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Immunocytochemical detection of insulin receptor substrate-1 (IRS-1) in rat brain: colocalization with phosphotyrosine

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Summary

In peripheral insulin-sensitive tissues, insulin receptor substrate (IRS-1) undergoes tyrosine phosphorylation immediately after cells are stimulated by insulin or insulin-like growth factor-1 (IGF-1), and may function as a molecular link between insulin/IGF-1 receptor tyrosine kinases and enzymes regulating cell growth and metabolism. A fundamental question pertaining to insulin/IGF-1 action in the brain is whether IRS-1 is expressed by neurons. In this study, the distribution of cells containing immunoreactivity to IRS-1 in the brain was determined by immunocytochemistry with polyclonal IRS-1 antiserum, and compared to the localization of immunostaining for phosphotyrosine using polyclonal phosphotyrosine antiserum. The immunostaining results with ABC-peroxidase method and cryostat sections showed the presence of IRS-1 immunoreactivity in many neuron cell bodies throughout the rat forebrain, particularly in the habenula, cerebral cortex and piriform cortex. In the hypothalamus, IRS-1 immunostaining was present in neurons of the paraventricular nucleus, supraoptic nucleus, and arcuate nucleus. The choroid plexus stained intensely for IRS-1. The populations of cells that stained for IRS-1 also showed strong immunostaining for phosphotyrosine. Studies at the cellular level are needed to verify coexpression of IRS-1 and receptors for insulin or IGF-1 by the same neurons, as well as in cells of the choroid plexus. The present results are the first demonstration of IRS-1 expression by neurons in adult mammalian brain. These findings are consistent with the hypothesis that insulin and IGF-1 actions in the brain involve signal transduction mechanisms common to those found in peripheral tissues.

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Introduction

Insulin receptor substrate (IRS-1) (previously identified as pp185) is a phosphoprotein which may function as a molecular link between insulin and insulin-like growth factor-1 (IGF-1) receptor tyrosine kinases and enzymes regulating cell growth and metabolism. It undergoes tyrosine phosphorylation immediately after cells are stimulated by insulin [1,2] or IGF-1 [3], and associates with phosphatidylinositol 3′-kinase [4–7]. The physiological significance of IRS-1 in insulin and IGF-1 signal transduction in vivo is uncertain since IRS-1 has been studied mainly in cultured cells. Numerous reports [3,4,7–9] suggest that IRS-1 functions in the responses of cells to insulin and IGF-1 in vivo. A cDNA encoding the protein structure of IRS-1 was isolated from rat liver cDNA libraries [4], and IRS-1 mRNA transcripts (9.5 kb) have been found by Northern blot analysis in a variety of insulin-sensitive rat tissues, including brain [1]. However, the types of cell that express IRS-1 in these organs and the extent to which cells expressing IRS-1 also express insulin receptors or IGF-1 receptors (type 1 IGF receptors) have not been reported. Moreover, no previous reports have documented IRS-1 expression by neurons of the adult mammalian brain.

Although the brain contains large numbers of specific receptors for insulin and IGF-1 [10–13], it is not generally viewed as an insulin-sensitive organ. Insulin does not exert effects on substrate uptake, transport, or metabolism on brain neurons that characterize the responses of classic insulin-sensitive tissues such as liver, adipose cells, and skeletal muscle [14]. The roles of insulin and IGF-1 in the adult brain are still somewhat undefined, although there is much evidence to suggest that they may function as growth factors that influence cell metabolism and gene expression [14]. Therefore, understanding postreceptor signaling in neurons is of critical importance for characterizing the nature of insulin and IGF-1 action in the brain.

Neurons expressing insulin and IGF-1 receptors and their mRNAs are concentrated in discrete regions, particularly in the olfactory bulb, hippocampus and hypothalamus. Within these brain areas, specific structures containing high densities of insulin and IGF-1 receptors are surrounded by cells with very low insulin and IGF-1 receptor expression [10–13]. The brain, therefore, provides a unique model for evaluating the specificity with which cells expressing receptors for insulin and IGF-1 also express IRS-1.

In preliminary work [15], we detected IRS-1 mRNA in populations of forebrain neurons that have been shown to express insulin receptor mRNA [10,16] and phosphotyrosine immunoreactivity [17,18]. Since IRS-1 is heavily phosphorylated on tyrosine residues following insulin and IGF-1 binding to their cognate receptors [1–3], we asked whether the IRS-1 protein can be detected by immunocytochemistry in the brain and if so, whether neuron populations that contain IRS-1 immunoreactivity also show phosphotyrosine immunoreactivity.

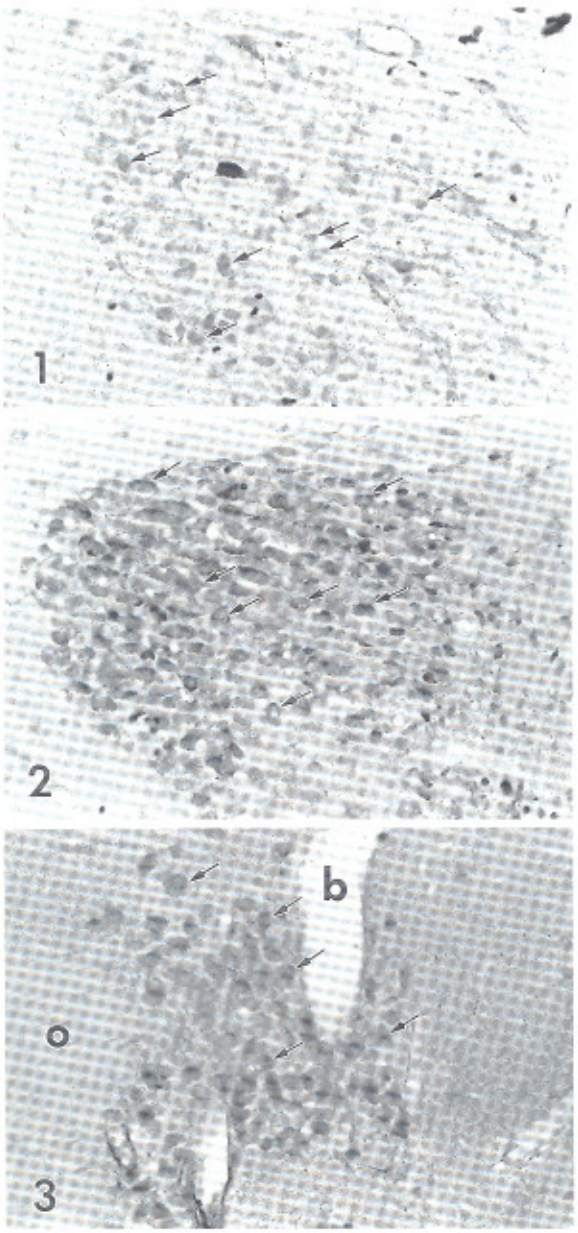
Materials and Methods

Tissues

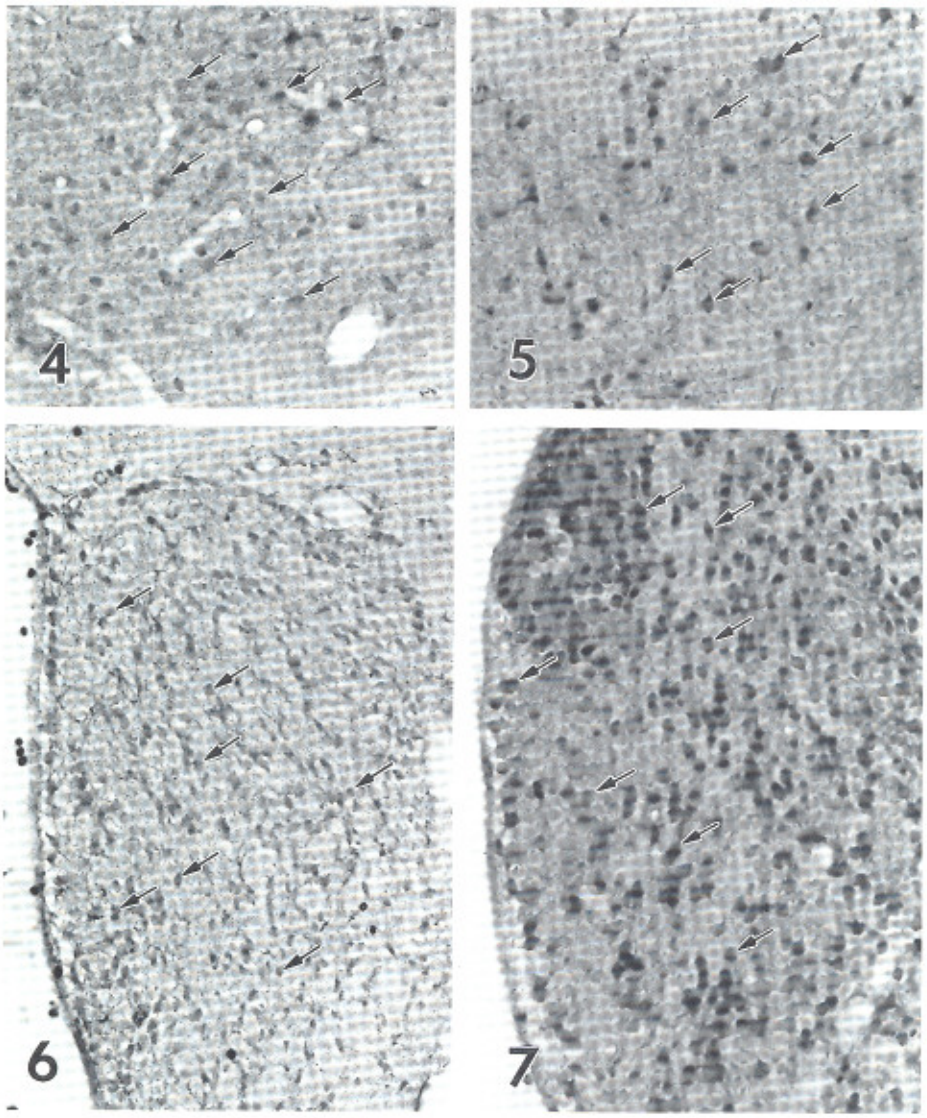
Brains were obtained from 260–300 g male Wistar rats anesthetized with Equithesin (3 ml/kg, i.p.). The brains were perfused via the left cardiac ventricle with cold 200 ml 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.3, for 15 min, then removed and immersed in 0.1 M phosphate buffer containing 25% sucrose for several days at 6°C and frozen in Freon 22. Cryostat sections (16 μm) were thaw mounted on gelatin-coated slides and stored at −80°C.

Antisera

IRS-1 polyclonal antiserum was raised in rabbits using a synthetic peptide sequence corresponding to the amino acid residues 489–507 of the IRS-1 protein and affinity purified [19]. The phosphotyrosine antibodies were raised in rabbits and affinity purified

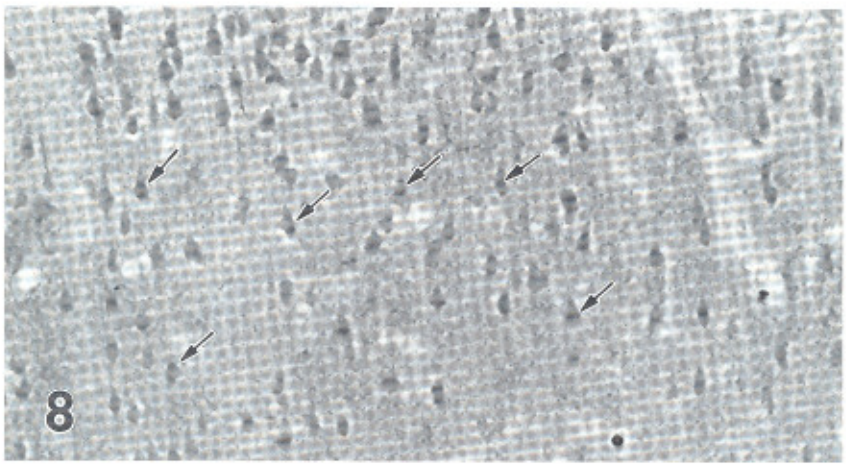


Figs. 1–3. Fig. 1. Immunostaining of paraventricular nucleus for IRS-1. Many stained cell bodies (some shown by arrows) are in the magnocellular portion of the nucleus. (Original magnification × 250.) Fig. 2. Immunostaining of paraventricular nucleus for phosphotyrosine, showing stained cell bodies (some shown by arrows) are in the magnocellular portion of the nucleus. (Original magnification × 250.) Fig. 3. Immunostaining of cell bodies (some shown by arrows) in the supraoptic nucleus for IRS-1. b, blood vessel; o, optic chiasm. (Original magnification × 250.)



Figs. 4–7. Fig. 4. Arcuate nucleus of hypothalamus with neuronal perikarya immunostained for IRS-1 (some shown by arrows). (Original magnification × 250.) Fig. 5. Arcuate nucleus of hypothalamus with neuronal perikarya immunostained for phosphotyrosine (some shown by arrows). (Original magnification × 250.) Fig. 6. IRS-1 immunostaining of cells (some shown by arrows) in the habenula. (Original magnification × 250.) Fig. 7. Phosphotyrosine immunostaining of cells (some shown by arrows) in the habenula. (Original magnification × 250.)

Figs. 8–10. Fig. 8. IRS-1 immunostained pyramidal neurons (some shown by arrows) in the cerebral cortex. (Original magnification × 250.)Fig. 9. Phosphotyrosine immunostained neurons (some shown by arrows) in the cerebral cortex. (Original magnification × 250.)Fig. 10. IRS-1 stained pyramidal neurons in the piriform cortex. Staining is also visible in synaptic layer I(s). (Original magnification × 250.)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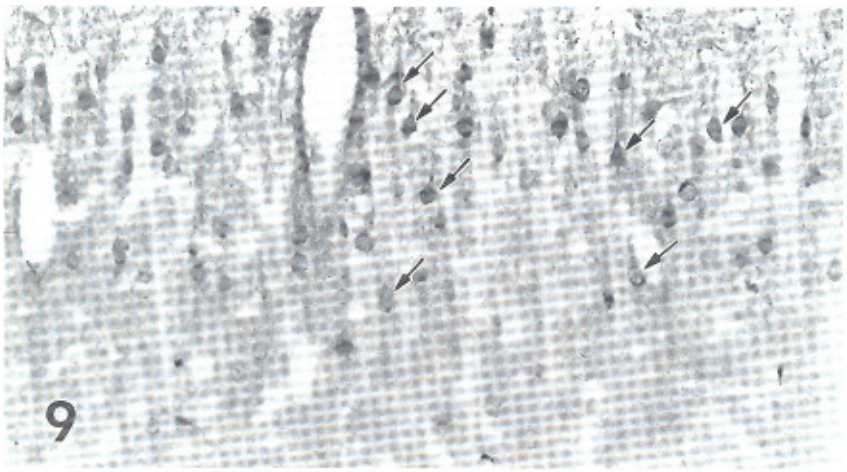
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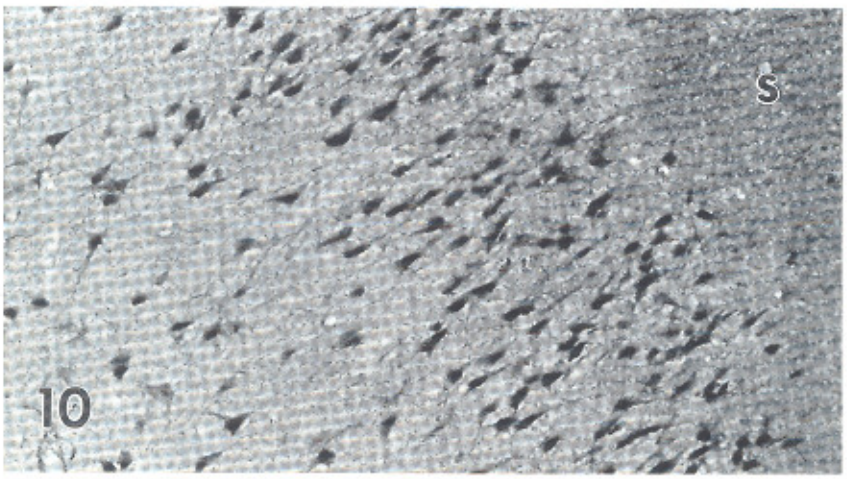
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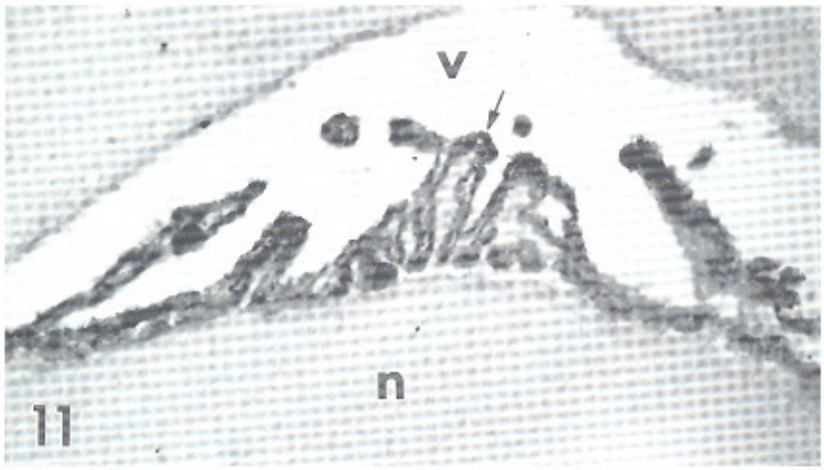
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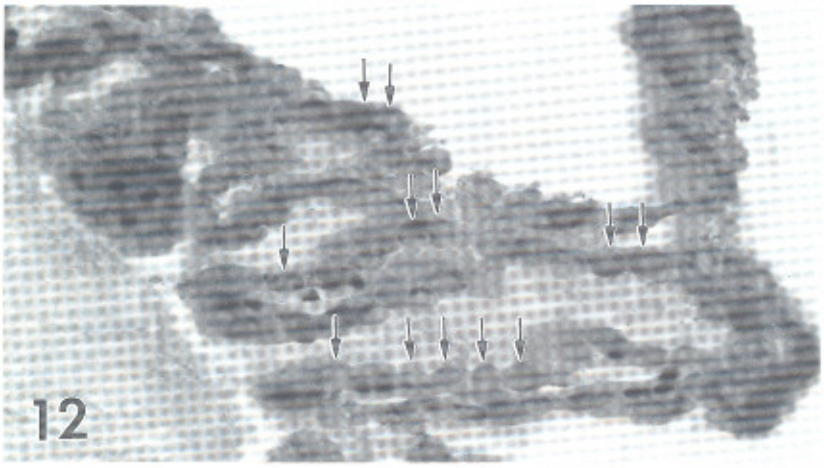
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on phosphotyrosine columns [20]. Both IRS-1 and phosphotyrosine antisera have been used for analysis of IRS-1 and phosphotyrosine immunoreactivity in cultured cells [1].

Immunocytochemistry

Sections were immunostained with the ABC peroxidase method, using the Elite Kit (Vector) and primary antiserum diluted 1:100–1:1000 in phosphate-buffered saline (PBS) containing 1% BSA and 0.05% Triton X-100. Except for primary antiserum incubations, all steps were done at room temperature; primary antisera incubations were at 4°C. All rinses were in cold PBS. After removal from freezer, sections were immersed in 0.01 M PBS for 1 h. followed by 1% hydrogen peroxide in 70% methanol for 10 min and then incubated in 3% normal goat serum in PBS containing 1% BSA and 0.05% Triton X-100, 1 h. They were then incubated in primary antiserum for 24–48 h followed by ABC reagents and development in diaminobenzidine-peroxide according to conventional protocols. Normal rabbit serum was used as a control for nonspecific staining.

Results

Immunostaining with IRS-1 antiserum revealed large numbers of neuronal cell bodies containing the peroxidase reaction in the forebrain. Glial cells and white matter regions showed relatively weak staining. Normal rabbit serum produced only background staining (not shown).

In the hypothalamus, IRS-1 immunostaining intensity was high in the paraventricular nucleus (Fig. 1). Almost all of the magnocellular neuronal cell bodies were stained, as were many of the parvocellular neurons, whereas neurons of the anterior and lateral hypothalamic regions were stained less intensely. The magnocellular neurons also showed strong staining for phosphotyrosine (Fig. 2). A similar pattern of immunostaining for IRS-1 (Fig. 3) and phosphotyrosine was present in the supraoptic nucleus. The arcuate nucleus contained numerous IRS-1 positive neuronal cell bodies (Fig. 4) and many neurons that stained for phosphotyrosine (Fig. 5). The IRS-1 staining in the arcuate nucleus was relatively weaker than seen in the paraventricular nucleus, however, and many arcuate nucleus perikaya showed no IRS-1 or phosphotyrosine staining.

The habenula, which flanks the third ventricle at its junction with the lateral ventricles, also showed numerous positively stained cells for IRS-1 (Fig. 6) and phosphotyrosine (Fig. 7). In the cerebral cortex many pyramidal neurons in layers II-III contained IRS-1 immunoreactivity (Fig. 8). These cells also stained for phosphotyrosine (Fig. 9). Pyramidal cells of layers II-III of the piriform (olfactory) cortex stained for IRS-1 (Fig. 10) and phosphotyrosine (not shown). IRS-1 immunostaining was also visible in the synaptic neuropile of layer I of the piriform cortex.

The choroid plexus showed strong IRS-1 immunostaining (Fig. 11), both in the third and lateral ventricles. This intense staining constrasted sharply with the adjacent brain neuropile, which showed very low IRS-1 immunostaining. A higher magnifications, the immunostaining of the choroid plexus was visibly seen to be associated with the cytoplasm of choroid plexus epithelial cells (Fig. 12), whereas the core of the choroidal villi showed very low staining. The choroid plexus epithelium also had very intense intense phosphotyrosine immunostaining (Fig. 13).

Figs. 11–13. Fig. 11. Choroid plexus (arrow) in lateral ventricle (v) immunostained for IRS-1. Note low background staining of brain neuropile parenchyma (n). (x125) Fig. 12. Choroid plexus in lateral ventricle, showing enlarged detail of villi indicated by arrow in Fig. 12. Note immunoreactive IRS-1 in choroid plexus epithelial cells (arrows) but absence in villi core. (Original magnification × 400.) Fig. 13. Choroid plexus (arrow) in lateral ventricle (v) in section near that shown in Figs. 11 and 12, immunostained for phosphotyrosine. (Original magnification × 250.)

Discussion

IRS-1 (pp185) has been detected in Western blots of numerous non-neural tissues [3,4,7–9], and IRS-1 mRNA transcripts have been isolated from a variety of rat tissues including the brain [1,4]. However, the identity of specific cells containing IRS-1 protein has not been previously demonstrated in vivo for any of these tissues. This paper, therefore, reports the first evidence for IRS-1 protein expression in the brain.

The major findings of the present study are: (a) that specific neurons in the adult rat brain contain immunoreactive IRS-1-like protein, (b) IRS-1 immunoreactivity is present in neuronal cell bodies in forebrain areas previously shown to contain insulin receptor mRNA, IGF-1 mRNA and IRS-1 mRNA based on in situ hybridization [10,25,16,21], and (c) neuronal populations which express IRS-1 generally contain high levels of phosphotyrosine immunoreactivity. These brain areas also exhibit binding sites for insulin and IGF-1 (based on receptor autoradiography) [10] and show insulin receptor immunoreactivity (based on immunostaining with insulin receptor antibodies) [18,22]. The intensity of the IRS-1 immunostaining in these cells paralleled the distribution and intensity of cells that immunostained with antisera to phosphotyrosine in this and previous reports [17,18].

In the hypothalamus, immunostaining for IRS-1 and phosphotyrosine was positive in the arcuate nucleus, which has a relatively dense concentration of insulin binding sites compared to IGF-1 receptors and is thought to be an important site of hypothalamic insulin action [11,13,23,24]. Recently, a role for insulin in the regulation of neuropeptide Y gene expression in the arcuate nucleus of the rat hypothalamus has been demonstrated [14,25,26], suggesting that signal transduction pathways leading to altered gene transcription are activated by insulin action on hypothalamic neuropeptide Y neurons. It is tempting to speculate that IRS-1 may mediate insulin postreceptor signal transduction events in neuropeptide Y neurons that result in changes in NPY gene expression following insulin binding in the arcuate nucleus. However, is not known specifically which cell types in these nuclei express IRS-1 or insulin and IGF-1 receptors. Since the arcuate nucleus has more insulin receptors compared to IGF-1 receptors [13,27], it offers a site where effects of insulin on IRS-1 could possibly be evaluated without interference from IGF-1 receptors. Finding IRS-1 immunoreactivity in the arcuate nucleus suggests that insulin action on these neurons involves similar signal transduction pathways as proposed for regulation of gene expression by insulin in non-neural tissues.

In addition, IRS-1 immunostaining was high in the paraventricular nucleus, which also has been shown to have abundant insulin binding sites [13]. Almost all of the magnocellular neurons in these sections stained for IRS-1 and phosphotyrosine, suggesting that these neurons may be major targets of insulin or IGF-1 action. Insulin has also been implicated as a regulator of NPY release in the paraventricular nucleus [28]. The present results suggest that most magnocellular neurons may have insulin or IGF-1 signal transduction pathways.

It is notable that neurons with relatively intense IRS-1 and phosphotyrosine staining were found in the habenula. The habenula is a major relay station for neurons between the periphery, amygdala, cerebral cortex and basal ganglia, and receives indolaminergic input from the dorsal raphe, catecholaminergic fibers from the pallidium, and dopamine axons from the substantia nigra [29]. It has a high density of insulin and IGF-1 binding sites [11–13,27]. There was also marked staining for IRS-1 and phosphotyrosine in pyramidal neurons in layers II-III of the piriform cortex. Many of these neurons project to the olfactory bulb and other limbic systems areas [29]. Layer I of the pyriform cortex, also showing IRS-1 and phosphotyrosine staining, receives afferent fibers from the main olfactory bulb mitral cells, and has insulin and IGF-1 binding sites [29]. Likewise, both IRS-1 and phosphotyrosine staining were prominent in pyramidal neurons of layers II-III of the cerebral cortex, which project extensively to diverse cortical

stuctures [29]. Finding IRS-1 in these locations, which also express insulin and IGF-1 receptors, suggests that these peptides modulate brain neural function by acting on receptors at major relay synapses.

Likewise, the choroid plexus was intensely stained for IRS-1 and phosphotyrosine in this study. This result is consistent with evidence that the choroid plexus has a very high density of receptors for insulin and IGF-1 [30], and suggests that insulin and IGF-1 actions on these cells results in activation of signal transduction pathways associated with altered gene expression.

IRS-1 immunoreactivity, therefore, is found among populations of brain cells previously shown to contain insulin and IGF-1 receptor mRNAs and to express insulin and IGF-1 receptors. Colocalization studies are needed to determine if IRS-1 is preferentially associated with cells expressing insulin receptors or IGF-1 receptors in these regions. In conclusion, the presence of IRS-1 in populations of neurons that express insulin and IGF-1 receptors as well as phosphotyrosine immunoreactivity suggests that these peptides play a role in the regulation of neuronal gene expression in the adult rat brain.

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