylsulfonyl fluoride, 40 μM leupeptin, 150 mM NaCl, 1% phosphatase inhibitor cocktail I and cocktail II). The crude homogenate was centrifuged at 4^{o}C for 15 minutes at 1,000 × *g* and the supernatants collected. After protein concentration was measured, the samples were heated at 99°C for 5 minutes and loaded onto a 4% stacking/7.5% separating SDS-polyacrylamide gel (Bio-Rad Laboratories). The proteins were then electrophoretically transferred onto a polyvinylidene difluoride membrane (Immobilon-P, Millipore). The membranes were blocked with 3% nonfat milk in Tris-buffered saline containing 0.1% Tween-20 for 1 hour and then incubated with primary antibodies overnight under gentle agitation. These antibodies included rabbit anti-p-mTOR-Ser^{2448} (1:1,000, Cell Signaling Technology), rabbit anti-p-S6K1-Thr^{421}/Ser^{424} (1:1,000, Cell Signaling Technology), rabbit anti-p-4E-BP1-Thr$^{37/46}$ (1:2,000, Cell Signaling Technology), rabbit anti-p-Akt-Ser^{473} (1:1,000, Cell Signaling Technology), rabbit anti-mTOR (1:2,000, Cell Signaling Technology), rabbit anti-S6K1 (1:2,000, Cell Signaling Technology), rabbit anti-4E-BP1 (1:2,000, Cell Signaling Technology),

rabbit anti-Akt (1:1,000, Cell Signaling Technology), rabbit anti-eIF4A (1:1,000, Cell Signaling Technology), rabbit anti-eIF4E (1:800, Cell Signaling Technology), rabbit anti-TSC2 (1:1,000, Cell Signaling Technology), mouse anti-PSD-95 (1:2,000, Millipore), rabbit anti-nNOS (1:1,000, BD Transduction Laboratories), rabbit anti-CaMKIIα (1:2,000, Santa Cruz Biotechnology), rabbit anti-PKCγ (1:2,000, Santa Cruz Biotechnology), rabbit anti-PKCβ (1:2,000, Santa Cruz Biotechnology), and mouse anti–β-actin (1:3,000, Sigma-Adrich). β-actin was used as a loading control. The proteins were detected by horseradish peroxidase-conjugated anti-mouse or anti-rabbit secondary antibody and visualized by chemiluminescence regents (ECL; Amersham Pharmacia Biotech, Piscataway, NJ) and exposure to film. The intensity of blots was quantified with densitometry. The blot density from the control group was set as 100%. The relative density values from the other groups were determined by dividing the optical density values from these groups by the control value after each was normalized to the corresponding β-actin.

Immunohistochemistry. After animals were perfused with 300 ml of 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4), the $L_{4/5}$ segments were harvested, postfixed at 4^{o}C for 4 hours, and cryoprotected in 30% sucrose overnight. The transverse sections were cut on a cryostat at a thickness of 25 μm (for single-labeling) or 10–15 μm (for double-labeling). Single-label immunofluorescence histochemistry was carried out as described previously (2). Briefly, after being blocked for 1 hour at 37^{o}C in PBS containing 5% goat serum and 0.3% Triton X-100, the sections were incubated with primary rabbit anti-p-mTOR-Ser^{2448} (1:500), rabbit anti-p-S6K1-Thr^{421}/Ser^{424} (1:500), or rabbit anti-p-4E-BP1-Thr$^{37/46}$ (1:500) overnight at 4^{o}C. The sections were then incubated with goat anti-rabbit IgG conjugated with Cy3 (1:300; Jackson ImmunoResearch) for 1 hour at 37^{o}C. Control experiments included pre-

absorption of the primary antiserum with excess of the corresponding antigen (Cell Signaling Technology), substitution of normal rabbit serum for the primary antiserum, and omission of the primary antiserum. Finally, the sections were mounted onto gelatin-coated glass slides. Double-label immunofluorescence histochemistry was carried out as described previously (2). The sections were incubated overnight at 4^{o}C with 1) a mixture of rabbit anti-p-mTOR and mouse anti-NeuN (1:600, Chemicon), anti-GFAP (1:500, Sigma-Aldrich), or anti–OX-42 (1:400, Sigma-Aldrich); 2) a mixture of rabbit anti-p-p70S6K and mouse anti-NeuN, anti-GFAP, or anti–OX-42; or 3) a mixture of rabbit anti-p-4E-BP1 and mouse anti-NeuN, anti-GFAP, or anti–OX-42. The sections were then incubated with a mixture of goat anti-rabbit IgG conjugated with Cy3 (1:300) and donkey anti-mouse IgG conjugated with Cy2 (1:300, Jackson ImmunoResearch) or FITC-labeled avidin D (1:200, Sigma-Aldrich) for 1 hour at 37^{o}C. Control experiments as described above were performed in parallel.

In situ hybridization histochemistry. In situ hybridization histochemistry was carried out as described previously (3). Briefly, full-length rat MOR plasmid provided by Dr. H. Akil (University of Michigan) was linearized with Acc65I or SacI to obtain the antisense and sense probes. The antisense and sense cRNA riboprobes were synthesized with a digoxigenin RNA labeling kit according to the manufacturer's instructions (Roche Diagnostics). Both probes were purified with MicroSpin™ G-50 Columns (GE Healthcare Life Sciences). After the sections were digested and prehybridized, they were hybridized with 2 ng/μl digoxigenin-labeled antisense riboprobe or 2 ng/μl digoxigenin-labeled sense riboprobe for 16 hours at 60°C. After being washed, the sections were incubated with alkaline phosphatase-conjugated anti-digoxigenin antibody (1:1,000) overnight at 4°C. The fluorescent signals were developed with

Fast Red (Roche). Immunohistochemistry for mTOR and p-mTOR was then carried out as described above. The sections were lastly incubated with goat anti-rabbit or anti-mouse IgG conjugated with Cy2 for 1 hour at 37^{o}C.

Dorsal horn neuronal culture. Primary dorsal horn neuronal culture was carried out as described previously (1;4). Briefly, spinal cord was harvested from rat embryos at gestational day 12–14. Under a surgical microscope, the dorsal and ventral parts of the spinal cord were separated. Only the dorsal part of the spinal cord was collected for digestion. Neurons were seeded onto 6-well plates at a density of 1.5×10^6 cells/ml. Cultures were treated with 10 µg/ml cytosine-β-arabinofuranoside (Sigma-Aldrich) on days 4 and 5 to prevent proliferation of non-neuronal cells. The experiments were performed on day 11 after plating.

Proliferation Assay. In a 96-well plate, 2×10^5 splenocytes were cultured in DMEM supplemented with 10% fetal bovine serum, 20 mM HEPES buffer, 2 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin and 1 × non-essential amino acids. Cells were stimulated with 1 µg/ml and 5 µg/ml of concanavalin A. 1 µCi of ^{3}H was added 16 - 18 hours prior to harvesting. Cells were harvested 24, 48 and 72 hours after stimulation. Proliferation index was calculated as counts per minute in the absence or presence of Con A.

References

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Supplemental Figure legends

Figure S1 Inhibition of spinal mTOR attenuates the development and maintenance of morphine-induced mechanical allodynia and thermal hyperalgesia in the right hind paw. (**A** and **B**) Rats were administered intrathecal injections of morphine twice daily for 6 days. Co-administration of rapamycin (10 μg daily), but not ascomycin (10 μg daily), significantly blocked reductions in paw withdrawal threshold (**A**) and latency (**B**) in response to mechanical and thermal stimuli, respectively, on day 7. (**C** and **D**) Rats were administered intrathecal injections of morphine twice daily for 11 days. Co-administration of rapamycin (10 μg daily), but not ascomycin (10 μg daily), beginning on day 7 significantly reversed reductions in paw withdrawal threshold (**C**) and latency (**D**) in response to mechanical and thermal stimuli, respectively, on day 12. S: saline; V: vehicle; M: morphine; R: rapamycin; A: ascomycin. **P* < 0.05, ***P* < 0.01 vs. baseline. #*P* < 0.05, ##*P* < 0.01 vs. morphine plus vehicle. n = 5-6 rats/group.

Figure S2 Representative Western blot showing an increase in phosphorylated mTOR (p-mTOR) in the fifth lumbar segment (L5), but not in the second cervical segment (C2), on day 7 after 6 days of twice-daily intrathecal morphine injections. Repeated intrathecal saline injections did not alter the basal level of p-mTOR at either segment. n = 3 rats.

Figure S3 Repeated intrathecal morphine injections do not alter expression of phosphorylated (p-) mTOR, p-S6K1, or p-4E-BP1 in L4/5 dorsal root ganglions. n = 5/group. (**A**) Representative Western blots. (**B**) Statistical summary of the densitometric analysis.

Figure S4 Morphine dose- and time-dependently increases expression of phosphorylated (p-) mTOR, p-S6K1, and p-4E-BP1 in cultured dorsal horn neurons. (**A**) Changes in expression of p-mTOR, p-S6K1, and p-4E-BP1 in dorsal horn neurons after exposure to different concentrations of morphine for 10 minutes. *P < 0.05 vs. corresponding control (0 μM). n = 4 repeats/dose. (**B**) Total protein expression of mTOR, S6K1, and 4E-BP1 did not change in dorsal horn neurons after exposure to different concentrations of morphine for 10 minutes. n=4 repeats/dose. (**C**) Time-dependent changes in expression of p-mTOR, p-S6K1, and p-4E-BP1 in dorsal horn neurons after exposure to 20 μM morphine. *P < 0.05, **P < 0.01 vs. corresponding control (0 μM). n = 4 repeats/dose. (**D**) Total protein expression of mTOR, S6K1, and 4E-BP1 did not change with time in dorsal horn neurons after exposure to 20 μM morphine. n = 4 repeats/dose.

Figure S5 κ and δ opioid receptors are not involved in morphine-stimulated activation of the mTOR pathway in dorsal horn neurons. Neither η-BNI (20 μM, **A**) nor naltrindole (Nalt, 20 μM, **B**) affected increases in phosphorylated (p-) mTOR, p-S6K1, or p-4E-BP1 in cultured dorsal horn neurons exposed to 20 μM morphine (M) for 10 minutes. n=4 repeats/dose. **P < 0.01 vs. corresponding control (naïve).

Figure S6 DAMGO dose- and time-dependently increases expression of phosphorylated (p-) mTOR, p-S6K1, and p-4E-BP1 in cultured dorsal horn neurons. (**A**) Changes in expression of p-mTOR, p-S6K1, and p-4E-BP1 in dorsal horn neurons after exposure to different concentrations of DAMGO for 5 minutes. *P < 0.05, **P < 0.01 vs. corresponding control (0 μM). n = 4 repeats/dose. (**B**) Total protein expression of mTOR, S6K1, and 4E-BP1 did not change in dorsal horn neurons after exposure to different concentrations of DAMGO for 5 minutes. n = 4

repeats/dose. (**C**) Time-dependent changes in expression of p-mTOR, p-S6K1, and p-4E-BP1 in dorsal horn neurons after exposure to 20 μM DAMGO. ***P* < 0.01 vs. corresponding control (0 μM). n = 4 repeats/dose. (**D**) Total protein expression of mTOR, S6K1, and 4E-BP1 did not change with time in dorsal horn neurons after exposure to 20 μM DAMGO. n = 4 repeats/dose.

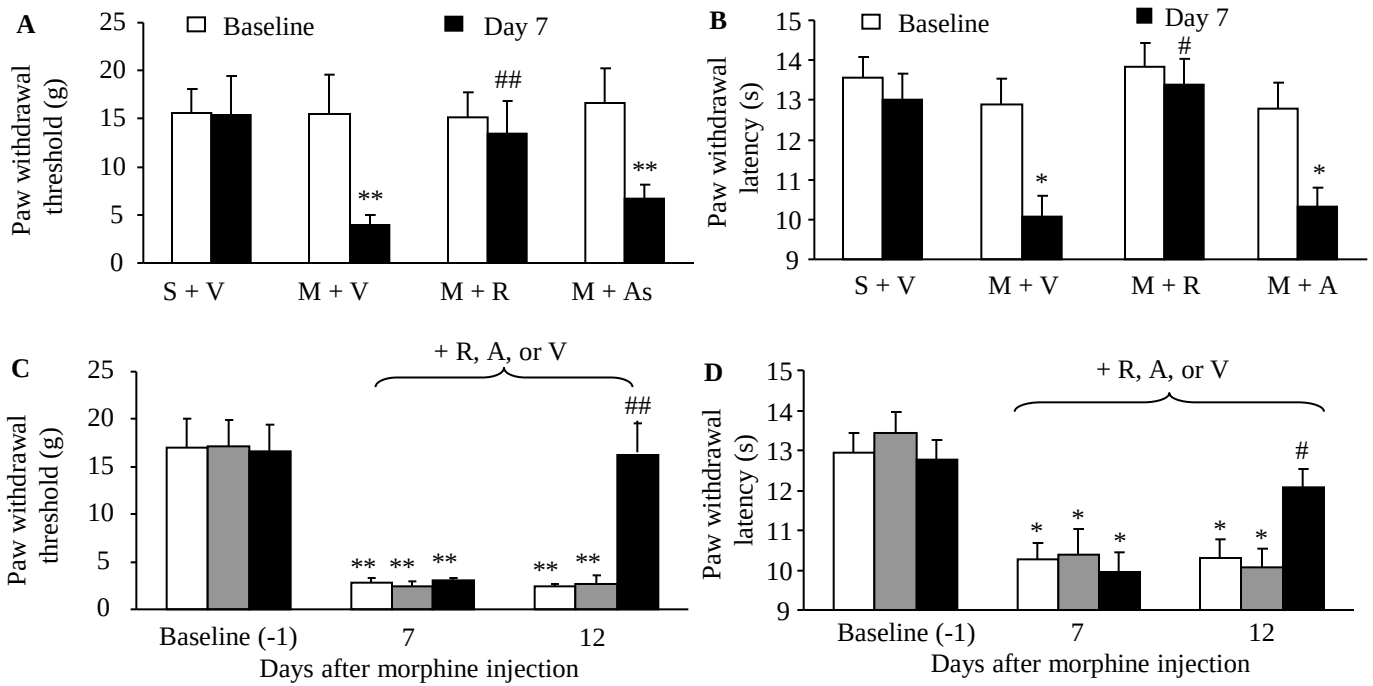
Figure S7 Spinal μ opioid receptor inhibition or knockout attenuates morphine tolerance and hyperalgesia. (**A**) Co-injection of CTOP (1 ng) significantly blocked the reduction of morphine's maximal potential analgesic effect (MPAE) on day 7 after 6 days of twice-daily intrathecal morphine injections (M, 10 μg). ***P* < 0.01 vs. morphine plus vehicle (V). n = 5 rats/group. S: saline. (**B** and **C**) Co-injection of CTOP (CT, 1 ng) significantly blocked the decreases in left (**B**) and right (**C**) hind paw withdrawal thresholds on day 7 after 6 days of twice-daily intrathecal morphine injections (10 μg). ***P* < 0.01 vs. saline plus vehicle. #*P* < 0.05 vs. morphine plus vehicle. n = 5 rats/group. (**D**) Compared to wild-type (WT) mice, μ opioid receptor knockout (KO) mice failed to display morphine analgesia on day 1 and failed to develop morphine tolerance on day 8 after repeated subcutaneous injections of morphine (20 mg/kg). n= 5 mice/group.

Figure S8 Distribution of μ opioid receptor mRNA in spinal cord dorsal horn. (**A**) The μ opioid receptor antisense (AS) probe shows very clear and intense fluorescent signals (developed with Fast Red) in dorsal horn. (**B**) A higher magnification of the boxed region in **A**. Cells labeled for μ opioid receptor mRNA are clearly visible. (**C**) The sense probe produced no signals in spinal cord dorsal horn. Scale bars: 50 μm.

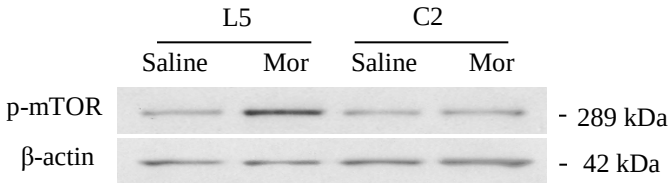
Figure S9 Spinal cord Akt is activated during chronic morphine exposure. Rats were injected intrathecally with 10 µl of morphine twice daily for the number of days shown. Top: Representative Western blot showing a time-dependent increase in phosphorylated Akt (p-Akt), but not total Akt, in rat dorsal horn after repeated intrathecal injections of morphine. Bottom: Statistical summary of the densitometric analysis expressed relative to the corresponding loading control (β-actin). n = 5 rats/group. *$P < 0.05$ vs. corresponding control (0 d).

Figure S10 Spinal mTOR inhibition does not affect morphine-induced spinal glia activation. (**A**) Representative images of glial fibrillary acidic protein (GFAP)-labeled cells in dorsal horn on day 7 after 6 days of twice-daily intrathecal injections of morphine (M; 10 µg) or saline (S) and once daily intrathecal injections of rapamycin (R; 10 µg) or vehicle (V). n = 4 rats/group. Scale bar: 100 µm. (**B**) Co-injection of rapamycin (10 µg daily) did not block the morphine-induced increase in GFAP in dorsal horn on day 7 after 6 days of twice-daily intrathecal morphine injections. Top: Representative Western blot. Bottom: Statistical summary of the densitometric analysis expressed relative to the corresponding loading control (β-actin). n = 4 rats/group. **$P < 0.01$ vs. saline plus vehicle. (**C**) Representative images of OX-42–labeled cells in dorsal horn on day 7 after 6 days of twice-daily intrathecal injections of morphine (10 µg) or saline and once daily intrathecal injections of rapamycin (10 µg) or vehicle. n = 4 rats/group. Scale bar: 50 µm.

Supplemental Fig. 1



Supplemental Fig. 2



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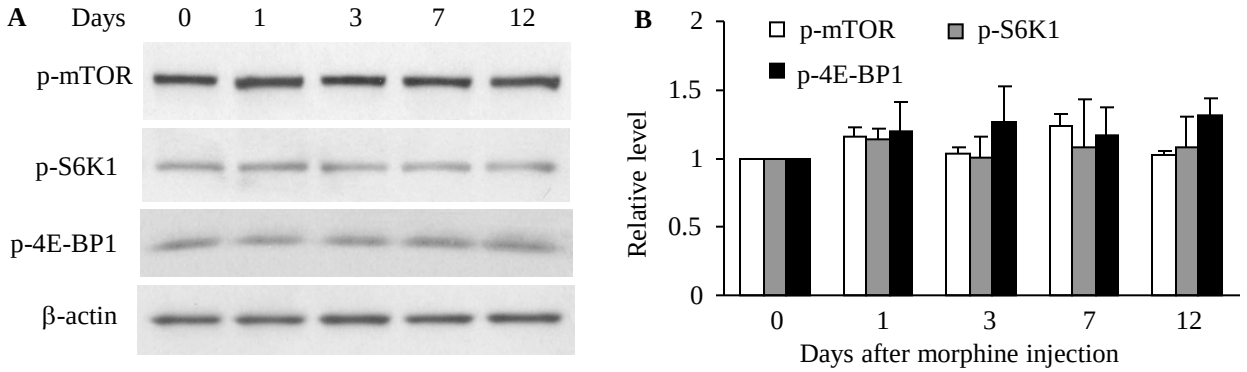

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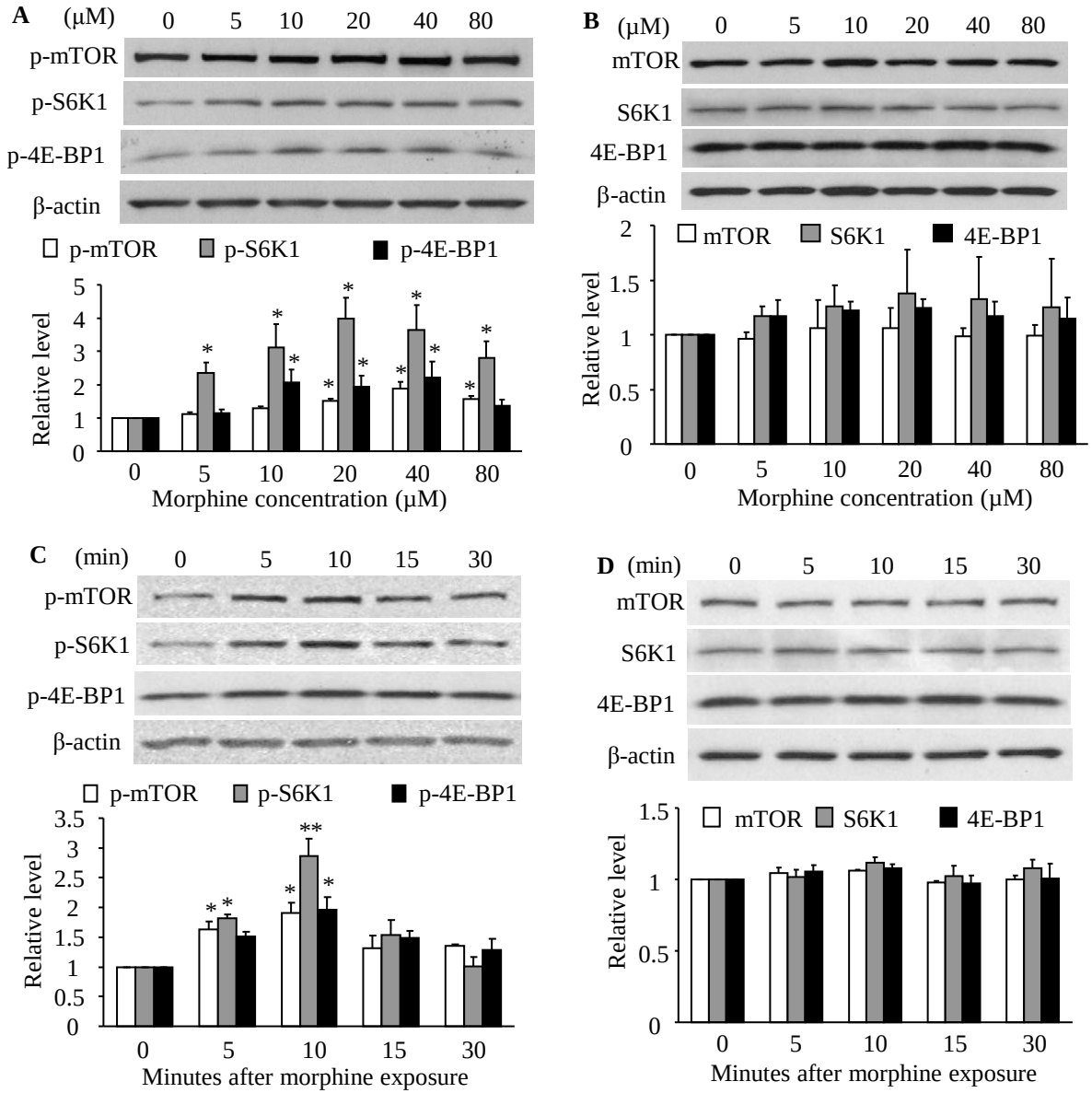

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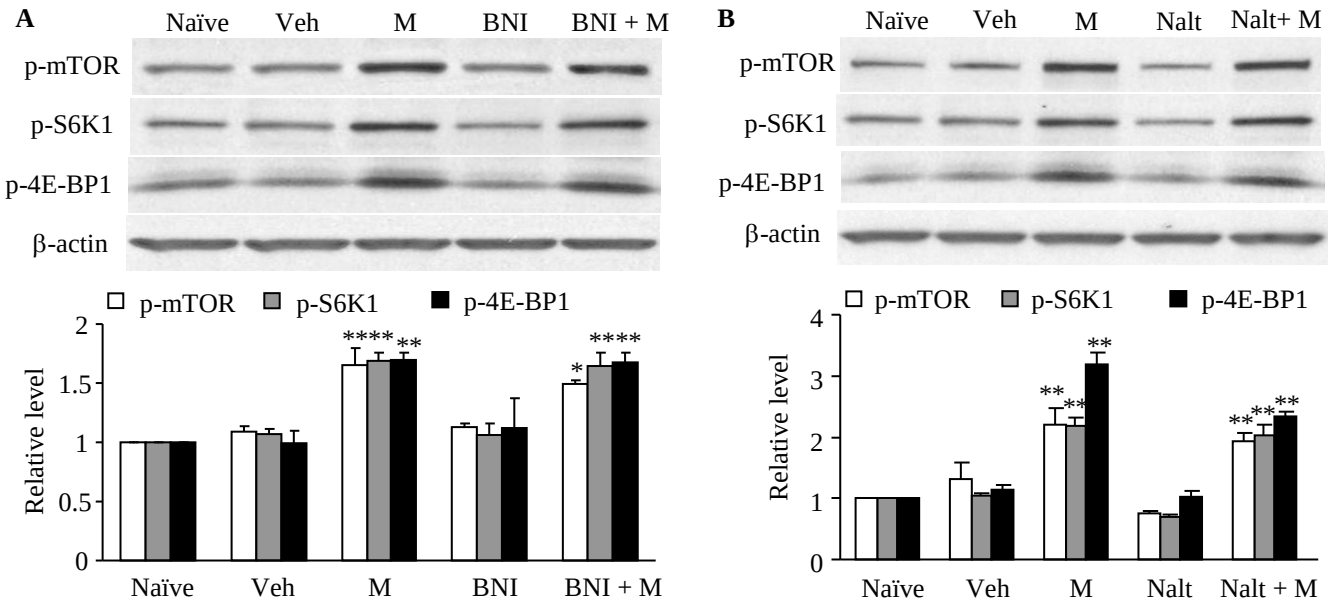

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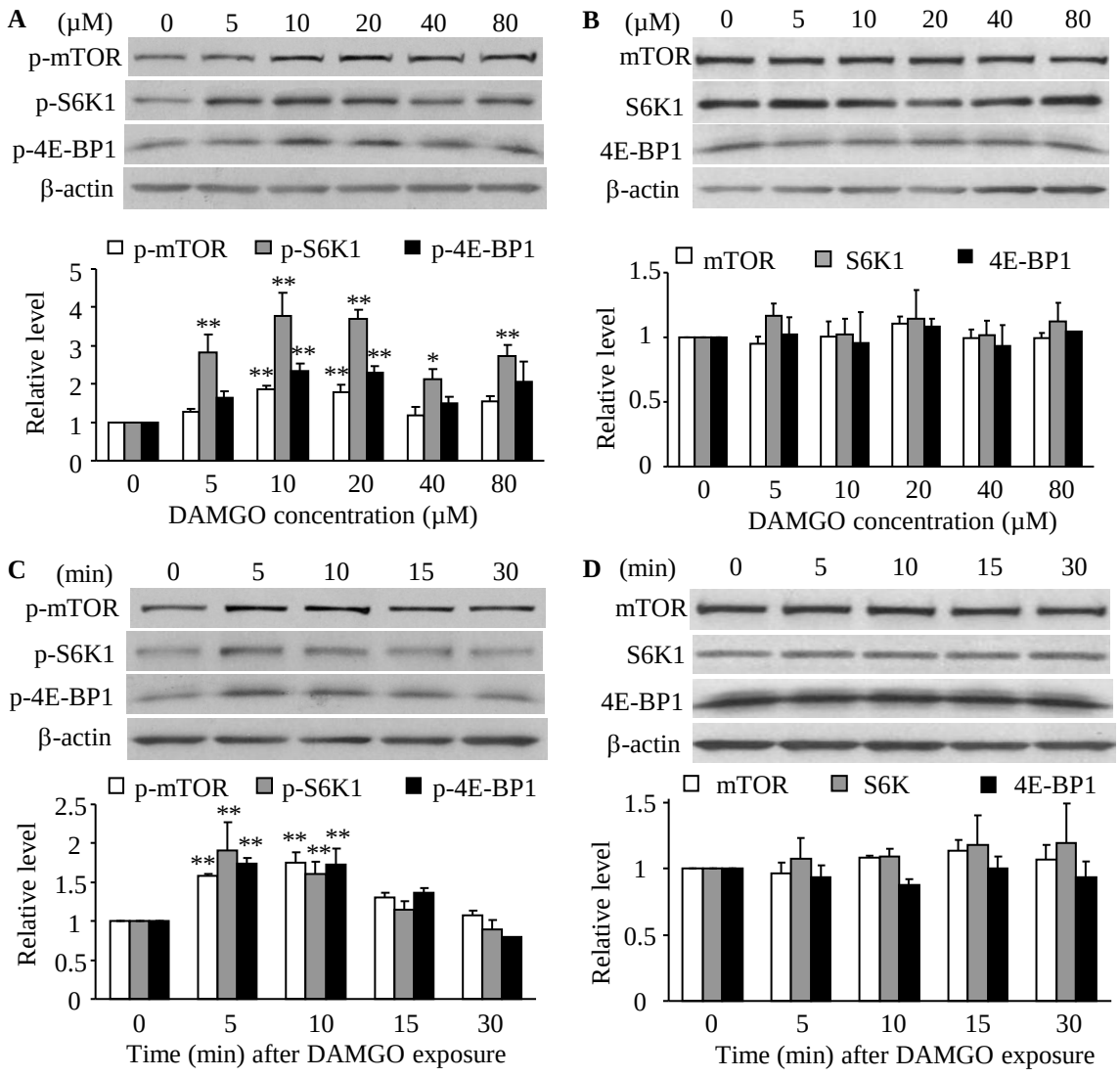

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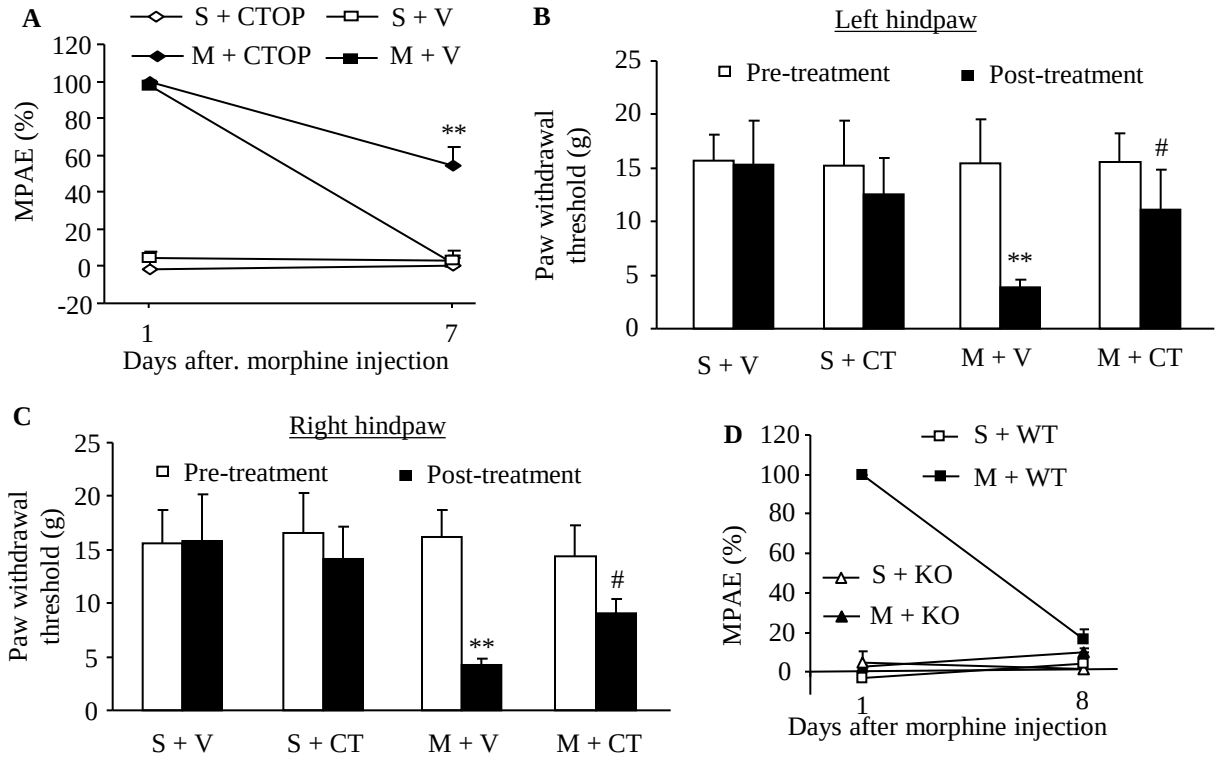

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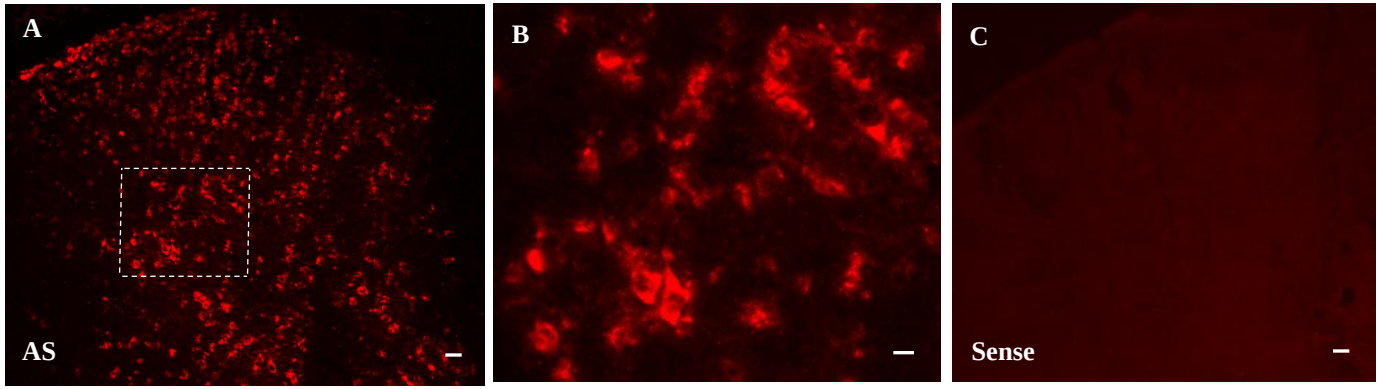

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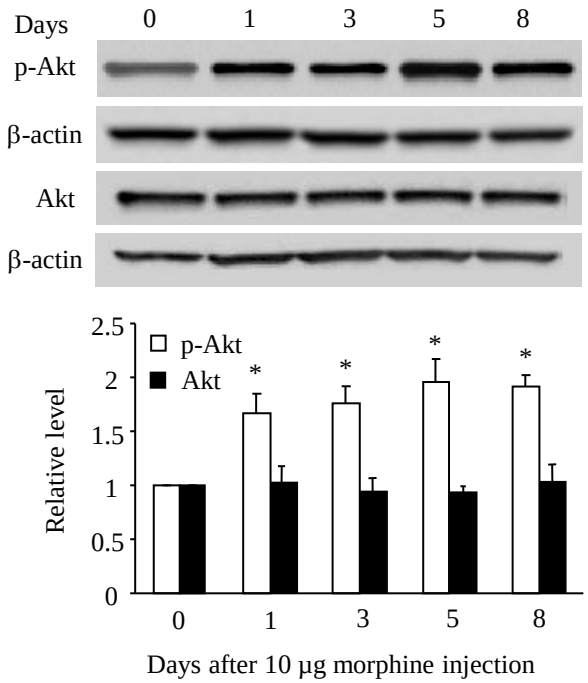
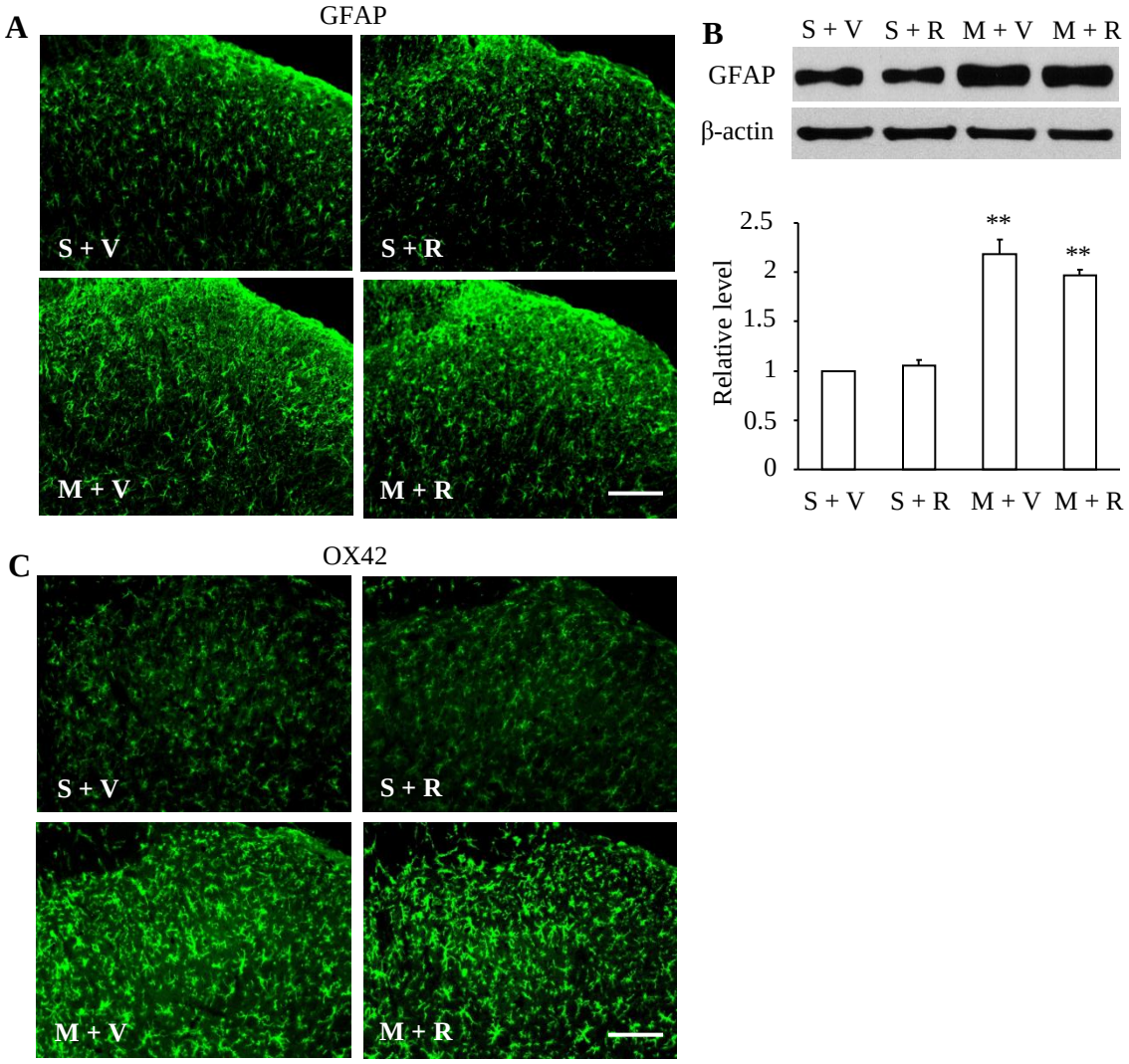


Supplemental Fig. 10



Supplemental Table 1. Mean (SEM) changes in locomotor test

Reagents	Placing	Grasping	Righting
Vehicle + saline	5 (0)	5 (0)	5 (0)
Vehicle + morphine	5 (0)	5 (0)	5 (0)
10 μg Rap + saline	5 (0)	5 (0)	5 (0)
10 μg Rap + morphine	5 (0)	5 (0)	5 (0)
10 μg Asc + morphine	5 (0)	5 (0)	5 (0)
Si + saline	5 (0)	5 (0)	5 (0)
Si + morphine	5 (0)	5 (0)	5 (0)
Sc + morphine	5 (0)	5 (0)	5 (0)

n = 6-7/group. 5 trials. Asc: ascomycin. Rap: rapamycin. Si: mTOR siRNA. Sc: scrambled siRNA.

ST 2

Supplemental Table 2. Effect of i.th. rapamycin on body weight, spleen weight, number of spleen cells, and number of blood cells

Treated Group	Body weight change (g) (post-baseline)	Spleen weight (g)	Relative spleen weight (spleen/body)	Red blood cells ($\times 10^6$)	White blood cells ($\times 10^3$)	Lymphocytes ($\times 10^3$)	Hemoglobin (g/dl)	Platelets ($\times 10^6$)	Splenocytes ($\times 10^7$)
Vehicle	35.7	1.17	0.0041	7.39	5.95	3.37	14.18	7.51	12
Rapamycin	33.4	1.10	0.0035	7.19	6.10	3.56	14.16	7.46	10.2

(Mean ± SEM. N = 5/group)

Supplemental Table 3. RT-PCR primers

Names	Sequences
Rat-μ opioid receptor-RT	5'- AACATCCCTCCACGGCTAATAC-3'
Rat-μ opioid receptor-F	5'- AACATCCCTCCACGGCTAATAC-3'
Rat-μ opioid receptor-R	5'- AGAGCCTCCCACACACCCTG-3'
Rat-PI3Kγ-RT	5'-ACCACCAGTCAAACCATCAAG-3'
Rat-PI3Kγ-F	5'-ACCACCAGTCAAACCATCAAG-3'
Rat-PI3Kγ-R	5'- GACCCGCAGCACAAAATCC-3'
Rat-Akt-RT	5'- CCCCACTCAACAACTTCTCAG-3'
Rat-Akt-F	5'- CCCCACTCAACAACTTCTCAG-3'
Rat-Akt-R	5'- CGTTTGAGTCCATCAGCCA-3'
Rat-mTOR-RT	5'- AGCTCCAGCACTATGTCACCA-3'
Rat-mTOR-F	5'- AGCTCCAGCACTATGTCACCA-3'
Rat-mTOR-R	5'- ATTCCCACCTTCCACTCCAA-3'
Rat-nNOS-RT	5’-GGGACCAGCCAAAGCAGAG-3’
Rat-nNOS-F	5’-GGGACCAGCCAAAGCAGAG-3’
Rat-nNOS-R	5‘-TTCCTTCTCTGAATACGGGTTG-3‘
Rat-PKCγ-RT	5‘- ACTTCATCTGGGGCATTGGA-3'
Rat-PKCγ-F	5‘- ACTTCATCTGGGGCATTGGA-3'
Rat-PKCγ-R	5'-TTGTGCTTGTTGCGAGGGTC-3‘
Rat-CaMKIIα-RT	5‘- CATCACCTGCACCCGATTCA-3'
Rat-CaMKIIα-F	5‘- CATCACCTGCACCCGATTCA-3'
Rat-CaMKIIα-R	5' –GCCTCGCGTTCCAACTTCTG-3'

RT: Reverse-transcription. F: Forward. R: Reverse.