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DISTRIBUTION OF INSULIN RECEPTOR-LIKE IMMUNOREACTIVITY IN THE RAT FOREBRAIN

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Abstract—Previous studies have suggested that insulin may play a role in the hormonal regulation of neurotransmitter metabolisms within the central nervous system. In order to provide additional information to support this hypothesis, we examined the distribution of insulin receptors within the forebrain of adult male rats. Insulin receptors were localized by immunocytochemistry, using an antibody directed against the carboxy-terminus of the beta-subunit of the insulin receptor. The antibody specificity was tested by immunoprecipitation of brain insulin receptors with antiserum and the purity of the receptor–antibody preparation was determined using hormone binding-assays with radiolabeled insulin and insulin-like growth factor-1. Insulin receptor-like immunoreactivity was found in a widespread, but selective, distribution on neurons throughout the rat forebrain. Double-labeling with glial fibrillary acidic protein did not demonstrate any detectable insulin receptor-like immunoreactivity on glial cells. Areas with the highest density of insulin receptor-like immunoreactivity were found in the olfactory bulbs, hypothalamus and median eminence, medial habenula, subthalamic nuclues, subfornical organ, CA 1/2 pyramidal cell layer of the hippocampus and piriform cortex. Double-staining of hypothalamic sections with somatostatin and vasopressin antisera revealed insulin receptor-like immunoreactivity on a subpopulation of somatostatin neurons in the periventricular region and on vasopressin neurons in the supraoptic nucleus. A moderately dense insulin receptor-like immunoreactivity was observed in layers II–IV of cerebral cortex, medial amygdala, reticular thalamic nucleus, zona incerta, and preoptic and septal regions, whereas a low density of insulin receptor-like immunoreactive neurons was found in basolateral amygdala and most thalamic regions. The basal ganglia and most parts of the thalamus were almost devoid of insulin receptor-like immunoreactivity.

Our findings provide morphological support for a direct action of insulin on selected regions of the rat forebrain and suggest that the insulin receptor may modulate synaptic transmission or the release of neurotransmitters and peptide hormones in the CNS.

Previous studies have suggested that insulin may play a role in the hormonal regulation of brain metabolism. Recently, it has been reported that the insulin binding properties of brain membranes are similar to those of typical target tissues[36] and that the insulin receptor from brain has the same ability as the peripheral receptor to undergo insulin-stimulated autophosphorylation and to phosphorylate tyrosines on exogenous protein substitutes.[47,61,63] However, the insulin receptor from the central nervous system differs from peripheral receptors by the degree of glycosylation.[24,25]

Recent studies have also demonstrated a relatively high receptor content in the olfactory bulb, hypothalamus, limbic system and cerebral cortex. However, much of the information about the presence of insulin receptors in the brain has been obtained from hormone binding studies and autoradiography.[4,5,11,22,23,27,35,37,55,56,58,63] Although these results describe the insulin receptor content of a particular brain area, they have not provided information regarding the distribution of receptors between glia and neurons, nor can they further characterize other features of the insulin receptor-positive cells, such as their neurotransmitter or neuropeptide content. In the present report, we have employed immunocytochemistry using an antibody against a specific amino acid sequence in the carboxy-terminus region of the insulin receptor beta-subunit to map the regional distribution of insulin receptor-like immunoreactivity (IR) in the adult rat forebrain. We have also compared the pattern of distribution of insulin receptors to specific neuropeptides present in areas of the hypothalamus, using double labeling of neurons for somatostatin and vasopressin.

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Abbreviations: EDTA, ethylene diaminetetro-acetate; GFAP, glial fibrillary acidic protein; HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethane sulphonic acid; IGF-1, insulin-like growth factor 1; IR, insulin receptor-like immunoreactivity; PAP, peroxidase–antiperoxidase; PBS, phosphate-buffered saline; S.E., standard error; SON, supraoptic nucleus; TPS, Tris–phosphate–saline.

EXPERIMENTAL PROCEDURES

Preparation and characterization of antiserum

The carboxy-terminus antibody (A-1314) was prepared by immunizing rabbits with a peptide that corresponds to

the unique amino acid sequence 1314–1324 from the carboxy-terminus of the human insulin receptor beta-subunit, i.e. sequence ArgSerTyrGluGluHisIleProTyrThr-His.[50] The peptide was coupled to keyhole limpet hemocyamin with bromoacetyl bromide as described by Pang *et al.*[41] Antiserum was subjected to affinity purification by passage over an Affi-gel column to which the peptide was coupled. Following extensive washing, the antibody was eluted by 100 mM glycine, pH 2.5 and then dialysed against 50 mm HEPES, pH 7.4. Absence of cross-reactivity with the receptor for insulin-like growth factor-1 was tested by insulin and insulin-like growth factor 1 (IGF-1) binding-assays on a brain receptor preparation, isolated by immunoprecipitation with the antibody to the beta-subunit (Fig. 1). Freshly dissected diencephalon from four rats was homogenized in 750 μl of a 50 mM HEPES buffer, pH 7.6, that contained 1% Triton X-100, 2 mM phenylmethylsulfonylfluoride, 5 mM EDTA, 1 mg/ml bacitracin and 0.1% bovine albumin. The homogenate was subjected to centrifugation (15,000 *g* for 30 min) and the supernatant diluted 1:10 with homogenization buffer in the absence of Triton X-100. Affinity purified A-1314 (1 μg protein) was added to 2 ml of the diluted receptor extract. Radiolabeled hormone ([^{125}I]insulin or [^{125}I]IGF-1, 50 pM final concentration) was added to 40-μl aliquots of the antibody and receptor extract in the presence or absence of the indicated concentration of unlabeled hormone, all contained in a total volume of 50 μl.[1] Following an overnight incubation at 4°C, immobilized protein A (Pansorbin, Segira Chemical Co.) was added and after 1 h the complex of protein A, antibody, receptor and radiolabeled ligand was collected by centrifugation. The pellet was washed once with the homogenization buffer without Triton X-100, and its radioactivity determined by gamma scintillation counting. The amount of hormone bound to receptor that was not immunoprecipitated was determined by subjecting the supernatant to polyethylene glycol (PEG) precipitation as described for small samples.[1]

Immunocytochemical staining of A-1314 was also compared to two other antibody preparations in order to further validate the specificity of the antibody staining reaction. The first was a polyclonal antibody, termed L-1, which was raised in rabbits against a highly purified preparation of rat liver insulin receptors. The ability of this antiserum to immunoprecipitate rat insulin receptors has been described previously.[2] The second antibody, termed alpha-IR$_1$, was a gift from Dr Steven Jacobs (Research Triangle Park, NC, U.S.A.). This monoclonal antibody is directed against the human insulin receptor and the characteristics of its specificity have been described previously.[34]

Immunocytochemistry

Six male Sprague–Dawley rats (body weight 150–200 g; Charles Rivers, Wilmington, MA) were anesthetized with pentobarbital (Nembutal) (50 mg/kg i.p.) and perfused through a cardiac canula with 100 ml ice-cold phosphate-buffered saline (PBS), pH 7.4, followed by 200 ml of a cold solution of 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4. The brain was removed and immersed in the same fixative for 2 h at 4°C. Following fixation, one set of three brains was cut on a Vibratome at a thickness of 40 μm in the coronal plane. Another set of three brains was immersed in increasing concentrations of sucrose in 0.1 M phosphate buffer, pH 7.4 for 48 h, and 40-μm frozen sections were cut on a sliding microtome. Vibratome and frozen sections were incubated free-floating in the antibody-dilutions.

In addition, two tissue blocks of human brains containing the hypothalamus (ages 47 and 63 years) were obtained at autopsy and fixed in a 1:1-solution of 4% paraformaldehyde/10% formalin for 1 week at 4°C. The times between death and immersion in fixative were 6 and 8 h, and neither case had any history of neurologic or psychiatric illness. After fixation, blocks containing the hypothalamus were placed in increasing concentrations of sucrose and 40-μm frozen sections were processed for immunocytochemistry with A-1314, L-1 as well as the alpha-IR$_1$ antibodies.

Tissue sections were processed according to the peroxidase–antiperoxidase (PAP) method described by Sternberger[49] or using the avidin–biotin method. In these studies the affinity purified A-1314 was commonly used at a dilution of 1:1000 (protein content of 46 ng/ml). The dilutions of the L-1 antisera were 1:1000–1:5000, whereas the monoclonal antibody alpha-IR$_1$ was used at a dilution of 1:200. The antisera, as well as PAP, were diluted in 0.1 M Tris–phosphate–saline (TPS), pH 7.8, that contained 1% normal sheep serum and 0.3% Triton X-100.

The sections were incubated for 24–48 h at 4°C with the indicated antibody. The sections were then rinsed several times in TPS, and incubated for 1 h at room temperature with sheep anti-rabbit IgG diluted 1:100 for the A-1314 and L-1 antibodies. The sections were again rinsed several times in TPS and then incubated with a 1:800 dilution of PAP (CAPPEL) for 1 h at room temperature. Immunostaining with the monoclonal alpha-IR$_1$ antibody was carried out by using the avidin–biotin method ("Vectastain ABC-Kit"). Following several rinses with TPS, sections were incubated for 5–10 min in a solution of 1 mg/ml diaminobenzidine (SIGMA) in TPS that contained 0.01% H_2O_2. Some sections were counterstained with Toluidine Blue to facilitate anatomical orientation before placing the coverslip.

Control staining was carried out by preincubation of the A-1314 antibody with the 11-amino acid antigenic peptide from the insulin receptor (20 μg/ml of diluted antibody) or with the same amount of a peptide that corresponds to a similar region in the IGF-1 receptor molecule (amino acid sequence 1294–1304 of the human IGF-1 receptor).[51] Additional controls were performed by omitting the first antibody or by replacing it with normal rabbit serum. These procedures resulted in complete absence of staining, except in the experiment of preincubation with the IGF-1 receptor peptide, which did not reduce staining (Fig. 2a–c).

Sections were examined with a Nikon microscope, equipped with bright-field and dark-field optics, or a Zeiss "Axiophot" microscope using Nomarski optics.

Double-staining procedure

In order to characterize the cell type or the neuropeptide content of cells staining positively for the insulin receptor, sections previously stained for IR were incubated in antisera directed against glial fibrillary acidic protein (GFAP), somatostatin or vasopressin. GFAP antiserum was provided by Dr L. Lapham (Department of Pathology, University of Rochester, NY), somatostatin antiserum by Drs A. Weindl and W. Lange (Department of Anatomy, University of Munich, F.R.G.) and vasopressin antiserum by Dr C. Sladek (Department of Neurology, University of Rochester, NY). GFAP-, somatostatin- and vasopressin-antibodies were used in dilutions of 1:500 and 1:1000 and the specificity of these antisera have been described previously.[16,48,52] The sections were incubated in either GFAP, somatostatin or vasopressin antisera for 24–48 h at 4°C. After rinsing three times in 0.1 mM PBS, pH 7.6, they were incubated in fluorescein isothiocyanate conjugated swine anti-rabbit immunoglobulin (DAKOPATTS; diluted 1:10 in PBS, containing 0.3% Triton X-100) for 45 min at 37°C.[30] After two rinses in PBS, the sections were mounted with glycerol/PBS (3:1) and examined under a Nikon epifluorescence microscope. Since IR forms a punctuate profile on the surface of cell bodies and processes, whereas peptide immunorectivities are distributed homogeneously throughout the cells, it was possible to identify cells that colocalized IR and either GFAP-, somatostatin- or vasopressin-immunoreactivity by combining epifluorescence with dark- or bright-field optics.

Abbreviations used in figures

3V	third ventricle
AAD	anterior amygdaloid area, dorsal
AAV	anterior amygdaloid area, ventral
Acb	accumbens nucleus
AcbC	accumbens nucleus, core
AcbSh	accumbens nucleus, shell
ACo	anterior cortical amygdaloid nucleus
AD	anterodorsal thalamic nucleus
AH	anterior hypothalamus
AHA	anterior hypothalamic area, anterior part
AHiAL	amygdalohippocampal area, anterolateral part
AI	agranular insular cortex
AM	anteromedial thalamic nucleus
AOD	anterior olfactory nucleus, dorsal part
AOL	anterior olfactory nucleus, lateral part
AOM	anterior olfactory nucleus, medial part
AOV	anterior olfactory nucleus, ventral part
Arc	arcuate hypothalamic nucleus
AVDM	anteroventral thalamic nucleus, dorsomedial part
AVVL	anteroventral thalamic nucleus, ventrolateral part
B	basal nucleus of Meynert
BLP	basolateral amygdaloid nucleus, posterior part
BMP	basomedial amygdaloid nucleus, posterior part
BSTMPL	bed nucleus of the stria terminalis, medial division, posterolateral part
BSTS	bed nucleus of the stria terminalis, supracapsular division
ca	anterior commissure
CA1–3	fields CA1–3 of Ammon's horn
cc	corpus callosum
Cg1	cingulate cortex, area 1
Cg2	cingulate cortex, area 2
Cg3	cingulate cortex, area 3
CL	centrolateral thalamic nucleus
Cl	claustrum
CM	central medial thalamic nucleus
CPu	caudate putamen
Cx	neocortex
CxA	cortex–amygdala transition zone
DEn	dorsal endopiriform nucleus
df	dorsal fornix
DG	dentate gyrus
DI	dysgranular insular cortex
DLG	dorsal lateral geniculate nucleus
DM	dorsomedial hypothalamic nucleus
D3V	dorsal third ventricle
FL	forelimb area of cortex
Fr1	frontal cortex, area 1
Fr2	frontal cortex, area 2
FStr	fundus striati
gcc	genu corpus callosum
Gem	gemini hypothalamic nucleus
GI	granular insular cortex
GP	globus pallidus
Gu	gustatory thalamic nucleus
Hb	habenula
HDB	nucleus of the horizontal limb of the diagonal band
HL	hindlimb area of the cortex
I	intercalated nuclei of the amygdala
IAD	interanterodorsal thalamic nucleus
ICj	islands of Calleja
ICjM	islands of Calleja, major island
IG	indusium griseum
IMD	intermediodorsal thalamic nucleus
InfS	infundibular stem
LA	lateroanterior hypothalamic nucleus
LaDL	lateral amygdaloid nucleus, dorsolateral part
LaVM	lateral amygdaloid nucleus, ventromedial part
LH	lateral hypothalamic area
LHbL	lateral habenular nucleus, lateral part
LHbM	lateral habenular nucleus, medial part
LO	lateral orbital cortex
LOT	nucleus of the lateral olfactory tract
LPLR	lateral posterior thalamic nucleus, laterorostral part
LPMR	lateral posterior thalamic nucleus, mediorostral part
LSD	lateral septal nucleus, dorsal part
LSI	lateral septal nucleus, intermediate part
LSV	lateral septal nucleus, ventral part
MCPO	magnocellular preoptic nucleus
MD	mediodorsal thalamic nucleus
MHb	medial habenular nucleus
MO	medial orbital cortex
MPA	medial preoptic area
MS	medial septal nucleus
OB	olfactory bulb
Oc2L	occipital cortex, area 2, lateral part
Oc2ML	occipital cortex, area 2, mediolateral part
Oc2MM	occipital cortex, area 2, mediomedial part
ox	optic chiasm
PaAP	paraventricular hypothalamic nucleus, anterior parvocellular part
Par1	parietal cortex, area 1
Par2	parietal cortex, area 2
Pe	periventricular hypothalamic nucleus
PF	parafascicular thalamic nucleus
Pir	piriform cortex
PLCo	posterolateral cortical amygdaloid nucleus
PMCo	posteromedial cortical amygdaloid nucleus
PMV	premammillary nucleus, ventral part
Po	posterior thalamic nuclear group
PoDG	polymorph layer of the dentate gyrus
PRh	perirhinal cortex
PVA	paraventricular thalamic nucleus, anterior part
PVP	paraventricular thalamic nucleus, posterior part
Py	pyramidal cell layer of the hippocampus
R	red nucleus
Re	reuniens thalamic nucleus
Rh	rhomboid thalamic nucleus
RSA	retrosplenial agranular cortex
RSG	retrosplenial granular cortex
Rt	reticular thalamic nucleus
SCh	suprachiasmatic nucleus
SFO	subfornical organ
SHi	septohippocampal nucleus
SI	substantia innominata
SM	nucleus of the stria medullaris
SO	supraoptic nucleus
SPF	subparafascicular thalamic nucleus
SStr	substriatal area
STh	subthalamic nucleus
Te	terete hypothalamic nucleus
Te1	temporal cortex, area 1
Te3	temporal cortex, area 3
Th	thalamus
TM	tuberomammillary nucleus
TMC	tuberal magnocellular nucleus
TS	triangular septal nucleus
TT	tenia tecta
Tu	olfactory tubercle
VDB	nucleus of the vertical limb of the diagonal band
VEn	ventral endopiriform nucleus
vhc	ventral hippocampal commissure
VLG	ventral lateral geniculate nucleus
VLO	ventrolateral orbital cortex
VMH	ventromedial hypothalamus
VO	ventral orbital cortex
VP	ventral pallidum
VPL	ventral posterolateral thalamic nucleus
VPM	ventral posteromedial thalamic nucleus
ZI	zona incerta
ZID	zona incerta, dorsal part
ZIV	zona incerta, ventral part

RESULTS

Specificity of antibody

The amino acid sequence used to synthesize the peptide for the generation of A-1314 was taken from the deduced sequence of the insulin receptor beta-subunit, beginning at Arg_{1314} and ending at His_{1324}.[50] This portion of the insulin receptor is unique among known proteins and is not present in the closely related receptor for IGF-1.[51] The ability of A-1314 to immunoprecipitate the brain insulin receptor is shown in Fig. 1. Since the insulin binding site of the receptor is in the alpha-subunit,[15] antibody binding to the beta-subunit does not block binding of the hormone. Consequently, it is possible to conduct a standard insulin binding assay with the receptor–antibody complex.

The competition curve with native insulin demonstrates that the binding activity immunoprecipitated by the antibody is one of high affinity. Furthermore, native IGF-1 at 5 ng/ml failed to compete with tracer insulin for binding and 50 ng/ml IGF-1 competed poorly when compared to the results gathered with the same insulin concentration, i.e. 50 ng/ml insulin inhibited tracer insulin binding by 74% ± 5% (standard error, S.E.) in comparison to an inhibition of 35% ± 8% by 50 ng IGF-1.

[^{125}I]IGF-1 (50 pM) failed to demonstrate any specific binding in the material immunoprecipitated by the antibody. Therefore, these data could not be placed on the graph in Fig. 1. The results strongly indicate that the receptors precipitated by the antibody are insulin receptors.

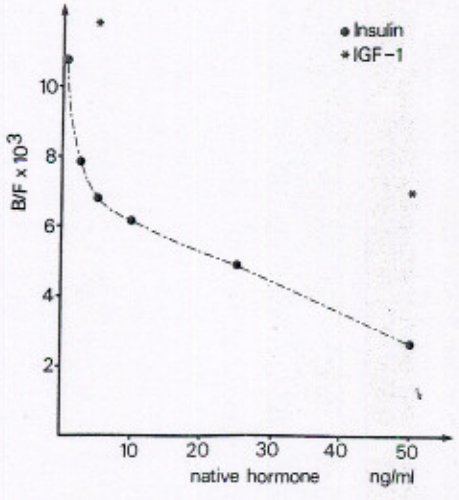

Fig. 1. Specific insulin binding by the immunoprecipitated insulin receptors from rat brain. The rat brain receptor extract was incubated with [^{125}I]insulin (50 pM) in the presence or absence of the indicated concentration of native insulin or native IGF-1 as described in Experimental Procedures. The amount of specifically bound [^{125}I]insulin immunoprecipitated by the antibody A-1314 is plotted as ratio of the bound versus free radiolabeled hormone.

The insulin binding left in solution after "clearing" with immobilized protein A has low affinity for insulin since the competition curve is scattered and has a shallow slope, i.e. half-maximal inhibition by insulin concentration greater than 50 ng/ml (data not shown). Because of the very low insulin binding affinity and the inability of 5 ng/ml or 50 ng/ml IGF-1 to inhibit tracer insulin binding, we could not identify this material as either the native insulin receptor or the receptor for IGF-1. The amount of this low affinity insulin binding activity left in the extract is about one-half that in the immunoprecipitate based on tracer binding activity, i.e. a B/F value of 12×10^3.

Although the experiment with [^{125}I]IGF-1 failed to demonstrate specific IGF-1 binding activity in the immunoprecipitate, specific IGF-1 binding activity was present in the brain extract. The B/F ratio for IGF-1 binding at the tracer concentration is 81×10^3, which is much higher than the 11×10^3 for specific insulin binding in the immunoprecipitate or the 12×10^3 insulin binding left in the PEG precipitate. Tracer binding of IGF-1 was inhibited by 50% ± 6% (S.E.) by 20 ng/ml native IGF-1 in this experiment. Therefore, even though a relatively large amount of IGF-1 binding activity was present in the brain extract, the A-1314 antibody did not cross-react with this receptor.

In similar experiments with the L-1 antiserum, there was a small amount of IGF-1 binding activity immunoprecipitated with the insulin receptor (data not shown). The degree of cross-reactivity with the IGF-1 receptor was approximately 10%, i.e. in a receptor extract that contained equal numbers of insulin and IGF-1 receptors, the immunoprecipitate prepared with this antibody will contain 90% insulin receptors and 10% IGF-1 receptors.

Immunocytochemistry

Immunocytochemistry with the two polyclonal antisera directed against the insulin receptor (A-1314, L-1) showed similar staining patterns of IR in adjacent sections throughout the forebrain of the rat. However, there was a slight difference in the appearance of the brown 3,3′-diaminobenzidine-reaction product on positively stained neurons and proximal processes. Staining with A-1314 resulted in fine, punctuate profiles on cells, while L-1 revealed a less distinct profile on the cells with large macroaggregates of reaction product. In addition A-1314 and L-1 showed immunoreactive structures in identical areas of the human hypothalamus (e.g. periventricular region) to that seen with the monoclonal alpha-IR_1 antibody, which is directed against the human insulin receptor (Fig. 2d–f). Immunohistochemical staining using A-1314 was completely abolished after preincubation with the antigenic peptide (Fig. 2b) and by omitting the first antibody or replacing it with non-immunserum. Staining with alpha-IR_1 was completely absent in the rat brain. These morphological findings further indicate the

ability of A-1314 to label specifically IR in the brain. Therefore, this antibody was used in the present mapping study.

Reaction product that localized the insulin receptor immunoreactivity was found in a widespread distribution throughout the forebrain of the adult rat, predominently on neuronal elements. On neurons, IR was observed as punctuate profiles on the perikarya and proximal processes. Overall, the densest IR was observed in hippocampus and hypothalamus, followed by habenula, olfactory regions, subthalamic nucleus, piriform cortex, neocortex and amygdala. The basal ganglia as well as most thalamic subnuclei contained very little IR. In addition to nerve cells, granular reaction product was present on non-neuronal structures, such as ependyma of the third ventricle, tanycytes in the median eminence and matrix cells of the choroid plexus. However, double-staining with GFAP in tissue sections through the rat forebrain showed no immunoreactivity on glial cells (e.g. astrocytes).

Because of the widespread distribution of IR, we have divided the results into three anatomical areas (rhinencephalon, telencephalon and diencephalon),

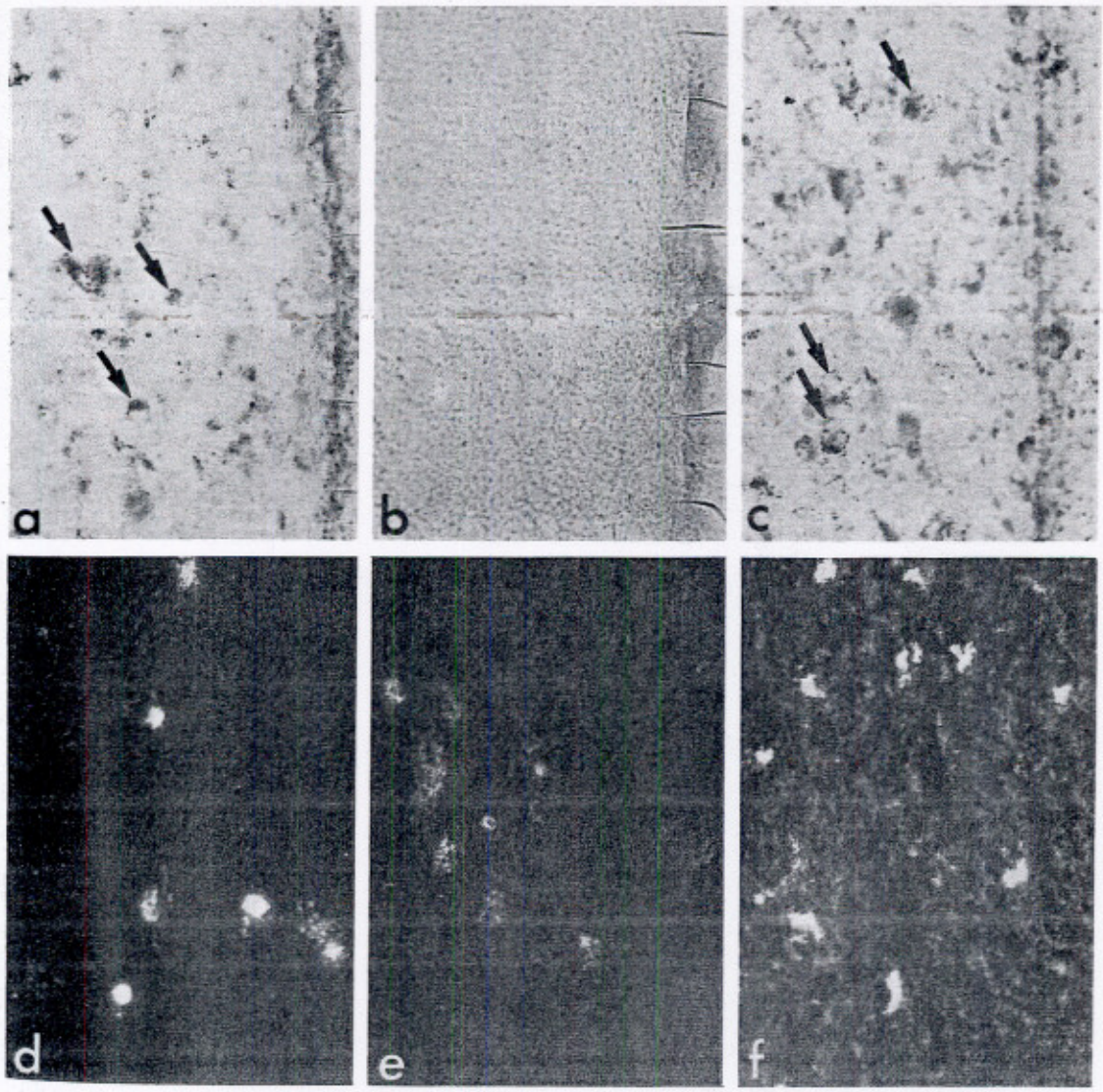

Fig. 2. Specificity test of the immunoperoxidase reaction in frontal sections through the periventricular region of the rat (a–c) and human (d–f) hypothalamus. a. IR, using A-1314, is found on neurons as well as ependymal cells of the third ventricle in rat forebrain. b. Preincubation of A-1314 with the antigenic peptide from the insulin receptor (20 μg/ml of diluted antiserum) eliminated all immunoreactivity in the hypothalamus. c. Preincubation with the peptide from the corresponding locus of the IGF-1 receptor does not reduce specific staining. d–f. Dark-field photomicrographs of human hypothalamus. All three antisera A-1314 (d), L-1 (e) and alpha IR-1 (f) revealed similar staining patterns with scattered IR cells in the periventricular area. (a–c, $\times$160; d–f, $\times$320).

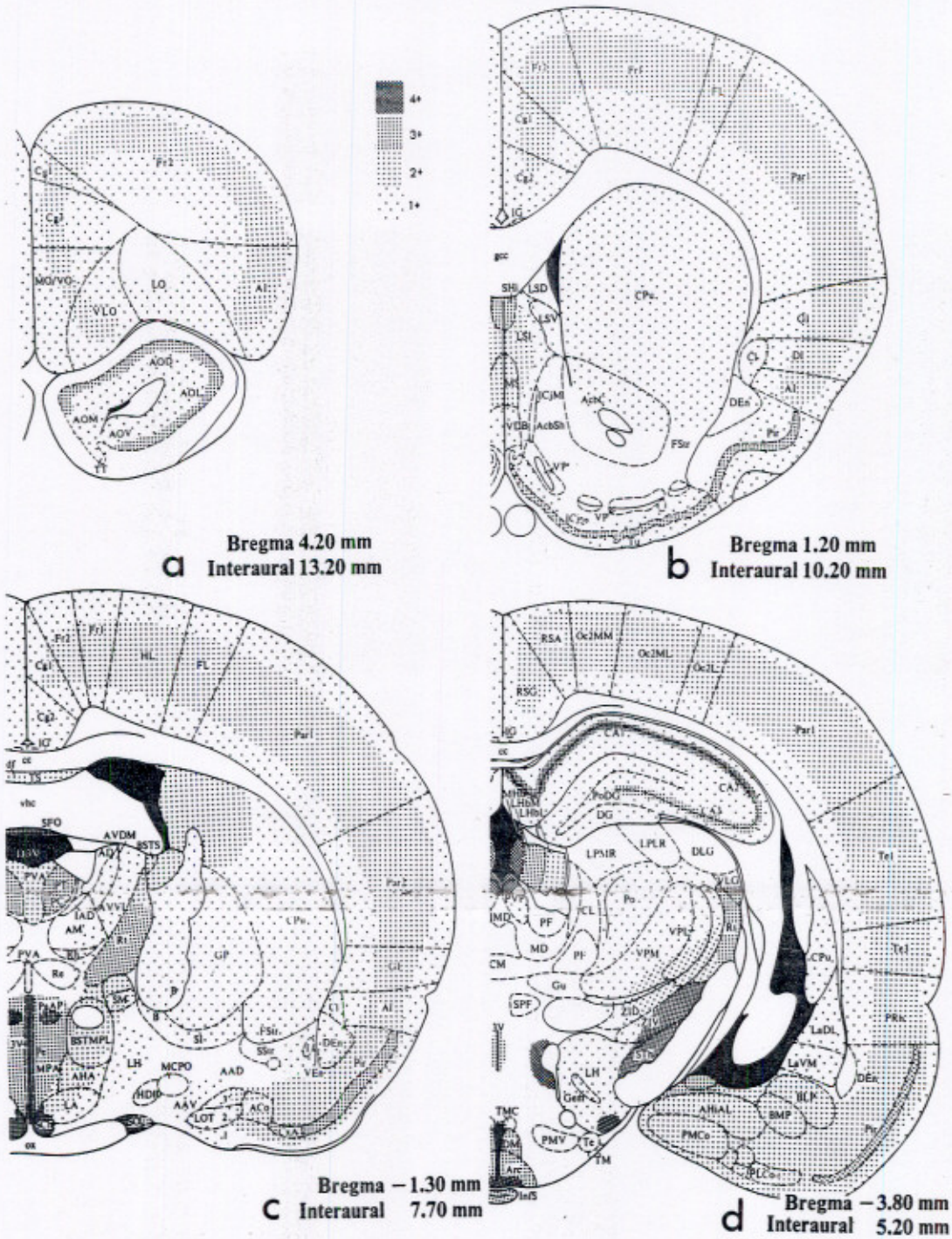

Fig. 3. Schematic coronal sections of the rat forebrain. a–d represent four different levels of the rat brain, indicated by stereotaxic coordinates (bregma, interaural line). Abbreviations are taken from the atlas of Paxinos and Watson.[42] The density of IR has been graded into four different categories: 4+ very high density; 3+ high density; 2+ moderate density; 1+ low density.

each with its own set of anatomical descriptions, schematics and photomicrographs.

Rhincencophalon, olfactory bulb

In the olfactory bulb the densest concentration of reaction product was found on neurons in the external plexiform layer and granule layer, whereas moderate dense staining was seen in the glomerular layer and anterior olfactory nucleus (Figs 3c and 4a). In contrast, the pars lateralis as well as the pars externa showed a low density of reaction product.

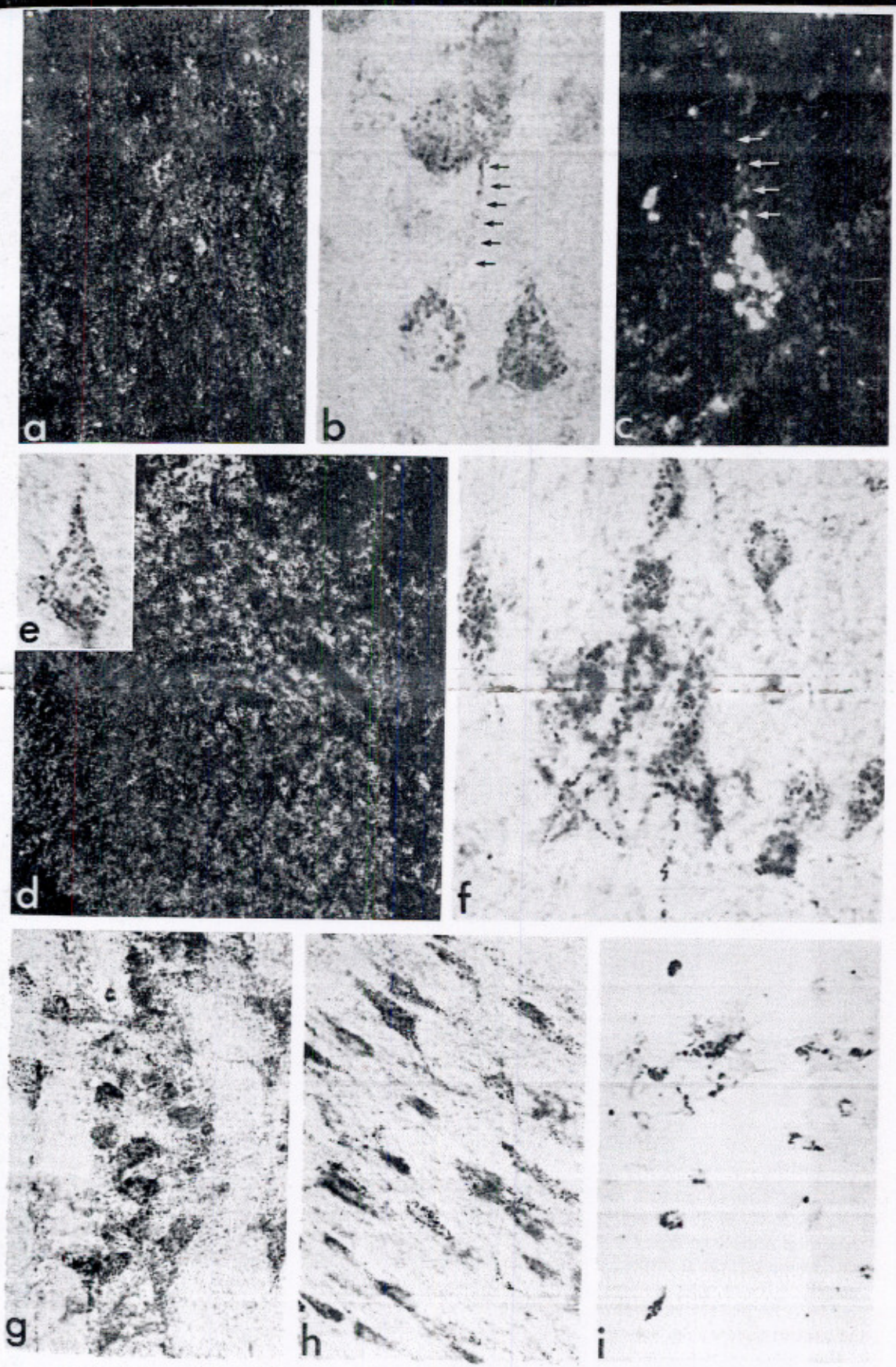

Fig. 4. Light- and dark-field photomicrographs, showing IR in different brain areas of the rat forebrain. a. High density of IR is present in the dorsal part of the anterior olfactory region (×160). b. IR on pyramidal neurons in layers III/IV of the neocortex. Note the fine granular profile of IR (×960). c. A single immunoreactive neuron in the white matter directly below the cortical mantle (×960). d and e. Very high density of IR is located on neurons throughout the medial habenula. d. Survey, dark-field (×160). e. Nomarski photomicrograph of an IR-positive neuron in the medial habenula (×800). f. A cluster of IR neurons in the lateral habenula. IR is present on perikaya, as well as on processes (×640). g. Scattered IR neurons are distributed throughout the reticular thalamic nucleus. Note the absence of reaction product on fiber tracts running between IR nerve cells (×320). h. IR on neurons of the subthalamic nucleus. (×320). i. A population of IR cells is found in the matrix of the choroid plexus (×320).

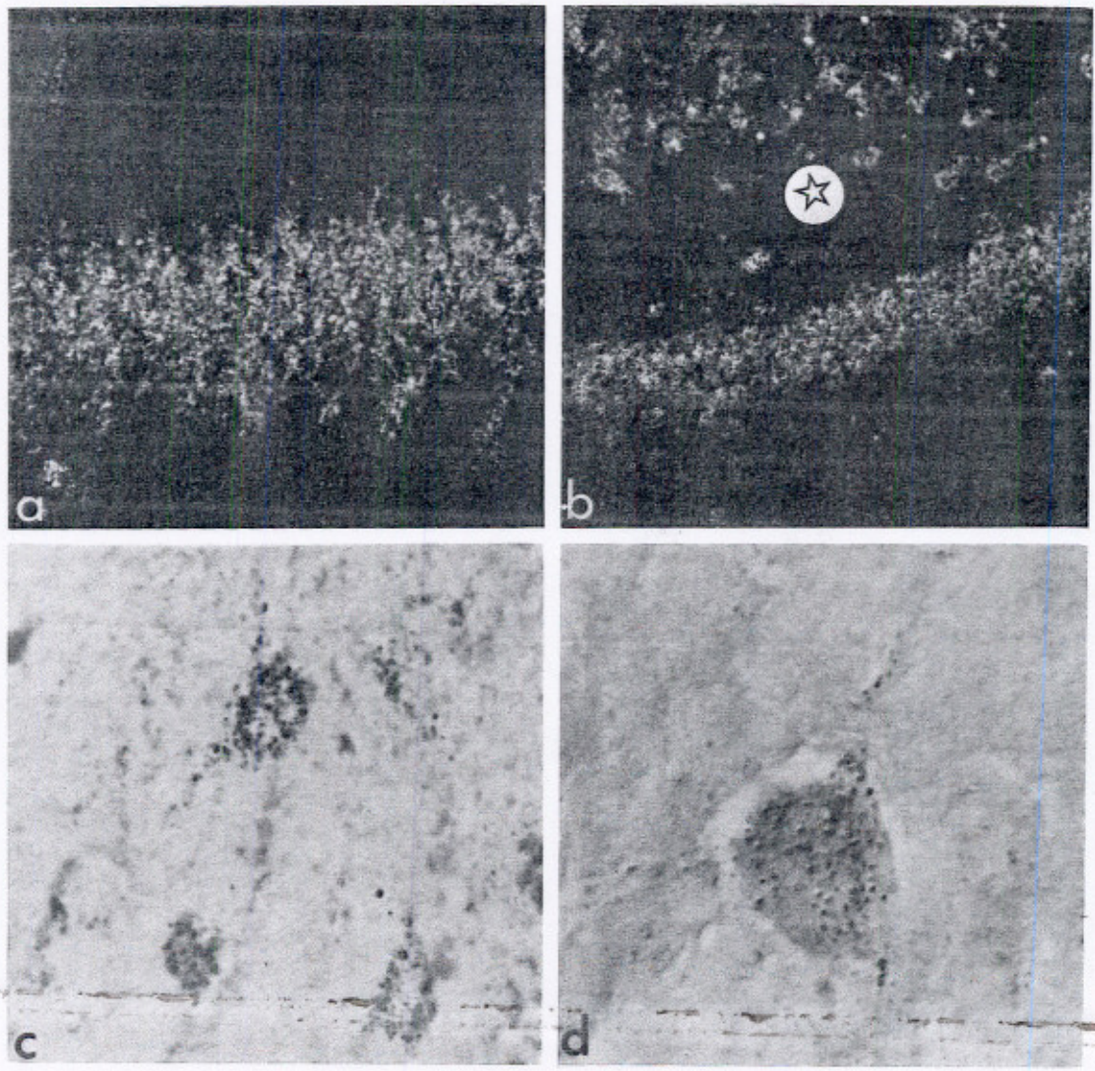

Fig. 5. Light- and dark-field photomicrographs of IR in the hippocampus. a. The highest density is present in the CA1 and CA2 pyramidal cell layer (×320). b. Moderate density of IR is present in the granule cell layer of the dentate gyrus, as well as on a group of neurons (☆) in the hilus of the dentate gyrus (×160). c and d. Single IR neurons are found throughout all hippocampal areas (c. ×960; d. Nomarski photomicrograph, ×1600).

Telencephalon

Cerebral cortex. All parts of the cortex contained dense IR. Receptor-positive cells were distributed throughout layers II–VI with the densest concentration on neurons in layers II–IV (Figs 3 and 4b), whereas the superficial cortical layer contained very little IR. A few receptor-positive neuronal cell bodies could also be found in the white matter directly below the cortical mantle (Fig. 4c).

Hippocampus. Reaction product localizing the receptor was found throughout the rostro-caudal extent of the hippocampal complex. The highest density of IR was observed in the pyramidal cell layer (Figs 3d and 5a) and moderate IR content was found in the granule cell layer of the dentate gyrus (Figs 3d and 5b). In addition, the density of fine granular reaction product revealed marked differences in areas of the cornu ammonis. Whereas CA1 and CA2 areas of the hippocampus showed a high density of reaction product, there was a significantly lower density in CA3 and CA4. In addition, the hilus of the dentate gyrus contained groups of insulin receptor-positive neurons. Single immunoreactive perikarya were found in the molecular layer of the dentate gyrus (Figs 5c and d).

Amygdala and piriform cortex. In the amygdala, granular reaction product was present with different densities in all regions of the amygdaloid complex. The differences in the distribution of IR within the amygdala did not completely follow subregional anatomical borders, but showed some overlap be-

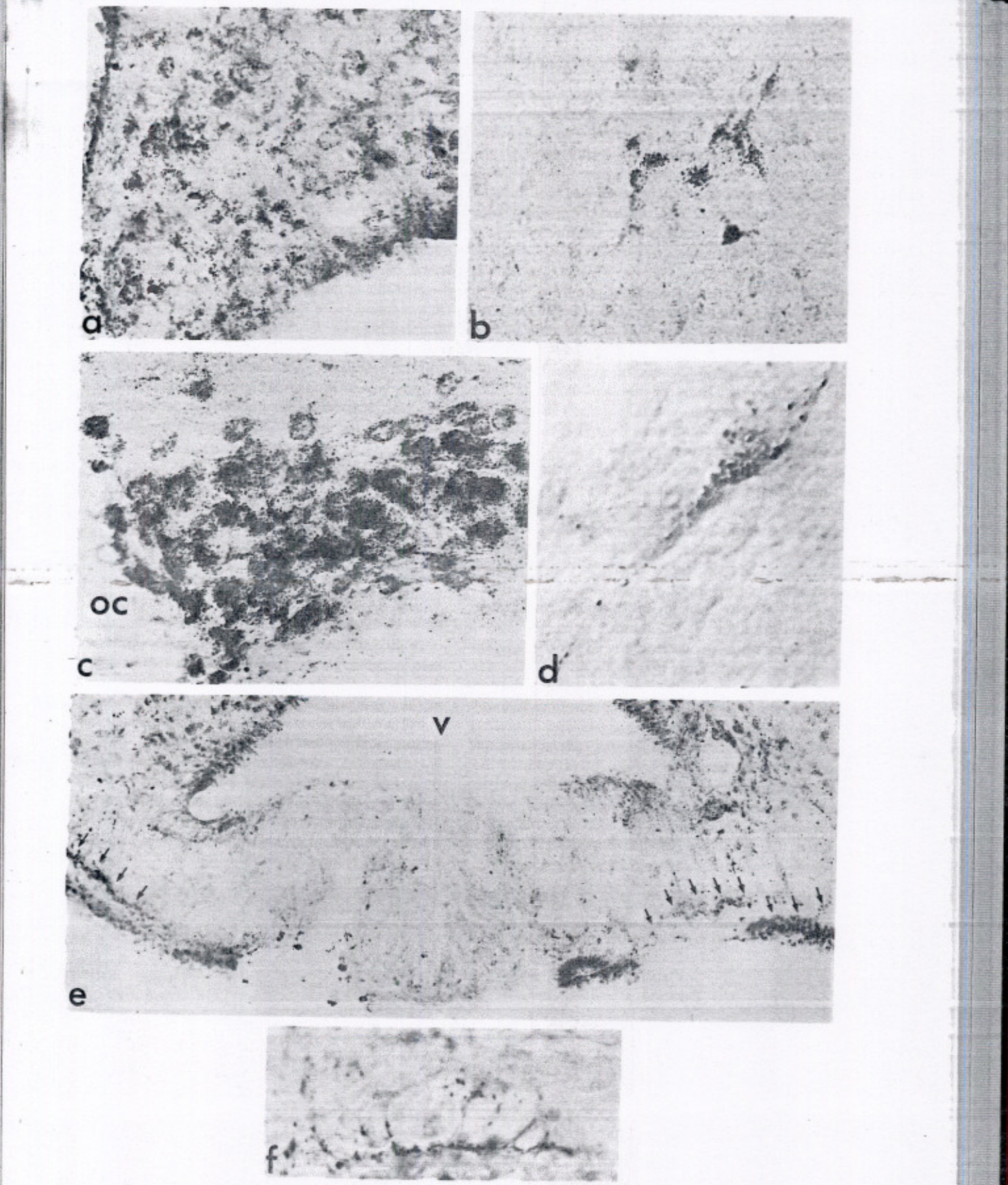

Fig. 6. Photomicrographs demonstrating IR in hypothalamic regions. a. Numerous receptor-positive neurons are located throughout the arcuate nucleus (×320). b. A distinct group of IR neurons is found in the posterior hypothalamic area with a few scattered cells extend into the lateral hypothalamus (×320). c. Frontal section through the ventromedial portion of the supraoptic nucleus, showing a high density of IR; oc, optic chiasm (×320). d. Example of an IR-positive neuron in the suprachiasmatic nucleus (Nomarski photomicrograph, ×1200). e. Frontal section through the median eminence. Note the bands of IR in the lateral parts of the zona externa (arrows), whereas moderate to low receptor density is localized in other parts of the median eminence; v, third ventricle (×160). f. In the median eminence, IR is found to be closely associated with tanycytes that surround blood vessels (×320).

tween subnuclei. However, neurons in the medial nucleus showed the densest aggregation of IR. A moderate density of immunoreactive perikarya was seen in the cortical nucleus of the amygdala and the basal nucleus (pars lateralis), whereas the remaining subregions of the amygdala exhibited a low density of IR. In the piriform cortex, a band of dense granular reaction product was demonstrated throughout its entire extent (Fig. 3b–d).

Diencephalon

Habenula and subfornical organ. Very dense IR was found throughout the medial habenular nucleus (Figs 3d, 4d and e), whereas the lateral habenula contained scattered groups of positive perikarya, primarily in its medial part (Figs 3d and 4f). The remaining habenular areas contained a moderate density of IR. Furthermore, a high density of IR was demonstrated in the subfornical organ.

Thalamus and geniculate nucleus. In general, the thalamus had very few IR structures. Only in the reticular thalamic nucleus was dense IR observed on neurons (Figs 3c and 4g). This population of immunoreactive cells extended into the ventral lateral geniculate nucleus, where moderately dense IR was observed.

Subthalamus. A large group of positive-staining cells was seen in the subthalamic nucleus (Figs 3d and 4h). These cells extended into the zona incerta, where single positive neurons were located.

Hypothalamus. Many hypothalamic areas contained high densities of IR. Numerous positive cells were found in the anterior hypothalamus, including high densities of IR in the suprachiasmatic and supraoptic nucleus (SON; Figs 3c, 6c and d). The reaction product formed punctuate profiles over the neurons predominently in a ventromedial part of the magnocellular SON. In addition, moderately dense IR was seen in the dorsolateral part of the SON. The medial preoptic area also showed moderate to low IR, while most of the remaining areas of the anterior hypothalamus contained a low density of reaction product.

A very high density of reaction product was localized close to the third ventricle in the periventricular region, in the dorsomedial nucleus and in the arcuate nucleus (Figs 3c and 6a). In addition, a high concentration of granular reaction product was found in the lateral part of the rostral median eminence with a dense profile in the external layer (Fig. 6e). However, IR was found to be less dense in caudal parts of the median eminence. Moderate dense IR was present in the ventromedial hypothalamic nucleus.

In the posterior hypothalamus a group of positive neurons was located in the posterior hypothalamic area with scattered immunoreactive perikarya extending into the lateral hypothalamic area (Fig. 6b).

Other receptor-positive brain structures

IR was identified on selective cells within the matrix of the choroid plexus in the lateral ventricles (Fig. 4i), as well as closely associated with the ependyma of the third ventricle (Fig. 2a). IR was also observed along the surface of tanycytes surrounding blood vessels in the lateral margin of the median eminence (Fig. 6f).

Colocalization with neuropeptides

Double-staining immunocytochemistry with somatostatin antibody revealed IR on somatostatin-immunoreactive neurons in the periventricular area of the hypothalamus (Fig. 7a and b). Approximately 30–50% of the somatostatin perikarya demonstrated immunostaining for the insulin receptor. Outside the hypothalamus, occasional colocalization of insulin receptors and somatostatin was observed in single neurons in the medial amygdala and cerebral cortex. No colocalization was detected in hippocampus. In addition, double-staining with vasopressin antibody

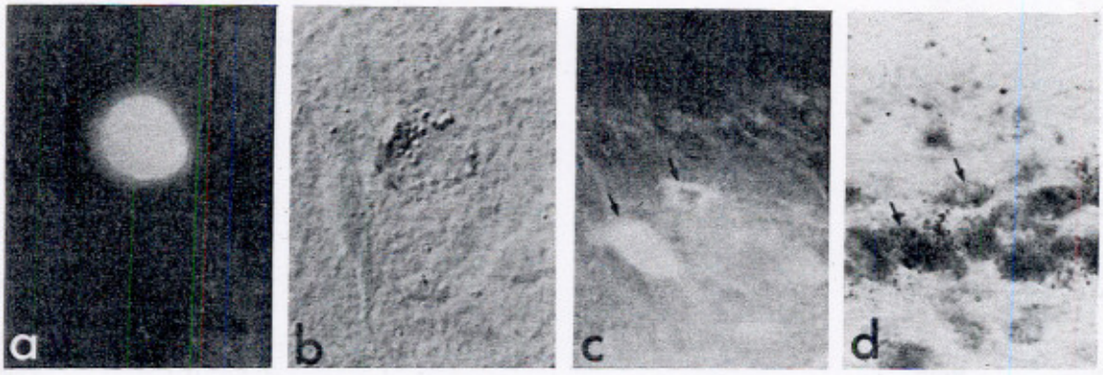

Fig. 7. Colocalization of IR with the neuropeptides somatostatin and vasopressin in rat brain. a. Somatostatin-immunofluorescence of a neuron in the periventricular area of the hypothalamus. b. Normaski photomicrograph of the same neuron, stained for IR. Note the inhomogenous distribution of IR over the cell surface in contrast to the homogenous fluorescence of somatostatin immunoreactivity (a and b ×640). c. Immunofluorescence for vasopressin in neurons of the supraoptic nucleus. d. Bright-field photomicrograph, showing fine granular reaction product of IR on identical vasopressin-immunoreactive cells as demonstrated in c (c and d ×320).

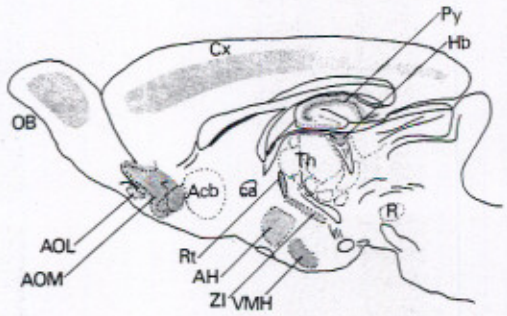

Fig. 8. Schematic overview of the IR distribution in areas of the rat forebrain.

showed dense insulin receptor-positive neurons in a ventromedial portion of the SON that contained vasopressin neurons (Fig. 7c and d).

DISCUSSION

We have used immunocytochemistry to describe the distribution of insulin receptors in discrete regions of the adult rat forebrain as well as selected neuronal populations, which were further characterized by double-labeling with antisera against specific neuropeptides. As with any immunocytochemical study, the validity of the data is based on the specificity of the primary antibody. The antiserum that we have used was affinity purified and characterized by its ability to immunoprecipitate exclusively insulin receptors from rat brain. Specificity of the staining and lack of cross-reactivity with the IGF-1 receptor has been further documented by immunoabsorption tests with the specific antigenic amino acid sequence of the insulin receptor molecule as well as a corresponding peptide from the IGF-1 receptor. Furthermore, immunocytochemistry using A-1314 revealed the same distribution of IR in human brain tissue as a monoclonal antibody against the human insulin receptor whose specificity has been previously documented.[34] In addition, recent autoradiographic studies have demonstrated that IGF-1 receptors are located in distinctly different regions of the rat brain than insulin receptors, e.g. low binding affinity for IGF-1[8] but a high density of insulin binding sites[11] were demonstrated in the arcuate nucleus of the hypothalamus. Since the immunohistochemically detected distribution of IR in the rat brain corresponds with receptor binding maps for insulin,[11] but not IGF-1,[8] we have further anatomical evidence for the specificity of the antibody A-1314. We also compared A-1314 to a polyclonal antibody (L-1) directed against the rat insulin receptor and, as expected, obtained similar but not identical results. We would suggest that the lack of identical results likely arises from the small amount of cross-reactivity of L-1 with the IGF-1 receptor. Also, since L-1 is a polyclonal antibody raised against the intact receptor, the differences in the characteristics of immunohistochemical staining between the two antibodies, i.e. punctuate profiles versus large macroaggregates, may reflect the differences between the recognition of a single antigenic site by the affinity purified antibody A-1314 versus the recognition of several antigenic sites on the receptor by the L-1 antibody. Overall, we conclude that the A-1314 antibody generated against a specific amino acid sequence of the carboxy-terminus of the beta-subunit of the insulin receptor is highly specific and provides an excellent means for identifying insulin receptors in rat tissue.

Anatomical aspects

Our findings, summerized in Fig. 8, demonstrate that insulin receptors are widely distributed throughout the rat forebrain and are consistent with previous biochemical studies that have described the general distribution of insulin receptors in extracts from certain brain regions.[21,37,44] Previous studies have reported a preferential localization of insulin receptors in the olfactory bulb, cerebral cortex, hypothalamus, hippocampus and cerebellum.[4–6,11,22,27,63] *In vivo* autoradiographic studies have also demonstrated binding of blood-borne insulin to receptors in circumventricular organs, such as the median eminence[53] and area postrema.[54] In addition, insulin binding by brain capillaries[20] and choroid plexus[4] has been shown. Recent studies using quantitative autoradiography demonstrated a widespread distribution of insulin binding sites in the rat brain, similar to our findings.[11,58] It is noteworthy that an immunohistochemical study on the distribution of insulin in the rat brain[3] demonstrated a localization and staining pattern of insulin-immunoreactivity that is similar to our findings that are based on direct immunohistochemical localization of the insulin receptor. This observation supports the view that immunostaining of insulin in the brain may actually represent insulin binding sites.[3]

Our findings also demonstrate that insulin receptors are localized predominently on perikarya and processes of specific neuronal cells in the intact, adult forebrain. Although it is not excluded that single glial cells (i.e. astrocytes) possess insulin receptors, our results using double-labeling immunocytochemistry with GFAP indicate that the vast majority of IR is localized on neurons. This is in contrast to previous biochemical studies in fetal cell cultures, which showed the presence of receptors on glial cells.[9,10,24] Recent immunohistochemical studies in cell cultures of fetal rat brain have demonstrated IR on neurons as well as on the background layer of dissociated nerve cells; however it was not determined whether this immunostaining occurred on glial cells or fibroblasts.[59,60] The present study suggests that the distribution of receptors may be different in fetal and adult brain, or alternatively that the expression of insulin receptors on glial cells in culture might be an artificial phenomenon of the environmental differences between culture and *in vivo* conditions.

Double-labeling of IR neurons with antibodies against somatostatin and vasopressin has also provided the first direct evidence for the identification of specific subpopulations of neuropeptide neurons that express IR. It is of particular interest that a subpopulation of somatostatin neurons in the periventricular nucleus of the hypothalamus shows IR. This finding is a morphological indication that some, but not all, somatostatin neurons may be modulated by insulin or insulin-related factors (e.g. IGF-1). Previous studies have shown that somatostatin neurons in the periventricular region project to the portal vessels of the median eminence, and the peptide is transported to the anterior pituitary, where it functions as the principle inhibitory factor in the regulation of growth hormone secretion.[12–14,33]

Functions of insulin receptors in the brain

We have found IR over the ependymal lining of the third ventricle as well as along the surface of tanycytes in the perivascular areas of the median eminence. Dense IR was also present in the external layer of the lateral margin of the median eminence. Although the precise cellular localization of insulin receptors in these regions needs to be further studied by ultrastructural analysis, recent *in vivo* autoradiographic investigations have suggested that insulin may be taken up from the cerebrospinal fluid into the brain after binding to the ependyma.[3] In addition, it has been demonstrated that peripheral insulin binds to axonal or synaptic receptors in the pars externa of the median eminence and is transported to neurons in the arcuate nucleus.[55] The pattern of staining for IR in the lateral part of the median eminence is closely related anatomically to the distribution of nerve terminals which originate from hypothalamic areas.[38,57] Although the functional significance of this relationship is presently unknown, it is well documented that the external layer of the median eminence is characterized by a dense accumulation of neuropeptide-containing axon terminals which regulate the secretion of hormones of the anterior pituitary by the secretion of releasing or inhibitory factors into the hypothalamus portal circulation.[57] Previous studies have demonstrated that increased insulin concentration in the cerebrospinal fluid leads to a satiety effect in rats[62] and that genetically obese Zucker rats are insensitive to the satiety effect of insulin.[29] In Zucker rats, insulin binding is reduced in hypothalamus and olfactory bulbs.[17,18,39] These findings suggest that peripheral insulin might interact directly with the central nervous system, especially the hypothalamus. This action may present an important physiological mechanism that allows rapid changes and adaptations in hypothalamic regulation of feeding behaviour (e.g. satiety signals) in response to changes in plasma insulin and glucose levels.

The finding that insulin receptors were not restricted to neuropeptide neurons of the hypothalamus, but were also present on other neuronal populations, suggests that insulin receptors may play an additional role in regulation of neuronal activity in these brain regions. Previous studies have shown that insulin may play a role in modulation of synaptic transmission, such as inhibition of electrophysiological activity of CA1 pyramidal neurons in the hippocampus,[40] as well as inhibiting norepinephrine uptake into isolated synaptosomes.[45] In addition, insulin may play a trophic or growth-promoting role during development of different brain areas, since its binding to brain structures changes during ontogeny and reaches highest levels around postnatal day 15.[31,32] Insulin plays a role in the regulation of neuronal maturation,[43] as well as promoting the neurite formation and outgrowth in cultured embryonic neurons of the mouse brain[7] and cultured human neuroblastoma cells.[46] It is unknown if insulin receptors mediate these trophic effects in the adult central nervous system. This function could be of importance of physiological growth and regeneration ("plasticity") as well as neuronal degeneration under pathological conditions.[19] Most noteworthy is the high density of receptors in distinct cytoarchitectonic areas of the limbic system, in particular the pyramidal layer and dentate gyrus of the hippocampus as well as the medial amygdala. CA1/2 has the densest IR, whereas CA3/4 shows a moderate density of receptor-positive structures. It is of interest that the distribution of highest receptor densities in the hippocampus and amygdala correlates with regional predilection for neuropathological changes in neurodegenerative disorders, in particular in Alzheimer's disease.[19,26,28,53] The reasons for the selective regional susceptibilities in Alzheimer's disease are still unknown, but neurocytologic features, such as the presence or absence of receptors for trophic agents, may provide some clues for the explanation.

CONCLUSION

In general, our findings are consistent with previous biochemical and autoradiographical studies. The identification of IR to specific cellular populations within selected regions of the rat forebrain should provide a basis for further investigations on the cellular locations of insulin receptors and their significance to potential mechanisms of action, i.e. relationship to neurotransmitters and neuropeptides, ultrastructural analysis of IR-positive neurons, ependymal cells, tanycytes and neurovascular terminals. Although the functional role of insulin receptors on neurons has not been determined, the widespread distribution of insulin receptors over a variety of physiologically distinct brain regions argues that the receptor may modulate multiple functions in the adult rat brain; e.g. insulin receptors in olfactory regions, amygdala and hypothalamus could modulate feeding behaviour and regulate release of peptide hormones from neuroendocrine neurons, such as

somatostatin and vasopressin. In addition, insulin receptors in other brain areas (e.g. hippocampal pyramidal cells) may participate in synaptic transmission of signals and, finally, insulin receptors may be of importance for the action of "growth factors" during ontogenetical development and maintenance of "neuronal plasticity" in the adult central nervous system.

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