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## Peripheral Administration of Translation Inhibitors Reverses Increased Hyperalgesia in a Model of Chronic Pain in the Rat

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### Abstract

Chronic pain is extremely difficult to manage, in part due to lack of progress in reversing the underlying pathophysiology. Since translation of mRNAs in the peripheral terminal of the nociceptor plays a role in the transition from acute to chronic pain, we tested the hypothesis that transient inhibition of translation in the peripheral terminal of the nociceptor could *reverse* hyperalgesic priming, a model of transition from acute to chronic pain. We report that injection of translation inhibitors, rapamycin and cordycepin, which inhibit translation by different mechanisms, at the peripheral terminal of the primed nociceptor, produce reversal of priming, in the rat, that outlasted the duration of action of these drugs to prevent the development of priming. These data support the suggestion that interruption of translation in the nociceptor can reverse a preclinical model of at least one form of chronic pain.

### Keywords

peripheral protein translation; hyperalgesic priming; sensory neuron; mechanical hyperalgesia; rat

### Introduction

Hyperalgesic priming, a model of the transition from acute to chronic pain, has been defined as a long-lasting latent hyperresponsiveness of nociceptors to inflammatory mediators subsequent to an inflammatory or neuropathic insult<sup>1,35,39</sup>. Priming, which can be detected as an enhanced and prolonged hyperalgesic response to the proinflammatory cytokine prostaglandin E<sub>2</sub> (PGE<sub>2</sub>), can be explained by a switch in the intracellular signaling pathway mediating PGE<sub>2</sub>-induced hyperalgesia<sup>1,23</sup>. Since hyperalgesic priming is still present, unattenuated, for a period of months<sup>1,10,35</sup>, it is likely associated with the formation of a type of molecular memory in primary afferent nociceptors. Given the continuous turnover of most cellular proteins it is difficult to conceive that a simple switch in the coupling of a G-protein coupled receptor to a different second messenger signaling pathway is sufficient to explain the endurance of the plasticity associated with the “primed” nociceptor.

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#### Disclosures

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We have recently shown that cytoplasmic polyadenylation element binding protein (CPEB), an RNA-binding molecule that regulates the translation of otherwise dormant mRNAs<sup>41,53</sup>, is not only almost exclusively expressed by isolectin B<sub>4</sub>-positive (IB<sub>4</sub>(+))-nociceptors, the subset of nociceptors in which hyperalgesic priming occurs<sup>22</sup> and can be co-immunoprecipitated with PKC $\epsilon$ , but that the downregulation of its level of expression by the intrathecal administration of antisense oligodeoxynucleotides (ODNs) targeting its mRNA can prevent, but not reverse, hyperalgesic priming<sup>5</sup>. Because our antisense only decreased CPEB by 28%, in peripheral nerves, we might not be inhibiting peripheral translation adequately to *reverse* hyperalgesic priming. That is, the degree of attenuation required to produce reversal of priming may be far greater than that needed to prevent the development of priming. In addition, local injection of anisomycin, a protein translation inhibitor, also prevented the induction of priming<sup>5</sup>, suggesting a role of local protein synthesis in our model. Therefore, in the present study, we evaluated the ability of peripheral administration of a translation inhibitor to reverse hyperalgesic priming. Our results are compatible with the idea that ongoing peripheral translation is necessary for maintenance of the hyperalgesic priming model of chronic pain.

## Materials and Methods

### Animals

All experiments were performed on adult male Sprague Dawley rats (220–400 g; Charles River Laboratories). Animals were housed, three per cage, under a 12 h light/dark cycle in a temperature- and humidity-controlled room in the animal care facility of the University of California, San Francisco. Food and water were available *ad libitum*. All nociceptive testing was done between 10:00 am and 5:00 pm and the experimental protocols were approved by the Institutional Animal Care and Use Committee at University of California at San Francisco and adhered to the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals. All effort was made to minimize the number of animals used and their suffering.

### Mechanical nociceptive threshold testing

Mechanical nociceptive threshold was quantified using an Ugo Basile Analgesymeter<sup>®</sup> (Randall-Selitto paw-withdrawal test, Stoelting, Chicago, IL), which applies a linearly increasing mechanical force to the dorsum of the rat's hind paw, as previously described<sup>10,11</sup>. The nociceptive threshold was defined as the force, in grams, at which the rat withdrew its paw, and baseline paw-pressure threshold was defined as the mean of the three readings taken before the test agents were injected. Each paw was treated as an independent measure and each experiment performed on a separate group of rats. Data are presented as mean change from baseline mechanical nociceptive threshold.

### Drugs and their administration

Cordycepin 5'-triphosphate sodium salt,  $\lambda$ -carrageenan and prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) were purchased from Sigma-Aldrich (St. Louis, MO), and rapamycin from EMD Chemicals (Gibbstown, NJ). The selection of the drug doses used in this study was based on dose-response curves determined during our previous studies<sup>1,34,35,48</sup>. Stock solutions of PGE<sub>2</sub> in absolute ethanol (1  $\mu$ g/ $\mu$ l) were diluted in 0.9% NaCl (1:50,  $C_{final}$ =0.2  $\mu$ g/ $\mu$ l) immediately before injection. The ethanol concentration of the final PGE<sub>2</sub> solution was ~2% and the injection volume was 5  $\mu$ l. Stock solutions of rapamycin (20  $\mu$ g/ $\mu$ l, dissolved in absolute DMSO) and cordycepin (10  $\mu$ g/ $\mu$ l, dissolved in a 1:1 mixture of 0.9% NaCl and absolute ethanol) were further diluted in distilled water or 0.9% NaCl, respectively, immediately before injection. The DMSO and ethanol concentrations in the final rapamycin and cordycepin solutions were ~10% and ~2%, respectively. Carrageenan was dissolved and

diluted in 0.9% NaCl ( $C_{final}=1\%$ ) immediately before injection. The injection volume was 5  $\mu$ l. Carrageenan was injected using a 27-gauge hypodermic needle. Intradermal injection of carrageenan induces a decrease in the mechanical threshold in 30–60 min, reaching a maximum between 2 and 6 h, returning to baseline values within 72 h<sup>1</sup>. All other drugs were administered via a beveled 30-gauge hypodermic needle attached to a Hamilton<sup>®</sup> microsyringe (Reno, NV, USA) by polyethylene tubing. Rapamycin, which inhibits the mammalian target of rapamycin (mTOR), a serinethreonine protein kinase involved in the local translation of mRNA, has been used to study peripheral<sup>12,32,37</sup> and spinal<sup>2,31,42,57</sup> mechanisms of inflammatory and neuropathic pain. Cordycepin, also a protein translation inhibitor, has a different mechanism of action than rapamycin<sup>13,24,30,40,46</sup>. These protein translation inhibitors were administered on the dorsum of the hind paw with a preceding hypotonic shock to facilitate cell permeability to these agents (2  $\mu$ l of distilled water, separated by a bubble of air to avoid mixing in the same syringe), to get compounds into the nerve terminal.

### Hyperalgesic priming

Hyperalgesic priming, a model of transition from acute to chronic pain, was produced using a procedure previously used in other studies<sup>1,33,35</sup>: to prime the sensory neuron terminals in the skin, carrageenan (1%) was intradermally injected on the dorsum of the hind paw. This induces mechanical hyperalgesia that resolves 3–5 days later, following which intradermal injection of PGE<sub>2</sub> at the same site produces enhanced and prolonged mechanical hyperalgesia that lasts more than 4 h and is still significant at 24 h, as opposed to the effect of intradermal injection of PGE<sub>2</sub> in intact skin, that lasts around 2 hours<sup>1</sup>.

### Statistics

In all experiments, the dependent variable was paw-withdrawal threshold, expressed as the percentage change from baseline. Average mechanical threshold immediately before the experiments was 115.1  $\pm$  1.2 g (N=96). Paired Student's *t*-test was used to compare the mechanical thresholds before and after the carrageenan injections, and as appropriate (as noted in the figure legends). When two different groups were compared (for instance, when comparing the mechanical thresholds of control and protein translation inhibitor treated groups 4 days after carrageenan treatment), unpaired Student's *t*-test was used. To compare the hyperalgesia induced by PGE<sub>2</sub> injection in different groups, two-way repeated-measures ANOVA, followed by Bonferroni post-test, was performed. Data are presented as mean  $\pm$  SEM.

## Results

### Rapamycin

To test the hypothesis that inhibiting peripheral translation can reverse hyperalgesic priming we injected rapamycin (1  $\mu$ g), which has been shown to inhibit peripheral translation<sup>20,28,31</sup>, at the site of nociceptive testing, both 30 minutes before and again with carrageenan. We then tested for hyperalgesic priming, by injection of PGE<sub>2</sub>, 4 days later, at which time hyperalgesic priming should be established<sup>1,5,33,39</sup>. In rats treated with rapamycin, before and again with carrageenan injection, priming, as indicated by prolongation of PGE<sub>2</sub> hyperalgesia measured at 4 hours, did not develop (Fig. 1). Next, to determine if rapamycin can also reverse priming, it was injected 7 days after carrageenan, and PGE<sub>2</sub> injected 30 minutes later. Again, PGE<sub>2</sub> failed to produce prolonged hyperalgesia (Fig. 2). Thus, local administration of a translation inhibitor in the vicinity of the peripheral terminal of nociceptors whose mechanical threshold was being monitored can also reverse hyperalgesic priming.

## Cordycepin

To further test the hypothesis that inhibitors of peripheral translation can reverse hyperalgesic priming, we injected a second translation inhibitor, cordycepin, which inhibits translation by a different mechanism than rapamycin. Cordycepin was also injected at the site of nociceptive testing, both 30 minutes before and again with carrageenan. We then tested for hyperalgesic priming, by injection of PGE<sub>2</sub>, 5 days after carrageenan administration (Fig. 3). In contrast with the vehicle-treated group, in the rats treated with cordycepin, before and again with carrageenan, hyperalgesic priming, as indicated by prolongation of PGE<sub>2</sub> hyperalgesia, at 4 hours, did not develop (Fig. 3). Next, to determine if cordycepin can also *reverse* priming, it was injected 4 days after carrageenan and PGE<sub>2</sub> injected 30 minutes later. Again, PGE<sub>2</sub> failed to produce prolonged hyperalgesia (Fig. 4). Thus, we have shown that a second translation inhibitor can reverse as well as prevent hyperalgesic priming.

To confirm that the translation inhibitor had actually *reversed* priming, we wanted to demonstrate that the effect of the translation inhibitor was no longer present at the time of testing for hyperalgesic priming. First, we determined if the reversal of priming was still present 10 days after injection of cordycepin. A group of primed rats that received cordycepin 4 days after carrageenan (shown in Fig. 4) was tested again for priming 10 and 20 days later (Fig. 5). In either case, PGE<sub>2</sub>-induced hyperalgesia, although present 30 min after injection, was significantly decreased at the 4 h time point (Fig. 5). Next, in order to determine if the action of cordycepin on translation was still present 20 days after cordycepin treatment (when we still observed inhibition of the prolonged PGE<sub>2</sub>-induced hyperalgesia, Fig. 5), we injected cordycepin (1 µg) 20 days before carrageenan, and, then, tested with PGE<sub>2</sub> 5 days after carrageenan (Fig. 6). In this control experiment, hyperalgesic priming did develop, as we observed prolongation of the PGE<sub>2</sub>-induced hyperalgesia (Fig. 6). Thus, at a time after cordycepin administration that it no longer prevented hyperalgesic priming, its ability to reverse priming was detectable.

## Local effect of the protein translation inhibitors

In order to evaluate if the effect of the protein translation inhibitors was restricted to the site of injection, we performed a control experiment, in rats that had their right paw primed by carrageenan injection 4 days before, by administering rapamycin or cordycepin in the left paw. When PGE<sub>2</sub> was injected in the right paw, it still induced prolonged hyperalgesia, i.e., there was no effect of the inhibitors injected in the contralateral paw on priming, confirming their lack of systemic effect (Fig. 7).

## Discussion

In this study we have examined the hypothesis that the persistence of chronic pain is dependent on ongoing translation in the peripheral terminal of the primary afferent nociceptor. We have previously shown, in a model of the transition from acute to chronic pain, hyperalgesic priming, that while the transition can be driven by activation of PKC $\epsilon$ , intrathecal treatment with antisense ODN for PKC $\epsilon$ , to decrease its level in the nociceptor, after the establishment of the chronic phase of hyperalgesic priming, could not permanently *reverse* the primed state (unpublished observation). In a subsequent study, to examine the signaling pathway downstream of PKC $\epsilon$  in the induction of hyperalgesic priming, we demonstrated a role of CPEB, which is involved in protein translation in axons and dendrites<sup>41,53</sup> and has been implicated in neuroplasticity in *Aplysia* sensory neurons<sup>4,44,45</sup>. However, while transient attenuation of CPEB, in the nociceptor, could prevent hyperalgesic priming, it was not able to reverse it<sup>5</sup>.

Given that the downstream consequences of translation may be amplified<sup>3,9,17,20,37,38,49,54</sup>, we considered the possibility that the intrathecal treatment with antisense ODN against CPEB mRNA would not adequately decrease CPEB to a level necessary to *reverse* the primed state. However, the fact that peripheral injection of anisomycin, a protein translation inhibitor, also prevented the development of priming<sup>5</sup>, suggested that local protein synthesis plays an important role in this model. Therefore, in the present study we used translation inhibitors injected directly into the peripheral receptive field of the primed nociceptor. Using two translation inhibitors, rapamycin, which acts on the initiation of protein biosynthesis by blocking mTOR, a serine/threonine kinase that triggers the process of mRNA translation<sup>15,43,55</sup>, and cordycepin, a derivative of the nucleoside adenosine shown to inhibit mRNA polyadenylation by preventing its incorporation into the poly-A tail, interrupting its elongation and, consequently, the initiation of protein translation<sup>25,30,55</sup>, we were able, for the first time, to *reverse* hyperalgesic priming. To confirm that the translation inhibitor was no longer blocking translation as the explanation for the loss of prolongation of PGE<sub>2</sub> hyperalgesia (i.e., the primed state) we determined how long after administration of a translation inhibitor, administration of a PKC $\epsilon$  activator, carrageenan, would again be able to produce priming. When PGE<sub>2</sub>-induced hyperalgesia was examined in rats primed the same amount of time, after cordycepin administration, we still detected reversal of hyperalgesic priming. The results of these experiments support the hypothesis that a stronger inhibition of translation in the peripheral terminal of the primary afferent nociceptor is able to *reverse* priming.

While our results do support the suggestion that at least some forms of chronic pain may be interrupted by the transient attenuation of translation in the peripheral terminal of the nociceptor, it also raises the question of what protein(s) is downstream of the peripheral translation, which led to the reversal of hyperalgesic priming. Richter and colleagues have enumerated mRNA species in axonal processes/neural tissue that have polyadenylation tails and are thus downstream targets of CPEB<sup>26,27,41,44</sup>. Amongst these, one, calcium/calmodulin-dependent protein kinase II (CaMKII), has also been implicated in neuroplasticity<sup>6,7,8,14,19,58</sup>. Of note, CaMKII can be both upstream, regulating the activation of CPEB<sup>3</sup> as well as downstream of CPEB<sup>18,56</sup>. Thus, CaMKII is a reasonable candidate as a potential positive feedback element that would mandate a stronger attenuation of mRNA translation in the peripheral terminal of the nociceptor, to reverse hyperalgesic priming. Cell biological experiments are currently in progress to test this hypothesis. To better understand the transition of acute to chronic pain it is useful to try to understand what is the normal function of peripheral translation of proteins in the peripheral terminal of a sensory neuron. Another approach is to consider the functions of the sensory neuron, over the lifespan of the organism. It has been suggested that mRNA translation in the peripheral terminal of sensory neurons is important in the developmental program and regeneration of these neurons<sup>16,29,36,47,50,59</sup>. This is of more than passing interest with respect to chronic pain, as it has been noted that young children and animals are much less likely to develop chronic pain, especially after nerve injury<sup>51,52</sup>. As neuropathic as well as inflammatory conditions produce hyperalgesic priming<sup>1,21,34,39</sup>, we propose that alterations in peripheral translation between the perinatal and adult animal might help explain this differential, age dependent, predisposition to undergo the transition from acute to chronic pain. Elucidation of age dependent differences in peripheral translation will be needed to address this important clinical observation. In summary, in the present study we provide evidence that ongoing translation of mRNA in the peripheral terminal of the primary afferent nociceptor may be involved in the neuroplasticity underlying persistence in a model of chronic pain, that is, the transition from acute to chronic pain<sup>1,33,39</sup>. Additional experiments will be required to elucidate the details of mechanism, which may involve mediators both upstream as well as downstream from the translation process in the peripheral terminal of the primary afferent nociceptor.



## Abbreviations

<b>CaMKII</b>	calcium/calmodulin-dependent protein kinase II
<b>CPEB</b>	cytoplasmic polyadenylation element binding protein
<b>IB<sub>4</sub>(+)</b>	isolectin B <sub>4</sub> -positive
<b>mTOR</b>	mammalian target of rapamycin
<b>ODN</b>	oligodeoxynucleotide
<b>PGE<sub>2</sub></b>	prostaglandin E <sub>2</sub>
<b>PKCε</b>	protein kinase C epsilon
<b>SEM</b>	standard error of the mean

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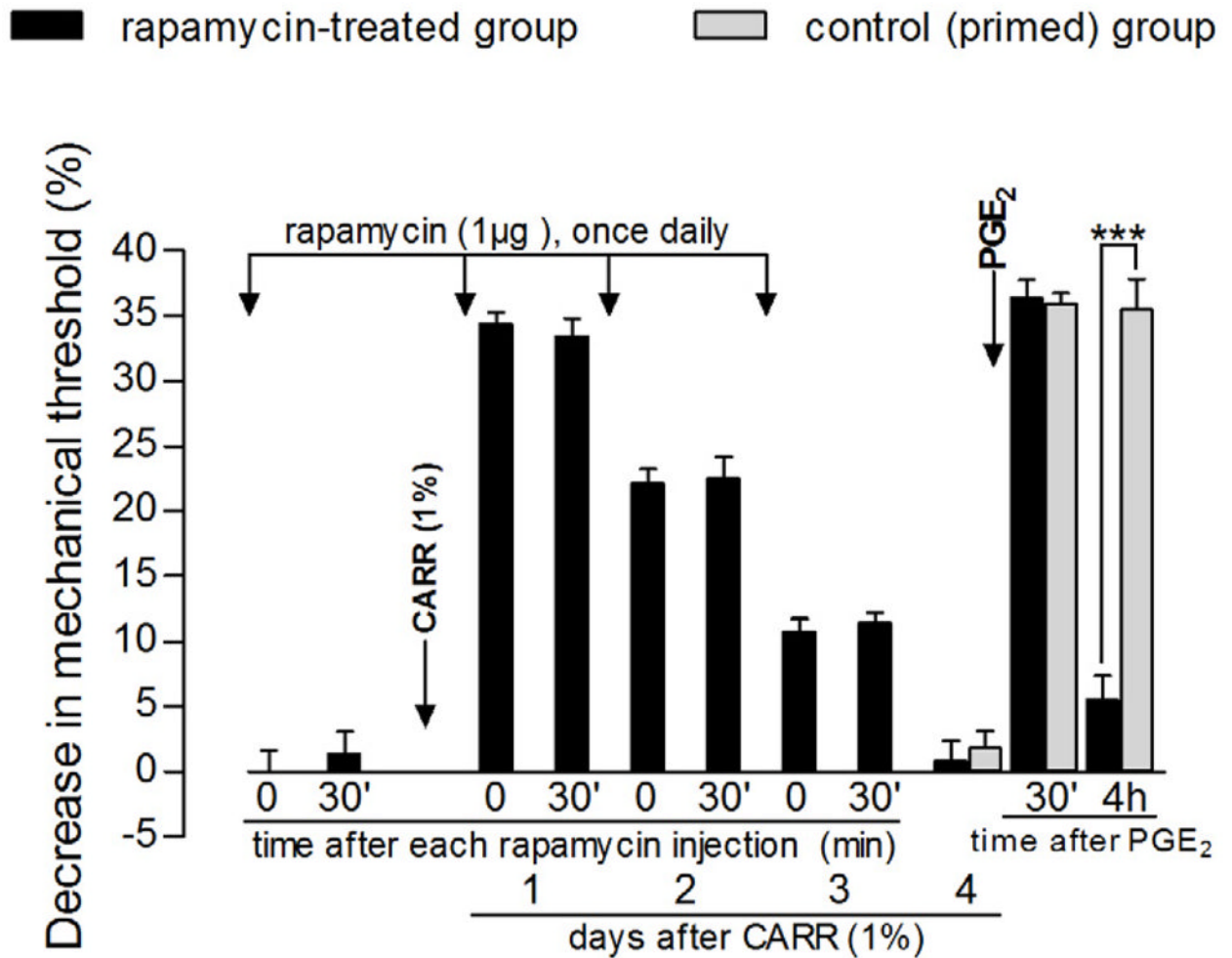


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### Perspectives

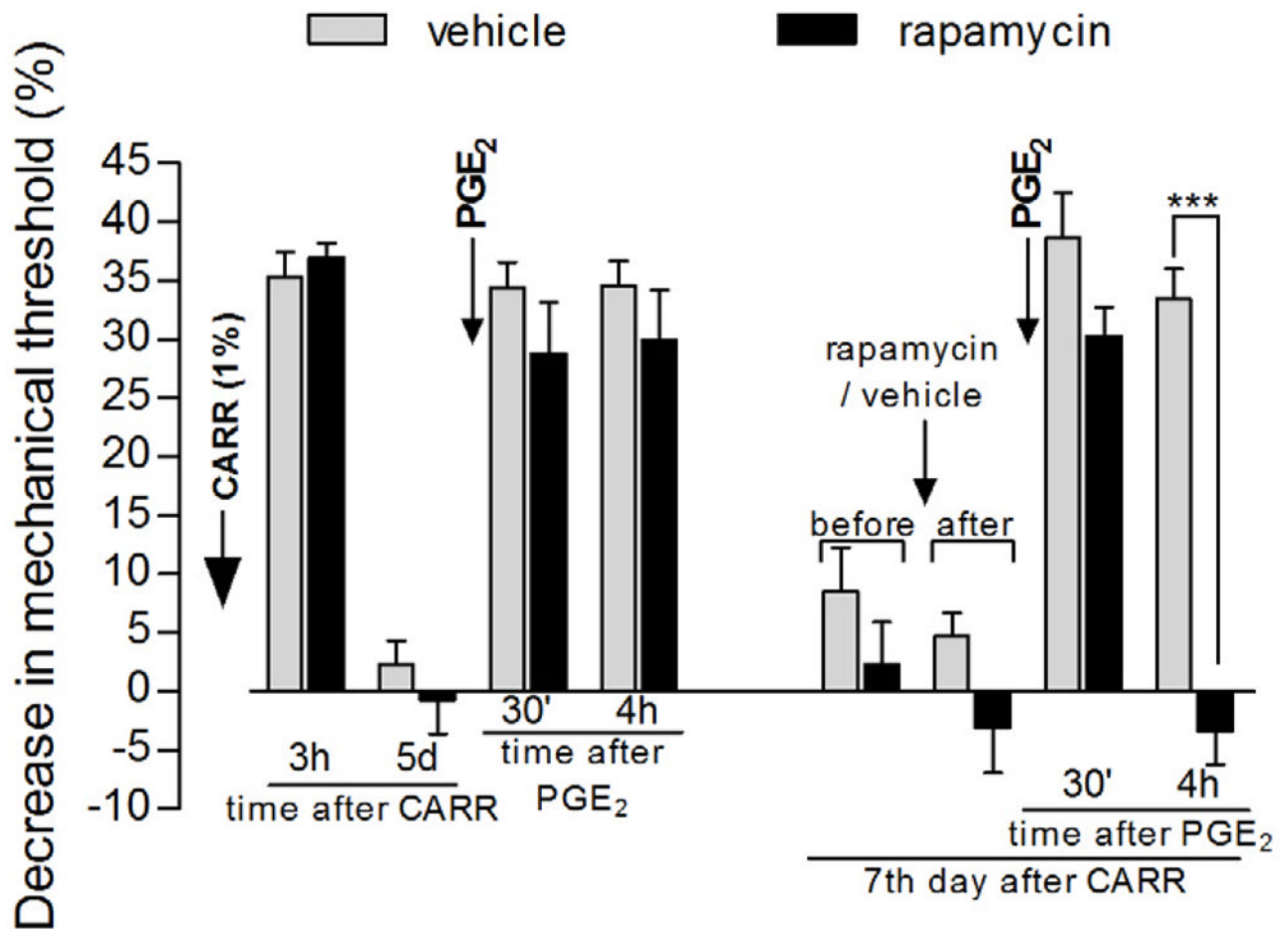
This study provides evidence that ongoing protein translation in the sensory neuron terminals is involved in pain chronification and local treatment that transiently interrupts this translation may be a useful therapy to chronic pain.



**Figure 1. Prevention of carrageenan-induced hyperalgesic priming by local injection of the mTOR inhibitor rapamycin**

Rats received intradermal injection of rapamycin (1 μg) on the dorsum of the hind paw. Evaluation of the mechanical nociceptive threshold, by the Randall-Selitto paw-withdrawal test, before and 30 min after injection, showed no effect of rapamycin by itself [average paw withdrawal thresholds before and 30 min after rapamycin injection were  $113.3 \pm 1.7$  g and  $111.6 \pm 1.5$  g, respectively (paired Student's *t*-test showed no significant difference between these values ( $t_5=0.8811$ ,  $p=0.4186$ ,  $N=6$ )). Carrageenan (CARR, 1%) was then injected at the same site and the mechanical threshold was evaluated daily, until the return to the baseline values (~4<sup>th</sup> day) [no significant difference on the mechanical nociceptive thresholds was observed immediately before and 4 days after injection of CARR ( $t_5=0.2911$ ,  $p=0.7827$ , paired Student's *t*-test)]. Daily injections of rapamycin (1 μg) were performed and readings taken before and 30 min after each injection showed no effect of rapamycin on the hyperalgesia induced by CARR (day 1 after CARR:  $t_5=0.5649$ ,  $p=0.5965$ ; day 2 after CARR:  $t_5=1.976$ ,  $p=0.1051$ ; day 3 after CARR:  $t_5=1.074$ ,  $p=0.3318$ , paired Student's *t*-test for each day). On the 4<sup>th</sup> day after CARR, test for priming was performed: PGE<sub>2</sub> (100 ng) was injected at the same site as CARR and rapamycin and the mechanical thresholds were evaluated 30 min and 4 h later. Two-way repeated measures ANOVA followed by Bonferroni post-test showed significant mechanical hyperalgesia 30 min after PGE<sub>2</sub> injection ( $p<0.001$ ), with decrease on the 4<sup>th</sup> h ( $p>0.05$ , when compared to thresholds before

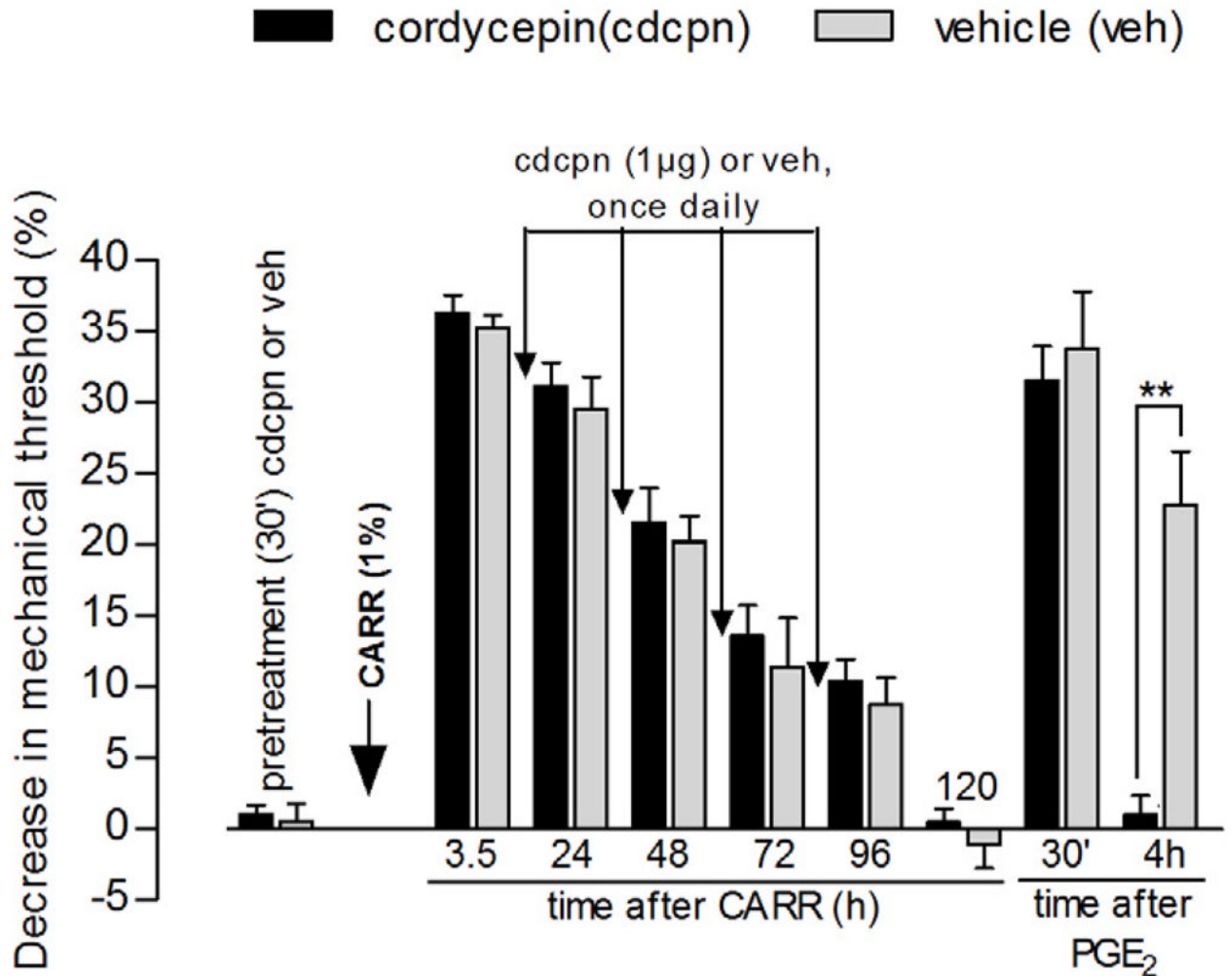
PGE<sub>2</sub>), indicating an inhibitory effect of rapamycin on the prolongation of PGE<sub>2</sub> hyperalgesia (black bars,  $F_{2,10}=196.98$ ,  $p<0.0001$ ). Of note, in a control group of rats that received CARR 4 days before (gray bars), the PGE<sub>2</sub>-induced hyperalgesia was still present at that time point [two-way repeated measures ANOVA followed by Bonferroni post-test showed significant difference between both groups ( $F_{2,30}=55.58$ ,  $p<0.0001$ ), with decrease in the mechanical hyperalgesia at the 4 h after PGE<sub>2</sub> in the rapamycin-treated group, when compared to the control group (\*\* $p<0.001$ ); N=6 paws].



**Figure 2. Reversal of carrageenan-induced hyperalgesic priming by local injection of the mTOR inhibitor rapamycin**

Different groups of rats received intradermal injection of carrageenan (CARR, 1%) on the dorsum of the hind paw. Mechanical nociceptive threshold evaluation, by the Randall-Selitto paw-withdrawal test, showed significant hyperalgesia 3 h after injection ( $p < 0.0001$ , for both groups when compared to baseline thresholds, paired Student's  $t$ -test,  $N = 6$  paws per group) and return to baseline values on the 5<sup>th</sup> day post CARR injection [average paw withdrawal thresholds before and on the 5<sup>th</sup> day after CARR injection:  $115.0 \pm 2.2$  g and  $116.6 \pm 2.1$  g, respectively, for the vehicle-treated group ( $t_5 = 0.4152$ ,  $p = 0.6952$ , gray bars), and  $108.3 \pm 3.0$  g and  $109.3 \pm 2.8$  g, respectively, for the rapamycin-treated group ( $t_5 = 0.3437$ ,  $p = 0.7451$ , black bars) (paired Student's  $t$ -test showed no significant difference between these values). Also, there was no difference between both groups 5 days after CARR injection ( $t_{10} = 2.086$ ,  $p = 0.0635$ , unpaired Student's  $t$ -test)]. Injection of PGE<sub>2</sub> at the same site as CARR induced hyperalgesia that was still present after 4 h, indicating the presence of hyperalgesic priming. Two days later (7 days after CARR injection), vehicle (gray bars) or the mTOR inhibitor rapamycin (black bars) was injected at the same site. No significant change in the mechanical nociceptive threshold was observed 30 min after rapamycin injection ( $t_5 = 1.126$ ,  $p = 0.3111$ , paired Student's  $t$ -test). However, when tested for priming with PGE<sub>2</sub>, no hyperalgesia was observed 4 h later in the rats treated with rapamycin when compared to the rats treated with vehicle (\*\* $p < 0.001$ , two-way repeated measures ANOVA followed by Bonferroni post-test), showing inhibition of CARR-induced priming by local injection of rapamycin ( $N = 6$  paws per group).

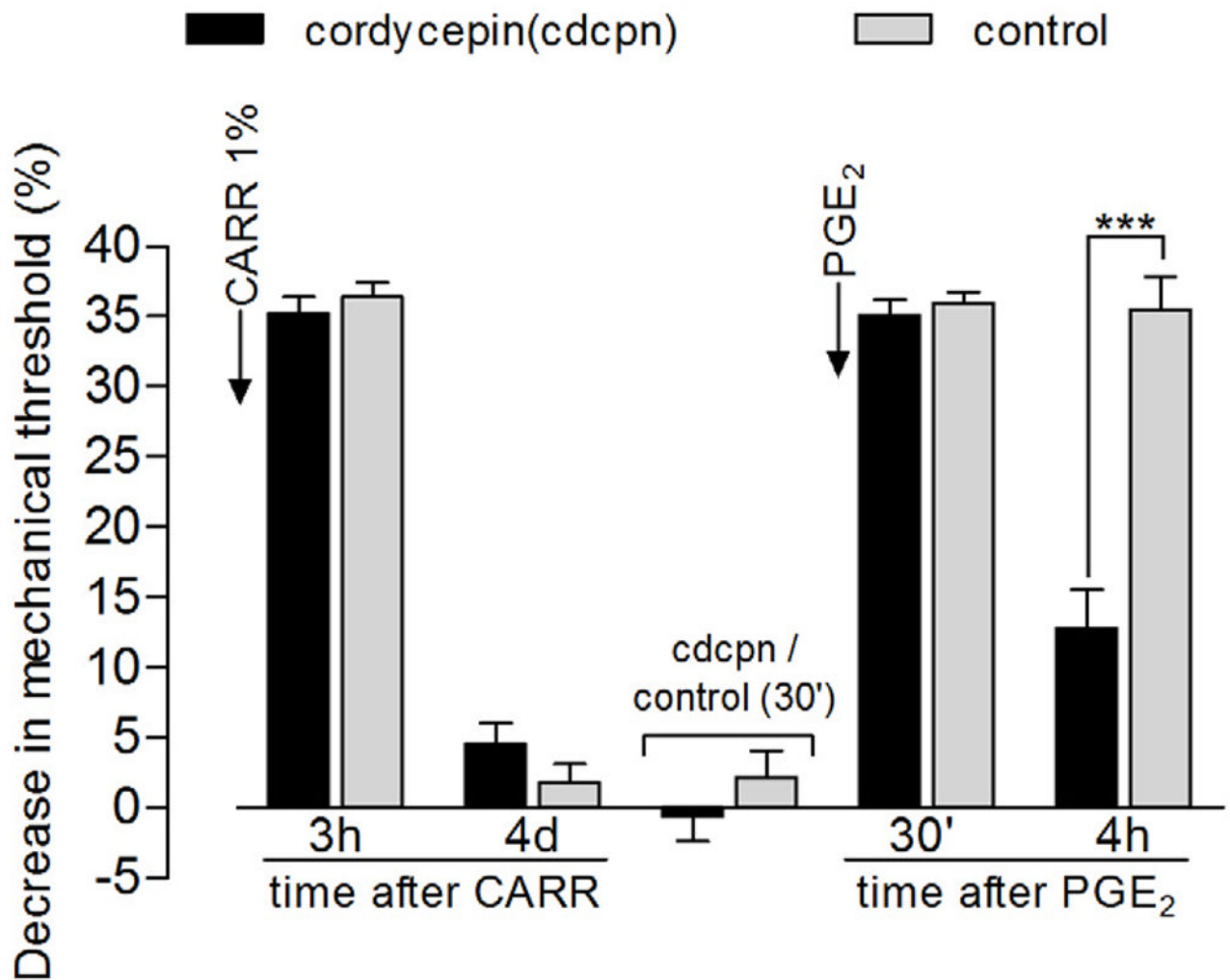




**Figure 3. Prevention of carrageenan-induced hyperalgesic priming by local injection of the protein translator inhibitor cordycepin**

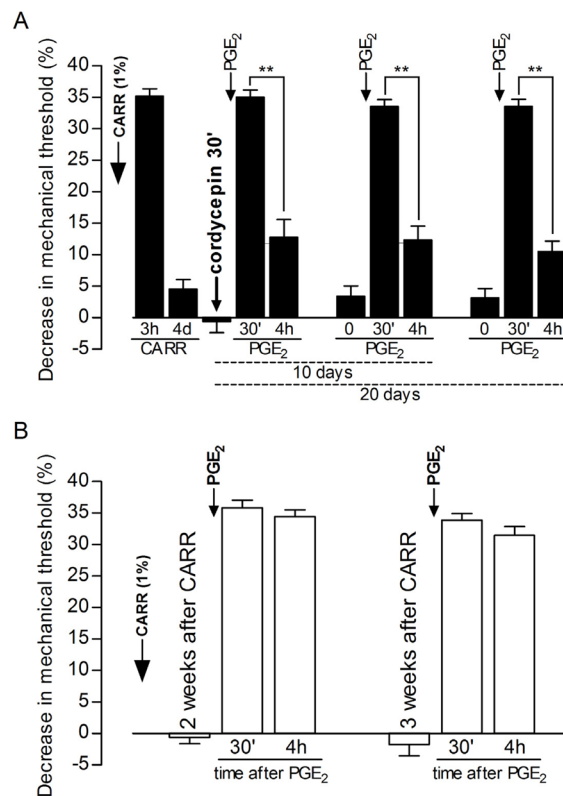
Different groups of rats received intradermal injection of vehicle (gray bars) or cordycepin (1 μg, black bars) on the dorsum of the hind paw. Evaluation of the mechanical nociceptive thresholds, by the Randall-Selitto paw-withdrawal test, before and 30 min after injection, showed no effect of cordycepin by itself [average paw withdrawal thresholds before and 30 min after injection:  $106.6 \pm 1.2$  g and  $105.6 \pm 1.6$  g, respectively, for the cordycepin group, and  $106.6 \pm 1.2$  g and  $106.0 \pm 1.8$  g, respectively, for the vehicle group (paired Student's *t*-test for each group showed no significant difference between these values); also, unpaired Student's *t*-test showed no difference between the groups after injections ( $t_{10}=0.1334$ ,  $p=0.8965$ )]. Carrageenan (CARR, 1%) was then injected at the same site and the mechanical thresholds were evaluated daily, until the return to the baseline values (~5<sup>th</sup> day) [no significant difference on the mechanical nociceptive threshold, for each group, was observed immediately before and 5 days after injection of carrageenan (cordycepin group:  $t_5=0.3071$ ,  $p=0.7711$ ; vehicle group:  $t_5=0.9325$ ,  $p=0.3939$ , paired Student's *t*-test)]. Daily injections of cordycepin (1 μg) or vehicle were performed; readings taken 30 min after each injection showed no difference in the hyperalgesia between both groups ( $F_{1,60}=0.67$ ,  $p=0.4321$ , two-way repeated measures ANOVA followed by Bonferroni post-test), i.e., no effect of cordycepin on the hyperalgesia induced by carrageenan. On the 5<sup>th</sup> day after carrageenan,

test for priming was performed: PGE<sub>2</sub> (100 ng) was injected at the same site as carrageenan and vehicle/cordycepin and the mechanical thresholds were evaluated 30 min and 4 h later. Two-way repeated measures ANOVA showed no hyperalgesia in the cordycepin-treated group, when compared to the vehicle-treated group, 4 h after PGE<sub>2</sub> injection ( $F_{2,20}=23.51$ , \*\* $p<0.0001$ , N=6 paws per group).



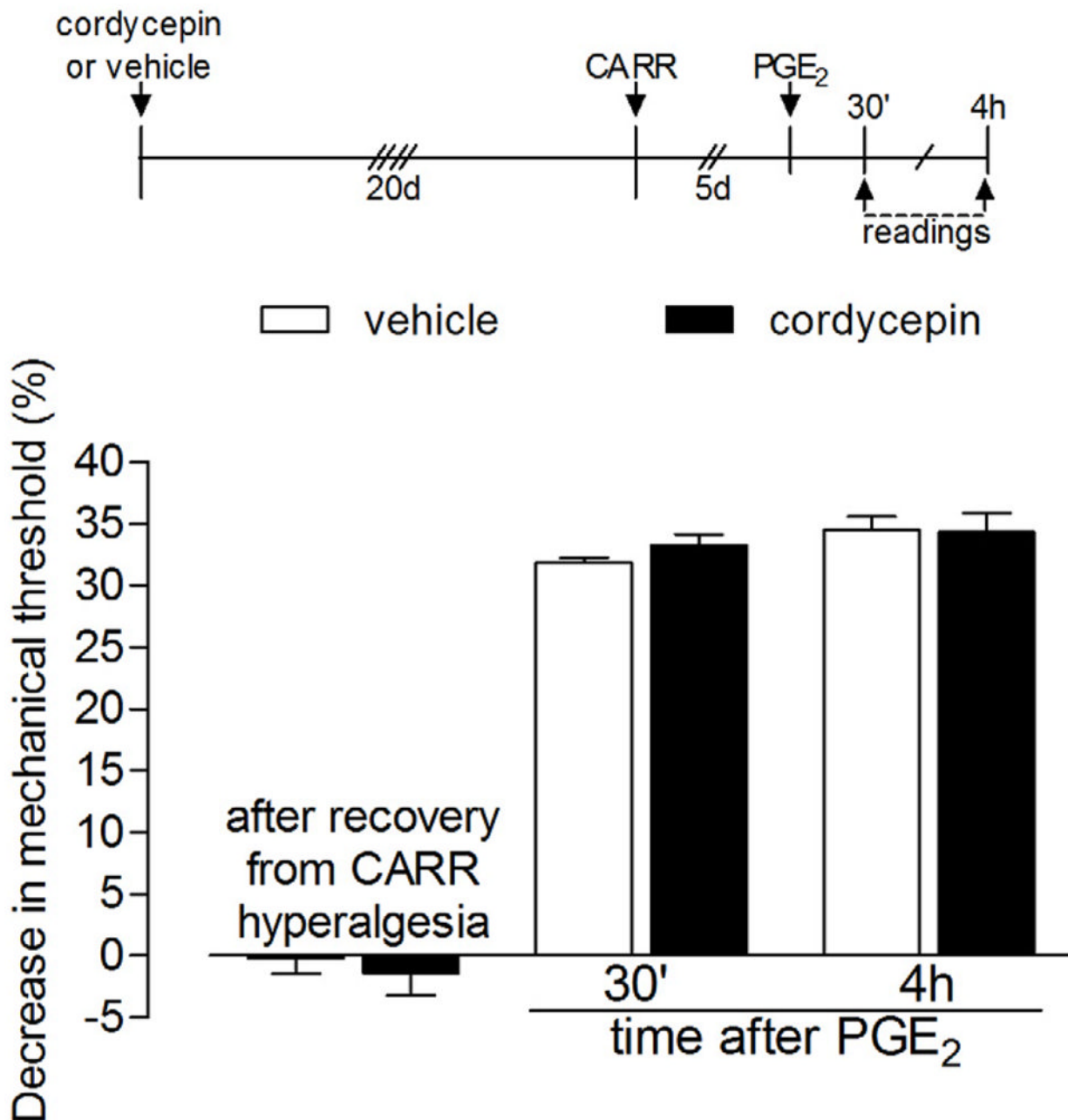
**Figure 4. Reversal of carrageenan-induced hyperalgesic priming by local injection of the protein translator inhibitor cordycepin**

Different groups of rats received intradermal injection of carrageenan (CARR, 1%) on the dorsum of the hind paw. Mechanical nociceptive thresholds evaluation, by the Randall-Selitto paw-withdrawal test, showed significant hyperalgesia 3 h after injection and return to baseline values on the 4<sup>th</sup> day post CARR injection [average paw withdrawal thresholds before and 4 d after injection - cordycepin group:  $116.8 \pm 1.5$  g and  $113.3 \pm 1.1$  g, respectively; control group:  $120.0 \pm 2.0$  g and  $117.6 \pm 1.7$  g, respectively (paired Student's *t*-test showed no significant difference between these values for each group: cordycepin:  $t_{11}=2.116$ ,  $p=0.0579$ ,  $N=12$  paws; control:  $t_5=1.400$ ,  $p=0.2204$ ,  $N=6$  paws); unpaired Student's *t*-test showed no difference between the groups on the 4<sup>th</sup> day after carrageenan injection ( $t_{16}=2.079$ ,  $p=0.0540$ )]. No significant difference in the mechanical threshold was observed 30 min after injection of cordycepin, when compared to the control group either (unpaired Student's *t*-test,  $t_{16}=1.038$ ,  $p=0.3148$ ). Injection of PGE<sub>2</sub> at the same site as carrageenan and cordycepin/vehicle induced significant hyperalgesia, evaluated 30 min and 4 h after injection (two-way repeated measures ANOVA followed by Bonferroni post-test,  $F_{2,32}=205.62$ ,  $p<0.0001$ ). However, at the 4<sup>th</sup> h post-PGE<sub>2</sub>, the magnitude of the hyperalgesia was significantly decreased in the cordycepin-treated group when compared to the control group (\*\*\*) ( $p<0.001$ ), indicating an effect of cordycepin on hyperalgesic priming.



**Figure 5. Duration of the effect of the protein translator inhibitor cordycepin on the carrageenan-induced hyperalgesic priming**

**Panel A:** Rats that received intradermal injection of carrageenan (CARR, 1%) on the dorsum of the hind paw were treated with cordycepin (1 μg) on the 4<sup>th</sup> day (after recovery from CARR-induced hyperalgesia). PGE<sub>2</sub> was injected 30 min after cordycepin and the mechanical nociceptive thresholds were evaluated, by the Randall-Selitto paw-withdrawal test, 30 min and 4 h later. Paired Student's *t*-test showed significant decrease in the PGE<sub>2</sub>-induced hyperalgesia on the 4<sup>th</sup> h after injection, indicating inhibition of hyperalgesic priming by cordycepin ( $t_{11}=8.748$ ,  $**p<0.0001$  when the magnitude of the hyperalgesia on 30 min and 4 h are compared,  $N=12$  paws). When PGE<sub>2</sub> was injected, again, 10 and 20 days later, at the same site where carrageenan/cordycepin were previously administered, it failed to induce prolonged hyperalgesia, evaluated at 4 h post injection [Paired Student's *t*-test comparing the magnitude of the hyperalgesia on 30 min and 4 h:  $t_{11}=8.799$ ,  $**p<0.0001$  (10 days after cordycepin injection),  $t_{11}=10.05$ ,  $**p<0.0001$  (20 days after cordycepin injection)] indicating the long-term reversal of the hyperalgesic priming by cordycepin ( $N=12$  paws); **Panel B: Hyperalgesic priming is still present 3 weeks after carrageenan injection:** rats that received carrageenan (CARR, 1%) on the dorsum of the hind paws were tested with PGE<sub>2</sub> (100 ng) 2 and 3 weeks afterwards. Readings were taken 30 min and 4 h after PGE<sub>2</sub> and, as expected, the magnitude of the hyperalgesia was still high at the 4<sup>th</sup> h, indicating the presence of hyperalgesic priming, induced by previous injection of CARR. This observation is important in order to evaluate the effect of cordycepin seen on Panel A [Paired Student's *t*-test comparing the magnitude of the hyperalgesia on 30 min and 4 h:  $t_5=1.101$ ,  $p=0.3211$  (2 weeks after CARR injection),  $t_5=1.849$ ,  $p=0.1237$  (3 weeks after CARR injection)] ( $N=6$  paws).

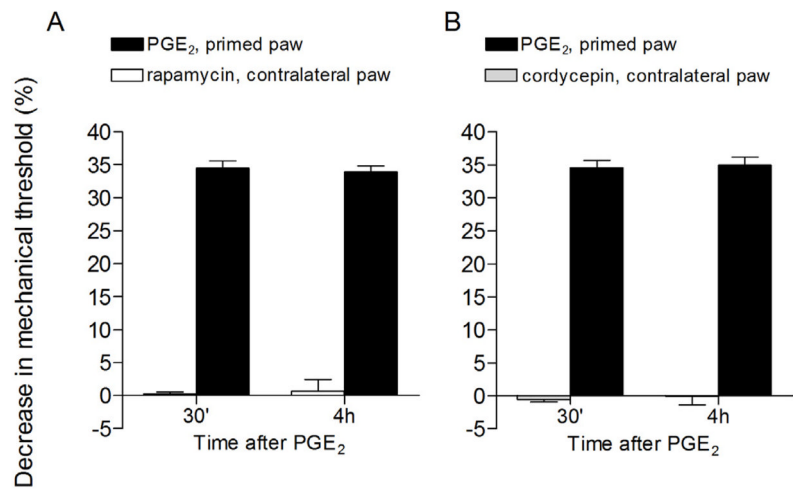


**Figure 6. Duration of the effect of the protein translator inhibitor cordycepin**

Rats received intradermal injection of vehicle (white bars) or cordycepin (1  $\mu$ g, black bars) on the dorsum of the hind paws 20 days before injection of carrageenan (CARR, 1%) at the same site. There was no difference in the carrageenan-induced hyperalgesia, evaluated by the Randall-Selitto paw-withdrawal test, between both groups (data not shown). 5 days later, when the mechanical thresholds were back to pre-carrageenan values (vehicle group:  $t_5=1.464$ ,  $p=0.2031$ ; cordycepin group:  $t_5=0.4152$ ,  $p=0.6952$ , paired Student's  $t$ -test), PGE<sub>2</sub> was injected at the same site and the mechanical thresholds were evaluated 30 min and 4 h later. Two-way repeated measures ANOVA followed by Bonferroni post-test showed no significant difference in the PGE<sub>2</sub>-induced hyperalgesia, both 30 min and 4 h after injection, between the vehicle- and the cordycepin-treated groups ( $F_{1,20}=0.01$ ,  $p=0.9067$ ), indicating

that, at that time point (20 days after injection), the effect of cordycepin was no longer present (N=6 paws per group).





**Figure 7. Local effect of the protein translation inhibitors rapamycin and cordycepin**  
Rats that had their right hind paw primed with carrageenan (1%) 4 days before received intradermal injection of the protein translation inhibitors rapamycin (1  $\mu$ g, **panel A**, white bars) or cordycepin (1  $\mu$ g, **panel B**, gray bars) on the dorsum of the contralateral paw. 30 min after injecting the inhibitors, PGE<sub>2</sub> was injected in the right hind paw and the mechanical nociceptive threshold was evaluated, by the Randall-Selitto paw-withdrawal test, 30 min and 4 h later (black bars, both panels). Paired Student's *t*-test showed no change in the magnitude of PGE<sub>2</sub>-induced hyperalgesia at 30 min and 4 h (panel A:  $t_5=0.5423$ ,  $p=0.6109$ ; panel B:  $t_5=0.7006$ ,  $p=0.5148$ ), indicating lack of effect of rapamycin or cordycepin injected in the contralateral paw on the hyperalgesic priming in the right paws (N=6 paws per group).