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Loss of insulin receptor immunoreactivity from the substantia nigra pars compacta neurons in Parkinson's disease

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Abstract Immunohistochemistry using both a newly developed polyclonal, and a commercially available monoclonal, anti-insulin receptor antibody was done on the midbrain from cases of idiopathic Parkinson's disease (PD), Alzheimer's disease, amyotrophic lateral sclerosis, vascular parkinsonism and non-neurological controls. Both antibodies gave indentical patterns of neuronal staining. The neurons of the oculomotor nucleus were immunopositive in all the brains. However, the neurons in the pars compacta of the substantia nigra, paranigral nucleus, parabrachial pigmental nucleus, tegmental pedunculopontine nucleus, supratrocheal nucleus, cuneiform nucleus, subcuneiform nucleus and lemniscus medialis, which were positive in other diseases and in non-neurological controls, were not stained by these antibodies in PD brains. These results suggest that, in PD, a dysfunction of the insulin/insulin receptor system may precede death of the dopaminergic neurons.

Key words Parkinson's disease · Substantia nigra
Insulin receptor · Immunochemistry

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Introduction

It is well known that both insulin and insulin receptors (IR) are present in the central nervous system [2, 16, 28]. Various functions for insulin in the central nervous system have been suggested, relating to neuronal growth and differentiation, the metabolism of neuronal and glial cells, feeding behavior and body weight [2–6, 19]. Insulin has been reported to cause increased release of catecholamines [22] and to have a neuroprotective effect in focal brain ischemia [14]. Insulin exerts its pleiotropic effects via an IR, a glycosylated heterotetrameric transmembrane glycoprotein (two extracellular α-subunits and two intracellular β-subunits) [8, 17, 26]. However, there appear to be some differences in the structure of the receptor expressed in neurons and in other cells [11, 21].

The distribution of IR has been studied in rat brain tissue, using autoradiography [7, 12] or immunohistochemistry [27, 29]. Dense IR immunoreactivity was found in limbic-hypothalamic neurons, as well as in brain stem nuclei including the substantia nigra [27–29]. No immunohistochemical data have so far been reported for human brain.

The loss of pigmented neuron in the substantia nigra pars compacta (SNC) and other brain stem nuclei is a characteristic pathology in Parkinson's disease (PD). However, the etiological factor causing massive cell death in the dopamine-containing cells is still unknown. Recently a loss of basic fibroblast growth factor, which has a trophic effect on mesencephalic dopaminergic neurons, from surviving dopaminergic neurons in the SNC has been found in PD [25], and a possible relationship with the disease process suggested.

We report here that the surviving SNC neurons in PD show no IR-like immunoreactivity, although such immunoreactivity was found in the SNC neurons in all the other brain examined, both those from cases of Alzheimer's disease (AD), amyotrophic lateral sclerosis (ALS), and vascular parkinsonism, as well as those of

non-neurological controls. All brains, including those of the PD cases, showed IR-like immunoreactivity in neurons of the oculomotor nucleus.

Materials and methods

Antibody preparation

In this study we used a newly developed, rabbit polyclonal antibody which was raised against a synthetic peptide with 16 amino acids (Gly-Lys-Lys-Asn-Gly-Arg-Ile-Leu-Thr-Leu-Pro-Arg-Ser-Asn-Pro-Ser); this sequence is from the C-terminal region of the β-subunit of human IR [8]. The peptide coupled to keyhole limpet hemocyanin was used to immunize rabbits.

Tissue preparation

The brains used in this study were from five neurologically normal controls (mean age, 73 years; range, 65–82 years), six cases of PD (mean age, 75.5 years; range 69–79 years), five cases of AD (mean age 74.4 years; range 66–86 years), one case of vascular parkinsonism (age 76 years), and two cases of ALS (ages 38 and 65 years). All of the neurological cases were diagnosed clinically premortem, with the diagnosis being confirmed at autopsy by pathologists. The control brains showed no evident pathology. All brains were obtained 2–20 h after death. The mean intervals of postmortem delay in each group were as follows: 8 h in neurologically normal controls, 9.3 h in PD, 9 h in AD, 15 h in vascular parkinsonism and 10 h in ALS. The mean postmortem delay of the PD group was not significantly different from that of the other cases ($P = 0.325$; Mann-Whitney U test).

The left half of each midbrain was dissected, fixed in 4 % paraformaldehyde for 2 days, and transferred to 15 % sucrose in 0.1 M phosphate-buffered saline (PBS; pH, 7.4) for maintenance in the cold prior to sectioning. The duration of storage for the samples was different in each case (from 2 weeks to 1 year).

Immunoblotting

To test the specificity of the antibodies [the polyclonal and a commercially available monoclonal (Immunotech)] against IR, immunoblots were run against extracts of the substantia nigra of two non-neurological cases. Each tissue was homogenized in 5 vol 20 mM TRIS-HCl buffer containing 1 mM EDTA, 1 mM EGTA, 10 μM leupeptin, 1 μM pepstatin and 0.3 μM aprotinin. Homogenate (100 μl) was saved as a crude whole fraction. The remaining solution was centrifuged at 15000 cpm at 4 °C for 20 min, and the supernatant was collected as a crude cytosolic fraction. The pellet was redissolved in the homogenization buffer and used as a crude membrane fraction. The protein content of each sample was determined before storage at -80 °C until used for immunoblots. An amount of each fraction containing about 50 μg of protein was mixed with sodium dodecyl sulfate (SDS) buffer (10 mM TRIS, pH 6.8, 1 % SDS, 20 % glycerol, and 0.02 % bromophenol blue), boiled for 5 min, and loaded onto a 7.5 % polyacrylamide gel along with BRL prestained standards as molecular weight markers. After 60 min electrophoresis, the proteins were transferred onto a PVDF membrane (Immobilon-P). Membranes were blocked for 1 h at room temperature with 5 % skimmed milk and 1 % fetal bovine serum in 25 mM TRIS-HCl buffer (pH 7.4) containing 150 mM NaCl and 0.1 % Tween 20 (TBST) before incubation with the antibodies. The first incubation was for 18 h at 4 °C in the monoclonal antibody at 1:1000 dilution or in the polyclonal antibody at 1:10000. The second incubation was for 2 h at room temperature with alkaline phosphatase-labeled anti-mouse IgG (BRL, 1:5000) for the monoclonal anti-IR antibody or with alkaline phosphatase-labeled goat anti-rabbit IgG (BRL, 1:5000) for the polyclonal anti-IR antibody. Incubations were carried out in 1 % skimmed milk in TBST. Washing between steps was with TBST. Immune complex bands were visualized by incubating the membrane for approximately 10 min in a solution of 0.33 mg/ml nitroblue tetrazolium and 0.165 mg/ml 5-bromo-4-chloro-3-indolyl phosphate (BRL) in TRIS-HCl (pH 9.5) containing 0.1 M NaCl and 50 mM $MgCl_2$.

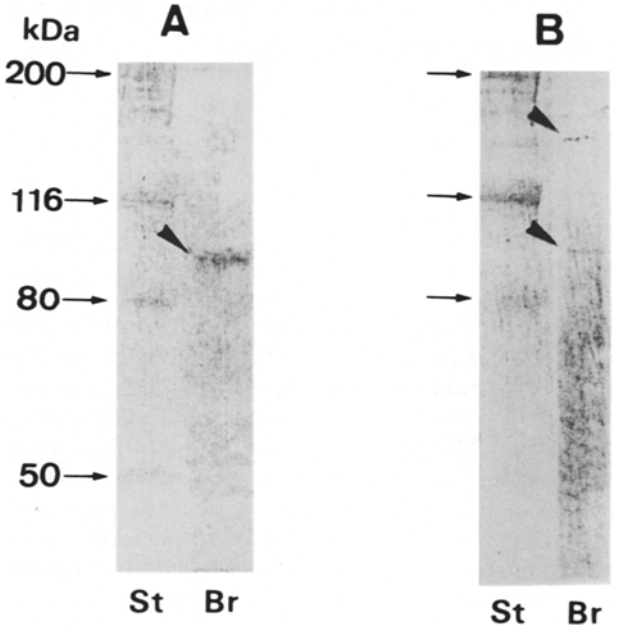

Fig. 1 A,B Immunoblot analyses using the membranous extract of the substantia nigra (SN) from a non-neurological control. The polyclonal antibody (**A**) recognized a protein of approximately 92 kDa (*arrowhead*) which corresponds to the β subunit of the insulin receptor (IR). The minor bands (45 and 55 kDa) were also seen. The monoclonal antibody (**B**) recognized both 135- and 92-kDa proteins (*arrowheads*) which correspond to the α and β subunits (*St* molecular standard, *Br* brain extract)

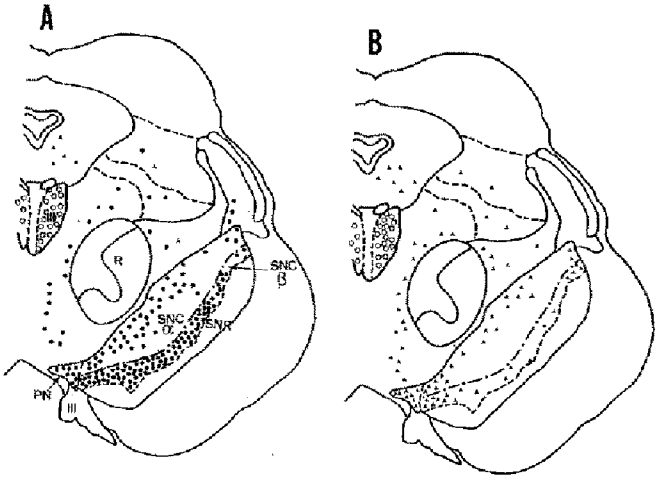

Fig. 2 Diagrams of the midbrain illustrating the distribution of only IR-containing (○), tyrosine hydroxylase (TH)-containing (▲), both IR- and TH-containing (●) neurons in control (**A**) and Parkinson's disease (PD) (**B**) brain *PN* paranigral nucleus (*R* red nucleus, *SNC α, β substantia nigra pars compacta α and β layers, respectively, SNR substantia nigra pars reticulata, III* oculomotor nucleus and nerve)

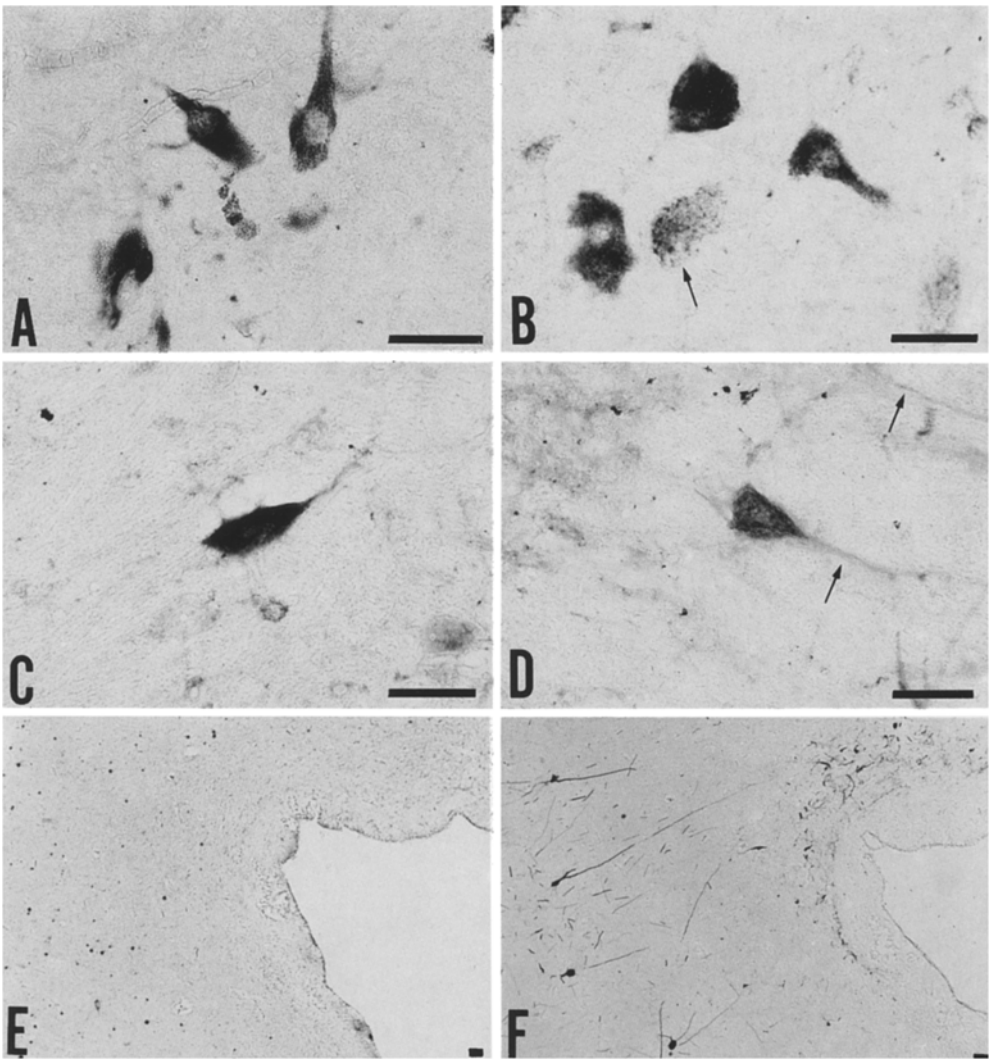

Fig. 3 A–F Immunohistochemistry in the midbrain of non-neurological controls. Positive neuronal staining with the polyclonal anti-IR antibody in the SNC (**A**) and the oculomotor nucleus (**B**). Punctate staining can be seen in a cell (*arrow*) showing a weak immunopositive reaction. Similar immunoreactivity for IR in the cuneiform nucleus (**C**) and double immunostaining of a neuron in the cuneiform nucleus with anti-IR and anti-TH antibodies (**D**). In **D** *arrows* indicate TH-positive process (*brown* in the original section). The doubly stained cell body and proximal process appeared purple in the section. **E, F** The polyclonal anti-IR antibody only stains corpora amylacea in the central gray (**E**), although there are TH-positive neurons in this area (**F**). In absence of the polyclonal antibody, no IR-positive structures were seen. *Bars* = 50 μm

Immunohistochemistry

Sections were cut on a freezing microtome at 30-μm thickness. For immunohistochemical staining, sections were rinsed for several hours in PBS containing 0.3 % Triton X-100 (PBST) before incubation for 48 h at 4 °C with the primary antibody (polyclonal at 1:10000, or monoclonal at 1:1000). Anti-tyrosine hydroxylase (TH) antibody (mouse monoclonal, 1:10000, Chemicon) was used for the identification of dopaminergic neurons.

After treatment with primary antibody, the sections were washed in PBST, and treated with biotinylated secondary antibody (Vector Lab, Burlingame, Calif.) diluted 1:1000 for 2 h at room temperature, followed by incubation in the avidin-biotinylated horseradish peroxidase complex (ABC Elite, Vector Lab) for 1 h at room temperature. After washing the sections in PBST, peroxidase labeling was visualized by incubating with a solution containing 0.001 % 3,3'-diaminobenzidine (DAB, Sigma), 0,6 % nickel ammonium sulfate (Fisher), 0.05 % imidazole and 0.0003 % H_2O_2. A dark purple product formed after a few minutes, and the reaction was terminated by transferring the sections to PBST. Sections were washed, mounted on glass slides, dehydrated with graded alcohols and coverslipped with Entellan (Merck). Three sections per case were stained for IR. Serial sections followed by IR staining were stained with anti-TH antibody.

In one normal control case, double immunostaining using anti-IR and anti-TH antibodies was done. Sections were treated for 30 min with 0.5 % H_2O_2 solution in PBST after the DAB reaction of the first cycle. The second immunohistochemical cycle was carried out in a similar manner to the first, except that nickel ammonium sulfate was omitted from the DAB solution, yielding a brown precipitate in the second cycle.

Results

Specificity of the IR polyclonal and monoclonal antibodies

Figure 1 shows the results obtained on Western blot examination of the membranous extract of a control substantia nigra (SN). The polyclonal IR antibody recognized a major band with a molecular mass of approximately 92 kDa, corresponding to that of the β subunit of IR. Two minor bands (55 and 45 kDa), which might be cleavage fragments, were also seen. On the other hand, the commercially supplied monoclonal antibody recognized two bands with molecular mass of 135 kDa and 92 kDa, corresponding to the α and β subunits, respectively.

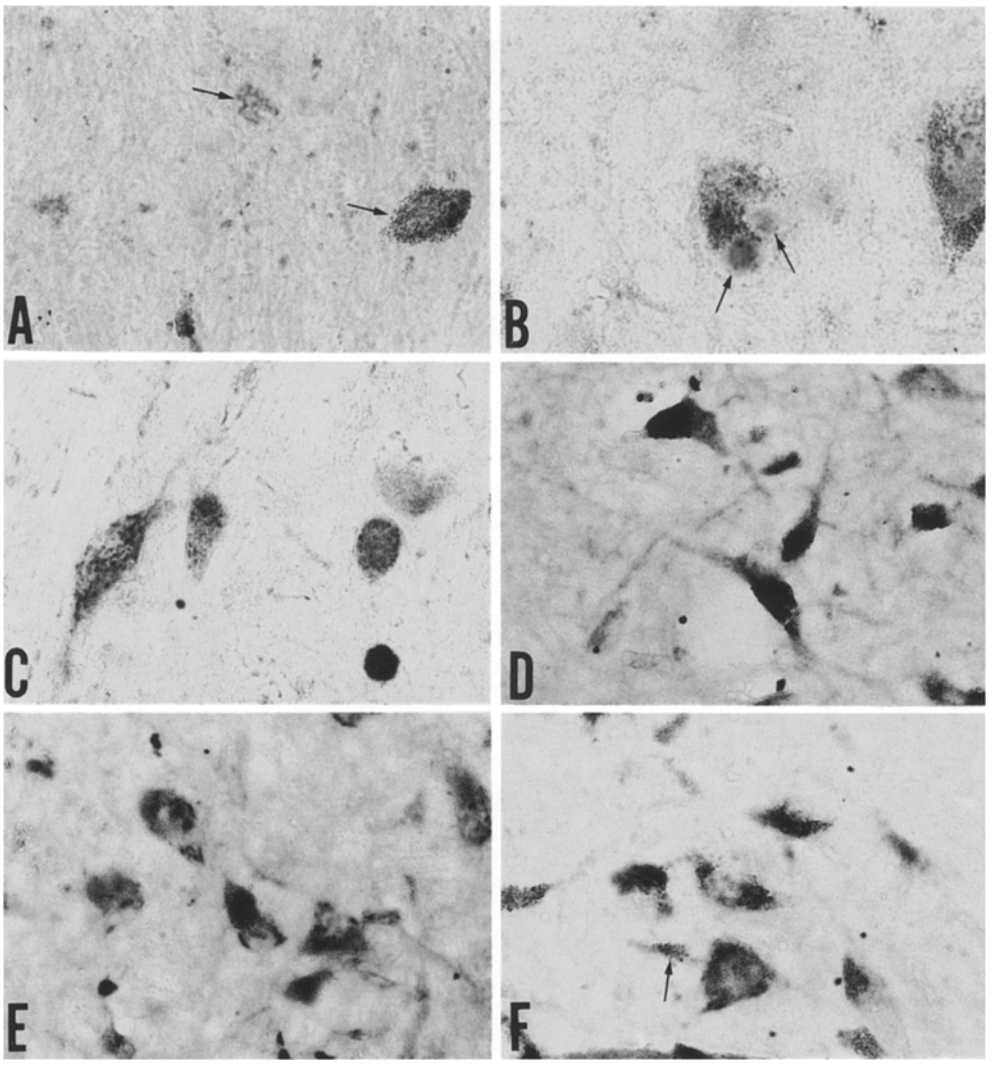

Fig. 4 Immunohistochemistry in the midbrain of PD (**A-C**), Alzheimer's disease (AD), amyotrophic lateral sclerosis (ALS) and vascular parkinsonism. **A** Lack of staining in the SNC in PD with the polyclonal anti-IR antibody. *Arrows* show melanin pigment. **B** Positive staining of a Lewy body (*arrow*) with the polyclonal anti-IR antibody. **C** In PD, neurons in the oculomotor nucleus stained by the polyclonal anti-IR antibody. **D-F** Staining of nigral neurons for IR in an AD case with the monoclonal antibody (**D**) and in ALS (**E**) and vascular parkinsonism (**F**) cases with the polyclonal antibody. An *arrow* in **F** shows melanin pigment.

Immunohistochemistry

The general distribution of IR-positive and TH-positive neurons in the SN of control and PD cases is shown in Fig. 2. All sections are approximately at the level of emergence of the oculomotor nerve ([18]; plate XXXVI).

The midbrains of the AD, ALS and vascular parkinsonism cases, as well as those of the non-neurological controls, showed very similar immunohistochemical results (Figs. 3A,B; 4D-F). Both polyclonal and monoclonal antibodies revealed almost the same staining pattern, except that the polyclonal antibody stained the corpora amylacea (Fig. 3E). In absence of the polyclonal antibody, no IR-positive structures were seen. Neurons stained positively with the anti-IR antibodies included both melanin-containing (Fig. 3A) and melanin-free cells of the SNC, paranigral nucleus, parabrachial pigmental nucleus, tegmental pedunculopontine nucleus, supratrocheal nucleus, cuneiform nucleus (Fig. 3C), subcuneiform nucleus, and lemniscus medialis and large cells in the oculomotor nucleus (Fig. 3B). No preferential staining in the SNC between medial and lateral or between dorsal and ventral was noted. No positive cells were seen in the SN pars reticularis, central gray or superior colliculus. Under high power light microscopy, the immunoreaction product could be seen as a fine granular product scattered over the perikarya and proximal processes, but absent from the nucleus (Figs. 3B, 4C).

Double staining of sections from a control brain showed that TH-positive cells in the SNC, the paranigral nucleus and most of the cells in the area surrounding the red nucleus (parabrachial pigmental, tegmental pedunculopontine, supratrocheal, cuneiform and subcuneiform nuclei) were also IR positive (Fig. 3D). However, in the central gray some TH-positive cells were seen, but there was no staining for IR (Fig. 3E, F).

All six PD brains showed the expected marked loss of pigmented neurons, especially in the mid and lateral division of the SNC, as well as fragmentation of melanin pigments. In the oculomotor nuclei, IR-immunopositive neurons were seen (Fig. 4C). However, the IR-immunopositive cells, which were seen in

other areas of the midbrain, including the SNC in non-PD brains, could not be shown in any of the PD cases (Fig. 4A). Serial sections showed that some TH-positive neurons remained in the SNC. Occasionally, however, Lewy bodies were positively stained by these anti-IR antibodies (Fig. 4B).

Discussion

In this study, we found that neurons in the SNC, paranigral nucleus and other TH-positive areas in the midbrain (with exception of the central gray) are immunopositive for IR in non-neurological, AD, ALS and vascular parkinsonism cases. Recent immunohistochemical data have shown the same positive immunoreactivity for IR in neurons of the rat SN [28, 29]. The fine granular staining pattern in the cytoplasm reported for the rat [27, 29] was also found here in the human. Since the anti-IR antibodies used in this study bind to the β subunit which is embedded in the plasma membrane and extends intracellularly, it is understandable that reaction products are found in the cytoplasm [28]. The localization of IR to TH-positive neurons in the midbrain is interesting, because one function of insulin may be regulation of catecholamine uptake or release [2, 4, 20].

Very recently, Tooyama et al. [25] reported that, while most pigmented neurons of the normal human SN are immunopositive for basic fibroblast growth factor (bFGF), only 8.2 % of the pigmented cells remaining in PD showed such immunopositivity. Staining of adjacent sections showed that the loss of bFGF preceded the loss of TH. Neurotrophic factors so far tested for dopaminergic neurons include bFGF [9, 24, 25], brain-derived neurotrophic factor [13, 23], epidermal growth factor [15], and insulin-like growth factors I and II [15]. Insulin and its receptor also appear to have neurotrophic functions [2, 3, 28], and Knusel et al. [15] have reported such effects on nigral dopaminergic neurons in vitro. In this study, we found an almost total loss of IR-positive neurons in SNC in PD where some TH-positive cells remained. The clear stainings for IR in the neurons of oculomotor nucleus in these same PD cases indicate the problem was not with the staining per se. Thus, as in the case of bFGF, there may appear to be a down-regulation of the insulin-IR system in these neurons before disappearance of the TH.

Several hypothesis have been proposed for the etiology of PD. Reduced trophic support on dopaminergic neurons is one candidate, and treatment using selected trophic factors has been proposed [1, 10]. Insulin and the IR system should be considered in this regard.

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