Chem. Senses 29: 463–471, 2004 doi:10.1093/chemse/bjh048

Rat Gustatory Neurons in the Geniculate Ganglion Express Glutamate Receptor Subunits

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Abstract

Taste receptor cells are innervated by primary gustatory neurons that relay sensory information to the central nervous system. The transmitter(s) at synapses between taste receptor cells and primary afferent fibers is (are) not yet known. By analogy with other sensory organs, glutamate might a transmitter in taste buds. We examined the presence of AMPA and NMDA receptor subunits in rat gustatory primary neurons in the ganglion that innervates the anterior tongue (geniculate ganglion). AMPA and NMDA type subunits were immunohistochemically detected with antibodies against GluR1, GluR2, GluR2/3, GluR4 and NR1 subunits. Gustatory neurons were specifically identified by retrograde tracing with fluorogold from injections made into the anterior portion of the tongue. Most gustatory neurons in the geniculate ganglion were strongly immunoreactive for GluR2/3 (68%), GluR4 (78%) or NR1 (71%). GluR1 was seen in few cells (16%). We further examined if glutamate receptors were present in the peripheral terminals of primary gustatory neurons in taste buds. Many axonal varicosities in fungiform and vallate taste buds were immunoreactive for GluR2/3 but not for NR1. We conclude that gustatory neurons express glutamate receptors and that glutamate receptors of the AMPA type are likely targeted to synapses within taste buds.

Key words: AMPA receptor, fluorogold tracing, NMDA receptor, sensory neurons, taste bud

Introduction

Primary gustatory neurons innervating taste receptor cells relay sensory information to the central nervous system. These neurons are located in the geniculate ganglion of the facial nerve, the petrosal ganglion of the glossopharyngeal nerve, and the nodose ganglion of the vagus nerve. Primary gustatory neurons do not form anatomically distinct neuronal groups in these ganglia but rather are interspersed among neurons of other sensory modalities. The majority of gustatory neurons in the geniculate ganglion innervate taste cells in taste buds in the anterior portion of the tongue and taste buds in the soft palate (Boudreau *et al.*, 1971; Gomez, 1978; Frank *et al.*, 1988; Harada and Smith, 1992). Gustatory neurons use glutamate as a neurotransmitter at their central synapses in the rostral portion of the nucleus of the solitary tract (Bradley *et al.*, 1996; Grabauskas and Bradley, 1996; Li and Smith, 1997). Recent studies suggest that glutamate might be a neurotransmitter in taste buds (Caicedo *et al.*, 2000a,b; Lawton *et al.*, 2000; Kim *et al.*, 2002), but whether glutamate mediates synaptic transmission between taste receptor cells and sensory axons of primary gustatory neurons is still unknown.

Glutamate is a major excitatory neurotransmitter in the central nervous system and is also the principal neurotransmitter at receptor cell/afferent nerve synapses in the cochlea, the vestibule, and the retina (Puel, 1995; Thoreson and Witkovsky, 1999; Usami *et al.*, 2001). Ionotropic glutamate receptors (iGluRs) mediate most fast and direct glutamate neurotransmission. iGluRs are commonly subdivided into three families according to their sensitivity to agonists. A first group is sensitive to NMDA, a second group consists of AMPA-sensitive receptors, and a third group is most sensitive to kainate. The receptor channels are heteromers composed of different subunits. Five subunits exist for NMDA receptors (NR1 and NR2A–D), four for AMPA receptors (GluR1–4), and five for kainate receptors (GluR5–7 and KA1–2). Sensory neurons of cranial and spinal ganglia express a variety of these subunits. For instance, primary auditory neurons of the cochlea express AMPA receptor subunits GluR2, 3 and 4, kainate receptor

subunits GluR 5 and 6, and KA 1 and 2, as well as NMDA receptor subunits NR1 and NR2A–D (e.g. Safieddine and Eybalin, 1992; Niedzielski and Wenthold, 1995). These subunits form functional receptors at afferent synapses with the inner hair cells (for a review see Puel, 1995). By analogy with other sensory neurons, primary gustatory neurons may also express iGluRs. We examined the presence of AMPA and NMDA receptor subunits in gustatory neurons of the geniculate ganglion that innervate the anterior portion of the tongue. We used immunohistochemistry with antibodies against GluR1, GluR2, GluR2/3, GluR4, and NR1 (Petralia and Wenthold, 1992; Wenthold *et al.*, 1992; Petralia *et al.*, 1994, 1997) combined with retrograde tracing with fluorogold. We found that most gustatory neurons express GluR3, GluR4 and NR1, some neurons express GluR2, and GluR1 is almost absent. In addition, sensory afferent axons in taste buds in fungiform and vallate papillae of the tongue were also immunoreactive for GluR2/3. These subunits may form functional glutamate receptors at sensory afferent axons in taste buds.

Materials and Methods

Tracer injection

Twelve Sprague–Dawley rats weighing 150–300 g were used. They were deeply anaesthetized with a mixture of ketamine (42.8 mg/ml), xylazine (8.6 mg/ml) and 1.4 mg/ml acepromazine (0.5 ml/kg i.m., prepared by the Department of Veterinary Resources, University of Miami). The tongue was gently retracted and tracer was microinjected into the lingual epithelium. The retrograde tracer fluorogold (2% dissolved in distilled water, Molecular Probes, Eugene, Oregon) was applied to most of the anterior half of the tongue in order to trace as many gustatory neurons in the geniculate ganglion as possible. Six injections of the tracer (1 µl) were applied with a 10 µl Hamilton microsyringe to three different antero-posterior regions on both sides of the tongue. Injections were placed beneath the epithelium as close as possible to the surface of the tongue. The tracer was pressure-injected for 5 min for each injection. Rats usually recovered from anesthesia within 40 min. Placement of the injections was confirmed histologically. All experimental procedures were approved by the animal care and use committee and conform to NIH guidelines.

Tissue preparation

Two days after the fluorogold injection, animals were again anesthetized (as described above) and perfused through the aorta with 0.9% saline, followed by ice-cold 4% paraformaldehyde (300 ml, 12 min) in 0.1 M phosphate buffer (pH 7.4). Geniculate ganglia were then exposed by carefully removing bones of the dorsal tympanic bulla and facial canal. The ganglia were removed and post-fixed for 2 h in the fixative used for perfusion. They were then immersed overnight in 0.1 M phosphate buffer containing 20% sucrose at 4°C. Sections (14 µm) were cut on a cryostat microtome in the horizontal plane and thaw-mounted on Superfrost Plus slides (VWR Scientific, West Chester, PA). For each geniculate ganglion, serial sections were collected on five different slides, each incubated with a different antibody. The brains were processed in parallel as a control. Tongue sections (20 µm) were also cut to determine the extent of tracer diffusion.

Immunohistochemistry

Sections were processed for immunohistochemistry using the indirect fluorescence method. First, the sections were rinsed in several changes of 0.01 M phosphate-buffered saline (PBS) and then incubated in a blocking solution containing 30% normal goat serum in PBS, pH 7.4. After 1 h, sections were transferred into the primary antiserum solution of either rabbit anti-GluR1 (1–1.5 µg/ml), anti-GluR2 (1–1.5 µg/ml), anti-GluR2/3 (1–1.5 µg/ml), anti-GluR4 (1–1.5 µg/ml) or anti-NR1 (1 µg/ml), diluted in PBS, and incubated for 72 h at 4°C. Thereafter, the sections were rinsed for 30 min in 3–5 changes of PBS, transferred to Cy3-conjugated anti-rabbit IgGs (1:500, Amersham Pharmacia Biotech, Piscataway, NJ) and incubated for 2 h at 20°C. Finally, the sections were thoroughly washed, mounted with aqueous mounting medium (Crystal Mount; Biomeda, Foster City, CA) and coverslipped.

Control experiments were carried out by incubating sections in the absence of primary antibodies. No immunostaining was detected under these conditions. Cross-reactivity among the antisera could be ruled out because their distribution patterns were distinctly different in the geniculate ganglion as well as in the cerebellum. The specificity of the antisera has been described previously in detail (Petralia and Wenthold, 1992; Wenthold *et al.*, 1992; Petralia *et al.*, 1994, 1997). Furthermore, staining patterns in the cerebellum were identical to those reported in those studies. The antibodies recognizing the different AMPA and NMDA receptor subunits were purchased from Chemicon (Temecula, CA).

For immunohistochemical detection of GluR subunits in the tongue we used tyramide signal amplification (Hunyady *et al.*, 1996; Wang *et al.*, 1999). To identify sensory axons in taste buds, we double immunostained tongue sections with antibodies against GluR subunits and against the synaptic protein SNAP-25 (Sternberger Monoclonals Incorporated, Lutherville, MD). SNAP-25 is an excellent marker of sensory afferent axons in taste buds (Pumplin and Getschman, 2000). Tongues were immersion fixed with 4% paraformaldehyde (30–60 min, 20°C), cryoprotected in 30% sucrose, and cut on a cryostat microtome. The short fixation time was necessary to visualize GluR immunostaining at synaptic sites, as has been reported for the retina (Grünert *et al.*, 2002). Tongue sections (25 µm) containing fungiform or vallate papillae were processed free-floating. Endogenous peroxidase activity was quenched in 3% H_2O_2 in methanol.

The sections were rinsed in several changes of 0.01 M phosphate-buffered saline (PBS) and then incubated in a blocking solution containing 30% normal goat serum in PBS, pH 7.4. After 1 h, sections were transferred into the primary antiserum solution of either rabbit anti GluR2/3 (1–1.5 μg/ml) or anti NR1 (1 μg/ml), and mouse anti SNAP-25 (1:10,000), diluted in PBS, and incubated for 72 h at 20°C. Thereafter, the sections were rinsed for 30 min in 3–5 changes of PBS, transferred to HRP-conjugated anti-rabbit IgGs (1:500, Molecular Probes, Eugene, OR) and Alexa488-conjugated anti-mouse IgGs (1:500, Molecular Probes), and incubated for 2 h at 20°C. Sections were rinsed for 30 min in 3–5 changes of PBS and incubated for 10 min in TSA amplification reagent labeled with Cy3 to visualize GluR2/3 or NR1 immunostaining. Sections were again rinsed for 30 min in 3–5 changes of PBS.

Data analysis

Geniculate ganglion sections were analyzed under a Zeiss Axioplan microscope using epifluorescent illumination. Labeled structures were photographed at ×10–40 magnification. Whenever possible, serial adjacent sections are shown in the figures to compare the staining patterns for the different AMPA receptor subunits. Fluorogold labeling was observed with a fluorescein filter set, Cy3 labeling with a rhodamine filter set. Double exposure photomicrographs were taken to document immunostaining in retrogradely traced neurons. Photographic slides were scanned (LS 1000, Nikon, Melville, NJ) and compiled using Adobe Photoshop 4.0.1 (Adobe Systems Inc., San Jose, CA). Figures were altered from the original scans only to adjust contrast and brightness for uniform tone within a single figure.

We calculated the percentage of retrogradely labeled gustatory neurons that were also positive for a particular AMPA receptor subunit. Sections were viewed at ×25 magnification and sequentially photographed with the two filter sets. The photographic slides were then projected onto a screen. We first counted the number of retrogradely labeled neurons and then the number of gustatory neurons that were double-labeled with both fluorogold and antibody. The number of double-stained neurons was divided by the total number of retrogradely traced neurons, and multiplied by 100. Neurons were counted in two sections. Because there were no differences between the distal and proximal portions of the geniculate ganglion, data were pooled. Ganglia were included in this study only if >50 retrogradely traced neurons were present in the two sections. Two investigators did all cell counts blindly and independently.

Determination of double staining of lightly immunoreactive neurons was often problematic. To prevent bias when identifying immunoreactive neurons, a gustatory neuron was only counted if it was clearly distinguishable from background (strong staining in the cytoplasm with discernible, less stained nuclei). Thus, it is likely that we have underestimated immunoreactive neurons because they could not be easily discerned from background staining.

Immunostaining in taste buds was examined with a dual fluorescence scanning confocal microscope (Olympus Fluoview, Melville, NY). Antibody concentrations and confocal microscope settings were adjusted to avoid fluorescence interference in the colocalization studies. Fluorescence images for the two fluorochromes were acquired sequentially with their specific settings and merged subsequently. For every section examined we determined that no red fluorescence (605 nm emission high pass filter) could be detected using the settings for green fluorescence (i.e. 488 nm excitation with the argon laser), and conversely that no green fluorescence (510–550 nm emission band pass filter) could be detected using the settings for red fluorescence (i.e. 568 nm excitation with the krypton laser). We examined thin optical sections (1 μm) to ensure that overlapping immunofluorescence was due to colocalization.

Results

AMPA receptor subunit staining in the geniculate ganglion

The different AMPA receptor subunits showed different immunostaining patterns in the geniculate ganglion. Whereas GluR1 and GluR2 staining was scarce, GluR2/3 and GluR4 immunoreactive cells were widespread (Figure 1). GluR1 staining was almost absent, but in some neurons the staining intensity was discernible above background levels (not shown). GluR2 staining was very strong only in a subset of neurons (Figures 1B and 2A,C). Other neurons showed low or moderate staining intensities or were not stained at all. Thus, of all immunoreactivities examined, that of GluR2 had the most heterogeneous staining pattern. By contrast, GluR2/3 staining was present throughout the geniculate ganglion, with most neurons intensely stained (Figures 1C and 2B,D). A similar staining pattern was seen for GluR4 (Figure 1D), but the staining intensity was moderate. There were no differences between the distal and proximal portions of the geniculate ganglion in the staining distribution of all four immunoreactivities (for GluR2 and GluR2/3, see Figures 1 and 2). Therefore, for our quantitative analysis, data were pooled from both regions. Parallel immunostaining on brain sections (positive controls) showed distribution patterns as described previously (see Materials and Methods), indicating that differences in patterns of immunostaining in geniculate ganglia were not due to intrinsic differences in the GluR antisera.

Major differences were observed between GluR2 and GluR2/3 immunostaining (Figures 1 and 2). GluR2 staining was present in fewer neurons, and was particularly found in large neurons throughout the ganglion (Figures 1B and 2A,C). Thus, it is likely that part of the GluR2/3 staining was exclusively due to the presence of GluR3 subunits in a subset of neurons.

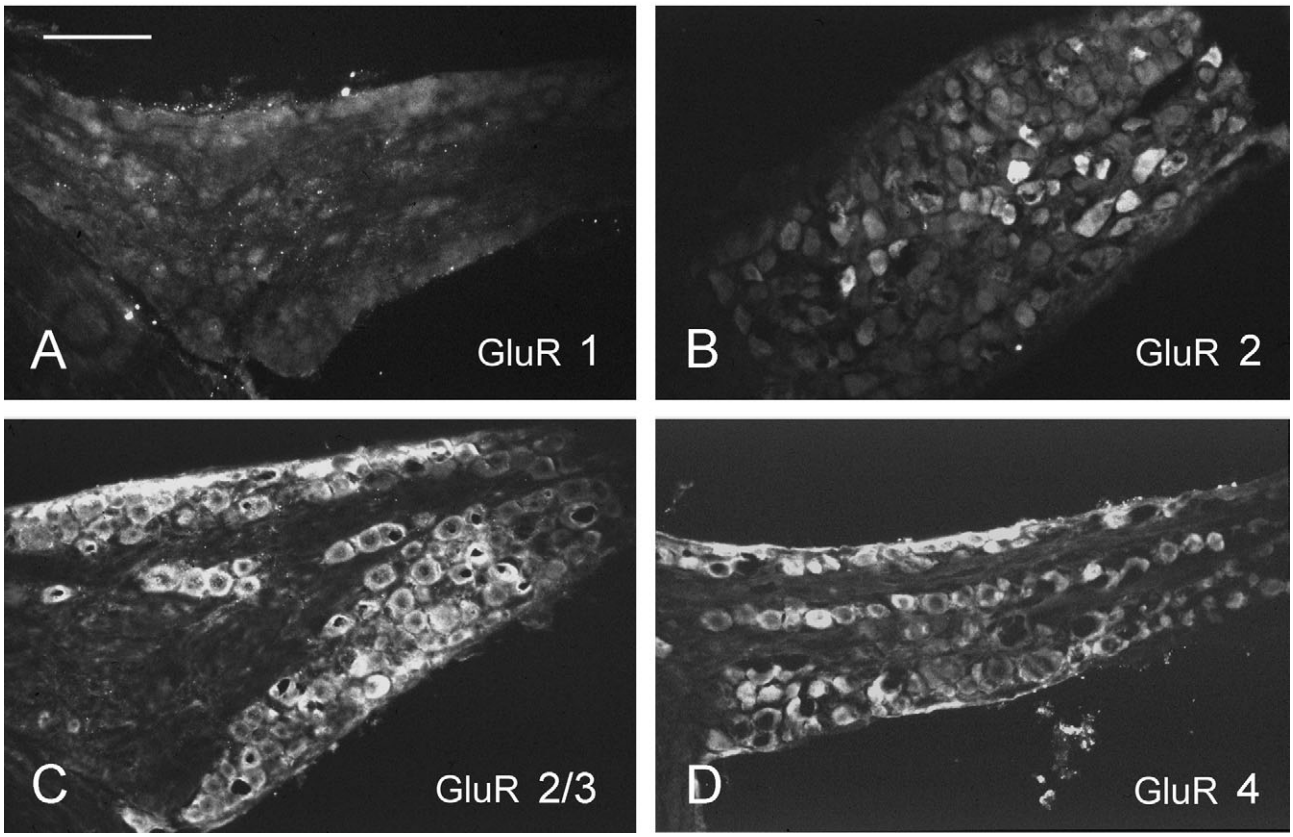

Figure 1 Photomicrographs showing the distribution of GluR1 **(A)**, GluR2 **(B)**, GluR2/3 **(C)** and GluR4 **(D)** immunoreactivities in the geniculate ganglion. GluR2/3 and GluR4 immunoreactivities were intense and widespread, whereas those of GluR1 and GluR2 were light. Note, however, that some neurons were intensely GluR2 stained. Distal is to the right. Scale bar = 100 μm (in A, applies to all panels).

AMPA receptor subunit staining in gustatory neurons

The geniculate ganglion is composed of a heterogeneous population of sensory neurons. Approximately one third of its neurons innervates the middle ear, and two thirds innervate gustatory regions of the anterior tongue and soft palate (Gomez, 1978). Thus, it is likely that many of the ganglion neurons that were immunoreactive for GluR2, 3 and 4 represented primary gustatory neurons. Nonetheless, to unambiguously identify gustatory neurons within the geniculate ganglion that innervate the fungiform papillae in the anterior tongue, we retrogradely labeled gustatory neurons by injecting tracer into the tongue (Krimm and Hill, 1998). After widespread fluorogold injections into the anterior portion of the tongue, neurons in the proximal and distal regions of the geniculate ganglion were labeled (Figure 3). Most labeled neurons were located in the periphery of the ganglion, with labeled neurons in the distal ganglion surrounding the greater superficial petrosal nerve (Figure 3C,D). When compared with the distribution of GluR2/3 staining, which was present in most neurons of the ganglion, fluorogold-labeled neurons appeared to be a minor fraction (~20% of GluR2/3-stained neurons; Figure 3A,B). However, the number of fluorogold-labeled neurons varied because of differences in the injection size and tracer uptake in the tongue. For the quantitative analysis, only ganglia with >50 fluorogold-stained neurons were used (as in Figure 3; see Material and Methods).

The immunostaining patterns for the different AMPA receptor subunits in the subpopulation of gustatory fluorogold-labeled neurons were similar to those seen for the whole ganglion. Most fluorogold-labeled neurons were GluR2/3 and GluR4 immunoreactive (68% and 78%, respectively; Figures 4 and 5), and a minority was immunostained for GluR1 and GluR2 (16% and 32%, respectively; Figures 4 and 5). However, the percentages of GluR2/3 and GluR4-stained neurons were probably underestimated because low intensity staining could not always be clearly discerned from background staining (see e.g. Figures 1D and 2B; see also Material and Methods). Fluorogold-labeled neurons that were not immunoreactive were found intermingled with double-labeled neurons and did not show any clear regional segregation (Figure 4C,I).

NMDA receptor staining in gustatory neurons

We further examined the presence of the NMDA receptor subunit NR1 within gustatory neurons of the geniculate ganglion. As with GluR2/3, NR1 immunoreactivity was widespread and intense in the geniculate ganglion (Figure 6).

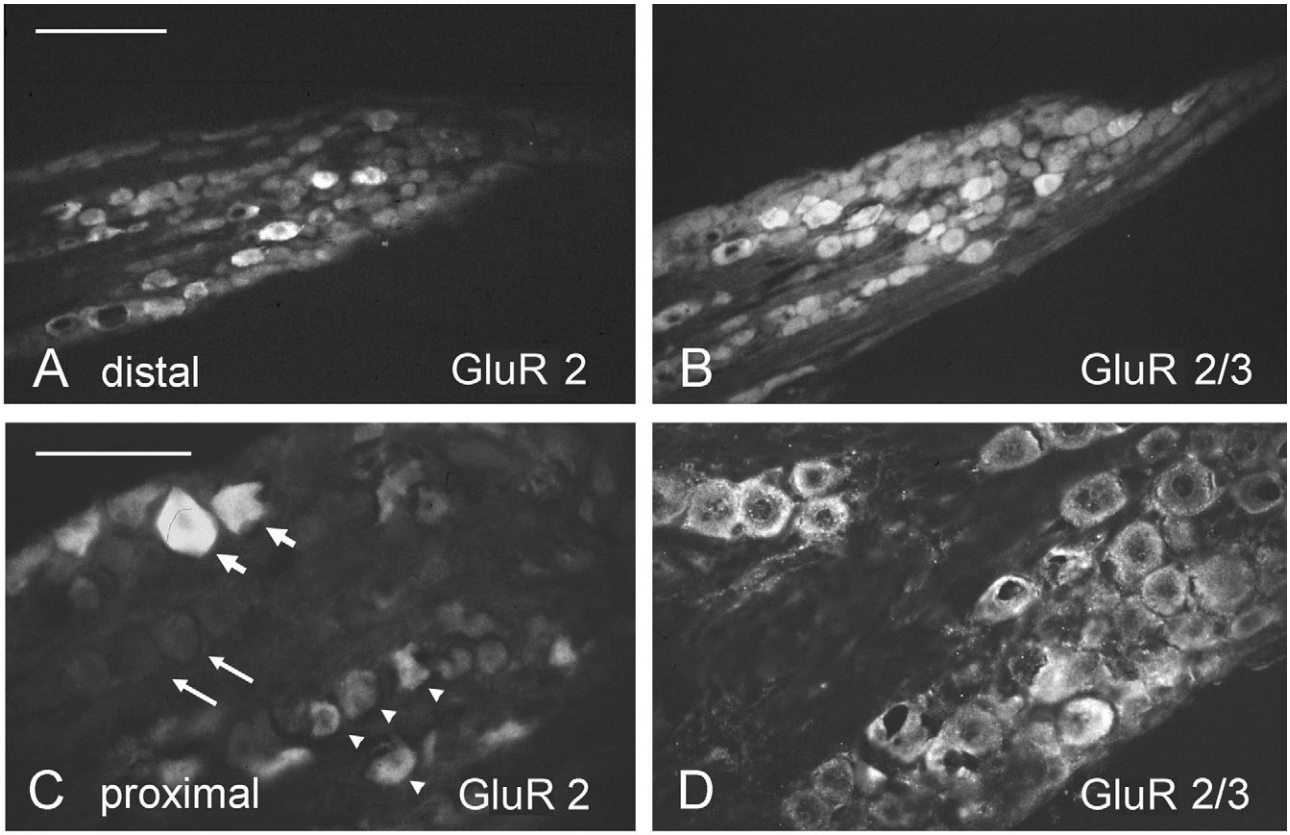

Figure 2 Photomicrographs showing differences between GluR2 **(A, C)** and GluR2/3 staining **(B, D)** in distal (A, B) and proximal portions (C, D) of the geniculate ganglion. Sections in A and B are adjacent. GluR2/3 staining was homogeneously distributed, with similar staining intensities throughout the ganglion. GluR2 staining was intense in some neurons (thick arrows), light in others (arrowheads), or absent (thin arrows, C). Distal is to the right. Scale bars = 200 μm (in A, also applies to B), 100 μm (in C, also applies to D).

Its presence within the subpopulation of gustatory neurons was also similar, with 71% being double-labeled (Figures 5 and 6C).

Glutamate receptor immunostaining in fungiform and vallate taste buds

We examined GluR2/3 and NR1 immunoreactivities in taste buds because these subunits were strongly expressed in gustatory neurons of the geniculate ganglion. Within the tongue epithelium GluR2/3 immunostaining was restricted to taste buds (Figures 7 and 8) and Remak's ganglion neurons (not shown). GluR2/3 immunostaining in fungiform and vallate taste buds had a punctate pattern (Figures 7 and 8). It has been shown in other sensory organs that these immunostained puncta represent GluR aggregates in postsynaptic sensory neurons (Demêmes et al., 1995; Grünert *et al.*, 2002). Most (~90%) of the GluR2/3 immunoreactive puncta colocalized with axonal varicosities visualized by the synaptic marker SNAP-25 (Figures 7C and 8C). As stated in the Materials and Methods section, SNAP-25 is an excellent marker for sensory afferent axons in taste buds. Thus, GluR2/3 immunostaining appeared to be confined to varicosities of sensory afferent axons. The thinner parts of the axons in the taste bud and the nerve plexus under the taste buds, however, were rarely GluR2/3 immunoreactive (Figures 7C and 8C). Indeed, GluR immunostaining in sensory axons is only detectable under special fixation conditions, nerve ligation, or with electron microscopy (Coggeshall and Carlton, 1998; Lu *et al.*, 2002). In addition to axonal varicosities, some taste cells were GluR2/3 immunoreactive in vallate taste buds, as recently reported (Kim *et al.*, 2002). In contrast to GluR2/3, NMDA immunostaining was scarce in axons but present in taste cells of fungiform papillae as well as of vallate papillae (not shown; Kim *et al.*, 2002).

Discussion

The main finding in the present study is that primary gustatory neurons innervating taste buds of the anterior tongue express GluRs. We detected GluRs of the AMPA type (i.e. GluR2/3 and GluR4) and of the NMDA type (i.e. NR1) in primary gustatory neurons in the geniculate ganglion. Importantly, GluR2/3 immunostaining was also present in sensory afferent axon terminals in taste buds. Thus, AMPA receptors expressed by primary gustatory neurons may be targeted to sensory afferent fibers postsynaptic to taste receptor cells. In analogy to other sensory organs (Puel, 1995; Thoreson and Witkovsky, 1999; Usami *et al.*, 2001),

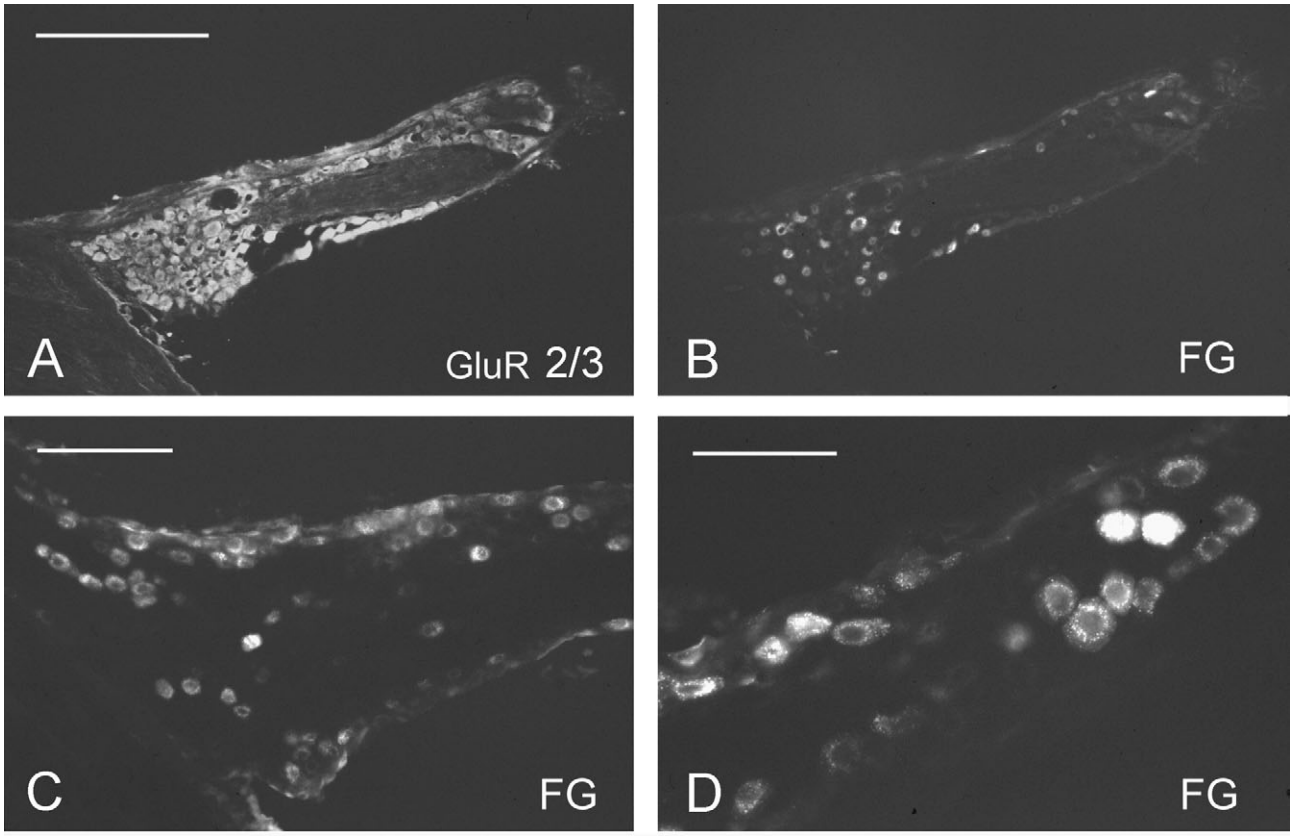

Figure 3 Photomicrographs showing the distribution of retrogradely labeled neurons in the geniculate ganglion after fluorogold injections in the anterior half of the tongue. When compared to the widespread GluR2/3 staining **(A)**, retrogradely labeled neurons represented a minor fraction **(B)**. Images are from same section but viewed with different filter sets. Retrogradely labeled neurons were present in proximal **(C)** as well as distal portions of the ganglion **(D)**. Distal is to the right. FG = fluorogold labeling. Scale bars = 500 μm (in A, also applies to B), 200 μm (C) and 100 μm (D).

these GluRs may mediate neurotransmission in taste buds. To our knowledge, this is the first identification of neurotransmitter receptors on gustatory afferent fibers in taste buds.

Most gustatory neurons in the geniculate ganglion were strongly immunoreactive for GluR2/3, GluR4 and NR1. Because only few gustatory neurons were GluR2 immunoreactive, GluR2/3 immunoreactivity is presumably due mainly to GluR3 antigenicity. Although immunostaining for AMPA receptor subunits does not necessarily reflect their incorporation into functional receptor complexes at synapses, our findings suggest that putative AMPA receptors in gustatory neurons are predominantly composed of GluR3 and GluR4 subunits. In a subset of neurons, GluR2 subunits may also be incorporated into glutamate receptor complexes. Primary gustatory neurons share with neurons in other sensory ganglia high levels of GluR2/3, GluR4 and NR1 immunostaining and low levels of GluR1 (Petralia and Wenthold, 1992; Tachibana *et al.*, 1994; Niedzielski and Wenthold, 1995). The presence of GluR immunostaining in cell bodies (e.g. as opposed to synaptic sites) may appear surprising, but it is well established that ganglion cell bodies are immunoreactive for receptors expressed at their terminals (Petralia, 1997). Furthermore, high cytoplasmic levels of GluR subunits have been widely reported in the brain (e.g. Petralia and Wenthold, 1992; for a review see Petralia, 1997).

Some technical issues deserve consideration in the interpretation of these results. It is possible that the antipeptide antisera against the different iGluR subtypes also recognize sequences of other unknown peptides. The specificity of the antisera has been described previously in detail (Petralia and Wenthold, 1992; Wenthold *et al.*, 1992; Petralia *et al.*, 1994, 1997) and staining patterns in the central nervous system were identical to those reported in those studies. Furthermore, cross-reactivity among the antisera could be ruled out because their distribution patterns were distinctly different in the geniculate ganglion as well as in the central nervous system. Thus, iGluR immunoreactivity is likely to represent iGluRs. Definite evidence for the lack of cross-reactivity, however, will require staining tissue in iGluR knockout mice.

Geniculate ganglion neurons that innervate taste buds in the anterior tongue project their central axons to the rostral portion of the nucleus of the solitary tract. Potential targets for GluR subunits expressed by gustatory neurons include their peripheral synapses in taste buds, at central processes (presynaptic receptors), and at cell bodies in the ganglion. At

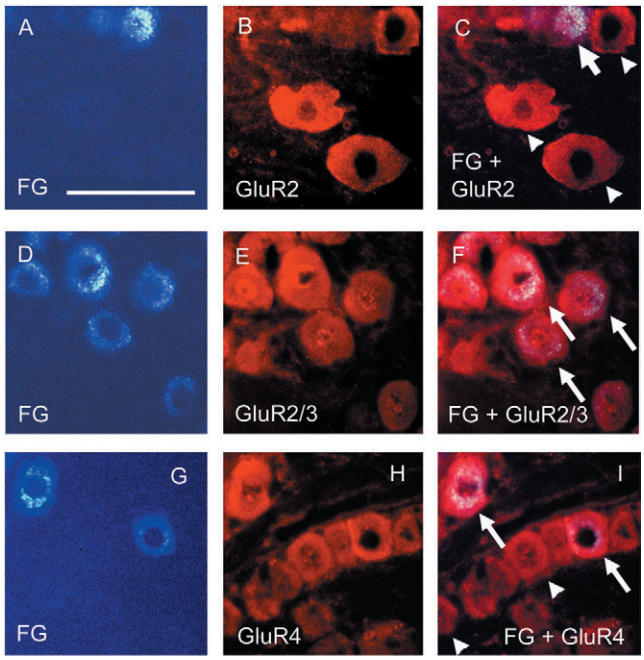

Figure 4 Photomicrographs showing AMPA receptor subunit staining in retrogradely traced gustatory neurons. The first row **(A–C)** shows colocalization (C) of fluorogold labeling (A) and GluR2 immunoreactivity (B); the second row **(D–F)**, colocalization (F) of fluorogold labeling (D) and GluR2/3 immunorteactivity (E); and the third row **(G–I)**, colocalization (I) of fluorogold labeling (G) and GluR4 immunoreactivity (H). Neurons showed colocalization (thin arrows), immunoreactivity only (arrowheads), or fluorogold labeling only (thick arrows). FG = fluorogold labeling. Scale bar = 100 μm (in A, also applies to all panels).

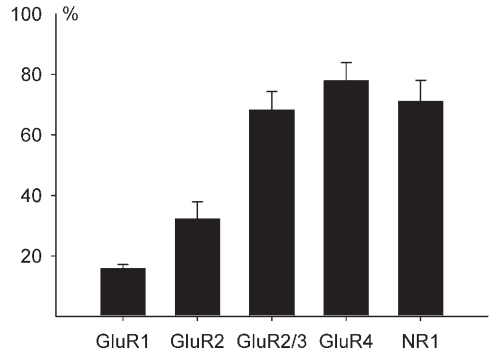

Figure 5 Histogram showing the percentage of retrogradely labeled neurons in which a given AMPA or NMDA receptor subunit was co-localized by immunostaining. While most of the retrogradely labeled neurons were immunoreactive for GluR2/3 (68%, $n = 8$), GluR4 (78%, $n = 7$), and NR1 (71%, $n = 3$), only a minority was immunoreactive for GluR1 (16%, $n = 4$) or GluR2 (32%, $n = 5$). Data are shown as mean ± SEM.

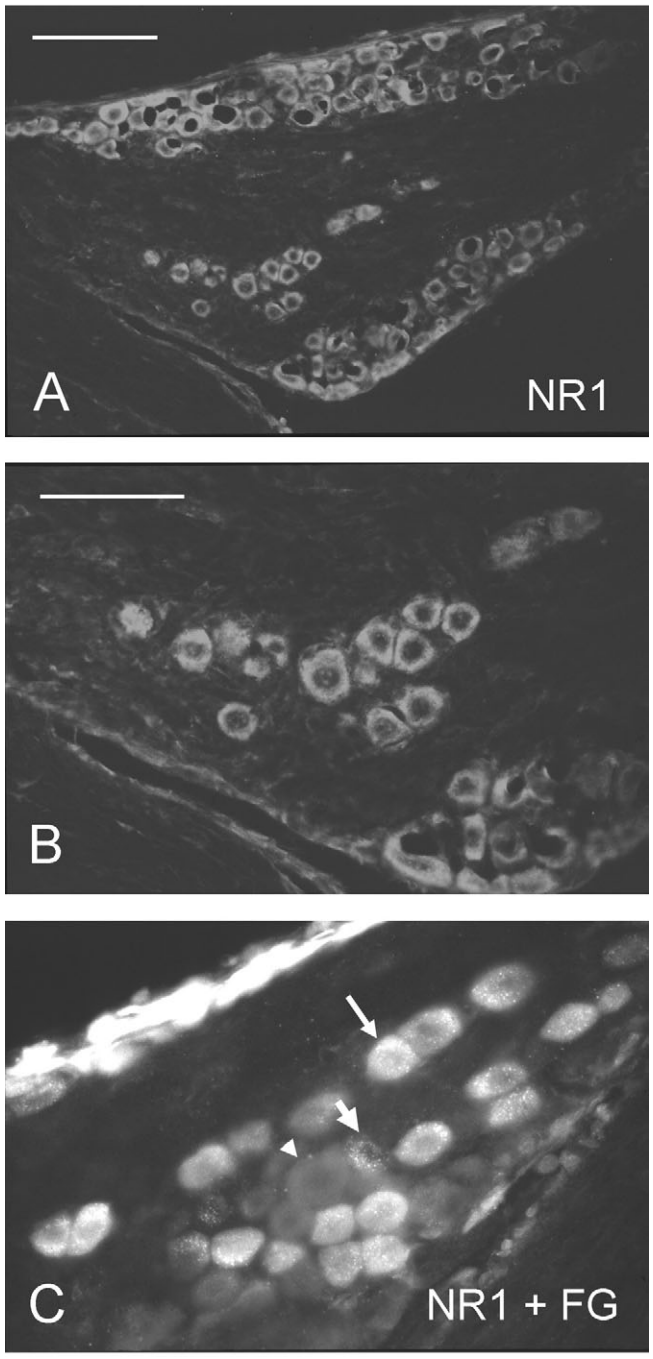

Figure 6 Photomicrographs showing NMDA receptor staining in retrogradely traced gustatory neurons. **(A)** NR1 immunoreactivity was strong and widespread in the geniculate ganglion. **(B)** A at higher magnification. **(C)** NR1 immunoreactivity colocalized with fluorogold labeling (thin arrow). Neurons also showed either fluorogold labeling only (thick arrow), or NR1 immunoreactivity only (arrowhead). FG = fluorogold labeling. Scale bars = 200 μm (A), 100 μm (B, also applies to C).

their central synapses in the nucleus of the solitary tract, gustatory neurons utilize glutamate as a transmitter (Bradley *et al.*, 1996; Li and Smith, 1997; Aicher *et al.*, 1999; King, 2003). In principle, these terminals could possess presynaptic glutamate autoreceptors that modulate neurotransmitter release, as shown elsewhere in the central nervous system (for a review see MacDermott *et al.*, 1999). This might be the case in particular for NMDA receptors expressed by gustatory neurons since we could not detect them in the peripheral axons in taste buds. NMDA receptors are present in afferent fibers in the nucleus of the solitary tract (Aicher *et al.*, 1999). GluR subunits could also be

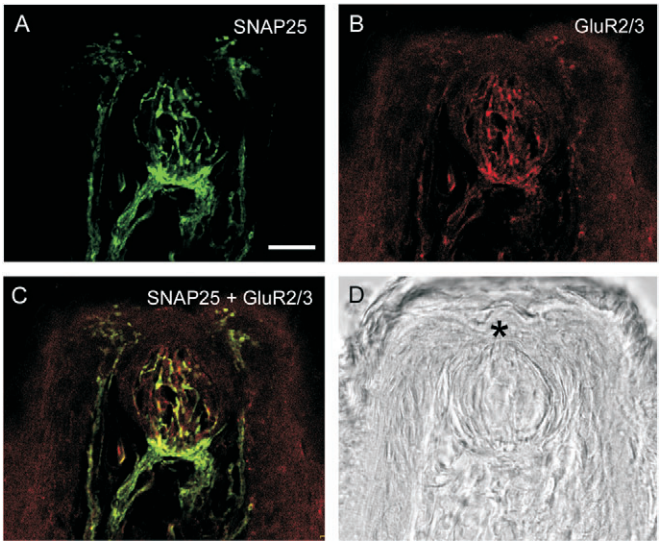

Figure 7 Confocal images showing GluR2/3 immunostaining in sensory afferent axons in fungiform taste buds of the anterior portion of the tongue. Sensory afferent axons were SNAP-25 immunoreactive **(A)**. GluR2/3 immunostaining was present in the taste bud **(B)** where it colocalized with SNAP-25 immunostaining **(C)**. A transmitted light image shows the morphology of the fungiform papilla and the location of the taste bud under the taste pore (asterisk, D). Scale bar = 20 μm.

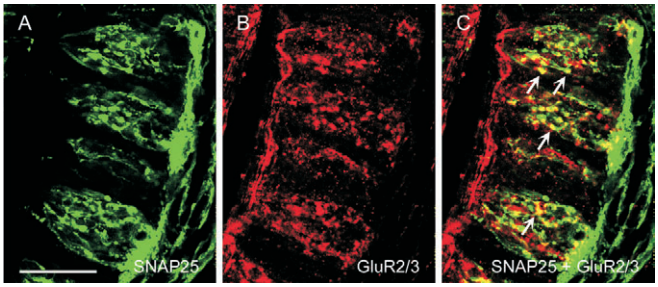

Figure 8 Confocal images showing GluR immunostaining in vallate papillae. In taste buds of vallate papillae, GluR2/3 immunostaining **(B)** was abundant in small puncta and extensively colocalized with SNAP-25 immunostaining **(A)** in axonal varicosities (arrows in **C**). The nerve plexus under the taste buds is strongly SNAP-25 immunoreactive. Scale bar = 50 μm (A, also applies to B, C).

targeted to cell bodies within the geniculate ganglion. Indeed, isolated gustatory neurons of the geniculate ganglion have been shown to respond to glutamate agonists (King and Bradley, 2000).

Studies in the central nervous system show that immunoreactivity for AMPA receptors is mainly postsynaptic (Petralia, 1997). Thus, a plausible role for AMPA receptors expressed by primary gustatory neurons is as receptors on afferent fibers postsynaptic to taste receptor cells. Using confocal microscopy we were able to visualize the AMPA receptor subunits GluR2/3 on sensory afferent axons in taste buds of the fungiform and vallate papillae. Our immunostaining results show that AMPA receptors are localized to axonal varicosities, which are postsynaptic sites on sensory afferent axons (Kinnamon *et al.*, 1988; Royer and Kinnamon, 1994). Whether AMPA receptors are present at synapses with taste cells has to be confirmed with immunohistochemical studies at the electron microscopic level.

There is as yet no compelling pharmacological or electrophysiological evidence for the presence of glutamatergic (or any other neurotransmitter) synapses between primary sensory afferents and taste bud cells. We have recently examined glutamatergic receptors in taste buds with physiological techniques and discovered that taste receptor cells express NMDA and non-NMDA GluRs (Caicedo *et al.*, 2000a,b), consistent with the findings presented here and by Kim *et al.* (2002). Furthermore, taste cells have been shown to express the glial glutamate transporter GLAST (Lawton *et al.*, 2000), suggesting that re-uptake mechanisms for synaptically released glutamate might exist in taste buds. Additional experiments with other approaches are required to clarify if AMPA and/or NMDA receptors are present at afferent synapses and if they are involved in mediation of afferent gustatory signals. Our results represent a first step in identifying glutamate as an afferent transmitter at synapses between taste cells and sensory afferent fibers.

Acknowledgements

Supported by NIDCD/NIH grants DC00374 (S.D.R.) and DC04525–01 (A.C.).

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Accepted April 9, 2004