

Immunohistochemical Investigation of the Nitrergic System in the Taste Organ of the Frog, *Rana esculenta*

G. Zaccone, C. Crescimanno¹, P. Lo Cascio, A. Mauceri, S. Fasulo and A. Sbarbati¹

Department of Animal Biology and Marine Ecology, Section of Cell and Evolutionary Biology, University of Messina and ¹Department of Morphological and Biomedical Sciences, Section of Anatomy and Histology, University of Verona, Italy

Correspondence to be sent to: Prof. Giacomo Zaccone, Department of Animal Biology and Marine Ecology, Section of Cell and Evolutionary Biology, University of Messina, Salita Sperone 31—S. Agata, I-98166 Messina, Italy. e-mail: gzaccone@unime.it

Abstract

We have studied by immunocytochemistry, the taste discs of the frog, *Rana esculenta*, with the aim of providing morphological and neurochemical data on the nitrergic system and of assessing the eventual presence of intrinsic neurons associated with the gustatory organs. In taste discs, antibodies against neuronal nitric oxide synthase (nNOS) revealed a positive immunoreaction in the taste receptor cell bodies and processes. The basal cells were also stained. All the fungiform papillae contained intragummal nerve fibers showing nNOS immunoreactivity; these fiber were mainly located in the basal plexus. Immunoreactive nerve fibers were also visible at the periphery of the papilla-contacting ciliate cells, which form a ring around the taste disc. In conclusion, the findings obtained in this study suggest that the occurrence of nNOS-immunoreactivity in basal cells, taste cells and nerves might reflect a role for nitric oxide in taste mechanisms of Amphibia. The results may also sustain the physiological implication of NO as a molecule involved in the local target function of maintaining the taste bud mucosal integrity and in regulating the blood flow to the epithelium.

Introduction

The free radical nitric oxide (NO) is a versatile signaling molecule that regulates a variety of cellular functions (Moncada and Higgs, 1993). This messenger molecule is produced by oxidation of L-arginine to NO and L-citrulline, a reaction catalysed by nitric oxide synthase (NOS) (Moncada *et al.*, 1991). Some studies have demonstrated that NO may contribute to the regulation of certain autonomic functions by acting as a non-adrenergic, non-cholinergic transmitter in the peripheral autonomic nervous system (De Man *et al.*, 1991; Toda and Okamura, 1991). This function has very recently also been argued for the visceral sensory system in lower vertebrates (Green and Campbell, 1994; Bodegas *et al.*, 1995; Brüning *et al.*, 1996; Mauceri *et al.*, 1999; Zaccone *et al.*, 2002).

In taste organs, an important role for nitrergic mechanisms has recently been suggested (Herness, 1996; Kretz *et al.*, 1998; Rosenzweig *et al.*, 1999; Sbarbati *et al.*, 1999). In the rat vallate papilla, these studies demonstrated that neurons of an intrinsic system are mainly nitrergic elements (Sbarbati *et al.*, 2000). These data suggested that in taste organs nitrergic ganglion cells are capable of sustaining and modulating local activities as described in integrative circuits of other organs, such as heart, airways and bladders (Burnstock *et al.*, 1987). However, to date, studies on nitrer-

gic mechanisms and intrinsic nervous systems associated with taste organs have only been performed in mammals and comparative data on other species of vertebrates are lacking.

Among the Amphibia, because of the peculiar characteristics of the taste disc, the taste organs of the frog have been considered by several authors to be a good model and used to obtain anatomical and physiological data. In this species, the taste organs consist of large discs located at the top of the fungiform papillae (Osculati and Sbarbati, 1995). In these structures four distinct cell types can be recognized. Cell types I, II and III reach the surface and contact the external environment. Type IV cells are found exclusively in the basal layer of the taste disc. These cells share many morphological and immunohistochemical characteristics with the Merkel cells of the skin (Tachibana, 1995; Zaccone *et al.*, 2001) and are different from the basal cells of mammalian taste buds. They are rich in the biogenic monoamine serotonin (Delay *et al.*, 1993; Zancanaro *et al.*, 1997), neuron-specific enolase (Toyoshima, 1989) and enkephalins (Zaccone *et al.*, 1995) and, moreover, they are considered as paraneurons.

The purpose of the present study is to provide morphological and neurochemical data on the nitrergic system and to assess the eventual presence of intrinsic neurons in the

taste discs of the frog, *Rana esculenta*, using immunocytochemistry for the neuronal isoform of nitric oxide synthase (nNOS). These data, compared to those recently obtained in mammals, could be useful in understanding the phylogenesis of the taste system.

Materials and methods

Specimens of *R. esculenta* were maintained in the laboratory at 10°C with access to tap water. Animals were killed by decapitation and their tongues removed. Lingual tissues were cut into 2–3 mm³ fragments. The specimens were fixed in 4% buffered formaldehyde for 5–6 h at 4°C, dehydrated by a series of graded ethanols and embedded in Paraplast. Immunostaining was performed with indirect immunoperoxidase visualization.

Serial sections (3–7 µm) were incubated in 10% normal goat serum for 2 h prior to incubation with primary antisera to reduce non-specific staining. Sections were next incubated in the polyclonal antibody against nNOS (Transduction Laboratories, Lexington) diluted 1:250 with 0.1 M phosphate buffered saline (PBS; pH 7.4). The tissues were subsequently washed in PBS and then incubated with secondary antiserum. The signals were developed by treatment with 0.05 M 3,3'-diaminobenzidine tetrahydrochloride.

After staining, sections were viewed and photographed in a Zeiss Axiophot microscope. In control experiments, the primary antibody was omitted or replaced with a non-immune serum. In addition, prior to the incubation of the sections, the antiserum was absorbed overnight at 4°C with 1 nmol of the respective antigen per millilitre of diluted antiserum.

Immunoblotting

The taste discs were cut free with small scissors under a dissection microscope and total number used was 25. The taste discs were then extracted by homogenization (Ultraturax) in 10–15 volumes of buffer A (2% SDS, 4 µM leupeptin, 4 µM aprotinin, 64 µM pepstatin A, 1 mM EGTA, 1 mM EDTA, 2 mM phenylmethylsulphonyl fluoride, 20 mM tetrahydrobiopterin). The homogenate was centrifuged at 106 000 g for 15 min at 4°C. The supernatant was removed and 5% β-mercaptoethanol, glycerol and bromophenol blue was added. Proteins were separated by SDS-PAGE and electrotransferred to nitrocellulose membrane (Hybond ECL; Amersham). The membrane was blocked for 1 h at room temperature with 5% bovine serum albumin (BSA) followed by incubation with nNOS antibody at a dilution of 1:50 at 4°C overnight. The membrane was incubated with horseradish-peroxidase-conjugated goat anti-rabbit immunoglobulin antibody (1/5000; Sigma) for 2 h at room temperature. The binding of primary antibody was detected by enhanced chemiluminescence (ECL; Amersham).

Results

Histology

Vertical sections of the taste organ show that it consisted of a taste disc located on a fungiform papilla. The papilla was surrounded by a ciliated epithelium (Figure 1c) and most of its surface was composed of glandular supporting cells. Apical processes of taste cells were seen between these cells (Figure 2b,c). Numerous basal cells were located in the peripheral basal portion of the papilla. These have been reported by earlier authors using immunohistochemistry and are reported here for the first time using nNOS antibodies (see below).

nNOS immunostaining

At low magnification, immunostaining was visible in fungiform papillae of the dorsal surface of the tongue. At higher magnification, in taste discs, antibodies against nNOS revealed a positive immunoreaction in the taste receptor cells bodies and processes (Figures 1c and 2b,c). In longitudinal sections, the immunoreactive taste cells have a spindle-shaped body with long, thin apical and basal processes lying between the glandular supporting cells (Figure 2b,c). The basal cells were also stained (Figures 1b and 3). All the fungiform papillae contained intragemmal nerve fibers showing nNOS immunoreactivity (IR). These fibers were mainly located in the basal plexus (Figures 1a,b and 2a). Immunoreactive nerve fibers were also visible at the periphery of the papilla contacting ciliate cells which form a ring around the taste disc (Figure 1c). Many nitrergic nerve fibers were seen in the connective tissues and were closely associated with the gland system. Some of the nitrergic nerves coursed among the secretory units in sections of the anterior, middle and posterior surface and the ventromedian region of the tongue (Figure 1d). Isolated nitrergic fibers or perivascular plexuses were detected along the walls of the lingual arteries. Numerous positive nerve fibers were demonstrated around the mucous glands. Nitrergic nerve fibers were also located in the connective tissue of the filiform papillae.

nNOS-IR cells bodies were not found in the connective tissue of fungiform papillae in the present study.

nNOS immunoblotting

The antibody directed against nNOS recognized a protein of molecular mass at least 160 kDa in extracts of frog taste discs (Figure 4).

Discussion

The present study demonstrates a wide presence of nitrergic structures in the taste organ of an amphibian species. These results are in agreement with data previously obtained in the gustatory system of mammals (Sbarbati *et al.*, 1999, 2000). However, some differences seem to exist between the

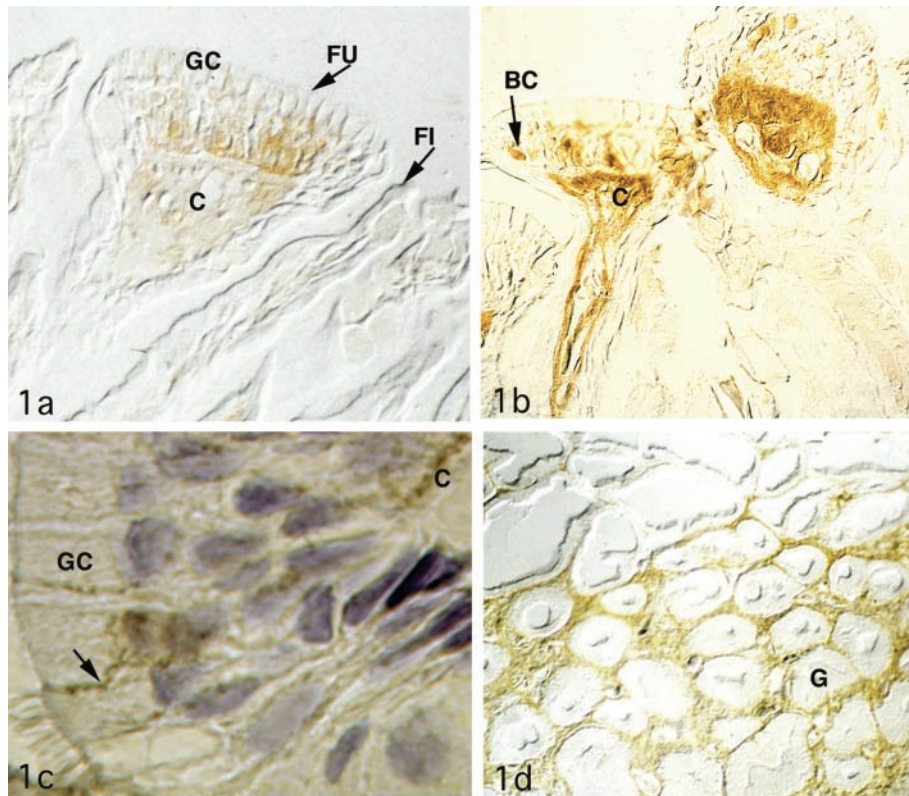


Figure 1 (a–d) nNOS immunoreactivity in frog fungiform papillae (FU). Immunoreactivity for n-NOS is mainly located among the intragemmal nerve fibers in the plexus at the basal periphery of the bud (a), in the extragemmal nerve bundles of the connective tissue (C) of lamina propria and a basal cell (BC; b), in the nitroergic fibers (arrows) contacting ciliated cells located in the peripheral portion of the taste disc and penetrating into the epithelium at the base of the bud (arrowed; c) and in nitroergic fibers around mucous glands (G). GC, glandular supporting cells; FI, filiform papilla (d). Final magnifications: a, 230 \times ; b, 460 \times ; c, 1150 \times ; d, 230 \times .

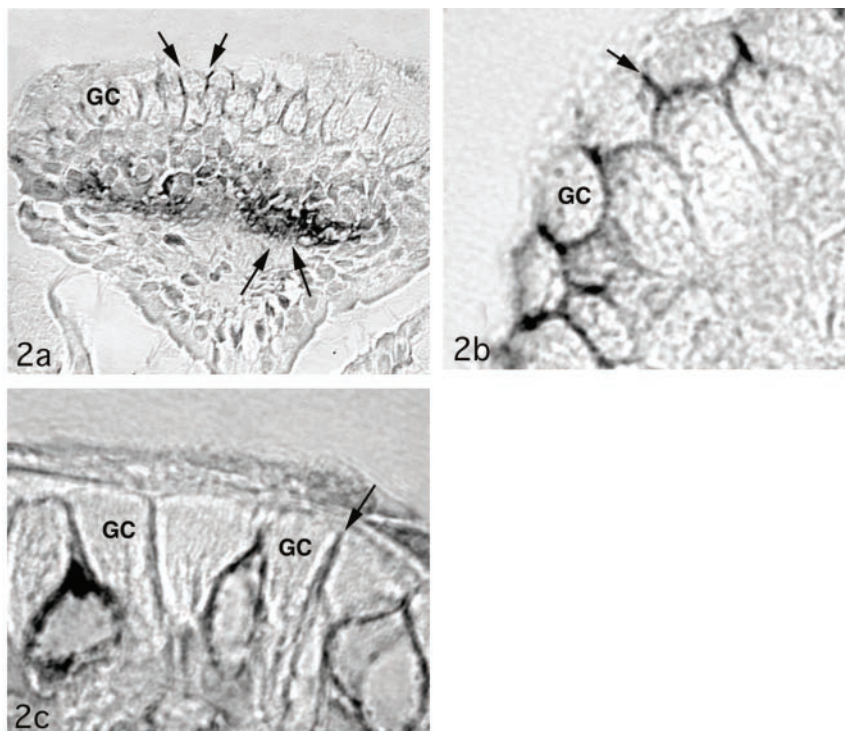


Figure 2 (a–c) nNOS positive immunoreaction is present in the nitroergic fibers at the base of the bud (arrowed) and taste cell processes (arrow) located at the apical surface of the taste disc. GC, glandular supporting cells. Final magnification: a, 335 \times ; b, 1100 \times ; c, 1100 \times .

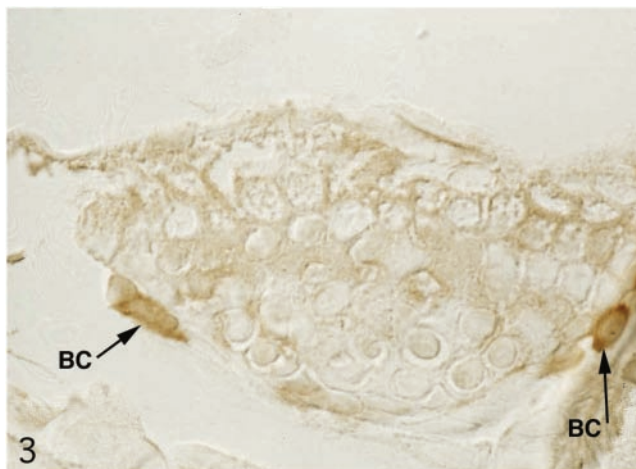


Figure 3 Oblique section of taste bud showing the presence of two nNOS immunoreactive basal cells (BC) in the basal periphery of the bud. Final magnification: 725x.

distribution of nitrergic structures in amphibians and mammals. In the frog, nNOS-IR has been detected in nerves and epithelial cells, while in the rat it seems to be restricted to nerves. In addition, the nervous intrinsic ganglia associated with taste organs in mammals are not described in the frog.

nNOS-IR in basal cells

In the present study we demonstrate the presence of nNOS-IR in basal cells of the taste disc. The neurotransmitter function of these cells has been postulated by several authors. They found, in immunohistochemical studies, that these cells are clearly serotonin positive (Zaccone, 1986; Delay *et al.*, 1993; Tachibana, 1995; Hamasaki *et al.*, 1998). Recently, immunoreactivity for leu-enkephalin has been reported in the basal cells of taste buds of *Ambystoma tigrinum* (Zaccone *et al.*, 1995). The significance of serotonin and of the bioactive substance in the basal cells of taste buds is still unknown. According to earlier workers (Delay *et al.*, 1993), these cells may be regarded as serotonergic neurons having also a trophic role in the maintenance of the morphological integrity of frog taste buds (Hamasaki *et al.*, 1998), but their function is still currently enigmatic.

nNOS-IR in taste cells

In the present study, nNOS-IR was also noticed in taste cells and their processes. To our knowledge, this is reported for the first time in amphibian taste buds. NO derived from nNOS is known to be an important signaling molecule regulating several neuroendocrine and behavioral functions (Gammie *et al.*, 2000). The occurrence of nNOS-IR in both basal and taste cells might reflect a role for NO in transmission mechanisms; in particular, this molecule may be useful for initiating the chemosensory stimulation of taste buds. It has been argued that basal cells are stimulated

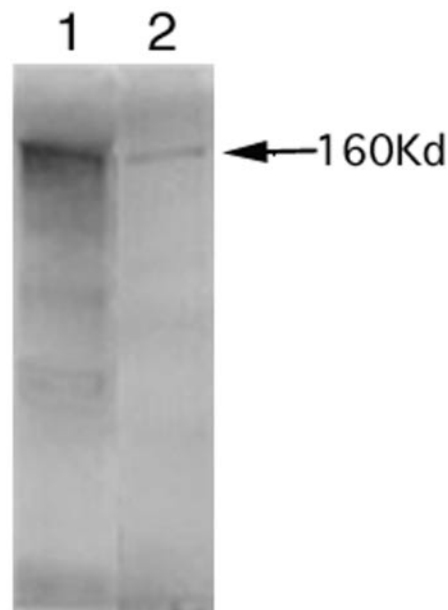


Figure 4 Immunoblotting of frog taste disc using antiserum to nNOS. Lane 1, 150 µg protein from taste disc; blot exposed to antiserum alone. Lane 2, blot exposed to antiserum plus 100 µg/ml peptide.

to release serotonin during chemostimulation of taste buds (Kim and Roper, 1995; Nagai *et al.*, 1996), although synaptic contacts between the taste cells and basal cells have not been ascertained (Osculati and Sbarbati, 1995). NO is regulated to occur under special circumstances and in neuronal processes, glutamate is the principal activator of NO release (Gammie *et al.*, 2000). NO also diffuses radially to affect surrounding synapses, carrying information in the opposite direction to neural transmission (Beckmann, 1996). So, the synthesis of NO by taste and basal cells may be indicative of the onset of functional activity in these taste cells and the activation of diverse afferent synaptic structures between the basal cells and/or the taste cells and nerve fibers.

nNOS-IR in nerves

The present study demonstrates the presence of nervous intragemmal and extragemmal nerve fibers immunostained with nNOS. Previous studies showed that the presence of nNOS enzymes corresponds with that in both central and peripheral nervous systems (Belai *et al.*, 1992; Hassal *et al.*, 1992). In absence of tracing and denervation experiments, we cannot demonstrate the extrinsic innervation of most of the immunohistochemically tested nerve fibers. Gustatory fibers usually arise from the geniculate ganglion innervating taste buds and the somatic nerve fibers distributing in the perigemmal epithelium, originate from the trigeminal ganglion (Hu *et al.*, 1996). In the dog and rat, nNOS-IR nerve fibers are thought to be of intrinsic origin, arising from the intralingual neurons (Hu *et al.*, 1996; Sbarbati *et al.*, 1999). The tongue of the examined species receives a

rich innervation of nitrergic fibers, which presumably provides a vasodilator and secretomotor action to the glands, but the origin of both intragemmal and extragemmal NO-producing fibers remains to be clarified by further investigations due to the failure to detect intralingual neurons. An interesting finding obtained from the present experiments is the relationship between ciliate cells and nitrergic nerves. The evidence seems to suggest that the ring of cilia surrounding the receptorial surface is controlled by a neural mechanism.

In conclusion, the findings obtained in previous research and in this study suggest that the occurrence of nNOS-IR in basal cells, taste cells and nerves might reflect a role for NO in the gustatory mechanisms of amphibians. The results may also sustain the physiological implication of NO as a cytoprotective molecule (Konturek and Konturek, 1995) in the local target function of maintaining taste bud mucosal integrity, in regulating the blood flow to the epithelium and in modulation of ciliary activity. NO plays multiple physiological roles in the regulation of the functions of numerous organs. Investigations continue to expand rapidly and further studies are indeed needed to clarify the NO function of the particular cell types of amphibian taste buds, responsible for their multifunctional roles.

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Accepted August 30, 2002