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Expression and distribution of mTOR, p70S6K, 4E-BP1, and their phosphorylated counterparts in rat dorsal root ganglion and spinal cord dorsal horn

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Abstract

Mammalian target of rapamycin (mTOR) controls protein translation and has an important role in the mechanism of pain hypersensitivity under persistent pain conditions. However, its expression and localization in pain-related regions of the nervous system is not completely understood. Here, we examined the expression and distribution of mTOR, eukaryotic initiation factor 4E-binding protein1/2 (4E-BP1/2), p70 ribosomal S6 protein kinase (p70S6K), and their phosphorylated (active) counterparts in two major pain-related regions, the dorsal root ganglion (DRG) and spinal cord dorsal horn. Reverse transcriptase-polymerase chain reaction showed that mTOR, 4E-BP1, and p70S6K mRNA are expressed in the DRG and dorsal horn. Western blot analysis further confirmed the existence of their protein products in these two regions, but expression of their phosphorylated counterparts was very low in dorsal horn and was not detected in the DRG. Immunohistochemistry revealed mTOR and p70S6K in the DRG neurons. Quantitative analysis showed that approximately 26.1% (± 3.2%) of DRG neurons were positive for mTOR and 19.1% (± 1.9%) were positive for p70S6K. Most of these neurons were small—less than 600 μm^2 in cross-sectional area—and some co-labeled with substance P or isolectin B4. Surprisingly, 4E-BP1 was observed only in the DRG satellite glial cells. In the dorsal horn, mTOR, p70S6K, and 4E-BP1 were detected in neurons, but not in astrocytes or microglia. They were distributed in the whole dorsal horn, especially in the superficial dorsal horn. Immunostaining for their phosphorylated counterparts was very low or undetectable in DRG and dorsal horn. Behavioral study showed that intrathecal mTOR inhibitor, rapamycin, did not affect acute nocicepetive transmission. The results indicate that although mTOR, p70S6K, and 4E-BP1 are highly expressed in the DRG and dorsal horn, their activate forms are very low in both regions under normal conditions. Our findings support the view that mTOR and its downstream effectors do not play a key role in acute pain.

Keywords

mTOR; p70S6K; 4E-BP1; dorsal root ganglion; dorsal horn; expression; distribution; rat

1. Introduction

Mammalian target of rapamycin (mTOR) is a serine/threonine protein kinase expressed in the mammalian nervous system. mTOR forms two heteromeric and functionally distinct protein complexes in cells (Hay and Sonenberg, 2004; Jaworski and Sheng, 2006; Swiech et al.,

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2008). When it binds to Raptor protein, it forms mTOR complex 1, which is sensitive to rapamycin (a specific inhibitor of mTOR complex 1) and controls protein translation by phosphorylating downstream effectors, such as eukaryotic initiation factor 4E-binding protein1/2 (4E-BP1/2), p70 ribosomal S6 protein kinase (p70S6K), and eukaryotic translation initiation factor 4G (Hay and Sonenberg, 2004). mTOR also interacts with Rictor to form rapamycin-insensitive mTOR complex 2, which regulates actin dynamics and phosphorylates protein kinase B/Akt and protein kinase C (Hay and Sonenberg, 2004). mTOR kinase activity can be modulated in response to a variety of stimuli, such as neurotransmitters, trophic factors, mitogens, hormones, cell energy status and cellular stress, ischemia, and heat shock (Swiech et al., 2008). Thus, the activation of mTOR and its downstream effectors influences numerous physiologic and pathologic processes in the nervous system.

Persistent pain is a major public health problem worldwide. It is characterized by pain hypersensitivity, including spontaneous ongoing or intermittent burning pain, an exaggerated response to painful stimuli, and pain in response to normally innocuous stimuli. The biochemical events that contribute to pain hypersensitivity in persistent pain involve pre- and post-translational changes in expression and function of receptors, enzymes, and voltage-dependent ion channels in primary afferent neurons of the dorsal root ganglion (DRG) and at synapses in the pathway for nociception in the central nervous system (Price and Geranton, 2009; Latremoliere and Woolf, 2009). As mTOR complex 1 controls protein translation (Hay and Sonenberg, 2004), it is not surprising that mTOR participates in pain hypersensitivity associated with persistent pain. Recent behavioral studies showed that intrathecal injection of rapamycin significantly attenuated pain hypersensitivity in the second phase of the formalin response (Price et al., 2007; Asante et al., 2009) and in the development and maintenance of neuropathic pain in rats and mice (Jimenez-Diaz et al., 2008; Price et al., 2007; Geranton et al., 2009). In addition, local perfusion of rapamycin onto the spinal cord of rats markedly reduced formalin-induced neuronal hyperexcitability in dorsal horn (Asante et al., 2009). Using tyramide signal amplification (TSA)-enhanced immunofluorescent staining, previous investigators observed mTOR, 4E-BP1/2, p70S6K, and their phosphorylated (active) counterparts to be abundantly present in cutaneous myelinated sensory fibers, dorsal root axons, and dorsal horn cells under normal conditions (Geranton et al., 2009; Jimenez-Diaz et al., 2008). However, intraplantar or intrathecal administration of rapamycin did not alter acute pain (that is, basal responses to mechanical and thermal stimuli) (Geranton et al., 2009; Jimenez-Diaz et al., 2008). Because the TSA-enhanced assay highly amplifies signals, it is very possible that low levels of mTOR, 4E-BP1/2, p70S6K, and their phosphorylated counterparts might have appeared to be in much greater abundance than is actually the case.

In the present study, we used reverse transcriptase-polymerase chain reaction (RT-PCR) to examine the expression of mTOR, p70S6K, and 4E-BP1 messenger RNAs (mRNAs) in two major pain-related regions, the DRG and spinal cord dorsal horn, of naïve rats. Furthermore, we used Western blot analysis and standard immunohistochemistry to observe the expression and distribution of total and phosphorylated (p-) mTOR, p70S6K, 4E-BP1 in these two regions. Finally, we examined whether spinal mTOR inhibition alters acute nociceptive transmission.

2. Results

2.1 Expression of mTOR, p70S6K, and 4E-BP1 mRNA in the DRG and spinal cord dorsal horn

RT-PCR analysis revealed expression of mTOR, p70S6K, and 4E-BP1 mRNA in the DRG, dorsal and ventral horns of spinal cord, and cortex (positive control) of adult rats (Fig. 1A). PCR products of the sizes predicted from the corresponding cDNA sequences were detected. Nucleotide sequence analysis by automatic DNA sequencing verified that the PCR products corresponded to the cDNA sequences. The PCR product of glyceraldehyde 3-phosphate

dehydrogenase (GAPDH), which was used as a loading control, was observed in all four regions (Fig. 1A).

2.2 Expression of mTOR, p70S6K, 4E-BP1, and their phosphorylated counterparts in the DRG and spinal cord dorsal horn

Western blot analysis was carried out with soluble fractions of DRG, dorsal horn, ventral horn, and cortex by using anti-peptide antibodies directed against mTOR, p70S6K, 4E-BP1, p-mTOR at serine 2448, p-p70S6K at threonine 421/serine 424, and p-4E-BP1 at serine 65. Consistent with the previous studies (Jimenez-Diaz et al., 2008; Li et al., 2005; Takei et al., 2004; Geranton et al., 2009), the primary antibodies used did not cross-react with other proteins or isoforms, as each antibody detected only one band, and its molecular weight was consistent with that of the corresponding protein predicted from analysis of its amino acid sequence (Fig. 1B and C). As expected, mTOR, p70S6K, and 4E-BP1 proteins were detected in all four regions, although the expression level in the DRG was lower than that in the other three regions (Fig. 1B). Their phosphorylated counterparts were detected in the dorsal and ventral horns (Fig. 1C), but it was necessary to load much more protein onto the gels (2.5-fold) and expose the membranes to film for a much longer time (10-fold) to achieve signals for the phosphorylated proteins as compared to the total proteins. None of the phosphorylated proteins were detected in the DRG (Fig. 1C). In addition, p70S6K phosphorylated at threonine 389 and 4E-BP1 phosphorylated at threonine 70 were detected at very low levels in the DRG, dorsal horn, and ventral horn (data not shown).

2.3 Cellular localization of mTOR, p70S6K, 4E-BP1, and their phosphorylated counterparts in the DRG

Immunofluorescent staining for mTOR, p70S6K, and 4E-BP1 was detected in the DRG, but signal for their phosphorylated counterparts was very weak or undetectable in this region (Figs. 2 and 4A). Staining for mTOR, p70S6K, and 4E-BP1 revealed distinct distribution patterns in DRG cells. mTOR and p70S6K were observed in DRG neurons (Fig. 2A). Approximately 26.1% (± 3.2%) of DRG neurons were mTOR-positive and 19.1% (± 1.9%) were p70S6K-positive. Although these mTOR- and p70S6K-positive neurons had a variety of sizes and shapes, most (62.8% of mTOR-positive neurons and 69.1% of p70S6K-positive neurons) were less than 600 μm^2 in cross-sectional area (Fig. 2B). Double-labeling studies showed that mTOR and p70S6K co-localized with isolectin B4 (IB4), a marker for small DRG non-peptidergic neurons (Tao et al., 2004), and substance P (SP), a marker for small DRG peptidergic neurons (Tao et al., 2004) (Fig. 3A and B). Approximately 27.1% (± 2.5%) of mTOR-positive neurons and 28.2% (± 1.8%) of p70S6K-positive neurons were positive for IB4, whereas approximately 22.2% (± 2.9%) of mTOR-positive neurons and 23.2% (± 3.2%) of p70S6K-positive neurons were positive for SP. In a few instances, mTOR and p70S6K were observed to co-localize with neurofilament-200 (NF-200), a marker for myelinated A-fibers (Obata et al., 2004) (Fig. 3A and B).

Unlike mTOR and p70S6K, 4E-BP1 was not detected in the DRG neurons (Fig. 4A). The staining for 4E-BP1 wrapped, but did not overlap with, IB4-, SP-, and NF-200-positive areas (Fig. 4B). Under high magnification, 4E-BP1 immunofluorescence was not localized in plasma membrane of the DRG neurons. To further identify in which type of DRG cell 4E-BP1 was localized, we performed a double-labeling study of 4E-BP1 with glial fibrillary acidic protein (GFAP), a satellite glial cell marker (Obata et al., 2004). Although GFAP is expressed at a low level in normal DRG, 4E-BP1 was found to co-localize with GFAP in some DRG cells (Fig. 4B), indicating that it is expressed in the DRG satellite glial cells.

In control experiments, no immunofluorescent staining was detected in the DRG when the primary antibodies were omitted, pre-absorbed with an excess of the corresponding antigen, or replaced with normal IgG during the staining procedure (Fig. 4A).

2.4 Cellular localization of mTOR, p70S6K, 4E-BP1, and their phosphorylated counterparts in spinal cord dorsal horn

In the dorsal horn of spinal cord, mTOR, p70S6K, and 4E-BP1 had similar expression patterns. The immunofluorescent staining of each was distributed throughout the whole dorsal horn, particularly in the superficial dorsal horn (Fig. 5A). Under high magnification, many cells positive for mTOR, p70S6K, or 4E-BP1 were seen in the dorsal horn (Fig. 5B–D). These cells were positive for NeuN, a specific marker for neural nuclei, but not for GFAP (an astrocyte marker) or OX-42 (a microglia marker) (Fig. 5B–D). In the superficial dorsal horn, some mTOR, p70S6K, and 4E-BP1 immunofluorescence appeared in patches or in a punctuate pattern. In contrast, throughout the whole dorsal horn, p-mTOR, p-p70S6K, and p-4E-BP1 immunofluorescence was very weak (Fig. 5A). Under high magnification, a few cells positive for p-mTOR, p-p70S6K, and p-4E-BP1 were observed.

Consistent with the control experiments in the DRG, no immunofluorescence was detected in the dorsal horn when the primary antibodies were omitted, pre-absorbed with an excess of the corresponding antigen, or replaced with normal IgG during the staining procedure (Fig. 5A).

2.5 Effect of spinal mTOR inhibition on acute nociceptive transmission

Acute nociceptive transmission was induced in rats by applying high-intensity radiant heat (thermal stimulus) and different intensities of von Frey monofilaments (mechanical stimuli) to the plantar sides of both hind paws or by applying high-intensity radiant heat to the tail. To block mTOR activity in the spinal cord and DRG, 10 μg of rapamycin (dissolved in 50% DMSO), a specific inhibitor of mTOR, was administrated intrathecally. Rapamycin at this dose has been reported to significantly attenuate formalin-induced pain behaviors and neuropathic pain (Jimenez-Diaz et al., 2008; Price et al., 2007; Asante et al., 2009; Geranton et al., 2009). This effect appears 25 min after injection and lasts for at least 50 min (Price et al., 2007; Asante et al., 2009). Thus, 30 min after rapamycin or vehicle (50% DMSO) injection, tail-flick latencies and paw withdrawal latencies and thresholds were measured. We observed no significant differences in paw withdrawal or tail-flick latencies (in response to thermal stimulus) or in paw withdrawal thresholds (in response to mechanical stimuli) between vehicle-treated and rapamycin-treated groups (Fig. 6). This finding indicates that acute nociceptive transmission is not significantly altered after intrathecal rapamycin administration.

3. Discussion

Using RT-PCR, Western blot analysis, and standard immunofluorescence techniques, we have characterized the expression levels of mTOR, p70S6K, 4E-BP1, and their activated counterparts in the DRG and dorsal horn. We observed mTOR, p70S6K, and 4E-BP1 mRNA and protein in the DRG and dorsal horn. In contrast, the levels of their phosphorylated counterparts were very low in dorsal horn and were undetectable in the DRG. Our findings indicate that mTOR and its downstream effectors are not highly activated in these two regions, especially in the DRG, under normal conditions, suggesting that these proteins might not participate in acute pain. This conclusion is strongly supported by our behavioral results (Fig. 6) and those from previous reports (Geranton et al., 2009;Jimenez-Diaz et al., 2008), which showed that intraplantar and intrathecal injections of rapamycin do not affect basal responses to mechanical and thermal stimuli. Interestingly, when the TSA-enhanced immunofluorescence technique was used, p-mTOR, p-p70S6K, and p-4E-BP1/2 were observed in myelinated peripheral fibers, dorsal roots, lamina I/II projection neurons, and

dorsal horn glial cells of naïve rats (Geranton et al., 2009;Jimenez-Diaz et al., 2008). However, because this technique highly amplifies the signals, the results may have overestimated the actual protein levels.

Our immunofluorescence study demonstrated that approximately 26.1% of DRG cells express mTOR and approximately 19.1% express p70S6K. Most of these mTOR- and p70S6K-positive neurons were small, and some also expressed SP or bound to IB4. Only 37.2% of mTOR-positive neurons and 30.9% of p70S6K-positive neurons were categorized as medium or large. However, in the peripheral and central fibers of DRG, mTOR and p70S6K were reported to express predominantly in myelinated peripheral fibers and myelinated dorsal roots (Geranton et al., 2009; Jimenez-Diaz et al., 2008). It is very likely that some of the mTOR and p70S6K in the medium and large (but not small) DRG cell bodies is quickly transported to myelinated peripheral and central fibers. This transportation might be related to the maintenance of thermal sensitivity of a subset of A-nociceptors, as intraplantar injection of rapamycin increased A- but not C-nociceptor-evoked paw withdrawal thresholds in naïve rats (Jimenez-Diaz et al., 2008). Since peripheral injection of rapamycin did not affect capsaicin-induced primary hyperalgesia (Jimenez-Diaz et al., 2008), the function of mTOR and p70S6K in small DRG neurons remains to be explored.

Unexpectedly, 4E-BP1 was detected in the satellite glial cells but not in the neurons of the DRG. This finding is inconsistent with recent reports that showed that p-4E-BP1 protein was present in myelinated peripheral and central fibers (Jimenez-Diaz et al., 2008; Geranton et al., 2009). It is very likely that most of the 4E-BP1 protein in DRG neurons is transported to the peripheral and central fibers, leaving too little in the cell bodies to be detected by the current immunofluorescence technique. Since the level of mTOR was low in the DRG satellite glial cells, it is unlikely a major upstream trigger of active 4E-BP1 in these cells. Rather, 4E-BP1 activity might be regulated by other, still unidentified kinases (Armengol et al., 2007). Recent studies demonstrated that the DRG satellite glial cells respond to peripheral nerve injury and inflammation by activation, proliferation, and release of various molecules that contribute to pain hypersensitivity in persistent pain (Dublin and Hanani, 2007; Huang et al., 2009; Xie et al., 2009). Phosphorylated 4E-BP1 is critical for initiating protein translation (Proud, 2007). The expression of 4E-BP1 in the DRG satellite glial cells suggests that 4E-BP1 might participate in activation, proliferation, and release of various molecules of the DRG satellite glial cells in persistent pain.

Interestingly, in dorsal horn, mTOR, p70S6K, and 4E-BP1 were detected in neurons but not in astrocytes or microglia with standard immunofluorescent staining, suggesting that the expression of these three proteins must be quite low in spinal glial cells, as the phosphorylated forms of these proteins were detected in both neurons and glial cells of dorsal horn with the TSA-enhanced immunofluorescence technique (Geranton et al., 2009). mTOR, p70S6K, and 4E-BP1 are expressed in the dorsal horn, especially in lamina I/II projection neurons (Geranton et al., 2009). This distribution pattern suggests that mTOR and its downstream effectors are involved in transmission and modulation of noxious information. Indeed, intrathecal rapamycin attenuated a capsaicin-induced increase in spinal phosphorylated S6 (a downstream effector of p70S6K) and secondary hyperalgesia (Geranton et al., 2009). Intrathecal rapamycin also reduced nerve injury-induced mechanical pain hypersensitivity (Geranton et al., 2009). Unexpectedly, increases in phosphorylated mTOR and S6 in dorsal horn and the dorsal root were not observed after peripheral nerve injury (Geranton et al., 2009). Thus, the role of spinal mTOR and its downstream effectors in persistent pain is still unclear.

In summary, here, we have demonstrated that, although the mRNA and protein for mTOR, p70S6K, and 4E-BP1 are highly expressed in both the DRG and dorsal horn, their phosphorylated counterparts are very low in these two regions under normal conditions.

Furthermore, behavioral experiments showed that spinal mTOR inhibition does not affect acute nociceptive transmission. Our findings support the view that mTOR and its downstream effectors in the DRG and dorsal horn do not play a key role in acute pain.

4. Experimental Procedures

4.1 Animals

Sprague-Dawley rats (250–300 g) were housed individually in cages on a standard 12 h-12 h light-dark cycle. Water and food were available *ad libitum* until rats were transported to the laboratory approximately 1 h before the experiments. All experiments were carried out with the approval of the Animal Care and Use Committee at the Johns Hopkins University and were consistent with the ethical guidelines of the National Institutes of Health and the International Association for the Study of Pain. All efforts were made to minimize animal suffering and to reduce the number of animals used.

4.2 Total RNA preparation and RT-PCR

The rats (n = 3) were euthanized with an overdose of isoflurane and decapitated. The dorsal and ventral portions of spinal cord, DRGs, and cortex (as positive controls) were dissected. Total RNA from these tissues was extracted by the Trizol method (Invitrogen, Carlsbad, CA), precipitated with isopropanol, and treated with DNase I (New England Biolabs, Ipswich, MA). The concentration and purity of RNA preparations were assessed by using a NanoDrop 1000 UV-Vis spectrophotometer (Midland, ON, Canada). Single strand cDNA was synthesized from total RNA by using the Omniscript RT kit (QIAGEN, Valencia, CA) with oligo-dT primer according to the manufacturer's instructions. Template (1 μL) was amplified by PCR with platinum Taq polymerase (1 unit) in 20 μL total reaction volume containing 0.5 μmol of each specific PCR primer. The primer sequences were chosen from the conserved part of the coding region for mTOR [5′-TTGAGGTTGCTATGACCAGAGAGAA-3′ (forward) and 5′-TTACCAGAAAGGACACCAGCCAATG-3′ (reverse)], p 70S6 K [5′-GGAGCCTGGGAGCCCTGATGTA-3′ (forward) and 5′-GAAGCCCTCTTTGATGCTGTCC-3′ (reverse)], and 4E-BP1 [5′-TAGCCCTACCAGCGATGAGCCT-3′ (forward) and 5′-GTATCAACAGAGGCA CAAGGAGGTAT-3′ (reverse)]. As a loading control, GAPDH cDNA was also amplified with primer sequences 5′-ACCACAGTCCATGCCATCAC-3′ (forward) and 5′-TCCACCACCCTGTTGCTGTA-3′ (reverse). PCR consisted of 30 cycles of 20 s at 94°C, 20 s at 55°C, and 30 s at 72°C. After amplification, the products were separated on a 1.2% agarose gel containing 0.025% ethidium bromide. Bands were then visualized under UV illumination, and gels were photographed with the BioDoc-It imaging system (Ultra-Violet Products Ltd, Upland, CA). The band density from dorsal horn was set as 100%. The relative density values from other tissues were determined by dividing the optical density values from these groups by the value from dorsal horn after they were normalized to the corresponding GAPDH.

4.3 Western blot analysis

Soluble proteins were prepared according to procedures described previously (Park et al., 2008; Park et al., 2009). In brief, after the rats (n = 7) were euthanized by an overdose of isoflurane, the tissues from the dorsal and ventral portions of lumbar spinal cord, DRGs, and cortex were dissected and rapidly frozen in liquid nitrogen. The tissues were then homogenized in the homogenization buffer [50 mM Tris-HCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, 1 μM leupeptin, 2 μM pepstatin A, 1:100 phosphatase inhibitor cocktail I/II (Sigma, St. Louis, MO), 1:100 protease inhibitor cocktail (Sigma)]. The crude homogenate was centrifuged at 4°C for 15 min at 1,000*g*, and the supernatants were collected. After measurement of protein concentration, the protein (20 μg for total protein and 50 μg for phosphorylated protein) was heated for 5 min at 98°C and loaded onto 4% stacking/7.5%

separating SDS-polyacrylamide gels. After separation, the protein was electrophoretically transferred onto a nitrocellulose membrane. The membrane was blocked with 3% non-fat dry milk and subsequently incubated for 2 h with polyclonal rabbit primary antibodies for mTOR (1:1,000, Cell Signaling Technology, Danvers, MA), p-mTOR (serine 2448, 1:1,000, Cell Signaling Technology), p70S6K (1:1,000, Cell Signaling Technology), p-p70S6K (threonine 421/serine 424, 1:1,000, Cell Signaling Technology), 4E-BP1 (1:1,000, Cell Signaling Technology), and p-4E-BP1 (serine 65, 1:1,000, Cell Signaling Technology) and with monoclonal mouse primary antibody for β-actin (1:2,000, Santa Cruz Biotechnology, Santa Cruz, CA). The specificity of the primary antibodies has been described previously (Jimenez-Diaz et al., 2008; Li et al., 2005; Takei et al., 2004; Geranton et al., 2009). The proteins were detected by horseradish peroxidase-conjugated anti-rabbit or anti-mouse secondary antibodies and visualized with chemiluminescence reagents provided with the ECL kit (Amersham Pharmacia Biotech, Piscataway, NJ) and exposure to film. The blot density from dorsal horn was set as 100%. The relative density values from other tissues were determined by dividing the optical density values from these groups by the value from dorsal horn after they were normalized to the corresponding β-actin.

4.4 Immunohistochemistry

The rats (n = 6) were deeply anesthetized and perfused transcardially with 100 mL of 0.01 M phosphate-buffered saline (PBS, pH 7.4) followed by 300 mL of 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). After the perfusion, the lumbar enlargement segments and bilateral L_5 DRGs were harvested, postfixed at 4°C for 4 h, 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-labeling immunofluorescence histochemistry was carried out as described previously (Tao et al., 2004). After being blocked for 1 h at 37°C in PBS containing 5% goat serum and 0.3% TritonX-100, the sections were incubated alone with primary rabbit polyclonal anti-mTOR (1:500, Cell Signaling Technology), anti-p-mTOR (serine 2448, 1:500, Cell Signaling Technology), anti-p70S6K (1:500, Cell Signaling Technology), anti-p-p70S6K (threonine 421/serine 424, 1:500, Cell Signaling Technology), anti-4E-BP1 (1:500, Cell Signaling Technology), or anti-p-4E-BP1 (serine 65, 1:500, Cell Signaling Technology) overnight at 4°C. The sections were then incubated with goat anti-rabbit IgG conjugated with Cy3 (1:300; Jackson ImmunoResearch, West Grove, PA) for 1 h at 37°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 rinsed in 0.01 M PBS and mounted onto gelatin-coated glass slides. Cover slips were applied with a mixture of 50% glycerin and 2.5% triethylene diamine in 0.01 M PBS.

Double-labeling immunofluorescence histochemistry was carried out as described previously (Tao et al., 2004). The sections were mounted onto gelatin-coated glass slides. The DRG sections were incubated overnight at 4°C with 1) a mixture of rabbit polyclonal anti-mTOR (1:500) and mouse monoclonal anti-NF-200 (1:400, Chemicon, Temecula, CA), biotinylated IB4 (1:100, Sigma), or mouse monoclonal anti-SP (1:100, R&D Systems, Minneapolis, MN); 2) a mixture of rabbit polyclonal anti-p70S6K (1:500) and mouse monoclonal anti-NF-200 (1:400), biotinylated IB4 (1:100), or mouse monoclonal anti-SP (1:100); or 3) a mixture of rabbit polyclonal anti-4E-BP1 (1:500) and mouse monoclonal anti-NF-200 (1:400), biotinylated IB4 (1:100), mouse monoclonal anti-SP (1:100), or mouse monoclonal GFAP (1:200, Sigma). Spinal cord sections were incubated overnight at 4°C with 1) a mixture of rabbit polyclonal anti-mTOR (1:500) and mouse monoclonal anti-NeuN (1:600, Chemicon), anti-GFAP (1:500, Sigma), or anti-OX-42 (1:400, Sigma); 2) a mixture of rabbit polyclonal

anti-p70S6K (1:500) and mouse monoclonal anti-NeuN (1:600), anti-GFAP (1:600), or anti-OX-42 (1:400); or 3) a mixture of rabbit polyclonal anti-4E-BP1 (1:500) and mouse monoclonal anti-NeuN (1:600), anti-GFAP (1:600), or anti-OX-42 (1:400). The sections were then incubated with a mixture of goat anti-rabbit IgG conjugated with Cy3 (1:300) and goat anti-mouse IgG conjugated with Cy2 (1:300, Jackson ImmunoResearch) or FITC-labeled avidin D (1:200, Sigma) for 1 h at 37°C. Control experiments as described above were performed in parallel. After the sections were rinsed in 0.01 M PBS, cover slips were applied as described above.

All immunofluorescence-labeled sections were viewed with an epifluorescence microscope under appropriate filter for Cy3 (excitation 550 nm; emission 570 nm) or for FITC and Cy2 (excitation 492 nm; emission 510–520 nm).

For size distribution analysis of mTOR-, p70S6K-, and 4E-BP1-positive cells within the DRG, approximately 36 sections from three rats (12–14 sections/rat) were randomly selected for each protein analysis. All labeled and unlabeled cells with nuclei were counted. Cell profiles were outlined and cell area was calculated by using the imaging software Image-Pro Plus (Media Cybernetics, Silver Spring, MD). To analyze the 10 groups of double-labeled sections from the DRG, sections from three rats (2 DRGs/rat; 10–11 sections/DRG) were randomly selected for each group. Single-labeled and double-labeled neurons were counted.

4.5. Behavioral testing

A tail-flick apparatus (Model 33B Tail Flick Analgesia Meter, IITC Life Science, Woodland Hills, CA, USA) with a radiant heat source connected to an automatic timer was used to assess the analgesic response (Liaw et al., 2008). A cut-off time latency of 10 s was used to avoid tissue damage to the tail. Tail-flick latencies were measured as the time required to induce a tail flick after applying radiant heat to the skin of the tail.

Thermal paw withdrawal latencies were measured with a Model 336 Analgesia Meter (IITC Life Science Instruments)(Park et al., 2008; Park et al., 2009). Each animal was placed in a Plexiglas chamber on a glass plate located above a light box. Radiant heat was applied by aiming a beam of light through a hole in the light box through the glass plate to the middle of the plantar surface of each hind paw. When the animal lifted its foot, the light beam was turned off. The length of time between the start of the light beam and the foot lift was defined as the paw withdrawal latency. Each trial was repeated five times at 5-min intervals for each paw. A cut-off time of 20 s was used to avoid paw tissue damage.

Mechanical paw withdrawal thresholds were measured with the up-down testing paradigm (Guan et al., 2009; Singh et al., 2009). Each rat was placed in a Plexiglas chamber on an elevated mesh screen. Von Frey hairs in log increments of force (3.61, 3.84, 4.08, 4.31, 4.56, 4.74, 4.93, 5.18 g) were applied to the plantar surface of the left and right hind paws. The 4.31-g stimulus was applied first. If a positive response occurred, the next smaller von Frey hair was used; if a negative response was observed, the next higher von Frey hair was used. The test was ended when (1) a negative response was obtained with the 5.18-g hair, (2) four stimuli were applied after the first positive response, or (3) nine stimuli were applied to one hind paw.

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Abbreviations

4E-BP1/2	eukaryotic initiation factor 4E-binding protein 1/2
DRG	dorsal root ganglion
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GFAP	glial fibrillary acidic protein
IB4	isolectin B4
mTOR	mammalian target of rapamycin
NF-200	neurofilament-200
p70S6K	p70 ribosomal S6 protein kinase
PBS	phosphate-buffered saline
RT-PCR	reverse transcriptase-polymerase chain reaction
SP	substance P
TSA	tyramide signal amplification

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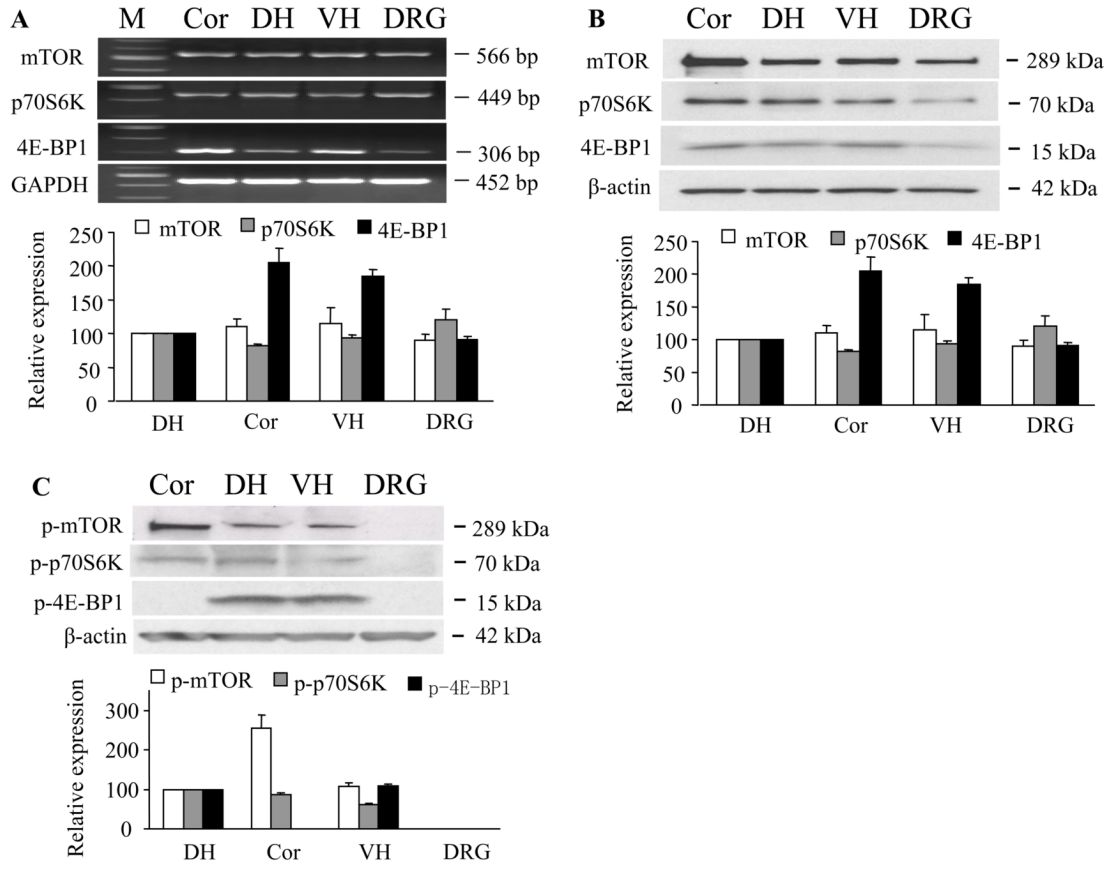

Fig. 1.

Expression of mTOR, p70S6K, 4E-BP1, and their phosphorylated counterparts in the DRG and spinal cord. (A) The upper panel depicts a representative RT-PCR gel that shows the segments of mTOR mRNA (566 bp), p70S6K mRNA (449 bp), and 4E-BP1 mRNA (306 bp) in the DRG, dorsal horn (DH), and ventral horn (VH). PCR products were verified by automatic DNA sequencing. Cortex (Cor) mRNA was used as a positive control and GAPDH mRNA as a loading control. M: molecular weight standard. The lower panel is the statistical summary of the densitometric analysis expressed relative to dorsal horn; n = 3. (B and C) The upper panels depict representative Western blots that show the protein expression of mTOR, p70S6K, 4E-BP1, and their phosphorylated counterparts in the DRG, dorsal horn, and ventral horn. Cortex is used a positive control and β-actin as a loading control. The lower panels show the statistical summary of the densitometric analysis expressed relative to dorsal horn; n = 7. The data are presented as mean ± SEM.

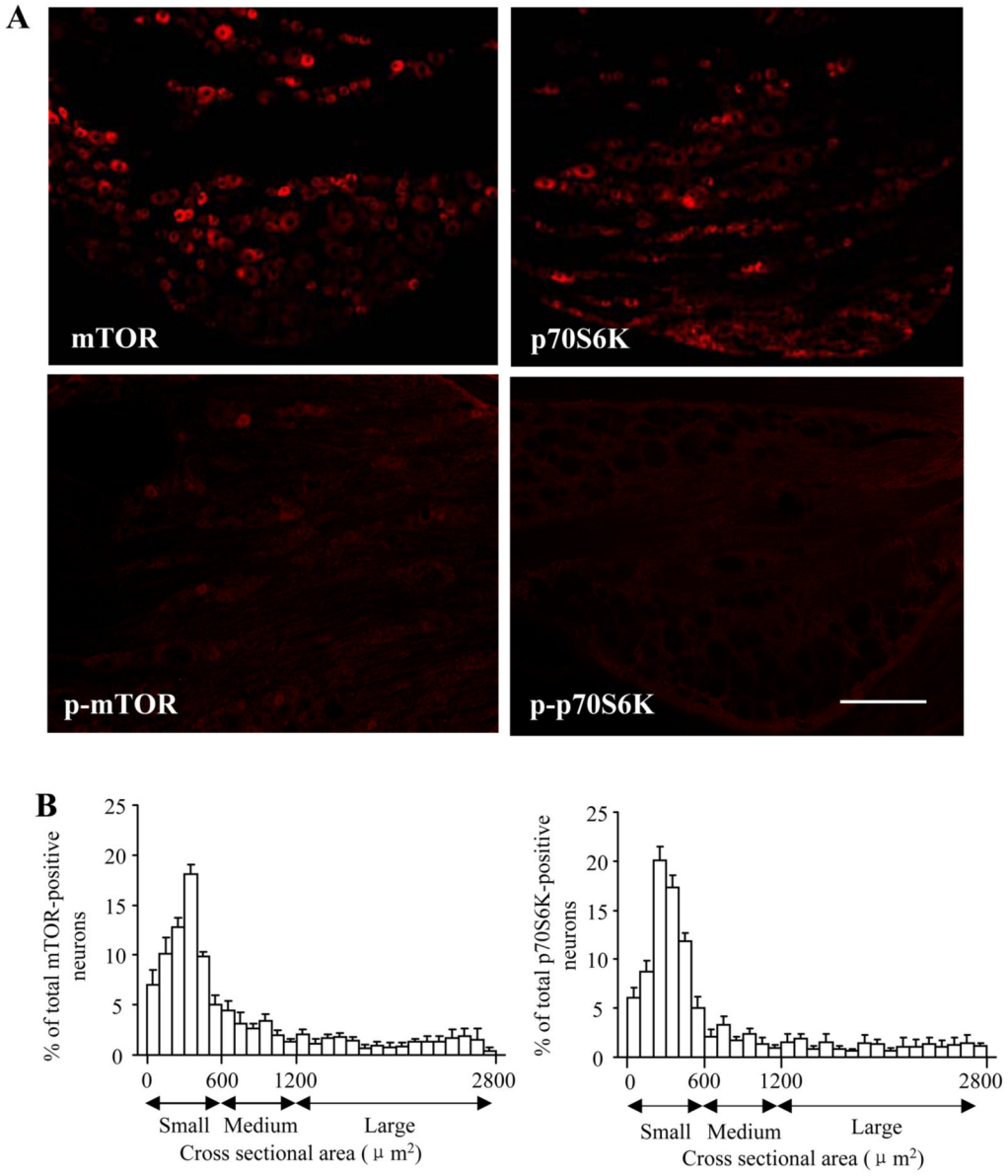

Fig. 2.

Localization and distribution of mTOR, p70S6K, and their phosphorylated counterparts in the DRG. (A) Distribution of mTOR-, p-mTOR-, p70S6K-, and p-p70S6K-immunofluorescent staining in the DRG. Scale bars: 100 μm. (B) Histograms showing the frequency of mTOR-labeled (left) and p70S6K-labeled (right) somata in the DRG by cross-sectional area.

Fig. 3A

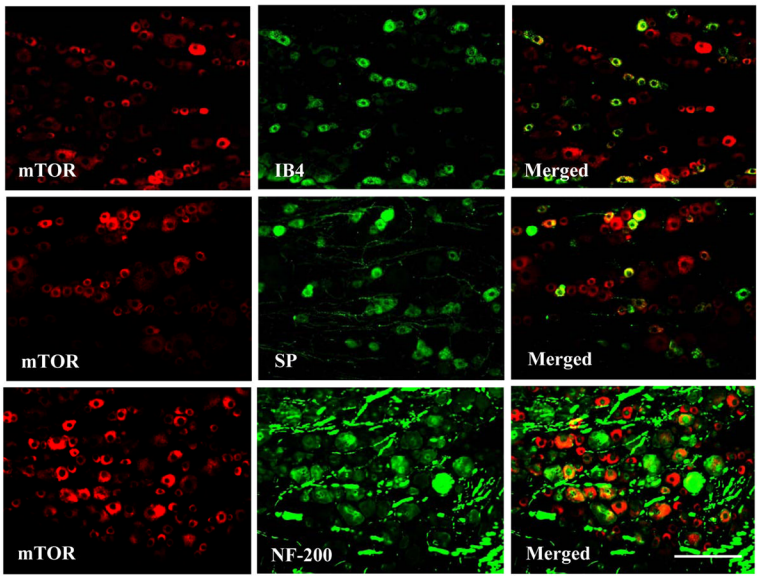

Fig. 3B

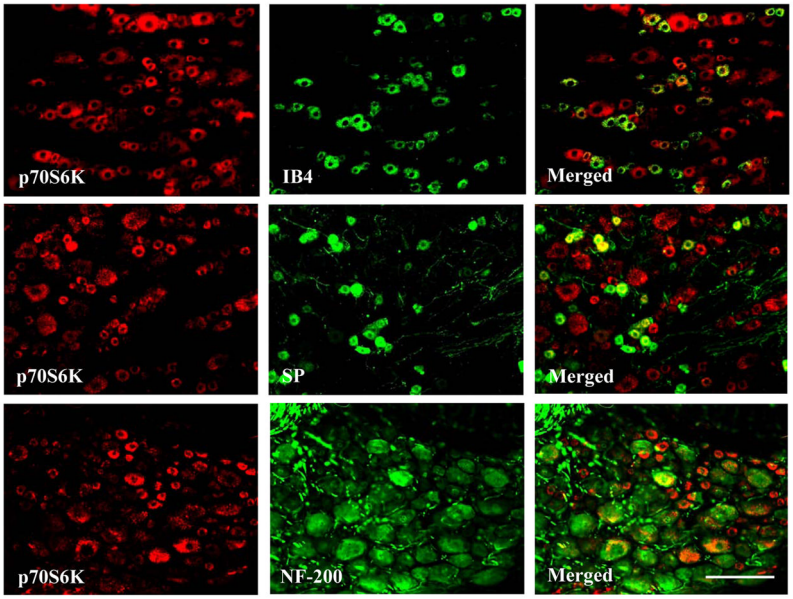

Fig. 3.

Co-localization of mTOR and p70S6K with IB4, SP, and NF-200 in DRG neurons. (A) Immunofluorescent co-localization of red reaction products for mTOR and green products for IB4, SP, and NF-200. (B) Immunofluorescent co-localization of red reaction products for p70S6K and green products for IB4, SP, and NF-200. Scale bars: 50 μm.

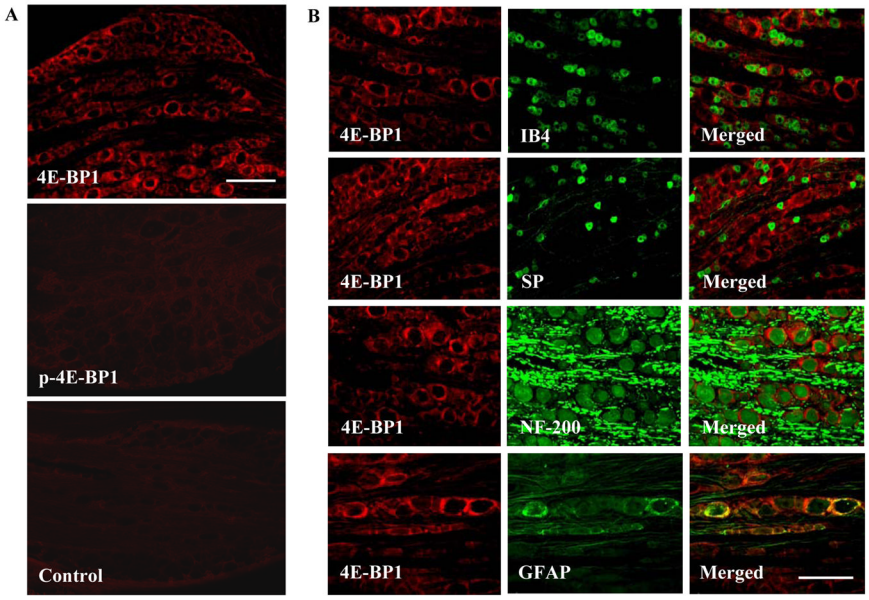

Fig. 4.

Localization and distribution of 4E-BP1 and p-4E-BP1 in the DRG. (A) Distribution of 4E-BP1 (top) and p-4E-BP1 (middle) immunofluorescent staining in the DRG. Bottom: negative control. Scale bar: 100 μm. (B) Double labeling of 4E-BP1 (red) with IB4 (green), SP (green), NF-200 (green), and GFAP (green), indicates co-localization of 4E-BP1 with GFAP, but not with IB4, SP, or NF-200. Scale bar: 50 μm.

Fig. 5A

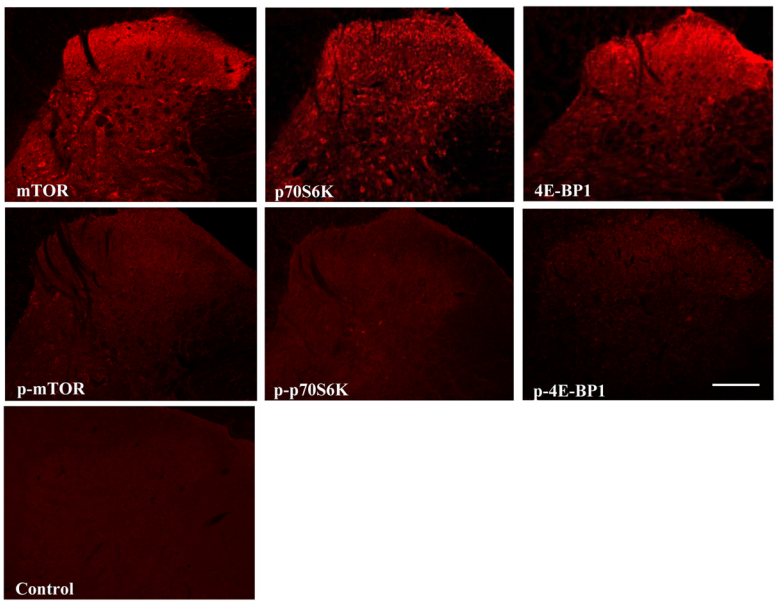

Fig. 5B

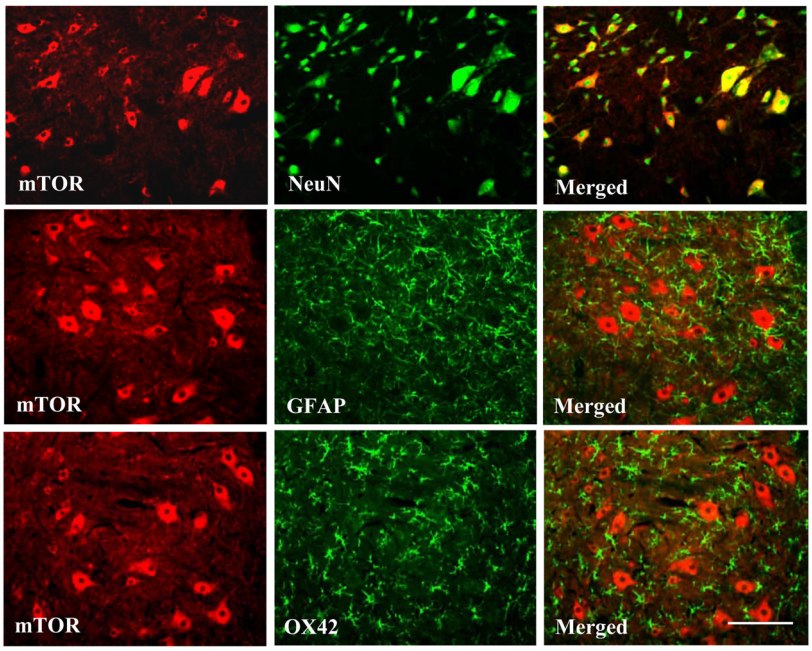

Fig. 5C

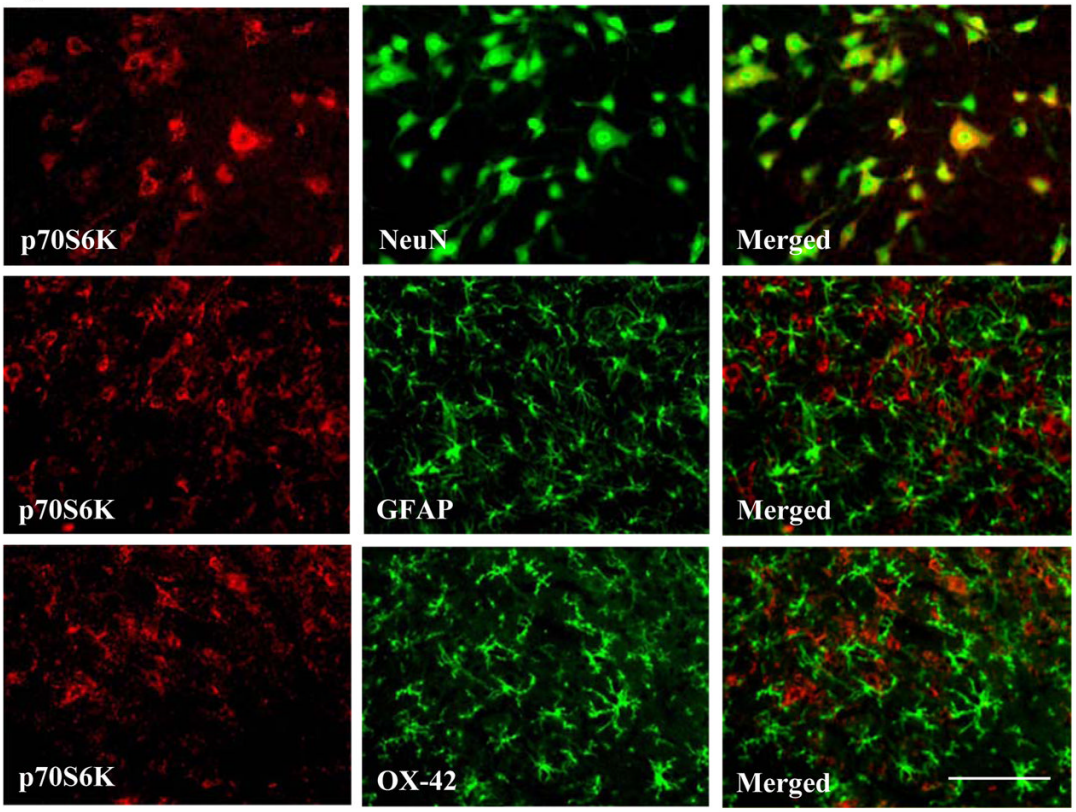

Fig. 5D

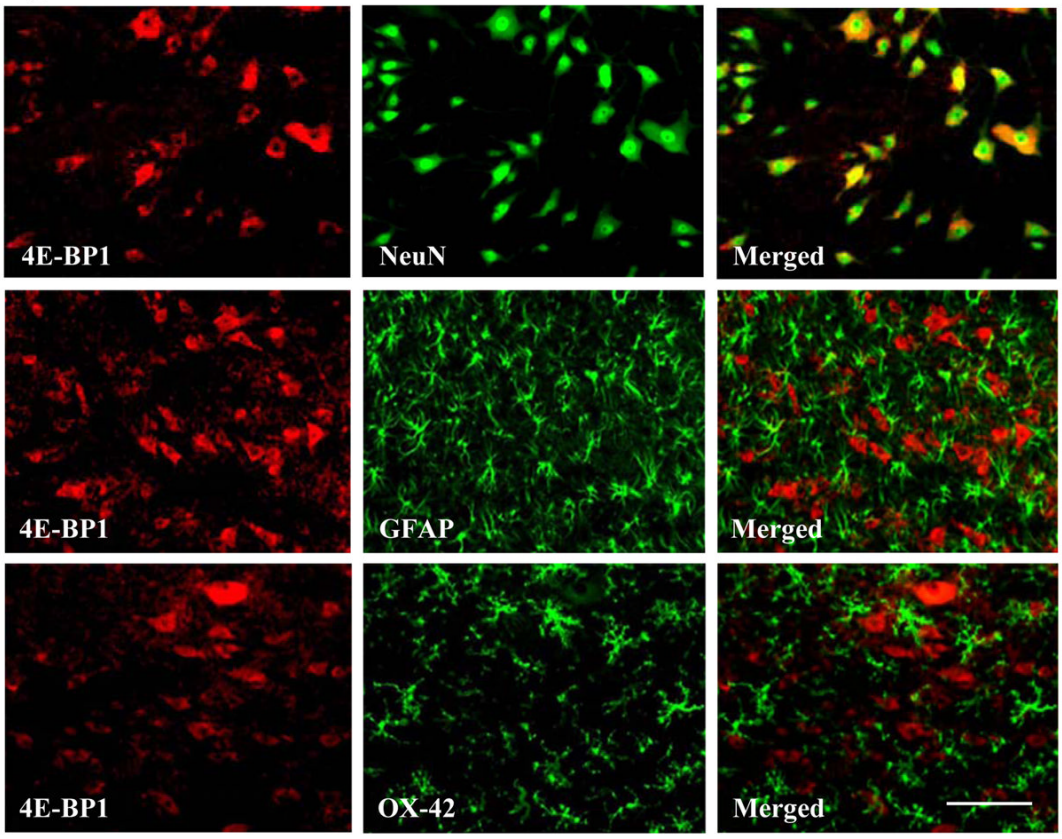

Fig. 5.

Localization and distribution of mTOR, p70S6K, 4E-BP1, and their phosphorylated counterparts in the dorsal horn. (A) Distribution of mTOR, p-mTOR, p70S6K, p-p70S6K, 4E-BP1, and p-4E-BP1 immunofluorescent staining in the dorsal horn. Bottom: negative control. Scale bar: 100 μm. (B-D) Double labeling of mTOR (red, B), p70S6K (red, C), or 4E-BP1 (red, D) with NeuN (green), GFAP (green), and OX-24 (green), indicates that mTOR, p70S6K, and 4E-BP1 each co-localize with NeuN, but not with GFAP or OX-42. Scale bars: 50 μm.

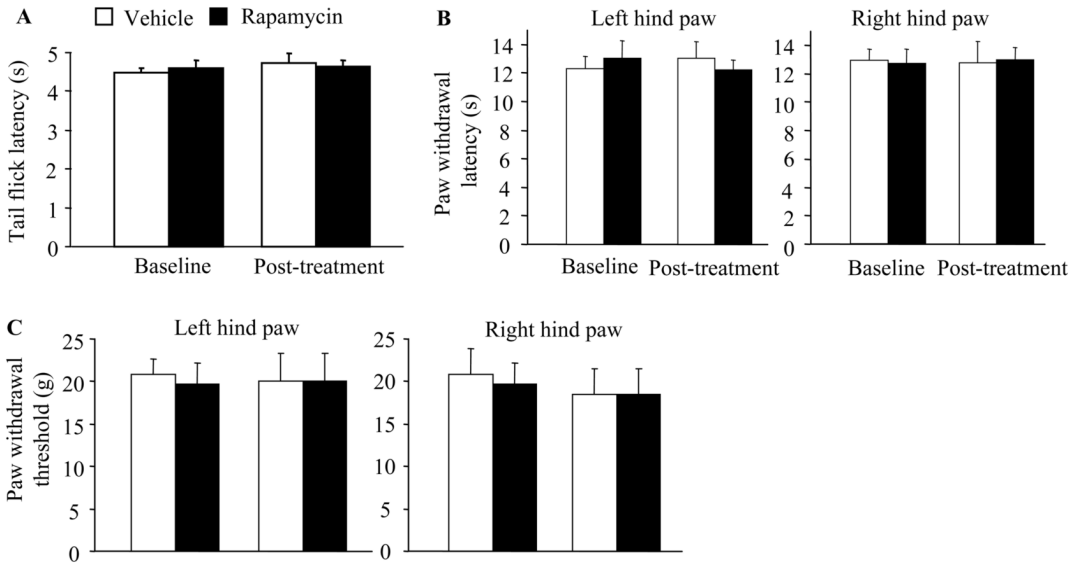

Fig. 6.

Intrathecal administration of rapamycin does not affect acute nociceptive transmission in rats. The effects of intrathecal injections of rapamycin (or vehicle) on tail-flick latency to heat (A; n = 5/group), on paw withdrawal latency to heat (B; n = 5/group), and on mechanical withdrawal threshold (C; n = 5/group) were measured by using the tail-flick test, the Hargreaves test, and von Frey hairs, respectively.