Overexpression of the nuclear factor kappaB inhibitor A20 is neurotoxic after an excitotoxic injury to the immature rat brain

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Background: The zinc finger protein A20 is an ubiquitinating/deubiquitinating enzyme essential for the termination of inflammatory reactions through the inhibition of nuclear factor kappaB (NF-kappaB) signaling. Moreover, it also shows anti-apoptotic activities in some cell types and proapoptotic/pronecrotic effects in others. Although it is known that the regulation of inflammatory and cell death processes are critical in proper brain functioning and that A20 mRNA is expressed in the CNS, its role in the brain under physiological and pathological conditions is still unknown.

Methods: In the present study, we have evaluated the effects of A20 overexpression in mixed cortical cultures in basal conditions: the *in vivo* pattern of endogenous A20 expression in the control and *N*-methyl-D-aspartate (NMDA) excitotoxically damaged postnatal day 9 immature rat brain, and the post-injury effects of A20 overexpression in the same lesion model.

Results: Our results show that overexpression of A20 in mixed cortical cultures induced significant neuronal death by decreasing neuronal cell counts by 45 ± 9%. *In vivo* analysis of endogenous A20 expression showed widespread expression in gray matter, mainly in neuronal cells. However, after NMDA-induced excitotoxicity, neuronal A20 was downregulated in the neurodegenerating cortex and striatum at 10–24 hours post-lesion, and it was re-expressed at longer survival times in reactive astrocytes located mainly in the lesion border. When A20 was overexpressed *in vivo* 2 hours after the excitotoxic damage, the lesion volume at 3 days post-lesion showed a significant increase (20.8 ± 7.0%). No A20-induced changes were observed in the astroglial response to injury.

Conclusions: A20 is found in neuronal cells in normal conditions and is also expressed in astrocytes after brain damage, and its overexpression is neurotoxic for cortical neurons in basal mixed neuron–glia culture conditions and exacerbates postnatal brain excitotoxic damage.

Keywords: Postnatal brain injury, Excitotoxicity, Gene therapy, Non-viral modular vector, A20, Nuclear factor kappaB, Neurodegeneration

Background

In the nervous system, nuclear factor kappaB (NF-kappaB) is involved in regulating responses of neurons and glial cells to activation, acting on several signalling pathways and in a variety of physiological and pathological situations.[1] In neuronal cells, NF-kappaB mediates cell survival and plasticity, and in glial cells, it contributes to the coordination of the inflammatory response upon damage or infection.[2,3]

The zinc finger protein A20 is a complex molecule essential for the termination of inflammatory reactions by its inhibitory properties on NF-kappaB signalling[4–6] based on its ubiquitinating/deubiquitinating activity.[7–9] Ubiquitination of proteins by the covalent ligation of one or several monomers of the ubiquitin protein is a major regulatory mechanism for a wide array of cellular processes, including

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endocytosis, vesicle trafficking, cell-cycle control, stress response, DNA repair, cell signalling, and gene transcription and silencing.[10] Interestingly, A20 is capable of removing polyubiquitin chains from a specific lysine of critical factors of the NF-kappaB signalling cascade-like TRAF6 and RIP1, but it can also polyubiquitinate RIP1 on another lysine, thereby inhibiting tumor necrosis factor-alpha receptors (TNF-alphaR) and Toll-like receptors activation of NF-kappaB.[5,7,8] Accordingly, A20 knockout mice develop severe inflammation and cachexia, are hypersensitive to inflammatory stimuli such as bacterial lipopolysaccharide and TNF-alpha treatment, and die prematurely.[4] Moreover, A20 has also been shown to participate in the regulation of cell death in a variety of cell types, showing anti-apoptotic activity in some cell types but pro-apoptotic/pro-necrotic effects in others. As an example, A20 protects pancreatic beta cells[11,12] and other cell types[4,13] from TNF-alpha, interleukin-1beta or interferon-gamma induced apoptosis. However, while protecting against TNF-alpha-induced apoptosis, A20 also sensitizes these cells to necrosis triggered by reactive oxygen species[14] and sensitizes smooth muscle cells to cytokine and Fas-induced apoptosis by a nitric oxide dependent mechanism.[15] Similarly, in a model of induced cell death in T lymphocyte hybridomas, A20 may promote pro or anti-apoptotic effects depending on the stimulus.[16]

Noteworthy, although it is known that A20 mRNA is expressed in the central nervous system (CNS),[17] and that it has been long established that NF-kappaB plays a key role in neuronal fate, no studies have analyzed the A20 protein cellular expression pattern in the CNS or the role of A20 in cell survival or death of CNS cells. In this sense, one aim of this study was to evaluate the effects of A20 overexpression on the survival of neuronal cells under basal conditions *in vitro* and to describe the *in vivo* brain expression of A20 in the control brain. Moreover, brain damage as a result of perinatal hypoxic–ischemic insult is a serious clinical problem with severe neurological consequences, where inflammation is known to play a fundamental role.[18,19] In fact, several lines of evidence suggest that the immature brain responds in a particular fashion to brain injuries,[18,19] at least in part due to the fact that many of the adult gene expression patterns, neural circuits organization, cell differentiation, or myelination have not yet been achieved.[20,21] For instance, the postnatal brain is more sensitive than the adult brain to the neurotoxic actions of *N*-methyl-D-aspartate (NMDA).[22] Thus, in this work, we also aimed to analyze the expression of A20 after a postnatal NMDA-induced excitotoxic injury and, finally, the effects of A20 overexpression after the mentioned excitotoxic postnatal injury using a previously described gene delivery approach.[23–26]

Methods

In vitro *studies*

Cell cultures and transfection

Primary cortical cultures were prepared from E17 Oncins France Strain A rat embryos by dissecting the cerebral cortices free of meninges. After dissociation, cells were plated at a density of 1.8×10^6 cells/ml in BME medium (04-25050; Pan Biotech) supplemented with 5% foetal horse serum (FHS; Invitrogen), 5% foetal calf serum (FCS; Invitrogen), glutamine 2mM, 0.6% glucose, 50 U/ml penicillin, and 50 μg/ml streptomycin on 10 μg/ml polylysine coated wells. At 7 days *in vitro*, mixed neuron–glia cultures were prepared by removing the medium with FCS and adding a total of 10% FHS and 10 μM cytosine arabinoside in the same medium. Under these conditions and as described earlier,[27] at 10–12 days *in vitro*, when mixed neuron–glia cultures were used, these contained an average of $21 \pm 2\%$ glial fibrillary acidic protein (GFAP)-positive astrocytes and $11 \pm 2\%$ NDPase-positive microglial cells. *In vitro* transfection of these cultures was performed by washing once in OPTIMEM medium, and incubating for 5 hours in 4.5–6 μg lipofectamine (Invitrogen) and 1 μg DNA per 24-well plate well, followed by the addition of BME supplemented with 10% FHS or DMEM supplemented with 10% FCS respectively as described elsewhere. Control cultures were incubated with lipofectamine alone in the same conditions. The recombinant transfection vehicle NLSCt, used in this study for *in vivo* delivery and overexpression of A20 gene, cannot be used *in vitro* under basal conditions as its mediated transfection efficiency is too low.[25]

The plasmids used for transgene overexpression were: (1) a negative control transgene, the red-shifted variant of jellyfish Aequorea Victoria green fluorescent protein (GFP) gene encoded into plasmid pEGFP-C1 (Clontech) under control of CMV promoter and SV40 enhancer element; and (2) the A20 transgene encoded into plasmid pCAGGS under the control of the chicken beta-actin/rabbit beta-globin hybrid promoter.[28]

Cell viability

Mixed neuron–glia cortical cultures were evaluated for overall cell viability using the spectrophotometric 3-[4,5-dimethylthiazol-2-yl]-2,5-dephenyl tetrazolium bromide (MTT) assay by incubating cultures for 45 minutes at 37°C with 5% CO_2 and 0.2 mg/ml MTT. Cells were then lysed in DMSO and the amount of MTT product (coloured formazan) was determined by measuring absorbance at 570nm and at 630nm. Neuronal cell viability was evaluated by immunocytochemical labelling using a mouse monoclonal primary antibody anti-microtubule-associated protein 2 (MAP2, 1 : 1500, MAB 3418; Chemicon) and anti-mouse HRP-linked (1 : 200, NA931V; Amersham

Bioscience) as secondary antibody. Visualization of immunostaining was performed using 3,3′-diaminobenzidine (DAB)-hydrogen peroxide as the developing procedure. Immunoreactive cells were counted in eight ×40 fields in each well. All experiments were performed at least three times in triplicate.

In vivo *studies*

Experimental animals

Experimental animal work was conducted according to Spanish regulations in agreement with European Union directives. Experimental procedures were approved by the ethical commission of the Autonomous University of Barcelona. A total of 34 Long–Evans black-hooded postnatal day 9 rats, distributed in several experimental groups, were used in this study.

Excitotoxic injury

Nine-day-old Long–Evans black-hooded rat pups (15–20 g, both sexes; Janvier, France) were used. Intracerebral injections were made into the right sensorimotor cortex at the level of the coronal suture (2 mm lateral of bregma and 0.5 mm depth) using a stereotaxic frame adapted for newborns (Kopf Instruments) under isoflurane (Baxter International Inc.) anesthesia. Excitotoxic lesions were performed as previously described,[29] by injecting 18.5 nmol of NMDA (Sigma-Aldrich, St Louis, MO, USA) diluted in 0.15 μl of saline solution (0.9% NaCl) (n=9) or the saline solution alone (vehicle controls) (n=6) at a rate of 0.05 μl/min using an automatic injector. The needle was left in place for 10 additional minutes to allow diffusion. After suture, pups were placed on a thermal pad for 2 hours at 36°C to maintain normothermia.

Endogenous A20 analysis by immunohistochemistry

At 10, 24, or 72 hours post-lesion, two saline-injected controls and three NMDA-injected brains for each survival time, together with intact-postnatal day 9 pups (n=3) were anesthetized and perfused intracardially with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). Brains were post-fixed in the same fixative for 2 hours and crioprotected overnight in a 30% sucrose solution before being frozen with dry CO_2. Coronal sections of the entire brain (30 μm thick) were obtained using a Leitz cryostat. Sections were processed for endogenous peroxidase inactivation by 10 minutes. incubation in 70% methanol + 2%H_2O_2, and then blocked for 1 hour in Tris-buffered saline (pH 7.4) plus 10% FCS and 1% Triton X-100. Sections were then incubated overnight at 4°C and 1 hour at room temperature in the same blocking solution containing a chicken polyclonal primary antibody anti-A20 (1 : 300, Genway biotech 15-288-21173). After several washes, sections were incubated for 1 hour at room temperature with a secondary biotinylated anti-chicken antibody (1 : 300, Genway biotech) and, subsequently with avidin-HRP (Dakopatts, PO364). Finally, DAB-hydrogen peroxide was used as developing procedure for immunolabelling visualization. Control sections were processed similarly, but omitting the primary antibody.

Transgene overexpression after excitotoxicity and sample processing

The effects exerted by A20 overexpression after excitotoxic damage was analyzed in another set of animals. For this *in vivo* gene therapy study, a previously generated recombinant gene delivery vehicle termed NLSCt was used (1 μl, 7 μM).[23] NLSCt protein and DNA complexes were formed by incubation of NLSCt and the plasmids detailed above for the *in vitro* experiment in 0.9% NaCl at room temperature for 1 hour at ratios of 0.03 μg DNA per μg of NLSCt protein, thereby obtaining NLSCt/EGFP or NLSCt/A20 complexes. Sixteen rat pups from 3 l were excitotoxically lesioned as described above and, 2 hours later, re-injected at 0.2 μl/min at the same coordinates, as previously described in detail,[25,26] with either NLSCt/EGFP (n=8) or NLSCt/A20 (n=8). Both treatments were made in each litter.

At 3 days post-lesion, animals transfected with the different transgenes were anesthetized and perfused intracardially with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) and processed for cryostat sectioning as described before in the immunohistochemistry section.

Lesion volume measurement

One series of slide-mounted sections (separated by 240 μm) per animal were stained for Nissl and used for the measurement of lesion volume and total ipsilateral hemisphere volume after high-resolution digitalization with a Nikon LS-1000 slide scanner. Using analySIS software tools and simultaneous microscope observation, the lesioned area, identified by its pale Nissl staining and unstructured citoarchitecture, was quantified in each section. To avoid miss-interpretations related to possible tissue edema or shrinkage, data are presented as the percentage of total ipsilateral hemisphere volume.

Analysis of astroglial response

For astroglial cell labelling, another series of parallel free-floating sections per animal were processed for endogenous peroxidase inactivation and blocked for 1 hour in Tris-buffered saline (pH 7.4) plus 10% FCS and 1% Triton X-100. Sections were then incubated overnight at 4°C plus 1 hour at room temperature in the same blocking solution containing a rabbit polyclonal primary antibody against GFAP (1 : 1000, Z-0334; Dakopatts, Glostrup, Denmark). After several washes, sections were incubated for 1 hour

with horseradish-linked anti-rabbit secondary antibody (1 : 200, NA934V; Amersham Biosciences, Piscataway, NJ, USA), and DAB-hydrogen peroxide was used as developing procedure for immunolabeling visualization. Control staining omitting the primary antibody was performed in order to analyze potential unspecific immunolabeling.

For densitometrical analysis of astroglial GFAP expression, three consecutive $\times 20$ images of each animal taken at the lesion core and at the lesion border conformed the region of interest (ROI) and were analyzed by using analySIS software tools. As previously described,[26,30] the mean gray value of ROI, and the area occupied by immunoreactive cells within ROI were obtained for each image, representing the total immunostaining intensity and the area of ROI occupied by immunoreactive cells respectively.

Data processing and statistical analysis

All results are expressed as mean $\pm$ standard error mean. Student's *t*-test was used to determine significant differences ($P<0.05$). *In vitro* experiments were performed al least three times in triplicates.

Results

In vitro *overexpression of A20*

In order to evaluate the effects of A20 overexpression in basal conditions, cortical mixed neuron–glia cultures were transfected with A20 or GFP transgenes by means of the widely used lipofectamine-mediated transfection method. Seventy-two hours after culture transfection and when compared to lipofectamine alone and GFP-transfected controls, overexpression of A20 in cortical cultures induced several effects, namely, significant cell death, as observed by phase contrast microscopy (Fig. 1A); a significant reduction in cell viability, as demonstrated by decreased levels in MTT reduction ($46 \pm 4\%$ reduction as compared to gene therapy control GFP-transfected cultures, Fig. 1B); a decrease in $MAP2^+$ neuronal cell number ($45 \pm 9\%$ reduction when compared to GFP-transfected cultures, Fig. 1A and C), and increased propidium iodide staining (data not shown).

In vivo *spatio-temporal expression pattern of A20 in the control and excitotoxically-damaged postnatal rat brain*

The non-lesioned immature brain showed widespread A20 staining, mainly in gray matter areas (Fig. 2). A20 staining appeared as a cytoplasmic fine punctuate in neuronal cells, mainly located in the neocortex, hippocampus, anterodorsal thalamus, septum and indusium griseum, caudate putamen, globus pallidus, pyriform cortex, and olfactory bulb. More specifically, in the striatum, weak immunoreactivity was observed between the fiber bundles, where few faintly stained neurons were seen (Fig. 2A). In the neocortex, the highest expression of A20 was seen in layer V pyramidal neurons, although staining was also observed in layers I, II, and III (Fig. 2B). Moreover, subplate neurons of the neonatal neocortex were also stained (Fig. 2B). Regarding the hippocampus, A20 was mainly observed in the CA2 region, but also in CA1 and the dentate gyrus, whereas CA3 showed very faint staining (Fig. 2C). The olfactory bulb showed intensely stained neurons in several layers (Fig. 2D). Finally, the ventricular walls also showed punctuate A20 staining, as did the choroid plexus.

Saline-injected control animals showed, at 10 hours post-injection, an identical pattern as non-injected animals, except for a slight bilateral increase in A20 immunoreactivity in some neurons of the septum (Fig. 3A and B). Both at 24 and 72 hours post-injection, a very restricted and slight upregulation of A20 was also noted in cortical neurons at the level of the injection (Fig. 3C).

NMDA injection into the sensorimotor cortex of the immature postnatal day 9 rat brain induced a previously characterized excitotoxic lesion that included a primary lesion involving the sensorimotor cortex and secondary lesioned areas like the caudal sub-plate neuronal layer, striatum, and hippocampus, as previously described in detail.[29,31]

Excitotoxically lesioned animals showed an increase in A20 immunoreactivity in the septum, similar to saline-injected animals, but in addition, they exhibited a decrease in neuronal A20 immunoreactivity within the neocortical lesion core at 10 hours post-lesion (cf. Fig. 3D and E). After 24 hours, neuronal decrease in A20 was seen throughout the lesioned neocortex and dorsal striatum (cf. Fig. 3G and H), but not in the lesioned hippocampus. At 72 hours post-lesion, decreased neuronal immunoreactivity for A20 was still observed in the cortical lesion core, which was now surrounded by $A20^+$ reactive astroglial-like cells in the lesion periphery of the cortex (cf. Fig. 3J and K), striatum and hippocampus (cf. Fig. 3I and L). Interestingly, A20 labeling in astroglial-like cells was more intense in the hippocampus than in the other neurodegenerating areas such as the cortex, not only in regard to the intensity of immunoreactivity, but also in the amount of $A20^+$ astroglial-like cells (cf. Fig. 3K and L). Finally, round cells resembling mast cells or macrophages showing intense intracellular $A20^+$ granular structures were observed in the fimbria (Fig. 3F).

In vivo *overexpression of A20 after an excitotoxic lesion to the immature rat brain*

Quantification of lesion volume on Nissl-stained coronal sections showed that experimentally induced A20 overexpression significantly increased excitotoxic lesion volume ($20.8 \pm 7.0\%$ increase in the percentage

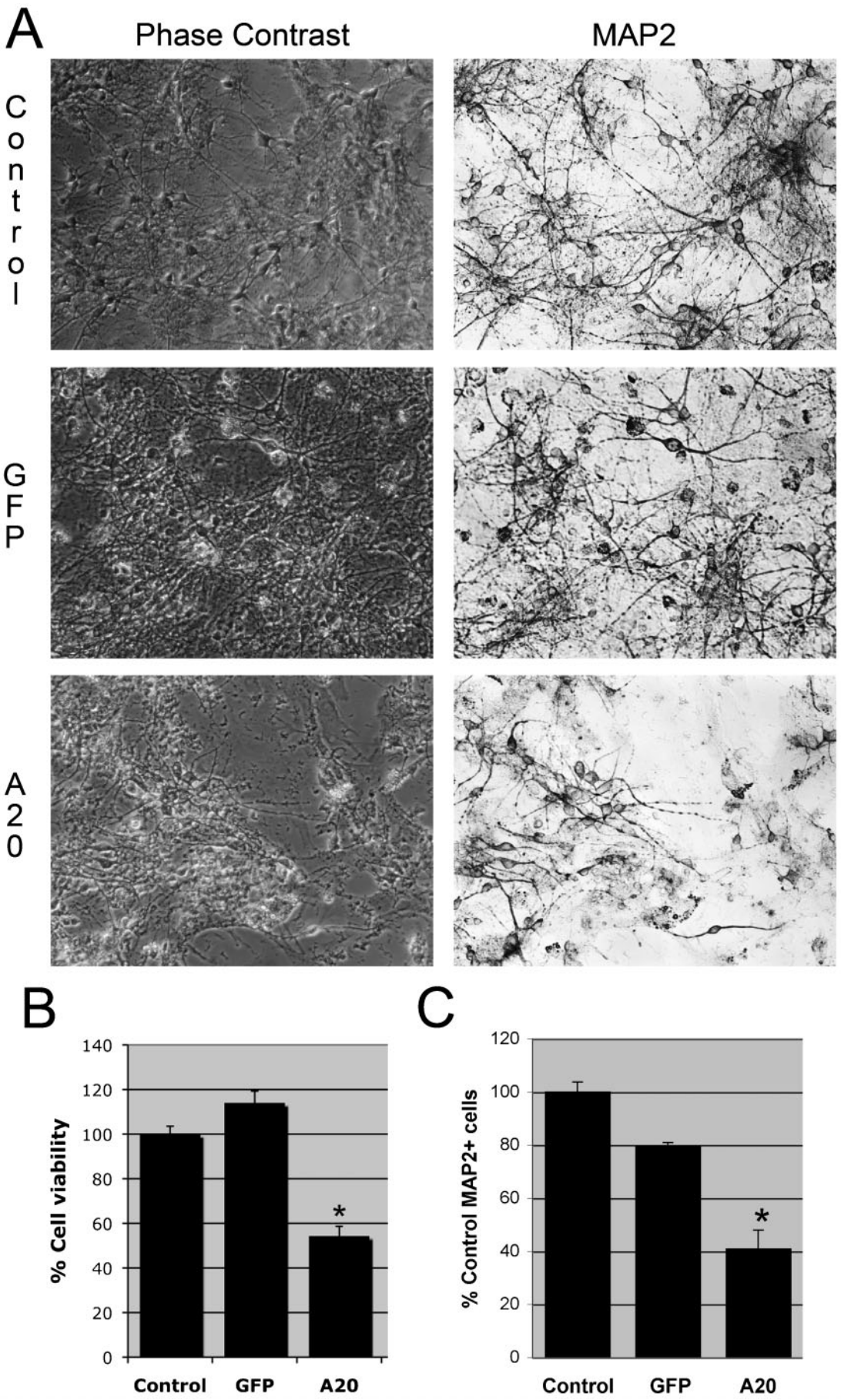

Figure 1 Overexpression of A20 in cortical mixed neuron-glia cultures. Cortical mixed cultures were incubated with lipofectamine alone or lipofectamine carrying the GFP or A20 plasmids. Seventy-two hours later, cell viability was analyzed by phase contrast microscopy (A), MTT (B), or direct cell counts of MAP2$^+$ cells to analyze neuronal cells (A and C). A20 overexpression induced a significant reduction in MTT levels (B), neuronal cell death, and neurite atrophy (A and C).

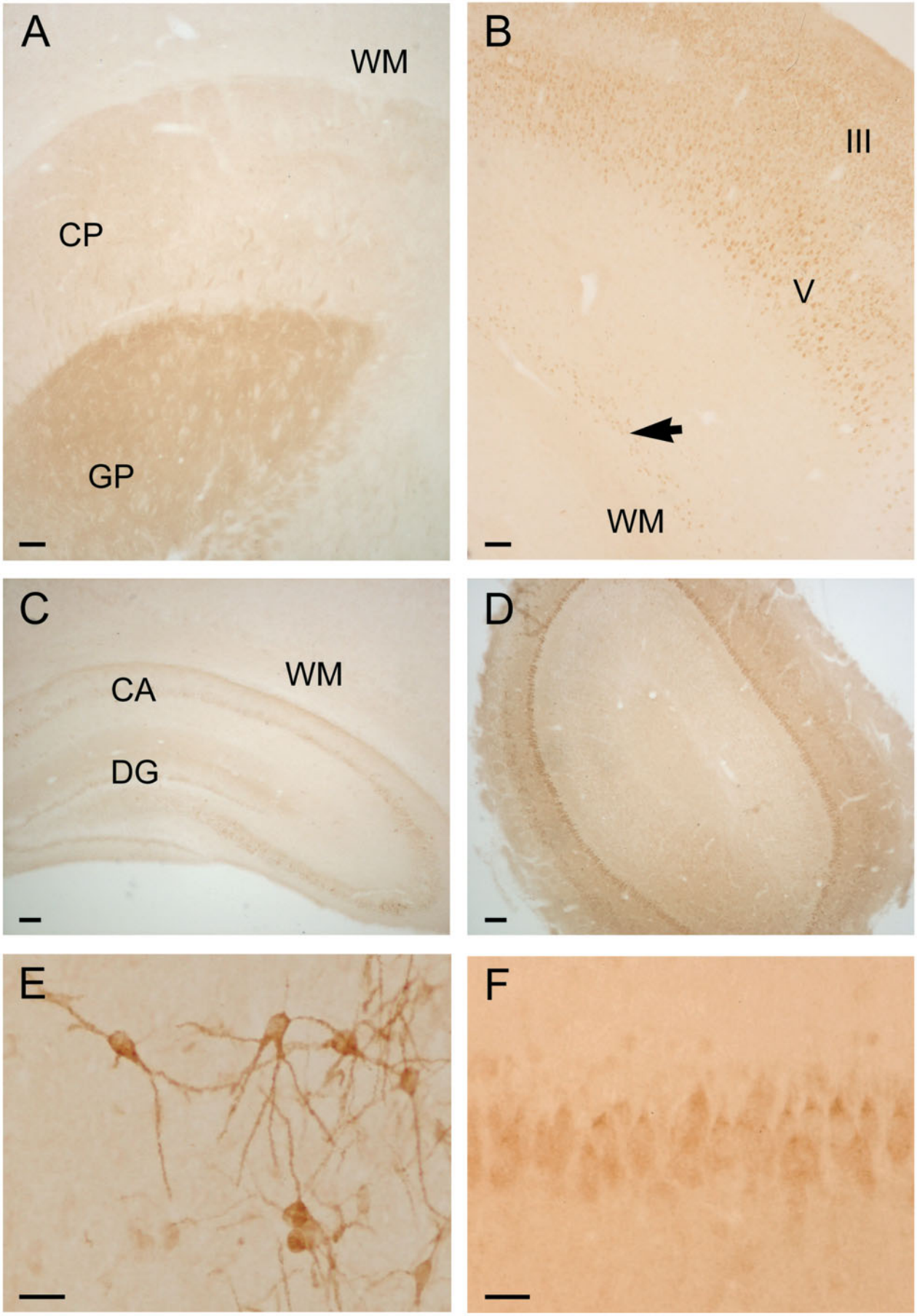

Figure 2 A20 expression in the normal immature brain. Control animals showed widespread A20 expression in the brain, including striatum (A; GP: globus palidus; CP: caudate putamen, WM: white matter), neocortex (B; arrow: subplate neuron layer), hippocampus (C; CA: cornu ammonis, DG: dentate gyrus), and olfactory bulb (D). The staining pattern appeared cytoplasmic and punctuated, staining also neurites (E; septum), but being generally only cytoplasmic (F; olfactory bulb). Scale bars: A, B, C, and D: 100 μM; E and F: 20 μM.

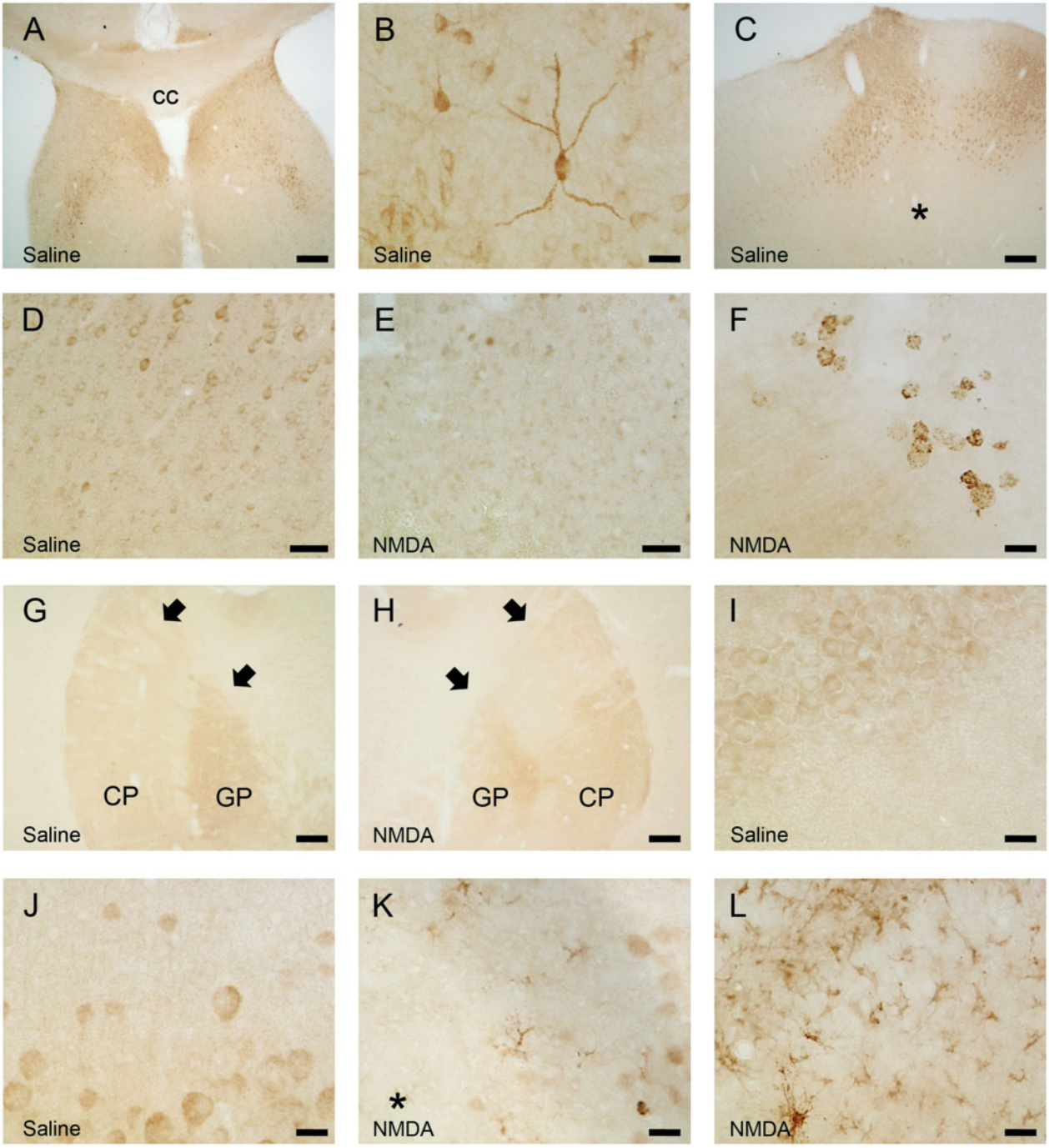

Figure 3 **A20 expression in the excitotoxically injured immature brain. Control animals injected intracortically with saline showed, 10 hours after the injection, slight bilateral upregulation of A20 in some neurons of the septum (A, B), and 24 hours after the saline injection, restricted upregulation of A20 was noted in cortical neurons of the injection point (C, asterisk: injection point). In contrast, NMDA injection induced a decrease in neuronal A20 immunoreactivity in the neocortical injection point at 10 hours (E) when compared to control (not shown) or saline injected neocortex (D). After 24 hours, neuronal decrease in A20 was also seen in the dorsal caudate putamen (CP) and globus pallidus (GP) (H, arrows) when compared to striatum of saline injected animals (G). Seventy-two hours after the lesion, A20-positive reactive astrocytes were observed in the cortical lesion periphery (K, asterisk: lesion core) and in the entire hippocampus (L), as compared to neuronal labelling in the neocortex (J) and hippocampus (I) of saline-injected animals. Rounded cells with irregular limits and very intense intracellular A20 positive granular structures were observed at the fimbria (F). Scale bars: A, C, G, and H: 200 μm; E and D: 100 μm; B, F, J, K, I, and L: 20 μm.**

of lesioned hemisphere in relation to the gene therapy control group NLSCt/GFP) (Fig. 4).

Moreover, as A20 was observed in reactive astrocytes after the lesion, we evaluated the effects of experimentally induced A20 overexpression on the astroglial response by GFAP immunoreactivity. At 72 hours after the lesion, in the gene therapy control GFP-transfected group, $GFAP^{+}$ astroglial cells showed a reactive morphology with strong GFAP expression and thick processes, as we have previously

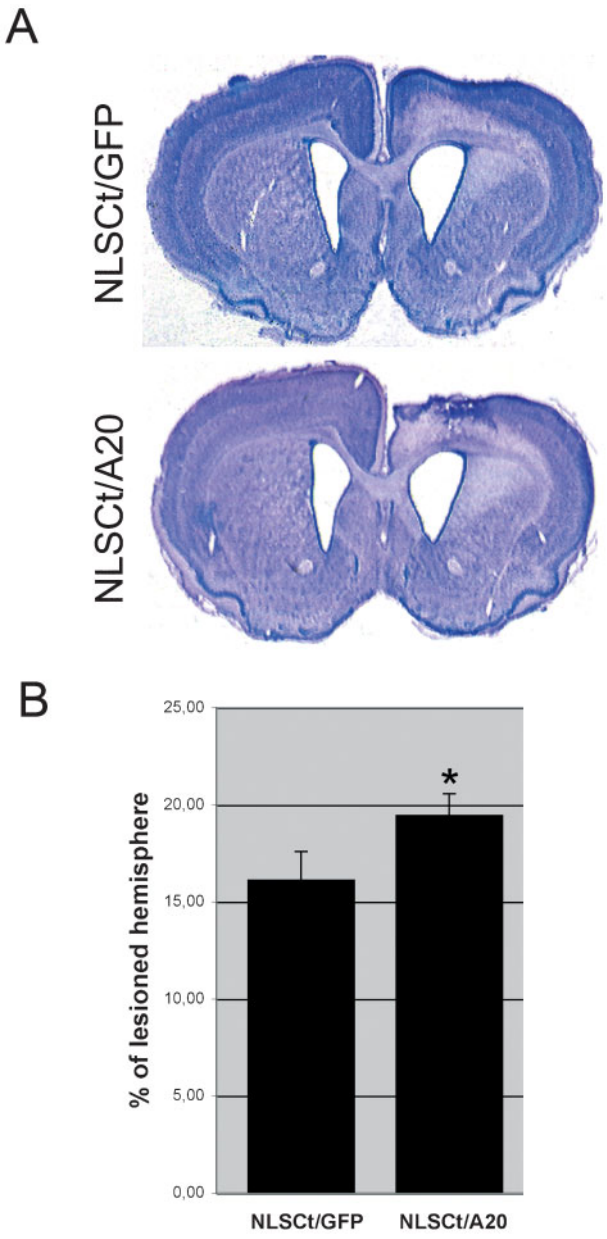

Figure 4 Lesion volume after overexpression of A20 in the excitotoxically injured immature brain. Two hours after NMDA-induced excitotoxic lesion, animals were injected at the same coordinates with the gene therapy vector NLSCt carrying the control transgene (NLSCt/GFP) or the A20 transgene (NLSCt/A20) (A). Image analysis of coronal sections stained with Nissl staining (A) showed that animals overexpressing A20 presented a significant increase in the percentage of lesioned hemisphere (*$P<0.05$ in relation to NLSCt/GFP injected animals) when compared to the GFP overexpressing transgene control animals (B).

described in the excitotoxically lesioned postnatal brain.[32] Similar distribution and astroglial morphology was observed after A20 overexpression (Fig. 5A). In this sense, quantitative densitometrical analysis of GFAP immunoreactivity (Fig. 5B) or analysis of the total area within ROI occupied by $GFAP^+$ astrocytes (Fig. 5C) confirmed that there were no significant differences in the astroglial response after A20 overexpression when compared to the control group transfected with GFP.

Discussion

The present study shows for the first time that the NF-kappaB binding molecule A20 is widely expressed on CNS neuronal cells under physiological conditions. After acute excitotoxic damage in the immature brain, neuronal A20 is rapidly downregulated in the lesion core, followed by a later upregulation in astrocytes. In addition, A20 overexpression, either *in vitro* under basal conditions or *in vivo* after the acute excitotoxic damage, induces an increase in neuronal cell death, contributing to the hypothesis that A20 plays a pivotal role in determining cell death/survival also in the CNS.

Expression of endogenous A20

A20 is a NF-kappaB binding molecule that was first described as an inhibitor of NF-kappaB signalling after activation of this transcription factor by inflammatory mediators acting on TNF-alphaR), interleukin-1beta receptors, and Toll-like receptors.[7–9] In addition, A20 is a NF-kappaB-regulated gene itself and therefore, its expression has been shown to be NF-kappaB-dependent.[33] In this regard, the previously reported presence of constitutive NF-kappaB activity in the CNS under physiological conditions[34–36] could account for the constitutive expression of A20 in the control brain described here. By using kappaB reporter mice, Bhakar and co-workers nicely described constitutive NF-kappaB activity mainly in neurons, particularly in the neocortex, pyriform cortex, hippocampus, amygdala, olfactory bulb, and olfactory tubercle (Islands of Calleja).[37] Accordingly, we here show that the immature rat brain showed widespread A20 expression in the gray matter, mainly in neuronal cells, with particularly high expression in mostly the same areas where constitutive NF-kappaB has been shown. Some differences exist, however, between the two expression patterns, which can easily be explained by variations on the κB binding sites used in the kappaB reporter mice and the one of the A20 gene. In fact, it is interesting to note that there is considerable diversity in the DNA binding properties of the different NF-kappaB-regulated genes, and the NF-kappaB consensus sequence has more than 60 variants with different binding properties. However, our study suggests that the overall basal expression of A20 in neuronal cells is probably regulated by the constitutive NF-kappaB transcription factor.

In contrast, after an acute excitotoxic lesion to the immature rat brain, neuronal A20 immunoreactivity decreased rapidly within 10 hours in the cortical lesion core, when neuronal death is an ongoing process,[38] and decreased neuronal A20 was still observed in secondary lesioned areas at 24 hours post-lesion. Noteworthy, we have previously shown that active NFκB is found in injured neurons located in the lesion core at 10 hours post-lesion,[39] correlating with

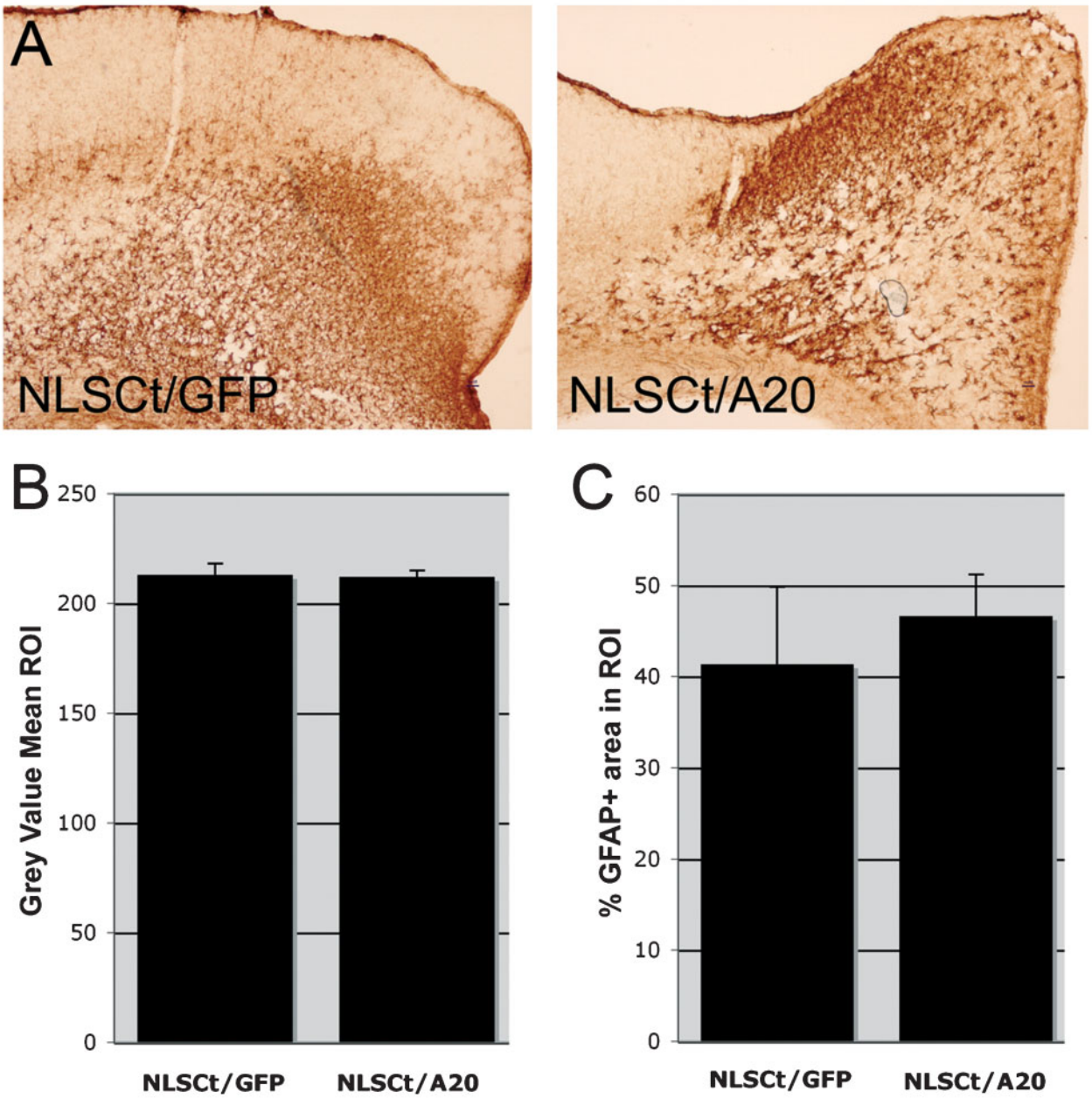

Figure 5 Astroglial reactivity after overexpression of A20 in the excitotoxically injured immature brain. Two hours after the NMDA lesion, animals were injected at the same coordinates with the gene delivery vector NLSCt carrying a control transgene (NLSCt/GFP) or the A20 transgene (NLSCt/A20) (A). There were no significant differences in the densitometrical analysis of GFAP immunoreactivity (B) nor in the percentage of GFAP immunoreactive area within ROI (C) between animals overexpressing GFP or A20.

neuronal expression of some NF-kappaB-target genes such as cyclooxygenase-2.[40] However, NF-kappaB activation does not seem to be enough to induce the neuronal expression of A20 under these circumstances, suggesting a complex regulation of A20 expression in damaged neurons. Alternatively, the reported decrease in A20 expression could also reflect an effect at the protein level, such as degradation of A20. Later on, 3 days after the excitotoxic damage, A20 expression was induced in some cells that due to their localization and morphology resemble reactive astrocytes, which were distributed in the lesion borders as has been previously described in this model using astroglial markers.[29,30,32] In relation to this, we have previously shown that astrocytes located in the neocortical lesion site in the same postnatal injury model, display activated NF-kappaB from a few hours after the lesion, showing maximum astroglial NF-kappaB at 24 hours post-lesion.[39] Therefore, it is likely that NF-kappaB may contribute to the induction of A20 in astrocytes, as we have observed expression of other NF-kappaB-regulated genes like iNOS[40] and Cu/Zn SOD[38] in this cell type. Thus, it is conceivable to think that NF-kappaB regulation of its target gene A20 is cell specific, pointing again to a complex regulation of its expression. Moreover, it should be noted that A20 mRNA levels increase in other neuropathological conditions like 6 days after brain infection by dengue

virus[41] and in total cortex homogenates of cryolesioned brain at 8 hours post-lesion.[17] However, relevant cell types were not identified in these studies.

Effects of A20 overexpression

To our knowledge, very few studies have evaluated the effects of A20 overexpression in CNS cells. We here report that A20 overexpression in cortical cultures induced significant neuronal cell death, in agreement with previous work demonstrating that A20 increases cell vulnerability to reactive oxygen species, triggering necrosis,[14] an effect that was dependent on the A20-mediated NF-kappaB inhibition. This effect could also explain, at least in part, why A20 overexpression 2 hours after the excitotoxic lesion to the immature brain enhanced lesion volume, as oxidative stress plays a critical role in promoting neurodegeneration in this postnatal lesion model.[26] In comparison, the overexpression of the NF-kappaB inhibitor IkappaBm both *in vitro* and *in vivo* showed a non-significant similar trend towards an increase in neurodegeneration. The lack of a significant effect of IkappaBm in this context could suggest other functions of A20 not mediated by NF-kappaB, or variable effects induced by different NF-kappaB inhibiting mechanisms.

A previous study analyzing the effects of A20 overexpression *in vitro* and *in vivo* has described a significant neuroprotective effect of A20 gene transfer when administered 2 days before middle cerebral artery occlusion in adult rats and in hippocampal cultures.[42] This neuroprotective effect was attributed to an A20-mediated inhibition of TNF-induced apoptosis by inhibiting the proteolytic cleavage of caspase-8 and -3,[42] pointing to a clear anti-apoptotic action of A20. Several reasons may account for the differences between the cited study and our results. First, the pre-treatment paradigm used by Yu and colleagues, which could induce NF-kappaB-mediated preconditioning effects, contrasts with our post-lesion treatment experimental design. Second, a different neuropathological process due to brain immaturity of CNS[18] occurs after postnatal excitotoxic damage, when activation of caspase-8 dependent pathway is not the main mechanism mediating neuronal cell death.[43] Finally, another reason behind these discrepancies could be attributed to the *in vitro* and *in vivo* transfection methods used, and their possible interaction or synergism with the physiological role of A20. However, here we used, on one hand, a previously characterized modular recombinant gene therapy vehicle for *in vivo* overexpression of A20 and, on the other hand, lipofectamine for the *in vitro* overexpression. Noticeably, in both cases, the result obtained was an increase in neurodegeneration, pointing against the existence of potential interactions between the transfection method used and the physiological role of A20.

An important number of studies have addressed in the last decade the effects of NF-kappaB inhibition in the CNS by using several mechanisms. In general, inhibition of NF-kappaB under physiological conditions *in vivo* does not seem to induce cell death[44,45] but causes learning disabilities.[46] Also, several *in vitro* studies have shown that NF-kappaB inhibition induced cell death in several cultured neuronal cell types[47,48] including cortical neurons,[37] in agreement with our results on A20 overexpression. Somewhat contradictory results have been reported on the effects of the inhibition of NF-kappaB signalling after acute injuries. NF-kappaB inhibition sensitizes cultured neurons to excitotoxic[49,50] and amyloid beta peptide-induced cell death[51]. *In vivo* NF-kappaB inhibition after acute injuries to the adult brain increases, in most cases, neurodegeneration.[46,49,52–54] although other studies demonstrated decreased lesion volume after transient focal ischemia.[55–58]

Taken together, these observations suggest that the level of A20 expression, and thus possibly of NF-kappaB activity within neurons should be preserved in a certain physiological range, which could be maintained by an autoregulatory loop including the expression of NF-kappaB inhibiting proteins such as IkappaB family members or deubiquitinating enzymes such as A20 or CYLD.[2] In accordance with these observations, a perturbation of physiological A20 expression could lead to neuropathological changes, which may include cell death. Moreover, as stated above, a NF-kappaB-independent effect of A20 cannot be ruled out.

Future experiments are necessary for a better understanding of the functions of A20 in the nervous system both under physiological and pathological conditions. Although A20 was described as a potent anti-inflammatory agent and modulator of inflammation, a parallel action of this molecule inducing neurotoxic effects is here described, precluding the use of A20 as a neuroprotective target gene in non cell-selective gene therapy applications.

Conclusions

Our results showed that A20 was normally expressed in neurons of the immature brain and that after an acute lesion, it was rapidly downregulated in neurons of the lesioned area and also upregulated in reactive astrocyte-like cells. Moreover, the overexpression of A20 in mixed cortical cultures and *in vivo* after an acute immature brain injury was neurotoxic, and thus cannot be used as an inflammatory modulating transgene for neuroprotective gene therapy applications.

Competing interests

Authors declare no competing interests.

Authors' contributions

HP participated in the design of the study, performed most of the *in vitro* and *in vivo* experiments, performed statistical analyses, and drafted the manuscript. PG participated in several experiments and helped to draft the manuscript. LA conceived and coordinated the study, and helped to design the study and to draft the manuscript. AA and AV designed and produced the modular vehicle for gene delivery used in this study. RB produced the A20 plasmids and helped to draft the manuscript. BG participated in the design and coordination of the study and helped to draft the manuscript. All authors read and approved the final manuscript.

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References

1 Mattson MP. NF-kappaB in the survival and plasticity of neurons. Neurochem Res. 2005;30(6–7):883–93.
2 Kaltschmidt B, Widera D, Kaltschmidt C. Signaling via NF-kappaB in the nervous system. Biochim Biophys Acta. 2005;1745(3):287–99.
3 Acarin L, Gonzalez B, Castellano B. Triflusal posttreatment inhibits glial nuclear factor-kappaB, downregulates the glial response, and is neuroprotective in an excitotoxic injury model in postnatal brain. Stroke. 2001;32(10):2394–402.
4 Lee EG, Boone DL, Chai S, Libby SL, Chien M, Lodolce JP, *et al.* Failure to regulate TNF-induced NF-kappaB and cell death responses in A20-deficient mice. Science. 2000; 289(5488):2350–4.
5 Boone DL, Turer EE, Lee EG, Ahmad RC, Wheeler MT, Tsui C, *et al.* The ubiquitin-modifying enzyme A20 is required for termination of Toll-like receptor responses. Nat Immunol. 2004;5(10):1052–60.
6 Silverman N, Fitzgerald K. DUBbing down innate immunity. Nat Immunol. 2004;5(10):1010–2.
7 Wertz IE, O'Rourke KM, Zhou H, Eby M, Aravind L, Seshagiri S, *et al.* De-ubiquitination and ubiquitin ligase domains of A20 downregulate NF-kappaB signalling. Nature. 2004;430(7000):694–9.
8 Heyninck K, Beyaert R. A20 inhibits NF-kappaB activation by dual ubiquitin-editing functions. Trends Biochem Sci. 2005;30(1):1–4.
9 Vereecke L, Beyaert R, van Loo G. The ubiquitin-editing enzyme A20 (TNFAIP3) is a central regulator of immunopathology. Trends Immunol. 2009;30(8):383–91.
10 Staub O, Rotin D. Role of ubiquitylation in cellular membrane transport. Physiol Rev. 2006;86(2):669–707.
11 Grey ST, Arvelo MB, Hasenkamp W, Bach FH, Ferran C. A20 inhibits cytokine-induced apoptosis and nuclear factor kappaB-dependent gene activation in islets. J Exp Med. 1999; 190(8):1135–46.
12 Liuwantara D, Elliot M, Smith M, Yam AO, Walters SN, Marino E, *et al.* Nuclear factor-kappaB regulates beta-cell death: a critical role for A20 in beta-cell protection. Diabetes. 2006;55(9):2491–501.
13 Daniel S, Arvelo MB, Patel VI, Longo CR, Shrikhande G, Shukri T, *et al.* A20 protects endothelial cells from TNF-, Fas-, and NK-mediated cell death by inhibiting caspase 8 activation. Blood. 2004;104(8):2376–84.
14 Storz P, Doppler H, Ferran C, Grey ST, Toker A. Functional dichotomy of A20 in apoptotic and necrotic cell death. Biochem J. 2005;387(Pt 1):47–55.
15 Patel VI, Daniel S, Longo CR, Shrikhande GV, Scali ST, Czismadia E, *et al.* A20, a modulator of smooth muscle cell proliferation and apoptosis, prevents and induces regression of neointimal hyperplasia. FASEB J. 2006;20(9):1418–30.
16 Malewicz M, Zeller N, Yilmaz ZB, Weih F. NF kappa B controls the balance between Fas and tumor necrosis factor cell death pathways during T cell receptor-induced apoptosis via the expression of its target gene A20. J Biol Chem. 2003;278(35):32825–33.
17 Quintana A, Giralt M, Rojas S, Penkowa M, Campbell IL, Hidalgo J, *et al.* Differential role of tumor necrosis factor receptors in mouse brain inflammatory responses in cryolesion brain injury. J Neurosci Res. 2005;82(5):701–16.
18 Ferriero DM. Neonatal brain injury. N Engl J Med. 2004;351(19):1985–95.
19 Vannucci SJ, Hagberg H. Hypoxia–ischemia in the immature brain. J Exp Biol. 2004;207(Pt 18):3149–54.
20 Romijn HJ, Hofman MA, Gramsbergen A. At what age is the developing cerebral cortex of the rat comparable to that of the full-term newborn human baby? Early Hum Dev. 1991;26(1):61–7.
21 Dobbing J, Sands J. Comparative aspects of the brain growth spurt. Early Hum Dev. 1979;3(1):79–83.
22 McDonald JW, Silverstein FS, Johnston MJ. Neurotoxicity of *N*-methyl-D-aspartate is markedly enhanced in developing rat central nervous system', Brain Res. 1988;459(1):200–3.
23 Aris A, Feliu JX, Knight A, Coutelle C, Villaverde A. Exploiting viral cell-targeting abilities in a single polypeptide, non-infectious, recombinant vehicle for integrin-mediated DNA delivery and gene expression. Biotechnol Bioeng. 2000;68(6):689–96.
24 Aris A, Villaverde A. Engineering nuclear localization signals in modular protein vehicles for gene therapy. Biochem Biophys Res Commun. 2003;304(4):625–31.
25 Peluffo H, Aris A, Acarin L, Gonzalez P, Villaverde A, Castellano B, *et al.* Nonviral gene delivery to the central nervous system based on a novel integrin-targeting multifunctional protein. Hum Gene Ther. 2003;14(13):1215–23.
26 Peluffo H, Acarin L, Aris A, Gonzalez P, Villaverde A, Castellano B, *et al.* Neuroprotection from NMDA excitotoxic lesion by Cu/Zn superoxide dismutase gene delivery to the postnatal rat brain by a modular protein vector. BMC Neurosci. 2006;7:35.
27 Peluffo H, Gonzalez P, Aris A, Saura J, Villaverde A, Castellano B, *et al.* RGD domains neuroprotect the immature brain by a glial-dependent mechanism. Ann Neurol. 2007;62(3):251–61.
28 Klinkenberg M, van Huffel S, Heyninck K, Beyaert R. Functional redundancy of the zinc fingers of A20 for inhibition of NF-kappaB activation and protein–protein interactions. FEBS Lett. 2001;498(1):93–7.
29 Acarin L, Gonzalez B, Castro AJ, Castellano B. Primary cortical glial reaction versus secondary thalamic glial response in the excitotoxically injured young brain: microglial/macrophage response and major histocompatibility complex class I and II expression. Neuroscience. 1999;89(2):549–65.
30 Acarin L, Gonzalez B, Castellano B, Castro AJ. Quantitative analysis of microglial reaction to a cortical excitotoxic lesion in the early postnatal brain. Exp Neurol. 1997;147(2):410–7.
31 Ikonomidou C, Mosinger JL, Salles KS, Labruyere J, Olney JW. Sensitivity of the developing rat brain to hypobaric/ischemic damage parallels sensitivity to *N*-methyl-aspartate neurotoxicity. J Neurosci. 1989;9(8):2809–18.
32 Acarin L, González B, Hidalgo J, Castro AJ, Castellano B. Primary cortical glial reaction versus secondary thalamic glial response in the excitotoxically injured young brain: astroglial response and metallothionein expression. Neuroscience. 1999;92(3):827–39.
33 Krikos A, Laherty CD, Dixit VM. Transcriptional activation of the tumor necrosis factor alpha-inducible zinc finger protein, A20, is mediated by kappa B elements. J Biol Chem. 1992;267(25):17971–6.
34 Bakalkin G, Yakovleva T, Terenius L. NF-kappa B-like factors in the murine brain. Developmentally-regulated and tissue-specific expression. Brain Res Mol Brain Res. 1993;20(1–2):137–46.

35 Rattner A, Korner M, Walker MD, Citri Y. NF-kappa B activates the HIV promoter in neurons. EMBO J. 1993; 12(11):4261–7.
36 Kaltschmidt C, Kaltschmidt B, Neumann H, Wekerle H, Baeuerle PA. Constitutive NF-kappa B activity in neurons. Mol Cell Biol. 1994;14(6):3981–92.
37 Bhakar AL, Tannis LL, Zeindler C, Russo MP, Jobin C, Park DS, *et al*. Constitutive nuclear factor-kappa B activity is required for central neuron survival. J Neurosci. 2002; 22(19):8466–75.
38 Peluffo H, Acarin L, Faiz M, Castellano B, Gonzalez B. Cu/Zn superoxide dismutase expression in the postnatal rat brain following an excitotoxic injury. J Neuroinflammation. 2005; 2(1):12.
39 Acarin L, Gonzalez B, Castellano B. STAT3 and NFkappaB activation precedes glial reactivity in the excitotoxically injured young cortex but not in the corresponding distal thalamic nuclei. J Neuropathol Exp Neurol. 2000;59(2):151–63.
40 Acarin L, Peluffo H, Gonzalez B, Castellano B. Expression of inducible nitric oxide synthase and cyclooxygenase-2 after excitotoxic damage to the immature rat brain. J Neurosci Res. 2002;68(6):745–54.
41 Sanchez-Burgos G, Hernandez-Pando R, Campbell IL, Ramos-Castaneda J, Ramos C. Cytokine production in brain of mice experimentally infected with dengue virus. Neuroreport. 2004;15(1):37–42.
42 Yu L, Miao H, Hou Y, Zhang B, Guo L. Neuroprotective effect of A20 on TNF-induced postischemic apoptosis. Neurochem Res. 2006;31(1):21–32.
43 Villapol S, Acarin L, Faiz M, Rohn TT, Castellano B, Gonzalez B. Distinct spatial and temporal activation of caspase pathways in neurons and glial cells after excitotoxic damage to the immature rat brain. J Neurosci Res. 2007;85(16):3545–56.
44 Fridmacher V, Kaltschmidt B, Goudeau B, Ndiaye D, Rossi FM, Pfeiffer J, *et al*. Forebrain-specific neuronal inhibition of nuclear factor-kappaB activity leads to loss of neuroprotection. J Neurosci. 2003;23(28):9403–8.
45 Sha WC, Liou HC, Tuomanen EI, Baltimore D. Targeted disruption of the p50 subunit of NF-kappa B leads to multifocal defects in immune responses. Cell. 1995;80(2):321–30.
46 Kassed CA, Willing AE, Garbuzova-Davis S, Sanberg PR, Pennypacker KR. Lack of NF-kappaB p50 exacerbates degeneration of hippocampal neurons after chemical exposure and impairs learning. Exp Neurol. 2002;176(2):277–88.
47 Fernyhough P, Smith DR, Schapansky J, van der Ploeg R, Gardiner NJ, Tweed CW, *et al*. Activation of nuclear factor-kappaB via endogenous tumor necrosis factor alpha regulates survival of axotomized adult sensory neurons. J Neurosci. 2005;25(7):1682–90.
48 Koulich E, Nguyen T, Johnson K, Giardina C, D'Mello S. NF-kappaB is involved in the survival of cerebellar granule neurons: association of IkappaBbeta [correction of Ikappabeta] phosphorylation with cell survival. J Neurochem. 2001;76(4):1188–98.
49 Yu Z, Zhou D, Bruce-Keller AJ, Kindy MS, Mattson MP. Lack of the p50 subunit of nuclear factor-kappaB increases the vulnerability of hippocampal neurons to excitotoxic injury. J Neurosci. 1999;19(20):8856–65.
50 Nijboer CH, Heijnen CJ, Groenendaal F, May MJ, van Bel F, Kavelaars A. A dual role of the NF-kappaB pathway in neonatal hypoxic–ischemic brain damage. Stroke. 2008; 39(9):2578–86.
51 Kaltschmidt B, Uherek M, Wellmann H, Volk B, Kaltschmidt C. Inhibition of NF-kappaB potentiates amyloid beta-mediated neuronal apoptosis. Proc Natl Acad Sci USA. 1999; 96(16):9409–14.
52 Lang H, Schulte BA, Zhou D, Smythe N, Spicer SS, Schmiedt RA. Nuclear factor kappaB deficiency is associated with auditory nerve degeneration and increased noise-induced hearing loss. J Neurosci. 2006;26(13):3541–50.
53 Duckworth EA, Butler T, Collier L, Pennypacker KR. NF-kappaB protects neurons from ischemic injury after middle cerebral artery occlusion in mice. Brain Res. 2006;1088(1):167–75.
54 Yu Z, Zhou D, Cheng G, Mattson MP. Neuroprotective role for the p50 subunit of NF-kappaB in an experimental model of Huntington's disease. J Mol Neurosci. 2000;15(1):31–44.
55 Schneider A, Martin-Villalba A, Weih F, Vogel J, Wirth T, Schwaninger M. NF-kappaB is activated and promotes cell death in focal cerebral ischemia. Nat Med. 1999;5(5):554–9.
56 Nurmi A, Lindsberg PJ, Koistinaho M, Zhang W, Juettler E, KarjalainenLindsberg ML, *et al*. Nuclear factor-kappaB contributes to infarction after permanent focal ischemia. Stroke. 2004;35(4):987–991.
57 Herrmann O, Baumann B, de Lorenzi R, Muhammad S, Zhang W, Kleesiek J, *et al*. IKK mediates ischemia-induced neuronal death. Nat Med. 2005;11(12):1322–9.
58 Zhang W, Potrovita I, Tarabin V, Herrmann O, Beer V, Weih F, *et al*. Neuronal activation of NF-kappaB contributes to cell death in cerebral ischemia. J Cereb Blood Flow Metab. 2005;25(1):30–40.