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Research Report

NOS-positive local circuit neurons are exclusively axo-dendritic cells both in the neo- and archicortex of the rat brain

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Abstract

Neuronal nitric oxide synthase (nNOS)-containing neurons and axon terminals were examined in the rat somatosensory and temporal neocortex, in the CA3/a–c areas of Ammon's horn and in the hippocampal dentate gyrus. In these areas, only nonpyramidal neurons were labeled with the antibody against nNOS. Previous observations suggested that all nNOS-positive nonpyramidal cells are GABAergic local circuit neurons, which form exclusively symmetric synapses. In agreement with this, nNOS-positive axon terminals in the hippocampal formation formed symmetric synapses exclusively with dendritic shafts. In the neocortex, in contrast, in addition to the nNOS-positive axon terminals that formed synapses with unlabeled spiny and aspiny dendrites and with nNOS-positive aspiny dendrites, a small proportion of the nNOS-positive axon terminals formed symmetric synapses with dendritic spines. These results suggest that nNOS-positive local circuit neurons form a distinct group of axo-dendritic cells displaying slightly different domain specificity in the archi- and neocortex. However, nNOS-positive cells show no target selectivity, because they innervate principal cells and local circuit neurons. Afferents to the NOS-positive cells display neither domain nor target selectivity, because small unlabeled terminals formed synapses with both the soma or dendrites of nNOS-positive neurons and an adjacent unlabeled dendrite or spine in both the hippocampal formation and in neocortex.

Theme: Neurotransmitters, modulators, transporters, and receptors
Topic: Other neurotransmitters

Keywords: Inhibitory neuron; Temporal cortex; Somatosensory cortex; Dentate gyrus; Ammon's horn

1. Introduction

The freely diffusible, reactive free radical gas, nitric oxide (NO), has been proposed to function as a biological intercellular signaling messenger, capable of communicating among neurons, glia, and the vasculature [9,31]. Nitric oxide is synthesized from arginine by cells containing the enzyme nitric oxide synthase (NOS) which exists in a variety of forms in different types of cells [7,14,18], and is released, by diffusion, into the extracellular space. Neuronal (nNOS), endothelial (eNOS), and inducible (iNOS) forms of the enzyme are present in the brain [20]. In the hippocampus, the eNOS is localized in pyramidal cells [5], whereas nNOS immunoreactivity is characteristic for interneurons [6,12,29,32]. In the hippocampal formation, nNOS- and NADPH-diaphorase (NADPHd)- positive neuronal populations are very similar, if not identical [17,29]. Neurons in the hippocampus and cuneate nucleus showing NADPHd or nNOS-reactivity are also immunoreactive for GABA, providing direct evidence that they are interneurons [13,29,30].

This study examined the postsynaptic targets of nNOS-positive axon terminals in the hippocampal formation and neocortex using nNOS immunocytochemistry. Although

Abbreviations: CA1-3, subregions of Ammon's horn (Cornu Ammonis 1–3); GABA, gamma amino butyric acid; nNOS, neuron-specific nitric oxide synthase

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NADPHd- or NOS-positive cells in the cortex and hippocampal formation have been described previously, their postsynaptic targets are known only in the CA1 area of Ammon's horn and in the subiculum [17,23]. The morphological features of NOS-positive nonpyramidal cells in hippocampus have not been extensively reported [29]. In other brain areas, these cells display the characteristic features of GABAergic neurons such as nuclear infoldings, intranuclear rods, and asymmetric axo-somatic synapses [11,30]. Moreover, in the cuneate nucleus, but not in the hippocampal formation or in the cortex, the postsynaptic targets of the axon terminals of nNOS-positive neurons have been identified as dendrites [30]. However, we do not know whether nNOS-positive local circuit neurons in CA2–3 areas of Ammon's horn, in the dentate gyrus or in the neocortex, give rise to perisomatic or axo-dendritic terminals or both. Nor do we know whether nNOS-positive axon terminals in these brain regions form exclusively symmetric synapses or whether, as in the subiculum, they also form asymmetric synapses.

2. Material and methods

Three male Wistar rats (200–300 g; Charles River, UK) were administered an overdose of Equithesin (0.3 ml/100 g). Once deep anesthesia had been verified by the absence of a toe-pinch response, the animals were transcardially perfused first with 50 ml of 0.9% saline followed by 300 ml of a fixative solution containing 3% paraformaldehyde and 0.1% glutaraldehyde in 0.1 M phosphate buffer (PB, pH 7.4). All experiments were conducted in accordance with the UK Animals (Scientific Procedures) Act of 1986 and the "Principles of laboratory animal care" were (NIH publication No. 86-23, revised 1985) followed.

After fixation, the brains were removed from the skull and placed in cold phosphate-buffered saline (PBS). A flat surface was prepared by removing the dorsal surface of the cortex and, starting from the ventral surface of the brain, horizontal sections, 100 μm thick, were cut through the hippocampal formation and the surrounding neocortical areas, using a vibrating microtome (Leica) and collected in PBS. To enhance penetration of immunoreagents, the sections were equilibrated in a cryoprotectant solution, frozen briefly in liquid nitrogen, and thawed in the same cryoprotectant solution [26].

Sections were washed thoroughly in PBS and neuronal nitric oxide synthase (nNOS) immunoreactivity was revealed using a sheep polyclonal antibody (a gift from Dr P.C. Emson; [10]) diluted 1:10,000 in PBS. Following incubation at 4 °C for 48 h, and with washes in PBS between steps, the sections were immersed in biotinylated anti-sheep IgG (Vector Laboratories, Peterborough, UK) diluted 1:100 in PBS for 2 h, followed by 2 h in ABC reagent (Vector Laboratories), both at room temperature. The bound peroxidase was revealed after incubation for 5 min in Vector SG (Slate Grey), prepared according to the manufacturer's instructions, with the optimal incubation time determined with test sections. The sections were then transferred to 0.1 M PB.

Further processing for electron microscopic evaluation involved flattening the sections onto a watch glass, followed by immersion in a solution of 1% osmium tetroxide in 0.1 M PB for 20–30 min, followed by a wash in water and dehydration through a graded series of ethanol concentrations. Uranyl acetate (1%) was included in the 70% ethanol stage (40 min) to enhance contrast. After the final incubation in 100% ethanol, the sections were transferred to propylene oxide before being placed into aluminum foil boats containing Durcupan resin (ACM Fluka, Gillingham, Dorset, UK) overnight. Sections were mounted on cleaned slides, a coverslip applied, and the resin cured for 48 h at 60 °C.

Areas of interest were photographed from well-stained sections of two animals, then cut and reblocked in Durcupan resin. The tissue areas were approximately 1 mm^2 containing either neocortical regions or part of the hippocampal formation. From the neocortex, the somatosensory and the temporal areas were chosen, because at the horizontal level that contained the hippocampal formation, these two areas could easily be identified. From the hippocampal formation, the dentate gyrus and the CA3 area were selected, because nNOS-positive nonpyramidal neurons have already been described in the CA1 area of Ammon's horn [23]. Serial ultrathin sections were collected on single-slot Collodion (Parlodion, Electron Microscopy Sciences, Fort Washington, PA, USA) coated, copper grids. Care was taken to collect the first few sections from the surface of the blocks, because tissue penetration of nNOS-antibody is relatively limited and axon terminals were rarely labeled more than 1.5 μm below the surface, although labeled dendrites and neuronal bodies could be examined in deeper parts of the tissue. The ultrathin sections were contrasted by uranyl acetate and lead citrate according to a standard procedure.

From two animals, five (three from one and two from the other) flat embedded sections were chosen and several blocks were prepared from each. Altogether, 12 nonconsecutive thin sections were systematically examined for each cortical area and 10 thin sections for both the dentate gyrus and the CA3 area of Ammon's horn in a JEOL 1200EX-II electron microscope. There was no reason to include more animals, because the two studied showed a staining pattern in the light microscope typical of that in other preparations and the terminal counts revealed no major differences. The number of immunostained axon-terminals varied greatly between the thin sections, mainly depending on the distance from the surface. Labeled terminals were not counted in consecutive thin sections in order to avoid counting the same terminals twice. In many instances, sections did not contain the entire width of the cortex, because the block was slightly curved in the Durcupan resin. Therefore, given the limited penetration of the nNOS antibody, the upper cortical layers were examined in different sections from deep layers of the same block.