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CHANGES IN EXPRESSION OF GFAP, APOE AND APP MRNA AND PROTEIN LEVELS IN THE ADULT RAT BRAIN FOLLOWING CORTICAL INJURY

NATAŠA LONČAREVIĆ-VASILJKOVIĆ, VESNA PEŠIĆ, N. TANIĆ, DESANKA MILANOVIĆ, ALEKSANDRA MLADENOVIĆ ĐORĐEVIĆ, MILKA PEROVIĆ, SELMA KANAZIR and SABERA RUŽDIJIĆ*,

Department of Neurobiology, Institute for Biological Research "Siniša Stanković", University of Belgrade, 11060 Belgrade, Serbia

Abstract – The recovery period following cortical injury (CI) is characterized by a dynamic and highly complex interplay between beneficial and detrimental events. The aim of this study was to examine the expressions of Glial Fibrillary Acidic Protein (GFAP), Apolipoprotein E (ApoE) and Amyloid Precursor Protein (APP), all of which are involved in brain plasticity and neurodegeneration. Our results reveal that CI strongly influenced GFAP, ApoE and APP mRNA expression, as well as GFAP and ApoE protein expression. Considering the pivotal role of these proteins in the brain, the obtained results point to their potential contribution in neurodegeneration and consequent Alzheimer's disease development.

Key words: Cortical injury, astrocytes, glial fibrillary acidic protein, amyloid precursor protein, apolipoprotein E, recovery, synaptic plasticity

INTRODUCTION

Traumatic brain injury (TBI) is a leading cause of death and disability. It is responsible for severe neurostructural damage and impairment of neurological functions. TBI shows many of the neuropathological characteristics of Alzheimer's disease (AD), including diffuse β-amyloid precursor protein (Aβ) plaques, neurofibrillary tangles and acetylcholine deficiency (Ikonomovic et al., 2004). Long-term disabilities resulting from TBI depend on the severity of the injury, location, age and general health of an individual. Recovery after TBI is varied and not fully explained by prognostic features, suggesting that additional factors modulate secondary injury and recovery (Masel and DeWitt, 2010).

Astrocytes are the most abundant glial cell type. They are dispersed throughout the brain tissue and they make essential contributions to many homeostatic functions that could directly influence neuronal survival and functional outcome after TBI. It has been established that astrocytes play a role in modulating neuron signaling (Volterra and Meldolesi, 2005). Astrocytes respond to injury by increasing the expression of the specific cellular marker GFAP by changing their cell surface and secreted molecules and by altering their morphology (Chen and Swanson, 2003; Middeldorp and Hol, 2011). There are pronounced changes in astrocyte morphology, as is shown by the upregulation of GFAP in the astrocytes around the injured site (Pekny and Pekna, 2004). They participate in glial scar formation and display both growth-promoting and growth-inhibiting properties in axon regeneration (Fawcett and Asher, 1999; Myer, et al. 2006).

Apolipoprotein E (ApoE) is a multifunctional protein with an expanding role in the neurobiology of

disease. Although originally described in the context of cholesterol metabolism, interest in the neurobiology of ApoE has intensified following the association between the ApoE genotype and the risk of developing Alzheimer's disease. Recent clinical observations also suggest that the ApoE genotype may influence recovery after a variety of neurological insults. ApoE distributes cholesterol and phospholipids to neurons after injury and is therefore an important mediator of the CNS response to brain injury (Hauser et al., 2011). In normal rodent brain, ApoE immunoreactivity is largely confined to astrocytes (Laskowitz et al., 1998; Smiljanic et al. 2010). After injury, its expression dramatically increases in both glial and neuronal cells. It has been shown that ApoE influences pivotal processes in the brain, such as neuronal repair, regeneration and survival (Horsburgh et al., 2000; White et al. 2001; Iwata et al., 2005). Although the neurobiology of ApoE in the injured brain remains incompletely defined, there is evidence to suggest neurotrophic, immunomodulatory and antioxidant effects. Considering all the important roles of ApoE listed above, one can assume that even small changes in the expression of this protein could have profound consequences.

Amyloid precursor protein (APP) is an integral membrane glycoprotein that is ubiquitously expressed and serves as a sensitive marker of axonal injury but may also have an important function following TBI (Selkoe et al. 1988; Bramlett et al. 1997). In the brain, APP is synthesized in the neuronal cell body. Following synthesis, APP undergoes rapid axonal transport and is targeted to synaptic sites, where can be detected in synaptic membranes (Card et al. 1988). Although the physiological role of APP in the brain is still unclear, studies have suggested a range of cellular functions for APP, including roles in neuroprotection, neurite outgrowth, synaptogenesis and synaptic plasticity (Van den Heuvel et al. 1999). APP is upregulated in injured neurons and reactive astrocytes following TBI, and this upregulation has been associated with increased Aβ peptide, the risk factor for the development of AD. Aβ peptide is a major constituent of senile plaques found in the brains of patients suffering from AD (Stephenson et al., 1992).

The clinical outcome after neurotrauma is considerably variable and can only partly be explained by known prognostic factors. There is converging evidence from genetic research that a number of genetic variants may contribute to this variability. Considering the pivotal roles of GFAP, ApoE and APP in regeneration and neurodegeneration in the CNS, the aim of this study was to examine the temporal alterations of their mRNA and protein levels in the ipsilateral cortex following cortical stab injury.

MATERIALS AND METHODS

Animals

A cohort of 55 adult male Wistar rats (6 months old; 300-350 g) was used. The animals were housed under standard conditions (23±2°C, 60-70% relative humidity, 12 h light/dark cycles (lights were switched on at 07:00); free access to food and water; n = 3 per cage) in the Institute for Biological Research, Belgrade. All the animal procedures were approved by the Committee for Ethical Animal Care and Use of the Institute for Biological Research, Belgrade, which acts in accordance with the Guide for the Care and Use of Laboratory Animals, published by the U.S. National Institute of Health (NIH Publication No. 85/23, revised in 1986).

Surgery

The animals were anesthetized with Nembutal (50 mg/kg, Serva), placed into a stereotaxic frame and their scalps shaved. An incision was made along the midline of the scalp and the bregma was exposed. Cortical focal injuries were performed unilaterally using a 1 mm-wide dental drill through the left somatosensory cortex. The drill was inserted 2 mm below the underlying cortical region. Lesions were performed in a triangular-shaped formation as previously described (Loncarevic-Vasiljkovic et al. 2009). After surgery, the animals were kept in isolation for 3 h until complete recovery from the anesthesia, and subsequently returned to their home cages in order to avoid social isolation stress (n= 3 per cage).

Experimental procedure

The animals were randomly divided into five groups (n = 11 per group). Physiological controls (designated as C) were decapitated without any treatment. The other animals were subjected to the surgery and groups were formed according to the times of the recovery period, at the end of which the animals were decapitated (after 2, 7, 14 and 28 days).

RNA isolation

Total RNA from pooled ipsilateral cortical tissues (n = 4 animals per each time point were examined) was isolated using Trizol reagent. The tissue samples were homogenized in Trizol reagent (Invitrogen) at a ratio of 1 ml Trizol:0.1 g tissue, and total RNA was prepared according to the manufacturer's instructions. RNA integrity was assessed by electrophoresis in 1% agarose gels. RNA concentrations were measured and calculated after spectrophotometry. Total RNA was treated with 10 U of RNase-free DNase I (Fermentas) and dissolved in diethylpyrocarbonate (DEPC)-treated water, according to the manufacturer's protocol.

Reverse transcription (RT)

The RT reactions were performed in 20 μl with a High-Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions. The reactions were carried out under RNase-free conditions at 25°C for 10 min, and at 37°C for 2 h. Each RT reaction was accompanied by an RT control in which the reverse transcriptase was replaced by DEPC-treated water. The cDNA was stored at -20°C until further use.

Real-time RT-PCR

TaqMan PCR reactions were performed with Assay-on-Demand Gene Expression Products (Applied Biosystems) for ApoE (Assay ID Rn00593680_m1), APP (Assay ID Rn00570673_m1), and GFAP (Assay ID Rn00566603_m1). The gene expression assay contained primers for amplification of the target gene and TaqMan MGB (minor groove binder) probe 6-FAM dye-labeled for the quantification step. Reactions were performed in a 25 μl reaction mixture containing 1×TaqMan Universal Master Mix with AmpErase UNG, 1×Assay Mix (Applied Biosystems) and the cDNA template (10 ng of RNA converted to cDNA). PCR reactions were carried out in the ABI Prism 7000 Sequence Detection System at 50°C for 2 min, at 95°C for 10 min, followed by 40 cycles at 95°C for 15 s and at 60°C for 1 min. The experimental threshold was calculated from the mean baseline fluorescence signal from cycles 3 to 15, plus 10 standard deviations. The point at which the amplification plot crosses this threshold is defined as Ct, which represents the cycle number at this point and is inversely proportional to the number of target copies present in the initial sample. Each sample was run in triplicate and a mean value of each Ct triplicate was used for further calculation. A reference, an endogenous control, was included in every analysis to correct the differences in inter-assay amplification efficiency, and the expression of each gene was normalized to GAPDH (glyceraldehyde-3-phosphate dehydrogenase) expression. For the quantification, validation experiments were performed as described previously (Tanic et al. 2007). Quantification was performed by the 2-ΔΔCt method. The obtained results were analyzed by RQ Study Add ON software for the 7000 v 1.1 SDS instrument (ABI Prism Sequence Detection System) with a confidence level of 95% (pb 0.05). For the assessment of the effects of cortical injury-on the recovery period (2-28 days), the fold-change in the mRNA level was expressed relative to the calibrator; day 0 was taken as a control value (100%).

Western blot analysis

For Western blot analysis, the rats were killed by rapid decapitation. The brains were removed and the ipsilateral cortex was collected (n = 4 animals per each time point examined). The tissue was homogenized with a Dounce homogenizer in 10 vol (w/v) of RIPA buffer (50 mM Tris-Cl, pH 7.5, 150mM-NaCl, 1% NP-40, 0.1% SDS, 10mMEDTA, pH 8.0, 10mMEGTA, pH 7.2, 0.5% Triton X-100), followed by centrifugation (21,000×g, 30 min, 4°C), and the

supernatant was collected. Protein concentrations were determined using a Micro BCA Protein Assay Kit (Pierce Biotechnology). Equal amounts of proteins (20 μg per lane) were loaded and separated by 10% SDS-PAGE and blotted onto nitrocellulose membranes (Amersham Bioscience). The membranes were blocked at room temperature for 1 h in 5% non-fat dry milk in Tris-buffered saline/0.1% Tween 20 (TBST). The membranes were incubated either with mouse anti-APP (22C11) (1:750, Chemicon rabbit anti-GFAP (1:2000, DAKO) or rabbit anti-ApoE (1:500, Biodesign) antibodies for 1 h at room temperature in 5% non-fat dry milk/TBST. The membranes were incubated with the HRP-labeled secondary anti-rabbit antibody (1:8000, ICN) or HRP-labeled secondary anti-mouse antibody (1:2000, Sigma) in TBST for 1 h at room temperature. Three subsequent washes with 0.1% TBST were performed between each step. Each blot was subsequently re-probed with rabbit anti-actin antibody (1:8000, Sigma) that served as an endogenous control. The signal was detected by enhanced chemiluminescence (ECL, Amersham Bioscience) and subsequent exposure on X-ray film. All films were analyzed by densitometry using the computerized image analysis program ImageQuant 5.0.

Immunohistochemical analysis

Rat brains for immunohistochemical analysis (n= 3 per group) were fixed in 4% paraformaldehyde for 24 h at 4°C and cryoprotected in a sucrose gradient (10-30%) in 0.01 M PBS (in each percentage of sucrose for 24 h in 4°C). The brains were frozen in isopentane on dry ice and stored at -70°C. Every fifth coronal section (18 μm thick) through the site of injury was mounted on a slide, allowed to dry overnight and stored at -20°C. Before the immune reaction, the sections were rinsed in 0.01 M PBS and treated with 0.3% hydrogen peroxide in methanol for 30 min at room temperature to block endogenous peroxidase. Three washes with 0.01 M PBS were performed between every step. Sections were immunostained with rabbit anti-GFAP antibody (1:2000, DAKO) in 0.1% Triton X-100/0.01 M PBS at 4°C overnight and washed. The immunoreactive signal was developed with the avidin-biotin-peroxidase complex (ABC kit, Dako) using DAB as a chromogen. Sections were cover-slipped and analyzed under a Leica (Leitz DMRB) microscope. A negative control slide for each tissue was incubated with non-immunized horse serum to replace the primary antibody.

Cresyl violet staining

Several coronal sections were also stained with the cellular stain cresyl violet to confirm typical neuronal and glial morphology. Briefly, the sections were incubated in 0.1% cresyl violet acetate (Sigma) for 15 min, rinsed in water for 5 min and dehydrated first in 95% and then in 100% ethanol, and observed under a light microscope.

Statistical analysis

Significant differences between the sets of data were determined using STATISTICA software, version 6.0, StatSoft. The relative changes in mRNA and protein levels are presented as percentages (mean ± SEM) of the control samples that were assumed to be 100%. Further statistical analysis was made with relative values. The data were analyzed by one-way analysis of variance (ANOVA), time after the injury as factor. *Post hoc* comparisons were performed with the Fisher LSD test. Statistical significance was set at $p<0.05$.

RESULTS

Lesion evaluation

The size of the lesion on the left unilateral cortex was assessed histologically by cresyl violet histological staining (Fig. 1). Apart from the lesion site, the other cortical regions did not exhibit any signs of damage. Typical neuronal polygonal morphology and astroglial cells with small, round and intensely-staining nuclei were verified by cresyl violet staining.

GFAP mRNA and protein levels

Cortical injury induced significant changes in GFAP

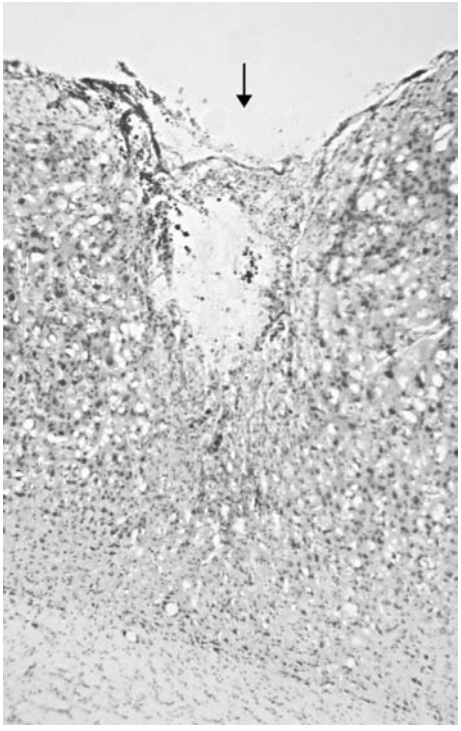

Fig. 1. Cresyl violet staining of the left ipsilateral cortex on the 2nd day after injury. The arrows show the size and localization of the cortical lesion that is surrounded by degenerating neurons with very dense nuclei.

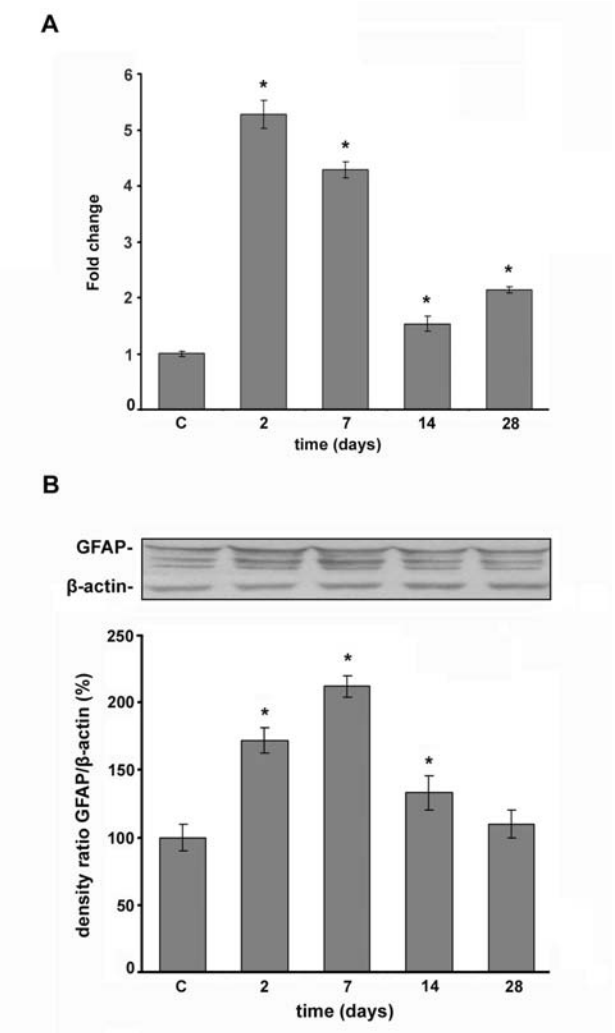

Fig. 2. (A) Expression of GFAP mRNA in the rat ipsilateral cortex following cortical focal injury, as revealed by Real-Time RT-PCR. **(B)** Expression of GFAP protein in the rat ipsilateral cortex following cortical focal injury. The results were obtained by Western blot analysis. The graph is accompanied by a representative immunoblot. *p<0.05 *vs*. control.

expression on both the mRNA and protein level in the ipsilateral cortex during recovery (Fig. 2A, B). The most prominent increase in the level of GFAP mRNA was observed on the 2nd and 7th days (5.6- to 4.7- fold, respectively), revealed by real time PCR (Fig. 2 A). Thereafter the mRNA declined progressively but remained above the control level. To examine whether the cortical injury affected the level of GFAP protein expression, we performed Western blot analysis (Fig. 2 B). One-way ANOVA revealed a significant effect of time after the injury as a factor ($F(4, 20) = 27.046$, $p = 0.000$). Compared to the control group, a significant increase in the content of GFAP protein was detected during days 2-14 after the injury (*$p<0.05$). By the 28th day, it returned to the control level.

To verify GFAP protein expression, we also performed an immunohistochemical analysis (Fig. 3). As expected, intense astroglial activation was detected around the injury site throughout the recovery period, with the most pronounced morphological changes observed on the 7th day after the injury (Fig 3B) compared to the control (Fig. 3A). The changes in astrocyte morphology pointed to the induction of reactive astrogliosis. By the 28th day of recovery, a glial scar was formed around the lesion site (Fig. 3C). The reactive astrocytes had enlarged cell bodies,

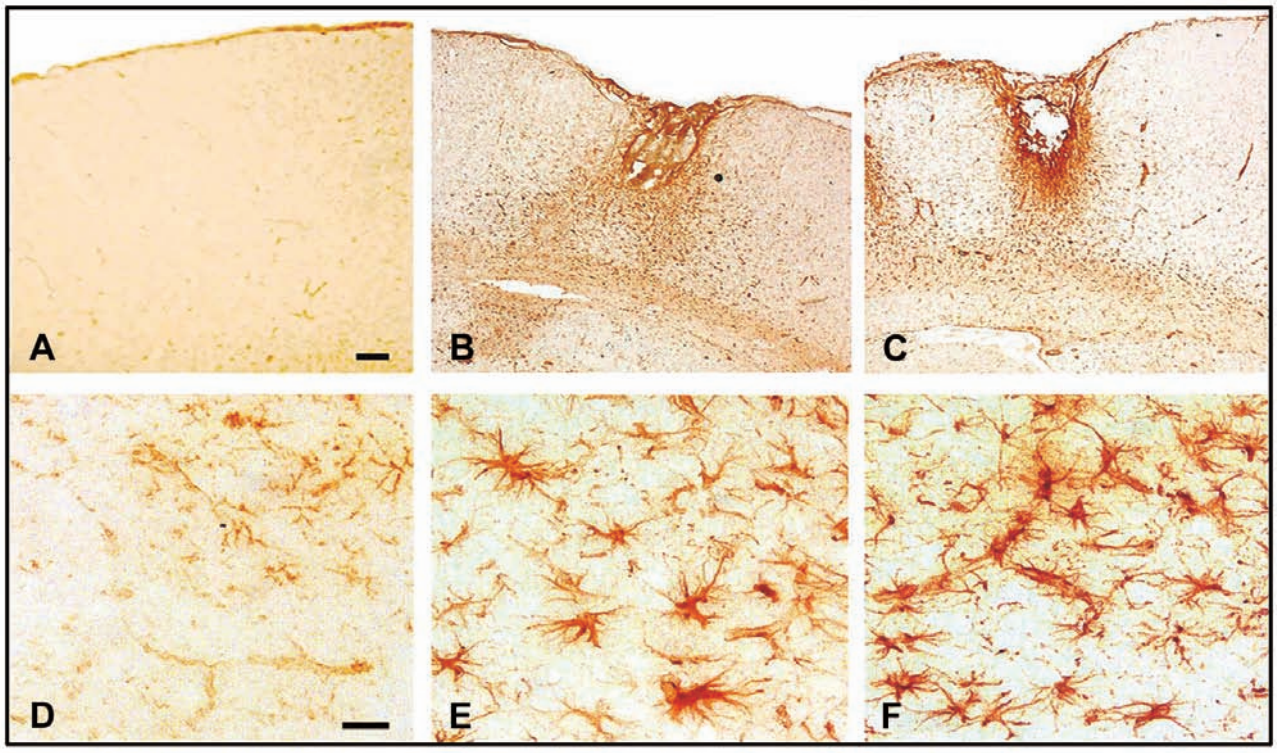

Fig. 3. GFAP immunostaining following cortical focal injury. Top row: representative images of GFAP immunostaining 2.5x magnification. Bottom row: representative images of GFAP immunostaining at 40x magnification. Scale bar for top row – 500 μm. Scale bar for bottom row – 40 μm.

with many very thick processes that branched in all directions, forming a dense net.

ApoE mRNA and protein levels

Real time PCR and Western blot analysis were also performed to examine the changes in levels of ApoE mRNA and protein expression during days 2-28 after the cortical lesion (Fig. 4A). Significant changes (40-50%) were observed in ApoE mRNA levels (2^{nd} 7^{th} and 14^{th} days) in comparison to the matching control.

Significant changes in ApoE protein levels in the ipsilateral cortex after the lesion were also observed (Fig. 4B). One-way ANOVA revealed the significant effect of time after injury as a factor ($F(4, 20)=13.62$, $p=0.000$). In comparison with the control group, the level of ApoE protein was significantly increased on the 2^{nd} and 7^{th} days after the injury ($^*p<0.05$).

APP mRNA and protein levels

Changes in APP mRNA and protein levels after the injury are presented in Fig. 5. The expression of APP mRNA in the cortex was significantly decreased (by 20-35%) on the 2^{nd}, 7^{th} and 14^{th} days after the lesion. Statistical analysis of the results obtained by Western blot analysis did not reveal significant changes in APP protein expression after the injury (Fig. 5B).

DISCUSSION

In the present study, we extended our previous investigations (Smiljanic et al., 2010) and examined the effect of cortical injury on the GFAP, ApoE and APP mRNA and protein levels during recovery after cortical insult. These gene products are involved in brain plasticity as well as in neurodegeneration (Arendt T., 2003). We examined the process of re-

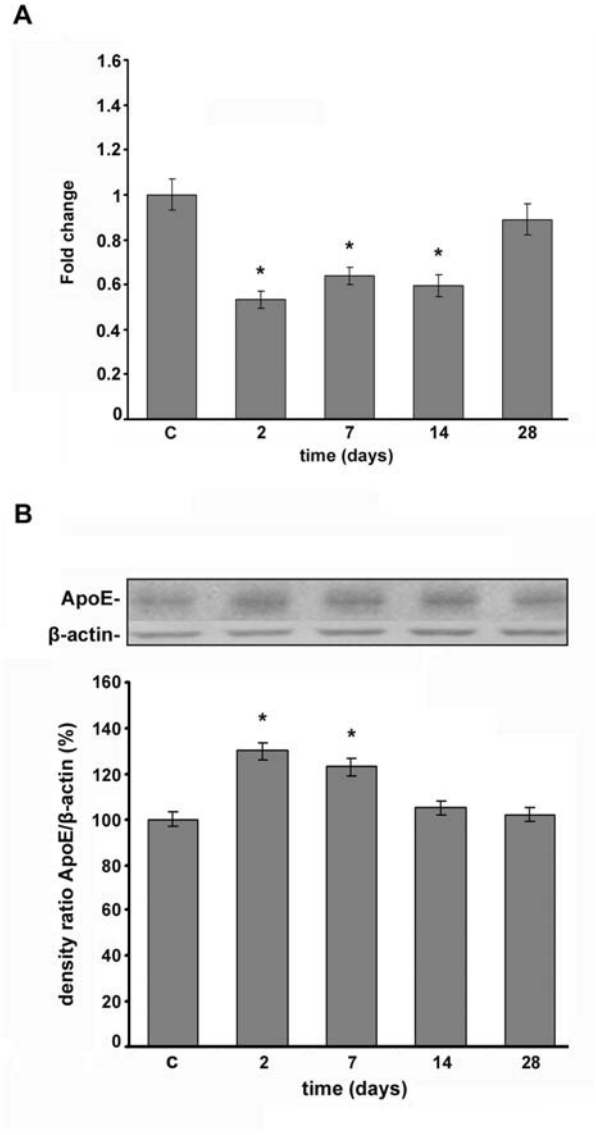

Fig. 4. (A) Expression of ApoE mRNA in the rat ipsilateral cortex following cortical focal injury, as revealed by Real-Time RT-PCR. **(B)** Expression of ApoE protein in the rat ipsilateral cortex following cortical focal injury. The results were obtained by Western blot analysis. The graph is accompanied by a representative immunoblot. *p<0.05 *vs*. control.

covery from the 2nd to the 28th day after injury since it covers the time of onset of axonal/terminal sprouting and the appearance of synaptogenesis. Our result is consistent with extensive literature and supports the hypothesis that a widespread glial reaction, as shown by GFAP immunopositive astrocytes, is considerably pronounced at the site of injury after 7 days of recovery (Sofroniew, 2009; Allmann et al., 2011). The fact that the observed astrocytes had enlarged cell bodies with numerous thick processes indicates that these cells are highly activated. By the 28th day of recovery, the activated astrocytes participated in the formation of the glial scar around the lesion site. The formation of a glial scar is a classic example of the complex interplay existing between neuroprotective and neurotoxic processes associated with reactive gliosis (Fawcett and Asher, 1999; Chen and Swanson, 2003). Reactive astrocytes release a wide array of mediators, including pro- and anti-inflammatory cytokines, neurotrophic factors, complement factors, reactive oxygen species (ROS), all of which potentially mediate neuroprotective and/or neurotoxic effects. However, although it has been shown that reactive astrocytes protect neurons from inflammatory processes after mild or moderate cortical injury (Sofroniew, 2009), it has also been documented that astroglial activation can have detrimental effects as well (Cafferty et al., 2007; Rolls et al., 2009). Namely, astroglial activation and increased GFAP expression are directly involved in the inhibition of axonal regrowth and regeneration (Pekny et al., 1999; Menet et al.,, 2003; Wilhelmsson et al., 2004). Considering that neuroregeneration, axonal regrowth and synaptogenesis are essential factors for successful recovery following injury, disruption of these processes could lead to neuropathology and neurodegeneration. In this context, the activation of astrocytes and excessive GFAP production described in the present study could lead to some neuropathology in the later time points.

Our study has also demonstrated that the upregulation of GFAP protein in the ipsilateral cortex was in fine correlation with ApoE protein expression on the 2nd and 7th day following injury. Unlike the protein, ApoE mRNA expression markedly declined from 2nd to 14th day following cortical injury; return to control level was achieved on day 28. The observed discrepancy between mRNA and protein levels could be due to post-transcriptional and post-translational mechanisms, which have been shown to temporally and spatially uncouple the ac-

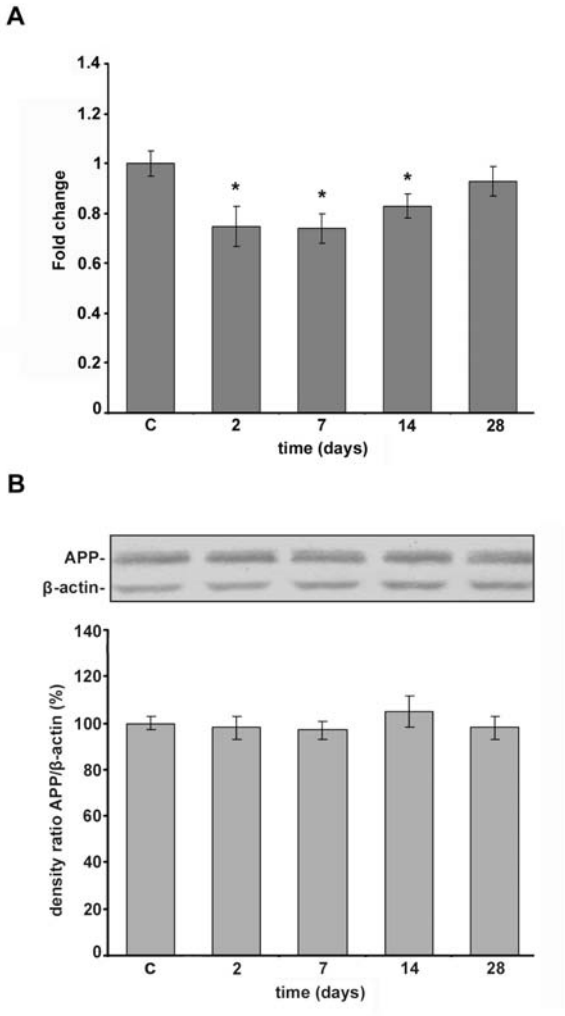

Fig. 5. (A) Expression of APP mRNA in the rat ipsilateral cortex following cortical focal injury, as revealed by Real-Time RT-PCR. **(B)** Expression of APP protein in the rat ipsilateral cortex following cortical focal injury. The results were obtained by Western blot analysis. The graph is accompanied by a representative immunoblot. *p<0.05 *vs*. control.

cumulation of mRNA and corresponding proteins (Petersen et al., 1999; Eastwood et al., 2006; Iwata et al., 2005). ApoE has a major role in lipid redistribution, which is important for membrane maintenance and repair in the brain, and in the regulation of synaptic remodeling following brain injury (Mahley RW, 1988; Poirier et al, 1993; Poirier et al, 1994; Horsburgh et al., 2000). Deregulated lipid metabolism may be of particular importance for CNS injuries and disorders, as this organ has the highest lipid concentration next to adipose tissue. It has also been hypothesized that the ApoE protein is involved in the clearance and redistribution of lipid and cholesterol debris from the site of injury for reutilization, and that it plays an important function in long-term alterations in plasticity following cortical injury (Horsburgh et al., 2000). Taking into account all essential processes in the cell that include ApoE, the upregulation of this protein observed in early time points following injury may indicate to its positive role in lipid redistribution and synaptic remodeling following injury. Considering that the upregulation of ApoE *per se* is not sufficient to claim that it is either a positive or negative event in the brain following injury, further investigations are necessary.

In the present study, APP mRNA and protein expression were also examined. The APP mRNA level was decreased (~35%) on the 2nd and 7th day and the lower level persisted until the 14th day after injury. Full recovery was not achieved after 28 days. However, the fact that there was no change in the APP protein level during recovery after cortical injury might point to one of two possibilities. Firstly, it could be due to the plastic properties of the brain that are capable of maintaining APP levels at the control level. Considering that it has been proposed that upregulation of APP is associated with increased Aβ peptide, the risk factor for the development of AD (Stephenson et al.,1992), and several other cerebral disorders (Zhang et al., 2011), the obtained result could mean that the brain itself is plastic enough to regulate a potentially detrimental protein following insult. Secondly, it might be due to the relatively short recovery period used in our experiment. Thus, there is a possibility that we are unaware of potential APP deregulations simply because they occur in later in time.

It was recently shown that alterations of cholesterol homeostasis in the brain by exogenous administration of dietary cholesterol, or through inhibition of cholesterol synthesis, markedly affect beta amyloid production and deposition and significant-

ly impair APP metabolism. It has been suggested that lipid homeostasis is controlled largely by the ApoE lipoprotein transport system in the extracellular space, whereas alterations in intracellular lipid homeostasis markedly affect APP processing, beta amyloid production and plaque formation *in vivo*. Convergence of the so-called amyloid cascade hypothesis and of the ApoE/lipid recycling cascade model is consistent with the notion that alterations in lipid homeostasis could serve as the common denominator for ApoE and beta amyloid dysfunctions in Alzheimer's disease (Poirier, 1994). It is also interesting to note that lipid homeostasis is a central feature of one of the most important neurotransmitter systems in thebrain, the cholinergic system. This system is unique in the CNS since it relies heavily on lipid bioavailability to locally synthesize acetylcholine. It is thus quite tempting to propose that two of the most common neuropathological landmarks of AD – namely, cholinergic dysfunction and amyloid deposition, may in fact depend on the integrity of local lipid homeostatic processes, which in turn are strongly dependent upon proper lipid delivery by the ApoE transport system.

Taken together, our findings highlight the complexity and heterogeneity of the molecular and cellular responses to cortical injury. Although our results showed that cortical injury affected GFAP, ApoE and APP mRNAs and protein expression, we can only hypothesize whether the observed changes have beneficial or detrimental consequences upon brain functioning. Further investigations are needed in order to answer this question.

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