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Ionotropic Glutamate Receptor Expression in Preganglionic Neurons of the Rat Inferior Salivatory Nucleus

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Abstract

Glutamate receptor (GluR) subunit composition of inferior salivatory nucleus (ISN) neurons was studied by immunohistochemical staining of retrogradely labeled neurons. Preganglionic ISN neurons innervating the von Ebner or parotid salivary glands were labeled by application of a fluorescent tracer to the lingual-tonsilar branch of the glossopharyngeal nerve or the otic ganglion respectively. We used polyclonal antibodies to glutamate receptor subunits NR1, NR2A, NR2B, (NMDA receptor subunits) GluR1, GluR2, GluR3, GluR4 (AMPA receptor subunits), and GluR5−7, KA2 (kainate receptor subunits) to determine their expression in ISN neurons. The distribution of the NMDA, AMPA and kainate receptor subunits in retrogradely labeled ISN neurons innervating the von Ebner and parotid glands was qualitatively similar. The percentage of retrogradley labeled ISN neurons innervating the parotid gland expressing the GluR subunits was always greater than those innervating the von Ebner gland. For both von Ebner and parotid ISN neurons, NR2A subunit staining had the highest expression and the lowest expression of GluR subunit staining was NR2B for von Ebner ISN neurons and GluR1 for parotid ISN neurons. The percentage of NR2B and GluR4 expressing ISN neurons was significantly different between the two glands. The percentage of ISN neurons that expressed GluR receptor subunits ranged widely indicating that the distribution of GluR subunit expression differs amongst the ISN neurons. While ISN preganglionic neurons express all the GluR subunits, differences in the percentage of ISN neurons expression between neurons innervating the von Ebner and parotid glands may relate to the different functional roles of these glands.

Keywords

salivary nucleus; glutamate receptors; immunohistochemistry; retrograde tracing; parasympathetic

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1. Introduction

Stimulation of taste buds results in reflex secretion of saliva (Schneyer and Kevin, 1955; Matsuo, 1999; Kerr, 1961; Kawamura and Yamamoto, 1978). Thus, afferent neural activity originating in taste buds synaptically drives brainstem secretomotor neurons controlling salivary secretion. These neurons are located in the salivatory nuclei which contain the cell bodies of preganglionic parasympathetic neurons innervating the salivary glands (Contreras et al., 1980). The salivatory nuclei are divided into inferior and superior subdivisions based on the cranial nerve distribution of the axons supplying the salivatory glands. Neurons of the inferior salivatory nucleus (ISN) innervate parotid and von Ebner (lingual) glands via the glossopharyngeal nerve while the superior salivatory nucleus (SSN) innervates submandibular, and sublingual glands via the chorda tympani branch of the facial nerve (Loewy, 1990).

Sensory information relayed from oral taste receptors via afferent fibers of the facial (VII), and glossopharyngeal (IX) nerves enter the brainstem and form the solitary tract and then synapse with nucleus of the solitary tract (NTS) neurons. NTS neurons involved in salivary secretion then project to preganglionic parasympathetic neurons of the salivatory nuclei. A number of investigators have demonstrated that the central terminals of the VII and IXth nerves use glutamate as their principle neurotransmitter (Li and Smith, 1997; Grabauskas and Bradley, 1996; Wang and Bradley, 1995). In addition, neurophysiological and immunohistochemical analysis of the synaptic properties of the salivatory neurons indicates that glutamate is the excitatory neurotransmitter involved in the reflex activation of salivary secretion (Bradley et al., 2005; Kobayashi et al., 1997; Mitoh et al., 2004; Bradley and Kim, 2006) mediated by both NMDA (N-methyl-D-aspartate), AMPA (α-amino-3-hydroxy-5-methyl-isoxazole-4-propionic acid) and kainate glutamate receptor (GluR) subtypes.

NMDA, AMPA and kainate receptors are composed of a number of receptor subunits (NR1, NR2A, NR2B; GluR1, GluR2, GluR3, GluR4, GluR5−7, KA2) (Hollmann and Heinemann, 1994; Monoaghan and Wenthold, 1997) which have been shown to be expressed in different brain areas (Petralia, 1997), and coexpressed in single neurons (Lambolez et al., 1992). Combinations of these receptor subunits provide for considerable variation in channel kinetics, dose-response characteristics, voltage dependencies and ion permeability (Hollmann and Heinemann, 1994; Dingledine et al., 1999). At present there is no information on GluR subunit immunoreactivity of the ISN neurons involved in salivary reflex excitation. The aim of the present study was to determine the distribution of the different glutamate receptors subunits using antibodies specific for the different subunits. ISN neurons were identified by retrograde neuronal tracing.

2. Materials and methods

2.1 Retrograde labeling

A total of 19 Sprague-Dawley rats (150−250g) were used in this study. Fifteen (mean weight 180 g) were used to study ISN neurons innervating the von Ebner glands and 5 (mean weight 165 g) were used to study the ISN neurons innervating the parotid glands. They were anesthetized with an intraperitoneal injection of a mixture of ketamine (10 mg/kg) and xylazine (2 mg/kg). Either the lingual-tonsillar branch of glossopharyngeal nerve or the otic ganglion was exposed. To label the ISN neurons, the fluorescent neuronal tracer Alexa Fluor 488 dextran (Molecular Probes) was applied to the cut end of the lingual-tonsillar branch of the glossopharyngeal nerve in 14 rats, as the nerve exited the posterior lacerated foramen. The nerve was isolated from surrounding tissue by placing it on a piece of Parafilm and crystals of the tracer applied to the cut end for 1 hr after which the nerve was wrapped with the Parafilm to prevent tracer spread. In 5 rats crystals of Alexa Fluor 488 were applied to the otic ganglion in the medial aspect of the mandibular division of the trigeminal nerve after several small

punctures were made to the ganglion with a microdissection needle. The skin wound was sutured closed and the rats recovered in an isolated cage on a heating pad and when ambulatory returned to their home cage. The procedures and animal care were done under National Institutes of Health and University of Michigan Animal Care and Use Committee approved protocols.

After 5 days survival, the rats were re-anesthetized and perfused with physiologic saline solution followed by 4% paraformaldehyde. The brains were removed, postfixed in 4% paraformaldehyde for two hours and placed in 0.1M phosphate buffered saline (PBS) at 4°C overnight. Brainstems were sectioned horizontally at 50 μm thickness on a Vibratome (Technical Products International). Seven to eight sections were cut to include all ISN neurons as previously described (Kim et al., 2004).

2.2 Immunohistochemistry

Tyramide signal amplification (TSA) (T20914 and T20924, Molecular Probes) was used for immunohistochemistry since it has been shown that this method results in significantly enhanced signal detection when combined with retrograde tracing (Kressel, 1998; Nagy et al., 2004). The sections were permeabilized with 0.1 % Triton X-100 for 10 minutes at room temperature and rinsed with PBS. To quench endogenous peroxidase activity, the sections were incubated in peroxidase quenching buffer for 1 hour at room temperature. A 1% blocking reagent was applied to the sections for 1 hr. Sections were then incubated in primary antibody for 48 hrs at 4°C. The primary antibodies to GluR subunits used in this study were all commercially available and the specificities of the antibodies and their respective antigens have been established in previous studies (Table 1). After rinsing, the sections were incubated in an HRP conjugated species-specific secondary antibody (1:100) for 1 hr at room temperature. All antibodies were diluted in 1% blocking reagent. After incubation with antibodies, the sections were rinsed three times with PBS. Alexa Fluor 568 (Red) conjugated Tyramide working solution (1:100) diluted in amplification buffer and 0.0015% H_2O_2 was applied to the sections for 10 min. After rinsing the sections were slide mounted and coverslipped with an antifade mounting medium (ProLong Gold, Molecular Probes).

Specificity of the staining was tested in several ways. Several sections were processed by omission of the primary antibody which always resulted in a complete absence of staining. In other tissue the TSA was omitted and CY3 conjugated antibodies used to visualize the immunoreactive neurons. This produced weaker staining but resulted in a similar distribution to the results using TSA. To compare the results of the present study with those of other published investigations, neurons of the dorsal motor nucleus of the vagus nerve and the hypoglossal motor nucleus were retrogradely labeled and immunoreacted for expression of glutamate receptor subunits. Comparison of our results with observations made on the vagal motor neurons (Corbett et al., 2003) and the hypoglossal motor neurons (García Del Caño et al., 1999) revealed no significant differences.

2.2 Image analysis

Confocal image stacks of the neurons labeled with the retrograde tracer (green) and antibodies to the GluR subunits (red) were captured with a Bio-Rad MRC-600 laser scanning confocal microscope. Images of the retrogradely and immunolabeled neurons were merged so that ISN neurons expressing a GluR subunit were yellow.

ISN neurons labeled with the retrograde tracer and antibodies to the GluR subunits were counted using the Neurolucida program (MicroBrightField). The counts were based on five ISN sections from two rats containing cell bodies innervating von Ebner's glands and three ISN sections from neurons in three rats controlling the parotid gland. The numbers of neurons

innervating von Ebner's and parotid glands labeled with retrograde tracer and antibodies as well as neurons that were retrogradely labeled but did not react with GluR subunit antibodies were counted. Neurons were considered to express the GluR subunit when the cell body, which included the nucleus, was labeled with both the retrograde tracer and the antibody in a merged projection image. This was confirmed by examining all optical sections in the image stack for both retrograde tracer and immunohistochemical reaction. This method of analysis usually resulted in a higher count of retrogradely labeled immunoreacted neurons than observed in the merged image of the confocal image stack. A Student's t-test was used to measure significant differences for each receptor subtype in parotid versus von Ebner's glands. Significance level was set at $p < 0.05$.

3. Results

3.1. General appearance of immunostaining and retrograde labeling

Fig. 1A is a low power micrograph of a horizontal section of the left lateral medulla to illustrate the extent of retrograde labeling of ISN neurons via the lingual-tonsilar branch of the glossopharyngeal nerve and immunostaining with one of the GluR subunits (NR2A). Because the lingual-tonsilar branch of the glossopharyngeal nerve contains both sensory afferent fibers from the posterior tongue as well as preganglionic parasympathetic efferent fibers innervating the von Ebner glands, the entering sensory fibers (IX) forming the descending solitary tract (ST) and terminal field (TF) as well as the ISN neurons are labeled in green. The retrograde tracer does not cross the synapse between the afferent fibers and the second order neurons in the NTS and therefore these neurons are unlabeled, but express the NR2A GluR subtype, that is immunostained red. In addition the NR2A subunit antibody intensely labels many other brainstem neurons.

At higher power ISN neurons identified by retrograde transport of label also immunoreact with antibodies to GluR subunits. By merging images of the retrogradely labeled neurons (Fig. 1Ba) with the same neurons immunoreacted with the NR2A subunit (Fig. 1Bb) the extent of ISN neurons that also immunoreact were apparent as yellow appearing neurons (Fig. 1Bc). Retrogradely labeled neurons that immunoreacted with NR2A antibodies have been indicated by arrows in Figs. 1Ba-c. As will be noted in this particular sample the majority of the ISN neurons also immunoreact with the NR2A receptor subunit antibodies. However, some do not, and there are a number of other neurons that are immunoreactive that possibly represent a second neuron type in the ISN that does not have efferent fibers in the IXth nerve. There was no apparent topographic orientation of retrogradely labeled neurons that also expressed NR2A GluR subunits.

3.2 GluR subunit labeling of identified ISN neurons

The distribution of the AMPA, NMDA and kainate receptor subunits in retrogradely labeled ISN neurons innervating the von Ebner and parotid glands was qualitatively similar. Examples of GluR subunit labels in retrogradely identified neurons innervating the von Ebner's glands are illustrated in Fig 2. ISN neurons immunoreacted strongly to NR1 and NR2A receptor subunits. The majority of the staining reaction with NR1 and NR2A antibodies strongly labeled the ISN cell bodies and proximal dendritic segments while NR2B intensely stained the nucleus. Neuron labeling with the GluR1and GluR3 antibodies also strongly labeled the soma and primary dendrites. Expression of GluR2 and GluR4 was confined mainly to the neuron soma. Label in ISN neurons expressing GluR5−7 and KA2 receptor subunits was concentrated in the cell bodies although considerable immunoreactivity was observed in fibers surrounding the labeled neurons.

3.3 Numbers of ISN neurons that express GluR subunits

The relative numbers of ISN neurons innervating the von Ebner and parotid glands that had label for receptor subunits were quantitatively different (Fig. 3 and Table 2). Percentage of retrogradely labeled ISN neurons innervating the parotid gland with GluR subunit expression was always greater than those innervating the von Ebner gland. These differences may relate to the different size of the neurons (Fukami and Bradley, 2005) as well as the functional roles of the glands they innervate.

The percentage of retrogradely identified parotid ISN neurons expressing GluR subunits ranged from a high of 90 ± 8 (NR2A subunit) to a low of 71 ± 4 (GluR1 subunit). The percentage of von Ebner ISN neurons expressing GluR subunits ranged from a high of 82 ± 11 (NR2A subunit) to a low of 36 ± 10 (NR2B subunit). Thus, for both von Ebner and parotid ISN neurons, NR2A subunit staining was the most commonly expressed but the lowest GluR subunit expression for von Ebner ISN neurons was with the NR2B subunit and GluR1 for parotid ISN neurons. However, when differences between subunit staining for the von Ebner and parotid ISN neurons were statistically tested, only the percentage of NR2B and GluR4 double labeled cells was significantly different between the two glands ($p<0.05$).

Although the percentage of retrogradely labeled ISN neurons expressing GluR subunits is high ranging from 43 to 87, a substantial percentage of retrogradely identified neuron do not immunoreact with a particular GluR subunit antibody. It is possible that ISN neurons that do not express a GluR subunit might be labeled with a different GluR subunit which would require double and triple immunofluorescence labeling. Thus the extent of colocalization of GluR subunits is unknown for ISN neurons.

4. Discussion

We have determined the subunit composition of ISN neuron glutamate receptors and demonstrated that all major NMDA, AMPA and kainite subunits are expressed in ISN. The subunits expressed are of the NMDA (NR1, NR2A, NR2B), AMPA (GluR1, GluR2, GluR3, GluR4) and kainate (GluR5−7, KA2) GluR types. Most retrogradely labeled ISN neurons were strongly immunoreactive to all of the GluR subtype antibodies used in this study. Differences in labeling percent of neurons innervating the parotid and von Ebner glands were significant for NR2B and GluR4 subunit expressing cells only.

The only previous study of glutamate immunoreactivity in salivatory parasympathetic secretomotor neurons concentrated on the SSN. In this investigation SSN neurons were retrogradely labeled and sections were immunostained for glutamate (Kobayashi et al., 1997). The retogradely labeled SSN neurons were described as being surrounded by glutamate immunoreactive axon varicosities. In a more recent study of the SSN, 88% the retrogradely labeled neurons expressed NR1 immunoreactivity. No other GluR subunits were examined in this study (Lin et al., 2003) but the percentage of identified SSN neurons immunopositive for NR1 subunit staining is similar to results of the present study.

Expression of GluR subunits has previously been reported in the dorsal motor nucleus of the vagus nerve (Corbett et al., 2003; Chen et al., 2002) which extends caudally from the ISN. The results are similar to the present study although GluR1 labeling was weak or absent, while labeling with antibodies directed to GluR2, GluR3, and GluR4 was moderate or intense. All neurons express the NR1 subunit and NR2A and NR2B expression resulted in strong labeling. Weak to moderate labeling was observed using antibodies to the GluR subunits KA2 and GluR5−7. However, the authors caution that labeling intensities for a particular receptor subunit may reflect factors such as antibody quality or affinity, or non-optimal tissue fixation conditions rather than indicating an absence of receptor expression. The relatively strong expression of

all the GluR subunit expression on the ISN neurons of the present investigation may reflect the use of tyramide amplification that optimized detection of the antibodies. Use of tyramide amplification and antigen-unmasking has resulted in detection of both NMDA and AMPA receptor subunits in the spinal cord that were not revealed using conventional immunocytochemistry (Nagy et al., 2004).

The fact that GluR subunits are expressed in the majority of the ISN neurons indicates they could be colocalized on these cells but because no attempt was made to double label the ISN neurons the extent of this colocalization is not known. In addition, none of the GluR subunits were expressed on all of the retrogradely labeled neurons. Thus, the exact composition of GluR subunit expression probably varies from neuron to neuron so that individual neurons could respond differently to afferent input mediated by glutamate. For example, a relative deficiency in the GluR2 permeable Ca^{2+} channel expression has been shown to results in loss of synaptic plasticity (Hartmann et al., 2004). It is possible, therefore, that the GluR receptors on ISN neurons may have other modulatory functions in addition to their role in fast synaptic transmission.

A significant difference was found in the percent of ISN neurons expressing NR2B and GluR4 subunits. The percentage of these two subunits was higher in ISN neurons innervating the parotid gland. In the hippocampus these subunits have been shown to be important in the mechanisms of synaptic plasticity and long term potentiation (Boehm and Malinow, 2005; Barria and Malinow, 2005). However, it is not clear if these subunits would play a similar role in the synaptic transmission between rNST and ISN neurons. In the brainstem NMDA and GluR4 AMPA receptor subunits have been shown to be involved in the development of a classical conditioned reflex (Keifer, 2001; Mokin and Keifer, 2004). Salivary secretion can be conditioned to various sensory stimuli (Hector and Linden, 1999) possibly mediated by upregulation of NR2B and GluR4 glutamate receptor subunits during conditioning.

The distribution of all three classes of ionotropic glutamate receptor subunits in ISN neurons is in agreement with electrophysiological studies of the synaptic properties of these and other parasympathetic preganglionic neurons in the brainstem and spinal cord. Excitatory inputs to these autonomic neurons are via glutamatergic inputs mediated by NMDA and AMPA glutamatergic receptors (Miura et al., 2003; Miura et al., 2001; Araki and De Groat, 1996; Travagli et al., 1991). All afferent excitatory transmission to neurons of the ISN and SSN is via NMDA and AMPA receptors since all synaptic currents and potentials evoked by stimulation of the solitary tract or surrounding tissue were eliminated by superfusion of glutamate receptor blockers(Suwabe and Bradley, 2005; Mitoh et al., 2004).

Glutamatergic input to the parasympathetic preganglionic neurons has variable synaptic latencies. Synaptic potentials elicited by tract stimulation can be of very short latency and unitary, suggesting that the electrical stimulation activates only a single neuron in contact with the electrode (Araki and De Groat, 1996). In other neurons synaptic latencies suggest a polysynaptic glutamatergic input (Suwabe and Bradley, 2005). Further evidence of divergent action of the glutamatergic input to the autonomic neurons was demonstrated by the use of glutamate receptor blockers. Spontaneous synaptic potentials recorded in the dorsal motor nucleus of the vagus could be eliminated using AMPA receptor blockers while synaptic currents elicited by vagal stimulation were reduced by NMDA receptor blockade (Travagli et al., 1991). Finally, while afferent excitatory input to ISN neurons is glutamatergic these neurons also receive input from forebrain structures mediated by peptidergic neurotransmitters (Suwabe and Bradley, 2007; Nemoto et al., 1995).

In conclusion, glutamate receptors on ISN neurons controlling the parotid and von Ebner salivary glands express NMDA, AMPA and kainate receptor subunits. Not all neurons

controlling these two salivary glands express all of the GluR subunits and there are some differences between the percentage of subunits expressed on neurons controlling either the parotid or von Ebner glands. The results are consistent with neurophysiological data indicating AMPA and NMDA responses in the parasympathetic preganglionic neurons controlling the salivary glands.

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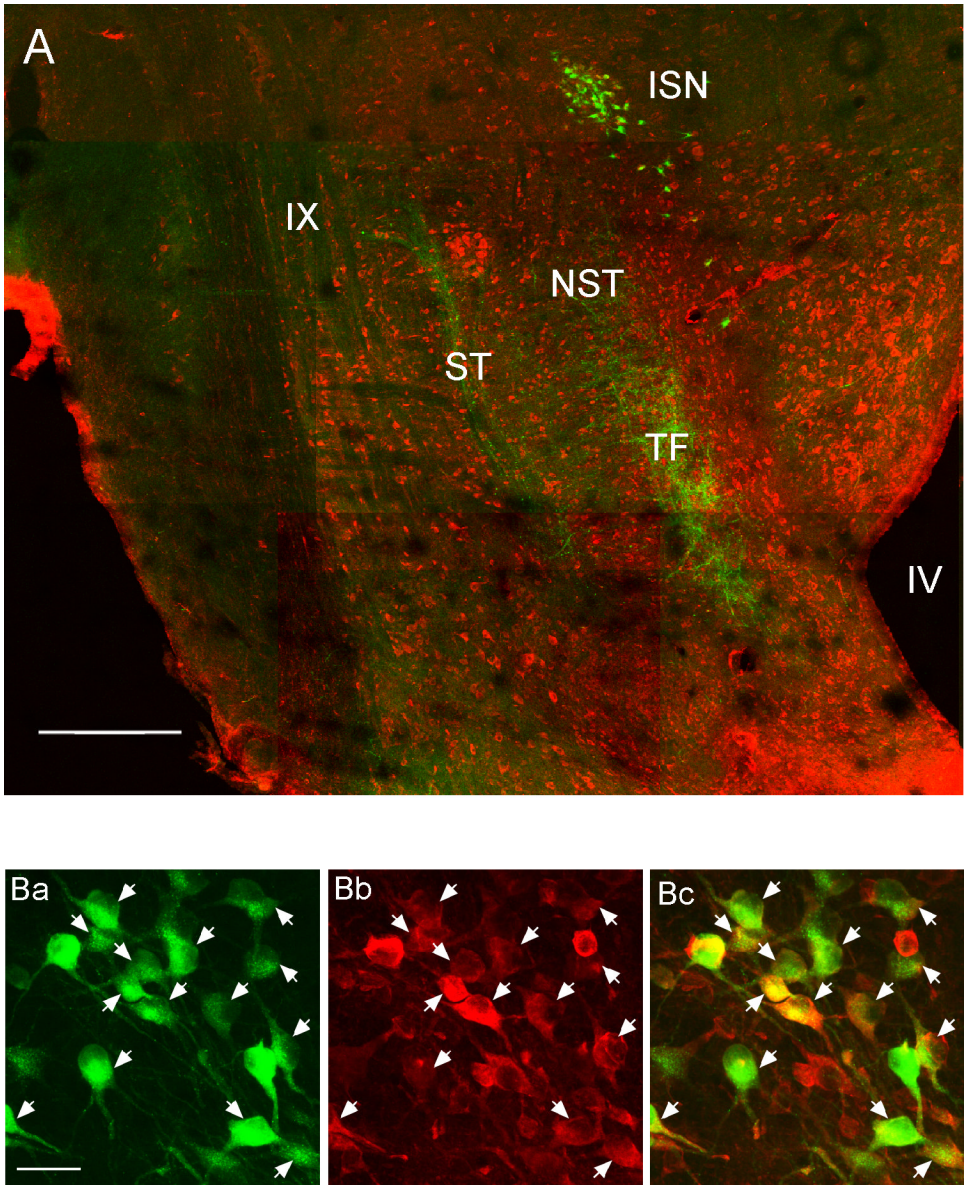

Fig. 1.

(A) Low power micrograph of a horizontal section of the left lateral medulla. The midline is to the right (IV – fourth ventricle) and caudal is towards the top of the figure. The lingual-tonsilar branch of the glossopharyngeal nerve has been labeled with Alexa Fluor 488 dextran (green fluorescence) to retrogradely label neurons of the ISN as well as anterogradely label the IXth nerve forming the solitary tract (ST) and terminal field in the NTS. The section was also immunoreacted with antibody to the GluR NR2A subunit. The NR2A subunit antibody intensely labels many other brainstem neurons. Scale bar = 400 μm. (Ba-c) Higher power ISN neurons identified by retrograde transport of label (Ba) also immunoreact with the NR2A subunit antibodies (Bb). (Bc) Merged images of the retrogradely labeled neurons and the same immunoreacted neurons. ISN neurons expressing NR2A subunits are indicated by arrows in Figs. 1Ba-c. The majority of the ISN neurons immunoreact with the NR2A receptor subunit antibodies but other neurons do not immunoreact. Scale bar = 50 μm.

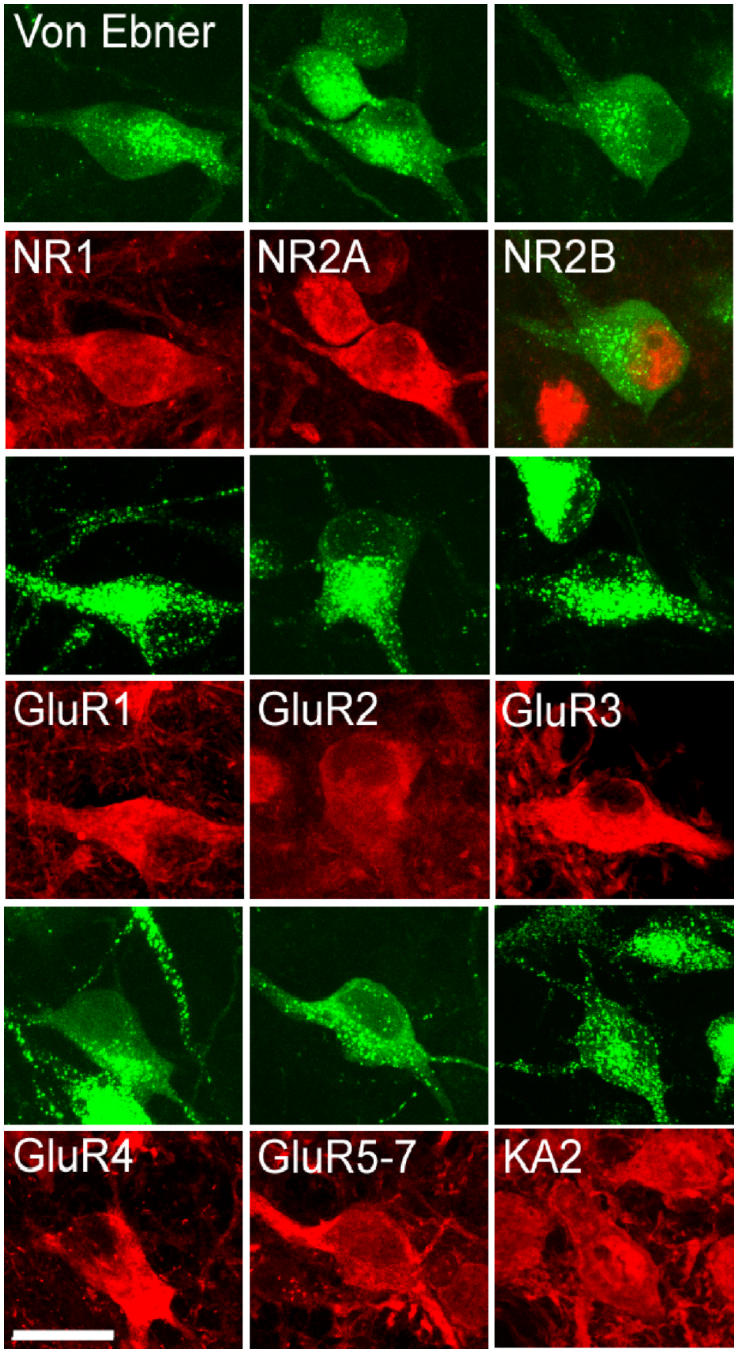

Fig. 2.

Examples of ISN neurons innervating von Ebner salivary glands immunoreacted with antibodies to GluR subunits. ISN neuron immunostained for NR1, NR2A, NR2B, GluR1, GluR2, GluR3, GluR4, GluR5−7 and KA2 are appear as red immunofluoresence. Above each panel of the immunoreacted neuron is the same neuron retrogradely labeled by tracer applied to the lingual-tonsilar branch of the IXth nerve (green). ISN neurons immunoreacted strongly to NR1 and NR2A receptor subunits. Neuron labeling with the GluR1and GluR3 antibodies also strongly labeled the soma and primary dendrites. Expression of GluR2 and GluR4 was confined mainly to the neuron soma. ISN neurons expressing GluR5−7 and KA2 receptor subunits antibodies were concentrated in the cell bodies although considerable

immunoreactivity was observed in fibers surrounding the labeled neurons. Immunoreactive puncta surrounded GluR1, GluR2, GluR3, GluR4 and GluR5−7 labeled ISN neurons. Some retrogradely labeled ISN neurons do not immunoreact with GluR subunit antibodies (e.g. GluR4 panel) and some immunoreacted neurons are not retrogradely labeled (e.g. GluR1, GluR2, GluR4 panels). Scale bar = 50 μm.

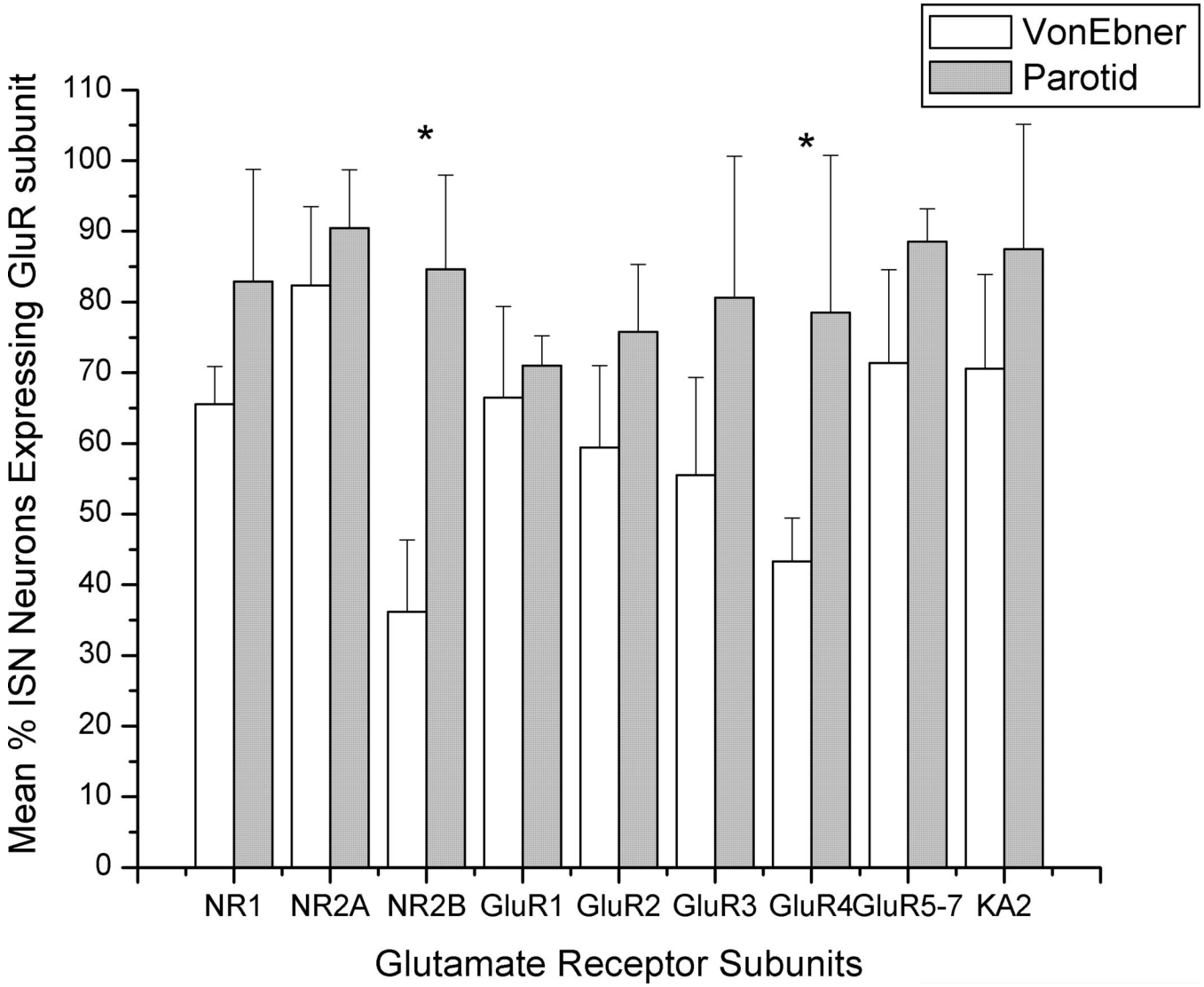

Fig. 3.

Histogram of percent of retrogradely labeled ISN neurons innervating the von Ebner and parotid glands that also express GluR subunits. Data is presented as means and SE of the mean. Differences between NR2B and GluR4 expressed in the Von Ebner and Parotid ISN neurons were significant at the < 0.05 level (*).

Table 1

Sources and specificities of the GluR subunit antibodies

Antibody	Antigenic sequence	Species	Source and catalog number	Dilution	Reference for production and specificity
NR1	660−811	Mouse clone 54.1	Pharmingen [a] 556308	1:50 10 μg/ml	(Siegel et al. 1994)
NR2A	1099−1213	Mouse clone A32D10	Chemicon [b] MAB5216	1:25 40 μg/ml	(Calon et al. 2005)
NR2B	1033−1161	Mouse clone 13A11	Chemicon MAB5220	1:25 36 μg/ml	(Calon et al. 2005)
GluR1	895−907	Rabbit	Chemicon AB1504	1:25 4 μg/ml	(Petralia & Wenthold, 1992)
GluR2	175−430	Mouse clone 6C4	Pharmingen 556341	1:50 10 μg/ml	(Vissavajjhala et al. 1996)
GluR3	245−451	Mouse clone 38.3	Chemicon MAB5416	1:50 8.6 μg/ml	(Moga et al., 2003)
GluR4	889−902	Rabbit	Chemicon AB1508	1:25 4 μg/ml	(Petralia & Wenthold, 1992)
GluR5−7	233−518	Mouse clone 4F5	Chemicon MAB379	1:50 (ascites)	(Huntley et al., 1993)
KA2	960−979	Rabbit	Upstate [c] 06~315	1:50 20 μg/ml	(Roche & Hugenir, 1995)

[a] Pharmingen, BD Biociences, Franklin Lakes, NJ.

[b] Chemicon International, Temecula, CA.

[c] Upstate, Charlottesville, VA

Table 2

Mean numbers of retrogradely labeled ISN neurons and ISN neurons labeled with retrograde tracer and glutamate receptor subunits

Glutamate Receptor Subunits	Von Ebner *		Parotid **		t	p
	ISN Neurons (mean)	Labeled Neurons (mean)	ISN Neurons (mean)	Labeled Neurons (mean)		
NR1	23	15	9	7	2.35	0.06
NR2A	28	24	7	6	0.92	0.39
NR2B	18	7	10	8	5.86	0.01
GluR1	11	7	10	7	0.58	0.59
GluR2	19	11	6	4	2.05	0.09
GluR3	27	15	7	5	2.13	0.08
GluR4	29	12	8	6	3.50	0.01
GluR5−7	28	20	10	9	2.11	0.08
KA2	25	18	5	4	1.80	0.12

* The counts are based on the mean number of retrogradely or double labeled ISN neurons innervating the Von Ebner gland in five sections from two rats.

** The counts are based on the mean number of retrogradely or double labeled ISN neurons innervating the parotid gland in three sections from three animals.