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Characterization of GFAP expression and cell proliferation in the rat median eminence following hypophysectomy

R. Vázquez, E. Blanco, F. Sánchez, J.A. Juanes, M. Rubio, M. Santos, G. Vázquez, E. Hernández, J.M. Riesco and J. Carretero

Department of Human Anatomy and Histology, Faculty of Medicine, University of Salamanca, Salamanca, Spain

Summary. To analyze whether the reorganization of the rat median eminence after hypophysectomy might be related to changes in glial fibrillary acidic protein (GFAP)- and cellular proliferation, the distribution of cells immunoreactive for GFAP and the proliferation rate of such cells were analyzed at 20, 40 and 60 days post-hypophysectomy. For this study, four rostro-caudal regions of the median eminence were differentiated: the retrochiasmatic, preinfundibular, infundibular and postinfundibular regions. In each of these regions, three layers were studied: the ependymal, the internal and the external. At 20 and 40 days after hypophysectomy, significant increases in cellular proliferation affecting all three layers studied in the preinfundibular and infundibular regions were found. At the same time points, increases in GFAP expression were also observed. However, after 60 days, GFAP and proliferative cellular nuclear antigen (PCNA) expression decreased. Although variations of PCNA and GFAP levels were evident, no colocalisation of PCNA and GFAP was found in the cells of the median eminence in untreated or hypophysectomized rats when sections were analyzed by double immunohistochemical staining. Our results suggest that reorganization of median eminence involves alterations (or modulation) of GFAP-immunoreactive cells together with a proliferation of cells that are not GFAP-immunoreactive. This study also demonstrates that this reorganization is completed within the first two months after hypophysectomy.

Key words: Median eminence, GFAP-cells, Cellular proliferation, Hypophysectomy, Immunohistochemistry

Offprint requests to: Prof. Dr. R. Vázquez, Departamento de Anatomía e Histología Humanas, Facultad de Medicina, Avda. Alfonso X el Sabio, s/n , E-37007 Salamanca, Spain. Fax: 34-923-294559. e-mail: rvr@gugu.usal.es

Introduction

Two unique regions of the adult mammalian central nervous system have regenerative potential and are capable of active regeneration following injury or structural compromise: the olfactory system and the neurohypophyseal system of the endocrine hypothalamus (Scott and Hansen, 1997). The median eminence is located in the ventral part of the hypothalamus. It is organized in different layers dorso-ventrally, from the ependymus of the third ventricle to the pial membrane in the most basal part. The median eminence is crossed by many fibers which, from different hypothalamic nuclei, arrive at the neurohemal space around the blood vessels of hypophyseal portal system and neurohypophyseal axons throughout the internal layer of the median eminence towards the blood vessels of the hypophyseal neural lobe. To regulate hormonal hypophyseal secretion, neurotransmitters are released from nerve endings to the portal blood in the median eminence, which is strongly related to the hypophyseal stalk. Throughout the intact median eminence, two main types of glial cells have been identified: tanycytes, immunoreactive to vimentin and slightly immunoreactive to GFAP, and classic astrocytes, which are immunoreactive to GFAP but vimentin-negative (Chauvet et al., 1995).

Hypophysectomy, which involves severing of the hypophyseal stalk, is a technique widely employed in reorganization and regeneration studies of the neurohypophyseal system. Based on this technique, many changes occurring at the level of the median eminence have been analysed to date. The reorganization of the hypophyseal stalk following hypophysectomy has long been known. This reorganization involves the formation of a structure resembling the neural lobe of the hypophysis and is accompanied by an increase in vascular density and in the connective tissue accompanying blood vessels (Polenov et al., 1981), and an increase in the number of mitoses of infundibular stalk glial cells during the first few days post-

hypophysectomy (Billenstein and Leveque, 1955; Polenov et al., 1981). The redistribution of several different neuropeptides in the median eminence such as vasopressin, oxytocin, galanin, VIP, cholecystokinin, dynorphin, CRH and adrenomedullin after hypophysectomy has been reported (Kawamoto and Kawashima, 1985a,b, 1987; Villar et al., 1994; Selvais et al., 1995; Polenov et al., 1997; Ueta et al., 1999).

The regeneration process is less characterized. Some axons of the tract that appear after cutting the hypophyseal stalk seem related to tanycyte or astrocyte processes (Chauvet et al., 1995). Scott and Hansen (1997) reported the involvement of primordial neuroblasts from the walls and floor of the third ventricle in this process of neural regeneration and suggested the occurrence of compensatory synaptogenesis involving nitric oxide synthase.

These studies were performed because it is important to determine the hypothalamic variations that occur immediately following hypophysectomy, possibly similar to those resulting from the neurosurgical treatment of pituitary adenomas. However, studies analyzing regenerative events and the reorganization of median eminence occurring one month after hypophysectomy have not been reported. Thus, it is not known whether hypophysectomy elicits a reorganization of glial GFAP-immunoreactive elements in the median eminence or whether this reorganization involves cellular proliferation in the final phases of this reorganization. To investigate these aspects of regeneration, here we studied the expression of GFAP (Eng, 1980; Mathewson and Berry, 1985; Takamiya et al., 1988; Calvo et al., 1991) and cellular proliferation based on expression of proliferating cell nuclear antigen (PCNA) in the rat median eminence following hypophysectomy.

Materials and methods

Animals and treatment

Thirty male Sprague-Dawley rats (125 g body weight) were studied; 15 were sham-operated as controls and 15 were hypophysectomized (Charles River®). All animals were housed under standard conditions and were divided into three groups of 10 animals each (5 controls and 5 hypophysectomized), which were sacrificed at 20, 40 and 60 days after hypophysectomy. The animals, whose weight had decreased visibly, were killed by decapitation under isofluorane anesthesia, the brains were dissected out and the hypothalamic block was removed and fixed in a 15% pycric acid solution in 4% paraformaldehyde in 0.1M phosphate buffer, pH 7.4, for 5 days. After embedding in paraffin, 5 μm serial coronal sections were obtained.

Selection of sections for study

Four regions of the median eminence were identified (Fig. 1): *the retrochiasmatic region*, which included the sections situated caudal to the optic chiasm until the visualization of the pars tuberalis of the hypophysis, which remained appended to the ventral limit of the hypothalamus; the *preinfundibular region*, which included the sections from the retrochiasmatic region extending to the visible appearance of the infundibular recess of the third ventricle; the *infundibular region*, which included all the sections of the median eminence in which the infundibular recess was evident, and finally the *postinfundibular regio*n, where the sections situated caudal to the infundibular recess were included. For the immunohistochemical study of the distribution of GFAP and/or PCNA in each of the regions of the median eminence, the following layers were differentiated dorso-ventrally: the ependymal layer; the internal layer, formed basically of the fiber layer; and the external layer, made up of the reticular layer and the palisade layer.

Single immunohistochemical staining

For immunohistochemical study of GFAP, the peroxidase anti-peroxidase (PAP) method was used while for PCNA the ABP method was employed. Endogenous peroxidase was blocked with H_2O_2 in methanol and non-specific reactions of the secondary serum were blocked by incubation with normal swine or goat serum (Dako diluted to 1:30). GFAP immunoexpression was determined with anti-b-GFAP rabbit serum (Dako, diluted 1:500 overnight at 4 °C), followed by incubation with anti-rabbit IgG swine serum (Dako, diluted 1:100, 30 minutes) and PAP complex (Dako, diluted 1:100, 30 minutes). PCNA immunoexpression was determined using monoclonal rat anti-PCNA serum (PC-10, Dako, diluted 1:3000 overnight at 4 °C), followed by biotinylated anti-mouse IgG goat serum (Dako, diluted 1:100, 40 minutes) and by avidin-biotinylated horseradish peroxidase complex (ABC Kit,

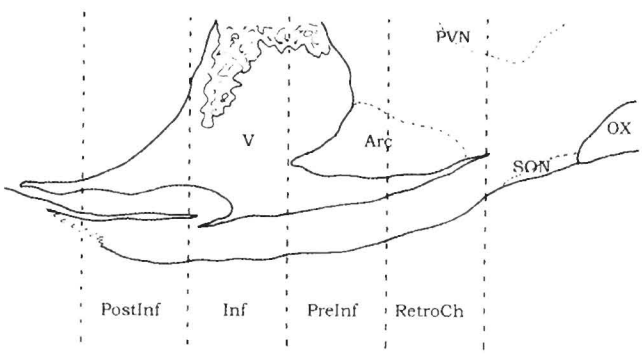

Fig. 1. Scheme depicting a sagittal section of the median eminence of the rat. Note the subdivisions studied in the present work. PNV: paraventricular nucleus; SON: supraoptic nucleus; OX: optic chiasm; Arc: arcuate nucleus; V: third ventricle; PostInf: post infundibular region; Inf: infundibular region; PreInf: preinfundibular region; RetroCh: retrochiasmatic region.

Dako, diluted 1:100, 30 minutes). Washes and dilutions were performed with TBS (0.05M, pH 7.4, with 0.8% NaCl). In both cases, the reactions were developed in freshly prepared 3,3'-diaminobenzidine (DAB) (0.025% in 0.05M Tris buffer, pH 7.4, containing 0.03% of H_2O_2).

Double immunohistochemical staining

To determine the PCNA-GFAP labeling index, a double label immunohistochemical method for PCNA and GFAP was carried out. Endogenous peroxidase was blocked in H_2O_2 in methanol and non-specific reactions of the secondary antibody were blocked by incubation in normal goat serum (Dako, diluted 1:30). Samples were incubated overnight at 4 °C with mouse anti-PCNA (PC10 mAb, Dako, diluted 1:3000), biotinylated goat anti-mouse IgG (Dako, diluted 1:100) and avidin-biotin peroxidase complex (ABC Kit, Dako, diluted 1:100) applied successively at room temperature for 40 min and 30 min, respectively. The reaction was developed in freshly prepared DAB (0.025% in Tris buffer containing 0.03% of H_2O_2). Following PCNA immunolabeling, the PAP reaction was performed for the detection of GFAP, using anti-GFAP rabbit serum (Dako, diluted 1:500), anti-rabbit swine serum (Dako, diluted 1:100) and rabbit-PAP complex (Dako, diluted 1:100). The reaction was developed in freshly prepared 4-chloro-1-naphthol ($1.7x10^{-3}$ M in 0.05M Tris buffer, pH 7.4 containing 0.3% of H_2O_2). Replacement of anti-GFAP or anti-PCNA by non-specific rabbit or mouse serum or by TBS abolished the reaction. By ELISA, the specificity of swine anti-rabbit IgG was lower than 1% for rat and mouse IgG and 100% for rabbit IgG. For the washes and dilutions of the sera, Tris buffer (0.05M, pH 7.4) containing 0.8% NaCl was used.

Quantification of PCNA immunoreactive cells

To determine the percentage of PCNA-immunoreactive cells, 10 sections per region of the median eminence (40 sections per animal) were chosen randomly. To avoid repeated studies of the same cells, because no nuclei larger than 15 μm in diameter were found in the median eminence, the sections studied were separated by 25 μm from each other rostro-caudally (1 out of every 5 sections was studied). Sections situated at the limits of the transition from one region to another were rejected. Using a Zeiss Axiophot microscope equipped with an optic grid at total enlargements of 400x, the nuclei in each of the three layers were counted in each of the sections, calculating the percentage of PCNA-immunoreactive nuclei from the total number of the nuclei present in each of the layers of each of the regions of the median eminence of each animal.

Statistical analysis

The results on PCNA quantification were processed statistically for each of the layers and regions studied in the different groups. Differences in arithmetic means were contrasted by analysis of variance (ANOVA), accepting values of $p<0.05$ for the Scheffé-F test as significant. The results are expressed as arithmetic means ± SEM.

Results

GFAP immunohistochemical expression

Control animals

GFAP-positive elements in the median eminence of the control animals were scantily dispersed throughout the median eminence (Fig. 2). In the retrochiasmatic region, isolated cells and GFAP-positive cellular prolongations were found (Fig. 2a). In the preinfundibular (Fig. 2b) and infundibular (Fig. 2c) regions, reactive cells appeared isolated, with small polygonal soma and small prolongations, mainly in the internal layer. In the lateral portion of the median eminence of the three regions (Fig. 2a-c), a stronger reaction was found at the limit of the median eminence and the adjacent neuropil. With the exception of scarce and isolated labeled cells situated ventrally in the ependymal layer -and so not always appreciable-, the postinfundibular region (Fig. 2d) displayed an evident GFAP reaction at the limit of the most basal pial membrane and in the external layer of the median eminence.

Hypophysectomized animals

GFAP immunoreaction was increased in the median eminence after hypophysectomy, although the variations observed affected the four regions differently and differed in relation to the time elapsed since surgery.

Retrochiasmatic region

Twenty days post-hypophysectomy, a strong increase in GFAP expression was observed in the retrochiasmatic region. The reaction was visible in the basal zone of hypothalamus and became more evident at 40 days after hypophysectomy (Fig. 2e). Thereafter, and up to 60 days, the reaction decreased but was accompanied by a marked presence of labeled elements around vessels (Fig. 2f).

Pre- and infundibular regions

The most noteworthy changes were found in the preinfundibular and infundibular regions; such changes were similar in both regions. In both, the reaction observed at 20 days post-hypophysectomy increased; by 40 days it was further increased, however at 60 days post-hypophysectomy it was decreased.

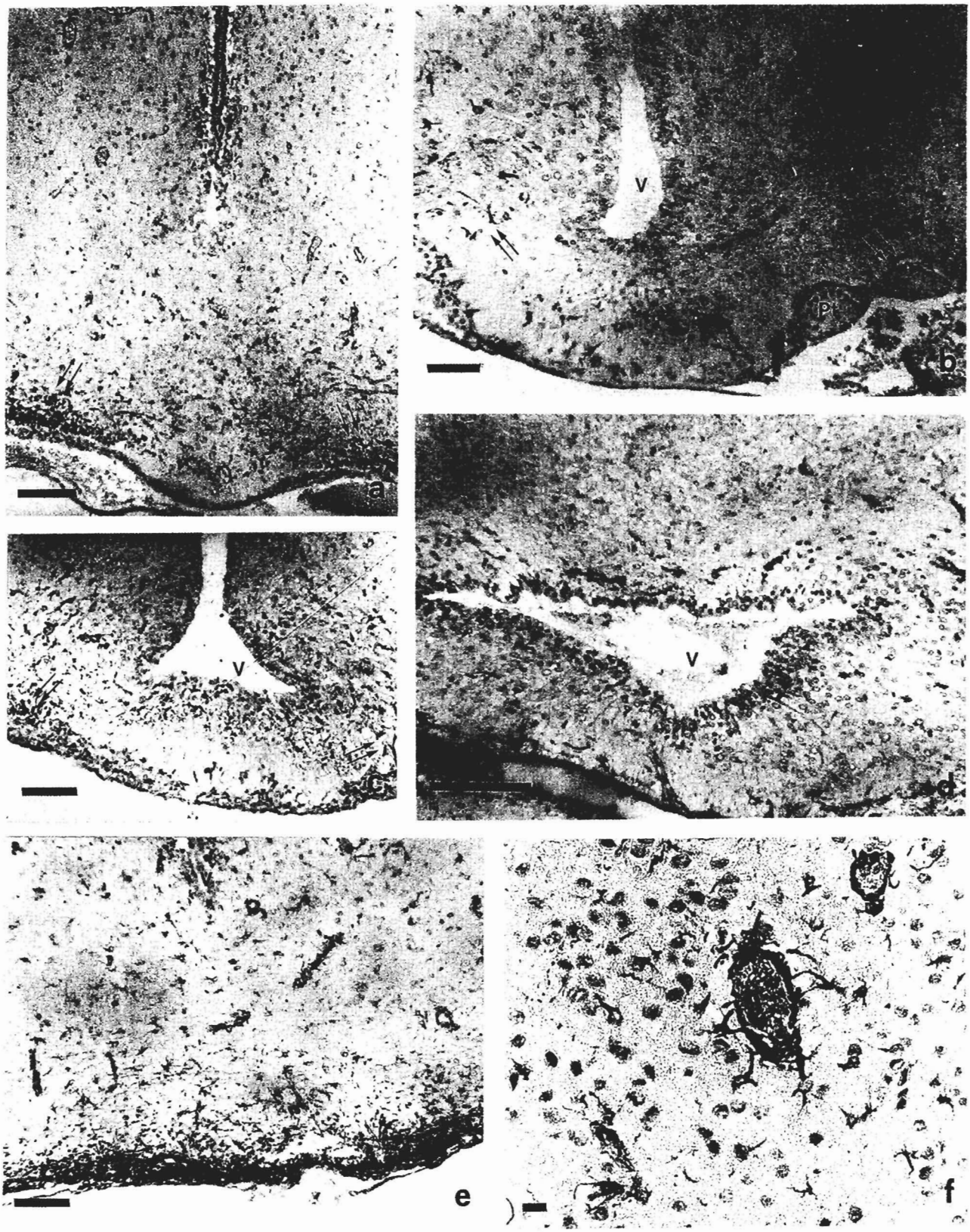

Fig. 2. Micrographs of GFAP-staining in regions of the median eminence. Sham-operated animals. Scarce reaction in the different regions of the median eminence is seen: retrochiasmatic region **(a)**, preinfundibular **(b)**, infundibular **(c)** and postinfundibular **(d)**. The reaction is most marked in the lateral portions, at the limit with the median eminence and the adjacent neuropil (arrows mark this reaction in the retrochiasmatic, preinfundibular and infundibular regions). Hypophysectomized animals. Retrochiasmatic region after 40 days; the increase in the reaction is considerable **(e)**, and is particularly clear around vessels **(f)**. V: Third ventricle; Pt: pars tuberalis. Bars: a-e, 100 μm; f, 10 μm.

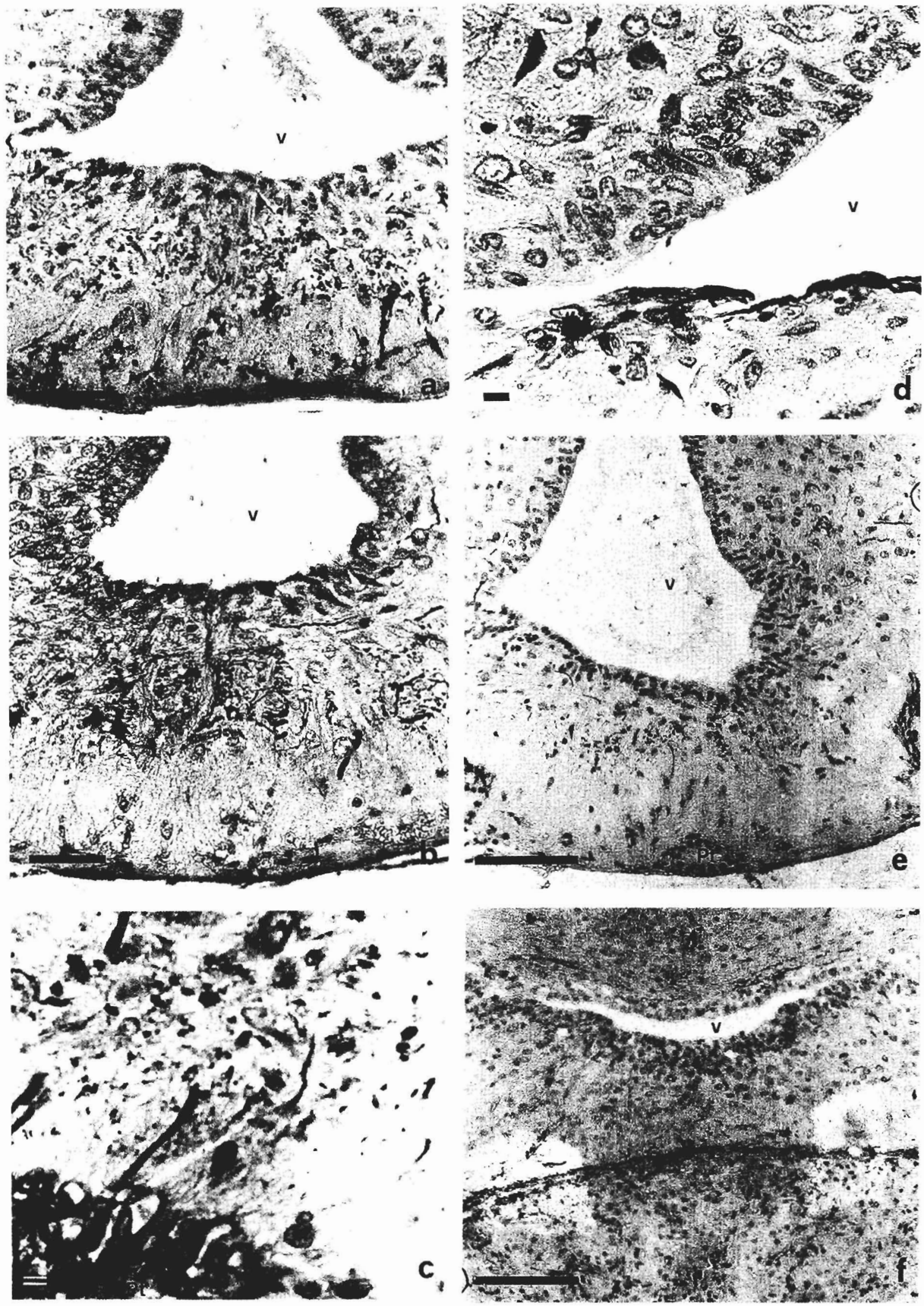

Fig. 3. Ultramicrographs of the infundibular region **(a, b, c, d and e)** and of the postinfundibular region **(f).** The GFAP immunoreactivity is evident at 20 days post-hypophysectomy **(a).** At 40 days, the reaction in the median eminence is well visible **(b)**, with a clear reaction in the lateral margins of the median eminence **(c)** and at supraependymal level **(d)**. The reaction decreases at 60 days post-hypophysectomy **(e)**. In the postinfundibular region **(f)** the reaction is always weak. V: Third ventricle; Pt: pars tuberalis. Bars: a,e,f, 100 μm; b, 50 μm; c,d, 10 μm.

The reaction was manifest at 20 days (Fig. 3a), when highly reactive GFAP glial cells were observed. Some of these cells were seen to send basal cytoplasmic prolongations towards the external layer, ending in the proximity of the post-hypophyseal system. At 40 days, the reaction was apparent in a higher number of cells and fibers (Fig. 3b), also located in the internal layer of the median eminence. At 20 and 40 days after hypophysectomy, a large number of reactive processes, which continued with the glial component of the basolateral hypothalamus, was observed in the lateral portion of the median eminence, affecting the internal and external layers (Fig. 3c). Ependymal and supraependymal reactive cells were also present, although not in large numbers (Fig. 3d). Sixty days after hypophysectomy, the reaction was decreased in comparison with that observed at 20 and 40 days, the prolongations and reactive cells being less evident (Fig. 3e).

Postinfundibular region

At 20 and 40 days after hypophysectomy, only some cells displaying small soma and fine prolongations were visible in the postinfundibular region; the only remarkable difference between the findings obtained at

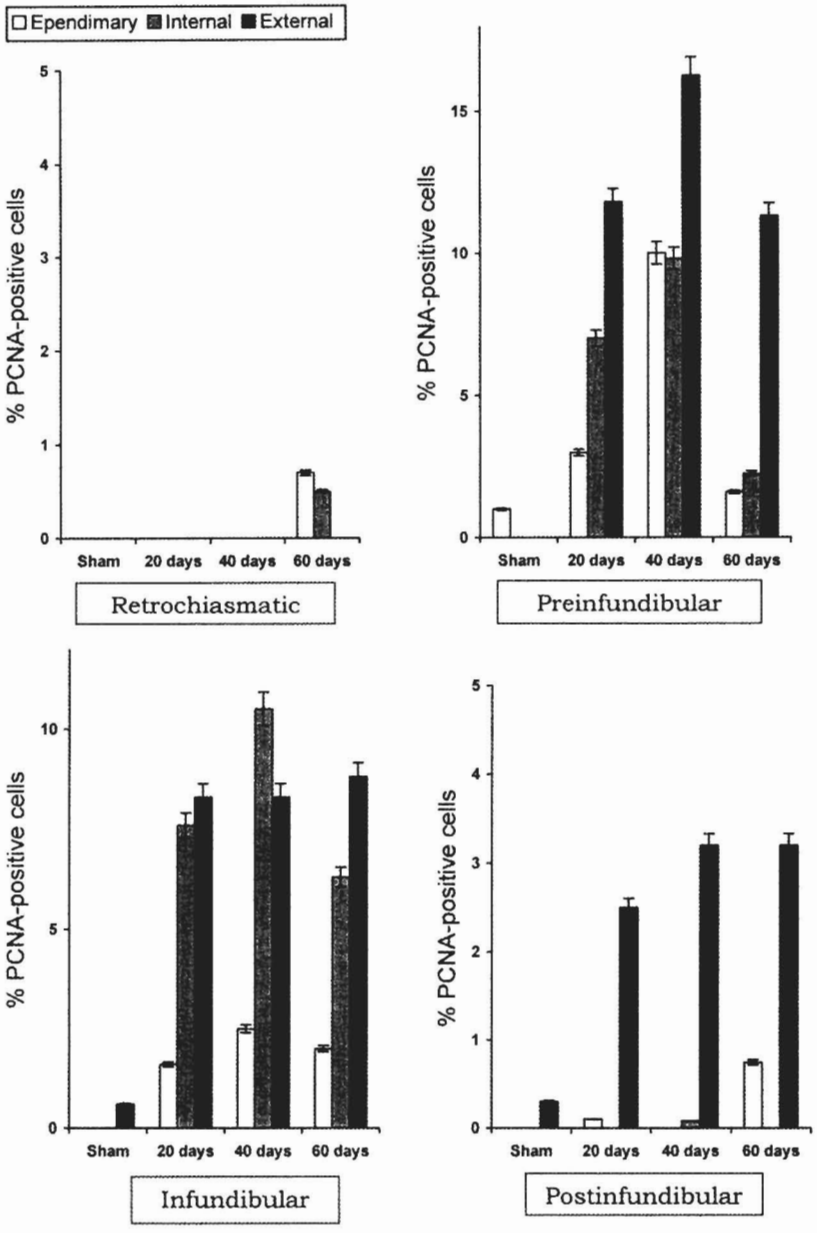

Fig. 4. Percentages of PCNA-immunoreactive cells in the different layers and regions of the median eminence studied in the different treatment groups. Sham: sham-operated animals; 20 days: animals studied at 20 days after hypophysectomy; 40 days: animals studied at 40 days after hypophysectomy; 60 days: animals studied at 60 days after hypophysectomy.

20 and 40 days was a stronger reaction intensity at 40 days. Regarding the remains of the hypophyseal stalk, a greater abundance of fine reactive fibers arranged parallel to the basal limit in the rostro-caudal direction was observed in the postinfundibular region. GFAP-immunoreactive cells were not observed (Fig. 3f). Sixty days after hypophysectomy, the pattern of immunoreactivity for GFAP in the postinfundibular region was very similar to that observed in the control animals.

Cellular proliferation in the median eminence

Control animals

Proliferating cells in the median eminence of the control animals were practically absent; only the external layer of the retrochiasmatic, infundibular and postinfundibular regions showed some PCNA-reactive nuclei, together with scarce nuclei in the ependymal layer of the preinfundibular region.

Hypophysectomized animals

Hypophysectomy significantly modified the percentages of PCNA-reactive nuclei at the three post-hypophysectomy time periods studied. Figure 4 summarizes the values found in the different groups, regions and layers studied.

In the retrochiasmatic region, the only noteworthy changes were observed at 60 days post-hypophysectomy; these affected only the ependymal and internal layers.

In the preinfundibular region, PCNA expression increased significantly ($p<0.01$ with respect to control animals) at 20 days after hypophysectomy in all three layers studied, maximum values being reached at 40 days ($p<0.05$ with respect to 40 days).

Twenty days after treatment, a significant increase ($p<0.01$ with respect to control animals) in PCNA-reactive nuclei was observed in all three layers of the infundibular region. The increase was more pronounced at 40 days in the ependymal and internal layers ($p<0.05$ with respect to 20 days) and decreased at 60 days ($p<0.05$ with respect to 40 days). In the external zone, the values observed at 40 and 60 days post-hypophysectomy were almost identical to those found 20 days after surgery.

In the postinfundibular region, an increase in PCNA-reactive nuclei was observed in the external layer at 20, 40 and 60 days ($p<0.01$ with respect to control animals), with no significant differences among these periods. A slight increase was also observed in the ependymal layer at 60 days ($p<0.05$ with respect to the other two periods).

Double immunocytochemical staining

Sections were double-labeled with antibodies to PCNA and GFAP. However, colocalisation of these markers was not observed in any of the layers nor in any of the regions of the median eminence.

Discussion

Knowledge of the responses of median eminence to hypophysectomy is important for different reasons: the clinical relevance of surgical ablation of the hypophysis, the fact that the hypothalamus is one of the two regions of the mammalian central nervous system capable of active regeneration following injury, and because hypophyseal ablation is a good experimental model to analyze the processes of secretion from the hypothalamus. Thus, in recent years several studies addressing the repercussions of hypophysectomy on the hypothalamus and median eminence have been performed (Villar et al., 1994; Chauvet et al., 1995; Sakamoto et al., 1996; Hara et al., 1997; Polenov et al., 1997; Scott, 1999).

In damaged regions of the central nervous system, astrocytes undergo a profound reaction and a glial scar appears. This scar is formed by the astrocytes themselves intermingled with a considerable amount of extracellular matrix (Windle et al., 1952; Clemente and Windle, 1954). In the glial scar, axons are prevented from growing due to the scar itself and also to the presence of cellular and molecular factors produced by astrocytes and oligodendrocytes (Fawcett, 1997; Fitch and Silver, 1997).

In the stalk adjacent to the hypophyseal area, interruption of the hypophyseal stalk by hypophysectomy, sectioning, or compression leads to a reorganization of the stalk in its attempt to substitute the absent neurohypophysis (Kawashima et al., 1966; Belenky et al., 1973; Raisman, 1973; Polenov et al., 1974; Antunes et al., 1980; Herman et al., 1986). It has been reported that one result of this reorganization is the accumulation of paraldehyde-fuschine-positive neurosecretory material formed by the neurohypophyseal hormones vasopressin and oxytocin (Ibata et al., 1983; Kawamoto and Kawashima, 1985a,b; 1986). At the same time, blood vessels increase and become dilated and the connective tissue increases (Raisman, 1973; Polenov et al., 1981). The number of mitotic glial cells of the hypophyseal stalk increases only in the first moments after hypophysectomy (Billenstein and Leveque, 1955; Polenov et al., 1981) and increases in vasopressin and oxytocin levels have been determined in the median eminence (Dohanics et al., 1992; Makara, 1993).

The presence of blood vessels and pituicytes is fundamental to this reorganization of the proximal stalk (Dellmann et al., 1988; Carithers and Dellmann, 1992; Dellmann and Carithers, 1992). Moreover, the meninges also seem to play an important role in this process. In support of this role, Ishikawa et al. (1995) have demonstrated *in vivo* and *in vitro* that the arachnoid and piamater membranes are necessary for the regeneration of vasopressinergic fibers following hypophysectomy.

Increases in the number and proliferation of

astrocytes located in reactive pathological situations accompanied by modifications in their cytoskeleton (O'Callaghan, 1988) have been described (Eng and Rubinstein, 1978; Latov et al., 1979; Bignani et al., 1980; Velasco et al., 1980; Browning and Ruina, 1984; Osborm et al., 1984; Ludwin, 1985; Miller et al., 1986; Schiffer et al., 1986; Takamiya et al., 1988). Therefore, it is plausible that the changes observed in the present study of the rat median eminence after hypophysectomy could reflect variations in astrocyte numbers and/or variations in their cytoskeletal organization that occur as a reaction to the lesion. However, it should be stressed that alterations of GFAP-immunoreactive elements have been found in intact cerebral areas (Juanes et al., 1989; Schiffer et al., 1993) and that variations appear in experimentally-induced states of endocrine insufficiency such as adrenalectomy (Sánchez et al., 1995) and following corticoid administration (O'Callaghan et al., 1989). Accordingly, the changes observed in our study could also result from the reaction of glial cells to an endocrine deficit.

As in the hypophyseal stalk, after hypophysectomy a reorganization occurs in the median eminence to compensate for the absence of the neural lobe. A similar process could take place as a result of the absence of the pars distalis of the adenohypophysis, even though parts of the pars tuberalis remain. In this sense, reports have been made of changes in the plasticity of the fibers driving various hypothalamic peptides, such as vasopressin and oxytocin (Kawamoto and Kawashima, 1985a,b, 1987; Villar et al., 1994), galanin (Villar et al., 1994; Selvais et al., 1995), VIP, cholecystokinin and dynorphin (Villar et al., 1994), and adrenomedullin (Ueta et al., 1999). Our results show that important changes occur in the GFAP-immunoreactive glial cells located in proximity of the fibers driving these neuropeptides. This observation suggests that these cells may participate in the functional reorganization of the median eminence. The fact that such changes are more pronounced initially and then tend to disappear two months after surgery, as observed here, seems to offer further support for our hypothesis.

In the reorganization of the median eminence at 40 days after hypophysectomy, the changes in GFAP-immunoexpression involve supraependymal and ependymal cells. Tanycytes, which are specialized ependymal cells, express GAFP (Roessmann et al., 1980; Basco et al., 1981). Evidence has been reported for the regeneration of transected axons of adult hypothalamo-neurohypophyseal neurons along tanycyte processes (Chauvet et al., 1995, 1996, 1998), probably because tanycytes may be involved in the regulation of neuroendocrine events since pituicytes (Vázquez et al., 1988) may exert brain-blood barrier functions or may be involved in transport mechanisms between the ventricles and the blood vessels of the portal system (Millhouse, 1975; Scott and Krobisch-Dudley, 1975; Carretero et al., 1987; Karhunen et al., 1995; El-Majdoubi et al., 1996; Fernández-Galaz et al., 1996; Diano et al., 1998; Eyigor and Jennes, 1998; Wittkowski, 1998). Scott (1999) reported that in the process of the regeneration of the neurohypophyseal system, cells and cell processes resembling neurons appear, and these migrate and emerge on the floor of the third ventricle and become localised over the ependymal cells of the median eminence. Although our studies do not exclude the possibility that supraependymal cells could be neuroblasts (Scott and Hansen, 1997; Scott, 1999), the current results demonstrate that GFAP-positive cells become supraependymal cells after hypophysectomy. Moreover, the differences in GFAP-expression observed between the control and hypophysectomized animals in the present study suggest the possibility that tanycytes could be involved in the reorganization of the median eminence since changes were observed in the ependymal layer.

This work is the first study of cellular proliferation in the rat median eminence using PCNA expression. PCNA is an auxiliary cyclin of delta DNA-polymerase that can be used as a marker of cellular proliferation and is considered to be the most sensitive for this type of study. As reported by Oishi et al. (1983) regarding the uptake of BrdU -comparable to thymidine uptake-, this marker is up to five-fold more sensitive for the detection of proliferating hypophyseal cells.

From the four regions analyzed in the median eminence, the present study demonstrates that main changes in cellular proliferation are seen in the preinfundibular and infundibular regions, in a similar way to variations of GFAP distribution in the median eminence.

Both these regions are responsible for maintaining a functional relationship, the release of hypophysotropic factors, between axonal projections from hypothalamic nuclei and the hypophyseal portal system and, after hypophysectomy, with the pars tuberalis. The fact that the main changes in cellular proliferation and in GFAP expression are found in both regions further supports the hypothesis of a restructuring of the median eminence after hypophysectomy. In favor of this hypothesis, Shinoda et al. (1996) have reported the presence of ß-2-microglobulin immunoreactivity in the median eminence of hypophysectomized rats. This is particularly evident in the internal area of the preinfundibular and infundibular regions, which seem to be related to the regeneration and possible redistribution of fibers from the magnocellular neurons of the supraoptic and paraventricular nuclei.

We did not detect the colocalization of GFAP and PCNA in any cells of median eminence after hypophysectomy. Thus, our results point -at least for the time periods studied- to the participation of GFAP-positive cells and proliferation, probably GFAP-negative glial cells or vascular endothelial cells, in the restructuration of the median eminence after hypophysectomy.

In conclusion, our results suggest the occurrence of a functional restructuring of the median eminence after

hypophysectomy. This reorganization is more evident in the preinfundibular and infundibular regions of the medial eminence where GFAP-positive cells are abundant, but show no proliferative activity.

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