Local Metabolism of Ethanol to Acetic Acid/Acetate in the Central Nucleus of Amygdala Elicits Sympathoexcitatory Responses through Activation of NMDAR in Sprague Dawley Rats

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ABSTRACT

Aim: Binge alcohol consumption elicits robust sympathoexcitation and excitatory neuronal output. However, the central mechanism that mediates these effects remains elusive. We investigated the effects of ethanol metabolism within the central nucleus of the amygdala (CeA) on sympathoexcitation, and elucidated the role of acetate in these excitatory responses.

Methods: *In vivo* arterial blood pressure, heart rate and sympathetic nerve activity responses to CeA microinjected ethanol or acetate with appropriate inhibitors/antagonists were tracked. *In vitro* whole-cell electrophysiology recording responses to acetate in CeA neurons with axon projecting to the rostral ventrolateral medulla (CeA-RVLM) were investigated, and cytosolic calcium responses in primary neuronal cultures were quantified.

Results: We demonstrate that in Sprague Dawley rats, local brain metabolism of ethanol in the CeA to acetic acid/acetate elicits sympathoexcitatory responses *in vivo* through activation of NMDAR. Aldehyde dehydrogenase inhibition using cyanamide and NMDAR antagonism using AP5 or memantine blunted these effects. Whole-cell patch-clamp recordings in brain slices containing autonomic CeA-RVLM neurons revealed a dose-dependent increase in neuronal excitability in response to acetate. The NMDAR antagonists AP5 and memantine suppressed the acetate-induced increase in CeA-RVLM neuronal excitability, as well as the direct activation of NMDAR-mediated inward currents by acetate in brain slices. We observed that acetate increased cytosolic Ca^{2+} in a time-dependent manner in primary neuronal cell cultures. The acetate enhancement of calcium signaling was abolished by memantine.

Conclusion: These findings suggest that within the CeA, ethanol is sympathoexcitatory through local brain metabolism, which generates acetic acid/acetate leading to activation of NMDAR.

Keywords: Acetate, Acetic Acid, Calcium, Central Nucleus of Amygdala, Ethanol, Glutamate, NMDA Receptors, RVLM, Sympathetic Nerve Activity.

INTRODUCTION

Alcohol is one of the most widely used and abused recreational substances globally. Ethanol metabolism generates acetic acid, which is the main component of vinegar and is produced in large quantities following ethanol ingestion. This leads to significantly elevated serum and cerebrospinal fluid (CSF) acetate concentrations of around 1-3 mM[1,2] and 2-5 mM[1,2], respectively. Concentrations of acetic acid/acetate in the circulation and CSF are reported to remain elevated long after ethanol has cleared the body.[2] It is therefore reasonable to suspect that acetic acid/acetate may be a player in the altered neuronal function following ethanol consumption and metabolism.

Precisely how ethanol produces excitatory effects in the CNS is largely unknown. On the one hand, increases in dopamine release from the ventral tegmental area[3] facilitate reward and possible excitatory actions on neurons post ethanol ingestion. However, this is partially contradicted by ethanol itself inhibiting NMDAR in slice recordings.[4-6] Secondly, studies involving NMDAR inhibition by ethanol are commonly done in brain slice preparation[4,5,7] with large circulating volumes of ethanol relative to small tissue samples, and the effects of in-slice metabolism of ethanol has yet to be reported. What is known is that ethanol consumption contributes to excitatory actions originating within the CNS[3,8] with some positive modulatory action on NMDAR.[9] Excitation is augmented following ethanol clearance, which can lead to hangover symptoms.[10,11] Acetate has been implicated in this response.[12] Unfortunately, literature investigating the influence of acetic acid/acetate on neuronal function remains scarce.

88 Our lab has been pursuing a line of thought that acetic acid/acetate is responsible for many
89 of the excitatory effects of ethanol metabolism on neurons. We have previously reported that
90 microinjection of ethanol and acetate into the central nucleus of amygdala (CeA), an area
91 containing pre-sympathetic neurons with axons projecting to the rostral ventrolateral medulla
92 (CeA-RVLM), induced sympathoexcitation and pressor responses mediated by local activation of
93 NMDAR.[13] Similarly, we have demonstrated that pathophysiological concentrations of acetate
94 from ethanol metabolism are cytotoxic in dopaminergic-like PC12 cells, at least partially through
95 NMDAR activation[14] and acetic acid increases excitability in medium spiny neurons of the nucleus
96 accumbens shell.[15] Given these parallel and converging sets of results, we hypothesized that
97 inhibition of ethanol metabolism (Fig. 1F) may attenuate the ethanol sympathoexcitatory response
98 by preventing the generation of acetic acid/acetate which we have found to activate NMDAR.
99 Similarly, we hypothesized that in brain slice recordings containing CeA-RVLM neurons, bath
100 application of acetate would increase neuronal excitability through activation of NMDAR.
101

102 **MATERIALS AND METHODS**

103 **Animals**

104 Male Sprague-Dawley rats purchased from Charles River Labs (Wilmington, MA, USA)
105 were individually housed in a temperature-controlled room (22-23 ^{o}C) with a 12:12 h light-dark
106 cycle. Chow and tap water were available *ad libitum* unless otherwise noted. All experimental and
107 surgical procedures were carried out under the guidelines of the National Institutes of Health Guide
108 for the Care and Use of Laboratory Animals with the approval of the Institutional Animal Care and
109 Use Committee at Michigan Technological University.
110

111 ***In vivo* whole animal recordings of arterial blood pressure (ABP), splanchnic sympathetic**
112 **nerve activity (SSNA) and lumbar sympathetic nerve activity (LSNA)**

113 Animal surgery was performed following the previously described protocol.[16] On the day
114 of the experiment, rats were anesthetized with an intraperitoneal injection containing a mixture of
115 α-chloralose (80 mg/kg) and urethane (800 mg/kg). Adequate depth of anesthesia was assessed
116 before surgery by the absence of the pedal withdrawal reflex and corneal reflexes. Animals were
117 instrumented with an arterial catheter inserted into the aorta through a femoral artery. The catheter
118 was connected to a pressure transducer to measure arterial blood pressure. Heart rate (HR) was
119 obtained from the R-wave of the electrocardiogram (ECG) (lead I). A catheter was also placed in
120 the left femoral vein to administer drugs. After tracheal cannulation, rats were paralyzed with
121 gallamine triethiodide (25 mg·kg−1·h−1 iv) and artificially ventilated with oxygen-enriched room
122 air. After paralysis, adequate depth of anesthesia was determined by lack of pressor responses to
123 noxious foot pinch. Supplemental doses of anesthesia equal to 10% of the initial dose were given
124 when needed. End-tidal PCO_2 was continuously monitored and maintained within normal limits
125 (35–40 mmHg) by adjusting ventilation rate (70–80 breaths/min) and/or tidal volume (2.0–3.0 ml).
126 Body temperature was held at 37°C with a water-circulating pad.

127 SSNA and LSNA recordings were performed according to previously described
128 protocols.[13,16,17] With the use of a left flank incision, a left lumbar and postganglionic splanchnic
129 sympathetic nerve bundle was isolated from surrounding tissue and mounted on a stainless steel
130 wire electrode (0.127-mm OD; A-M Systems), and covered with a silicon-based impression
131 material (Coltene, Light Body) to insulate the recording from body fluids. The recorded signal was
132 directed to an AC amplifier (P511; Grass Technologies) equipped with half-amplitude filters (band
133 pass: 100-1,000 Hz) and a 60-Hz notch filter. The processed signal was rectified, integrated (10-

134 ms time constant), and digitized at a frequency of 5,000 Hz using a 1401 Micro3 analog-to-digital
135 converter and Spike 2 software (7.04 version; Cambridge Electronic Design, Cambridge, UK).
136 The background noise was determined by a bolus injection of hexamethonium (30 mg/kg iv), a
137 ganglionic blocker, at the end of the experiment and was subtracted from all the integrated values
138 of sympathetic nerve activity.
139

140 **CeA microinjection**

141 CeA injections were performed as previously described.[13] Animals were placed in a
142 stereotaxic head frame, and the skull was leveled between bregma and lambda. A section of skull
143 was removed so that a single-barreled glass microinjector pipette could be lowered vertically into
144 the CeA. The following stereotaxic coordinates were used: CeA, caudal to bregma, −2.4 ~ −2.5
145 mm; lateral to midline, 4.8~5.0 mm; and ventral to dura, 7.7~8.0 mm. All animals were allowed
146 to stabilize at least 2 h following surgery. To test whether inhibition of ALDH prevented ethanol
147 elicited sympathoexcitatory responses, ethanol (1.7 μmol) or a cocktail of ethanol (1.7 μmol) and
148 cyanamide (0.24 nmol) were microinjected into the CeA in separate groups of animals. Similarly,
149 the effects of acetate and the involvement of NMDAR on acetate evoked sympathoexcitatory
150 response were also explored through CeA injection of acetate (0.1 μmol and 0.20 μmol) and a
151 cocktail containing acetate (0.20 μmol) and memantine (3.0 nmol), or acetate (0.2 μmol) and AP5
152 (3.0 nmol) in separate groups of animals. All compounds were microinjected in a volume of 100
153 nL with a pneumatic pump (WPI). The volume of each injection was determined by measuring the
154 movement of the fluid meniscus within the microinjector pipette using a dissecting microscope
155 equipped with an eyepiece reticule. At the end of each experiment, Chicago blue dye solution (2%
156 in saline, 100 nL) was injected into the CeA to mark the site of each injection. Brains were removed

157 and post fixed for 5 days at room temperature in 4% paraformaldehyde. Brain coronal sections
158 containing the CeA were cut, and microinjection sites were identified under light microscopy. Rats
159 with injection site(s) not inside the CeA were excluded from data analysis.
160

161 **Retrograde labeling**

162 Retrograde labeling of CeA-RVLM neurons[13]: Male Sprague-Dawely rats (350-450 g)
163 were retrograde labeled from the RVLM to the CeA. Rats were anesthetized with an intraperitoneal
164 injection (i.p.) of sodium pentobarbital (50 mg/kg), placed in a stereotaxic frame, leveled between
165 bregma and lambda and the skull exposed with a midline incision. A small, unilateral burr hole
166 was drilled to expose the cerebellum in the caudal section of the skull and a glass micropipette
167 lowered into the pressor region of the RVLM (coordinates: −12.7 mm caudal to bregma, 1.8 mm
168 lateral to midline and 8.9 mm below the skull).[18] 100 nL fluospheres (Life Technologies) was
169 injected into the RVLM using a pneumatic pump (WPI) and left in place for approximately 5
170 minutes to prevent tracer backflow. The midline incision was closed, and the rats were
171 administered a subcutaneous (s.c.) injection of penicillin G (30,000 units) and Metacam (1 mg/kg,
172 NSAID) in 0.5 mL saline for three days post-surgery to prevent infection and reduce pain.
173

174 **Whole-cell patch clamp recordings**

175 Five to seven days post labeling, rats were anesthetized with isoflurane (3% in O_2) and
176 decapitated. The brain was rapidly removed and chilled in ice cold cutting solution, containing (in
177 mM): 206 sucrose, 2 KCl, 2 $MgSO_4$, 1.25 NaH_2PO_4, 26 $NaHCO_3$, 1 $CaCl_2$, 1 $MgCl_2$, 10 d-glucose,
178 and 0.4 ascorbic acid, osmolarity 295-302 mosmol L^{-1} measured with an osmometer (Wescor), pH
179 7.3-7.4, continuously gassed with 95:5 O_2:CO_2 to maintain pH and pO_2. A brain block was cut

180 including the CeA region and affixed to a vibrating microtome (Leica VT 1000S; Leica, Nussloch,
181 Germany). Coronal sections of 250 µm thickness were cut, and the slices transferred to a holding
182 container of artificial cerebral spinal fluid (ACSF) maintained at 30 °C, continuously gassed with
183 95:5 O_2:CO_2, containing (in mM): 125 NaCl, 2 KCl, 2 $MgSO_4$, 1.25 NaH_2PO_4, 26 $NaHCO_3$, 2
184 $CaCl_2$, 10 d-glucose, and 0.4 ascorbic acid (osmolality: 295–302 mosmol L^{-1}; pH 7.3-7.4) and
185 allowed to recover for 1 hr. Following recovery, slices were transferred to a glass-bottomed
186 recording chamber and viewed through an upright microscope (E600FN, Nikon) equipped with
187 DIC optics, epi-fluorescence, an infrared (IR) filter and an IR-sensitive video camera (C2400,
188 Hamamatsu, Bridgewater, NJ).

189 Slices transferred to the glass-bottomed recording chamber were continuously perfused
190 with ACSF, gassed with 95:5 O_2:CO_2, maintained at 30 °C and circulated at a flow of 2 mL min^{-}
191 ^{1}. Patch electrodes were pulled (Flaming/Brown P-97, Sutter Instrument, Novato, CA) from
192 borosilicate glass capillaries and polished to a tip resistance of 4–8 MΩ. Electrodes were filled
193 with a solution containing (in mM) 135 K-gluconate, 10 HEPES, 0.1 EGTA, 1.0 $MgCl_2$, 1.0 NaCl,
194 2.0 Na_2ATP, and 0.5 Na_2GTP (osmolality: 280–285 mosmol L^{-1}; pH 7.3). Once a GΩ seal was
195 obtained, slight suction was applied to break into whole-cell configuration. The cell was allowed
196 to stabilize; this was determined by monitoring capacitance, membrane resistance, access
197 resistance and resting membrane potential (V_m).[18] Records were not corrected for a liquid junction
198 potential of -15 mV. Cells that met the following criteria were included in the analysis: action
199 potential amplitude ≥50 mV from threshold to peak, input resistance (R_{input}) >0.3 GΩ (determined
200 by injection of −20 pA from a holding potential of −80 mV), resting V_m negative to −50 mV, and
201 <20% change in series resistance during the recording. Recordings were made using an Axopatch
202 200B amplifier and pCLAMP software (v10.2, Axon Instruments, Union City, CA). Signals were

filtered at 1 kHz, digitized at 10 kHz (Digidata 1400A, Axon Instruments), and saved on a computer for off-line analysis. To study the mAHP, recordings were made from a potential of −60 mV, and +150 pA current injections (500 ms) were made followed by a return of V_m to −60 mV for 5 s. To study single action potential AHP, the 7th action potential was selected from our mAHP protocol train. AHP was determined from the change in greatest depression in AHP of the 7th action potential relative to 10 ms post action potential.

The effect of acetate on the excitability of CeA-RVLM neurons and the role of NMDAR (current-clamp): With V_m adjusted to -80 mV by continuous negative current injection, a series of square-wave current injections was delivered in steps of +25 pA, each for a duration of 800 ms. To determine the action potential voltage threshold (Vt) and depolarizing R_{input} below V_t, ramp current injections (0.2 pA/ms, 1,000 ms) were made from a potential of -80 mV. Square-wave and ramp current injections were made in the same neurons. Effects of acetate on neuronal excitability were determined by comparing current evoked spike frequency responses under control conditions with responses recorded from neurons exposed to bath application of acetate. To determine the dose-dependent response, the effect of various concentration of acetate (0.0, 3.75, 7.5, 37.5 and 75.0 mM) on the excitability was determined in separate groups of neurons, respectively. To test whether the activation of NMDAR was involved in the increased neuronal excitability elicited by acetate, the effect of bath application of AP5 (60 μM, NMDAR competitive blocker) or memantine (30 μM, NMDAR pore blocker), on the excitability was determined in the presence of acetate (37.5 mM) in a separate group of neurons.

Acetate evoked inward current and the role of NMDAR (voltage-clamp) in CeA-RVLM neurons: Brain slices were continuously perfused with modified, Mg^{2+} free ACSF containing (in mM): 127 NaCl, 2 KCl, 1.25 NaH_2PO_4, 26 $NaHCO_3$, 2 $CaCl_2$, 10 d-glucose, and 0.4 ascorbic acid

226 (osmolality: 295–302 mosmol L^{-1}; pH 7.3-7.4), gassed with 95:5 O_2:CO_2, maintained at 30 °C and
227 circulated at a flow of 2 mL min^{-1}. Tetrodotoxin (TTX, voltage gated sodium channel blocker, 0.5
228 μM) and picrotoxin (GABA-A blocker, 100 μM) were added into the circulating extracellular,
229 Mg^{2+} free ACSF. Cells were voltage clamped at V_m = -80 mV and allowed to stabilize by
230 monitoring baseline current. Once cells were stable and baseline recording in the absence of acetate
231 ~1.5 min was observed, acetate (37.5 mM) was added to the circulating bath and recorded until a
232 plateau was observed > 15 min. At this point, washout and recovery was observed. To test whether
233 the activation of NMDAR was involved in the inward current evoked by acetate, the effect of
234 memantine (30 μM) or AP5 (60 μM) on the inward current was determined. In separate groups of
235 neurons, acetate (37.5 mM) and memantine (30 μM) or acetate (37.5 mM) and AP5 (60μM) were
236 added to the circulating bath and the inward current was observed. To exclude the possibility that
237 the inward current evoked by sodium acetate was due to the increase in extracellular sodium
238 concentration, equimolar sodium gluconate (37.5 mM) was substituted for sodium acetate and the
239 inward current was recorded.

240 Note that current- and voltage-clamp recordings were made from separated groups of CeA-
241 RVLM neurons.

242

243 **Preparation of neuronal cultures**

244 Whole brain neuronal cultures were made from 1 day old SD rats according to our previous
245 publication.[19,20] Briefly, rats were anesthetized with pentobarbital (50 mg/kg) and the brains were
246 extracted and dissected. Dissected brain areas were combined, and brain cells were dissociated
247 before being plated on poly-L-lysine precoated culture dishes. Two days following plating,
248 cultures were treated for two days with cytosine arabanoside (ARC, 10 μM) to kill off rapidly

249 dividing cells (astroglia and astrocytes) followed by fresh ARC free media for the remainder of
250 cultures. Neuronal cultures contained > 90% neurons with the remainder primarily astroglia.
251 Neuronal cell cultures were grown for 10-14 days before use in cell culture experiments.
252

253 **Real-time calcium imaging**

254 Primary neuronal cultures 12-14 days post isolation were incubated for 30 min with Fluo-
255 4AM (Thermofisher, 3 μM final concentration) according to the manufactures instructions in
256 artificial cerebral spinal fluid (ACSF), containing (in mM): 125 NaCl, 2 KCl, 2 $MgSO_4$, 1.25
257 NaH_2PO_4, 26 $NaHCO_3$, 2 $CaCl_2$, 10 d-glucose, and 0.4 ascorbic acid (osmolality: 295–302 mosmol
258 L^{-1}; pH 7.3-7.4) in a 5% CO_2 humidified incubator at 37 °C. The staining solution was aspirated
259 and fresh ACSF (0.5 mL) added to the cells. Baseline calcium fluorescence were taken followed
260 by the addition of acetate and/or acetate and memantine in ACSF (0.5 mL additional volume
261 added) to reach the desired concentrations (e.g., 0.5 mL ACSF initial, then add 0.5 mL acetate (75
262 mM) in ACSF. Final concentration acetate (37.5 mM)). Images were captured for each well in a
263 24 well plate using a cell imager equipped with the correct LED lights (ZOE, Bio-Rad).
264 Fluorescence intensity was analyzed and quantified using ImageJ software[21] on 10-13 randomly
265 selected cells for each treatment and corrected fluorescence intensity (CFIT) compared between
266 treatment groups prior to adjusting backgrounds. CFIT was calculated based on individual
267 background fluorescence within each well, prior to batch background normalization for image
268 preparation. Duplicates of each treatment were compared, and all showed similar trends. Negative
269 controls were run for ACSF alone on time course for alterations in baseline calcium and there was
270 no significant change in baseline values compared to 25 minutes post ACSF only.
271

272 **Justification on doses/concentrations**

273 To verify the effects of ethanol in the CeA, we selected a dose of ethanol (1.7 µmole or
274 100 nL of 17.15 M) which was a dose we found to yield a maximum response *in vivo* as previously
275 described.[13] Previous studies of targeted brain delivery of ethanol to the CeA in rats (75-350 mg%,
276 consisting of 8,000-10,000 nL infusions or a total dose of 0.761 µmole ethanol)[22] have been
277 reported, and our maximum dose utilized is close to this dosing regimen. The following acetate
278 doses were used for *in vivo* CeA microinjections: 0.1 and 0.2 µmole or 100 nL of 1 M and 2 M
279 sodium acetate. Previous intraventricular infusions (0.8-2.8 µmole) of acetate have been reported[23]
280 and we are within acceptable limits.

281 For our electrophysiology experiments, we utilized the current ethanol metabolism profile
282 (roughly 90% of ethanol is metabolized to acetic acid/acetate).[24] That is to say, ethanol metabolism
283 to acetic acid/acetate is a 1:1 conversion and apparent ethanol concentrations measured would in
284 theory produce equivalent acetic acid/acetate concentrations (e.g., 50 mM ethanol should equal 50
285 mM acetate). We constructed a dose-dependent response curve to acetate and found an EC_{50} of
286 5.90 mM and an E_{max} of 37.5 mM. We utilized 37.5 mM for our electrophysiology studies because
287 it yielded the most robust response.

288

289 **Chemicals**

290 All chemicals were obtained from Sigma-Aldrich (St Louis, MO, USA) with the exception
291 of tetrodotoxin (Tocris Bioscience, UK) and tetraethylammonium sodium (Fluka BioChemika,
292 Switzerland). Glacial acetic acid was obtained from Sigma-Aldrich (St. Louis, MO) and ultra-pure
293 water (> 18 MΩ) obtained in-house by filtration and purification of house distilled water from an
294 EasyPure II water filtration system (Barnstead).

295

296 **Statistical analysis**

297 Data values were reported as mean ± SD. Depending on the experiments, group means
298 were compared using either paired, unpaired Student's *t*-test, a one-way or a two-way ANOVA.
299 Differences between means were considered significant at $p < 0.05$. Where differences were
300 found, Bonferroni post hoc tests were used for multiple pair-wise comparisons. All statistical
301 analyses were performed with a commercially available statistical package (GraphPad Prism,
302 version 9.0).

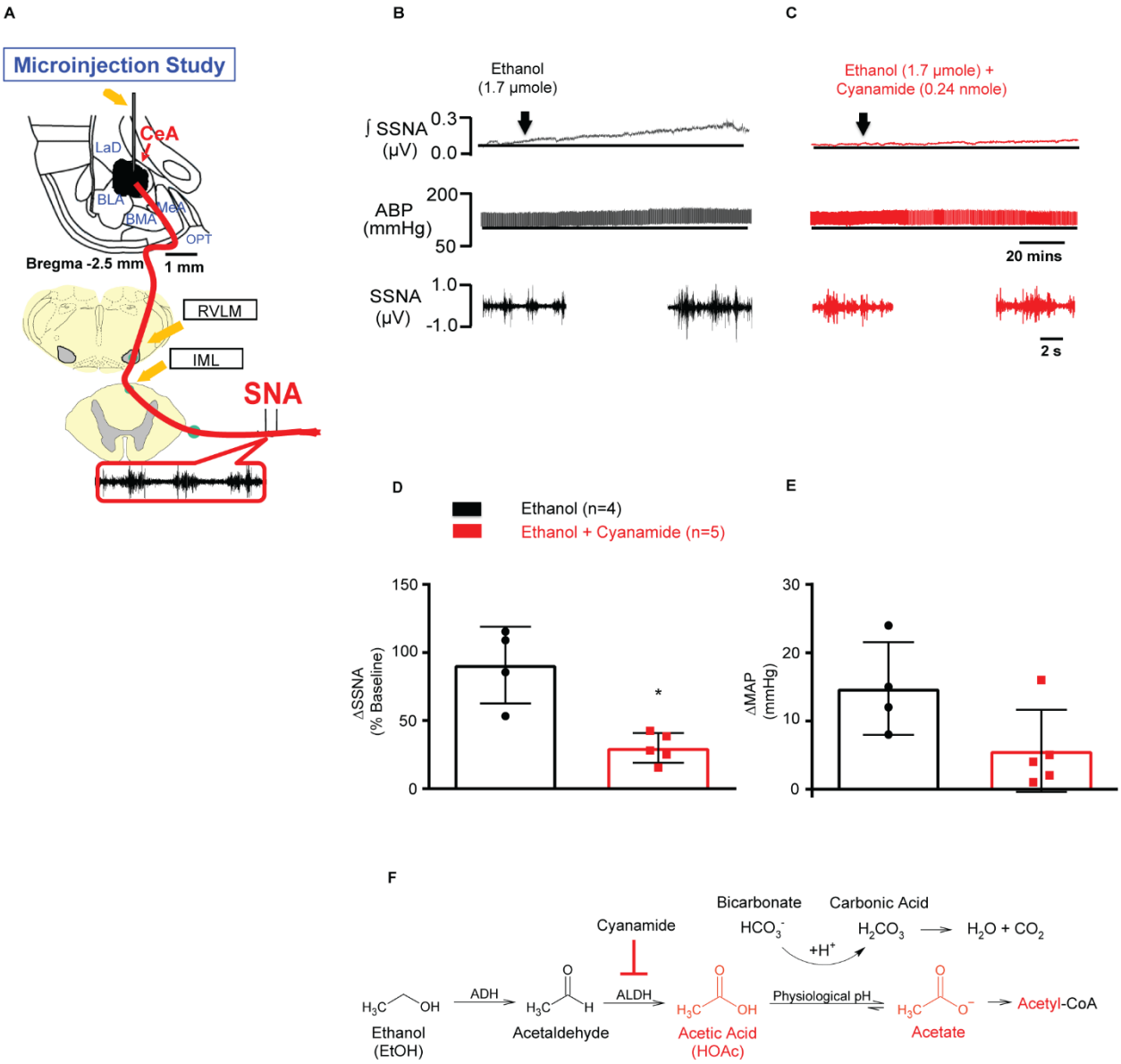
303

304 **RESULTS**

305 **Local metabolism of ethanol to acetic acid/acetate in the CeA activates NMDAR *in vivo***

306 Our lab previously reported that ethanol and acetate microinjected into the CeA of
307 anesthetized rats resulted in a sympathoexcitatory effect and speculated that it was due to local
308 metabolism of ethanol to acetate.[13] Consistent with our previous findings, microinjection of
309 ethanol into the CeA of anesthetized rats elicited a sympathoexcitatory and pressor response (Fig
310 1B,D,E). Because we speculated that this sympathoexcitatory response was due to local
311 metabolism of ethanol to acetate, we co-applied ethanol with an aldehyde dehydrogenase inhibitor
312 (ALDH), cyanamide,[25] into the CeA (Fig 1C,D,E). Blockade of ALDH with cyanamide
313 significantly blunted the sympathoexcitatory response from ethanol (ethanol SSNA: + 90.83 ±
314 28.11 % baseline, ethanol MAP: 14.75 ± 6.8 mmHg vs ethanol & cyanamide SSNA: +29.89 ±
315 10.88 % baseline, ethanol & cyanamide MAP: 5.6 ± 6.03 mmHg), indicating that neither ethanol
316 nor acetaldehyde was likely a causitive agent in the excitatory response. Cyanamide (0.24 nmole)
317 alone had no significant effect on SSNA (pre: 0.039 ± 0.016 vs post: 0.051 ± 0.020, p=0.06, paired

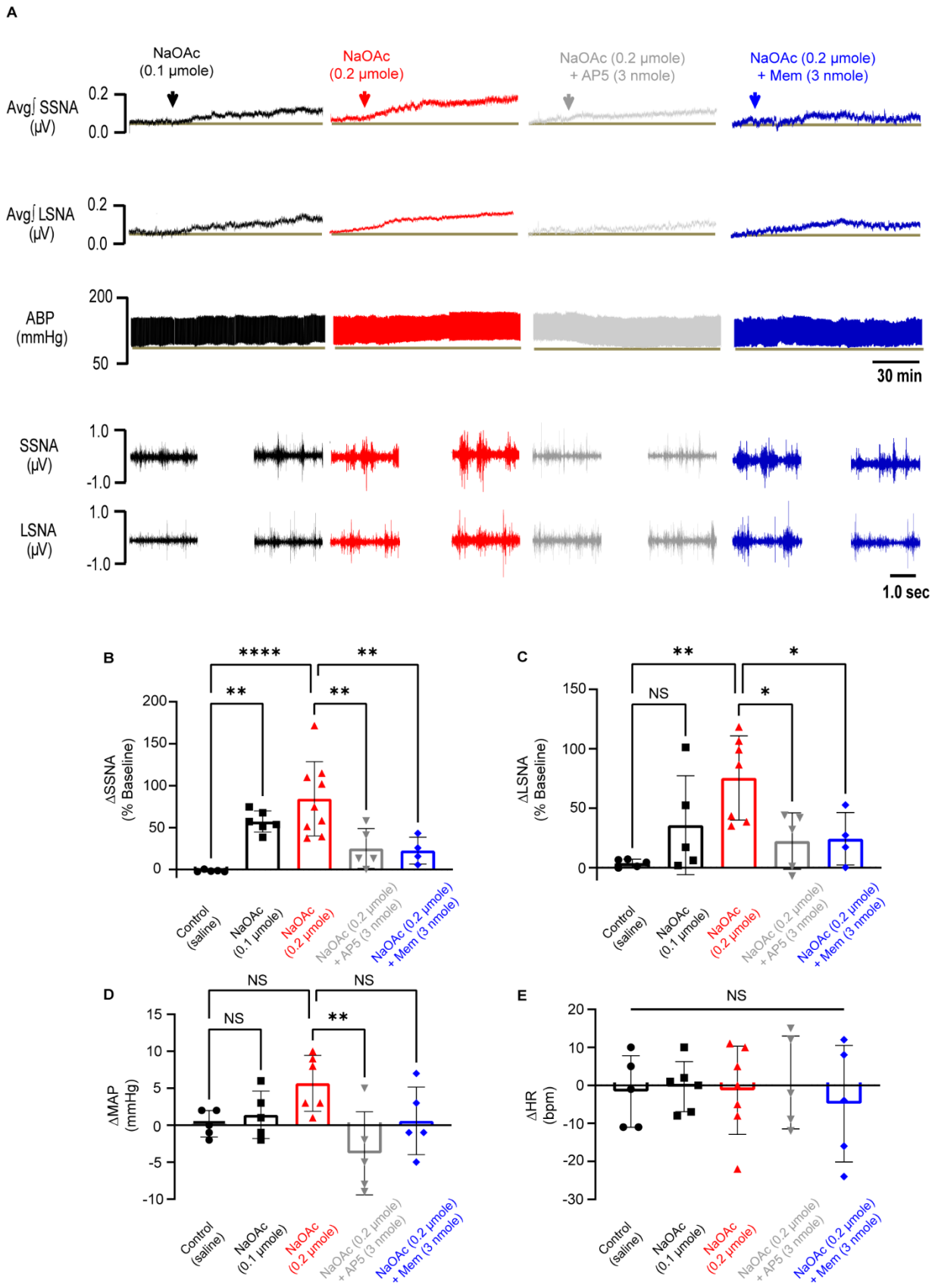
318 t-test) and LSNA (pre: 0.015 ± 0.007 vs post: 0.020 ± 0.009, p=0.10, paired t-test) but caused a
319 significant decrease in the MAP (pre: 120 ± 9.5 vs post: 109 ± 6.6, p =0.03, paired t-test) (n=3
320 rats).



322 **Figure 1. Local CeA brain metabolism of ethanol is sympathoexcitatory. (A)** Graphical
323 depection of CeA microinjection study for sympathetic nerve recordings. **(B)** Representative raw
324 traces displaying SSNA and arterial blood pressure (ABP) from CeA microinjected ethanol. **(C)**
325 Representative raw traces displaying SSNA and ABP from CeA microinjected ethanol with ALDH

326 inhibitor, cyanamide. **(D)** Summary data of SSNA for ethanol (black) and ethanol & cyanamide
327 (red) (*p=0.028 vs ethanol). **(E)** Summary data of change in mean aterial pressure (MAP) for
328 ethanol (black) and ethanol & cyanamide (red), (n=number of rats). **(F)** Major metabolic pathway
329 for ethanol metabolism (abbreviations: ADH, alcohol dehydrogenase; ALDH, aldehyde
330 dehydrogenase).
331
332 Next, we verified that acetate was the bioactive metabolite from ethanol metabolism.
333 Microinjection of acetate into the CeA of anesthetized rats increased sympathetic nerve activity
334 (saline SSNA: -1.61 ± 1.25, acetate (0.1 μmol) SSNA: +57.15 ± 12.76, acetate (0.2 μmole) SSNA:
335 +94.60 ± 44.35 % baseline) (saline LSNA: +3.84 ± 3.26, acetate (0.1 μmol) LSNA: +35.79 ±
336 41.65, acetate (0.2 μmol) LSNA: +67.02 ± 38.91 % baseline) and mean arterial blood pressure
337 (MAP) (saline MAP: 0.2 ± 1.79, acetate (0.1 μmol) MAP: 1.4 ± 3.2, acetate (0.2 μmol) MAP: 5.67
338 ± 3.78 mmHg) in a dose-dependent manner (Fig 2). For NMDAR antagonism, memantine has seen
339 utilization in alcohol studies[26,27] to prevent cognitive decline and as a possible therapeutic for
340 excitotoxicity.[28] Furthermore, it is an FDA approved NMDAR blocker for the treatment of
341 Alzhiemer's disease,[29] which would give more powerful treatment options clinically compared to
342 utilizing AP5 as previously described[13] or MK-801 (Olney's lesions).[30] Blockade of NMDAR
343 within the CeA using memantine or AP5 significantly attenuated the acetate induced
344 sympathoexcitatory and pressor responses (acetate (0.2 μmol) SSNA: +94.60 ± 44.35 vs acetate
345 (0.2 μmol) & memantine (3.0 nmol) SSNA: +22.55 ± 16.04 vs acetate (0.2 μmol) & AP5 (3.0
346 nmol) SSNA: +25.1 ± 23.7 % baseline), (acetate (0.2 μmol) LSNA: +67.02 ± 38.91 vs acetate (0.2
347 μmol) & memantine (3.0 nmole) LSNA: +24.37 ± 21.98 vs acetate (0.2 μmol) & AP5 (3.0 nmol)
348 LSNA: +22.4 ± 23.8 % baseline), (acetate (0.2 μmol) MAP: 5.67 ± 3.78 vs acetate (0.2 μmol) &

349 memantine (3.0 nmol) MAP: 0.6 ±4.56 vs acetate (0.2 µmol) & AP5 (3.0 nmol) MAP: -3.8 ± 5.6
350 mmHg) suggesting that increases in SNA were due to acetate activation of NMDAR (Fig 2).
351 Memantine (3.0 nmole) alone had no significant effect on baseline MAP (pre: 112 ± 13.2 vs post:
352 110.3 ± 15.0, p =0.58, paired t-test), SSNA (pre: 0.023 ± 0.014 vs post: 0.024 ± 0.015, p=0.37,
353 paired t-test) and LSNA (pre: 0.018 ± 0.003 vs post: 0.019 ± 0.004, p=0.14, paired t-test) (n=3
354 rats). Likewise, our previous study utizling AP5 saw no significant difference to CeA
355 microinjected AP5 on baseline parmeters.[13]


A
NaOAc (0.1 µmole)
NaOAc (0.2 µmole)
NaOAc (0.2 µmole) + AP5 (3 nmole)
NaOAc (0.2 µmole) + Mem (3 nmole)
Avg∫ SSNA (µV)
Avg∫ LSNA (µV)
ABP (mmHg)
30 min
SSNA (µV)
LSNA (µV)
1.0 sec
B
ΔSSNA (% Baseline)
C
ΔLSNA (% Baseline)
D
ΔMAP (mmHg)
E
ΔHR (bpm)
NS
Control (saline)
NaOAc (0.1 µmole)
NaOAc (0.2 µmole)
NaOAc (0.2 µmole) + AP5 (3 nmole)
NaOAc (0.2 µmole) + Mem (3 nmole)

357 **Figure 2. CeA microinjected acetate dose-dependently increases sympatheic nerve activity**
358 **and is NMDAR dependent. (A)** Representative raw traces displaying sympathetic nerve activity
359 (SSNA and LSNA) and ABP from CeA microinjected acetate. **(B)** Summary SSNA data
360 (**p=0.0093, **** p<0.0001). **(C)** Summary LSNA data (*p=0.045, **p=0.018). **(D)** Summary
361 MAP data (**p=0.003). **(E)** Summary change in HR data (NS=not significant), (individual
362 points=number of animals).
363
364 **Estimating final CeA tissue concentrations from microinjection**
365 Estimation of brain microinjected compounds is a notoriously difficult task. Small
366 volumes, effects of anesthesia, and blood flow can drastically alter what local brain area tissues
367 see in terms of final concentrations. The ethanol literature reports ~90% ethanol metabolism to
368 acetic acid/acetate, and this conversion is 1:1.[24] Reported intoxicating blood alcohol concentrations
369 in humans and rodents typically fall in the range of 17-100 mM,[31-33] however brain slice patch
370 clamp recordings can sometimes utilize ethanol concentrations in excess of 200 mM.[34-36] To
371 estimate brain tissue concentrations to compounds tested, we microinjected 100 nL of ethanol or
372 saline loaded with Chicago blue dye within the CeA, collected the stained tissue and recorded the
373 weight. CeA estimated concentrations were determined by [x µmole compound/average tissue
374 volume (mL)]. These concentrations represent the upper theoretical concentration limit and are
375 likely an overestimate as diffusion is greater than the visualized stained tissue excised.
376 Nevertheless, the upper theoretical concentration for 1.7 µmole ethanol is 283 mM, for 0.1 µmole
377 acetate is 16.6 mM and for 0.2 µmole acetate is 33.3 mM.

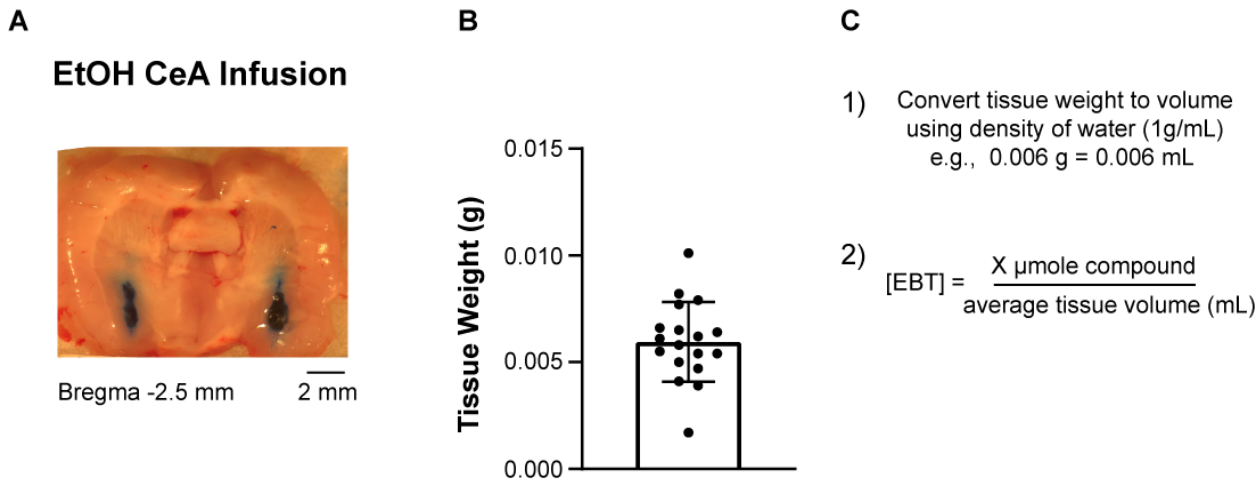
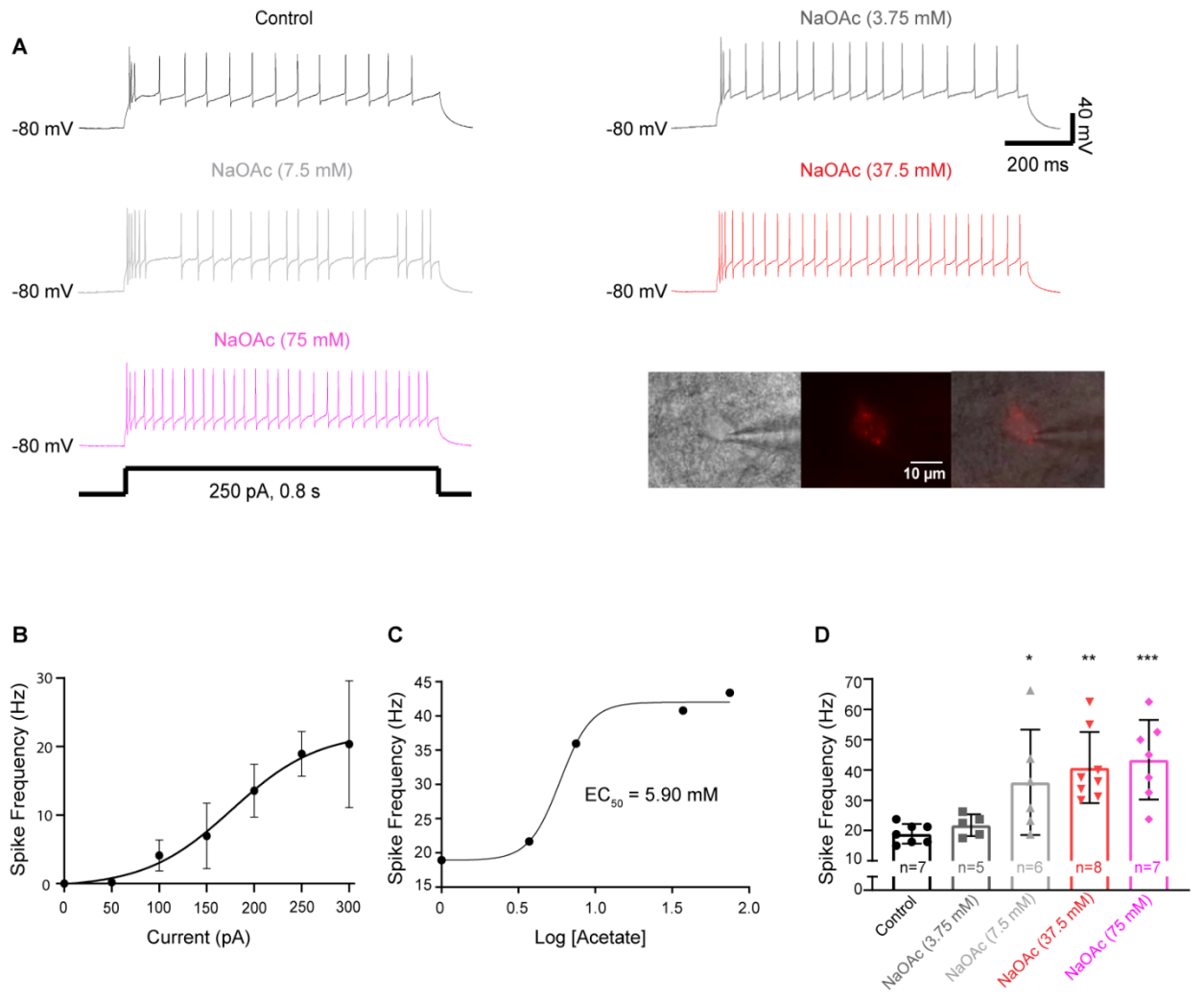


Figure 3. CeA brain tissue concentrations. (A) Representative image of CeA microinjection of 100 nL ethanol loaded with chicago blue dye. **(B)** Excised CeA tissue weights of microinjected ethanol or saline. **(C)** Method for estimating upper theoretical limit of brain tissue concentrations (n=18 CeA tissue samples from N=9 rats). Abbreviations: Estimated brain tissue = EBT.

Bath application of acetate increases CeA-RVLM neuronal excitability through activatation of NMDAR

Our previous *in vivo* microinjection study demonstrated ethanol and acetate induced sympathoexcitation by activation of NMDAR in the CeA through a projection to the rostral ventraolateral medulla (CeA-RVLM).[13] We therefore examined whether acetate was capable of increasing neuronal excitability of pre-sympathetic CeA-RVLM neurons in brain slice preparation. The RVLM as we and others have previously reported[13,37,38] is a key source of sympathoexcitatory drive that can contribute to elevated sympathetic outflow and heavily regulates cardiovascular function. What we found was that extracellular acetate consistently increased CeA-RVLM neuronal excitability in a dose-dependent manner (0.0 mM: 19 ± 3.3 Hz, n=7; 3.75 mM: 21.8 ± 3.6 Hz, n=5, 7.5mM: 36 ± 17.4 Hz, n=6; 37.5 mM: 41 ± 11.7, n=8; 75 mM: 44 ± 13.1, n=7), with

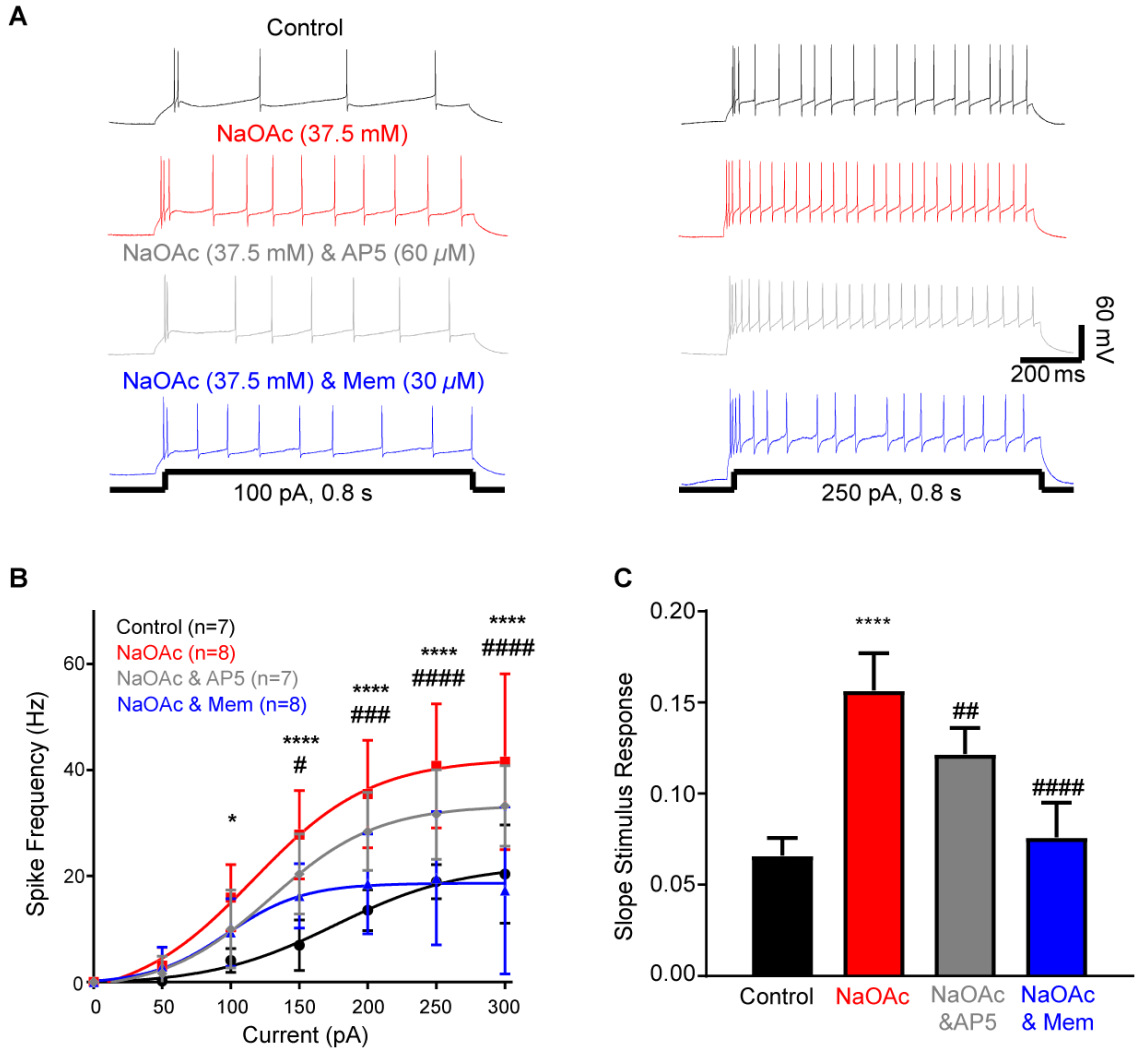
395 an EC_{50} value of 5.90 mM (Fig 4) and an E_{max} of 37.5 mM. The EC_{50} value is close to peak acetate
396 concentrations of ~4.60 mM measured by Wang and colleagues in ethanol and acetate infused rats[1]
397 and is also similar to cytotoxic doses we observed in dopaminergic-like PC12 cells.[14] Our E_{max}
398 value of 37.5 mM was utilized to elicit maximum responses to both intrinsic excitability as well
399 as NMDAR mediated inward currents. A one-way ANOVA with Bonferroni post-hoc test revealed
400 differences ($F_{(4,28)}$ = 6.41, p=0.0009) in firing frequency observed between control vs 7.5 mM,
401 37.5 mM acetate and control vs 75 mM acetate.



403 **Figure 4. Acetate increases CeA-RVLM neuronal excitability in a dose-dependent manner.**
404 **(A)** Representative raw excitability traces for varying doses of acetate to +250 pA current injection

405 and representative CeA-RVLM neuron; differential interference contrast (DIC, left),
406 epifluorescence of the same neuron from retrograde RVLM fluorescent labeling (middle), merged
407 DIC/epifluorescence indicating CeA neuron with projection to RVLM (right). **(B)** Stimulus
408 response curve for CeA-RVLM neurons (n=7 neurons, N=5 rats). Maximum firing occurred at
409 +250 pA current injection. **(C)** Dose-dependent response curve for sodium acetate (NaOAc) on
410 firing frequency at +250 pA current injection. The EC_{50} value for NaOAc was 5.90 mM. **(D)**
411 Summary data for spike frequency at +250 pA current injection in control, and NaOAc (0, 3.75,
412 7.5, 37.5 and 75 mM) treated CeA-RVLM neurons (*p=0.0474, **p=0.0036, ***p=0.0016 vs
413 control): (n=7 neurons, N=5 rats), (n=5 neurons, N=2 rats), (n=6 neurons, N=3 rats), (n=8 neurons,
414 N=3 rats), (n=7 neurons, N=3 rats).
415
416 To verify that NMDAR were involved in the acetate induced increase in CeA-RVLM
417 neuronal excitability, we used two NMDAR blockers, AP5 (primary synaptic NMDAR
418 competitive antagonist)[39-41] and memantine (a synaptic and extrasynaptic non-competitive pore
419 blocker at 30 μM).[42] Co-application of acetate and AP5 was only able to slightly reduce neuronal
420 excitability (Fig 5A-C) while co-application of acetate and memantine (Fig 5A-C) was able to
421 completely abolish the acetate induced increase in CeA-RVLM neuronal excitability. The stark
422 differences in effectiveness of AP5 vs memantine likely highlights the capabilities of acetate to
423 activate both synaptic (AP5 & memantine) and extrasynaptic (memantine) NMDAR. Synaptic
424 blockade of acetate via AP5[43] partially reduced CeA-RVLM neuronal excitability (Fig 5A-C),
425 whereas synaptic and extrasynaptic blockade via memantine completely abolished the acetate
426 induced increase in intrinsic excitability (Fig 5A-C). A two-way ANOVA of the current stimulus
427 response (Fig 5B) showed a significant group by current interaction ($F_{(18,168)} = 2.947$, $p = 0.0005$).

428 To understand the net effects of acetate on intrinsic excitability and the involvement of NMDAR
429 over the range of the stimulus response, we analyzed the slope (gain) of each group's curve (0-250
430 pA). Acetate significantly increased the slope of the stimulus response curve compared to control
431 (0.157 ± 0.02 vs 0.066 ± 0.009 Hz/pA). Acetate and AP5 or acetate and memantine reduced the
432 slopes to 0.1217 ± 0.014 Hz/pA and 0.0761 ± 0.019 Hz/pA respectively (Fig 5C). A one-way
433 ANOVA with Bonferroni post-hoc test revealed differences ($F_{(3,24)} = 47.81$, $p < 0.0001$) between
434 control vs acetate, acetate vs acetate + AP5, and acetate vs acetate + memantine.



436 **Figure 5. Effect of NMDAR antagonists on acetate induced excitability. (A)** Representative raw
437 traces for control (black), NaOAc (37.5 mM, red), NaOAc & AP5 (grey) and NaOAc & memantine

438 (blue) at +100 pA (left) and +250 pA (right). **(B)** Current stimulus response for control (black),
439 NaOAc (red), NaOAc & AP5 (grey) and NaOAc & memantine (blue). (*p<0.05, ****p< 0.0001
440 NaOAc vs control, # p<0.05, ### p<0.001, ### p<0.0001 NaOAc vs NaOAc & memantine). **(C)**
441 Slope of the stimulus response in control (black), NaOAc (red), NaOAc & AP5 (grey) and NaOAc
442 & memantine (blue). (****p< 0.0001 vs control, ##p=0.0012, ####p< 0.0001 vs NaOAc) (n=7
443 neurons, N=5 rats), (n=8 neurons, N=3 rats), (n=6 neurons, N=2 rats) (n=6 neurons, N=3 rats).
444

445 **Bath application of acetate induces NMDAR mediated inward currents in CeA-RVLM**
446 **neurons**

447 Next we examined whether external acetate application resulted in NMDAR mediated
448 inward currents in CeA-RVLM neurons. We again used both AP5 and memantine as our choice
449 NMDAR antagonists. What we found consistent with our excitability study was that bath
450 application of acetate significantly increased NMDAR mediated inward currents, -22.5 ± 5.2 pA
451 (Fig 6). Co-application of acetate and memantine or acetate and AP5 was able to completely
452 abolish NMDAR mediated inward currents, 7.3 ± 13.5 pA and 29.3 ± 18 pA, respectively (Fig 6).
453 Since we were applying acetate as a sodium salt, we wanted to verify that the increased NMDAR
454 mediated inward currents were not due to increased sodium concentration. As such, we used
455 sodium gluconate as a sodium ion control and found that 37.5 mM sodium gluconate was unable
456 to trigger any NMDAR mediated inward currents, -1.1 ± 3.0 pA (Fig 6), suggesting acetate was
457 the bioactive compound.

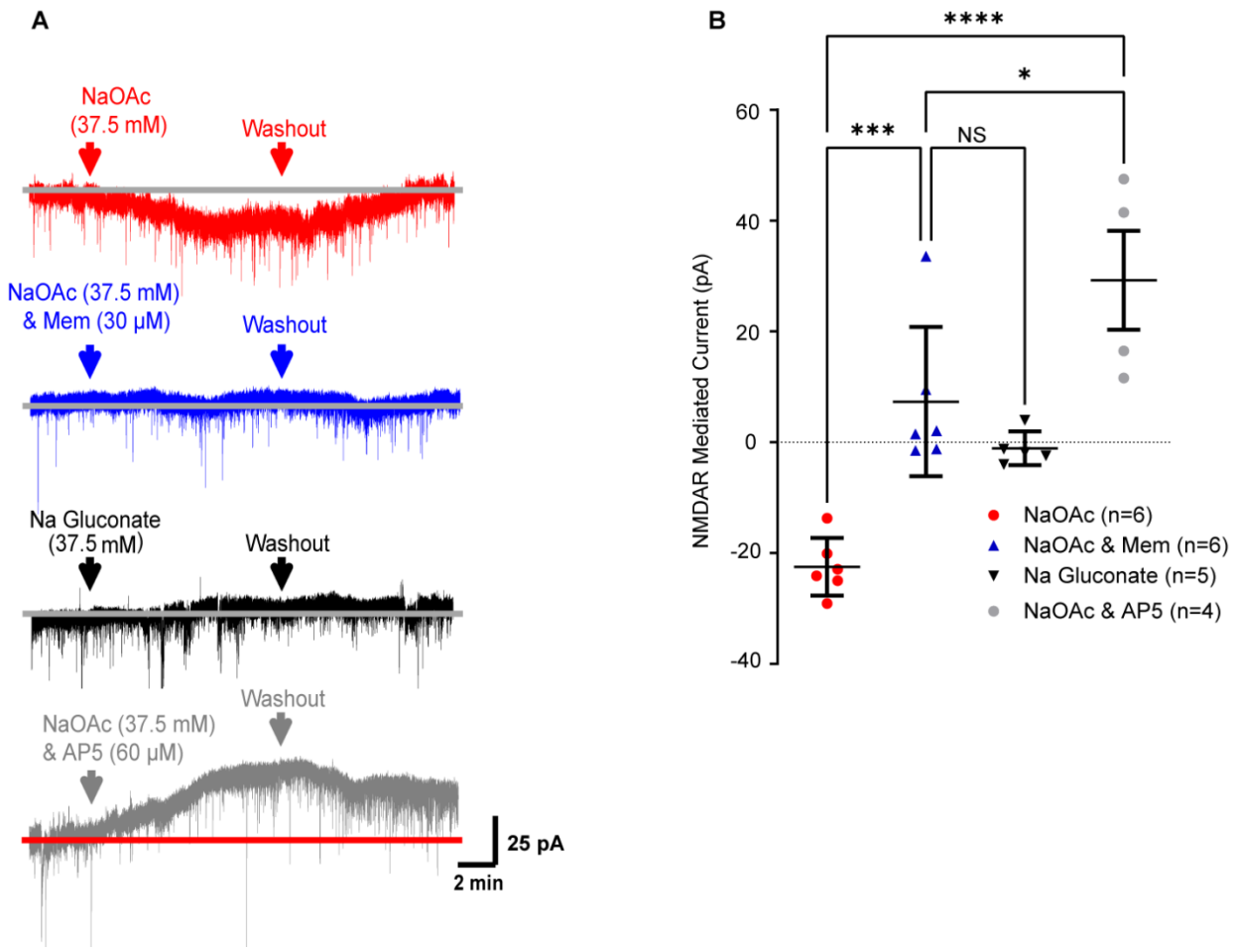


Figure 6. Acetate mediated NMDAR currents. (A) Representative raw traces for NMDAR mediated currents in CeA-RVLM neurons. **(B)** Summary data for NMDAR mediated currents in CeA-RVLM neurons. (****p<0.0001,***p=0.0004, **p=0.005 vs NaOAc) (n=6 neurons, N=4 rats), (n=6 neurons, N=2 rats), (n=5 neurons, N=3 rats) and (n=3 neurons, N=3 rats).

Bath application of acetate increases depolarizing input resistance (R_{input}) in CeA-RVLM neurons

To further understand the mechanistic effects of external acetate on CeA-RVLM neuronal excitability, neurons were subjected to a ramp protocol aimed to explore voltage threshold for firing an action potential (V_t) and to determine depolarizing input resistance (Depolarizing R_{input})

(Fig 7A-C). Input resistance (impedance) by definition is the measure of the opposition to current in an electrical network (ie, a greater depolarizing R_{input} suggests a greater resistance to positive current injection). Acetate increased the depolariing R_{input} compared to control (0.75 ± 0.1 GΩ vs 0.53 ± 0.12 GΩ) (Fig 7B), but had no statistically significant effect on alterations to V_t (Fig 7D). From an excitability standpoint, increased depolarizing R_{input} suggests an increase of positive ion influx in acetate treated neurons compared to control as they are depolarized. The effect of NMDAR blockers significantly reduced depolarizing R_{input}, ($F_{(3,25)}$ = 4.00, P=0.0186) (Fig 7B).

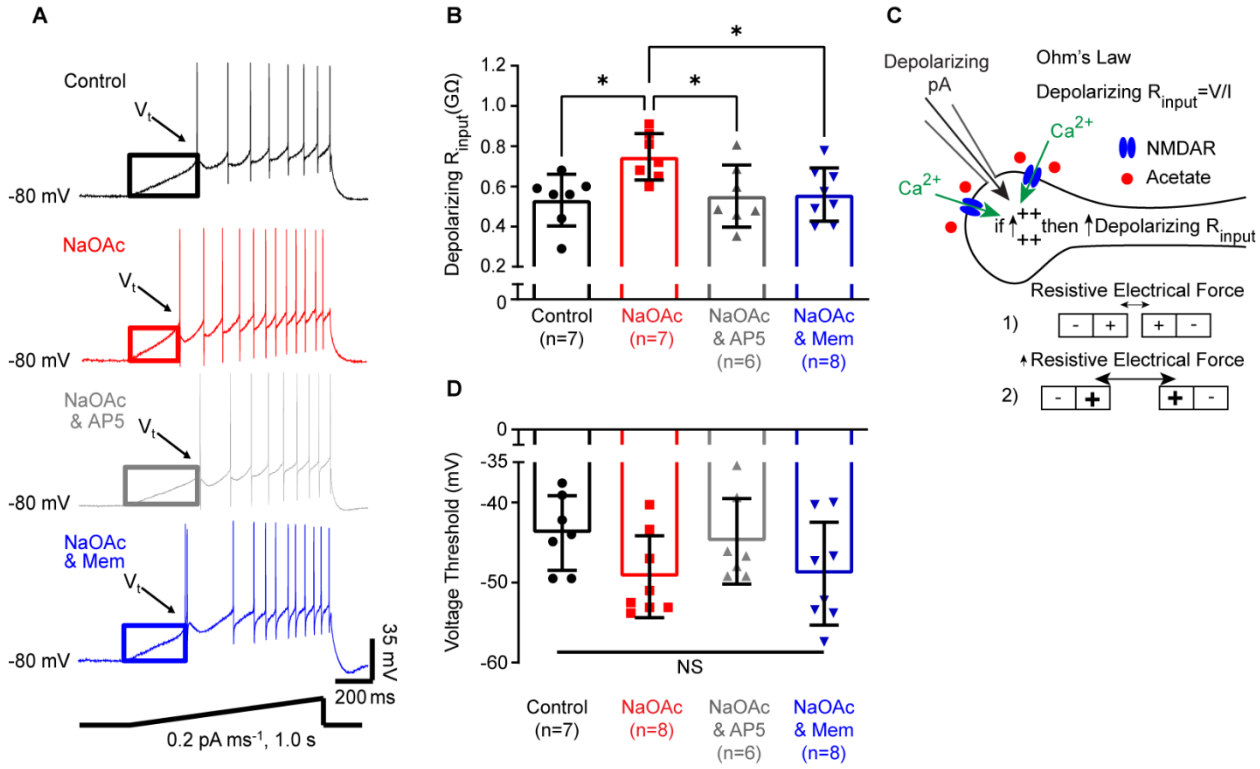


Figure 7. Effect of acetate on depolarizing input resistance (Depolarizing R_{input}) and voltage threshold (V_t) to firing an action potential. (A) Representative raw traces for ramp excitability protocol for control (black), NaOAc (red), NaOAc & AP5 (grey) and NaOAc & memantine (blue). **(B)** Summary data for depolarizing R_{input}. (* $p < 0.05$ vs control). **(C)** Graphical summary of depolarizing input resistance. Increased depolarizing current injection and activation of NMDAR results in an increase in positive charge buildup measured as a change in overall resistance via

483 Ohm's law. 1) Baseline electrical charge resistive force, 2) increased calcium enhanced electrical
484 charge resistive force. **(D)** Summary data of voltage threshold to firing an action potential.
485

486 **No apparent alterations to the magnitude of afterhyperpolarization (AHP) at -60 mV**

487 To assess the impact of acetate on the magnitude of AHP, neurons were subjected to a
488 specific AHP protocol as we have previously reported.[44] While there was a trend for acetate to
489 increase the magnitude of AHPs, a one-way ANOVA among groups was not statistically
490 significant ($F_{(3,27)} = 1.50$, p=0.069) (Fig 8).
491

492 **Acetate increases the magnitude of single action potential AHP which is abolished by**
493 **memantine**

494 Our second method for AHP analysis was from a single action potential. Within the same
495 set of trains as our AHP protocol (Fig 8), we analyzed the seventh action potential AHP. Our
496 rationale for this additional analysis was to compare NMDAR contributions. At -60 mV,
497 magnesium block of the NMDAR channel has been reported[45] and this effect may mask the effects
498 of NMDAR interaction with SK and BK channels, both channels activated by increased cytosolic
499 Ca^{2+}. Analyzing a single action potential in a train ensures magnesium unblock from the NMDAR
500 channel and allows for examination of the above mentioned interactions. A one-way ANOVA of
501 single action potential AHP magnitudes revealed an acetate induced increase on the magnitude of
502 AHP (Fig 9 A,B) compared to control. This response was abolisehd by co-application of
503 memantine ($F_{(3,26)} = 5.36$, p=0.0052). AP5 had a non-statistical trend to reduce the AHP magnitude,
504 however memantine was more effective (Fig 9 B) (acetate vs acetate & mem).
505

506 **Unexpected combined effect of memantine and acetate on shaping neuronal action potentials**

507 In the process of examining the effects of NMDAR antagonists, AP5 (competitive blocker)
508 and memantine (non-competitive pore blocker), an interesting phenomenon was observed.
509 Memantine in combination with acetate was found to differentially shape action potentials when
510 compared to all other groups (Fig 9, A,C). This was seen through a reduction in the decay-slope
511 of action potentials and an overall increase in half-width, likely implicating modulation of voltage-
512 gated/Ca^{2+} activated potassium channels such as the BK channel which as been found to be
513 activated by the ethanol metabolite, acetic acid/acetate.[46]

514

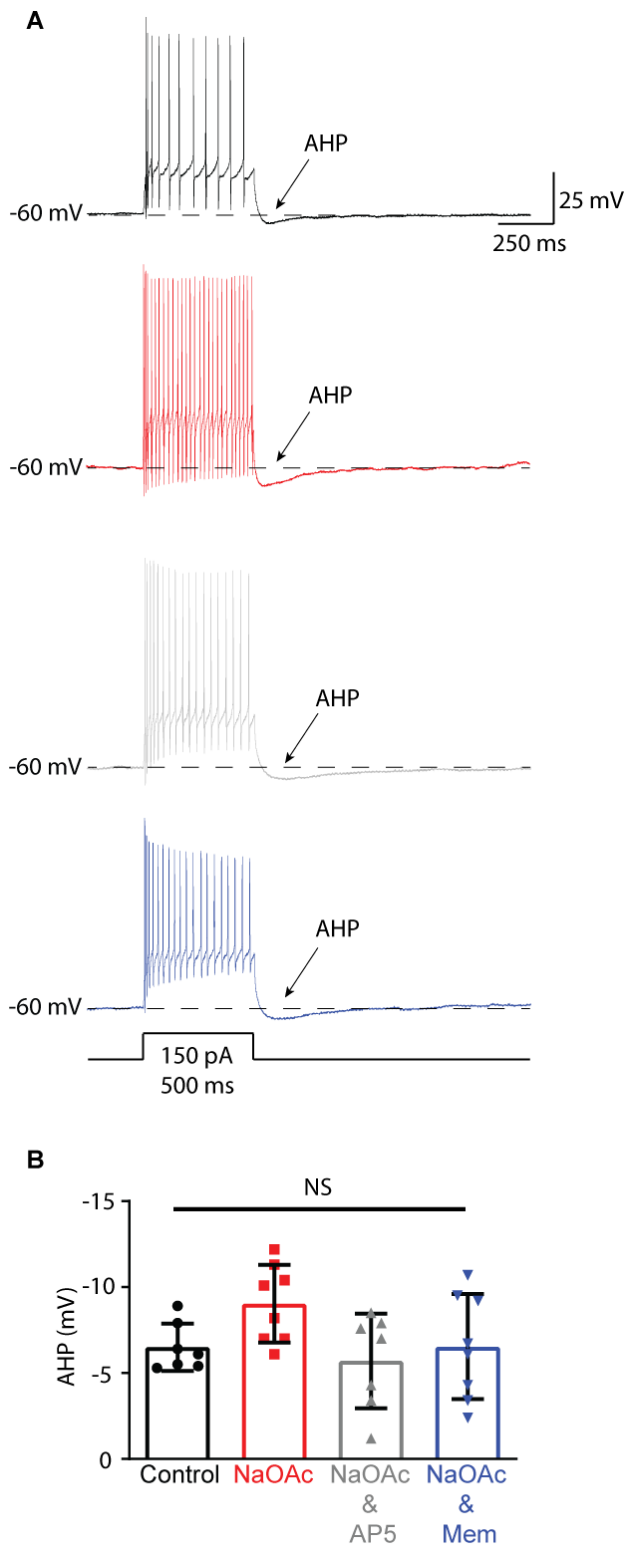
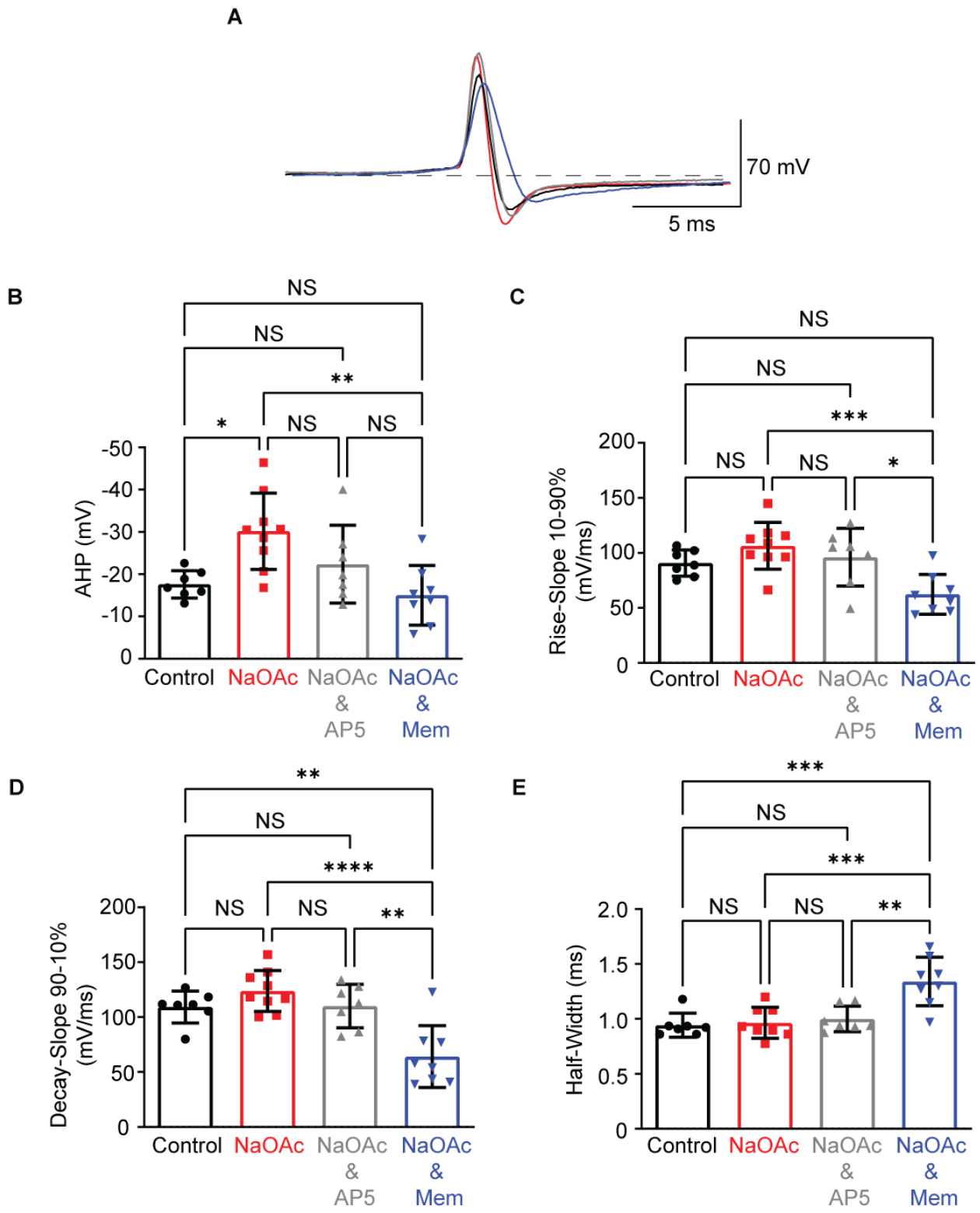


Figure 8. No effect of acetate on AHP measured at -60 mV. (A) Representative raw traces obtained from AHP protocol. **(B)** Summary data for AHP among different groups.



519 **Figure 9.** Effect of acetate on AHP from single action potential. **(A)** Representative single action

520 potential trace overlay of control (black), acetate (red), acetate & AP5 (gray) and acetate &

521 memantine (blue). **(B)** Summary data for the magnitude of AHP following a single action potential.
522 **(C)** Summary data for magnitude in rise-slope during single action potential. **(D)** Summary data
523 for magnitude in decay-slope during single action potential. **(E)** Summary data for half-width
524 during single action potential.
525

526 **Bath application of acetate to primary neuronal cultures increases cytosolic calcium and is**
527 **NMDAR dependent**

528 Our lab previously reported that bath application of acetate to dopaminergic-like PC12
529 cells increased cytosolic calcium[14] which was attenuated by co-application of acetate and
530 memantine. We wanted to corroborate our previous findings in primary neuronal cultures. Bath
531 application of acetate (37.5 mM, exact as those used in patch clamp recordings) increased cytosolic
532 calcium fluorescence intensity in primary neurons in a time-dependent manner (baseline, 1 min
533 and 25 min), 3,759 ± 1,552, 6,046 ± 1,805 and 6,775 ± 1,862 CFIT, respectively ($F_{(2,36)} = 10.58$,
534 $p = 0.0002$) (Fig 10 A-C, G). Co-application of acetate (37.5 mM) and memantine (30 μM)
535 completely abolished any acetate induced increase in cytosolic calcium in neurons ($F_{(2,27)} = 0.21$,
536 $p = 0.81$) (Fig 10 D-F, H).

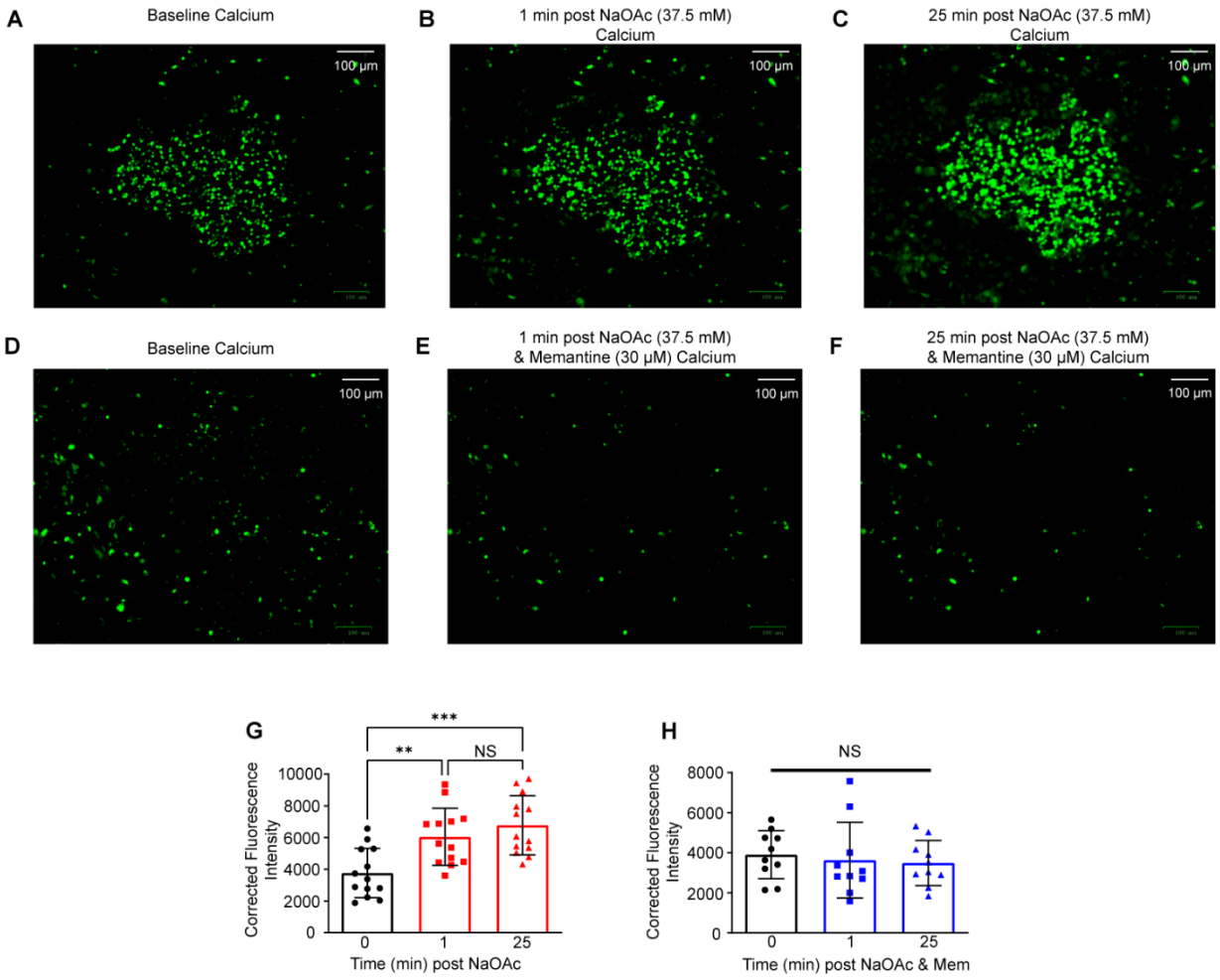


Figure 10. Acetate increases cytosolic calcium in neurons. (A) Baseline calcium fluorescence. **(B)** 1-minute post NaOAc. **(C)** 25 minutes post NaOAc. **(D)** Baseline calcium fluorescence. **(E)** 1-minute post NaOAc & memantine. **(F)** 25 minutes post NaOAc & memantine. **(G)** Neuronal calcium time-course summary data with NaOAc (**p<0.01, ***p<0.001 vs baseline). **(H)** Neuronal calcium time-course summary data with NaOAc & memantine (Mem). Note **A-C** and **D-F** images were acquired from different samples.

DISCUSSION

In the present study, we evaluated how local brain metabolism of ethanol within the CeA elicits sympathoexcitatory responses *in vivo*. We find in a similar fashion to our previously

548 reported work that microinjection of ethanol within the CeA increases SNA and MAP.[13] Inhibiting
549 local CeA ethanol metabolism with cyanamide (ALDH inhibitor) significantly blunts the ethanol
550 induced sympathoexcitatory response. CeA microinjection of the ethanol metabolite, acetate, was
551 found to increase SNA and MAP in a dose-dependent manner. These responses were attenuated
552 by NMDAR antagonists, AP5 or memantine. In brain slice recordings containing CeA autonomic
553 neurons with axon projecting to the RVLM (CeA-RVLM), bath application of acetate dose-
554 dependently increased neuronal excitability with a calculated EC_{50} =5.90 mM and an E_{max} of 37.5
555 mM. Increased depolarizing input resistance was apparent in acetate treated CeA-RVLM neurons.
556 Co-application of NMDAR antagonists attenuated these acetate induced responses, with
557 memantine proving more effective. Similarly, NMDAR mediated inward currents in the presence
558 of acetate were abolished by co-application with NMDAR antagonists. Finally, in primary
559 neuronal cell cultures, bath application of acetate increased cytosolic calcium in a time-dependent
560 manner, and this response was abolished by co-application of acetate and memantine. These
561 findings suggest that ethanol-mediated inhibition of NMDAR *in vitro* is counteracted by acetate-
562 mediated activation of the same receptor.
563
564 **Ethanol and neural control of cardiovascular function**
565 Human studies have consistently demonstrated that acute ethanol consumption elicits
566 sympathoexcitatory effects.[47-49] While these effects are likely a combination of both peripheral and
567 central mechanisms,[50] centrally mediated mechanisms remain unclear. Furthermore, only a
568 handful of studies have explored metabolic based mechanisms.[13,51] In our whole-animal SNA
569 recordings from targeted brain delivery, we measure SSNA as well as LSNA. The SSNA closely
570 corresponds with venous blood return and redistribution,[52] while LSNA can be inferred as a close

571 proxy for muscle SNA.[53] While microinjection of acetate in the CeA increases both SSNA and
572 LSNA, a key finding of increased LSNA may be linked to an effect noted in human studies. Acute
573 ethanol was found to increase muscle SNA in humans.[47,48] Our results of CeA acetate on increased
574 LSNA supports this previously mentioned finding in humans,[47,48] suggesting that increased muscle
575 SNA in humans from ethanol may at least be partially mediated by centrally acting acetate. Future
576 studies investigating the effects of microinjected acetate in other cardiovascular regulatory centers
577 in the brain may delineate diverging, mechanistic and temporal output responses from acetate.
578

579 **Synaptic versus extrasynaptic acetate induced NMDAR activation**

580 Our study used two types of NMDAR antagonists to investigate the role of acetate in CeA-
581 RVLM neuronal excitability, AP5 (classical competitive antagonist)[39-41,43] and memantine (non-
582 competitive pore blocker). Our results demonstrate very different responses between the two
583 different antagonists in reducing the acetate induced increase in intrinsic excitability (Fig 5). AP5
584 appears to be less effective compared to memantine. A possible explanation for this difference
585 may lie in synaptic vs extrasynaptic activation of NMDARs. Co-application of acetate and AP5
586 slightly blunted the acetate induced excitatory effect, whereas memantine and acetate completely
587 normalized the response relative to control. Previous work in neurosecretory autonomic neurons
588 has identified extrasynaptic NMDAR currents under astrocytic glutamate control.[40,41] NMDAR
589 mediated inward currents (-70 mV) produced robust outward currents[41] which appeared to be
590 larger using AP5 compared with those involving the use of memantine (30 μM).[40] Similarly to the
591 these studies, our CeA-RVLM NMDAR mediated inward currents in the presence of acetate and
592 AP5 show robust outward currents compared to acetate and memantine. AP5 NMDAR synaptic
593 blockade of acetate but excluding extrasynaptic NMDAR blockade could account for both this

594 outward current as well as the inability for AP5 to abolish the acetate induced increase in intrinsic
595 excitability. Conversely, memantine produces very little NMDAR mediated outward current in the
596 presence of acetate and abolished the acetate induced increase in neuronal excitability. This is in
597 agreement with the effects of memantine (30 μM) antagonism synaptically and extrasynaptically
598 at NMDAR.[42] Furthermore, acetate induced incorporation into GABA[54] and glutamate[55] pools
599 through astrocytic uptake of acetate has been demonstrated. This lends further support to our
600 speculation that NMDAR modulation occurs both synaptically and extrasynaptically, either
601 directly through acetate and/or indirectly through astrocytic modulation. However, we cannot rule
602 out the possibility that the true source of the AP5 and acetate induced outward current is outward
603 K^+ current through BK-channels induced by extrasynaptic NMDAR activation. Future studies will
604 be needed to delineate these mechanistic effects.
605

606 **Possible involvement of large-conductance Ca^{2+} and voltage-gated K^+ channels (BK-**
607 **channels) in acetate response**

608 Through the course of our single action potential analyses, it was revealed that acetate
609 increased the magnitude of the single action potential AHP relative to control neurons. Given the
610 acetate induced response at NMDAR, calcium activation of BK-channels is a likely mediator in
611 this response and NMDAR-induced-BK-channel activation has been demonstrated.[56] While BK-
612 channels can contribute to spike repolarization and AHP to reduce neuronal excitability[57-59] they
613 can oppositely increase neuronal excitability as well.[59-62] Ethanol[63,64] and its metabolite, acetic
614 acid/acetate[46] have both been found to activate BK-channels, however the former involving
615 ethanol in accumbens slice preparation lacked a control of inhibiting in-slice ethanol metabolism.
616 The ethanol enhancement of repolarization and shaping of action potentials in accumbens

617 neurons[64] may be due to in-slice metabolism and production of acetic acid/acetate. Our effects of
618 acetate here support this notion. Furthermore, bath application of memantine (synaptic and
619 extrasynaptic NMDAR blocker) with acetate abolished the acetate induced increase in single
620 action potential AHP magnitude while also decreasing the slope of action potential decay as well
621 as increasing the half-width of the action potential (Fig 9). Memantine blockade of NMDAR
622 calcium would be anticipated to reduce BK-channel activation via a reduction in cytosolic calcium
623 and would likely display the action potential shaping we find here: normalization of AHP with
624 peak widening and slower repolarization, thus contributing to overall reduced neuronal firing (Fig
625 5). While we cannot rule out acetate induced modulation of other voltage-gated K^+ channels or
626 small conductance calcium activated K^+ channels (SK-channels), SK-channel involvement is
627 highly unlikely as SK-channel loss of function has been demonstrated to increase neuronal
628 excitability.[44,65,66]
629

630 **Potential role of dopamine and serotonin in the acetate induced excitatory response within**
631 **the CeA**

632 In addition to glutamate,[67] the CeA also contains dopaminergic[68] as well as serotoninergic[69]
633 inputs, and both are enhanced by alcohol.[69-71] Increased dopamine release in the CeA has been
634 found to reduce inhibitory synaptic currents[68] while serotoninergic activation was found to
635 increase GABAergic release in naïve rats.[69] NMDAR pore-blockers (memantine and ketamine)
636 have off targets such as serotonin receptors and dopamine receptors,[72,73] while MK-801 has off
637 targets of dopamine and serotonin transporters.[74,75] Our difference in effectiveness of AP5 vs
638 memantine may highlight a possibility of non-NMDAR based mechanisms, which we cannot
639 exclude. Acetate has been demonstrated to increase dopamine release[76] and if this occurs within

640 the CeA, loss of inhibitory input may potentiate increases in neuronal excitability, effects blunted
641 by memantine but not AP5. The effects of serotonin on GABAergic strength are far less clear,
642 especially with ethanol.[69] Nevertheless, both the dopaminergic and serotoninergic contributions
643 within the CeA are potential modulation sites of acetic acid/acetate that deserve future
644 consideration.
645
646 **Other mechanisms for acetate induced increase in excitability within the CeA**
647 While our present study suggests a major involvement of CeA NMDAR in the acetate
648 induced effects on neuronal excitability and sympathoexcitatory responses, there are other
649 potential contributing mechanisms. Acetate incorporation into glutamatergic[77,78] as well as
650 GABAergic[2,54,77] pools is likely a reflection of astrocytic metabolism. As such, astrocytic influence
651 on glutamate cannot be ruled out. Previous work has demonstrated that acute acetic acid increased
652 mEPSC frequency but not amplitudes, suggesting an increase in presynaptic glutamate.[15] Whether
653 this is from astrocytic generation or presynaptic synapses remains to be determined. Similarly, in
654 the present study, we cannot rule out this astrocytic based mechanism, and a portion of our data
655 (NMDAR mediated outward currents) support the notion of extrasynaptic NMDAR activation via
656 acetate, either directly or indirectly via astrocyte-mediated glutamate release.
657 Another potential contribution to increased CeA excitability in the presence of acetate is
658 AMPAR activation. We did see an ethanol induced AMPAR component in our previous
659 microinjection study within the CeA,[13] which is likely mediated via metabolism to acetate,
660 however the NMDAR component appeared larger and thus we chose to focus our attention there.
661 However, this AMPAR component may be a contributing factor seen in lack of apparent
662 effectiveness of AP5. While we have strong evidence to suggest synaptic vs extrasynaptic

663 NMDAR activation for this difference, we also cannot rule out the impact of AMPARs in this
664 response, especially as previous studies demonstrate an increase in mEPSC frequencies (likely
665 AMPAR mediated events) in response to acute acetic acid in the accumbens shell.[15] The fact that
666 ethanol (44 mM) increased mEPSC frequencies with no apparent effect on intrinsic excitability
667 supports the notion that this linkage to increased intrinsic excitability is not necessarily
668 straightforward given the dissociation between the two.[15] Nevertheless, these two previous studies
669 demonstrate an inherent effect of acetic acid/acetate on AMPAR and this contribution deserves
670 more attention in future studies.

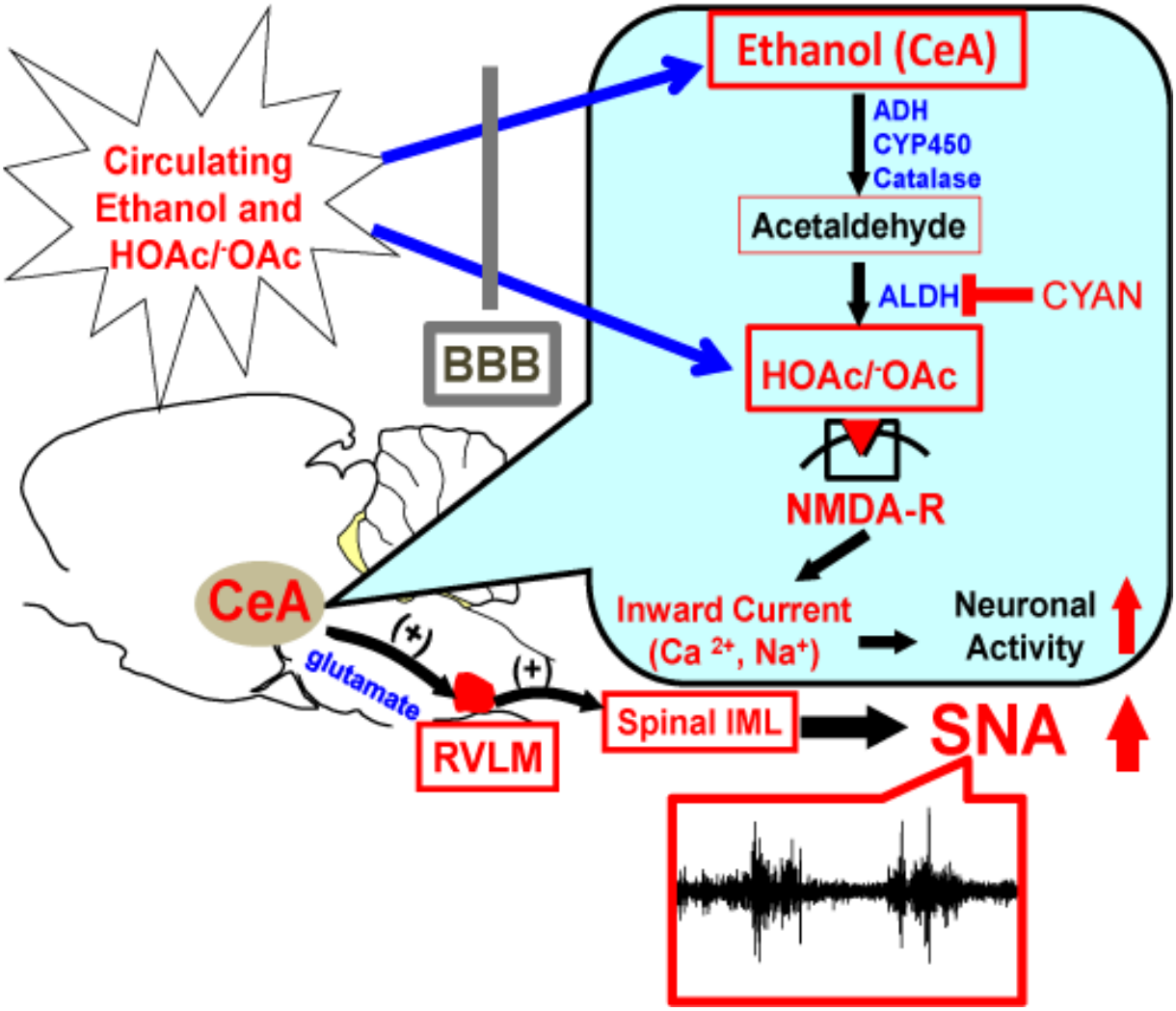
671 Given the nature of ethanol in regulating NMDAR function, acutely inhibiting[4,79] and
672 chronic upregulation,[79] indirect modulation of these receptors via alternative signaling
673 mechanisms seems likely. The Src family kinase, Fyn, likely plays a role in the gain of function
674 of NMDAR via phosphorylation of tyrosine residues.[80,81] Ethanol modulation of NMDAR via Fyn
675 in the dorsal striatum was found to increase alcohol intake.[82] Fyn can also be directly activated via
676 nitric oxide (NO) and other free radicals,[83] leading to gain of function of NMDAR.[80,81] Previous
677 work out of our lab exploring cellular effects of acetate have revealed an acetate induced increase
678 in cytosolic reactive oxygen species[14] which in addition to being cytotoxic may also play a role in
679 Fyn regulation of NMDAR function. Our finding of memantine only being slightly effective at
680 reducing acetate induced toxicity in cell culture,[14] combined with the above mentioned potential
681 contributing mechanisms suggest multiple signaling cascades evoked by acetate.

682

683 **Perspectives and future directions**

684 To the best of our knowledge, we are the first to report the effects of acetate/acetic acid on
685 intrinsic exctiability of neurons, particularly autonomic CeA-RVLM neurons. As such, there is

686 limited availability of literature to compare our findings with. What is within the literature
687 regarding acetate/acetic acid regulating neuronal function seems supportive of the notion that both
688 have robust effects in the CNS[2,54,76,84-86] which we also report here. In summary, more studies are
689 needed to determine the effects of centrally acting acetic acid/acetate on neuronal function and
690 behavioral outputs.



692 **Graphical Figure.** Proposed mechanisms for ethanol and acetate induced increases in
693 sympathoexcitation within the central nucleus of the amygdala (CeA). Abbreviations: Acetic acid
694 (HOAc), acetate (⁻OAc), ADH (alcohol dehydrogenase), ALDH (aldehyde dehydrogenase), BBB

695 (blood brain barrier), CYAN (cyanamide), CYP450 (cytochrome P450), IML (intermediolateral
696 nucleus), RVLM (rostral ventrolateral medulla), SNA (sympathetic nerve activity).
697
698 **Table 1. Membrane properties of CeA-RVLM neurons**
699
700

Table 1. Membrane Properties of CeA-RVLM neurons

Group	n	V_m (mV)	C_m (pF)	HR_{input} (GO)	V_t (mV)	Rats (n)
Control	7	-58.0 ± 3.0	47.4 ± 2.5	0.47 ± 0.07	-43.8 ± 1.8	5
NaOAc (7.5 mM)	6	-58.5 ± 2.6	45.3 ± 4.5	0.52 ± 0.08	-42.4 ± 2.7	3
NaOAc (37.5 mM)	8	-62.3 ± 2.2	34.6 ± 2.5	0.58 ± 0.03	-49.3 ± 1.8	3
NaOAc (37.5 mM) & AP5	7	-67.9 ± 4.3	36.3 ± 2.0	0.45 ± 0.07	-45.0 ± 2.5	2
NaOAc (37.5 mM) & memantine	8	-63.0 ± 3.4	39.0 ± 3.5	0.54± 0.10	-47.5 ± 1.7	3
NaOAc (75 mM)	7	-59.9 ± 1.8	42.2 ± 1.6	0.40 ± 0.04	-47.7 ± 1.1	3

Values are mean ± SE, n = number of neurons, V_m, resting membrane potential; C_m, membrane capacitance; HR_{input}, hyperpolarizing input resistance; V_t, sub-threshold of membrane potential to fire action potential, NaOAc = acetate, HOAc = acetic acid.

702 **Authors' Contribution:**

703 A.D.C., M.J.H., K.M.D., J.E.B. and R.A.L. performed experiments. A.D.C., M.J.H., K.M.D.,
704 J.E.B., R.A.L., Z.S., L.Z. and Q.H.C. analyzed data. A.D.C., Q.H.C. and L.Z. prepared figures.
705 A.D.C., Q.H.C. and L.Z. drafted the manuscript. All authors edited and revised manuscript. All

706 authors approved the final version of the manuscript. A.D.C. and Q.H.C. conceptualized and
707 designed the research.
708

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