

# Receptor-mediated internalization of insulin-like growth factor-I in neurons and glia of the central nervous system of the adult rat

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## SUMMARY

Insulin-like growth factor-I is produced in the developing brain where it acts as a trophic signal for different neuronal and glial populations. While its synthesis declines in most brain areas with adulthood, expression of its receptor persists, together with a broad distribution of cells immunoreactive for the peptide. In the adult brain, insulin-like growth factor-I acts as a neuromodulator and a neuroendocrine messenger and is also involved in neurodegenerative processes, reducing neuronal loss after brain injury. To determine whether adult brain cells are able to uptake insulin-like growth factor-I from the cerebrospinal fluid, the peptide was labeled with digoxigenin and injected into the lateral cerebral ventricle. A large population of ependymal cells, glial cells and neurons located throughout the entire central nervous system accumulated labeled peptide. Their distribution corresponded to that of cells expressing insulin-like growth factor-I receptors and also to the distribution of cells immunoreactive for the peptide in the adult rat brain. Accumulation of the growth factor was receptor-mediated, since it was blocked by simultaneous intracerebroventricular injection of unlabeled peptide and by JB1, a specific antagonist of insulin-like growth factor-I receptors. Insulin, at a concentration known to interact with insulin-like growth factor-I receptors also blocked accumulation of the growth factor. These results indicate that insulin-like growth factor-I is internalized by its target cells in the adult central nervous system and that intracerebroventricular injections are a useful mean to dispense the growth factor to its target cells.

**Key words:** Insulin-like growth factor I - Insulin - Growth factors - Cerebrospinal fluid.

## INTRODUCTION

Insulin-like growth factor-I (IGF-I) is a peptide of the insulin family that is widely expressed in developing brain tissue, where it exerts a variety of trophic effects (De Pablo and De la Rosa, 1995). Levels of expression of IGF-I mRNA decline in most areas of the adult brain, while expression of IGF-I receptors remains high in many different cell populations (Andersson et al., 1988; Bach et al., 1991; Bondy, 1991; Bondy et al., 1992; García-Segura et al., 1991, 1997; Lee et al., 1992; Rotwein et al., 1988; Werther et al., 1990). This suggests that IGF-I is still able to exert trophic or neuromodulatory effects in the adult brain, even if its local production is low under normal circumstances. Furthermore, IGF-I expression is enhanced in areas of the adult brain injured or suffering pathological disturbances (Breese et al., 1996; García-Estrada et al., 1992; Gehrman et al., 1994; Komoly et al., 1992; Lee et al., 1992; Yamaguchi et al., 1991), suggesting that IGF-I may participate in brain repair processes. Indeed, IGF-I regulates neuronal and glial responses to injury in the adult CNS (Fernández et al., 1997), preventing neuronal loss (Guan et al., 1996; Li et al., 1994; Fernández et al., 1998) and promoting functional recover (Fernández et al., 1998).

The differences observed in the distribution of IGF-I mRNA and IGF-I receptors in the adult brain indicate that IGF-I may act in areas located at a certain distance from its site of synthesis. In

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Submitted: June 26, 1998  
Accepted: October 30, 1998

recent years the concept of volume transmission in the brain has been developed (Agnati et al., 1995). Volume transmission refers to those forms of communication involving signal diffusion within the brain extracellular fluid and the cerebrospinal fluid (CSF) and includes both short and long-distance signals that act on specific neurons or glial cells endowed with the appropriate receptors. The concept of volume transmission is not of exclusive theoretical interest since it opens the possibility for therapeutical administration of trophic factors into the CSF.

To better understand the physiological and physiopathological implications of the cerebral uptake of IGF-I from the CSF, it was first necessary to determine the anatomical distribution of the cells that accumulate exogenous IGF-I. For this purpose, we used intraventricular injections of IGF-I labeled with digoxigenin, a marker that allows precise cellular localization and identification, to determine whether cells of the central nervous system are capable of concentrating IGF-I from CSF.

## MATERIALS AND METHODS

### Labeling of peptides

IGF-I and bovine serum albumin (BSA, Sigma) were labeled with digoxigenin by using the digoxigenin labeling kit of Boehringer. IGF-I (0.5 mg in 0.5 ml of phosphate buffered saline, 0.1 M, pH 7.5) was mixed with 0.235 mg of digoxigenin-3-O-succinyl-e-aminocaproic acid-N-hydroxy-succinimide ester (D-NHS) in 11.7  $\mu$ l of DMSO. BSA (1 mg in 1 ml of phosphate buffered saline, 0.1 M, pH 7.5) was mixed with 0.107 mg of D-NHS in 5.34  $\mu$ l of DMSO. The mixtures were incubated for 2 h at room temperature under gentle stirring. Labeled peptides and non-reacted labels were separated by gel filtration on a Sephadex G-25 column. The presence of labeled peptides in the corresponding molecular weight band was ascertained by western immunoblotting (Fig. 1a). The binding of digoxigenin labeled IGF-I (D-IGF-I) to IGF-I receptor was ascertained as described previously for  $^{125}$ I-IGF-I (Pons and Torres-Aleman, 1992), by cross-linking to hypothalamic membranes with dimethyl-pimelidate and digoxigenin immunodetection after Western blotting (Fig. 1b, left). The binding of D-IGF-I to IGF binding proteins (IGFBPs) was ascertained by Western ligand (Pons and Torres-Aleman, 1992) of rat serum samples and digoxigenin immunodetection (Fig. 1b, right).

### Animals and administration of peptides

Wistar albino rats from our in-house colony were used. Animals were kept in a 12h dark-light cycle and received food and water *ad libitum*.

Animals were handled following the European Union (86/609/EEC) guidelines and special care was taken to minimize animal suffering and to reduce the number of animals used. Intra-cerebro-ventricular (icv) administration of peptides was performed in adult male rats (250-300 g) under tribromoethanol anaesthesia (250 mg/100 g b.w.). Rats were placed in a stereotaxic frame and peptides were injected in the lateral cerebral ventricle (coordinates: AP, -0.8; L, 1.6; DV 3.5 mm) with a perfusion pump (Carnegie) at 1  $\mu$ l/min fluid rate. The amount injected corresponded to approximately either 1 or 6  $\mu$ g of D-IGF-I or 6  $\mu$ g of digoxigenin-labeled BSA (D-BSA). In displacement experiments, rats received a simultaneous icv injection of 20  $\mu$ g of unlabeled IGF-I, 10 mg of unlabeled insulin or 50  $\mu$ g of JB1, a specific IGF-I receptor antagonist (Pietrzowski et al., 1992). At 15, 30, 60, 120, 240 or 1,000 min after the injection, the animals were anesthetized with sodium pentobarbital and the brain fixed by intra-cardiac perfusion.

Table 1 shows a summary of all the animals studied, the peptide(s) injected and the time elapsed between the injection of the peptides and the perfusion. Those animals showing the injection path outside the lateral cerebral ventricle were omitted from the study and are not shown in the table.

### Immunohistochemistry

Anesthetized rats were perfused through the left cardiac ventricle, first with 50 ml of 0.9% NaCl and then with 250 ml of 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4 at room temperature. After perfusion, the brains were removed and placed at 4°C in the same fixative for an additional 24 hours. After washing in several changes of 0.1 M phosphate buffer, the

Labeled peptide <sup>1</sup>	unlabeled peptide <sup>1,2</sup>	time of survival	number of animals
IGF-I (6)	—	15 min	1
IGF-I (6)	—	30 min	3
IGF-I (6)	—	1 h	3
IGF-I (1)	—	1 h	6
IGF-I (6)	—	2 h	3
IGF-I (6)	—	4 h	3
IGF-I (6)	—	17 h	1
IGF-I (6)	IGF-I (20)	30 min	1
IGF-I (6)	IGF-I (20)	4 h	2
IGF-I (6)	insulin (10)	1 h	1
IGF-I (1)	JB1 (50)	1 h	2
BSA (6)	—	1 h	1

<sup>1</sup> The amount injected ( $\mu$ g) is indicated in brackets.

<sup>2</sup> Both unlabeled and labeled peptides were administered simultaneously by icv injection

**Table 1.**— List of animals studied indicating the peptides injected.

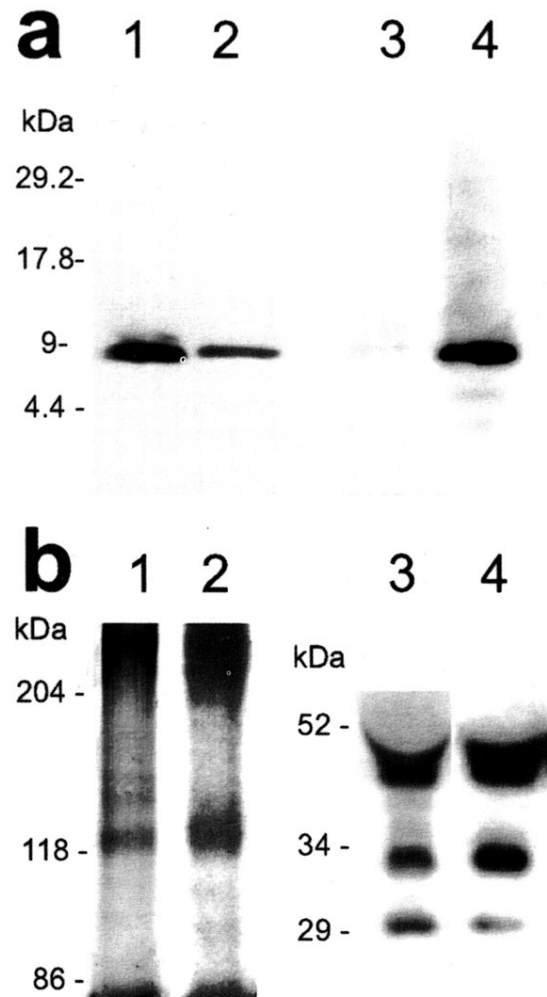
brains were soaked in 0.1 M phosphate buffer containing 30% sucrose and stored in this solution until they sank. All washes and incubations were done on free-floating coronal cryostat sections under moderate shaking. Sections (35  $\mu$ m thick) were first incubated with 0.5% hydrogen peroxide in 100% methanol for 20 min to remove endogenous peroxidase activity. Following this treatment, the sections were washed twice for 20 min in 0.1 M phosphate buffer with 0.1% bovine serum albumin and 0.1% Triton X-100. This buffer was used in subsequent washes and incubations. Sections were incubated overnight at 4°C with a sheep anti-digoxigenin antiserum (Boehringer) at a dilution of 1:200. The sections were then washed twice in buffer, incubated for 2 h at 22°C with the secondary antibody (biotinylated anti-sheep IgG, Vector, diluted 1:250) and again washed 3 times with buffer. The avidin-biotin system of Vector (ABC kit, elite) was then used following the manufacturer's instructions. Peroxidase activity was revealed with the metal enhanced diaminobenzidine kit of Pierce. For confocal microscopy, the secondary biotinylated antibody was recognized with streptavidin labeled with Cy3 (Amersham; 1:500). Sections were observed in a Leica TCS4D confocal scanner installed in a Leitz DMIRB microscope. Confocal images are displayed as individual optical sections. Since the number of positive cells and processes probably can be modified by differences in antiserum penetration into the sections, special care was taken to incubate all the sections for exactly the same period and under the same conditions. With all injected animals, a control rat, which was not injected with any labeled peptide, was perfused and its brain processed in parallel for digoxigenin immunocytochemistry.

## RESULTS

### Characterization of D-IGF-I

Biochemical and biological characterization of D-IGF-I was necessary to ascertain that the labeling procedure was not deleterious. Western blot analysis of D-IGF-I indicated that the label did not alter its electrophoretic behavior since D-IGF-I migrated in parallel to unlabeled IGF-I (Fig. 1a). In addition, D-IGF-I is able to bind to IGF-I receptors since after cross-linking to hypothalamic membranes, a 116 kDa band corresponding to the alpha subunit of the IGF-I receptor plus the peptide (LeRoith et al., 1995; Pons and Torres-Aleman, 1992), was obtained using either D-IGF-I or  $^{125}$ I-IGF-I (Fig. 1b, left). Finally, full biological activity of D-IGF-I was ascertained by its ability to bind to circulating IGF-BPs. The Western ligand pattern of both iodinated and D-IGF-I using

serum samples was identical, showing bands corresponding to at least the 3 major forms of circulating IGF-BPs (Ocrant et al., 1990; Pons and Torres-Aleman, 1992) (Fig. 1b, right).



**Fig. 1.**— Characterization of D-IGF-I. **a)** Western immunoblot of D-IGF-I and unlabeled IGF-I after migration on a 15% polyacrylamide-tricine gel. Lanes 1 and 3: unlabeled IGF-I. Lanes 2 and 4: D-IGF-I. Lanes 1 and 2 were immunostained with an IGF-I polyclonal antibody followed by a biotinylated secondary antibody and ABC detection. Lanes 3 and 4 were immunostained with an antidigoxigenin antibody labeled with alkaline phosphatase. **b)** Binding of D-IGF-I to IGF-I receptor and IGF-BPs. Lanes 1 and 2: Western blot of hypothalamic membrane extracts cross linked to D-IGF-I (lane 1) or  $^{125}$ I-IGF-I (lane 2) shows bands corresponding to the IGF-I receptor alpha subunit. Lanes 3 and 4: Western ligand of serum samples using D-IGF-I and digoxigenin immunodetection (lane 3) or  $^{125}$ I-IGF-I and autoradiographic detection (lane 4). Binding to the 3 main IGF-BPs was found.

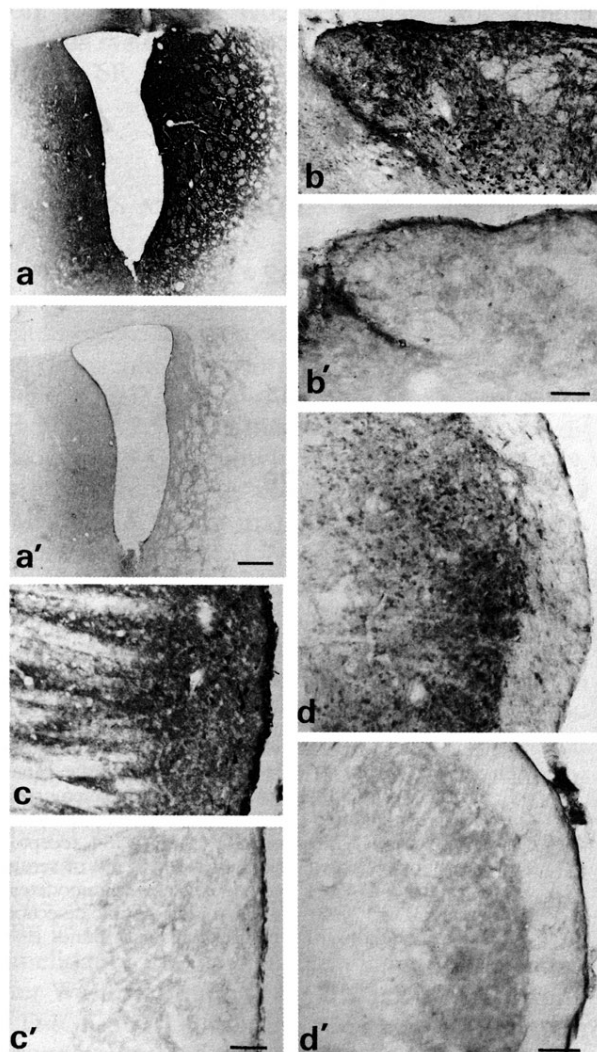
### Specificity of labeling

Labeled cells were observed in the central nervous system of animals injected with D-IGF-I. Cellular labeling was dramatically reduced in the animals that received a simultaneous icv injection of D-IGF-I and unlabeled insulin or unlabeled IGF-I (Fig. 2). The icv administration of D-IGF-I and the specific IGF-I receptor antagonist JB1, resulted in the complete absence of

staining. No staining was observed in the brain of the animal that was icv injected with D-BSA, except along the borders of the tract of the canula. Staining was always absent in sections from not injected animals.

#### *Distribution of labeled cells*

Accumulation in neural tissue of labeled IGF-I injected into the lateral cerebral ventricle showed a progressive displacement from rostral structures, located near the injection site, to caudal areas, such as the spinal cord, located far from the injection site. By 15 min after icv injection, accumulation of labeled IGF-I was observed in the choroid plexus, in ependymal cells and in neurons and glial cells located around the ventricular cavities and at brain borders in contact with the subarachnoid space (i.e., in the proximity of the interfaces between CSF and cerebral parenchyma). By



**Fig. 2.**— Labeling in the brain following injection into the lateral ventricle of either D-IGF-I alone (**a,b,c,d**), D-IGF-I together with unlabeled IGF-I (**a'**), or unlabeled insulin (**b',c',d'**). All rats were killed 1 h after the injection. **a**) and **a'**), Panoramic view of the injected lateral ventricle. Bar, 0,5 mm. **b** and **b'**), Nucleus gracilis. Bar, 75  $\mu$ m. **c**) and **c'**), Septum. Bar, 75  $\mu$ m. **d**) and **d'**), Spinal trigeminal nucleus. Bar, 75  $\mu$ m.

1 h after icv injection, the number of IGF-I accumulating cells was increased, in particular in caudal CNS areas located far from the injected cerebral ventricle. The number of stained cells decreased 2 h after icv injection of labeled IGF-I and only some positive cells remained by 4 h, mainly in caudal areas of the CNS. No staining was observed in the rat killed 17 h after the icv injection of D-IGF-I. A detailed description of the distribution of stained cells is presented below based in the animals killed 60 min after administration of D-IGF-I, the time of maximal extension of labeling. Similar results were observed after injection of either 1 or 6 mg of the peptide. A summary of the location of stained cells is provided in Table 2.

#### *Cells in contact with the CSF*

Choroid plexi of lateral, third and fourth ventricles.  
Ependymal cells in the walls of the cerebral ventricles, Sylvian aqueduct and central canal of the spinal cord.  
Tanyocytes in the walls of the third ventricle.  
Marginal glia.

#### *Telencephalon*

Olfactory nerve fibers  
Olfactory glomeruli (F,N)  
Olfactory bulb (N,G)  
Olfactory cortex (N)  
Cingulate gyrus layers II/III (N)  
Frontoparietal cortex layer VI (N)  
Amygdala (N)  
Horizontal limb diagonal band (N)  
Hippocampus: CA1, CA2, CA3, dentate gyrus (N)  
Striatum (N)  
Septum (N)

#### *Diencephalon*

Habenula (F)  
Hypothalamus (N)

#### *Midbrain*

Central gray (N)  
Substantia nigra (N)

#### *Brain stem*

Spinal trigeminal nucleus (N)  
Gracilis and cuneatus (N)  
Cochlear nuclei (N)  
Vestibular nuclei (N)  
Prepositus hypoglossal nucleus (N)  
Superior olivary complex (N)  
Lateral and medial nuclei of the trapezoid body (N)  
Locus coeruleus (N)  
Gigantocellular reticular, lateral paragigantocellularis, lateral reticular nuclei (N)  
Inferior olivary nucleus (F)  
Cochlear ganglion (N)  
Motor nuclei (N)  
Pyramidal tract (G)

#### *Cerebellum*

Central cerebellar nuclei (N,F)  
Cerebellar cortex: Bergman glia, Purkinje cells, Basket and stellate cells. A few cells in the granule cell layer.

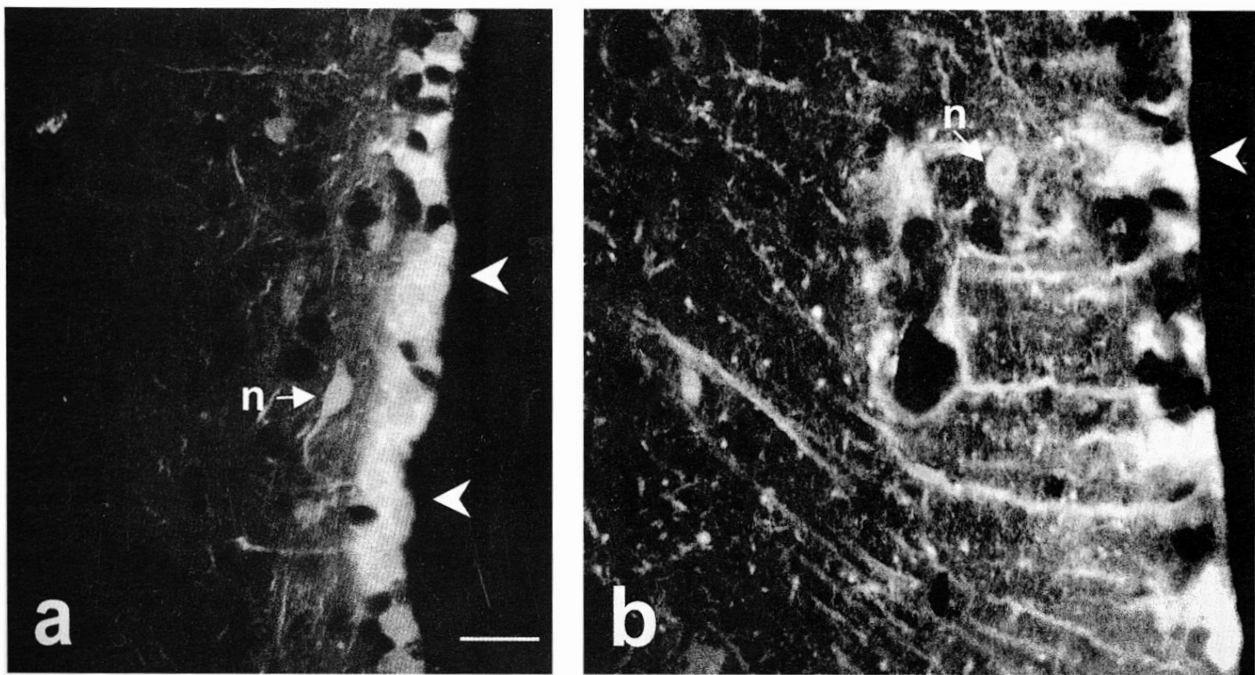
#### *Spinal cord*

White matter (G)  
Dorsal horn (N)  
Ventral horn (N)

N, neurons; G, glia; F, neuronal processes.

**Table 2.**— Summary of the distribution of digoxigenin immunoreactivity in animals killed 1 h after administration of D-IGF-I in the lateral cerebral ventricle.





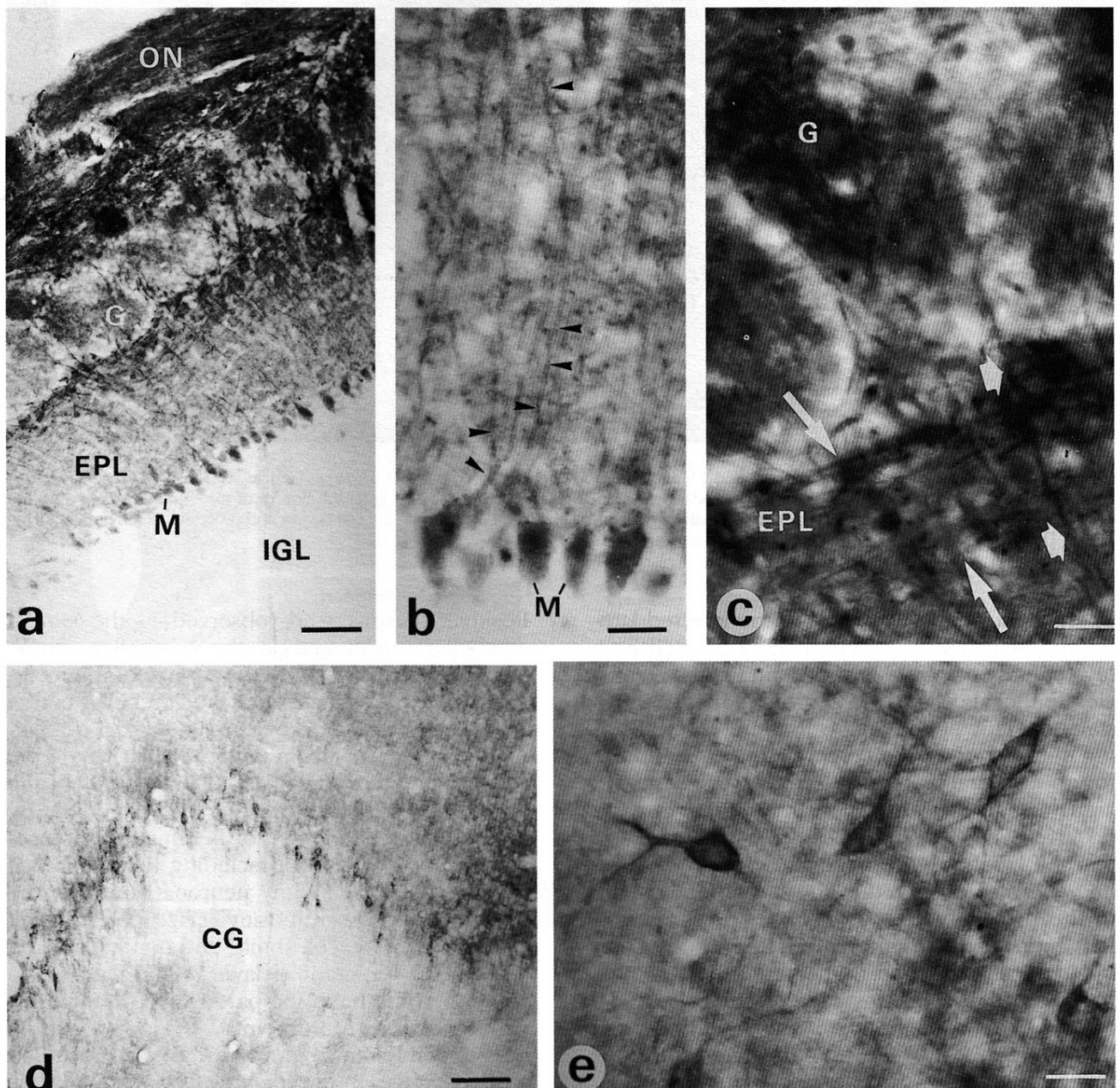
**Fig. 3.**— Labeling in the hypothalamus of rats killed 1 h after injection of D-IGF-I in the lateral cerebral ventricle visualized by confocal microscopy. **a)** Dorsal hypothalamus showing labeling in the octoplasm of ependymal cells in the border of the third ventricle (arrowheads). N, Neuron, Bar, 20  $\mu$ m. **b)** Ventromedial hypothalamus. Labeling is observed in the cell bodies and processes of tanyocytes (arrowheads). N, neuron. Both figures are at the same magnification.

Ependymal cells in the walls of the injected lateral cerebral ventricle were stained as well as a few ependymal cells in the contralateral ventricle. Epithelial cells of the choroid plexi were also stained. Many ependymal cells along the walls of the third ventricle, the cerebral aqueduct and the fourth cerebral ventricle and tanyocytes in the arcuate nucleus and median eminence were positive (Fig. 3). Confocal microscopy revealed that the labeling was intracellular (Fig. 3). Labeling was also observed in the brain parenchyma in the proximity of the ventricles. This may in part represent non-internalized IGF-I present in the extracellular space. Some neurons in the proximity of the ventricles were labeled (Fig. 3). However, the majority of the neuronal cell bodies were negative, even if they were surrounded by a strongly labeled parenchyma or by stained glial processes. Furthermore, most axons in myelinated fiber tracts were negative. Large myelinated fiber tracts were a barrier for the diffusion of D-IGF-I. Only a few labeled axons were observed in the fornix and the corpus callosum (not shown).

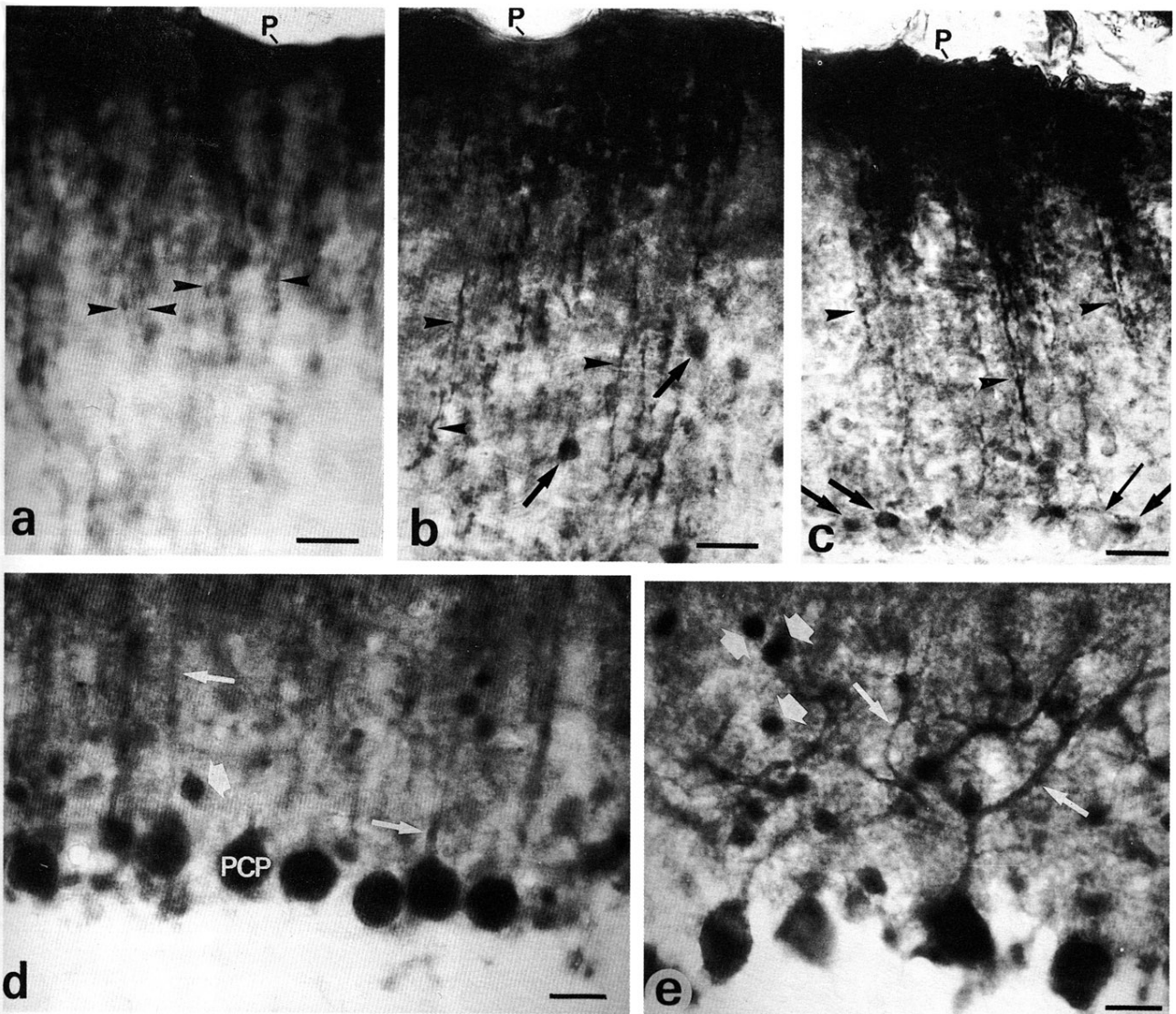
In the olfactory bulb a strong labeling was observed in the olfactory nerve fibers, the olfactory glomeruli, in the periglomerular neurons and in the apical dendrites and somas of mitral cells (Fig. 4 a-c). Positive neurons were observed in layers II/III of the cingulate gyrus, layer VI of the frontoparietal cortex (Fig. 4 d), the olfactory cortex, the hippocampal formation (Fig. 4 e), septal area, striatum, amygdala and the horizontal limb of the diagonal band (not shown).

Positive neurons were observed in the central cerebellar nuclei and in the cerebellar cortex. Staining was restricted to the surface of the molecular layer in most areas of the cerebellar cortex 15 min after injection of D-IGF-I (Fig. 5 a). Staining appeared in fibers perpendicular to the pia that had the morphological characteristics of the apical processes of Bergmann glia (Fig. 5 a). By 30 min, the entire length of Bergmann processes were stained (Fig. 5 b), including Bergmann cell somas. By 60 min, many neuronal somas in the molecular layer were positive (Fig. 5 b,c) as well as many Purkinje cell somas and dendrites and a few cells in the granular layer (Fig. 5 d,e).

Strong staining in the parenchyma was observed around the fourth ventricle (Fig. 6 a), while the subcommissural organ and the area postrema were negative. In the midbrain, positive neurons were located in the central gray and in the substantia nigra. Positive neurons and dendrites were observed in centers of the brain stem related with somatosensory afferents such as the spinal trigeminal nucleus and the gracilis and cuneate nuclei, in primary sensory nuclei related to sensory organs such as the cochlear and vestibular nuclei (Fig. 6 b,c) and in second order relay stations such as the prepositus hypoglossal nucleus, the superior olivary complex (Fig. 6 d) and the lateral and medial nuclei of the trapezoid body. Positive neurons were also observed in the locus coeruleus and in the reticular formation. The parenchyma of the inferior olivary nucleus was strongly labeled, although neuronal cell bodies were negative. Primary sensory neurons, such as

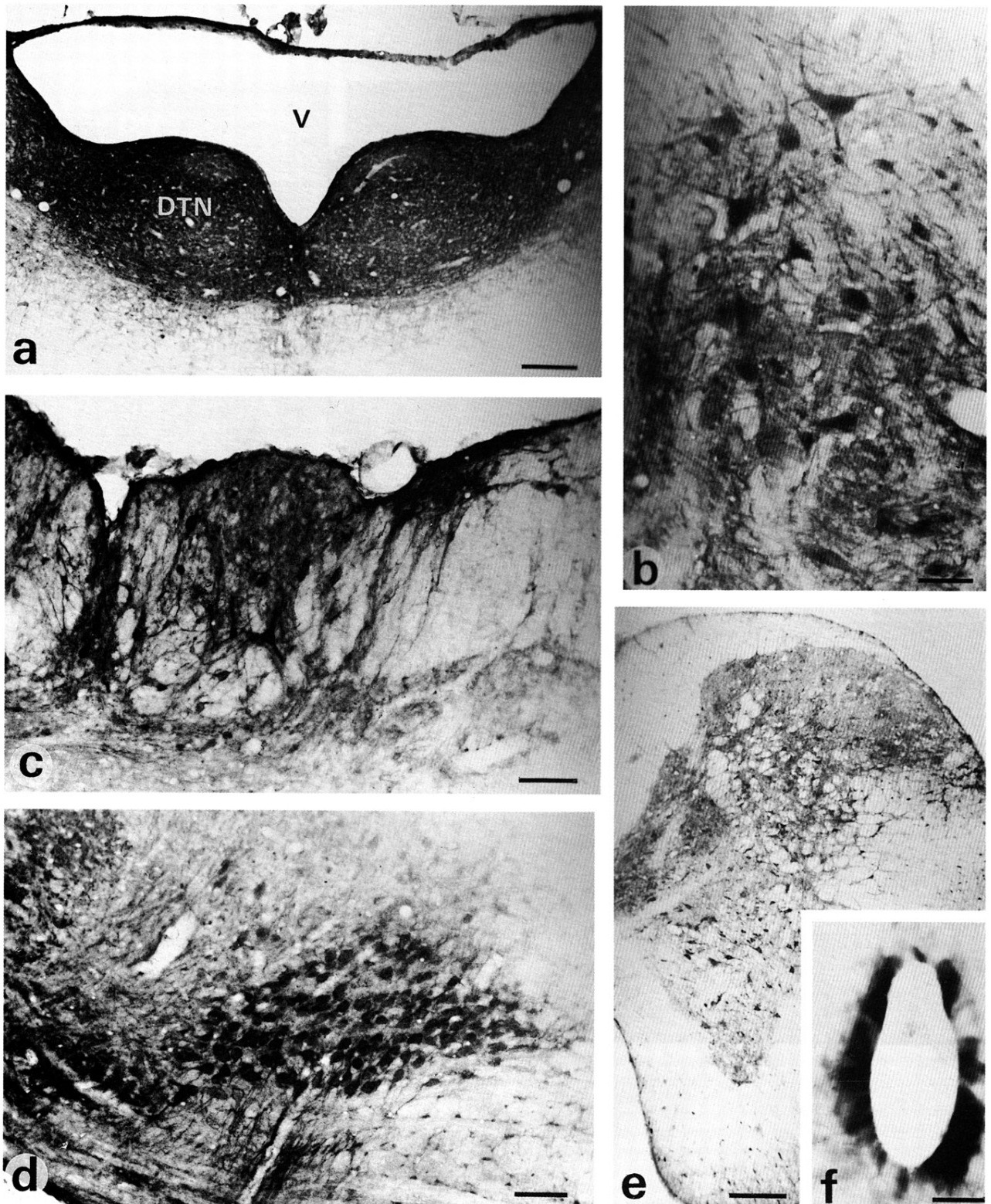


**Fig. 4.**— Labelling in the telencephalon of rats killed 1 h after injection of D-IGF-I in the lateral cerebral ventricle. **a)** Panoramic view of the olfactory bulb showing staining in the olfactory nerve fibers (ON), the olfactory glomeruli (G), the external plexiform layer (EPL) and the mitral cells (M). The internal plexiform layer and the internal granular layer (IGL) are negative. Bar, 70  $\mu$ m. **b)** High magnification of the inner part of the external plexiform layer of the olfactory bulb showing staining in the dendrites of mitral cells (arrowheads). M, Mitral cell perikarya. Bar, 20  $\mu$ m. **c)** High magnification of the limit between the external plexiform layer (EPL) and the glomerular layer (G) showing positive dendrites from mitral cells reaching the olfactory glomeruli (short arrows). Horizontal fibers in the external plexiform layer are also positive (long arrows). Bar, 20  $\mu$ m. **d)** Positive neurons in layer VI of the frontoparietal cortex. CG, cingulum of the corpus callosum. Bar, 80  $\mu$ m. **e)** Examples of labeled neurons in the area CA3 of the hippocampal formation. Bar, 20  $\mu$ m.



**Fig. 5.**— Labelling of the molecular layer of the cerebellar cortex after injection of D-IGF-I into the lateral cerebral ventricle. **a)** High magnification of the upper portion of the molecular layer of a rat killed 15 min after the injection. Labeling is observed in vertical processes (arrowheads) emerging from the pia (P). These processes have the morphological aspect of Bergmann fibers. Bar, 20  $\mu$ m. **b)** Rat killed 30 min after the injection. The staining of vertical processes (arrowheads) is increased compared to the rats killed 15 min after the injection. Stained processes now reach deeper levels in the molecular layer. There is also increased staining of cell somas (arrows). (P), Pia. Bar, 20  $\mu$ m. **c)** Panoramic view of the molecular layer of a rat killed 30 min after the injection of D-IGF-I to show that the staining of vertical processes (arrowheads) reach the Purkinje cell layer. Cell bodies of small size (large arrows) in the Purkinje cell layer, corresponding to Bergmann cell somas, are stained. Purkinje cell somas are negative or slightly stained (thin arrow). P, Pia. Bar, 30  $\mu$ m. **d)** High magnification of a frontal section of the inner portion of the molecular layer showing the staining of Purkinje cell dendrites (long arrows) and Purkinje cell perikarya (PCP) in a rat killed 60 min after the injection of D-IGF-I. Cell bodies in the molecular layer are also positive (short arrow). Bar, 20  $\mu$ m. **e)** High magnification of a sagittal section of the inner portion of the molecular layer showing the staining of dendritic cell arbor (long arrows). Numerous cell somas of the molecular layer are also stained (short arrows). Rat killed 60 min after the injection of D-IGF-I. Bar, 20  $\mu$ m.





**Fig. 6.**— Labeling in the brain stem and spinal cord of rats killed 1 h after injection of D-IGF-I into the lateral cerebral ventricle. **a)** Labeling in the walls of the fourth ventricle at the level of the pons. Staining is observed in the central gray and in the dorsal tegmental nucleus (DNT). Bar, 180  $\mu$ m. **b)** High magnification of the superior vestibular nucleus showing labeling in neuronal somas and processes. Bar, 60  $\mu$ m. **c)** Labeling of neurons and fibers in the nucleus cuneatus. Bar, 75  $\mu$ m. **d)** Neurons in the superior olive. Bar, 60  $\mu$ m. **e)** Panoramic section of the cervical spinal cord. Labeling is observed in the dorsal and ventral horns of the gray matter. Large motoneurons in the ventral horn are clearly visible as well as some glial cells in the white matter, Bar, 175  $\mu$ m. **f)** Central canal of the cervical spinal cord showing labeling in the ependymal cells. Bar, 30  $\mu$ m.



cochlear ganglion cells, and the motoneurons of the motor cranial nerves were positive. Positive glial cells in the pyramidal tract were also observed. Staining was also observed in the spinal cord (Fig. 6 e,f). Many ependymal cells in the central canal were positive (Fig. 6 f), as well as many neurons in the dorsal horn, motoneurons in the ventral horn and many glial cells in the white matter (Fig. 6 e).

## DISCUSSION

Receptor-mediated internalization of trophic factors and their subsequent lysosomal degradation is a general event that is involved in receptor down-regulation (Sorkin and Waters, 1993) and that may also be required to trigger distinct intracellular signaling pathways and cellular responses (Vieira et al., 1996). As in other cell types, IGF-I has been shown to be internalized and degraded by fetal rat brain neurons and glial cells (Auletta et al., 1992; Nielsen et al., 1991). The internalization and intracellular processing of IGF-I is receptor mediated. In this study we have labeled IGF-I with digoxigenin to follow the distribution of the peptide after its icv administration. Labeling with digoxigenin did not affect the ability of IGF-I to bind to IGF-I receptor and IGF-BPs and allowed for a precise cellular localization. Our results indicate that ependyma and various subsets of neurons and glial cells throughout the CNS of adult rats are able to transiently accumulate IGF-I when administered in the CSF, suggesting that IGF-I is internalized and degraded by such cells. The intracellular localization of the staining observed by confocal microscopy indicates that labeled IGF-I was internalized. The time course of cellular labeling after IGF-I administration *in vivo* is similar to that observed *in vitro* (Auletta et al., 1992; Nielsen et al., 1991). Furthermore, the rapid turnover of IGF-I accumulation may explain previous failing to detect incorporation of  $^{125}\text{I}$ -IGF-I in the brain 18 h after its icv administration (Ferguson et al., 1991). In our hands, staining was strongly reduced 4 h after the injection and all the CNS structures were completely negative after 17 h.

The accumulation of labeled IGF-I *in vivo* was substantially decreased by simultaneous administration of unlabeled IGF-I or unlabeled insulin at a concentration that acts on IGF-I receptors (LeRoith et al., 1995), and was blocked by JB1, a specific IGF-I receptor antagonist (Pietrzowski et al., 1992). Furthermore, labeling was not observed after icv administration of D-BSA. In addition, the pattern of accumulation of labeled IGF-I in the CNS after its icv injection differs from the pattern of accumulation observed for other peptides, such as  $^{125}\text{I}$ -NGF (Ferguson et al., 1991; Lapchak et al., 1993), and correlates

well with the distribution of IGF-I receptors detected by autoradiographic localization of radioligand binding sites, with the expression of IGF-I receptor mRNA and with the immunocytochemical localization of IGF-I receptor immunoreactivity (Baura et al., 1993; Bondy et al., 1992; Garcia-Segura et al., 1997; Kar et al., 1993; Lesniak et al., 1988; Werther et al., 1989, 1990). Together this evidence indicates that the accumulation by CNS cells of IGF-I originating from CSF is receptor-mediated and specific.

The expression of IGF-I is maintained in specific CNS areas during adulthood, including the olfactory bulb, hippocampus, spinal cord and cerebellum (Aguado et al., 1992; Andersson et al., 1988; Bondy, 1991; Garcia-Segura et al., 1991; Lee et al., 1992; Rotwein et al., 1988; Werther et al., 1990). Cells in these areas show receptor-mediated accumulation of IGF-I, raising the question of the functional significance of the occurrence of accumulation of a growth factor which is already synthesized by brain cells. One example of this apparent redundancy is the Purkinje cells in the cerebellar cortex. These neurons synthesize IGF-I (Lee et al., 1992), are affected by IGF-I transported by climbing fiber afferents (Nieto-Bona et al., 1993; Torres-Aleman et al., 1991), and as our results now indicate, accumulate IGF-I from CSF in a highly specific fashion. In addition, Purkinje cells express IGF-I receptors and are targets of IGF-I trophic activity (Lee et al., 1992; Torres-Aleman et al., 1992; Garcia-Segura et al., 1997). Since manipulation of IGF-I levels in the cerebellum compromise Purkinje cell function (Castro-Alamancos and Torres-Aleman, 1993), it seems reasonable to assume that this growth factor is so important for the normal function of these neurons that redundant mechanisms are needed to assure a permanent supply of IGF-I to the Purkinje cell. This may also be true for other neurons throughout the brain. Furthermore, since IGF-I can directly control its own synthesis by nerve cells (unpublished observations), variations in IGF-I levels in CSF can eventually lead to changes in its local synthesis in IGF-I accumulating neurons.

IGF-I internalization was not limited to brain areas in which IGF-I producing cells are located. The broad distribution of cells that accumulate IGF-I after its administration in the lateral cerebral ventricle together with the reduced local IGF-I synthesis in most areas of the adult CNS suggest that volume transmission (Agnati et al., 1995) may be involved in the actions of this factor. It is of interest to note that the cells that accumulate labeled IGF-I after icv administration have been previously shown to be IGF-I immunoreactive (Aguado et al., 1992; Andersson et al., 1988; Dueñas et al., 1994; Garcia-Segura et al., 1991). It should be noted also that IGF-I immunoreactive cells are widely distributed in the adult rat brain, contrasting with the

restricted distribution of IGF-I mRNA expression. However, the distribution of IGF-I immunoreactive cells is very similar to the distribution of IGF-I receptor expressing cells. It is therefore possible that the distribution of IGF-I immunoreactive cells in the brain may in part reflect the distribution of IGF-I accumulating cells, further suggesting that IGF-I may be acting in the adult brain in areas located far from its place of synthesis. Some evidence in support of this possibility has been obtained in relation to the neuroendocrine actions of IGF-I. This factor is involved in the regulation of growth hormone secretion by affecting the synthesis or the release of growth hormone releasing hormone and somatostatin by hypothalamic neurons (Bereulowitz et al., 1981; Tannenbaum et al., 1993). Interestingly, IGF-I levels increase in the CSF of growth hormone-deficient human patients after treatment with recombinant growth hormone (Johansson et al., 1995), suggesting that IGF-I present in CSF may be involved in the feedback regulation of growth hormone under normal circumstances.

In addition to play a role in volume transmission of signals between different brain areas, CSF may also be involved in the transmission of signals of peripheral origin. It is well proven that insulin, a peptide structurally related to IGF-I, is able to cross the blood brain barrier (Baura et al., 1993; Reinhardt and Bondy, 1994; Schwartz et al., 1992). Insulin is accumulated in the CSF after injection in plasma (Baura et al., 1993; Schwartz et al., 1990), and is taken up by brain parenchyma from the CSF (Ferguson et al., 1991; Manin et al., 1990). Passage from blood to the brain parenchyma has been demonstrated for other neurotrophic peptides as well, such as nerve growth factor (Loy et al., 1994). Since in the brain IGFs are synthesized locally and their levels are not affected by hypophysectomy, such as it occurs in all other IGF-producing tissues, it has been thought that brain IGFs are regulated independently from blood-borne IGFs. However, recent evidence indicates that peripherally-injected  $^{125}\text{I}$ -IGF-I can be specifically taken up by the brain (Reinhardt and Bondy, 1994). This opens the possibility that peripheral changes in IGF-I levels may ultimately impinge on their brain levels. Indeed, several evidences suggest that IGF-I plasma levels may influence IGF-I brain content. For instance, brain IGF-I synthesis is decreased after fasting, in parallel by a similar decrease in other organs and serum IGF-I levels (LeRoith et al., 1990). Furthermore, IGF-I protein levels are decreased in the cerebellum of diabetic rats, in parallel to the decrease in plasma IGF-I levels, while cerebellar IGF-I synthesis is unaffected (Busiguina et al., 1996). Moreover, peripheral IGF-I regulates the onset of female puberty acting directly on

the hypothalamus (Dueñas et al., 1994; Hiney et al., 1996) and subcutaneous administration of IGF-I restores motor coordination in a rat model of cerebellar ataxia (Fernandez et al., 1998). All these examples reflect the communication between the central and systemic IGF-I systems. Based on our present results, it is tempting to speculate that IGF-I released in CSF from circumventricular organs lacking blood-brain barrier, such as the median eminence, may be involved in such communication.

In conclusion, the present findings indicate that epithelial cells from the choroid plexus, ependymal cells, some glial cell types and a large population of neurons that are widely distributed through the entire CNS are able to specifically uptake and accumulate IGF-I in the adult rat. The wide distribution of IGF-I accumulating cells contrast with a restricted IGF-I synthesis in most areas of the adult brain. These results suggest that volume transmission may be involved in trophic and neuromodulatory effects of IGF-I in the adult CNS.

#### ACKNOWLEDGEMENTS

We would like to acknowledge J.A. Chowen for critical reading of the manuscript. This work was sponsored by Comunidad de Madrid (08.5/0009/1997) and Fundación Endocrinología y Nutrición.

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