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Title Page

Article Type: Original Research

Title: Indirect AMPK activators prevent incision-induced hyperalgesia and block hyperalgesic priming while positive allosteric modulators only block priming in mice.

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Running Title Page

Running Title: AMPK modulation of incisional pain

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Abstract

AMP activated protein kinase (AMPK) is a multifunctional kinase that negatively regulates mechanistic target of rapamycin (mTOR) and mitogen activated protein kinase (MAPK) signaling, two signaling pathways that are linked to pain promotion after injury, such as surgical incision. AMPK can be activated directly using positive allosteric modulators as well as indirectly through the upregulation of upstream kinases such as liver kinase B1 (LKB1) which is a mechanism of action of metformin. Metformin's anti-hyperalgesic effects have been shown to occur only in male mice, raising questions about how metformin regulates pain sensitivity. We used metformin as well as other structurally distinct AMPK activators narciclasine, ZLN-024 and MK8722 to treat incision-induced mechanical hypersensitivity and hyperalgesic priming in male and female mice. We found that metformin was the only AMPK activator to have sex specific effects. We also found that indirect AMPK activators metformin and NCLS were able to reduce mechanical hypersensitivity and block hyperalgesic priming while direct AMPK activators, ZLN-024 and MK8722 only blocked priming. Direct and indirect AMPK activators stimulated AMPK in dorsal root ganglion (DRG) neuron cultures to a similar degree. However, incision decreased phosphorylated AMPK (p-AMPK) in DRG. Because AMPK phosphorylation is required for kinase activity, we interpret our findings as evidence that indirect AMPK activators are more effective for treating pain hypersensitivity after incision because they are able to drive increased p-AMPK through upstream kinases like LKB1. These findings have important implications for the development of AMPK-targeting therapeutics for pain treatment.

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Significance Statement

Non-opioid treatments for post-surgical pain are needed. Our work focused on whether direct or indirect AMPK activators would show greater efficacy for inhibiting incisional pain and also tested for potential sex differences. We conclude that indirect AMPK activators are likely to be more effective as potential therapeutics for post-surgical pain because they inhibit acute pain caused by incision and also prevent long-term neuronal plasticity that is involved in persistent post-surgical pain. Our work points to the natural product, indirect AMPK activator, narciclasine, as an excellent starting point for development of therapeutics.

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Introduction

Chronic pain is a rapidly growing international health issue that contributes substantially to morbidity, mortality, disability, demands on health care systems, and economic burdens for societies (Institute of Medicine Committee on Advancing Pain Research and Education, 2011). New classes of analgesics are needed to combat this growing problem (Finnerup NB, 2015). One of the most common and significant forms of chronic pain is chronic post-surgical pain (Haroutiunian S, 2013) which causes major functional impairment in 5-10% of surgery patients (Kehlet H, 2006). Post-surgical pain medicines that can provide pain relief acutely and also diminish the probability of developing chronic pain would improve health care (Price TJ, 2018). Adenosine monophosphate-activated protein kinase (AMPK) is a cellular fuel-sensing enzyme present in most cells across species (Hardie, 2007). This kinase detects changes in the AMP/ATP ratio to regulate anabolic processes when cellular energy status is low (Kahn BB, 2005). Several studies have shown that AMPK plays a key role in nociceptive sensitization (Song H, 2015; Bullón P, 2016). AMPK activation causes a decrease in mTOR and MAPK signaling leading to many cellular effects including a suppression of protein synthesis mediated by activation of mTOR and MAPK pathways (Hardie, 2007). In animal models of incisional and nerve injury pain, MAPK and mTOR signaling pathways are turned on in dorsal root ganglion (DRG) neurons, including nociceptors, and these signaling events are a causative factor in increased nociceptor excitability and behavioral nociceptive hypersensitivity (Sato and Ohshima, 2000; Banik et al., 2005). Many groups have shown that AMPK activators can be effective in attenuating this cellular hyperexcitability and behavioral hypersensitivity (Tillu et al., 2012; Mejia et al., 2016). We have also shown that

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AMPK activators prevent the development of hyperalgesic priming, a preclinical model mimicking aspects of the transition from acute to chronic pain.

Metformin is a very commonly prescribed drug for type II diabetes that activates AMPK indirectly in a liver kinase B1 (LKB1) dependent fashion (Shaw RJ, 2005). While the exact mechanism of the drug remains an area of active inquiry, metformin is safe and could potentially be repurposed as a pain-relieving drug (Rena et al., 2017; Wu et al., 2018). We have previously shown that metformin is effective in reversing SNI-induced neuropathic pain (Melemedjian OK, 2011) as well as reducing mechanical hypersensitivity following surgical incision without reducing the rate of wound healing (Burton et al., 2017). Interestingly, the anti-hyperalgesic effects of metformin seem to occur specifically in male mice (Inyang et al., 2018).

Various other AMPK activators have emerged in recent years and could be better potential analgesics than metformin (Cao et al., 2018; Qin et al., 2018). It is also unknown whether these drugs could show sex-specific effects. Narciclasine is a natural compound from the plant *Amaryllidaceae* (Dumont et al., 2007) and indirectly activates AMPK (Julien SG, 2017) through a mechanism that may involve cAMP (Zhang et al., 2009). Narciclasine and related compounds are also known to have anti-inflammatory effects (Yui et al., 2001; Lubahn et al., 2012). While indirect AMPK activators stimulate upstream kinases that phosphorylate AMPK, another way to activate AMPK is via direct positive allosteric mechanisms. Several positive allosteric modulators of AMPK, including ZLN024 (Zhang et al., 2013) and MK8722 (Feng et al., 2018; Weihrauch and Handschin, 2018), have recently been described and have favorable pharmacokinetics for *in vivo* dosing.

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The goal of the experiments described here was to establish whether the sex-specificity seen with metformin treatment following spared nerve injury (SNI) was also found in the incisional pain model. We also sought to determine whether other AMPK activators can be effective in alleviating incision-induced nociceptive hypersensitivity and hyperalgesic priming in male and/or female mice. Our experiments demonstrate that metformin is effective in reducing hypersensitivity following incision as well as hyperalgesic priming in male but not female mice. On the other hand, narciclasine reduced acute pain caused by incision and blocked development of hyperalgesic priming in male and female mice. Direct AMPK allosteric modulators ZLN024 and MK8722 both only attenuated hyperalgesic priming but were effective in both sexes. We conclude that indirect AMPK activators have the best potential for future development as pain therapeutics.

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Materials and Methods

Laboratory Animals

Animal procedures were approved by The University of Texas at Dallas Institutional Animal Care and Use Committee and were in accordance with National Institutes of Health Guidelines. All the experiments were performed on male or female ICR outbred mice obtained from Envigo at 4 weeks of age or bred at University of Texas at Dallas. Mice were housed in the University of Texas at Dallas Animal Care Facility for at least one week prior to the start of behavior testing and surgery. Animals had *ad libitum* access to food and water and were on a 12 hr non-inverted light/dark cycle. Experimenters were blinded to treatment groups in behavioral experiments. Mice were randomized to treatment groups using a random number generator and in such a manner that multiple treatment groups were always found within any individual cage of animals. Male and female mice were housed separately in groups of 4 per cage.

Behavioral Testing

The plantar incision model was used to induce post-surgical pain in mice as described previously (Banik RK, 2006). Mechanical sensitivity was assessed using stimulation of the hindpaw of the mouse with calibrated von Frey filaments from Stoelting. We used 0.6, 1.0 and 1.4-gram filaments and measured the response frequency to 10 consecutive stimulations of the hindpaw with each filament with stimulations spaced by at least 5 sec following 45 minutes of habituation to the testing boxes. The number of responses for each filament force was recorded. Following baseline testing, mice were treated systemically with an AMPK activator for 7 consecutive days. Immediately following day 3

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of treatment, plantar incision surgery was performed by making a 5 mm longitudinal incision with a number 11 scalpel blade in the skin of the left hindpaw and the underlying muscle tissue 2 mm below the heel. The wound was closed using a 5-mm silk suture followed by a 200 µL subcutaneous injection of gentamicin (Sigma-Aldrich, 5 mg/mL). For the sham surgery, mice were put under isoflurane for 5 minutes followed by subcutaneous gentamicin. Mice were tested for mechanical hypersensitivity periodically until response frequency returned to baseline levels. Following the return to baseline, hyperalgesic priming was tested by giving each animal an intraplantar injection of prostaglandin E_2 (PGE_2) (100 ng/ 25 µL). Response frequency following PGE_2 was tested 3 and 24 hrs post-injection. The different AMPK activators used in this experiment were metformin i.p. (LKT Laboratories, 200 mg/kg), ZLN-024 hydrochloride i.p. (Tocris, 30 mg/kg) (Zhang et al., 2013), narciclasine p.o. (Santa Cruz Biotech, 3 mg/kg) (Julien SG, 2017) and MK-8722 p.o. (a kind of gift from Merck, 30 mg/kg) (Feng et al., 2018). Metformin and ZLN were dissolved in 0.9% saline and narciclasine was made in (2-Hydroxypropyl)-β-cyclodextrin (Sigma Aldrich). MK-8722 was administered in 0.25% methyl cellulose (MC), 5% Tween-80, and 0.02% sodium dodecyl sulfate (SDS)(Feng et al., 2018).

Rotorod testing was done using a series 8 rotorod device from IITC Life Science. Mice were placed on the rotorod with the setting of 4 rotations per minute to start with an increase to 40 rotations per minute over the course of 108 seconds. The latency to fall was measured with the device. Testing was done before drug treatment and again on the last day of drug treatment.

Metformin Pharmacokinetics

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This study was done at Sai Life Science Limited at Hinjewadi, India. Eighteen ICR mice weighing between 20 and 25 g were used, 9 male, and 9 female. Blood samples of approximately 60 μL were collected under light isoflurane anesthesia from sets of 3 male and female mice at 0.5, 1 and 4 hrs after dosing. Plasma was harvested by centrifugation of blood and stored at -70 C until analysis. After blood collection, brain samples were isolated at each time point from the same animals. Brain was dipped 3 times in ice-cold phosphate buffered saline, blotted dry and weighed. Brain samples were homogenized using ice-cold phosphate buffered saline with twice the volume of brain weight and then stored at -70 until analysis. Plasma and brain samples were quantified by fit-for-purpose LC-MS/MS methods. Data are shown as ng/mL in plasma and ng/g brain weight.

Neuron Culture

DRGs were extracted aseptically from 4-week old male ICR mice in Hank's Buffered Salt Solution (HBSS, Invitrogen) on ice. The DRGs 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 μg/ml, Roche) for 20 min. Afterward, a trypsin inhibitor (1 mg/ml, Roche) that contained bovine serum albumin (BSA, Fisher, 1 mg/ml) was applied and the ganglia were mixed to allow for further dissociation with a polished Pasteur pipette. The tissue was then filtered through 70-μm nylon cell strainer (Falcon) and re-suspended in DMEM F-12 GlutaMax media (Invitrogen) that contained 10% fetal bovine serum (FBS, Hyclone) and 1× penicillin streptomycin (Pen-Strep). The media also contained NGF (10 ng/ml, Millipore) and 5-fluoro-2′-deoxyuridine + uridine (FRDU-U, 3.0 μg/ml + 7.0 μg/ml, Sigma) to reduce proliferation of glia and fibroblasts. Neurons were cultured for 7 days on 12-mm glass coverslips (#1 thickness, Chemglass) in a 24-well tissue culture plate

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(Falcon) coated with poly-d-lysine (Sigma) at 37 °C with 95% air and 5% CO2. On the day of the experiment, drugs were diluted into DMEM F-12 plus GlutaMax media and added directly onto the neurons at concentrations indicated in the results for 1 hr. All DRGs from 1 mouse were used to generate approximately 4 coverslips of primary cells. Coverslips from multiple independent animals were used in each experiment.

Immunocytochemistry (ICC) and digital image analysis

Following AMPK activator treatment, the cells were washed with phosphate buffered saline (PBS) and fixed with 10% formalin in phosphate buffered saline (PBS) for 30 minutes. Cells were blocked with 10% normal goat serum and labelled with Anti-peripherin, mouse monoclonal (Sigma, 1:500) and phospho-ACC (Ser 79) (p-ACC, 1:1000; Cell Signaling Technologies catalog # 3661) overnight at 4° C. Next, cells were washed and incubated with flurochrome-conjugated secondary antibodies (Alexa Fluor, anti-rabbit 488 and anti-mouse 568, invitrogen) and counterstained with a DNA stain, 4',6-diamidino-2-phenylindole (DAPI) (Invitrogen) and mounted with Prolong Gold (Invitrogen). Images were taken on an Olympus Fluoview FV1200 laser scanning confocal microscope and analyzed using the co-localization tool within Olympus' FV software. The intensity of each channel was adjusted so that only areas that contained a strong signal of 488 nm and 405 nm were visible. This adjusted imaged contained distinct puncta that could then be counted and analyzed using Graphpad Prism. Results were reported as the average percent area of p-ACC signal in neurons labelled with peripherin for all representative pictures.

Western blotting

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Male mice were anesthetized with isoflurane and spinal cords and DRGs innervating the hindpaw (L3 – L5) were removed. Fresh tissues were placed in ice cold lysis buffer (50 mM Tris pH 7.4, 150 mM NaCl, 1 mM EDTA pH 8.0, and 1% Triton X-100) containing protease and phosphatase inhibitors cocktails (Sigma–Aldrich) and homogenized using beaded homogenization tubes (Bertin Corp). Samples were centrifuged at 14,000 rpm for 15 min at 4°C and the supernatant containing protein extracts was collected. Protein concentrations were assessed using the Pierce BCA protein assay kit (ThermoFisher Scientific) as directed. A total of 10 µg of protein was mixed with Laemmli sample buffer (Bio-Rad) and 2-mercaptoethanol and was heated at 95°C for 5 min. Samples were loaded into each well of a 10% SDS–PAGE gel along with 15 µL of Precision plus protein kaleidoscope prestained protein standards (Bio-Rad). Proteins were transferred to a 0.45 PVDF membrane (Millipore, Billierca, MA, United States) at 100 V for 1 h. Membranes were blocked using 5% non-fat dry milk in 1× Tris Buffer Saline-Tween (TTBS) for 2 hrs prior to primary antibody incubation. Primary antibodies used for this experiment were pAMPKα (Thr 172) (1:1000, Cell Signaling catalog # 2535) and total AMPKα (1:1000, Cell Signaling, catalog # 2532). Bands were visualized using a Bio-Rad ChemiDoc Touch. Analysis was performed using Image Lab version 6.0.

Statistics

Data are shown as mean +/- standard error of the mean (SEM) and the number of animals or samples used in each analysis are given in figure legends. GraphPad Prism 7 was used to analyze data and to do curve fitting and other statistical tests are given in figure legends. Two-way ANOVAs were used to analyze von Frey data. Post-hoc tests used

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were the Bonferroni multiple comparisons test. Significance level was set at $\alpha < 0.05$. Details on test statistics are given in Supplementary Table 1.

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Results

Metformin decreases incision-induced mechanical hypersensitivity and blocked hyperalgesic priming in male but not female mice.

To assess the effects of metformin on incision-evoked mechanical hypersensitivity in male mice, we first obtained baseline responses for von Frey filament strengths of 0.6, 1.0 and 1.4 grams. Following baseline, mice were allocated into metformin (200 mg/kg) and vehicle (0.9% saline) i.p. treatment groups for 7 days. On day 3 of treatment, plantar incision surgery was performed on all of the mice. These mice were tested ~~1, 2, 4, 7, 10 and 14 days post surgery~~ periodically over the next 2 weeks until they returned to baseline. Once the mice returned to baseline level sensitivity, animals received 100 ng PGE_2 into the affected hindpaw to assess hyperalgesic priming. These mice were tested 3 and 24 hrs post-injection. In male mice, metformin reduced acute mechanical hypersensitivity following plantar incision. Metformin also prevented the development of hyperalgesic priming (**Fig 1A-C**). These findings are consistent with our previously published data using the up-down von Frey testing method (Burton et al., 2017). A separate group of sham surgery male mice were allocated into metformin and vehicle treatment groups and baselined for von Frey and rotarod testing to assess possible effects on motor behavior. These mice were treated for 7 days and given a sham surgery on day 3. The mice were tested periodically for von Frey responses and no differences were observed based on treatment (**Supplemental figure 1A-C**). Following the last day of drug treatment, these mice were tested on the rotarod again to assess the effects of metformin on motor behavior. Metformin had no effect on locomotive behavior in male mice **(Supplemental figure 1D)**.

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To test whether similar effects would be observed in female mice, we obtained baseline mechanical sensitivity measurements and randomized them into metformin and vehicle treatment groups under similar conditions as with males. On day 3 of treatment, plantar incision surgery was performed on these mice. Mechanical hypersensitivity was tested 1, 2, 4, 7, 10- and 14-days post-surgery. Once the mice returned to baseline level sensitivity, animals received 100 ng PGE_2 into the affected hindpaw. Unlike with male mice, metformin had no effect on the acute hypersensitivity caused by incision. Metformin also failed to block hyperalgesic priming in female mice (**Fig 1D-F**). These findings are consistent with our observations in female SNI mice where metformin only relieved mechanical hypersensitivity in male mice. While a pronounced difference in mechanical hypersensitivity was seen between male and female metformin treated mice after plantar incision, we did not observe any sex difference in the duration or magnitude of mechanical hypersensitivity in vehicle treated mice (**Fig 2**). To test the possibility that lower doses of metformin could be effective in female mice in the plantar incision test we did treatments with 100 and 50 mg/kg metformin. These doses also failed to impact acute mechanical hypersensitivity or to block hyperalgesic priming in female mice **(Supplemental figure 2)**.

We next sought to determine whether this sex difference with metformin could be explained by pharmacokinetics. Nine male and 9 female ICR mice were given a single i.p. dose of 200 mg/kg metformin. A third of the mice were euthanized 30 min post-injection, another third 1 hr post-injection and the remaining 4 hrs post-injection with brain and plasma being taken at each time point. Metformin was measured in the brain and plasma of each animal and compared for each timepoint and across sexes. Surprisingly,

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we found that plasma levels of metformin were higher in female mice at 0.5 and 1 hr post i.p. injection (**Supplemental figure 3A**). Brain levels were higher in female mice at all time points (**Supplemental figure 3B**). Brain to blood ratios did not differ between sexes (**Supplemental figure 3C, Supplementary Table 2**). Differences in pharmacokinetics for plasma or blood levels of metformin do not explain sex differences in efficacy seen in the incisional model.

Narciclasine decreased incision-induced mechanical hypersensitivity and blocked priming in male and female mice.

We then tested whether a structurally distinct AMPK activator would show similar sex-specific effects. We obtained baseline mechanical sensitivity measurements from male mice the sorted them into narciclasine (1, 3 or 6 mg/kg) and vehicle ((2-Hydroxypropyl)-β-cyclodextrin) p.o. groups. These mice were treated orally for 7 consecutive days and plantar incision surgery was performed on day 3 of treatment. These mice were tested for von Frey responses under the same time course as in the metformin experiments. Once the mice returned to baseline sensitivity, animals received PGE_2 into the hindpaw and were tested 3 and 24 hrs post-injection. In male mice, narciclasine reduced mechanical hypersensitivity following plantar incision in a dose-dependent fashion. All narciclasine doses completely blocked development of hyperalgesic priming **(Fig 2A-C)**. Because the 3 mg/kg dose showed full efficacy in male mice, we chose to continue our experiments in female mice using only this dose. The male experiment was repeated in its entirety in a group of female mice over the same time course. Once the females returned to baseline, they also received PGE_2 into the affected paw and were tested 3 and 24 hrs post-surgery. Similar to the experiment with

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males, narciclasine prevented acute mechanical hypersensitivity in female mice and blocked development of hyperalgesic priming **(Fig 2D-F)**. Therefore, indirect AMPK activators can be effective in reducing incision-evoked mechanical hypersensitivity and hyperalgesic priming in both male and female mice. A separate group of sham surgery male mice were allocated into narciclasine and vehicle treatment groups and baselined for von Frey and rotarod testing to assess possible effects on motor behavior. These mice were also treated for 7 days but given a sham surgery treatment on day 3. The mice were tested periodically for von Frey responses and no differences were observed based on treatment (**Supplemental figure 4A-C**). Narciclasine also had no effect on rotorod behaviors (**Supplemental figure 4D**).

While narciclasine has been shown to activate AMPK in other cell types, it has never been tested for AMPK activity in DRG neurons. To directly assess this, DRGs were cultured from naïve male mice and treated with vehicle, 100 nM, 1 μM narciclasine for 1 hr. We observed a significant increase in p-ACC intensity with narciclasine treatment at 1 μM demonstrating that narciclasine induces AMPK activation in DRG neurons **(Fig 3A-D)**. This concentration is higher than previous demonstrations of AMPK activation with 20 nM narciclasine in skeletal muscle cells, but those experiments were done over 48 hr treatment and ours was done with a 1 hr treatment time. The discrepancy in concentration of narciclasine needed to activate AMPK may be cell type- or time course-dependent. We conclude from this biochemical study that narciclasine is a potent activator of AMPK in DRG neurons.

In addition to these *in vitro* findings, we used western blotting to confirm that narciclasine can activate AMPK in the DRG in males and females *in vivo*. Mice of both

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sexes were given a single dose of NCLS (3 mg/kg) and DRGs and livers were taken 1 hr post-injection and homogenized for Western blotting. Primary antibodies used were p-AMPK and total-AMPK to assess the effects of narciclasine on AMPK signaling in these tissues. We observed a significant increase in p-AMPK/total-AMPK ratio in both the male and female DRGs with narciclasine treatment compared to vehicle **(Fig 4)**. There was no difference in p-AMPK/total-AMPK ratio in male or female liver with narciclasine treatment, which is consistent with previously published data with this compound (Julien SG, 2017).

Direct, allosteric AMPK activators reduce hyperalgesic priming in male and female mice without impacting acute incision-induced mechanical hypersensitivity.

Metformin and narciclasine activate AMPK via upstream signaling mechanisms. ZLN024 and MK8722 are structurally distinct AMPK activators that bind directly to the kinase to allosterically increase kinase activity, albeit via distinct mechanisms. We used these compounds because they have thoroughly described pharmacokinetics. After establishing baseline mechanical sensitivity measurements from male mice, we then assigned them to ZLN-024 (30 mg/kg) and vehicle (0.9% saline) i.p. treatment groups. These mice received i.p. ZLN-024 for 7 consecutive days and plantar incision surgery was performed on day 3 of treatment. Once the mice returned to baseline sensitivity, animals received PGE_2 into the hindpaw and tested 3 and 24 hrs post-injection. In male mice, ZLN-024 did not significantly decrease mechanical hypersensitivity following plantar incision surgery, but 7-day treatment did block hyperalgesic priming **(Fig 5A-C)**. For the female cohorts, ZLN-024 also had no effect on the initial surgery-induced mechanical hypersensitivity but did block hyperalgesic priming following PGE_2 just as with the male cohort **(Fig 5D-F).** Using the same paradigm for testing in male mice, MK8722

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did not significantly decrease mechanical hypersensitivity following plantar incision surgery, except a small effect at day 7 after incision. However, 7-day treatment did block hyperalgesic priming **(Fig 6A-C)**. In the female cohorts, MK8722 again had no effect on the initial incision-induced mechanical hypersensitivity but also blocked hyperalgesic priming following PGE_2 just as with the male cohort **(Fig 6D-F).** These experiments demonstrate that direct activators of AMPK have a strong effect on hyperalgesic priming, but do not influence pain hypersensitivity caused by acute incision. A separate group of male mice were allocated into MK8722 and vehicle treatment groups and baselined for von Frey and the rotarod. These mice were treated for 7 days and given a sham surgery on day 3. The mice were tested in the von Frey test throughout this time and, following treatment, they were tested on the rotarod again to assess the effects of MK8722 on locomotive behavior. As with metformin and narciclasine on sham mice, MK8722 had no effect on von Frey or locomotive behavior in male mice (**Supplemental figure 5A-D**).

We have previously given 2 local injections of AMPK activators (one at the time of incision and another 24 hrs later) to demonstrate that local activation of AMPK can attenuate incision-evoked mechanical hypersensitivity (Tillu et al., 2012; Burton et al., 2017). We assessed whether MK8722 (10 µg dose per injection) would have an effect on incision-evoked pain. We found that in both males **(Fig 7A-C)** and females **(Fig 7D-F)** MK8722 had no effect on acute mechanical hypersensitivity but local treatment with this direct AMPK activator did attenuate hyperalgesic priming **(Fig 7A-F)**. This effect is consistent with the systemic dosing experiment with MK8722 but is in contrast to our previous findings with local injection of indirect AMPK activators (e.g. resveratrol) that

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blocked acute mechanical hypersensitivity and hyperalgesic priming (Tillu et al., 2012; Burton et al., 2017).

Like narciclasine, MK8722 activates AMPK in many cell types but has not previously been tested on DRG neurons. DRGs were cultured from naïve male mice and treated with vehicle, 100 nM or 10 μM MK8722 for 1 h. We observed a significant increase in p-ACC intensity with MK8722 treatment at 100 nM and 10 μM demonstrating that MK8722 induces AMPK activation neurons **(Fig 8A-D)**. Quantification was done by measuring the maximum florescence **(Fig 8D)**. Based on this result we conducted a full concentration response curve for MK8722 and obtained an approximate EC_{50} of 900 nM (95% confidence interval 253 nM – 2.62 μM) for AMPK activation in DRG neurons (**Fig 8E**). This EC_{50} for MK8722 is similar to observations in rat primary hepatocytes for AMPK activation (Myers et al., 2017).

Plantar incision decreases pAMPK in the DRG ipsilateral to incision injury.

Male mice went through the same behavioral battery described above and then plantar incision surgery was performed. Two hrs post-surgery, lumbar DRGs were taken on the ipsilateral and contralateral sides from these mice and homogenized for western blotting. Primary antibodies used were p-AMPK and total-AMPK to assess the effects of injury on AMPK signaling. We saw a significant decrease in p-AMPK/total-AMPK ratio in ipsilateral DRGs compared to contralateral DRGs **(Fig 9).** This finding is consistent with previously published data demonstrating that pain-inducing stimuli cause a decrease in AMPK signaling in DRG (Atef et al., 2018). Given that many direct AMPK activators require AMPK phosphorylation for their pharmacological activity, this potentially explains why those compounds are less efficacious for acute incisional pain.

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Discussion

One of the key findings of this study is that while metformin's sex differential effect on neuropathic pain (Inyang et al., 2018) can also be observed in the incisional pain model, other AMPK activators did not have a sex-specific effect on pain in our experiments. Narciclasine, ZLN-024 and MK 8722 all had the same level of efficacy on hyperalgesic priming in male and female mice. Metformin had a robust initial anti-hyperalgesic effect and blocked hyperalgesic priming in male mice but showed no effect in female mice. These sex differences were not readily explained by pharmacokinetics of metformin. In fact, female mice had higher plasma and brain levels of metformin than did male mice. While we do not have an explanation for sex differences in metformin's efficacy in incisional or neuropathic pain models in mice, our work does illustrate that other AMPK activators can be effective in the incisional model in both sexes. A possible mechanism that can be explored in future work is sexual dimorphic expression of transporters that are required for metformin to enter cells. Previous studies have demonstrated that this is an important aspect of metformin action, and is controlled by sex hormones (Cai et al., 2019).

Another key finding of this study is our demonstration that indirect AMPK activators are more efficacious in the acute phase of mechanical hypersensitivity following incisional injury. While the positive allosteric modulators ZLN-024 and MK 8722 showed a robust ability to block hyperalgesic priming, these drugs did little to attenuate the initial mechanical hypersensitivity caused by incision pain. In contrast, pretreatment with narciclasine (in males and females) and metformin (in males) caused a large initial anti-hyperalgesic effect and more rapid resolution of surgery-induced hypersensitivity in

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addition to blocking priming. Despite this behavioral difference, we did not see a difference *in vitro* in the ability of these compounds to activate AMPK. Both narciclasine as well as MK 8722 induced significant AMPK activation in DRG neurons culture with an EC_{50} for MK 8722 that was consistent with values previously reported in the literature (Myers et al., 2017). It is unlikely that these differences occurred due to pharmacokinetic or pharmacodynamic issues in the DRG because we used these compounds at *in vivo* doses that have been thoroughly vetted previously for AMPK activation. In line with this, we show here that narciclasine activates AMPK in the DRG, but not liver, of male and female mice

How, then, can the difference in acute effects of these different modes of AMPK activation be explained? One potential explanation for the behavioral difference we saw between the indirect AMPK activators and positive allosteric modulators is the decrease in pAMPK in the DRG caused by incision injury, and has been shown previously in diabetic neuropathic pain (Atef et al., 2018). A decrease in DRG neuron levels of phosphorylated AMPK would theoretically substantially decrease the ability of allosteric modulators to activate AMPK. This is because these drugs are thought to rely on phosphorylated AMPK to be able to increase AMPK activity. If AMPK phosphorylation is decreased by a preceding injury, these drugs would achieve a smaller amount of enhanced AMPK activation unless upstream AMPK phosphorylation is increased via some other mechanism. On the other hand, indirect AMPK activators increase AMPK phosphorylation because they act via upstream kinases that then phosphorylate AMPK. A limitation of this idea is that the existing data on MK8722 has not directly assessed

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whether AMPK phosphorylation is needed for AMPK activation with this compound but it is clearly a direct activator of the enzyme (Myers et al., 2017).

As previously mentioned, metformin activates AMPK indirectly through the upstream kinase LKB1 (Shaw RJ, 2005). Narciclasine is thought to increase AMPK phosphorylation via a cAMP- and ADP/ATP ratio-dependent mechanism which may act similarly to resveratrol (Julien SG, 2017). Importantly, resveratrol also inhibits acute incision-evoked pain and blocks the development of hyperalgesic priming (Burton et al., 2017). Therefore, from the perspective of therapeutic development of AMPK activators for the treatment of post-surgical pain, our findings support a focus on potent indirect AMPK activators. Given that narciclasine is far more potent than metformin, this molecule may represent an excellent starting point for further refinement in this space.

Another question arising from our work is why direct allosteric modulators of AMPK are effective in blocking the development of hyperalgesic priming but not acute incision-evoked hypersensitivity. Previous studies have made it clear that hyperalgesic priming can be completely reversed without having any effect at all on the acute pain phase (Asiedu et al., 2011; Price and Inyang, 2015) as we have shown here with ZLN024 and MK8722. While we have not investigated this directly, we favor the hypothesis that these compounds block mTOR and MAPK-dependent protein synthesis sufficiently to attenuate gene expression of proteins that are needed for the development of hyperalgesic priming. This could include the synthesis of proteins like CREB that act as retrograde signaling factors from the site of injury to then regulate transcriptional programs that are needed for the transition to a chronic pain state (Melemedjian, 2014). Our findings would suggest that the inhibition of translation of these proteins requires less AMPK activation than the

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translation of proteins that are required for the acute sensitization of nociceptors. This hypothesis can be explored in future studies.

There are several shortcomings to our study that should be addressed in the future. First, while we have done a large number of pharmacological manipulations in this study, we have not used genetic approaches to directly determine whether AMPK is responsible for the effects we have observed. Second, narciclasine's pharmacology is potentially complex and may involve signaling pathways that are independent of AMPK. We cannot rule this out based on our current experiments. Another caveat is that we have relied on evoked testing for pain assessment and we have not used other methods like paw guarding or mouse grimace scale. Our future work will focus on non-evoked pain measures. Finally, whether the hyperalgesic priming paradigm truly models development of chronic post-surgical pain is controversial, however, we are not aware of a widely accepted model of chronic post-surgical pain.

In conclusion, direct and indirect AMPK activators block the transition of acute to chronic pain that is potentially modeled preclinically by the hyperalgesic priming paradigm, and they can do this in males and females. However, indirect AMPK activators have an acute anti-hyperalgesic effect that is not seen with direct positive allosteric modulators. Based on these findings, we conclude that indirect AMPK activators, like narciclasine, should be further pursued as a treatment option for post-surgical pain that could achieve analgesia and a blockade of development of chronic pain in both sexes. More work is needed to ultimately determine the selectivity of narciclasine at AMPK but given the favorable pharmacological profile that has already been described for this

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compound, we propose that it has excellent potential as a starting point for further development.

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Author contributions:

Participated in research design: Inyang, Burton, Dussor and Price

Conducted experiments: Inyang, Szabo-Pardi, Wentworth, McDougal, Ramirez, and Pradhan

Performed data analysis: Inyang, Burton and Price

Wrote or contributed to the writing of the manuscript: Inyang, Dussor and Price

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Figure Legends

Figure 1: Metformin treatment attenuates incision-induced mechanical hypersensitivity and blocks hyperalgesic priming in male mice: A-C. Metformin treatment starting immediately after baseline measurements, which were done 3 days prior to plantar incision, decreased mechanical hypersensitivity following plantar incision surgery and prevented plantar incision-induced hyperalgesic priming precipitated by PGE_2 injection. D-F: In female mice, metformin treatment failed to decrease mechanical hypersensitivity compared to vehicle treated mice. Metformin also failed to prevent plantar incision-induced hyperalgesic priming precipitated by PGE_2 injection. ***$p < 0.001$; treatment effect; N= 6 for the metformin groups and N= 5 for the vehicle groups.

Figure 2: Male and female mice recover from plantar incision surgery at the same rate. A-C. Plantar incision surgery in male and female mice caused an increase in mechanical hypersensitivity that resolved at the same rate with no significant differences in mechanical hypersensitivity. N= 21 males and N= 18 females.

Figure 3: Narciclasine treatment attenuates surgery-induced mechanical hypersensitivity and blocks hyperalgesic priming in male and female mice: A-C. Narciclasine treatment starting immediately after baseline measurements dose-dependently decreased mechanical hypersensitivity following plantar incision surgery and prevented plantar incision-induced hyperalgesic priming precipitated by PGE_2 injection at all doses. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; N= 4 for the NCLS groups and N= 3 for the vehicle group. D-F. Narciclasine treatment also decreased mechanical hypersensitivity following plantar incision surgery and prevented surgery-induced hyperalgesic priming

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precipitated by PGE_2 injection. *p < 0.05; **p < 0.01; ***p < 0.001; N= 6 for the NCLS group and N= 6 for the vehicle group.

Figure 4: Narciclasine induces AMPK activity in male DRG neurons *in vitro*. Male neuron cultures were treated with vehicle A), 100 nM (B) or 1 μM narciclasine (NCLS) (C) for 1 hr. Representative immunohistochemistry images of the DRG neurons at 40X magnification. Quantification of images shown in D. 1 μM NCLS increased p-ACC intensity in neuron cultures. Only neurons that were positive for peripherin staining were analyzed. Maximum florescence refers to the maximum florescence intensity per neuron analyzed. **p < 0.01. N= 39 images analyzed per group.

Figure 5: Narciclasine increases AMPK signaling in male and female DRGs. A single dose of NCLS (3 mg/kg) caused a significant increase in AMPK signaling in DRGs for male (A) and female (C) mice 1 hr post-injection while not effecting AMPK signaling in liver for male (B) or females (D). NCLS treatment increased the ratio of p-AMPK to total-AMPK in male (E) and females (G) DRGs but not in male (F) or female (H) liver. *p < 0.05; students t-test; N= 4 per condition in male mice; N= 4 for female NCLS treatment and N=3 for female vehicle.

Figure 6: ZLN-024 treatment blocks hyperalgesic priming in male and female mice but had no effect on acute incision-induced mechanical hypersensitivity: A-F. While ZLN-024 treatment starting immediately after baseline measurements had no effect on initial mechanical hypersensitivity following plantar incision surgery, drug treatment was effective in prevention of plantar incision-induced hyperalgesic priming precipitated by PGE_2 injection in male (A-C) and female (D-F) mice. *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001; N= 6 for the ZLN-024 group and N= 6 for the vehicle group.

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Figure 7: MK8722 treatment blocks hyperalgesic priming in male and female mice but had no effect on acute incision-induced mechanical hypersensitivity: A-F. While MK8722 treatment starting immediately after baseline measurements had no effect on initial mechanical hypersensitivity following plantar incision surgery, drug treatment was effective in prevention of plantar incision-induced hyperalgesic priming precipitated by PGE_2 injection in male (A-C) and female (D-F) mice. *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001; N= 6 for the MK8722 group and N= 5 for the vehicle group.

Figure 8: Local MK8722 treatment attenuates hyperalgesic priming in male and female mice but had no effect on acute incision-induced mechanical hypersensitivity: Local injection of MK8722 at the time of incision and again 24 hrs later (red arrows) had no effect on initial mechanical hypersensitivity following plantar incision surgery in males or females. However, drug treatment was effective in prevention of plantar incision-induced hyperalgesic priming precipitated by PGE_2 injection in male (A-C) and female (D-F) mice. *p < 0.05; **p < 0.01; ***p < 0.001; N= 4 for the MK8722 group and N= 4 for the vehicle group in both males and females.

Figure 9: MK8722 induces AMPK activation in DRG neurons *in vitro*. Neuron cultures were treated with vehicle (A), 100 nM (B) or 10 μM MK 8722 (C) for 1 hr. Representative immunohistochemistry images of the DRG neurons at 40X magnification. Quantification of images shown in D. 100 nM and 10 μM NCLS increased p-ACC intensity in male neuron cultures. Only neurons that were positive for peripherin staining were analyzed. Maximum florescence refers to the maximum florescence intensity per neuron analyzed. **p < 0.01; ***p < 0.001. N= 39 images analyzed per

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group. E) Shows a full concentration-response curve of MK8722 with an approximate EC_{50} of 900 nM (95% confidence interval 253 nM – 2.62 μM).

Figure 10: Incision decreases AMPK signaling in injured lumbar DRGs: A) Plantar incision caused a decrease in p-AMPK signaling in ipsilateral lumbar DRGs 2 hrs post-surgery measured by Western blot. B) Incision decreased the ratio between p-AMPK and total-AMPK in the ipsilateral DRGs compared to the side contralateral from the injury. **p < 0.01; students t-test; N= 5 per condition in male mice.

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FIGURE 1

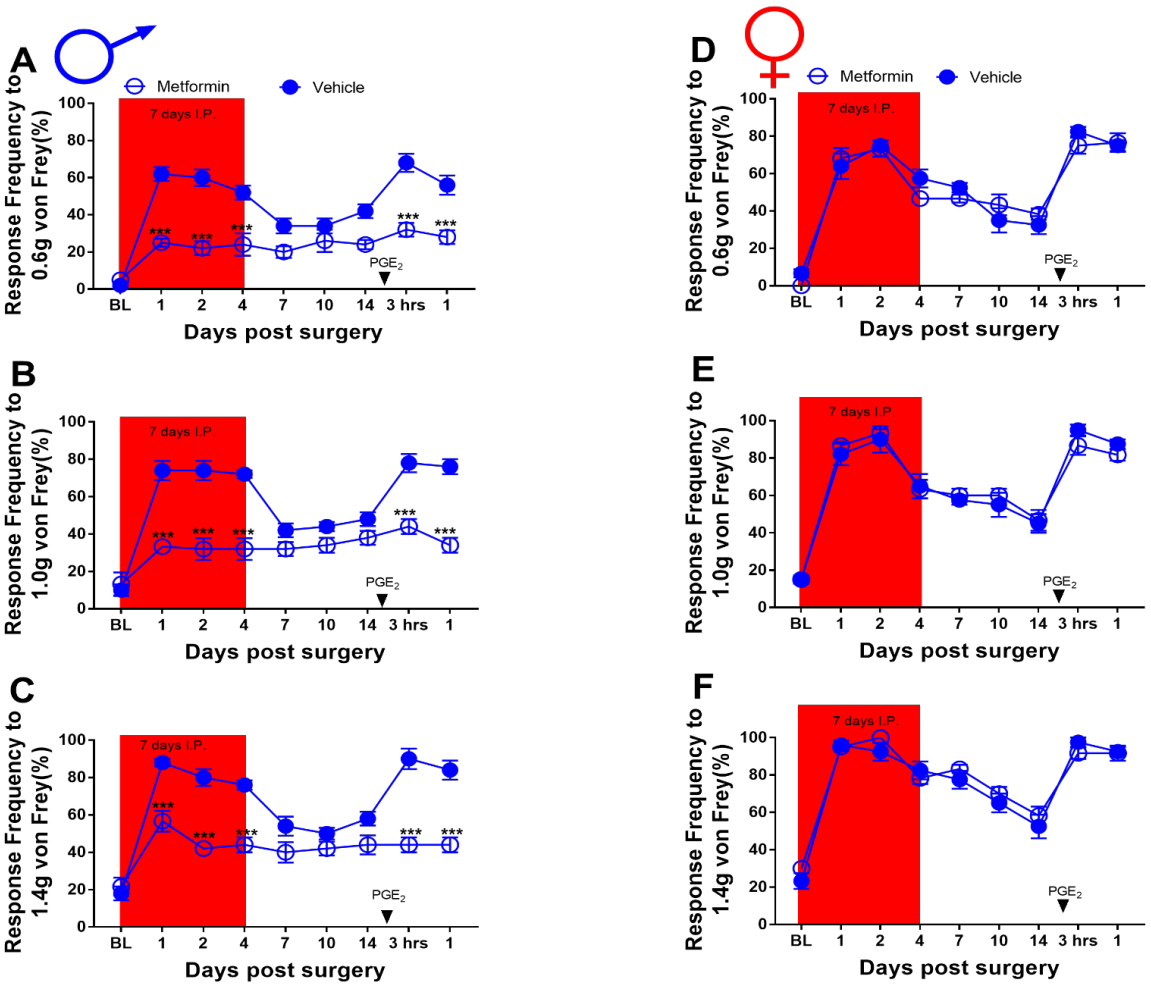

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FIGURE 2

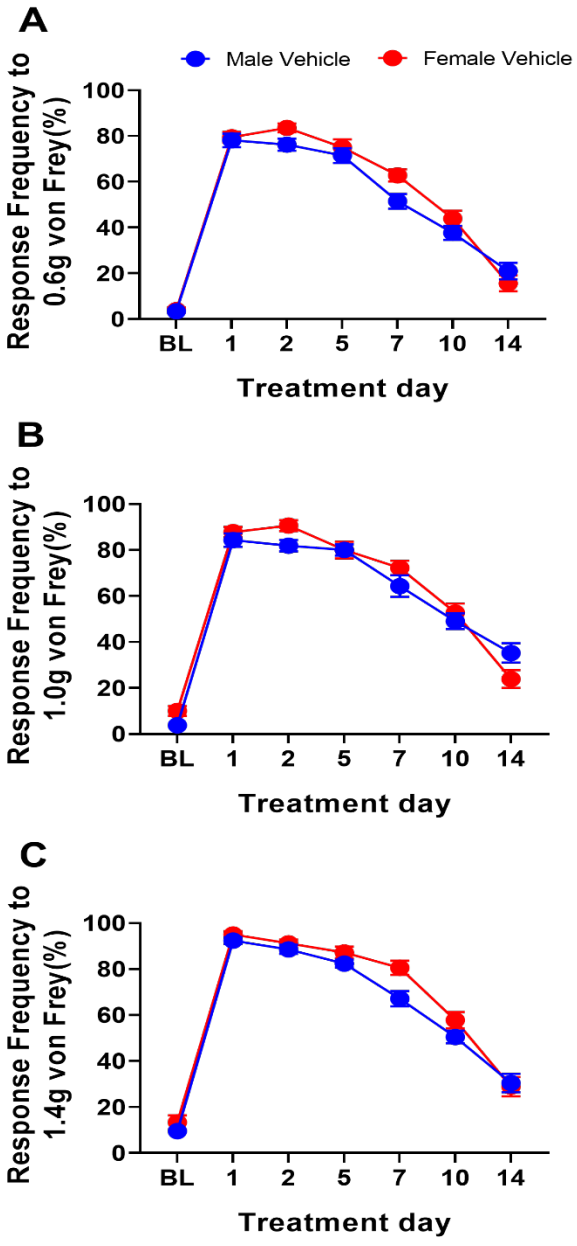

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FIGURE 3

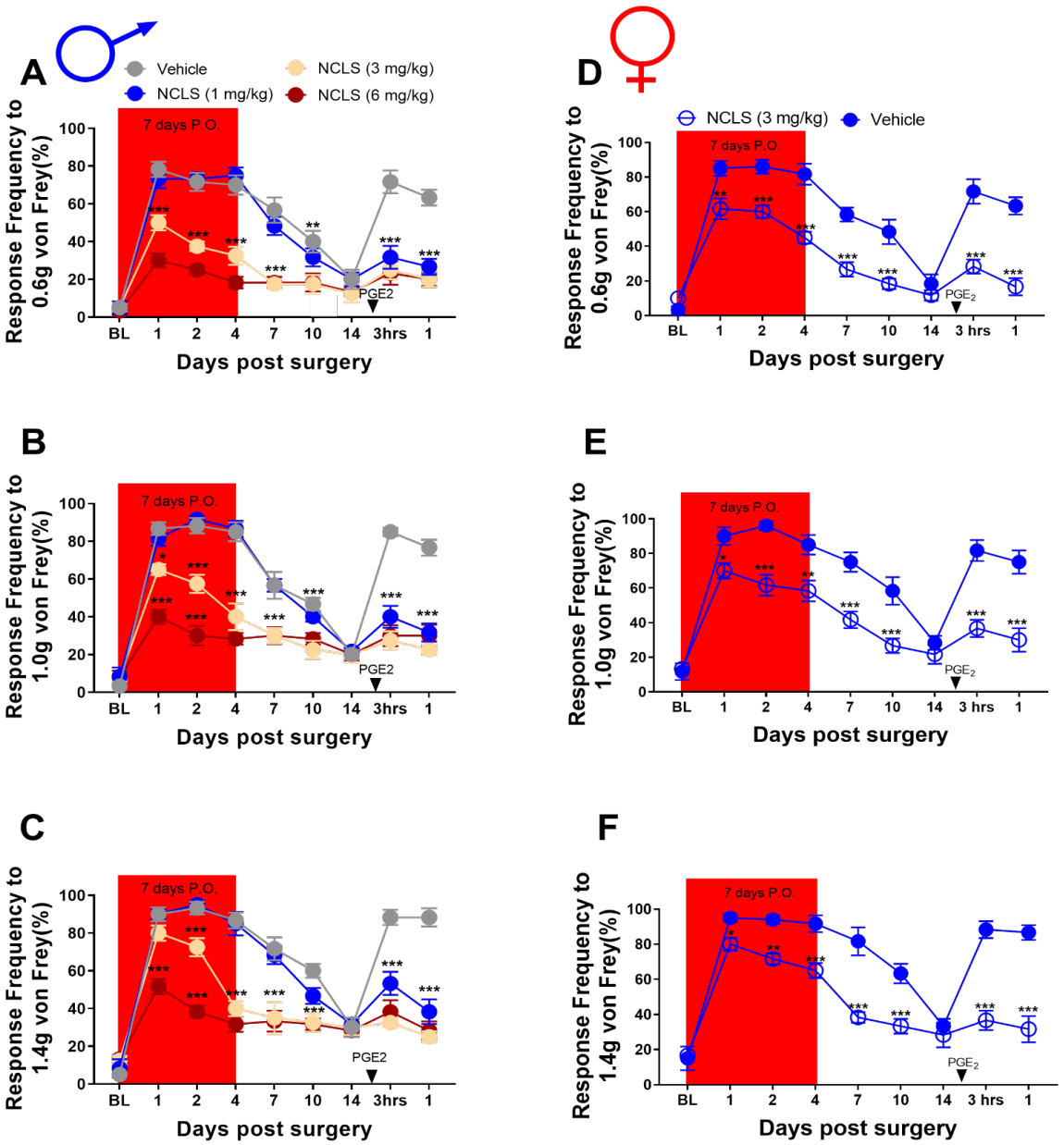

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FIGURE 4

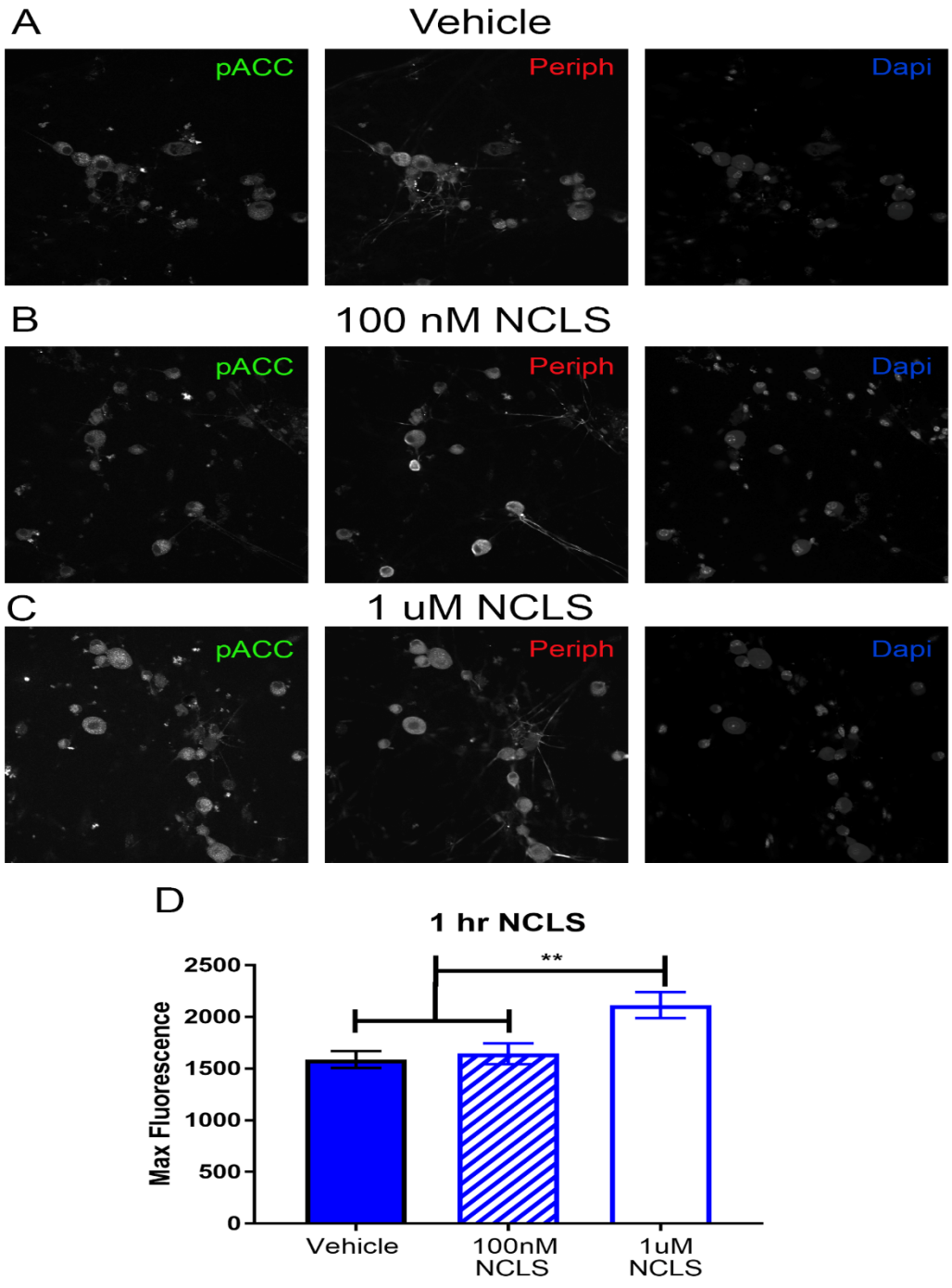

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FIGURE 5

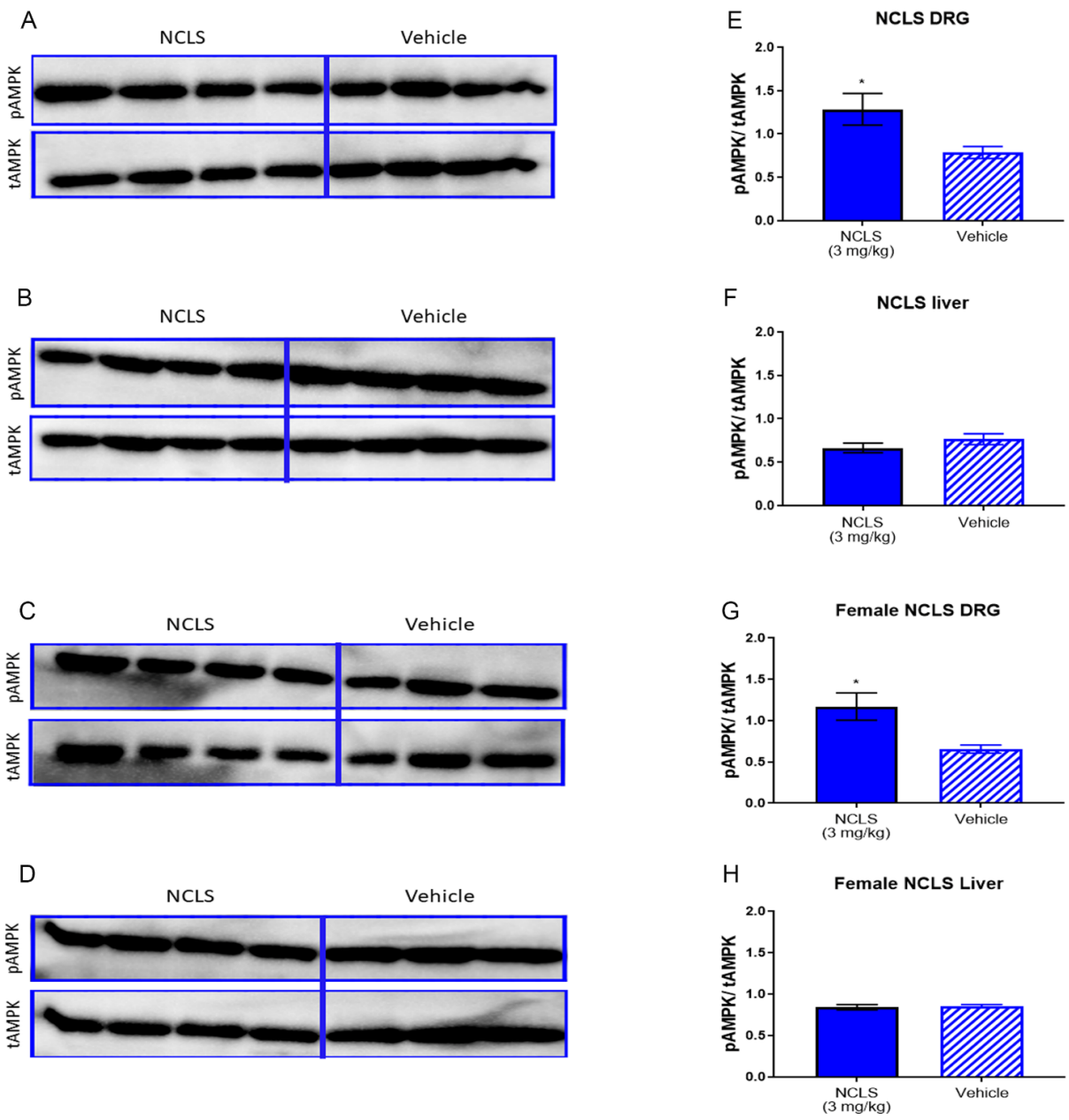

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FIGURE 6

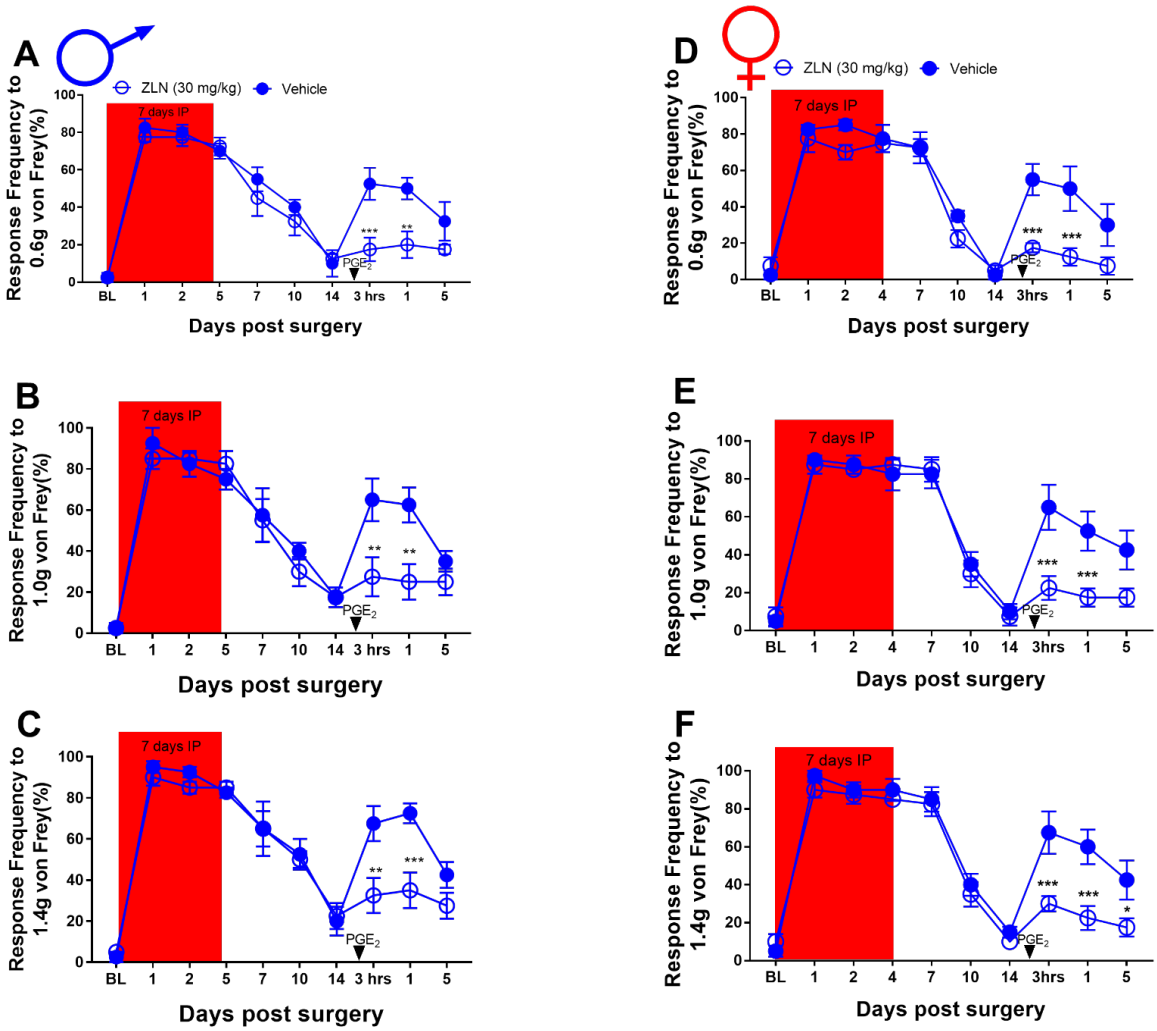

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FIGURE 7

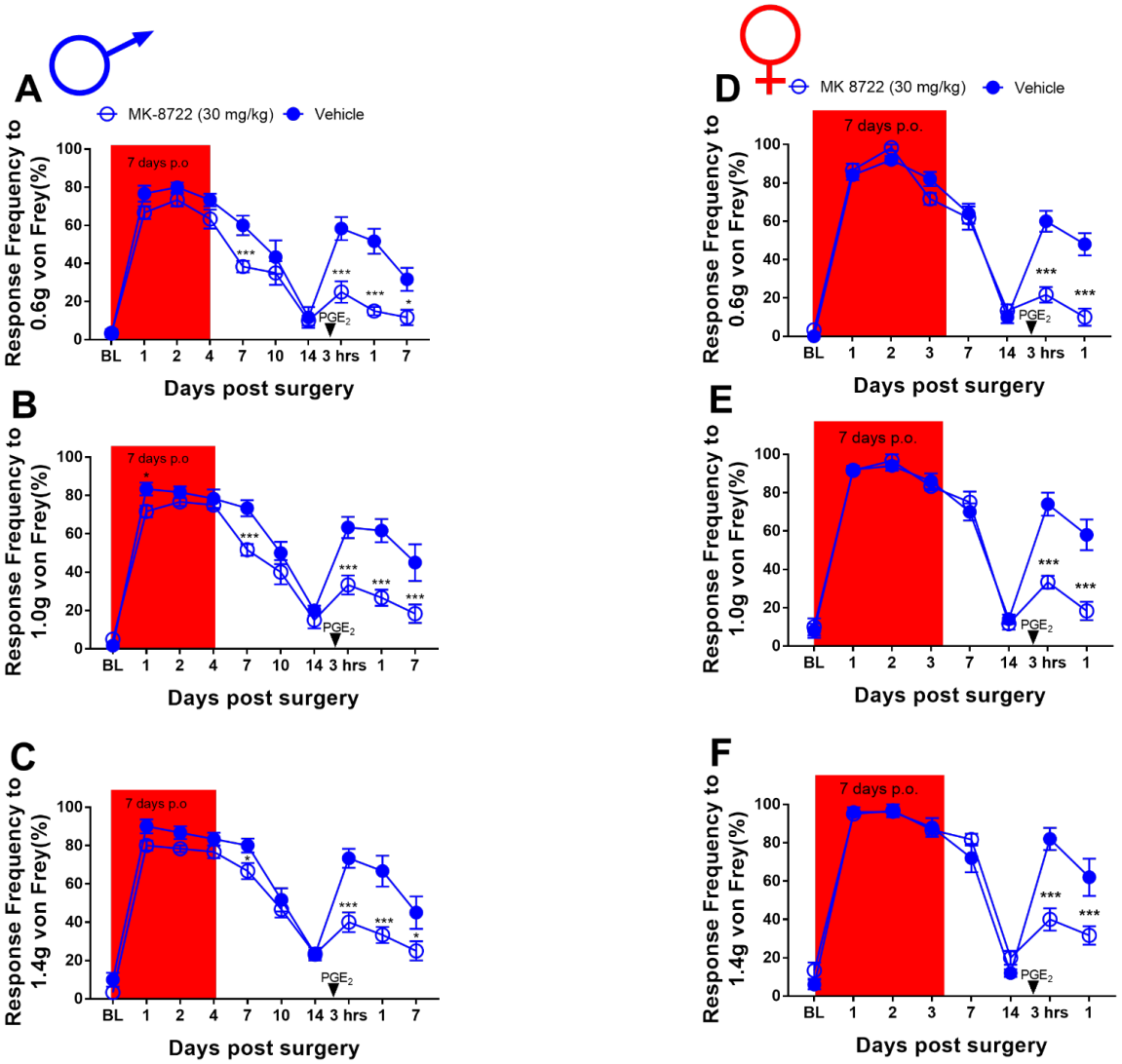

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FIGURE 8

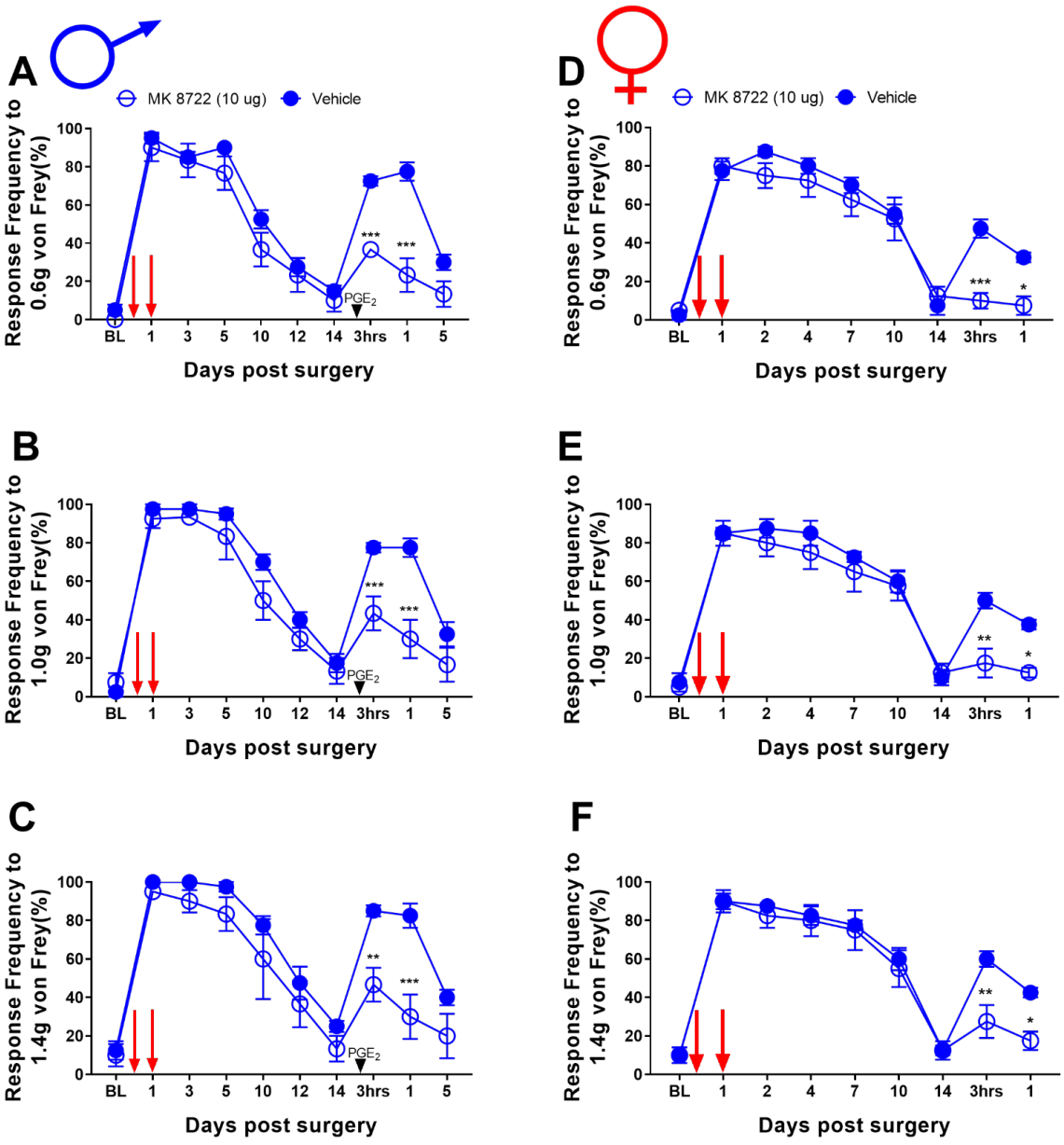

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FIGURE 9

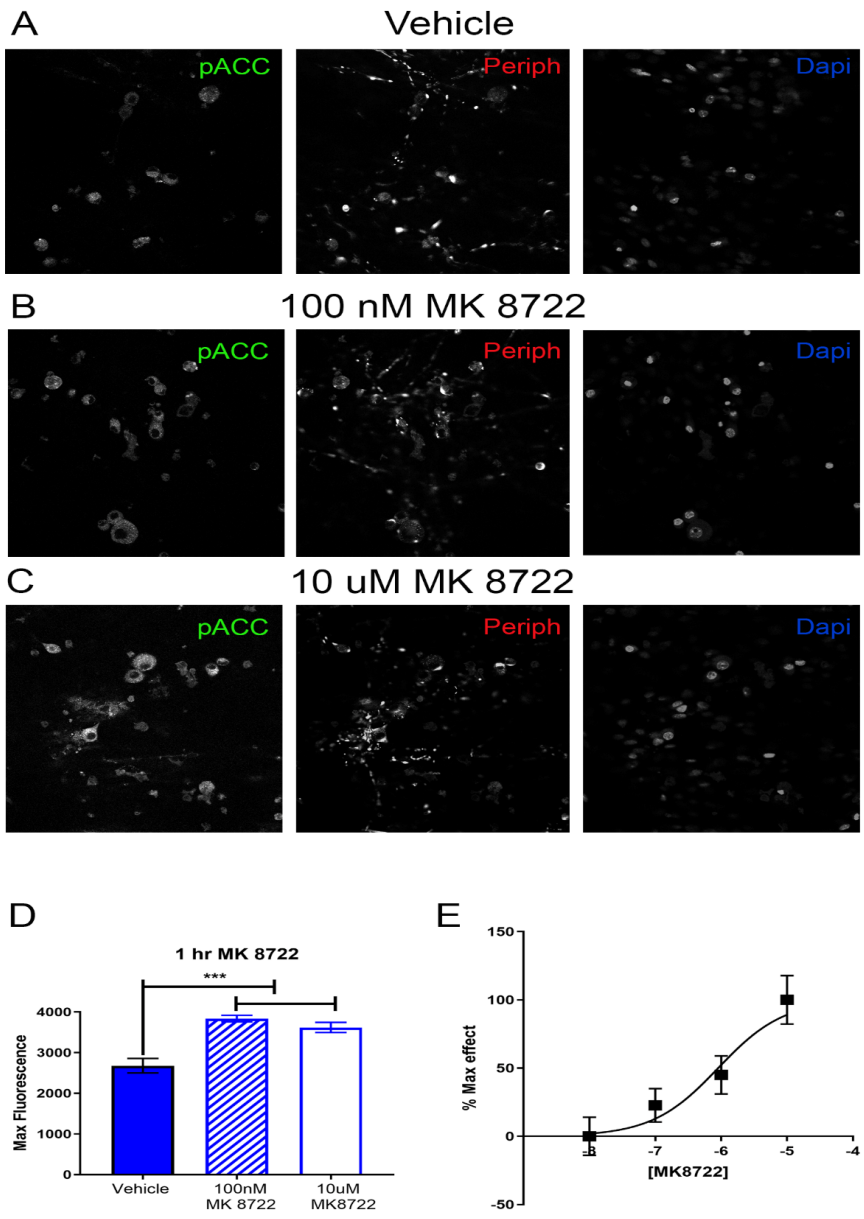

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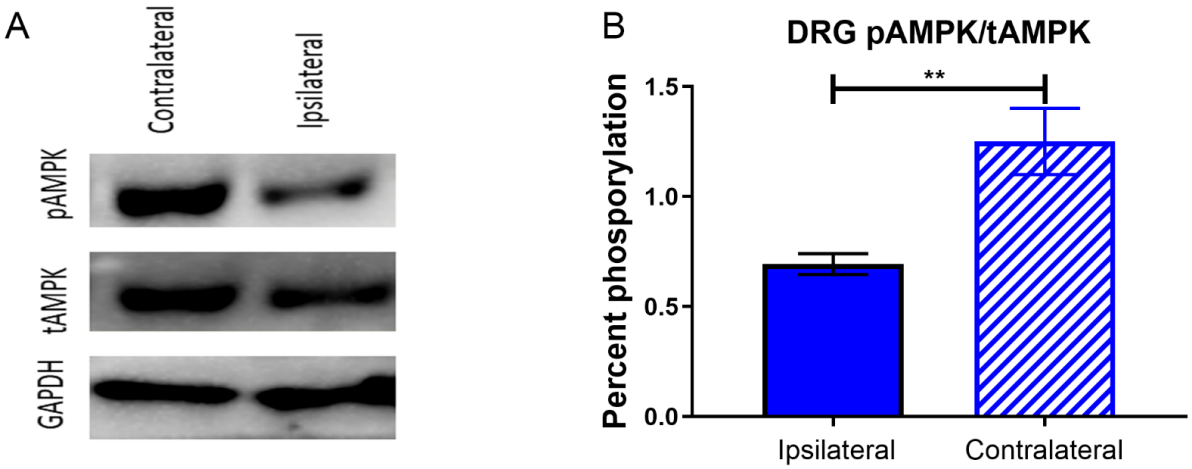
FIGURE 10



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