Neurobiology of Pain 5 (2019) 100026

Contents lists available at ScienceDirect

Neurobiology of Pain

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

AMPK activation regulates P-body dynamics in mouse sensory neurons *in vitro* and *in vivo*

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ARTICLE INFO

Keywords:
Processing bodies
AMPK
Metformin
Spared-nerve injury
Translation control

ABSTRACT

Increased mRNA translation in sensory neurons following peripheral nerve injury contributes to the induction and maintenance of chronic neuropathic pain. Metformin, a common anti-diabetic drug and an activator of AMP-activated protein kinase (AMPK), inhibits cap-dependent mRNA translation and reverses mechanical hypersensitivity caused by a neuropathic injury in both mice and rats. P-bodies are RNA granules that comprise sites for metabolizing mRNA through the process of de-capping followed by RNA decay. These RNA granules may also sequester mRNAs for storage. We have previously demonstrated that induction of cap-dependent translation in cultured trigeminal ganglion (TG) neurons decreases P-body formation and AMPK activators increase P-body formation. Here we examined the impact of AMPK activation on protein synthesis and P-body formation *in vitro* and *in vivo* on mouse dorsal root ganglion (DRG) neurons. We demonstrate that AMPK activators inhibit nascent protein synthesis and increase P-body formation in DRG neurons. We also demonstrate that mice with a spared-nerve injury (SNI) show decreased P-bodies in the DRG, consistent with increased mRNA translation resulting from injury. Metformin treatment normalizes this effect in SNI mice and increases P-body formation in sham animals. These findings indicate that P-bodies are dynamically regulated by nerve injury *in vivo* and this effect can be regulated via AMPK activation.

Introduction

Neuropathic pain is a debilitating disease for which there are few effective treatments (Finnerup et al., 2015; Price et al., 2018). Injury to the peripheral nervous system causes altered gene expression that is partially driven by increased transportation of mRNAs to distal sites in neurons as well as enhanced mRNA translation (Zheng et al., 2001; Yudin et al., 2008; Melemedjian et al., 2011; Terenzio et al., 2018). This increased mRNA translation is part of a regeneration response (Terenzio et al., 2018) but there is strong evidence that it also increases the excitability of nociceptors causing neuropathic pain (Melemedjian et al., 2011; Khoutorsky and Price, 2018). Signaling pathways that increase mRNA translation in DRG neurons after injury include the mechanistic target of rapamycin complex 1 (mTORC1) (Jimenez-Diaz et al., 2008; Geranton et al., 2009; Megat et al., 2018) and the mitogen activated protein kinase interacting kinase (MNK) – eukaryotic initiation factor (eIF) 4E pathways (Moy et al., 2017). Importantly, both of these signaling pathways can be inhibited by activation of the AMPK pathway. This occurs via multiple phosphorylation events that impinge on mTORC1 and MNK-eIF4E signaling (Hardie, 2014). Previous work from our group and others has demonstrated that activation of AMPK can reverse mechanical allodynia in many pain models (Melemedjian et al., 2011; Tillu et al., 2012; Price and Dussor, 2013; Russe et al., 2013; Ma et al., 2015; Burton et al., 2017) and cognitive deficits caused by neuropathic pain (Shiers et al., 2018). While the precise mechanism of action for anti-neuropathic pain effects of AMPK activators is not known, existing data supports the conclusion that these drugs target AMPK in DRG neurons to decrease mTORC1 and MNK-eIF4E signaling resulting in decreased mRNA translation and a decrease in sensory neuron excitability (Price and Dussor, 2013; Price and Inyang, 2015; Asiedu et al., 2016).

Processing bodies (P-bodies) are RNA granules that comprise sites for metabolizing and/or sequestering mRNA (Decker and Parker, 2012). P-bodies contain high concentrations of RNA decapping enzymes which are frequently used to label these RNA granules in cells (Aizer et al., 2008; Ramaswami et al., 2013). We have previously demonstrated that Interleukin (IL)-6 and Nerve Growth Factor (NGF), both of which induce cap-dependent translation in sensory neurons (Melemedjian et al., 2010), decrease the P-body marker Rck/p54 in DRG neurons. Conversely, metformin increases Rck/p54-containing

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https://doi.org/10.1016/j.ynpai.2018.100026
Received 26 September 2018; Received in revised form 30 November 2018; Accepted 30 November 2018
Available online 01 December 2018

RNA granules in TG neurons in culture (Melemedjian et al., 2014), suggesting that P-body dynamics are regulated by signaling to mRNA translation mechanisms in sensory neurons (Tan, 2014). Here we examined the impact of AMPK activators on protein synthesis and P-body formation on mouse DRG neurons *in vitro* and demonstrate that they inhibit nascent protein synthesis and increase P-body formation, consistent with our previous work on TG neurons (Melemedjian et al., 2014). We also demonstrated that animals with a spared-nerve injury (SNI) have decreased P-body formation in the affected DRG and treatment with metformin reverses this effect. From these findings we conclude that P-bodies in DRG neurons are dynamically regulated by injury and drug treatment and that these RNA granules may be an important site of action for the anti-neuropathic pain effects of drugs that decrease mTORC1 and MNK-eIF4E signaling in sensory neurons, like metformin.

Methods and materials

Animals

All animal procedures were approved by the Institutional Animal Care and Use Committee at the University of Texas at Dallas. For each experiment naïve, 4 week old ICR mice were purchased from Envigo (Indianapolis, IN). Only male mice were used in all experiments. We made this choice because our previous work has not demonstrated any sex differences in translation regulation signaling in pain models (Moy et al., 2017; Megat et al., 2018) with the exception of metformin effects, which occur only in males (Inyang et al., 2018). Therefore, we chose to focus only on males for these studies. Groups of 2–4 animals were housed together on a 12:12 hr light/dark cycle. Food and water were available *ad libitum*. For drug treatment experiments animals were randomly assigned to their experimental groups, with a minimum of one animal per drug treatment in each housing group.

Surgery and metformin administration

SNI was induced in mice as previously described (Decosterd and Woolf, 2000). Twenty-one days following nerve injury mice were given IP injections of 200 mg/kg metformin (Melemedjian et al., 2011; Shiers et al., 2018) or vehicle every day for 4 days. A separate group of sham surgery animals were given metformin at the same dose for 4 days. The average weight of the animals was 40 g at time of treatment. One hour after the final injection of metformin or vehicle, L3 and L4 DRGs were dissected and immediately flash frozen on dry ice in optimum cutting temperature (OCT) gel and stored at −20 C. We used these DRGs because they are the ones that are injured in the SNI model in mice (Laedermann et al., 2014). The DRGs were cut using a cryostat into 40 µm thick sections and mounted on charged slides. Slides were then stored at -20 C.

Neuronal culture

Dorsal root ganglia (DRG) were extracted aseptically from 4-week old male ICR (CD-1) mice (Harlan/Envigo) and placed in Hank's Buffered Salt Solution (HBSS, Invitrogen) on ice. The ganglia were dissociated enzymatically at 37 C; first with collagenase A (1 mg/ml, Roche) for 25 min, then collagenase D (1 mg/ml, Roche) that included papain (30 ug/ml, Roche) for 20 min. Afterwards, a trypsin inhibitor (1 mg/ml, Roche) that contained bovine serum albumin (BSA, Fisher, 1 mg/ml) was used to homogenize the ganglia. The tissue was then filtered through 70 µm nylon filters (Falcon) and re-suspended in DMEM F-12 GlutaMax media (Invitrogen) that contained 10% fetal bovine serum (FBS, Hyclone) and 1x penicillin/streptomycin. The media also contained nerve growth factor (NGF, 10 ng/ml, Millipore) and cytosine arabinoside (Ara-C, 2.4ug/ml, Sigma). Neurons were cultured for seven days on 12 mm glass coverslips (#1 thickness, Chemglass) in a 24-well tissue culture plate (Falcon) at 37 C with 95% air and 5% CO2. On day 4, Ara-c was removed from the media and excluded for the remaining 3 days. On the day of the experiment, drugs were diluted into DMEM F-12 GlutaMax media and added directly into the neurons without any wash.

Table 1
Key Reagents List.

Reagent or resource	Concentration used	Source	Identifier
Antibodies			
anti-βIII – Tubilin Rabbit	1:1000	Cell Signaling Technology	5568
Anti-Puromycin Mouse	1:5000	Millipore	MABE343
Anti-Rck/p54/p54 Rabbit	1:2000	Cell Signaling Technology	9407
Goat Anti Mouse 546	1:1000	ThermoFisher Scientific	A-11030
Goat Anti Rabbit 488	1:1000	Life Technologies	A11034
Drugs			
A769662	200 µM	Tocris	3336
AICAR	2 mM	Cayman Chemical	10010241
Cyclohexamide	350 µM	Tocris	0970
HHT	50 µM	Tocris	1416
Metformin	200 mg/kg	LKT Labs	1115-70-4
Reagents			
Puromycin	1 µM	Alomone Labs	P-540
Digitonin	0.00036%	Sigma-Aldrich	D141

Drugs

Cyclohexamide (350 µM, Cat No. 0970), HHT (50 µM, Cat No. 1416), and A769662 (200 µM, Cat No. 3336) were all obtained from Tocris (Minneapolis, MN). AICAR (2 mM, Cat No. 10010241) was obtained from Cayman Chemical Company (Ann Arbor, MI). Metformin (200 mg/kg, intraperitoneal (IP) dose) was obtained from LKT labs. The vehicle for CHX, HHT, AICAR, and A769662 used in culture was sterile 1X PBS. The vehicle for IP metformin dosing was sterile 0.9% saline. For a complete list of reagents and sources see Table 1.

Immunofluorescence

Immediately following drug treatment for *in vitro* experiments or after cutting 40 µm sections for DRG tissue, coverslips or slides, respectively, were placed in ice cold 10% formalin for 1 h then washed three times with 1x PBS for five minutes per wash. They were then moved to a permeabilization solution consisting of 10% Normal Goat Serum (NGS) and 0.1% Triton 100× in 1× PBS for 30 min. After this the slides were washed three times again with 1× PBS for 5 min. The slides were then blocked for 2 h in 10% NGS in 1× PBS. After blocking, the primary antibody was applied overnight. The primary antibodies used were anti-Puromycin (1:5000), anti-βIII-Tubulin (1:1000), and anti-Rck/p54/p54 (1:2000) as described previously (Melemedjian et al., 2014; Barragan-Iglesias et al., 2018). The next day, the slides were washed with 1x PBS three times and the secondary antibody was added. These were goat anti-rabbit alexafluor-488 and goat anti-mouse alexfluor-546 both at a concentration of 1:1000. The slides were left in secondary for 1 h then cover-slipped using Prolong Gold and stored at 4 C until confocal imaging.

Surface sensing of translation (SUnSET) assay

We performed the SUnSET assay on primary DRG neurons, which were cultured for 7 days (Schmidt et al., 2009). This assay measures nascent protein synthesis through incorporation of puromycin into nascent polypeptide chains that can then be detected by an antibody. On the 7th day drugs were added to the cultures for 1 h and then 1 µM

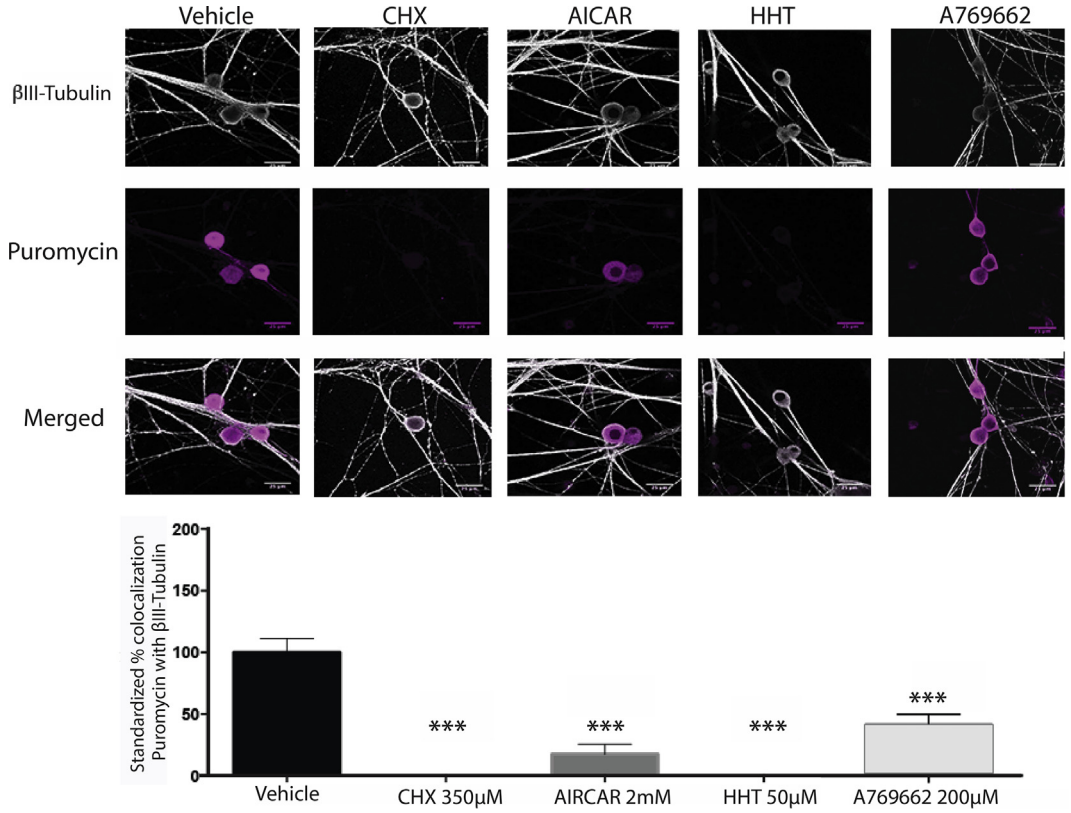

Fig. 1. The SUnSET assay was used to measure nascent protein synthesis in mouse DRG neurons that were treated with cycloheximide (CHX), AICAR, homoharringtonine (HHT), and A769662 for 1 h. Puromycin incorporation into proteins was measured with an anti-puromycin antibody and assessed in βIII tubulin-positive DRG neurons, where CHX, AICAR, and HHT all blocked nascent protein synthesis. Differences between groups were measured using a one-way ANOVA with Bonferroni's post hoc test, $^{***}p < 0.0005$. Three independently generated cultures were made for each experimental condition.

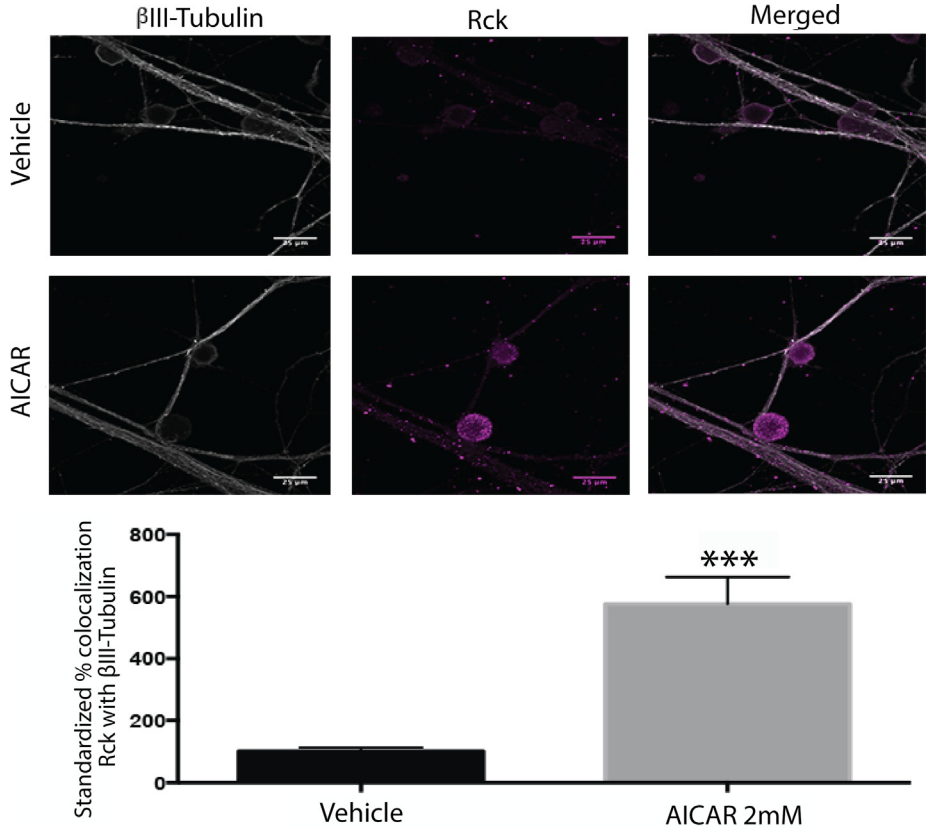

Fig. 2. Mouse DRG neurons were treated with AICAR for 1 h. Following treatment, Rck-positive puncta were co-localized to βIII tubulin, which revealed an increase in P-body formation following AICAR treatment. Differences between groups were measured using an unpaired *t*-test, $^{***}p < 0.0005$. Three independently generated cultures were made for each experimental condition.

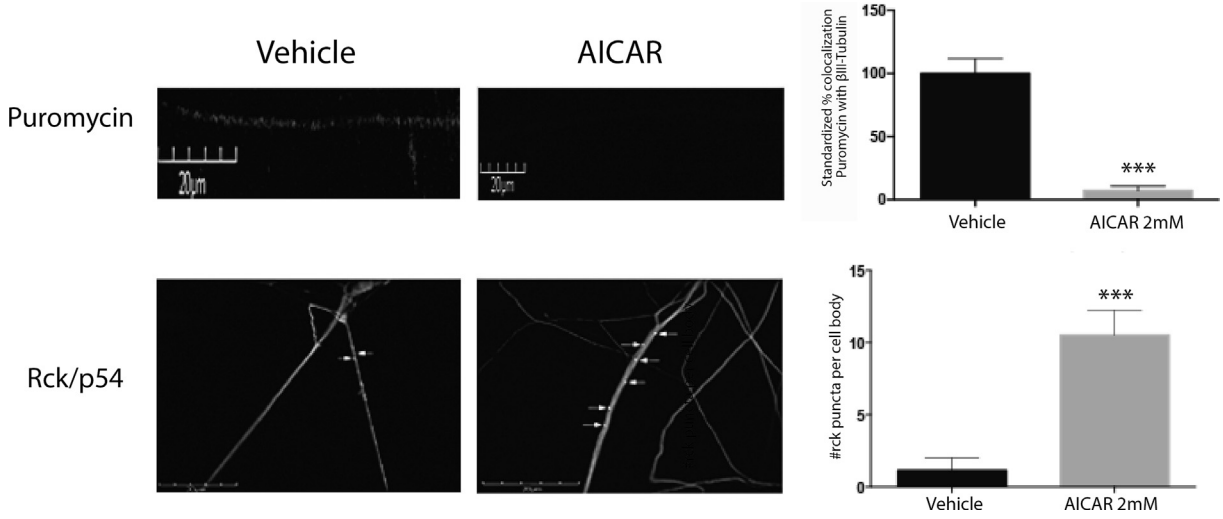

Fig. 3. SUnSET assay was used to measure nascent protein synthesis in axons from mouse DRG neurons that were treated with AICAR for 1 h. A decrease in puromycin incorporation into proteins was measured with a puromycin antibody and assessed in βIII tubulin-positive DRG neurons. An increase of Rck-positive puncta was found in axons from mouse DRG neurons that were treated with AICAR for 1 h. Differences between groups were measured using an unpaired *t*-test, $^{***}p < 0.0005$. Three independently generated cultures were made for each experimental condition.

puromycin was added and allowed to incubate for an additional 15 min. Following this incubation neurons were washed in HBSS containing 0.00036% digitonin during a single 2-minute wash. Immunocytochemistry was performed as described above, where neurons were stained for anti-puromycin and anti-βIII-tubulin antibodies followed by the secondary antibodies.

Microscopy and image analysis

Images were taken on an Olympus Fluoview FV1200 laser scanning confocal microscope and analyzed using the colocalization tool within Olympus' FV software. The intensity of each channel was adjusted so that only areas that contained a strong signal of both 488 nm and 568 nm were visible. This adjusted imaged containes distinct puncta that could then be counted and analyzed with ImageJ software (NIH). The Just Another Co-localization Plugin (JACoP) for ImageJ was used to analyze puromycin incorporation or Rck/p54-containing puncta with neuronal markers and the Mander's overlap coefficient (M1) was calculated for images obtained as we have done previously (Melemedjian et al., 2010). Scale bar in all images is 20 μm.

Statistics

All statistical analysis was performed using Graphpad Prism version 6. Data are displayed with the mean ± the standard error of the mean (SEM) with a level of significance set at $\alpha < 0.05$.

Results

AMPK activators block nascent protein synthesis in cultured DRG neurons

DRG cultures were treated for 1 h with either vehicle, the general translation inhibitors CHX or HHT, or the AMPK activators AICAR or A769662. A769662 is a direct AMPK activator that binds to the β subunit of the enzyme (Cool et al., 2006) while AICAR is an indirect AMPK activator that is converted in cells to an AMP mimetic molecule (Hardie et al., 2012). Using the SUnSET assay, levels of nascently synthesized proteins were measured. CHX and HHT blocked all new protein synthesis when compared to cultures treated with the vehicle (Fig. 1). The indirect AMPK activator AICAR also significantly decreased nascent protein synthesis when compared to cultures treated with vehicle (Fig. 1). A769662 also significantly inhibited nascent protein synthesis in cultured DRG neurons (Fig. 1)

AICAR increases P-body formation in DRG neuron cell bodies and axons

Our previous work demonstrated that metformin and A769662, as well as the mTORC1 inhibitor rapamycin, increase P-body formation in TG neurons in culture (Melemedjian et al., 2014). Because we have used these compounds before on TG neurons and because the effect of AICAR on nascent protein synthesis was comparatively larger than A769662, we focused on AICAR in these experiments. DRG cultures were treated with AICAR for 1 h. Following this incubation immunocytochemistry was performed to quantify Rck/p54 puncta as a marker of P-bodies (Coller and Parker, 2005; Aizer et al., 2008). Neurons treated with AICAR showed a significant increase in Rck/p54 puncta when compared to cultures treated with vehicle (Fig. 2).

To determine if these changes also occur in axons we focused specifically on imaging DRG neuron axons. We examined nascent protein synthesis and P-body formation in response to AICAR treatment. Levels of axonal nascent protein synthesis were significantly lower in axons treated with AICAR than in those treated with vehicle (Fig. 3). Additionally, Rck/p54 puncta were significantly increased in axons of DRG neurons treated with AICAR (Fig. 3). Therefore, AICAR-mediated activation of AMPK decreases nascent protein synthesis in DRG neuron cell bodies and axons and this is inversely correlated with P-body formation in both of these sub-cellular compartments.

SNI regulates P-body formation in mouse DRG neurons in vivo – effects of metformin treatment

Mice received SNI surgery and 21 days later DRGs on both the ipsilateral and contralateral side to injury were removed. Immunohistochemistry was done to examine levels of Rck/p54 puncta in DRG neurons following injury. In these animals, Rck/p54 staining was significantly reduced on the side ipsilateral to injury when compared to the contralateral side (Fig. 4A). This finding is consistent with previous work demonstrating that nerve injury and neuropathic pain is associated with increased mRNA translation in DRG neurons (Geranton et al., 2009; Melemedjian et al., 2011).

We then aimed to determine the effect metformin on P body dynamics in SNI and sham mice. We used metformin for these experiments because many previous studies have shown positive effects of metformin in chronic pain models (Melemedjian et al., 2013; Russe

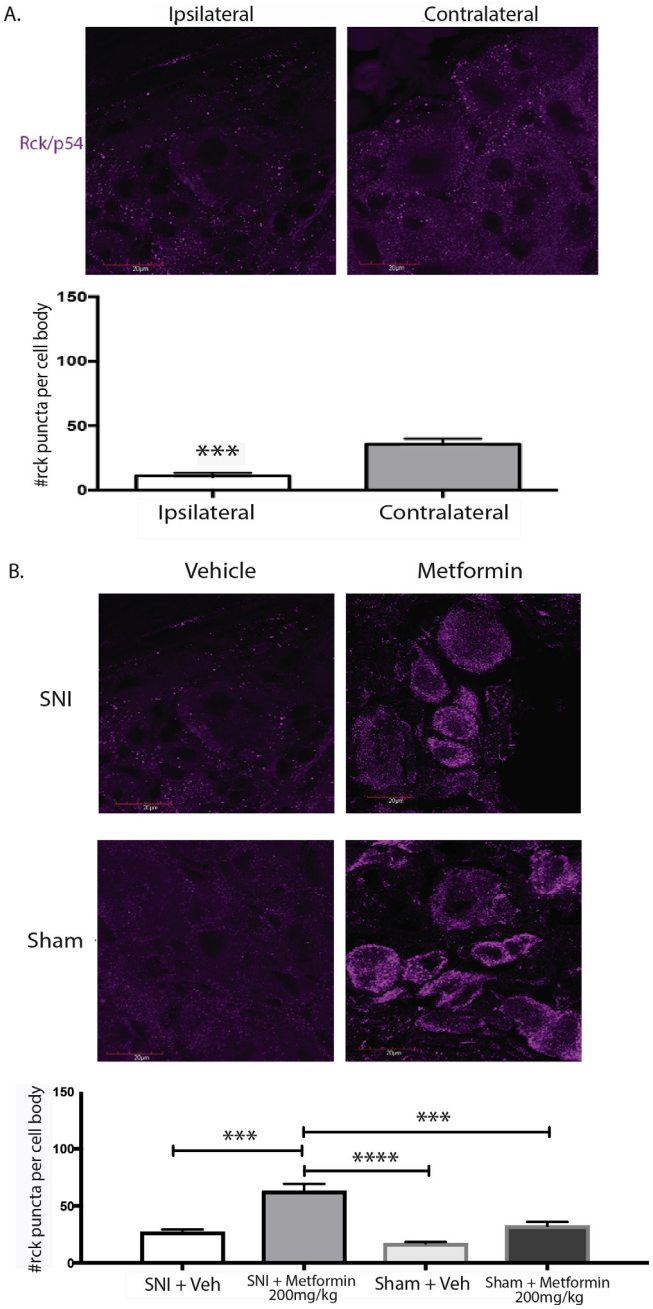

Fig. 4. Rck-positive puncta were visualized and counted in mouse DRG neurons from SNI mice 21 days after injury (A), from SNI mice treated with metformin (200 mg/kg) or vehicle for 4 days (B), or sham mice treated with metformin (200 mg/kg) or vehicle for 4 days (B). A. P body formation was decreased on the ipsilateral side of injury following SNI surgery. B. SNI animals that were treated with metformin for 4 days showed an increase in P body formation compared to animals treated with vehicle. There was an increase in P body formation following 200 mg/kg of metformin treatment in sham mice. Differences between groups were measured using an unpaired *t*-test(A) or a two-way ANOVA with Bonferroni's *post hoc* test, $^{***}p < 0.0005$, $^{****}p < 0.0001$. All experimental groups were comprised of 4 mice.

et al., 2013; Mao-Ying et al., 2014; Alcocer-Gomez et al., 2015; Bullon et al., 2015; Burton et al., 2017; Ge et al., 2018; Shiers et al., 2018). Twenty-one days after surgery, animals were split into two groups, where one group of animals received metformin (200 mg/kg, IP) treatment and the other group received saline vehicle (IP) daily for 4 days. After treatment DRGs were collected, sliced, and stained for Rck/p54. Compared to vehicle treated SNI mice, animals that received metformin had increased levels of Rck/p54 punta (Fig. 4B). Moreover, in sham mice, treatment with metformin did not significantly increase P-body formation in DRG neurons compared to vehicle treated mice (Fig. 4B). These experiments demonstrate that the AMPK activator metformin induces P body formation in both SNI and sham mice in the DRG.

Discussion

Using the SUnSET assay we determined that A769662 and AICAR, two structurally distinct AMPK activators, were both able to block nascent protein synthesis in DRG neurons from mice. AMPK activators also increase Rck/p54 puncta in DRG neurons, an effect which is a marker for P-body formation. From this we are able to conclude that AMPK activation causes a decrease in protein synthesis and an increase in P-body formation and that this effect occurs in DRG neuron cell bodies and axons. Our interpretation of these findings is that AMPK activation induces a sequestration of mRNAs in P-bodies. At the very least this decreases nascent protein synthesis because mRNAs are not available for translation. This is in addition to changes in phosphorylation of translation initiation factors like eIF4E and 4EBP that is also known to occur with AMPK activation. An interesting, yet unanswered question is whether mRNAs that move into P-bodies in DRG or TG neurons are sequestered there but eventually released for translation or if they are decapped and degraded. Gaining insight to this question can be done with recently described experimental approaches that allow for RNA sequencing on specific RNA granules isolated from cells (Hubstenberger et al., 2017). Interestingly, this technology suggests that although P bodies are enriched with decapping enzymes, they are primarily sites of RNA sequestration and translational repression rather than mRNA decay (Hubstenberger et al., 2017).

Importantly, our work suggests an *in vivo* role for P-body formation following metformin treatment in the reversal of established neuropathic pain by this drug (Melemedjian et al., 2011; Melemedjian et al., 2013; Shiers et al., 2018). Peripheral nerve injury in both rats and mice up-regulates activity for factors associated with cap-dependent translation such as mTORC1-4EBP and MNK-eIF4E (Geranton et al., 2009; Melemedjian et al., 2011; Khoutorsky and Price, 2018). In sensory neurons from rats that have undergone spinal nerve ligation (SNL), nascent protein synthesis is increased by 50% and there is enhanced eIF4F complex formation in the DRG and sciatic nerve (Melemedjian et al., 2011). Metformin inhibits this eIF4F complex formation and blocks nascent protein synthesis when applied *in vitro*. Additionally, metformin administered *in vivo* alleviates mechanical allodynia in rats with SNL and mice with SNI (Melemedjian et al., 2011; Melemedjian et al., 2013) and the antinociceptive effects of metformin are likely due to AMPK because they are lost when AMPK is specifically deleted from sensory neurons (Russe et al., 2013). We have recently shown that in addition to reversing mechanical hypersensitivity in mice with SNI, metformin also reverses cognitive deficits induced by neuropathic pain (Shiers et al., 2018) as well as microglial activation in the dorsal horn (Inyang et al., 2018). This suggests a powerful anti-neuropathic pain effect of this drug, in male mice. While the precise impact of metformin-induced P -body formation in DRG neurons was not tested in our experiments, we speculate that increased P-body formation in DRG neurons as a result of metformin treatment is involved in the normalization of translational control pathways. This is consistent with previous observations that nascent protein synthesis is increased in the DRG in neuropathic pain (Melemedjian et al., 2011) and that metformin reverses this effect. It is also consistent with our observation in the present experiments that SNI induces a decrease in P-bodies in DRG neurons. Collectively these results suggest an intricate coordination of RNA trafficking, translation and sequestration in response to nerve injury that is likely critical for neuropathic pain and that can be targeted by specific pharmacological mechanisms such as AMPK activation.

In conclusion, our current work enriches knowledge on the

mechanism by which AMPK activation works to curtail chronic pain by examining the *in vitro* cell biology of P-body dynamics in DRG neuron and the *in vivo* effects of metformin on P-body formation. To our knowledge, these are the first data to demonstrate the positive correlation between AMPK activation and P-body formation *in vivo*. Our work on P-body dynamics in SNI mice is consistent with a previous study suggesting dysregulation of RNA granules in neuropathic pain using an RNA sequencing approach (Hirai et al., 2017). Given the emerging evidence that RNA granule pathobiology is crucial for many neurological disorders (Ramaswami et al., 2013), it is likely that additional therapeutic opportunities will emerge from this field of enquiry.

Conflict of interest statement

The authors declare no conflicts of interest

Acknowledgements

This work was supported by NIH grants R01NS065926 (TJP), R01GM102575 (TJP and GD).

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