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Source / Izvornik: **Neuroscience Letters, 2015, 597, 176 - 82**

Journal article, Published version
Rad u časopisu, Objavljena verzija rada (izdavačev PDF)

https://doi.org/10.1016/j.neulet.2015.04.042

Permanent link / Trajna poveznica: https://urn.nsk.hr/urn:nbn:hr:105:897203

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Download date / Datum preuzimanja: **2024-05-15**

Repository / Repozitorij:

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Neuroscience Letters 597 (2015) 176–182

Contents lists available at ScienceDirect

Neuroscience Letters

journal homepage: www.elsevier.com/locate/neulet

Research article

Increased expression and colocalization of GAP43 and CASP3 after brain ischemic lesion in mouse

Dunja Gorup[a], Ivan Bohaček[a], Tena Miličević[a], Roland Pochet[a,b], Dinko Mitrečić[c,*], Jasna Križ[d], Srećko Gajović[a]

[a] Laboratory for Neurogenetics and Developmental Genetics, Croatian Institute for Brain Research, School of Medicine, University of Zagreb, Šalata 12, Zagreb HR-10000, Croatia
[b] Laboratory of Histology, Neuroanatomy and Neuropathology, Faculty of Medicine, Université Libre de Bruxelles, 808 Route de Lennik, Brussels B-1070, Belgium
[c] Laboratory for Stem Cells, Croatian Institute for Brain Research, School of Medicine, University of Zagreb, Šalata 12, Zagreb HR-10000, Croatia
[d] Research Centre of Institute universitaire en santé mentale and Department of Psychiatry and Neuroscience, Laval University, Quebec City G1J2G3a, Canada

HIGHLIGHTS

- GAP43 and CASP3 are primarily present in neurons after stroke.
- Expression of Gap43 and Casp3 increased after onset of stroke.
- GAP43 and CASP3 colocalized after onset of stroke.

ARTICLE INFO

Article history:
Received 7 January 2015
Received in revised form 15 April 2015
Accepted 16 April 2015
Available online 27 April 2015

Keywords:
GAP43
CASP3
Brain ischemia
Bioluminescence
tMCAO

ABSTRACT

GAP43 is a protein involved in neurite outgrowth during development and axon regeneration reflecting its presynaptic localization in developing neurons. Recently, it has been demonstrated that GAP43 is a ligand of CASP3 involved in receptor endocytosis and is also localized post-synaptically. In this study, by using a transgenic mouse strain carrying a bioluminescent reporter for GAP43 combined with an in vivo bioluminescence assay for CASP3, we demonstrated that one day after brain ischemic lesion and, even more pronounced, four days after stroke, expression of both *CASP3* and *Gap43* in neurons increased more than 40 times. The in vivo approach of CASP3 and GAP43 colocalization imaging was further validated and quantified by immunofluorescence. Importantly, in 82% of GAP43 positive cells, colocalization with CASP3 was present. These findings suggested that one and four days after stroke *CASP3* expression, not necessarily associated with neuronal death, increased and suggested that CASP3 and GAP43 might be part of a common molecular pathway involved in early response to ischemic events occurring after onset of stroke.

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1. Introduction

GAP43 (growth-associated protein-43) is a protein associated with neurite growth during development and regeneration of the nervous system. It promotes accumulation of F-actin in neurites, contributing to the formation of the cytoskeleton [1,2]. *Gap43* expression is highest during critical periods of neural system development [3,4]. Perinatally it is expressed in all neurons, but its expression diminishes with decrease of axonal arborization and synaptogenesis and remains only in high-plasticity areas, such as the hippocampus and the olfactory bulb in mice [5]. Recently it has been shown that GAP43 in neurons is a substrate for CASP3 (caspase 3) and that mutation of *Gap43* causes decreased endocytotosis of the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor leading to impaired long term depression on the level of postsynaptic membrane [6]. This suggested new roles for both CASP3 and GAP43 in the nervous system.

* Corresponding author. Tel.: +385 14566792.
E-mail addresses: dgorup@hiim.hr (D. Gorup), ibohacek@hiim.hr (I. Bohaček), tenamilicevic@gmail.com (T. Miličević), rpochet@ulb.ac.be (R. Pochet), dinko.mitrecic@mef.hr (D. Mitrečić), jasna.kriz@fmed.ulaval.ca (J. Križ), srecko.gajovic@hiim.hr (S. Gajović).

http://dx.doi.org/10.1016/j.neulet.2015.04.042
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After onset of stroke, one of the most prominent regenerative events is axonal sprouting in the penumbra, which is accompanied by high expression of GAP43 [7–10]. Since CASP3 is a protease, not only involved in apoptosis but also in the fine tuning of formation of new synaptic contacts [11], and since GAP43 is a newly discovered postsynaptic substrate for CASP3 [6], we hypothesized that GAP43 and CASP3 together might be part of a recently suggested GAP43/CASP3 common molecular pathway and that it could be involved in tissue response after stroke.

To visualize levels of GAP43 after stroke, we used transgenic mice bearing the luciferase (*luc*) and green fluorescence protein (*gfp*) reporter genes under the control of the murine *Gap43* promoter (C57Bl/6-*Tg(Gap43-luc/gfp) 10Kri*) [12]. By using the luciferase substrate VivoGlo™ Caspase 3/7 Substrate (Promega), we were able to detect a subpopulation of CASP3 positive cells among GAP43 cells.

Our results revealed that expression of both CASP3 and GAP43 after stroke significantly increased (more than 40 times increase by optical density measurements and more than 200 times increase by counting positive cells). Moreover, in 82% of GAP43 positive cells, colocalization with CASP3 was detected. This colocalization further suggests that after stroke CASP3 activity in association with GAP43 could be non-apoptotic.

2. Materials and methods

2.1. Animals

A transgenic C57Bl/6-*Tg(Gap43-luc/gfp) 10Kri* mouse line was generated by Gravel and co-workers and genotyped by polymerase chain reaction (PCR) detection of the luciferase reporter gene as previously described [12,13]. For in vivo imaging 4 animals were used, while ex vivo experiments included 3 groups of animals: baseline ($N=4$), 24 h ($N=5$) and 4 days ($N=5$) with mortality of 5% accounting for 1 animal that reached an ethical endpoint before the experimental time point (2 days) in 4 days group. Total number of animals used was 19.

2.2. Transient middle cerebral artery occlusion (tMCAO)

In order to visualize neuronal responses to injury and repair, we chose the tMCAO method. It is based on unilateral transient focal cerebral ischemia by intraluminal filament occlusion of the left middle cerebral artery for 1 h followed by a reperfusion period previously described [14]. It has been shown that this method gives the highest level of reproducibility and it is the most often used approach in research of brain ischemia/stroke.

2.3. In vivo bioluminescence imaging

As previously described the images were gathered using the IVIS 200 Imaging System (PerkinElmer, Waltham, MA, USA) [13]. The validity of the Gap43 transgenic mice to mirror *Gap43* expression by reporter genes was described previously by one of us [12]. The luciferase substrate VivoGlo™ Caspase 3/7 Substrate (Z-DEVD-Aminoluciferin Sodium Salt, Promega, Madison, WI, USA) was injected intraperitoneally 20 min prior to the imaging session. The mice were anesthetized with 2% isoflurane in 100% oxygen at a flow rate of 2 L/min, placed in the heated light-tight imaging chamber, and maintained anesthetized by constant delivery of the 2% isoflurane–oxygen mixture at 1 L/min through an IVIS anesthesia manifold. To obtain baseline expression measurements, all animals ($N=4$) were imaged before and then first and fourth day following the injury.

The light output was quantified by determining the total number of photons emitted per second (p/s) using the Living Image 4.0 acquisition and imaging software (PerkinElmer, Waltham, MA, USA). Region of interest measurements on the images were used to convert surface radiance (p/s/cm^2/sr) to source flux or total flux of photons expressed in photons/seconds.

2.4. Tissue collection

Animals were anesthetized by an intraperitoneal injection of 2.5% Avertin (Sigma–Aldrich, St. Louis, Mo, USA) and then transcardially perfused with 0.9% saline, followed by 4% paraformaldehyde (PFA) at pH 7.4 dissolved in phosphate-buffered saline (PBS). Isolated brains were postfixed overnight in 4% PFA, equilibrated in PBS/30% sucrose for 48 h, embedded into Tissue-Tek (O.C.T. compound, Sakura, USA), frozen at −20 °C, cut into 30 μm thick coronal section with a Cryostat (Leica, Wetzlar, Germany), and stored at −20 °C.

2.5. Immunofluorescence

Brain sections were blocked for 30 min in PBS containing 10% goat serum and 0.25% Triton X-100. Sections were then incubated overnight at room temperature with 1:250 primary antibody rabbit polyclonal anti-cleaved CASP3 (Cell Signaling, Danvers, MA, USA), 1:125, 1:250 primary antibody rabbit polyclonal anti-GFP (Merck Millipore, Billerica, MA, USA) used for GAP43 visualization, 1:1000 primary antibody chicken polyclonal anti-GFAP (Abcam, Cambridge, UK) and 1:250 primary antibody mouse monoclonal anti-Neu-N (Merck Millipore, Billerica, MA, USA). Afterwards, sections were incubated for 2 h at room temperature in corresponding secondary Alexa-Flour antibody 1:500 (Invitrogen, Eugene, OR, USA). After both primary and secondary antibody application, slides were given five 10-min washes in PBS with 0.1% Triton X-100 detergent. Two additional washings of 1 min each were done before mounting the slides with Dako Fluorescence Mounting Medium (Dako, Denmark). Before microscopy, slides were left to dry off over night at room temperature.

2.6. Quantification of the immunofluorescent signal

For the quantification of the immunofluorescent signal (Cleaved CASP3, GAP43) four fields of view per section (every sixth section, all ischemic lesion area), ten sections per animal were acquired on a fluorescent microscope (Axiovert 200, Zeiss, Oberkochen, Germany) from the perilesional ipsilateral and corresponding contralateral cortex. Immunoreactivity was quantified with the ImageJ software by measuring the integrated optical density (intensity of fluorescence per unit of surface area), and number of maxima. The results were expressed in arbitrary units as previously described [13]. For the quantification of cells positive for either of markers, the number of positive cells in a field of view was counted manually.

2.7. Confocal imaging and reconstruction

The microscope used was IX81 Olympus microscope equipped with a confocal scan unit FV500 with 3 laser lines: Ar–Kr (488 nm), He–Ne red (646 nm) and He–Ne (532 nm) and UV diode (Olympus, Tokyo, Japan). Images were taken sequentially in Laser Scanning Microscopy (LSM) modality, using a step size as close as possible to pixel size for *z*-axis. Acquired multi-page TIF files were uploaded to Imaris software (Bitplane AG, Zuerich, Switzerland), and processed as described previously [15].

2.8. Statistical analysis

For in vivo imaging results the unpaired Student's *t*-test was used to compare the total photon emission in two different regions

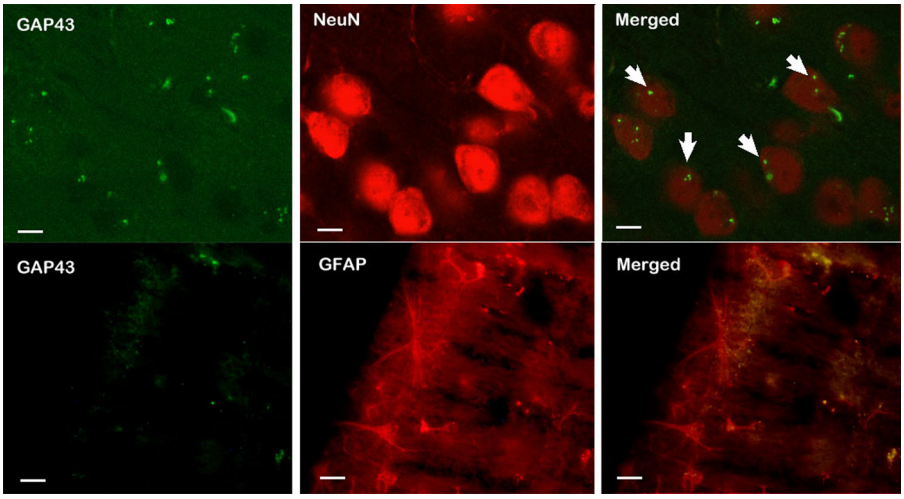

Fig. 1. Immunohistochemistry of GFP signal, which was driven by *Gap43* promoter in our transgenic mice showing colocalization with neuronal marker NeuN. In contrast, when compared with GFAP, a marker of astrocytes, no colocalization was observed in the ischemic penumbra. Size marker 20 μm.

of interest (ROI) in the same mouse at the same time point. As confirmed by *F* test, the variances were equivalent between the two groups.

The data from quantification of immunofluorescent signal for both IOD and cell counts were averaged and analyzed by 1 way analysis of variance of the 6 groups followed by Bonferonni's multiple comparison test.

Using data averaged per given animal, correlation tables were assembled observing the R^2 value of correlation coefficient.

3. Results

3.1. GAP43 and CASP3 were primarily present in neurons after ischemic lesion

To determine which cells contained GAP43 and CASP3, we performed double immunohistochemistry for proteins markers of the astrocytes and neurons on ischemic mouse brain tissue. GAP43/NeuN double immunohistochemistry revealed that GAP43 was exclusively expressed in neurons. Based on GAP43/GFAP, no presence of GAP43 in astrocytes was found (Fig. 1).

CASP3 was also found primarily in neurons; a signal was present in astrocytes as well, to a lesser degree (Fig. 2).

3.2. Expression of Gap43 and Casp3 significantly increased after onset of ischemic lesion

To analyze how stroke influenced the expression of *Gap43* and *Casp3*, we induced it using the MCAO approach. We performed immunohistochemical and bioluminescence analyses and quantified expression by measuring optical density and cell counting.

Immunohistochemistry revealed that already one day after ischemic lesion and, even more pronounced, four days after ischemic lesion, expression of GAP43 and CASP3 significantly increased (Fig. 3) as detected by one-way ANOVA test ($P < 0.0001$). Optical density method revealed that expression of GAP43 increased 27 fold one day after ischemic lesion, while 4 days after ischemic lesion, the increase was 47 fold. The cell count method revealed that, one day after ischemic lesion, the number of GAP43

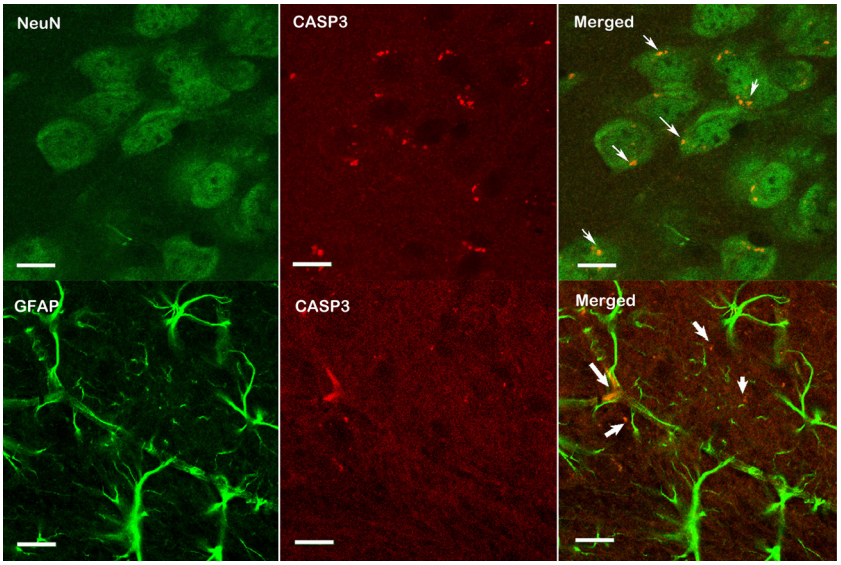

Fig. 2. CASP3 was predominantly present in NeuN positive cells, but also at lower level in GFAP positive cells 4 days after tMCAO. Arrows are pointing to regions of clearly visible double immunohistochemistry positive signal. The figures correspond to penumbra, peri-infarct region of cortex. Size marker 10 μm.

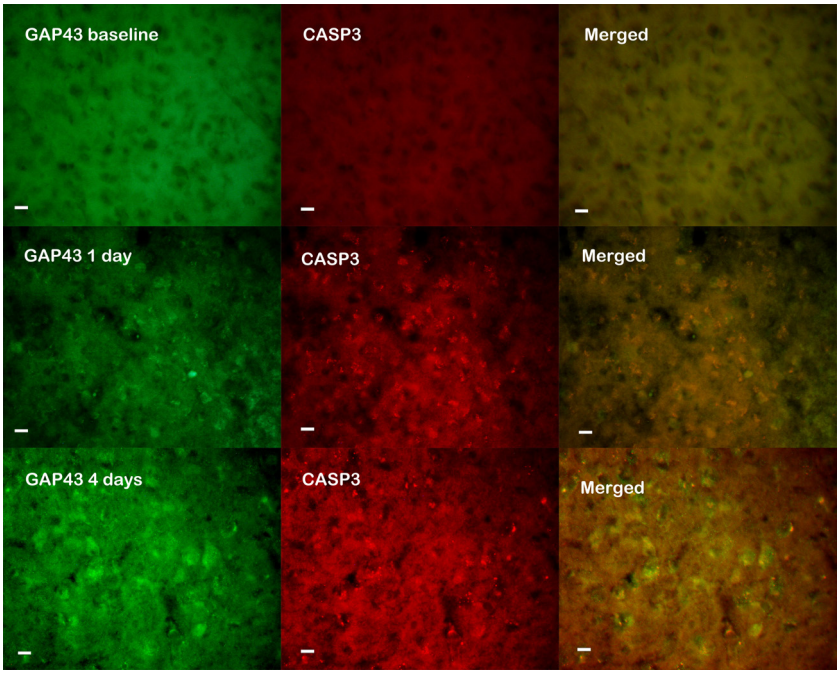

Fig. 3. Immunohistochemistry of GFP signal, which was driven by *Gap43* promoter, and CASP3 at baseline, 1 and 4 days after stroke in ipsilateral cortex. The increase in both GFP and CASP3 intensities after stroke is clearly visible. The figures correspond to penumbra, peri-infarct region of cortex. Size marker 20 μm.

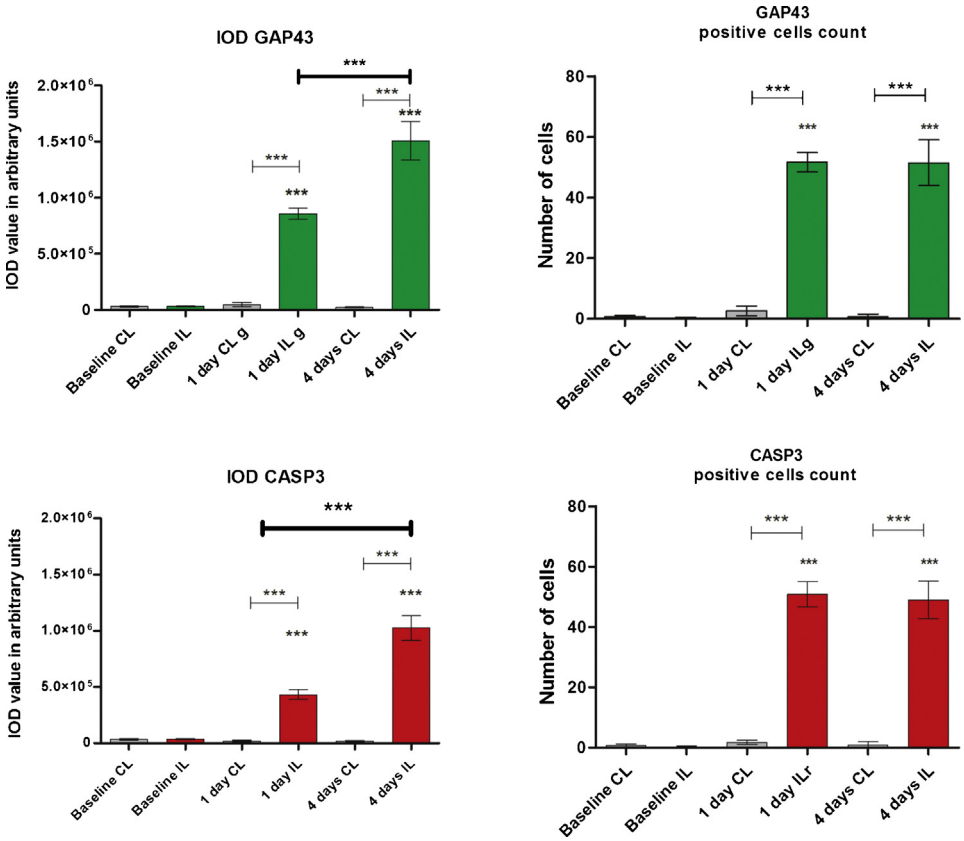

Fig. 4. Statistically significant increase for both GAP43 and CASP3 signal was found when both 1 day and 4 days after tMCAO were compared to their contralateral (healthy) hemisphere brain baselines. This increase was found when both integrated optical density and cell count methods were applied. Moreover, when integrated optical density was applied, a statistical significant increase was found for both GAP43 and CASP3 when signals were compared between 1 and 4 days inside ischemia affected hemisphere. There was no statistically significant difference when cell count method compared GAP43 and CASP3 signal between 1 and 4 days inside ischemia affected hemisphere. Average values are represented by columns heights and error bars represent standard error. Data collected correspond to penumbra, peri-infarct region of cortex.

positive cells increased 206 fold and 4 days after ischemic lesion the number of GAP43 positive cells increased 205 fold (Fig. 4). The optical density method (IOD) revealed that expression of CASP3 increased 24 fold one day after ischemic lesion, while 4 days after ischemic lesion, the increase was 42 fold. The cell count method indicated that one day after ischemic lesion number of CASP3 positive cells increased 203 fold and 4 days after ischemic lesion the number of CASP3 positive cells increased 205 fold (Fig. 4). The increase of GAP43 signal after tMCAO correlated with CASP3 signal intensity ($R^2 = 0.79$, Supplementary Fig. S1).

Previous work on this transgenic animal model showed that there is a low basal level of endogenous GAP-43 protein present in fibrillary/punctate formations, and high-magnification immunofluorescence analysis of the different brain regions showed colocalization of endogenous protein and the GAP-43-driven transgene GFP in the CA1 and CA3 pyramidal cells of the hippocampus [12].

Since the signal is present in the brain structures affected by MCAO, including the frontal and parietal cortex, striatum, and hippocampus, depending on the lesion size, we focused on perilesional cortex as a representative region of neuronal activation.

The number of GAP43 positive cells increased 1 and 4 days after tMCAO compared to the baseline and to the contralateral hemispheres of each time point. However, the number of GAP43 positive cells did not significantly differ when comparing 1 and 4 days post-ischemia.

Similar findings were detected for cleaved CASP3 showing a dramatic increase the first day after ischemic lesion compared to the baseline and contralateral hemisphere levels. This increase persisted even 4 days after the lesion.

When counted percentage of neurons positive for CASP3, their number increased from less than 1% in healthy cortices to 33% and 42% of CASP3 positive neurons in peri-infarct cortices 1 and 4 days after tMCAO, respectively (Fig. S4). In the same region percentage of neurons positive for GAP43 was 1% in controls, which increased to 26% and 25% 1 and 4 days after tMCAO, respectively (Fig. S4).

Regarding astrocyte marker GFAP, it colocalized with CASP3 in 4% of cells in healthy brain and 13% and 14% cells 1 day and 4 days after tMCAO, respectively (Fig. S4). As mentioned above, there was no expression of GAP43 in astrocytes, neither before nor after the stroke.

3.3. GAP43 and CASP3 significantly colocalized after onset of ischemic lesion

To test the in vivo coexpression of GAP43 and CASP3 we used in vivo bioluminescence imaging using VivoGlo™ Caspase 3/7 substrate (Promega). Since Caspase7 is exclusively activated following activation of CASP3, and since CASP3 is much more abundant protein and represents far the biggest portion of the signal in this system, this substrate allowed us to measure expression of luciferase driven by GAP43 promoter in CASP3 positive cells.

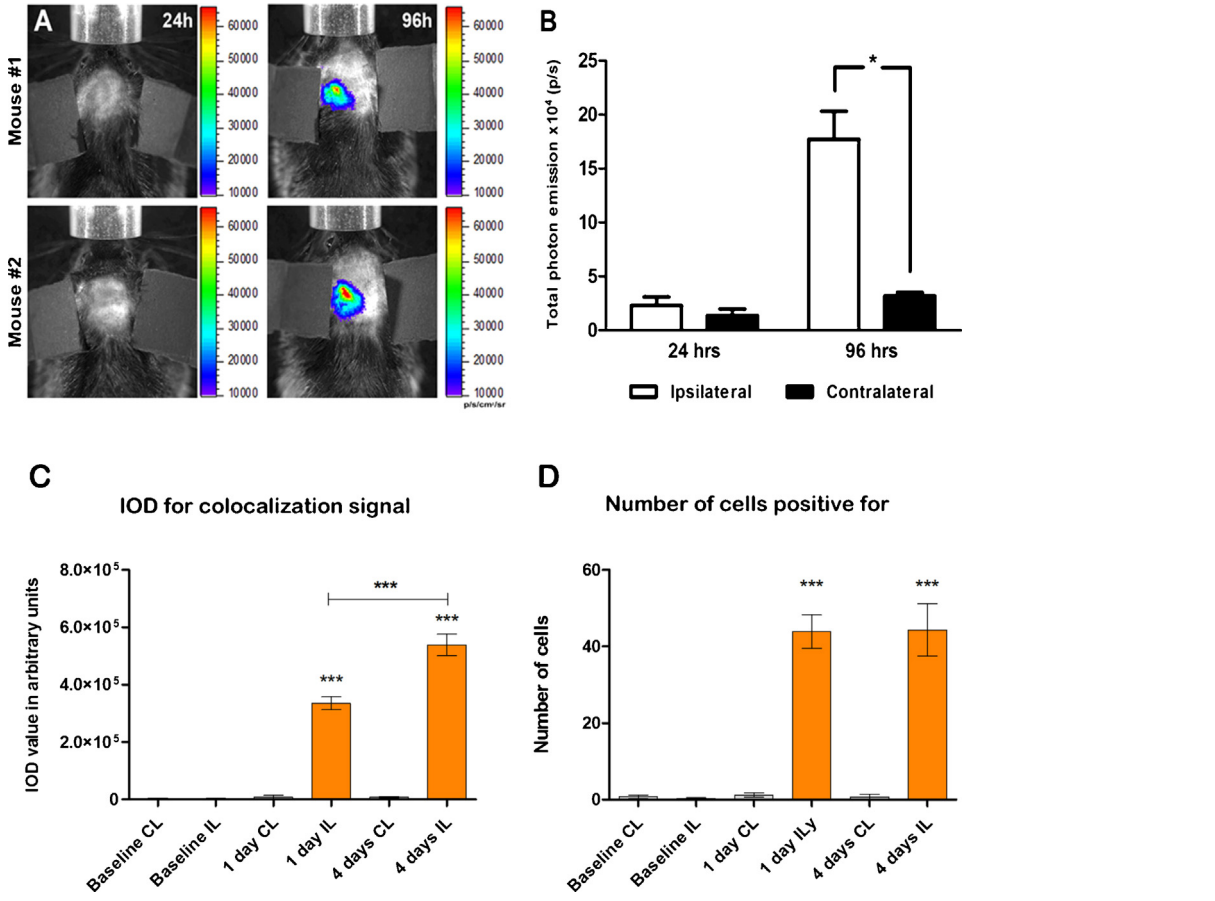

Fig. 5. Increase of GAP43/CASP3 colocalization signal using Vivo-Glo substrate for Caspase3/7 measured by bioluminescence (A and B), integrated optical density (C) and cells count (D). (A and B) Bioluminescent in vivo imaging detecting GAP43/CASP3 colocalization was visible 4 days after stroke, as presented in columnar view. (C) Integrated optical density method performed on slides revealed a significant increase in GAP43/CASP3 colocalization between 1 and 4 days after onset of brain ischemia. (D) Cells count method revealed a significant GAP43/CASP3 colocalization signal for both 1 and 4 days after onset of brain ischemia. The figures correspond to penumbra, peri-infarct region of cortex.

Analysis of the bioluminescent signal revealed GAP43/CASP3 colocalization after one day and a significantly higher signal 4 days after MCAO (Fig. 5). In our hands, baseline values of the signals in healthy GAP43 animals regularly correspond to signals in healthy hemispheres of ischemia affected animals (also described in [12]). Therefore, in this work we used contralateral hemispheres for baseline values, thus following the recommendation of our ethical board and sparing the animals unnecessary stress.

Using immunofluorescence, IOD showed increased overlap signal although no difference in the number of cells exhibiting GAP43/CASP3 colocalization was detected between days 1 and 4. Colocalization signal was also confirmed using confocal microscopy and 3D reconstruction (Supplementary Figs. S2 and S3).

4. Discussion

The main events occurring after onset of ischemic lesion include activation of molecular pathways associated with inflammation and tissue regeneration [16,17]. Adult cortex in mammals responds to injury with axonal sprouting, with GAP43 being one of the key players in the outgrowth of new axons [7–9,18]. Other genes involved in this process are mostly linked to formation of neurofilaments, microtubules or myelin. In rodents affected by tMCAO, GAP43 is present during the early phase in the focus of ischemic lesion, followed by expression in penumbra [18]. This suggested an early role for GAP43 in the rescue and regeneration of tissue. In permanent MCAO, where tissue is more and irreversibly damaged, there is no GAP43 in the lesion focus, but its expression is increased in penumbra, reaching its maximum at day 7 [19]. Elucidation of the role of GAP43 has evolved with the recent discoveries that GAP43 is also present on the postsynaptic membrane and can be a substrate for CASP3 [5,10]. Discovery of the postsynaptic location of this protein shifted understanding of its role from mechanical elongation of cell projections toward more complex tuning of the intercellular networks needed for proper tissue regeneration. The next important step towards elucidation of the role of GAP43 and CASP3 came with recent findings which reported that CASP3 can act on 56 substrates in neurons, and apart from GAP43, it is involved in pathways associated with the control of neuronal cytoskeletal components and their regulators (e.g., actin, MAP2, GAP43, Dbn1, and calmodulin) [6]. This added a new dimension to the understanding of regeneration after ischemic lesion: cell death is tightly correlated to elongation of new cell processes and linked to fine tuning of newly formed intercellular connections. By integrating the recent findings, we hypothesized that CASP3 and one of its major ligands, GAP43, are involved in a common pathway in the early response following stroke.

Our results show that both GAP43 and CASP3 increase rapidly and dramatically after stroke and this increase occurred in a linear correlation. Since neuroinflammation is a very complex, partly individual and not always easily repeatable process, it was important to minimize variability as much as possible. Our noninvasive in vivo bioluminescence tracking allowed repetitive assessment of localization and quantification of the cell signal on the same animals. The observed signal increase of both GAP43 and CAP3 using bioluminescence was further analyzed in detail using histological slides. Increase in the signal was quantified using both established methods for quantification of bioluminescence signal and of histological slides. The analysis revealed that the increase in the signal intensity of both GAP43 and CASP3 after onset of stroke was not due to an increase in the number of cells expressing those genes. This leads to conclude that both GAP43 and CASP3 belong to genes activated in the earliest stage after onset of stroke and that it is the cells involved in the regenerative processes that trigger the expression of these two genes, which is in opposition to an apoptotic process. A similar finding was obtained with colocalization analysis which showed that GAP43 exhibits relatively stronger expression on the first day after stroke as compared to CASP3, followed by an increased expression of CASP3 after 4 days, but without an increase in cell number, resulting in an increase of colocalization. Although the observed events, here described for the first time, are still not completely understood, we can propose the model in which GAP43 reacts as an early signal for axonogenesis, followed by an accelerated increase of CASP3 not acting as a pro-apoptotic molecule, but more as to control its substrate GAP43. Colocalization within the same cells highly suggests their importance in fine tuning of axonal elongation and cytoskeletal reorganization, as major elements of regeneration of the nervous system.

5. Conclusion

Here we have shown using bioluminescent imaging and immunohistochemistry that expression of GAP43 and CASP3 following MCAO were linked to each other. Following their recently described presence in both pre- and post-synaptic compartments and the link with cytoskeletal proteins, here we propose that these two proteins may act in a coordinated way in the regulation of brain response and repair after stroke.

Acknowlegdements

We thank Stefano Fumagalli and Ivan Alić for help in visualization of slides using confocal microscopy and Gabriel Borden for proofreading the manuscript. DM thanks to Foundation Adris for financial support. This work has been supported by EU-FP7-Regpot Glowbrain project (REGPOT-2012-CT2012-316120) awarded to SG.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.plantsci.2004.08.011.

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