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

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Running title: Paradoxical Potentiation of the EPR

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Abstract

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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

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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

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

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

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Introduction

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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).

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

106

107 ASIC3 on the endings of group IV afferents are stimulated by hydrogen ions dissociated
108 from both lactic and phosphatic acid. The threshold pH that opens ASIC3 *in vitro* is 7.2
109 (3, 21), a hydrogen ion concentration that is routinely achieved in hindlimb muscles
110 when they contract (37). Recently, endomorphin 2, a naturally produced mu opioid
111 receptor agonist, has been shown to potentiate the sustained inward ASIC3 current
112 evoked by acidic solution (pH 6.0) in isolated dorsal root ganglion cells innervating the
113 gastrocnemius muscles of rats (13). More recently, this finding has been extended to
114 prescription-based mu opioid agonists, including oxycodone and fentanyl (42).

115

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

130

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

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Methods

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Ethical Approval

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

154

In vivo experimental procedures

155

Surgical procedures

156
157 On the day of the experiment, rats were anesthetized with a mixture of 3% isoflurane
158 and 100% oxygen as described previously (10). The trachea was cannulated, and lungs
159 were mechanically ventilated (model 683; Harvard Apparatus, Holliston, MA). The left
160

161 and right common carotid arteries were cannulated, one of which was used to measure
162 blood pressure (P23XL; Gould-Statham Instruments, Los Angeles, CA). Similarly, the
163 right jugular vein was cannulated to inject fluid and drugs into the systemic circulation.
164 The left superficial epigastric artery was cannulated; the tip of the cannula was
165 advanced so that it was near the junction of the superficial epigastric artery and the
166 femoral artery. A snare was placed around the femoral artery and vein approximately
167 one centimeter proximal to the cannulated superficial epigastric artery. When tightened,
168 the snare occluded the femoral circulation to the hindlimb muscles. The pelvis was
169 clamped to hold the rat securely in place. The left popliteal fossa was opened to expose
170 and isolate the tibial nerve, which was hooked with bipolar stainless-steel electrode. The
171 triceps surae muscles were isolated. The hind paw was secured to the experimental
172 table, and the head of the rat was secured using a customized stereotaxic unit. The
173 calcaneus bone was severed, and with its attached Achilles tendon, was connected to a
174 force transducer (FT03; Grass Instrument, Quincy, MA). A precollicular decerebration
175 was performed (9, 34) and all neural tissue rostral to the plane of section was removed.
176 The isoflurane anesthesia was then terminated, and the lungs were subsequently
177 ventilated with room air. Body temperature was maintained around 37°C using a heating
178 lamp. Blood arterial PO₂ (100-150 mmHg), PCO₂ (35-40 mmHg), and pH (7.35-7.45)
179 were regularly monitored and kept within the physiological range. The experimental
180 protocol was initiated after the lungs were ventilated with room air for 45-60 min. At the
181 end of the experiment, the decerebrated rats were killed by intravenous injection of 3
182 mL of a supersaturated KCl solution and the chest was opened.

183

184 *Experimental protocols*

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

192 repeated to verify that the pressor response was reproducible. The responses evoked
193 by the second contraction comprised the control data. Next, the snare on the femoral
194 artery and vein was tightened and naloxone (a mu opioid receptor antagonist, 100
195 $\mu\text{g}/\text{kg}$, Sigma-Aldrich), followed by endomorphin 2 (an endogenous mu opioid receptor
196 agonist, 10 μM , Sigma-Aldrich), were infused with a pump (10 min each) into the arterial
197 supply of the hindlimb via the superficial epigastric artery catheter. The snare was then
198 released and after 5 min the static contraction was repeated as described above. After a
199 recovery period of 10 min, APETx2 (200 $\mu\text{g}/\text{kg}$), an ASIC3 antagonist and endomorphin
200 2 (10 μM) were infused in the same manner and the contraction maneuver was
201 repeated. This protocol is shown schematically in Figure 1. To provide further evidence
202 of the role played by ASIC3 we repeated this protocol on six ASIC3 KO rats except that
203 APETx2 was not infused.

204

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

220

221 *Data Analysis*

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

236

237 *Statistical Analysis*

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

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247 **Results**

248

249 *Responses to static contraction in presence of endomorphin 2*

250

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

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

263
264 In additional three rats not used in the above experiments, we measured the peak and
265 integrated pressor (i.e., BPI) responses to contraction of the triceps surae muscles
266 before and after intravenous infusions of naloxone followed by endomorphin 2. The
267 protocol was identical to that used when we infused these agents into the superficial
268 epigastric artery. We found that intravenous infusion of naloxone followed by
269 endomorphin had no statistically significant effect on the exercise pressor reflex.
270 Specifically, the peak pressor response to contraction averaged 23.3 ± 5.7 mmHg
271 before infusion of these agents and 25.3 ± 8.6 mmHg after infusion ($p= 0.368$).
272 Likewise, the BPI averaged 474 ± 164 mmHg*s before infusion and 282 ± 193 mmHg*s
273 afterwards ($p= 0.159$). These findings suggest that infusion of naloxone and
274 endomorphin 2 did not circulate to the spinal cord or brain to exert their potentiating
275 effects.

276
277 When ASIC3 KO rats were used, there was no significant increase in pressor response
278 and blood pressure index following naloxone and endomorphin 2 injection (Figure 2A,
279 B), a finding which supports the hypothesis that the mu opioid agonist, endomorphin 2,
280 directly potentiated ASIC3 activity. Tension time indices (TTIs) were significantly
281 different only between ASIC3 KO rats ($p=0.0205$, Figure 2C), but the increase was
282 small and did not cause an increase in the pressor responses to contraction. None of
283 the infusions altered the cardioaccelerator responses to contraction (Figure 2C, D). At

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

290

291 *Responses to static contraction in presence of oxycodone*

292

293 In another group of 9 rats, the exogenous mu opioid receptor agonist, oxycodone, was
294 substituted for endomorphin 2. In these rats, the cardiovascular reflex responses to
295 static contraction were assessed before and after infusion of naloxone followed by
296 oxycodone, and then after APETx2 followed by oxycodone. Overall, a significant
297 increase over control contraction levels was observed for the peak pressor ($p=0.0035$)
298 and BPI responses ($p=0.0286$) after infusion of naloxone and oxycodone (Figures 4A,
299 4B and 5A). Subsequent infusion of APETx2 and endomorphin 2 reduced both the peak
300 pressor and BPI responses to contraction but the effect was not significantly from the
301 responses evoked after naloxone and oxycodone injections. Analysis of the time course
302 of these pressor responses revealed that the exercise pressor reflex evoked from
303 triceps surae muscles in presence of naloxone and oxycodone was significantly greater
304 than the exercise pressor reflex evoked from triceps surae muscles in presence of
305 APETx2 and oxycodone (Figure 5B). There were no differences in the tension time
306 indices (TTIs) and in the cardioaccelerator responses (Figure 4C, D). At the end of each
307 experiment, there was no change in arterial pressure when the tibial nerve was
308 stimulated after pancuronium bromide injection, confirming that the pressor responses
309 to contraction were not caused by direct electrical stimulation of group III and IV axons
310 within the tibial nerve.

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313 **Discussion**

314

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

337

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

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

347

348 ASIC1a channels, in contrast to ASIC3 channels, play an important role in evoking the
349 exercise pressor reflex when the contracting muscles are freely perfused (11). This
350 contrast is surprising because the threshold pH needed to open either ASIC1a or ASIC3
351 are similar (2, 3). This conundrum may be explained by the number of channels
352 distributed on the interstitial endings of group III and IV muscle afferents. Specifically,
353 we speculate that when muscles are freely perfused that the number of ASIC1a
354 channels is greater than the number of ASIC3 channels. We further speculate that when
355 the muscles are ischemic for a prolonged period of time, the number of ASIC1a
356 channels decreases and the number of ASIC3 channels increases. This speculation is
357 supported by both Liu et al. (25) and Farrag et al. (13) who found that femoral arterial
358 occlusion for three days increased ASIC3 channels in the lower lumbar dorsal root
359 ganglia. Despite their important role in evoking the exercise pressor reflex when the
360 contracting muscles are freely perfused, the inward current passed by ASIC1a channels
361 when exposed to acidic saline was not potentiated by the presence of endomorphin 2
362 (13).

363

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

375

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

399

400 We used two approaches to demonstrate that ASIC3 was responsible for the
401 paradoxical potentiation of the exercise pressor reflex by endomorphin 2. The first
402 approach was pharmacological and used APETx2 to block ASIC3 (19). Although
403 APETx2 discriminates well between ASIC3 and ASIC2 and ASIC1, it has potential off
404 target effects because it can block voltage-gated $Na_v1.8$ sodium channels in slightly
405 higher concentrations than that needed to block ASIC3 (4). To overcome this potential
406 problem, we used a second approach, namely CRISPR-Cas9 technology to genetically

407 alter ASIC3 so that these channels were not functional. Both approaches showed that
408 when ASIC3 were rendered non-functional by either pharmacological or genetic means,
409 (13, 42) endomorphin 2 did not potentiate the exercise pressor reflex.

410

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

425

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

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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

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

593

594 **Figure 2.** The authors mentioned in the methods that the control contraction was
595 performed twice to ensure reproducibility of the pressor response to muscle contraction.
596 Which response is shown in the figures and results? The first contraction, the second, or an
597 average of the two? 2. Effect of the endogenous mu opioid receptor agonist endomorphin
598 2 (10 µM), in the presence of naloxone (100 µg/kg) or APETx2 (200 µg/kg) on the
599 pressor response to static contraction. Data are presented as individual (black dots) and
600 group means (open bars for wild type rats and gray bars for ASIC3 knock out rats) for
601 the peak increase in mean arterial blood pressure (MAP; A), blood pressure index (BPI;
602 B), time-tension index (TTI; C), and peak heart rates (HR; D) evoked by static
603 contraction. Baseline mean values and standard deviation (parentheses) for MAP and
604 HR are shown below the columns. Contractions were evoked before (control; n = 10),
605 after naloxone and endomorphin 2 (N+E2; n = 10) and after APETx2 and endomorphin
606 2 (A+E2; n = 10) infusion into the superficial epigastric artery. $p < 0.05$, significant
607 difference between before and after the treatments.

608

609 **Figure 3.** Representative traces and time course of the pressor response to static
610 contraction before and after naloxone (100 µg/kg) and endomorphin 2 (10 µM) or
611 APETx2 (200 µg/kg) and endomorphin 2 (10 µM) infusions. A: representative traces of
612 the pressor responses to contraction before (control), after naloxone and endomorphin
613 2 and then after APETx2 and endomorphin 2 injections in the same rat. B: averaged
614 time course of the pressor response to contraction, which was evoked before (control,
615 open dots), and after naloxone and endomorphin 2 (black dots) or APETx2 and
616 endomorphin 2 (black triangles) injections (n=10).

617

618 **Figure 4.** Effect of the exogenous mu opioid receptor agonist, oxycodone (10 μ M), in
619 the presence of naloxone (100 μ g/kg) or APETx2 (200 μ g/kg) on the pressor response
620 to static contraction. Data are presented as individual (black dots) and group means
621 (open bars) for the peak increase in mean arterial blood pressure (MAP; A), blood
622 pressure index (BPI; B), time-tension index (TTI; C), and peak heart rates (HR; D)
623 evoked by static contraction. Baseline mean values and standard deviation
624 (parentheses) for MAP and HR are shown below the columns. Contractions were
625 evoked before (control; n = 9), after naloxone and oxycodone (N+Oxy; n = 9) and after
626 APETx2 and oxycodone (A+Oxy; n = 9) infusion into the superficial epigastric artery. $p <$
627 0.05, significant difference between before and after the treatments.

628
629 **Figure 5.** Representative traces and time course of the pressor response to static
630 contraction before and after naloxone (100 μ g/kg) and oxycodone (10 μ M) or APETx2
631 (200 μ g/kg) and oxycodone (10 μ M) infusions. A: representative traces of the pressor
632 responses to contraction before (control), after naloxone and oxycodone and then after
633 APETx2 and oxycodone infusions in the same rat. B: averaged time course of the
634 pressor response to contraction, which was evoked before (control, open dots), and
635 after naloxone and oxycodone (black dots) or APETx2 and oxycodone (black triangles)
636 infusions (n=9).

637

Figure 1

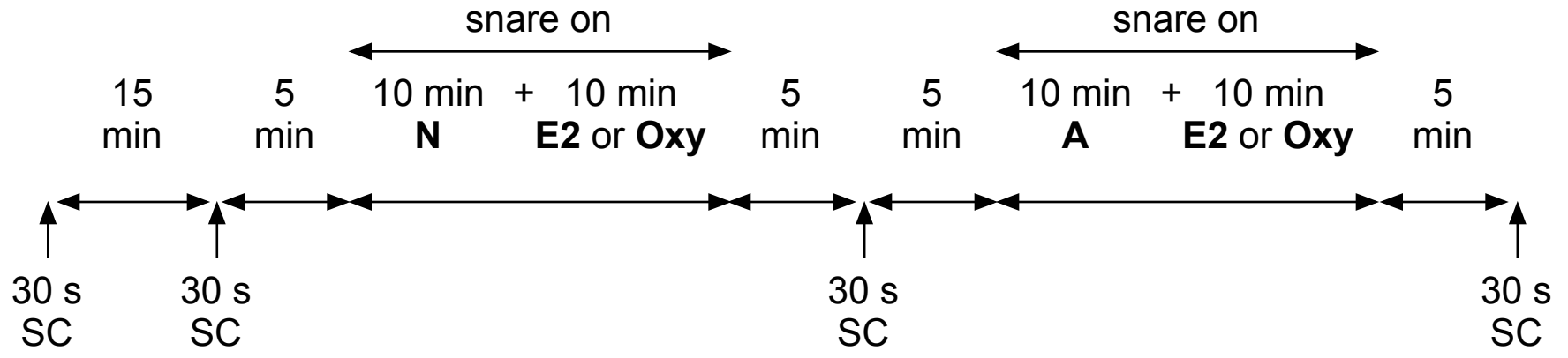
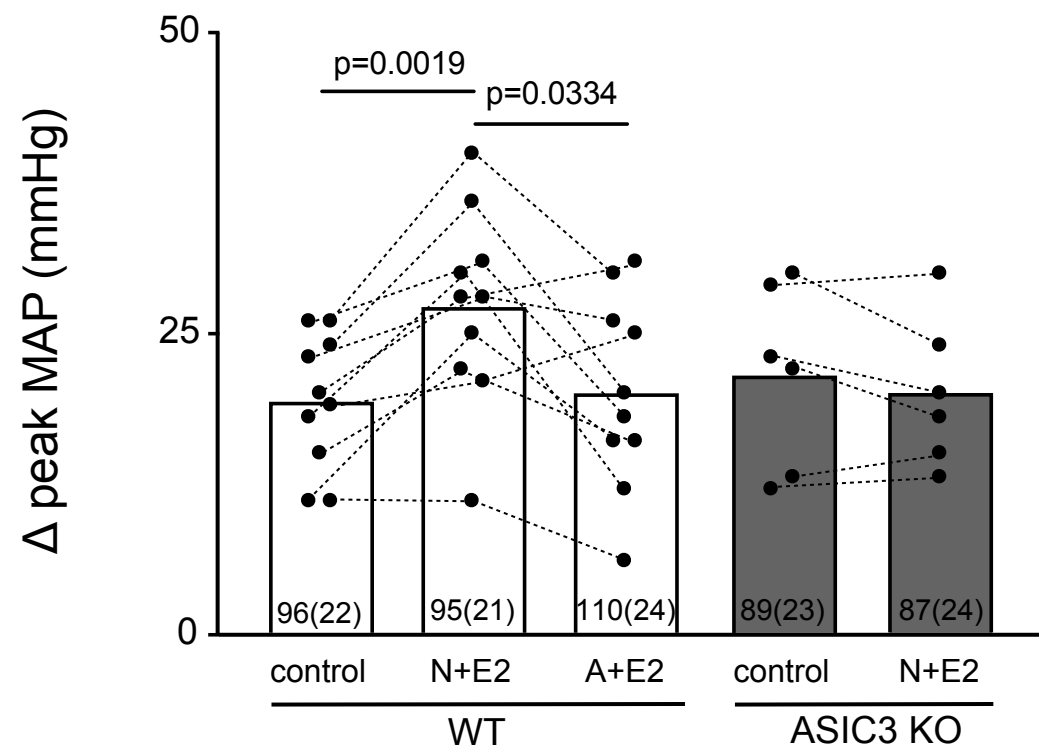


Figure 2

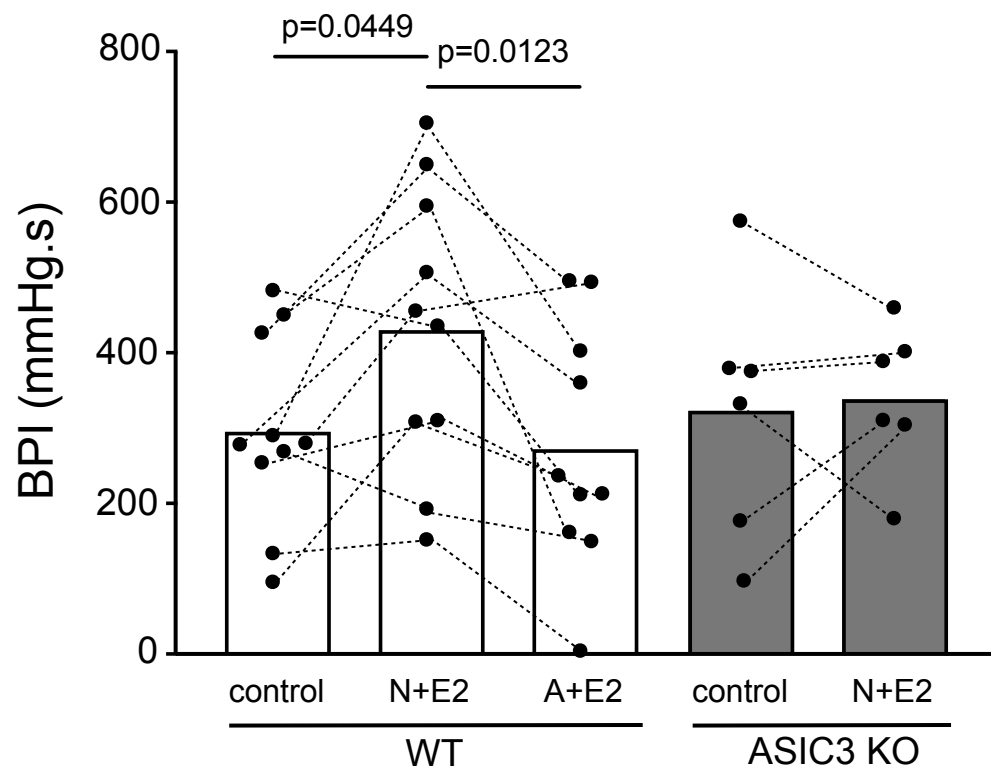
A

treatment effect (WT): $p=0.0029$
treatment effect (KO): $p=0.3124$



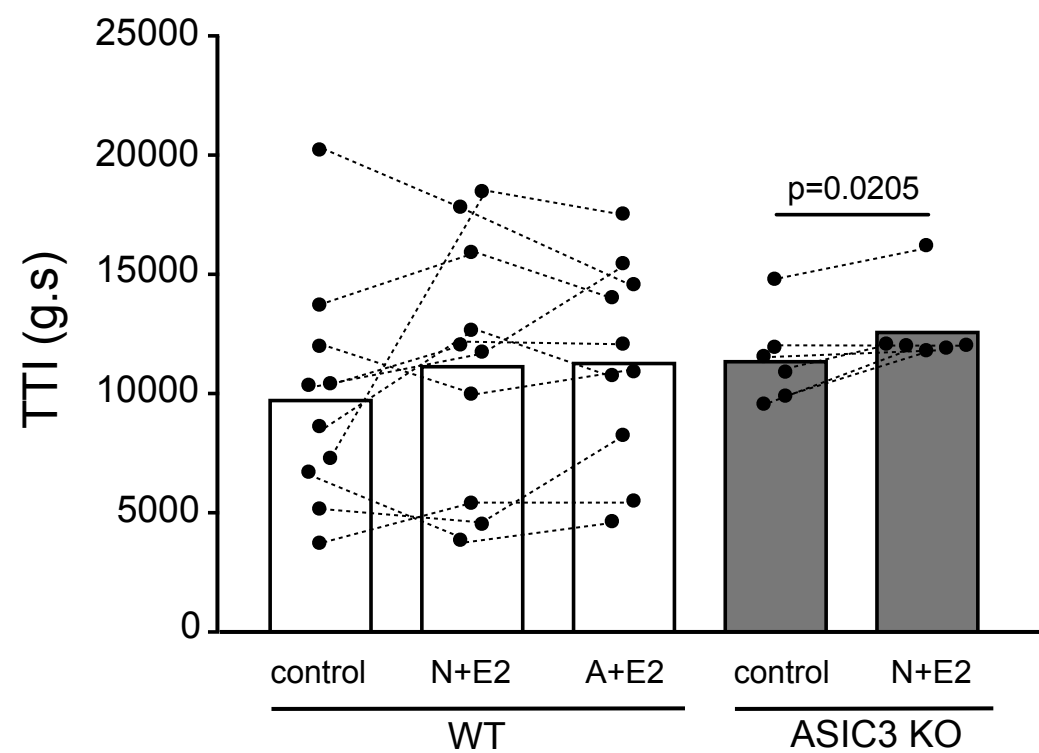
B

treatment effect (WT): $p=0.0114$
treatment effect (KO): $p=0.7798$



C

treatment effect (WT): $p=0.3407$
treatment effect (KO): $p=0.0205$



D

treatment effect (WT): $p=0.2612$
treatment effect (KO): $p=0.6462$

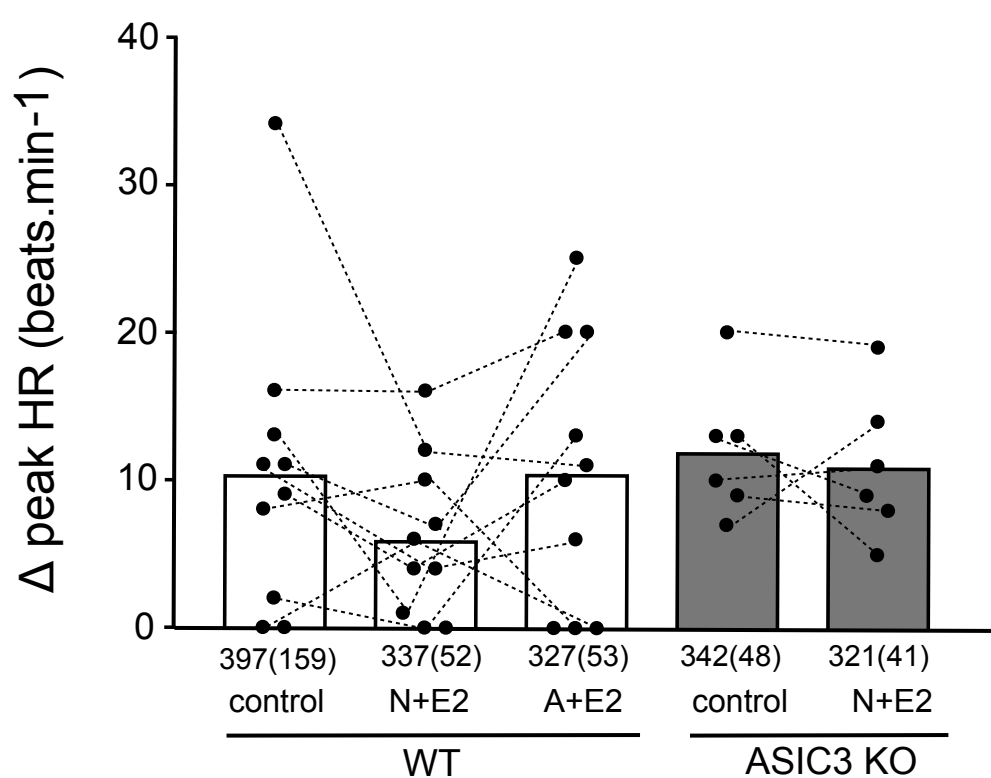
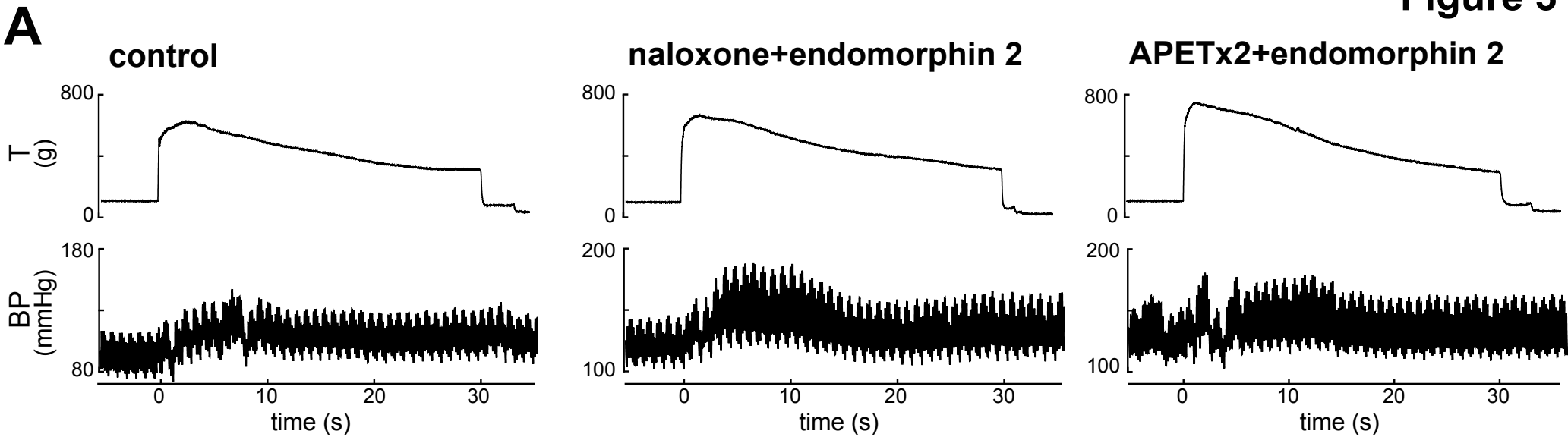


Figure 3



B

time effect: $p < 0.0001$
treatment effect: $p = 0.0108$
interaction: $p = 0.2354$

○ control
● naloxone + endomorphin 2
▲ APETx2 + endomorphin 2

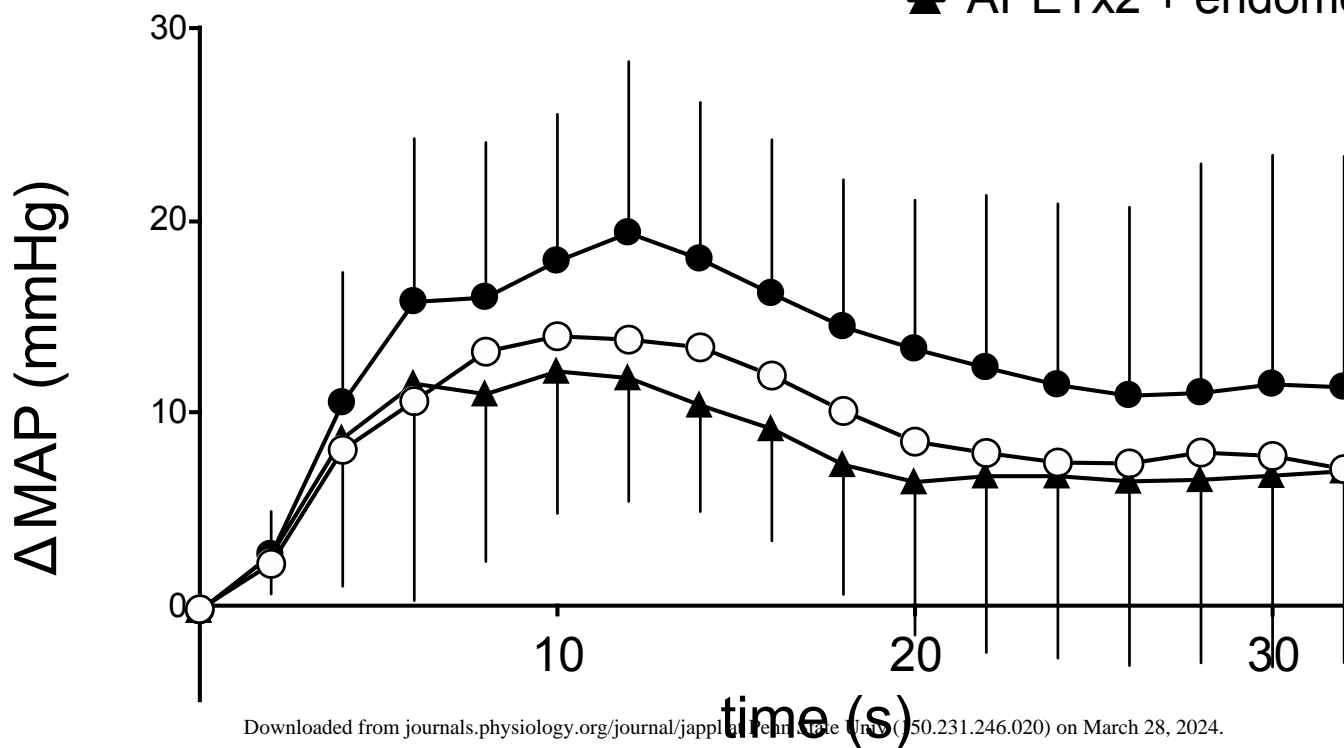
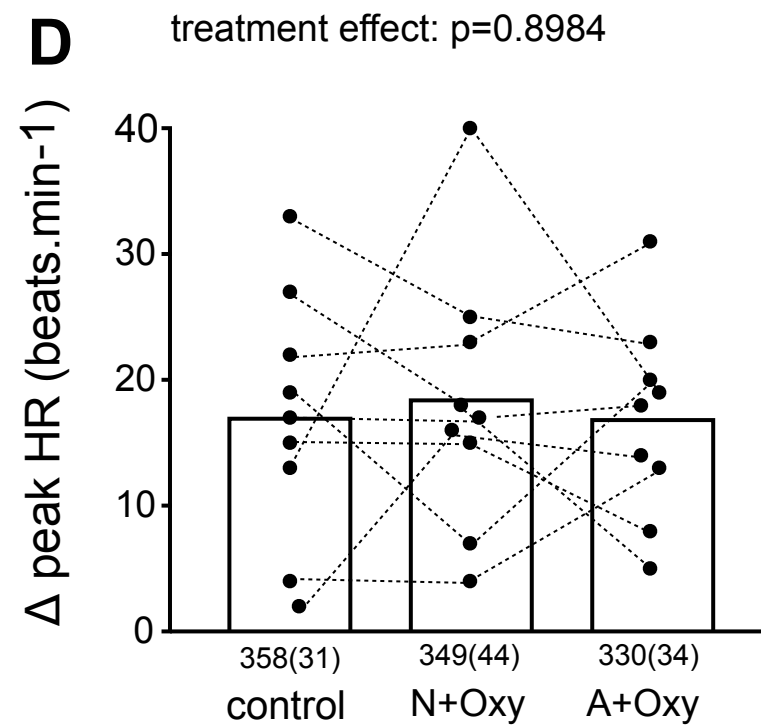
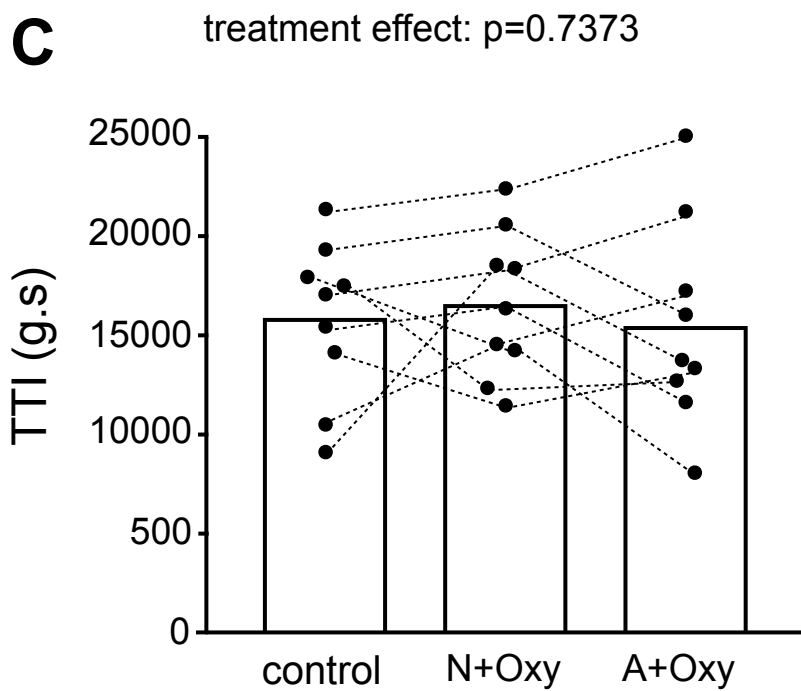
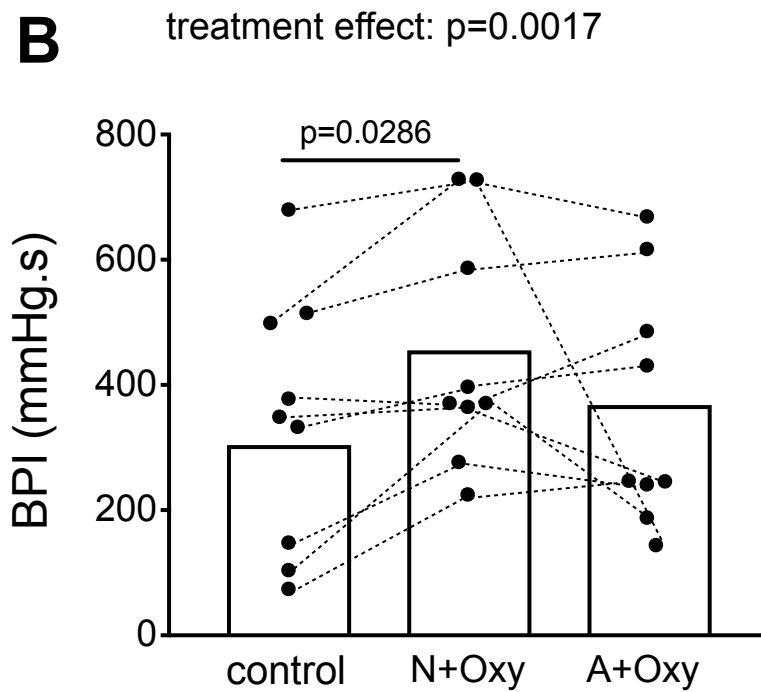
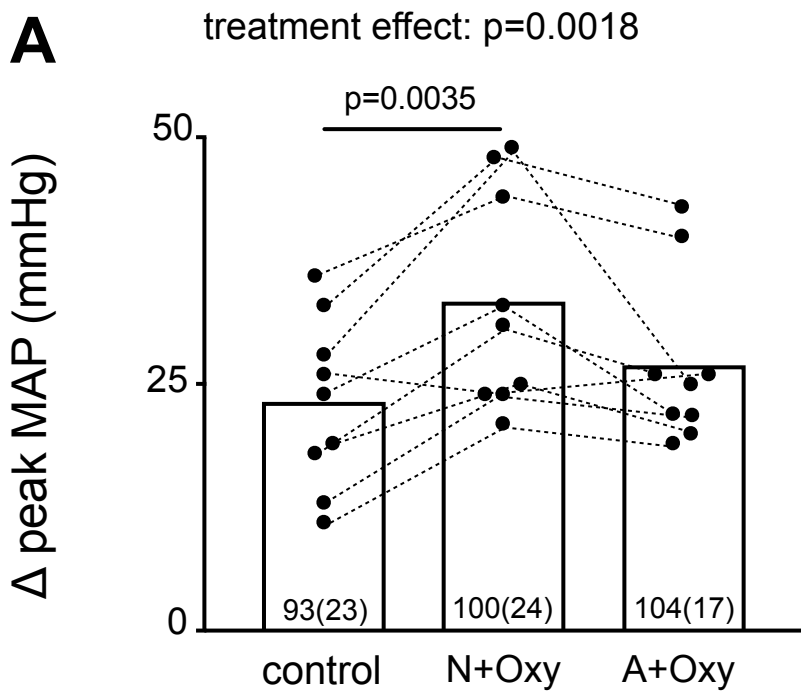
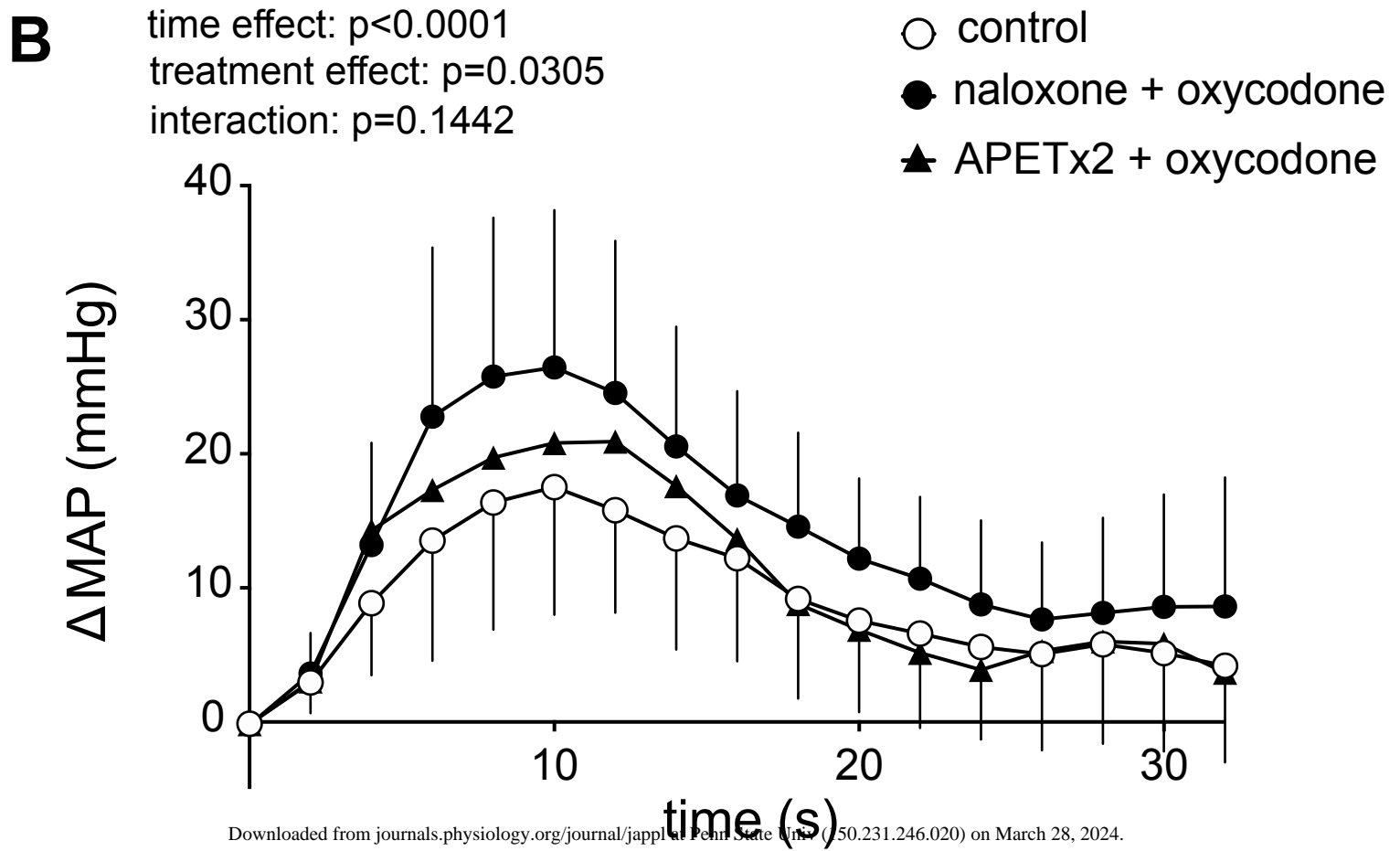
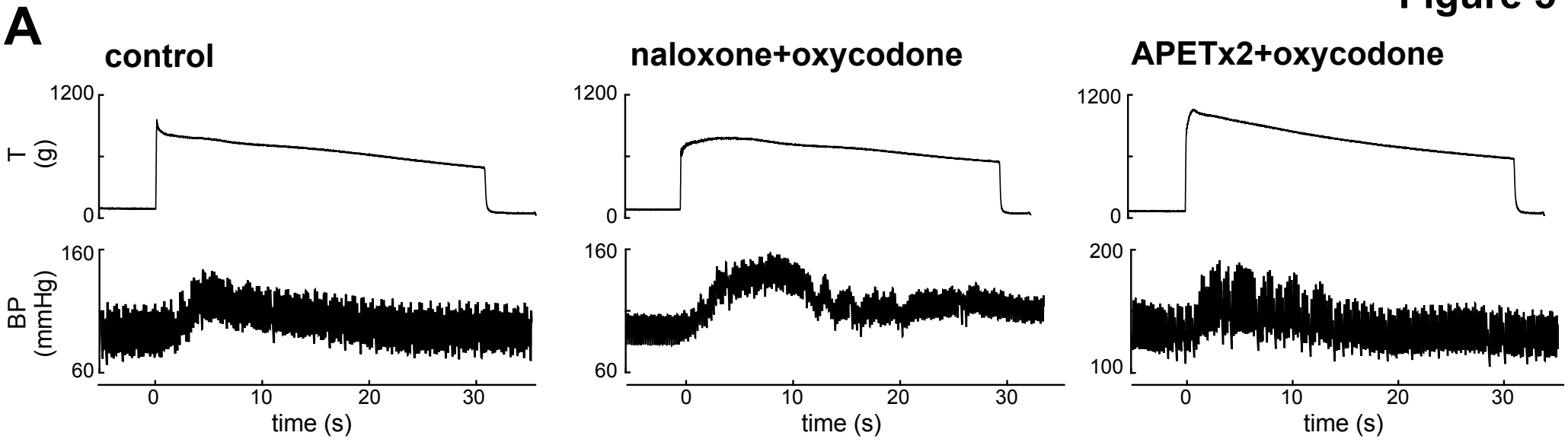


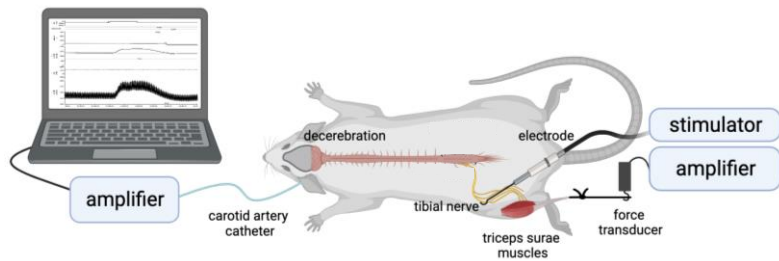
Figure 4





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

METHODS

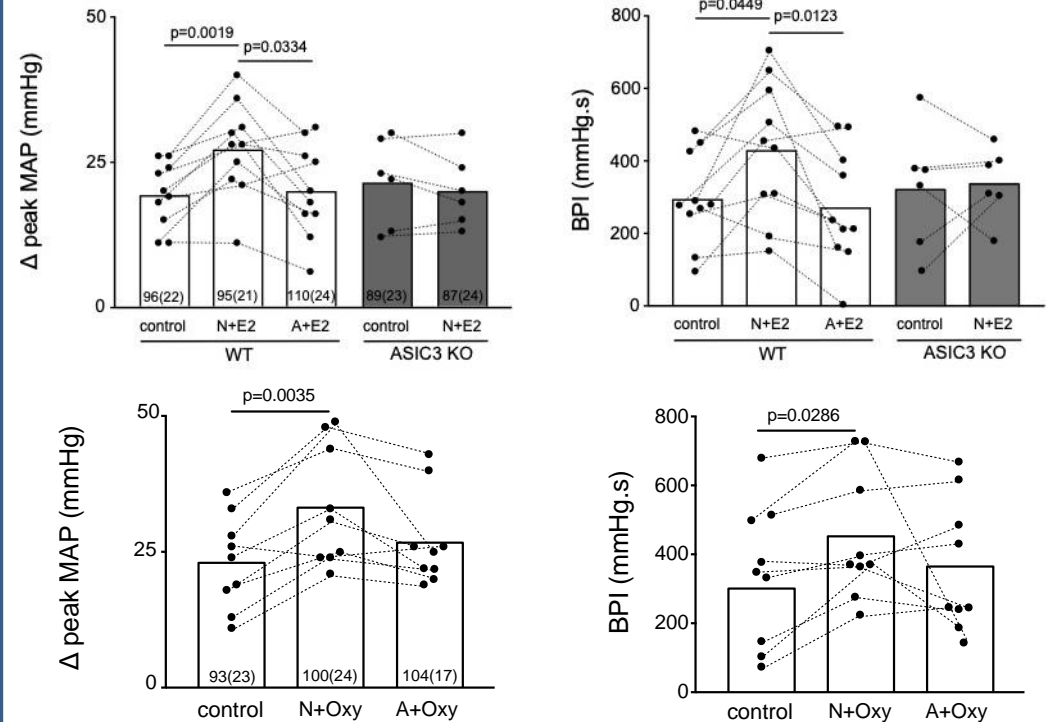


EXERCISE PRESSOR REFLEX was evoked by electrical stimulation of the tibial nerve to statically contract the triceps surae muscles

CONCLUSION

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

OUTCOME



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