Published in final edited form as:

Brain Res. 2013 June 4; 1513: 17–25. doi:10.1016/j.brainres.2013.04.003.

mTOR and its downstream pathway are activated in the dorsal root ganglion and spinal cord after peripheral inflammation, but not after nerve injury

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Abstract

Protein translation controlled through activation of mammalian target of rapamycin (mTOR) participates in many physiological and pathological processes. However, whether such activation is required for chronic pain is still unknown. Here, we examined activation of the mTOR signaling pathway during complete Freund's adjuvant (CFA)-induced chronic inflammatory pain and L5 spinal nerve ligation (SNL)-induced neuropathic pain in rats. Western blot analysis showed significantly increased levels of phosphorylated mTOR (p-mTOR) and phosphorylated p70S6 kinase 1 (p-S6K1, a downstream effector of mTOR) in the ipsilateral L4/5 spinal cord 2 h, 1 day, 3 days, and 7 days after intraplantar CFA injection and in the ipsilateral L4/5 dorsal root ganglions (DRGs) 1 and 3 days after CFA injection. Immunohistochemistry also demonstrated increases in number of p-mTOR-labeled neurons in the ipsilateral L4/5 DRGs and in density of p-mTOR-labeled immunoreactivity in the ipsilateral L4/5 superficial dorsal horn 1 day after CFA injection. Moreover, intrathecal administration of rapamycin, a selective inhibitor of mTOR, significantly blocked CFA-induced mechanical allodynia and thermal hyperalgesia 1 day post-CFA injection. Interestingly, expression of neither p-mTOR nor p-S6K1 was markedly altered on days 3, 7, or 14 after L5 SNL in L5 spinal cord or DRG. These findings indicate that in DRG and spinal cord, mTOR and S6K1 are activated during chronic inflammatory pain, but not during neuropathic pain. Our results strongly suggest that mTOR and its downstream pathway contribute to the development of chronic inflammatory pain.

Keywords

mTOR; inflammatory pain; neuropathic pain; spinal cord; dorsal root ganglion

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The authors do not have any conflicts of interest.

1. Introduction

Chronic pain, usually caused by inflammation or tissue or nerve injury, is a major public health problem worldwide. The development of optimal treatments for such pain has remained elusive because of the complex pathologic mechanisms involved. Changes in neuronal plasticity in spinal cord and dorsal root ganglion (DRG) are thought to contribute to the development and maintenance of chronic pain. Understanding the molecular mechanisms that underlie these changes could be helpful for developing novel pain treatments.

Mammalian target of rapamycin (mTOR) is a serine/threonine protein kinase expressed in the mammalian nervous system. Once mTOR binds to Raptor protein, it forms mTOR complex 1 and phosphorylates downstream effectors, such as p70 ribosomal S6 protein kinase 1 (S6K1), to govern translation of mRNA into protein (Swiech et al., 2008; Jaworski and Sheng, 2006; Hay and Sonenberg, 2004). The activity of mTOR 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; Jaworski and Sheng, 2006). In turn, the activation of mTOR and its downstream effectors can influence numerous physiologic and pathologic processes in the nervous system (Swiech et al., 2008; Jaworski and Sheng, 2006). Recent studies showed that mTOR and its downstream effectors might be implicated in neuropathic pain, inflammatory pain, and cancer pain. Systemic or local administration of rapamycin, a specific inhibitor of mTOR, has been shown to alleviate mechanical hypersensitivity induced by intraplantar injection of carrageenan or capsaicin (inflammatory pain), peripheral nerve injury (neuropathic pain), and injection of prostate cancer cells into tibia (bone cancer pain) (Shih et al., 2012; Xu et al., 2011; Obara et al., 2011; Norsted et al., 2010; Geranton et al., 2009; Jimenez-Diaz et al., 2008; Price et al., 2007). Although mTOR and S6K1 are highly expressed in small DRG neurons and spinal cord dorsal horn neurons, their active (phosphorylated) forms are expressed at only very low levels under normal conditions (Xu et al., 2010). We and others have shown that peripheral noxious insults caused by intraplantar carrageenan or bone cancer lead to increases in phosphorylation of mTOR (p-mTOR) and S6K1 (p-S6K1) in rat spinal dorsal horn but not in DRGs (Shih et al., 2012; Xu et al., 2011). However, little is known about the role of mTOR in chronic inflammatory pain. Moreover, whether mTOR and its downstream effectors are activated in spinal cord and DRG after peripheral nerve injury is controversial (Melemedjian et al., 2011; Asante et al., 2010; Geranton et al., 2009).

In the present study, we compared the expression and activity of mTOR and S6K1 in DRG and spinal cord of rats during chronic inflammatory pain induced by intraplantar injection of complete Freund's adjuvant (CFA) and during chronic neuropathic pain caused by spinal nerve ligation (SNL).

2. Results

2.1. mTOR and S6K1 activity in DRG and spinal cord under chronic inflammatory pain conditions

Consistent with previous studies (Yaster et al., 2011; Park et al., 2009; Zhang et al., 2003), CFA injection produced mechanical hypersensitivity, as evidenced by a reduction in paw withdrawal threshold (PWT), and thermal pain hypersensitivity, as evidenced by a reduction in paw withdrawal latency (PWL). Hypersensitivity developed only on the ipsilateral side, was present by 2 h after CFA injection, reached a peak level around day 1, and lasted for at least 7 days (Figs. 1a and 1b). Time-dependent increases in the activation levels of mTOR and S6K1 were evident in the ipsilateral L4/5 spinal cord after CFA injection. Compared

with expression in control rats (0 h), expression levels of p-mTOR and p-S6K1 were increased at 2 h after CFA injection (p-mTOR: 2.58 ± 0.52 fold that of control rats; p-S6K1: 2.49 ± 0.03 fold that of control rats; $P < 0.05$) and were maintained for at least 7 days (Figs. 1c and 1d; $n = 3/\text{time point}$). As expected, the expression levels of p-mTOR and p-S6K1 in the contralateral L4/5 spinal cord were not markedly altered during the observation period (Fig. 1e). mTOR was also activated in the ipsilateral L4/5 DRGs (Fig. 2). The level of p-mTOR was significantly increased compared to that in control rats beginning 1 day after CFA injection (2.09 ± 0.05 fold that of control rats; $P < 0.05$) and remained elevated for at least 3 days (1.81 ± 0.25 fold that of control rats on day 3; $P < 0.05$; Fig. 2a). L4/5 DRG p-mTOR expression was not significantly different from that of control rats at 2 h (1.73 ± 0.44 fold that of control rats; $P > 0.05$) or 7 days (1.95 ± 0.47 fold that of control rats; $P > 0.05$) post-CFA. Consistent with our previous report (Xu et al., 2010), p-S6K1 was not detected in L4/5 DRGs from either CFA-injected or control rats. Saline injection did not change the basal level of p-mTOR in L4/5 spinal cord or DRGs or the basal amount of p-S6K1 in L4/5 spinal cord on either side (data not shown). These results indicate that CFA-induced activation of mTOR and S6K1 in spinal cord and DRG correlates with CFA-induced development and maintenance of pain hypersensitivity.

We also examined whether CFA injection affects total expression of mTOR and S6K1 proteins in spinal cord and DRG. Quantitative Western blot analysis indicated that CFA injection did not produce significant changes in the levels of total mTOR or S6K1 in spinal cord or DRG within the 7-day observation period (Figs. 1 and 2). Thus, CFA-induced inflammation alters phosphorylation status of mTOR and S6K1 but not total protein expression.

Immunohistochemical analysis also demonstrated that the number of p-mTOR-labeled neurons was significantly increased in the ipsilateral L4/5 DRGs on days 1 (Fig. 3a) and 3 (data not shown) after CFA injection. On day 1 after CFA injection, $12.03 \pm 1.24\%$ of L4 DRG neurons and $13.07 \pm 0.92\%$ of L5 DRG neurons were positive for p-mTOR on the ipsilateral side ($n = 3$; Fig. 3b). On the contralateral side, the corresponding values were only $6.73 \pm 1.0\%$ and $5.4 \pm 1.22\%$, respectively (Fig. 3b). In spinal cord dorsal horn, the density of p-mTOR immunofluorescent staining in the ipsilateral superficial dorsal horn was higher than that of naïve rats on day 1 after CFA injection (Fig. 3c). However, in the contralateral dorsal horn, p-mTOR immunofluorescence was very weak (Fig. 3c), as reported previously for naïve rats (Xu et al., 2010). We were unable to obtain a detailed cellular distribution of p-S6K1 in spinal cord and DRG because the p-S6K1 antibody is inadequate for immunohistochemistry. Taken together, our findings indicate that mTOR and its downstream effectors are activated in DRG and spinal cord under chronic inflammatory pain conditions.

2.2. Effect of intrathecal rapamycin on CFA-induced pain hypersensitivities

The data described above suggest that activation of the mTOR pathway in spinal cord and DRG participate in the development of CFA-induced inflammatory pain. To further test this hypothesis, we examined the effects of intrathecally administered rapamycin on CFA-induced mechanical and thermal pain hypersensitivities on day 1 post-CFA injection. As expected, on day 1 after CFA injection, ipsilateral PWT (3.82 ± 0.23 g) of the vehicle-treated rats was significantly reduced compared to basal PWT (23.86 ± 1.51 g; $n = 5$; $P < 0.01$; Fig. 4a) in response to mechanical stimuli. Treatment with intrathecal rapamycin increased the ipsilateral PWT by 5.25-fold compared to vehicle treatment ($P < 0.01$, $n = 5$; Fig. 4a). Similarly, on day 1 after CFA injection, ipsilateral PWL (3.59 ± 0.26 s) of the vehicle-treated rats was significantly reduced compared to basal PWL (10.28 ± 0.60 s; $n = 5$; $P < 0.01$; Fig. 4b) in response to thermal stimulation. Treatment with intrathecal rapamycin increased the ipsilateral PWL by 1.6-fold compared to vehicle treatment ($P < 0.05$, $n = 5$;

Fig. 4b). Neither rapamycin nor vehicle alone altered basal paw withdrawal responses to mechanical and thermal stimuli in the saline-injected rats ($n = 5$, Fig. 4). These results suggest that mTOR in the spinal cord and DRG participates in the induction of CFA-induced inflammatory pain.

2.3. mTOR and S6K1 activity in DRG and spinal cord under chronic neuropathic pain conditions

Finally, we examined whether the mTOR pathway was activated in DRG and spinal cord after peripheral nerve injury. Expression levels of p-mTOR and p-S6K1 in L5 spinal cord and L5 DRG were measured at 3, 7, and 14 days after SNL surgery. Unexpectedly, the level of neither p-mTOR nor p-S6K1 was significantly altered during the observation period on either side (Fig. 5).

3. Discussion

Peripheral nerve injury and inflammation produce chronic pain hypersensitivities in rodent animal models that mimic clinical chronic neuropathic pain and chronic inflammatory pain, respectively. Understanding the mechanisms that cause pain hypersensitivity may lead to novel therapeutic strategies for its prevention and/or treatment. Although chronic neuropathic pain and inflammatory pain share some intracellular signaling pathways (Latremoliere and Woolf, 2010), each type of pain may also have unique characteristics in pathogenesis, central mechanisms, and treatment. Here, we report that CFA-induced inflammatory insult significantly increases the activity of mTOR and its downstream effector S6K1 in DRG and spinal cord dorsal horn, whereas SNL-induced nerve injury fails to do so. These findings suggest that the activation of mTOR and its downstream signals are causally linked to chronic inflammatory pain development and maintenance.

In our study, immunohistochemical staining showed that the increase in p-mTOR occurred in DRG neurons, but not in DRG satellite glial cells. In the superficial dorsal horn, it is difficult to discern cellular location of p-mTOR because its expression is low on the contralateral side and immunostaining density is high on the ipsilateral side after CFA injection. However, the CFA-induced increase in p-mTOR in the superficial dorsal horn likely occurs in neurons because mTOR protein is detected exclusively in dorsal horn neurons in naïve rats (Xu et al., 2010), and CFA injection does not change the level of total mTOR in spinal cord (Fig. 1). The level of p-S6K1 in DRG is too low to be detected by Western blot analysis or immunohistochemistry in either naïve (Xu et al., 2010) or CFA-injected rats (data not shown). Although we are able to detect a CFA-induced increase in p-S6K1 in spinal cord by Western blot analysis, whether spinal p-S6K1 undergoes changes in distribution is unknown and remains to be determined when an antibody against p-S6K1 becomes available for immunostaining.

It should be noted that CFA-induced increases in p-mTOR and p-S6K1 are time-dependent. Increases were observed at 2 h and on days 1, 3, and 7 in the ipsilateral L4-5 dorsal horns and on days 1 and 3 in the ipsilateral L4/5 DRGs after CFA injection. It is evident that these increases, especially in spinal cord, coincide temporally with induction and maintenance of CFA-induced mechanical and thermal pain hypersensitivities, suggesting the potential linkage of mTOR and S6K1 activity with inflammatory pain. We further found that intrathecal administration of the mTOR inhibitor reduced CFA-induced mechanical and thermal pain hypersensitivities. Our findings indicate that the mTOR pathway may participate in the development and maintenance of chronic inflammatory pain.

Whether mTOR and p-S6K1 are activated in DRG and spinal cord after peripheral nerve injury is still controversial. Geranton et al. (Geranton et al., 2009) first reported that spared

nerve injury did not alter the expression of p-mTOR and p-S6K1 in dorsal roots or in the superficial dorsal horn 7 days after surgery. Asante et al. (Asante et al., 2010) then showed that the level of p-S6K1 was reduced in the superficial dorsal horn of L5 spinal cord but unchanged in the L4 DRG on the ipsilateral side 14 days after SNL. However, Melemedjian et al. (Melemedjian et al., 2011) revealed that SNL upregulated expression of both p-mTOR and mTOR in L4 DRG, L5 DRG, and sciatic nerves on the ipsilateral side 17 days post-surgery. Interestingly, the ratios of p-mTOR to mTOR were increased in the ipsilateral L4 DRG and sciatic nerve, but not in the ipsilateral L5 DRG (Melemedjian et al., 2011). In our study, SNL did not alter the level of p-mTOR or p-S6K1 in the ipsilateral L5 spinal cord or the level of p-mTOR in the ipsilateral L5 DRG within the first 2 weeks after surgery. The reason for the discrepancies among these studies is unknown but may be related to variances in observation time points post-surgery, different primary antibodies, and dissimilar animal models. Pharmacologic studies showed that intraplantar injection of rapamycin significantly attenuated neuropathic pain without changing basal responses to noxious stimuli (Geranton et al., 2009; Jimenez-Diaz et al., 2008). Such findings indicate that mTOR activity may be increased in peripheral afferents. This conclusion is supported by the observation that p-mTOR was increased in the sciatic nerves of the uninjured L4 DRG after SNL (Melemedjian et al., 2011). Thus, the role of mTOR and its downstream signaling pathway in neuropathic pain is still a mystery waiting to be solved.

In conclusion, our findings strongly support the role of mTOR and its downstream effectors in chronic inflammatory pain. mTOR inhibitors are FDA-approved drugs that have been used clinically for organ transplantation and phase II trials of cancer treatment (Al-Batran et al., 2012; Slomovitz et al., 2010; Garcia and Danielpour, 2008; Dean et al., 2008; Yee et al., 2006). Our recent work showed that intrathecal rapamycin reduced cancer-related pain, but did not produce significant systemic side effects such as immunosuppression (Shih et al., 2012). Therefore, local spinal mTOR inhibition may represent a promising novel treatment for chronic inflammatory pain and cancer pain.

4. Experimental Procedures

4.1. Animal preparation

Adult, male Sprague Dawley rats (250–300 g) were housed in cages on a standard 12:12 h light/dark cycle. Water and food were available *ad libitum* until rats were transported to the laboratory for experiments. The animals were used in protocols approved by the Animal Care and Use Committee at the Johns Hopkins University. Animal procedures were consistent with the ethical guidelines of the National Institutes of Health, the International Association for the Study of Pain, and the ethical guidelines to investigate experimental pain in a conscious animal. Efforts were made to minimize animal suffering and to reduce the number of animals used.

For intrathecal drug delivery, the rats were implanted with an intrathecal catheter under isoflurane anesthesia as described previously (Obata et al., 2004). Briefly, a laminectomy of the L5 vertebra was performed and the dura cut. A polyethylene-10 catheter was inserted into the subarachnoid space of the spinal cord at the level of the L4/5 spinal cord. The animals were allowed to recover for a week before being used experimentally. Rats that showed neurologic deficits postoperatively were discarded from the study. The location of the intrathecal catheter was confirmed after completion of the experiments.

4.2. CFA-induced inflammatory pain model

Chronic peripheral inflammation was induced in rats by injecting 100 μ l of CFA (*Mycobacterium tuberculosis*; Sigma-Aldrich, St. Louis, MO), suspended in an oil-saline

(1:1) emulsion, subcutaneously into the plantar side of one hind paw as described previously (Yaster et al., 2011; Park et al., 2009; Zhang et al., 2003). Age-matched rats were injected with saline (0.9%; 100 μ l) for use as controls.

To examine the role of mTOR in the development of CFA-induced inflammatory pain, we administered rapamycin (20 μ g/10 μ l; dissolved in 50% DMSO) or vehicle (50% DMSO; 10 μ l) intrathecally 1 h before intraplantar injection of saline or CFA and again the day after saline or CFA injection. The rats were divided into 4 groups ($n = 5$ /group): saline + vehicle, saline + rapamycin, CFA + vehicle, and CFA + rapamycin. Mechanical and thermal tests as described below were carried out prior to rapamycin or vehicle administration and 2 h after the second rapamycin or vehicle administration. The dosage of rapamycin was based on those used in previous studies (Shih et al., 2012; Xu et al., 2010; Norsted et al., 2010; Jimenez-Diaz et al., 2008; Price et al., 2007).

4.3 SNL-induced neuropathic pain model

L5 SNL was carried out according to our previously published protocol (Lee et al., 2011; Wang et al., 2011; Singh et al., 2009; Guan et al., 2009). Briefly, rats were anesthetized with isoflurane and placed in a prone position. A dorsolateral skin incision was made on the lower back. The sixth lumbar transverse process was identified and freed of its muscle attachments and then removed. The underlying fifth lumbar nerve root was isolated, ligated with a 3-0 silk suture, and transected just distal to the ligature. The surgical procedure for the sham group was identical, except that the fifth lumbar spinal nerve was not ligated and transected.

4.4. Behavioral testing

For thermal testing, PWL was measured with a Model 336 Analgesia Meter (IITC Life Science Instruments, CA). 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 withdrew its paw in response to the beam, 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 PWL. 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.

For mechanical testing, PWT was measured with the up-down testing paradigm (Lee et al., 2011; Wang et al., 2011; Singh et al., 2009; Guan et al., 2009). Each rat was placed in a Plexiglas chamber on an elevated mesh screen. Von Frey filaments in log increments of force (0.407, 0.692, 1.202, 2.041, 3.63, 5.495, 8.511, and 15.136 g) were applied to the plantar surface of the left and right hind paws. The 2.041-g stimulus was applied first. If the rat exhibited a positive response (abrupt paw withdrawal, licking, or shaking) occurred, the next smaller von Frey hair was used; if a negative response was observed, the next higher force was used. The test was continued until (1) the responses to five stimuli were assessed after the first crossing of the withdrawal threshold or (2) the upper or lower end of the von Frey hair set was reached before a positive or negative response had been obtained.

4.5. Western blot analysis

After the rats were anesthetized with an overdose of isoflurane, L4/5 DRGs and spinal cord were dissected out and quickly frozen in liquid nitrogen. The tissues were homogenized in lysis buffer [10 mM Tris-HCl, 5 mM MgCl₂, 5 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol, 40 μ M leupeptin, 1:100 phosphatase inhibitor cocktail II (Sigma-Aldrich), 1:100 protease inhibitor cocktail III (Sigma-Aldrich)]. All procedures were carried out on ice. The crude homogenates were centrifuged at 1000 $\times$ g for 15 min (4°C),

and the supernatants were collected. Protein concentration was measured by Bio-Rad protein assay (Bio-Rad, Hercules, CA). After the samples were combined with loading buffer and heated for 5 min at 99°C, equal amounts of protein were loaded onto 4–20% Tris-HCl polyacrylamide gels. Separated proteins were transferred to a nitrocellulose membrane electrophoretically. Membranes were first incubated in Tris-buffered saline containing 0.1% Tween 20 and 3% non-fat dry milk for 2 h at room temperature (RT). The membranes were then incubated with primary antibodies (4°C overnight) and finally with anti-rabbit or anti-mouse secondary antibodies conjugated with horseradish peroxidase (1:3000, Jackson ImmunoResearch, West Grove, PA) for 2 h at RT. The primary antibodies used included rabbit anti-p-mTOR polyclonal antibody (Ser2448, 1:1000, Cell Signaling Technology, Danvers, MA), rabbit anti-mTOR polyclonal antibody (1:1000, Cell Signaling Technology), rabbit anti-p-S6K1 polyclonal antibody (1:1000, Cell Signaling Technology), rabbit anti-S6K1 polyclonal antibody (1:1000, Cell Signaling Technology), and mouse anti- β -actin monoclonal antibody (1:1000, Santa Cruz Biotechnology, Santa Cruz, CA). Signal was detected by enhanced chemoluminescence (Denville, Metuchen, NJ). After exposure of membranes, films were scanned and the blot densities quantified. The blot density from normal control tissue was set as 100%. After normalization to the corresponding β -actin (a loading control), the relative density values from other groups were calculated by dividing the optical density values from these groups by the value from the control value.

4.6. Immunohistochemistry

Under deep anesthesia with isoflurane, the rats were perfused intracardially with normal saline followed by cooled 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4, 4°C). L4/5 DRGs and spinal cord were harvested, post-fixed, and dehydrated. The tissues were frozen-cut at a thickness of 20 μ m for DRG and at a thickness of 30 μ m for spinal cord. Two sets of DRG sections (4–5 sections/DRG/set) were collected by grouping every third serial section. All sections were blocked with 10% goat serum in 0.01 M phosphate-buffered saline and 0.3% Triton X-100 for 2 h at RT. Then the spinal cord sections and one set of DRG sections were incubated with rabbit anti-p-mTOR polyclonal antibody (1:1000, Cell Signaling Technology) at 4°C overnight and then with goat anti-rabbit antibody conjugated with Cy3 (1:200, Jackson ImmunoResearch) for 2 h at RT. Control experiments, which were carried out in some spinal cord sections and the second set of DRG sections, included pre-absorption of the primary antibodies with excess of the corresponding antigens, substitution of normal serum, and omission of the primary antibodies. Finally, the sections were covered with Fluoromount™ Aqueous Mounting Medium (Sigma-Aldrich) and viewed with an epifluorescence microscope under appropriate filter for Cy3 (excitation 550 nm; emission 570 nm). For quantitative analysis in DRG, 12–15 sections from three rats were randomly selected. All labeled and unlabeled cells with nuclei were counted.

4.7. Statistical analysis

All statistical analysis was carried out with SigmaStat 2.03 software. Data were analyzed by Student's *t*-test or one-way or two-way ANOVA followed by the Tukey post hoc test. In each group, five to six rats were included for behavioral tests and three to six rats were used for Western blots or immunohistochemistry. The results are presented as mean $\pm$ SEM. *P* < 0.05 was considered statistically significant.

Acknowledgments

The authors thank Claire F. Levine, MS, for her editorial assistance.

This work was supported by grants (NS072206, NS058886, DA033390) from the National Institutes of Health; Mr. David Koch and the Patrick C. Walsh Prostate Cancer Research Fund; the Rita Allen Foundation; and the Blaustein Pain Research Fund.

Abbreviations

CFA	complete Freund's adjuvant
DRG	dorsal root ganglion
mTOR	mammalian target of rapamycin
PWL	paw withdrawal latency
PWT	paw withdrawal threshold
RT	room temperature
S6K1	p70 ribosomal S6 protein kinase 1
SNL	spinal nerve ligation

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Fig. 1.

Time-dependent pain hypersensitivity and activation of mTOR and S6K1 in spinal cord after intraplantar CFA injection. **a and b** Intraplantar injection of CFA produced mechanical allodynia (**a**) and thermal hyperalgesia (**b**) on the ipsilateral, but not contralateral, side. $n = 5$. $*P < 0.05$, $**P < 0.01$ vs contralateral side, two-way ANOVA followed by the Tukey post hoc test. **c and d** The levels of phosphorylated (p-) mTOR (**c**) and p-S6K1 (**d**) were significantly increased in the ipsilateral L4/5 spinal cord at 2 h, 1 day, 3 days, and 7 days after CFA injection. CFA injection did not alter the expression of total mTOR (**c**) or S6K1 (**d**) in the ipsilateral L4/5 spinal cord at these time points. **(e)** The levels of neither p-mTOR nor p-S6K1 were altered in the contralateral L4/5 spinal cord after CFA injection during the observation period. For **c–e**, the upper panels depict representative Western blots, and the

lower panels show the statistical summary of the densitometric analysis expressed relative to basal level (0 h) after normalization to the corresponding β -actin. $n = 3/\text{time point}$. $*P < 0.05$ vs the corresponding naïve rats (0 h), one-way ANOVA followed by the Tukey post hoc test.

Fig. 2.

Time-dependent activation of mTOR in dorsal root ganglion (DRG) after intraplantar CFA injection. **(a)** The level of p-mTOR was significantly increased in the ipsilateral L4/5 DRGs on days 1 and 3, but not at 2 h or on day 7, after CFA injection. The amount of total mTOR was not changed in the ipsilateral L4/5 DRGs during the observation period. **(b)** p-mTOR expression did not change in the contralateral L4/5 DRGs after CFA injection during the observation period. In **a** and **b**, the upper panels depict representative Western blots, and the lower panels show the statistical summary of the densitometric analysis expressed relative to basal level (0 h) after normalization to the corresponding β -actin. $n = 3/\text{time point}$. $*P < 0.05$ vs the corresponding naïve rats (0 h), one-way ANOVA followed by the Tukey post hoc test.

Fig. 3. p-mTOR immunoreactivity increases in L4/5 DRGs and L4/5 spinal cord after intraplantar CFA injection. (a) Representative images for p-mTOR-labeled neurons in the ipsilateral (Ipsi) and contralateral (Contra) L5 DRG 1 day after CFA injection. The number of p-mTOR-labeled neurons was significantly greater in the ipsilateral L4/5 DRGs than in the contralateral L4/5 DRGs. Scale bar: 50 μ m. (b) Statistical summary of the percentages of p-mTOR-labeled neurons in the ipsilateral and contralateral L4/5 DRGs. $n = 3$. * $P < 0.05$, ** $P < 0.01$ vs the corresponding contralateral side by Student's t -test. (c) Representative images depict the distribution of p-mTOR immunoreactivity in ipsilateral and contralateral L5 dorsal horn 1 day after CFA injection. Scale bar: 30 μ m.

Fig. 4.

Effect of mTOR inhibition on CFA-induced mechanical and thermal pain hypersensitivities.

(a) Intrathecal injection of rapamycin (Rapa; 20 μ g dissolved in 50% DMSO) markedly reversed the CFA-induced decrease in paw withdrawal threshold on day 1 after intraplantar CFA injection. Intrathecal injection of rapamycin in the absence of CFA injection had no effect on basal paw withdrawal threshold. (b) Intrathecal injection of rapamycin significantly reversed the CFA-induced reduction in paw withdrawal latency on day 1 after CFA injection. Intrathecal injection of rapamycin in the absence of CFA injection did not alter basal paw withdrawal latency. For **a** and **b**, $n = 5/\text{group}$; ** $P < 0.01$ vs the corresponding baseline; # $P < 0.05$, ## $P < 0.01$ vs the group injected with CFA and treated with vehicle (50% DMSO), two-way ANOVA followed by the Tukey post hoc test.

Fig. 5.

Time course of pain hypersensitivity and expression of p-mTOR and p-S6K1 in the ipsilateral spinal cord and DRG after spinal nerve ligation (SNL). **a and b** L5 spinal nerve ligation produced mechanical allodynia (**a**) and thermal hyperalgesia (**b**) on the ipsilateral, but not contralateral, side. $n = 5$. * $P < 0.05$, ** $P < 0.01$ vs contralateral side, two-way ANOVA followed by the Tukey post hoc test. **(c)** The levels of p-mTOR and p-S6K1 in the ipsilateral L5 spinal cord were not significantly altered on days 3, 7, or 14 post-SNL. $n = 3$ /time point. **(d)** The level of p-mTOR in the ipsilateral L5 DRG was not significantly altered on days 3, 7, or 14 post-SNL. $n = 3$ /time point. For **c** and **d**, the upper panels depict representative Western blots, and the lower panels show the statistical summary of the densitometric analysis expressed relative to basal level (0 d) after normalization to the corresponding β -actin by one-way ANOVA followed by the Tukey post hoc test.