

1 **Title:** Adenosine modulates extracellular glutamate levels via adenosine A<sub>2A</sub> receptors in the delayed-ethanol induced  
2 headache.

3 **Abbreviated Title:** Adenosinergic Mechanisms of Hangover Headache

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## 35 **Abstract**

36 Identifying the mechanism behind delayed ethanol-induced headache (DEIH), otherwise known as the hangover  
37 headache, may provide insight into the mechanisms behind common headache triggers. Acetate was previously shown to  
38 be the key ethanol metabolite behind DEIH in the recurrent inflammatory stimulation (IS) rat model of headache. The  
39 reversal of trigeminal sensitivity following ethanol exposure with caffeine previously suggested a role of adenosine in  
40 DEIH. To characterize this, behavioral analysis and measurement of brainstem adenosine and glutamate with  
41 microdialysis and HPLC was performed while pharmacologically manipulating adenosine signaling in the IS and  
42 Spontaneous Trigeminal Allodynia (STA) rat models of headache. Blocking adenosine  $A_{2A}$  receptor activation with  
43 istradefylline or acetate transport into astrocytes with the monocarboxylate transporter competitive inhibitor, alpha-cyano-  
44 4-hydroxycinnamate (4-CIN), prevented acetate-induced trigeminal sensitivity. Blocking adenosine  $A_1$ ,  $A_{2B}$ , and  $A_3$   
45 receptor signaling did not prevent trigeminal sensitivity. Compared to control rats, IS rats had greater increases in  
46 extracellular adenosine and glutamate within the trigeminal nucleus caudalis (TNC) of the brainstem during local acetate  
47 perfusion. Blocking transport of acetate into astrocytes with 4-CIN prevented the increase in adenosine and glutamate.  
48 Blocking  $A_{2A}$  receptor activation prevented the increase in extracellular glutamate, but not adenosine in the TNC. These  
49 data are the first to demonstrate the physiological consequence of acetate on adenosinergic systems within trigeminal pain  
50 by suggesting that acetate-induced trigeminal sensitivity in DEIH is mediated by adenosine  $A_{2A}$  receptor activation which  
51 modulates extracellular glutamate levels in the TNC.

## 52 **Significance Statement**

53 It is unknown how several common headache triggers induce headache pain. Since migraineurs are more sensitive to these  
54 triggers, studying the mechanisms behind their effects may reveal unique migraine pathophysiology. In this study, we  
55 explored the common headache trigger, ethanol, which migraineurs are particularly sensitive to. When ethanol is ingested,  
56 its quickly metabolized to acetaldehyde and subsequently into acetate. We find that acetate increases brainstem adenosine  
57 and causes trigeminal sensitivity, which is exacerbated in the rat headache model. Blocking either acetate uptake or  
58 adenosine signaling prevents trigeminal sensitivity and brainstem glutamatergic signaling, suggesting that adenosine is  
59 involved in the hangover headache and that differences in acetate metabolism may account for the increased sensitivity to  
60 ethanol in migraineurs.

62 **Keywords:** headache, migraine, adenosine, ethanol, trigeminal, hangover headache, acetate, glutamate

63 **Abbreviation:** Inflammatory stimulation (IS), inflammatory stimulation model (IS rats), spontaneous trigeminal allodynia  
64 model (STA rats), Delayed ethanol-induced headache (DEIH), Trigeminal nucleus caudalis (TNC)

## 65 INTRODUCTION

66 Alcohol's capacity to induce the delayed ethanol-induced headache (DEIH), or hangover headache, has been  
67 recognized for at least nineteen-hundred years, yet the mechanism behind this headache trigger is poorly understood  
68 (Dueland, 2015; Leith, 2015; Panconesi, 2016). Migraineurs experience more severe headaches with much less alcohol  
69 than non-migraineurs, suggesting that while the physiology responsible for DEIH is common, underlying pathophysiology  
70 is uniquely present in migraine patients (Anon, 2004). Understanding these mechanisms may reveal pathological systems  
71 unique to amongst migraineurs and potentially targetable. We used two rat models of trigeminal sensitivity with migraine-  
72 like features to study this common form of headache.

73 It was previously shown that a rat model of trigeminal sensitivity is susceptible to ethanol-induced trigeminal  
74 sensitivity (Maxwell et al., 2010). Similar to DEIH in humans, these rats experience increased trigeminal sensitivity 4-6  
75 hours following ethanol treatment (Anon, 2004). Ethanol is metabolized into acetaldehyde which is then quickly  
76 metabolized into acetate (Zimatkin et al., 2006; Jiang et al., 2013). Since ethanol and acetaldehyde are both absent during  
77 the onset of pain, it was suggested and later confirmed that acetate was the key ethanol metabolite responsible for DEIH in  
78 this rat model (Tsukamoto et al., 1989). This study also illustrated that caffeine, a non-specific adenosine receptor  
79 antagonist, could prevent ethanol-induced trigeminal sensitivity, suggesting that adenosine played a role in DEIH.

80 In the brain, acetate is preferentially utilized by astrocytes where its converted into acetyl-coenzyme A in an ATP-  
81 dependent reaction, resulting in a residual AMP molecule that's readily converted into adenosine and released to act at  
82 purinergic receptors throughout the brain (Carmichael et al., 1991; Pascual et al., 2005; Panatier et al., 2011; Zorec et al.,  
83 2012; Jiang et al., 2013). Serum-adenosine levels increase after ethanol ingestion in humans (Nagy, 1992) and  
84 extracellular adenosine concentrations increase in rats during microdialysis perfusion of ethanol in the brain (Sharma et  
85 al., 2010). In fact, the purinergic system has been hypothesized to play a role in migraine and pain (Cieślak et al., 2015;  
86 Fried et al., 2017).

87 Similar to migraine headache, DEIH includes throbbing pain with phono- and photophobia that is exacerbated  
88 with physical movement (García-Azorín et al., 2020). The headache phase of migraine is thought to be caused by  
89 activation of dural C and A $\delta$  nociceptors of the trigeminal ganglion that project to the trigeminal nucleus caudalis (TNC)  
90 in the brainstem (Burstein et al., 2011). Two rat models of chronic trigeminal sensitivity with migraine-like characteristics  
91 currently exist to study migraine pathophysiology. The recurrent inflammatory stimulation model (IS rats) is induced by  
92 infusing inflammatory agents onto the dura through an affixed cannula 3x/week for four weeks (Oshinsky and  
93 Gomonchareonsiri, 2007). These animals transition to a steady-state of chronic trigeminal sensitivity that outlasts the final  
94 infusion for months. The spontaneous trigeminal allodynia model (STA rats) features inheritable primary trigeminal  
95 allodynia (Oshinsky et al., 2012; Munro et al., 2018). Similar to migraineurs who experience a combination of headache-  
96 free and headache days with fluctuating intensity, these animals feature episodically fluctuating trigeminal sensitivity. The  
97 goal of using two different rodent models of headache, featuring different sources of trigeminal pain/sensitization, is to  
98 demonstrate that the role of adenosine in DEIH is independent of the particular features of these individual models.

99 We investigated acetate modulation of the adenosinergic system in the IS and STA rat models to explore the  
100 mechanisms behind DEIH. To determine the adenosine receptor subtype responsible for this form of trigeminal  
101 sensitivity, we pharmacologically manipulated adenosine receptor signaling during acetate treatment while measuring  
102 trigeminal sensitivity behaviorally. We then used microdialysis and HPLC to measure adenosine, glutamate, and  
103 glutamine in response to perfusion of acetate in the TNC. Our data support the hypothesis that acetate derived from  
104 ethanol metabolism induces accumulation of extracellular adenosine which produces trigeminal sensitivity through  
105 modulation of glutamateric signaling via adenosine A<sub>2A</sub> receptors within the TNC. This study is the first to identify  
106 physiological consequences of acetate on adenosinergic systems within the trigeminal pain system.

## 107 **MATERIALS AND METHODS**

108 A combination of male Sprague Dawley rats from Charles River (used to induce the IS model) and an in-house  
109 colony originating from Charles River (STA rats) (250-300 g, n = 168) were housed individually in a temperature-  
110 controlled environment under a 12-hour light/dark cycle. Animals were allowed access to food and water ad libitum. All  
111 procedures performed on the animals were approved by the Thomas Jefferson University Institutional Animal Care and  
112 Use Committee. Efforts were made to minimize animal numbers and suffering.

### 113 **Induction of the Inflammatory Soup Rats**

114 **Cannula implantation:** Surgical procedures for the inflammatory stimulation model were previously described  
115 (Oshinsky and Gomomchareonsiri, 2007). Briefly, two weeks of habituation and training were performed prior to putting  
116 the rats under isoflurane anesthesia (3% induction, 1.5% maintenance) mixed with compressed air. A 3 mm wide  
117 craniotomy was performed above the junction of the superior sagittal and transverse sinuses to expose the dura. A plastic  
118 cap and stainless steel cannula (26 gauge, Plastics One Inc., Roanoke, VA, USA) was secured to the skull with a  
119 combination of small screws and dental cement. This was then sealed with an obturator that was custom cut to extend just  
120 beyond the internal end of the cannula above the dura to prevent scar tissue formation that could obstruct the flow of  
121 compounds onto the dura. Rats recovered from surgery for one week, during which trigeminal pressure thresholds and  
122 weight were monitored to ensure the return to baseline levels.

123 **Inflammatory stimulation:** Inflammatory stimulation of the dura with 0.1 mM prostaglandin E2 in 0.9% sterile  
124 saline (Sigma Aldrich, St Louis, MO) was performed in their home cage. Polyethylene tubing (PE50) was connected to a  
125 30  $\mu$ l Hamilton syringe and the exposed cannula. 25  $\mu$ l of the IS was steadily infused over 5 minutes at an approximate  
126 rate of 5  $\mu$ l per minute. Infusions were performed 3x/wk for a total of 12 infusions. Rats that develop chronic trigeminal  
127 sensitivity (< 4 g thresholds) during this infusion period that outlasts the final infusion for at least 1 week were used in  
128 these studies.

### 129 **Spontaneous Trigeminal Allodynia Rats**

130 The spontaneous trigeminal allodynia rats in this paper are an in-house colony originating from Charles River  
131 representing the 17<sup>th</sup> and 18<sup>th</sup> generation of an inbred line of rats (Oshinsky et al., 2012). These animals experience  
132 spontaneous trigeminal allodynia similar to how migraineurs experience headache and headache-free days without the  
133 need for surgical or experimental manipulation. STA rats do not experience sensitivity in any area outside of the  
134 trigeminal region. These rats are monitored each day to assess their morning periorbital thresholds. Experiments are  
135 conducted on days when rats have morning thresholds less than 4 g.

### 136 **Tactile Sensory Testing**

137 Rats, tested during the day, were trained and acclimated to a plastic tube restraint (inner diameter 8 cm, length 25  
138 cm) before and after cannula implantation. Rats entered uncoaxed into this atraumatic restrainer to prevent the rats from  
139 walking away during sensory testing.

140 Periorbital pressure thresholds were assessed by applying von Frey monofilaments (Stoelting Co., Wood Dale, IL,  
141 USA) to both the left and right sides of the face over the medial portion of the eye. These von Frey hairs are calibrated  
142 nylon monofilaments that generate a reproducible buckling stress. Each monofilament is identified by manufacturer-  
143 assigned force values (10, 8, 6, 4, 2, 1.4, 1, 0.6, 0.4, 0.16, 0.07, 0.04, 0.02, 0.008 grams). The higher the value on the  
144 monofilament, the stiffer and more difficult it is to bend. For each time point, the right and left threshold data are recorded  
145 individually. The von Frey stimuli were presented in sequential descending order to determine the threshold of response.  
146 Several behaviors presented by the rat were considered a positive response: vigorously stroking its face with the ipsilateral  
147 forepaw, quickly recoiling its head away from the stimulus, or vocalizing. A force value is considered positive if the rat  
148 responds in a positive way to 2 of 3 trials of the von Frey monofilament. After a positive response, a weaker stimulus was  
149 presented. The threshold is considered the lowest force value that produces a positive response (2 out of 3 trials). Results  
150 are presented as the proportional change in periorbital threshold from baseline levels. Rats that did not respond to the 10 g  
151 stimulus were assigned 10 g as their threshold for analysis.

## 152 **Drug Treatment**

153 After rats were confirmed to have developed chronic trigeminal sensitivity for at least 1 week following the last  
154 infusion of IS, animals were treated with ethanol, acetate, adenosine, adenosine antagonists/agonists,  $\alpha$ -Cyano-4-  
155 hydroxycinnamic acid, or combination thereof at effective concentrations found throughout the literature. Rats had free  
156 access to water and standard rodent chow throughout the duration of all experiments. Periorbital thresholds were assessed  
157 prior to each treatment to establish baseline sensitivity. Periorbital thresholds were assessed hourly for 8 hours following  
158 ethanol (300 mg/kg i.p. Sigma, St Louis, MO) administration as previously performed (Maxwell et al., 2010). Periorbital  
159 thresholds were assessed each hour for 5 hours following acetate (60 mg/kg i.p. Sigma, St Louis, MO) administration.  
160 Caffeine (50 mg/kg i.p. Sigma, St Louis, MO) was administered 1.5 hours after acetate treatment as previously performed  
161 (Maxwell et al., 2010).

162 Adenosine receptor subtype-specific antagonists were administered [3mg/kg DPCX p.o., adenosine A<sub>1</sub> receptor  
163 antagonist, Tocris, Bristol, UK] [0.1 mg/kg istradefyline p.o., adenosine A<sub>2A</sub> receptor antagonist, Tocris, Bristol, UK]  
164 [12.5 mg/kg alloxazine p.o., adenosine A<sub>2B</sub> receptor antagonist, Sigma, St Louis, MO] [1 mg/kg MRS-1523 p.o.,  
165 adenosine A<sub>2b</sub> receptor antagonist, Sigma, St Louis, MO] 1.5 hours after acetate treatment in individual experiments.  $\alpha$ -

166 Cyano-4-hydroxycinnamic acid o.g. (4-CIN) (100 mg/kg) was administered 30 minutes prior to acetate treatment to  
167 ensure this competitive inhibitor was at the site of action before acetate.

168 To determine the local effects of adenosine receptor signaling on trigeminal pain, compounds were directly  
169 injected into the cisterna magna, an opening of the subarachnoid space which allows for direct access to the region of the  
170 brainstem which contains the trigeminal nucleus caudalis (TNC). Intracisternal injection of capsaicin induces c-Fos  
171 activation primarily in the TNC, validating the use of these injections to target the region of the brainstem containing the  
172 TNC (Mitsikostas et al., 1998, 1999). 10  $\mu$ l of 12.5  $\mu$ M adenosine (Sigma, St Louis, MO) or aCSF was injected into the  
173 cisterna magna. To target centrally acting  $A_{2A}$  receptors within the TNC 10  $\mu$ l of 300  $\mu$ M CV-1808 [adenosine  $A_{2A}/A_{2B}$   
174 receptor agonist, Tocris, Bristol, UK] was injected into the cisterna magna 30 minutes following administration of  
175 alloxazine (12.5 mg/kg p.o.). Dose and time-points for each compound were selected based on literature and pilot studies.

#### 176 **Acetate perfusion and microdialysis sampling of the trigeminal nucleus caudalus**

177 EiCOM CX-I-12-01 brain probes (EiCOM USA, San Diego, CA) with a 12 mm guide cannula length, 1 mm  
178 artificial cellulose membrane length, 50,000 Dalton cutoff, and a 0.22 mm outer diameter had their individual recovery  
179 rates determined prior to the experiment with adenosine, glutamate and glutamine standards (Sigma Aldrich, St Louis,  
180 MO). Recovery rates varied between 5-10% for adenosine. Probes were placed into the trigeminal nucleus caudalis (TNC)  
181 of anesthetized rats -2.6 to -2.9 mm from obex and 1.7 to 1.9 mm lateral to the midline. The probe's inlet was connected  
182 with PE10 tubing to a CMA 110 Liquid Switch and then to a 2.5 mL glass syringe mounted on a CMA/100 microinjection  
183 pump. The probe's outlet was connected to a CMA 170 refrigerated fraction collector (CMA Microdialysis AB, North  
184 Chelmsford, MA, USA) with PE10 tubing. The dialysis system was perfused at 0.7  $\mu$ L/min with sterile, pyrogen-free  
185 artificial extracellular spinal fluid (aCSF; composition in mM/L: 135 NaCl; 3 KCl; 1 MgCl<sub>2</sub>; CaCl<sub>2</sub>; pH = 7.2). Probes  
186 were inserted into anesthetized animals 2.5 hours prior to experiment to allow neurotransmitters to settle to baseline  
187 levels. Baseline levels of adenosine, glutamate, and glutamine were confirmed to occur after 2 hours following probe  
188 insertion with no statistical change in neurotransmitter levels for the next 6 hours (n = 4). Samples were collected  
189 continuously every 20 minutes and collection began immediately after probe insertion.

190 Experimental concentrations were calibrated for each probe by using the recovery rate to calculate the acetate  
191 concentration needed to obtain an extracellular concentration of 2 mM, 10 mM, 20 mM, or 40 mM acetate in the TNC  
192 during perfusion (i.e., if 5% recovery rate, then perfusate contained 40 mM of acetate to achieve 2 mM of acetate in the



193 TNC). The average recovery rate across all probes used was  $6.1 \pm 0.4\%$ . Syringes of aCSF containing these calculated  
194 levels of acetate (Sigma-Aldrich, St Louis, MO) were mounted onto the microinjection pump. Three samples (i.e. 60  
195 minutes of collection) were obtained in a sequentially step-wise increasing manner for each concentration by switching  
196 the inlet lines with the CMA 110 Liquid Switch, accounting for dead space of tubing.

197 Similar calculations using the recovery rate for each probe were used for experiments containing 4-CIN (Sigma-  
198 Aldrich, St Louis, MO) and istradefylline (Tocris, Bristol, UK) within the perfusate. For these experiments, aCSF  
199 containing sufficient concentrations to achieve 200  $\mu\text{M}$  4-CIN or 5 nM of istradefylline was perfused for 40 minutes prior  
200 to acetate exposure. These concentrations of 4-CIN and istradefylline were sustained throughout the entire experiment as  
201 they were included with acetate concentrations necessary to obtain 10 mM and 40 mM local acetate concentrations in the  
202 TNC.

203 At the end of the experiment, animals were administered 0.5 mL of Euthasol via i.p. injection. The probe was  
204 removed and stored in distilled water. The brain and spinal cord were removed and stored in 4% paraformaldehyde for  
205 later analysis to check the position of the probe via cryosectioning.

### 206 **HPLC Measurement of Amino Acids and Adenosine**

207 The amino acid content (glutamate and glutamine) of each sample was analyzed via high-performance liquid  
208 chromatography (HPLC) using a binary gradient and pre-column derivatization of O-phthal aldehyde (OPA) with  
209 fluorescence detection.

210 Samples were diluted (4  $\mu\text{L}$  aCSF + 6  $\mu\text{L}$  dialysate) and a 1:2 sample to reagent ratio was used (10  $\mu\text{L}$  sample +  
211 20  $\mu\text{L}$  OPA). After a 60 second reaction, 20  $\mu\text{L}$  of the sample-OPA mixture was auto-injected into an Agilent Zorbax  
212 Eclipse AAA column (150 x 4.6 mm; 5  $\mu\text{m}$  particle size). A binary gradient of mobile phase A (40 mM sodium phosphate  
213 monobasic; pH = 7.4) and mobile phase B (45% acetonitrile; 45% methanol; 10% water) with a flow rate of 1.5 mL/min  
214 was used for separation.

215 Adenosine content of each sample was separated via HPLC and analyzed with a UV detector using previously  
216 developed methods (Sharma et al., 2010). 10  $\mu\text{L}$  of dialysate sample was injected into the HPLC system containing a  
217 mobile phase of 8 mM  $\text{NaH}_2\text{PO}_4$  and 8% methanol (pH = 4) at a flow rate of 80  $\mu\text{L}/\text{min}$ . Adenosine was separated with a



218 microbore column (1 × 100 mm; MF-8949; BASi, West Lafayette, IN) and detected with a UV detector (Model SPD-  
219 10Avp/10AVvp, Shimadzu Scientific Instruments, Columbia MD) at a 258 nm wavelength.

220 Column temperatures were maintained at 30°C. EZChrom Elite version 3.1.6 software was used to determine  
221 concentrations of extracellular neurotransmitters by comparing retention time and area under the peak to known amounts  
222 of standards

## 223 **Experimental Design and Statistical Analysis**

224 All statistical analyses were performed using SPSS version 19. Sufficient power was confirmed with a power  
225 analysis for these studies using PASS software (NCSS, Kaysville, UT) to limit the number of animals needed for these  
226 experiments. Periorbital thresholds at individual time points were analyzed using a one-way ANOVA to determine drug  
227 effects on trigeminal sensitivity. One-way repeated measure ANOVA was used to compare the effect of acetate on  
228 neurotransmitter release and a one-way ANOVA was used at each acetate concentration to compare groups. Bonferroni  
229 post hoc tests were performed where applicable. A t-test was used to compare basal levels of neurochemicals.

## 230 **Results**

231 We used two rat models of trigeminal sensitivity that feature several migraine-like traits: the recurrent  
232 inflammatory stimulation (IS) rat model and the spontaneous trigeminal allodynia (STA) rat model. Both systems model  
233 the trigeminal sensitivity (IS- chronic sensitivity, STA- episodic sensitivity), phonophobia, sensitivity to migraine triggers,  
234 and similar efficacious responses to migraine treatments (Oshinsky and Luo, 2006; Maxwell et al., 2010; Fried et al.,  
235 2014; Oshinsky et al., 2014; Munro et al., 2018). Trigeminal sensitivity was measured in male STA (n = 54) and IS rats (n  
236 = 107) in response to acetate treatment (60 mg/kg i.p.) while pharmacologically manipulating adenosine receptor  
237 signaling to determine the adenosine receptor subtype that is responsible for acetate-induced trigeminal sensitivity in the  
238 delayed ethanol-induced headache (DEIH). Extracellular adenosine, glutamate, and glutamine were measured with  
239 microdialysis and HPLC in the trigeminal nucleus caudalis (TNC) in response to local acetate perfusion to assess the  
240 mechanisms behind acetate-induced trigeminal sensitivity in DEIH.

### 241 **Acetate induces sensitivity in two rat models of trigeminal sensitivity.**

242 Ethanol treatment (300 mg/kg i.p.) of IS rats caused a biphasic effect on trigeminal pain thresholds (Fig. 1A).  
243 Initially, an analgesic effect was produced 1-2 hr post ethanol treatment as expressed by significantly higher periorbital

244 thresholds in comparison to saline treated animals at the 1 hr ( $p = 0.011$ ) and 2 hr ( $p = 0.031$ ) time points. Animals then  
245 experienced an increase in trigeminal sensitivity as expressed by significantly lower periorbital thresholds during the 4 hr  
246 ( $p = 0.013$ ) and 5 hr ( $p = 0.008$ ) time points in comparison to saline treated animals (Fig. 1A). Ethanol is metabolized into  
247 acetaldehyde by alcohol dehydrogenase which is subsequently metabolized to acetate by aldehyde dehydrogenase.  
248 Previous pharmacological studies illustrated that acetate is the key pro-nociceptive metabolite of ethanol. Bypassing  
249 unrelated effects of ethanol by directly treating IS rats with acetate (60 mg/kg i.p.) increased trigeminal sensitivity as  
250 expressed by significantly lower periorbital thresholds during the 1 hr ( $p = 0.032$ ), 2 hr ( $p < 0.001$ ), 3 hr ( $p < 0.001$ ) and 4  
251 hr ( $p = 0.014$ ) hour time points in comparison to saline treated animals (Fig. 1B). These treatment concentrations (60  
252 mg/kg i.p. acetate and 300mg/kg i.p. ethanol) result in similar serum-acetate levels in the rat (approximately 1 mM)  
253 (Maxwell et al., 2010).

254 We confirmed that ethanol and acetate also cause increased trigeminal sensitivity in the STA rat model of  
255 trigeminal pain. In this model, ethanol (300 mg/kg i.p.) also produced a biphasic effect on trigeminal sensitivity. An  
256 analgesic effect initially occurred as seen by significantly higher periorbital thresholds at the 1 hr ( $p = 0.043$ ) and 2 hr ( $p =$   
257  $0.045$ ) time points in comparison to the saline treated animals. This was followed by increased trigeminal sensitivity as  
258 expressed by significantly lower periorbital thresholds at the 4 hr ( $p = 0.042$ ) and 5 hr ( $p = 0.039$ ) time points in  
259 comparison to saline treated animals (Fig. 1C). Acetate treatment (60 mg/kg i.p.) caused trigeminal sensitivity as seen by  
260 significantly lower periorbital thresholds during the 1 hr ( $p = 0.007$ ), 2 hr ( $p < 0.001$ ), 3 hr ( $p < 0.001$ ), and 4 hr ( $p =$   
261  $0.034$ ) time points in comparison to saline treated animals (Fig. 1D).

### 262 **Competitive inhibition of monocarboxylate transport prevents acetate-induced trigeminal sensitivity.**

263 Acetate is primarily utilized as an energy substrate by astrocytes within the brain since neurons do not import it  
264 (Waniewski and Martin, 1998; Wyss et al., 2011). To determine whether acetate entry into astrocytes is required for  
265 acetate's effect on trigeminal sensitivity, STA and IS rats were treated with alpha-cyano-4-hydroxycinnamate (4-CIN), a  
266 monocarboxylate transporter (MCT) competitive inhibitor, thirty minutes prior to acetate treatment. Treatment of IS rats  
267 with 4-CIN (100 mg/kg p.o.) attenuated the acetate-induced trigeminal sensitivity as seen by a significantly higher  
268 threshold in the 4-CIN co-treated animals in comparison to the acetate only animals at the 1 hr ( $p = 0.003$ ), 2 hr ( $p <$   
269  $0.001$ ), 3 hr ( $p < 0.001$ ), and 4 hr ( $p = 0.03$ ) time points (Fig. 2A).

270 Treatment with 4-CIN (100 mg/kg p.o.) also attenuated the acetate-induced trigeminal sensitivity in STA rats as  
271 seen by a significantly higher threshold in the 4-CIN co-treated animals in comparison to the acetate only animals at the 1  
272 hr ( $p = 0.003$ ), 2 hr ( $p < 0.001$ ), 3 hr ( $p < 0.001$ ), and 4 hr ( $p = 0.03$ ) time points (Fig 2B). 4-CIN administered to IS or  
273 STA rats in the absence of acetate had no effect on thresholds across 5 hours (Sup. Fig. 1). These data suggest that  
274 acetate-induced trigeminal sensitivity requires acetate entry, and possibly metabolism, in astrocytes.

### 275 **Adenosine A<sub>2A</sub> receptor activation is necessary for acetate-induced trigeminal sensitivity.**

276 Caffeine treatment, a non-specific adenosine receptor antagonist, prevents ethanol-induced trigeminal sensitivity  
277 in IS rats (Maxwell et al., 2010). To confirm adenosine receptor involvement in acetate-induced trigeminal sensitivity, IS  
278 rats were treated with caffeine (50 mg/kg i.p.) 1.5 hour following acetate treatment. This timing was used due to caffeine's  
279 relatively short half-life in rats (1.2 hr) to ensure sufficient antagonism during the peak of acetate-induced trigeminal  
280 sensitivity (Nehlig, 1999). This also allowed us to confirm acetate's initial effect on trigeminal thresholds during the 1 hr  
281 time point prior to drug treatment. Similar to caffeine's effects on ethanol-induced trigeminal sensitivity, caffeine  
282 treatment attenuated acetate-induced trigeminal sensitivity as seen by a significantly higher threshold in the caffeine co-  
283 treated animals in comparison to the acetate only animals at the 2 hr ( $p = 0.009$ ), 3 hr ( $p < 0.001$ ), and 4 hr ( $p = 0.006$ )  
284 time points (Fig. 3A). Animals receiving caffeine co-treatment had similar thresholds to acetate-only treated animals at  
285 the 1 hr time point, prior to caffeine treatment ( $p = 0.654$ ). These data suggest that adenosine signaling plays a role in  
286 acetate-induced trigeminal sensitivity.

287 To determine which adenosine receptor subtype (A<sub>1</sub>, A<sub>2A</sub>, A<sub>2B</sub>, and A<sub>3</sub>) is primarily responsible for acetate-  
288 induced trigeminal sensitivity, IS rats were treated 1.5 hours after acetate administration with adenosine receptor subtype-  
289 specific antagonists and also compared to the animals treated only with acetate. For easier visualization of these  
290 comparisons, we separated this experiment into two figure panels (Fig. 3B and Fig. 3C) and thus the acetate group is the  
291 same as that seen in Fig. 3A, but all groups in Fig. 3A, 3B, and 3C were compared with a single one-way repeated  
292 measures ANOVA. Co-treatment with the adenosine A<sub>1</sub> receptor antagonist (DPCX 3 mg/kg p.o.), the A<sub>2B</sub> receptor  
293 antagonist (alloxazine 12.5 mg/kg p.o.), or the A<sub>3</sub> receptor antagonist (MRS-1527 1 mg/kg p.o.) did not attenuate the  
294 acetate-induced trigeminal sensitivity in IS rats as seen by no significant differences in periorbital thresholds between the  
295 antagonist treated groups and the acetate-only group (Fig. 3B) (Jacobson and Gao, 2006).

296 Treatment with an A<sub>2A</sub> receptor antagonist (istradefylline 0.1 mg/kg p.o.), however, attenuated acetate-induced  
297 trigeminal sensitivity as seen by a significantly higher threshold in the istradefylline co-treated animals in comparison to  
298 the acetate only animals at the 2 hr (p = 0.009) and 3 hr (p < 0.001) time points in the IS rats (Fig. 3C). Animals receiving  
299 istradefylline co-treatment had similar thresholds to acetate-only treated animals at the 1 hr time point, prior to  
300 istradefylline treatment (p = 0.826). We confirmed similar effects of istradefylline co-treatment in the STA rats where the  
301 istradefylline co-treated animals had significantly higher thresholds than then acetate-only group at the 2 hr (p < 0.001),  
302 3hr (p = 0.002), and 4 hr (p = 0.03) time points (Fig. 3D). Istradefylline administered to IS or STA rats in the absence of  
303 acetate had no effect on thresholds across 5 hours (Sup. Fig. 1). These data suggest that acetate is inducing trigeminal  
304 sensitivity via adenosine A<sub>2A</sub> receptors.

305 Following ethanol ingestion, acetate is produced throughout the entire body (Tsukamoto et al., 1989). Thus,  
306 acetate's impact on trigeminal pain via adenosine receptor signaling may occur peripherally at primary afferents or  
307 centrally within the TNC, containing second order neurons. To determine if central adenosine receptor signaling is  
308 sufficient for trigeminal pain, we directly injected adenosine into the cisterna magna, an opening of the subarachnoid  
309 space, which allows for noninvasive access to the region of the brainstem containing the TNC. Intercisternal injections  
310 have previously been used to specifically target the TNC (Mitsikostas et al., 1998, 1999). For easier visualization of these  
311 comparisons, we separated this experiment into two figure panels (Fig. 3E and Fig. 3F) and thus the aCSF group is the  
312 same in both panels, but all groups in Fig. 3E and 3F were compared with a single one-way repeated measures ANOVA.

313 Intracisternal injection of adenosine (10 µl, 12.5 µM) in IS rats induced trigeminal sensitivity as seen by  
314 significantly lower thresholds in the adenosine treated animals in comparison to aCSF treated animals at the 0.25 hr (p =  
315 0.034), 0.5 hr (p = 0.029), and 1.5 hr (p = 0.002) time points (Fig. 3E). This was confirmed in STA rats where  
316 significantly lower thresholds were seen in the adenosine treated animals in comparison to aCSF treated animals at the 1.5  
317 hr (p < 0.001) time point (Fig. 3E).

318 To confirm the role of the A<sub>2A</sub> receptors within the TNC, an adenosine A<sub>2A</sub> /A<sub>2B</sub> receptor agonist (CV-1808; 10  
319 µl, 300 µM) was directly injected into the cisterna magna of IS rats thirty minutes following administration of the A<sub>2B</sub>  
320 receptor antagonist (alloxazine 12.5 mg/kg p.o.) since no reliable A<sub>2A</sub> exists. This allowed for selective stimulation of  
321 centrally-acting A<sub>2A</sub> receptors within the TNC. This selective targeting of A<sub>2A</sub> receptors induced trigeminal sensitivity as

322 seen by significantly lower thresholds in the “A<sub>2A</sub>-agonist” animal group in comparison to aCSF treated animals at the  
323 0.25 hr (p = 0.006), 0.5 hr (p < 0.001), and 1.5 hr (p = 0.001) time points (Fig. 3F).

324 **Acetate perfusion in the TNC induces dose-dependent increase in extracellular adenosine, glutamate, and**  
325 **glutamine concentrations.**

326 Microdialysis and HPLC was used to quantify the effect of central acetate perfusion on extracellular adenosine  
327 concentrations within the TNC of naive and IS rats. Local perfusion of acetate into the TNC with the use of microdialysis  
328 as opposed to systemic i.p. administration allowed for acetate exposure directly to the local brain parenchyma of the TNC.  
329 Use of acetate instead of ethanol allowed us to bypass the complicating factors of ethanol effects unrelated to pain.  
330 Calculating the *in vitro* recovery of each microdialysis probe, acetate concentrations were estimated in the perfusate for  
331 each experiment to obtain 0 mM, 2 mM, 10 mM, 20 mM, or 40 mM acetate around the vicinity of the probe tip in the  
332 TNC. After obtaining a stable baseline for 2-3 hours following probe insertion (this baseline is used to calculate fold  
333 change in each neurochemical), each concentration of acetate was sequentially perfused for 1 hour at a flow rate of 0.7  
334  $\mu$ l/min with 14  $\mu$ l of microdialysate collected every 20 minutes (i.e., following baseline, 2 mM acetate is perfused for 1  
335 hr, followed by 10 mM for 1 hr, followed by 20 mM for 1 hr, followed by 40 mM for one hr). 10  $\mu$ l of each  
336 microdialysate was separated with HPLC and analyzed with a UV detector to measure extracellular adenosine  
337 concentrations (Sharma et al., 2010). The remaining 4  $\mu$ l was separated with HPLC and used to determine extracellular  
338 concentrations of glutamate and glutamine via OPA-conjugated fluorescence detection. Fold change is calculated as the  
339 average fold change for each acetate concentration (i.e., the reported fold change is the average of the three samples  
340 collected during each acetate concentration).

341 Acetate perfusion induced a dose-dependent increase of extracellular adenosine in both IS and naive rats. The IS  
342 rats, however, experienced significantly larger increases of extracellular adenosine with perfusion of 2 mM ( $1.5 \pm 0.2$  fold  
343 in IS rats vs  $0.9 \pm 0.1$  fold in naive rats, p = 0.035), 10 mM ( $2.7 \pm 0.5$  fold in IS rats vs  $1.4 \pm 0.1$  fold in naive rats, p =  
344 0.041), and 40 mM acetate ( $8.1 \pm 1.8$  fold in IS rats vs  $2.5 \pm 0.6$  fold in naive rats, p = 0.025) (Fig. 4A). Perfusion of 20  
345 mM acetate increased extracellular adenosine to similar levels in the IS rats ( $4.7 \pm 1.1$  fold) and naive rats ( $2.3 \pm 0.5$  fold)  
346 (Fig. 4A). Using each microdialysis probe’s individual recovery rate, estimation of basal adenosine concentrations within  
347 the TNC were calculated and were not statistically different between IS rats ( $159 \pm 31$  nM) and naive rats ( $188 \pm 54$  nM)  
348 (Fig. 4B).

349 Extracellular glutamate within the dorsal horn or the TNC has been used as a neurochemical marker for pain, thus,  
350 we quantified changes in glutamate levels in response to acetate perfusion (Tao et al., 2005; Oshinsky and Luo, 2006;  
351 Oshinsky et al., 2014). A dose-dependent increase of extracellular glutamate in response to acetate concentration was  
352 observed in the both IS and naive rats. IS rats, however, experienced a significantly higher level of extracellular glutamate  
353 with perfusion of 2 mM ( $2.2 \pm 0.5$  fold in IS rats vs  $0.76 \pm 0.2$  fold in naive rats,  $p = 0.035$ ), 10 mM ( $2.7 \pm 0.5$  fold in IS  
354 rats vs  $1.1 \pm 0.3$  fold in naive rats,  $p = 0.035$ ), 20 mM ( $4.5 \pm 0.7$  fold in IS rats vs  $1.5 \pm 0.4$  fold in naive rats,  $p = 0.009$ ),  
355 and 40 mM ( $8.7 \pm 1.5$  fold in IS rats vs  $3.5 \pm 0.8$  fold in naive rats,  $p = 0.02$ ) acetate (Fig. 4C). Estimated basal glutamate  
356 concentrations within the TNC were not statistically different between IS rats ( $1.1 \pm 0.1$   $\mu\text{M}$ ) and naive rats ( $1.4 \pm 0.2$   
357  $\mu\text{M}$ ) (Fig 4D).

358 Upon acetate utilization as an energy substrate by astrocytes, acetyl Co-A is formed which enters the Krebs cycle,  
359 producing  $\alpha$ -ketoglutarate that is used to produce glutamate. This glutamate is converted to glutamine and released from  
360 astrocytes for neuronal uptake as part of the glutamate-glutamine cycling between astrocytes and neurons (Jiang et al.,  
361 2013). Measurement of extracellular glutamine, therefore, can serve as a surrogate marker of acetate utilization.  
362 Glutamine levels were quantified in response to acetate perfusion in IS and naive rats. Although a dose-dependent  
363 increase of extracellular glutamate in response to acetate concentration was observed, no significant difference was found  
364 with perfusion of 2 mM ( $1.5 \pm 0.1$  fold in IS rats,  $1.6 \pm 0.4$  fold in naive rats), 10 mM ( $2.0 \pm 0.2$  fold in IS rats,  $2.5 \pm 0.5$   
365 fold in naive rats), 20 mM ( $2.9 \pm 0.4$  fold in IS rats,  $3.5 \pm 0.3$  fold in naive rats), or 40 mM ( $3.8 \pm 0.7$  fold in IS rats,  $4.5 \pm$   
366  $0.8$  fold in naive rats) acetate between IS and naive rats (Fig. 4E). This suggested that both groups metabolize similar  
367 levels of acetate within the TNC during microdialysis perfusion. Estimated basal glutamine concentrations within the  
368 TNC were not statistically different between IS rats ( $5.2 \pm 1.5$   $\mu\text{M}$ ) and naive rats ( $5.1 \pm 1.3$   $\mu\text{M}$ ) (Fig. 4F).

#### 369 **4-CIN co-perfusion prevents the increase in adenosine, glutamate, and glutamine during acetate perfusion.**

370 To determine whether acetate entry into astrocytes is required for the production of adenosine, 4-CIN (200  $\mu\text{M}$ )  
371 was included in the acetate-containing perfusate during microdialysis of IS rats. To allow 4-CIN to sufficiently block  
372 MCT transport, 4-CIN containing aCSF was perfused 20 minutes prior to exposure of perfusate containing both acetate  
373 (10 mM or 40 mM) and 4-CIN (200  $\mu\text{M}$ ). The presence of 4-CIN prevented the acetate-induced increase in extracellular  
374 adenosine concentrations as seen by a significantly lower fold change during perfusion of 10 mM ( $1.0 \pm 0.1$  fold with 4-  
375 CIN vs.  $2.7 \pm 0.5$  fold without 4-CIN,  $p = 0.042$ ) but not 40 mM ( $3.9 \pm 0.1$  fold with 4-CIN,  $8.1 \pm 1.8$  fold without 4-CIN)

376 acetate (Fig. 5A). A similar effect was seen in extracellular glutamate and glutamine. The presence of 4-CIN prevented  
377 the acetate-induced increase in extracellular glutamate concentrations as seen by a significantly lower fold change during  
378 perfusion of 10 mM ( $1.2 \pm 0.2$  fold with 4-CIN vs.  $2.9 \pm 0.4$  fold without 4-CIN,  $p = 0.001$ ) but not 40 mM ( $6.6 \pm 1.7$  fold  
379 with 4-CIN,  $9.9 \pm 1.1$  fold without 4-CIN) acetate (Fig. 5C). The presence of 4-CIN prevented the acetate-induced  
380 increase in extracellular glutamine concentrations as seen by a significantly lower fold change during perfusion of 10 mM  
381 ( $1.0 \pm 0.1$  fold with 4-CIN vs.  $2.7 \pm 0.5$  fold without 4-CIN,  $p = 0.009$ ) but not 40 mM ( $2.0 \pm 0.2$  fold with 4-CIN,  $3.8 \pm$   
382  $0.7$  fold without 4-CIN) acetate (Fig. 5E).

### 383 **Istradefylline co-perfusion prevents dose-dependent increase in glutamate but not adenosine or glutamine during** 384 **acetate perfusion.**

385 To further determine the neurochemical mechanism behind acetate-induced trigeminal sensitivity and to  
386 determine if the increase in glutamate levels is a result of adenosine interaction with adenosine  $A_{2A}$  receptors,  
387 istradefylline (5 nM) was included in the perfusate during microdialysis in the TNC of IS rats. To allow sufficient  
388 antagonism of adenosine  $A_{2A}$  receptors, istradefylline was perfused through the microdialysis probe 20 minutes prior to  
389 exposure of perfusate containing both acetate and istradefylline (5 nM). Inclusion of istradefylline did not affect the  
390 acetate-induced increase in extracellular adenosine or glutamine during perfusion of 10 mM or 40 mM acetate, but it did  
391 significantly prevent acetate-induced increases in glutamate concentrations. Istradefylline presence did not significantly  
392 prevent the increase in extracellular adenosine concentrations during perfusion of 10 mM ( $2.8 \pm 0.4$  fold with  
393 istradefylline vs.  $2.7 \pm 0.5$  fold without istradefylline) and 40 mM ( $6.1 \pm 1.6$  fold with istradefylline vs.  $8.1 \pm 1.8$  fold  
394 without istradefylline) acetate (Fig. 5B). Istradefylline presence did significantly prevent the increase in extracellular  
395 glutamate concentrations during perfusion of 10 mM ( $1.0 \pm 0.2$  fold with istradefylline vs.  $2.9 \pm 0.4$  fold without  
396 istradefylline,  $p = 0.005$ ) and 40 mM ( $2.0 \pm 0.3$  fold with istradefylline vs.  $9.9 \pm 1.1$  fold without istradefylline,  $p < 0.001$ )  
397 acetate (Fig. 5D). Inclusion of istradefylline did not significantly prevent the increase in extracellular glutamine  
398 concentrations during perfusion of 10mM ( $1.8 \pm 0.5$  fold with istradefylline vs.  $2.0 \pm 0.2$  fold without istradefylline) and  
399 40mM ( $3.7 \pm 0.7$  fold with istradefylline vs.  $3.8 \pm 0.7$  fold without istradefylline) acetate (Fig. 5F).

## 400 **DISCUSSION**

401 We identified adenosine as a critical component of the nociceptive effects of acetate in delayed ethanol-induced  
402 headache (DEIH). Adenosine  $A_{2A}$  receptor activation was essential for this effect in both rodent models, but the three



403 other known adenosine receptor subtypes ( $A_1$ ,  $A_{2B}$ , and  $A_3$ ) were not involved. This mechanism relies on MCT transport  
404 of acetate, likely into astrocytes which preferentially transport and metabolize acetate (Waniewski and Martin, 1998).  
405 Intracisternal injection of adenosine to target the TNC induced trigeminal sensitivity, suggesting that the mechanism  
406 behind DEIH is centrally-mediated (Panconesi, 2016). Acetate perfusion within the TNC induced a dose-dependent  
407 increase in adenosine, glutamate, and glutamine. In the IS rats, this effect was significantly greater for adenosine and  
408 glutamate, but not glutamine. This suggests that acetate utilization is similar in both groups, but that a physiological  
409 difference downstream of acetate conversion to acetyl-coA in the TNC of rats enhances the production of adenosine and  
410 glutamate. 4-CIN, an MCT competitive inhibitor, prevented this increase in adenosine, glutamate, and glutamine,  
411 supporting that acetate-induced release of these neurochemicals occur downstream of acetate transport. Istradefylline, an  
412 adenosine  $A_{2A}$  receptor antagonist, prevented the rise in glutamate but not adenosine or glutamine, suggesting that the  
413 increase in glutamate is downstream of adenosine  $A_{2A}$  receptor activation. Together, these data suggest a model for DEIH  
414 where adenosine derived from astrocytic acetate utilization activates adenosine  $A_{2A}$  receptors, which then modulates  
415 extracellular glutamate within the TNC to produce trigeminal sensitivity following ethanol exposure.

416 We find that adenosine  $A_{2A}$  receptor activation is responsible for modulation of extracellular glutamate levels in  
417 the TNC following systemic acetate treatment. Since glutamate is the principle excitatory neurotransmitter, its  
418 concentration is closely regulated via GLT1 (glutamate transporter 1) which is exclusively expressed on astrocytes to  
419 transport glutamate out of the synaptic cleft (Lehre and Danbolt, 1998; Anderson and Swanson, 2000). This is driven by  
420 a  $Na^+$  gradient maintained by the energetically-demanding  $Na^+/K^+$  ATPase (NKA) on the cell surface (Zerangue and  
421 Kavanaugh, 1996; Rose et al., 2009). The reliance of this  $Na^+$  gradient and the process of maintaining it is so energetically  
422 demanding that co-compartmentalization of GLT-1, NKA, and mitochondria exists to allow rapid restoration of this  
423 critical  $Na^+$  gradient (Rose et al., 2009; Genda et al., 2011). Interestingly, modulation of this functional unit by inhibition  
424 of NKA activity, mitochondria, GLT1, or the  $Na^+$  concentration can affect glutamate transport (Keller et al., 1997; Li and  
425 Stys, 2001; Kawahara et al., 2002; Veldhuis et al., 2003; Köfalvi et al., 2020). Adenosine  $A_{2A}$  receptors physically  
426 associate with the  $\alpha_2$  subunit of NKAs and their activation decreases NKA activity, modulating GLT1 function to decrease  
427 glutamate transport and increase its concentrations in the extracellular space levels (Matos et al., 2012, 2013). This  
428 mechanism may be responsible for the observation that extracellular glutamate concentrations can be modulated by the  
429 inhibition of adenosine  $A_{2A}$  receptors during acetate perfusion. An alternative mechanism may involve the excitatory  
430 effects of adenosine  $A_{2A}$  receptors on presynaptic neurons which can enhance synaptic transmission (Marchi et al., 2002).

431 Adenosine's modulation of extracellular glutamate may play a key role in trigeminal pain. Extracellular glutamate  
432 within the TNC of IS rats has been used as a neurochemical marker of trigeminal pain (Oshinsky et al., 2014). Glutamate  
433 also plays a role in the spinal dorsal horn where modulation of glutamate is associated with other forms of chronic pain  
434 (Tao et al., 2005). Centrally-acting glutamate receptor antagonists are effective in reducing pain in animal models and  
435 humans while blocking excitatory amino acid transporters, increasing synaptic glutamate concentrations, produces pain  
436 (Hewitt, 2000; Liaw et al., 2005). Excess glutamate can lead to over-excitation of neuronal circuits within the dorsal horn  
437 or TNC, contributing to the development and maintenance of chronic pain states (Niederberger et al., 2003; Tao et al.,  
438 2005). Since adenosine A<sub>2A</sub> receptor antagonism prevents the rise in glutamate and acetate-induced trigeminal sensitivity,  
439 adenosine likely modulates trigeminal pain via its effect on extracellular glutamate concentrations in the TNC during  
440 DEIH.

441 4-CIN, a MCT inhibitor, prevented acetate-induced trigeminal sensitivity in both models and prevented the  
442 increase in adenosine, glutamate, and glutamine in IS rats. This suggests that transport of acetate through MCTs is  
443 essential to the mechanisms behind acetate-induced trigeminal sensitivity in DEIH. Although monocarboxylic acid  
444 transport modulation can occur at the blood-brain barrier (BBB), we believe it is transport directly into astrocytes that is  
445 being modulated in this system (Muir et al., 1986; Terasaki et al., 1991; Waniewski and Martin, 1998; Hatazawa et al.,  
446 2010; Patel et al., 2010; Wyss et al., 2011; Jiang et al., 2013; DosSantos et al., 2014). Although MCTs play a vital role in  
447 transport of other monocarboxylic acids at the BBB, acetate is believed to not be regulated at the BBB, crossing by simple  
448 diffusion and is instead regulated at the cell surface of astrocytes (Terasaki et al., 1991; Waniewski and Martin, 1998;  
449 Patel et al., 2010). Thus, changes in BBB permeability seen in the IS model would not affect acetate transport, suggesting  
450 that 4-CIN regulation of acetate transport occurs at the astrocyte surface (Fried et al., 2018). 4-CIN does have affinity for  
451 other MCTs that modulate pyruvate or lactate transport in other cell types (Granja et al., 2013). We found, however, that  
452 4-CIN alone does not change baseline trigeminal sensory thresholds, suggesting that its interaction with other MCT  
453 isoforms likely does not play a role in this process. 4-CIN's ability to prevent acetate transport into astrocytes is further  
454 confirmed in that it prevented the formation of glutamine which is formed during acetate utilization.

455 We find that baseline adenosine concentrations within the TNC similar to concentrations observed in the rat  
456 hippocampus (120-200 nM), striatum (40-210 nM), and cortex (120 nM) (Latini and Pedata, 2001). Similar to human  
457 migraineurs who are more susceptible to DEIH than non-migraineurs, the acetate-induced adenosine rise is exacerbated in  
458 the IS rats in comparison to control animals. In fact, control animals need 20 mM of acetate perfused in the TNC to

459 produce similar extracellular glutamate levels as IS rats experience with only 2 mM of acetate. This suggests that a  
460 physiological difference exists within the TNC of IS rats that may make them more susceptible to adenosine formation  
461 when exposed to acetate. Since there was no difference in glutamine production during acetate perfusion, this  
462 physiological difference is likely downstream of acetate conversion to acetyl-coA. This may be due to a decreased  
463 mitochondrial spare respiratory capacity that was identified in the mitochondria of brain slices from the TNC in both the  
464 IS and STA rat models (Fried et al., 2014; Fried and Oshinsky, 2015). Although this dysfunction does not impact basal  
465 levels of mitochondrial function, it may lower downstream processes involved in substrate utilization in active neuronal  
466 tissue which is already functioning at a level requiring 80% of the maximum capacity of mitochondrial output (Desler et  
467 al., 2012). Interestingly, mitochondrial dysfunction is associated with an increase in adenosine formation (Watanabe et al.,  
468 1983; Eltzhig et al., 2004; Duley et al., 2011). If this decrease in spare capacity within the TNC is present in astrocytes,  
469 the metabolic processes which control adenine nucleotide balance within the cell may be disrupted while those controlling  
470 acetate entry into the citric acid cycle (resulting in glutamine production) are not. Since DEIH can occur in non-  
471 migraineurs, it suggests that the mechanism of acetate-induced adenosine formation and subsequent modulation of  
472 extracellular glutamate is not unique to migraineurs or sensitized animals, but that it is exacerbated in these animals;  
473 potentially due to mitochondrial dysfunction or other physiological changes.

474 These results contribute to the migraine headache and alcohol fields at large. Adenosine plays a significant role in  
475 neurogenic inflammation, neuronal modulation, glial function, other forms of pain, changes in the blood-brain barrier and  
476 many other physiological processes that affect migraine (Guieu et al., 1998; Sawynok and Liu, 2003; Carman et al., 2011;  
477 Pedata et al., 2014). Notably, there is mounting evidence of adenosine as a critical player in the pathophysiology of  
478 migraine and headache (Fried et al., 2017). There is growing evidence suggesting that chronic pain and alcoholism may  
479 involve similar neurophysiological changes in overlapping brain regions involved in both disorders (Egli et al., 2012).  
480 Similar to DEIH, alcohol can produce hyperalgesia during the withdrawal period and conversely, chronic pain conditions  
481 are associated with alcohol misuse and dependence (Castillo et al., 2006; Gatch, 2009; Edwards et al., 2012). Adenosine is  
482 heavily involved in the development of alcoholism and may serve as the key neurophysiological modulator for the  
483 interplay between chronic pain and alcoholism (Ruby et al., 2010).

484 These studies expand on our previous work that identified acetate as the key metabolite of DEIH by providing  
485 further evidence that acetate's effect on trigeminal pain is induced via adenosinergic mechanisms. We also provide  
486 evidence that the mechanism behind this effect likely involves modulation of extracellular glutamate levels via adenosine

487 A<sub>2A</sub> receptors. These data present, for the first time *in vivo*, evidence that supports adenosine A<sub>2A</sub> receptor modulation of  
488 astrocytic glutamate transport (Genda et al., 2011; Matos et al., 2013). Additionally, these data illuminate adenosine as a  
489 potential key element to headache pathophysiology.

## 490 AUTHOR CONTRIBUTIONS

491 NTF designed studies, acquired and analyzed data and the drafted manuscript. CRM designed and acquired data.  
492 JBH critically revised the manuscript. MBE made experimental, data interpretation and editorial contributions to the  
493 manuscript. MLO designed, analyzed, supervised the study and critically revised the manuscript. Each author gave final  
494 approval of the version to be published.

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## 640 **FIGURE LEGENDS**

### 641 **Figure 1: Ethanol and acetate treatment of IS and STA rat models.**

642 Comparison of the effect of ethanol or acetate vs saline on periorbital von Frey hair withdrawal thresholds in the IS and  
643 STA rat models of trigeminal pain as measured by proportional change in threshold from baseline. A) Ethanol treatment  
644 (300 mg/kg i.p., n = 9) vs saline (n = 9) in IS rats. B) Acetate treatment (60 mg/kg i.p., n = 7) vs saline (n = 5) in IS rats.  
645 C) Ethanol treatment (300 mg/kg i.p., n = 10) vs saline (n = 10) in STA rats. D) Acetate treatment (60 mg/kg i.p., n = 7)  
646 vs saline (n = 6) in STA rats. (\*,  $p < 0.05$ ) (\*\*,  $p < 0.01$ ) (!,  $p < 0.001$ ).

### 647 **Figure 2: 4-CIN treatment of acetate-treated IS and STA rats.**

648 Measurement of the effect of  $\alpha$ -Cyano-4-hydroxycinnamic acid (4-CIN), a competitive MCT inhibitor, on acetate-induced  
649 trigeminal sensitivity as measured by proportional change in periorbital von Frey hair withdrawal thresholds from  
650 baseline. IS rats (A) and STA rats (B) were administered 4-CIN (100 mg/kg p.o.) 30 minutes prior to acetate treatment (60  
651 mg/kg i.p.) and compared to the acetate-only group. Each group consists of 6 animals. (\*,  $p < 0.05$ ) (\*\*,  $p < 0.01$ ) (!,  $p <$   
652  $0.001$ ).

### 653 **Figure 3: Adenosine receptor subtype involvement in acetate-induced trigeminal sensitivity in the IS and STA rats.**

654 Measurement of the effect of adenosine receptor modulation on acetate-induced trigeminal sensitivity as measured by  
655 proportional change in periorbital von Frey hair withdrawal thresholds from baseline in IS and STA rats. A) The effect of  
656 treatment with caffeine (50 mg/kg i.p.) 1.5 hours after acetate treatment (60 mg/kg i.p.) compared to acetate-only in IS  
657 rats. B) The effect of treatment with the adenosine A<sub>1</sub> receptor antagonist (DPCX 3 mg/kg p.o.), the A<sub>2B</sub> receptor  
658 antagonist (alloxazine 12.5 mg/kg p.o.), or the A<sub>3</sub> receptor antagonist (MRS-1527 1 mg/kg p.o.) 1.5 hours after acetate  
659 treatment (60 mg/kg i.p.) compared to acetate-only in IS rats. C) The effect of treatment with istradefylline (0.1 mg/kg

660 p.o.), an A<sub>2A</sub> antagonist, 1.5 hours after acetate treatment (60 mg/kg i.p.) compared to acetate-only in IS rats. D) The  
661 effect of treatment with istradefylline (0.1 mg/kg p.o.), an A<sub>2A</sub> antagonist, 1.5 hours after acetate treatment (60 mg/kg i.p.)  
662 compared to acetate-only in STA rats. E) The effect of intracisternal injection of adenosine (10 μl, 12.5 μM) compared to  
663 aCSF in IS and STA rats. F) The effect of intracisternal injection of an adenosine A<sub>2A</sub>/A<sub>2B</sub> receptor agonist (CV-1808; 10  
664 μl, 300 μM) 30 minutes following administration of the A<sub>2B</sub> receptor antagonist (alloxazine 12.5 mg/kg p.o.) compared to  
665 aCSF in IS rats. Solid arrow in A, B, C, and D indicates the time when the antagonist is administered. To increase visual  
666 clarity for comparison of groups, the acetate-only groups in A, B, and C are the same data, but all groups were compared  
667 with a one-way repeated measures ANOVA at the same time. For the same reasons, the aCSF groups in E and F are the  
668 same, but all groups were compared with a single repeated measures ANOVA. Acetate groups consist of 10 animals. All  
669 other groups consist of 6 animals each. (\*,  $p < 0.05$ ) (\*\*,  $p < 0.01$ ) (!,  $p < 0.001$ ).

670 **Figure 4: Measurement of extracellular adenosine, glutamate, and glutamine in response to acetate perfusion in the**  
671 **TNC.**

672 Microdialysis and HPLC analysis of acetate perfusion within the trigeminal nucleus caudalis (TNC) of IS rats induces  
673 greater increases in extracellular adenosine and glutamate, but not glutamine, in comparison to naive rats. A) Average fold  
674 change from baseline in extracellular adenosine levels in the TNC of IS and naive rats in response to 2 mM, 10 mM, 20  
675 mM, and 40 mM acetate perfusion. B) Calculated basal levels of extracellular adenosine in the TNC of IS and naive rats.  
676 C) Average fold change from baseline in extracellular glutamate levels in the TNC of IS and naive rats in response to 2  
677 mM, 10 mM, 20 mM, and 40 mM acetate perfusion. D) Estimated basal levels of extracellular glutamate in the TNC of IS  
678 and naive rats. E) Average fold change from baseline in extracellular glutamine levels in the TNC of IS and naive rats in  
679 response to 2 mM, 10 mM, 20 mM, and 40 mM acetate perfusion. F) Estimated basal levels of extracellular glutamine in  
680 the TNC of IS and naive rats. Each group consists of 7 animals. (\*,  $p < 0.05$ ) (\*\*,  $p < 0.01$ ).

681 **Figure 5: Measurement of acetate-induced changes in extracellular adenosine, glutamate, and glutamine in**  
682 **response to istradefylline or 4-CIN perfusion in the TNC.**

683 Microdialysis and HPLC analysis of acetate perfusion within the trigeminal nucleus caudalis (TNC) on extracellular  
684 concentrations of adenosine, glutamate, and glutamine of IS rats is modulated by istradefylline and 4-CIN. A) Average  
685 fold change from baseline in extracellular adenosine levels in the TNC of IS rats in response to 2 mM, 10 mM, 20 mM,  
686 and 40 mM acetate and 4-CIN (200 μM) perfusion. B) Average fold change from baseline in extracellular adenosine

687 levels in the TNC of IS rats in response to 2 mM, 10 mM, 20 mM, and 40 mM acetate and istradefylline (5 nM) perfusion.  
688 C) Average fold change from baseline in extracellular glutamate levels in the TNC of IS rats in response to 2 mM, 10  
689 mM, 20 mM, and 40 mM acetate and 4-CIN (200  $\mu$ M) perfusion. D) Average fold change from baseline in extracellular  
690 glutamate levels in the TNC of IS rats in response to 2 mM, 10 mM, 20 mM, and 40 mM acetate and istradefylline (5 nM)  
691 perfusion. E) Average fold change from baseline in extracellular glutamine levels in the TNC of IS rats in response to 2  
692 mM, 10 mM, 20 mM, and 40 mM acetate and 4-CIN (200  $\mu$ M) perfusion. F) Average fold change from baseline in  
693 extracellular glutamine levels in the TNC of IS rats in response to 2 mM, 10 mM, 20 mM, and 40 mM acetate and  
694 istradefylline (5 nM) perfusion. Each group consists of 5 animals. (\*,  $p < 0.05$ ) (\*\*,  $p < 0.01$ ) (!,  $p < 0.001$ ).

695 **Supplemental Figure 1: 4-CIN and istradefylline alone treatments of IS and STA rats.**

696 Von Frey Hair periorbital thresholds (g) of IS and STA rats in response to 4-CIN and istradefylline treatment alone. IS  
697 rats (A) (n = 5) and STA rats (B) (n = 4) were administered either 4-CIN (100 mg/kg p.o.) or istradefylline (0.1 mg/kg  
698 p.o.) and periorbital thresholds were tracked over 5 hrs.

699

Figure 1

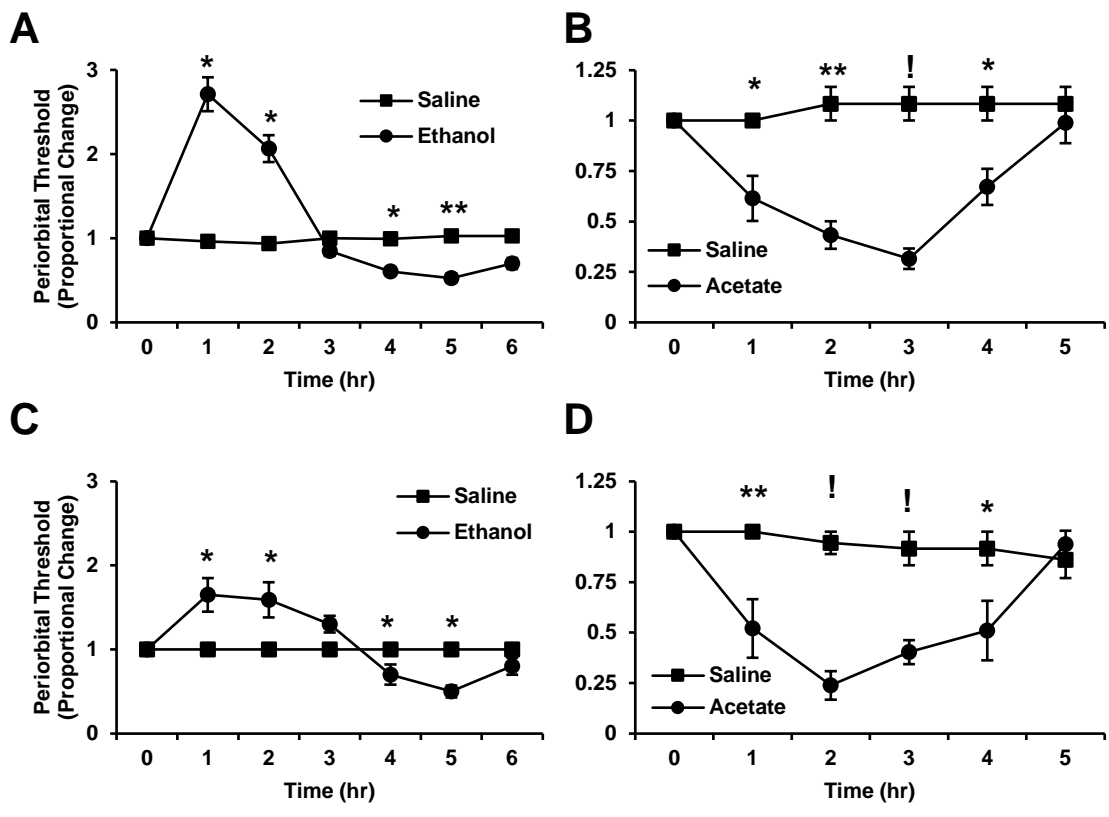
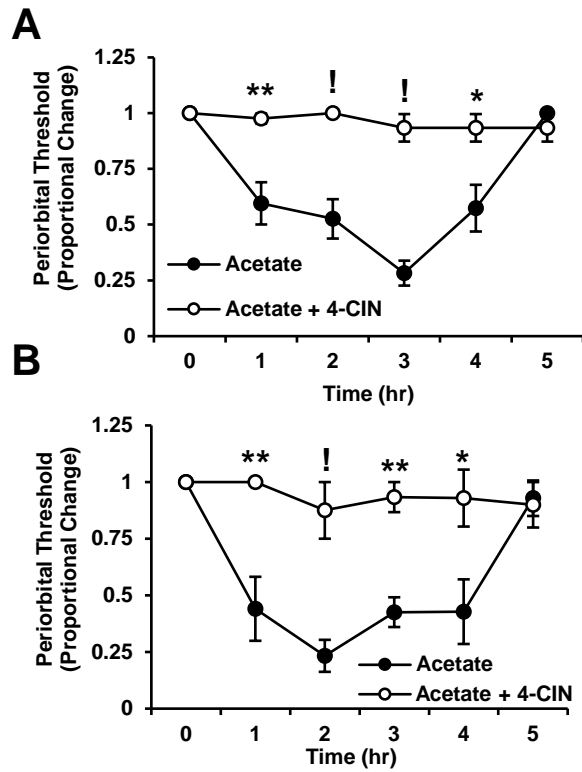
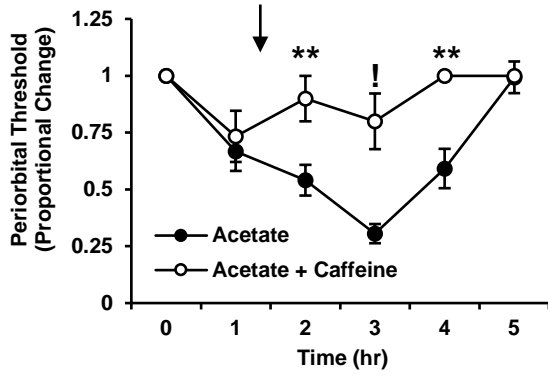


Figure 2

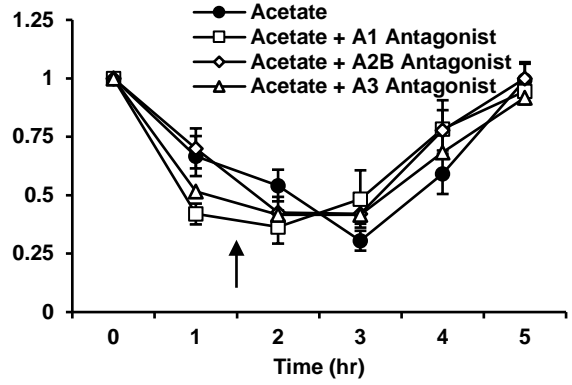


**Figure 3**

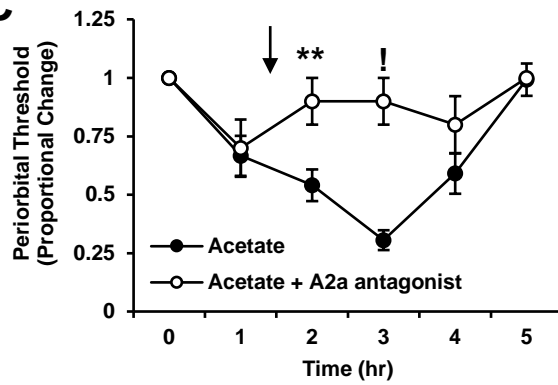
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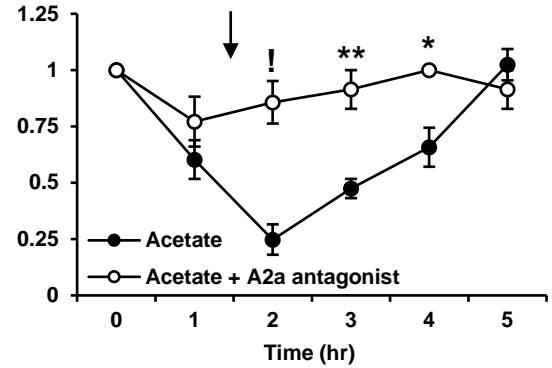
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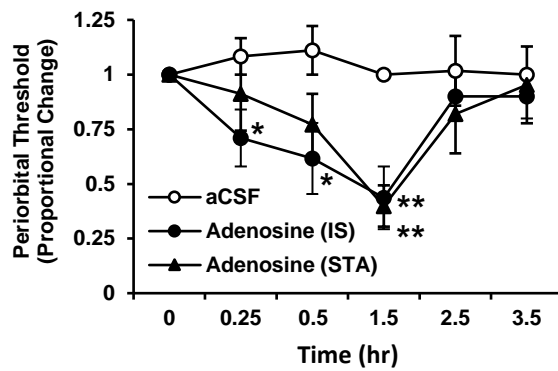
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**D**



**E**



**F**

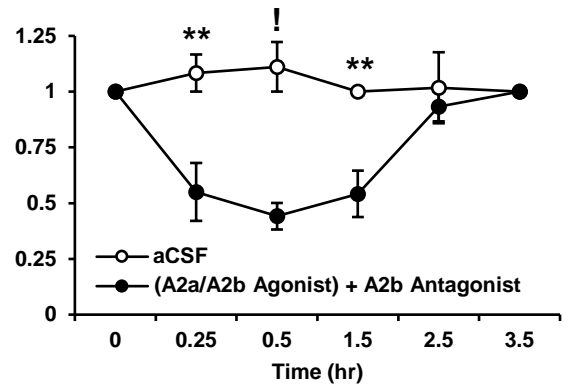


Figure 4

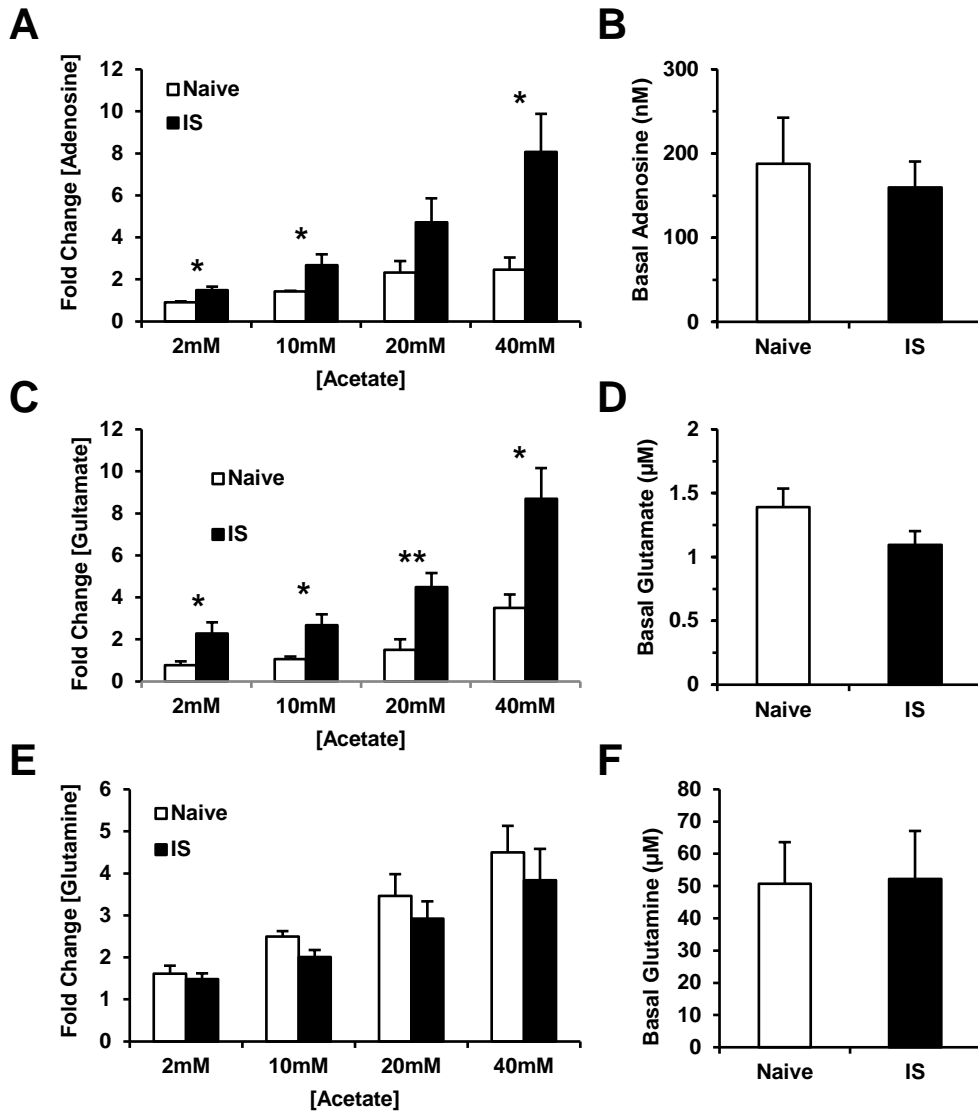
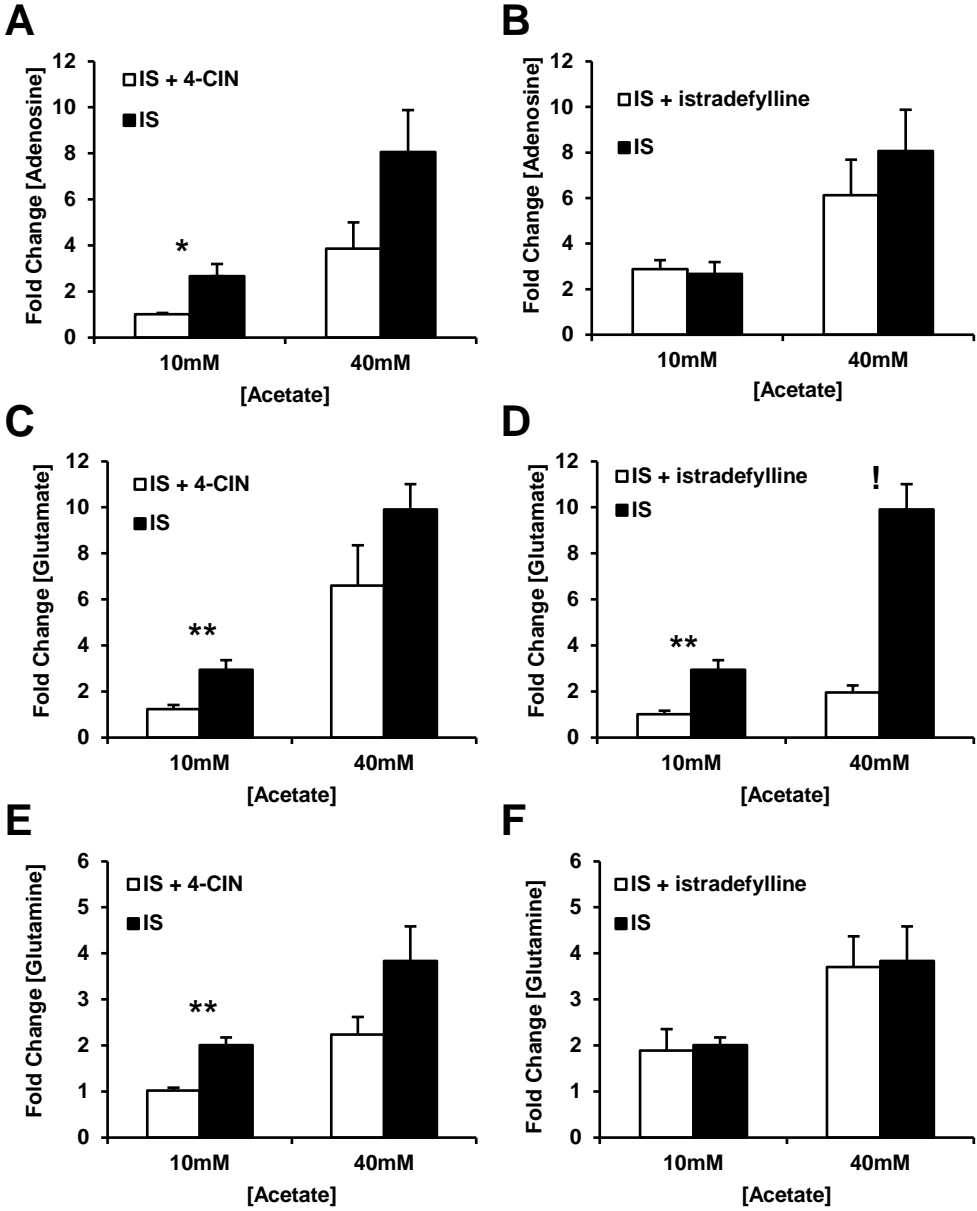


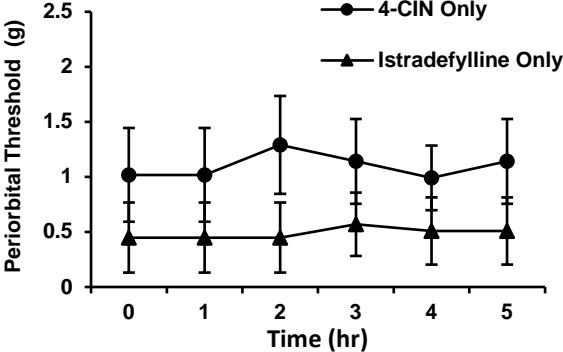


Figure 5



# Supplemental Figure 1

**A**



**B**

