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DANCE ROUND

WE DANCE ROUND *IN A RING AND SUPPOSE,*
BUT THE SECRET SITS IN THE MIDDLE AND KNOWS.
ROBERT FROST

DETECTION OF PHOSPHOTYROSINE, INSULIN RECEPTOR SUBSTRATE-1 AND GROWTH FACTOR RECEPTOR-BOUND PROTEIN-2 IN THE MAGNOCELLULAR FOREBRAIN SYSTEM AND HYPOTHALAMUS OF CAT AND MAN

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• Insulin action initiated by insulin binding to its cognate receptor is performed *via* phosphorylation of tyrosines on substrate proteins by the receptor tyrosine kinase domain. This process involves autophosphorylation of tyrosine residues in the cytoplasmic domain of the receptor (1). A comparable action is mediated by nerve growth factor (NGF) and epidermal growth factor (EOF) receptors (2,3). Few articles have been directed to the morphological regional distribution in the brain of phosphotyrosine, using antibodies. The first extensive description that proved a topographical distribution for phosphotyrosine in the rat brain was conducted by Marani and Maassen (4). It was shown that alternating areas positive and negative for phosphotyrosine could be described. These areas showed different localizations that were in good agreement with the biochemical results obtained by others (5,6). Moreover, fetal and postnatal series confirmed the results (6) that phosphotyrosine content is extremely high in the developing brain as compared to the mature brain. In the mature brain, the phosphotyrosine localization is also found in the neuropil, not only in neurons. High concentrations of phosphotyrosine in a regional distribution are found in the rat rhinencephalon, the cortex, the basal ganglia (mainly in neostriatum and substantia nigra), hypothalamus and the habenular nuclei. In the hippocampus, the positivity for phosphotyrosine can be detected in the pyramidal cells and the neuropil. The hippocampal subdivisions of CA-1 and CA-3 can be weakly discerned (4). Topographical studies of the distribution of insulin receptor substrate-1 (IRS-1), growth factor receptor-bound protein-2 (GRB-2) or its adaptor molecule and substrate of insulin receptor kinase (She) that complexes to GRB-2 and conducts insulin action towards the *ras* complex (Fig.l) (7) are absent for the brain.

This *Dance Round* deals with the presence of signaling intermediates involved in insulin action as studied by antibodies against phosphotyrosine, IRS-1 and GRB-2 in the cat and human basal forebrain and hypothalamus.

• The basal forebrain contains the cholinergic neurons designated as the groups Chl-Ch4, also known as the magnocellular forebrain system (8-14). This system is known for its involvement in Alzheimer's disease (13,15-30), especially the Ch4 group, corresponding to the nucleus of Meynert (31-33). The basal nucleus of Meynert undergoes pathologic changes also in several other severe disorders, like Parkinson's disease (16,17,26), Korsakoff s disease (16,26), Pick's disease (34), Down's syndrome (35), olivopontocerebellar atrophy (36) and in Huntington's disease (37). In the Huntington's chorea also an increase of CD15 (3-fucosyl-N-acetyl-lactosamine) (38,39) epitope positivity in astrocytes in the magnocellular basal forebrain system was described (40). Here we report that these magnocellular neurons demonstrated in the human brain have a relatively high expression of IRS-1 and GRB-2, indicating that these cells are phosphotyrosine activated not only by the NGF receptor but also by the insulin receptor.

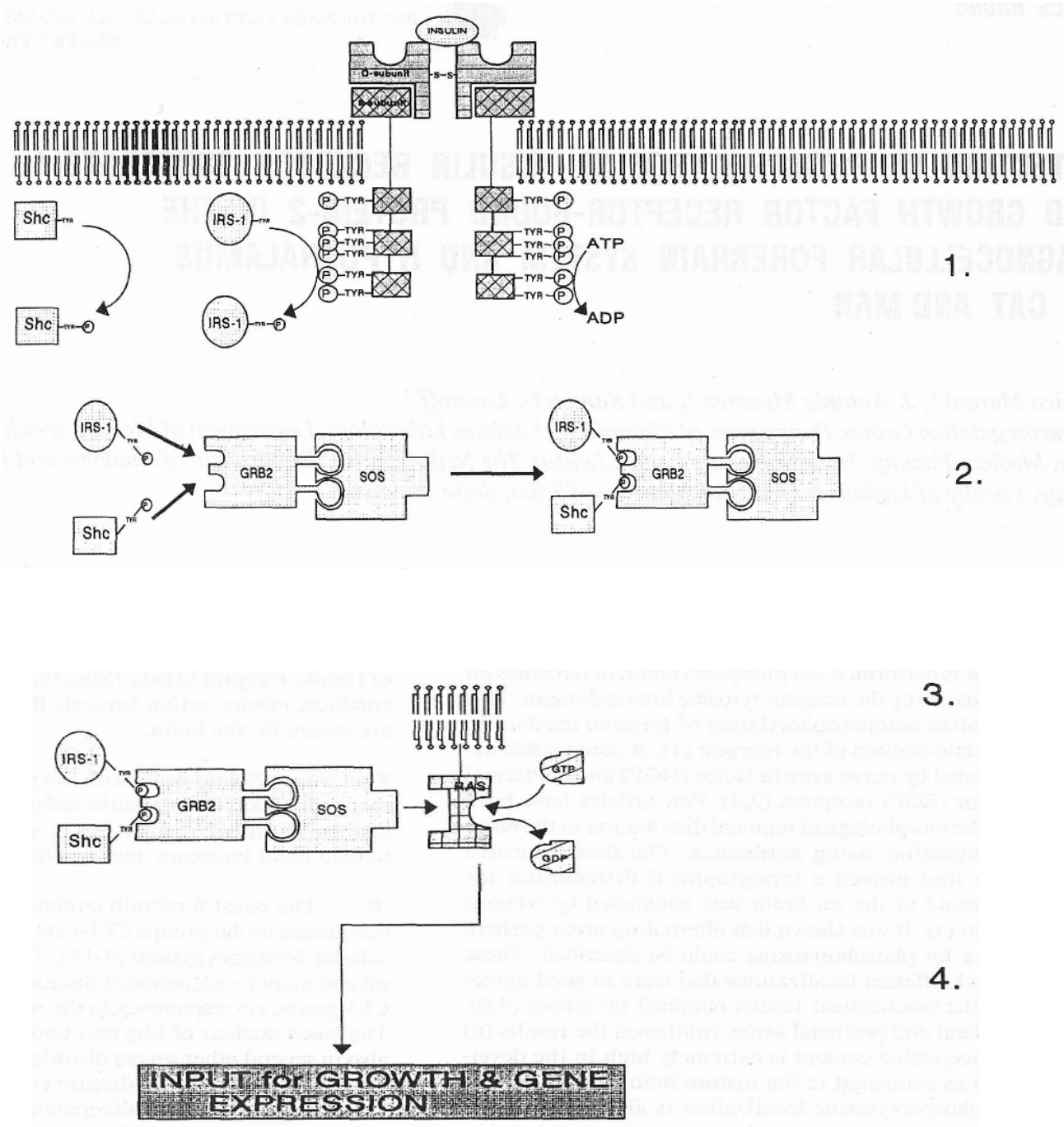


Figure 1. *The insulin action pathway (simplified after Ref.7, and prepared by Ms Simone Mulder). After stimulating the receptor tt-subunits, the fi-subunits are phosphorylated. (1.) This leads to phosphorylation ofIRS-1. (2.) GRB-2, which is linked to son-of-sevenless (SOS) protein, links to IRS-1 and to She. (3.) This process activates SOS and links it to GRB-2, thus activating ras by replacing GDP with GTP. (4.) The ras starts the pathway to modulate the growth and gene expression. The a-subunit is indicated by horizontal lines, and the fl-subunit by diamonds. . ,.--.-, -. ,., .*

IRS-1 and GRB-2 were expressed in *E. coli* as fusion protein, and antibodies against these proteins were raised in rabbits as described earlier (41). Their specificity was tested (Fig. 2). For the preparation and the specificity of the rabbit phosphotyrosine-binding antibody, see (4).

Human brains without any detectable neurological and psychiatric diseases were obtained from the Leiden Universiy Department of Pathology and Department of Anatomy. Three normal brains from patients (64-70 years old) were used, from which the basal forebrain was removed. Left and right parts were separately sectioned. All brain sections were collected and 1 to 5 sections stained forNissl, Kliiver-Barrera (42), IRS-1 ,GRB-2 and phosphotyrosine, respectively. Previously prepared dense series of coronal sections from two human brains (C3305, C3495) stained alternately with the Nissl and Kliiver-Barrera methods were used for the cytoarchitectonic orientation.

Mature male mongrel cats used for C7 nerve avulsion experiments (43) were perfused with a Karnovsky fixative. The brain and spinal cord were dissected free and the brains were stored for three to six months in the same fixative. The same antibodies were used as in human material.

Frozen sections were incubated for 24 hrs with the first antibody in a moist chamber. The second antibody, peroxidase conjugated, was used for two hours, followed by a DAB/peroxide incubation for 15 minutes. For an extensive description of the used immunocytochemistry, see (4).

• In cats, the magnocellular cholinergic forebrain system is prominent and one might definitely distinguish the Chl-Ch4 groups (11; Usunoff *el al,* unpublished data). However only part of their cells display a moderate to weak immunopositivity for IRS-1, GRB-2 and phosphotyrosine. On the other hand, strong immunoreaction is present in both neurosecretory magnocellular hypothalamic nuclei: *nucleus paraventricularis* and *nucleus supraopticus* (Fig.3,4). From the lateral wedge of the supraoptic nucleus a strand of immunopositive neurons extends laterally, ventral to the entopeduncular nucleus (the internal pallidum in subprimate species) and dorsal to the optic tract. There is mainly a perikaryal localization of finely granulated reaction products for IRS-1, GRB-2 and phosphotyrosine. The reaction product is followed into the proximal dendrites, and occasional immunopositive axons are encountered too.

The normal distribution of the magnocellular neurons in human basal forebrain shows the same appearance in our series as in previous descriptions (8,12,14,31,33,40). Briefly, the magnocellular basal complex starts rostrally with quite few neurons located in the medial septal nucleus (Chl group), and ventral to them, within the vertical limb of the Broca's diagonal band is the more substantial Ch2 group. The latter merges imperceptibly with the more loosely arranged Ch3 group, which however contains larger neurons than the Chl and Ch2 groups. The largest neurons are found in the most prominent group, i.e. the Ch4, comprising five subgroups: anteromedial (Ch4am), anterointermediate (Ch4ai), anterolateral (Ch4al), intermediate (Ch4i), and posterior (Ch4p). Rostromedially, Ch4am is continuous with the Ch3 group and the Ch4 neurons spread caudolaterally ventral to the internal and external pallidal segments, within the *substantia innominata.* The bulk of the Ch4 group is encountered at the level of the *pars tecta columnae fornicis* and from the level of the anterior thalamic pole diminishes in caudal direction. Ch4p disappears at the level of the mamillary bodies. Few Ch4 neurons invade the medial and lateral pallidal laminae.

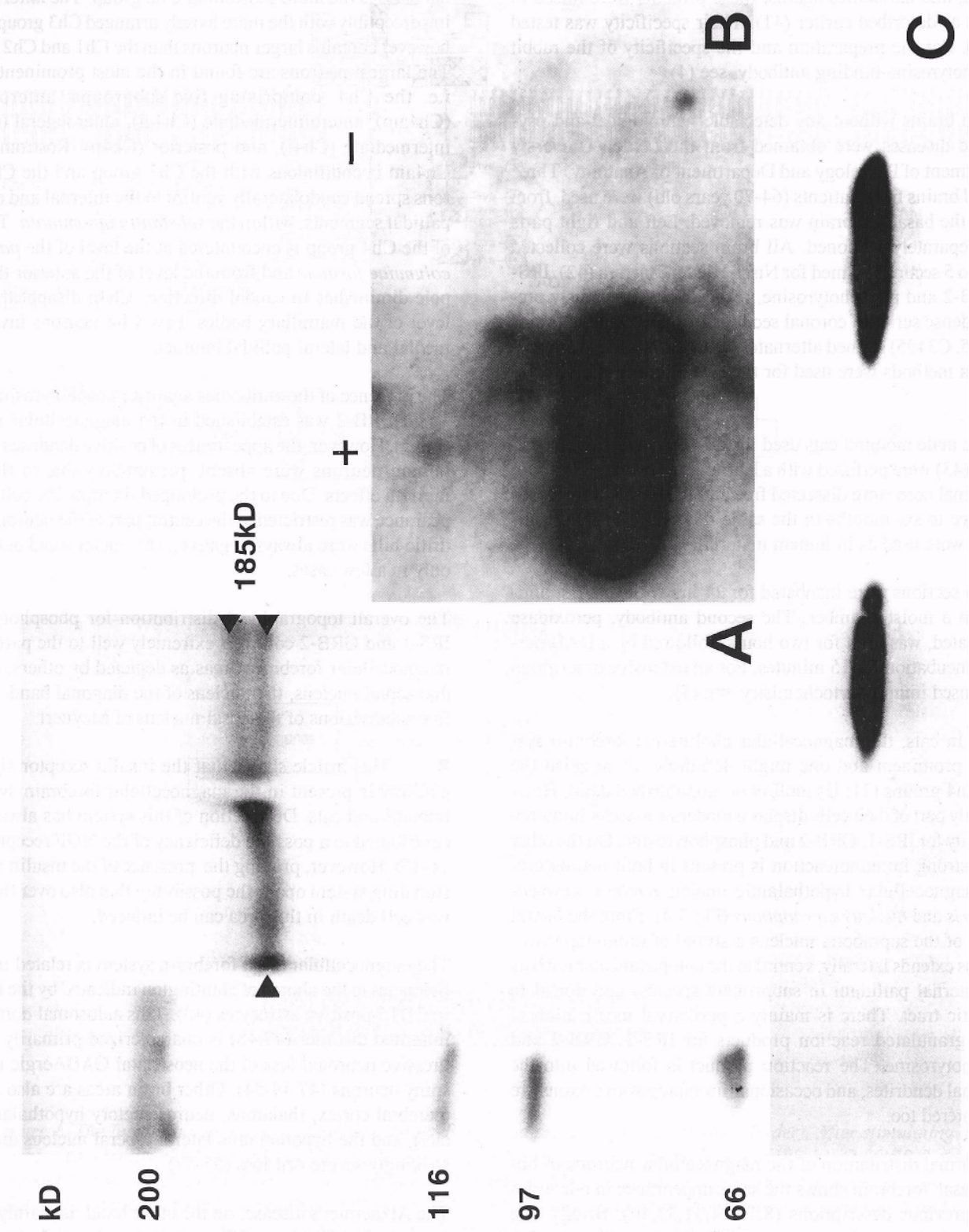
The presence of the antibodies against phosphotyrosine, IRS-1 and GRB-2 was established in the magnocellular neurons (Fig.5). However, the appearances of positive dendrites in these human neurons were absent, presumably due to the post-mortem effects. Due to the prolonged fixation, the cellular appearance was restricted to the central part of the neurons. Dendritic hills were always negative. The nuclei stand out clearly only in a few cases.

The overall topographical distribution for phosphotyrosine, IRS-1 and GRB-2 confined extremely well to the parts of the magnocellular forebrain areas as depicted by others: the medial septal nucleus, the nucleus of the diagonal band, and the five subdivisions of the basal nucleus of Meynert.

• This article shows that the insulin receptor signaling pathway is present in the magnocellular forebrain system in humans and cats. Destruction of this system has always been contributed to a possible deficiency of the NGF receptors (13, 44-46). However, proving the presence of the insulin receptor signaling system opens the possibility that also over this pathway cell death in this area can be induced.

The magnocellular basal forebrain system is related in its deficiencies to the chorea of Huntington indicated by the increase in CD15-positive astrocytes (40). This autosomal dominantly inherited disorder (47,48) is characterized primarily by progressive neuronal loss of the neostriatal GABAergic medium spiny neurons (47,49-54). Other brain areas are also affected (cerebral cortex, thalamus, neurosecretory hypothalamic nuclei), and the hypothalamic lateral tuberal nucleus displays a strikingly severe cell loss (55-57).

The Alzheimer's disease, on the other hand, is mainly related to cortical cholinergic differentiation and to a destruction of the magnocellular forebrain area, especially the nucleus of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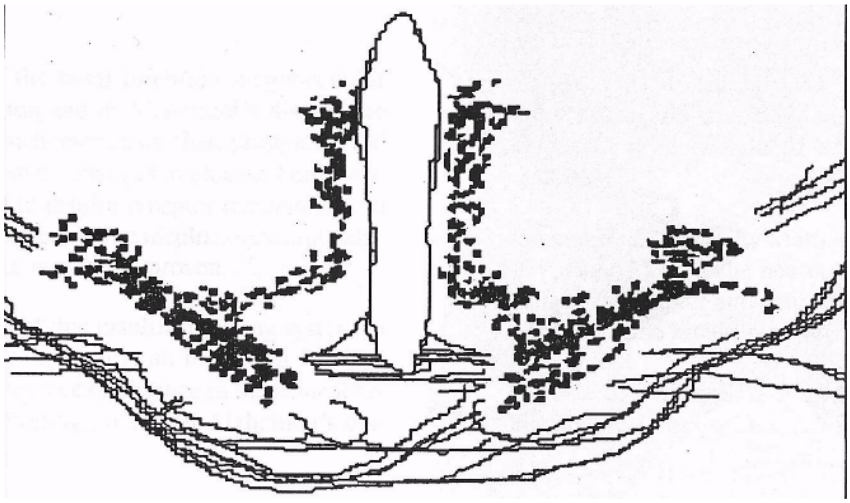

Figure 3. *3D-reconstruction of the overall localization of the magnocellular neurons positive for IRS-1 within the cat hypothalamic neurosecretory nuclei.*

Meynert (15-30), where the corticopetal cholinergic magnocellular neurons are located (8-14). But, again, milder changes are to be found in many brain regions (57-61). Therefore, as Kremer (57) stated, "it is hard to pinpoint the "cause" of the disruption to a single hypothalamic structure", or to a basal forebrain structure. As to endocrine aspects of hypothalamic and basal forebrain structures, the same holds. Impaired glucose tolerance and increased levels of circulating insulin in patients with chorea of Huntington have been described (62-65), but also denied (57,66-68). None of these patients showed clinical signs of diabetes mellitus.

Figure *2. Panel A: Immune staining of IRS-1 in total cell lysate after Western blotting. A14 cells, a NIH 3T3 derived cell line overexpressing insulin receptors, were stimulated with 1 jlM insulin for 10 minutes (lane 3) or kept unstimulated (lane 2). Cells were lysed and 30 fig of protein was applied onto an SDS-polyacrylamide gel and proteins were transferred to nitrocellulose filters. The filter was incubated with antibody against IRS-1 (rabbit) at a dilution of 1:2000. Visualization of the antibody was by peroxidase conjugated anti-rabbit IgG (goat, supplier Promega) followed by enhanced chemical luminescence (ECL, supplier Amersham). Before insulin stimulation, IRS-1 was stained as a single band at 180 kD. Insulin induces phosphorylation of a fraction of IRS-1 on multiple Tyr-residues, leading to a mobility shift of a fraction of the protein. (Lane 1 contains molecular weight markers). Panel B: Immune precipitation of Shc-GRB-2 complex by She-antibodies. A14 cells were kept unstimulated (lane 2) or stimulated with l^M insulin (lane 1). The protein She complexes to GRB-2 after insulin stimulation were lysed and She was immune precipitated with She-antibodies (rabbit, dilution 1:50) Immune complexes were isolated by protein A sepharose beads. The immune complex was analyzed for coprecipitating GRB-2 as outlined in panel C. It is demonstrated that insulin incubation induces coprecipitation of GRB-2. Panel C: Detection of GRB-2 in total cell lysates ofA14 cells. 30 [lg of total cell lysate was applied onto a SDS-polyacrylamide gel and the protein was transferred to nitrocellulose filters. The filter was incubated with GRB-2 antibodies (rabbit) at dilutions of 1:5000, 1:2500 and 1:1000, respectively, from left to right. Bound antibodies were visualized by ECL as described in panel A.*

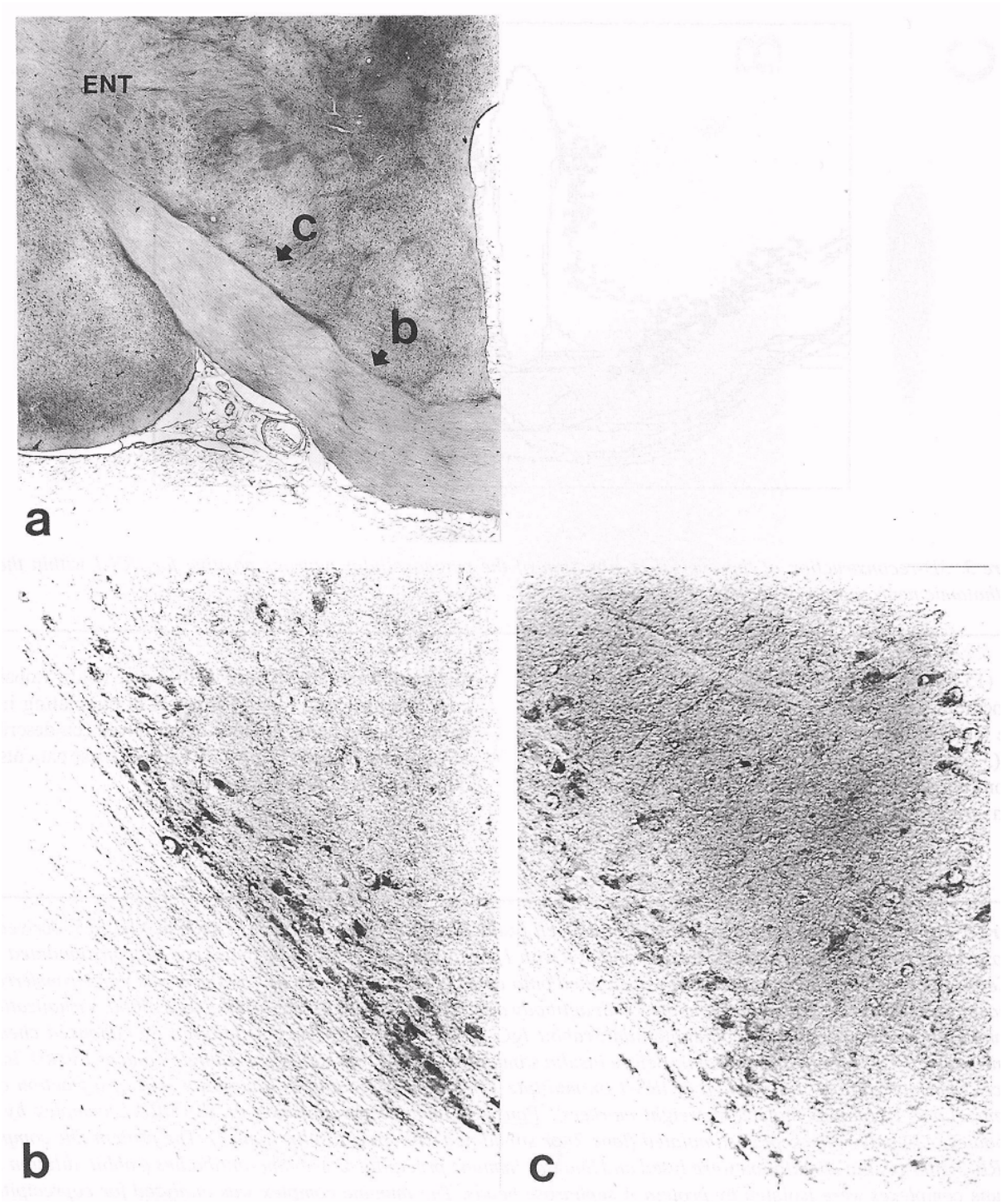

Figure 4. *Photomicrograph of cat magnocellular GRB-2- positive neurons near the optic tract, (a) Nissl overview of half hypothalamic area, with arrows indicating regions ilustrated in b and c. ENT - entopenduncular nucleus, (b) medial part of the supraoptic nucleus with GRB-2-positive neurons, (c) more lateral part of the supraoptic nucleus with GRB-2-positive neurons.*

In Alzheimer's disease patients, lowered fasting and postprandial glucose values (69) as well *as* an altered correlation between glucose and insulin levels after 24 hours fasting were reported (70).

Although disturbances of the basal forebrain magnocellular area in chorea of Huntington and in Alzheimer's disease are related to changes in glucose homeostasis, their presence could not firmly be proven. Moreover, changes in glucose homeostasis are till now not related to insulin receptor mechanisms in the brain, while the involvement of the insulin signaling pathway in Alzheimer's disease is also not proven.

Nevertheless, the existence of this insulin signaling system in the magnocellular forebrain system is an important finding, which requires check its presence or absence in the same forebrain areas in chorea of Huntington and in Alzheimer's disease.

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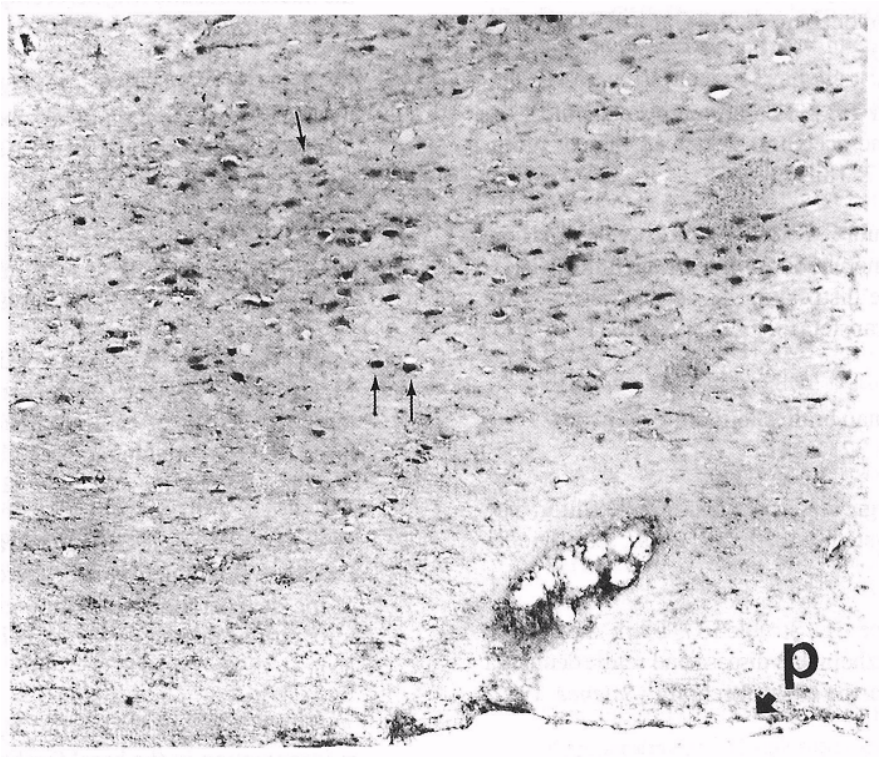

Figure 5. *Photomicrograph of the IRS-1 positivity (arrows) in the magnocellular forebrain neurons, subgroup Ch4ai in the human. P - pial surface.*

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