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Running title: Paradoxical Potentiation of the EPR

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Abstract

When contracting muscles are freely perfused, the acid-sensing ion channel 3 (ASIC3) on group IV afferents plays a minor role in evoking the exercise pressor reflex. We recently showed in isolated dorsal root ganglion neurons innervating the gastrocnemius muscles that two mu opioid receptor agonists, namely endomorphin 2 and oxycodone, potentiated the sustained inward ASIC3 current evoked by acidic solutions. This *in vitro* finding prompted us to determine if endomorphin 2 and oxycodone, when infused into the arterial supply of freely perfused contracting hindlimb muscles, potentiated the exercise pressor reflex. We found that infusion of endomorphin 2 and naloxone in decerebrated rats potentiated the pressor responses to contraction of the triceps surae muscles. The endomorphin 2-induced potentiation of the pressor responses to contraction were prevented by infusion of APETx2, an ASIC3 antagonist. Specifically, the peak pressor response to contraction averaged 19.3 ± 5.6 mmHg for control (n=10), 27.2 ± 8.1 mmHg after naloxone and endomorphin 2 infusion (n=10), and 20 ± 8 mmHg after APETx2 and endomorphin 2 infusion (n=10). Infusion of endomorphin and naloxone did not potentiate the pressor responses to contraction in ASIC3 knockout rats (n=6). Partly similar findings were observed when oxycodone was substituted for endomorphin 2. Oxycodone infusion significantly increased the exercise pressor reflex over its control level, but subsequent APETx2 infusion failed to restore the increase to its control level (n=9). The peak pressor response averaged 23.1 ± 8.6 mmHg for control (n=9), 33.2 ± 11 mmHg after naloxone and oxycodone were infused (n=9), and 27 ± 8.6 mmHg after APETx2 and oxycodone were infused (n=9). Our data suggest that after opioid receptor blockade, ASIC3 stimulation by the endogenous mu opioid, endomorphin 2, potentiated the exercise pressor reflex.

New & Noteworthy

This paper provides the first *in vivo* evidence that endomorphin 2, an endogenous opioid peptide, can paradoxically increase the magnitude of the exercise pressor reflex

by an ASIC3 dependent mechanism even when the contracting muscles are freely perfused.

Key Words: group III and IV muscle afferents, sympathetic nervous system, mu opioid receptors, naloxone, hyperalgesia

Introduction

The exercise pressor reflex is evoked by the contraction-induced stimulation of group III and IV muscle afferents (7, 20, 26, 29). The reflex is manifested by increases in the sympathetic outflow to the vasculature of the kidneys (41), the skeletal muscles (18, 32), and the heart (39). These increases in the sympathetic outflow as well as decreases in the parasympathetic outflow to the heart (16, 28) results in increases in arterial blood pressure, heart rate and cardiac output (6, 26, 31). The exercise pressor reflex is evoked by both mechanical and metabolic stimuli, and as a result is often described as having a mechanical component, termed the muscle mechanoreflex, and a metabolic component termed the muscle metaboreflex. During whole body exercise, the exercise pressor reflex counters the metabolic vasodilation arising in contracting muscles, thereby functioning to maintain arterial blood pressure (1, 38). In addition, the metabolic component of the exercise pressor reflex functions to increase arterial blood flow to the contracting muscles (1, 31).

The sensory endings of the group III and IV muscle afferents are located in the interstitial space between myocytes, in the walls of their accompanying vessels and at the junction of the muscles and their tendons (12, 35). The receptor proteins on the afferents' endings that are stimulated by mechanical and metabolic stimuli have been investigated. So far, Piezo 2 and Transient Receptor Potential Vanilloid (TRPV) 4 channels have been shown to transduce mechanical stimuli (8, 15). These mechanosensitive channels are believed to be located on the sensory endings of thinly myelinated group III muscle afferents. The purinergic 2X3 (P2X3) channel and the Acid Sensing Ion Channel (ASIC) 1a, among others, transduce metabolic stimuli generated by freely perfused contracting muscles. These metabosensitive channels are located on the endings of unmyelinated group IV muscle afferents (11, 27). Group III afferents, though primarily mechanosensitive, are considered to be polymodal because they sometimes respond to metabolic stimuli. Likewise, Group IV afferents, though primarily metabosensitive, are also considered to be polymodal because they often respond to noxious mechanical stimuli (20).

The evidence so far has shown that ASIC3 on the endings of group IV afferents play a minor role in evoking the metaboreflex when the contracting muscles are freely perfused. In contrast, ASIC3 play a major role in evoking the metaboreflex when the contracting muscles are ischemic (22, 36, 40), a state where the arterial blood supply to the contracting muscles is not meeting the muscles' metabolic demand. Last, ASIC3 play no role in evoking the muscle mechanoreflex (40).

ASIC3 on the endings of group IV afferents are stimulated by hydrogen ions dissociated from both lactic and phosphatic acid. The threshold pH that opens ASIC3 *in vitro* is 7.2 (3, 21), a hydrogen ion concentration that is routinely achieved in hindlimb muscles when they contract (37). Recently, endomorphin 2, a naturally produced mu opioid receptor agonist, has been shown to potentiate the sustained inward ASIC3 current evoked by acidic solution (pH 6.0) in isolated dorsal root ganglion cells innervating the gastrocnemius muscles of rats (13). More recently, this finding has been extended to prescription-based mu opioid agonists, including oxycodone and fentanyl (42).

The finding that mu opioid agonists potentiated ASIC3 currents in isolated dorsal root ganglion cells has important implications for the phenomenon of opioid-induced hyperalgesia, a paradoxical state in which opioid treatment increases the perception of pain rather than decreases it (24). Opioid-induced hyperalgesia is most frequently seen in patients after sustained opioid use, which causes mu opioid receptors to be "internalized." In other words, mu opioid receptors are transported away from the extracellular membrane and into the cytoplasm of sensory nerve endings. Internalized mu opioid receptors cannot be opened by agonists released into the interstitial space and, consequently, the responses of group IV afferents to hydrogen ions cannot be buffered by functioning mu opioid receptors (17). Once mu opioid receptors have been internalized, release of mu opioid agonists from immune cells could increase the responses of group IV nociceptors to acidic stimuli by potentiating the sustained inward current generated by membrane bound ASIC3 channels located on group IV nociceptors (13).

These *in vitro* findings suggesting that ASIC3 stimulation by mu opioid agonists is responsible for opioid-induced hyperalgesia prompted us to attempt to determine *in vivo* if ASIC3 stimulation by mu opioid agonists could potentiate the exercise pressor reflex. In the experiments to be described, we tested the hypothesis that administration of either a naturally produced opioid, namely endomorphin 2, or a prescription opioid, namely oxycodone, potentiated the exercise pressor reflex in decerebrated rats. In humans, opioid-induced hyperalgesia occurs when mu opioid receptors are internalized by repetitive opioid use, rendering them non-functional. In rats, mu opioid receptors were rendered non-functional by giving naloxone, a mu opioid receptor antagonist.

Methods

Ethical Approval

All procedures were conducted in accordance with the National Institutes for Health guidelines, with the approval of the Pennsylvania State University College of Medicine Institutional Animal Care and Use Committee (IACUC), and according to journal policies and regulations on animal experimentation. Both adult male Sprague-Dawley rats and adult male Wistar-Kyoto ASIC3 KO rats were used. All rats were housed within the central animal facility of the Pennsylvania State University College of Medicine, had access to food and water *ad libitum*, and were exposed to a 50:50 light/dark cycle. All attempts were made to minimize animal discomfort and pain.

In vivo experimental procedures

Surgical procedures

On the day of the experiment, rats were anesthetized with a mixture of 3% isoflurane and 100% oxygen as described previously (10). The trachea was cannulated, and lungs were mechanically ventilated (model 683; Harvard Apparatus, Holliston, MA). The left

and right common carotid arteries were cannulated, one of which was used to measure blood pressure (P23XL; Gould-Statham Instruments, Los Angeles, CA). Similarly, the right jugular vein was cannulated to inject fluid and drugs into the systemic circulation. The left superficial epigastric artery was cannulated; the tip of the cannula was advanced so that it was near the junction of the superficial epigastric artery and the femoral artery. A snare was placed around the femoral artery and vein approximately one centimeter proximal to the cannulated superficial epigastric artery. When tightened, the snare occluded the femoral circulation to the hindlimb muscles. The pelvis was clamped to hold the rat securely in place. The left popliteal fossa was opened to expose and isolate the tibial nerve, which was hooked with bipolar stainless-steel electrode. The triceps surae muscles were isolated. The hind paw was secured to the experimental table, and the head of the rat was secured using a customized stereotaxic unit. The calcaneus bone was severed, and with its attached Achilles tendon, was connected to a force transducer (FT03; Grass Instrument, Quincy, MA). A precollicular decerebration was performed (9, 34) and all neural tissue rostral to the plane of section was removed. The isoflurane anesthesia was then terminated, and the lungs were subsequently ventilated with room air. Body temperature was maintained around 37°C using a heating lamp. Blood arterial PO_2 (100-150 mmHg), PCO_2 (35-40 mmHg), and pH (7.35-7.45) were regularly monitored and kept within the physiological range. The experimental protocol was initiated after the lungs were ventilated with room air for 45-60 min. At the end of the experiment, the decerebrated rats were killed by intravenous injection of 3 mL of a supersaturated KCl solution and the chest was opened.

Experimental protocols

In a first group of 10 Sprague-Dawley rats, cardiovascular responses to static contraction of the triceps surae muscle were examined. The motor threshold was determined by progressively increasing the current of a single pulse (0.01 ms) applied to the tibial nerve until a muscle twitch was observed. The stimulator output was then set at a current intensity that was 1.5 times motor threshold. The tibial nerve was stimulated for 30 s at 40 Hz (0.01 ms pulse duration) to statically contract the hindlimb muscles. After a recovery period of at least 10 min, the contraction procedure was

repeated to verify that the pressor response was reproducible. The responses evoked by the second contraction comprised the control data. Next, the snare on the femoral artery and vein was tightened and naloxone (a mu opioid receptor antagonist, 100 μg/kg, Sigma-Aldrich), followed by endomorphin 2 (an endogenous mu opioid receptor agonist, 10 μM, Sigma-Aldrich), were infused with a pump (10 min each) into the arterial supply of the hindlimb via the superficial epigastric artery catheter. The snare was then released and after 5 min the static contraction was repeated as described above. After a recovery period of 10 min, APETx2 (200 μg/kg), an ASIC3 antagonist and endomorphin 2 (10 μM) were infused in the same manner and the contraction maneuver was repeated. This protocol is shown schematically in Figure 1. To provide further evidence of the role played by ASIC3 we repeated this protocol on six ASIC3 KO rats except that APETx2 was not infused.

In a second group of 9 Sprague-Dawley rats, we repeated the same experimental protocol described above except that the exogenous mu opioid receptor agonist, oxycodone (10 μM) was substituted for endomorphin 2. At the end of each experiment, the rats were paralyzed by intravenous injection of pancuronium bromide (1 mg/mL; 0.2 mL) and the tibial nerve was stimulated with the same parameters as those used to induce static contraction. This was done to control for the possibility that tibial nerve stimulation electrically activated the axons of the group III and IV afferents, which evoke the exercise pressor reflex. Stimulation of the tibial nerve after pancuronium bromide injection did not increase blood pressure or HR in any of the experiments in this study. Naloxone, APETx2, endomorphin 2 and oxycodone were dissolved with saline. In every rat studied, the triceps surae muscles were contracted while the femoral artery was freely perfused. At the end of every experiment, we infused 0.2 ml of Evans Blue dye into the superficial epigastric artery. We considered that the infusion reached the triceps surae muscles if they turned blue. If the muscles did not turn blue, we excluded the data from the study.

Data Analysis

Tension developed by the contracting triceps surae muscles and arterial blood pressure were amplified (Gould Universal and Pressure Processors; Gould-Statham Instruments, Los Angeles, CA) and recorded at 1 kHz with an analog-to-digital converter (Micro1401 MKII; Cambridge Electronic Design, Cambridge, UK). These signals were subsequently displayed using commercially available software (Spike2; Cambridge Electronic Design). Heart rate was calculated electronically beat by beat from the arterial pressure pulse and expressed as beats per minute. To determine the pressor response to 30 s of static contraction, we calculated the difference between the peak mean arterial pressure and its corresponding baseline value. The blood pressure index (BPI) was calculated by integrating the area under the curve during the contraction period and then subtracting from this value the area under the curve measured 30 s before the contraction. Using a similar method, we calculated the change in peak tension produced by contraction, as well as the tension-time index (i.e., the equivalent of the blood pressure index for tension).

Statistical Analysis

All data are expressed as mean ± standard deviation (SD). Normality was verified with the Kolmogorov-Smirnoff test. For the data collected from the ASIC3 wild-type animals, within-group comparisons were performed with a one-way ANOVA with repeated measures. If an overall significant difference was found, Tukey post-hoc tests were performed to assess significance between any of the two mean values. For the data collected from the ASIC3 knock out animals, comparisons were performed using paired Student's *t*-test. The criterion for statistical significance was $p<0.05$.

Results

Responses to static contraction in presence of endomorphin 2

In 10 rats, we measured the reflex pressor and cardioaccelerator responses to static contraction of the triceps surae muscles before (i.e., control) and after infusions into the

superficial epigastric artery of naloxone followed by endomorphin 2. We found that the peak pressor response to contraction and its accompanying blood pressure index (BPI) were both significantly increased by the infusion of naloxone followed by endomorphin 2 ($p=0.0019$ and $p=0.0449$, respectively; Figures 2A, 2B and 3A). Analysis of the time course of these pressor responses revealed that the exercise pressor reflex evoked from triceps surae muscles in presence of naloxone and endomorphin 2 was significantly greater than the exercise pressor reflex evoked from triceps surae muscles in presence of APETx2 and endomorphin 2 (Figure 3B). Subsequent infusion of endomorphin 2 and APETx2, an ASIC3 antagonist, restored the pressor and BPI responses to contraction to their control levels.

In additional three rats not used in the above experiments, we measured the peak and integrated pressor (i.e., BPI) responses to contraction of the triceps surae muscles before and after intravenous infusions of naloxone followed by endomorphin 2. The protocol was identical to that used when we infused these agents into the superficial epigastric artery. We found that intravenous infusion of naloxone followed by endomorphin had no statistically significant effect on the exercise pressor reflex. Specifically, the peak pressor response to contraction averaged 23.3 ± 5.7 mmHg before infusion of these agents and 25.3 ± 8.6 mmHg after infusion ($p= 0.368$). Likewise, the BPI averaged 474 ± 164 mmHg*s before infusion and 282 ± 193 mmHg*s afterwards ($p= 0.159$). These findings suggest that infusion of naloxone and endomorphin 2 did not circulate to the spinal cord or brain to exert their potentiating effects.

When ASIC3 KO rats were used, there was no significant increase in pressor response and blood pressure index following naloxone and endomorphin 2 injection (Figure 2A, B), a finding which supports the hypothesis that the mu opioid agonist, endomorphin 2, directly potentiated ASIC3 activity. Tension time indices (TTIs) were significantly different only between ASIC3 KO rats ($p=0.0205$, Figure 2C), but the increase was small and did not cause an increase in the pressor responses to contraction. None of the infusions altered the cardioaccelerator responses to contraction (Figure 2C, D). At

the end of each experiment the neuromuscular blocker, pancuronium bromide, was used and the tibial nerve was electrically stimulated with the same currents, pulse width and frequency as those used to contract the triceps surae muscles. No effect on arterial pressure was observed. This finding indicated that the pressor responses to contraction were not caused by direct electrical stimulation of group III and IV axons within the tibial nerve.

Responses to static contraction in presence of oxycodone

In another group of 9 rats, the exogenous mu opioid receptor agonist, oxycodone, was substituted for endomorphin 2. In these rats, the cardiovascular reflex responses to static contraction were assessed before and after infusion of naloxone followed by oxycodone, and then after APETx2 followed by oxycodone. Overall, a significant increase over control contraction levels was observed for the peak pressor ($p=0.0035$) and BPI responses ($p=0.0286$) after infusion of naloxone and oxycodone (Figures 4A, 4B and 5A). Subsequent infusion of APETx2 and endomorphin 2 reduced both the peak pressor and BPI responses to contraction but the effect was not significantly from the responses evoked after naloxone and oxycodone injections. Analysis of the time course of these pressor responses revealed that the exercise pressor reflex evoked from triceps surae muscles in presence of naloxone and oxycodone was significantly greater than the exercise pressor reflex evoked from triceps surae muscles in presence of APETx2 and oxycodone (Figure 5B). There were no differences in the tension time indices (TTIs) and in the cardioaccelerator responses (Figure 4C, D). At the end of each experiment, there was no change in arterial pressure when the tibial nerve was stimulated after pancuronium bromide injection, confirming that the pressor responses to contraction were not caused by direct electrical stimulation of group III and IV axons within the tibial nerve.

Discussion

Endomorphin 2, a tetrapeptide, is a potent and selective mu opioid agonist that is released in the periphery by immune cells under inflammatory conditions (14). Under normal circumstances, one would expect that administration of endomorphin 2, or for that matter, oxycodone, would inhibit the responses of group III and IV muscle afferents to static contraction (17), an effect which, in turn, would decrease the magnitude of the exercise pressor reflex. After blocking mu opioid receptors with naloxone, we found that endomorphin 2, injected into the arterial supply of contracting muscles, paradoxically increased the magnitude of the reflex. We presented two lines of evidence suggesting that the paradoxical increase was due to the mu opioid-induced stimulation of ASIC3. The first line of evidence was our finding in genetically intact rats that APETx2, an ASIC3 antagonist, reduced the magnitude of the exercise pressor reflex to a level seen before endomorphin 2 was injected into the arterial supply of the triceps surae muscles. The second line of evidence was that endomorphin 2, injected into the arterial supply of the triceps surae muscles, did not potentiate the exercise pressor reflex in ASIC3 knockout rats. Our *in vivo* finding that mu opioid agonists potentiated the exercise pressor reflex by an ASIC3 dependent mechanism extends the *in vitro* findings of Farrag et al. (13) and Zaremba & Ruiz-Velasco (42). These investigators reported that both endomorphin 2 and oxycodone potentiated the responses of dorsal root ganglion cells innervating the rat's gastrocnemius muscles to acidic solution having a pH of 6.0. They also reported that endomorphin 2, infused into the arterial supply of the hindlimb, potentiated the pressor reflex evoked by bolus injections of 12 mM lactic acid having a pH of 2.81 (13).

In decerebrated rats, ASIC3 channels appear to play little, if any role, in evoking the exercise pressor reflex when contracting muscles are freely perfused. In contrast, ASIC3 channels, play a significant role in generating the exaggerated exercise pressor reflex when the contracting muscles are chronically ischemic. These conclusions are based both on findings using APETx2 to block ASIC3 channels and on findings using CRISPR-Cas9 technology to functionally inactivate ASIC3 channels (22, 36, 40). The present findings are the first to show that when mu opioid receptors are blocked that

ASIC3 channels play a role in evoking the exercise pressor reflex when the contracting muscles are freely perfused.

ASIC1a channels, in contrast to ASIC3 channels, play an important role in evoking the exercise pressor reflex when the contracting muscles are freely perfused (11). This contrast is surprising because the threshold pH needed to open either ASIC1a or ASIC3 are similar (2, 3). This conundrum may be explained by the number of channels distributed on the interstitial endings of group III and IV muscle afferents. Specifically, we speculate that when muscles are freely perfused that the number of ASIC1a channels is greater than the number of ASIC3 channels. We further speculate that when the muscles are ischemic for a prolonged period of time, the number of ASIC1a channels decreases and the number of ASIC3 channels increases. This speculation is supported by both Liu et al. (25) and Farrag et al. (13) who found that femoral arterial occlusion for three days increased ASIC3 channels in the lower lumbar dorsal root ganglia. Despite their important role in evoking the exercise pressor reflex when the contracting muscles are freely perfused, the inward current passed by ASIC1a channels when exposed to acidic saline was not potentiated by the presence of endomorphin 2 (13).

When exposed to acidic solutions, the current passed by heterologous cells transfected with the ASIC3 protein has two components, the first being a peak current and the second being a sustained current (13, 42). Exposure of these heterologous cells to either endomorphin 2 or oxycodone in the presence of acidic solutions had inconsistent effects on the peak ASIC3 current, sometimes increasing it (13), sometimes decreasing it (5, 13), and at other times having no effect on it (13). In contrast, exposure of these heterologous cells to either endomorphin 2 or oxycodone consistently increased the sustained current evoked by acidic solutions (13, 42). These findings strongly suggest that the ASIC3 sustained current potentiated by mu-opioids is the mechanism whereby endomorphin 2 and oxycodone potentiated the exercise pressor reflex in our *in vivo* experiments.

Interpretation of our findings has four limitations. First, the relationship between our findings showing that opioids can amplify the exercise pressor reflex by an ASIC3 mechanism and opioid-induced hyperalgesia needs to be viewed cautiously. In our experiments in decerebrated rats, we could not use behavioral measures to assess hyperalgesia. Nevertheless, our findings allowed us to extend the concept of opioid-induced potentiation of ASIC3 channels to the control of the circulation during exercise. Second, opioid-induced hyperalgesia is thought to occur when mu opioid receptors are internalized and, therefore, do not have access to their agonists. In our experiments, these receptors were not internalized, an effect which occurs after repetitive opioid use. Instead, we simulated internalization of mu opioid receptors by giving naloxone, an opioid receptor antagonist. Both receptor internalization and naloxone administration yield the same result, namely rendering mu opioid receptors inoperative. Third, the concentration of endomorphin 2 in the muscles' interstitial space is not known, although immunohistochemical experiments have revealed the presence of endomorphin 2 released by immune cells after treatment with inflammation causing agents (30). Consequently, we do not know if the concentration of endomorphin 2 created in the muscles' interstitial space approximated that created by inflammation or ischemia. Likewise, we do not know how the concentration of oxycodone in the muscles' interstitial space in our experiments relates to the concentration of oxycodone in the muscles' interstitial space of patients ingesting this opioid. Fourth, our findings are applicable to only male rats. We restricted our experiments to males in order to reduce variability in the magnitude of the exercise pressor reflex, which is well documented between the sexes in rats (23).

We used two approaches to demonstrate that ASIC3 was responsible for the paradoxical potentiation of the exercise pressor reflex by endomorphin 2. The first approach was pharmacological and used APETx2 to block ASIC3 (19). Although APETx2 discriminates well between ASIC3 and ASIC2 and ASIC1, it has potential off target effects because it can block voltage-gated $Na_V1.8$ sodium channels in slightly higher concentrations than that needed to block ASIC3 (4). To overcome this potential problem, we used a second approach, namely CRISPR-Cas9 technology to genetically

alter ASIC3 so that these channels were not functional. Both approaches showed that when ASIC3 were rendered non-functional by either pharmacological or genetic means, (13, 42) endomorphin 2 did not potentiate the exercise pressor reflex.

In our experiments, we found that both endomorphin 2 and oxycodone potentiated both the peak and the integrated (i.e., BPI) pressor responses to static contraction of the triceps surae muscles. In addition, the potentiating effect of endomorphin 2 on these pressor effects was attenuated significantly by APETx2, a selective ASIC3 antagonist. In contrast, the potentiating effects of oxycodone were attenuated by APETx2, but the effects for both the peak pressor response and the BPI were not statistically significant. The concentrations of endomorphin 2 and oxycodone in our experiments were equimolar so differences in the number of opioid molecules capable of binding to ASIC3 cannot explain the difference. One explanation, though speculative, might be that endomorphin 2 and oxycodone bind to different locations on the ASIC3 molecule, sites which, in turn, cause differences in how they interact with the binding with APETx2 on the ASIC3 molecule. In addition, we cannot exclude the possibility that oxycodone potentiates the exercise pressor reflex through its stimulation of another receptor besides ASIC3.

ASIC3 on the endings of group IV afferents have been implicated in transducing pain arising from inflamed, ischemic, or damaged skeletal muscles (33). Likewise, ASIC3 appear responsible for evoking opioid-induced hyperalgesia (13, 42). ASIC3, moreover, have been implicated in evoking the exaggerated metaboreflex arising when arterial blood flow to exercising muscle does not meet its metabolic demand (22, 40). In each of these pathological states, ASIC3 are believed to be stimulated by hydrogen ions released from muscle. Our findings are the first to show that mu opioid receptor agonists can exaggerate the exercise pressor reflex by an ASIC3 dependent mechanism even when the contracting muscles are freely perfused. This finding may be particularly relevant in situations where the mu opioid receptor is translocated away from the cell membrane into the cytoplasm, a situation which can occur in either addiction or persistent opioid use in the treatment of surgical pain.

Data Availability

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Disclosures

No conflicts of interest, financial or otherwise, are declared by the authors.

Author Contributions

L.A., G.P.D., J.S.K., V.R.-V.and M.P.K. conceived and designed research; L.A., J.S.K. and P.B.H. performed experiments; L.A., G.P.D., and M.P.K. analyzed data; L.A., G.P.D., V.R.-V. and M.P.K interpreted results of experiments; L.A., G.P.D. and M.P.K. prepared figures; L.A. and M.P.K. drafted manuscript; L.A., G.P.D., J.S.K., V.R.-V.and M.P.K. edited and revised manuscript; L.A., G.P.D., J.S.K., P.B.H., V.R.-V. and M.P.K. approved final version of manuscript.

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Figure Legends

Figure 1: Schematic protocol describing the timing of contractions and drug injections. SC=static contraction; N=naloxone (100 μg/kg); E2=endomorphin 2 (10 μM); Oxy=oxycodone (10 μM); A=APETx2 (200 μg/kg).

Figure 2. The authors mentioned in the methods that the control contraction was performed twice to ensure reproducibility of the pressor response to muscle contraction. Which response is shown in the figures and results? The first contraction, the second, or an average of the two?2**.** Effect of the endogenous mu opioid receptor agonist endomorphin 2 (10 μM), in the presence of naloxone (100 μg/kg) or APETx2 (200 μg/kg) on the pressor response to static contraction. Data are presented as individual (black dots) and group means (open bars for wild type rats and gray bars for ASIC3 knock out rats) for the peak increase in mean arterial blood pressure (MAP; A), blood pressure index (BPI; B), time-tension index (TTI; C), and peak heart rates (HR; D) evoked by static contraction. Baseline mean values and standard deviation (parentheses) for MAP and HR are shown below the columns. Contractions were evoked before (control; n = 10), after naloxone and endomorphin 2 (N+E2; n = 10) and after APETx2 and endomorphin 2 (A+E2; n = 10) infusion into the superficial epigastric artery. $p < 0.05$, significant difference between before and after the treatments.

Figure 3. Representative traces and time course of the pressor response to static contraction before and after naloxone (100 μg/kg) and endomorphin 2 (10 μM) or APETx2 (200 μg/kg) and endomorphin 2 (10 μM) infusions. A: representative traces of the pressor responses to contraction before (control), after naloxone and endomorphin 2 and then after APETx2 and endomorphin 2 injections in the same rat. B: averaged time course of the pressor response to contraction, which was evoked before (control, open dots), and after naloxone and endomorphin 2 (black dots) or APETx2 and endomorphin 2 (black triangles) injections (n=10).

Figure 4. Effect of the exogenous mu opioid receptor agonist, oxycodone (10 μM), in the presence of naloxone (100 μg/kg) or APETx2 (200 μg/kg) on the pressor response to static contraction. Data are presented as individual (black dots) and group means (open bars) for the peak increase in mean arterial blood pressure (MAP; A), blood pressure index (BPI; B), time-tension index (TTI; C), and peak heart rates (HR; D) evoked by static contraction. Baseline mean values and standard deviation (parentheses) for MAP and HR are shown below the columns. Contractions were evoked before (control; n = 9), after naloxone and oxycodone (N+Oxy; n = 9) and after APETx2 and oxycodone (A+Oxy; n = 9) infusion into the superficial epigastric artery. $p < 0.05$, significant difference between before and after the treatments.

Figure 5. Representative traces and time course of the pressor response to static contraction before and after naloxone (100 μg/kg) and oxycodone (10 μM) or APETx2 (200 μg/kg) and oxycodone (10 μM) infusions. A: representative traces of the pressor responses to contraction before (control), after naloxone and oxycodone and then after APETx2 and oxycodone infusions in the same rat. B: averaged time course of the pressor response to contraction, which was evoked before (control, open dots), and after naloxone and oxycodone (black dots) or APETx2 and oxycodone (black triangles) infusions (n=9).

Figure 1

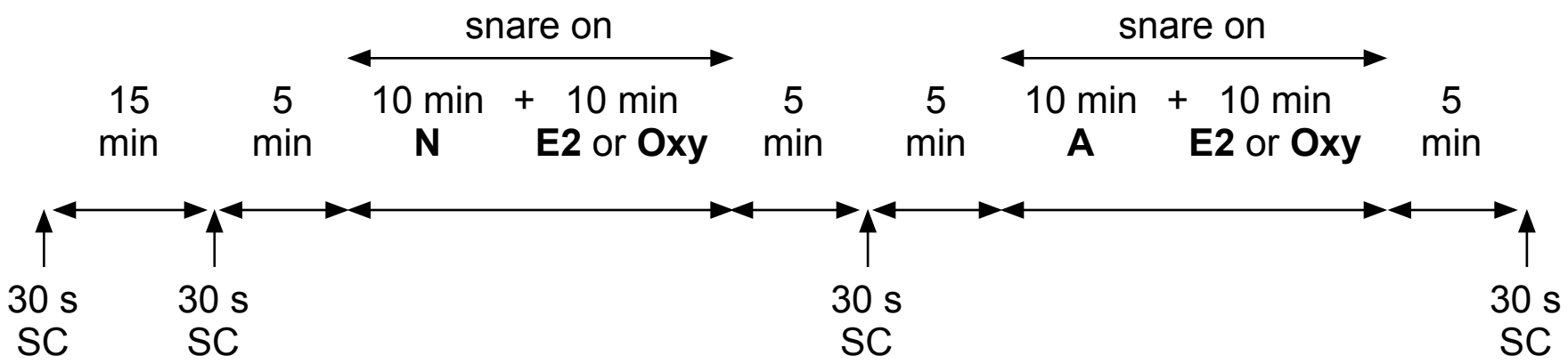


Figure 2

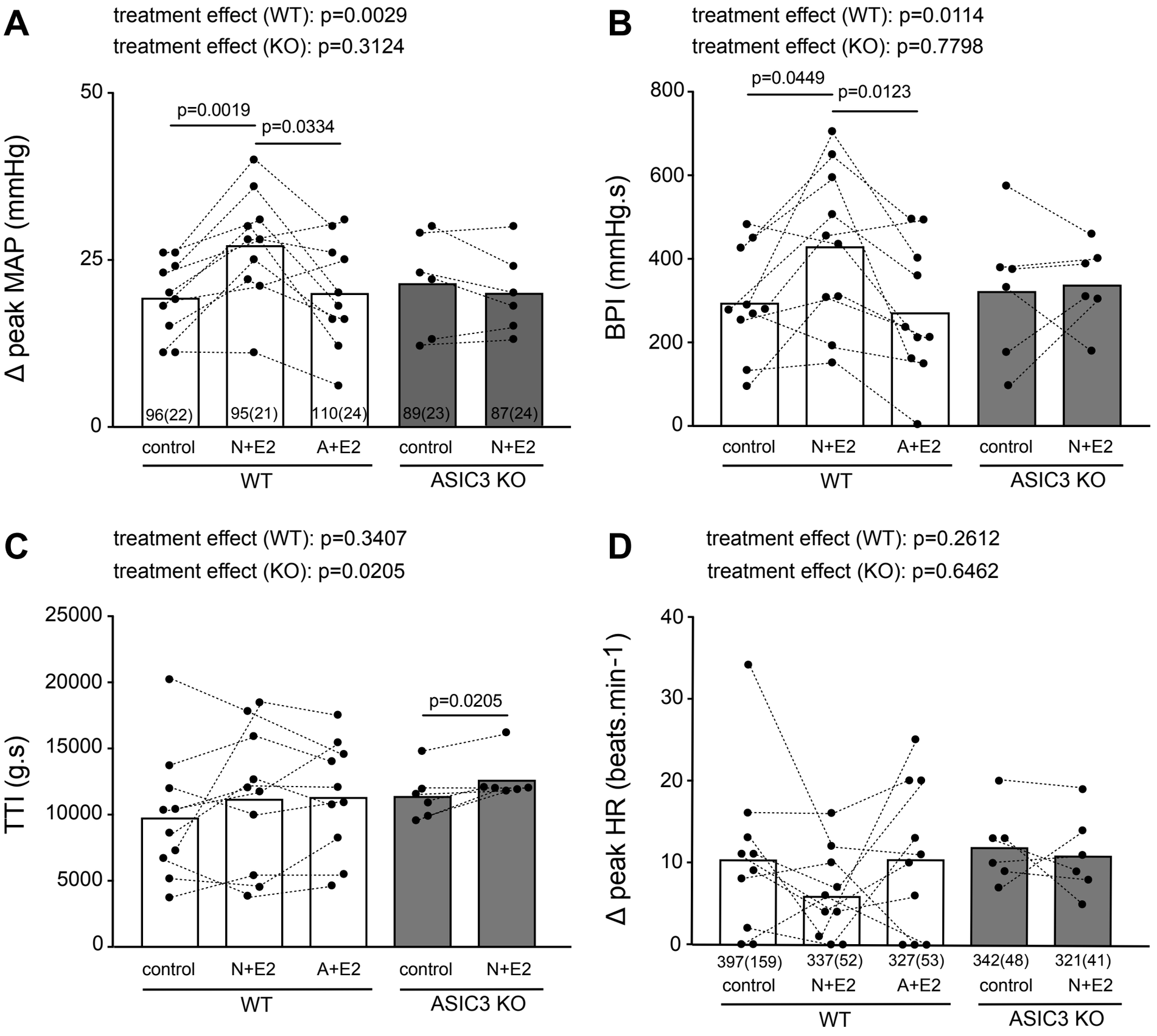
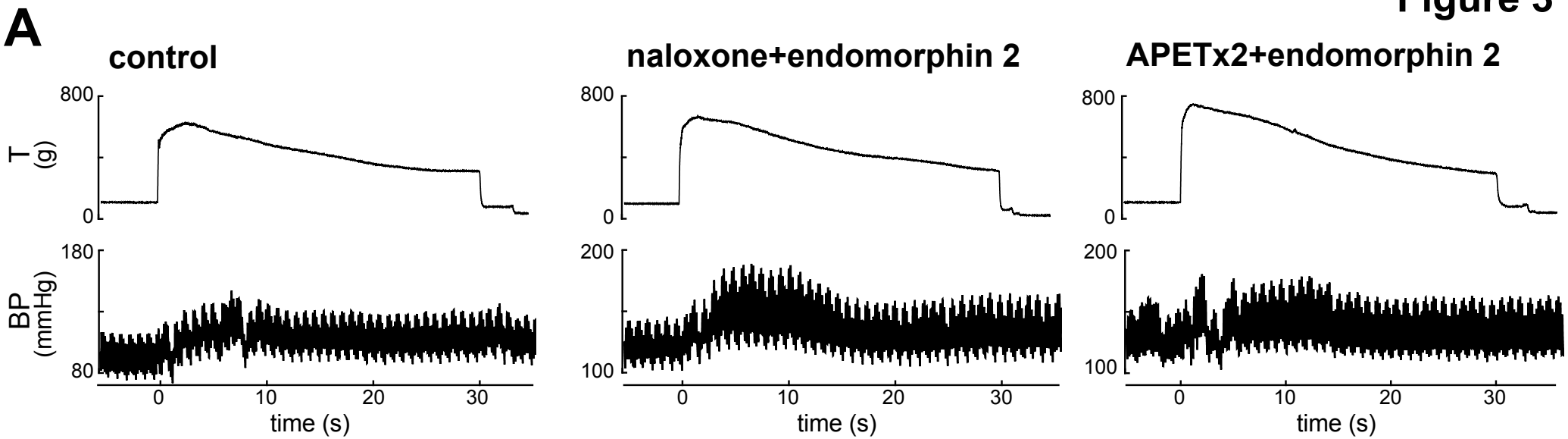


Figure 3



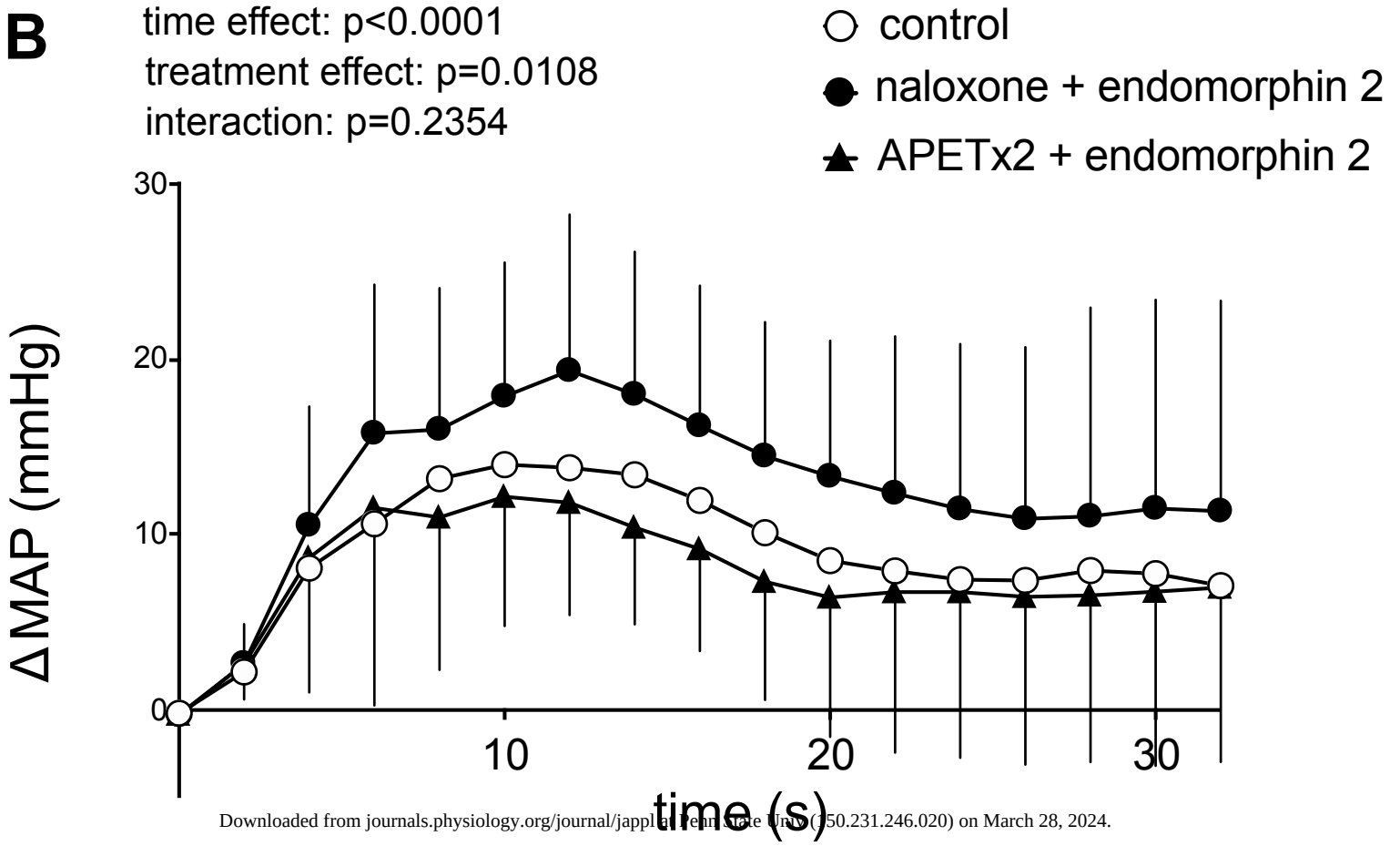


Figure 4

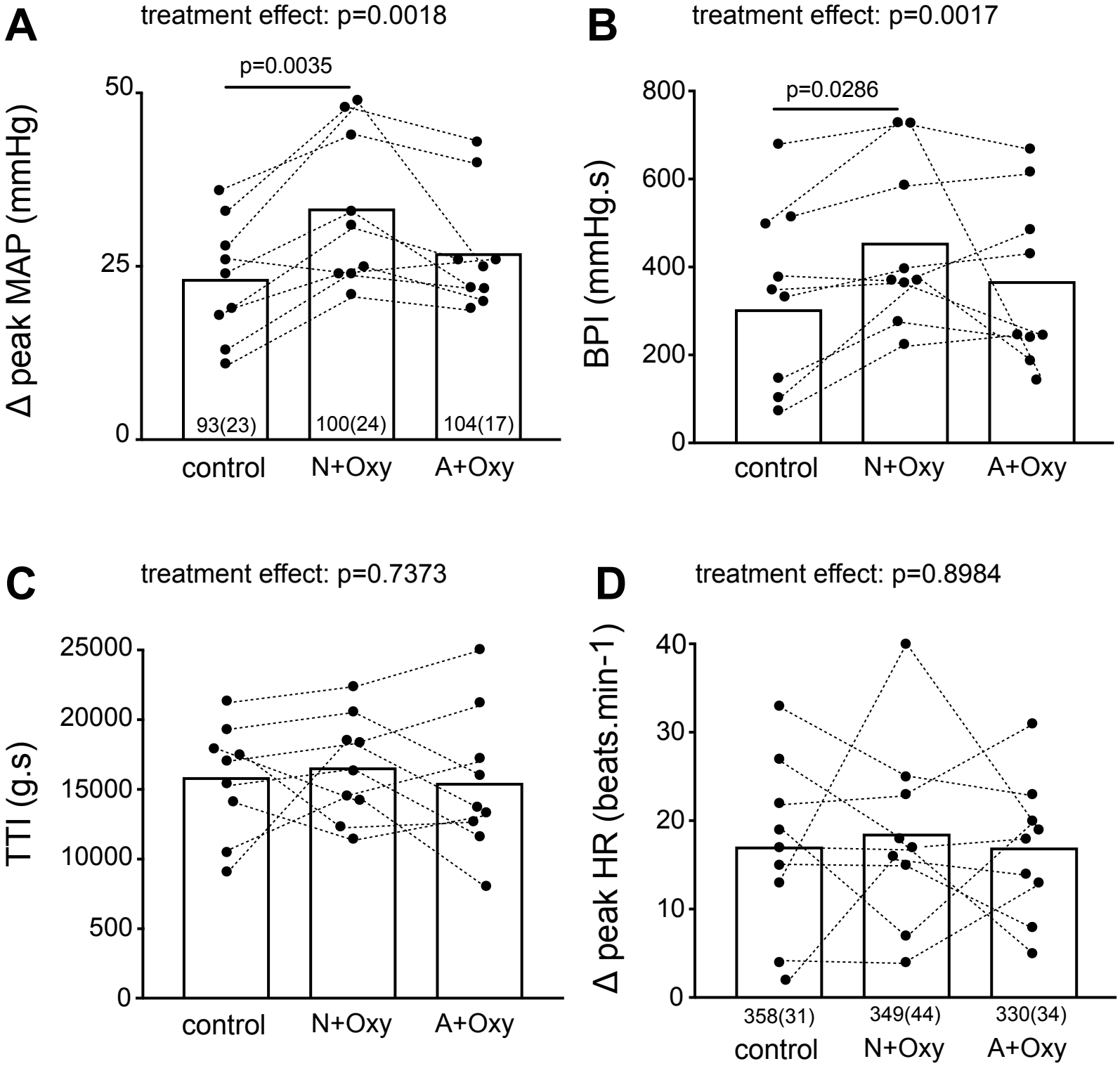
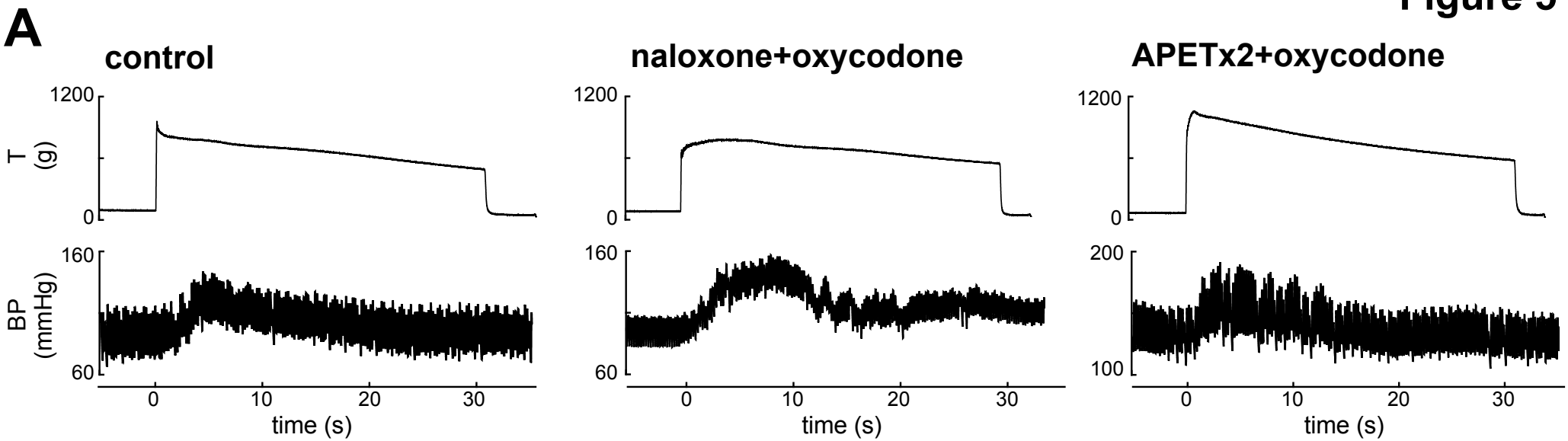
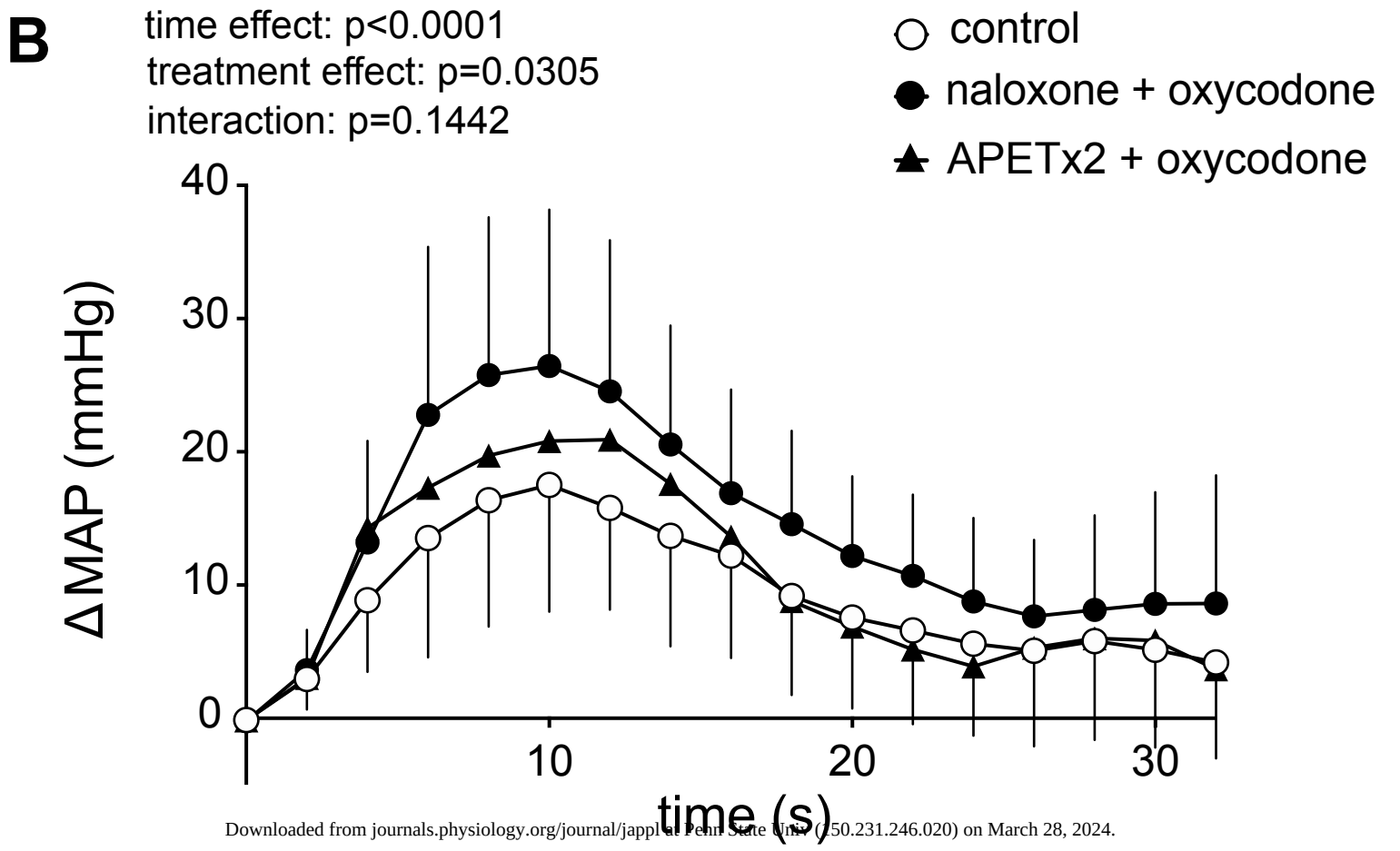


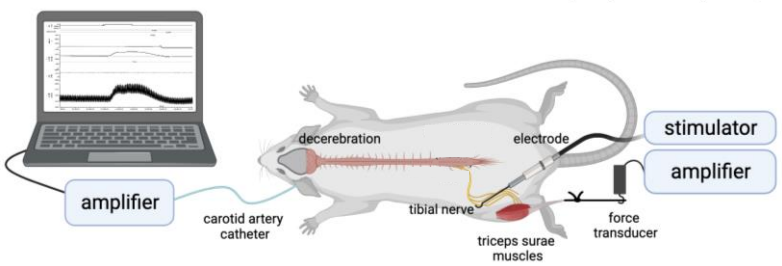
Figure 5





Paradoxical Potentiation of the Exercise Pressor Reflex by Endomorphin 2 in the Presence of Naloxone

METHODS

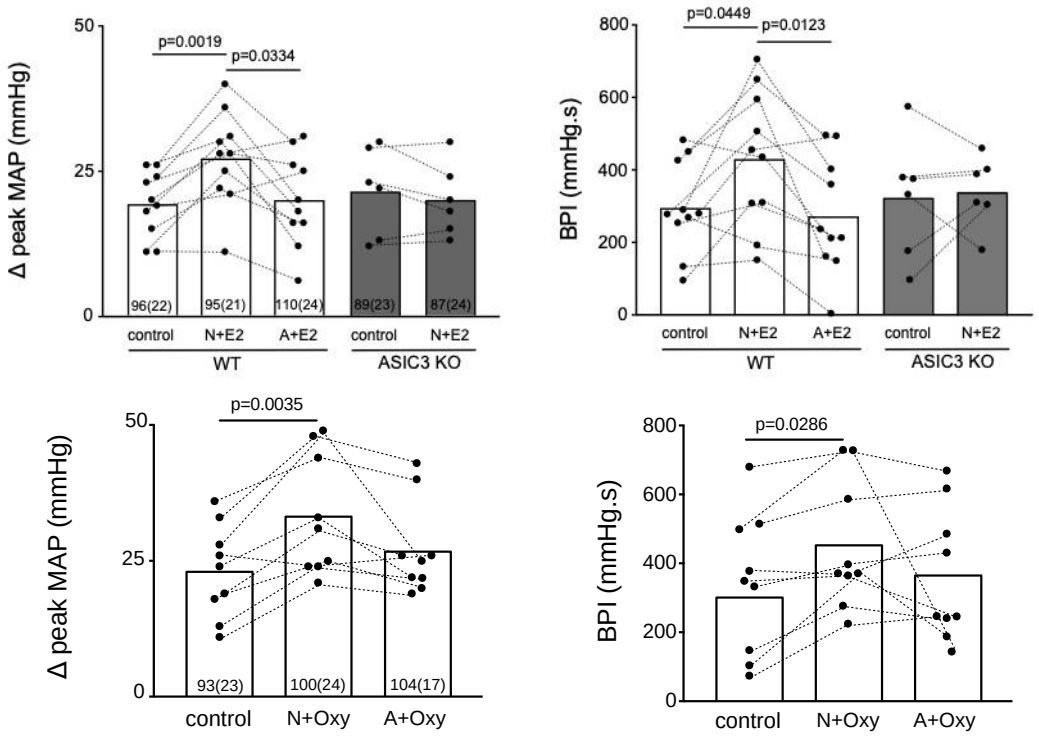


EXERCISE PRESSOR REFLEX was evoked by electrical stimulation of the tibial nerve to statically contract the triceps surae muscles

CONCLUSION

Endomorphin 2 paradoxically exaggerated the exercise pressor reflex by ASIC3 mechanism.

OUTCOME



N=naloxone; E2=endomorphin 2; Oxy=oxycodone ; A=APETx2