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Restoration of quinine-stimulated Fos-immunoreactive neurons in the central nucleus of the amygdala and gustatory cortex following reinnervation or cross-reinnervation of the lingual taste nerves in rats

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Abstract

Remarkably, when lingual gustatory nerves are surgically re-routed to inappropriate taste fields in the tongue, some taste functions recover. We previously demonstrated that quinine-stimulated oromotor rejection reflexes and neural activity (assessed by Fos-immunoreactivity) in subregions of hindbrain gustatory nuclei were restored if the posterior tongue, which contains receptor cells that respond strongly to bitter compounds, was cross-reinnervated by the chorda tympani nerve. Such functional recovery was not seen if instead, the anterior tongue, where receptor cells are less responsive to bitter compounds, was cross-reinnervated by the glossopharyngeal nerve, despite that this nerve typically responds robustly to bitter substances. Thus, recovery depended more on the taste field being reinnervated than on the nerve itself. Here, the distribution of quinine-stimulated Fos-immunoreactive neurons in two taste-associated forebrain areas was examined in these same rats. In the central nucleus of the amygdala (CeA), a rostrocaudal gradient characterized the normal quinine-stimulated Fos response, with the greatest number of labeled cells rostrally situated. Quinine-stimulated neurons were found throughout the gustatory cortex but a ‘hot spot’ was observed in its anterior-posterior center in subregions approximating the dysgranular/agranular layers. Fos neurons here and in the rostral CeA were highly correlated with quinine-elicited gaps. Denervation of the posterior tongue eliminated, and its reinnervation by either nerve restored, numbers of quinine-stimulated labeled cells in the rostral-most CeA and in the subregion approximating dysgranular gustatory cortex. These results underscore the

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CONFLICT OF INTEREST:

All authors declare no conflict of interest.

ROLE OF AUTHORS:

All authors had full access to all the data in the study, and they both take responsibility for the integrity of the data and the accuracy of the data analysis. Acquisition of data: CTK, MG; Statistical Analysis: CTK, ACS; Analysis and interpretation of data: ACS, CTK; Drafting of the manuscript: CTK, ACS; Critical Revision: ACS, CTK, MG; Obtained funding: ACS; Administrative, technical, and material support: MG; Study supervision: ACS

remarkable plasticity of the gustatory system and also help clarify the functional anatomy of neural circuits activated by bitter taste stimulation.

Keywords

bitter taste; insular cortex; taste reactivity; hedonic; palatability; neural plasticity; brain mapping

Introduction

Taste receptor cells on the tongue are continually replaced, yet characteristic taste response profiles of the gustatory nerves which innervate them remain stable and support proper taste function (Beidler and Smallman, 1965; Farbman, 1980), a fact that highlights the natural capacity of the central gustatory system to adapt to normal changes in peripheral input. Studies examining the consequences of gustatory nerve transection and regeneration on taste function have served to further illuminate the extent of the plasticity of the gustatory system. For instance, bilateral transection of the glossopharyngeal nerve (GL), which innervates the taste buds of the posterior tongue, severely attenuates stereotypical oromotor rejection behaviors (e.g. gaping) elicited in response to intraorally delivered bitter substances like quinine (Grill and Norgren, 1978a; Travers et al., 1987; Grill and Schwartz, 1992; Grill et al., 1992; King et al., 2000). However, upon regeneration of the GL into the posterior tongue, taste buds are re-innervated, and full recovery of gaping behavior ensues (King et al., 2000, King et al., 2008). Because bilateral transection of the chorda tympani nerve (CT), which innervates taste buds of the anterior tongue, has little effect on gaping (Travers et al., 1987; Grill and Schwartz, 1992; Grill et al., 1992), the above findings underscore the necessity of the posterior tongue and/or the GL in maintaining gaping behavior, but they cannot specify whether the restored behavior depends upon the extrinsic input stemming from the posterior receptor field, and/or something intrinsic to the GL itself or its central nervous system targets.

Investigations of the effects of cross-regeneration of sensory nerves in the visual system and in the taste system have, however, provided strong support for the hypothesis that function following the regeneration of a nerve relies upon input stemming from specific receptor fields. In a remarkable series of experiments in the visual system, Sur and his colleagues redirected inputs from the retina to the auditory cortex in neonatal ferrets (Sharma et al. 2000; von Melchner et al., 2000). The cross-wired projections induced visual orientation columns in the auditory cortex that successfully mediated normal visual behavior. That is, the animals responded to a light stimulus presented to the cross-wired visual field as though they perceived a visual rather than an auditory stimulus despite the fact that information from the photoreceptors had been re-routed to the auditory cortex. Although the von Melchner et al. (2000) study involved a nervous system still in development, in a now classic experiment in the field of taste, Oakley (1967) found that adult rat taste nerves, when made to regenerate into non-native receptor fields, acquired taste properties that were characteristic of the new location in the tongue. Specifically, using whole nerve electrophysiology, he showed that when CT nerve fibers were cross-wired into the posterior tongue instead of their normal target in the anterior tongue, the CT responded well to

quinine and poorly to NaCl, response characteristics typical of the GL but not the CT. The reverse held true when he redirected the GL into the anterior tongue; the response properties of the cross-wired GL were more similar to those of a normal CT. Oakley's findings confirmed that the response profiles of the nerves relied on their peripheral targets in the tongue, a finding that later received support from another electrophysiological study on the cross-reinnervation of gustatory nerves in rats (Nejad and Beidler, 1994, but see also, Ninomiya, 1998).

To examine the effects of the cross-wiring of these gustatory nerves on oromotor reflexes, King et al. (2008) more recently cross-wired the GL to the anterior tongue, leaving the posterior tongue denervated. In other animals, the CT was cross-wired to the posterior tongue, leaving the anterior tongue without gustatory innervation. Animals in which the non-native CT was re-routed to the posterior tongue displayed relatively normal oromotor reflexes in response to intraorally delivered quinine. On the contrary, the animals in which the GL was re-routed to the anterior tongue did not. In fact, these animals behaviorally responded to the intraoral infusion of quinine as though they were receiving water. These data substantiate the hypothesis that function following the regeneration of a taste nerve relies upon the receptor field in the tongue innervated rather than the taste nerve itself.

In the rat, the CT and GL project in a roughly topographic fashion to the nucleus of the solitary tract (NST) with the CT terminating more rostrally in the nucleus than the GL, though overlap in their terminal fields exists (Hamilton and Norgren, 1984; King and Hill, 1991; May and Hill, 2006; Corson and Hill, 2011; Corson et al., 2012). From the standpoint of this topographic correspondence between the tongue and the NST, cross-reinnervation of these nerves necessarily "miswires" the brain, yet somehow the neural structures involved in eliciting oromotor reflexes in response to quinine are sufficiently activated - provided the neural signal originates in the posterior tongue. Several studies have demonstrated a robust and characteristic distribution of Fos-immunoreactive (FI) neurons, a marker of neuronal activation (Dragunow and Faull, 1989) in both the NST and the parabrachial nucleus (PBN, the second synaptic relay in the rat central gustatory system) following the infusion of quinine (Yamamoto et al., 1994; Harrer and Travers, 1996; King et al., 1999, 2000, 2003; Yamamoto and Sawa, 2000; Travers, 2002; Chan et al., 2004). As with the gaping behavior, the quinine-stimulated Fos response in these gustatory structures resembled the pattern observed in water-stimulated animals if the GL was severed. Moreover, when either the native GL or the non-native CT reinnervated the posterior tongue, the numbers of quinine-stimulated neurons in critical subregions within the NST and PBN returned to normal, providing support for the role of these hindbrain structures in the processing of bitter taste information (King et al., 2008).

Because the intraoral infusion of quinine elicits gapes (and other oromotor reflexes) in chronic supracollicular decerebrate rats, in which the forebrain has been disconnected from the hindbrain (Grill and Norgren 1978b; Flynn and Grill 1988; Travers et al., 1999), the hindbrain is apparently sufficient for maintaining unconditioned rejection responses to aversive taste stimuli and calls into question the necessity of forebrain taste regions. On the other hand, quinine does activate neurons in critical gustatory structures in the forebrain. The central nucleus of the amygdala (CeA), a structure known to be involved with the

regulation of motivated behavior, is a major recipient of gustatory projections from the PBN (Norgren 1976; Saper and Loewy, 1980; Voshart and van der Kooy, 1981), sends efferent fibers to the brainstem (Veening, et al. 1984), and responds robustly to quinine stimulation relative to other taste stimuli (Yasoshima et al., 1995; Nishijo et al., 1998, 2000). Moreover, Zhao et al. (2012) recently demonstrated a large population of quinine-stimulated FI-neurons in the CeA. Collectively, these findings suggest that the CeA is involved in processing information related to the presence of this bitter substance. But, because neurons responsive to non-taste oral stimulation are distributed throughout the amygdala (Nishijo et al., 1998, 2000), and because the CeA receives visceral input from the gastrointestinal tract as well as gustatory input (Cechetto, 1987; Cechetto and Saper, 1987), the Zhao et al. (2012) finding could potentially reflect, in part, the contribution of non-taste sensory signals to the Fos response observed. Accordingly, examination of the effects of taste nerve transection, regeneration, and cross-regeneration may shed light on the source of activation of CeA neurons in response to quinine and may provide insight into the role of the CeA in bitter taste.

Taste information from the PBN also ascends to specific portions of insular cortex, principally via the thalamus (Wolf, 1968; Norgren and Wolf, 1975; Norgren, 1976; Moga et al., 1980; Lasiter et al., 1982; Fulwiler and Saper, 1984; Kosar, et al., 1986b; Cechetto and Saper, 1987). It is presumed that the gustatory cortical area plays an important role in taste processing but its functional architecture remains unresolved. Historically, the gustatory cortex has been considered “broadly tuned”, its neurons responding “best” to one stimulus but also responding to other taste stimuli (for reviews see Spector and Travers, 2005; Simon et al., 2006; Carleton et al., 2010). Accolla et al. (2007) used *in vivo* intrinsic optical imaging, a technique that allows the examination of the activity of many neurons simultaneously, and reported that some primary tastes (e.g. bitter, sweet) were represented by distinctive spatial patterns in the gustatory cortex but their patterns were overlapping. That is, no region in the gustatory cortex was specific to a single taste modality. In contrast, Chen et al. (2011), using two-photon calcium imaging, reported very distinct “hot spots” for several taste modalities that were located more caudally in the gustatory cortex compared to sucrose stimulation, which activated a region rostradorsal to the bitter field. We are unaware of any published reports that have quantified the distribution of FI-neurons in the gustatory cortex following oral administration of quinine. The current study was conducted to examine the consequences of cross-regeneration of the CT and GL on the numbers of quinine-stimulated FI-neurons in the CeA and insular cortex to help elucidate their roles in bitter taste processing and to examine further the extent of plasticity within the central gustatory system.

Materials and Methods

Subjects

The forebrain structures analyzed for the current study are from the same brain tissues from an earlier report examining the effects of cross-regeneration on quinine-stimulated FI-neurons in the NST and PBN (King et al., 2008). The subjects were male Sprague-Dawley rats (Charles River Breeders: Wilmington, MA) weighing 250–275 g at the time of nerve

surgery. They were individually housed in hanging wire mesh cages where light cycle (lights on 6 a.m. – 6 p.m.), temperature, and humidity were automatically controlled. All manipulations were performed during the light phase. Laboratory chow (5001; PMI: St Louis, MO) and distilled water were available ad libitum. All animal procedures were performed in accordance with National Institutes of Health (NIH) guidelines for humane handling of animals and all protocols were approved by the Institutional Animal Care and Use Committees at the authors' institutions.

Surgical Procedures

The bilateral cross-anastomosis procedures, modified from those described by Oakley (1967), are described in detail elsewhere (King et al., 2008). The different surgical manipulations provided for 1) reinnervation of the posterior tongue taste receptor field by the native GL nerve with no reinnervation of anterior tongue (GL→PT, N = 5); 2) reinnervation of the anterior taste receptor field by the native CT nerve with no reinnervation of the posterior tongue (CT→AT, N = 4); 3) cross-reinnervation of the posterior tongue by the non-native CT nerve with no reinnervation of the anterior tongue (CT→PT, N = 5); 4) cross-reinnervation of the anterior tongue by the non-native GL with no reinnervation of the posterior tongue (GL→AT, N = 7); or 5) no reinnervation of the posterior or anterior tongue by either the GL or CT as these nerves were prevented from regenerating into the tongue (No Reg, N = 4). In addition, two sham-surgical groups - one stimulated with quinine (Sham-Q, N = 5), the other stimulated with water (Sham-W, N = 6) - were included, for a total of seven groups. All nerve surgeries were conducted between 149–231 days prior to behavioral testing to allow time for successful regeneration of the nerves. Because taste buds in the rat tongue degenerate and taste pores disappear when their nerve supply is interrupted (Guth, 1957; Ganchrow and Ganchrow, 1989), the taste pores in hemotoxylin and eosin-stained paraffin-embedded sections (10 μ m) of the circumvallate and foliate papillae, and in methylene blue-stained anterior tongues were counted by an experimenter unaware of the specific nerve-condition of the animals (King et al., 2008). The subjects included in the current analyses (the above noted numbers of subjects) had histologically confirmed denervation or reinnervation of taste buds.

Taste stimuli were delivered into the oral cavity via intraoral cannulae that were implanted two weeks prior to the beginning of the behavioral procedures for each rat (See Grill and Norgren, 1978a; King et al., 1999 for details of the procedure). The cannulae were cleaned daily to maintain patency and prevent infection. After the 14 day recovery period, behavioral procedures commenced.

Behavioral Procedures

As described by King et al. (1999, 2008), subjects were habituated to both the behavioral arena (a cylindrical Plexiglas chamber) and the infusion process (with distilled water) for 3 days prior to the test day. During the 30-min infusion period, 7 ml of distilled water or 3.0 mM quinine-hydrochloride (on test day only) were infused through the cannula at a rate of 0.233ml/min. During the first min, an experimenter videotaped the oromotor responses of each rat for subsequent scoring of taste reactivity behaviors. After 30 min, the infusion

pump was turned off but the animal was left in the behavioral arena for 45 min before either being returned to its home cage (habituation days) or anesthetized for perfusion (test day).

Brain Histology: Tissue Preparation

Immediately following the 45 min post-infusion period on the test day, animals were deeply anesthetized with an overdose of sodium pentobarbital (> 80 mg/kg bodyweight) and perfused intracardially with heparinized 0.15 M NaCl (~200 ml), followed by sodium phosphate buffered 4% paraformaldehyde, pH=7.3 (~500 ml). Brains were removed and postfixed overnight at 4°C. Each brain was cut in the coronal plane (75 µm) with the use of a Vibratome. Every other brain section was processed for Fos-immunoreactivity. Free-floating sections were pretreated for 20 min in a sodium borohydride solution (1% in potassium phosphate buffered saline, KPBS) to block endogenous peroxidase activity and then rinsed several times in KPBS before being placed in the primary antibody solution.

Antibody Characterization

The primary antibody (Table 1), a rabbit polyclonal antibody (c-Fos (4): sc-52, Santa Cruz Biotechnology, Santa Cruz, CA), was used at a dilution of 1:10,000 in 0.4% Triton X-100 in KPBS) for 72 hr at 4°C. The anti c-Fos antibody was generated against the epitope corresponding to the N-terminus residues 3–16 of the protein encoded by human *c-fos* and its specificity was verified by preabsorption and by Western analysis (see Gaszner et al., 2007).

Brain Histology: Immunohistochemistry

After several rinses in KPBS, the free floating sections were placed in biotinylated goat anti-rabbit IgG (Zymed, San Francisco, CA) at a dilution of 1:600 in 0.4% Triton X-100 solution for 4 hrs at room temperature. The tissues were rinsed again in KPBS before being placed in an avidin-biotinylated peroxidase complex solution (ABC kit: Vector Laboratories, Burlingame, CA) overnight at 4°C. Following several rinses in KPBS, the sections were reacted with 0.03% diaminobenzidine, 0.008% nickel ammonium sulfate, and 0.0075% hydrogen peroxide in sodium phosphate buffer. The reacted sections were mounted on chrome-alum subbed slides, dehydrated, and coverslipped. The alternate sections were mounted, stained with 0.1% thionin, dehydrated and coverslipped.

Microscopic Analysis of Brain Tissues

A Zeiss Axioscope microscope (equipped with a digital video camera coupled to a video monitor and computer) and AxioVision software by Zeiss were used to capture images of the brain regions of interest. All FI-neurons, regardless of staining intensity, within these regions were visually identified using 2.5X–40X objectives, individually tagged on the digital images, and counted by an experimenter who was unaware of the experimental condition of the animal. Our study was intended to determine similarities or differences in FI-neurons among groups and was not designed to ascertain absolute numbers of cells per brain area, thus, no comprehensive stereology method was employed (Saper, 1996). Because relatively accurate profile counts can be achieved if small objects are counted in thick sections (Guillery, 2002), we did not correct for the possibility of overcounting.

Identification of the CeA and the gustatory insular cortex was aided by the use of a stereotaxic atlas (Paxinos and Watson, 2007) and the Nissl stained sections.

CeA

The entire CeA, divided into four regions (Rostral, Intermediate-Rostral, Intermediate-Caudal, and Caudal), was examined. Three to four brain sections per animal comprised each of these brain regions, for a total of 10–14 Fos-immunoreacted brain sections per animal that contained the CeA. The most rostral of these sections occurred ~ -1.50 from Bregma. The Rostral region was defined as the portion of the CeA where the commissural stria terminalis (cst) was positioned ventral to the nucleus or just abutted its ventral border (Fig. 1A). For Intermediate-Rostral and Intermediate-Caudal regions, the cst was positioned in the ventral or dorsal half of the nucleus, respectively (Fig. 1B, 1C). The Caudal region typically began where the cst and the optic tract nearly fused (Fig. 1D). Because the number of sections per region (Rostral, Intermediate-Rostral, Intermediate-Caudal, and Caudal) was not identical in each subject, the mean number of FI-neurons per section per region was calculated and used for analysis.

Gustatory and Peri-Gustatory Insular Cortex

The delimitation of gustatory cortex was based on our assessment of the collective literature detailing the location of taste-responsive neurons and the pattern of projections arising from the gustatory zone of the thalamus in rats (e.g., Benjamin and Pfaffman 1955; Norgren and Wolf 1975; Yamamoto et al. 1980, 1984, 1985, 1989; Kosar et al., 1986a, b; Cechetto and Saper 1987; Ogawa et al. 1990, 1992; Hanamori et al., 1998). The anterior boundary of gustatory insular cortex was defined as the section in which the corpus callosum first joins at the midline; the posterior boundary was defined as the section just rostral to the one in which the anterior commissure first joins at midline (approximate level of Bregma). The brain sections within these anterior–posterior boundaries were divided into roughly equal thirds: Anterior, Middle, Posterior. For the majority (27) of subjects, Fos-immunoreacted brain sections within these boundaries totaled 12–14. For five subjects, the number of sections was 10–11 and for four subjects, the number was 15–16. In the Anterior sections, the anterior commissure was positioned approximately midway between the external capsule and the lateral ventricle (Fig. 1E). In the Middle sections it approached the lateral ventricle (Fig. 1F) and in the Posterior sections its tip was positioned ventromedial to the lateral ventricle (Fig. 1G). Two brain sections, roughly in the center of each region, were selected for acquisition. If a section identified for acquisition contained tissue that was damaged, the adjacent section on the slide was acquired. The total number of FI-neurons in the two sections per region was summed and used for analysis.

In each of the brain sections acquired for the cortex analysis, the insular cortex was divided into four dorsal-ventral ‘rectangular’ subregions: Dorsal (D), Intermediate-Dorsal (ID), Intermediate-Ventral (IV), Ventral (V), which collectively incorporate gustatory cortex and the insular cortex surrounding it. These subregions roughly approximate the granular, dysgranular, and dorsal and ventral agranular insular cortex as delineated in Paxinos and Watson (2007). Fig. 1E–H describes and depicts these subregions.

Statistical Analyses

Separate one-way ANOVAs, one for each major region identified in the CeA (Rostral, Intermediate-Rostral, Intermediate-Caudal, and Caudal) and in the gustatory insular cortex (Anterior, Middle, Posterior), as well as for each cortical subregion (Dorsal, Intermediate-Dorsal, Intermediate-Ventral, Ventral) were conducted using the seven groups to assess main effects. If the ANOVA revealed significant differences, then post hoc Bonferroni tests were performed. Pearson's correlation procedure (with Bonferroni corrections) was used to assess significant relationships between quinine-stimulated gapes and the number of FI-neurons in the identified regions and subregions. The statistical null hypothesis rejection criterion used for significance was $P = 0.05$.

Results

CeA: Rostrocaudal Analysis

As reported recently (Zhao et al., 2012), many FI-neurons were observed in the CeA after intraoral stimulation with quinine (Figs. 2, 3). Our analysis revealed that these FI-neurons are not distributed equally throughout the rostrocaudal extent of the nucleus. In the Rostral CeA ($F_{(6,29)} = 33.69$, $P < 0.00001$) and the Intermediate-Rostral CeA ($F_{(6,29)} = 7.54$, $P < 0.0001$), intraoral stimulation with quinine produced significantly more FI-neurons than did water stimulation (P 's < 0.01 , Figs. 2, 3). Although the Fos response in the Intermediate-Caudal CeA differed among the groups ($F_{(6,29)} = 2.75$, $P < 0.04$), none of the post hoc comparisons involving the Sham-Q and the Sham-W groups reached statistical significance (Figs. 2, 3). In the Caudal CeA, the fewest quinine-stimulated FI-neurons were elicited and there were no statistically significant differences detected among the groups ($F_{(6,29)} = 1.68$, $P = 0.16$, Figs. 2, 3). Collectively, these data provide support for a rostrocaudal gradient of quinine-stimulated FI-neurons in the CeA. This gradient can be visualized in Fig. 4 in which the quinine-stimulated Fos response is represented in a temperature scale as a percent increase over the water-stimulated Fos response across the rostrocaudal levels of the CeA examined. In Sham-Q animals, the largest percent increase ($>300\%$) occurred in the Rostral region and progressively decreased in the other three CeA regions (201%, 162%, and 35%, respectively). Unfortunately, this pattern could not be statistically confirmed because it is based on percentages formed from the group averages, but Fig. 4 does provide a useful map of quinine-stimulated relative to water-stimulated neuronal activity, as determined by Fos expression, across the regions examined.

Preventing CT and GL innervation of the tongue (No Reg group) eliminated the normal quinine-stimulated Fos response in the Rostral CeA (Figs. 2, 5). In fact, the number of quinine-stimulated FI-neurons in the No Reg group was not statistically different from the number of water-stimulated FI-neurons in nerve-intact animals (Sham-W group). In the Intermediate-Rostral CeA, the Fos response in the No Reg group fell midway between the Sham-Q and Sham-W responses; the mean number of quinine-stimulated neurons in this group was not statistically different from the values in either of the control groups (Fig. 2).

When the gustatory nerves reinnervated the tongue, the quinine-stimulated Fos response was differentially affected depending on the field reinnervated. Reinnervation of the posterior

tongue by either the native GL nerve (GL→PT) or the non-native CT nerve (CT→PT) restored quinine-stimulated FI-neurons in the Rostral CeA to a mean value comparable to that observed in quinine-stimulated controls (Sham-Q) and significantly greater than that found in water-stimulated controls (Sham-W; $P < 0.01$, Figs. 2, 5). In the Intermediate-Rostral CeA, reinnervation of the posterior tongue (GL→PT) likewise yielded numbers of quinine-stimulated FI-neurons that were comparable to those observed in quinine-stimulated control animals (Sham-Q) and different from those in water-stimulated control animals (Sham-W, $P < 0.01$, Fig. 2). The number of FI-neurons in the Intermediate-Rostral CeA in animals with cross-reinnervation of the posterior tongue (CT→PT) however was not statistically different from either quinine- or water-stimulated animals (Figs. 2, 5).

The similarity of the pattern of the Fos response of the Sham-Q group with those of animals with reinnervation or cross-reinnervation of the posterior tongue can be seen in Fig. 4. A similar rostrocaudal gradient of quinine-stimulated, relative to water-stimulated, FI-neurons can be seen across the four CeA levels in the Sham-Q, GL→PT, and CT→PT groups. While the general pattern is the same, the magnitude of the response appears to be weaker in the latter two groups (especially in the CT→PT group).

In contrast, reinnervation of the anterior tongue by either its native CT nerve (CT→AT) or the non-native GL (GL→AT) did not restore the quinine-stimulated Fos response; significantly fewer quinine-stimulated FI-neurons in both the Rostral- and Intermediate-Rostral CeA were observed in these animals as compared with quinine-stimulated controls (Sham-Q; P 's = 0.02, Figs 2, 5). In fact, the numbers of quinine-stimulated FI-neurons in these animals was no different from those observed in the water-stimulated control group (Sham-W). Moreover, the rostrocaudal gradient of the quinine-stimulated Fos response observed in the Sham-Q, GL→PT, and CT→PT groups, was not seen in the groups in which the posterior tongue remained denervated (No Reg, CT→AT, GL→AT; Fig. 4).

Gustatory and Peri-Gustatory Insular Cortex: Anterior-Posterior Analysis

Significant differences in the number of FI-neurons among groups were observed in Anterior ($F_{(6,29)} = 7.65$, $P < 0.001$), Middle ($F_{(6,29)} = 9.32$, $P < 0.001$), and Posterior ($F_{(6,29)} = 8.17$, $P < 0.001$, Fig. 6) regions of the gustatory cortex and the surrounding insular cortex. At each of these anterior-posterior levels, intraoral stimulation with quinine in control animals (Sham-Q) elicited more FI-neurons than did water (Sham-W; P 's < 0.002, Figs. 6, 7). Although there appeared to be a greater number of quinine-stimulated FI-neurons in both the Anterior and Middle regions (as compared with the Posterior region) in the Sham-Q group, when these numbers were considered as a percentage increase over the mean response of the Sham-W group, the peak quinine-stimulated activation appeared to be positioned in the core (Middle sections) of gustatory cortex. The average percent increase across the four dorsal-ventral subregions in the Middle region of gustatory and peri-gustatory cortex was 234%, which is large, relative to the average percent increase in the Anterior (152%) and Posterior (158%) regions. As mentioned earlier, this difference could not be statistically confirmed because it is based on percentages formed from the group averages, but the map in Fig. 8 does provide a useful comparison of the quinine- relative to water-stimulated Fos responses across the regions examined.

Throughout the anterior-posterior plane of the insular cortex significantly fewer quinine-stimulated FI-neurons were observed in animals without lingual innervation by the GL and CT (No Reg, Figs. 6, 9). And, when the posterior tongue remained denervated, regardless of the whether the anterior tongue was reinnervated by the CT or cross-reinnervated by the GL, there was no significant increase in quinine-stimulated FI-neuron number relative to the Sham-W group (Figs. 6, 9).

Interestingly, only in the Middle region of insular cortex did reinnervation of the posterior tongue by the GL (GL→PT) fully restore the quinine-stimulated Fos response to values that were both similar to those in quinine-stimulated controls (Sham-Q) and different from those in water-stimulated controls (Sham-W; $P < 0.02$, Figs. 6, 9). When the non-native CT nerve cross-reinnervated the posterior tongue (CT→PT), the Fos response in these Middle sections was only partially restored in that the number of FI-neurons was similar to that found in quinine-stimulated controls (Sham-Q) but the comparison with water-stimulated controls (Sham-W) just missed the statistical significance criterion ($P = 0.065$, Figs. 6, 9). As was the case in the Sham-Q animals, the greatest average percent increase in quinine-stimulated, relative to water-stimulated FI-neurons, was also observed in the Middle gustatory and peri-gustatory region in animals that had the posterior tongue reinnervated (GL→PT) or cross-reinnervated (CT→PT), albeit that the magnitude of the response was reduced; a result that emulates what was found in the CeA (Fig. 8).

Gustatory and Peri-Gustatory Insular Cortex: Dorsal to Ventral Analysis

In ID ($F_{(6,29)} = 15.63$, $P < 0.001$), IV ($F_{(6,29)} = 10.59$, $P < 0.001$) and V ($F_{(6,29)} = 5.08$, $P < 0.002$), quinine-stimulation yielded significantly more FI-neurons than water stimulation in animals with intact nerves (Sham-Q vs Sham-W, all P 's, $P < 0.005$, Figs. 7, 10). However, in the dorsal-most subregion, D ($F_{(6,29)} = 4.06$, $P < 0.005$), the comparison between the Sham-Q and the Sham-W group did not yield a statistically significant difference ($P = 0.06$, Figs. 7, 10). The average percent increases (across the three anterior-posterior levels) in quinine-stimulated over water-stimulated FI-neurons were large in ID and IV (244% and 196%, respectively) as compared with the D (124%) and V subregions (161%). As can be seen in Fig. 8, the largest percent increases in ID and IV occurred in the core (Middle sections) of gustatory cortex (316% and 290%, respectively).

Denervation of the tongue (No Reg) severely attenuated the normal quinine-stimulated Fos response in all subregions except D (Figs. 9, 10). When the gustatory nerves reinnervated the tongue, quinine-stimulated activity was restored only in ID and IV, but once again, that recovery depended on the region of the tongue being reinnervated. In particular, in ID and IV, which roughly correspond to the dysgranular and dorsal agranular insular cortex subdivisions, respectively, reinnervation of the posterior tongue by the GL (GL→PT) restored the Fos response to values that were both similar to those in quinine-stimulated controls (Sham-Q) and different from those observed in water-stimulated controls (Sham-W; P 's < 0.009 , Figs. 9, 10). Likewise, upon reinnervation of the non-native CT into the posterior tongue (CT→PT), the quinine-stimulated Fos response was very similar to that observed in quinine-stimulated controls for these two subregions (ID and IV) and significantly different from the Fos response observed in water-stimulated animals in ID (P

< 0.02, Figs. 9, 10). In IV however, the comparison with water-stimulated animals failed to reach statistical significance ($P = 0.16$, Figs. 9, 10). As was generally observed in the anterior-posterior analyses of the insular cortex subregions and the CeA, when the posterior tongue remained denervated the quinine-stimulated Fos response resembled that obtained with water-stimulation regardless of whether the anterior tongue was reinnervated (CT→AT, GL→AT, Figs. 8, 9, 10).

Behavior & Brain Activity Correlations

The gaping responses (and other oromotor behaviors) of these same animals were scored previously (King et al., 2008). To assess the relationship between behavior and activity of neurons in the forebrain structures examined, the number of FI-neurons in animals receiving quinine (regardless of nerve condition) and the number of gapes they elicited were statistically compared in a series of correlation tests. In the CeA, the strongest correlation between the number of gapes elicited by quinine and number of FI-neurons was observed in the Rostral CeA ($r = 0.83$, $P < 0.00005$). A moderate and significant correlation was also observed between quinine-stimulated gapes and FI-neurons in the Intermediate-Rostral CeA ($r = 0.60$; $P < 0.003$). On the contrary, in the two caudal CeA subregions, the correlations were weaker and not statistically significant (Intermediate-Caudal, $r = 0.44$, $P = 0.056$; Caudal, $r = 0.07$, $P > 1.00$). Thus, in general, the correlations were consistent with a rostrocaudal organization of quinine responsiveness in the CeA.

In the gustatory and peri-gustatory insular cortex, interestingly, the strongest correlations between gapes to quinine and numbers of FI-neurons were observed in both ID and IV in the Anterior (ID: $r = 0.78$, $P < 0.001$; IV: $r = 0.70$, $P = 0.002$), Middle (ID: $r = 0.70$, $P = 0.001$; IV: $r = 0.65$, $P = 0.007$), and Posterior (ID: $r = 0.75$, $P < 0.001$; IV: $r = 0.77$, $P = 0.001$) sections in all animals receiving quinine. All other correlations were weaker, ranging between $r = 0.47$ to 0.62 , but were nonetheless statistically significant, with one exception (the Dorsal subregion in the Middle region, $P = 0.096$).

Discussion

The current findings first and foremost underscore once again the extraordinary ability of the central gustatory system to adapt to peripherally reorganized input. As we have shown previously in both the NST and PBN (King et al, 2008), we report here that quinine-stimulated neural activity (as assessed by Fos-immunoreactivity) in specific regions of the CeA and gustatory portions of insular cortex appears remarkably normal when the GL is severed and allowed to reinnervate its native taste receptor field in the posterior tongue. Moreover, when the CT, which normally innervates the anterior tongue, is re-routed into the posterior tongue the Fos response in some of these subregions is also either fully or partially restored. Notably, the rewired or even ‘miswired’ central gustatory system can support normal oromotor rejection behaviors (King et al., 2008). Our study of the distribution of quinine-stimulated FI-neurons in these re-wired and cross-wired animals has provided valuable insight into the functional architecture of the NST and the PBN (King et al., 2008). The current observations in the CeA and the gustatory cortex likewise may further our

understanding of the role these structures play in the neural circuit underlying bitter taste processing.

CeA

Although this study is not the first to report increased numbers of FI-neurons in the CeA in response to quinine stimulation (Zhao et al., 2012), it is the first to show a rostrocaudal gradient of quinine-induced FI-neurons in this nucleus with the greatest Fos response generated rostrally (Fig. 4). Furthermore, results from the nerve-manipulations, coupled with the data from the water control group, provide strong evidence that the quinine-stimulated Fos response observed in the rostral portions of the CeA is related primarily to “taste” rather than post-ingestive events or non-taste oral factors. In fact, the data indicate that quinine-stimulated FI-neurons in the Rostral CeA depended specifically upon taste input arising from the posterior tongue because reinnervation of this taste field by either the GL or the CT restored the Fos response to control values while quinine-stimulated activity arising from the anterior tongue receptor field, regardless of the nerve innervating that field, was neither necessary nor sufficient for the expression of a quinine-stimulated Fos response above that observed in water-stimulated controls here or at any other rostrocaudal level of the CeA (Fig. 4).

The distribution of taste-related FI-neurons in the CeA receives some support from an anatomical tract-tracing study that showed that the projections from the gustatory thalamus (specifically the parvocellular part of the ventral posteromedial thalamic nucleus) terminate mainly in the rostral part of the lateral CeA (Nakashima et al., 2000). Though we did not conduct a quantitative analysis of the numbers of FI-neurons in the various subnuclei of the CeA, qualitatively, they appeared predominately distributed in the lateral half of the CeA, an observation that is in line with the projection data (Nakashima et al., 2000). Moreover, it is worth mentioning that in the NST of these same animals, the number of quinine-stimulated FI-neurons in rats with cross-reinnervation of the posterior tongue (CT→PT) was only comparable with that seen in control rats within the rostral-most portion of the NST (King et al., 2008). At more caudal levels of the NST, the number of quinine-stimulated FI-neurons decreased and was significantly less than the values observed in control rats stimulated with quinine. These data, in conjunction with the current CeA findings, suggest, albeit mildly, a possible functional topography between the NST and CeA regarding bitter taste processing. In this regard, it would be instructive to assess the distribution of FI-neurons in the CeA as a function of other non-bitter aversive taste stimuli (e.g., high concentrations of acids or NaCl) as well as preferred taste stimuli (e.g., sucrose, polycose) to determine whether the rostrocaudal organization simply reflects general taste input or is specific to the quality or hedonic value of the oral stimulus.

The strong correlation ($r = 0.83$) observed between the numbers of quinine-stimulated gapes previously measured in these same animals (King et al., 2008) and quinine-stimulated FI-neurons in the Rostral CeA suggests a role for the CeA in this oromotor rejection response. However, that decerebrate rats (Grill and Norgren 1978b; Flynn and Grill 1988; Travers et al., 1999) and rats with lesions of the CeA itself (Galaverna et al., 1993) are capable of eliciting normal rejection responses excludes the necessity of the CeA in the expression of

such responses. Nonetheless, in light of the bidirectional connectivity the CeA has with brainstem gustatory nuclei (Norgren, 1976; Voshart and van der Kooy, 1981; van der Kooy et al., 1984; Bernard et al., 1993; Nakashima et al., 2000; Lundy Jr and Norgren, 2001, 2004; Kang and Lundy, 2009, 2010; Zhang et al., 2011), as well as its connections with motor circuitry in the reticular formation involved in jaw and tongue movements (Sasamoto and Ohta, 1982; Yasui et al., 2004; Zhang et al., 2011), it is reasonable to speculate a modulatory contribution of the CeA to taste-related behaviors in the rat. Touzani et al. (1997), for example, reported that bilateral lesions of the CeA increased the aversive potency of intraorally delivered taste stimuli, including quinine. Accordingly, the CeA, perhaps specifically its rostral portion, may play a significant role, along with other forebrain structures, in mediating taste-related behaviors that discourage (or promote) consumption (Lundy, 2008; Zhang et al., 2011).

Because it is likely that some quinine or water was swallowed during the 30-min infusion period, it is possible that some of the FI-neurons were generated from neural activity stemming from the gut. Visceral relay nuclei in the brainstem send many projections to the amygdala, especially the CeA (for review, see Cechetto, 1987). Recently, Hao et al. (2009) showed a strong Fos response in the CeA following intragastric gavage of a bitter mixture, which included quinine. These data may help to explain the Fos response in the CeA observed in animals with complete denervation of the tongue (i.e., No Reg group). Alternatively, other orosensory factors could also explain the non-taste related Fos response in the CeA. Nonetheless, by virtue of the gustatory nerve manipulations and the inclusion of a water-stimulated control group, the findings here strongly suggest that the number of quinine-stimulated FI-neurons in the Rostral CeA is related to gustatory signals arising from the posterior tongue and that this region of the amygdala likely contributes to behaviors associated with taste stimulation arising from bitter tasting ligands.

Insular Gustatory Cortex: Anterior-Posterior Plane

The current results are also, to our knowledge, the first to characterize the distribution of FI-neurons in the gustatory insular cortex in response to intraorally delivered quinine. A strong and significant Fos response was observed throughout the entire anterior-posterior extent of the insular cortex examined. However, peak activation appeared to occur in the heart of the conventionally defined gustatory cortex in an area where, on average, the middle cerebral artery intersects the rhinal fissure (although see Hashimoto and Spector, 2014). These findings match well with the results of an anatomical tract tracing study of gustatory thalamic projections to the insular cortex (Kosar et al., 1986b) showing labeled fibers across the entire rostrocaudal extent of the region where taste-responsive neurons are situated (Kosar et al., 1986a). In a similar vein, Accolla et al. (2007) showed that the rostrocaudal spatial representation of quinine stimulation in the rat gustatory cortex was large, greater than 1.5 mm, overlapping with the cortical activation sites elicited by other tastants. Although the quinine representation was positioned more caudally in the gustatory cortex, the peak of activation flanked the middle cerebral artery slightly above the rhinal fissure, an area which corresponds well with our Middle gustatory cortex region.

It is worthwhile to mention also that Cechetto and Saper (1987) and others (Hanamori et al., 1998) reported that taste responsive neurons were located more anteriorly in the gustatory cortex, whereas neurons responding to general visceral input were positioned more posteriorly, which may help to explain the abundance of quinine-stimulated neurons in our Anterior region. In contrast, a recent two-photon optical imaging study of calcium responses to taste stimulation in the mouse cortex revealed anatomically distinct clusters of cells that were selectively tuned to respond to a qualitative perceptual class of compounds (e.g., “sweeteners”, “bitters”) and the cluster activated by quinine and other bitter-tasting compounds was located in the most posterior regions of the overall taste response zone of insular cortex (Chen et al., 2011). Interestingly, the rostrocaudal extent of the taste-responsive area of insular cortex in the Chen et al. (2011) study was approximately 2.5 mm which, given the smaller size of the mouse brain, is quite more extensive than what has been reported in the rat, but the general location between the two species, surrounding the middle cerebral artery slightly above the rhinal fissure, was similar. The fact that we did not test other taste compounds in our study precludes any direct comparisons with the findings of Chen et al (2011), but any potential anatomical differences between their results and our FI-neuron distribution might be related to species differences or to the fact that our rats were awake and behaving during the taste stimulation.

Data obtained from the quinine-stimulated control animals alone cannot specify the source (gustatory vs orosensory vs visceral) of the Fos response, while data from the nerve-manipulated animals are more useful in that regard. When the posterior tongue remained denervated (i.e., in animals with only reinnervation of the anterior tongue), the quinine-stimulated Fos response, above that elicited by water, was weak in all three anterior-posterior regions (Fig. 8, bottom three panels), a finding implying that neural input from the anterior tongue is insufficient to drive a strong quinine-stimulated Fos response in the gustatory cortex. Interestingly, in animals in which the posterior tongue was reinnervated by its native GL, or cross-reinnervated by the non-native CT, the greatest quinine-stimulated Fos response above that elicited by water occurred in the core (Middle sections) of the gustatory cortex (Fig. 8, top three panels). Based on these data, it appears that taste signals originating in the posterior tongue are necessary for eliciting quinine-stimulated FI-neurons in the gustatory cortex, in particular, in its central core region.

Insular Gustatory Cortex: Dorsal-Ventral Plane

The borders of the dorsal-ventral subregions we identified, while reliably reproducible, could have idiosyncratically included more or less granular or dysgranular cortex; accordingly, the dorsal-ventral analysis must be viewed with this caveat in mind. Throughout the dorsal-ventral extent of insular cortex, quinine- as opposed to water-stimulation elicited a strong Fos response, but in the dorsal-most subregion the Fos response was not statistically different ($P = 0.06$) from that observed following water-stimulation. Studies using electrophysiological (Yamamoto et al., 1984; Kosar et al., 1986a) and optical imaging techniques (Accolla et al., 2007) have shown that mechanical stimulation of the tongue activates neurons located more dorsally in insular cortex compared to those showing gustatory sensitivity. In addition, Cechetto and Saper (1987) found gastric mechanoreceptor-responsive cells located more dorsally in the insular cortex as compared with taste-

responsive neurons. As such, it is certainly possible some of the FI-neurons generated in our dorsal-most subregion may be related to mechanical or gut stimulation.

The greatest number of quinine-stimulated FI-neurons in control rats was observed in ID. This subregion, as well as IV were the only two subregions for which the quinine-stimulated Fos response appeared normal (and significantly different from the response elicited by water) in animals in which the posterior tongue was reinnervated by the native GL nerve. The cross-wired CT was sufficient to maintain quinine-stimulated activity only in ID. Moreover, in quinine-stimulated animals with either intact nerves or with reinnervation of the posterior tongue, quinine-stimulated neural activity appeared to peak, relative to water, in ID and IV (Fig. 8). These data indicate that the generation of quinine-stimulated FI-neurons in these two subregions, which roughly correspond to dysgranular and dorsal agranular gustatory cortex, is dependent on taste signals arising from the posterior tongue. Interestingly, among the cortical subregions demarcated, the strongest correlations between quinine-stimulated gaping behavior and the number of FI-neurons were observed in ID and IV throughout the anterior-posterior extent of the cortical regions examined. Nonetheless, it remains to be seen to what extent these relationships are causal especially in light of the finding that quinine is able to elicit gapes in the decerebrate preparation (Grill and Norgren 1978b).

Closing Remarks

In summary, the distribution of quinine-stimulated FI-neurons in the CeA and gustatory insular cortex was characterized and found to be differentially altered by gustatory nerve transection and regeneration. In the CeA, a rostrocaudal gradient of quinine-stimulated neurons was observed with the greatest Fos response occurring rostrally in the nucleus (Fig. 4). Throughout the anterior-posterior extent of the gustatory cortex, more quinine- than water stimulated FI-neurons were elicited, although in subregions ID and IV, which approximate dysgranular and agranular insular cortex, respectively, in the center of gustatory cortex, a “hotspot” of quinine-generated activity was revealed (Fig. 8). Moreover, the effects of the gustatory nerve manipulations on the quinine-stimulated Fos response in the rostral regions of the CeA and the ID and IV subregions in the central core of the gustatory cortex match the outcomes obtained in upstream nuclei of the gustatory neuraxis, specifically the medial dorsal NST and the waist area of the PBN, and also bear a strong relation to quinine-stimulated gaping (King et al, 2008).

Such results implicate these subregions as part of a neural circuit involved in processing bitter taste, and perhaps, more generally, in hedonic evaluation of tastants. This does not rule out the contribution of other areas of the brain from contributing to such processing as well. Indeed, although the focus of our study was the CeA and the gustatory cortex, we noticed that oral stimulation with quinine generated increases in FI-neurons in nearby regions, including the amygdalostriatal transition area, the claustrum, and endopiriform nucleus. Interestingly, the amygdalostriatal transition area has been proposed to be part of an interconnected neural circuitry involved in motivated behaviors and in translating emotionally salient stimuli into behavioral responses (Jolkkonen et al, 2001). Though the exact function of the claustrum remains uncertain, its proposed functions include the binding

of information within and between sensory and motor systems, serving as a synchrony integrator and/or a salience detector (Majak et al., 2002; Crick and Koch, 2005; Remedios et al., 2010; Smythies et al., 2012). Finally, the Fos-response in the endopiriform nucleus may relate to its role as a potential site of integration of gustatory and olfactory information (e.g., Fu et al., 2004). Because no quantitative analysis of areas outside our regions of interest was conducted, no firm conclusions can be reached regarding potential effects in the amygdalostriatal transition area, the claustrum, and endopiriform nucleus, but the apparent quinine-stimulated Fos response in these brain sites justifies further experimental scrutiny.

Although our interpretations are parsimonious with many studies on bitter taste processing and palatability, one must keep in mind that the expression of the Fos protein merely indicates neural activation of a subpopulation of cells (Dragunow and Faull, 1989); the functional significance of that activation must be cautiously interpreted. These animals were receiving quinine for the first and only time, thus we cannot rule out the possibility that the Fos response in the CeA or gustatory cortex was due to the novelty of the tastant (Dunn and Everitt, 1988; Koh et al., 2003). In a similar vein, the generation of FI-neurons in the CeA or gustatory cortex may also be related to neural activity resulting from associating the bitter taste with other forms of sensory stimulation, for example visceral input (Nishijo et al., 1998), and/or to storing memories of the taste experience (e.g., Yasoshima and Yamamoto, 1997; Berman et al., 1998). Nevertheless, the above interpretative caveats notwithstanding, the effects of the gustatory nerve manipulations reported here provide firm evidence that the Fos response observed in the CeA and the gustatory cortex, regardless of its specific functional role, is based on taste input.

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Figure 1.

Photomicrographs of Nissl-stained sections depicting the rostrocaudal levels of the central nucleus of the amygdala (CeA) and the anterior-posterior levels of insular cortex examined. In Rostral CeA (A), Intermediate-Rostral CeA (B), Intermediate-Caudal CeA (C), and Caudal CeA (D), the borders of the CeA, drawn with the aid of a stereotaxic atlas (Paxinos and Watson, 2007) are shown. In Anterior (E), Middle (F), and Posterior (G) sections of the portion of insular cortex examined, the dashed lines outline the regions of interest. A higher power image (H) of the Middle section shown in panel F provides an example of the delineation of the four dorsal-ventral subregions. To create these subregions, a line was drawn starting at the dorsal tip of the claustrum and was extended laterally such that it was roughly perpendicular to the tangent of the brain surface. A second line, parallel and ventral to the first, was drawn such that it rested directly atop layer 2 of piriform cortex and extended from the lateral surface of the brain toward the ventral part of the claustrum and the dorsal endopiriform nucleus. The distance between these two parallel lines was measured and divided by four. Three more lines were drawn in between the two parallel lines to yield the four roughly rectangular subregions, Dorsal (D), Intermediate-Dorsal (ID), Intermediate-Ventral (IV), and Ventral (V), which approximate granular, dysgranular, dorsal agranular, and ventral agranular insular cortex (Paxinos and Watson, 2007). Abbreviations: aca, anterior commissure, anterior; ASt, amygdalostratial transition area; BLA, basolateral nucleus; CeA, central nucleus of the amygdala, cst, commissural stria terminalis; CPu,

caudate putamen; DeN, dorsal endopiriform cortex; DCl, dorsal part of the claustrum; I, intercalated amygdala; iv, layer 4 of cortex; IM, main intercalated amygdala, LaDL, lateral amygdaloid nucleus, dorsolateral division; LV, Lateral ventricle; opt, optic tract; Pir2, piriform cortex, layer 2; rf, rhinal fissure; VCl, ventral part of the claustrum. Scale bars = 500 μ m. Scale bar in panel D applies to panels A–G.

Figure 2.

The mean numbers (+ standard errors) of Fos-immunoreactive (FI) neurons per brain section in each region of the CeA elicited by intraoral administration of 3.0 mM quinine or distilled water in intact or nerve-manipulated rats. Significantly more quinine-stimulated (Sham-Q) vs water-stimulated (Sham-W) Fos-immunoreactive (FI) neurons were elicited in the Rostral (A) and Intermediate-Rostral (B) CeA regions. This statistical difference was not observed in the Intermediate-Caudal (C) or Caudal regions (D), yielding a rostrocaudal gradient of quinine-stimulated FI-neurons in the CeA in control animals. Following nerve-transection, reinnervation of the posterior tongue by the glossopharyngeal nerve (GL, GL→PT, solid red bars) restored the Fos response to quinine in the rostral half of the nucleus (A, B). Cross-regeneration of the chorda tympani nerve (CT) into the posterior tongue (CT→PT, cross-

hatched red bars) successfully maintained the quinine-stimulated Fos response only in the rostral-most portion of the CeA (A); in the Intermediate-Rostral CeA (B), the Fos response was partially restored. On the contrary, reinnervation of the anterior tongue by either the CT (CT→AT, solid blue bars) or cross-reinnervation by the GL (GL→AT, cross-hatched blue bars) was neither necessary nor sufficient to maintain quinine-stimulated neural activity in any region of the CeA (A–D). Filled circles represent data from individual animals. An asterisk (*) or a pound sign (#) denotes a statistically significant difference from the Sham-W group or Sham-Q group, respectively.

Figure 3.

Photomicrographs illustrating the Fos response elicited by quinine (A–D) or water (E–H) stimulation in each rostrocaudal region of the CeA in nerve-intact animals. In rostral regions (A, B), a robust quinine-stimulated response (as compared with the water-stimulated response, E, F), was observed. In more caudal regions (C, D), the quinine-stimulated Fos response was much weaker and appeared similar to the water-stimulated response (G, H). Scale bar = 500 μ m.

Figure 4.

Temperature scale representation of the percent increases in quinine-stimulated FI-neurons over water-stimulated FI-neurons in the rostrocaudal regions of the CeA for different nerve conditions. Warmer shades (reds) indicate greater increases above the water-stimulated condition. Note the rostrocaudal pattern of quinine-stimulated neural activity above that elicited by water in control animals (Sham-Q). Remarkably, reinnervation (GL→PT) or cross-reinnervation (CT→PT) of the posterior tongue produced a similar, albeit weaker, neural response. The quinine-stimulated neural responses over that elicited by water in animals with no reinnervation of the tongue (No Reg) or with only anterior tongue reinnervation (CT→AT and GL→AT) were modest at best in comparison and lacked any rostrocaudal pattern.

Figure 5.

Photomicrographs illustrating the quinine-stimulated Fos response in the Rostral CeA in nerve manipulated rats. As compared with Sham-Q animals (A, Photomicrograph from Fig. 3A is replicated here), a very similar, robust quinine-stimulated Fos response was observed in animals with either reinnervation (B; GL→PT), or cross-reinnervation (C; CT→PT) of the posterior tongue receptor fields. A much weaker Fos response was apparent in animals without lingual nerve reinnervation (D; No Reg), or with only reinnervation of the anterior tongue (E; CT→AT, or F; GL→AT). Compare with Sham-W animals (Fig. 3E). Scale bar = 500 μ m.

Figure 6.

The mean sum (+ standard errors) of FI-neurons in each anterior-posterior region of the gustatory and peri-gustatory insular cortex elicited by intraoral administration of 3.0 mM quinine or distilled water in intact or nerve-manipulated rats. In Anterior (A), Middle (B) and Posterior (C) regions, quinine-stimulated FI-neurons in control animals (Sham-Q) were significantly greater in number than those elicited by stimulation with water (Sham-W). Only in the core region (B, Middle) did the nerve manipulations yield a significant effect. Specifically, when the posterior tongue was reinnervated by the GL (GL→PT, solid red bars), the number of quinine-stimulated FI-neurons was fully restored while the Fos response following cross-wiring (CT→PT, cross-hatched red bars) was only partially restored; the comparison with the Sham-W group only tended toward significance. When only the anterior tongue was reinnervated, regardless of the gustatory nerve (CT→AT, solid

blue bars; or GL→AT, cross-hatched blue bars), quinine-stimulated FI-neuron numbers were comparable to water-stimulated FI-neuron numbers in each region. Filled circles represent data from individual animals. An asterisk (*) or a pound sign (#) denotes a statistically significant difference from the Sham-W group or Sham-Q group, respectively.

Figure 7.

Photomicrographs illustrating the Fos response elicited by quinine (A–C) or water (D–F) stimulation in the dorsal-ventral subregions of the gustatory and peri-gustatory insular cortex in Anterior (A,D), Middle (B,E) and Posterior (C,F) sections in nerve intact animals. Quinine, as compared with water, yielded a strong Fos response throughout the anterior-posterior extent of the insular cortex, in particular in the Intermediate-Dorsal and Intermediate-Ventral subregions in Anterior and Middle regions. Arrows denote approximate subregion borders. Scale bar = 500 μm.

Figure 8.

Temperature scale representation of the percent increase in quinine-stimulated FI-neurons over water-stimulated FI-neurons in gustatory and peri-gustatory insular cortex regions for different nerve conditions. Warmer shades (reds) indicate greater increases above the water-stimulated condition. Animals with intact gustatory nerves and stimulated with quinine (Sham-Q) showed the greatest percent increases in FI-neuron number in Intermediate-Dorsal and Intermediate-Ventral subregions in the core (Middle sections) of the gustatory cortex. This pattern was also observed in animals with regeneration of the GL into the posterior tongue (GL→PT). Remarkably, cross-regeneration of the non-native CT into the posterior tongue (CT→PT) also yielded strong increases in Intermediate-Dorsal and Intermediate-Ventral subregions, though the greatest increase over water stimulation occurred in the Dorsal subregion in the Middle sections in this surgical group. A dramatically different picture emerged in animals in which only the anterior tongue was reinnervated by either its native nerve (CT→AT) or cross-reinnervated (GL→AT). Much smaller increases in quinine-stimulated neural activity above that elicited by water were observed. In fact, these neural response patterns resembled those in found in animals with complete denervation of the tongue (No Reg). Abbreviations: Ant, Anterior; Mid, Middle; Post, Posterior

Figure 9.

Photomicrographs illustrating the labeling of quinine-stimulated FI-neurons in the core (Middle region) of gustatory cortex and peri-gustatory insular cortex in nerve manipulated rats. A Fos response similar to the one observed in Sham-Q animals (A, Photomicrograph from Fig. 7B is replicated here) was apparent in animals in which the posterior tongue was reinnervated (B; GL→PT). Even cross-reinnervation of the posterior tongue (C; CT→PT) elicited a strong Fos response following stimulation with quinine. On the contrary, the quinine-stimulated neural response in animals with complete denervation (D; No Reg) or with only anterior tongue reinnervation by either the CT (E, CT→AT) or GL (E, GL→AT) resembled that observed in water-stimulated rats (Compare with Fig. 7E). Arrows denote approximate subregion boundaries. Scale bar = 500 μ m.

Figure 10.

The mean sum (+ standard errors) of Fos-immunoreactive (FI) neurons at each dorsal-ventral level of gustatory cortex and peri-gustatory insular cortex elicited by intraoral administration of 3.0 mM quinine or distilled water in intact or nerve-manipulated rats. In the Dorsal (A), the Intermediate-Dorsal (B), the Intermediate-Ventral (C) and the Ventral (D) subregions, many quinine-stimulated FI-neurons were elicited in Sham-Q rats, but in the Dorsal subregion the comparison with Sham-W rats only approached significance. Only in the Intermediate-Dorsal subregion did reinnervation (GL→PT, solid red bars) or cross-reinnervation (CT→PT, cross hatched red bars) of the posterior tongue restore numbers of quinine-stimulated FI-neurons to values similar to those in Sham-Q rats and different from those in Sham-W rats. In the Intermediate-Ventral subregion, reinnervation of the posterior tongue by the GL (but not the CT) fully restored the Fos response. Reinnervation of the

anterior tongue by either nerve did not restore the quinine-stimulated Fos response in any dorsal-ventral subregion. Filled circles represent data from individual animals. An asterisk (*) or a pound sign (#) denotes a statistically significant difference from the Sham-W group or Sham-Q group, respectively.

Table 1

Primary Antibody

Antibody	Antigen	Manufacturer, species, type, catalog number	Dilution	Reference for the specificity
c Fos	Synthetic peptide mapping at the N-terminus residues 3–16 of the protein encoded by human <i>c-fos</i>	Santa Cruz Biotechnology, rabbit, polyclonal, #sc-52	1:10,000	Manufacturer's specification (western blot) Gaszner et al., 2007