Parvalbumin-immunoreactive neurons in the entorhinal cortex of the rat: localization, morphology, connectivity and ultrastructure

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Summary

We studied the distribution, morphology, ultrastructure and connectivity of parvalbumin-immunoreactive neurons in the entorhinal cortex of the rat. Immunoreactive cell bodies were found in all layers of the entorhinal cortex except layer I. The highest numbers were observed in layers II and III of the dorsal division of the lateral entorhinal area whereas the lowest numbers occurred in the ventral division of the lateral entorhinal area. Most such neurons displayed multipolar configurations with smooth dendrites. We distinguished a type with long dendrites and a type with short dendrites. We also observed pyramidal immunoreactive neurons. A dense plexus of immunoreactive dendrites and axons was prominent in layers II and III of the dorsal division of the lateral entorhinal area and the medial entorhinal area. None of the parvalbumin-immunoreactive cells became retrogradely labelled after injection of horseradish peroxidase into the hippocampal formation. By electron microscopy, immunoreactivity was observed in cell bodies, dendrites, myelinated and unmyelinated axons and axon terminals. Immunoreactive dendrites and axons occurred in all cortical layers. We noted many myelinated immunoreactive axons. Immunoreactive axon terminals were medium sized, contained pleomorphic synaptic vesicles, and established symmetrical synapses. Both horseradish peroxidase labelled and unlabelled immunonegative cell bodies often received synapses from immunopositive axon terminals arranged in baskets. Synapses between immunoreactive axon terminals and unlabelled dendritic shafts and spines were abundant. Synapses with initial axon segments occurred less frequently. In addition, synaptic contacts were present between immunopositive axon terminals and cell bodies and dendrites. Thus, the several types of parvalbumin-containing neurons in the entorhinal cortex are interneurons, connected to one another and to immunonegative neurons through a network of synaptic contacts. Immunonegative cells projecting to the hippocampal formation receive axo-somatic basket synapses from immunopositive terminals. This connectivity may form the morphological substrate underlying the reported strong inhibition of cells in layers II and III of the entorhinal cortex projecting to the hippocampal formation.

Introduction

The entorhinal cortex is intimately interconnected with several association cortices and with the hippocampal formation. It is considered to mediate the flow of cortical information to and from the hippocampal formation (for reviews see Witter et al., 1986, 1989; Swanson et al., 1987). Detailed knowledge of the connectivity and the neuronal organization of the entorhinal cortex is essential in order to understand the functional associations between the entorhinal cortex and the hippocampal formation. The connectivity of the entorhinal cortex has been subjected to detailed analysis (for review see Witter et al., 1989). Although some data on intrinsic entorhinal connectivity are available (Köhler, 1986, 1988a), our knowledge of the morphology, chemical signature and synaptic connectivity of the involved entorhinal cell types is limited. Light and electron microscopic studies have demonstrated that entorhinal projection and intrinsic neurons receive synaptic input directly from extrinsic sources (Wouterlood & Nederlof, 1983; Wouterlood et al., 1985; Buhl et al., 1989; Germroth et al., 1991; Wouterlood, 1991; Caballero-Bleda & Witter, 1994). Further information on the synaptic links between the axon input and the projection neurons, as well as to
what degree interneurons are involved, is unavailable. Lorente de Nó (1933) pioneered the study of interneurons by categorizing Golgi-silver impregnated entorhinal cells. More recently, the morphological diversity of chemically identified entorhinal neurons has been determined in studies using antibodies against several neuroactive substances present in interneurons (glutamic acid decarboxylase, gamma-aminobutyric acid (GABA), somatostatin, neuropeptide Y and CCK) (Köhler & Chan-Palay, 1983; Köhler et al., 1985; Wouterlood et al., 1985; Köhler, 1988b).

New insights concerning the intrinsic circuitry of the entorhinal cortex have emerged from physiological studies. Recent data suggest that entorhinal neurons in layer II that project to the hippocampal formation are under a strong inhibitory influence (Jones, 1993). Buhl and Jones (1993) have shown via a combined physiological-anatomical approach that basket neurons are among the interneurons inhibiting these projection neurons. Basket cells bind antibodies against parvalbumin (Hendry et al., 1989). This calcium-binding protein (Celio & Heizmann, 1981; Andressen et al., 1993) coexists with the inhibitory neurotransmitter GABA in populations of non-pyramidal cortical cells (Celio, 1986; Hendry et al., 1989; Sánchez et al., 1992). Since parvalbumin appears to be fairly homogeneously distributed in the perikarya and nearly all the processes of neurons that express the marker (Celio, 1990; Nitsch et al., 1990), it is possible to compare the morphology of immunoreactive neurons directly with neurons stained according to Golgi-silver impregnation procedures. The first aim of the present study was to perform such a comparison in the entorhinal cortex of the rat. The second aim was to determine whether immunoreactive entorhinal cells are true interneurons by combining retrograde tracing with parvalbumin immunocytochemistry. The third aim was to study immunopositive structures at the ultrastructural level. A preliminary report of this study has been published (Wouterlood, 1993).

**Materials and methods**

We used 20 female Wistar rats (body weight 200–250 g); seven and six animals for light and electron microscopic immunocytochemistry respectively, and seven for retrograde tracing combined with immunocytochemistry.

**Immunocytochemistry: perfusion fixation**

The rats were deeply anaesthetized with sodium pentobarbital (Nembutal, 60 mg kg⁻¹ body weight, intraperitoneally administered) and perfused transcardially under a constant hydrostatic pressure (Jonkers et al., 1984) and under artificial respiration with 95% oxygen and 5% carbon dioxide, with 100 ml of physiological saline solution (pH 6.9; 38°C). This vascular rinse was immediately followed by 1000 ml of a mixture of 4% freshly depolymerized parafformaldehyde, 0.25%–0.1% glutaraldehyde and 0.2% picric acid in 125 mM phosphate buffer (pH 7.3) (room temperature). One hour after the perfusion, the brains were removed and stored in 125 mM phosphate buffer in a refrigerator.

**Light microscopy**

The brains were immersed in dimethyl sulfoxide (DMSO) in 125 mM phosphate buffer (pH 7.4) (1 h in 10% DMSO and 1 h in 20% DMSO), frozen onto a sliding microtome and cut into 40 µm thick frontal or horizontal sections (1:10 series). The sections were collected in 20% DMSO in buffer and stored at −40°C until further processing.

Immunocytochemistry was conducted according to two protocols. The first included standard indirect peroxidase–antiperoxidase (PAP) immunocytochemistry on free-floating sections. The sections were thawed and rinsed with a medium consisting of 50 mM Tris, 0.875% sodium chloride, and 0.5% Triton X-100 (Sigma, St Louis, USA) (pH 8.0) (TBS-T). Unless stated otherwise, all rinses and incubations were done in TBS-T, and at room temperature (20°C). Before and after each incubation step the sections were rinsed 3 x 10 min with TBS-T. Incubation in the primary antisera was conducted overnight with a monoclonal mouse antibody against parvalbumin (Sigma, St Louis, USA, 1:500). Incubation in the secondary (goat anti-mouse IgG, DAKO, Copenhagen, Denmark, 1:50) and tertiary (rat monoclonal peroxidase–antiperoxidase, Sternberger Monoclonals, Baltimore, USA, 1:100) antisera lasted 1.5 h. The sections were next transferred to 50 mM Tris-HCl (pH 7.6), and for 20–60 min with a solution of 0.04% 3,3′-diaminobenzidine tetrahydrochloride (DAB, Sigma) and 0.015% H₂O₂ in 50 mM Tris-HCl (pH 7.6). The sections were then mounted from a 0.2% gelatin solution in 50 mM Tris buffer onto glass slides and dried. Series of sections were directly dehydrated through ethanol to xylene, or first counterstained with Cresyl Violet, and coverslipped with Entellan (Merck, Darmstadt, Germany). In the second protocol we used biotinylated primary antisera. Biotinylation of the (Sigma) mouse anti-parvalbumin was done in our own laboratory (see Härtig et al., 1994). Sections were rinsed with TBS-T and incubated overnight in biotinylated mouse anti-parvalbumin (1:500). Next, they were incubated for 2 h with horseradish peroxidase conjugated to streptavidin (streptavidin-HRP, Jackson Immunoresearch Labs, West Grove, USA, 1:200), and incubated for 2 h with an avidin–biotin–peroxidase complex (ABC-kit, Vectastain, Sigma; solutions prepared according to the manufacturer’s instructions). The immunoreaction and further processing were performed as described above.

**Tracing experiments**

In seven rats large mechanical injections were made of HRP bilaterally into the hippocampal formation. The injections were placed approximately halfway along the septotemporal axis. The rats were deeply anaesthetized with 1 ml kg⁻¹ body weight of a mixture of four parts ketamine (Aesco, Bockel, The Netherlands) and three parts xylazine (Rompun, Bayer, Brussels, Belgium) and mounted in a stereotaxic frame. One microlitre of a 50% aqueous solution of HRP (Boehringer, Mannheim, Germany) was injected over a 10 min period.