Ultrastructural localization of nitric oxide synthase immunoreactivity in the cat ventrobasal complex

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Summary

This study describes the ultrastructural localization of nitric oxide synthase (NOS) immunoreactivity in the cat ventrobasal complex. NOS immunoreactivity was found in the cell bodies and dendrites of local circuit neurons and in vesicle-containing profiles. The vesicle-containing profiles could be divided into two classes, those of dendritic origin (presynaptic dendrite boutons) and those of axonal origin. The NOS labelled axon terminals varied in size and packing density and were principally located in the extra-glomerular neuropil. These boutons presented a range of morphologies and it was not possible to determine the probable source based on morphological criteria. The NOS immunoreactive presynaptic dendrite boutons were found both within and outside glomeruli and established both pre- and post-synaptic relationships with other elements. Post-embedding GABA immunocytochemistry showed that some NOS immunoreactive axonal boutons and presynaptic dendrites were also immunopositive for GABA. This finding suggests that some of the NOS labelled axonal boutons are of local circuit neuron origin. These results suggest that local circuit neurons in the cat ventrobasal complex might be involved in specific, short range interactions using GABA and longer, more global interactions using nitric oxide.

Introduction

The cat thalamic ventrobasal (VB) complex contains two classes of neuron, the thalamocortical projection neuron (TCP) and the local circuit neuron (LCN; Jones, 1985; Ralston et al., 1988). These neurons receive afferents from a number of sources that include the cerebral cortex, spinal cord, dorsal column nuclei, thalamic reticular nucleus and a number of brain stem nuclei. The two cell classes and the various afferents interact in the VB through a variety of synaptic connections that have been described anatomically in considerable detail (Ralston & Herman, 1969; Jones, 1985; Ohara et al., 1987; Ralston et al., 1988). The intrinsic and extrinsic sources of synaptic connections utilize several different transmitters although our knowledge of transmitters and receptors in the VB is less complete than our knowledge of the anatomical components. Most recently it has been shown that nitric oxide (NO) is present in the thalamus and the distribution of enzymes associated with the production of NO has been shown to vary between species (Mizukawa et al., 1989; Bickford et al., 1993; Gabbott & Bacon, 1994; Meng et al., 1996). In the cat dorsal lateral geniculate nucleus (LGNd) the sole source of NO appears to be parabrachial afferents (Bickford et al., 1993) while in the rat LGNd the synthetic enzymes for NO appear to be present in LCNs (Gabbott & Bacon, 1994). Evidence from pharmacological and microdialysis studies of the rat VB (Do et al., 1994; Williams et al., 1997) has led to the speculation that NO may act in a positive-feedback manner to enhance the responsiveness of thalamic relay neurons.

Recently, we showed in a confocal scanning laser microscopic (CSLM) study, that LCNs in the cat VB contain nitric oxide synthase (NOS, Meng et al., 1996), a synthetic enzyme in the NO pathway and an accepted marker for NO. We found that approximately 17% of the LCNs contained NOS co-localized with GABA. It has been known for some time that thalamic LCNs are GABAergic and that their dendrites possess complex appendages that contain synaptic vesicles and constitute the major source of synaptic output of these neurons (Ralston, 1971; Lieberman, 1973; Jones, 1985; Ohara & Lieberman, 1993). Our CSLM study (Meng et al., 1996) suggested that the VB might possess two classes of LCN distinguished by the presence or absence of NOS. The CSLM study was, however, unable to whom correspondence should be addressed.

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to provide information on two aspects of LCN connectivity. First, NOS immunoreactivity was only seen in the soma and primary dendrites of the LCNs. Due to the resolution limits of the CSLM and the complex ramifications of the LCN dendrites, we were unable to determine whether or not NOS was present in the smaller diameter dendrites and dendritic appendages. The presence of NOS in the vesicle filled dendritic appendages would suggest that NO is released from these sites together with GABA. Second, it is not known if the synaptic connectivity of the NOS-positive and NOS negative LCNs is different. Differences in synaptic connectivity might suggest different functions for the two types of LCN and provide further evidence for different classes of LCN.

Their small size, lack of projecting axon, complex dendritic arbor and complex synaptic interactions have resulted in LCNs proving resistant to study by many current neurobiological methods. Electron microscopy combined with immunocytochemistry remains one of the most useful means of studying these neurons. In this study we have used the electron microscope to examine the ultrastructural localization of NOS to determine whether NOS is present in LCN dendritic appendages and whether the synaptic interactions of NOS containing LCNs are the same as those of NOS-negative LCNs.

Materials and methods

Three adult male cats, each weighing 2.3–3.2 kg, were used in this study. The animals were anaesthetized deeply with sodium pentobarbital (60 mg kg⁻¹, i.v.) and perfused transcardially through the ascending aorta with 150 ml of 0.1 M phosphate buffered saline (PBS; pH 7.4, room temperature) followed by 1600 ml of fixative (4 °C). Three different fixatives (all in 0.1 M phosphate buffer, pH 7.4) were used; 4% paraformaldehyde/0.1% glutaraldehyde, 4% formaldehyde/0.3% glutaraldehyde or 2% formaldehyde/2% glutaraldehyde. Immediately after perfusion-fixation, the brains were removed and placed in the same fixative overnight at 4 °C. Coronal 50 μm sections were cut serially through the rostro-caudal extent of the VB using a Vibratome and collected into 0.1 M phosphate buffer (PB).

Light microscopy

For light microscopy (LM), free-floating sections were preincubated for 1 h in normal goat serum (NGS; 10% in PBS) with 0.5% Triton X-100 and then incubated for 3 h at room temperature in a rabbit polyclonal NOS primary antibody (b NOS 1:6000, Product number N31030, Transduction Laboratories, Lexington, KT). The sections were rinsed in PBS and then incubated in biotinylated goat anti-rabbit IgG (Vector Laboratories, 1:200) in PBS with 10% NGS for 2 h room temperature or overnight at 4 °C. Subsequently, sections were rinsed in PBS, incubated in avidinbiotin-peroxidase complex (ABC, Vector Laboratories) for 30 min, washed several times in PBS, and then the NOS immunoreactivity was revealed by a nickel-intensified diaminobenzidine reaction (n-DAB; Llewellyn-Smith et al., 1993). The sections were mounted onto chrome-alum gelatin treated slides, dehydrated, and coverslipped with DePeX (G. T. Gurr, England).

Details of the specificity of the NOS antibody have been previously reported (Mitchell et al., 1990). Control sections were processed as described above, except that the primary antibody was omitted. No staining was present in these sections.

Electron microscopy: pre-embedding immunocytochemistry

For pre-embedding electron microscopy (EM), 50 μm vibratome sections were pretreated in 50% ethanol for 30 min to increase antibody penetration, then processed as detailed above for LM with the exception that Triton X-100 was omitted from the protocol. Following the n-DAB reaction, the sections were thoroughly rinsed with PBS, osmicated with 1% (w/v) OsO₄ in 0.1 M PB for 1 h, dehydrated in a graded series of alcohols followed by propylene oxide, and embedded in Epon 812. Blocks containing the VB or LGNd were trimmed from the vibratome sections and thin sections were cut from the surface. The sections were then stained with uranyl acetate and Reynolds’ lead citrate and examined with a JEOL 100CX II electron microscope.

Electron microscopy: post-embedding immunogold double labelling

Some ultrathin sections from different animals processed by the pre-embedding method for the visualization of NOS immunoreactivity were subsequently processed for the demonstration of GABA immunoreactivity by a standard post-embedding immunogold method (Milroy & Ralston, 1995) using a rabbit polyclonal GABA antiserum (Incastar, Stillwater, MN). Briefly, ultrathin sections collected on nickel grids were rinsed in Tris-buffered saline and incubated at room temperature with the primary antiserum (1:750, overnight). After extensive rinses, grids were incubated for 1 h in a solution of goat anti-rabbit IgG coupled to 15 nm gold particles (1:30, Amersham, UK). Following thorough washing, the sections were counterstained with uranyl acetate and lead citrate. Specificity of the immunogold labelling was evaluated by the application of rabbit non-immune serum as well as by processing series of sections omitting various stages of the regular staining sequence.

Results

Light microscopy

The CSLM appearance of the NOS-immunoreactive (NOS-ir) and GABA-immunoreactive neurons visualized with fluorescent tagged antibodies has been reported in detail previously (Meng et al., 1996). In brief, NOS-ir neurons were found to be a subset of the GABA-ir neurons and were identified as thalamic local circuit neurons. All NOS-ir neurons seen in the VB complex were also GABA immunoreactive. The NOS-ir positive neurons were among the largest LCNs and labelling was most clearly visible in the soma and proximal dendrites. Although there were small labeled elements scattered throughout the neuropil that