

Anatomical basis for the trigeminal autonomic cephalalgia-like response produced by 2 formalin injection into the facial cheek in rats

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1 **Anatomical basis for the trigeminal autonomic cephalalgia-like response produced by**
2 **formalin injection into the facial cheek in rats**

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16

17 **Abstract**

18 Trigeminal autonomic cephalalgias (TACs) comprise cluster headaches and are characterized
19 by unilateral neuralgiform headache attacks associated with autonomic responses and somatic

20 responses. An animal model for evaluating the anatomical basis of the TAC-like response is
21 not currently available. Twenty-five rats weighing 550-650 g were anaesthetized with
22 urethane. The TAC-like response was produced either by subcutaneous injection of formalin
23 into the unilateral facial cheek or by electrical stimulation of the unilateral intact trigeminal
24 nerve. The induced TAC-like response, which included ipsilateral common carotid arterial
25 flow (iCCAF) and other autonomic responses, was studied in intact nerves or after cutting
26 either the ipsilateral trigeminal nerve or the ipsilateral facial nerve. The formalin injections
27 produced concentration-dependent iCCAF increases accompanied by ipsilateral autonomic
28 responses of rhinitis-like nasal congestion, nasal mucus, meiosis, lacrimation, red eye, and
29 eyelid oedema. The formalin (5% or 10%, 0.5 cc)-induced responses were nearly abolished
30 by sectioning of either the facial or trigeminal nerve. The electrical stimulation (15 V, 60 Hz,
31 and 0.4 ms) of the intact trigeminal nerve or its ophthalmo-maxillary branch also produced
32 stimulation strength-dependent iCCAF increases as well as autonomic responses; however,
33 the electrical stimulation-induced iCCAF increases and other autonomic responses could still
34 be induced by electrical stimulation of the central but not the peripheral end of the
35 ophthalmo-maxillary branch (n = 8) or the trigeminal nerve (n = 2). Thus, an animal model
36 for inducing the TAC-like response by subcutaneous formalin injection into the rat facial
37 cheek was established. The TAC-like response could be sequentially mediated via the
38 afferent trigeminal nerve, trigeminal nucleus, dorsal facial nucleus, and efferent facial nerve.

39

40 **Keywords:** Trigeminal autonomic cephalalgia-like responses, Formalin pain test,
41 Ophthalmic-maxillary branch of the trigeminal nerve, Facial nerve, Common carotid arterial
42 flow, Autonomic responses

43

44 **Background**

45 Primary headaches such as cluster headaches are thought to be caused by acute inflammation
46 of the trigeminal nerve [1-3]. Cluster headaches, which are regarded as vascular headaches [4,
47 5], have been attributed to vascular inflammation that dilates the intracranial internal carotid
48 artery [1]. Trigeminal autonomic cephalalgias (TACs) comprise cluster headaches and consist
49 of short-lasting unilateral neuralgiform headache attacks that are associated with autonomic
50 responses, including cerebral blood flow increases, conjunctival injection and tearing
51 syndrome [6-8]. There are few models of the trigeminal autonomic cephalalgia; however,
52 there is a study reporting the influence of capsaicin application to facial mucosa on dural
53 blood flow [9], and another study examined the effect of trigeminal nerve stimulation on
54 carotid artery blood flow in cat [10] and in monkey [11]. Whether cerebral blood flow
55 increases and autonomic responses can be evoked by trigeminally related facial pain or
56 trigeminal nerve stimulation remains unknown.

57 The trigeminal sensory nucleus, which receives afferents from the trigeminal nerve,

58 gives rise to projections to the nucleus reticularis parvocellularis [12-15] of the dorsal facial
59 area (DFA) [16]. Either glutamate (Glu) injection or electrical stimulation of the DFA
60 produces a marked increase in ipsilateral common carotid arterial flow (iCCAF), which
61 ipsilaterally supplies both intracerebral and extracranial blood flow [16]. Electrical or Glu
62 stimulation of the trigeminal sensory nucleus also elicits increases in iCCAF [17]. The
63 findings described above suggest that DFA-induced increases in iCCAF can be related to the
64 trigeminal sensory nucleus. In other words, increases in iCCAF elicited by electrical or Glu
65 stimulation of the trigeminal sensory nucleus can be mediated through the DFA. Whether the
66 iCCAF increases and the associated autonomic responses are related to the trigeminal and
67 facial nerves has not been explored.

68 Our preliminary study in Lanyu pigs demonstrated that a TAC-like response,
69 characterized by increases in iCCAF with autonomic responses, was induced by injecting
70 formalin into the facial cheeks innervated by the trigeminal nerve (afferent site), suggesting
71 involvement of the trigeminal nerve [18]. Whether the afferent site of the TAC-like response
72 is mediated by the trigeminal nerve was further evaluated in the rats in this study. In addition,
73 whether the efferent site of the response was mediated by the facial nerve (efferent site) was
74 determined. The present investigation reports a novel rat model for the TAC-like response,
75 demonstrating the involvement of the trigeminal and facial nerves in the TAC-like response
76 in a rat model.

77

78 **Results**

79

80 *Cardiovascular responses induced by formalin injection in the facial cheek*

81 Adopting the oral-facial formalin pain test described by [19], we subcutaneously injected
82 formalin into the facial cheek innervated by the afferent trigeminal nerve.

83 A marked increase in ipsilateral common carotid arterial flow (iCCAF) was observed.
84 The iCCAF max increase was obtained at 3-5 min after formalin injection, while the iCCAF
85 increase could last for 20 min after formalin injection, accompanied by slight changes in the
86 SAP, MSAP, and HR induced by formalin (5%, 0.5 cc) injection into the facial cheek (Fig. 1a
87 and b). Since the marked iCCAF increase was accompanied by slight changes in the systemic
88 arterial pressure (SAP), meanSAP (MSAP), and heart rate (HR), this increase could not have
89 been caused by the increases in SAP, MSAP, or HR [16].

90 Concentration-dependent iCCAF increases were induced by 0.5 cc of formalin at
91 concentrations of 1%, 2.5%, 5%, and 10% (Fig. 1c, n=6).

92 The duration of the iCCAF increase was correlated with the concentration of formalin
93 (1%, 2.5%, 5%, and 10%, 0.5 cc) injected into the facial cheek. These injections elicited
94 iCCAF increases that lasted for durations of 19 ± 11 min, 22 ± 9 min, 30 ± 15 min, and $74 \pm$
95 14 min (n=6), respectively. The duration for maintaining the iCCAF increase was positively

96 correlated with the formalin concentration (Fig. 1d, $r = 0.87$, $r^2 = 0.75$, $P < 0.05$).

97

98 ***iCCAF increases were associated with autonomic responses***

99 As shown in Fig. 2, injections with 0.5 cc of formalin at concentrations of 1% (n=6), 2.5%
100 (n=6), 5% (n=8), and 10% (n=11) into the same facial cheek area produced a
101 concentration-dependent % increase in autonomic responses in all rats. Among the
102 autonomic responses, the concentration-dependent % increases in nasal mucus secretion
103 were 17%, 17%, 9%, and 27%, respectively. The % increases in rhinitis-like nasal
104 congestion were 17%, 33%, 36%, and 73%, and those in lacrimation (tearing) were 33%,
105 50%, 45%, and 91%, respectively. The rhinitis-like nasal congestion was evaluated by
106 taking pictures and listening to breathing sounds (with a stuffy nose). The rats could breathe
107 when their noses were congested. Rhinitis-like nasal congestion induced by formalin (10%,
108 0.5 cc; 5%, 0.5 cc; 2.5%, 0.5 cc; 1%, 0.5 cc) into the facial cheek returned to normal in 3
109 hours, 1 - 2 hours, 30 min, and 10-15 min, respectively. Whether the marked iCCAF
110 increase was affected by the accompanying increases in autonomic responses (Fig. 2) has
111 not been addressed.

112

113 ***The stimulation parameters for the ophthalmo-maxillary nerve to induce the maximum***

114 ***increase in iCCAF***

115 The iCCAF increases were dependent on the stimulation parameters (voltage, frequency, and
116 duration) of the nerve. The electrical stimulation parameters that induced the maximum
117 increase in iCCAF were 15 V, 60 Hz, and 0.4 ms (Fig. 3). Nevertheless, we used
118 approximately 12-15 V, 40-60 Hz, and 0.2-0.4 ms to induce the optimal responses that
119 appeared to be repeatable in the present investigation, as shown in Fig. 4. The intervals
120 between stimulation were 3-5 min, which allowed repeatable results.

121

122 ***Effects of sections of the ophthalmic-maxillary or facial nerve on formalin-induced iCCAF***
123 ***increases***

124 Injections of formalin (5% and 10%, 0.5 cc) into the facial cheek produced a marked increase
125 in iCCAF [(1.93±0.54 cc to 4.57±1.13 cc, n=7) and (1.8±0.82 cc to 4.1±2.25 cc, n=6)], which
126 was nearly abolished by the cutting of the ophthalmic-maxillary branch of the trigeminal
127 nerve (1.5±0.63 cc to 1.8±0.98 cc, n=6) (Fig. 5a) or the facial nerve (2.6±1.6 cc to 3±2.03 cc,
128 n=6) (Fig. 5b).

129 The interruption of the facial nerve also markedly reduced the autonomic responses of
130 ipsilateral rhinitis-like nasal congestion (73% to 0%), nasal mucus, meiosis (27% to 0%),
131 lacrimation (tearing) (91% to 33%), red eye (100% to 20%), and eyelid oedema (100% to
132 20%).

133 Fig. 5b shows that basal flow in iCCAF increased after cutting the facial nerve. To

134 compare and distinguish between the two, it is necessary to double the dosage of formalin 2
135 times, which makes it easier to identify differences.

136

137 ***Comparison among iCCAF increases induced by electrical stimulation of the intact***
138 ***trigeminal nerve or of its central or peripheral end***

139 Electrical stimulation (15 V, 60 Hz, and 0.4 ms for 15 s) of the intact or central end of the
140 ophthalmic-maxillary branch of the trigeminal sensory nerve produced a significantly similar
141 increase in iCCAF, while that of the peripheral end did not, indicating that retrograde
142 excitation of the peripheral end cannot induce an increase in iCCAF (Fig. 4). The iCCAF
143 increase was maximal 0.25 min after the stimulation and lasted for approximately 3 min.

144

145 **Discussion**

146 This paper demonstrated the following: 1. A marked increase in iCCAF, accompanied by
147 slight changes in the SAP, MSAP, and HR, was induced by formalin (5%, 0.5 cc) injection
148 into the facial cheek (Fig. 1a and b). 2. The cheek injection of 0.5 cc of formalin at
149 concentrations of 1%, 2.5%, 5%, and 10% in 0.5 cc of saline induced a
150 concentration-dependent increase in iCCAF (Fig. 1c) and an increase in the duration of
151 iCCAF (Fig. 1d). 3. Similar injections produced concentration-dependent % increases in
152 iCCAF as well as autonomic responses (Fig. 2). 4. The marked increase in iCCAF and

153 autonomic responses induced by the injection of formalin (5%, 0.5 cc) was markedly reduced
154 by cutting of the trigeminal nerve or its ophthalmic-maxillary branch (Fig. 5a) or of the facial
155 nerve (Fig. 5b). 5. The electrical stimulation parameters that induced the maximum iCCAF
156 increase were 15 V, 60 Hz, and 0.4 ms (Fig. 3). 6. The stimulation of the intact or central end
157 of the trigeminal sensory nerve produced a significantly similar increase in iCCAF, while that
158 of the peripheral end did not (Fig. 4). We propose that the induced iCCAF increase and the
159 associated autonomic responses may be mediated by the afferent trigeminal nerve and may be
160 ultimately mediated by the efferent facial nerve (Fig. 6). Thus, we successfully established a
161 rat TAC-like response model.

162 This rat TAC-like response model is novel, reliable, and convenient. First, formalin
163 was injected into the facial cheek, which is the centre of the trigeminal-innervating area;
164 second, the flow meter probe was directly hooked onto the common carotid artery of SD rats
165 weighing 550-650 grams, which are commonly used small lab animals, so that we directly
166 measured iCCAF.

167 Our findings are the first to demonstrate that a marked iCCAF increase, accompanied
168 by slight changes in the SAP, MSAP, and HR (Fig. 1a and b) but with increases in other
169 autonomic responses (Fig. 2), was induced by subcutaneous formalin (5%, 0.5 cc) injection
170 into the facial cheek. Previously, subcutaneous oral-facial injections of formalin (0.5 cc every
171 5 min) at concentrations of 1%, 2.5%, 5% and 10% have been performed to induce the

172 so-called trigeminal autonomic cephalalgia (TAC)-like response [19, 21]. However, these
173 investigations did not address the neuro-anatomical mechanisms. We clearly demonstrated
174 that the TAC-like response was mediated through the trigeminal afferent and facial efferent
175 nerves.

176 To substantiate and establish a reliable, standard and precise protocol for future
177 experiments, we further demonstrated that the subcutaneous facial-cheek injection of 0.5 cc
178 of formalin at concentrations of 1%, 2.5%, 5%, and 10% induced concentration-dependent
179 increases in iCCAF (Fig. 1c) and increases in the duration of iCCAF (Fig. 1d). In addition,
180 this formalin injection produced % increases in autonomic responses (Fig. 2). These findings
181 confirm that repeated formalin stimulations are possible, although formalin is detrimental to
182 tissues at higher response concentrations or induces neuroinflammation in the hemilateral
183 trigeminal inflammatory pain model [22]. More importantly, formalin injections not only
184 mimic TAC-like but also reveal iCCAF increases and other autonomic responses.

185

186 *Afferent sensory pathway*

187 For future electrical stimulations, although the electrical stimulation parameters that induced
188 the maximum iCCAF increase were 15 V, 60 Hz, and 0.4 ms (Fig. 3), we suggest using lower
189 values such as 12-15 V, 40-60 Hz, and 0.2-0.4 ms, as shown in Fig. 4. In particular, for
190 formalin injections, the interval between each injection was approximately 50 min so that

191 repeatable results could be obtained. For electrical stimulations, the interval between each
192 stimulation was approximately 3-5 min.

193 The iCCAF increase induced by 5% formalin into the facial cheek was markedly
194 reduced by interruption of the sensory ophthalmic-maxillary branch of the trigeminal nerve
195 (Fig. 5a) but was induced by electrical stimulation of the intact trigeminal nerve or the central
196 end of the cut ophthalmic-maxillary nerve (or the trigeminal nerve) and was not induced by
197 stimulation of the peripheral end of the cut ophthalmic-maxillary nerve (Fig. 4).

198

199 ***Mimicking trigeminal autonomic cephalalgias by formalin pain test in the facial cheek***

200 Injections of formalin into the facial cheek produced concentration-dependent iCCAF
201 increases and slight decreases or no change in SAP and HR. The duration for maintaining the
202 CCAF increase (Fig. 1c) and % increases of accompanying autonomic and somatic responses
203 is positively correlated with the formalin concentration (Figs. 1 and 2). In general, trigeminal
204 afferent inputs, especially nociceptive input, induce the increase in SAP in rat [21, 23] and
205 cause no change in SAP [24] and decrease the SAP in cat [10]. Why does this discrepancy
206 occur? (1) The discrepancy can be explained by the following description: In the literature,
207 electrically stimulating the anterior ethmoidal nerve (AEN), a small nerve of the trigeminal
208 ophthalmic division innervating the mucosa and nose, elicited alterations in cardiorespiratory
209 behaviour, including increases in arterial blood pressure, bradycardia, and apnoea [25, 26].

210 On the other hand, stimulation of the trigeminal input or TAC-like response (please refer to
211 lines 1 – 6 of the first paragraph, INTRODUCTION) induced primarily an increase in the
212 cerebral blood flow, consistent with our findings. Our present experiment showed that
213 electrical stimulation of the ophthalmic-maxillary nerve (though containing AEN) and of the
214 trigeminal nerve induced trigeminal autonomic headache-like responses, which is mainly the
215 result of exciting the VII parasympathetic nerve and suppressing the sympathetic nerve
216 (rostral ventrolateral medulla, RVLM, or dorsal medulla, DM). However, either glutamate
217 (Glu) injection or electric stimulation of the DFA also possibly caused only an increase in
218 iCCAF (vasodilatation) and did not interact with other systemic cardiovascular parameters
219 (SAP, MSAP, HR, dPdt (cardiac constriction), superior mesenteric arterial flow (SMAF),
220 renal arterial flow (RAF) and femoral arterial flow (FAF) [16, 17].

221 These responses and our previous results in Lenya pigs, which mimic maximum pain
222 in cluster headache attacks via noxious stimulations by injecting 20% formalin (0.5 cc) into
223 the facial cheek, innervated by the afferent trigeminal nerve, produce an iCCAF increase
224 (16 ± 4 to 50 ± 24 cc, $n=6$) that lasts approximately 35 min, accompanied by an autonomic
225 response including ipsilateral nasal stuffiness, nasal mucus, marked salivation, and
226 sometimes a licking response. These responses look similar to the cluster headache induced
227 in humans [18]. These responses are often short-lasting attacks of unilateral pain associated
228 with prominent autonomic symptoms, such as conjunctival injection, lacrimation, nasal

229 congestion rhinorrhoea, ptosis or eyelid oedema, and are characterized by activation of both
230 sensory and parasympathetic cranial nerve fibres [6].

231 Trigeminal autonomic cephalalgias (TACs) comprise cluster headaches, which are
232 shorting-lasting unilateral neuralgiform headache attacks with conjuction injection and
233 tearing (SUNCT) syndrome [6]. However, previous studies investigating the change in
234 cerebral blood flow (CBF) in cluster headache are few in number. Most have been done
235 with single-photon emission computed tomography (SPECT), and the results of this
236 semiquantitative method have been heterogeneous, with some reporting an increase [7, 8],
237 some a decrease [27], and some no differences in cortical blood flow [28, 29], probably
238 because of methodologic differences. Taken together, the data suggest that neurovascular
239 activation in the trigeminal system is a function of its afferent role in any form of pain and
240 is highly potent and somatotopically organized.

241

242 ***Inflammation resulted from CCA vasodilatation through a neurovascular mechanism***

243 In this experiment, after formalin (10%, 0.5 cc) was injected into the cheek area to cause
244 maximal CCAF dilatation (CCAF increase), we took a section of CCA for
245 cyclooxygenase-2 (COX-2) immunohistochemistry (IHC) staining. We found that the outer
246 membrane of the CCA tube wall had obvious IHC staining COX-2 (n = 6), suggesting that
247 CCA showed inflammation (unpublished data) (supplement materials-additional file 2),

248 accompanied by autonomic responses such as oedema, red eyes, tearing, and congestive
249 rhinitis. CCA has an inflammatory response that is roughly the same as the onset time
250 associated with spontaneous reactions such as ocular oedema, red eyes, tearing, and
251 congestive rhinitis [30]. Therefore, we reasonably inferred that the effects of facial
252 nociceptive stimulation on autonomic function are a physiopathological reaction.

253 Studies have reported that neurogenic inflammation caused by peripheral events
254 involves the release of neuropeptides, such as substance P, neurokinin A, and CGRP. These
255 neuropeptides cause a series of events characterized by oedema formation, vasodilation, and
256 proinflammatory mediators (such as bradykinin, prostaglandins, and protons) [3]. The
257 activation of parasympathetic pain fibres is attributed to the results of intracranial carotid
258 artery expansion mediated by the neuroinflammatory mechanical action of the vessel wall [1],
259 which may play a possible role in the production of migraine or cluster headaches. Electrical
260 stimulation of the sphenopalatine ganglia (SPG) induces plasma protein extravasation (PPE)
261 in the dura mater, indicating that the parasympathetic nervous system can trigger neurogenic
262 inflammation in the dura mater through muscarinic cholinergic receptors. Sensory C fibres
263 cause pain and inflammation [31].

264

265 *Possible central nuclei of the reflex centre*

266 It is not known whether the vasodilatation or vasoconstriction of the CCAF in the DFA

267 evoked by some neurotransmitters is released afferently from possible neural pathways by
268 cluster headache attacks. Two possible routes could be suggested. The first route is activation
269 of a brain stem reflex, the afferent arc of which is the trigeminal nerve, projected to the
270 trigeminal sensory nucleus, which elicits significant vasodilatation of the CCA by stimulation
271 of electrical and Glu [17] and solitary nucleus (NTS) and then innervates to the DFA. The
272 efferent outflow containing vasoactive intestine peptides (VIP), nitric oxide synthases (NOS)
273 and acetylcholine (Ach) [12] from the DFA, which is the preganglionic parasympathetic
274 neurons of the sphenopalatine ganglia of the VIIth [32], is mediated by the VIIth [16, 33] and
275 IXth [16] nerves to the extracranial and intracranial carotid arteries. Our previous
276 experiments in support of the present study show that pretreatment with removal of the facial
277 nerve can significantly reduce the formalin-induced CCAF increase and other autonomic
278 responses (Fig. 5b). Other authors found that VIP release is abolished by trigeminal nerve
279 lesion [33], thus suggesting a reciprocal connection between the DFA and/or superior
280 salivatory nucleus and the trigeminal complex. In the second route, the posterior
281 hypothalamic areas (PHA), the periaqueductal grey (PAG) that expresses calcitonin
282 gene-related peptide (CGRP) and CGRP receptors [12], and the trigeminal sensory complex
283 that contains CGRP fibres and CGRP receptors [12, 13] innervate the nucleus reticularis
284 parvocellular (Pc) in the DFA, and the nucleus reticularis Pc in the DFA receives the P-like
285 and methionine-enkephalin-like afferents from the PAG [14]. The observed activation in

286 migraine and in several trigeminal-autonomic headaches is involved in the pain process;
287 therefore, the PHA could be a central triggering cause of acute or chronic cluster headache
288 [13, 34]. Microinjection of morphine into the PHA and PAG elicits powerful suppression of
289 nociceptive behaviours in the formalin test, an animal model of injury-produced pain.
290 Stimulation of the PHA in a patient with intractable cluster headache led to a complete relief
291 of attacks [35].

292

293 ***The DFA, PAG and/or trigeminal sensory complex induced an iCCAF increase that could***
294 ***play a role in the pathophysiology of the trigemino-vascular reflex in cluster***

295 ***headache/migraine***

296 The iCCAF increase induced by 5% formalin injection into the facial cheek was abolished
297 after cutting of the sensory ophthalmomaxillary nerve branch of the trigeminal nerve (Fig.
298 5a). In addition, it was significantly induced by electrical stimulation of the central end of
299 the ophthalmomaxillary nerve and the trigeminal nerve. However, the iCCAF increase was
300 slightly induced by electrical stimulation of the peripheral end of the ophthalmomaxillary
301 nerve (Fig. 4). Therefore, the central area could be responsible for the iCCAF increase.

302 Microinjection of sodium glutamate (Glu, 0.1M, 400 nl) into the DFA in SD rats cause the
303 left common carotid blood flow (LCCAF) increases (3 ± 1 cc to 5 ± 1 cc, n=9), accompanied by
304 tearing (unpublished data) (supplement materials-additional file 3). The DFA could give

305 rise to parasympathetic efferent fibres of the facial nerve innervating the CCAF; Glu
306 chemical stimulation of DFA induces the iCCAF increase without a change in SAP and HR
307 involving partially muscarinic and non-muscarinic mechanisms in our previous studies [16,
308 17]; non-muscarinic mechanisms may include vasoactive intestine peptides (VIP) [36] and
309 calcitonin gene-related peptide (CGRP) [37], which play important roles in the vasodilator
310 action for the extra- and intracranial vessels [16, 17]. The changes observed in CGRP and
311 VIP levels during the chronic paroxysmal hemicrania (CPH) [12, 38, 39] suggest that some
312 aspects of the pathophysiology resembling those of a cluster headache are characterized by
313 activation of both sensory and parasympathetic cranial nerve fibres. Activation of the
314 parasympathetic pain fibres is attributed to the results of dilation of the intracranial internal
315 carotid artery mediated by the neuroinflammatory mechanic effect of the vessel wall [1],
316 and these factors play a possible role in the generation of a migraine or cluster headache.

317 Indeed, (1) we were also unsure if headache was elicited by formalin injection and did
318 not particularly mention “the headache is elicited by the formalin injection”. (2) Certainly,
319 there is no evidence regarding this matter. (3) The present investigation reports a novel rat
320 model for the TAC-like response, demonstrating the involvement of the trigeminal and facial
321 nerves in the TAC-like response in a rat model. This description is similar to what the Referee
322 mentioned: “the influence of facial nociceptive stimulation on autonomic function as a
323 physiological reaction.”

324

325 *Efferent motor pathway*

326 Therefore, the formalin-induced iCCAF increase could relay to the central nuclei and
327 possibly the trigeminal nerve nucleus, which receives afferents from the trigeminal nerve [11,
328 17], and then to the DFA, which receives afferent projections from the trigeminal nerve
329 nucleus (Fig. 6). The anatomical location of the DFA [16] is consistent with that of the rostral
330 inferior salivary nucleus [40-42] and caudal superior salivary nucleus [43-46]. The DFA in
331 turn projects to the preganglionic parasympathetic neurons of the sphenopalatine ganglia of
332 the VII [16, 20, 32, 33, 47] and IX [16] cranial nerves innervating the extracranial and
333 intracranial carotid arteries.

334 The present study demonstrated that interruption of the facial nerve could
335 significantly reduce the formalin-induced iCCAF increase and other autonomic responses
336 (Fig. 5b). These findings further indicate that for formalin-induced responses in the facial
337 cheek, both the VII and IX cranial nerves may be the final pathway to the extracranial and
338 intracranial carotid arteries as well as to other autonomic responses.

339 Based on the discussion in the last paragraph, the schematic drawing of Fig. 6 shows
340 the anatomical basis of TAC-like responses induced by formalin injection into the facial
341 cheek in the rat.

342

343 **Conclusions**

344 In conclusion, we propose that the formalin-induced iCCAF increase and the associated
345 autonomic responses could be mediated by the pathway from the afferent trigeminal nerve to
346 the trigeminal sensory nucleus. In addition, this response may likely relay to the trigeminal
347 sensory nucleus and the DFA and may finally mediate the autonomic efferents of the VII and
348 IX cranial nerves (Fig. 6). The latter notion needs further investigation in the future. We
349 believe that this model represents an appropriate tool for the study of TAC, including cluster
350 headaches and migraines, with the use of a facial cheek formalin pain test. Thus, these
351 investigations are worth further study in the future.

352

353 **Methods**

354

355 *Animal ethics*

356 The preparation of the animal, including the use of anaesthesia throughout the entire course
357 of the experiment, was performed according to the affidavit of approval of animal use
358 protocol listed below. This affidavit was reviewed and approved (Approval number:
359 vghks-2011-A003) by the Institutional Animal Care and Use Committee (IACUC) of
360 Kaohsiung Veterans General Hospital and conformed with the guidelines for the care and
361 use of laboratory animals issued by the Chinese society for laboratory animal science,

362 Taiwan, R.O.C. [48] and clinical laboratory animal medicine [49], which conform with
363 international standards.

364 In rats, urethane produces a suitable level of surgical anaesthesia. In fact, urethane is
365 recommended for acute experiments studying reflex responses because it only slightly
366 affects the sensitivity of neurons in both the central and peripheral nervous systems [50, 51].
367 Proper anaesthesia during surgery was maintained with urethane (1100-1200 mg/kg, i.p.).
368 We assessed the anaesthetic depth by evaluating the loss of four reflexes: the pinnae reflex,
369 the pedal withdrawal reflex in the forelimbs and hind limbs, the tail pinch reflex, and the
370 eyelid reflex; we also assessed the loss of muscle tone reflected in the loss of purposeful
371 movements and the twitching of whiskers. However, during the experiment but after the
372 surgery, lighter anaesthesia was required so that stimulation could induce reflex reactions,
373 namely, the autonomic response of the TAC-like response. These responses are just a reflex
374 response that requires a brainstem centre; these responses are not purposeful movements
375 that largely require supra-brainstem levels.

376 The physiological indices of stable blood pressure, HR, and respiration were elicited
377 and carefully monitored to ensure adequate anaesthesia throughout the experiment (not just
378 during surgery).

379

380 *Monitoring of cardiovascular responses*

381 Twenty-five male SD rats, weighing 550-650 g and approximately 1-1.5 years old, were
382 cared for and fed by the animal caregivers from the Laboratory Animal Center of Kaohsiung
383 Veterans General Hospital, and then were anaesthetized with urethane (1200 mg/kg, i.p.)
384 supplemented with halothane inhalation or 0.1 cc of urethane during surgical procedures;
385 however, halothane was terminated after the surgery to maintain spontaneous respiration. The
386 femoral vein and abdominal aortic artery were cannulated for infusing fluid and for
387 monitoring SAP, respectively, and HR was monitored by a tachometer. As previously
388 described, the left common carotid artery (CCA) was isolated for monitoring blood flow. The
389 CCA was placed into an appropriately sized electromagnetic probe that constricted the CCA
390 diameter to 85-90%. The probe was then connected to an SP2204B flowmeter (Spectramed
391 Inc., Oxnard, CA 93030, USA). All physiological parameters were recorded on a Gould
392 2800S (Cleveland, OH, USA) polygraph [52-54].

393

394 *The injection of formalin into the facial cheek*

395 The reason why the authors used two kinds of stimulation methods is that peripheral nerve
396 electrical stimulation can be repeatedly stimulated to obtain repeatable responses, which
397 return to normal in a relatively shorter time, approximately 1 - 5 min. On the other hand,
398 chemical stimulation by formalin inevitably causes injury to the tissue so that repetitive
399 experiments are limited to 3-5 times. The response takes longer to return to normal,

400 approximately 30-180 min. In fact, in rats, formalin can also activate central neurons of the
401 amygdala in the central nervous system in a hemilateral trigeminal inflammatory pain model
402 [22].

403 Trigeminal autonomic cephalalgias (TACs) comprise cluster headaches and are
404 characterized by short-lasting unilateral neuralgiform headache attacks. This attack induces
405 an increase in CCAF accompanied by conjunctiva injection and tearing. Although the eyes of
406 an SD rat are red, we could still visually observe the changes in redness and tearing to
407 evaluate conjunctival vascular congestion and exudates sufficiently, as well as the presence
408 of a little blood exudation.

409 The TAC-like response was produced by subcutaneous injections of formalin (0.5 cc
410 every 5 min) at concentrations of 1%, 2.5%, 5% and 10% into the facial cheek in mice or rats
411 [19, 21, 55]. According to these papers, the order of injections usually started from the lower
412 to higher concentrations.

413

414 *Stimulations of the trigeminal nerve or ophthalmomaxillary nerve*

415 An incision of 1.5 to 2 cm was made over the skin between the eye and ear. Then, all
416 connective tissues and temporal muscles of the parietal bone were removed until the floor of
417 the anterior cranial fossa was exposed, showing two branches of the trigeminal ganglion: (1)
418 the ophthalmomaxillary nerve, which passes through the foramen orbitorotundum; and (2)

419 the mandibular nerve. The exposed trigeminal nerve was hooked with an IC clip line
420 carrying a platinum wire for electrical stimulation. The reference electrode was placed on
421 the temporal muscle. Schemas of the abovementioned experiment of electrical stimulation
422 of the ophthalmic-maxillary nerve are shown in Fig. 7. Monopolar electrical stimulation was
423 administered with rectangular pulses of 15 V, 60 Hz, and 0.5 ms for 15 s from a Grass S88
424 stimulator (Grass Instrument Co., Quincy, MA, USA).

425

426 ***Stimulation of the facial nerve***

427 An incision approximately 1 cm behind the left ear was made with a scalpel. The upper part
428 of the neck muscles was separated to expose the centre of the facial nerve near the stem of
429 the stylomastoid foramen. Then, IC clip line hook sets on the facial nerve were used to
430 conduct monopolar electrical stimulation. The reference electrode was placed in the neck
431 muscles. Electrical stimulation was then delivered as described above. Schemas of the
432 abovementioned experiment of electrical stimulation of the facial nerve are shown in Fig. 8.

433

434 ***Statistics***

435 Percent changes of the induced cardiovascular responses were calculated with the following
436 formula: **[(response value - control value)/(control value)] × 100%**. For comparison of
437 the responses before and after the injection of formalin, data were analysed with Student's *t*

438 test. Data are presented as the means \pm SEM. A *p* value (*) less than 0.05 was considered
439 statistically significant. The statistical formula for the incidence (%) of autonomic reactions
440 associated with the CCAF vasodilatation induced by formalin stimulation of the facial
441 cheek is the number of each autonomic reaction divided by the number of all CCAF
442 vasodilatations induced by formalin stimulations of the facial cheek.

443 The correlation between the duration of maintained increases in iCCAF and formalin
444 concentration (1%, 2.5%, 5%, and 10%, 0.5 cc) was analysed by simple linear regression for
445 calculating the correlation coefficient (*r*). R^2 indicates the square of the correlation coefficient
446 and the regression coefficient. Significant testing of r^2 was performed by analysis of variance
447 and Fisher's distribution.

448 Each of the formalin concentration test groups only counted the increased iCCAF
449 response with the occurrence of each autonomic response, so the incidence of each
450 accompanying autonomic response (%) was calculated in each group. The statistical formula
451 for the incidence (%) of autonomic responses associated with the CCAF vasodilatation
452 induced by formalin stimulation of the facial cheek is the number of each autonomic response
453 divided by the number of all CCAF vasodilatations induced by formalin stimulation of the
454 facial cheek. Measurement of the substances produced by the autonomic response has
455 experimental limitations. Because of the small amount of substances and difficulty of
456 obtaining measurement tools, such measurements are occasionally recorded by photography

457 and video recording methods. The author used an iPhone 6 plus camera and photography
458 functions to record substances released from the cheek by formalin injection, including CCA
459 vasodilatation, meiosis, tearing, runny nose, red eyes, and congestive rhinitis; to observe and
460 evaluate the magnified image and obtain experimental evidence; and to record data in the
461 experimental notebook.

462

463 **List of abbreviations**

464 *Ach*: Acetylcholine

465 *AEN*: Anterior ethmoidal nerve

466 *CCA*: Common carotid artery

467 *CBF*: Cerebral blood flow

468 *CGRP*: Calcitonin gene-related peptide

469 *COX-2*: Cyclooxygenase-2

470 *DFA*: Dorsal facial area

471 *DM*: Dorsal medulla

472 *dPdt*: Cardiac constriction

473 *ECV*: Extracranial vessel

474 *FAF*: Femoral arterial flow

475 *Glu*: Glutamate

- 476 **HR**: Heart rate
- 477 **Hz**: Hertz
- 478 **IC**: Integrated circuit
- 479 **iCCAF**: Ipsilateral common carotid arterial flow
- 480 **ICV**: Intracranial vessel
- 481 **IHC**: Immunohistochemistry
- 482 **min**: Minute
- 483 **ms**: Millisecond
- 484 **MSAP**: Mean systemic arterial pressure
- 485 **NOS**: Nitric oxide synthesis
- 486 **NTS**: Solitary nucleus
- 487 **PAG**: Periaqueductal grey
- 488 **PHA**: Posterior hypothalamic area
- 489 **PPE**: Plasma protein extravasation
- 490 **r**: Pearson product-moment correlation coefficient
- 491 **RAF**: Renal arterial flow
- 492 **RVLM**: Rostral ventrolateral medulla
- 493 **SAP**: Systemic arterial pressure
- 494 **SD**: Sprague-Dawley

495 *sec*: Second

496 *SEM*: Standard error of the mean

497 *SMAF*: Superior mesenteric arterial flow

498 *SPECT*: Single-photon emission computed tomography

499 *SPG*: Sphenopalatine ganglia

500 *SUNCT*: Shorting-lasting unilateral neuralgiform with conjuction and tearing headache

501 *TAC*: Trigeminal autonomic cephalalgias

502 *TSN*: Trigeminal sensory nucleus

503 *V*: Volt

504 *VIP*: Vasoactive intestine peptides

505

506 **Declarations**

507

508 *Ethical approval and consent to participate*

509 All applicable guidelines for the care and use of animals, including the use of anaesthesia

510 throughout the entire course of the experiment, were performed according to the affidavit of

511 approval of the animal use protocol listed below. This affidavit was reviewed and approved

512 (Approval number: vghks-2011-A003) by the Institutional Animal Care and Use Committee

513 (IACUC) of Kaohsiung Veterans General Hospital and conformed with the guidelines for

514 the care and use of laboratory animals issued by the Chinese society for laboratory animal
515 science, Taiwan, R.O.C. and clinical laboratory animal medicine, which conform with
516 international standards.

517

518 ***Consent for publication***

519 Not applicable.

520

521 ***Availability of data and materials***

522 The datasets used and/or analysed during the current study are available from the
523 corresponding author upon reasonable request.

524

525 ***Competing interests***

526 The authors declare that they have no competing interest.

527

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532

533 *Authors' contributions*

534 Conception and design: M.R.W.; completion of the experiments and collection of the data:
535 M.R.W.; data analysis and interpretation: M.R.W.; revision of the manuscript: M.R.W., C.J.T.
536 and J.S.K. All authors approved the final version of the manuscript and agreed to be
537 accountable for all aspects of the work, ensuring that questions related to the accuracy or
538 integrity of any part of the work are appropriately investigated and resolved. All persons
539 designated as authors qualify for authorship, and all those who qualify for authorship are
540 listed.

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564 **Additional information**

565

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721

722 **Figure legends**

723 **Fig. 1.** Cardiovascular changes induced by formalin injections into the facial cheek area of
724 anaesthetized rats. (a) Authors used the solid arrow marker (↑) to indicate when formalin
725 (5%, 0.5 cc) was injected. (b) Data obtained from seven anaesthetized rats. (c) iCCAF
726 increases induced by injections of formalin (1%, 2.5%, 5%, and 10%, 0.5 cc) into the facial
727 cheek of anaesthetized rats. They reached maximal increases at 5 min. (d) Correlation
728 between the increase in duration of iCCAF and the concentration of formalin (1%, 2.5%, 5%,
729 and 10%, 0.5 cc) injected into the facial cheek of anaesthetized rats. “The time (duration) for
730 maintaining iCCAF increases” is expressed as the time required for a 75% reduction of the
731 maximal iCCAF increase.

732 **Fig. 2.** Concentration-dependent % increases in autonomic (nasal mucus, rhinitis-like nasal
733 congestion and lacrimation) responses induced by injections of 0.5 cc of formalin at
734 concentrations of 1%, 2.5%, 5%, and 10% into the facial cheek of anaesthetized rats. Each of
735 the formalin concentration test groups only counted the increased iCCAF response with the
736 occurrence of each autonomic response, so the incidence of each accompanying autonomic
737 response (%) was calculated in each group. The rate (%) was equal to the total number of
738 times the increased iCCAF response was initiated, and the number of occurrences of each
739 autonomic response was divided by the total number of occurrences in the experimental
740 group. The measurement of the substances produced by the autonomic response has
741 experimental limitations. Because of the small amount of substance and difficulty in

742 obtaining measurement tools, this measurement is occasionally recorded by photography and
743 video recording methods.

744 **Fig. 3.** Induction of iCCAF increases with stimulation of the afferent (sensory)
745 ophthalmic-maxillary nerve. The stimulation was made on the left nerve, and iCCAF was
746 recorded. Note that the iCCAF increases in a manner dependent on stimulation parameters
747 (voltage, frequency, and duration). The modest electrical parameters of voltage, frequency
748 and pulse duration for data have been filled in the top, middle and bottom panels,
749 respectively.

750 **Fig. 4.** The iCCAF increase induced by electrical stimulation (15 V, 60 Hz, and 0.4 ms) of
751 the intact ophthalmic-maxillary nerve (◆), as well as its central and peripheral ends. The
752 iCCAF increase was first induced by stimulation of the intact ophthalmic-maxillary nerve,
753 and then the ophthalmic-maxillary nerve was cut for stimulation of its peripheral (▲) and
754 central (■) ends. A p value (*) less than 0.05 was considered statistically significant for the
755 comparison of the response of the ophthalmic-maxillary nerve-intact with the nerve-central,
756 and other p values (θ) less than 0.05 were considered statistically significant for comparison
757 of the response of the ophthalmic-maxillary nerve-intact and the nerve-peri. Another p value
758 (§) less than 0.05 was considered statistically significant for the comparison of the response
759 of the ophthalmic-maxillary nerve-central and the nerve-peri.

760 **Fig. 5.** Effects of sections of the ophthalmic-maxillary nerve (a) or facial nerve (b) on the

761 iCCAF increase induced by 0.5 cc of 5% (a) or 10% (b) formalin injection into the facial
762 cheek area. The formalin injection was made into the left facial cheek, and the
763 ophthalmic-maxillary and facial nerves were cut on the same side. (a) shows the comparison
764 between sections of the ophthalmic-maxillary nerve responses and the injection of formalin,
765 and (b) shows the comparison between cutting facial nerve responses and the injection of
766 formalin. Data were analysed with Student's *t* test. Data are presented as the means \pm SEM.
767 A *p* value (*) less than 0.05 was considered statistically significant.

768 **Fig. 6.** Anatomical basis of TAC-like responses induced by formalin injection into the facial
769 cheek rats. Formalin injection into the facial cheek induces nociceptive or inflammatory
770 stimulation of the sensory endings of the ophthalmic-maxillary branch of the trigeminal nerve.
771 The trigeminal nerve then excites the neurons of the trigeminal sensory nucleus (TSN) in the
772 medulla, which project fibres to the dorsal facial area (DFA). The DFA is a parasympathetic
773 nucleus that gives rise to preganglionic parasympathetic fibres projecting to the
774 sphenopalatine ganglion of the facial nerve, and the postganglionic fibres innervate
775 intracranial and extracranial vessels (ICVs and ECVs) or branches of the common carotid
776 artery (CCA). Ipsilateral excitations of the DFA and its related facial nerve are responsible
777 for iCCAF [16, 20]. Therefore, formalin-induced nociceptive or inflammatory stimulation of
778 the sensory endings of the ophthalmic-maxillary branch of the trigeminal nerve elicits an
779 increase in iCCAF. Since interruption of the facial nerve also markedly reduces other

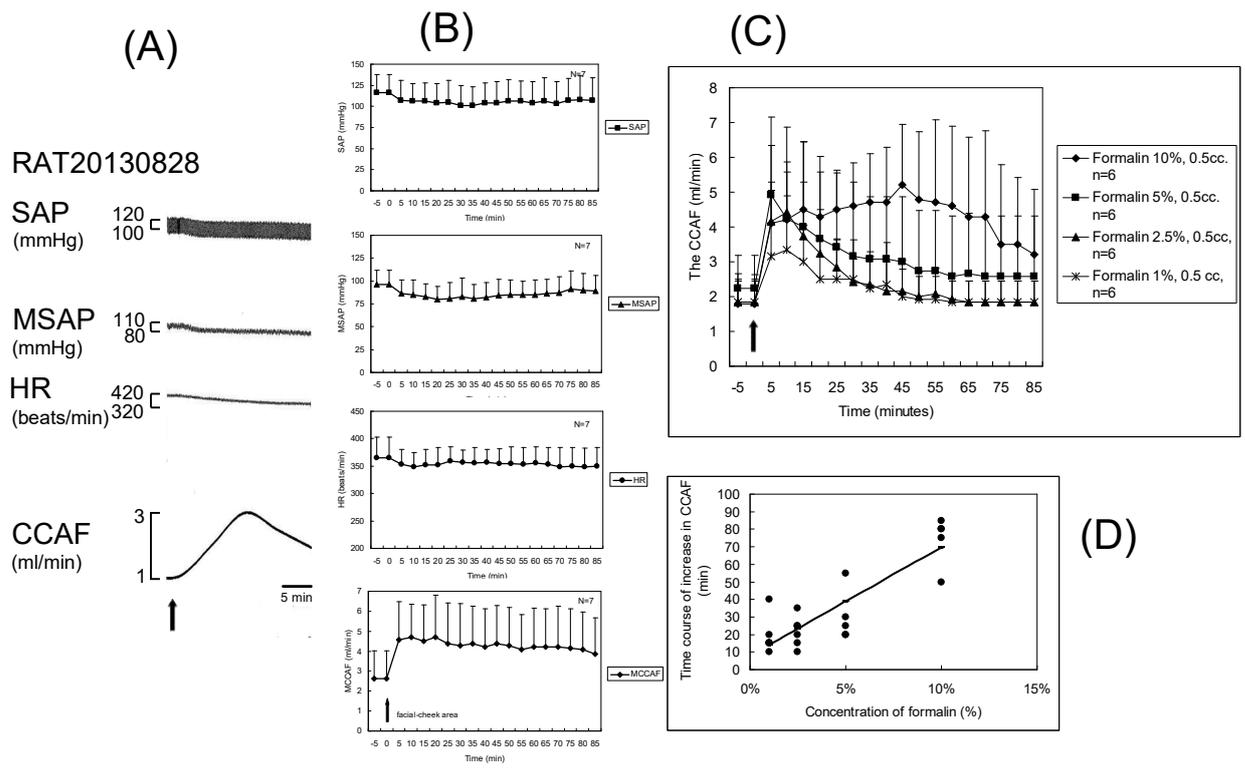
780 formalin-induced autonomic responses, these responses may also be mediated through the
781 facial nerve, which contains both autonomic-components. We propose that the induced
782 iCCAF increase and the associated autonomic responses can be mediated by the afferent
783 trigeminal nerve and can ultimately be mediated by the efferent facial nerve (Fig. 6). Thus,
784 we successfully established a rat TAC-like response model. Further investigation is needed.

785 **Fig. 7.** Schemas of the experiment involving electrical stimulation of the ophthalmomaxillary
786 nerve. All connective tissues and temporal muscles of the parietal bone were removed until
787 the floor of the anterior cranial fossa was exposed, showing two branches of the trigeminal
788 ganglion: (1) the ophthalmo-maxillary nerve, which passes through the foramen
789 orbitorotundum; and (2) the mandibular nerve. The exposed trigeminal nerve was hooked
790 with an IC clip line carrying a platinum wire for electrical stimulation. The reference
791 electrode was placed on the temporal muscle.

792 **Fig. 8.** Schemas of the experiment involving electrical stimulation of the facial nerve. The
793 upper part of the neck muscles was separated to expose the centre of the facial nerve near the
794 stem of the stylomastoid foramen. Then, the IC clip line hook sets on the facial nerve were
795 used to conduct monopolar electrical stimulation. The reference electrode was placed in the
796 neck muscles.

797

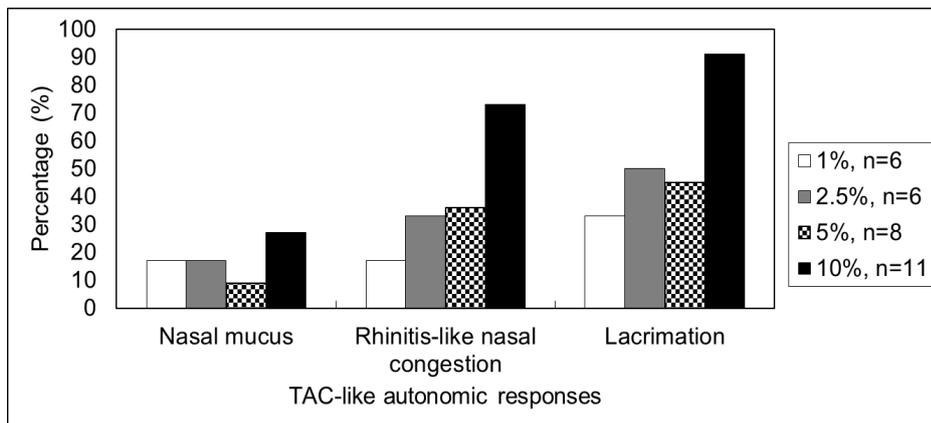
798 **Figures**



799

800 **Fig. 1**

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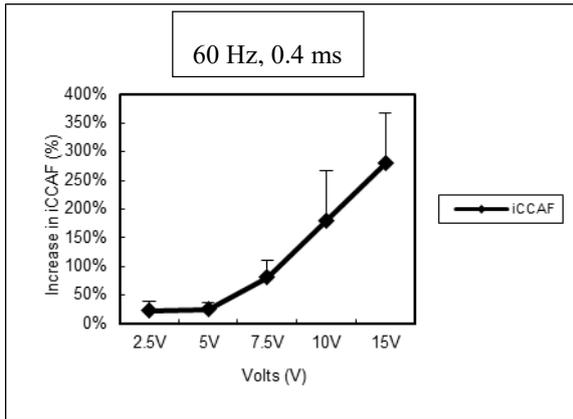


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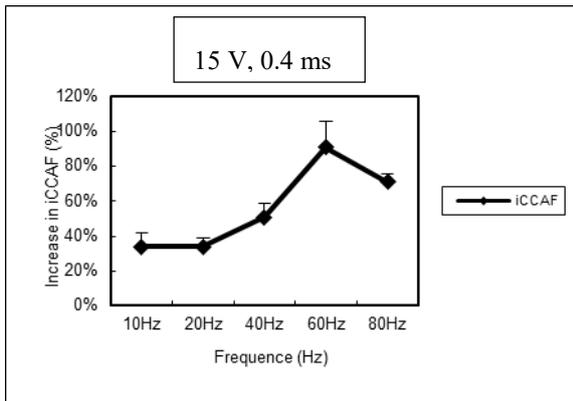
803 **Fig. 2**

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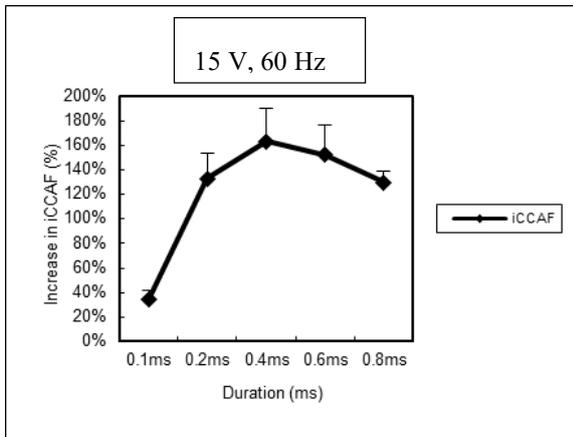
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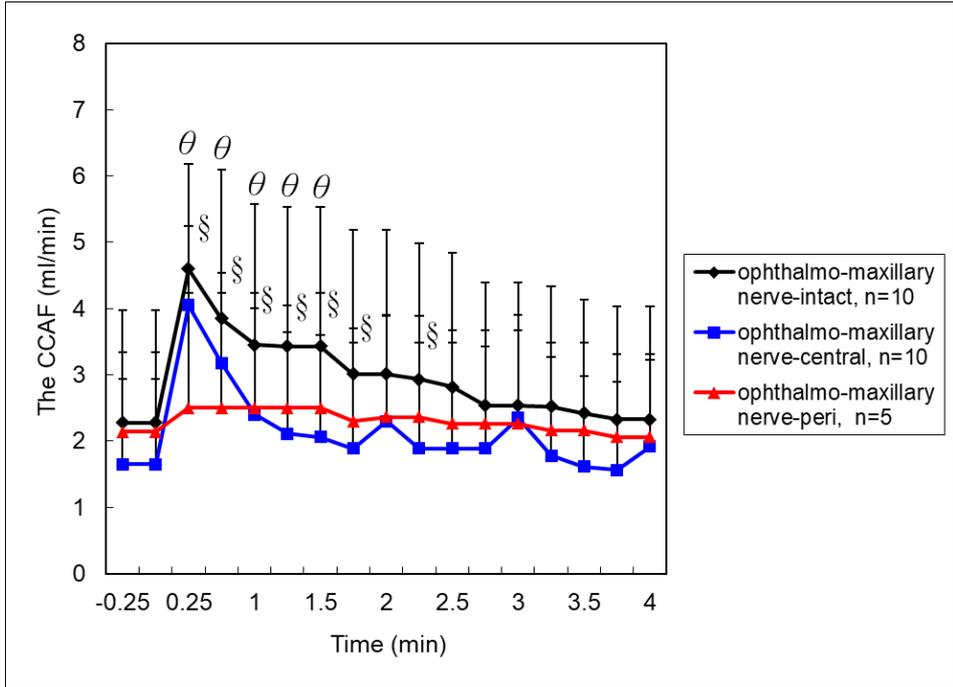


807



808 **Fig. 3**

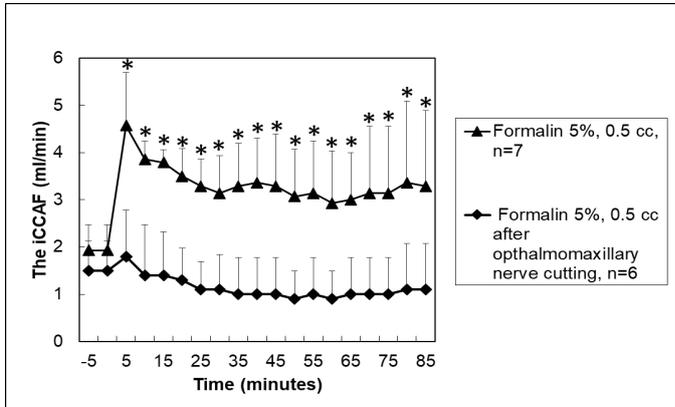
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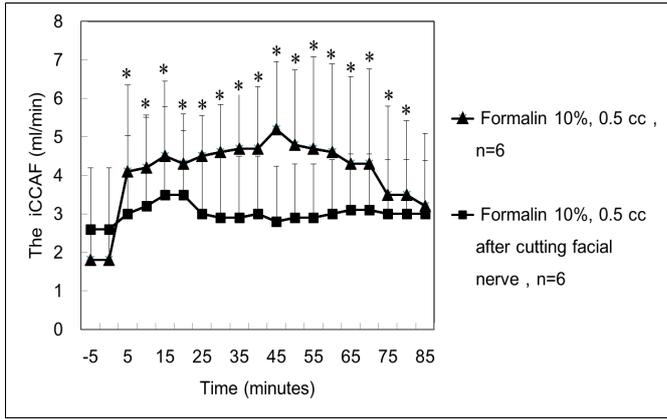
811 **Fig. 4**

812



813

814 (a)

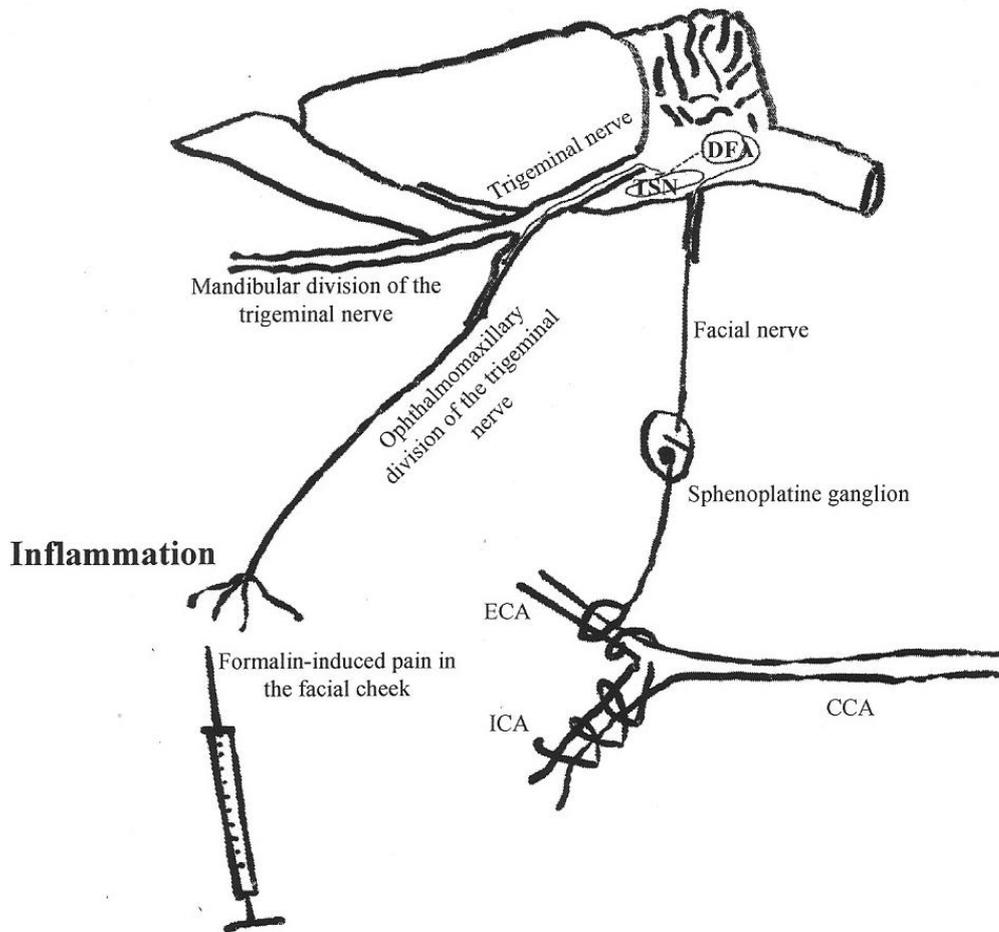


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816 (b)

817 **Fig. 5**

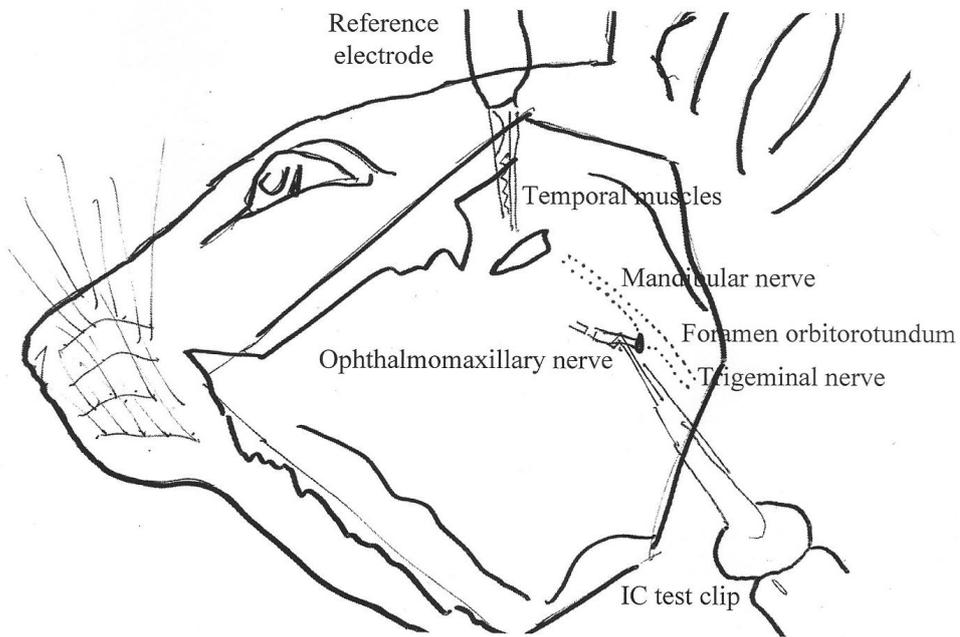
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820 **Fig. 6**

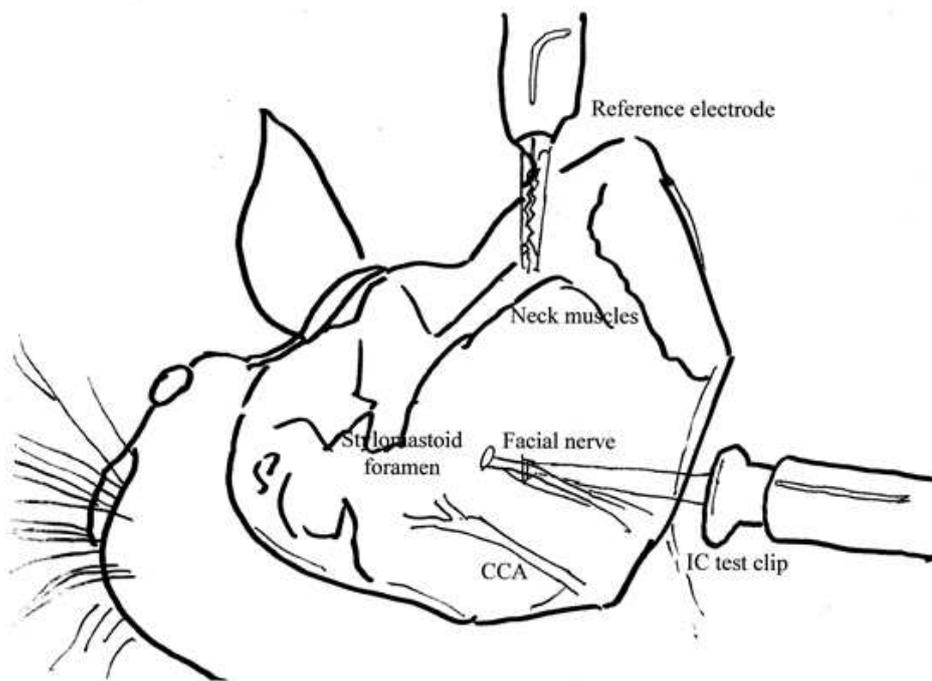
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823 **Fig. 7**

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825

826 **Fig. 8**

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828 **Additional information**

829 **Additional file 1:** Fig. S4 Increase in iCCAF and autonomic responses (nasal mucus, eyelid
830 oedema, red eye, meiosis and lacrimation) induced by injections of 0.5 cc of formalin into the
831 facial cheek of anaesthetized rats.

832 **Additional file 2:** Fig. S4 COX-2 dense staining (brown colour) in the outer membrane of the
833 CCA, which is a section of the same CCA at approximately 180 min after a marked increase
834 (vasodilatation) in the CCAF was induced by formalin at 10%, 0.5 cc into the facial cheek in
835 SD rats.

836 **Additional file 3:** Fig. S4 Microinjection of sodium glutamate (Glu, 0.1M, 400 nl) into the
837 dorsal facial area (DFA) in SD rats cause the left common carotid blood flow (LCCAF)
838 increases (3.3 to 6.5 cc), and mean LCCAF (3 ± 1 cc to 5 ± 1 cc, n=9).

Figures

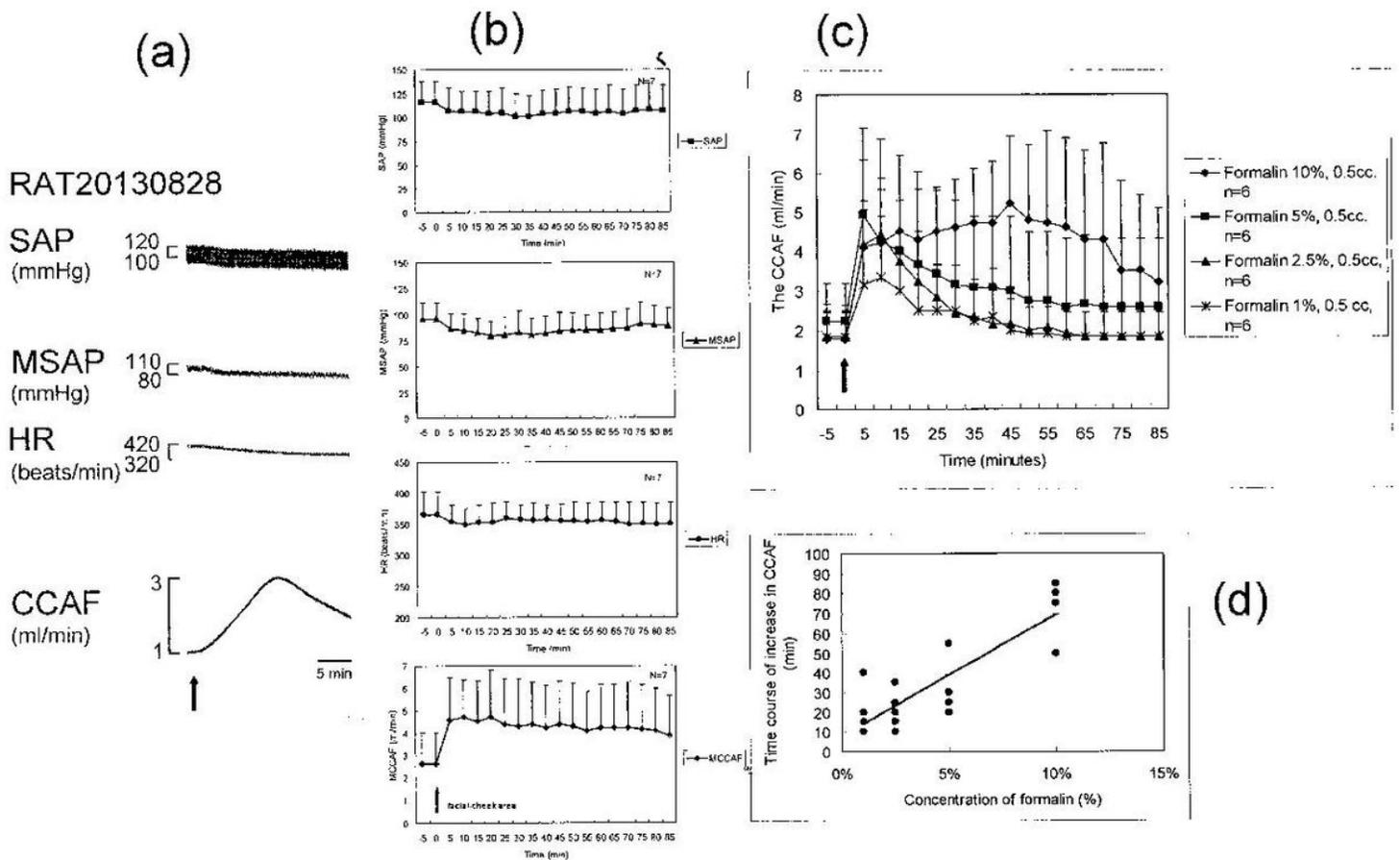


Fig. 1

Figure 1

Cardiovascular changes induced by formalin injections into the facial cheek area of 724 anaesthetized rats. (a) Authors used the solid arrow marker () to indicate when formalin 725 (5%, 0.5 cc) was injected. (b) Data obtained from seven anaesthetized rats. (c) iCCAF 726 increases induced by injections of formalin (1%, 2.5%, 5%, and 10%, 0.5 cc) into the facial 727 cheek of anaesthetized rats. They reached maximal increases at 5 min. (d) Correlation 728 between the increase in duration of iCCAF and the concentration of formalin (1%, 2.5%, 5%, 729 and 10%, 0.5 cc) injected into the facial cheek of anaesthetized rats. "The time (duration) for 730 maintaining iCCAF increases" is expressed as the time required for a 75% reduction of the 731 maximal iCCAF increase.

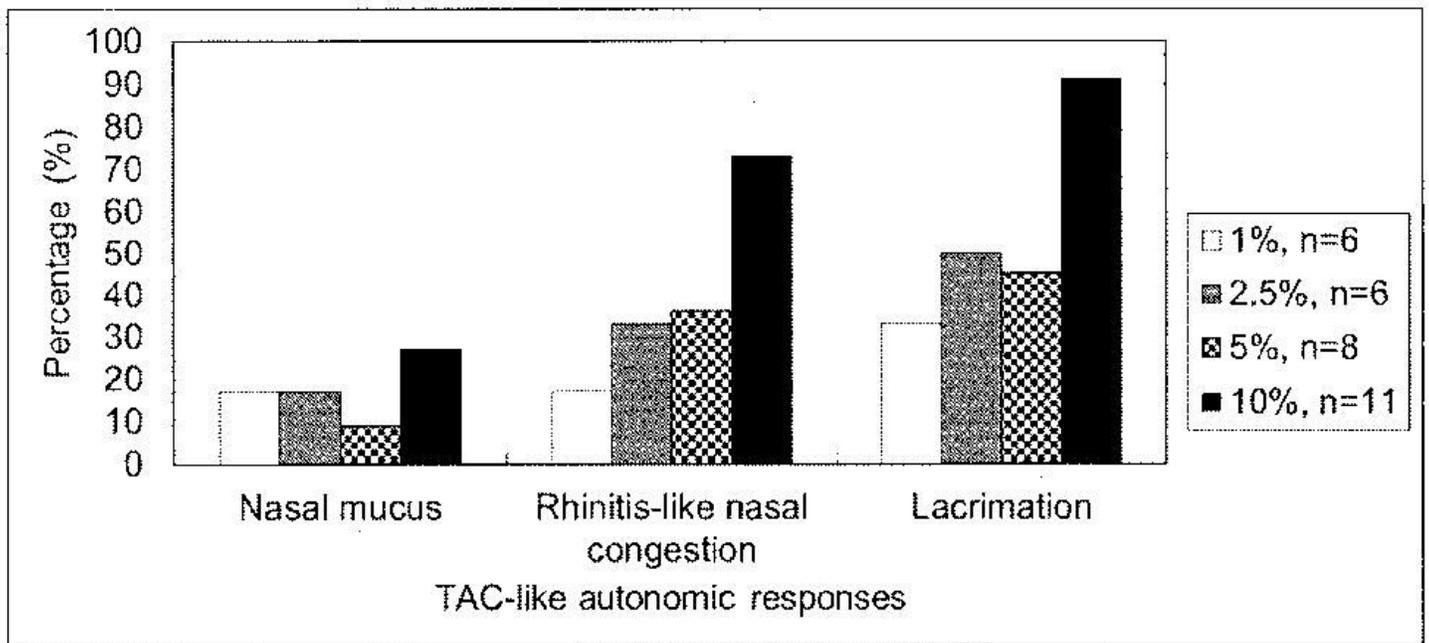


Fig. 2

Figure 2

Concentration-dependent % increases in autonomic (nasal mucus, rhinitis-like nasal 733 congestion and lacrimation) responses induced by injections of 0.5 cc of formalin at 734 concentrations of 1%, 2.5%, 5%, and 10% into the facial cheek of anaesthetized rats. Each of 735 the formalin concentration test groups only counted the increased iCCAF response with the 736 occurrence of each autonomic response, so the incidence of each accompanying autonomic 737 response (%) was calculated in each group. The rate (%) was equal to the total number of 738 times the increased iCCAF response was initiated, and the number of occurrences of each 739 autonomic response was divided by the total number of occurrences in the experimental 740 group. The measurement of the substances produced by the autonomic response has 741 experimental limitations. Because of the small amount of substance and difficulty in 40 742 obtaining measurement tools, this measurement is occasionally recorded by photography and 743 video recording methods.

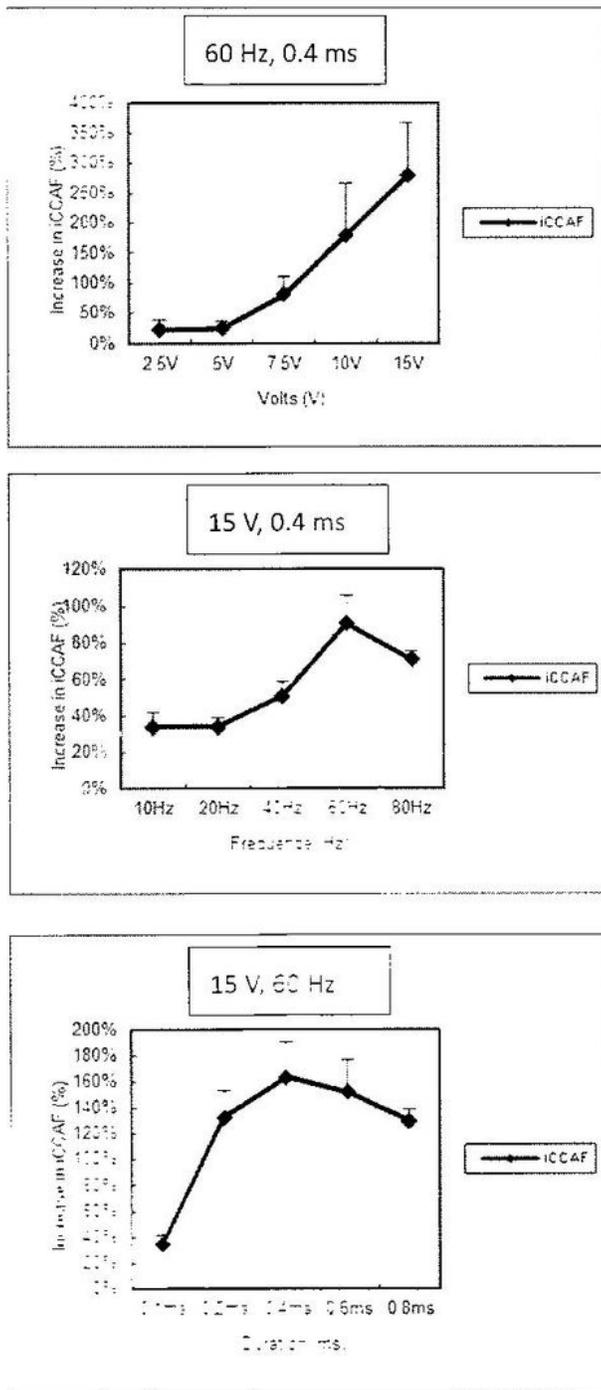


Fig. 3

Figure 3

Induction of iCCAF increases with stimulation of the afferent (sensory) 745 ophthalmo-maxillary nerve. The stimulation was made on the left nerve, and iCCAF was 746 recorded. Note that the iCCAF increases in a manner dependent on stimulation parameters 747 (voltage, frequency, and duration). The modest electrical parameters of voltage, frequency 748 and pulse duration for data have been filled in the top, middle and bottom panels, 749 respectively.

Figure 5

Effects of sections of the ophthalmomaxillary nerve (a) or facial nerve (b) on the 41 761 iCCAF increase induced by 0.5 cc of 5% (a) or 10% (b) formalin injection into the facial 762 cheek area. The formalin injection was made into the left facial cheek, and the 763 ophthalmomaxillary and facial nerves were cut on the same side. (a) shows the comparison 764 between sections of the ophthalmomaxillary nerve responses and the injection of formalin, 765 and (b) shows the comparison between cutting facial nerve responses and the injection of 766 formalin. Data were analysed with Student's t test. Data are presented as the means \pm SEM. 767 A p value (*) less than 0.05 was considered statistically significant.

Figure 6

Anatomical basis of TAC-like responses induced by formalin injection into the facial 769 cheek rats. Formalin injection into the facial cheek induces nociceptive or inflammatory 770 stimulation of the sensory endings of the ophthalmomaxillary branch of the trigeminal nerve. 771 The trigeminal nerve then excites the neurons of the trigeminal sensory nucleus (TSN) in the 772 medulla, which project fibres to the dorsal facial area (DFA). The DFA is a parasympathetic 773 nucleus that gives rise to preganglionic parasympathetic fibres projecting to the 774 sphenopalatine ganglion of the facial nerve, and the postganglionic fibres innervate 775 intracranial and extracranial vessels (ICVs and ECVs) or branches of the common carotid 776 artery (CCA). Ipsilateral excitations of the DFA and its related facial nerve are responsible 777 for iCCAF [16, 20]. Therefore, formalin-induced nociceptive or inflammatory stimulation of 778 the sensory endings of the ophthalmomaxillary branch of the trigeminal nerve elicits an 779 increase in iCCAF. Since interruption of the facial nerve also markedly reduces other 42 780 formalin-induced autonomic responses, these responses may also be mediated through the 781 facial nerve, which contains both autonomic components. We propose that the induced 782 iCCAF increase and the associated autonomic responses can be mediated by the afferent 783 trigeminal nerve and can ultimately be mediated by the efferent facial nerve (Fig. 6). Thus, 784 we successfully established a rat TAC-like response model. Further investigation is needed.

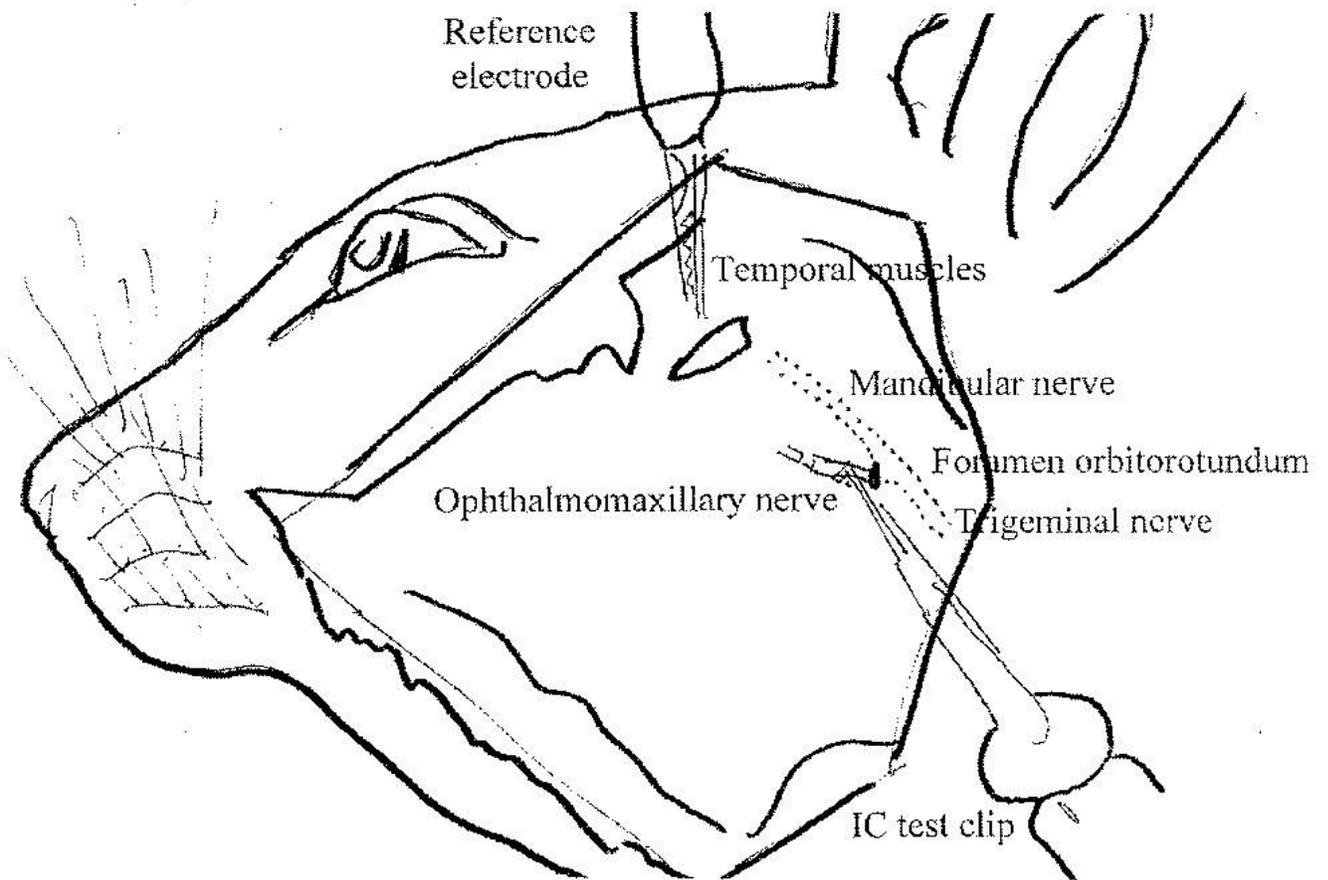


Fig. 7

Figure 7

Schemas of the experiment involving electrical stimulation of the ophthalmomaxillary 786 nerve. All connective tissues and temporal muscles of the parietal bone were removed until 787 the floor of the anterior cranial fossa was exposed, showing two branches of the trigeminal 788 ganglion: (1) the ophthalmo-maxillary nerve, which passes through the foramen 789 orbitorotundum; and (2) the mandibular nerve. The exposed trigeminal nerve was hooked 790 with an IC clip line carrying a platinum wire for electrical stimulation. The reference 791 electrode was placed on the temporal muscle.

Figure 8

Schemas of the experiment involving electrical stimulation of the facial nerve. The 793 upper part of the neck muscles was separated to expose the centre of the facial nerve near the 794 stem of the

stylomastoid foramen. Then, the IC clip line hook sets on the facial nerve were used to conduct monopolar electrical stimulation. The reference electrode was placed in the neck muscles.

Supplementary Files

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