.Morphometric analysis of neurons and astrocytes from postnatal cerebral cortex by specific protein markers

Análisis morfométrico de neuronas y astrocitos derivados de corteza cerebral postnatal mediante marcadores proteicos específicos

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Resumen

Los cultivos celulares son una herramienta para estudiar los mecanismos biológicos y bioquímicos implicados en la diferenciación, la supervivencia y la muerte celular, no obstante, el uso de cocultivos ha sido poco explorado. El objetivo de este estudio fue establecer las condiciones de un cultivo primario mixto, neuronal y astrocitario, procedente de células de rata en etapa postnatal (P3), describiendo las características morfométricas de estas células y el comportamiento general del cocultivo en un curso de tiempo de 6, 7, 8 y 9 días. Se determinaron los cambios morfológicos en neuronas (MAP-2) y astrocitos (GFAP) con contratinción nuclear (Hoechst) y se evaluaron ocho parámetros cuantitativos para cada linaje usando el software Image J-1.49 (NIH). La densidad celular, perímetro y área de neuronas y astrocitos aumentaron al día 8 ($p<0.0001$), comparado con el día 6 del cultivo mixto. El recuento celular, área, número y longitud de las neuritas ($p>0.05$) no mostraron diferencias. La proporción de astrocitos / neuronas fue de 1:1, sin diferencias entre los días de cultivo. En conclusión, el cultivo mixto neurona-astrocito en condiciones descritas en este estudio favorece la caracterización morfométrica de estas células y constituye una herramienta en estudios de neuroprotección y neurotoxicidad en tejido nervioso in vitro.

Palabras Clave: *Morfometría, cultivo neural, inmunofluorescencia, MAP-2, GFAP.*

Abstract

Cell cultures is a tool to study biological and biochemical mechanisms involved in differentiation, cell survival and death; however, the use of mixed cultures has rarely explored. The aim of this study was to establish the conditions of a mixed culture (Neurons and astrocytes) from postnatal rat cells (P3) describing the morphometric characteristics of these cells and the general behavior of coculture in a time course of 6, 7, 8 and 9 days. Morphologic changes in neurons (MAP-2) and astrocytes (GFAP) with nuclear counterstain (Hoechst) were determined by immunocytochemistry and eight quantitative parameters for each linage were assessed by using Image-J 1.49 software (NIH). Cell density, perimeter, area of neurons and astrocytes increased on day 8 ($p<0.0001$) compared to day 6 from the mixed culture. Cell count, area, number and length of neurites ($p>0.05$) did not show differences. The ratio astrocytes / neurons was 1.1, without differences between days of culture. In conclusion, the mixed cell culture neuron-astrocyte under the conditions described in this study allow the morphometric characterization of those cells and constitute an important tool in studies of neuroprotection and neurotoxicity in nerve tissue in vitro.

Keywords: *Morphometry, neural culture, immunofluorescence, MAP-2, GFAP*

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The interaction between neurons and glial cells in the central nervous system (CNS), is vital to preserve cerebral homeostasis during embryonic development and adulthood (Liu *et al.*, 2012). Alteration of the relationship between neurons and glial cells causes reduced cell viability, changes in cell density, morphology and distribution, leading to neurodegeneration and cell death; for this reason, it is important to recognize the relationship between these nerve cells and when the cell structure is altered (Liu *et al.*, 2012; Stipursky *et al.*, 2011).

In most regions of the nervous system, progenitor cells become neurons and glia; particularly, glial cells are substantially numerous and play

an important role in the synthesis, release and reuptake of various neurotransmitters than neurons (Cimadevilla *et al.*, 1997; Tiwari *et al.*, 2014). Most of the non-neuronal glial cells modulate the type of neurodevelopment, including the support awarded by astrocytes, migration through the radial glial cells, regulating the number of neurons and connections among functional cells that favor the maturation of the nerve system (Colognato and ffrench-Constant, 2004; Freeman, 2006).

The mammalian neural stem cells can give rise to both neurons and glia; however, whereas the neurogenesis is almost completed immediately after birth, the gliogenesis remains active and evolve during the postnatal period (Sizonenko *et al.*, 2008).Therefore, evaluation of the dynamic events occurring in a mixed culture of neurons and astrocytes obtained from rat brain cortex postnatal (day 3) (Majumdar *et al.*, 2011). It is necessary and important to establish an alternative experimental model to assess pharmacological substances and their potential biological effects.

Appropriate function of neurons and glial cells requires intrinsic activation of genes which express proteins with defined specificities (Sauvageot, 2002). In addition, extrinsic factors such as neurotrophic factors are also necessary for cellular differentiation and development (Alfonsi *et al.*, 2008; Verkhratsky *et al.*, 2011) and play a critical role in the induction, specification, survival and neuronal maturation. Certain neurotrophic factors also function in the adult brain for supporting and protecting mature cell populations (Sullivan and Toulouse, 2011). Proteoglycans, interleukin 6 (IL-6) and some cytokines have been shown to have a central role in the development of the nervous system, as modulators of the proliferation, differentiation and cell migration, as well as axonal growth and synapse formation (Alfonsi *et al.*, 2008; Araujo *et al.*, 2010) generation of the facial nucleus involves complex developmental processes that will lead to the formation of a structure composed of motor neurons, astrocytes and oligodendrocytes. The implication of LIF-related cytokines in the development of this nucleus came to light with the analysis of mice mutant for the receptor of these cytokines, LIFR beta, which exhibit a massive loss of facial branchiomotor (fbm. Exploration of the potential effects of those factors early in the postnatal period may offer new possibilities to modulate the function of the CNS.

Advances in neural cell culture have strengthened biology knowledge, particularly in the structure, morphology and development of different cell types, as well as neuroprotective or neurotoxic effects of various substances; however, the interaction between neuronal and glial cells such as astrocytes, which are critical in the physiological interdependence mediated by chemical signals such as trophic factors, in the formation of synapses, and providing structural support, homeostasis and other functions that support the development, growth and neuronal activity, are rarely taken into account (Fu *et al.*, 2013; Hatton, 2002).

Considering cellular interactions, mixed cultures and co-cultures, are proposed as a valuable tool for studying *in vitro* the interaction between neurons and astrocytes, simulating what occurs *in vivo*, as opposed to individual cell cultures or single cell type that only provide information on specific responses and whose survival depends exclusively on the conditions of the culture medium. The cell interaction that occurs in a mixed cell culture provides a trophic support, structural and physical contact that cannot be replaced by any medium (Stoppelkamp *et al.*, 2010)however, divergent results are a likely consequence of media modifications and culture composition. Using Fura-2 based imaging techniques, we here set out to compare calcium responses of rat hippocampal neurones and glia to excitatory stimulation with l-glutamate in different culture types and media. Neurones in neurone-enriched cultures had increased responses to 10 \u03bcM and 100 \ u03bcM l-glutamate (+43 and 45%, respectively; p's< 0.001,and may be a more accurate way to evaluate the interaction and responses between those cells.

Studies with mixed cell cultures have supported the view that glial cells play a neuroprotective role by reducing the effect of toxicity on neurons (Lee et al., 2004; (Zuo *et al.*, 2014) Rosenfeld's staining and immunofluorescence staining of microtubule-associated protein 2 (MAP2 particularly glutamate that has been reduced toxicity in mixed cultures than in pure neuronal cultures (Perez, 2006); however, in order to understand the morphological and functional changes of nerve cells in the development and relationship with other cells in an integrated microenvironment, *in vitro* models under controlled conditions of mixed cultures are required to simulate the developmental process that occurs *in vivo*. The system should

be reproducible and applicable to study cellular responses versus time of postnatal development.

Materials and Methods

Primary Cortical Culture

Wistar rats were maintained and handled according to the ethical principles to use laboratory animals in research, Colombian standards (Law 84 of 1989) and guidelines described in the "Guide for de Care and Use of Laboratory Animals". Rat primary neurons and astrocytes cultures were obtained from cerebral cortices of newborn rats (P.3) from the animal facility of the School of Science at the University of Tolima upon approval of the Local Ethics Committee. The cortices were collected and put on ice-cold Hanks' balanced salt solution (HBSS) containing 10 mM HEPES, with penicillin 100 IU/ml, and streptomycin 100 µg/ml (LABG&M).

After chemical dissociation with a solution containing 0.25% trypsin EDTA (0.5% 10X Gibco) and 1% DNAse I (Roche Labs.), the tissue was incubated for 25 minutes at 37°C and the reaction stopped with the same volume of medium DMEM /F12 1:1 (1X Gibco) + 5% FBS (LABG&M). Also, it was mechanically dissociated with gradually finer pipettes. The cell package was centrifuged at 1700 r.p.m. per five minutes. After staining with 0.4% Trypan blue stain (15250-Gibco) only viable cells were counted and plated at a density of 340,000 cells/cm^2 onto poly-L-lysine coated coverslips located in Petri plates (P-60). Two days after plating half of the cell culture medium volume was changed with new Neurobasal medium (1X Gibco) supplemented with 2% B27, 100 U/ml penicillin, and 100 µg/ml streptomycin, (all from Gibco) and dextrose (5% 1:10 v/v). Every 2 days, the one half of medium was changed with new one and the cultures were observed in inverted phase contrast microscope (Advanced ™ XD-202). Cell cultures were kept at 37°C in a humidified atmosphere containing 5% CO_2. Typically, this procedure yields more than 75% neurons with 25% of astrocytes at four days of plating. This culture will be referred to as neuron-astrocyte cortical culture without exposure to cytosine arabinoside (Ara-C inhibitor of astrocytic proliferation). The medium was changed, and then it remained the same thereafter. The population of cells will be referred to as mixed cortical culture.

The culture conditions were slightly modified in order to provide optimal medium for growth, development and maintenance of neurons and astrocytes simultaneously, based on others validated protocols (Viviani, 2006). Primary cortical cells were seeded onto Petri plates (P60) at similar conditions a density of 2.5 or 5.0×10^4 cells/mm^2, corresponding to 250,000 and 500,000 cell/cm^2 and cultured in DMEM plus 2.5% fetal bovine serum (FBS) for the first two days. The medium was then replaced with neuronal cell culture medium (Neurobasal ™) supplied with B27 (Gibco Supplement 50X) and reduced level of FBS (2.5%) plus dextrose 5% but L-Glutamine free to permit growth and differentiation of neuron and astrocytes simultaneously, different to the procedure of a pure neuronal culture. Cells were fixed at different times (6, 7, 8 and 9 days) of the mixed culture with paraformaldehyde 4% before staining.

Immunocytochemistry

The immunocytochemistry protocol followed the Current Protocols (Donaldson, 2001) with slight modifications. Briefly, cells grown on poly-L-lysine coated slides were washed with PBS 0.1 M and fixed by adding paraformaldehyde 4% for 25 minutes at 4°C. To saturate unspecific binding sites, cells were incubated for 30 minutes at room temperature (RT° C) with a blocking solution containing 2% Bovine Serum Albumin (BSA) and 0.1% Triton X-100 in PBS 0.1M. Cells were then incubated at RT for 60 minutes with anti-GFAP (1:200, rabbit, Sigma) and anti-microtubule-associated protein 2 (MAP2; 1:500, mouse, Sigma) antibodies. All antibodies were diluted in blocking solution and after two consecutive washes (5 minutes each one) with PBS 1X, cells were incubated for 1 hour at RT °C with Alexa Fluor 546-conjugated anti-rabbit (1:1,000 Life Technologies) and with Alexa Fluor 488-conjugated anti-mouse (1:1,000. Life Technologies) antibodies. In some experiments, the incubation with secondary antibodies was followed by a 30-minutes incubation with Hoechst 33258 (Life Technologies) diluted 1:5,000 in PBS. Coverslips of circulars glass were mounted in anti-bleaching medium (Fluoromount F4680 Sigma) and analyzed by fluorescence microscopy Zeiss AXIO Lab.A1 and fluorescent images were acquired using a Zeiss Axio cam (ERc 5s software, Germany) connected to PC workstation and processed by Optical Digital Morphometry (ODM). Fluorescence microscope was equipped with 3 fluorescent channels (blue, green and red) of Light Emitting Diode (LED) and plan

Fluor/apochromatic objectives. The studies were performed using 20X objectives (0.75). All slides were photographed under the same conditions for exposure time, magnification, lamp intensity and camera gain. Quantitative analysis was performed on fluorescent images generated in 3 fluorescent colors (Stained for MAP-2, GFAP and Hoechst 33258 Life Technology). Although, the entire surface of the sample was quickly scanned, the central region of the coverslip (Excluding the edges to eliminate some cell aggregation and fluorescence saturation) was used for morphometric analysis.

An unbiased quantification of astrocytes and neurons were made by staining the cells (Positive cells) with anti-GFAP and anti-MAP-2 antibodies respectively and counterstained with Hoechst 33258. The cells were evaluated by stereological and morphometric method at 20X magnification and processed with Fiji Image-J 1.49 software (NIH).

Cell counting (Neurons and astrocytes),neurons/ astrocytes ratio, numbers and length of primary neurites, total cell perimeter, shape factor (%), fraction area and simple density, and cell morphology (phenotype description) were quantified in a time course (6, 7, 8 and 9 days) after seeding. Two coverslips were processed per treatment where three photos in four different fields were taken for a total of twenty four fields per treatment. In all cases, the digital images were quantified. Cell counting was made by manual method and in all cases a standardized protocol adapted of Image-J NIH system was used.

The following parameters were set for specific morphometric analysis: Longest neurite length, minor neurite length and numbers of neurites/cell were evaluated at least in ten cells per field (40 X). The counting frame was made by manual method using Fiji Image-J and the statistic Graph Pad Prism 5 software.

Nonparametric Kruskal-Wallis and post-hoc Dunn's test for multiple comparisons among the treatments were used. Data was previously subjected to Shapiro-Wilk for normality test. All values are expressed as mean ± S.E.M. Statistical analysis was performed using Graph-Pad Prism 5 software and for all tests, p <0.05 was considered significant.

Results and Discussion

In this study, the establishment of a primary mixed culture of rat cortical cells in postnatal age (P.3) was established for simultaneous morphometric analysis of neurons and astrocytes; in addition to, the changes in these cells in a controlled culture medium as it favored the process of cell maturation; specifically, quantification of two population and the proportion of both cell types (Neurons and astrocytes). Neurites development without differentiating between dendrites and axons, the morphological changes of the lineages each day of cultivation and the changes by growing cells during the development. The data provided constitute an important tool for future studies on neuron-astrocyte cell function.

Primary rat cortical mixed cultures

The mixed cultures were prepared from cerebral cortex of neonatal Wistar rats (Postnatal day 3) as previously described and similar to other reports (Ould-Yahoui *et al.*, 2009; Pool *et al.*, 2008; Zuo *et al.*, 2014). On the 6th day, the cultures were fed with medium containing FSB (2.5 mg/ml) to permit the development of astrocytes, keeping them in a similar proportion to neurons (Figure 1 B-C). The neurons were cultured 9 days for experimental use. The conditioned medium for the mixed culture allowed the growth, development and survival of both linages (Neurons and astrocytes) which was evidenced in cells with typical and unique shape for the days of the culture (Figure 4 A), in terms of size, shape, number of neurites (Data not shown) and volume (Figure 4 B) was similar to those reported by others (Stoppelkamp *et al.*, 2010).

The algorithm allowed the determination of total neurites length, the number of neurons and also, integrated density, relative area, perimeter and volume of cells. The algorithm allows control of thresholds for immunofluorescence staining intensity and cell/nuclei size. This experiment represents a useful tool for quantification of cells (Neurons and astrocytes) and changes in density or size from a variety of cells with applications that include potential identification of molecules that can block or stimulate neurites outgrowth in conventional or modified culture media or the identification of agents that can overcome neurites outgrowth inhibition by inhibitory substrates (Mitchell *et al.*, 2007).

The microfluidic cell co-culture platform developed by Majumdar *et al.*, (2011), permits individual manipulation of the microenvironment of different cell types, make them ideal for studying central nervous system interactions on both cellular and molecular level (Majumdar *et al.*, 2011). However, the manipulation of the membranes, nutrient media and the establishment of communicating the two types of cells plated, differs from the model used in this study, in which the neuron-glia interactions are allowed from the first day of cultivation; thus, the ratio and cellular dynamics is different.

Morphometric parameters of neurons

Based on stereological and morphometric principles, previously described in the literature (Pool *et al.*, 2008; Price *et al.*, 2006; Ramm *et al.*, 2003; Ronn *et al.*, 2000)L, of neurites per cell was subsequently estimated from the number of neurite intersections, I, per cell by means of the equation L = (??d/2 at least eight (8) variables to neurons in mixed culture (Table 1) were quantified.

Table 1. Morphometric parameters of neurons analyzed in a mixed cell culture

Parameters / mean ± S.E.M.	Sixth day	Seventh day	Eighth day	Ninth day
Number of neurons per field	48.0 ± 6.90	54.0 ± 4.50	55.0 ± 4.30	69.0 ± 3.50
Number of neurites per neuron	3.9 ± 0.46	3.7 ± 0.37	3.5 ± 0.34	3.5 ± 0.62
Neurons area (μm^2)	51.1 ± 5.02	71.4 ± 6.83	97.3 ± 13.40	60.0 ± 3.65
Neurons perimeter (μm)	67.3 ± 5.65	96.9 ± 7.52	130.7 ± 18.29	64.6 ± 4.54
Integrated density neurons (R.U.)	116.7 ± 15.1	85.8 ± 20.7	123.8 ± 19.8	92.7 ± 13.1
Neurons relative area 2D (μm^2)	176 ± 49	197 ± 61	141 ± 38	119 ± 37
Neurons volume (μm^3-3D)	16.1 ± 1.82	23.3 ± 2.83	18.46 ± 1.96	14.0 ± 0.92
Neurons surface (μm^2)	71.7 ± 8.10	104 ± 12.58	82.0 ± 8.69	62.3 ± 4.07

Much of the analysis of neurites outgrowth has been done in rat embryonic hippocampal culture; however, analysis in other systems does not always correspond to hippocampal culture and sometimes are even in contradiction with the results obtained in culture (Khodosevich and Monyer, 2010). That is why, the cortical cultures differ from hippocampus, even under conditions of co-culture or mixed culture from cells in early postnatal stage.

The results indicate that neuron numbers remained stable during development, with no significant differences throughout the evaluated time period. It is possible that the absence of differences in length and number of neurites between different days of culture will be the result of an already differentiated post natal neuron. The manual cell counting and measurement of neurites length provided similar results to those obtained in other studies who used an automatized system (Price *et al.*, 2006)currently available techniques for measuring neurite outgrowth are either time or resource intensive. The authors established a system in which chronic treatment of a subcloned SH-SY5Y cell line with aphidicolin and various concentrations of nerve growth factor (NGF without differences between two system.

In the present study, the length of the longest neurites were 52.26 ± 6.93 um (Day 6), 56.58 ± 4.92 μm (day 7), 51.61 ± 6.24 μm (day 8) and 22.12 ± 3.20 μm (Day 9); by which it can be inferred that high seeding density (350,000 cell / cm^2) and low input of FSB (2.5%) and glucose (5%) in the culture medium, influenced the extent of neuronal dendritic processes, as it was observed from the first day of culture (Data not shown) until day nine; however, in another study (Correa, G; Lombart, M. 2010) length of dendrites was of 93.90 ± 5.01 μm (24 h) much higher than that reported in the present work even with less time of culture. In our report, the measurements of longest neurites length were around 50 μm, with the exception of day 9 where the longest neurites was 22.12 ± 3.20 μm with significant differences (p <0.05) compared to days 7, 8 and 6 of culture.

Neuronal cell culture may be affected by different factors that can induce stress and/or apoptotic responses and alter the growth and development

of neurites. A number of active compounds at sublethal concentrations may affect the biochemical mechanisms involved in neurites extension (Yeyeodu *et al*., 2010)lengthy sample preparation and handling, costly reagents and slow rates of data acquisition and analysis. We have developed a high throughput screen (HTS; thus, changes in neurite extension after exposure make them feasible indicators of cell stress in healthy mixed cultures. In the other hand, studies in cultured PC12 cells have shown that other substances such as donepezil could induce neurites growth through the enhancement of growth factors such as NGF by activating ERK (Oda *et al*., 2007) whereby the increased length of neurites in culture can be associated with increased availability of trophic factors in the culture medium. Mature neurotrophins (NTs) including nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF) and NT-3, NT-4/5 are well characterized positive signals promoting neurites outgrowth (Sun *et al*., 2012).

The Neurobasal™ is a commercial medium for neuronal culture; meanwhile, MNB27 presents the conditions that favor the development of neuronal over glial cells such as optimal concentrations of some amino acids, zinc sulfate, vitamin B12 development, among others, in comparison with other culture media as DMEM and DMEM / F12 (Correa &, Lombart, 2010) used for the mixed culture in this research and may be responsible for some of the morphological or structural differences.

The number of neurons per field had a mean value of 56.5 ± 4.8 for the four days of culture with a slight increase from day 7 but no significant differences (p>0.05) were observed for neuronal counts (Data not shown) between days of culture (Figure 1 B). The number of neurites per neuron was 3.6 ± 0.4 without significant differences (p>0.05) between days of mixed culture, similar to that reported by Correa & Longart (2010) which ranged between 3-6 neurites per cell throughout the culture period (21 days) demonstrating that neurites number tend to be constant in the cultures. In the cell lines, the number of neurites per neuron is on average 3.5 in several experiments (Price *et al*., 2006) currently available techniques for measuring neurite outgrowth are either time or resource intensive. The authors established a system in which chronic treatment of a subcloned SH-SY5Y cell line with aphidicolin and various concentrations of nerve growth factor (NGF. In other study that used nerve growth factor (NGF) and neurotrophin-3 (NT-3) showed a dose dependent outgrowth of neurites. These responses suggest that neurites area measurement using image analysis provides a robust method to assess neurotrophic activity of growth factors and neurotrophin mimetics in vitro (Bilsland *et al*., 1999).

On the other hand, significant differences were observed for neuronal area between days six-seven (p <0.05) and highly significant between days six eight (p <0.0001) and eight-nine (p <0.01) (Figures 2 C). Similarly, highly significant differences were evidenced for integrated density of neurons between days six-seven (p <0.01), six-eight (p<0.0001) and six-nine (p<0.0001) (Figure 2 B). In relation to the perimeter of neurons, highly significant differences between days seven-nine (p <0.01) and also between days eight-nine (p <0.0001) were observed but no differences were observed in relation to the neuronal surface and volume of neurons (p>0.05) as seen in figure 4 B.

Morphometric parameters of astrocytes

Like neurons, analyzing variables of astrocytes were based on the stereological and morphometric quantification methods previously described by several authors and the results are presented in the table 2.

Table 2. Morphometric parameters of astrocytes and neurons in a time course of cell mixed culture.

Parameters / mean ± S.E.M.	Sixth day	Seventh day	Eighth day	Ninth day
Number of astrocytes per field	42.5 ± 1.56	53.50 ± 3.12	55.75 ± 1.93	64.75 ± 2.46
Astrocytes area (μm^2)	277.9 ± 17.5	263.8 ± 28.6	171.2 ± 16.0	166.9 ± 14.4
Astrocytes perimeter (µm)	131.2 ± 7.84	170.3 ± 16.0	132.6 ± 9.6	173.7 ± 15.2
Integrated density astrocytes (R.U.)	173.7 ± 15.2	305.7 ± 28.6	395.7 ± 35.1	396.4 ± 32.4
Astrocytes relative area 2D (μm^2)	302 ± 54	233 ± 13	242 ± 14	257 ± 23
Astrocytes volume (μm^3-3D)	54.0 ± 3.14	45.1 ± 3.71	38.2 ± 4.69	34.07 ± 3.48
Astrocytes surface (μm^2)	240.0 ± 13.9	200.7 ± 16.5	170.1 ± 20.8	151.4 ± 15.4
Number of astrocytes per neuron	0.95 ± 0.13	1.03 ± 0.15	1.05 ± 0.12	0.95 ± 0.04

The acquired images of each of the three different fields were analyzed and the number of astrocytes cell counted based in the immunostaining of GFAP protein. Regarding the morphology, flat and elongated forms with fibrillar characteristic and large nuclei were observed (Figure 3 A). The number of astrocytes was significantly higher (p <0.01) at day nine of culture compared to day six; nevertheless, no significant differences at other days (p> 0.05) were observed. Concerning astrocytes density in the field, highly significant differences (p <0.0001) between days six and seven, six eight, six and nine were observed, but not between days seven-eight, seven-nine and eight-nine (p <0.05) as shown in figure 3 B. Lastly, highly significant differences among the six to eight days (p <0.0001) and between six-nine days (p <0.01) (Data not shown) were found in the surface of astrocytes.

On the other hand, highly significant differences (p<0.0001) between days six to seven, six to eight, seven to nine of the culture, but not between six-nine days and seven to eight (p>0.05) were perceived in relative area, as shown in figure 3 C and respect to perimeter between six-seven days, six-nine day (p<0.0001) and six-eight (p<0.05) (Data not shown). In terms of volume of astrocytes in culture, highly significant differences were observed between days six-eight and seven-eight (p<0.0001), but not between days six to nine (p>0.05) as shown in Figure 4 C.

It has been reported that GFAP is present at high levels in rat astrocytes during the first 7 days after birth. The intensity of the GFAP immunoreactivity varied within the culture (Fig. 2A–D) probably reflecting differences in the maturity of the cells (Ahlemeyer *et al*., 2013)grown for up to 42 days in vitro (DIV which corresponds to the increase in density and area observed in astrocytes in culture on days 7 and 8 in relation to the sixth day. As regards the numerical relationship between astrocytes and neurons (ratio), the number of cells was similar for both linages during the course of culture at a ratio of 0.99 ± 0.11 very close to one (Figure 1 C) indicating that the number of neurons and astrocytes were maintained in a ratio of 1: 1 under the specified conditions of the culture medium; conversely, similar to neurons, astrocytes showed a tendency to increase in number from seven day to nine day of culture (Figure 1 B) without significant difference (p>0.05).

Relationship between astrocytes and neurons in postnatal mixed culture

The phenotype of cells in primary cultures of neurons and astrocytes is mostly determined by manual counting after immunostaining by an immunocitochemical method. In a study by flow cytometry, the utility and precision was greatly demonstrated to be used for phenotypic characterization of neurons and astrocytes, even in heterogeneous cultures (Sergent-Tanguy *et al*., 2003). In this research, the use of GFAP and MAP-2 markers allowed to discriminate astrocytes and neurons by conventional immunofluorescence and the staining pattern was consistent between slides and allowed the evaluation of morphological parameters in a confident manner.

About the relationship between astrocytes and neurons in mixed culture, both populations (Neurons and astrocytes) were maintained during

the time course of the culture (6-9 days) particularly, between 7 and 9 days, with no significant differences in the two lineages ($p > 0.05$) as seen in figure 1 A-B. This suggests that survival of neurons and astrocytes depended mainly on the proportion between them in the medium and possibly denoting culture stability.

Neurons at P.3 appeared completely differentiated and did not increase in numbers during the assessed time period; whereas, astrocytes won its peak of proliferation at day 7 of culture; nevertheless, the restriction of SFB (2.5%) avoided overpopulation of astrocytes and keep the appropriate proportion to neurons, being very close to 1:1 during the evaluated days (Figure 1 C) and without the appearance of morphologic changes in the astrocyte populations.

Conclusions

A quantitative analysis of the length of neurites, numbers of neurons and astrocytes, area, density and other morphometric parameters of neurons and astrocytes in primary mixed cultures is provided and constitute a valuable tool to study neuron-astrocyte interaction and response. The analysis was performed in photomicrographs of immunofluorescent labeled cells by using the Fiji Image J software. Measurements have long been an accepted endpoint for assessment of regenerative potential in neurons, but the role of astrocytes appear to be very important and this cell type need to be assessed in this interaction. The conditions established in this study will be great value to study the effects of different therapeutics or toxics substances and open a new avenue to neuroscience field.

In conclusion, the present study represents an important advance in the simultaneous characterization of morphometric parameters of neurons and astrocytes in mixed cultures that allows the quantitative analysis of the main aspects of those differentiated cells. The measurement of those morphological parameters will help to identify and describe more precisely the different stages of development of neural cells in mixed postnatal culture conditions.

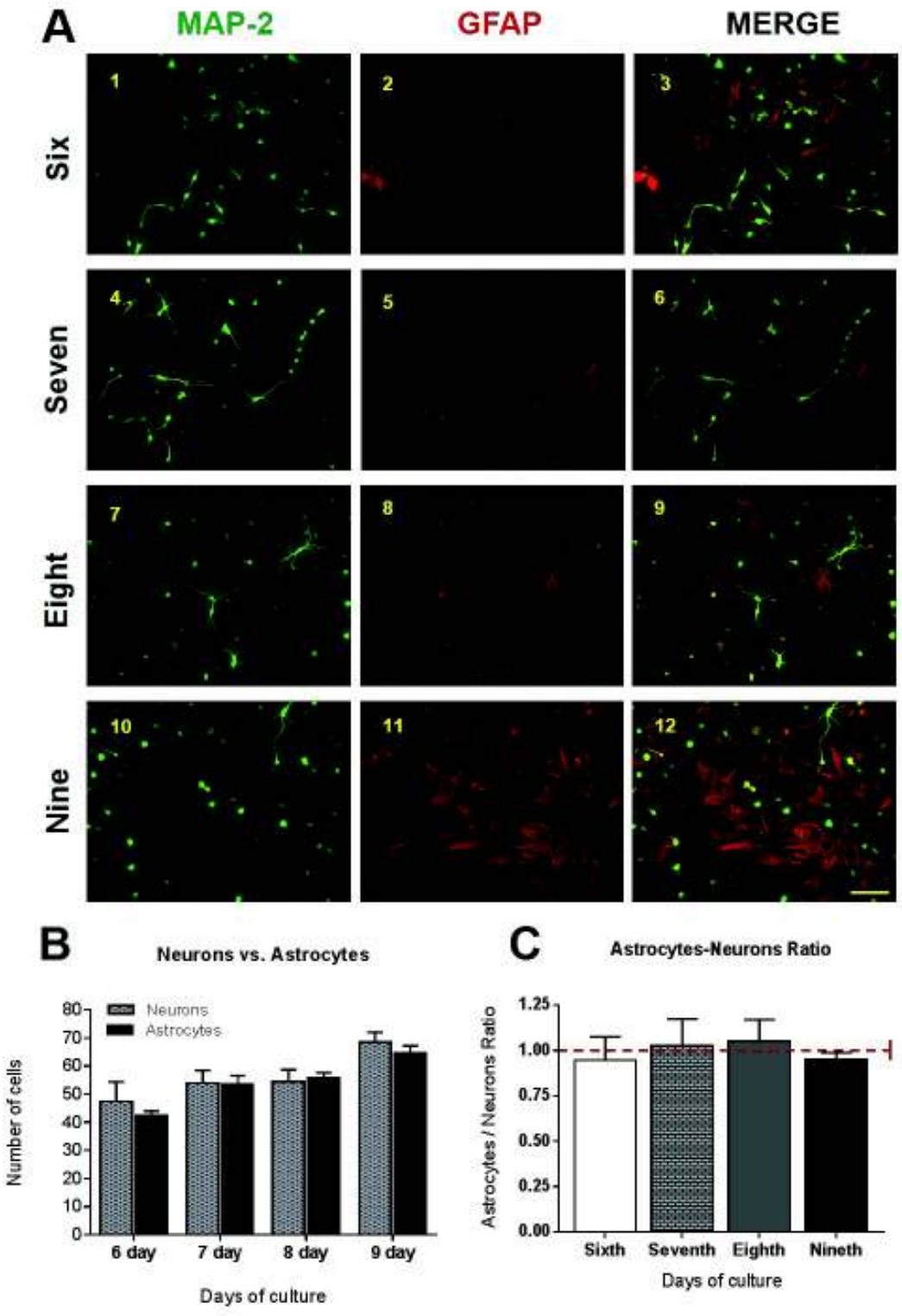

Figure 1. A. Fluorescent microphotographs of cortical neurons and astrocytes in mixed cultures showing MAP-2 (Green), GFAP (Red) and the merged images (1-A 3, 6, 9, 12) by immunostaining at 6, 7, 8 and 9 days after seeding. B. Quantification of neurons and astrocytes. C. Relative proportion of neuron and astrocytes are expressed in mean ± SEM. Figure is representative of at least six optic fields from three independent dishes. n=10 neurons/day. Scale bar, 50 μm.

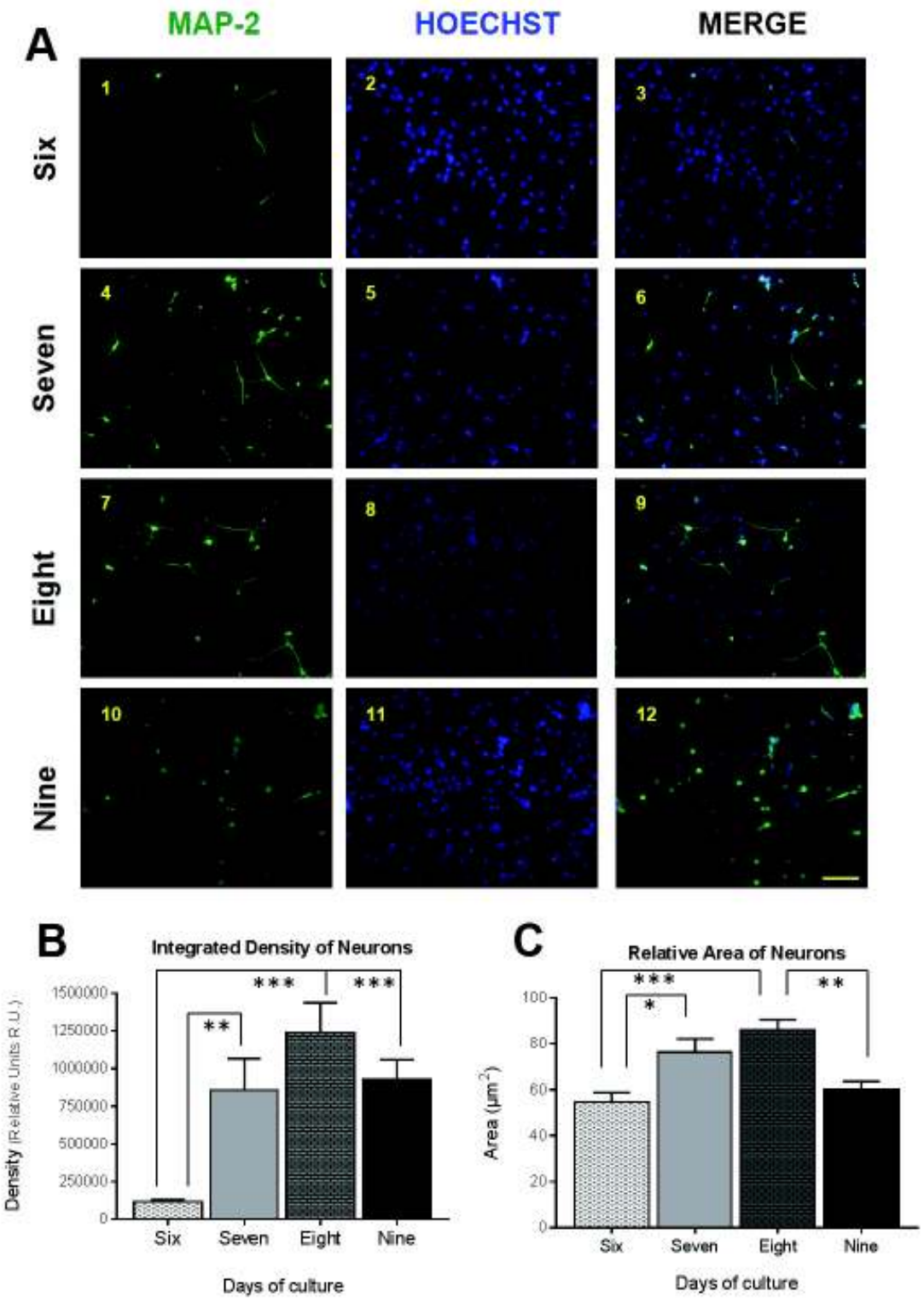

Figure 2. A. Fluorescent microphotographs of cortical neurons in mixed cultures showing MAP-2 (Green) co-labelled with the nuclear marker Hoechst (Blue) and the merged images (2-A 3, 6, 9,12) by immunostaining at 6, 7, 8 and 9 days after seeding. B. Integrated Density and C. Relative Area were evaluated and expressed in mean ± SEM. Figure is representative of at least six optic fields from three independent dishes. *p<0.05, **p<0.001 and ***p<0.0001, compared by one-way ANOVA with least-significant difference post hoc test. n=10 neurons/day. Scale bar, 50 µm.

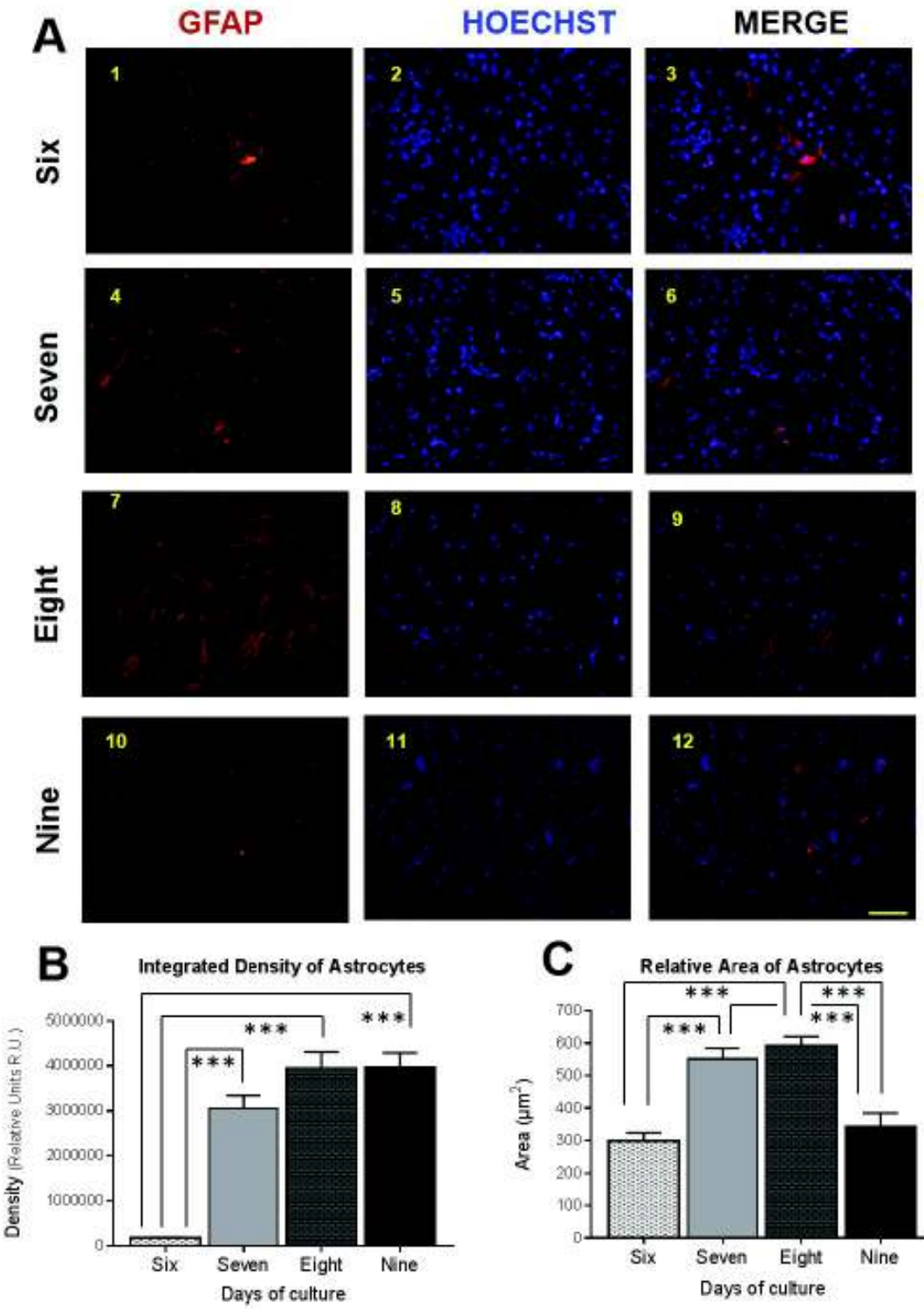

Figure 3. Time-course analysis of neurons and astrocytes from rat in a primary mixed cultures (P.3). Fluorescent microphotographs of cortical astrocytes showing GFAP (Red), nuclear Hoechst (Blue) staining and merged (3-A 3, 6, 9, 12) images obtained by immunostaining of cells at 6, 7, 8 and 9 days after seeding (A). Integrated Density (B) and Relative Area (C) expressed as mean ± SEM. Figure is representative of at least six optic fields from three independent co-cultures. ***p<0.0001, compares by one-way ANOVA with least-significant difference in a post hoc test. n=10 neurons/day. Scale bar, 50 µm

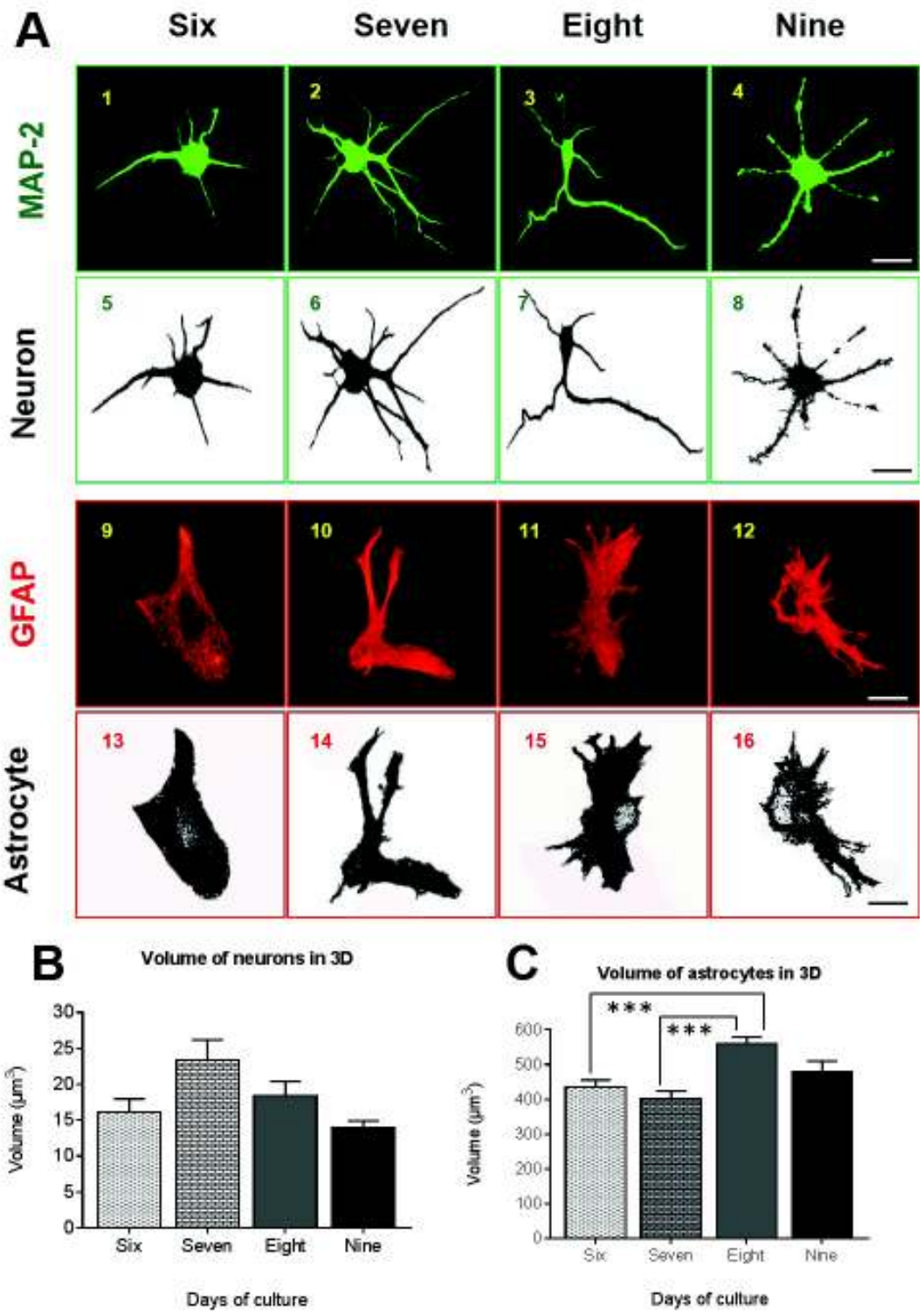

Figure 4. Developmental morphological changes of neuron and astroglial cells in primary mixed cultures (Six to nine days). Evolution of phenotypic characteristics by immunostaining of neurons (4 A 1-4) with anti-MAP-2 antibody. Images were processed by Fiji Image-J software (4 A 5-8). Similarly, the astrocyte morphotypes obtained by anti-GFAP staining (4A 9-12) and its images were processed (4 A 13-16) as previously described. Figures 4B and 4C correspond to statistic graphics of neuronal and astrocytic volume respectively. ***p<0.0001, compares by one-way ANOVA with least-significant difference in a post hoc test. n=10 neurons/day. Scale bar, 25 µm.

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