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Distribution of Fos-immunoreactive neurons in the gustatory cortex elicited by intra-oral infusion of taste solutions in conscious rats

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Abstract

The location of neurons in the gustatory cortex (GC) activated by intra-oral infusion of solutions in conscious rats was mapped using Fos immunohistochemistry. Groups of adult male Wistar rats ($N=5$) received an infusion of one of the following: dH₂O, 0.1 or 1.0M NaCl, 0.1 or 1.0M sucrose, 0.32M MSG (with 100 μ M amiloride and 2.5M inosine 5'-monophosphate), 0.03M HCl, or 0.003M QHCl delivered via an intra-oral cannula (0.233ml/min for 5 min). Unstimulated control rats received no infusion. Taste reactivity (TR) behaviors were videotaped and scored. The number of Fos-immunoreactive (Fos-IR) neurons was counted in eight sections throughout the anterior-posterior extent of the GC in the medial and lateral halves of the granular (GI), dysgranular (DI), and dorsal (AID) and ventral (AIV) agranular insular cortices. Intra-oral infusion of dH₂O, NaCl, or sucrose altered the number of Fos-IR neurons in only specific subareas of the GC and the effects of these tastants were concentration-dependent. For example, 1.0M NaCl increased Fos-IR neurons in the posterior lateral AID and DI and elicited more aversive TR responses than 0.1M NaCl. Compared to dH₂O, infusions of HCl or QHCl increased the total number of Fos-IR neurons in many subareas of the GC throughout its anterior-posterior extent and increased aversive TR behaviors. Linear regression analyses suggested that neurons in the medial AID of the posterior GC may influence aversive behavioral responses to HCl and QHCl while neurons in the posterior lateral AID and DI may play a role in aversive TR responses to 1.0M NaCl.

Graphical Abstract

Schematic depiction of the medial and lateral parts of each subarea along the anterior-posterior extent of the GC (distances are from bregma) indicating the tastants that altered the number of Fos-IR neurons compared to intra-oral infusion of dH₂O ($p < 0.05$). Darkly shaded subareas had more Fos-IR neurons elicited by intra-oral infusion of QHCl. Subareas within which the number of Fos-IR neurons changed due to intra-oral infusion of HCl, MSG, 1.0M NaCl (HN), and 1.0M sucrose (HS) are indicated by letters within that subarea. In these cases, HCl, MSG, and HN increased while HS decreased the number of Fos-IR neurons compared to dH₂O.

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A. 1.7 mm		B. 1.4 mm	
	medial lateral		medial lateral
GI		GI	HCl
DI		DI	
AID	HS	AID	
AIV	HS MSG	AIV	HS HS

C. 1.1 mm		D. 0.8 mm	
	medial lateral		medial lateral
GI	HCl	GI	
DI	HCl HN HCl	DI	HCl
AID		AID	
AIV		AIV	

E. 0.5 mm		F. 0.2 mm	
	medial lateral		medial lateral
GI		GI	HCl
DI		DI	HCl
AID		AID	HN HCl
AIV		AIV	

G. -0.1 mm		H. -0.4 mm	
	medial lateral		medial lateral
GI	HCl	GI	
DI	HCl	DI	
AID		AID	
AIV		AIV	HCl

Keywords

behavior; bitter; gustatory; immunohistochemistry; insular cortex; taste stimuli

1. Introduction

The gustatory cortex (GC) is located within the insular cortex, extending from about 2.0 mm anterior to just posterior to bregma in the adult rat (Norgren, 1995). For about 1.0 mm posterior to bregma taste-responsive neurons intermingle with neurons that receive visceral and baroreceptor input (Cechetto and Saper, 1987; Hanamori et al., 1998). In the dorsal-ventral plane, the insular cortex is split into three subareas based on cytoarchitecture: the granular (GI), dysgranular (DI), and agranular (AI) insular cortices (Norgren, 1995; Paxinos and Watson, 2007; Fig. 1). Although anatomical and electrophysiological data as well as a more recent imaging study in rats have suggested a functional topography within the GC (Accolla et al., 2007; Cechetto and Saper, 1987; Kosar et al., 1986a; Yamamoto et al., 1981; 1989), a definitive map of the neurons in the rat GC activated by taste inputs remains elusive.

In the rat, the ascending gustatory pathway from the thalamus terminates in the ventral GI and dorsal DI cortices with the core of this terminal field near the intersection of the middle cerebral artery and rhinal fissure (Cechetto and Saper, 1987; Kosar et al., 1986b; Nakashima et al., 2000; Norgren and Wolf, 1975). Electrophysiological recordings verify that neurons in this region respond to stimulation of gustatory nerves and taste input (Kosar et al., 1986a; Ogawa et al., 1990; Yamamoto et al., 1980) and suggest a general topography of sensory inputs with taste-responsive neurons located primarily within DI and ventral to neurons that respond to orotactile and thermal input (Kosar et al., 1986a; Norgren, 1995; Yamamoto et al., 1981). With respect to the anterior-posterior plane of the rat GC, neurons responding to sucrose have been reported to be positioned anterior to those responding to NaCl and HCl with quinine-responsive neurons occupying the more posterior regions of the GC (Yamamoto et al., 1989; Yoshimura et al., 2004). This topography is supported by an imaging study of the rat GC that found overlapping, but unique, regions activated by tastants with different hedonic value (Accolla et al., 2007). However, imaging studies in mice have produced differing results. One study (Fletcher et al., 2017) reported no spatial organization of neurons in the central part of the GC that respond to different tastes while another reported discrete 'hot spots' of neurons responding to bitter, salty, sweet, and umami (but not sour) tastes (Chen et al., 2011).

To extend the findings from the recording and imaging studies in rat, the current investigation used immunohistochemistry for the Fos protein to identify the distribution of neurons within the GC activated by intra-oral infusion of taste solutions in conscious rats. Fos-immunoreactive (Fos-IR) neurons have been identified within central gustatory structures following taste nerve stimulation (Harrison, 2001), intra-oral infusion of tastants (DiNardo and Travers, 1997; Harrer and Travers, 1996; King et al., 1999; 2014; Mungarndee et al., 2008; Tokita et al. 2007; Travers et al., 1999; Travers, 2002; Yamamoto et al., 1993; 1994), and stimulation of brain structures (Morganti et al., 2007; Riley and King, 2013). Furthermore, following intra-oral stimulation with citrate Fos-IR neurons were located in the same forebrain regions identified as active using PET scan imaging (Kobayashi et al., 2013). Therefore, Fos immunohistochemistry may provide an accurate way to identify the populations of GC neurons that respond to taste stimuli in conscious rats. Only one study to date has reported the distribution of Fos-IR neurons throughout the rat GC in response to intra-oral stimulation, but this study only used dH₂O and quinine hydrochloride (QHCl) as stimuli (King et al., 2014). To more completely discern the distribution of taste-responsive neurons in the rat GC and thereby address the controversy related to the location and distribution of GC neurons that respond to different tastes, the five prototypical tastants and dH₂O were used as stimuli in the current study and Fos-IR neurons were mapped throughout the anterior-posterior and dorsal-ventral extent of the GC. In addition, relatively low (0.1M) and high (1.0M) concentrations of sucrose and NaCl were used to determine if any changes in the number and location of activated neurons in the GC occur as normally appetitive tastants become aversive as suggested by an imaging study in rats during conditioned taste aversion (Accolla and Carleton, 2008). Preliminary data from the current study have been presented in abstract form (King M. et al., 2014).

2. Results

2.1. Distribution of Fos-IR Neurons in the Gustatory Cortex

Even in the absence of oral stimulation many Fos-IR neurons were present throughout the GC (Figs. 2 and 3A). In these unstimulated control rats, the number of labelled neurons generally increased when moving lateral to medial within each GC subarea and when moving posterior to anterior (with the exception of GI). In addition, the ventral subareas (ventral and dorsal AI) tended to contain more Fos-IR neurons than the dorsal subareas (DI and GI). This general pattern of distribution of Fos-IR neurons within the GC was maintained in the experimental groups (Figs. 3 and 4). However, with respect to the total number of Fos neurons in the GC, only following oral infusion of QHCl or HCl was the number increased significantly over the unstimulated controls (Fig. 2; $p's < 0.02$). Two-way ANOVAs revealed a significant main effect of both treatment and subarea ($p's < 0.001$) in each section analyzed as well as an interaction (treatment x subarea) in the posterior 3 sections of the GC (Table 1). Therefore, the number of Fos-IR neurons in specific subareas was altered by taste stimuli. For example, although the introduction of dH₂O into the oral cavity did not increase the total number of Fos-IR neurons counted in the whole GC (Fig. 2), it did increase the number of labelled neurons in specific sections of the lateral DI and lateral ventral AI (AIV, Fig. 3B) as well as overall within the posterior lateral AID (Fig. 7B) compared to the control group ($p's < 0.017$).

Compared to dH₂O, the number and distribution of Fos-IR neurons within the GC was not altered by intra-oral infusion of 0.1M sucrose (Figs. 2 and 3). However, 0.1M sucrose did increase the number of labelled neurons in the posterior lateral AID compared to control rats ($p < 0.05$, Fig. 7A). On the other hand, 1.0M sucrose decreased the number of Fos-IR neurons in the anterior ventral GC compared to dH₂O (Fig. 3D). Specifically, intra-oral infusion of 1.0M sucrose decreased the number of labelled neurons throughout the anterior medial AIV compared to dH₂O and 0.1M sucrose ($p's < 0.05$, Fig. 8B). Similar to sucrose, intra-oral infusion of 0.1M NaCl did not alter the number or distribution of Fos-IR neurons within the GC compared to dH₂O while 1.0M NaCl had more influence on the number of labelled neurons in specific subareas (Figs. 2 and 3). For 1.0M NaCl the main effects on the number of Fos-IR neurons were in the lateral DI and AID subareas of the GC (Figs. 3F, 6A and 7A) where this solution increased the number of labelled neurons compared to the control group and dH₂O-infused animals ($p's < 0.05$). 1.0M NaCl also increased the number of Fos-IR neurons in the posterior lateral DI and AID compared to 0.1M NaCl ($p's < 0.017$, Fig. 6A and 7A). Intra-oral infusion of MSG (with amiloride and IMP) significantly decreased the number of labelled neurons in only one section of the anterior lateral AIV compared to dH₂O (Fig. 4B).

Intra-oral infusion of HCl or QHCl had the most consistent and significant effects on the distribution and number of Fos-IR neurons in the GC (Figs. 1, 2, 4–8). In particular, HCl increased the number of labelled neurons throughout the GC compared to dH₂O with the most pronounced effects occurring in the lateral DI and AID subareas ($p's < 0.017$). Intra-oral infusion of QHCl had similar but more widespread effects as HCl causing an increase in the number of Fos-IR neurons in more than half of the areas examined (36 of the 64 subareas in

specific sections) compared to dH₂O (p 's<0.05). These effects were specific to the GC because the intra-oral infusion of HCl or QHCl did not alter the number of Fos-IR neurons in the perirhinal cortex 2.0 mm posterior to bregma.

2.2 Relationship between Fos-IR Neurons and Taste Reactivity Behaviors

Intra-oral infusion of each of the solutions elicited ingestive TR behaviors (Fig. 9, p 's<0.001). Although infusion of QHCl caused ingestive TR responses, all other solutions elicited more ingestive behaviors than this tastant (p 's<0.05). Compared to dH₂O, a significant increase in aversive TR behaviors occurred following intra-oral infusion of QHCl, HCl, and 1.0M NaCl (p 's<0.05). In addition, 1.0M NaCl and sucrose elicited more aversive responses than 0.1M NaCl and sucrose, respectively (p 's<0.05).

Linear regression analyses revealed that, in some cases, the number of behaviors performed during intra-oral infusion of a solution might be related to the number of Fos-IR neurons located in specific regions of the GC subareas (Fig. 10). For example, the ingestive TR responses to intra-oral infusion of dH₂O were positively related to the number of Fos-IR neurons elicited by dH₂O in the posterior lateral AID ($R=0.781$, $p=0.029$), an area within which dH₂O increased the number of Fos-IR neurons compared to the control group. Also, the number of aversive TR behaviors performed during infusion of sucrose (both concentrations combined) showed a negative relationship with the number of Fos-IR neurons in the anterior medial AIV subarea of the GC ($R=-0.762$, $p=0.011$), an area in which 1.0M sucrose elicited fewer Fos-IR neurons than 0.1M sucrose. The number of aversive behaviors elicited by intra-oral infusion of NaCl (both concentrations combined) was positively related to the number of labelled neurons in the posterior lateral DI ($R=0.725$, $p=0.002$). This GC subregion, as well as the posterior lateral AID, was one of a few locations where the higher concentration of NaCl elicited more Fos-IR neurons in the GC than the lower concentration (Fig. 7). Finally, the aversive TR responses to QHCl and HCl showed a strong positive relationship with the number of Fos-IR neurons in the posterior medial AID ($R=0.842$, $p=0.002$). No such relationship was found between aversive TR responses to these tastants and the number of labelled neurons in the anterior part of this GC subarea.

3. Discussion

There is some evidence of a functional topography within the rat GC (Accolla et al., 2007; Kosar et al., 1986a; Norgren, 1995; Yamamoto et al., 1981; 1989) however; a precise and complete distribution of the neurons activated by dH₂O and the five prototypical tastants has not been established. Therefore, the main goal of the current study was to describe the distribution of the neurons in GC subareas along their entire anterior-posterior axis that are activated by intra-oral infusion of dH₂O or tastants in conscious rats using immunohistochemistry to the Fos protein. The main findings were that only intra-oral infusion of HCl or QHCl significantly increased the total number of Fos-IR neurons in the GC and that these increases were not confined to one GC location. In addition, intra-oral infusion of dH₂O, NaCl, or sucrose altered the number of Fos-IR neurons only in specific subareas of the GC and the effects of these tastants were concentration-dependent. Another goal of the current study was to identify GC areas that may be involved in eliciting TR

behaviors. Linear regression analyses suggested that neurons in the medial dorsal AI of the posterior GC may influence aversive behavioral responses to HCl and QHCl while neurons in the posterior lateral dorsal AI and DI may play a role in aversive TR responses to 1.0M NaCl. Interestingly, neurons in the lateral dorsal AI in the posterior GC also were identified as potentially playing a role in ingestive responses to intra-oral infusion of dH₂O. Finally, the number of Fos-IR neurons in the medial ventral AI in the anterior GC was found to be related to the behavioral responses to sucrose.

3.1 Technical Considerations

Before interpreting the results of the current study it is important to consider some potential limitations of the techniques employed. The Fos immunohistochemistry technique allows the number and specific location of individual neurons activated by a particular treatment to be identified in brain tissue. This technique was employed in the current study to determine the number and distribution of neurons in specific subareas of the GC activated by intra-oral infusion of dH₂O or tastants in conscious rats. It is important to consider that only a subset of active neurons are labeled using this technique (Dragunow and Faull, 1989), so the number of Fos-IR neurons reported undoubtedly is lower than the total number activated by each treatment and therefore some potential differences caused by the treatments may have been missed.

Although the concentrations of tastants used in the current study were similar to those used in previous work (DiNardo and Travers, 1997; Harrer and Travers, 1996; King et al., 1999; Travers, 2002) and have been shown to elicit TR behaviors and Fos expression in the gustatory system (Harrer and Travers, 1996; Spector et al., 1988; Tokita et al., 2007), differences in concentration could significantly influence the results. To address this issue, both relatively low and high concentrations of NaCl and sucrose were used in the current study. Because the higher concentrations of these tastants elicited aversive TR behaviors, a potential change in the distribution of Fos-IR neurons that may accompany the hedonic change in NaCl or sucrose was addressed. Although the volume and rate of the intra-oral infusions (0.233mL/min for 5 min) were chosen to stimulate gustatory receptors adequately with minimal ingestion to prevent effects of visceral input on the behavioral and Fos-IR responses, it is likely that some of the palatable stimuli were consumed. Therefore, visceral input could have influenced Fos expression and behavioral responses. However, it should be noted that the volume and/or rate used in the current study were similar to or considerably less than those used in previous studies (DiNardo and Travers, 1997; Harrer and Travers, 1996; King et al., 1999; Tokita et al., 2007; Travers, 2002; Yamamoto et al., 1994).

Novel tastes have been shown to influence activity of neurons in the GC as detected using Fos immunohistochemistry (Koh et al., 2003; Lin et al., 2012). Therefore, it is possible that the novelty of the taste stimuli presented in the current study influenced the number of Fos-IR neurons counted. However, the effect of most taste stimuli on the number of Fos-IR neurons did not differ from dH₂O (which was not novel), and HCl and QHCl significantly increased the number of labelled neurons over the other novel treatments.

3.2 Effects of Intra-Oral Infusions on Fos-IR neurons in the GC

Many Fos-IR neurons were present in control rats even though they did not receive an intra-oral infusion. This result suggests that neural activity within the GC occurs without the introduction of a solution into the oral cavity and is consistent with previous studies using the Fos technique that examined brainstem gustatory structures (DiNardo and Travers, 1997; Harrer and Travers, 1996; Travers et al., 1999; Travers, 2002). In the unstimulated control rats in the current study, there was a distinct pattern of Fos-IR neurons with more labelled neurons tending to be located within the anterior, medial, and ventral GC. Intra-oral infusion of dH₂O had only minor effects on this pattern of Fos expression by causing an increase in Fos-IR neurons in one part of the anterior ventral AI and in the posterior lateral dorsal AI. The latter location also was identified as containing dH₂O-responsive neurons in a previous study (Accolla et al., 2007). The effects of intra-oral infusion of taste stimuli on active neurons within the GC were measured as changes in the number and location of Fos-IR neurons compared to the unstimulated control rats as well as the dH₂O-infused group. Only intra-oral infusion of HCl or QHCl increased the total number of Fos-IR neurons in the GC compared to the control group and consistently increased Fos expression throughout the GC compared to rats receiving dH₂O intra-orally. These data suggest that these two tastants, particularly QHCl, increase the number of active neurons throughout the anterior-posterior and dorsal-ventral extent of the GC and not in a particular GC region. These results for QHCl are consistent with a previous study that reported a robust increase in Fos-IR neurons throughout the anterior-posterior plane of the GC to intra-oral infusion of this tastant (King et al., 2014). It is possible that the bitter-activated neurons in different subareas and at different anterior-posterior levels of the GC may serve different functions as previously suggested (Cechetto and Saper, 1987; Hanamori et al., 1998; Sowards, 2004). For example, the QHCl-responsive neurons in anterior GC may be important for discrimination of this bitter tastant, those in the middle of the GC may promote gaping behavior (King et al., 2014), while neurons in posterior GC regions may be more important in behavioral responses influenced by visceral input, like conditioned taste aversions (Schier et al., 2014; 2016). So, although the current data confirm that GC areas previously identified to respond to bitter and sour tastes contain Fos-IR neurons following intra-oral infusion of QHCl or HCl (Accolla et al., 2007; Chen et al., 2011; Kobayashi et al., 2013; Yamamoto et al., 1989) they also suggest that neurons responsive to these tastants are not confined to these specific regions.

The effects of sucrose, NaCl, and MSG on the expression of Fos within the GC were much more restricted than the effects of HCl and QHCl. In particular, intra-oral infusion of MSG changed the number of Fos-IR neurons in only one section of the anterior lateral ventral AI compared to dH₂O. Interestingly, this change in Fos-IR neurons was a decrease and this area is not similar to the ventral central location proposed for umami taste in mice (Chen et al., 2011). Infusion of 0.1M sucrose did not change the pattern of Fos expression within the GC compared to dH₂O but 1.0M sucrose significantly reduced the number of Fos-IR neurons in the anterior ventral GC. Previous studies have identified the anterior GC as an area that contains neurons responding to intra-oral infusion of sucrose (Accolla et al., 2007; Chen et al., 2011; Kobayashi et al., 2010; Sugita and Shiba, 2003; Yamamoto et al., 1989; Yoshimura et al., 2004) however the current study suggests that the number of active neurons in this

region actually is reduced by sweet taste as the concentration increases. Clearly, many Fos-IR neurons remain in the anterior GC during 1.0M sucrose infusion and their activity may account for the previous results. Similarly, introduction of 0.1M NaCl into the oral cavity did not alter Fos expression in the GC compared to dH₂O however 1.0M NaCl did increase the number of Fos-IR neurons in the lateral dorsal AI and lateral DI in the middle and posterior GC regions. Previously, the middle of the GC was identified as responding to salt taste (Accolla et al., 2007, Yamamoto et al., 1989; Yoshimura et al., 2004). The inclusion of the posterior GC in the area activated by NaCl is different from the concentration effects reported by Accolla et al. (2007) and may be due to the high concentration of salt becoming aversive.

Although some GC areas more responsive to particular tastes were identified in the current study, the overlap of the position of Fos-IR neurons following intra-oral infusion of different tastants does not support the existence of areas with neurons that respond only to a specific taste in the rat GC. Instead, the current findings are more consistent with the idea that GC neurons respond to multiple tastants (Scott and Plata-Salamán, 1999; Stapleton et al., 2006; Yamamoto et al., 1985) and that populations of neurons that respond to different tastants overlap significantly within the rat GC (Accolla et al., 2007; Schoenfeld et al., 2004; Scott and Plata-Salamán, 1999). It is likely that other aspects of GC neural responses (e.g., temporal patterns), that would not be detected with Fos immunohistochemistry, are vital for coding taste quality (Katz et al., 2001).

3.3 Relationship between Fos-IR Neurons in GC and TR Behaviors

Due to the limited number of animals in each group, and therefore, the potential of a few outliers strongly influencing the relationship between the number of Fos-IR neurons in the GC and TR behaviors performed, care should be taken when interpreting the results of the linear regression analyses. Nevertheless, these analyses suggested a few potential relationships between active GC neurons and the behaviors performed. Even when data were combined for these analyses (across concentrations of NaCl and sucrose, and HCl with QHCl) the number of Fos-IR neurons in only a few GC areas was suggested to be related to the TR behaviors performed. Specifically, the data suggested that neurons in the posterior medial dorsal AI may play a role in the aversive TR behaviors performed during intra-oral infusion of QHCl and HCl. This suggestion is consistent with previous reports that neurons in the posterior GC preferentially respond to aversive tastes (Accolla et al. 2007; Yamamoto et al. 1989), are involved in gaping behavior (King et al. 2014), and promote avoidance behaviors when stimulated in mice (Peng et al. 2015). In addition, the current analyses suggest that neurons in the posterior lateral DI may play a role in the aversive responses to 1.0M NaCl. It appears, then, that aversive behaviors to a high concentration of salt are influenced by different GC neurons than aversive behaviors to sour and bitter. Interestingly, this general cortical area has been shown to become active when palatable tastes are avoided after taste aversion (Accolla and Carelton, 2008), so the observed increase in Fos-IR neurons in posterior GC may account for the increase in aversive behaviors performed to intra-oral infusion of 1.0M NaCl compared to 0.1M NaCl. More work is necessary to confirm that aversive behaviors to HCl and QHCl are due to activation of different neuronal populations than are aversive responses to other tastants. The suggested relationship between the number

of neurons in the posterior lateral dorsal AI and the number of ingestive TR behaviors to dH₂O suggests a possible functional role for this region of the GC. Although this area has been shown to contain water-responsive neurons (Accolla et al. 2007), there has been no other report of an association of this area with ingestive behaviors; therefore this finding is novel and warrants further investigation. Finally, the decrease in the number of Fos-IR neurons in the ventral medial AI in the anterior GC may be related to the increase in aversive responses to the high compared to the low concentration of sucrose. This finding is consistent with the anterior GC being responsive to sweet tastes (Accolla et al. 2007; Yamamoto et al., 1989; Yoshimura et al., 2004) and activity in that area influencing ingestive responses (Peng et al. 2015).

3.4 Conclusions

Using Fos immunohistochemistry, the current study mapped the location of neurons in the GC that respond to intra-oral infusion of taste solutions and dH₂O in conscious rats. Because the Fos technique identifies individual active neurons, details about the number and location of neurons that respond to intra-oral infusion of solutions and are potentially involved in the behavioral responses to these solutions were revealed. It was found that bitter and sour tastes activated neurons throughout the GC while other tastes altered the number of Fos-expressing neurons in fewer GC areas. Specifically, intra-oral infusion of dH₂O activated neurons in the posterior lateral dorsal agranular insula, umami-responsive neurons were limited to the anterior lateral ventral agranular insula, a high concentration of salt increased Fos-IR neurons in the middle and posterior lateral agranular and dysgranular cortices, and intra-oral infusion of a strongly sweet solution reduced the number of active neurons in the anterior ventral GC. In addition, the current findings suggest that neurons in the medial dorsal agranular insula in the posterior GC are important in the aversive TR responses to bitter and sour, neurons in the posterior lateral dorsal agranular insula may influence aversive behaviors to high salt concentrations, and neurons in the anterior medial agranular insula may play a role in behavioral responses to sweet. Additional studies combining behavioral with anatomical techniques are necessary to confirm these possibilities.

4. Experimental Procedure

4.1 Animals

Male Wistar rats (Charles River Laboratories) were housed individually in standard hanging stainless steel cages in a secluded room with a 12h light-12h dark cycle and constant access to water and standard block rodent food (Harlan Teklad). The housing conditions and procedures that were performed during this study conform to the guidelines of the National Institutes of Health and were approved by the Stetson University Animal Care and Use Committee. Data from 45 rats (270–340g) are included in this report (N=5 in each group).

4.2 Surgical Implantation of Intra-Oral Cannulas

Each rat had intra-oral cannulas implanted bilaterally. Only one cannula was used to infuse taste solutions during experimentation but two were implanted just in case one became non-functional. The cannulas were formed from approximately 1.0 cm of PE-100 tubing that had a Teflon washer threaded onto one end that was then heat flanged to secure the washer. One

side of the washer was cut flat to allow it to sit beside the gum comfortably when in place. The surgical procedure to implant the cannulas was similar to that previously described (Grill and Norgren, 1978; King et al., 1999; Morganti et al., 2007; Riley and King, 2013). Briefly, rats were anesthetized by i.p. injection of 60 mg/kg sodium pentobarbital and placed in a stereotaxic device with non-traumatic ear bars (Stoelting Co.) so that the top of the skull was horizontal. The scalp was shaved and cleaned with a betadine solution and a 1–2 cm incision was made in the scalp. The tubing of the intra-oral cannula was connected to a 20-gauge syringe needle that allowed it to be inserted through the temporal muscle just anterolateral to the first maxillary molar and brought up the side of the skull, under the skin, to exit the incision in the scalp. On the top of the skull the PE tubing was cut and connected to about 1.0 cm of 19-gauge stainless steel tubing, tied with suture, and secured in place with three small screws embedded into the skull and dental acrylic. Finally, a topical antibiotic was applied, the skin sutured shut, and each rat placed back into its home cage after a brief recovery on a heated pad.

4.3 Behavioral Testing

The rats were given one week to recover from surgery before behavioral testing. On each day during recovery the wound was examined for infection, the rats weighed to assess recovery, and the intra-oral cannulas flushed with dH₂O. For three days prior to behavioral testing, each rat was placed into the behavioral arena for thirty minutes without stimulation to allow for acclimation to the testing environment. The behavioral arena was located in an isolated room and consisted of an opaque cylinder (26 cm tall and 26 cm diameter) mounted on a Plexiglas stand with a mirror underneath the platform to allow visualization of the rats from below. On testing day, one intra-oral cannula was attached to tubing connected to a 10 ml syringe that was held within a syringe pump (Harvard Apparatus) and the rat was placed into the arena for 30 min before testing. Then, intra-oral infusion of dH₂O, 0.10 and 1.0M NaCl, 0.10 and 1.0M sucrose, 0.03M HCl, 0.003M QHCl, or 0.32M monosodium glutamate (MSG) plus 100μM amiloride hydrochloride and 2.5mM inosine 5'-monophosphate (Sigma-Aldrich) occurred over 5 min (0.233mL/min). The concentrations of tastants used were based upon the range employed by Grill and Norgren (1978). The amiloride hydrochloride and inosine 5'-monophosphate were included with the MSG to block the Na⁺ component and to enhance the glutamate effect, respectively, as demonstrated in a previous study (Smith and Spector, 2014). The same procedures were followed for unstimulated control rats but they did not receive an intra-oral infusion during the test period. During the 5 min test period TR behaviors were videotaped with S-VHS equipment.

4.4 Histology and Fos Immunohistochemistry

The histological techniques employed were adopted from work published by other researchers (Rowland et al. 1995; Harrer and Travers, 1996) and included in our previous publications (Morganti et al., 2007; Riley and King, 2013). Following the test period and 45 min to allow for expression of the Fos protein, the rats were sacrificed with an overdose of sodium pentobarbital (80 mg/kg). Once unresponsive to toe pinch, the rats were perfused intra-cardially with about 200 ml of cold heparinized 0.15M sodium chloride followed by about 500 ml of sodium phosphate-buffered 4% paraformaldehyde. The brains then were removed and post-fixed overnight at 4°C and then cut into 75 μm coronal sections using a

vibratome. Every other section was treated with 1% sodium borohydride in potassium phosphate-buffered saline (KPBS) for 20 min. Following rinses in KPBS, the brain sections were incubated in a Fos primary antibody raised in rabbit (Santa Cruz Biotech) diluted at 1:10,000 in KPBS with 0.4% Triton X-100 for 72 h at 4°C. After incubation in the primary antibody, the sections were rinsed with KPBS and incubated in biotinylated goat anti-rabbit IgG (Vector Labs) at 1:600 in KPBS with 0.4% Triton X-100 for 4 h at room temperature. The sections then were rinsed using KPBS and incubated in the reagents of an ABC kit (Vector Labs) overnight at 4°C. Finally, the sections were rinsed in KPBS and reacted in 0.1 M sodium phosphate buffer containing 0.03% diaminobenzidine, 0.008% nickel ammonium sulfate, 0.008% cobalt chloride, and 0.0075% H₂O₂ for 9 min at room temperature. Following a final rinse in KPBS, the sections were mounted on gelatin- and chrome alum-coated glass slides, let to dry overnight, and then coverslips mounted using Permount (Fisher Scientific). The alternate sections that were not processed for the Fos protein were mounted on slides and Nissl-stained with 0.1% thionin.

4.5 Data Analysis

Fos-IR neurons were counted in the right GC in 8 brain sections extending from approximately 1.7 mm anterior to bregma to 0.4 mm posterior to bregma (Paxinos and Watson, 2007). The anterior-most section (section 1) was the first in which the corpus callosum crossed the midline, the fourth section was where the medial edge of the anterior commissure just reached the ventral edge of the lateral ventricle, and the posterior-most section (section 8) was where the anterior commissure no longer crossed the midline (King et al., 2014). Within each section analyzed, the number of Fos-IR neurons in the medial and lateral halves of the GI, DI, AID, and AIV subareas was counted (Fig. 1A). The GC subareas were outlined in Nissl-stained tissue viewed on a Zeiss AxioPlan 2 light microscope equipped with a video camera using Paxinos and Watson (2007) as a guide. The ventral-most border of the GC was determined by drawing a line from the lateral edge of the brain just dorsal to the piriform cortex to the ventral edge of the claustrum (Fig. 1A). The dorsal-most border of the GC was at the dorsal edge of the claustrum and dorsal to the point where layer 4 was no longer defined. The rhinal fissure was used as a landmark for the border between the DI and AID subareas. In each section analyzed the distance from the dorsal to the ventral edge of the GC always was the same but varied a bit along the anterior-posterior axis of the GC. To improve the accuracy of the description of the location of Fos-IR neurons, each GC subarea was split into equal medial and lateral halves (Fig. 1A). The line separating medial from lateral always was within cortical layer 5 (the granular cell layer was in the lateral half of GI). Once the GC subareas were outlined in the Nissl-stained tissue, the outlines were superimposed onto the corresponding Fos-labeled sections on the computer. These outlines were visible as the number of Fos-IR neurons in each subarea were counted manually on the microscope while being viewed at 200x magnification. The neuron counts were performed by an investigator who was unaware of the behavioral response outcomes. As a control for the technique of counting Fos-IR neurons, labelled neurons were counted in the same 8 subareas described above in a section of the perirhinal cortex 2.0 mm posterior to bregma in rats that received intra-oral infusion of dH₂O, HCl or QHCl.

TR behaviors were viewed frame by frame and counted for the entire 5 min test period using previously described criteria (Grill and Norgen, 1978; Spector et al., 1988) by an investigator who was unaware of the tape sequence being analyzed. Only one investigator reviewed the videotapes as in previous studies (King et al., 2008; Riley and King, 2013). Ingestive behaviors counted were mouth movements, lip flares, tongue protrusions, and lateral tongue protrusions. Mouth movements included all movements of the mouth when closed as well as small openings of the mouth. Lip flares were an indentation or splitting of the upper lip. Tongue protrusions were when the tongue protruded from the mouth in the midline while during lateral tongue protrusions the tongue exited the mouth to one side. Aversive behaviors counted were gapes, chin rubs, head shakes, and forelimb flails. Gapes were large openings of the mouth during which the upper and lower incisors were visible. Chin rubs were extensions of the neck that brought the chin into contact with the Plexiglas floor or a side of the behavioral arena. Head shakes were rapid side to side movements of the head and forelimb flails were when either one or both of the forepaws were moved back and forth rapidly. The number, type, and timing of each behavior were recorded. Total ingestive and aversive scores reflect the sum of the occurrences of each individual oromotor behavior.

Statistical analyses were accomplished by first performing separate two-way analysis of variance tests (ANOVA) for each section with treatment and subarea as factors and the number of Fos-IR neurons as the dependent variable. If the two-way ANOVAs revealed a significant effect of treatment or an interaction between treatment and subarea ($p < 0.05$), then post-hoc tests (Fisher's Least Significance Difference (LSD) tests) were used to determine differences among treatment groups. The statistically significant results of post-hoc tests on data where the two-way ANOVA did not show an interaction between treatment and subarea are included as a descriptive tool but should be interpreted cautiously. Single factor ANOVAs with LSD post-hoc tests were used to determine if behavioral responses and the total number of Fos-IR neurons in the GC were different among groups receiving different tastes. To specifically address concentration effects, differences between 1.0 and 0.1M NaCl and 1.0 and 0.1M sucrose were determined using Bonferroni corrected Student's T-tests. Potential relationships between the number of TR behaviors performed and the number of Fos-IR neurons in a GC subarea at particular anterior-posterior positions under each stimulation condition were investigated using linear regression analysis. For some of the linear regression analyses, data from the 0.1M and 1.0M sucrose, 0.1M and 1.0M NaCl, and HCl and QHCl groups were combined to increase the number of data points from 5 to 10. In addition, the data for each subarea over 4 adjacent sections were combined for these analyses so that data from the anterior, middle, and posterior 4 sections were analyzed together. In this analysis, data from the middle 4 sections were represented twice; once in the 'middle 4' calculation and then again in the 'anterior 4' (sections 3 and 4) and 'posterior 4' (sections 5 and 6) calculations.

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- Fos immunohistochemistry was used to map neurons in the rat gustatory cortex (GC).
- dH₂O, NaCl, sucrose, MSG, HCl, or QHCl was infused intra-orally in conscious rats.
- HCl and QHCl caused increases in Fos-IR neurons throughout the GC.
- NaCl and sucrose had discrete and concentration-dependent effects on Fos neurons.
- Neurons in the posterior medial agranular GC may influence aversive behaviors.

Fig. 1.

Images of coronal sections through the middle of the gustatory cortex (~0.7 mm anterior to bregma). A. Nissl-stained section outlining the granular (GI), dysgranular (DI), and dorsal and ventral agranular (AID and AIV) insular cortices. Each subarea is split into medial and lateral halves. B.-D. Fos-stained section of the same region from rats that received intra-oral infusion of dH₂O (B), HCl (C), or QHCl (D). Other abbreviations are: Cl, claustrum; ec, external capsule, Pir, piriform cortex, and rf, rhinal fissure.

Fig. 2.

Graph of the mean total number of Fos-IR neurons in the whole GC for each treatment group. # indicates a significant difference from all other treatments except dH₂O and QHCl, while * indicates a significant difference from all other treatments except HCl ($p < 0.02$).

Fig. 3.

Graphs of the mean number of Fos-IR neurons in the medial and lateral halves of each subarea in each of the eight sections examined along the anterior-posterior axis of the GC in unstimulated (none) control rats (A) as well as in rats that received an intra-oral infusion of dH₂O (B), 0.1M sucrose (C), 1.0M sucrose (D), 0.1M NaCl (E), and 1.0M NaCl (F). A circle on the top front face or top edge of a bar indicates a significant difference from the corresponding bar on the adjacent graph (comparing dH₂O to none, 0.1M sucrose to 1.0M sucrose, and 0.1M NaCl to 1.0M NaCl). An asterisk (*) on a bar indicates a significant difference ($p < 0.05$) from dH₂O.

A. dH₂O **B. MSG** **C. HCl** **D. QHCl** **Fig. 4.**

Graphs of the mean number of Fos-IR neurons in the medial and lateral halves of each subarea in each of the eight sections examined along the anterior-posterior axis of the GC in rats that received an intra-oral infusion of dH₂O (A), 0.32M MSG plus 100μM amiloride and 2.5 mM IMP (B), 0.03M HCl (C), and 0.003M QHCl (D). An asterisk (*) on the top front face or top edge of a bar indicates a significant difference ($p < 0.05$) from dH₂O.

Fig. 5.

Graphs of the mean number of Fos-IR neurons in different anterior-posterior regions of the lateral (A) and medial (B) halves of the GI subarea of the GC. Data in the 'anterior 4' group were from sections 1-4 and the 'posterior 4' group includes data from sections 5-8. Data in the 'middle 4' group were collected from sections 3-6. Each triplet of bars represents the results for the different treatment groups indicated along the x-axes. An asterisk (*) above a bar indicates a significant difference ($p < 0.05$) from the corresponding bar for the control group (none) while a 'w' indicates a difference from the dH₂O group. New abbreviations are: LS (0.1M sucrose), HS (1.0M sucrose), LN (0.1M NaCl), and HN (1.0M NaCl).

Fig. 6.

Graphs of the mean number of Fos-IR neurons in different anterior-posterior regions of the lateral (A) and medial (B) halves of the DI subarea of the GC. Data were combined as described in Methods and the caption for Fig. 5. Statistics are indicated as in Fig. 5 with the addition of brackets joining two bars from above indicating a significant difference between those bars. Abbreviations are as in Fig. 5.

Fig. 7.

Graphs of the mean number of Fos-IR neurons in different anterior-posterior regions of the lateral (A) and medial (B) halves of the AID subarea of the GC. Data were combined as described in Methods and the caption for Fig. 5. Statistics and abbreviations are indicated as in Figs. 5 and 6.

Fig. 8.

Graphs of the mean number of Fos-IR neurons in different anterior-posterior regions of the lateral (A) and medial (B) halves of the AIV subarea of the GC. Data were combined as described in Methods and the caption for Fig. 5. Statistics and abbreviations are indicated as in Figs. 5 and 6.

Fig. 9.

Graph of the mean number of ingestive and aversive taste reactivity (TR) behaviors performed during the 5 min behavioral trial in the control group (none) as well as in rats receiving an intra-oral infusion of dH₂O and each taste solution. An asterisk (*) above a bar indicates a significant difference from all other groups ($p < 0.05$), while the other symbols indicate a difference from the rats that received an intra-oral infusion of dH₂O (w), 0.1M sucrose (LS), and 0.1M NaCl (LN).

Fig. 10.

Results of linear regression analyses of the relationship between the mean number of Fos-IR neurons in a GC subarea within 4 sections along the anterior-posterior axis of the GC and the number of taste reactivity (TR) behaviors performed. A. The relationship between the number of Fos-IR neurons in the posterior four sections of the lateral AID subarea and the number of ingestive TR behaviors performed in rats that received intra-oral infusion of dH₂O. B. The relationship between the number of Fos-IR neurons in the anterior four sections of the medial AIV subarea and the number of aversive TR behaviors performed in rats that received intra-oral infusion of 0.1M and 1.0M sucrose. (The 4 data points above 10 aversive behaviors represent 4 of the 5 responses to 1.0M sucrose.) C. The relationship between the number of Fos-IR neurons in the posterior four sections of the lateral DI subarea and the number of aversive TR behaviors performed in rats that received intra-oral infusion of 0.1M and 1.0M NaCl. (The 5 data points above 20 aversive behaviors represent responses to 1.0M NaCl.) D. The relationship between the number of Fos-IR neurons in the posterior four sections of the medial AID subarea and the number of aversive TR behaviors

performed in rats that received intra-oral infusion of 0.03M HCl and 0.003M QHCl. (The 5 data points above 90 aversive behaviors represent responses to QHCl.)

Table 1

P values resulting from the two-way ANOVAs to discern the effect of treatment and subarea on the number of Fos-IR neurons in each section of the GC studied.

Section *	Treatment main effect	Subarea main effect	Treatment x Subarea
1	<0.001	<0.001	0.984
2	<0.001	<0.001	0.997
3	<0.001	<0.001	0.712
4	<0.001	<0.001	0.972
5	<0.001	<0.001	0.064
6	<0.001	<0.001	0.003
7	<0.001	<0.001	0.002
8	<0.001	<0.001	0.013

* section 1 is the anterior-most