Neuroscience Letters 718 (2020) 134742

Contents lists available at ScienceDirect

Neuroscience Letters

journal homepage: www.elsevier.com/locate/neulet

Research article

mTOR signaling intervention by Torin1 and XL388 in the insular cortex alleviates neuropathic pain

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ARTICLE INFO

Keywords:
Neuropathic pain
Insular cortex
mTORC1/2
Torin1
XL388

ABSTRACT

Signaling by mammalian target of rapamycin (mTOR), a kinase regulator of protein synthesis, has been implicated in the development of chronic pain. The mTOR comprises two distinct protein complexes, mTOR complex 1 (mTORC1) and mTORC2. Although effective inhibitors of mTORC1 and C2 have been developed, studies on the effect of these inhibitors related to pain modulation are still lacking. This study was conducted to determine the inhibitory effects of Torin1 and XL388 in an animal model of neuropathic pain. Seven days after neuropathic surgery, Torin1 or XL388 were microinjected into the insular cortex (IC) of nerve-injured animals and behavioral changes were assessed. Administration of Torin1 or XL388 into the IC significantly increased mechanical thresholds and reduced mechanical allodynia. At the immunoblotting results, Torin1 and XL388 significantly reduced phosphorylation of mTOR, 4E-BP1, p70S6K, and PKCα, without affecting Akt. These results strongly suggest that Torin1 and XL388 may attenuate neuropathic pain via inhibition of mTORC1 and mTORC2 in the IC.

1. Introduction

Pain signals transmitted via nociceptive neurons are comprehensively projected to several brain regions [1,2]. The insular cortex (IC), one of the pain-related brain regions, can be activated upon nociceptive stimulation and is associated with pain memory and emotional processing of pain [3]. Several studies have shown that neural plasticity also forms in the IC under conditions of neuropathic pain [3–5]. For example, pain behaviors were diminished when blocking protein kinase Mζ, a key factor in maintaining long-term potentiation (LTP) in the IC [6].

Our previous report demonstrated that neural plasticity could be induced by nerve injury and altered through mTOR modulation in the IC [7]. Mammalian target of rapamycin (mTOR) is a protein serine/threonine kinase that regulates cell proliferation, cell growth, motility, and cell survival [8]. It serves as an integral component of two complexes, mTOR complex (mTORC) 1 and mTORC2 (mTORC1/2) [9–11]. Active mTORC1 phosphorylates downstream effectors such as eukaryotic translation initiation factor 4E binding protein 1 (4E-BP1) and p70 ribosomal S6 kinase (p70S6K), which serve as crucial factors in the initiation of mRNA translation [12–14]. Downstream effectors of mTORC2 include protein kinase B (Akt/PKB) and protein kinase C alpha (PKCα), which play a role in cell survival, cytoskeletal rearrangement, and actin regulation [15].

The mTORC1-blocking allosteric mTOR inhibitors, including rapamycin, rapalogs, and their derivatives, have been widely studied in oncogenic research owing to their efficacy and limited side effects [5–7]. Recently, it has been studied that mTOR inhibitors may affect cancer-related pain, in addition to inhibiting cancer itself [16,17]. Furthermore, mTOR inhibitors have also been studied in relation to inflammatory pain and neuropathic pain [8,9]. The association between mTOR signaling and neuropathic pain is still being investigated in animal studies [4,10,11]. However, inhibiting only mTORC1 activates the feedback loop associated with the mTOR pathway and makes it difficult to complete suppression of the mTOR complex [18]. Consequently, several drugs targeting both mTOR complexes, such as the ATP-competitive mTOR inhibitor and dual phosphatidylinositol 3-kinase (PI3K)/mTOR inhibitor, are currently under development [18,19].

Abbreviations: mTOR, mammalian target of rapamycin; IC, insular cortex; p70S6K, p70 ribosomal S6 kinase; 4E-BP1, eukaryotic translation initiation factor 4E binding protein 1; Akt, protein kinase B; PKCα, protein kinase C alpha; PI3K, phosphatidylinositol 3-kinase; PDK1, phosphatidylinositol 3-dependent kinase 1; PIKK, phosphatidylinositol 3-kinase related kinase

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https://doi.org/10.1016/j.neulet.2020.134742
Received 11 September 2019; Received in revised form 19 December 2019; Accepted 3 January 2020
Available online 07 January 2020

Torin1 and XL388 are newly developed ATP-competitive inhibitors that suppress the activation of both mTOR complexes [20–22]. A few studies have confirmed the pain relieving effect of novel mTOR inhibitors, which have been improved over rapamycin and rapalogs in the spinal cord [23,24]. However, there has been no report about attenuating pain via direct injection of these novel mTOR inhibitors into the parenchyma of the brain.

The pain attenuating effect of simultaneous inhibition of the two mTOR complexes in the IC was not investigated so far. Therefore, this study was conducted to assess the pain alleviation effects of Torin1 and XL388 by direct administration into the IC. Specifically, we assessed the phosphorylation levels of mTOR and of the respective downstream effectors in the IC following Torin1 or XL388 administration. The findings of this study may provide the potential therapeutic intervention for pain management using mTORC1/2 inhibition.

2. Materials and methods

2.1. Experimental animals

All experimental procedures adhered to the guidelines of the National Institute of Health, and were approved by the Institutional Animal Care and Use Committee of Yonsei University Health System (permit no. 2017-0148). Male Sprague-Dawley rats (200 − 250 g; Harlan, Koatec, Pyeongtaek, Korea) were used for all experiments.

2.2. Cannula implantation

Rats were anesthetized with sodium pentobarbital (50 mg/kg, i.p.) for deep anesthesia and placed in a stereotaxic frame (David KOPF instruments, Los Angeles, CA, U.S.A.). After local anesthesia with lidocaine to minimize the pain from surgery, the scalp was cut to expose the bregma. The guide cannulae (28-gauge) were bilaterally implanted into the rostral anterior IC (anterior: 1.0 mm from bregma; lateral: ± 4.9 mm from the midline; depth: -5.9 mm from the surface of the skull). The cannulae were fixed to the bone with dental cement, and the dummy cannulae were inserted into the guide cannulae to prevent clogging caused by coagulation.

2.3. Neuropathic surgery

Neuropathic surgery was conducted as described previously [25] following a 7-day recovery period after cannula implantation. Rats were reanesthetized with isoflurane using a 5 % vaporizer prior to neuropathic surgery. The tibial and sural nerves were tightly ligated with 5-0 black silk and sectioned distal to the ligation, whereas the common peroneal nerve was left intact. Rats in the sham group were subjected to the same surgical procedure for exposing the sciatic nerve, without the injury procedure.

2.4. Mechanical allodynia assessment

To assess changes in the mechanical threshold, the mechanical allodynia test was performed one day prior to nerve injury and on postoperative days (PODs) 1, 4, and 7. On POD 7, additional behavioral tests were performed from 30 min up to 48 h following microinjection. Each rat was habituated for 15 min in an acrylic cage. The mechanical allodynia test was performed using an electronic von Frey filament (no. 38450; UGO Basile, Varese, Italy). The measurements were repeated seven times at 2- to 3-min intervals, and the data were averaged, excluding the maximum and minimum values.

2.5. Microinjection of Torin1 and XL388 into the IC

All injected solutions were saline-based and contained 0.06 % DMSO. Torin1 (Tocris Bioscience, Bristol, UK.) and XL388 (Tocris Bioscience) were diluted in base solution at 400 nM and 500 nM, respectively. These doses of Torin1 and XL388 were used as the optimal concentration for our experiment, in accordance with previous studies [20,26]. The vehicle, Torin1, or XL388 was injected into the IC bilaterally at the same rate of 0.5 μl/min. After the microinjection, the injection cannula was placed additional 1 min to minimize reflux along the cannula.

2.6. Western blot analysis

On POD7, targeted IC regions were collected 4 h after microinjection and immediately frozen in liquid nitrogen and stored at −70 °C. For protein extraction, phosphatase inhibitors (PhosSTOP; Roche, Mannheim, Germany) were added to the lysis buffer (PRO-PREP; Intron Biotechnology, Pyeongtaek, Korea) and samples were homogenized and centrifuged at 15,000 rpm for 10 min. Protein samples were denatured and separated then transferred onto a membrane (Merck Millipore, Darmstadt, Germany). The membrane was incubated in a 5 % bovine serum albumin solution for 1 h at room temperature, and then incubated with primary antibody overnight at 4 °C. The following primary antibodies were used : mTOR (1:500 dilution; no.2972), p-mTOR (1:500; no.2971), p70S6K (1:1000; no.2708), p-p70S6K (1:500; no.9205), 4E-BP1 (1:1000; no.9644), p-4E-BP1 (1:500; no.2855), Akt (1:3000; no.4691) and p-Akt (1:1000; no.4058) antibodies were from Cell Signaling Technology (Beverly, MA, USA); PKCα (1:1000; ab4124) and p-PKCα (1:1000; ab23512) antibodies were from Abcam (Cambridge, UK); β-actin (1:15,000; LF-PA0207) was from ABFrontier (Seoul, Korea). The membrane was then incubated with secondary anti-rabbit antibody (1:5000; no.7074, CST) for 2 h at 20 °C. Six animals per group were used for western blot analysis. Samples of each group were pooled and replicated more than four times. The band intensity was quantified using the LAS system (LAS 4000, Fuji Film Inc., Tokyo, Japan). β-actin was used as an internal loading control.

2.7. Statistical analyses

Statistical analyses were performed using the SPSS 20.0 software (IBM Corporation, Armonk, NY, USA). Behavioral data were analyzed using a two-way repeated measures ANOVA followed by the Bonferroni's post-hoc multiple comparison test. Western blotting data were analyzed using a one-way ANOVA followed by Bonferroni's post-hoc multiple comparison test. All values are expressed as mean ± standard error of the mean (SEM). P values less than 0.05 were considered statistically significant.

3. Results

3.1. Nerve injury induces mechanical allodynia

Following neuropathic surgery, the mechanical threshold was measured on PODs 1, 4, and 7 (Fig. 1A). Prior to surgery, animals in the neuropathic (NP) and sham-injured (Sham) groups exhibited no differences in the threshold. However, the mechanical threshold of the NP group was significantly decreased following surgery relative to the sham group (Fig. 1B).

3.2. Microinjection of Torin1 or XL388 into the IC attenuates neuropathic pain

The pain-relieving effects of Torin1 and XL388 were assessed. On POD 7, the vehicle (0.06 % DMSO in saline, 0.5 μl), Torin1 (400 nM, 0.5 μl), or XL388 (500 nM, 0.5 μl) was administered into the IC of neuropathic rats. Mechanical thresholds were measured 0.5, 1, 2, 4, 8, 12, 24, and 48 h following injection (Fig. 2). The most substantial attenuation effects of Torin1 and XL388 were observed 4 h following the microinjection. Significant analgesic effects of Torin1 and XL388 were

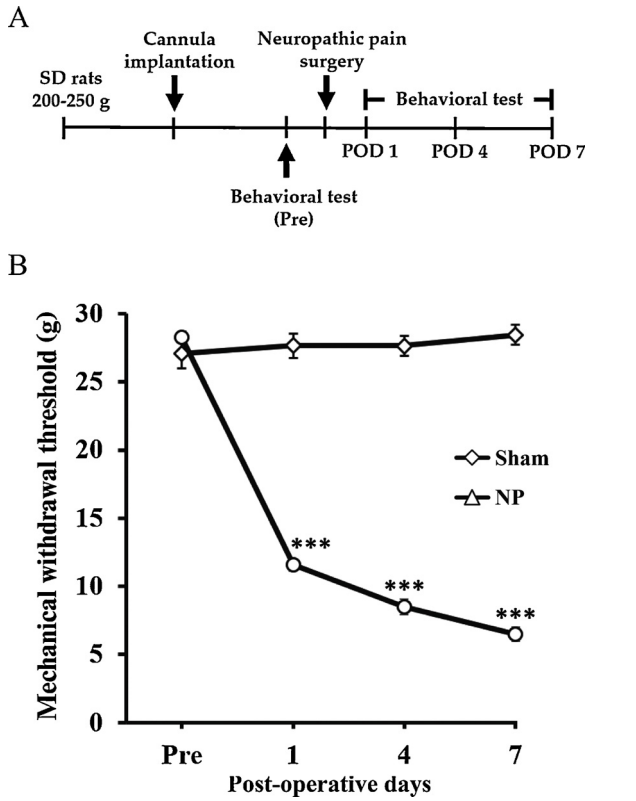

Fig. 1. Assessment of hind-paw mechanical withdrawal threshold following nerve injury. (A) Schematic diagram of the experimental procedure, indicating timing of behavioral testing and neuropathic surgery. (B) Changes in the withdrawal threshold in neuropathic (NP; n = 24) and sham-injured (Sham; n = 8) groups, as measured prior to the surgical procedures and on post-operative days (PODs) 1, 4, and 7. Data are presented as means ± SEM, *** $p < 0.001$ vs. sham, as determined using a two-way repeated measures analysis of variance (ANOVA) followed by the Bonferroni's post-hoc multiple comparison test.

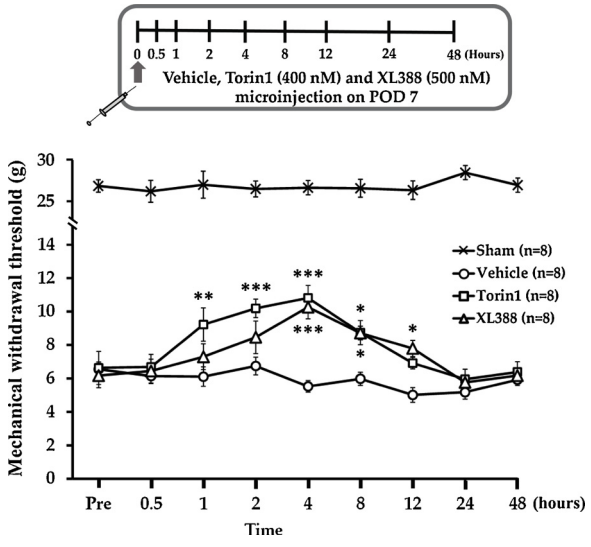

Fig. 2. Changes in hind-paw withdrawal thresholds after microinjection of vehicle (n = 8), Torin1 (n = 8), or XL388 (n = 8) on POD 7. Data are presented as means ± SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. vehicle, as determined using a two-way ANOVA followed by the Bonferroni's post-hoc multiple comparison test.

observed at 1–8 h and 4–12 h after microinjection, respectively (Fig. 2). By 24 h after the microinjection, the withdrawal thresholds of the Torin1- and XL388-injected groups had decreased and resembled those of the vehicle group.

3.3. Torin1 and XL388 downregulate mTORC1 downstream targets

Immunoblotting was performed using samples collected 4 h following microinjection, corresponding to the timing of the highest pain alleviation effect. The vehicle control group exhibited increased levels of phosphorylated mTOR (p-mTOR) relative to the sham controls (Fig. 3A). Administration of Torin1 or XL388 reversed this effect, decreasing p-mTOR abundance to levels below those observed in the vehicle group. Similarly, the levels of phosphorylated 4E-BP1 (p-4E-BP1) were significantly increased in the vehicle-injected group (Fig. 3B). The Torin1- and XL388-treated groups exhibited lower levels of p-4E-BP1 relative to the vehicle-injected group. Neuropathic injury was also associated with elevated levels of phosphorylated p70S6K (p-p70S6K), with this increase suppressed by Torin1 or XL388 administration (Fig. 3C).

3.4. Microinjection of Torin1 and XL388 into the IC alters mTORC2 downstream targets

We measured the phosphorylation levels of PKCα and Akt in neuropathic rats following Torin1 or XL388 administration. Levels of phosphorylated PKCα (p-PKCα) were increased in the vehicle-injected group relative to the sham group. However, rats injected with Torin1 or XL388 showed a significant decrease in p-PKCα levels compared to rats injected with the vehicle control (Fig. 4A). The levels of phosphorylated Akt (p-Akt) showed a significant increase in the vehicle group compared to the sham injury group. However, no significant differences were observed in p-Akt expressions in the Torin1- and XL388-treated groups compared to the vehicle (Fig. 4B).

4. Discussion

In this study, we demonstrated the pain alleviation effect of Torin1 and XL388. We examined the inhibition of mTORC1 as well as of mTORC2 to confirm the pain alleviation effect of the mTOR signaling pathway in the IC. After the administration of Torin1 or XL388, the mechanical allodynia was significantly decreased and the activity of mTOR complexes and their downstream effectors was significantly reduced.

In the process of pain-related neural plasticity, mTOR kinase is one of the key factors related to protein synthesis [10]. In the present study, a significant increase in p-mTOR was shown in the nerve-injured group. This result is consistent with other studies, including our previous studies, showing increased phosphorylation of mTOR following nerve injury [7,23,27], and it indicates that the protein synthesis involved in neural plasticity causing neuropathic pain is increased. The decline in mechanical allodynia after administration of Torin1 or XL388 suggests that the mTOR-mediated plasticity was decreased.

We have examined the changes in the phosphorylation of mTOR complexes by measuring activated individual substrates. Each mTOR complex phosphorylates corresponding downstream effectors [28,29]. Activated 4E-BP1 and p70S6K can trigger *de novo* proteins synthesis [14]. One of the processes that require *de novo* protein synthesis is the alteration of neuroplasticity that develops in chronic neuropathy conditions [30]. Local protein synthesis via 4E-BP1 and p70S6K has been reported to affect nociceptive function [31], and deletion of the mTORC1 downstream effectors has resulted in a deficiency of long-term memory [32,33]. mTORC1 related to translation factors such as 4E-BP1 and p70S6K has been studied in some cortical regions involved in pain processing in our previous studies [7,27]. p-4E-BP1 and p-p70S6K were increased under chronic pain conditions, and mechanical allodynia was

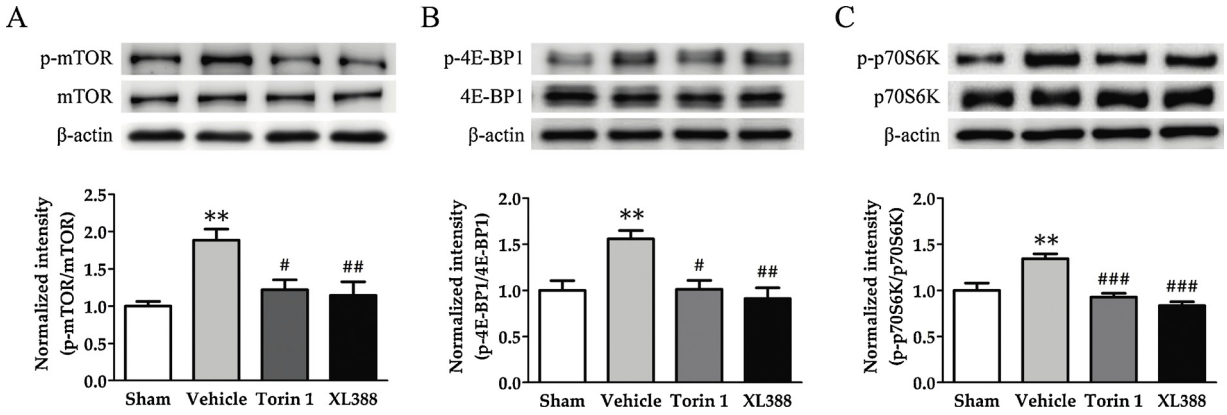

Fig. 3. Microinjection of Torin1 and XL388 into the IC reverses upregulation of p-mTOR and of mTORC1 downstream signaling. Levels of phosphorylated (p-) mTOR (A), p-4E-BP1 (B), and p-p70S6K (C) relative to the total form were assessed with immunoblotting. Data are presented as means ± SEM. ** $p < 0.01$ vs. Sham, # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$ vs. vehicle, as determined using a one-way ANOVA followed by the Bonferroni's post-hoc multiple comparison test.

attenuated when these translation factors were inhibited. In line with our previous studies [7,27], we observed an increase in p-4E-BP1 and p-p70S6K in the vehicle group, implying that protein synthesis required for LTP accompanied by the development of neuropathic pain was elevated. Conversely, a significant decrease in p-4E-BP1 and p-S6K in the Torin1- and XL388-treatment groups was attributed to the relief of neuropathic pain by inhibiting protein synthesis involved in neural plasticity.

In our results, p-PKCα was increased in the nerve-injured group and was reversed in the group that was treated with Torin1 or XL388. Many studies have confirmed activation of PKCα, which follows mTORC2 phosphorylation [29,34]. PKCα is known to play a critical role in the cytoskeleton rearrangement induced by mTORC2 [15]. Moreover, down-regulation of the PKCα generates an abnormal cell shape or excessive actin cytoskeleton and it alters neurons by rearranging their configuration, volume, or length [28]. Such changes in the shape or dimensions of existing neurons could affect neuronal plasticity [35]. Based on previous studies, our results suggest that the cytoskeleton-associated plasticity was impeded by Torin1 and XL388.

Akt, another important factor related to mTORC2, is a crucial factor involved in both mTORC1 and mTORC2 signaling [17,30]. Akt has been reported to be involved in neuronal plasticity [36], and Akt-mediated hypersensitivity studies have been conducted in various hyperalgesia animal models such as spinal nerve ligation-induced hyperalgesia or carrageen- and capsaicin-induced hypersensitivity [36–39]. In our results, p-Akt was increased in the vehicle-treated group compared to the sham group. This result was consistent with the significant increase in p-Akt in the spinal dorsal horn (SDH) and dorsal root ganglion observed on the spinal nerve ligation-induced neuropathic pain model [37]. Therefore, we speculated that the increased p-Akt in the vehicle group was due to the upregulated phosphorylation of mTORC1/2 caused by nerve injury. In the Torin1- and XL388-treated groups, p-Akt tended to decrease compared to the vehicle group, but the difference was not statistically significant. According to studies on mTORC1/2 inhibition, Torin1 and XL388 decrease p-Akt in addition to arresting pathological cell growth [22,23]. These results, which differ from those of our study, might be due to the different cell types in *in vitro* studies in which abnormal cell growth and cell activity were suppressed in tumor cells, but not in nerve tissues [22,24]. Among other possibilities, the inhibition of p-Akt, which was increased by hyperalgesia, might be time-dependently changed. After spinal nerve ligation, p-Akt was significantly increased in the SDH. However, the inhibition of p-Akt on 3 or 7 days after nerve ligation had no pain alleviation effect [37]. In line with this finding, we speculated that the downregulation of p-Akt may not be significant in Torin1 and XL388-treated groups, since the drugs were injected 7 days after nerve injury. As another possibility, various feedback loops of Akt may have influenced p-Akt expression in the drug-treated groups with Torin1 and XL388. Akt is associated with a feedback loop through phosphatidylinositol 3-dependent kinase-1 (PDK1) that is enhanced by inhibiting the phosphorylation of mTOR [34,40], and may also be affected by its own feedback loop which regulates the phosphorylation of Akt depending on the levels of phosphorylation at different phosphate sites such as Ser 473 and Thr308 [41,42]. Furthermore, Torin1 and XL388 may not significantly reduce p-Akt due to the limitation of

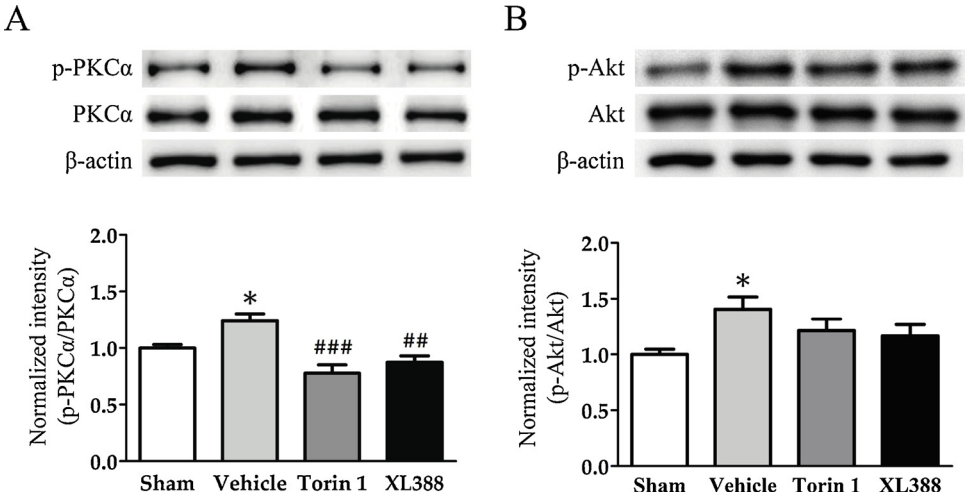

Fig. 4. Microinjection of Torin1 and XL388 into the IC modulates mTORC1/2 downstream effectors. Levels of phosphorylated (p-) PKCα (A) and p-Akt (B) relative to the total form were assessed with immunoblotting. Data are presented as means ± SEM. * $p < 0.05$ vs. sham, ## $p < 0.01$, ### $p < 0.001$ vs. vehicle, as determined using a one-way ANOVA followed by the Bonferroni's post-hoc multiple comparison test.

potency that can only partially inhibit PI3K [22,43]. In line with previous studies, Torin1 and XL388 could inhibit the activation of mTORC2. However, the expression levels of p-Akt may be affected by factors other than mTORC2 in neuropathic pain rats. Further studies are required to shed light on the alleviation of neuropathic pain that involves Akt signaling.

Torin1 and XL388 mainly inhibit phosphorylation of both mTOR complexes. However, they differ in their molecular shapes and affect different factors such as PI3K and phosphatidylinositol 3-kinase-related kinase (PIKK) family, which are related to the mTOR pathway [44]. We tried to assess the potential differences in the effects of Torin1 and XL388 on pain alleviation and mTOR downstream effectors. However, we could not observe significant difference in the extent of pain attenuation effect between Torin1 and XL388, and there were no significant differences in the expression levels of mTOR substrates.

In this study, we extended the research on pain-related mTOR signaling in the brain by studying the effect of mTORC1/2 inhibition. Compared to our previous study, which demonstrated a pain alleviation effect when rapamycin was injected directly into the IC [7], simultaneous inhibition of mTORC1/2 with Torin1 or XL388 did not appear to be more effective. The reversed mechanical withdrawal thresholds as well as the changes in expressions of downstream targets of mTORC1 in the two studies were comparable. Moreover, pain attenuation effect was observed without a significant decrease in p-Akt, which is a pivotal sub-factor of mTORC2. A recent study [23] examined the pain alleviation effect by using an mTORC1 inhibitor and Torin1. Two groups treated by intraperitoneal administration of an mTORC1 inhibitor or Torin1 were found to show effective inhibition of mechanical and cold hypersensitivity. However, the analgesic effect of Torin1-injected group did not have a superior effect compared to that of the mTORC1 inhibitor group [23]. In line with this research and our findings, relieving neuropathic pain by suppressing both mTOR complexes seemed clearly effective. However, we speculate that mTORC2 may not be a key attributor in the transient pain alleviation. Drugs that can chemically inhibit only mTOC2 phosphorylation have not yet been developed, and it is difficult to confirm the role of individual complexes that modulate pain. To determine the role of mTORC2 in pain control, more detailed future studies that target mTORC2 and rictor-specific inhibition affecting mTORC2 functions are needed.

5. Conclusion

We investigated the pain-relieving effects of mTOR inhibition using Torin1 and XL388 in the IC. We found that inhibition of mTORC1/2 reduces mechanical allodynia and modulates the downstream effectors of mTORC1/2 in the IC. Our behavioral and molecular data may contribute to establishing novel approaches for pain control in chronic neuropathy.

Conflicts of interest

The authors declare no conflict of interest.

CRediT authorship contribution statement

Songyeon Choi: Methodology, Validation, Formal analysis, Investigation, Writing - original draft, Writing - review & editing. **Kyeongmin Kim:** Methodology, Validation, Formal analysis, Writing - original draft, Data curation, Writing - review & editing. **Myeounghoon Cha:** Conceptualization, Methodology, Validation, Investigation, Supervision, Project administration, Funding acquisition. **Minjee Kim:** Formal analysis, Data curation. **Bae Hwan Lee:** Conceptualization, Supervision, Project administration, Funding acquisition.

Acknowledgements

This work was supported by a National Research Foundation of Korea (NRF) grant funded by the Korea government (MSIT) (No. 2017R1A2B3005753).

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