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Nitroxidergic system in human trigeminal ganglia neurons: A quantitative evaluation

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Summary

The trigeminal ganglia are involved in transmission of orofacial sensitivity. The free radical gas nitric oxide (NO) has recently been found to function as a messenger molecule in both central and peripheral trigeminal primary afferent neurons. NO is produced within neurons mainly by two enzymes: a constitutive (neuronal) form of NO synthase (nNOS) or an inducible form of NOS (iNOS). The aim of the study was to evaluate the distribution of trigeminal neurons according to size (small, medium and large neurons) and to correlate the percentage of NOS-immunopositive neurons with regard to neuronal size. The results showed a significant relationship between the percentage of nNOS-immunopositive neurons and the size of neurons. Evaluation of the percentage of nNOS-immunopositive neurons showed that they constitute about 50% of the total number of neurons and that they are represented mainly as large-sized neurons. The iNOS immunolabelling was very faint in all neuronal types. Since the nitroxidergic system is well represented in human trigeminal ganglia, this study indicates that it could play a relevant role in trigeminal neurotransmission.
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Introduction

The trigeminal ganglion (TG), also known as the semilunar ganglion, is found within the inferolateral aspect of Meckel's cave. The anatomical characteristics of the TG have been described by means of anatomical dissection, computed

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tomography and unenhanced high-resolution magnetic resonance imaging (Daniels et al., 1986; Rubinstein et al., 1994; Downs et al., 1996). The shape of the TG varies from thin and regular to thick and nodular, with the convex margin always oriented inferolaterally (Downs et al., 1996). The trigeminal nerve consists of numerous small fibers within the posterior and superior aspect of Meckel's cave. In all cases, the TG is continuous with the mandibular division of the trigeminal nerve inferiorly at the level of the *foramen ovale*. The trigeminal sensory system is very complex and is characterized by the presence of two distinct populations of primary afferent neurons. Most of the neuronal cell bodies (perikarya) are located in the TG, but some of them are found in the mesencephalic trigeminal nucleus (MTN). Primary sensory neurons conduct somatosensory information to neurons of the central nervous system from a variety of peripheral sensory receptors. TG neurons innervate mainly mechanoreceptors, thermoreceptors and nociceptors in the face, oral cavity and nasal cavities (Davies, 1988). The TG neurons are unipolar (pseudounipolar) with a single axon, which divides into a peripheral and a central branch. The perikarya are completely surrounded by a layer of small glial cells known as satellite cells, so they are free of synaptic contacts (Pannese, 1981).

It is currently believed that sensitisation of the trigeminal system, including the neurons of trigeminal ganglia, is involved in the pathway leading to migraine pain (Malick and Burstein, 2000). Furthermore, trigeminal nerve fibers extend from the trigeminal ganglia to the trigeminal *Nucleus caudalis* located in the brain stem, which is responsible for the transmission of nociceptive information to higher brain centres, where pain is perceived (Kaube et al., 1993; Goadsby and Hoskin, 1997). Several neurotransmitters are located in trigeminal neurons including calcitonin gene-related peptide, substance P, neurokinin A, pituitary adenylate cyclase activating peptide (PACAP), amylin and nitric oxide (NO) (Tajti et al., 1999).

The free radical gas, nitric oxide, functions as a major messenger molecule in both central and peripheral trigeminal primary afferent neurons (Schuman and Madison, 1991; Snyder, 1992). Rather than acting via traditional receptors on postsynaptic membranes, this molecule exerts its effects by diffusion into the adjacent neuron to activate soluble guanylyl cyclase. NO is produced in neurons from L-arginine by a constitutive (neuronal) form of NO synthase (nNOS), an enzyme localized in neurons and which requires activation by intracellular calcium of a calmodulin-sensitive site, or by an inducible form of NOS (iNOS) localized predominantly in the glia, which requires activation by endotoxins and cytokines. A third form is endothelial NOS (eNOS), which generates NO in blood vessels and plays a role in regulating vascular function. The NO acts as a second messenger by activating the soluble guanylate cyclase leading to increased levels of cyclic guanosine monophosphate.

nNOS acts as a neural transmitter with several important functions in memory (Bult et al., 1990; Schuman and Madison, 1991) and pain transmission (Martucci et al., 2008). It is present in trigeminal primary afferent neurons, both peripherally within the TG and centrally in the MTN (Lazarov and Dandov, 1998; Lazarov et al., 1998). Moreover, its histochemical marker is generally considered to be NADPH-d and parallel application of nNOS and NADPH-d immunohistochemical localization results in similar, though not identical, distributional patterns (Traub et al., 1994). iNOS is expressed only in pathological conditions (Jenkins et al., 1994) and is induced by pro-inflammatory cytokines and/or endotoxins (Jenkins et al., 1994).

NOS immunoreactivity of trigeminal neuronal bodies and its colocalization with a wide range of neurotransmitters has led to the suggestion that NO is a key molecule involved in modulation of sensitive pathways (Yasuhara et al., 2007; Martucci et al., 2008).

To the best of our knowledge, there is little information on the presence of NOS isoforms in neurons of trigeminal ganglia in humans. The aim of this study was to describe the morphology and nNOS/iNOS immunolocalization in trigeminal ganglia using immunohistochemical techniques. The information obtained may provide an important tool to improve our understanding of the role of NO in neural transmission and modulation.

Materials and methods

Tissue processing

The trigeminal ganglia were removed, with Ethic Commitee permission, bilaterally from six cadavers (three females and three males) with an average age of 71 years (60–82 years) used in the dissection courses for medical students at Vienna University. None of the donors had suffered from any nervous system disease. Tissue was collected within 48 h of death, frozen on dry ice and stored at −20 °C. Serial frozen sections were cut at 12 μm thickness using a cryostat and mounted on poly-L-lysine-coated glass

slides (Sigma–Aldrich, St. Louis, MO, USA); alternate sections were then processed for morphological staining and for NOS immunohistochemistry.

Morphological analysis

The distribution of the different classes of neurons in human trigeminal ganglia was assessed using toluidine blue staining, a useful method to visualize the cytoarchitecture of the nervous system (Ricci et al., 2006; Rodella et al., 2008). All analyses were performed in a 'blinded' manner and were measured in six sections for each ganglion: two sections of the anteromedial portion, two sections of the middle portion and two sections of the posterolateral portion (Jannetta, 1967; Marfurt, 1981). The neurons were subdivided into three groups according to perikaryonal diameter: small cells (under 30 μm), medium-sized cells (30–60 μm) and large-sized cells (over 60 μm) (according to Hou et al., 2003). Digital images of the slides at 200 × magnification were analyzed using an optical microscope (Olympus BX50 upright microscope for transmitted light brightfield, Hamburg, Germany) equipped with image analysis software (Image-Pro Plus™ 4.5.1, Milan, Italy). Only the neurons with a clearly visible nucleus were considered. The percentage of neurons for each class was evaluated in 10 randomly selected fields with identical areas examined for each section.

nNOS and iNOS immunohistochemistry

Unfixed cryostat sections were washed in Tris-buffered saline (TBS), fixed in cold acetone for 10 min and treated with 3% hydrogen peroxide (H_2O_2) in methanol (1:1) for 10 min. The sections were incubated in normal goat serum (Vector Laboratories, Burlingame, CA, USA −10% in TBS containing 0.1% Triton X-100) for 30 min and then incubated in rabbit polyclonal primary antiserum directed against nNOS (Cayman 160870, Ann Arbor, MI, USA – diluted 1:200) or iNOS (Santa Cruz Biotechnology sc-651, Santa Cruz, CA, USA – diluted 1:500) prepared in phosphate-buffered saline containing 3% normal goat serum and 0.1% Triton X-100, for 24 h at 4 °C. After incubation in the primary antiserum, the sections were subsequently incubated in biotinylated goat anti-rabbit immunoglobulins (Vector Laboratories, – IgG, 7.5 μg/ml) for 30 min and avidin–biotin peroxidase complex for 30 min, using an ABC kit according to the manifacturer's protocol (Vector Laboratories). The reaction product was visualized using 0.0006% hydrogen peroxide and 0.5 μg/ml diaminobenzidine (Sigma–Aldrich, St. Louis, MO, USA) as a chromogen, for 5 min. Specificity of the immunohistochemical labelling was confirmed by omitting the primary antiserum, using sections incubated with normal serum alone, and also using isotype controls incubated with the same concentration of primary antibodies. The sections were counterstained with methyl green, dehydrated in ethanol, cleared in xylene, mounted in DPX (Sigma–Aldrich) and finally observed using an Olympus BX50 microscope. The immunolabelling intensity was computed as integrated optical density (IOD) using image analysis software as described below.

Quantitative analysis of labelling

We evaluated immunolabelling in human trigeminal ganglia. All analyses were performed ''blind'' (on coded sections) and measurements made on six sections for each ganglion: that is, two sections of the anteromedial portion, two sections of the middle portion and two sections of the posterolateral portion considering the somatotopic organization of trigeminal ganglia (Jannetta, 1967; Marfurt, 1981). Digital (frame-grabbed) images at 200 × magnification were analyzed using an optical microscope (Olympus BX50) equipped with an image analysis program (Image-Pro Plus™ ver.4.5.1, Milan, Italy). Only the neurons with a clearly visible nucleus were considered. Background determined from an empty surface of the slide was subtracted from the immunolabelling intensity measure.

The percentages of NOS-immunopositive neurons with respect to the total number of neurons for each class were determined counting 10 randomly collected fields of identical areas (0.86 mm^2) for each section and the results were compared with the total number of neurons for each class found in the toluidine blue stained sections.

The IOD for NOS immunohistochemistry was calculated for arbitrary areas, measuring the neurons in 10 randomly collected fields with the same area for each section. Labelling intensity was computed as IOD according to Goettl et al. (2003). The data were pooled to represent a mean value and a statistical analysis was applied to compare the results obtained from the samples.

Statistical evaluation

Distribution of neuronal population with respect to size

The data were analyzed and compared by analysis of variance (ANOVA) and by a Bonferroni

multiple comparison test. The p value threshold was 0.05.

Distribution of NOS-immunopositive neurons

This parameter was evaluated using a log-linear model.

Relationship between intensity of NOS immunolabelling and neuronal size

This parameter was evaluated using a multilevel model. IOD and neuronal size were considered in a logarithmic scale.

Results

Distribution of neuronal population with respect to size

We evaluated the percentage of neurons with respect to their perikaryonal size. We observed that the neuronal population in ganglia is rich in medium-sized neurons (81–85%) in comparison with small-sized (9.75%) and large-sized (8.39%) neurons (Figure 1).

Distribution of NOS-immunopositive neurons

Positive immunolabelling was visible in the cytoplasm of the neurons as a diffuse positivity (Figure 2). We estimated that the total number of nNOS-immunopositive neurons constitute about 50% of the overall number of neurons. The percentage of immunopositive small-sized neurons was about 48%; with about 56% of the medium-sized neurons labelling positively, and the percentage of large-sized immunopositive neurons was about 69%. This shows that the number of large-sized neurons labelling positively was statistically ($p<0.05$) higher than the small and medium-sized neurons (Figure 3).

The immunolabelling intensity of iNOS was very weak and only seen in a limited number of neurons, so it was not possible to undertake a quantitative evaluation.

Relationship between intensity of NOS immunolabelling and neuronal size

The intensity of nNOS immunolabelling in neurons was significantly associated with the neuronal size. The IOD values were statistically different between small/medium and large neurons with the small and medium-sized neurons presenting higher IOD values than the large-sized neurons (Figure 4).

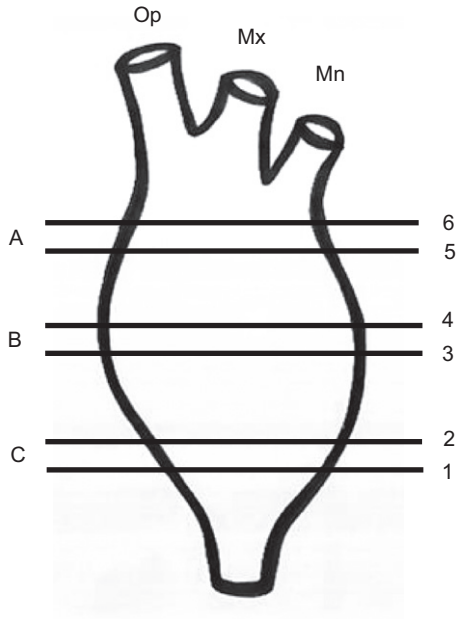

Figure 1. Schematic drawing of the human trigeminal ganglia. Op, ophthalmic division; Mx, maxillary division; Mn, mandibular division; A, anteromedial portion; B, middle portion; C, posterolateral portion; 1–6, site of the six sections.

Discussion

In this study, we describe the distribution of the neuronal population in human trigeminal ganglia with respect to neuronal size and NOS immunolocalization. According to previous reported data, the analysis of trigeminal ganglion neuronal size in the human has revealed three distinct groups. The small ganglion cells with a conduction velocity corresponding to the C fiber category are considered to be nociceptive with respect to their sensory modality (Lazarov, 2002). The medium- to large-sized ganglion cells have conduction velocities in the A-beta and -delta fiber categories, which are representative of low-threshold mechanoreceptors, thermoreceptors and nociceptors (Galeano et al., 2000; Hou et al., 2003). NOS is localized in neurons of both the central and peripheral nervous systems (Bredt et al., 1990). Information regarding cellular localization of NOS has been obtained by NOS-immunohistochemistry and in situ hybridization (Bredt et al., 1991; Verge et al., 1992) and by the histochemical technique for demonstration of NADPH-diaphorase (Dawson et al., 1991; Hope et al., 1991). Using NADPH-diaphorase staining and NOS immunohistochemistry, several studies have underlined the occurrence of NOS in sensory neurons (Aimi et al., 1991; Terenghi et al., 1993; Stoyanova and Lazarov, 2005) suggesting its role in the neuronal transmission of sensory information in

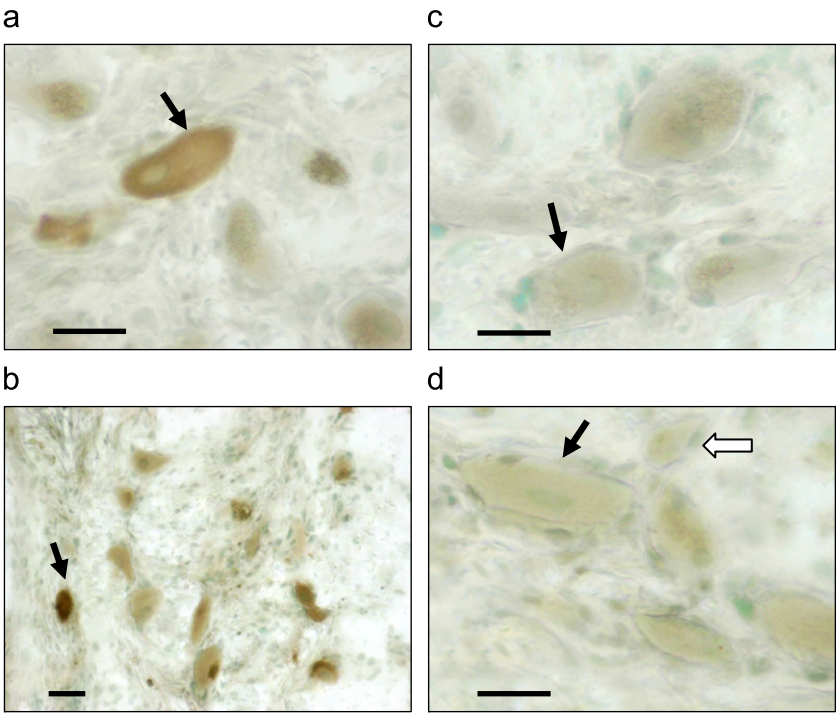

Figure 2. nNOS immunolabelling of trigeminal ganglia (counterstained with methyl green): (a) arrow indicates strongly immunopositive medium-sized neuron; (b) arrow indicates strongly immunopositive small-sized neuron; (c) arrow indicates weakly immunopositive medium-sized neuron and (d) black arrow indicates faintly immunopositive large-sized neuron and white arrow indicates faintly immunopositive small-sized neuron. Bar: 30 μm.

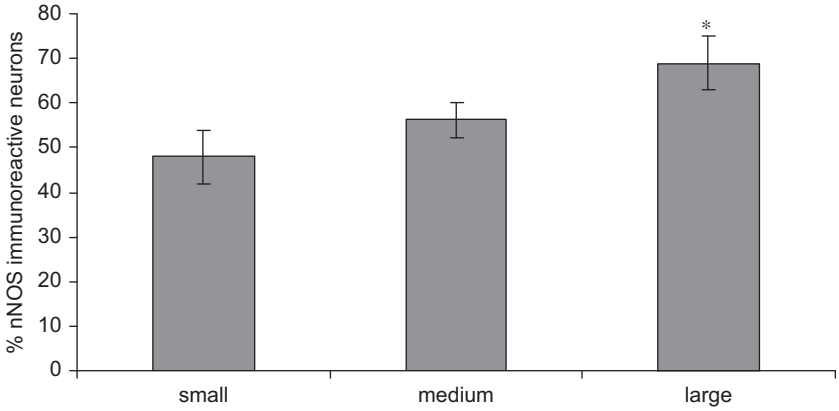

Figure 3. Distribution of nNOS-immunoreactive neurons. The distribution of nNOS-immunoreactive neurons was expressed as a percentage of positive neurons respect to the total neuronal population in each class of neurons (small- medium- and large-sized neurons) ($n = 6$). The values are expressed in percentage as mean ± SD. *$p < 0.05$ vs. small.

TG. Several studies have revealed widespread nNOS-immunoreactivity and NADPH-diaphorase activity in the TG in different animal species (humans: Tajti et al., 1999; pigs: Sienkiewicz et al., 1995; cats: Lohinai et al., 1997; rats: Aimi et al., 1991; Alm et al., 1995; chicks: Brüning et al., 1994a; quail: Panzica et al., 1994; turtles: Brüning et al., 1994b).

Using nNOS-immunohistochemistry, we found that nNOS immunolabelling was detectable in about 50% of trigeminal neurons and they were mainly large-sized neurons. This result is different from some other previous studies, but this is not surprising since the data in the literature also differ from each other (Tajti et al., 1999; Hou et al., 2003; Gottanka et al., 2005). Tajti et al. (1999) showed about 15% NOS-immunoreactive perikarya, Gottanka et al. (2005) reported 18% of nNOS-immunoreactive perikarya and Hou et al. (2003) indirectly reported about 35% of nNOS-positive neurons. These discrepancies could be due to different fixation protocols and sensitivity of the

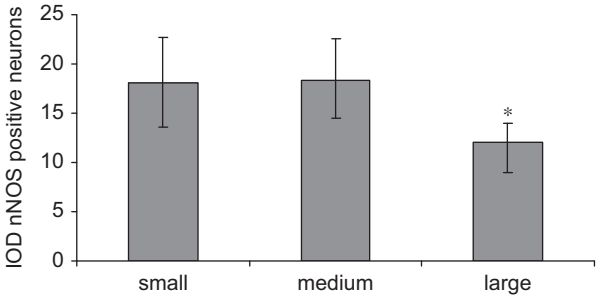

Figure 4. Relationship between intensity of nNOS immunopositivity and neuronal size. Evaluation of intensity of nNOS immunolabelling as IOD in small, medium and large neurons ($n = 6$). The values are expressed in percentage as mean $\pm$ SD. *$p < 0.05$ vs. small.

primary antibody. For this reason, in this study, we did not use aldehydic fixation. In addition, in all the previous studies, the cutting protocol was not specified and this is important information considering the somatotopic organization of trigeminal ganglia and neccessity to identify the portions of the ganglia examined (Jannetta, 1967; Marfurt, 1981). Finally, as reported in this paper, the immunolabelling was not homogeneous in all kinds of neurons. In particular, the large-sized neurons were not heavily labelled. They are relatively more sensitive to fixation and may not have been counted as immunopositive neurons by some authors.

The presence of NOS-immunoreactive trigeminal neuronal bodies and the colocalization of NOS with a wide range of neurotransmitters has led to the suggestion that NO is a key molecule for modulating sensory pathways (Yasuhara et al., 2007; Martucci et al., 2008). It appears that neurons of the trigeminal ganglia provide a somewhat richer source of NO immunoreactive neurons in humans as compared with cats and rats (Nozaki et al., 1993; Edvinsson et al., 1998; Rodella et al., 2000).

The intensity of nNOS immunopositivity was statistically different between the different classes of neurons. We found a higher immunolabelling intensity in the small- and medium-sized neurons. These data agree with some observations in cats that the neuronal cell bodies showing higher intensities of NOS immunolabelling were predominantly of small to medium size (Lohinai et al., 1997; Edvinsson et al., 1998; Lazarov et al., 1998), probably of a nociceptive nature (Wang et al., 1996, 1997; Martucci et al., 2008).

iNOS immunolocalization was not observed and this is probably because, according to the literature, this isoform is only expressed in pathological conditions (Jenkins et al., 1994). iNOS expression is induced by pro-inflammatory cytokines and/or endotoxins (Jenkins et al., 1994). Administration of the NO donor, glyceryl trinitrate to rats causes neurogenic inflammation of the dura mater with increased levels of iNOS 4–6 h after the infusion (Reuter et al., 2001). NO is also involved in the sensitization of sensory nerve endings (Malick and Burstein, 2000) where NO may act in concert with prostaglandins, histamine, bradykinins and neuropeptides (Strassman et al., 1996; Ebersberger et al., 1997). In primary cultures of rat trigeminal cells, data indicate that iNOS expression may involve a molecular mechanism mediating the adaptive responses of trigeminal ganglia cells to the serum free stressful stimulus found in tissue culture environments. It may act as a cellular signalling molecule that is expressed after cell activation (Jansen-Olesen et al., 2005).

In conclusion, it appears that NO is a key molecule that may modulate the transmission of sensitivity from the periphery to the neocortex and its high expression in different subpopulations of human trigeminal neurons may indicate its pivotal role in neurotransmission.

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