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Localization of insulin-like immunoreactive neurons in the rat gracile nucleus

S.T. Dheen, S.S.W. Tay and W.C. Wong

Department of Anatomy, Faculty of Medicine, National University of Singapore, Kent Ridge, Singapore

Summary. An insulin-like immunoreactivity (ILIR) was localized in the neuronal somata, dendrites and myelinated axons in the gracile nucleus of the male Wistar rat. In the neuronal somata, the reaction product was dispersed in the cell nucleus and cytoplasm. In the cell nucleus, the reaction product was scattered throughout the nucleoplasm, but not within the nucleolus. In the cytoplasm, the reaction product was evenly distributed, mainly in the vicinity of the cisternae of the rough endoplasmic reticulum. In labelled dendrites, the reaction product was closely associated with the parallel arrays of neurotubules and postsynaptic densities. Most of these labelled dendrites were postsynaptic to unlabelled axon terminals. A labelled dendrite often formed the central element of a synaptic glomerulus with several unlabelled axon terminals. Numerous labelled myelinated axons were also present in the neuropil. However, axon terminals appeared to be unlabelled. It is hypothesized that insulin-like substance(s) may be modulating nuclear activities as well as neurotransmission at the synapse in the gracile nucleus.

Key words: Rat, Gracile nucleus, Insulin-like immunoreactivity, Neurons

Introduction

The presence of insulin and insulin receptors in the central nervous system (CNS) was first reported by Havrankova et al. (1978a,b). It has been further reported that the insulin concentration in the CNS is independent of peripheral insulin levels (Havrankova et al., 1979). Since then, insulin-like immunoreactivity (ILIR) has been localized in several nuclei of the CNS of various animal species (Dorn et al., 1981, 1982; LeRoith et al., 1983; Tay and Wong, 1992; Dheen et al., 1994a). Dorn et al. (1981) have shown the presence of insulin-like immunoreactive neurons in the hypothalamus, thalamus, hippocampus and amygdala of the mouse brain using immunofluorescence. More recently, electron microscopic studies have shown insulin-like immunoreactive neuronal profiles in the cervical, thoracic, lumbar and sacral segments of the spinal cord in the rat and the hypothalamic paraventricular as well as supraoptic nuclei (Tay and Wong et al., 1992; Dheen et al., 1994a). However, there appears to be no report on the ILIR in the brainstem dorsal column nuclei. Hence, the present study was undertaken to localize ILIR in the gracile nucleus of the rat.

Offprint requests to: Dr. S.S.W. Tay, Department of Anatomy, Faculty of Medicine, National University of Singapore, Kent Ridge, Singapore 119260

Materials and methods

A total of 10 adult male Wistar rats (200-250 g body wt) was used in the present study. Before perfusion, each rat was anaesthetized with 1-1.5 ml of chloral hydrate (70 mg/ml). Tracheostomy was carried out and artificial respiration maintained with a Harvard rodent ventilator (model 683). One thousand units of heparin and 2 ml of 1% sodium nitrite per kg body wt were given by intracardiac injection just before perfusion.

Conventional electron microscopy

Each of the 2 rats used was rapidly perfused through the left cardiac ventricle with 100 ml of Ringer's solution (pH 7.4) followed by 500 ml of fresh fixative (2% paraformaldehyde + 3% glutaraldehyde) in 0.1M phosphate buffer (pH 7.4). The brainstem was dissected out immediately and immersed in the same fixative for 4 h at 4 °C. Tissue blocks measuring 0.5 mm in thickness were taken from the level of the obex to 5 mm caudal to it. They were then trimmed to include the gracile nucleus. After overnight rinsing in ice-cold 0.1M phosphate buffer (pH 7.4) containing 20% sucrose, the tissue blocks were postfixed in 1% osmium tetroxide for 2 h, dehydrated in an ascending series of ethanol and embedded in an Araldite mixture. Ultrathin sections were cut with a Reichert E ultramicrotome, stained with uranyl acetate and lead citrate and viewed in a Philips 400T electron microscope.

Immunoelectron microscopy

Each of the 8 rats used was rapidly perfused through the left cardiac ventricle with 100 ml of Ringer's solution (pH 7.4) followed by 500 ml of fresh fixative (4% formaldehyde + 0.2% glutaraldehyde, pH 7.4). The brainstem was dissected out immediately and immersed in the same fixative for 4 h at 4 ºC and kept overnight in 0.1M phosphate buffer containing 20% sucrose. Coronal sections (40 µm thick) were cut through the dorsal column nuclei using an Oxford vibratome and collected in incubation wells containing phosphate buffered saline (PBS, pH 7.4). The sections were processed for immunocytochemistry using the free floating technique with avidin-biotin peroxidase immunostaining (Hsu et al., 1981). The free sections were rinsed in PBS and incubated in 4% normal goat serum (NGS) for 1 h at room temperature to reduce non-specific staining. The sections were washed for 30 min with 3 changes of PBS and subsequently incubated for 40 h at 4 ºC with insulin antiserum (Incstar Corp., Minnesota, USA) at 1:500 dilution in 1% NGS. This was followed by incubation of the sections in a biotinylated secondary antibody against guinea pig IgG and the avidin peroxidase complex (PK 4007, Vector Lab., Calif., USA). Subsequently, the antigen sites were made visible by the addition of 3,3'-diaminobenzidine tetrahydrochloride as a peroxidase substrate. The sections were osmicated for 15 min, dehydrated in a graded series of ethanol and flat-embedded in an Araldite mixture. Areas containing the gracile nucleus were trimmed out from the embedded sections and remounted on Araldite-filled capsules for ultrathin sectioning. Ultrathin sections were cut using a Reichert E ultramicrotome, stained with lead citrate only and viewed in a Philips 400T electron microscope. Immunostaining was abolished by incubating the sections in 1% NGS without antiserum, or by preincubating the antiserum with 50 µg/ml of synthetic insulin (Chemicon, Calif., USA).

Results

Conventional electron microscopy

The gracile nucleus contained a prominent cell cluster lying posterolateral to the central canal and medial to the cuneate nucleus in the brainstem. Two types of neurons have been observed in the cell clusters: a small-sized neuron (average size: 12x7 µm) and a predominant medium-sized neuron (average size: 20x12 µm). The small-sized neurons had an indented nucleus with a prominent nucleolus (Fig. 1) and sparse cytoplasmic organelles. The medium-sized neurons had a round or oval nucleus (Fig. 2), and its cytoplasm displayed a distinct Golgi apparatus, widely distributed cisternae of rough endoplasmic reticulum, mitochondria and some lipofuscin granules. Numerous dendrites, axons and axon terminals were also found in the neuropil of the gracile nucleus (Figs. 1, 2). Dendrites were characterized by a pale cytoplasm containing neurotubules, cisternae of endoplasmic reticulum and mitochondria. Axon terminals containing pleomorphic, small spherical or flattened agranular vesicles made synaptic contacts with neuronal somata, dendrites as well as other axon terminals. Frequently, axon terminals and dendrites from centro-axonic and centro-dendritic synaptic glomeruli. Distributed amongst the axon terminals and dendrites were numerous myelinated and unmyelinated axons (Figs. 1, 2).

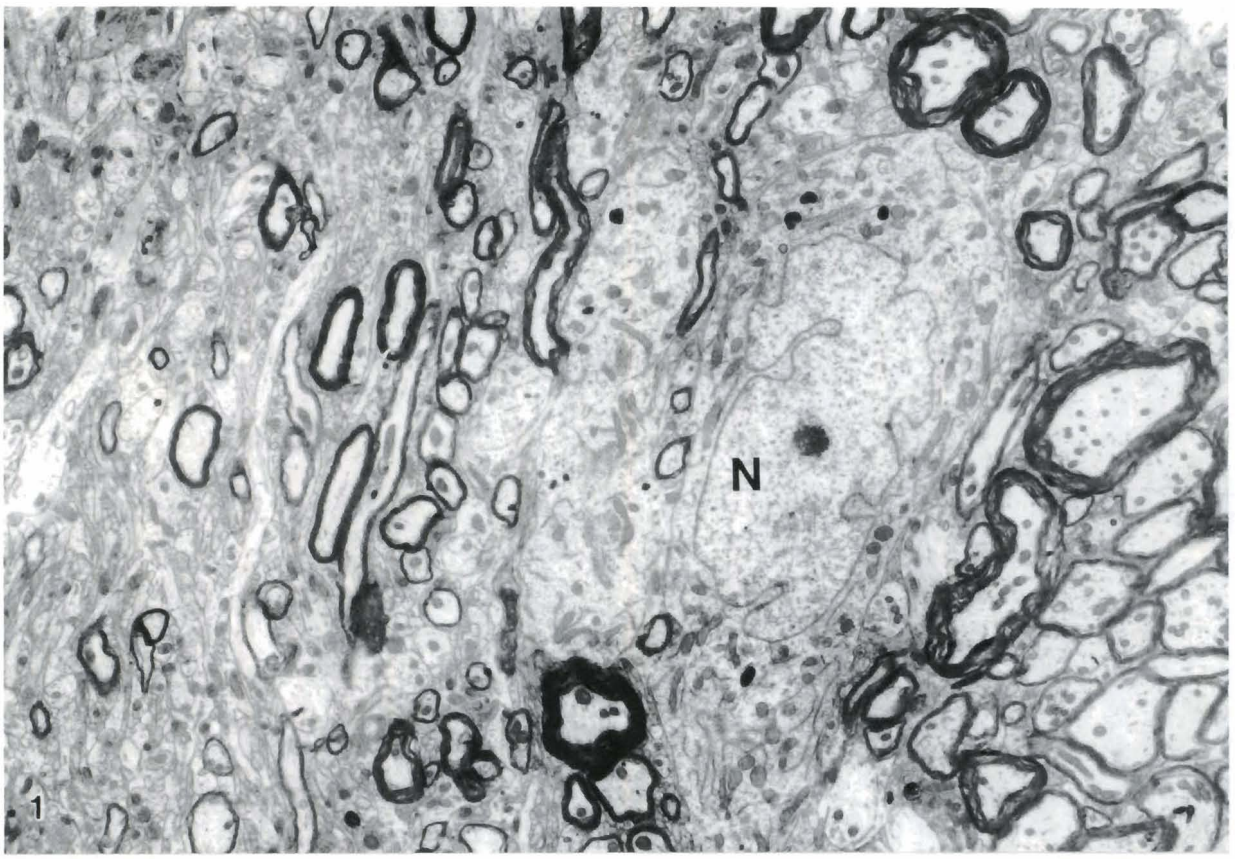
Immunoelectron microscopy

ILIR was localized in the neuronal somata, dendrites and axons of the gracile nucleus. In the neuronal somata, the reaction product was mainly localized in the cytoplasm and nucleoplasm (Fig. 3). In the cell cytoplasm, the reaction product was localized in the entire cytoplasm, often closely associated with the cisternae of the rough endoplasmic reticulum and outer nuclear membrane. In the cell nucleus, the reaction product was localized throughout the matrix of the nucleoplasm, but not within the nucleolus.

In the neuropil of the gracile nucleus, numerous insulin-like immunoreactive dendrites and axon profiles were observed (Fig. 4). However, most of the pre-synaptic axon terminals were not labelled. In insulin-like immunoreactive dendrites, the reaction product was closely associated with the neurotubules and postsynaptic densities (Fig. 5). Majority of these labelled dendrites were postsynaptic to unlabelled axon terminals containing small spherical or flattened agranular vesicles and mitochondria (Fig. 5). Very often, insulin-like immunoreactive dendrites formed the central elements of synaptic glomeruli with several unlabelled axon terminals which contained small spherical or flattened agranular vesicles and a few mitochondria in their axoplasm (Fig. 4). Some of the myelinated axons in the neuropil were also labelled for ILIR (Fig. 6). The reaction product was randomly scattered in the axoplasm of these myelinated axons.

Fig. 1. Electron micrograph of a small-sized neuron (N) from the gracile nucleus. Note the large indented nucleus and its prominent central nucleolus. There is a paucity of organelles present in the cytoplasm. In the neuropil are numerous myelinated and unmyelinated axons, axon terminals and dendrites. x 4,375

Fig. 2. A medium-sized neuron (N) from the gracile nucleus. Note the large pale nucleus with a rather smooth outline and the centrally placed nucleolus. In the neuropil are numerous myelinated and unmyelinated axons, axon terminals and dendrites. x 4,375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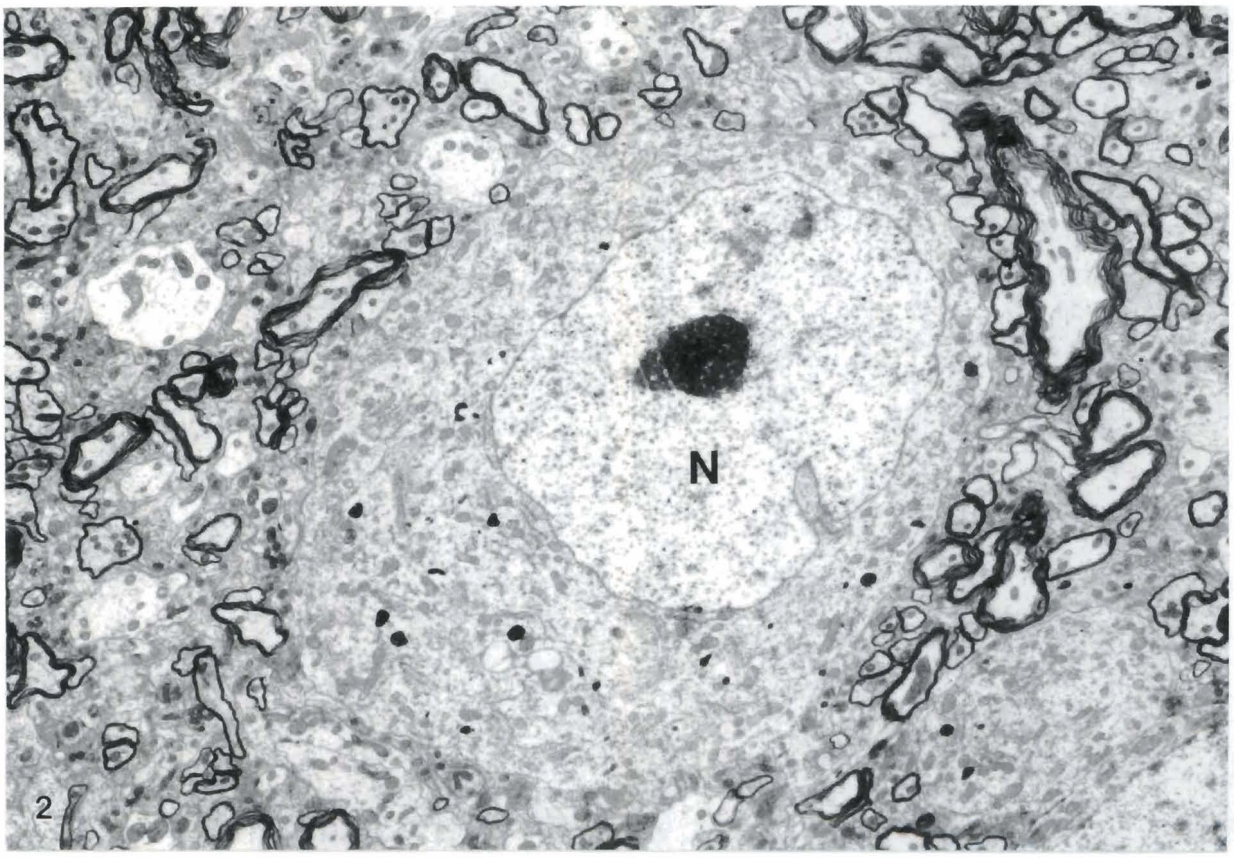
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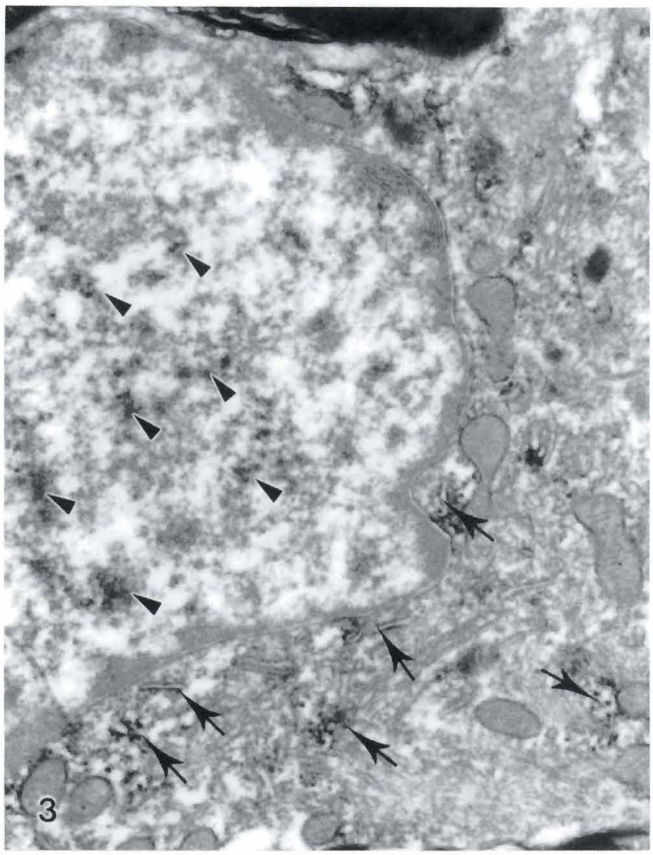
2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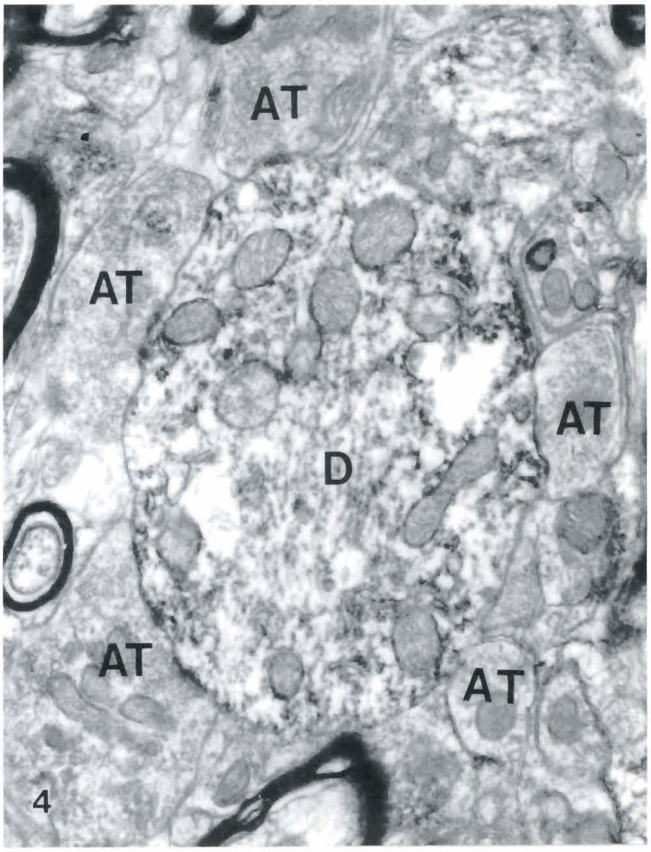
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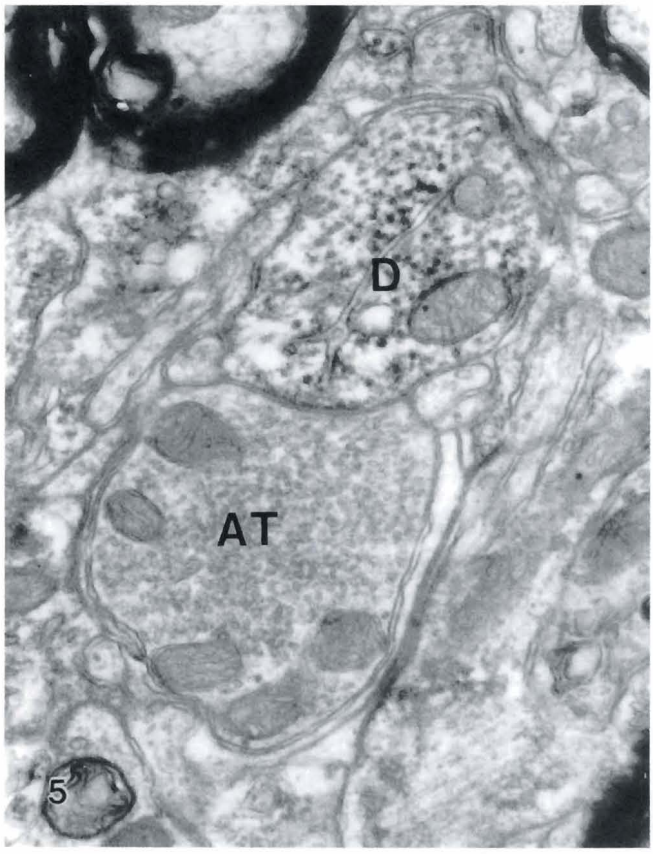
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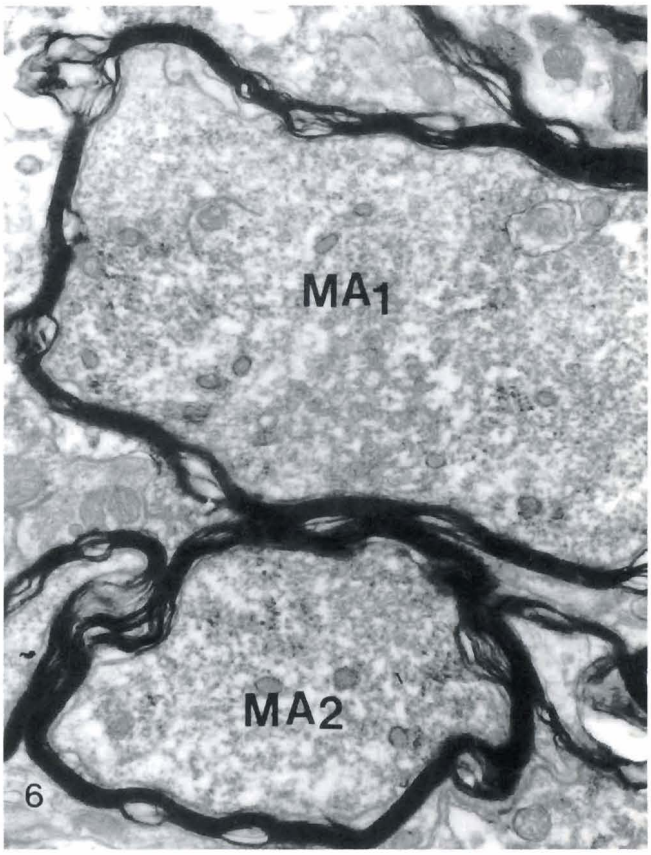
MA1
MA2
6

Fig. 3. Electron micrograph showing a portion of an insulin-like immunoreactive neuron from the gracile nucleus. Note that the reaction product is localized in the cell cytoplasm (arrows) and its nucleus (arrowheads). x 6,900

Fig. 4. An insulin-like immunoreactive dendrite (D) forms the central element of a synaptic glomerulus with several non-immunoreactive axon terminals (AT). The reaction product is closely associated with the neurotubules in the labelled dendrite. x 10,120

Fig. 5. An insulin-like immunoreactive dendrite (D) is postsynaptic to a non-immunoreactive axon terminal (AT) containing flattened agranular vesicles. The reaction product is closely associated with the neurotubules and the outer mitochondrial membranes. x 10,120

Fig. 6. Two insulin-like immunoreactive myelinated axons (MA_1, MA_2) in the neuropil of the gracile nucleus. The reaction product is localized as fine particles throughout the axoplasm. x 6,900

Discussion

Traditionally, insulin has been considered to be an unique product of the vertebrate pancreas. Recent studies have indicated that there may be extra-pancreatic sources of insulin and that insulin may act on tissues other than its classic target tissues (LeRoith et al., 1987). Although insulin has been localized in various nuclei of the CNS (Havrankova et al., 1978a,b; Pacold and Blackard, 1979; Dorn et al., 1982; Baskin et al., 1983; LeRoith et al., 1983; Hill et al., 1986; Tay and Wong, 1992; Dheen et al., 1994a), the exact origin of the peptide remains unclear. In the present study, ILIR has been localized in the neuronal somata, dendrites and axons of the gracile nucleus. Most of the labelled dendrites made synaptic contact with unlabelled axon terminals and some of them formed the central element of a synaptic glomerulus. It is believed that most of the immunoreactive neuronal profiles were probably derived from neurons within the gracile nucleus and that the unlabelled axon terminals could be derived from different sources.

The origin of insulin localized in the gracile nucleus is obscure. Several authors have hypothesized that insulin may be synthesized by the neurons themselves (Havrankova et al., 1978b; Villa-Komaroff et al., 1984; Boyd et al., 1985). Recently, Villa-Komaroff et al. (1984) have provided evidence that insulin mRNA is present in the brain, thereby providing the essential biochemical basis for the local synthesis of insulin by the neuronal cells. On the other hand, other workers have contended that peripheral insulin has access to the brain (Woods et al., 1985) by a receptor mediated transport system present in the vascular endothelial cells (Van Houten et al., 1979). Frank et al. (1985) have also demonstrated that insulin is able to cross the neonatal rabbit blood brain barrier. In another study, Baskin et al. (1983) have reported that insulin uptake in the rat hypothalamus could be derived from the cerebrospinal fluid.

It is interesting to note that the ILIR has been localized only in some specific regions of the CNS. In the present study, ILIR has been localized in the neuronal somata, dendrites and some axon profiles in the gracile nucleus. This selective localization of ILIR indicates that insulin may play a homeostatic role in specific regions of the CNS. This seems to be more probable in view of the observed degenerative changes in certain CNS nuclei of insulin deficient diabetic rats (Tay and Wong, 1991; Dheen et al., 1994b,c,d). It has been reported that insulin does not stimulate glucose utilization by the brain acutely, but there are indications that it has longer-term growth promoting (Puro and Agardh, 1984) and signalling (Unger et al., 1991) functions. Postnatal brain protein rates have been found to correlate positively with the number of insulin receptors (Kappy et al., 1984). Insulin has also been shown to stimulate protein synthesis in cultured chick embryonic forebrain neurons and this was mediated by the brain subtype of insulin receptors (Heidenreich and Toledo, 1989). Hence, it is suggested that in diabetic condition, the hypoinsulinaemia may alter the functional mechanism of the CNS insulin which could lead to inhibition of neuronal protein synthesis thereby resulting in neuronal degeneration, as reported in some CNS nuclei including the gracile nucleus (Tay and Wong, 1991). Further work is in progress employing *in situ* hybridization to elucidate the origin and role of insulin-like substance(s) in the neurons of the gracile nucleus.

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