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.

37 INTRODUCTION

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

48 In mice lacking a functional taste system (P2X2/3-dbl-KO mice), the chorda tympani
49 nerve, which provides only intragemmal innervation to taste buds (Silverman and Kruger 1990,
50 Ohman-Gault, Huang et al. 2017), shows no responses to any tastants including acids although
51 tactile and thermal responses remain (Finger, Danilova et al. 2005). Consistent with this, these
52 mice show no preference for sweeteners or avoidance of bitter substances in brief-access taste
53 tests. Despite the apparent lack of taste responses to acids, the P2X2/3-dbl-KO mice do exhibit
54 normal avoidance of citric acid in similar brief access tests (Hallock, Tatangelo et al. 2009). This
55 avoidance may be mediated not by taste, which is non-functional in these mice, but by acid-
56 responsive fibers in the trigeminal, glossopharyngeal or laryngeal nerves which do show
57 residual low level activity in the P2X2/3-dbl-KO mice (Ohkuri, Horio et al. 2012).

58 Polymodal nociceptors respond to acidification as well as other potentially painful stimuli
59 (Bessou and Perl 1969). Many small caliber polymodal nociceptors that innervate the oral cavity

60 (Kichko, Neuhuber et al. 2018, Wu, Arris et al. 2018) express one or both of the pH-sensitive
61 transient receptor potential (Trp) channels, TrpA1 (Wang, Chang et al. 2011) and TrpV1
62 (Tominaga, Caterina et al. 1998). TrpA1 has been implicated especially in responsiveness to
63 weak acids capable of penetrating cell membranes to produce intracellular acidification (Wang,
64 Chang et al. 2011). TrpV1 is implicated in responses of the glossopharyngeal and vagus nerves
65 to acidification of the oral and pharyngeal epithelium (Arai, Ohkuri et al. 2010). Gating of either
66 of these channels by acids can directly activate the nociceptive fiber resulting in a noxious
67 sensation suitable for driving avoidance behavior. Ablation of the ganglion cells expressing
68 TrpV1 in mice lacking sour taste receptors does result in loss of acid-avoidance behavior (Zhang,
69 Jin et al. 2019) but it is not clear whether this is due to loss of TrpV1 itself or other ion-sensing
70 mechanisms of these neurons. We tested whether genetic deletion of both TrpA1 and TrpV1
71 affected either acid-induced avoidance behaviors or the pattern of neuronal activation in the
72 brainstem taste (nuc. solitary tract, nTS) or trigeminal oral (dorsomedial trigeminal nucleus,
73 DMSp5) sensory nuclei.
74
75
76

77 # Materials and Methods

78 **Animals**

79 TrpA1V1-double KO mice, on a C57BL6 background (TRPA1/V1$^{Dbl-/-}$) were a generous
80 gifts from Diana Bautista, University of California Berkeley (Gerhold and Bautista 2008). In
81 these mice, deletion of TrpA1 had been accomplished by elimination of residues 901–951
82 including most of exon 23 – a region encoding the putative pore and part of the sixth
83 transmembrane domain (Bautista, Jordt et al. 2006). The VR1 gene was disrupted (Caterina,
84 Leffler et al. 2000) by deleting part of the fifth and all of the sixth putative transmembrane
85 domains, together with the intervening pore-loop region. The TRPA1/V1$^{Dbl-/-}$ mice were
86 genotyped by PCR to ensure quality control of the breeding. C57BL6/J mice were purchased
87 from The Jackson Laboratory (Bar Harbor, ME). For brief access lickometer behavioral assays,
88 2-6 months old male (n=9) and female (n=6) TRPA1/V1$^{Dbl-/-}$ mice and 2-10 month old male
89 (n=8) and female (n=3) C57BL6/J mice were used. For immunohistochemical staining, we used
90 TRPA1/V1$^{Dbl-/-}$ (2 to 8 mo, n=10, 7 male and 3 female)) and C57BL6/J mice (4 to 7 mo, n=6, all
91 male). In an addition to the C57 cases, we utilized 2 mice (1 male, 1 female) of the line B6.Cg-
92 Tg(Fos-tTA,Fos-EGFP*)1Mmay/J which carry 2 transgenes associated with c-Fos: cfos-tTA and
93 cfos-shEGFP. In these mice, on a C57BL6/J background, the randomly inserted transgenes
94 utilize the c-Fos promotor to drive expression of respectively tTA and a short-lived (two-hour
95 half-life) GFP. We did not find close correlation between GFP expression and c-Fos
96 immunostaining and so the GFP results were ignored in our analysis. Nonethelss, the c-Fos
97 counts from these animals were entirely consistent with those obtained from the wildtype
98 C57BL6/J mice and so were included in those results.
99

100 All animal procedures were performed in accordance with NIH guidelines and were approved by
101 the Institutional Animal Care and Use Committee at the University of Colorado School of
102 Medicine.
103
104 **Brief access preference tests**
105 Davis Rig Lickometers (MS-160; Dilog Instruments and Systems) were used for training
106 and brief-access testing of mice. The animals underwent a 4-day water training before acid
107 testing. Mice were placed on water deprivation 20 hours before the first day of training or acid
108 testing. On the first day of the 4-day training, water-deprived animals were allowed access to a
109 single water spout for a 30-min period. In the following three days of water training, 2-4 bottles
110 of water were given in random sequences for 15 min. Mice had 5 seconds to lick at each spout
111 after their first lick before the door closed and the bottle was switched to the next position with
112 an inter-trial interval of 7.5 s. The mice were considered well-trained if they lick more than 30
113 times consistently during each trial for the first 15 trials. No mice lost more than 15% of body
114 weight during any phase of the experimental procedure. All mice were given 2 day recovery
115 period with ad libitum access to water after every 4 day block of testing.
116 For the acid testing days, six bottles of tastants including 4 concentrations of acetic or
117 citric acid (1 mM, 3 mM, 10 mM and 30 mM), 1 concentration of artificial sweetener SC45647
118 (100 μM or 300μM) and H_2O were presented in a testing block with a set up similar to the
119 training session. Testing periods lasted for 15 min, with the opportunity for the mice to sample
120 from the 6 solutions a total of 30 times. Licks were measured using InstaCal software. Each
121 block of 6 tastant trials featured each solution in random order. Only data from completed blocks
122 were used in the calculation of preference. Preference was calculated by averaging the licks per

123 tastant block of each solution relative to those for water (i.e., a 'lick ratio'). The same mice that
124 were assessed for citric acid preference were then tested for acetic acid preference again with the
125 same paradigm. The solutions used in acetic acid testing were as follows: 100 or 300 μM
126 SC45647, H_2O, 1, 3, 10 and 30 mM acetic acid. Testing for each series of tastants was repeated
127 at least twice. Because there was no statistically significant difference in licking across each
128 testing day for each mouse [$F(1,2) = 0.42$, $p = 0.66$], the number of licks for each tastant were
129 averages across these testing days for each mouse.
130
131 **Citric acid stimulation for c-Fos via intraoral cannula**
132 Both bilateral intraoral cannulae implantation and stimulation methods were adopted
133 from (Stratford and Thompson 2016). Briefly, mice were anesthetized with an intramuscular
134 injection of a combination of medetomidine hydrochloride (Domitor; 0.4 mg/kg; Pfizer) and
135 ketamine hydrochloride (40 mg/kg; Bioniche Pharma). Intraoral cannulae were inserted via a
136 midline incision immediately caudal to the pinnae. Then a sterile 2-3 cm stainless steel needle
137 (19 gauge; Hamilton) with a flared end polyethylene tube (50 gauge; Becton Dickinson) was
138 inserted from behind the pinna and guided subcutaneously into the oral cavity, lateral to the first
139 maxillary molar. The needle was then withdrawn, leaving the polyethylene cannula and washer
140 in place in the rear of the oral cavity. These mice were given 4 days for recovery prior to
141 training.
142 For training and testing, liquids were delivered into intraoral cannula via a 5-cc syringe
143 connected to a syringe pump (Model R99-E, Razel Scientific Instruments). To train the mice for
144 the acclimation of liquid stimulation through the cannulae, they were water deprived for 23 hour
145 / day, and given 3 ml of deionized water as a constant pulsatile flow of 0.1 ml/min through one

146 of the two intraoral cannulae over the course of 30 min. We trained all the mice with deionized
147 water for 2 days followed by stimulation with either deionized water (n=4 [all M] for C57BL6/J,
148 n=5 [2 F, 3 M] for TrpA1V1-dbl KO), or 30 mM citric acid (n=4 [1 F, 3 M] for C57BL6/J, n=5
149 [1F, 4M] for TrpA1V1-dbl KO). Animals exposed to the stimulus were left undisturbed for an
150 additional 45 min prior to sacrifice.
151
152 **c-Fos immunohistochemistry**
153 Seventy-five minutes after onset of taste stimulation through cannula, animals were
154 deeply anesthetized with Fatal-Plus® (50 mg/kg intraperitoneally; MWI), and then perfused
155 transcardially with saline followed by 4% paraformaldehyde (PFA) in 0.1 M pH 7.2 phosphate
156 buffer (PB). The brains were post fixed for 3 hours at room temperature, and then cryo-protected
157 overnight in 20% sucrose in PB at 4 °C. After cyroprotection, brainstems were cut and embedded
158 in OCT, frozen and sectioned at 40 μm using a cryostat. Free-floating sections were collected in
159 PBS.
160 For immunostaining, sections were washed in PBS 3 times and then processed for antigen
161 retrieval in sodium citrate (pH 6) at 85 °C for 10 min. After tissues cooled, non-specific protein
162 binding was blocked in a medium consisting of 2% normal donkey serum (Jackson
163 ImmunoResearch) in antibody medium (AB medium: 0.3% TritonX100, 0.15 M NaCl, and 1%
164 BSA in PB) for 1 hour at room temperature.
165 We utilized Mouse c-Fos antibody (1:1000, PhosphoSolutions; Cat#: 309-cFos; RRID
166 RRID:AB_2632380; Lot#: GS117P) and Rabbit P2X2 antibody (1:1000, Alomone Labs;
167 Cat#:APR003; RRID AB_2040054; Lot# APR003AN1002). These antibodies were diluted in
168 AB medium accordingly and were used to incubate the sections for 4 days at 4 °C. After 3 10-

169 min washes in PBS, sections were transferred to secondary antibody cocktail (Alexa Fluor 568
170 donkey anti mouse, 1:500; Alexa Fluor 488 donkey anti rabbit, 1:500; NeuroTrace Nissl
171 640/660, 1:500; all from Life Technologies) for 2-hour incubation at room temperature.
172 Following another 3 10-min washes in PBS, free-floating tissue sections were mounted onto
173 Microscope Slides (Tanner scientific, #TNR WHT90AD), and then coverslipped using
174 Fuoromount-G (Southern Biotech).

175 According to the manufacturer's data sheet, the PhosphoSolutions Mouse c-Fos antibody
176 shows a broad band of reactivity in Western blots of HeLa cells centered at 50kDa. We tested
177 whether this antibody stains similarly in fixed mouse brain tissues by comparing
178 immunoreactivity of this antibody to the one we have utilized previously: we allowed a wildtype
179 mouse to drink 150 mM MSG for 30min and perfused the mouse with 4% buffered
180 paraformaldehyde after an additional 45 mins. The olfactory bulb & brainstem were removed,
181 cryoprotected in 20% sucrose and frozen free-floating sections were cut at 40 µm.
182 Representative sections were incubated in sodium citrate buffer pH6 at 85 °C for 10 min. After
183 cooling to room temperature for 20 min, the sections were rinsed 3 X 5min in PBS, then
184 incubated with 2%NDS+AB media for 1hr at room temperature. The free-floating sections then
185 were incubated with a mixture of primary antibodies: rabbit a-cFos (lot.D00148958) 1:500
186 / mouse a-cFos (lot. GS418y) 1:1000 for 4 nights at 4 °C. After 3 X 10 min rinses in PBS,
187 sections were incubated with a mixture of secondary antibodies: A488 donkey-a-rabbit 1:500/
188 A568 donkey-a-mouse 1:500 / Nissl 1:500 for 2 hrs at room temperature. The sections then were
189 rinsed in PBS followed by 0.05M PB prior to coverslipping. The distribution of labeled cells
190 within and around the nTS was similar to that observed in single label cases and nearly all cells
191 were labeled by both antibodies. To quantitatively analyze the degree of co-localization, we

192 counted cells labeled by one or both antibodies in the olfactory bulb where the density of labeled
193 cells permits quantitative assessment. In randomly selected fields through the granule cells layer
194 of 3 sections, we counted the number of single and double-label cells. Of 153 labeled cells, 149
195 were double-labeled; 4 were labeled by only the PhosphoSolutions antiserum and none were
196 labeled only by the rabbit antibody. We conclude that the 2 antibodies label nearly identical
197 populations and that results from the PhosphoSolutions antibody should be comparable to those
198 obtained previously with the rabbit antibody.
199
200 **c-Fos activity determination**
201 Brainstem sections were observed under an Olympus BX41microscope. Representative
202 levels (r1, r2, r3, r4, i1, i2) of the nucleus of the solitary tract (nTS) were chosen under a 10X
203 magnification objective according to Stratford et al. (Stratford, Thompson et al. 2017) and
204 photographed at 20X magnification using CellSense software with a XM10 camera. Boundaries
205 of the nTS were drawn using P2X2 and Nissl staining as reference and according to Stratford et
206 al. (2017) in ImageJ. Boundaries of the DMSp5 were not distinct based on the images but the
207 approximate boundaries were drawn using nTS boundaries and other brainstem nuclei as
208 reference (Corson, Aldridge et al. 2012). The DMSp5 was taken as the area between the lateral
209 edge of nTS and the spinal trigeminal nucleus.
210 The area of nTS was further divided into 6 parts following the system described in
211 Stratford & Finger (Stratford and Finger 2011) using a horizontal line dividing the area into
212 dorsal and ventral tiers and two vertical lines dividing the medial-lateral extent into thirds. The
213 six parts of nTS were named DM, DI, DL, VM, VI, VL, and the cFos signals in these areas as

214 well as DMSp5 were counted using ImageJ Cell counter plugin. The identity, stimulus and
215 genotype of all cases were blinded to the person performing the cell counts.
216

217 ## Statistical Analysis

218 Behavioral data are presented as group means with individual data points indicated.
219 Immunohistochemical data are presented as group means ± SD. Data were first analyzed for
220 normality using an Anderson-Darling test (AD). Because all data were deemed normally
221 distributed based on the results of the AD test, appropriate two- and three-way analysis of
222 variance (ANOVA)s were conducted (Statistica; StatSoft, Tulsa, OK). Tukey's honest significant
223 difference tests were used to assess statistically significant ($p < 0.05$) main effects or interactions
224 (see Results for details).
225

226 RESULTS

227 **Behavioral Assessment**

228 For the acetic acid experiments, behavioral preferences were not statistically different
229 between males and females across each genotype (WT and TRPA1/V1$^{Dbl-/-}$), (genotype x sex
230 interaction, $F(1, 14) = 0.00$, $p = 0.99$). However, behavioral preferences were statistically
231 different between different taste solutions, (main effect of taste, $F(4, 56) = 24.89$, $p < 0.0001$,
232 Fig. 1A).

233 For the citric acid experiments, behavioral preferences were not statistically different
234 between males and females, $F(1, 22) = 2.80$. $p = 0.11$. Moreover, the overall preference curves
235 for both WT and TRPA1/V1$^{Dbl-/-}$ animals appeared nearly identical, (taste x genotype interaction,
236 $F(4,88) = 0.34$, $p = 0.85$, Fig. 1B).

237

238 **c-Fos Induction**

239 Injection of 30 mM citric acid in the mouth via intraoral cannulae reliably induced c-Fos
240 within particular regions of the nTS as compared to similar injection of water. As calculated
241 previously, raw counts of c-Fos positive neurons in sub-compartments of the nTS were compared
242 between water-stimulated and citric acid-stimulated animals to produce a measure of citric acid-
243 specific c-Fos induction. In our previous study (Stratford, Thompson et al. 2017), acid-induced
244 c-Fos was highest in the central portion of rostral-intermediate nTS (r3-i2) and in ventrolateral
245 subdivisions in intermediate nTS (i1-i3). These regions did not show such activation in the
246 P2X2/P2X3 dbl KO mice which lack taste function. However, in P2X2/P2X3 dbl KO animals,
247 significant levels of activation to citric acid did remain in the DMSp5, which receives substantial
248 input from polymodal nociceptor (CGRP+) fibers of the trigeminal and glossopharyngeal nerves.
249 Accordingly, we especially focused on possible changes in c-Fos activation in this trigeminal-
250 recipient area. Fig.2 shows a representative image of c-Fos activation in relation to the lateral
251 nTS and the DMSp5 of TRPA1/V1$^{Dbl-/-}$ mice following stimulation with citric acid.

252 <u>'Raw' Tastant-Evoked c-Fos Counts</u>. For all animals (i.e. both WT and TRPA1/V1$^{Dbl-/-}$
253 mice), the total number of c-Fos positive cells evoked by citric acid oral stimulation was
254 significantly greater than water stimulation within the nTS overall (water: 39.48 ± 3.59; citric
255 acid: 71.03 ± 5.65, main effect of tastant: $F(1, 5) = 49.53$, $p < 0.001$; see Table 1). Moreover, the
256 total number of water-evoked and citric acid-evoked c-Fos positive cells were significantly
257 higher in WT as compared to TRPA1/V1$^{Dbl-/-}$ mice, regardless of tastant (For WT: 73.50 ± 7.17;
258 For TRPA1/V1$^{Dbl-/-}$: 58.25 ± 5.57; main effect of genotype: $F(1, 5) = 6.67$, $p < 0.05$. However,
259 there was no genotype x tastant interaction: $F(1, 82) = 0.05$, $p = 0.83$, see Table 1 and Fig. 3).

260 Finally, there was no significant three-way tastant x genotype x nTS level interaction (F (1, 82) =
261 0.28, p = 0.92), nor was there a four-way tastant x genotype x nTS level x nTS subregion
262 interaction (F (25, 410) = 0.55, p = 0.96).
263
264 Table 1. Average (+/-S.D.) c-fos positive cells per region in
265 the nTS and DMSp5 in WT and TrpA1/V1$^{Dbl-/-}$ mice following
266 water and citric acid stimulation.

	Water	Citric Acid
WT	5.28 ± 1.58	9.83 ± 1.59
TRPA1/V1$^{Dbl-/-}$	13.72 ± 2.50	19.17 ± 2.54

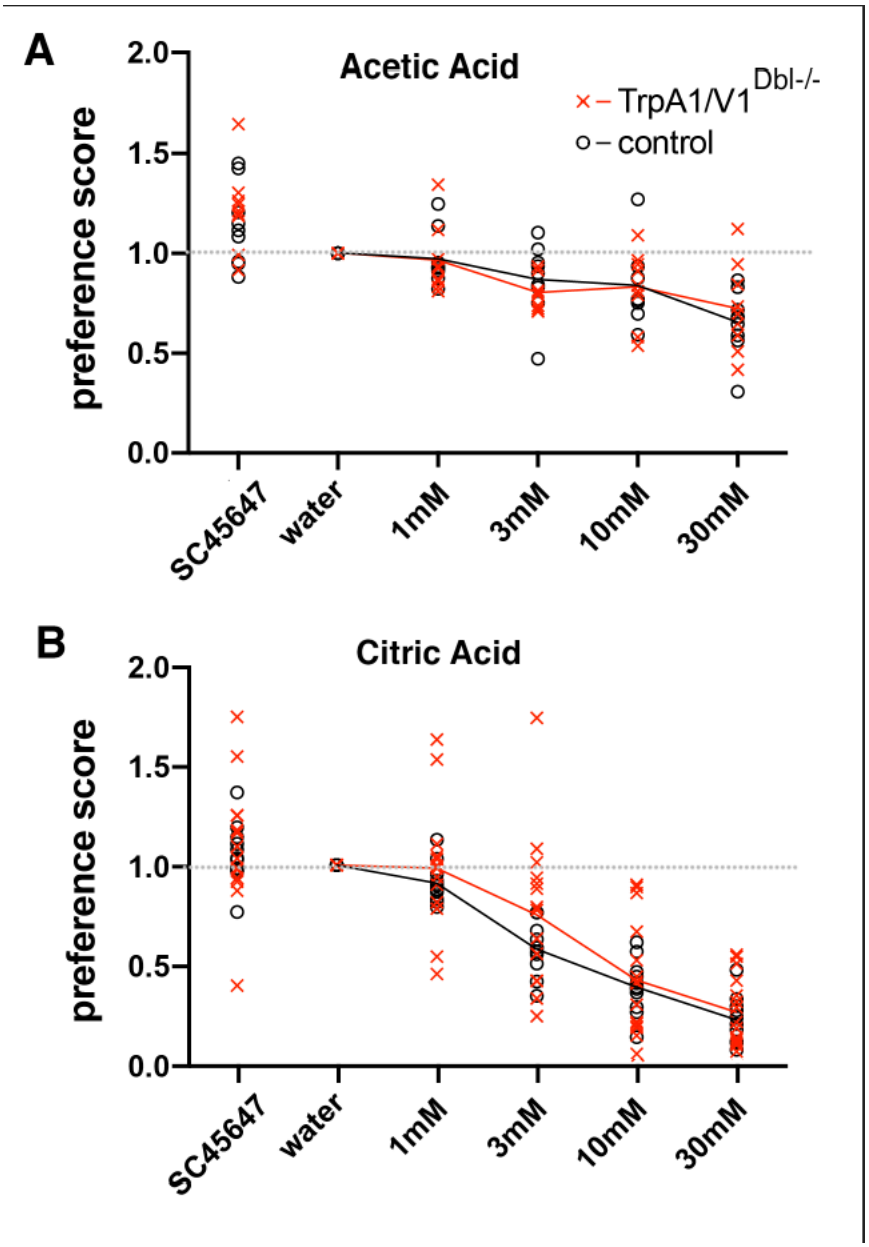
267
268 Citric Acid- Specific c-Fos Activation. Because the number of c-Fos positive cells was
269 lower in TRPA1/V1$^{Dbl-/-}$ mice as compared to WT nice (regardless of tastant), we calculated citric
270 acid-specific activation for each region by taking the number of 'raw' citric acid-evoked c-Fos
271 positive cells minus the average number of water-evoked c-Fos positive cells for each region for
272 each genotype. Overall, citric-acid specific activation in the nTS did not differ between
273 genotypes (main effect of genotype: F (1,5) = 0.08, p = 0.78). Furthermore, as shown in Fig. 3,
274 the amount of citric acid-specific c-Fos was significantly different between nTS subregions and
275 levels regardless of genotype, interaction between nTS subregion x nTS level: F (25, 210) =
276 4.24, $p < 0.001$. In particular, citric acid evoked significant activity in the central subregion of
277 rostrointermediate nTS (r2-i2), in the lateral subregion of intermediate nTS (i1-2) and in the
278 DMSp5 of both WT and TRPA1/V1$^{Dbl-/-}$ mice as compared to all other nTS subregions (all p's <
279 0.05). This pattern is similar to that reported previously for the mixed background (Ola-
280 C57/BL6) controls in the previous study (Stratford, Thompson et al. 2017).
281 In summary, the TRPA1/V1$^{Dbl-/-}$ mice show no differences in the pattern of neural
282 activation by citric acid compared to the WT animals. This suggests that neither TrpA1 nor

283 TrpV1 plays a significant role in detection and avoidance of 30 mM concentrations of citric acid,
284 although this concentration is readily avoided by both TRPA1/V1$^{Dbl-/-}$ and WT animals.
285
286 DISCUSSION
287 Acidity is the underlying chemical feature of sour substances. When acids are taken into
288 the mouth, they stimulate sour-sensitive taste cells which depolarize, then release
289 neurotransmitter to activate the taste fibers innervating them. This signaling between taste cells
290 and nerve fibers requires functional P2X2 and P2X3 receptors; genetic deletion or
291 pharmacological blockage of these receptors essentially eliminates taste-mediated neural activity
292 (Finger, Danilova et al. 2005, Vandenbeuch, Anderson et al. 2013). Similarly, taste driven
293 acceptance of sweet and umami, and taste driven avoidance of bitter is lost in mice lacking
294 functional P2X2 and P2X3 receptors. In contrast, behavioral avoidance of intraoral acids remains
295 intact despite the loss of taste-related neural activity to these stimuli. Similarly, avoidance of
296 acids persists even after genetic deletion of Otop1, the ion channel receptor for H^+ underlying
297 sour detection (Teng, Wilson et al. 2019, Zhang, Jin et al. 2019). These findings strongly suggest
298 that not just taste, but another sensory modality drives the avoidance of acids in this context.
299 The oropharynx is innervated by chemically-sensitive free nerve endings arising from the
300 trigeminal, glossopharyngeal and vagus nerves. These nerves include populations of polymodal
301 nociceptors that express acid-sensitive ion channels including TrpA1 and TrpV1 (Tominaga,
302 Caterina et al. 1998, Wang, Chang et al. 2011), and the TrpV1-expressing fibers are necessary
303 for non-taste mediated avoidance of citric acid (Zhang, Jin et al. 2019). Yet these studies do not
304 demonstrate that TrpV1 itself is the necessary receptor. It is likely that other acid-sensitive
305 channels and receptors exist in these TrpV1-expressing fibers. Further, TrpV1 fully activates at a

306 pH around 5 at body temperature (Tominaga, Caterina et al. 1998), whereas avoidance of citric
307 acid begins at a pH near 3. It is likely that the tissue overlying the TrpV1 sensory terminals
308 provides some buffering of acids applied to the surface of the epithelium, but weak acids, such as
309 citric acid, effectively acidify the epithelium deep into the tissue (Richter, Caicedo et al. 2003),
310 well beyond the region in which the nerve terminals lie. Taken together, these results suggest
311 that TrpV1 itself may not be entirely responsible for responses to citric acid. Accordingly, we
312 tested whether either TrpV1 or TrpA1 channels contribute to either the behavioral avoidance
313 response, or the activation of brainstem neurons by citric acid. We found that neither the
314 behavior nor the pattern and degree of neural activation was altered by genetic deletion of these
315 channels.

316 If neither TrpV1 nor TrpA1 channels underlie avoidance of acidic substances in the
317 absence of taste, what other mechanisms might be responsible? Likely candidates are one or
318 more members of the ASIC (acid sensing ion channel) family, which are gated by decreases in
319 extracellular pH. While many members of this family open at pH values near 7, others activate
320 at lower pH values (Deval, Gasull et al. 2010). In particular, ASIC3 is expressed widely in
321 polymodal nociceptors including those expressing TrpV1 (Trigeminal ganglion clusters 7 & 10
322 in (Nguyen, Wu et al. 2017)) and plays a role in responses to pH5.0 (Price, McIlwrath et al.
323 2001). Further, amiloride, a non-specific blocker of ASIC channels, decreases the irritation of
324 citric acid (albeit at a much higher concentration) measured psychophysically (Dessirier,
325 O'Mahony et al. 2000), supporting the role of ASICs in this response. Conversely, inclusion of
326 amiloride, a blocker of ASICs, in orally-applied solutions in rats neither decreased the trigeminal
327 response to citric acid nor attenuated acid-induced c-Fos in brainstem trigeminal nuclei (Sudo,

328 Sudo et al. 2003). Whether ASICs or some other acid-responsive mechanisms play a role in non-
329 gustatory behavioral avoidance of weak acids then is unresolved.
330
331
332



333

334 Fig. 1: Brief access tests showing similar avoidance of acetic acid and citric acid for
335 TRPA1/V1$^{Dbl-/-}$ mice and wildtype controls of the same background. ANOVA shows no
336 significant difference between the groups.

337

338

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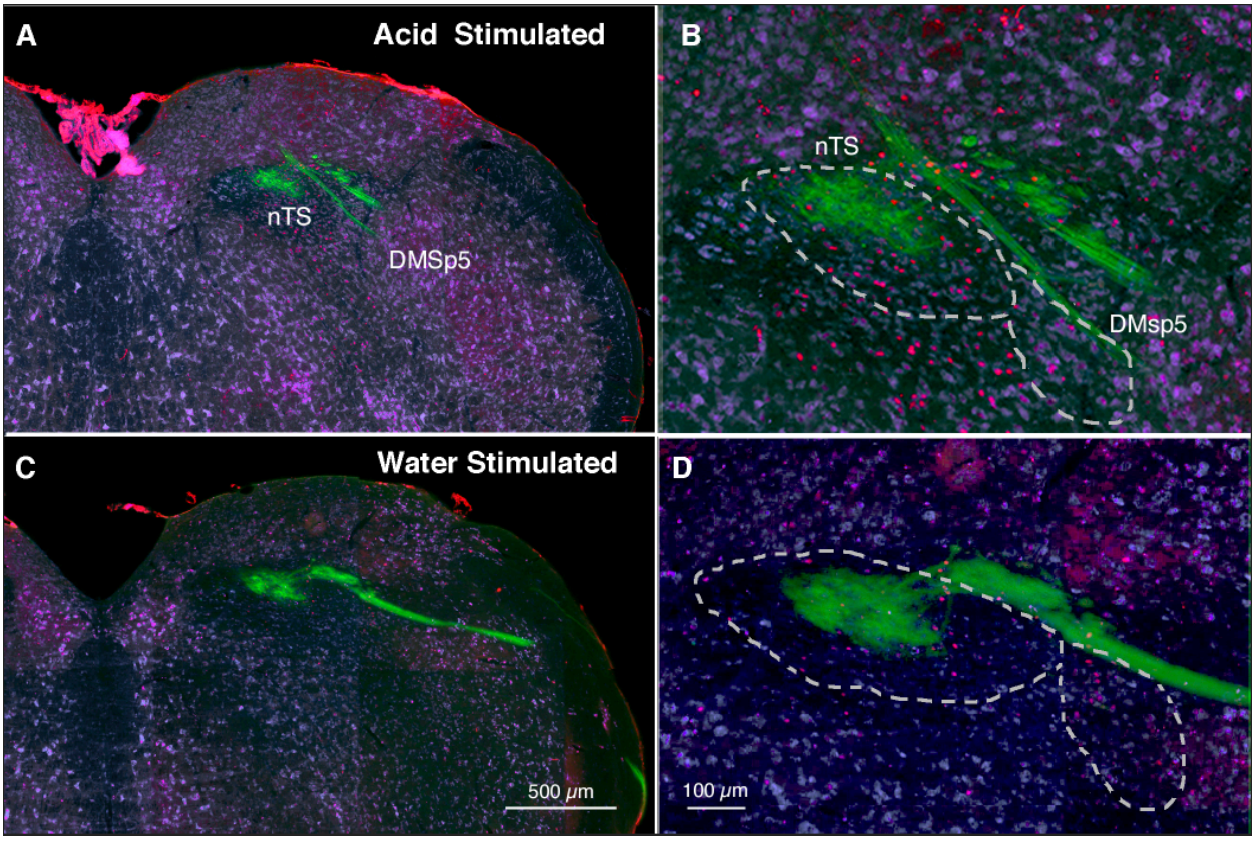

341

342 Fig. 2. C-Fos staining (red dots) in nTS and DMsp5 of the TRPA1/V1$^{Dbl-/-}$ mouse at

343 approximately the R3 level comparing a mouse stimulated by 30 mM citric acid (A, B) to one

344 stimulated only by water (C, D). The number and location of c-fos positive cells is similar in

345 WT and TRPA1/V1$^{Dbl-/-}$ strains. Note the higher number of c-fos positive cell nuclei (red) in the

346 acid stimulated compared to the water-stimulated animal. Green staining shows the distribution

347 of P2X3-immunoreactive fibers which terminate largely in the dorsocentral part of the nTS. Most

348 acid-stimulated cells lie in the ventral tier of the nucleus and in the DMSp5 region ventrolateral

349 to the nTS. Blue counterstain from FluoroNissl.

350

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

352 Fig. 3.Heat maps of c-Fos expression in nTS and DMSp5 regions comparing wildtype (left) and
353 KO (TRPA1/V1$^{Dbl-/-}$) mice, stimulated by intraoral injection of water (top row and by citric acid
354 (middle row). The numbers indicate the aaverage number of c-fos-positive neurons in that
355 compartment. See Suppl. Table 1 for means and standard deviations for each location for each
356 genotype. Bottom row shows acid-specific activation, i.e. citric acid counts minus the counts
357 produced by water alone. No significant differences occur either in the pattern or absolute level
358 of expression across subregions (see text for details).
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363 Supplemenatry Table 1: Means and standard deviations of counts of c-fos positive cells in

364 DMSp5 and each subnucleus of the NTS for water and citric acid stimulation by intraoral

365 cannula for WT and TrpA1/V1$^{-/-}$ mice.

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WT H2O		DM	DI	DL	VM	VI	VL	DMSp5
r1		1.5	2.3	2.3	2.1	2.0	1.1	2.9
	stdev	1.1	1.8	1.0	0.8	1.1	1.1	2.7
r2		3.3	2.9	2.9	2.1	1.0	1.9	3.0
	stdev	1.4	1.5	2.0	1.1	0.5	1.0	1.8
r3		5.6	12.6	9.8	4.1	13.4	9.6	8.5
	stdev	4.3	6.3	5.9	1.9	7.6	6.0	9.5
r4		4.3	16.6	10.9	4.5	14.8	12.9	10.6
	stdev	3.0	6.7	7.0	2.1	4.4	6.4	6.9
i1		15.0	15.8	15.1	7.3	9.5	12.1	12.3
	stdev	6.2	8.7	6.3	5.3	4.5	6.8	8.2
i2		11.2	14.2	7.8	5.5	8.2	12.2	18.2
	stdev	10.6	8.0	4.7	3.7	5.5	7.5	14.4

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WT Cit Acid		DM	DI	DL	VM	VI	VL	DMSp5
r1		3.4	6.9	4.6	2.9	2.3	2.6	4.9
	stdev	1.8	1.8	3.3	2.6	1.3	1.6	3.4
r2		4.5	9.8	3.5	5.0	5.8	4.0	2.8
	stdev	1.6	3.4	2.0	2.7	4.3	2.1	2.9
r3		7.0	21.4	8.1	5.9	18.9	12.3	11.1
	stdev	3.6	8.3	3.4	2.7	2.6	6.8	9.4
r4		9.6	27.9	13.3	8.3	24.6	20.3	22.6
	stdev	3.4	9.4	3.3	3.9	6.0	7.4	14.3
i1		22.9	31.0	13.6	10.8	22.4	26.4	20.3
	stdev	10.7	9.6	7.3	4.8	4.9	7.8	8.5
i2		13.3	22.9	11.6	9.5	16.6	28.1	26.4
	stdev	9.0	4.8	5.4	5.4	7.5	9.8	18.8

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TrpA1/V1$^{-/-}$ H2O	DM	DI	DL	VM	VI	VL	DMSp5
r1	2.3	5.0	1.9	1.4	0.6	0.9	2.1
stdev	3.4	4.6	2.0	1.5	0.7	1.7	2.9
r2	2.4	8.0	5.4	1.6	2.6	4.0	5.1
stdev	1.6	5.0	5.8	1.5	4.3	5.8	7.8
r3	4.1	10.9	3.8	3.0	5.3	2.9	2.4
stdev	2.3	4.8	1.9	2.7	5.2	2.7	2.1
r4	1.6	14.3	6.5	1.5	11.9	4.5	5.3
stdev	1.8	8.9	4.1	2.2	6.8	3.7	8.2
i1	11.6	9.9	9.9	6.1	6.8	9.2	9.1
stdev	6.1	5.9	8.0	3.4	5.2	7.2	11.6
i2	9.5	8.6	4.6	5.8	6.3	6.7	9.9
stdev	9.1	5.4	3.3	5.3	3.4	5.3	12.9

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TrpA1/V1$^{-/-}$ Cit Acid	DM	DI	DL	VM	VI	VL	DMSp5
r1	3.7	5.0	2.3	2.4	2.8	1.7	3.4
stdev	2.8	2.0	1.1	0.8	2.4	1.7	2.2
r2	5.9	13.4	5.3	6.4	6.6	4.4	5.2
stdev	3.2	5.8	3.1	5.6	4.2	2.9	5.2
r3	6.2	17.2	8.3	6.1	19.0	11.0	6.3
stdev	3.9	8.0	5.3	4.4	9.2	8.7	4.7
r4	9.5	23.1	13.7	7.0	24.8	13.4	10.9
stdev	7.6	8.4	7.7	2.7	7.8	6.2	5.5
i1	10.6	22.9	18.0	5.8	19.2	27.9	17.3
stdev	5.3	18.3	9.2	3.2	9.8	12.0	10.9
i2	10.0	12.3	7.5	8.3	15.1	22.6	17.3
stdev	5.1	17.4	8.6	3.9	6.9	8.9	13.1

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