Ultrastructural identification of cholinergic neurons in the external cuneate nucleus of the gerbil: acetylcholinesterase histochemistry and choline acetyltransferase immunocytochemistry

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Summary

Using acetylcholinesterase histochemical and choline acetyltransferase immunocytochemical localization methods, this study has provided conclusive evidence for the existence of cholinergic neurons in the external cuneate nucleus of gerbils. By light microscopy, both acetylcholinesterase and choline acetyltransferase labelling was confined to the rostral portion of the external cuneate nucleus. Ultrastructurally, acetylcholinesterase reaction products were found in the nuclear envelope, cisternae of rough endoplasmic reticulum and Golgi saccules of some somata and large dendrites as well as in the membranes of small dendrites, myelinated axons and axon terminals. These neuronal elements were also stained for choline acetyltransferase; immunoreactivity was associated with nuclear pores, nuclear envelope, perikaryal membrane and all the membranous structures within the cytoplasm. Of the total choline acetyltransferase-labelled neuronal profiles analysed, 79% were myelinated axons, 15% dendrites, 4% somata and 2% axon terminals. The immunostained axon terminals consisted of two types containing either round (Rd type; 62.5%) or pleomorphic (Pd type; 37.5%) vesicles. Both were associated directly with choline acetyltransferase-positive dendrites. In contrast to the paucity of choline acetyltransferase-positive axon terminals, numerous choline acetyltransferase-positive myelinated axons were present. It may thus be hypothesized that most, if not all, of the external cuneate nucleus cholinergic neurons are projection cells; such cells may give rise to axonal collaterals which synapse onto their own dendrites for possible feedback control. Choline acetyltransferase-positive dendrites were contacted by numerous unlabelled presynaptic boutons, 60% of which contained round or spherical synaptic vesicles (Rd boutons) and 40% flattened vesicles (Fd boutons), suggesting that these neurons are under strong inhibitory control. The preferential concentration of cholinergic components in the rostral external cuneate nucleus may be significant in the light of the highly organized somatotopy in the external cuneate nucleus and its extensive efferent projections to medullary autonomic-related nuclei. Our results suggest that the cholinergic neurons may be involved in somatoautonomic integration.

Introduction

It is well established that the external cuneate nucleus (ECN) in the rostral medulla gives rise to the cuneocerebellar tract for the relay of proprioceptive information from muscles of the upper extremity and cervical axis to the cerebellum (Carpenter, 1991). Recent evidence, however, also suggests the involvement of this nucleus in cardiovascular and respiratory control. There is evidence from horseradish peroxidase (HRP) and autoradiographic studies that in ferrets (Fitzakerley & Lucier, 1988), cats (Ciriello et al., 1981a,b) and monkeys (Beckstead & Norgren, 1979) the ECN receives cardiovascular and visceral afferents from the vagus and glossopharyngeal nerves. Following electrical stimulation of the carotid sinus, vagal, or cardiopulmonary sympathetic afferents, it has been shown that glucose utilization in the ECN of dogs and cats is increased (Kostreva, 1983). Ciriello and Calaresu (1978, 1979) have demonstrated electrophysiologically that vagal bradycardia is elicited by stimulation of the ECN in cats; this was mediated by stimulation of the carotid sinus nerve. A recent study by Dean and Kostreva (1987) using electrical and chemical stimulation methods suggested that the ECN of cats contained cell bodies which may modulate
various components of cardiac, respiratory and renal reflexes. Furthermore, efferent projections from the ECN to various medullary nuclei in the gerbil (Meriones unguiculatus) have been demonstrated recently by us in fresh living brainstem slices using an in vitro anterograde tracing method (Lan et al., 1994d). Other than the traditional known function as a relay centre for proprioceptive transmission (Lan et al., 1994a,b,c), we postulated, therefore, that the ECN may also be involved in the mediation of autonomic functions (Lan et al., 1994d). The results of these studies suggest a possible existence of cholinergic neurons in the ECN since acetylcholine (ACh) is known to contribute to the central regulation of autonomic and behavioural processes including gastric (Ishikawa et al., 1982; Okuma et al., 1983) and cardiorespiratory functions (Philippu, 1981; Brezenoff & Giuliano, 1982; Willette et al., 1987), neuroendocrine control (Ganong, 1975; Kisch et al., 1986), sleep-wake cycle integration (Gnadt & Pegram, 1986; Shiromani & McGinty, 1986; Shiromani et al., 1986) and arousal/defense response (Warburton, 1981). So far, studies seeking the identification of ECN cholinergic neurons by means of acetylcholinesterase (AChE) histochemistry and choline acetyltransferase (ChAT) immunohistochemistry have been carried out at the light microscopic (LM) level only and have produced conflicting results. Mizukawa and colleagues (1986) demonstrated that some of the neuronal cell bodies, dendrites and fibre network of the human ECN were AChE-positive but ChAT-negative. In a recent study by Tago and colleagues (1989), the presence of a few ChAT-positive neurons in the ECN of rats was reported. Contrary to this was the findings of Ruggiero and colleagues (1990) and Henderson and Sherriff (1991) in rats and ferrets in which the authors found only a sparse distribution of ChAT-positive punctate varicosities resembling axon terminals in the ECN. Other studies that dealt with AChE and/or ChAT-containing structures in the CNS of rats (Palkovits & Jacobowitz, 1974; Eckenstein & Sofroniew, 1983), cats (Jones & Beaudet, 1987; Vincent & Reiner, 1987), monkeys (Mesulam et al., 1984) and baboon (Satoh & Fibiger, 1985) were focused primarily on five neuronal groupings in the CNS, namely, the basal forebrain, neostriatal, parabrachial, reticular and cranial motor nuclei, containing conspicuous cholinergic cells. In contrast to these intensely labelled cholinergic cell groups, the sensory nuclei – the ECN in particular – have received relatively little attention with respect to their constituent cholinergic structures. The latter have been shown to exhibit only a weak staining for AChE and/or ChAT but the results were equivocal (Mizukawa et al., 1986; Tago et al., 1989; Ruggiero et al., 1990; Henderson & Sherriff, 1991).

In the present study we have examined the distribution of AChE and ChAT in the ECN of gerbils.

Materials and methods

A total of 19 young adult gerbils of both sexes and weighing about 60–70 g were used, 16 for AChE histochemistry, and 13 for ChAT immunocytochemistry.

Acetylcholinesterase histochemistry

Following anaesthesia with an intraperitoneal injection (i.p.) of chloral hydrate (0.49 g kg⁻¹ body weight), the animals were killed by transcardiac perfusion which began with a prewash of 50 ml of Ringer's solution (pH 7.4), followed by a 300 ml mixture of 4% paraformaldehyde, 2% glutaraldehyde, 4% sucrose and 2 mm calcium acetate in 0.05 M cacodylate buffer (pH 7.4). Prior to perfusion-fixation, all animals were given an injection of heparin (1000 unit kg⁻¹) and sodium nitrite (0.02 g kg⁻¹) directly through the left cardiac ventricle with a 1-ml syringe. The entire perfusion lasted 30 min. After fixation, the rostral medulla containing the ECN was removed, kept overnight at 4 °C in 0.05 M cacodylate buffer (pH 7.0) containing 2 mm calcium acetate and isotonic sodium sulphate. Half of the animals were used for LM observations and the remaining for electron microscopic (EM) studies. For LM study, serial transverse or horizontal 30 μm sections were cut with a freezing microtome. For EM study, transverse 50-μm thick sections were cut with a Vibratome (Oxford Instruments, UK) and processed for AChE histochemistry at 4 °C according to the method of Lewis and Shute (1969). To prepare the incubation medium, 100 mg acetylthiocholine iodide was dissolved in 4 ml of distilled water to which 7 ml of 0.1 M copper sulphate was added drop by drop. The solution was then centrifuged and to each 4 ml of the supernatant was added 25 mg glycine, 4 ml of isotonic sodium sulphate, 1 ml of 2 mM tetraisopropyl pyrophosphoramide (iso-OMPA) and 0.5 ml of 0.5 sucinic acid. The pH of the mixture was adjusted to 5.3 with 0.1 N NaOH. In this preparation, acetylthiocholine iodide acted as the substrate for localization of true cholinesterase (i.e., AChE) while iso-OMPA served as a blocking agent for inhibition of butyrylcholinesterase (BChE), a false hydrolysing enzyme for ACh. Tissue sections were incubated in the above-mentioned medium for 4 or 3 h for LM and EM studies, respectively. For control, some sections were incubated either in butyrylthiocholine iodide (BChE substrate) and iso-OMPA or in acetylthiocholine iodide with both cholinesterase inhibitors (iso-OMPA and BW284C51). After incubation, the sections were washed for 1 h in two changes of the washing medium of Lewis and Shute (1969) and developed twice (30 min each time) in fresh, filtered sodium sulphide solution of Lewis and Shute (1969). The sections were finally rinsed in the washing medium at room temperature. For LM study, the sections were lightly counterstained with 1% Cresyl Violet and coverslipped with Permount. For EM study, the sections were trimmed down to small blocks which included only the ECN and were osmicated in Dalton's fixative for 1.5 h. The tissue blocks were then dehydrated in ethanol and embedded in Epon-Araldite. Ultrathin sections were mounted on 150-mesh grids, stained with lead citrate and uranyl acetate and examined under a JEOL-2000 EX II electron microscope. Acetylcholinesterase and BChE activities were absent in all the control specimens. From the electron micrographs, the cross-sectional areas of AChE-labelled somata were outlined.