Insulin-like growth factor system in human central nervous system, multiple sclerosis and amyotrophic lateral sclerosis
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Insulin-like growth factor II receptors in human brain and their absence in astrogliotic plaques in multiple sclerosis

Nadine Wilczak, Pieter de Bleser, Paul Luiten, Albert Geerts, Albert Teelken, and Jacques de Keyser

Abstract

Insulin-like growth factor (IGF) II receptors were studied in human adult brain by using autoradiography with $^{[125I]}$IGF-II. Receptors were found to be widely distributed throughout all neuronal regions. The highest densities were found in plexus choroideus, granular layer of the cerebellar cortex, gyrus dendatus and pyramidal layer of the hippocampus, striatum, and cerebral cortex. White matter was devoid of IGF-II receptors. We also examined $^{[125I]}$IGF-II binding in six plaques of multiple sclerosis, which were characterised by a dense network of astrocytes. We were unable to detect IGF-II receptors in any of the astrogliotic plaques, suggesting that IGF-II receptors in human brain are not involved in astrogliosis. The regional variations in neuronal distribution of IGF-II receptors suggest involvement of IGF-II in functions associated with specific neuronal pathways.
Introduction

The insulin-like growth factors (IGFs), IGF-I and IGF-II, are mitogenic polypeptides with structural homology to insulin. Two major types of receptors recognise the IGFs and mediate their physiological effects: IGF-I and IGF-II receptors \(^6^,^8^,^9\). The IGF-I receptor is a heterotetrameric glycoprotein composed of two alpha and two beta subunits linked by disulphide bounds. Signals are transmitted by means of receptor autophosphorylation and tyrosine kinase activation \(^6\). The IGF-II receptor has a completely different structure. It is a monomeric receptor with a striking extracellular domain made up almost exclusively of 15 cysteine-based repeats, and is identical to the cation-independent mannose-6-phosphate receptor \(^14^,^18\). In fact, these receptors display two distinctive binding sites: one for IGF-II and another for mannose-6-phosphate containing glycoproteins, which are mainly lysosomal enzymes that are endocytosed into endosomes.

IGF-I receptors have been studied extensively in the central nervous system in both rodents \(^3^,^11^,^16\) and humans \(^1^,^7\). The distribution of IGF-II receptors has been investigated in rodent brain \(^11^,^16^,^20\). However, there is only one report dealing with the presence of IGF-II binding sites in brain homogenates from a single individual \(^19\). Here we present the first autoradiographic study in human brain.

Materials and methods

**Brain tissues**

Brain tissue was obtained at autopsy from six patients (three females and three males), aged 65 to 78 years, who had died without evidence of neurologic or psychiatric disease. Slices of hemispheric white matter containing chronic plaques with dense astrogliosis, identified by glial fibrillary acidic protein (GFAP) immunoperoxidase staining, were obtained from four patients with multiple sclerosis (three females and one male; aged 59 to 78 years). Post mortem delay, defined as the time elapsed between death and freezing of the brain sections, ranged between 4 and 12 hours. Tissue blocks of approximately 0.5 cm thick were frozen rapidly by immersion in isopenthanedry-ice or liquid nitrogen, and stored at -80°C until further processing.
Materials
Human recombinant $^{125}\text{I}]$IGF-II (specific activity 2000 Ci/mmol), $^{125}\text{I}$ standards, and $^{3}\text{H}]$Ultrofilms were obtained from Amersham (Buckinghamshire, UK). Human recombinant IGF-I and IGF-II were purchased from Gropep (Adelaide, Australia). Goat-anti-human IGF binding proteins (IGFBP)-1, -2, -3, -5 and -6 antibodies were purchased from Gropep. All other chemicals were of the highest grade commercially available.

Radioligand binding studies
Frozen tissue blocks were mounted on a cryostat chuck coated with embedding medium (OCT compound, Lab-Tek Products), and serial sections of 15 $\mu$M thickness were cut at -20°C using a microtome-cryostat (Reichert-Jung cryostat Frigocut 2800), mounted on gelatine-coated glass slides, and dried overnight under vacuum. Sections were preincubated for 15 min at 20°C in 25 mM Tris-HCl (pH 7.4), containing 10 mM MgCl$_2$ and 0.1 % bovine serum albumin (BSA). Incubation experiments with $^{125}\text{I}]$IGF-II were done in duplicate at 20°C in the same buffer composition.

For saturation and competition binding studies the sections were incubated for 120 min with $^{125}\text{I}]$IGF-II. For saturation binding experiments nine concentrations of $^{125}\text{I}]$IGF-II between 0.01 and 2 nM were used. Competition binding experiments with IGF-I and IGF-II were done with a concentration of 0.1 nM $^{125}\text{I}]$IGF-II. After incubation, the sections were washed three times for one minute in ice-cold buffer to remove unbound ligand, wiped from the slides with Whatman GF/B glass fiber filters, and radioactivity was determined in a gamma-counter.

Binding isotherms were analysed by non-linear least-square curve fitting. $K_D$ values were calculated from analysis of the Scatchard plots. The dissociation constants ($K_i$ values) of IGF-I and IGF-II were calculated from the corresponding IC$_{50}$ values according to the equation of Cheng and Prusoff $^4$. Non-specific binding was obtained in the presence of 0.5 $\mu$M IGF-II. Specific binding was obtained by subtracting non-specific binding from the total binding.

For autoradiography, the sections were dried under a stream of cold air, placed in X-ray cassettes together with $^{125}\text{I}]$standards, and apposed to $^{3}\text{H}]$Ultrofilm for four days and analysed as described previously $^7$. The regions of interest were sampled and mean optical densities determined, and converted into fmol/mg protein, based on the experimentally determined relation between polymer and brain paste standards.
**Immunohistochemistry**

Frozen sections were fixed in 3% paraformaldehyde in 0.01 M phosphate buffered saline (PBS) for 15 min at room temperature and three times washed with PBS for 5 minutes. After blocking of non-specific background staining with 5% normal rabbit serum, the avidin-biotin-peroxidase complex was used. Sections were incubated in primary antibody solution (1/200) goat-anti-human IGFBPs-1, -2, -3, -5 and -6 (1/200) in PBS, overnight at 4 °C. The secondary antibody was biotinylated, sections were incubated for 90 min at room temperature in rabbit-anti-goat (1/200) in PBS. Finally the sections were incubated in horseradish peroxidase-(HRP)-conjugated streptavidin (1/200) in PBS for 90 min at room temperature and processed by the dianinobenzidin (DAB)/H₂O₂ reaction (30 mg DAB and 0.01 % H₂O₂/100 ml Tris buffer). Between all steps the sections were rinsed thoroughly with PBS.

**Results**

**Characteristics of the binding of [¹²⁵I]IGF-II and regional distribution of [¹²⁵I]IGF-II receptors**

The binding of [¹²⁵I]IGF-II to human cerebellum was saturable. Scatchard analysis of the saturation binding data revealed a $K_D$ value (mean ± S.E.M., n=3 for each regions) of 0.79 nM ± 0.06 nM (not shown). Competition curves with IGF-I and IGF-II were best fitted to a one-site binding model (Figure 1). The calculated $K_i$ value (mean ± S.E.M., n=3) was 2.4 ± 0.3 nM for IGF-II and 25.6 ± 2.1 nM for IGF-I.

By using immunohistochemistry on unwashed slices of frontal cortex we were able to identify IGFBPs-1, -2, -3, -5 and –6 on neuronal cell bodies. However, after preincubating the slices for 15 min at 20°C in the same buffer used by autoradiographic experiments, IGFBPs were no longer detectable in the slices (Figure 2).

The regional distribution of the IGF-II receptors is shown in Table 1. Representative autoradiographs are shown in Figures 3 and 4. The highest density was observed in choroid plexus. Relatively high levels of IGF-II receptors were found in the different layers of the cerebral cortex and in the neostriatum (caudate nucleus, putamen, and accumbens nucleus). In the hippocampal formation, IGF-II receptors were concentrated in the granular layer of the dentate gyrus and the pyramidal layers of the CA1-CA3 subfields. In the cerebellar cortex, highest labelling was observed in the granular layer and low levels were seen in the molecular
layer. In the midbrain, IGF-II receptors were mainly detected in substantia nigra and periaqueductal gray. Low binding was also observed in the red nucleus. In the pons moderate levels of IGF-II receptors were associated with the pontine nuclei. Moderate levels of IGF-II receptors were also observed in the pallidium, amygdala, and thalamus. We found no IGF-II receptors in the white matter.

Chronic plaques of multiple sclerosis were identified by myelin staining (Figure 4). The plaques contained a dense astrogliosis as visualised by GFAP immunohistochemistry (not shown). Similar to normal appearing white matter, we could not detect specific binding of $[^{125}\text{I}]$IGF-II to IGF-II receptors in any of these plaques. IGF-II receptor concentrations in cerebral cortex of MS patients ($31 \pm 3$ fmol/mg protein) were not significantly different from those in controls ($29 \pm 7$ fmol/mg protein; Mann-Whitney test).

**Figure 1**

Representative competition curves for IGF-II (○) and IGF-I (●) inhibition of 0.1 nM $[^{125}\text{I}]$IGF-II specific binding on microtome sections of cerebellum. Computer analysis of the binding data revealed that the competition curves were best described by a one-component binding model. $K_i$ values are given in Table I.
Figure 2

Photomicrographs of IGFBP-3 in frontal cortex Immunocytochemistry with anti-IGFBP-3 in non-preincubated (A) and preincubated (B) slices of frontal cortex. IGFBP-3 is located on neuronal cell bodies (arrows). After preincubation in Tris-HCl, MgCl₂, and 0.1 % bovine serum albumin buffer, IGFBP-3 is no longer detectable.
Bar = 50 μm.

Table 1  Regional distribution of IGF-II receptors in human brain

<table>
<thead>
<tr>
<th>Region</th>
<th>IGF-II receptor concentration (fmol/mg protein, mean ± S.E.M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Frontal, parietal, occipital, and temporal cortex</td>
<td>29 ± 7</td>
</tr>
<tr>
<td>Cerebral white matter</td>
<td>ND</td>
</tr>
<tr>
<td>Corpus callosum</td>
<td>ND</td>
</tr>
<tr>
<td>Caudate nucleus, putamen, accumbens</td>
<td>27 ± 5</td>
</tr>
<tr>
<td>Pallidium</td>
<td>13 ± 3</td>
</tr>
<tr>
<td>Thalamus</td>
<td>10 ± 3</td>
</tr>
<tr>
<td>Amygdala</td>
<td>22 ± 3</td>
</tr>
<tr>
<td>Claustrum</td>
<td>15 ± 4</td>
</tr>
<tr>
<td>Hippocampus</td>
<td></td>
</tr>
<tr>
<td>pyramidal cell layer</td>
<td>28 ± 2</td>
</tr>
<tr>
<td>dentate gyrus</td>
<td>16 ± 3</td>
</tr>
<tr>
<td>Choroid plexus</td>
<td>58 ± 9</td>
</tr>
<tr>
<td>Cerebellum</td>
<td></td>
</tr>
<tr>
<td>molecular layer</td>
<td>13 ± 1</td>
</tr>
<tr>
<td>granular layer</td>
<td>33 ± 6</td>
</tr>
<tr>
<td>white matter</td>
<td>ND</td>
</tr>
<tr>
<td>Midbrain</td>
<td></td>
</tr>
<tr>
<td>substantia nigra</td>
<td>21 ± 4</td>
</tr>
<tr>
<td>red nucleus</td>
<td>7 ± 1</td>
</tr>
<tr>
<td>periaqueductal gray</td>
<td>15 ± 4</td>
</tr>
<tr>
<td>Pons</td>
<td></td>
</tr>
<tr>
<td>pontine nuclei</td>
<td>26 ± 3</td>
</tr>
</tbody>
</table>

Data were generated in brain samples from six individuals. ND, not detectable.
Figure 3

Autoradiographs of 0.1 nM $[^{125}]$IGF-II binding in the absence = total binding (T) and the presence of 0.5 µM unlabelled IGF-II = non-specific binding (N) to microscope slide-mounted sections at the level of (A) frontal cortex (co) and white matter (wm), (B) caudate nucleus (cd)/putamen (put) = neostriatum and nucleus accumbens (acc), (C) claustrum (clau), pallidium (pal) and putamen (put), (D) amygdala (am), (E) hippocampus: dentate gyrus (dg)/pyramidal cell layer (pc), and parahypocampal gyrus (pg), and (F) midbrain: substantia nigra (sn)/colliculus superior (cs)/ red nucleus (rn) and periaqueductal gray (pg). Bar = 1cm.
IGF-II receptors in MS

Figure 4

Autoradiographs of 0.1 nM $[^{125}\text{I}]$IGF-II binding in the absence = total binding (T) and the presence of 0.5 µM unlabelled IGF-II = non-specific binding (N) to microscope slide-mounted sections at the level of (A) pons: pontine nuclei (pn), (B) cerebellum: molecular layer (ml)/ granular layer (gl), white matter (wm), and (C) normal appearing white matter (wm), demyelinated astrogliotic plaque (p) and cerebral cortex (co) from a patient with multiple sclerosis. Section (M) shows the myelin staining with luxol fast blue revealing a plaque (p). Bar = 1cm.
Discussion

Our study demonstrates that similar to rodent brain, IGF-II receptors were found to be widely expressed throughout the neuronal regions of the human brain. In many brain areas, such as the striatum, cerebral cortex, and hippocampus, the distribution pattern of IGF-II receptors was comparable to that of IGF-I receptors. The most striking difference was that in the cerebellar cortex highest labelling was observed in the granular cell layer, whereas IGF-I receptors predominated in the molecular cell layer.

The finding that discrete neuronal regions contain higher levels of IGF-II receptors may suggest a more specialised role of IGF-II as trophic/survival factor or perhaps as neuromodulator for well-defined neuronal systems. The role of the IGF-II receptors in the different neuronal regions is not well understood. In vitro, IGF-II mimics the mitogenic actions of IGF-I, through stimulation of IGF-I receptors. In human brain IGF-I has a ten times higher affinity than IGF-II for the IGF-I receptor. On the other hand, we found in this study that IGF-II was ten times more potent than IGF-I in displacing $^{125}$IIGF-II. These findings indicate that in human brain, under physiological conditions, IGF-I preferentially interacts with IGF-I receptors, and IGF-II with IGF-II receptors. In vitro observations suggest that IGF-II receptors may function in diverse biologic processes. IGF-II may act as a modulator for the trafficking of mannose 6-phosphate bearing lysosomal enzymes into the cell. Binding of IGF-II to its receptor may also activate a signalling mechanism through interaction with a GTP-binding protein, the physiological role of which is not yet understood. Konishi and co-workers found that IGF-II enhanced choline acetyltransferase activity in a dose-dependent manner in cultures of embryonic mouse septal neurons. Part of this effect was related to a specific interaction with IGF-II receptors, and suggested a role of IGF-II receptors in the differentiation of cholinergic neurons. Further evidence for a role of IGF-II in the cholinergic neurotransmitter system was the observation that IGF-II potentiated the $K^+$-evoked release of acetylcholine from hippocampal slices. Recently, Kang et al., found that retinoic acid also binds to the IGF-II receptor and suggested that the biological consequence of this interaction appears to be the suppression of cell proliferation and/or the induction of apoptosis. Studies in different neurodegenerative disorders may help to clarify the functional roles of IGF-II receptors in human brain.

It has been previously reported that IGF-I receptors are present in white matter and astrogliotic plaques from patients with multiple sclerosis, which is consistent with a well
recognised role of IGF-I in oligodendrocyte survival, myelination, and stimulation of astrocytes. The absence of IGF-II receptors in the astrogliotic plaques indicates that IGF-II receptors are not involved in the mechanism of astrogliosis.

The brain also contains IGFBPs, of which at least six types have been identified. These proteins can bind both IGF-I and IGF-II, and regulate their access to the IGF receptors. We found that IGFBPs were removed from the tissue slices during the preincubation procedure, indicating that in the experimental conditions that we used, [125I]IGF-II binding occurred to IGF-II receptors.

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