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

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Research Article

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Abstract

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

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

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

Background

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

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.

Results

Cardiovascular responses induced by formalin injection in the facial cheek

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

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

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

The duration of the iCCAF increase was correlated with the concentration of formalin (1%, 2.5%, 5%, and 10%, 0.5 cc) injected into the facial cheek. These injections elicited iCCAF increases that lasted for durations of 19 ± 11 min, 22 ± 9 min, 30 ± 15 min, and 74 ± 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 ophthalmo-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 ophthalmo-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

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

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

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

Discussion

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

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

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

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

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

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

Afferent sensory pathway

For future electrical stimulations, although the electrical stimulation parameters that induced the maximum iCCAF increase were 15 V, 60 Hz, and 0.4 ms (Fig. 3), we suggest using lower values such as 12-15 V, 40-60 Hz, and 0.2-0.4 ms, as shown in Fig. 4. In particular, for 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 ophthalmo-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 ophthalmo-maxillary nerve (or the trigeminal nerve) and was not induced by
197 stimulation of the peripheral end of the cut ophthalmo-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].

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

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

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

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

Inflammation resulted from CCA vasodilatation through a neurovascular mechanism

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

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

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

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

The iCCAF increase induced by 5% formalin injection into the facial cheek was abolished after cutting of the sensory ophthalmomaxillary nerve branch of the trigeminal nerve (Fig. 5a). In addition, it was significantly induced by electrical stimulation of the central end of the ophthalmomaxillary nerve and the trigeminal nerve. However, the iCCAF increase was slightly induced by electrical stimulation of the peripheral end of the ophthalmomaxillary nerve (Fig. 4). Therefore, the central area could be responsible for the iCCAF increase. Microinjection of sodium glutamate (Glu, 0.1M, 400 nl) into the DFA in SD rats cause the left common carotid blood flow (LCCAF) increases (3±1cc to 5±1cc, n=9), accompanied by 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.”

Efferent motor pathway

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

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

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

Conclusions

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

Methods

Animal ethics

The preparation of the animal, including the use of anaesthesia throughout the entire course of the experiment, was performed according to the Animal Research: Reporting in Vivo Experiments (ARRIVE) guiding principle and the affidavit of approval of animal use protocol listed below. This affidavit was reviewed and approved (Approval number: vghks-2011-A003) by the Institutional Animal Care and Use Committee (IACUC) of Kaohsiung Veterans General Hospital and conformed with the guidelines for the care and

use of laboratory animals issued by the Chinese society for laboratory animal science, Taiwan, R.O.C. [48] and clinical laboratory animal medicine [49], which conform with international standards.

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

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

Monitoring of cardiovascular responses

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

The injection of formalin into the facial cheek

The reason why the authors used two kinds of stimulation methods is that peripheral nerve electrical stimulation can be repeatedly stimulated to obtain repeatable responses, which return to normal in a relatively shorter time, approximately 1 - 5 min. On the other hand, chemical stimulation by formalin inevitably causes injury to the tissue so that repetitive 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, 52]. 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 ophthalmo-maxillary 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 ophthalmo-maxillary 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 ophthalmo-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 ± 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

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

List of abbreviations

Ach: Acetylcholine

AEN: Anterior ethmoidal nerve

CCA: Common carotid artery

CBF: Cerebral blood flow

CGRP: Calcitonin gene-related peptide

COX-2: Cyclooxygenase-2

DFA: Dorsal facial area

DM: Dorsal medulla

dPdt: Cardiac constriction

ECV: Extracranial vessel

FAF: Femoral arterial flow

Glu: Glutamate

HR: Heart rate

Hz: Hertz

IC: Integrated circuit

iCCAF: Ipsilateral common carotid arterial flow

ICV: Intracranial vessel

IHC: Immunohistochemistry

min: Minute

ms: Millisecond

MSAP: Mean systemic arterial pressure

NOS: Nitric oxide synthesis

NTS: Solitary nucleus

PAG: Periaqueductal grey

PHA: Posterior hypothalamic area

PPE: Plasma protein extravasation

r: Pearson product-moment correlation coefficient

RAF: Renal arterial flow

RVLM: Rostral ventrolateral medulla

SAP: Systemic arterial pressure

SD: Sprague-Dawley

sec: Second

SEM: Standard error of the mean

SMAF: Superior mesenteric arterial flow

SPECT: Single-photon emission computed tomography

SPG: Sphenopalatine ganglia

SUNCT: Shorting-lasting unilateral neuralgiform with conjunction and tearing headache

TAC: Trigeminal autonomic cephalalgias

TSN: Trigeminal sensory nucleus

V: Volt

VIP: Vasoactive intestine peptides

Declarations

Ethics declarations

Ethical approval and consent to participate

Animal procedures was conducted under the National Institutes of Health's Guide for Care and Use of Laboratory Animals and the Animal Research: Reporting in Vivo Experiments (ARRIVE) guiding principle. Also, all experimental and surgical procedures of this study were confirmed by the Institutional Animal Care and Use Committee (IACUC) of Kaohsiung Veterans General Hospital (Approval number: vghks-2011-A003).

514

515 ***Consent for publication***

516 Not applicable.

517

518 ***Availability of data and materials***

519 The datasets used and/or analysed during the current study are available from the

520 corresponding author upon reasonable request.

521

522 ***Competing interests***

523 The authors declare that they have no competing interest.

524

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530 ***Authors' contributions***

531 Conception and design: M.R.W.; completion of the experiments and collection of the data:

532 M.R.W.; data analysis and interpretation: M.R.W.; revision of the manuscript: M.R.W., C.J.T.

and J.S.K. All authors approved the final version of the manuscript and agreed to be accountable for all aspects of the work, ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. All persons designated as authors qualify for authorship, and all those who qualify for authorship are listed.

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561 **Additional information**

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709

710 **Figure legends**

711 **Fig. 1.** Cardiovascular changes induced by formalin injections into the facial cheek area of
712 anaesthetized rats. (a) Authors used the solid arrow marker (⬆) to indicate when formalin
713 (5%, 0.5 cc) was injected. (b) Data obtained from seven anaesthetized rats. (c) iCCAF
714 increases induced by injections of formalin (1%, 2.5%, 5%, and 10%, 0.5 cc) into the facial
715 cheek of anaesthetized rats. They reached maximal increases at 5 min. (d) Correlation
716 between the increase in duration of iCCAF and the concentration of formalin (1%, 2.5%, 5%,
717 and 10%, 0.5 cc) injected into the facial cheek of anaesthetized rats. “The time (duration) for
718 maintaining iCCAF increases” is expressed as the time required for a 75% reduction of the
719 maximal iCCAF increase.

720 **Fig. 2.** Concentration-dependent % increases in autonomic (nasal mucus, rhinitis-like nasal
721 congestion and lacrimation) responses induced by injections of 0.5 cc of formalin at
722 concentrations of 1%, 2.5%, 5%, and 10% into the facial cheek of anaesthetized rats. Each of

723 the formalin concentration test groups only counted the increased iCCAF response with the
724 occurrence of each autonomic response, so the incidence of each accompanying autonomic
725 response (%) was calculated in each group. The rate (%) was equal to the total number of
726 times the increased iCCAF response was initiated, and the number of occurrences of each
727 autonomic response was divided by the total number of occurrences in the experimental
728 group. The measurement of the substances produced by the autonomic response has
729 experimental limitations. Because of the small amount of substance and difficulty in
730 obtaining measurement tools, this measurement is occasionally recorded by photography and
731 video recording methods.

732 **Fig. 3.** Induction of iCCAF increases with stimulation of the afferent (sensory)
733 ophthalmo-maxillary nerve. The stimulation was made on the left nerve, and iCCAF was
734 recorded. Note that the iCCAF increases in a manner dependent on stimulation parameters
735 (voltage, frequency, and duration). The modest electrical parameters of voltage, frequency
736 and pulse duration for data have been filled in the top, middle and bottom panels,
737 respectively.

738 **Fig. 4.** The iCCAF increase induced by electrical stimulation (15 V, 60 Hz, and 0.4 ms) of
739 the intact ophthalmo-maxillary nerve (◆), as well as its central and peripheral ends. The
740 iCCAF increase was first induced by stimulation of the intact ophthalmo-maxillary nerve,
741 and then the ophthalmo-maxillary nerve was cut for stimulation of its peripheral (▲) and

central (■) ends. A *p* value (*) less than 0.05 was considered statistically significant for the comparison of the response of the ophthalmo-maxillary nerve-intact with the nerve-central, and other *p* values (θ) less than 0.05 were considered statistically significant for comparison of the response of the ophthalmo-maxillary nerve-intact and the nerve-peri. Another *p* value (§) less than 0.05 was considered statistically significant for the comparison of the response of the ophthalmo-maxillary nerve-central and the nerve-peri.

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

Fig. 6. Anatomical basis of TAC-like responses induced by formalin injection into the facial cheek rats. Formalin injection into the facial cheek induces nociceptive or inflammatory stimulation of the sensory endings of the ophthalmo-maxillary branch of the trigeminal nerve. The trigeminal nerve then excites the neurons of the trigeminal sensory nucleus (TSN) in the medulla, which project fibres to the dorsal facial area (DFA). The DFA is a parasympathetic

761 nucleus that gives rise to preganglionic parasympathetic fibres projecting to the
762 sphenopalatine ganglion of the facial nerve, and the postganglionic fibres innervate
763 intracranial and extracranial vessels (ICVs and ECVs) or branches of the common carotid
764 artery (CCA). Ipsilateral excitations of the DFA and its related facial nerve are responsible
765 for iCCAF [16, 20]. Therefore, formalin-induced nociceptive or inflammatory stimulation of
766 the sensory endings of the ophthalmo-maxillary branch of the trigeminal nerve elicits an
767 increase in iCCAF. Since interruption of the facial nerve also markedly reduces other
768 formalin-induced autonomic responses, these responses may also be mediated through the
769 facial nerve, which contains both autonomic-components. We propose that the induced
770 iCCAF increase and the associated autonomic responses can be mediated by the afferent
771 trigeminal nerve and can ultimately be mediated by the efferent facial nerve (Fig. 6). Thus,
772 we successfully established a rat TAC-like response model. Further investigation is needed.
773 **Fig. 7.** Schemas of the experiment involving electrical stimulation of the ophthalmomaxillary
774 nerve. All connective tissues and temporal muscles of the parietal bone were removed until
775 the floor of the anterior cranial fossa was exposed, showing two branches of the trigeminal
776 ganglion: (1) the ophthalmo-maxillary nerve, which passes through the foramen
777 orbitorotundum; and (2) the mandibular nerve. The exposed trigeminal nerve was hooked
778 with an IC clip line carrying a platinum wire for electrical stimulation. The reference
779 electrode was placed on the temporal muscle.

Fig. 8. Schemas of the experiment involving electrical stimulation of the facial nerve. The upper part of the neck muscles was separated to expose the centre of the facial nerve near the 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.

Figures

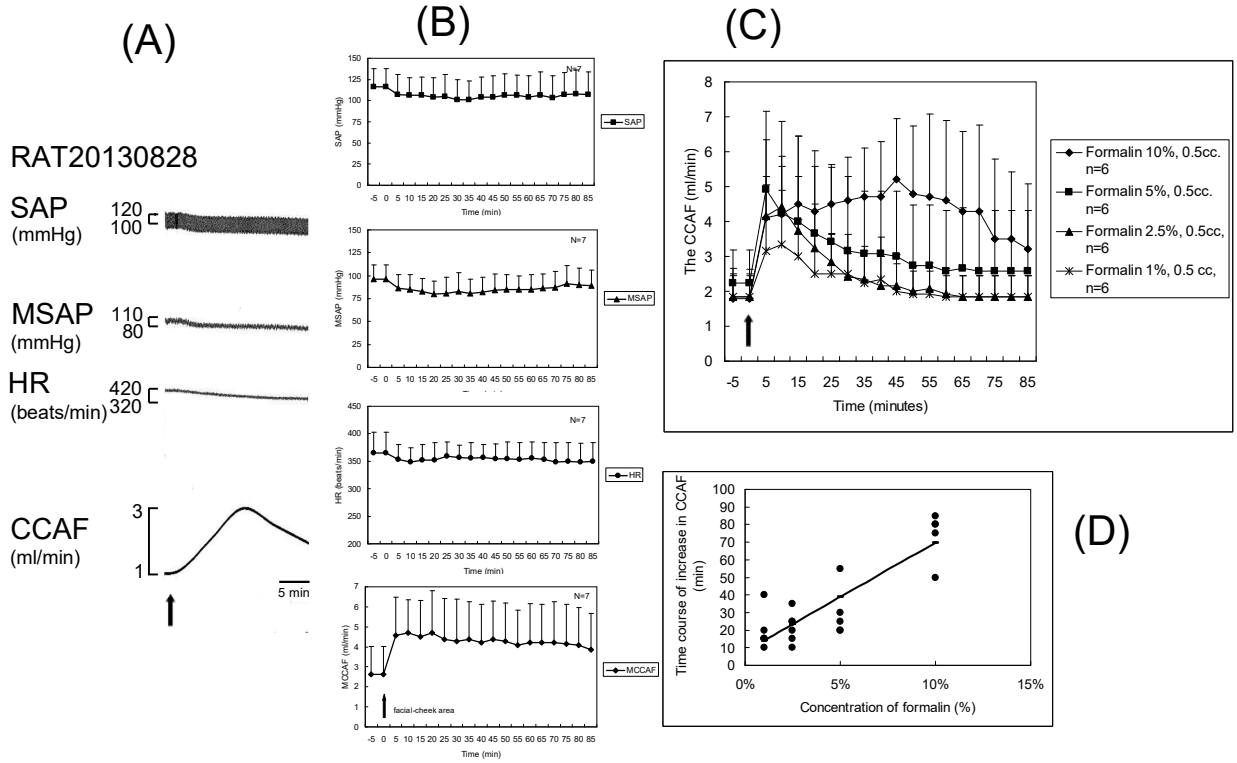
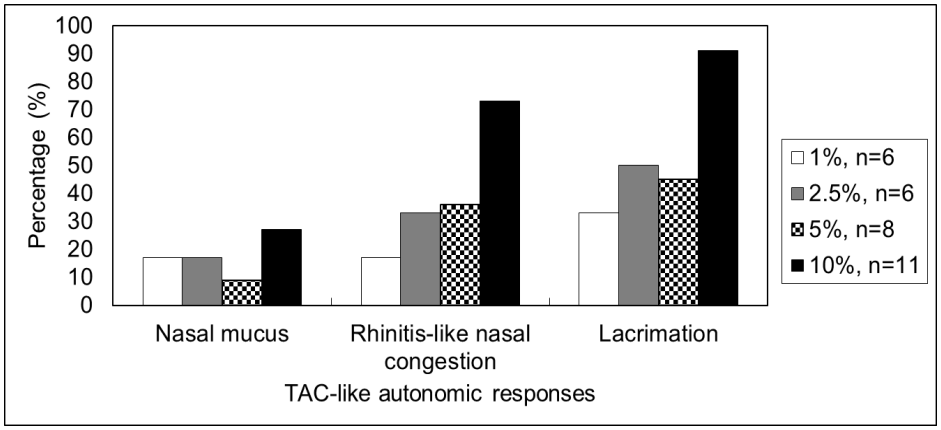


Fig. 1



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791 **Fig. 2**

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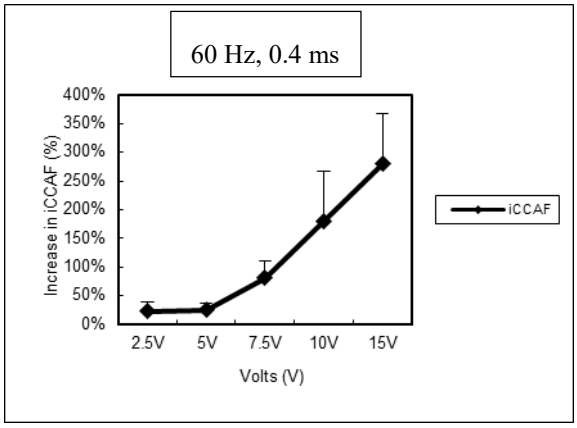

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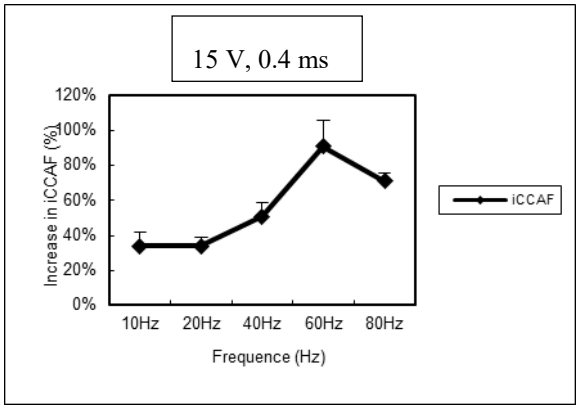

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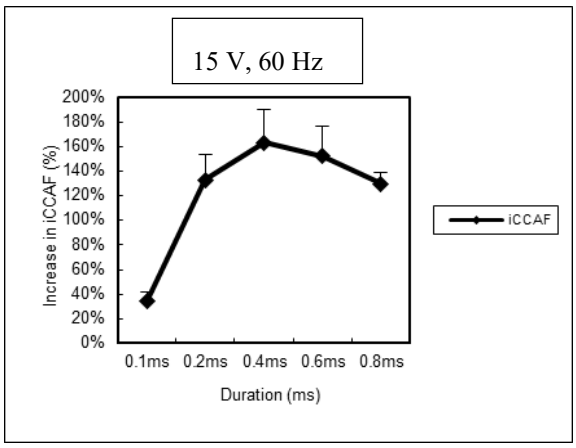

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796 **Fig. 3**

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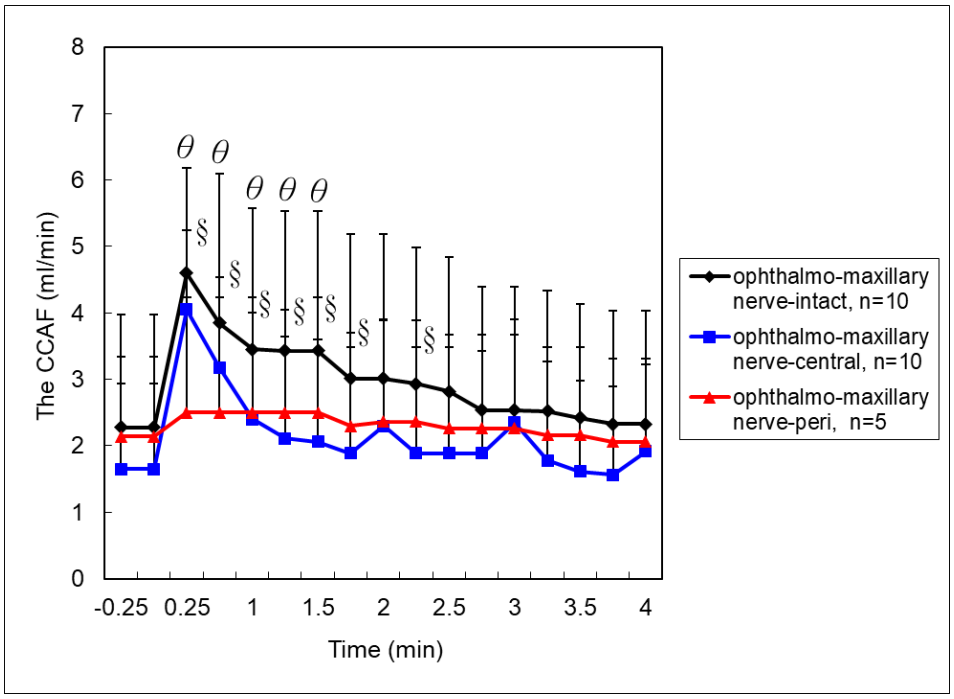

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799 **Fig. 4**

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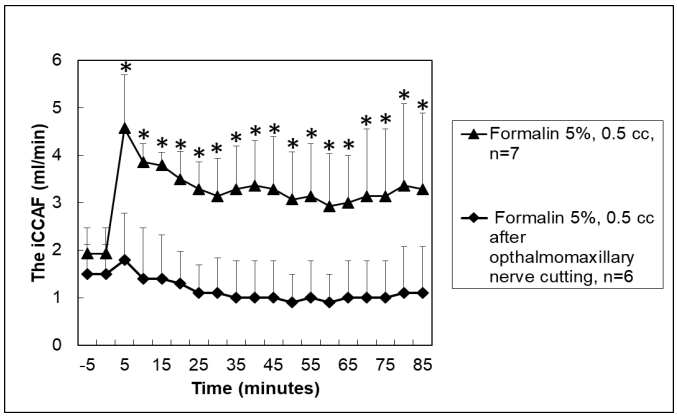
801 

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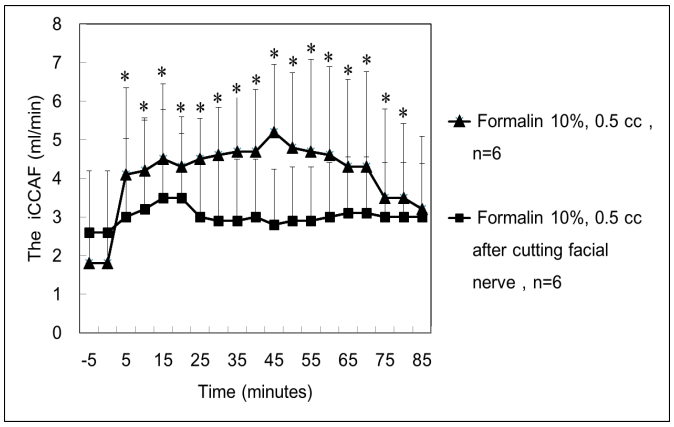
803 

804 (b)

805 **Fig. 5**

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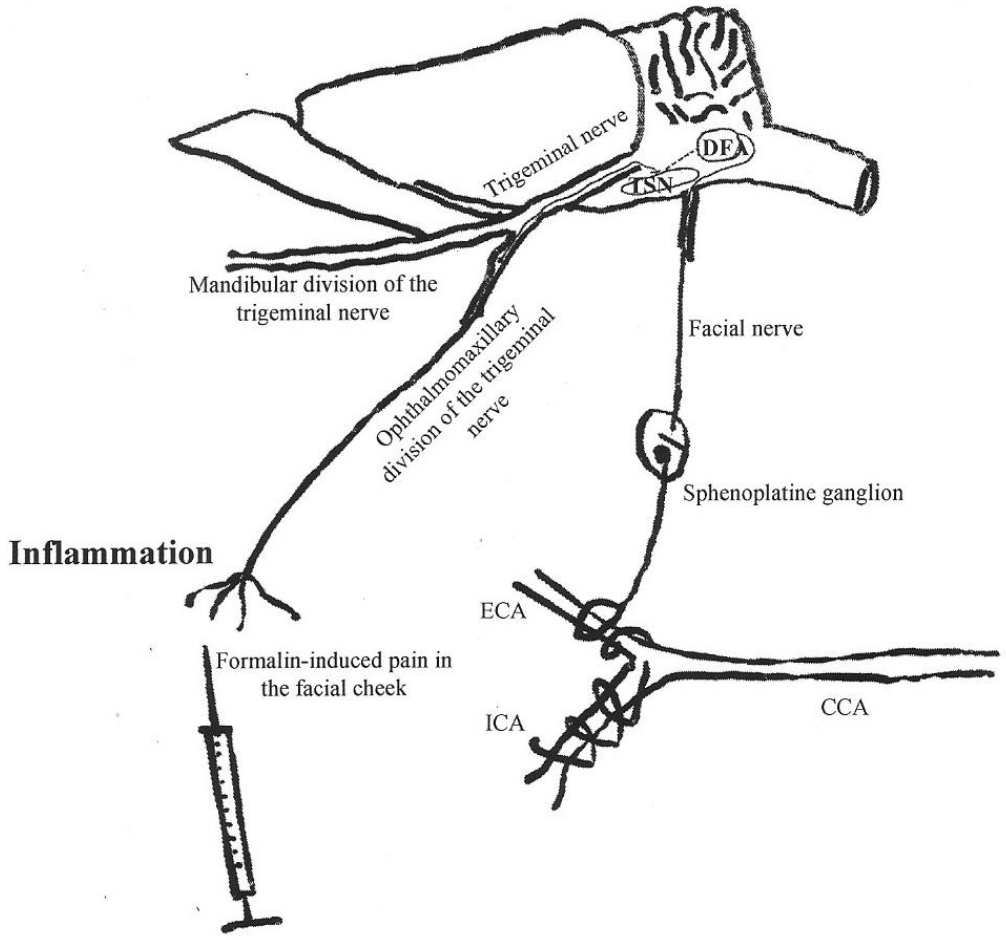

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808 **Fig. 6**

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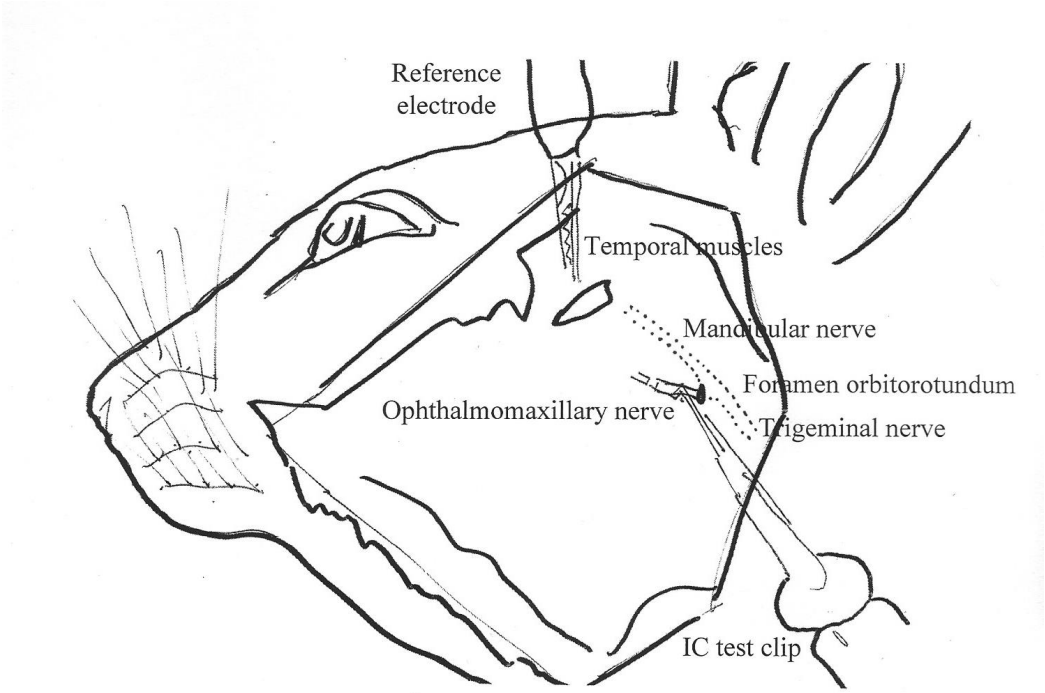

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811 **Fig. 7**

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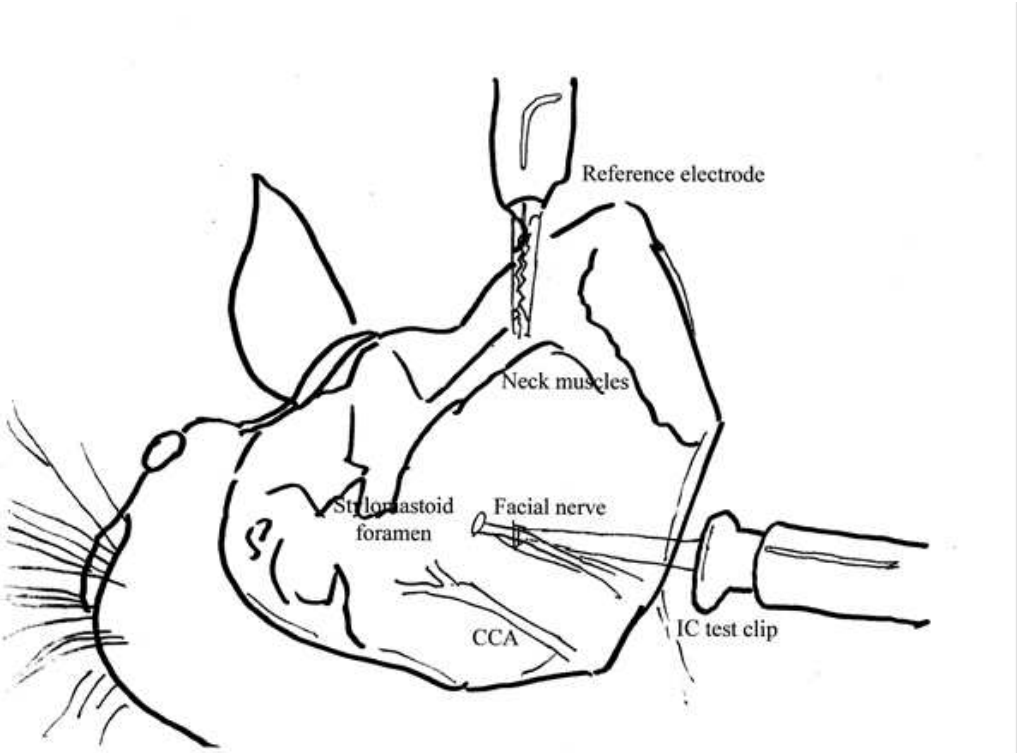

Fig. 8

Additonal information

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

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

824 **Additional file 3:** Fig. S4 Microinjection of sodium glutamate (Glu, 0.1M, 400 nl) into the

825 dorsal facial area (DFA) in SD rats cause the left common carotid blood flow (LCCAF)

826 increases (3.3 to 6.5 cc), and mean LCCAF (3±1cc to 5±1cc, n=9).

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Additionalfile1autonomicresponses.pdf
- Addditionalfile2COX2stained.pdf
- AdditionalfileiincreaseiCCAFinDFAinrat.pdf