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A Functional Role for IL-1 in the Injured Peripheral Taste System

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Abstract

The peripheral taste system presents an excellent model for studying the consequences of neural injury, for the damaged nerve and sensory cells and the neighboring, intact neural cells. Sectioning a primary afferent nerve, the chorda tympani (CT), rapidly recruits neutrophils to both sides of the tongue. The bilateral neutrophil response induces transient functional deficits in the intact CT. Normal function is subsequently restored as macrophages respond to injury. We hypothesized that macrophages produce the proinflammatory cytokine, interleukin (IL)-1, which contributes to the maintenance of normal taste function after nearby injury. We demonstrate that IL-1 β protein levels are significantly increased on the injured side of the tongue at day 2 after injury. Dietary sodium deficiency, a manipulation which prevents macrophage recruitment, inhibits the elevation in IL-1 β . IL-1 β was expressed in several cell populations, including taste receptor cells and infiltrating neutrophils and macrophages. To test whether IL-1 modulates taste function after injury, we blocked signaling with an IL-1 receptor antagonist (IL-1 RA) and recorded taste responses from the intact CT. This treatment inhibited the bilateral macrophage response to injury, and impaired taste responses in the intact CT. Cytokine actions in the taste system are largely unstudied. These results demonstrate that IL-1 has a beneficial effect on taste function after nearby injury, in contrast to its detrimental role in the injured central nervous system (CNS).

Keywords

gustatory; chorda tympani nerve; taste bud; degeneration; cytokine; nerve injury; neural-immune interactions

INTRODUCTION

The peripheral taste system remains functionally plastic, even in adulthood. Unilateral chorda tympani nerve (CT) injury is especially effective in revealing this plasticity, which occurs in both the regenerated and uninjured nerves (Hill and Phillips, 1994; Hendricks et al., 2002; Wall and McCluskey, 2008). Early functional changes after sectioning depend on bilateral infiltration of the taste receptor fields by leukocytes and their interaction with sensory receptor cells. In the current study, we focus on the role of IL-1 in maintaining taste function after neighboring injury.

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Sensory receptor cells in the peripheral taste system are bilaterally innervated by distinct chorda tympani nerves but exist within a continuous epithelium. Soon after one CT is sectioned, neutrophils invade the denervated and uninjured side of the tongue (Steen et al., 2010a). Though neutrophils clear tissue debris and prevent infection (Nathan, 2006), they can be detrimental to neural function (Taoka et al., 1997; Carlson et al., 1998; Perkins and Tracey, 2000; Profyris et al., 2004). The peripheral taste system is no exception, since both lingual inflammation and CT nerve injury attract neutrophils which induce deficits in taste function. Specifically, neural responses to sodium are reduced in the uninjured, neighboring CT. Normal taste responses are restored when the neutrophil response ends or when neutrophils are experimentally depleted (Steen et al., 2010b).

During the next post-injury phase, chemokines and adhesion molecules are upregulated and macrophages invade both sides of the tongue (McCluskey, 2004; Cavallin and McCluskey, 2007a, 2007b). Activated macrophages are likely beneficial to taste function, as their entry parallels the recovery of normal taste function in the uninjured nerve (McCluskey, 2004; Cavallin and McCluskey, 2005; Wall and McCluskey, 2008). Moreover, treatments that inhibit macrophage entry provoke abnormal taste responses (McCluskey, 2004; Cavallin and McCluskey, 2005; Guagliardo et al., 2009).

Peripheral taste function and leukocyte responses can be perturbed by manipulating the dietary environment. Animals on a sodium-deficient diet exhibit continued functional impairment in the intact CT nerve after contralateral sectioning (Hill and Phillips, 1994), while normal responses recover by day 2 post-injury in control-fed rats (Wall and McCluskey, 2008). The low-sodium diet also amplifies and extends the bilateral neutrophil response to nerve injury (Steen et al., 2010b), downregulates vascular cell adhesion molecule (VCAM)-1 expression (Cavallin and McCluskey, 2007a), and prevents macrophage infiltration (McCluskey, 2004; Cavallin and McCluskey, 2005) in addition to its long-term effects on the regenerated and intact CT nerves (Hill and Phillips, 1994; Hendricks et al., 2002).

We propose that leukocytes invade the injured peripheral taste system and release cytokines that modulate taste receptor cell function. IL-1 is a particularly attractive candidate given its prominence in innate immune responses and injured central and peripheral nervous systems (Schneider et al., 1998; Allan and Rothwell, 2003). IL-1 is generally considered harmful to the wounded brain (Allan et al., 2005) but may have a positive influence in degenerating peripheral nerves (Shamash et al., 2002; Perrin et al., 2005). Importantly, IL-1 β can modulate the epithelial sodium channel (ENaC), which is proposed to be the site of leukocyte-induced changes in taste receptor cells (Barmeyer et al., 2004; Roux et al., 2005; Choi et al., 2007).

We tested the hypothesis that IL-1 plays a beneficial role in the injured taste system by administering an IL-1 receptor antagonist (IL-1RA) after CT sectioning, then recording neural responses from the intact CT. This recombinant form of a naturally occurring antagonist prevents IL-1 α and IL-1 β signaling through the IL-1 receptor 1 (IL-1 RI). We also analyzed IL-1 β expression and regulation by nerve injury and dietary sodium deficiency, since little is known about cytokine influences on the injured peripheral taste system.

METHODS

Animals

The Animal Care and Use Committee at the Medical College of Georgia approved all protocols, which followed guidelines set by the National Institutes of Health and the Society

for Neuroscience. Female specified pathogen-free (SPF) Sprague Dawley rats (Charles River, Wilmington, MA) were 40–60 days old at the time of treatment. Rats were housed in cages with barrier tops and received autoclaved food, bedding, and water.

CT sectioning and sodium depletion

Rats received the following treatments: 1) Unilateral CT section or sham sectioning; 2) Dietary sodium restriction or a control diet; and 3) IL-1 RA or vehicle injection. Rats receiving nerve section were injected with atropine sulfate [0.5 mg/ml; intraperitoneal (i.p.)] followed by a mixture of ketamine (40 mg/kg; i.p.) and xylazine (10 mg/kg; i.p.). The right CT nerve was exposed by a ventral dissection and transected after its bifurcation from the lingual nerve, before entering the tongue, as in previous work (Hill and Phillips, 1994; McCluskey, 2004; Cavallin and McCluskey, 2005). Following CT sectioning, rats in sodium-restricted groups received two injections of the diuretic, furosemide (10 mg each within 24 hours, i.p.; Sigma), to promote urinary excretion of sodium, and were maintained on low-sodium chow (0.03% NaCl, MP Biomedicals, Solon, OH) and distilled water. Control-fed groups were given normal food (0.25% NaCl; Purina, St. Louis, MO) and tap water.

Rats were injected daily with 1 mg of the human, recombinant IL-1 RA (in 0.1 ml; i.p.; a generous gift from Amgen; Thousand Oaks, CA) or the same volume of sterile vehicle (0.82% sodium chloride; 0.19% sodium citrate, 0.02% EDTA, 0.01% TWEEN in water). Similar systemic doses of IL-1 RA block peripheral IL-1 signaling in rats (Kent et al., 1992; Avitsur et al., 1997; Ehses et al., 2009) and are used clinically for the treatment of rheumatoid arthritis (Mertens and Singh, 2009). Additional animals were euthanized 6 hr after LPS injection (100 µg/kg i.p. in sterile saline) to provide control tissue (Nguyen et al., 1998).

Tissue collection and ELISAs

Rats (n=56 total) received unilateral CT sectioning and tissues were collected 6 hr (n= 7), 12 hr (n=7), 24 hr (n=6), 2 days (n=6) or 4 days (n=8) later for ELISAs. Sham-sectioned control rats (n=9) and sodium-restricted rats were sacrificed at 2 days (n=6) or 4 days (n=7) post-sectioning. Rats were deeply anesthetized with sodium pentobarbital (80 mg/kg; i.p.) and perfused through the heart with a solution of proteinase inhibitors in ice-cold PBS. Longitudinally-sectioned tongue halves were flash-frozen, pulverized, weighed and homogenized as previously described (Cavallin and McCluskey, 2007a, 2007b). Protein samples from control tissues such as hippocampus, cerebellum, and spleen were similarly prepared.

We measured IL-1 β protein levels in tongue homogenates with enzyme-linked immunosorbent assay (ELISA) kits (R & D Systems; Minneapolis, MN; detection limit 5 pg/ml) according to the manufacturer's directions. Samples were analyzed in duplicate on an EL800 (Bio-Tek Instruments, Winooski, VT) microplate reader for absorbance at 450 nm and a reference wavelength of 540 nm. Four-parameter logistic curve-fit analysis (KC Junior Software, Bio-Tek Instruments) was used to generate standard curves based on specified concentrations of recombinant rat IL-1 β . The data was normalized to tissue weight (pg IL-1 β /g total protein) as in previous work (Cavallin and McCluskey, 2007a, 2007b).

Immunohistochemical localization of IL-1 β and its receptor

IL-1 β was localized within the peripheral taste system of animals receiving: 1) CT sectioning (n=8); 2) CT sectioning and a sodium-deficient diet (n = 8); and 3) sham sectioning (n=8). Rats were euthanized at day 2 post-sectioning, as described above. This time was chosen based on the peak IL-1 β levels detected by ELISA. Tissues were collected

and cryosectioning performed as previously described (McCluskey, 2004). Double immunofluorescent staining was performed with a rabbit polyclonal antibody to IL-1 β (1:50; Abcam, Cambridge, MA) (Maddahi and Edvinsson, 2010) in conjunction with antibodies against the markers shown in Table 1.

Results from control experiments are shown in Supplementary Fig. 1. The specificity of the IL-1 β antibody was confirmed by (1) identifying IL-1 β ⁺ leukocytes in spleen as a positive control (Abbas and Lichtman, 2003); (2) performing negative control staining with pre-immune rabbit serum or primary antibody preabsorbed with rat recombinant IL-1 β peptide (5X) (Vogt et al., 2008); (3) demonstrating minimal and increased IL-1 β expression in the brain of control and LPS-injected rats, respectively (Nguyen et al., 1998; Vitkovic et al., 2000); and (4) Western blotting using increasing concentrations of rIL-1 β protein (R & D Systems; Minneapolis, MN) to confirm that the antibody recognizes 17kD bands of increasing intensity (Vogt et al., 2008). Cytokine transcription can be uncoupled from release and functional involvement in neural injury (Shamash et al., 2002; Dinarello, 2009). Since our hypothesis is that macrophage-derived IL-1 β has a paracrine effect on taste receptor cell function, we chose to identify IL-1 β protein rather than mRNA.

Tissue sections were incubated in primary antibodies (Table 1) and then in Alexa Fluor secondary antibodies (1:1000; Molecular Probes/Invitrogen; Carlsbad, CA) for one hour at room temperature. Non-specific staining in the absence of primary antibody, in the presence of equal concentration of preimmune serum, after incubation in peptide-preabsorbed antibody, or in brain was found to be minimal (Supp. Fig. 1). Images showing fluorescent staining were captured with a digital camera (Cool Snap; Roper Scientific, Tucson, AZ), with the exception of images showing IL-1 β ⁺ macrophages which were obtained with a Zeiss LSM 520 confocal microscope (Zeiss, Göttingen, Germany).

Neurophysiology

CT recordings were performed at day 4 post-injury to determine whether the IL-1 RA modulates neural responses in the intact CT after contralateral sectioning (n=23 total). At this post-sectioning period, taste responses to sodium are reduced in the intact CT of animals receiving sectioning in combination with a low sodium diet (Hill and Phillips, 1994). While sodium taste function remains low for several weeks, this early time point allowed us to minimize the amount of IL-1 RA used. Control groups received sham sectioning and the IL-1 RA ("Sham + IL-1 RA"; n=8) or CT sectioning and injection of vehicle ("Cut + Vehicle"; n=8), as described above. The experimental group received CT sectioning and daily injections of the IL-1 RA ("Cut + IL-1 RA"; n=7).

Rats were anesthetized with chloral hydrate (525 mg/kg, i.p.) prior to neurophysiological recordings. Additional injections were given as necessary to maintain anesthesia at a surgical level. Dissection of the CT nerve and multifiber recordings were conducted as described in previous work (Hill and Phillips, 1994; Wall and McCluskey, 2008). Summated electrical activity was monitored and analyzed using PowerLab software (AD Instruments, Inc., Colorado Springs, CO).

Taste stimuli included concentration series (0.05–0.50M) of NaCl, sodium acetate (NaAc), and KCl salts. Stimulation with 0.01M quinine, 0.01N HCl, 0.10M monosodium glutamate (MSG), and 1.0M sucrose was used to measure non-salt responses. Ammonium chloride (0.50M NH₄Cl) was applied to the tongue at the beginning and end of each stimulus series (Hill and Phillips, 1994). Stable series were bracketed by NH₄Cl responses within 10% of each other. The magnitude of steady-state taste responses was measured at 20 sec after stimulus application. Response magnitudes were analyzed by expressing them as a ratio of the average of bracketing NH₄Cl responses. A concentration series of NaCl in 50 μ M

amiloride was recorded at the end of each experiment to assess epithelial sodium channel (ENaC) function in taste receptor cells. Rats were then euthanized with sodium pentobarbital (80 mg/kg, i.p.) and tissues harvested.

Leukocyte labeling and analysis

Since macrophages or neutrophils could influence function in the intact CT nerve (McCluskey, 2004; Steen et al., 2010b), we tested whether the IL-1 RA affects leukocyte infiltration of the injured peripheral taste system. Macrophage and neutrophil responses to nerve section were analyzed in groups given the same treatment as in neurophysiological experiments: 1) Sham + IL-1 RA (n=9); 2) Cut + Vehicle (n=14); or 3) Cut + IL-1 RA (n=9). Rats were overdosed with sodium pentobarbital (80 mg/kg; i.p.) on day 1 or 2 post-sectioning. This period corresponds to the peak neutrophil and macrophage responses to nerve injury, respectively (McCluskey, 2004; Wall and McCluskey, 2007). We also analyzed macrophage responses in a subset of animals following recordings at day 4 post-injury (n=17), to determine whether the IL-1 antagonist delayed macrophage recruitment.

The ED1 antibody was used to identify activated macrophages, and the MPO antibody to detect neutrophils (Table 1). Biotinylated goat anti-mouse IgG (1:100; Jackson ImmunoResearch, West Grove, PA) or goat anti-rabbit IgG (1:100; Jackson) was used as the secondary antibody for ED1 or MPO labeling, respectively. Incubation in avidin-biotin complex (Vector; Burlingame, CA) was followed by detection with diaminobenzidine (Sigma). Nonspecific staining was assessed in each assay by omitting the primary antibody.

Immunolabeled leukocytes were analyzed with a computer imaging system with MetaMorph software (Universal Imaging Corporation/Molecular Devices; Downingtown, PA) and a digital color camera (Cool Snap; Roper Scientific). Images were captured at 50X and were used to quantify leukocytes in four regions per coronal section: 1) the denervated epithelium and lamina propria; 2) the denervated submucosa and muscle; 3) the intact, contralateral epithelium and lamina propria; and 4) the intact submucosa and muscle. A standard-sized area (12.9 mm²) was placed within each region to encompass the most immunopositive label. Thus, the total area counted was 206.4 mm²/section. The process used to select sections from the anterior, mid, and posterior fungiform field for imaging was standardized (McCluskey, 2004). The percentage of stained pixels/standard area was calculated and used to quantify ED1⁺ macrophages (McCluskey, 2004; Cavallin and McCluskey, 2005). Since neutrophils are round and lack elaborate processes, we counted these cells to determine the number of cells/standard area (Steen et al., 2010b).

Statistical analyses

Results from ELISAs were not normally distributed. Therefore, a square root transformation ($y=y^2$) was first performed. This treatment is appropriate for skewed, ratiometric data (i.e. normalized protein values) (Howell, 1987). Lingual levels of IL-1 β in sham-sectioned controls were compared with those receiving CT sectioning by ANOVAs followed by Dunnett's Multiple Comparison test where appropriate. IL-1 β levels were compared in control-fed vs. sodium-restricted groups at day 2 and 4 post-sectioning using unpaired Student's *t* tests.

Mean relative CT response ratios were compared among treatment groups with analyses of variance (ANOVAs) followed by Student Newman-Keuls post-tests where appropriate. ANOVAs were also used to compare mean percentages of stained pixels/standard area (macrophages) and mean numbers of neutrophils/standard area on the denervated and intact side of the tongue in treatment groups. Newman-Keuls posttests were used to determine the

source of group differences. Statistical analyses were done with Prism 3.0 (GraphPad Software, La Jolla, CA) software. The α level was set at $p \leq 0.05$.

RESULTS

Modulation of lingual IL-1 β levels by nerve section and diet

We measured IL-1 β levels in the injured peripheral taste system and control samples by ELISA (Fig. 1A, Supp. Fig. 1). Even in sham-sectioned controls, lingual IL-1 β levels were approximately four-fold higher than the detection limit of the assay. IL-1 β was maintained at sham-like levels for 6–24 hr post-sectioning on both the injured and intact sides of the tongue. At day 2 post-sectioning, there was a dramatic increase in IL-1 β expression on the denervated side of the tongue ($p < 0.001$ vs. sham-sectioned controls) that returned to baseline by day 4. On the neighboring, uninjured side of the tongue, IL-1 β was slightly, though not significantly, elevated ($p > 0.05$).

A sodium-deficient diet inhibits VCAM-1 expression (Cavallin and McCluskey, 2007a), dysregulates leukocyte responses to nerve injury (McCluskey, 2004; Cavallin and McCluskey, 2005; Guagliardo et al., 2009; Steen et al., 2010b), and impairs sodium taste function in the uninjured nerve (Hill and Phillips, 1994; Phillips and Hill, 1996; Wall and McCluskey, 2008). Therefore, we also determined the effects of the diet on IL-1 β at day 2 post-sectioning, when levels spike in control-fed animals (Fig. 1A). As shown in Fig. 1B, sodium-restriction prevented the increase in IL-1 β on the sectioned side of the tongue ($p = .04$). The elevation in IL-1 β was blocked, rather than merely delayed, as cytokine levels remained low at day 4 post-sectioning in sodium-deficient rats ($p > 0.05$; Fig. 1C).

Localization of IL-1 β in the peripheral taste system

At day 2 post-injury, IL-1 β is upregulated on the denervated side of the tongue. Our next step was to determine where IL-1 β is expressed in the peripheral taste system of rats treated with sham sectioning, unilateral CT sectioning, or CT sectioning in combination with a sodium-deficient diet. As shown in Fig. 2, IL-1 β expression is robust in the peripheral taste system of normal and nerve-sectioned animals. ED1+ activated macrophages are among the IL-1 expressing cells, as expected (Shamash et al., 2002; Allan and Rothwell, 2003). IL-1 β + activated macrophages increase on both sides of the tongue in control-fed rats after nerve sectioning, consistent with previous work (McCluskey, 2004). Few macrophages are present in the lingual mucosa of sham-sectioned controls or sodium-deficient animals after CT sectioning. However, ED1+ macrophages are IL-1 β + regardless of surgical or dietary treatment. These results suggest that IL-1 β levels remain low in sodium-restricted animals, at least in part, because macrophages expressing the cytokine fail to infiltrate the injured peripheral taste system (McCluskey, 2004).

An unexpected finding was that taste buds identified by morphology, location, and the CK-19 marker are strongly IL-1 β + (Fig. 3). Moreover, IL-1 β staining appeared stronger in taste receptor cells of control-fed animals receiving CT section, especially on the injured side of the tongue. This finding suggests that taste receptor cells could modulate themselves or neighbors by autocrine or paracrine IL-1 β signals, respectively. Thus, we asked whether taste receptor cells also express the IL-1 RI, which would suggest the potential for downstream signaling. The type I receptor is functionally relevant, compared to the IL-1 type II receptor which acts as a decoy receptor due to the lack of an intracellular binding domain (Allan et al., 2005). As shown in Fig. 4, most, if not all taste receptor cells demonstrate IL-1 RI immunoreactivity. Moreover, epithelial cells and endothelial cells express the IL-1 RI.

Non-gustatory cells in the tongue also express IL-1 β , as confirmed by cell-specific markers (Table 1; Fig. 5) and distinctive morphologies. For example, non-gustatory epithelial cells surrounding fungiform taste buds are IL-1 β ⁺ (Fig. 3). In sham and CT sectioned rats, nerve bundles identified by neurofilament expression express IL-1 β . These IL-1⁺ nerves, including neurons and Schwann cells, were found on both sides of the tongue regardless of diet. Qualitatively, IL-1 β ⁺ nerves did not appear to be more (or less) prominent on the denervated side of the tongue. However, our intent was to survey cytokine-positive cell types, and CT fibers were not selectively labeled. IL-1 β ⁺ endothelial cells forming lingual vessels were also identified by morphology and colocalization with von Willebrand factor. Finally, our recent work points to the importance of neutrophils in regulating taste function (Steen et al., 2010b), and virtually all neutrophils express IL-1 β . While these double-positive leukocytes are sparse in sham-sectioned animals (Fig. 5), they are more prevalent after CT sectioning, as expected.

Blocking IL-1 alters taste function in the intact nerve after contralateral injury

We hypothesized that IL-1 β plays a beneficial role in maintaining sodium taste function in the intact CT after neighboring injury. To test this hypothesis, we performed unilateral CT sectioning and administered an IL-1 RA to block IL-1 signaling. On day 4 post-injury, we recorded neural responses to taste stimuli in the uninjured CT to determine whether responses to sodium stimuli were inhibited. In other words, does blocking IL-1 after injury mimic the low sodium diet and induce functional deficits in the neighboring, uninjured nerve?

As shown in Fig. 6A, the IL-1 RA did not alter responses to NaCl in sham-sectioned animals, even though considerable levels of IL-1 β are found in the normal peripheral taste system. Control animals receiving CT sectioning and vehicle injection also exhibited normal responses to NaCl in the contralateral nerve. In contrast, rats given CT sectioning and the IL-1 RA had significantly reduced responses to 0.25 M NaCl ($p < 0.05$ vs. sham-sectioned controls). Responses to 0.50M NaCl were significantly decreased in the experimental group vs. both control groups ($p < 0.01$). Lingual application of amiloride, which blocks ENaC, abolished group-related differences ($p > 0.05$).

The effects of the IL-1 RA on neural responses were largely specific to sodium stimuli. CT responses to 0.50M NaAc, another sodium stimulus, were significantly decreased by IL-1 RA after sectioning (Fig. 6B; $p < 0.05$). Responses to concentration series of KCl (Fig. 7A) and NH₄Cl (Fig. 7B); and single concentrations of QHCl, MSG, and sucrose (Fig. 7C) were not significantly altered by surgical or pharmacological treatment ($p > 0.05$). There were two exceptions to the sodium-specificity of the changes in taste responses after CT sectioning and IL-1 RA. Responses to 0.50M KCl and 0.10N HCl were significantly reduced by this treatment compared to one or more control groups (Fig. 7A and 7C; $p < 0.05$).

Taken together, these findings show that blocking IL-1 signaling inhibits sodium responses in the uninjured nerve. These functional deficits mimic the effects of dietary sodium restriction, and suggest that IL-1 has beneficial effects in maintaining normal taste responses after nearby injury.

Effects of blocking IL-1 on leukocyte responses to nerve injury

Previous work shows that the activated macrophage response to injury parallels the recovery of normal sodium taste function in the neighboring, uninjured CT (McCluskey, 2004; Cavallin and McCluskey, 2005; Wall and McCluskey, 2008; Guagliardo et al., 2009). Since responses to NaCl decreased in rats treated with the IL-1 RA after nerve sectioning, we tested whether this treatment also blocks macrophage responses.

Fig. 8 shows macrophage responses to nerve injury at day 2 post-sectioning, when infiltration peaks (McCluskey, 2004). ED1⁺ activated macrophages are sparse in sham-sectioned animals injected with the IL-1 RA. More macrophages are observed on both sides of the tongue after CT sectioning and vehicle injection. In contrast, blocking IL-1 signaling reduces the bilateral macrophage response to injury. Image analysis (Fig. 8J) confirms that ED1 levels are significantly elevated on both the sectioned ($p < 0.05$) and intact ($p < 0.05$) sides of the tongue in the group given CT sectioning and daily vehicle injection. IL-1 RA inhibits the bilateral macrophage response to nerve injury ($p < 0.05$). By day 4 post-injury, macrophage levels returned to baseline in each treatment group ($p > 0.05$; not shown), consistent with previous work (McCluskey, 2004). Thus, the IL-1 RA blocked the macrophage response to CT sectioning rather than delayed macrophage entry to the tongue.

Neutrophils respond to CT nerve injury before macrophages, and impair sodium taste function in the neighboring nerve (Steen et al., 2010b). Since the IL-1 RA reduces sodium taste function in the intact CT, we asked whether the antagonist might also affect neutrophil recruitment. On day 1 post-sectioning, MPO⁺ neutrophils are increased on the denervated side of the tongue in Cut + Vehicle animals compared to sham-sectioned rats injected with the IL-1 RA (Fig. 9; $p < 0.001$). The IL-1 RA significantly decreased the neutrophil response to CT sectioning on the injured side of the tongue ($p < 0.01$ vs. Cut + Vehicle). In contrast, the number of neutrophils on the intact side of the tongue did not significantly differ between groups ($p > 0.05$). Thus, neutrophils were not likely responsible for functional deficits in the intact population of taste receptor cells at day 4 after CT injury and IL-1 RA treatment.

DISCUSSION

Nearly all nucleated cells have the potential to express IL-1, and we show that the cytokine is widely expressed in the normal and injured peripheral taste system. Endothelial cells, epithelial cells, nerve bundles, and infiltrating inflammatory cells are IL-1 β +, as described in other tissues and models (Lim and Brunjes, 1999; Copray et al., 2001; Ruohonen et al., 2002; Shamash et al., 2002; Abbas and Lichtman, 2003; Pineau and Lacroix, 2007). Our current findings show that taste buds also express IL-1 β . At day 2 after unilateral CT sectioning, IL-1 β levels surge on the injured side of the tongue, consistent with the dynamics in the injured sciatic nerve (Shamash et al., 2002). Two sources likely account for the dramatic increase. First, IL-1 β expression is elevated in denervated taste receptor cells and surrounding epithelial cells after nerve section. Second, IL-1 β + activated macrophages invade both sides of the tongue. Dietary sodium deficiency inhibits both the increase in lingual IL-1 β and the entry of IL-1 β + macrophages, though the few macrophages observed in sodium-restricted animals are IL-1 β +. The diet also dysregulates the immune response to injury and perpetuates abnormal taste function in the neighboring taste receptor cells (Hill and Phillips, 1994; McCluskey, 2004; Steen et al., 2010b).

Results from negative and positive control tissues support the specificity of antibody binding (Supp. Fig. 1). IL-1 β expression was below the limits of detection by ELISA in hippocampus and cerebellum, but upregulated by LPS as previously reported (Nguyen et al., 1998). IL-1 β is synthesized as a pro-hormone before cleavage by caspase-1 to produce the biologically active form. The 17kD cytokine is then secreted and binds the IL-1 RI to elicit paracrine or autocrine effects (Allan et al., 2005). IL-1 β antibodies used in ELISA and immunohistochemical analyses may bind the intracellular pro-peptide. However, as others note (Nguyen et al., 1998), administration of LPS elevates bioactive IL-1 β in the brain (Fontana et al., 1984; Coceani et al., 1988). This treatment also increased IL-1 β levels in our positive control samples from hippocampus and cerebellum (Supp. Fig. 1). Furthermore, the neurophysiological changes induced by the IL-1RA after neural injury demonstrate IL-1 β

signaling by active cytokine. Interestingly, ATP, which is released by taste receptor cells upon stimulation (Finger et al., 2005), also causes the cleavage and release of active IL-1 β (Allan et al., 2005).

We used a recombinant form of the endogenous IL-1 RA to show that IL-1 can maintain normal taste function after contralateral nerve injury. Specifically, the prevention of receptor-specific IL-1 signaling decreased sodium taste function in the intact CT. Differences between treatment groups were eliminated by applying the ENaC blocker, amiloride, to the tongue. Given the amiloride sensitivity of neurophysiological changes, ENaC is the likely target for the effects of the IL-1 RA on taste function. In other words, the amiloride-insensitive portion of the sodium response (which persists after amiloride treatment) was similar between groups. In contrast, the amiloride-sensitive component of the neural response to sodium was reduced by CT section and IL-1 RA vs. control groups.

Strong behavioral (Spector and Glendinning, 2009) and physiological evidence (DeSimone and Lyall, 2006) indicates that one pathway for sodium transduction is through ENaC in taste receptor cells. Recent work directly demonstrates that α -ENaC is critical for sodium transduction in murine taste receptor cells (Chandrashekar et al., 2010). Importantly for the current study, IL-1 β modulates ENaC expression and/or function in other sodium-sensing epithelia. For example, the cytokine inhibits ENaC in distal colon (Barmeyer et al., 2004), epithelial cells from lung (Roux et al., 2005) and middle ear (Choi et al., 2007). In fetal lung, IL-1 β upregulates ENaC- α expression and fluid absorption (Li et al., 2009). Thus, IL-1 β can enhance or inhibit ENaC depending on the tissue and stage of development.

The effects of the IL-1 RA on taste function were largely limited to sodium stimuli. However, responses to 0.50M KCl and 0.01N HCl were also significantly reduced by the antagonist. This suggests that IL-1 may bind to taste receptor cells sensitive to high concentrations of KCl and NaCl as well as acid (i.e. taste cells with low sodium selectivity) (Chandrashekar et al., 2010). Another possibility is that IL-1 modulates presynaptic taste receptor cells receiving converging input from narrowly tuned receptor cells in the taste bud (Roper, 2007). Regardless of the identity of sodium-sensitive taste cells, blocking IL-1 signaling after neighboring nerve injury significantly alters gustatory input to the CNS.

Though IL-1 β is extensively expressed in the normal peripheral taste system, the antagonist did not affect CT responses in control animals. We suggest that leukocyte recruitment to the tongue by injury or inflammation is a key step in inducing functional changes through IL-1 signaling. Neutrophils, which are the first invaders after either CT nerve section or an inflammatory stimulus, cause a transient decrease in sodium taste function in the intact nerve (Steen et al., 2010b). In the current study, the IL-1 RA only inhibited neutrophil responses on the denervated side of the fungiform field. However, macrophages were inhibited on both sides of the tongue by this treatment. The macrophage response to injury parallels the recovery of normal taste function in the intact CT after neighboring nerve injury (McCluskey, 2004; Cavallin and McCluskey, 2005; Guagliardo et al., 2009). We propose that macrophages contribute to changes in neural responses recorded at day 4 post-injury in the current work, since the IL-1RA prevented their infiltration of the intact fungiform taste field.

IL-1 is a potent inducer of leukocyte infiltration. This is particularly well-studied in the CNS, where IL-1 β injection (Allan et al., 2005) or chronic expression by a transgene (Shaftel et al., 2007) stimulates leukocyte entry. Conversely, pharmacological or genetic knockdown of IL-1 β or its receptor reduces leukocyte recruitment in response to cytokine injection or neural injury (Basu et al., 2002; Ching et al., 2007). IL-1 β recruits macrophages to the CNS by upregulating endothelial levels of macrophage chemotactic protein (MCP)-1, intracellular

adhesion molecule (ICAM)-1, and vascular cell adhesion molecule (VCAM)-1 (Basu et al., 2002; Shaftel et al., 2007). Expression levels of these same recruitment factors increase in parallel with macrophage entry to the injured peripheral taste system (Cavallin and McCluskey, 2007a, 2007b). Here, the IL-1 RA may act in part by blocking the upregulation of vascular molecules that stimulate macrophage entry to the tongue.

We propose a sequence of events where vascular adhesion molecules upregulated by CT sectioning stimulate macrophage recruitment. Activated macrophages then invade both sides of the tongue and release IL-1 β , which binds its receptor on taste receptor cells to promote ENaC expression and/or function. Blocking this pathway with the IL-1RA results in decreased sodium taste function in intact taste receptor cells. In vitro studies are in progress to resolve whether IL-1 β can directly regulate taste receptor cell function. Given the redundancy of proinflammatory cytokine cascades, however, it is critical to demonstrate the role of IL-1 in vivo, as shown here. Indeed, TNF- α and other cytokines are likely upregulated after nerve injury in the peripheral taste system and may have either positive or negative effects on taste function after trauma (Shamash et al., 2002).

We demonstrate that a proinflammatory cytokine is beneficial for taste function after neighboring neural injury. IL-1 β appears to have both positive and negative effects after damage to other peripheral nerves. IL-1 β protein is upregulated in the sciatic nerve of wild-type mice after injury, but not in mutants with delayed degeneration and impaired regeneration (Shamash et al., 2002). Furthermore, axonal regeneration is improved by administering IL-1 β after sciatic nerve sectioning, as evident by morphology and the recovery of sensory function (Temporin et al., 2008). However, IL-1 β signaling increases spontaneous activity in nociceptors and associated neurons, and contributes to hyperalgesia (Liu et al., 2006; Binshtok et al., 2008). Many studies in the injured brain indicate that IL-1 β exacerbates damage, making the IL-1 RA an attractive candidate for treating neurologic disease (Allan et al., 2005). Clearly, IL-1 β has different roles in the injured CNS and PNS, and even among tissues within the PNS.

The release of IL-1 β in damaged neural systems results in stereotyped intracellular events. The cytokine binds membrane-bound IL-1 RI and triggers the activation of mitogen-activated protein kinases (MAPKs) and nuclear factor (NF)- κ B. Diverse secondary mediators, including proinflammatory cytokines, adhesion molecules, chemokines, growth factors, and matrix metalloproteinases, are then synthesized (Allan et al., 2005). IL-1 β downregulates ENaC expression in alveolar epithelial cells by inhibiting ENaC- α promoter activity via p38 MAPK (Roux et al., 2005). P38 MAPK is enriched in fungiform papillae during embryonic development (Liu et al., 2008), but the role of this kinase in adult taste transduction has not (to our knowledge) been studied.

The peripheral taste system faces a barrage of mechanical and microbial challenges. However, surprisingly little is known about the lingual cytokine environment and regulation of taste function. An exception is recent work demonstrating the expression of interferon (IFN) and members of its signaling pathways in normal lingual epithelium (Wang et al., 2007). Systemic treatment with IFN- α or IFN- γ upregulated several IFN-inducible genes in taste receptor cells, and increased apoptotic taste cells. This group did not find an increase in IL-1 β mRNA in taste buds on the posterior tongue (i.e. circumvallate and foliate) at 6 hrs after systemic LPS, though additional time points were not examined (Cohn et al., 2010). Thus, it appears that cytokine expression and regulation of events in the peripheral taste system depends on the molecule of interest, stimulus (e.g. injury or infection), taste cell population, and timing.

We demonstrate that the proinflammatory cytokine, IL-1, has a beneficial role in maintaining normal taste input to the brain following nearby injury. Understanding the complex effects of cytokines on taste function will likely be a fertile area for future studies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

Lingual IL-1 β is upregulated by unilateral CT nerve sectioning. ELISA were used to measure IL-1 β in protein lysates from the sectioned or intact sides of the tongue (means of n=6–9 rats/group). Optical density values are normalized to tissue weight (pg/g). **A.** IL-1 β is present in sham-sectioned control rats. Control-like IL-1 β levels are maintained through 24 hr post-injury, and then increase significantly on the sectioned side of the tongue at day 2. **B.** Dietary sodium restriction prevents the upregulation of IL-1 β levels on the sectioned side of the tongue at day 2 post-injury. **C.** IL-1 β levels do not differ with dietary treatment at day 4 post-injury. * $p < 0.05$; *** $p < 0.001$

Figure 2. IL-1 β expression in ED1 $^{+}$ activated macrophages at day 2 after unilateral CT sectioning. **A–C.** The few ED1 $^{+}$ macrophages (red) present in sham-sectioned animals are immunopositive for IL-1 β . **D–I.** Following nerve injury, many IL-1 β $^{+}$ macrophages are observed on both sides of the tongue. **J–L.** Animals receiving CT sectioning and a low-sodium diet exhibit few macrophages, but they are IL-1 β $^{+}$ on both the sectioned and intact (not shown) sides of the tongue. Scale bar in L = 30 μ m.

Figure 3.

IL-1 β expression in fungiform taste buds at day 2 following nerve sectioning. IL-1 β (green) is double-labeled with Cytokeratin 19 (CK-19; red) which selectively stains taste receptor cells. **A–C.** IL-1 β is expressed in sham-sectioned taste buds and surrounding epithelial cells. **D–F.** On the sectioned, cut side of the tongue, IL-1 β staining appears stronger in taste buds and surrounding epithelium compared to shams. **G–I.** Robust IL-1 β expression is observed in uninjured taste buds. **J–L.** Denervated (not shown) and intact CK-19 $^{+}$ taste buds are also IL-1 β^{+} in animals receiving nerve sectioning and dietary sodium deficiency. Scale bar in L = 20 μ m.

Figure 4. IL-1 β receptor expression in fungiform taste buds at day 2 post-sectioning. **A–C.** IL-1 β receptor immunoreactivity is present within CK-19⁺ taste receptor cells and surrounding epithelium in sham-sectioned control animals. **D–F.** IL-1 β receptor is also present in denervated (not shown) and intact fungiform taste buds in control-fed and **(G–I)** sodium-deficient rats. Scale bar in I = 30 μ m.

Figure 5.

Lingual expression of IL-1 β in non-gustatory cells. Images are sham-sectioned control rats, though similar patterns are observed after CT sectioning as described in the text. **A–C.** IL-1 β is localized to neurofilament (NF)-positive nerve bundles. **D–F.** IL-1 β is present in vessels, identified by von Willebrand (von Will) factor, in the submucosa, lamina propria, and within fungiform papillae. **G–I.** Neutrophils identified by staining for myeloperoxidase (MPO) are IL-1 β ⁺. Scale bars = 40 μ m in C and F; 15 μ m in I.

Figure 6.

Mean relative responses to sodium stimuli recorded from the intact CT four days after contralateral sectioning. **A.** Responses to 0.25M and 0.50M NaCl are significantly reduced in the group receiving the IL-1 receptor antagonist (RA) and sectioning compared to controls. The epithelial sodium channel blocker, amiloride, reduces neural responses from each group to the same magnitude. **B.** Responses to 0.50M sodium acetate (NaAc) are significantly decreased by the IL-1 RA in combination with neighboring nerve injury vs. control groups. **p < 0.01; *p < 0.05.

Figure 7.

Mean relative responses to non-sodium stimuli recorded from the intact CT four days after contralateral section. **A.** Responses to 0.50M KCl were reduced by the IL-1 receptor antagonist (RA) and neighboring nerve injury compared to control groups. Note that while the response to 0.25M NaAc appears reduced by antagonist treatment in combination with nerve sectioning, the change was not statistically different. **B.** Responses to NH₄Cl did not differ among groups. **C.** Neural responses to QHCl, monosodium glutamate (MSG), and sucrose (Suc) were similar across groups, indicating that the surgical and/or pharmacological treatment did not affect bitter, umami, or sweet transduction, respectively. Trends suggest that HCl responses, however, were lower in animals receiving contralateral sectioning and IL-1 RA compared to the sham-sectioned group injected with antagonist. * $p < 0.05$.

Figure 8.

Lingual ED1⁺ activated macrophages (brown) at day 2 after CT nerve section. **A–C.** Few macrophages are present in sham-sectioned animals treated with the IL-1 receptor antagonist (RA). **D–E.** More ED1⁺ macrophages are found on the denervated “Cut Side” of the tongue, the intact side, and in fungiform papillae housing taste buds (grey star). **G–I.** Macrophages are sparse in each tissue compartment of animals treated with the IL-1 RA after nerve sectioning. **J.** Quantification of ED1⁺ staining demonstrates that CT sectioning (“Cut + Vehicle”) significantly increases macrophage levels on both sides of the tongue compared to sham-sectioned controls (“Sham + IL-1 RA”). The bilateral macrophage response is prevented by treatment with the IL-1 RA (“Cut + IL-1 RA”). Scale bar in I = 30 μ m. * p < 0.05

Figure 9. Lingual MPO⁺ neutrophils (brown) at day 1 after CT nerve section. **A–C.** Few neutrophils are present in the submucosa (A–B) or fungiform papilla (C) with taste bud (grey star) after sham CT sectioning and treatment with the IL-1 receptor antagonist (RA). **D–F.** More neutrophils are observed after CT sectioning, especially on the denervated “Cut Side” of the tongue. **G–I.** MPO⁺ neutrophils are observed in each tissue compartment after nerve sectioning and IL-1 RA injection. **J.** Quantification of neutrophils in the tongue. The number of neutrophils significantly increases on the injured side of the tongue after CT sectioning. In the group given the IL-1 RA after nerve sectioning, neutrophils remain at sham-like levels. There is no significant difference in the number of neutrophils on the contralateral, intact side of the tongue. Scale bar in I = 30 μ m. * $p < 0.05$

Table 1

Summary of primary antibodies used

Cell Identified	Immunogen/Clone	Dilution	Manufacturer	References
Multiple	IL-1 β	1: 50	Abcam; Cambridge, MA	(Maddahi and Edvinsson, 2010)
Multiple	IL-1 β Receptor	1:100	Abcam; Santa Cruz Biotechnology, Inc.; Santa Cruz, CA	(Wang et al., 2002; Wang et al., 2006; Bletsa et al., 2009)
Taste receptor cells	Cytokeratin 19/K4.62	1:400	Sigma; St. Louis, MO	(Wong et al., 1994; McCluskey and Hill, 2002)
Neurons	Neurofilament 200/N52	1:500	Sigma	(Debus et al., 1983; Franke et al., 1991)
Endothelial cells	vonWillebrand factor	1:100	Cedarlane; Hornby, Ontario, Canada	(Jin et al., 2006)
Neutrophils	Myeloperoxidase	1:50	Abcam	(Carlson et al., 1998; Perkins and Tracey, 2000; Klebanoff, 2005; Fleming et al., 2006)
Activated macrophages	Lysosomal membranes/ED1	1:400	Serotec; Raleigh, NC	(Dijkstra et al., 1985; Damoiseaux et al., 1994; McCluskey, 2004; Cavallin and McCluskey, 2005; Guagliardo and Hill, 2007)