Genetic Deletion of TrpV1 and TrpA1 Does not Alter Avoidance of or Patterns of Brainstem Activation to Citric Acid in Mice

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ABSTRACT:

Exposure of the oral cavity to acidic solutions evokes not only a sensation of sour, but also of sharp or tangy. Acidic substances potentially stimulate both taste buds and acid-sensitive mucosal free nerve endings. Mice lacking taste function (P2X2/P2X3 double-KO mice) refuse acidic solutions similarly to wildtype mice and intraoral infusion of acidic solutions in these KO animals evokes substantial c-Fos activity within orosensory trigeminal nuclei as well as of the nucleus of the solitary tract (nTS) (Stratford et. al 2017). This residual acid-evoked, non-taste activity includes areas that receive inputs from trigeminal and glossopharyngeal peptidergic (CGRP-containing) nerve fibers that express TrpA1 and TrpV1 both of which are activated by low pH. We compared avoidance responses in wildtype (WT) and TrpA1/V1 double KO ($TRPA1/V1^{Dbl-/-}$) mice in brief-access behavioral assay (lickometer) to 1, 3, 10, 30 mM citric acid, along with 100 μM SC45647 and H_2O. Both WT and $TRPA1/V1^{Dbl-/-}$ show similar avoidance, including to higher concentrations of citric acid (10 and 30 mM; pH 2.62 and pH 2.36 respectively), indicating that neither TrpA1 nor TrpV1 is necessary for the acid avoidance behavior in animals with an intact taste system. Similarly, induction of c-Fos in the nTS and dorsomedial spinal trigeminal nucleus was similar in the WT and $TRPA1/V1^{Dbl-/-}$ animals. Taken together these results suggest non-TrpV1 and non-TrpA1 receptors underlie the residual responses to acids in mice lacking taste function.

Keywords: taste bud, trigeminal, solitary nucleus, sour, c-fos, irritation

22 INTRODUCTION

23 When acidic solutions (e.g. lemon juice) are taken into the mouth, we describe the
24 resulting perception as "sour" and consider it to be an aversive basic taste. However, the
25 sensations arising from acidic substances in the oral cavity are a compound sensation of sour (a
26 taste originating from taste buds) and direct activation of acid-sensitive general mucosal afferents
27 of the oropharynx including the trigeminal, glossopharyngeal and superior laryngeal nerves.
28 Indeed, a dictionary definition of sour (thefreedictionary.com) includes descriptors such 'sharp,'
29 'tart', or 'tangy' which are not taste modalities, but rather chemesthetic ones corresponding to
30 activation of acid-sensitive mucosal nerves likely including polymodal nociceptors. Thus,
31 aversion to acidic solutions might be due to activation of these nociceptors rather than of the
32 taste system alone.

33 In mice lacking a functional taste system (P2X2/3-dbl-KO mice), the chorda tympani
34 nerve, which is a pure taste nerve, shows no responses to any tastants including acids (Finger,
35 Danilova et al. 2005). Consistent with this, these mice show no preference for sweeteners or
36 avoidance of bitter substances in brief-access taste tests. Despite the apparent lack of taste
37 responses to acids, the P2X2/3-dbl-KO mice do exhibit normal avoidance of citric acid in similar
38 brief access tests (Hallock, Tatangelo et al. 2009). This avoidance may be mediated not by taste,
39 which is non-functional in these mice, but by acid-responsive fibers in the trigeminal,
40 glossopharyngeal or laryngeal nerves which do show residual low level activity in the P2X2/3-
41 dbl-KO mice (Ohkuri, Horio et al. 2012).

42 Polymodal nociceptors respond to acidification as well as other potentially painful stimuli
43 (Bessou and Perl 1969). Many small caliber polymodal nociceptors that innervate the oral cavity
44 (Kichko, Neuhuber et al. 2018, Wu, Arris et al. 2018) express one or both of the pH-sensitive

45 transient receptor potential (Trp) channels, TrpA1 (Wang, Chang et al. 2011) and TrpV1
46 (Tominaga, Caterina et al. 1998). TrpA1 has been implicated especially in responsiveness to
47 weak acids capable of penetrating cell membranes to produce intracellular acidification (Wang,
48 Chang et al. 2011). TrpV1 is implicated in responses of the glossopharyngeal and vagus nerves
49 to acidification of the oral and pharyngeal epithelium (Arai, Ohkuri et al. 2010). Gating of either
50 of these channels by acids can directly activate the nociceptive fiber resulting in a noxious
51 sensation suitable for driving avoidance behavior. Ablation of the ganglion cells expressing
52 TrpV1 in mice lacking sour taste receptors does result in loss of acid-avoidance behavior (Zhang,
53 Jin et al. 2019) but it is not clear whether this is due to loss of TrpV1 itself or other ion-sensing
54 mechanisms of these neurons. Peptidergic polymodal nociceptor fibers, most of which express
55 one or both of TrpV1 and TrpA1 (Bae, Oh et al. 2004, Nguyen, Wu et al. 2017, Kim, Kim et al.
56 2018), terminate centrally in both the lateral portion of the nuc. solitary tract (nTS) as well as in
57 adjacent trigeminal brainstem nuclei including the dorsomedial trigeminal nucleus (DMSp5)
58 (Corson, Aldridge et al. 2012, Stratford, Thompson et al. 2017). We tested whether genetic
59 deletion of both TrpA1 and TrpV1 affected either acid-induced avoidance behaviors or the
60 pattern of neuronal activation in the nTS or adjacent DMSp5.
61
62
63

64 # Materials and Methods

65 **Animals**

66 TrpA1V1-double KO mice, on a C57BL6 background (TRPA1/V1$^{Dbl-/-}$) were a generous
67 gifts from Diana Bautista, University of California Berkeley (Gerhold and Bautista 2008). In
68 these mice, deletion of TrpA1 had been accomplished by elimination of residues 901–951
69 including most of exon 23 – a region encoding the putative pore and part of the sixth
70 transmembrane domain (Bautista, Jordt et al. 2006). The VR1 gene was disrupted (Caterina,
71 Leffler et al. 2000) by deleting part of the fifth and all of the sixth putative transmembrane
72 domains, together with the intervening pore-loop region. C57BL6/J mice were purchased from
73 The Jackson Laboratory (Bar Harbor, ME). For brief access lickometer behavioral assays, 4-6
74 months old male TRPA1/V1$^{Dbl-/-}$ mice (n=7) and 10 month old male C57BL6/J mice (n=3) were
75 used. For immunohistochemical staining, we used both male and female mice: TRPA1/V1$^{Dbl-/-}$
76 (2 to 8 mo, *n*=10); C57BL6/J mice (4 to 7 mo, n=8). In an addition to the C57 cases, we utilized
77 mice of the line B6.Cg-Tg(Fos-tTA,Fos-EGFP*)1Mmay/J which carry 2 transgenes associated
78 with c-Fos: cfos-tTA and cfos-shEGFP. In these mice, on a C57BL6/J background, the randomly
79 inserted transgenes utilize the c-Fos promotor to drive expression of respectively tTA and a
80 short-lived (two-hour half-life) GFP. We did not find close correlation between GFP expression
81 and c-Fos immunostaining and so the GFP results were ignored in our analysis. Further, the c-
82 Fos immunostaining counts from these animals were entirely consistent with those obtained from
83 the wildtype C57BL6/J mice and so were included in those results.

84

85 All animal procedures were performed in accordance with NIH guidelines and were approved by
86 the Institutional Animal Care and Use Committee at the University of Colorado School of
87 Medicine.
88
89 **Brief access preference tests**
90 Davis Rig Lickometers (MS-160; Dilog Instruments and Systems) were used for training
91 and brief-access testing of mice. The animals underwent a 4-day water training before acid
92 testing. Mice were placed on water deprivation 20-23 hours before the first day of training or
93 acid testing. No extra water was given after the training and testing days. On the first day of the
94 4-day training, water-deprived animals were allowed access to a single water spout for a 30-min
95 period. In the following three days of water training, 2-4 bottles of water were given in random
96 sequences for 15 min. Mice had 5 seconds to lick at each spout after their first lick before the
97 door closed and the bottle was switched to the next position with an inter-trial interval of 7.5 s.
98 The mice were considered well-trained if they lick more than 30 times consistently during each
99 trial for the first 15 trials.
100 For the acid testing days, six bottles of tastants including 4 concentrations of acetic or
101 citric acid (1 mM, 3 mM, 10 mM and 30 mM), 1 concentration of artificial sweetener SC45647
102 (100 μM) and H_2O were presented in a testing block with a set up similar to the training session.
103 Testing periods lasted for 15 min, with the opportunity for the mice to sample from the 6
104 solutions a total of 30 times. Licks were measured using InstaCal software. Each block of 6
105 tastant trials featured each solution in random order. Only data from completed blocks were used
106 in the calculation of preference. Preference was calculated by averaging the licks per tastant
107 block of each solution relative to those for water (i.e., a 'lick ratio'). The same mice that were

108 assessed for citric acid preference were then tested for acetic acid preference again with the same
109 paradigm. The solutions used in acetic acid testing were as follows: 100μM SC45647, H_2O, 1, 3,
110 10 and 30mM acetic acid. Testing for each series of tastants was repeated at least twice. Because
111 there was no statistically significant difference in licking across each testing day for each mouse
112 [$F(1,2) = 0.42$, $p = 0.66$], the number of licks for each tastant were averages across these testing
113 days for each mouse.
114
115 **Citric acid stimulation for c-Fos via intraoral cannula**
116 Both bilateral intraoral cannulae implantation and stimulation methods were adopted
117 from (Stratford and Thompson 2016). Briefly, mice were anesthetized with an intramuscular
118 injection of a combination of medetomidine hydrochloride (Domitor; 0.4 mg/kg; Pfizer) and
119 ketamine hydrochloride (40 mg/kg; Bioniche Pharma). Intraoral cannulae were inserted via a
120 midline incision immediately caudal to the pinnae. Then a sterile 2-3 cm stainless steel needle
121 (19 gauge; Hamilton) with a flared end polyethylene tube (50 gauge; Becton Dickinson) was
122 inserted from behind the pinna and guided subcutaneously into the oral cavity, lateral to the first
123 maxillary molar. The needle was then withdrawn, leaving the polyethylene cannula and washer
124 in place in the rear of the oral cavity. These mice were given 4 days for recovery prior to
125 training.
126 For training and testing, liquids were delivered into intraoral cannula via a 5-cc syringe
127 connected to a syringe pump (Model R99-E, Razel Scientific Instruments). To train the mice for
128 the acclimation of liquid stimulation through the cannulae, they were water deprived for 23 hour
129 / day, and given 3 ml of deionized water through one of the two intraoral cannulae over the
130 course of 30 min. We trained all the mice with deionized water for 2 days followed by

131 stimulation with either deionized water (n=4 [all M] for C57BL6/J; n=5 [2 F, 3 M] for
132 TRPA1/V1$^{Dbl-/-}$) or 30 mM citric acid (n=4 [1 F, 3 M] for C57BL6/J; n=5 [1F, 4M] for
133 TRPA1/V1$^{Dbl-/-}$). Animals exposed to the stimulus were left undisturbed for an additional 45 min
134 prior to sacrifice.
135
136 **c-Fos immunohistochemistry**
137 Seventy-five minutes after onset of taste stimulation through cannula, animals were
138 deeply anesthetized with Fatal-Plus® (50 mg/kg intraperitoneally; MWI), and then perfused
139 transcardially with saline followed by 4% paraformaldehyde (PFA) in 0.1 M pH 7.2 phosphate
140 buffer (PB). The brains were post fixed for 3 hours at room temperature, and then cryoprotected
141 overnight in 20% sucrose in PB at 4 °C. After cryoprotection, brainstems were cut and embedded
142 in OCT, frozen and sectioned at 40 μm using a cryostat. Free-floating sections were collected in
143 PBS.
144 For immunostaining, sections were washed in PBS 3 times and then processed for antigen
145 retrieval in sodium citrate (pH 6) at 85 °C for 10 min. After tissues cooled, non-specific protein
146 binding was blocked in a medium consisting of 2% normal donkey serum (Jackson
147 ImmunoResearch) in antibody medium (AB medium: 0.3% TritonX100, 0.15 M NaCl, and 1%
148 BSA in PB) for 1 hour at room temperature.
149 We utilized Mouse c-Fos antibody (1:1000, PhosphoSolutions; Cat#: 309-cFos; RRID
150 RRID:AB_2632380; Lot#: GS117P) and Rabbit P2X2 antibody (1:1000, Alomone Labs;
151 Cat#:APR003; RRID AB_2040054; Lot# APR003AN1002). These antibodies were diluted in
152 AB medium accordingly and were used to incubate the sections for 4 days at 4 °C. After 3 10-
153 min washes in PBS, sections were transferred to secondary antibody cocktail (Alexa Fluor 568

154 donkey anti mouse, 1:500; Alexa Fluor 488 donkey anti rabbit, 1:500; NeuroTrace Nissl
155 640/660, 1:500; all from Life Technologies) for 2-hour incubation at room temperature.
156 Following another 3 10-min washes in PBS, free-floating tissue sections were mounted onto
157 Microscope Slides (Tanner scientific, #TNR WHT90AD), and then coverslipped using
158 Fuoromount-G (Southern Biotech).

159 According to the manufacturer's data sheet, the PhosphoSolutions Mouse c-Fos antibody
160 shows a broad band of reactivity in Western blots of HeLa cells centered at 50kDa. We tested
161 whether this antibody stains similarly in fixed mouse brain tissues by comparing
162 immunoreactivity of this antibody to the one we have utilized previously: we allowed a wildtype
163 mouse to drink 150mM MSG for 30min and perfused the mouse with 4% buffered
164 paraformaldehyde after an additional 45 mins. The olfactory bulb & brainstem were removed,
165 cryoprotected in 20% sucrose and frozen free-floating sections were cut at 40um. Representative
166 sections were incubated in sodium citrate buffer pH6 at 85 °C for 10min. After cooling to RT for
167 20min, the sections were rinsed 3 X 5min in PBS, then incubated with 2%NDS+AB media for
168 1hr at RT. The free-floating sections then were incubated with a mixture of primary antibodies:
169 rabbit a-cFos (lot.D00148958) 1:500 / mouse a-cFos (lot. GS418y) 1:1000 for 4 nights at 4 °C.
170 After 3 X 10min rinses in PBS, sections were incubated with a mixture of secondary antibodies:
171 A488 donkey-a-rabbit 1:500/ A568 donkey-a-mouse 1:500/ Nissl 1:500 for 2hrs at RT. The
172 sections then were rinsed in PBS followed by 0.05M PB prior to coverslipping. The distribution
173 of labeled cells within and around the nTS was similar to that observed in single label cases and
174 nearly all cells were labeled by both antibodies. To quantitatively analyze the degree of co-
175 localization, we counted cells labeled by one or both antibodies in the olfactory bulb where the
176 density of labeled cells permits quantitative assessment. In randomly selected fields through the

177 granule cells layer of 3 sections, we counted the number of single and double-label cells. Of 153
178 labeled cells, 149 were double-labeled; 4 were labeled by only the PhosphoSolutions antiserum
179 and none were labeled only by the rabbit antibody. We conclude that the 2 antibodies label
180 nearly identical populations and that results from the PhosphoSolutions antibody should be
181 comparable to those obtained previously with the rabbit antibody.
182
183 **c-Fos activity determination**
184 Brainstem sections were observed under an Olympus BX41microscope. Representative
185 levels (r1, r2, r3, r4, i1, i2) of the nucleus of the solitary tract (nTS) were decided under a 10X
186 magnification objective according to Stratford et al. (Stratford, Thompson et al. 2017) and
187 photographed at 20X magnification using CellSense software with a XM10 camera. Boundaries
188 of the nTS were drawn using P2X2 and Nissl staining as reference and according to Stratford et
189 al. 2017 in ImageJ. Boundaries of the DMSp5 were not distinct based on the images but the
190 approximate boundaries were drawn using nTS boundaries and other brainstem nuclei as
191 reference (Corson, Aldridge et al. 2012). The DMSp5 was taken as the area between the lateral
192 edge of nTS and the spinal trigeminal nucleus.
193 The area of nTS was further divided into 6 parts following the system described in
194 Stratford et al (Stratford and Finger 2011) using a horizontal line dividing the area into dorsal
195 and ventral tiers and two vertical lines dividing the medial-lateral extent into thirds. The six parts
196 of nTS were named DM, DI, DL, VM, VI, VL, and the cFos signals in these areas as well as
197 DMSp5 were counted using ImageJ Cell counter plugin. The identity, stimulus and genotype of
198 all cases were blinded to the person performing the cell counts.
199

200 **Statistical Analysis**

201 Behavioral data are presented as group means with individual data points indicated.
202 Immunohistochemical data are presented as group means ± SEM. Data were analyzed using
203 appropriate two- and three-way analysis of variance (ANOVA)s (Statistica; StatSoft, Tulsa, OK).
204 Tukey's honest significant difference tests were used to assess statistically significant ($p < 0.05$)
205 main effects or interactions (see Results for details).

206

207 RESULTS

208 **Behavioral Assessment**

209 The overall preference curves for both WT and TRPA1/V1$^{Dbl-/-}$ animals appeared nearly
210 identical with significant aversion at 3mM but not significant aversion at 1mM concentration of
211 citric acid (Fig. 1). For the acetic acid experiments, behavioral preferences were statistically
212 different across different acetic acid concentrations, [$F(5,10) = 26.82$, $p < 0.001$]. In particular,
213 the preference for 10 mM and 30 mM acetic acid were significantly lower than the preferences
214 for water, 1 mM and 3 mM acetic acid (all p's < 0.05). Also, the preference for 100µM SC45647
215 was significantly higher than the preference for all other tastants (all p's < 0.05). Moreover,
216 overall, there was no statistical differences in the behavioral preference for the acetic acid
217 concentrations tested between WT and TrpA1/V1$^{Dbl-/-}$ animals, $F(5,50) = 1.75$, $p = 0.14$. For
218 citric acid experiments, behavioral preferences were statistically different between different taste
219 solutions, $F(5, 15) = 111.83$, $p < 0.0001$, with the amount of licking to 3, 10 and 30 mM citric
220 acid being significantly lower than to that of 1 mM citric acid and water (all 'p's < 0.05).
221 However, there were no statistically significant differences between Trp channel knockouts and
222 controls for any tastant, $F(5,75) = 1.25$, $p = 0.30$.

223

224 **c-Fos Induction**

225 Injection of 30 mM citric acid in the mouth via intraoral cannulae reliably induced c-Fos
226 within particular regions of the nTS as compared to similar injection of water. As calculated
227 previously, raw counts of c-Fos positive neurons in sub-compartments of the nTS were compared
228 between water-stimulated and citric acid-stimulated animals to produce a measure of citric acid-
229 specific c-Fos induction. In our previous study (Stratford, Thompson et al. 2017), acid-induced
230 c-Fos was highest in the central portion of rostral-intermediate nTS (r3-i2) and in ventrolateral
231 subdivisions in intermediate nTS (i1-i3). These regions did not show such activation in the
232 P2X2/P2X3 dbl KO mice which lack taste function. However, in P2X2/P2X3 dbl KO animals,
233 significant levels of activation to citric acid did remain in the DMSp5, which receives substantial
234 input from polymodal nociceptor (CGRP+) fibers of the trigeminal and glossopharyngeal nerves.
235 Accordingly, we especially focused on possible changes in c-Fos activation in this trigeminal-
236 recipient area.

237 Fig. 2 shows a representative image of c-Fos activation in relation to the lateral nTS and
238 the DMSp5 of WT and TRPA1/V1$^{Dbl-/-}$ mice following stimulation with citric acid. The number
239 of c-Fos positive cells was significantly greater with citric acid stimulation than water
240 stimulation, $F(1, 5) = 49.53$, $p < 0.05$. However, there was no statistical difference in the number
241 of c-Fos positive cells between WT and TRPA1/V1$^{Dbl-/-}$ mice for either water or citric acid, $F(1,$
242 $82) = 0.05$, $p = 0.83$.

243 As shown in Fig. 3, the amount of citric acid-specific c-Fos was significantly different
244 between nTS subregions and levels regardless of genotype, $F (25, 210) = 4.24$, $p < 0.001$. In
245 particular, citric acid evoked significant activity in the central subregion of rostrointermediate

246 nTS (r2-i2), in the lateral subregion of intermediate nTS (i1-2) and in the DMSp5 of both WT
247 and TRPA1/V1$^{Dbl-/-}$ mice as compared to all other nTS subregions (all p's < 0.05). This pattern is
248 similar to that reported previously for the mixed background (Ola-C57/BL6) controls in the
249 previous study (Stratford, Thompson et al. 2017).

250 Moreover, the TRPA1/V1$^{Dbl-/-}$ mice show overall levels of acid-induced c-Fos similar to
251 the WT controls in all nTS subregions, $F(25, 210) = 0.87$, $p = 0.65$, (average difference 0.06
252 cells/compartment ± 0.64 SEM.; 95% C.I. -1.32 -1.20). Likewise, the TRPA1/V1$^{Dbl-/-}$ mice show
253 similar levels of acid-induced c-Fos in DMSp5 compared to WT controls, $F (5, 25) = 0.31$, $p =$
254 0.91 (average difference 0.32 cells/region ± 0.89 SEM.; 95% C.I. -1.42-2.06).

255 In summary, the TRPA1/V1$^{Dbl-/-}$ mice show no differences in the pattern of neural
256 activation by citric acid compared to the WT animals. This suggests that neither TrpA1 nor
257 TrpV1 play a significant role in detection and avoidance of 30 mM concentrations of citric acid,
258 although this concentration is readily avoided by both TRPA1/V1$^{Dbl-/-}$ and WT animals.

259

260 DISCUSSION

261 Acidity is the underlying chemical feature of sour substances. When acids are taken into
262 the mouth, they stimulate sour-sensitive taste cells which depolarize, then release
263 neurotransmitter to activate the taste fibers innervating them. This signaling between taste cells
264 and nerve fibers requires functional P2X2 and P2X3 receptors; genetic deletion or
265 pharmacological blockage of these receptors essentially eliminates taste-mediated neural activity
266 (Finger, Danilova et al. 2005, Vandenbeuch, Anderson et al. 2013). Similarly, taste driven
267 acceptance of sweet and umami, and taste driven avoidance of bitter is lost in mice lacking
268 functional P2X2 and P2X3 receptors. In contrast, behavioral avoidance of intraoral acids remains

269 intact despite the loss of taste-related neural activity to these stimuli. Similarly, avoidance of
270 acids persists even after genetic deletion of Otop1, the ion channel receptor for H^+ underlying
271 sour detection (Zhang, Jin et al. 2019). These findings strongly suggest that not just taste, but
272 another sensory modality drives the avoidance of acids in this context.

273 The oropharynx is innervated by chemically-sensitive free nerve endings arising from the
274 trigeminal, glossopharyngeal and vagus nerves. These nerves include populations of polymodal
275 nociceptors that express acid-sensitive ion channels including TrpA1 and TrpV1 (Tominaga,
276 Caterina et al. 1998, Wang, Chang et al. 2011), and the TrpV1-expressing fibers are necessary
277 for non-taste mediated avoidance of citric acid (Zhang, Jin et al. 2019). Yet these studies do not
278 demonstrate that TrpV1 itself is the necessary receptor. It is likely that other acid-sensitive
279 channels and receptors exist in these TrpV1-expressing fibers. Further, TrpV1 fully activates at a
280 pH around 5 at body temperature (Tominaga, Caterina et al. 1998), whereas avoidance of citric
281 acid begins at a pH near 3. It is likely that the tissue overlying the TrpV1 sensory terminals
282 provides some buffering of acids applied to the surface of the epithelium, but weak acids, such as
283 citric acid, effectively acidify the epithelium deep into the tissue (Richter, Caicedo et al. 2003),
284 well beyond the region in which the nerve terminals lie. Taken together, these results suggest
285 that TrpV1 itself may not be entirely responsible for responses to citric acid. Accordingly, we
286 tested whether either TrpV1 or TrpA1 channels contribute to either the behavioral avoidance
287 response, or the activation of brainstem neurons by citric acid. We found that neither the
288 behavior nor the pattern and degree of neural activation was altered by genetic deletion of these
289 channels.

290 If neither TrpV1 nor TrpA1 channels underlie avoidance of acidic substances in the
291 absence of taste, what other mechanisms might be responsible? Likely candidates are one or

292 more members of the ASIC (acid sensing ion channel) family. Ion channels in this family are
293 gated by decreases in extracellular pH. While many members of this family open at pH values
294 near 7, others activate at lower pH values (Deval, Gasull et al. 2010). In particular, ASIC3 is
295 expressed widely in polymodal nociceptors and plays a role in responses to pH5.0 (Price,
296 McIlwrath et al. 2001). Further, amiloride, a non-specific blocker of ASIC channels, decreases
297 the irritation of citric acid (albeit at a much higher concentration) measured psychophysically
298 (Dessirier, O'Mahony et al. 2000), supporting the role of ASICs in this response. Conversely,
299 inclusion of amiloride, a blocker of ASICs, in orally-applied solutions in rats neither decreased
300 the trigeminal response to citric acid nor attenuated acid-induced c-Fos in brainstem trigeminal
301 nuclei (Sudo, Sudo et al. 2003). Whether ASICs or some other acid-responsive mechanisms play
302 a role in non-gustatory behavioral avoidance of weak acids then is unresolved.
303

304 **CONFLICTS OF INTEREST**

305 The authors declare no conflicts of interest in relation to this work.
306

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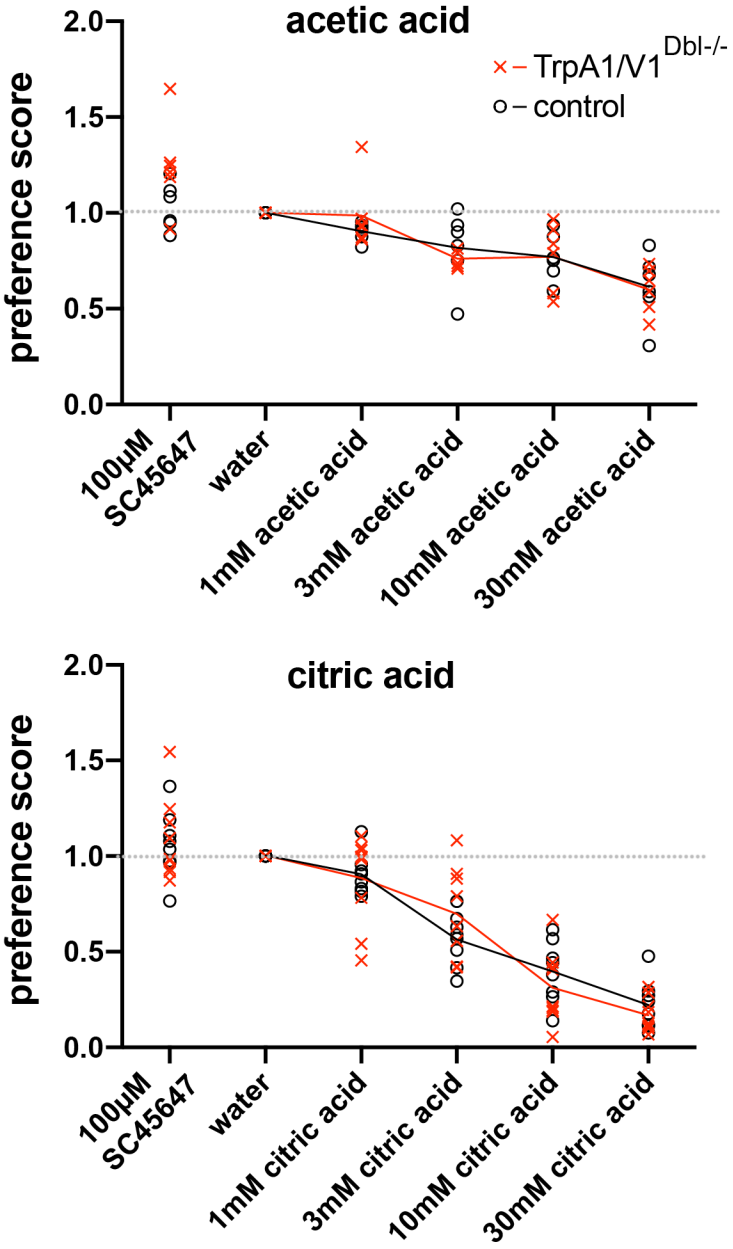
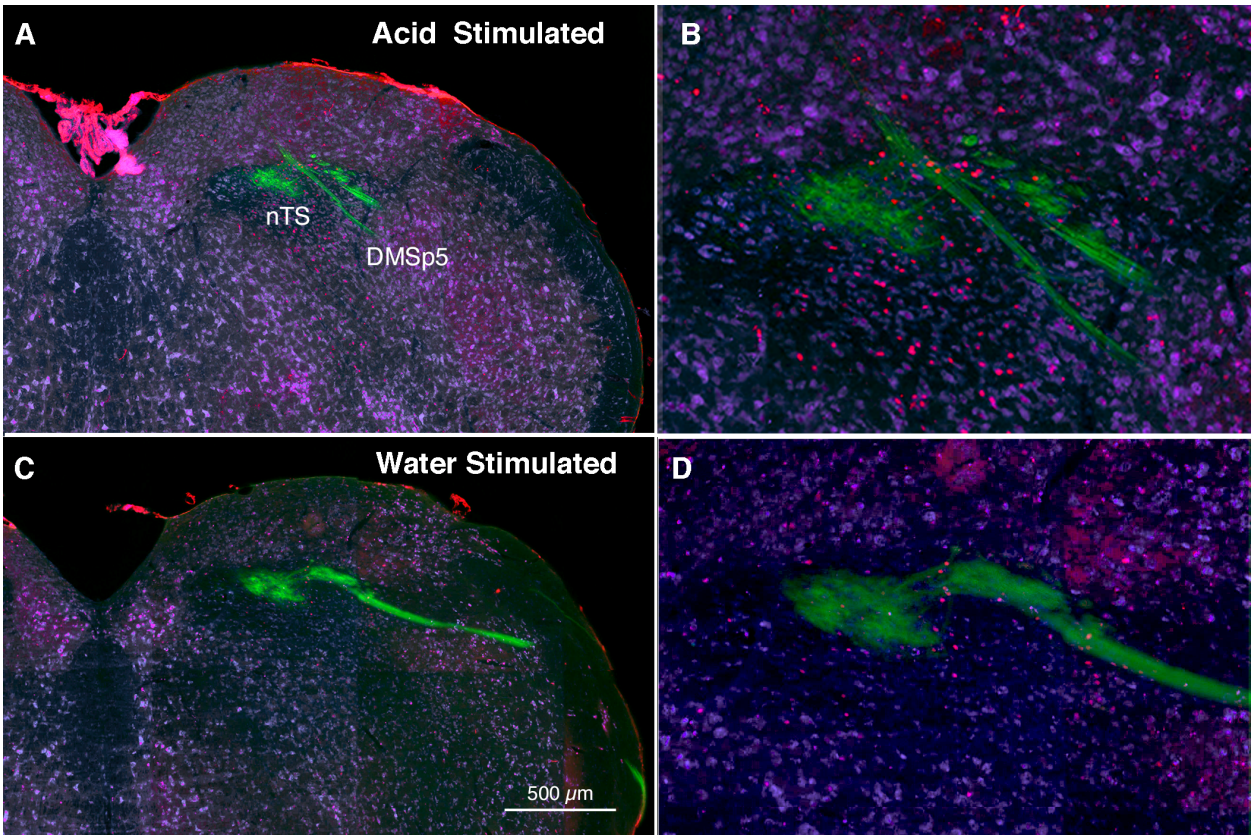


Fig. 1: Brief access tests showing similar avoidance of citric acid and acetic acid for TRPA1/V1$^{Dbl-/-}$ mice and wildtype controls of the same background. ANOVA shows no significant difference between the groups. Preference scores represent the average number of licks relative to those for water. Preference scores higher than 1 indicate a preference for the taste stimulus, while preference scores lower than 1 indicate an avoidance.

397 

398 Fig. 2. C-Fos staining (red dots) in nTS of the TRPA1/V1$^{Dbl-/-}$ mouse at approximately the R3
399 level comparing a mouse stimulated by 30mM citric acid (A, B) to one stimulated only by water
400 (C, D). Note the higher number of c-Fos positive cell nuclei (red) in the acid stimulated
401 compared to the water-stimulated animal. Green staining shows the distribution of P2X3-
402 immunoreactive fibers which terminate largely in the dorsocentral part of the nTS. Most acid-
403 stimulated cells lie in the ventral tier of the nucleus and in the DMSp5 region ventrolateral to the
404 nTS. Blue counterstain from FluoroNissl.
405

WT Mice

Water Stimulated

		Medial	I	Lateral	DMSp5
r1	D	1.5	2.3	2.3	
	V	2.1	2.0	1.1	2.9
r2	D	3.3	2.9	2.9	
	V	2.1	1.0	1.9	3.0
r3	D	5.6	12.6	9.8	
	V	4.1	13.4	9.6	8.5
r4	D	4.3	16.6	10.9	
	V	4.5	14.8	12.9	10.6
i1	D	15.0	15.8	15.1	
	V	7.3	9.5	12.1	12.3
i2	D	11.2	14.2	7.8	
	V	5.5	8.2	12.2	18.2

Citric Acid Stimulated

		Medial	I	Lateral	DMSp5
r1	D	3.4	6.9	4.6	
	V	2.9	2.3	2.6	4.9
r2	D	4.5	9.8	3.5	
	V	5.0	5.8	4.0	2.8
r3	D	7.0	21.4	8.1	
	V	5.9	18.9	12.3	11.1
r4	D	9.6	27.9	13.3	
	V	8.3	24.6	20.3	22.6
i1	D	22.9	31.0	13.6	
	V	10.8	22.4	26.4	20.3
i2	D	13.3	22.9	11.6	
	V	9.5	16.6	28.1	26.4

Acid Specific (C.A. - water)

		Medial	I	Lateral	DMSp5
r1	D	1.9	4.6	2.4	
	V	0.8	0.3	1.5	2.0
r2	D	1.3	6.9	0.6	
	V	2.9	4.8	2.1	-0.3
r3	D	1.4	8.8	-1.6	
	V	1.8	5.5	2.6	2.6
r4	D	5.4	11.3	2.4	
	V	3.8	9.9	7.4	12.0
i1	D	7.9	15.3	-1.5	
	V	3.5	12.9	14.3	8.0
i2	D	2.1	8.7	3.8	
	V	4.0	8.5	16.0	8.2

KO Mice

Water Stimulated

		Medial	I	Lateral	DMSp5
r1	D	2.3	5.0	1.9	
	V	1.4	0.6	0.9	2.1
r2	D	2.4	8.0	5.4	
	V	1.6	2.6	4.0	5.1
r3	D	4.1	10.9	3.8	
	V	3.0	5.3	2.9	2.4
r4	D	1.6	14.3	6.5	
	V	1.5	11.9	4.5	5.3
i1	D	11.6	9.9	9.9	
	V	6.1	6.8	9.2	9.1
i2	D	9.5	8.6	4.6	
	V	5.8	6.3	6.7	9.9

Citric Acid Stimulated

		Medial	I	Lateral	DMSp5
r1	D	3.7	5.0	2.3	
	V	2.4	2.8	1.7	3.4
r2	D	5.9	13.4	5.3	
	V	6.4	6.6	4.4	5.2
r3	D	6.2	17.2	8.3	
	V	6.1	19.0	11.0	6.3
r4	D	9.5	23.1	13.7	
	V	7.0	24.8	13.4	10.9
i1	D	10.6	22.9	18.0	
	V	5.8	19.2	27.9	17.3
i2	D	10.0	12.3	7.5	
	V	8.3	15.1	22.6	17.3

Acid Specific (C.A. - water)

		Medial	I	Lateral	DMSp5
r1	D	1.5	0.0	0.4	
	V	1.0	2.2	0.8	1.3
r2	D	3.5	5.4	-0.1	
	V	4.8	4.0	0.4	0.1
r3	D	2.1	6.3	4.5	
	V	3.1	13.7	8.1	3.9
r4	D	7.9	8.8	7.2	
	V	5.5	12.9	8.9	5.6
i1	D	-1.0	13.0	8.1	
	V	-0.3	12.4	18.7	8.2
i2	D	0.5	3.7	2.9	
	V	2.5	8.8	15.9	7.4

407 Fig. 3.Heat maps of c-Fos expression in nTS and DMSp5 regions comparing wildtype (left) and
408 TRPA1/V1$^{Dbl-/-}$ mice, stimulated by intraoral injection of water (top row and by citric acid
409 (middle row). Bottom row shows acid-specific activation, i.e. citric acid counts minus the counts
410 produced by water alone. No significant differences occur either in the pattern or absolute level
411 of expression across subregions (see text for details). For each panel, the maximum value was
412 assigned as red; zero was assigned light yellow, and negative values shades of blue.
413
414