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Location of phosphotyrosine-containing proteins by immunocytochemistry in the rat forebrain corresponds to the distribution of the insulin receptor

A. M. MOSS*†, J. W. UNGER‡, R. T. MOXLEY§, AND J. N. LIVINGSTON*

Departments of *Medicine and §Neurology, University of Rochester School of Medicine, Rochester, NY 14642; and ‡Department of Anatomy, University of Munich, Munich, Federal Republic of Germany

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ABSTRACT **Cellular regulation by certain growth factor receptors and protooncogene products involves tyrosine kinase activity with the resultant tyrosine phosphorylation of protein substrates. In the present report we describe the distribution of phosphotyrosine-containing material detected by immunocytochemistry (ICC) in the rat forebrain. Specificity of the affinity-purified antibody against phosphotyrosine used in the ICC technique was demonstrated by the ability of phosphotyrosine and *p*-nitrophenyl phosphate but not phosphoserine, phosphothreonine, or L-tyrosine to inhibit the immunostaining reaction. With ICC, relatively high amounts of phosphotyrosine-positive material were observed in neurons in specific structures that included the supraoptic, paraventricular, and arcuate nuclei; the median eminence; medial habenula; subfornical organ; and piriform cortex. Moderate to high amounts were present in the cerebral cortical layers II–IV and in the pyramidal cell layer of the hippocampus. Small to moderate amounts were detected in a few other locations. Glial elements showed minimal staining. Other areas of the rat forebrain failed to react with this antibody. Importantly, the distribution of the areas positive for phosphotyrosine agreed to a remarkable extent with the distribution of the brain insulin receptor, which itself has tyrosine kinase activity. These findings suggest a relationship between the insulin receptor and the increased phosphotyrosine content of these neurons and support the concept that the brain insulin receptor is active *in vivo*.**

A number of growth factor receptors and protein products of retroviral oncogenes and protooncogenes are protein-tyrosine kinases (1). This enzymatic activity apparently plays an important role in the mediation of the actions of these regulatory proteins (1, 2). Although much of the work on tyrosine kinases has focused on growth regulation, recent findings in the nonproliferating cells of the central nervous system demonstrate the presence of relatively large amounts of tyrosine kinase activity (2, 3). For example, the c-*src* gene product, pp60 src, is widely distributed in the adult rat brain (4) and is expressed throughout brain development (5–8).

Receptors for insulin and insulin-like growth factor 1 (IGF-1) are also present in specific brain regions (9–13). Both receptors demonstrate ligand-controlled autophosphorylation on specific tyrosines and tyrosine phosphorylation of endogenous proteins (14–17), although the distribution of the two receptor types differs in the rat brain (12, 13, 18).

Little information is available on the *in vivo* activation of these kinases in the central nervous system. Previous work has used brain tissue extracts to examine tyrosine kinase activity (19), an approach that does not provide direct evidence of *in vivo* tyrosine kinase activity nor does it establish the precise location of areas rich in tyrosine phosphorylation.

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In the present investigation, we have examined the rat forebrain with an immunocytochemical technique (ICC) that detects phosphotyrosine. This method identifies specific neuronal clusters and nonneuronal cells that contain elevated phosphotyrosine levels. The specific pattern and intensity of staining for phosphotyrosine provides an index of the relative degree of tyrosine phosphorylation of endogenous protein substrates in discrete brain regions.¶,‖ With ICC, we find highly specific immunostaining of phosphotyrosine-containing material in specific brain areas. Interestingly, this material is distributed in the same areas of the rat forebrain that contain the insulin receptor (20).

METHODS

Preparation of Antisera. The anti-phosphotyrosine antibody used for the majority of the studies, goat anti-Tyr(*P*), was prepared by immunizing a goat with phosphotyramine coupled to keyhole limpet hemocyanin supplied by Morris White (Joslin Diabetes Center, Boston, MA). Affinity purification of goat anti-Tyr(*P*) was performed by passage of the goat serum over a 7 × 1 cm column of phosphotyramine coupled to Affi-Gel 10. The column was extensively washed, and goat anti-Tyr(*P*) was eluted with 100 mM *p*-nitrophenyl phosphate as described (21). After extensive dialysis against phosphate-buffered saline (PBS) to remove *p*-nitrophenyl phosphate, goat anti-Tyr(*P*) was stored at −20°C at a 2 mg/ml concentration of IgG protein.

An affinity-purified rabbit antibody against phosphotyrosine, rabbit anti-Tyr(*P*), also was supplied by Morris White. The characteristics of rabbit anti-Tyr(*P*) for the immunoprecipitation of phosphotyrosine-containing proteins have been described (22, 23).

ICC. Male Sprague–Dawley rats weighing 200–250 g were anesthetized with 50 mg of pentobarbital per kg of body weight given i.p. and were perfused with 100 ml of cold PBS

Abbreviations: ICC, immunocytochemistry; IGF-1, insulin-like growth factor 1; anti-Tyr(*P*), anti-phosphotyrosine.

†To whom reprint requests should be addressed at: Department of Medicine, University of Rochester School of Medicine, Box 693, Rochester, NY 14642.

¶The antibody used in the ICC technique detects phosphotyrosine. However, since the brain is fixed and the tissue sections undergo an extensive wash protocol during the immunostaining procedure and free phosphotyrosine has not been reported in cells, the phosphotyrosine detected by ICC must be a part of a macromolecule. Because only proteins have been described to contain phosphotyrosine, we have used phosphotyrosine and phosphotyrosine-containing proteins interchangeably.

‖It is possible that low levels of phosphotyrosine phosphatase activity could contribute to increased levels of phosphotyrosine-immunoreactive material. However, increases in the amount of phosphotyrosine-containing proteins by such a mechanism has not been described *in vivo*. Therefore, we have elected to discuss only the possibility that elevated phosphotyrosine content is indicative of an elevated level of tyrosine kinase activity.

through a cardiac cannula. This perfusion was followed by perfusion with 200 ml of chilled 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4), and the brain was removed and immersed in the fixative for 2 hr at 4°C. After fixation the brain was placed in 10% sucrose in the phosphate buffer for 24 hr, which was followed by 30% sucrose for an additional 48 hr. Frozen sections (40 μm) were prepared by using a sliding microtome.

In some studies, the phosphatase inhibitor, sodium orthovanadate (final concentration, 1 mM) was included in the perfusion and fixative solutions. However, the presence of this inhibitor did not change either the amount of phosphotyrosine or its distribution in the brain sections.

ICC was performed by the avidin–biotin method with biotinylated peroxidase or with the peroxidase-antiperoxidase method (24). Tissue sections were incubated free-floating with a 1:1000 dilution of goat anti-Tyr(*P*) in PBS that contained 1% rabbit serum. Unless specified, all treatments and the tissue rinses were conducted in PBS. The sections were incubated for 16 hr at 4°C with goat anti-Tyr(*P*), rinsed three times, then incubated at 21°C for 1 hr with biotinylated anti-goat IgG (Vectastain ABC kit) according to the manufacturer's instructions. After three rinses, the sections were incubated for 1 hr at 21°C with the avidin–biotin–peroxidase complex (Vectastain), rinsed once in PBS, and then rinsed twice in 10 mM imidazole/50 mM acetate buffer, pH 7.4. The sections were then incubated with diaminobenzidine at 0.4 mg/ml in the acetate/imidazole buffer that contained nickel(II) sulfate at 25 mg/ml and 0.005% H_2O_2. Color development was stopped after 5 min.

ICC with rabbit anti-Tyr(*P*) was conducted under the same conditions except in the presence of 1% sheep serum. A 1:100 dilution of goat anti-rabbit IgG and a 1:800 dilution of peroxidase-antiperoxidase (Cappel Laboratories) was used as described above for the Vectastain ABC procedure. The sections were developed with the nickel-containing substrate in the presence of 0.01% H_2O_2.

Specificity of the antibodies for phosphotyrosine was evaluated by the incubating goat or rabbit anti-Tyr(*P*) for 16 hr at 4°C with 500 μM concentrations of phosphotyrosine, phosphoserine, phosphothreonine, L-tyrosine, or *p*-nitrophenyl phosphate. The antibodies were then used in the ICC procedures described above. Additional control studies were conducted by omitting the primary antibody or replacing it with nonimmune serum.

Sections were examined and photographed with a Nikon Optiphot microscope.

RESULTS

Specificity of Goat Anti-Tyr(*P*) for Phosphotyrosine in Brain Tissue Slices. ICC with goat or rabbit anti-Tyr(*P*) generated very fine granules on positive neurons, the development of which depended on the presence of the antibody (Fig. 1*a*). Preincubation of goat or rabbit anti-Tyr(*P*) with phosphotyrosine (Fig. 1*b*) or *p*-nitrophenyl phosphate (Fig. 1*f*) abolished the staining reaction, whereas preincubation of goat anti-Tyr(*P*) with phosphoserine, phosphothreonine, or L-tyrosine did not affect the formation of the antigen–antibody product (Fig. 1 *c–e*). Similar results were obtained with rabbit anti-Tyr(*P*) (data not shown). These findings demonstrate the specificity of goat anti-Tyr(*P*) for the phosphotyrosine moiety present in macromolecules in brain tissue.

Distribution of Phosphotyrosine Immunoreactivity. A detailed study of phosphotyrosine distribution in the rat forebrain was conducted with goat anti-Tyr(*P*) as schematically illustrated in Fig. 2. These results were obtained with an antibody dilution of 1:1000 and were consistent regardless of the concentration of goat anti-Tyr(*P*) (1:250 to 1:3000; data not shown). Also, rabbit anti-Tyr(*P*) provided the same results as goat anti-Tyr(*P*) (data not shown).

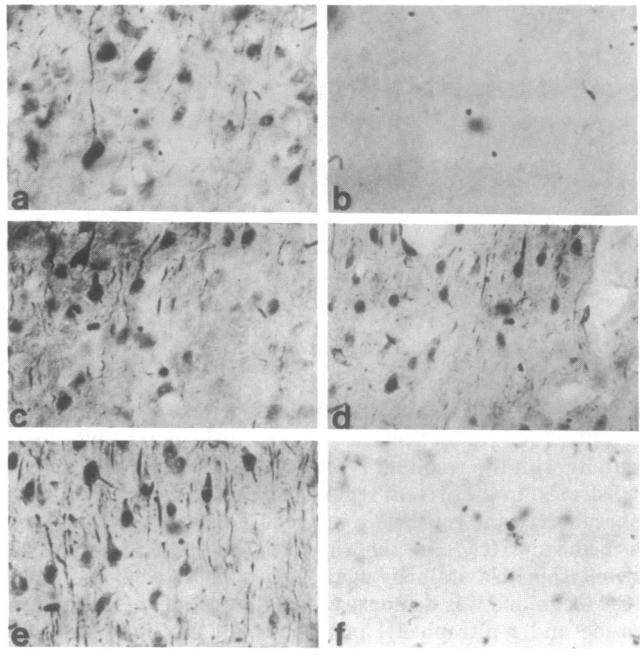

FIG. 1. Specificity of goat anti-Tyr(*P*) demonstrated in the rat piriform cortex. (*a*) Neurons immunostained with goat anti-Tyr(*P*). (*b–f*) Preincubation of goat anti-Tyr(*P*) with 500 μM each of phosphotyrosine (*b*), phosphothreonine (*c*), phosphoserine (*d*), L-tyrosine (*e*), or *p*-nitrophenyl phosphate (*f*) prior to ICC.

Positive ICC with goat anti-Tyr(*P*) was present in neurons and neuronal processes in specific anatomical locations. However, most areas of the forebrain showed little or no immunostaining. The majority of glial cells also demonstrated little or no detectable staining. The distribution of phosphotyrosine in the rat forebrain is described below according to anatomical subdivisions.

Rhinencephalon. A moderately dense concentration of reaction product was present in the external plexiform and granular cell layers and in the anterior olfactory nucleus. The remainder of the olfactory bulb contained occasional positive cells.

Telencephalon. A moderate concentration of reaction product was present in the cerebral cortical layers II–IV among all anatomic subdivisions. Layers V and VI contained a few positive cells, whereas cells in layer I contained no visible reaction product.

The hippocampus contained a moderate amount of immunoreactivity in all fields of Ammon's Horn and in the dentate gyrus. Most of the reaction product was found in the pyramidal cell layer and was evenly distributed in CA 1-4.

Dense reaction product was present throughout the entire piriform cortex. Most amygdaloid areas were lightly to moderately immunostained with the greatest amounts in the lateral nuclei.

Diencephalon. The medial habenular nucleus contained a relatively large amount of positive material in contrast to the lateral habenula, which contained much smaller amounts. Neuronal cell bodies and processes within the subfornical organ contained a high concentration of reaction product. Of these, the cells nearest the third ventricle were the most intensely stained.

The majority of the thalamus contained only scattered phosphotyrosine-positive cells. An exception was the reticulo-thalamic nucleus, which contained a moderate number of positive neurons. The subthalamic nucleus demonstrated

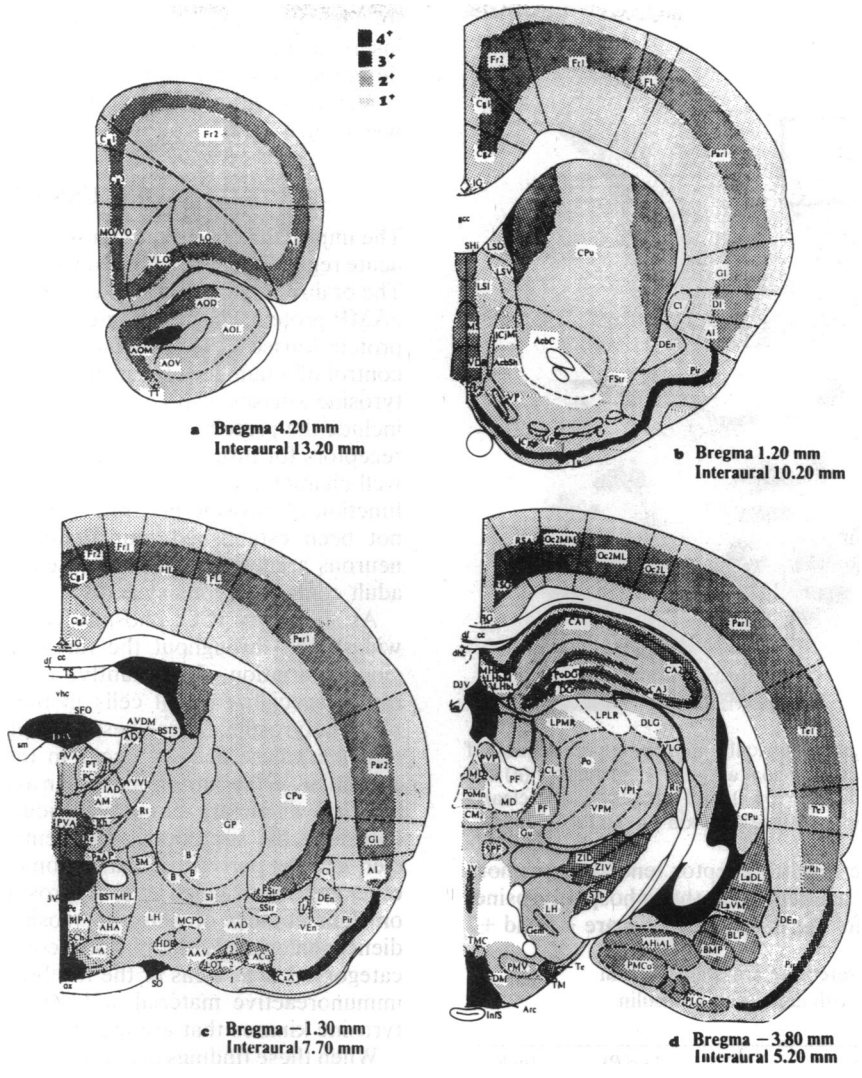

FIG. 2. Schematic coronal sections of the rat forebrain. (*a–d*) Four different levels of the rat brain indicated by stereotaxic coordinates. Abbreviations are taken from the atlas of Paxinos and Watson (25). (See Table 1 for a definition of abbreviations for selected regions positive for phosphotyrosine.) The density of phosphotyrosine has been graded as follows: 4+, very high density; 3+, high density; 2+, moderate density; 1+, low density.

staining of moderate intensity, and a large number of positive cells were evident throughout the zona incerta.

Many regions in the hypothalamus had large numbers of densely stained cells. The supraoptic nucleus was intensely stained as was the suprachiasmatic nucleus. Only scattered positive cells were present in the remainder of the anterior hypothalamus.

A large number of densely stained cells were present in the periventricular region of the third ventricle. The paraventricular nucleus contained densely stained cells, especially in the magnocellular areas. In a pattern similar to that seen in the magnocellular supraoptic nucleus, neuronal cell bodies were clearly delineated by reaction product (Fig. 3). A high concentration of reaction product was found in the pericapillary areas and in the external zone of the median eminence. The arcuate nucleus was moderately and uniformly stained throughout its extent. Controls showed the absence of reaction product.

Other Phosphotyrosine-Positive Regions. Other areas of staining in the rat forebrain included the matrix and fibrocytes of the choroid plexus. The matrix was stained in long, fibrous networks. Fibrocytes and the ependymal cells that line the third ventricle contained moderate amounts of positive material. In addition, moderately dense staining was seen on tanycytes, the specialized glial cells that line portal capillaries in the median eminence.

Comparison of Phosphotyrosine-Positive Regions with Regions That Contain the Insulin Receptor. Table 1 provides a comparison of the brain subregions for the presence of phosphotyrosine and for the insulin receptor. Data for the insulin receptor is taken from a previous study in which we used ICC with a specific affinity-purified antibody to the insulin receptor (20). The methods used for ICC of the insulin receptor were similar to those used in the present work, which facilitates making a direct comparison between immunostaining of phosphotyrosine and staining of the insulin receptor.

As illustrated in this table, a positive correlation exists between the regions stained with the two antibodies. In all instances, regions positive for the insulin receptor were positive for phosphotyrosine. Conversely, all regions positive for phosphotyrosine were positive for the insulin receptor—i.e., no region was positive for one in the absence of a positive reaction for the other. Moreover, the correlation extends to the intensity of staining as well. Thus, for example, all areas judged +3 or +4 for phosphotyrosine content were also designated +3 or +4 for the insulin receptor. This degree of correlation includes the choroid plexus, which is +4 for both immunoreactive materials.

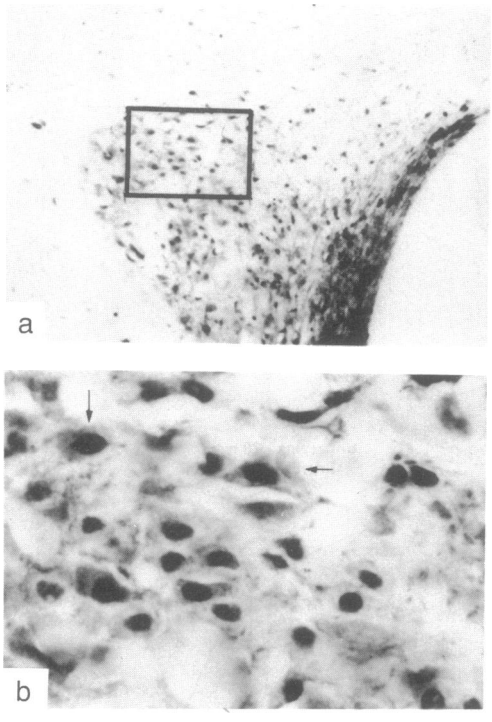

FIG. 3. Hypothalamic paraventricular nucleus from a section of rat forebrain immunostained with goat anti-Tyr(*P*) under low (*a*) and high (*b*) power. Cell bodies (arrows) and processes are clearly delineated by the immunoperoxidase reaction product.

In relative terms, the insulin receptor tended to be more enriched in many of the positive areas than phosphotyrosine. Most +4 and +3 areas for the insulin receptor are +3 and +2 for phosphotyrosine. This trend is maintained in the zona inserta and the subthalamic nucleus, the two areas where the difference is the most pronounced. In both areas the insulin receptor was graded as +4 and the phosphotyrosine content was judged +2.

Table 1. Comparison of forebrain areas enriched in phosphotyrosine [Tyr(*P*)] with areas rich in insulin receptors (InsR)

Brain area	Tyr(*P*)	InsR
Subfornical organ (SFO)	4+	4+
Supraoptic nucleus (SO)	4+	4+
Medial habenula (MHb)	3+	4+
Hypothalamus		
Paraventricular nucleus (PaAP)	3+	4+
Periventricular region (Pe)	3+	4+
Suprachiasmatic nucleus (SCh)	3+	4+
Arcuate nucleus (Arc)	3+	4+
Piriform cortex (Pir)	3+	3+
Olfactory bulb		
Anterior olfactory nucleus (AOM)	3+	2+
External plexiform layer	3+	2+
Granular layer	1+	1+
Glomerular layer	1+	1+
Zona incerta (ZID, ZIV)	2+	4+
Amygdala (ACo, AAV)	2+	2+
Pyramidal layer of hippocampus		
CA 1-2 (CA1, CA2)	2+	3+
CA 3-4 (CA3, CA4)	2+	2+
Dentate gyrus (DG)	2+	2+
Cortex layers II–IV	2+	2+
Reticulothalamic nucleus (Rt)	2+	3+
Subthalamic nucleus (STh)	2+	4+
Paraventricular nucleus of thalamus (PVP, PVA)	2+	3+
Septohippocampal nucleus (SHi)	2+	3+
Caudate putamen (Pu)	1+	1+
Globus pallidus (GP)	1+	1+
Choroid plexus	4+	4+

DISCUSSION

The importance of protein phosphorylation reactions for the acute regulation of cell function is well established (2, 26, 27). The brain is no exception as evidenced by its high content of cAMP protein kinases, non-cAMP–dependent kinases, and protein kinase C, all of which have been implicated in the control of a number of neuronal processes (28–30). Protein-tyrosine kinases also have been demonstrated in brain and include the protein product of c-*src* (4–8, 31), the hormone receptors for insulin and IGF-1 (9–13), as well as other less well-characterized tyrosine kinases (32–33). Although the function of tyrosine phosphorylation of neural proteins has not been established, our finding of phosphotyrosine in neurons argues that these kinases are active *in vivo* in the adult central nervous system.

As shown by ICC, phosphotyrosine immunoreactivity is widespread throughout the rat forebrain with marked regional variation. Importantly, only neurons and certain highly specialized glial cells in the positive areas contain phosphotyrosine. These results suggest that high levels of tyrosine kinase activity occur in the diencephalon and, in particular, in the subfornical organ and the median eminence, both of which are circumventricular organs. The medial habenula; the supraoptic, paraventricular, and arcuate nuclei; and the periventricular region of the hypothalamus are other areas with a high level of tyrosine kinase activity, based on their content of phosphotyrosine. In areas outside the diencephalon, only the piriform cortex can be placed in this category. Large areas of the forebrain contained little or no immunoreactive material and thus do not appear to have tyrosine kinases that are highly active *in vivo*.

When these findings are compared with *in vitro* analysis of tyrosine kinase activity of extracts from forebrain subregions, some differences and similarities are evident. The *in vitro* assays show high levels of kinase activity in the hippocampus, the olfactory bulb, and the piriform cortex, followed by regions in the diencephalon (19). As listed in Table 1, the hippocampus and olfactory bulb contain moderate amounts of phosphotyrosine, whereas the piriform cortex has moderate to high levels. Moderate to low levels are present in the basal ganglia by both ICC and the *in vitro* measurements.

There are several possible reasons for the differences in the results from ICC and the *in vitro* measurements. The most likely reason is that the kinases are silent or only partially active *in vivo* but are activated by the conditions used in the *in vitro* analysis. Moreover, it is difficult to make a direct comparison of the results from the two types of studies. ICC allows an evaluation of smaller and more discrete brain regions than the tissue dissection and extraction protocol used in the *in vitro* assays. Regardless of the differences, both approaches provide valuable information needed to characterize the tyrosine kinases in the central nervous system and to understand their function.

Although immunoreactive phosphotyrosine indicates the presence of *in vivo* tyrosine kinase activity, these results do not identify the kinase(s) responsible for generating the tyrosine phosphorylated proteins. However, some insight can be gained by comparing the distribution of phosphotyrosine with the distribution of tyrosine kinases in the central nervous system. As shown in Table 1, the distribution of the insulin receptor correlates closely with the distribution of phosphotyrosine.

The reported locations of other tyrosine kinases do not correlate as precisely (4, 12, 13, 18). Thus, the two forms of c-*src* that exist in neural tissue (4–6, 31, 35) are distributed throughout the central nervous system, with the highest amounts in the hippocampus, the piriform cortex, and the neocortex and with smaller amounts in the diencephalon (4). This distribution pattern does not match the pattern of distribution for phosphotyrosine.

A tyrosine kinase that has a central nervous system distribution pattern that matches in several areas the distribution of phosphotyrosine is the IGF-1 receptor. This receptor is structurally related to the insulin receptor, and both are present in several regions of the rat forebrain (12, 13, 17). There are regions, however, that contain primarily only one or the other receptor. The arcuate nucleus is rich in insulin receptors as well as phosphotyrosine but has only a barely detectable number of IGF-1 receptors. In contrast, cortical layer VI is rich in IGF-1 receptors but is devoid of insulin receptors and phosphotyrosine. No area positive for phosphotyrosine contains only the IGF-1 receptor. Therefore, the distribution of phosphotyrosine follows the distribution of the insulin receptor more closely than it follows the distribution of the IGF-1 receptor.

Other tyrosine kinases detected in brain tissue (32–34) have not been localized to brain subregions and thus cannot be included in this analysis. It is possible that one or more of these kinases, like the insulin receptor, will codistribute with phosphotyrosine.

It is also possible that other phosphotyrosine-containing proteins are present that are not detected by goat or rabbit anti-Tyr(*P*). Thus, the distribution of phosphotyrosine found in the present study should be viewed as a minimal estimate of areas rich in this material.

Interestingly, the distribution of the insulin receptor has been reported to correspond with the distribution of neuropeptides (36). We were able to show in previous colocalization studies that insulin receptors are present on vasopressin-containing neurons in the supraoptic nucleus and on somatostatin-containing neurons in the periventricular nucleus (20). Since these regions also contain large amounts of phosphotyrosine, it is tempting to speculate that the brain insulin receptor is active *in vivo*, as other evidence suggests (9, 37–39) and that its tyrosine kinase activity helps to regulate the synthesis or secretion of neuropeptides by neuroendocrine neurons.

In summary, the present report shows phosphotyrosine-containing material in neurons of specific regions in the adult rat forebrain and shows that these regions correlate with the presence of the insulin receptor. Because tyrosine phosphorylation is important in the signaling pathways for certain growth factor receptors and products of protooncogenes, this information should prove useful in deciphering their regulatory roles in the central nervous system.

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