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IMMUNOCYTOCHEMICAL AND MORPHOLOGICAL EFFECTS OF SHORT-TERM STIMULATION OF CULTURED ASTROCYTES

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Abstract Both image analysis at light microscopy level and ultrastructural characterization by transmission electron microscopy were employed to evaluate the differentiation stage in young cultured mouse astrocytes after 1-day exposure to dBcAMP, a chemical compound known to induce cell activation. The aim was to validate an experimental model of stimulated astrocytes preserving the properties of recently seeded cells, thus avoiding the overlapping effects of *in vitro* aging. Differentiated astrocytes, as evidenced by GFAP labeling by streptavidin-peroxidase, doubled their number in treated cultures (45%) *versus* controls (23%). In addition, a significant increase in process-bearing astrocytes (elongated and ramified forms) to the detriment of immature polygonal astrocytes, was recorded. No noticeable changes were found in cell perimeter, but cell area displayed a significant reduction in labeled surface of astrocytes undergoing morphological differentiation. Concomitantly, electron microscopy showed that radially organized bundles of numerous intermediate filaments compatible with GFAP replaced the few scattered structures observed in control cultures. However, methodological caution is advisable as regards the relevance of this *in vitro* counterpart of *in situ* reactive astrocytes, since cell plasticity is recognized to depend on culture conditions. At any rate, present quantitative results demonstrate that GFAP-positive cell percentage and cell area measurement are adequate parameters of early immunocytochemical and morphological differentiation, respectively, and thus contribute to a better histometric characterization of an easily available substrate to discriminate the wide variety of factors involved in CNS response to injury.

Resumen ***Efectos inmunocitoquímicos y morfológicos de la estimulación a corto plazo de cultivos astrocitarios.*** Se recurrió al análisis de imagen a nivel de microscopía óptica y a la caracterización ultraestructural por microscopía electrónica de transmisión, para evaluar la diferenciación celular en jóvenes cultivos astrocitarios de ratón expuestos a dBcAMP durante 1 día. Los astrocitos diferenciados, así definidos por la tinción de GFAP mediante estreptavidina-peroxidasa, doblaron su número (45%) con respecto a los controles (23%). Además, y a expensas de las células inmaduras poligonales carentes de prolongaciones, se registró un aumento significativo de astrocitos elongados y ramificados. No se observaron cambios apreciables en el perímetro celular, pero sí en el área celular, ya que la superficie inmunomarcada estaba significativamente reducida. Concomitantemente, la microscopía electrónica visualizó en el citoplasma a numerosos filamentos intermedios de distribución radial y de características compatibles con GFAP, lo que contrastaba con su escasez y menor organización en los cultivos controles. Si bien la reconocida plasticidad evidenciada por el astrocito ante las diversas condiciones de cultivo impone cautela en su empleo como contraparte de la célula reactiva *in vivo,* los cultivos astrocitarios activados representan una herramienta valiosa para la discriminación de factores inherentes a la respuesta del SNC a la injuria. Cabe concluir que en un modelo experimental de astrocitos activados pero carentes de los efectos agregados del envejecimiento celular, los presentes resultados han posibilitado una más precisa caracterización cuantitativa, ya que han permitido establecer que el porcentaje de células GFAP-positivas y la medida del área celular son adecuados parámetros de la temprana diferenciación inmunocitoquímica y morfológica, respectivamente.

Key words: astrocyte, cell differentiation, dBcAMP, image analysis, electron microscopy

Cultured astroglial cells from perinatal rodents are widely used for neurobiological research. Since most neurons are fully developed at this stage, the great majority of brain cells able to multiply *in vitro* arise from active astroglial precursors[1], originally present in the proliferative subventricular zone[2]. Once in culture, dissociated cells from newborn brain form a predominantly glial monolayer, whereas few neurons are able to survive. A shaking procedure is then effective to discard any contaminating neuron and selectively detach oligodendrocytes grown on top of the remaining adherent astroglial cell monolayer, which in turn may be subcultured once to obtain more purified cells[3].

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In both primary of first passaged cultures, transformation from an initial flattened undifferentiated morphology to stellate process-bearing astrocytes takes place. Such conversion, slow in basal conditions or fast after stimulation, seems likely to depend on incorporation of growth factors and chemical agents to the nutrient medium[4,5], rather than on the persistence of any particular phenotype. Among the chemical compounds, an analog of cyclic adenosine monophosphate (cAMP) such as dibutyryl cyclic AMP (dBcAMP), has been extensively employed to stimulate immature cultured cells into reactive-like astrocytes. It is generally accepted that such effects are probably exerted by raising intracellular cAMP levels by stimulating adenylate cyclase or by inhibiting phosphodiesterase activity[6]. In either case, in a confluent monolayer initially made up of predominantly flat polygonal cells, dBcAMP addition to nutrient medium promotes formation of cytoplasmic processes[7,8], increases GFAP content[9,10], and induces ultrastructural changes characteristic of *in situ* astrocytes[11,12]. Therefore, such cell cultures represent a useful tool to attempt defining and localizing biochemical events underlying neurotrophic or inhibitory potential of reactive astrocytes, notwithstanding the limitations of an *in vitro* model[5,13].

According to described routine procedures, 1 mM of dBcAMP has been the most commonly employed concentration for *in vitro* studies, while exposure times vary from 2-5 days to 2-3 weeks[13]. In this connection, we have found that 1-day treatment seemed enough to induce immunocytochemical and morphological differentiation of first subculture of rodent astrocytes, as evidenced by an increased number of GFAP-positive cell foci and greater perimeter/area ratios[14]. As suggested by our preliminary results, short-term stimulation effects were not dissimilar to those achieved at longer exposure time, so that the availability of a monolayer made up of young cells affords an obvious advantage over a culture where astrocyte differentiation could be overlapped by cell aging[15,16].

Therefore, in the present study, we carried out both image analysis at light microscopy level and ultrastructural characterization by transmission electron microscopy to evaluate the differentiation stage in young cultured astrocytes (2 days in vitro) harvested following 24 hs of dBcAMP treatment. Our aim was to validate an *in vitro* model of differentiated astrocytes preserving the properties of recently seeded cells, thus providing a feasible approach to discriminate between molecular events preceding and triggering astrocyte activation. *In situ* cell response has been detected as early as 24 hs after experimental infection with neurotropic viruses by means of classic histological techniques[17], and even within 1 hour of a focal mechanical trauma through the identification of GFAP-mRNA transcripts[18]. Although intracellular activation mechanisms still await clarification, it is hoped that elucidation of neurotrophic or inhibitory potential of such astrocytes will lead to the development of new therapies for CNS viral infections as well as for immune-mediated neurodegenerative disorders.

Materials and Methods

Cell cultures. Mouse brain cell monolayers were obtained as previously described for rat tissues[19]. Briefly, cortical hemispheres were harvested from newborn BALB/c mice, neural tissues digested by 0.25% trypsin, and dissociated cells grown in MEM plus 10% fetal calf serum. Three weeks later, the confluent cell culture was shaken for 2 hs at 37 °C, and the supernatant discarded; the remaining adherent astroglial cell monolayer was then subcultured by trypsinization and cells seeded onto glass coverslips of Leighton tubes and plastic flasks. After 1 day *in vitro,* growth medium was removed and already confluent cell monolayers subjected to 2 hs exposure to 1 mM of dBcAMP (Sigma LAb, USA) in PBS, while control cultures received an equivalent amount of PBS alone. Subsequently, maintenance medium (MEM plus 5% fetal calf serum) was added. Twenty-four hs later, cell monolayers were processed by techniques either for light or electron microscopy.

Light microscopy. dBcAMP-treated and non-treated cultures grown in glass coverslips of Leighton tubes were fixed with chilled methanol plus 5% acetic acid for 5 min, and then incubated overnight with 1/1600 dilution of rabbit polyclonal antibody, followed by successive 30-min incubation with 1/100 dilution of goat anti-rabbit antibody and 1/100 dilution of streptavidin-peroxidase. All three reagents were purchased from Dako Lab, USA. Reaction development was achieved by 10 min exposure to 0.03% diaminobenzidine tetrahydrochloride (DAB) obtained from Fluka Lab, USA, plus 0.02% hydrogen peroxide. Finally, light counterstaining with Mayer's hematoxylin was performed for nuclear counting of whole monolayer. For more thorough analysis, a suitable software (IMAGE PRO-PLUS 1.1, Media Cybernetics, Silver Spring, USA) was employed. Hardware consisted of a black and white CCD video camara coupled to a Zeiss microscope. Under standardized conditions using a 500/510 nm green filter, which was chosen because DAB absorption proves maximal at such wavelength, final image resolution was then fixed at 768 x 512 pixels with 8-bit depth, representing 256 grey levels. Accordingly, 20 fields for each coverslip were digitalized at 250 X magnification. In each field, the number and percentage of GFAP-labeled and unlabeled cells were recorded. Resorting to GraphPad Instant software (GraphPad Software Inc, USA) values were analyzed statistically by non-paired t test and ANOVA. For morphometric characterization, GFAP-positive astrocytes were discriminated by cursory inspection as polygonal (plentiful cytoplasm devoid of processes), elongated (scarce cytoplasm and at most two processes) and ramified (scarce cytoplasm and at least three processes). At 30 X final magnification, the number and percentage of each form (polygonal, elongated or ramified) of GFAP-positive astrocytes were recorded. Besides, 30 cells of each form were evaluated according to their perimeter and area in digitalized images at a final magnification of 400 X. Depending of the eyepiece employed, the equipment was previously calibrated by means of a scalemeter (Carl Zeiss, Germany), and the value obtained incorporated with a 0.1 µm error.

Electron microscopy. Cell monolayers grown in plastic flasks were washed three times with 0.1 M sodium cacodylate buffer (pH 7.4), then fixed in a solution containing 4% parafor-

maldehyde and 2% glutaraldehyde in 0.1 M sodium cacodylate buffer, for 2 hs at room temperature. Cell monolayers were then harvested with a rubber policeman, and pelleted at 400 x g for 10 min. Pellets were post-fixed for 1 hr in 1% osmium tetroxide. After dehydration with 70, 96 and 100% ethanol, pellets were clarified in acetone and embedded in Epon 812. Slides were obtained with glass knives, and stained with uranyl acetate and lead citrate. Grids were observed in a Zeiss EM-109-T transmission electron microscope at 80 kv.

Results

Light microscopy. After 1-day treatment with dBcAMP, cell monolayer appeared less dense than control, subsequently to progressive cell body retraction of former large polygonal astrocytes that became widely separated from one another.

Besides, while non-stimulated cell monolayers, predominantly made up of polygonal cells, showed a limited degree of GFAP labeling in perinuclear cytoplasm, dBcAMP-treated cultures exhibited a more uniform staining of cell bodies as well as their cytoplasmic processes. This enhanced immunocytochemical differentiation was confirmed by counting GFAP-positive astrocytes (Fig. 1), since their percentage increased almost two-fold *versus* control cultures (45 vs 23%). Further-more, when such percentage of labeled astrocytes was discriminated according to their polygonal, elongated or ramified appearance (Fig. 2), a significant increase in ramified process-bearing cells, to the detriment of immature polygonal forms, was recorded in dBcAMP-stimulated cultures.

As regards morphological changes evaluated by cell perimeter (Table 1) and area (Table 2), no marked alterations were found in the former, but the latter displayed a significant reduction in labeled surface in both elongated ($p < 0.05$) and in ramified ($p < 0.001$) stained astrocytes.

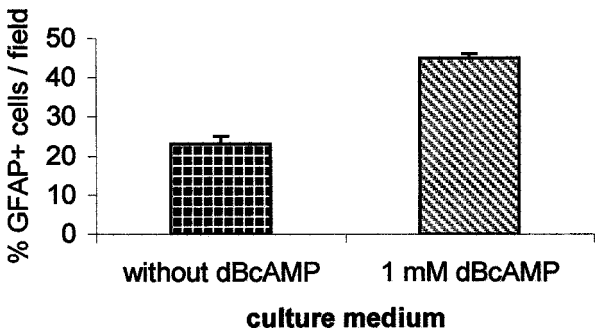

Fig. 1.– Percentage of GFAP-positive astrocytes counted after 1-day treatment. Values are means ± SEM in 60 microscopic fields (total manification 100 X) corresponding to 3 coverslips of Leighton tubes. In dBcAMP-treated cultures, percentage of labeled astrocytes is greater than in non-stimulated cultures (t test, $p < 0.001$).

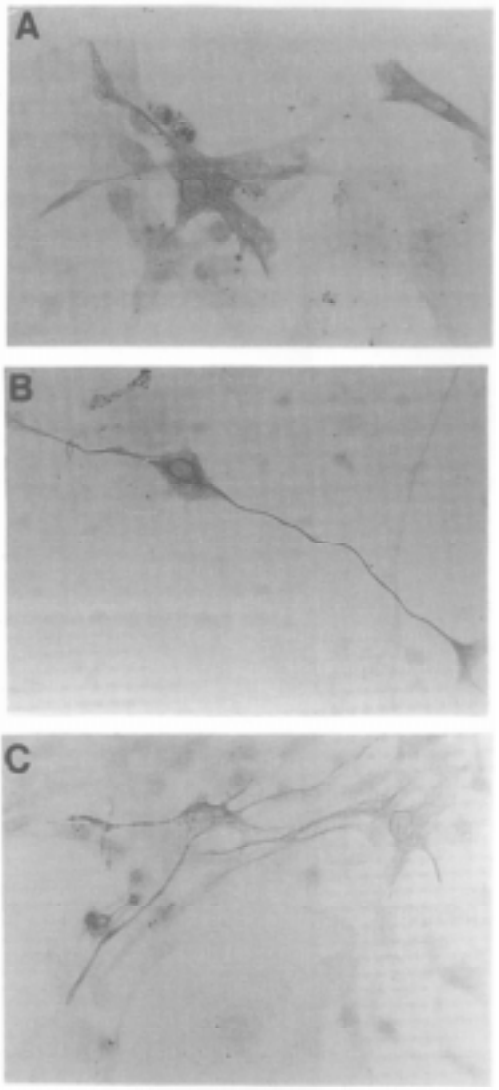

Fig. 2.– GFAP labeling of cultured mouse astrocytes after 1 day-treatment with dBcAMP. Streptavidin-peroxidase. 40 X. GFAP-positive astrocytes undergoing progressive morphological changes, from polygonal (A) to process-bearing cells whether elongated (B) or ramified (C).

Electron microscopy. Bunches of intermediate filaments (8.0-9.0 nm of average diameter) were observed in greater number in cytoplasm of dBcAMP-(Fig. 4, C) than in non-stimulated (Fig. 4, A) cells. At higher magnification, random distribution of intermediate filaments in control cultures (Fig. 4, B) contrasted with the more tightly radial pattern following dBcAMP exposure (Fig. 4, D). No other major differences could be detected

Table 1.– *Perimeter of three different forms shown by cultured mouse astrocytes after 1-day treatment. Values are means ± SEM in µm from 30 randomly chosen GFAP-positive cells. No significant differences were disclosed by t test*

Culture medium	Astrocytes		
	Polygonal	Elongated	Ramified
without dBcAMP	356.2 ± 27.3	316.7 ± 18.2	451.4 ± 29.0
1 mM dBcAMP	256.7 ± 48.6	269.7 ± 45.2	368.9 ± 39.0

Table 2.– *Area of three different forms shown by cultured mouse astrocytes after 1-day treatment. Values are means of ± SEM in µm² from 30 randomly chosen GFAP-positive cells. Following dBcAMP stimulation, values are significantly lower in elongated and ramified astrocytes (t test, * p < 0.05 and ** p < 0.01, respectively)*

Culture medium	Astrocytes		
	Polygonal	Elongated	Ramified
without dBcAMP	1426.2 ± 185.3	553.9 ± 70.9	896.5 ± 105.0
1 mM dBcAMP	851.8 ± 225.7	352.7 ± 62.5*	519.9 ± 48.8**

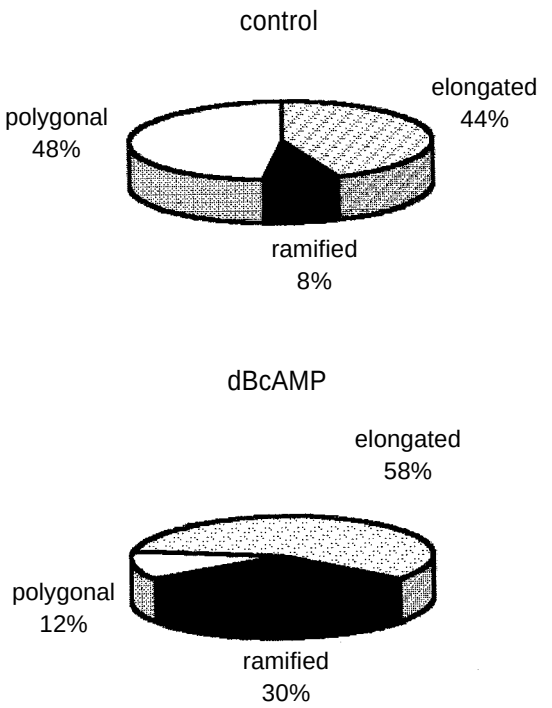

Fig. 3.– GFAP labeling of cultured mouse astrocytes after 1 day exposure to nutrient medium with or without dBcAMP. For each labeled astrocyte form (polygonal, elongated or ramified), cell counts in 20 microscopic fields at 30 X were expressed as percentages of total number. Subsequent to dBcAMP stimulation, polygonal cell percentage decreases while that of process-bearing cells increases.

between both groups, except for more plentiful organelles in the denser cytoplasm of untreated cultures.

Discussion

Following the first descriptions of morphological and biochemical changes induced by dBcAMP in cultured astrocytes[20-20], interest in this compound as stimulus to cell differentiation waned, given the concurrent discovery and later characterization of so-called glia maturation factors obtained from brain extracts[23-25], as well as the development of serum-free defined media for growth and differentiation of glial cells in culture[26, 27].

Nevertheless, further characterization of dBcAMP effects by demonstrating its capacity to inhibit cell cycle and DNA in cultured astrocytes[6], together with the pressing need to develop an *in vitro* counterpart for both quiescent and activated astrocytes in the whole organism, has led to growing application of such a readily handled chemical agent. Although most studies traditionally resorted to more than 7-day treatment[28-31], comparable results have been described with lower exposure[8, 32-34], mainly dealing with up- or down-regulation of structural and enzymatic protein synthesis.

In our case, we were spurred by the goal of achieving a fast differentiation of astrocyte culture as a substrate for a neurotropic virus exerting such an early cytolytic effect as Theiler-GDVII strain[35]. In this connection, it became evident that only a relatively spared cell monolayer could enable a comparative approach

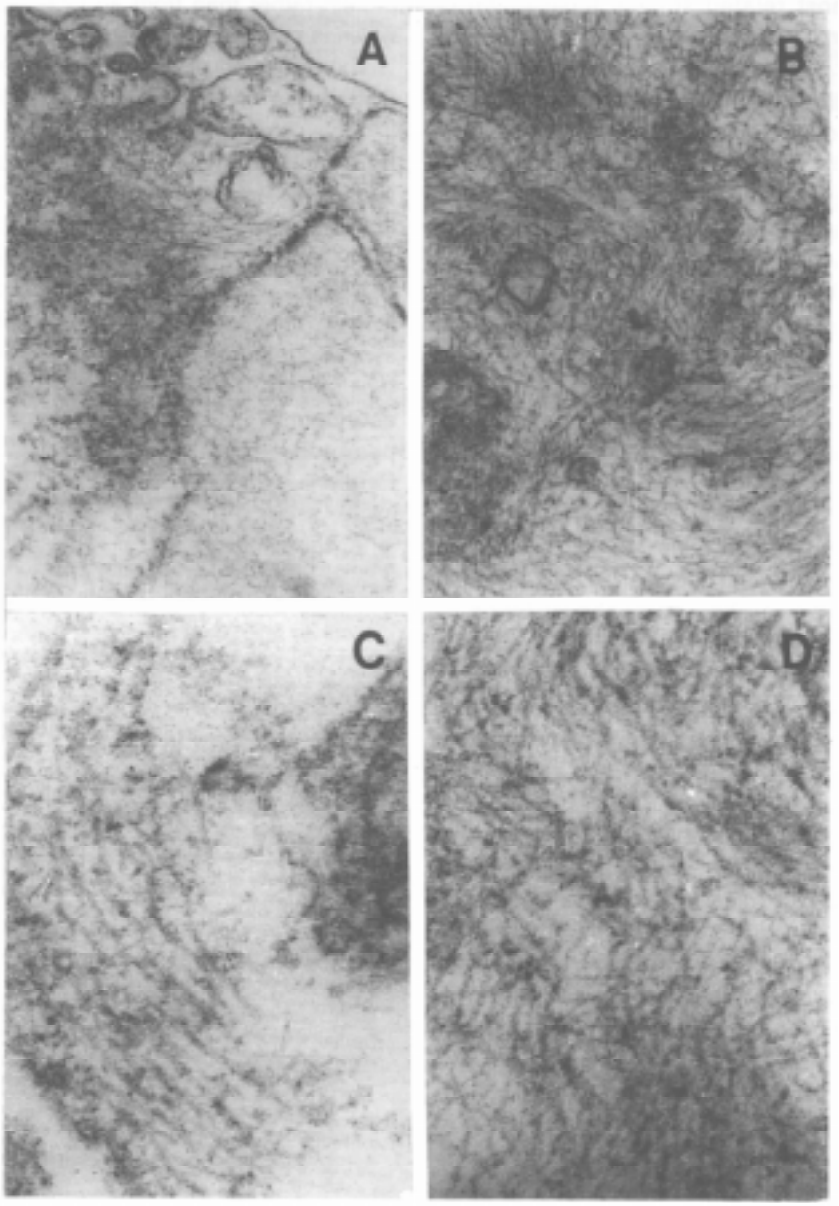

Fig. 4.– Ultrastructure of cultured mouse astrocytes after 1-day exposure to nutrient medium with or without dBcAMP. In both cases, bundles of intermediate filaments characteristic of astrocytes are present. Non-stimulated astrocytes (A and C) contain a higher density of organelles but a low number of intermediate filaments. Following stimulation (B and D), an increased number of intermediate filaments arranged in tightly packed bundles may be observed. A and B: 25,000 X; C and D: 125,000 X.

through image analysis which implied discriminating the potential changes exerted by viral infection *per se* on the activated astrocyte. However, the recognized plasticity of the cultured astrocyte requires methological caution when interpreting data[13], since chemical agents (mono or dibutyryl cyclic AMP, theophylline), as well as nutrient medium composition (serum withdrawal, hormones, brain extracts) may enhance the spontaneous morphological and immunocytochemical differentiation evolving as a function of *in vitro* ageing. Therefore, overall changes must be quantified to allow histometric characterization of treated *versus* untreated cultured astrocytes, a crucial goal when evaluating the effects of biologically active agents such as viruses. So far, quantitative parameters of both spontaneous and induced *in vitro* astrocyte differentiation have been

lacking, since most related studies have relied on traditional visual microscopic examination.

As regards our quantitative results, the percentage of GFAP-positive cells represented a suitable parameter to assess immunocytochemical differentiation, since significant differences were observed in astroglial cultures subjected to 1-day exposure to dBcAMP, even though such treatment decreased total cell number due to the mitosis inhibition accompanying cell differentiation.

The finding that following dBcAMP stimulation, GFAP-labeled astrocytes showed reduction in cell area with maintenance of perimeter can be explained by the mechanism according to which cell processes develop in cultured astrocytes: indeed, they are not projected from cell periphery but result from a peculiar cytoplasmic retraction characterized by the presence of cavities adjacent to GFAP-positive radial bundles becoming sharply demarcated. It is the subsequent reabsorption of such cell cavities which gives rise to preformed astrocyte processes, as has been shown by phase contrast microscopy[11] and controlled silver impregnation[36]. Therefore, this ongoing morphological transformation would be characterized by maintenance of cell periphery values with reduction in cell area, the latter proving an adequate parameter to evaluate dBcAMP-induced changes.

Increased astrocyte differentiation assessed by light microscopy was confirmed by ultrastructural findings, given the remarkable change in the intermediate filament pattern observed after dBcAMP treatment, as radially organized bundles progressively replaced the few scattered structures in control cultures. Although immunoelectron microscopy was not performed to determine whether GFAP was the main component of such organized intermediate filaments, their size, shape and cytoplasmic distribution agreed with the recognized GFAP profile. In support, gold immunolabeling procedures carried out by Abd-El-Basset et al[37] have shown that the composition of intermediate filaments varies with the degree of astrocyte differentiation and the nature of functional demands, since GFAP gradually replaces vimentin as cells mature.

To sum up, histometric parameters as validated by the present results, appear adequate to evaluate cell differentiation stage in stimulated cultured astrocytes. A model of *in vitro* astrogliosis, whether early or chronic, enables experimental manipulation of factors involved in one of the earliest and most remarkable cellular reponses subsequent to CNS injury. As a counterpart of *in situ* activated astrocytes[12], cell culture subjected to short-term dBcAMP stimulation would allow accurate analysis of the wide variety of hormones, cytokines and growth factors that regulate initial GFAP expression, presumably resulting from post-translational modifications of the protein rather than from an increase in GFAP-mRNA, which is only found after a longer treatment[38].

Extrapolation of valid information from an *in vitro* model to the whole organism may provide a further insight into the *in vivo* biological relevance of glial activation. Although it is known that astrocytes have beneficial supportive functions within the CNS[39], their potential detrimental role has received increasing attention, since astrogliosis is a hallmark of several neurodegenerative[40] as well as infectious and immune-mediated[41] diseases. In this connection, attempts to inhibit or delay the synthesis and/or polymerization of GFAP[42] represent a fascinating therapeutic approach to control the development of a physical and/or chemical glial barrier perpetuating the impairment of CNS structures and functions.

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