Pilocarpine-Induced Status Epilepticus in Rats Involves Ischemic and Excitotoxic Mechanisms

Paolo Francesco Fabene1*, Flavia Merigo1, Mirco Galiè1, Donatella Benati1, Paolo Bernardi1, Paolo Farace1, Elena Nicolato1, Pasquina Marzola2, Andrea Sbarbati1

1 Section of Anatomy and Histology, Department of Morphological and Biomedical Sciences, University of Verona, Verona, Italy, 2 Center of Experimental. Magnetic Resonance Imaging, University of Verona, Verona, Italy

The neuron loss characteristic of hippocampal sclerosis in temporal lobe epilepsy patients is thought to be the result of excitotoxic, rather than ischemic, injury. In this study, we assessed changes in vascular structure, gene expression, and the time course of neuronal degeneration in the cerebral cortex during the acute period after onset of pilocarpine-induced status epilepticus (SE). Immediately after 2 hr SE, the subgranular layers of somatosensory cortex exhibited a reduced vascular perfusion indicative of ischemia, whereas the immediately adjacent supragranular layers exhibited increased perfusion. Subgranular layers exhibited necrotic pathology, whereas the supergranular layers were characterized by a delayed (24 h after SE) degeneration apparently via programmed cell death. These results indicate that both excitotoxic and ischemic injuries occur during pilocarpine-induced SE. Both of these degenerative pathways, as well as the widespread and severe brain damage observed, should be considered when animal model-based data are compared to human pathology.

Citation: Fabene PF, Merigo F, Galiè M, Benati D, Bernardi P, et al (2007) Pilocarpine-Induced Status Epilepticus in Rats Involves Ischemic and Excitotoxic Mechanisms. PLoS ONE 2(10): e1105. doi:10.1371/journal.pone.0001105

INTRODUCTION
Ischemic and excitotoxic injuries involve a variety of mechanisms that produce different types of neuronal death [1]. Although seizure-induced neuronal injury was originally called “ischemic injury”, pioneering studies by Meldrum and colleagues demonstrated that when ischemia was prevented, seizure activity still produced irreversible neuronal injury [2]. Subsequent studies demonstrated that seizure activity itself can be neurotoxic, and that the primary mechanisms involve excitatory amino acid receptor mediation of excitotoxic insults [3–6]. However, recent studies have suggested that prolonged status epilepticus (SE) in rats, whether induced by pilocarpine or kainate, also involves profound vascular changes that cause ischemic, as well as excitotoxic injury [7,8]. Thus, precisely which pathological changes observed after prolonged SE in animals is ischemic in nature, and which are excitotoxic, is unclear. This is an important issue to resolve because prolonged SE is commonly used to induce a chronic epileptic state, and it is generally assumed that all of the SE-induced changes in animals are excitotoxic in nature [9], and closely related to the pattern of cell death exhibited in human temporal lobe epilepsy [2]. The possibility that prolonged SE also involves ischemic injury has significant implications for the interpretation of results generated by pilocarpine-induced epilepsy.

We recently showed that pilocarpine-induced SE produced significant vascular/ischemic effects in the cingulate and somatosensory cortices of Wistar rats [7]. Given the profound vascular effects subsequently reported in the hippocampi of pilocarpine- and kainate-treated rats [8], prolonged SE may be an insult more severe than that observed in other experimental epilepsy models, or in humans who exhibit a highly selective and relatively limited pattern of neuron loss [2]. In our previous study, we reported that vascular alterations, characterized by hyperemia and degenerating neurons, were evident in the supragranular layers of the somatosensory cortex and in the layers II/III of the cingulate cortex of Wistar rats 12 h after SE lasting 4 h, whereas immediately adjacent subgranular layers exhibited edema and necrotic cell death [7]. These results suggested that cortical injury caused by pilocarpine-induced SE might involve different mechanisms in adjacent structures because of differences in blood vessels organization and regulation between the two areas. In the present study, we addressed anoxic/hypoxic/ischemic alterations and excitotoxic injury in the immediate post-SE period (2 h post-SE) to evaluate vascular changes, neurodegenerative patterns, and possible differences in the expression of genes that may mediate different mechanisms of SE-induced neuronal death. In this study, we used a multidisciplinary approach, based both on in vivo (MRI) and ex vivo (immunohistochemistry, electron microscopy, super-arrays) analysis.

MATERIALS AND METHODS

Animals
Male adult Wistar rats (80–90 days of age), were kept under controlled environmental parameters and veterinarian control (Fig. 1). The animals were habituated to the experimenters for at least two weeks prior to the procedures employed in the present study. The experiments received authorization from the Italian Ministry of Health, and were conducted following the principles of the NIH Guide for the Use and Care of Laboratory Animals, and the European Community Council (86/609/EEC) directive. All efforts were made to minimize the number of animals used and avoid their suffering. Seizures were induced by pilocarpine injections as reported in previous studies [7]. Briefly, to minimize
In this experiment an USPIO particle (Sinerem®, kindly supplied by Guerbet, Aulnay-Sous-Bois, France) was used as a contrast agent. Sinerem® is constituted by an iron-oxide core of about 6 nm diameter coated by dextran (coated particle dimensions of about 20 nm) and is characterized by a blood half-time longer than 2 h in rats [12]. Sinerem® (6 mg iron/kg) was dissolved in saline and injected in the tail vein.

rCBV acquisition protocols and image analysis techniques were as described previously [7,13]. Briefly, transversal multislice gradient echo images were acquired before and two minutes after administration of Sinerem® with the following parameters: TR = 350 ms, TE = 15 ms, flip angle = 30°. Field of view 4×4 cm², matrix size 256×256, slice thickness = 2 mm. Five continuous slices were acquired.

The dynamic data were then analyzed to calculate the peak enhancement and especially the relative TTP. The latter parameter is related to mean transit time and blood flow. TTP was evaluated on the signal intensity plot as the time point of maximal signal reduction. Relative TTP was obtained as a difference between TTP and time of arrival TA: rTTP = TTP − TA, where the time of arrival is the time for contrast material to arrive in the brain [14].

Peak enhancements were calculated by the relation: (SImin−SImpre)/SImpre, where SImin is the signal intensity at the time of maximal signal drop and SImpre is the average signal intensity of three points before contrast administration.

Histochemical analysis
Free floating sections were washed in PBS at room temperature and permeabilized for 1 hour in PBS containing 0.3% Triton X-100, 1% bovine serum albumin and 2% normal goat serum, the same solution was used to dilute the antibodies. Subsequently, sections were incubated overnight in a mixture of rabbit polyclonal anti-Agrin with mouse monoclonal anti-Glial Fibrillary Acidic Protein (GFAP; Cymbus Biotechnology LTD, Chemicon International, CA, USA). For ultrastructural analysis, dissected tissue blocks from 16 animals (4 animals per group) were fixed by immersion in 2% glutaraldehyde (4 hours at 4°C), postfixed in 1% OsO4 in PB for 1 hour, dehydrated in graded ethanol (100% for 2 hours at 4°C), and embedded in Epon 812. For transmission electron microscopy, 1 μm thick sections were cut, stained with uranyl acetate and lead citrate, and observed on a Zeiss EM 1110 electron microscope. Sections treated as above, but in the absence of the primary or secondary antibody, were used as control. To label injured neurons, the Dark Neuron stain method was used as described by others [15].

Transmission Electron Microscopy
For ultrastructural analysis, dissected tissue blocks from 16 animals (4 animals per group) were fixed by immersion in 2% glutaraldehyde in 0.1 M phosphate buffer (PB), pH 7.4, for 2 hours at 4°C. After rinsing in 0.1 M PB, the specimens were postfixed in 1% OsO4 in PB for 1 hour, dehydrated in graded concentrations of acetone and embedded in a mixture of Epon and Araldite (Electron Microscopic Sciences, Fort Washington, PA, USA). Semithin sections, at 1 μm thickness, were stained with toluidine blue. Ultrathin sections were cut at 70 nm thickness on an Ultracut-E ultramicrotome (Reichert-Jung), stained with lead citrate and uranyl acetate and observed on a Zeiss EM 10 electron microscope (Zeiss, Oberkochen, Germany).
Vascular Casts
Vascular casts were obtained as described in our previous work [16]. For the vascular cast preparation, two rats per group were anesthetized with sodium pentobarbital and thoracotomized. A catheter was introduced into the arch of the aorta through the left ventricle and the right atrium was opened. The circulatory system was rinsed with phosphate-buffered saline and a freshly prepared solution of a low-viscosity resin, Mercox CL-2B (Dainippon Ink & Chemicals, Tokyo, Japan) was injected along the same route. Maceration of the brain tissue was performed using a 10% solution of potassium hydroxide until only the resin casts of blood vessels remained. The specimens were rinsed by several passages of distilled water, and then frozen. The casts were freeze-dried (Modulyo, Edwards-Kniese, Marburg, Germany), fixed to stubs with colloidal silver, sputter-coated with gold (MED 010, Balzers), and examined under a SEM (DSM 690, Zeiss).

MicroArrays
mRNA was isolated from the cingulate cortex of 4 rats in each group. Tissue was selected in supragranular and subgranular layers. The mRNA was used as the template for generating a cDNA library. cRNA labeled with dUDP-biotin (Enzo Roche Molecular Biochemicals, Mannheim, Germany) has been retro-transcribed. dUDP-biotin-cRNA was purified by and hybridized on different OligoGEArrays containing probes specific for genes implicated in signal transduction pathways (95 genes, 2 blanks, 6 negative controls, 9 positive controls) (Oligo GEArray Rat Apoptosis Microarray ORN-014, SuperArray Bioscience Corporation), apoptosis (96 genes, 1 blanks, 6 negative controls, 9 positive controls) (Oligo GEArray Rat Apoptosis Microarray ORN-012, SuperArray Bioscience Corporation) and DNA-damage response (113 genes, 2 blanks, 6 negative controls, 9 positive controls) (Oligo GEArray Rat Apoptosis Microarray ORN-029, SuperArray Bioscience Corporation), apoptosis (96 genes, 2 blanks, 6 negative controls, 9 positive controls) (Oligo GEArray Rat Apoptosis Microarray ORN-012, SuperArray Bioscience Corporation) and DNA-damage response (113 genes, 2 blanks, 6 negative controls, 9 positive controls) (Oligo GEArray Rat Apoptosis Microarray ORN-029, SuperArray Bioscience Corporation). The hybridization pattern was revealed by CDP-Star® substrate fluorescence using Chemiluminescent Detection Kit and recorded on a X-ray film.

The film was acquired using a desktop scanner and saved as a grayscale TIFF file. Data from array were analyzed using GEArray Analyzer software (Superarray Bioscience Corporation).

Statistical evaluation
For MRI data analysis, the difference between T2W and rCBV values obtained in control vs pilocarpine-treated rats was evaluated with one-way analysis of variance (ANOVA), followed by the LSD post-hoc test, setting the significance at p<0.05. The same statistical approach was used for immunohistochemical and vascular cast analysis.

For microarrays analysis, genes with values <0.20 were considered to be “not expressed”. Differences of expression of 1.5-fold or more between the lineages under analysis were considered significant. A cross-validation between different arrays is provided.

RESULTS
Cerebral blood volume and rTTP
Compared to controls, animals pilocarpine-treated showed a specific laminar pattern in rCBV distribution, with increased values in the more superficial layers (control: 0.32±0.13 a.u.; epileptic: 0.63±0.16 a.u.; p<.0001) and decreased in rCBV values in the deeper layers (control: 0.21±0.07 a.u.; epileptic: 0.11±0.05 a.u.; p<.0001). The decreased rCBV values indicate an ischemic area (Fig. 1).

rTTP was used as a surrogate measure of mean transit time [14], to evaluate blood flow alterations in epileptic vs control brains. rTTP maps show a generalized increase (about 0.4 secs) in blood flow rate in epileptic vs control brains, with a spatially distinct pattern (Fig. 2). Maximal signal drop values, obtained by first passage technique, resemble those obtained by images acquired at the equilibrium distribution of USPIO (all rCBV maps reported in Fig. 2).

Vascular cast
Vascular changes were evident in animals injected by pilocarpine versus controls, both in the principal vessels (Fig. 3A-B and D-E) and in the microcirculation (vessels diameter <100 μm, Fig. 3C, F). Principal arteries and veins were flattened and veins were 20% larger in diameter than in control animals (diameter: control: 149.03 μm±4.5; exp: 207.54 μm±6.1; p<0.02). Microvessels were clearly altered in the deeper layers where flattened vessels were observed (control: 7.67 μm±0.9; exp: 3.51 μm±0.2; p<.0001).

Endothelial permeability, reactive gliosis and neuronal death
Agrin expression was dramatically increased 2 h after SE in the Wistar rat cerebral cortex (Fig. 4; densitometric evaluation: control: 2.13±0.2 a.u.; 2 h: 5.27±1.1 a.u.; p<.0001). This increase was more evident in the supergranular than in the subgranular layers (Fig. 4). Reactive astrogliosis paralleled agrin expression (Fig. 4; densitometric evaluation: control: 4.22±0.9 a.u.; exp: 6.56±1.7 a.u.; p<.001), as well as dark neuron staining (cell number/41,400 μm² evaluation: supragranular layers: control: 0±0; 2 h: 0±0; 24 h: 3.2±1.4. Subgranular layers: control: 0±0; 2 h: 12.2±2.8; 24 h: 68.1±5.3; p<.0001).

Ultrastructural analysis
The ultrastructural analysis demonstrated the histological and cytological alterations occurring after pilocarpine-induced SE (Fig. 5). Two hours after SE supragranular layers appeared almost normal, with only a few morphological indicators of histopathology (i.e.: the perivascular edema in Fig. 5A), whereas subgranular layers at this early time-point exhibited a severe parenchymal changes and vacuolisation (Fig. 5B). This pattern were changed 24 h post-SE, when histopathological alterations were apparent in both the superficial (Fig. 5A') and inferior (Fig. 5B') layers.

Gene arrays analysis
Gene array data were analyzed comparing mRNA expression of 2 h versus control and 24 h versus 2 h. We cross-validated gene array by the mean of correlation index of 12 genes present in two or more different arrays (signal transduction vs apoptosis: r = 0.956; apoptosis vs DNA-damage: r = 0.986; signal transduction vs DNA-damage: r = 0.999).

In the subgranular layers, Bel2/bel, Bel10 expression was upregulated and Bax expression was downregulated. DNA-repairing (upregulation of PCNA, Xrcl1, Xrcl3), was noted 2 h after SE (Table 1). This activation was paralleled by neuroprotective mechanisms (upregulation of NGEF) (Table 1). In these early stages of cortical alteration, in the supragranular layers a different and more complex gene-expression pattern was evident. Signals of cellular stress (upregulation of Hhexokinase2 and Ubiquitin conjugating enzyme), paralleled by a downregulation of heat shock protein 27 were observed in these layers at 24 h compared to 2 h after SE levels (Table 1).

Twenty-four h after SE, in the subgranular layers, several genes involved in the DNA-repairing processes (Erec1, Rad23a, Rad23b,
Ddit3, Atrx, Mre11a, Mgmt, Tdg, Rad23a, as well as neuroprotective (NGF) and anti-apoptotic genes (Bcl2, Birc7, Bnip3, Beclin1) were upregulated (Table 1). Conversely, in the supragranular layers, a more composite pattern was found. Evidences of apoptotic cell death, mediated by an upregulation of Bax and a downregulation of Birc1, as well as DNA-repairing genes (Ercc1, Trex1, Ercc3, Ddit3, PCNA) and inflammatory processes (IL-4R, Chemokine ligand 2) were noted (Table 1) 24 h after SE. An indication of protein-degradation alteration (upregulation of Cathepsin D, Hsp27, Ubiquitin conjugating enzymes) was also evident (Table 1).

**DISCUSSION**

The results of this study indicate that a two different patterns of vascular and neurodegenerative phenomena can be identified in the Wistar rat neocortex after pilocarpine-induced SE. First, in the subgranular layers (cortical layer V and VI), 2 h after SE-onset, ischemic (anoxic) alterations are followed by necrotic cell death and edema. Second, the supragranular area (cortical layers II/III), is characterized by hyperperfusion and delayed apoptotic cell death. Thus, prolonged SE-induced brain damage appears to involve both ischemic and excitotoxic mechanisms.

![Figure 2. Peak enhancement in control (A, C) and pilocarpine-treated animals (B, D). Compared to controls, in rats during SE, sub-granular layers showed a decreased contrast medium peak concentration (D, blue dots), indicating a relative ischemic core, whereas supra-granular layers were characterized by hyperemia (D, red dots). Overlay rTTP maps on source images showing a generalized increase in blood flow rate in pilocarpine-treated (B) versus control brain (A). These alterations in the cerebral cortex of pilocarpine-treated rats present a specific spatial distribution (supra-(red arrow) versus sub-granular layers (blue arrow)). doi:10.1371/journal.pone.0001105.g002](#)
Vascular alterations

Cerebral blood flow and cerebral metabolic rate alterations during prolonged epileptic seizures in rats were extensively studied in the 70’s [17,18] less frequently recently [19,20]. In early studies, a marked increase in CBF substantially exceeding the increase in cerebral oxygen consumption, was noted within a few seconds of the onset of a generalized epileptic seizure and lasted for several hours [18]. In this study, we report for the first time that vascular alterations are not homogeneous in the cerebral cortex after 2 h pilocarpine-induced SE. Vascular cast and MRI observations of superficial arteries and veins in the supragranular layers indicate increased blood volume, whereas in the deeper layers of the cortex (subgranular layers), microcirculation studies showed a reduced diameter, resulting in an ischemic core. Furthermore, our TTP study confirmed the CBF alterations previously reported [17], demonstrating a specific spatial distribution (supra- versus sub-granular layers). Histopathological analysis, based on Nissl staining and agrin detection strongly suggest the vascular alteration and endothelial changes due to pilocarpine-induced SE. In facts, agrin is supposed to mediated the formation and maintenance of cerebral microvascular impermeability [21]. As a component of the brain microvascular basal lamina, agrin may impact pathological processes in which the vascular permeability barrier is defective [21]. The correlation between vascular alterations and neurodegeneration have been obtained by dark neuron (DN) staining, a silver-impregnation techniques used to label the cell body and dendritic processes of degenerating neurons. It has long been recognized that many dying and dead neurons, seen in a variety of diseases, exhibit an increased affinity for various silver stains (argyrophilia) [22]. This technique has been used in comparison with the anionic fluorochrome Fluoro Jade B (FJB) in pilocarpine-treated rats [23]; a significant increased percentage of silver-stained profiles at earlier time points (<24 h) compared with Fluoro-Jade positive cells stained at the same time in adjacent brain sections was reported [23].

Anoxic/hypoxic damage and neurodegeneration

Our results indicate that SE-induced neurodegeneration in the cerebral cortex of Wistar rats involves vascular insult, that presumably results in an alteration of the intra- and extra-cellular ionic homeostasis due to release of excitotoxic amino acids (e.g: glutamate) in the extracellular compartment, with a consequent cellular integrity loss and the presence of initial necrotic phases [24]. One of the most common consequences of ischemic insult is oxygen free radical-accumulation, which causes extensive damage [24,25]. In the ischemic area, necrotic mechanisms are frequently detected as well as expression of anti-excitotoxic, anti-inflammatory and anti-apoptotic mechanisms [24]. The efficacy of such protective mechanisms may be dependent on the severity of the insult.

Our results indicate that the subgranular layers 2 h after SE are morphologically characterized by a massive necrotic degeneration and tissue alteration, while the transcriptional profile reveals an activation of anti-apoptotic (Bcl2 and Bcl10 up-regulation paralleled by a downregulation of Bax), neuroregenerative (NGF and egr-1 upregulation) and DNA-reparative (PCNA, Xrcc1 and Xrcc3 up-regulation) pattern. Members of the Bcl2 family are thought to be proteins involved in regulation of neurodegenerative phenomena. The expression of Bcl2 has been found to inhibit apoptosis after various types of neuronal injuries [26], as well as to
inhibit necrosis [27]. In order to inhibit apoptosis, Bcl2 forms a heterodimer with Bax [28]. Bax can also dimerise with itself, and appears to induce apoptosis when overproduced [29]. It has been proposed that the balance of Bax/Bcl2 in a cell is a critical factor determining whether a cell will undergo apoptosis [30].

Several studies reported a neuroprotective and reparative role of NGF in neurodegenerative diseases [31]. In particular, the increased production of NGF and other trophic factors in CNS during neurodegenerative disease may suppress inflammation by switching the immune response to an anti-inflammatory, suppressive mode [32].

Ischemia-related DNA alterations have been extensively reported [24]; activation of DNA-repairing mechanisms (e.g.: Xrcc family) has been considered as an extreme response also in other experimental model of epilepsy [33].

Excitotoxic cell death

The supragranular layers exhibited a delayed programmed cell death temporally and spatially distinct from the fast neuronal and glial cell death (pannecrosis, minute to hours) in the deeper layers [34]. In fact, 24 h after SE, the supragranular layers exhibited an upregulation of Bax, suggesting a pro-apoptotic switch. We also found an upregulation of DNA-repairing enzymes (for example, PCNA, Ercc1, Ercc3, Trex1) and neurodegeneration-associated proteins (e.g.: fibronectin1, Igf).

The neuroprotective molecule Hsp 27 is constitutively expressed in mammalian rat brain [35] and is upregulated after ischemic insults [36] or lesions [37]. Interestingly, Plumier and colleagues [38] demonstrated that this gene is increased 2.5 fold after kainic acid-induced SE. In agreement with these data, we have found a moderate expression of Hsp27 both in supragranular and
Table 1. MicroArray analysis of cortical sample from supragranular vs subgranular layers at different time-points.

|                             | 2 h versus control | 24 h versus 2 h |
|-----------------------------|--------------------|-----------------|
|                             | Upregulation       | Upregulation    |
| Supragranular layers (II/III) |                    |                 |
| c-fos (9.6)                 | FBJ murine osteosarcoma viral oncogene homolog | Apex 1 (689.5) Apurinic/apyrimidinic endonuclease 1 |
| Ube 2d3 (2.85)              | Ubiquitin-conjugating enzyme E2D 3 | Bax (2.0) (2.54) Bcl-2-associated X protein |
| Ube 2l (2.0)                | Ubiquitin-conjugating enzyme E2l | Cib 1 (86.8) Calcium and integrin binding protein 1 |
| TNF R sf1a (5.25)          | Tumor necrosis factor receptor 1a | Becn1 (3.74) Beclin 1 |
| Cdkn2a (33.7)               | Cyclin dependent kinase inhibitor 2a | Ube2i (2.7) Ubiquitin-conjugating enzyme E2l |
| Cdk 2 (2.1)                 | Cyclin dependent kinase 2 | Hspb1 (2.9) Heat Shock 27 KDa Protein |
| Hk2 (5.5)                   | Hexokinase 2 | Ctsd (2.5) Cathepsin D |
| Igf bp3 (2.09)              | Insulin-like growth factor binding protein 3 | Fn1 (5.2) Fibronectin 1 |
|                             | IL-4 R (2.2) Interleukin 4 receptor |
|                             | Cdc2 (7.5) Chemokine (C-C motif) ligand 2 |
|                             | a2m (9.0) Alpha 2 microglobulin |
|                             | bmp4 (5.7) Bone morphogenetic protein 4 |
|                             | Igfbp3 (2.26) Insulin-like growth factor, bin prot 3 |
|                             | Csn2 (4.6) Casein beta |
|                             | Cdk inhib 1b (4.7) Cyclin dependent kinase inhibitor 1B |
|                             | PCNA (3.09) Proliferating cell nuclear antigen |
|                             | Ddit3 (2.97) DNA-damage inducible transcript 3 |
|                             | Smc11 (4.75) Structural maintenance chromosomes |
|                             | Ercc1 (5.8) Similar to excision repair |
|                             | Mre11a (3.0) Meiogenic recombination 11 homolog A |
|                             | Mgmt (2.0) O-6-methylguanine-DNA methyltransferase |
|                             | Tdg (13.8) Thymidine-DNA glycosylase |
|                             | Atf2 (4.6) Activating transcription factor 2 |
|                             | Rad 23 a (0.5) Similar to UV excision repair protein RAD23 homolog A |
|                             | Pnkp (4.56) Similar to polynucleotide kinase 3-phosphate |
| Downregulation              | Downregulation     |
| Hsb1 (0.48)                 | Heat shock 27 KDa protein | TNFsF13 (0.59) Tumor necrosis factor ligand 13 |
| Bax (0.36)                  | Bcl-2-associated X protein | Birc1 (0.25) Baculoviral IAP repeat-containing 1b |
| Csf2 (0.48)                 | Colony stimulating factor 2 | C-fos (0.236) FBJ murine osteosarcoma viral oncogene homolog |
| Bmp4 (0.288)                | Bone morphogenetic rotein 4 | E24 (0.42) Similar to E24 |
| Csn2 (0.418)                | Casein beta         |
| Rad 23 (0.22)               | Similar to excision repair protein RAD23 homolog A |
| IL-2 (0.26)                 | Interleukin 2       |
| Hoxb1 (0.160)               |                        |
| Subgranular layers (V/VI)   | Upregulation        | Upregulation    |
| c-fos (9.6)                 | Early growth response 1 | Ercc1 (2.0) Similar to excision repair cross-complementing rodent repair deficiency, complgroup1 |
| V-Jun (3.7)                 |                        | Rad23a (3.6) Similar to UV excision repair protein RAD23 homolog A |
| NGF (2.0)                   | Nerve growth factor | Rad23b (8.0) Similar to UV excision repair protein RAD23 homolog A |
| TNFR1a (2.9)                | Tumor necrosis factor receptor 1a | Csgp6 (7.45) Caspase 6 |
| Bcl 10 (16.4)               | B-cell leukemia/lymphoma 10 | Cib 1 (4.8) Calcium and integrin binding protein 1 |
| Bcl 2 a 1 (5.6)             | B-cell leukemia/lymphoma 2 related protein A1 | Dclre1a (4.5) Similar to SNM1 protein |
subgranular layers of control animals, with a dramatic upregulation 24 h after SE [7], probably as a response of the brain against neurodegeneration and misfolded protein accumulation.

**Summary**

Taken together, the data obtained in this study, point out that a) the cerebral cortex responds in different ways to pilocarpine stimulation, with ischemic, necrotic processes in the subgranular layers and presumably excitotoxic, apoptotic cell death in the supragranular layers; b) these two degenerative pathways take place at different time points, with a delayed degeneration in the supragranular layers. Furthermore, the present findings, pointing out the different pathways involved in the degenerative alterations following SE and the different time-window of the occurrence of such mechanisms, could prompt future studies for putative treatments that can prevent or limiting epileptogenic changes following SE or repeated seizures.

**ACKNOWLEDGMENTS**

The Authors would like to thank Prof. Marina Bentivoglio for the fruitful suggestions during the experimental work and the precious help during the preparation of the manuscript.

**Author Contributions**

Conceived and designed the experiments: PF AS. Performed the experiments: PF MG DB PB EN PM FM. Analyzed the data: PF PF EN PM AS. Contributed reagents/materials/analysis tools: PF MG DB PB FM. Wrote the paper: PF.

**REFERENCES**

1. Dirnagl U, Ladeoca C, Moskowitz MA (1999) Pathobiology of ischaemic stroke: an integrated view. Trends Neurosci 22: 391–397.
2. Meldrum BS, Bruton CJ (1992) Epilepsy. In: Adams JH, Duchen LW, eds (1992) Greenfield's Neuropathology. New York: Oxford University Press. pp 1246–1283.
3. Nadler JV, White WF, Vaca KW, Perry BW, Cotman CW (1978) Biochemical correlates of transmission mediated by glutamate and aspartate. J Neurochem 31: 147–155.
4. Schwoeb JE, Fuller T, Price JL, Olney JW (1980) Widespread patterns of neuronal damage following systemic or intracerebral injections of kainic acid: a histological study. Neuroscience 5: 991–1014.
5. Sloviter RS, Damiano BP (1981) On the relationship between kainic acid-induced epileptiform activity and hippocampal neuronal damage. Neuropharmacology 20: 1003–1011.
6. McIntyre DC, Nathanson D, Edson N (1982) A new model of partial status epilepticus based on kindling. Brain Res 250: 53–63.
7. Fabene PF, Marzola P, Sharbati A, Bentivoglio M (2003) Magnetic resonance imaging of changes elicited by status epilepticus in the rat brain: diffusion-weighted and T2-weighted images, regional blood volume maps, and direct correlation with tissue and cell damage. Neuroimage 18: 375–389.
8. Sloviter RS (2005) The neurobiology of temporal lobe epilepsy: too much information, not enough knowledge. C R Biol 328: 143–153.
9. Leroy C, Roch C, Koning E, Namer IJ, Nehlig A (2003) In the lithium-pilocarpine model of epilepsy, brain lesions are not linked to changes in blood-brain barrier permeability: an autoradiographic study in adult and developing rats. Exp Neurol 182: 361–372.
10. Racine RJ (1972) Modification of seizure activity by electrical stimulation. II. Motor seizure. Electroencephalogr Clin Neurophysiol 32: 261–294.
11. Fabene PF, Sharbati A (2004) In vivo MRI in different models of experimental epilepsy. Curr Drug Targets 5: 629–636.
weighted, conventional T2-weighted, and gadolinium-enhanced T1-weighted MR images in rats with experimental autoimmune encephalomyelitis. AJNR 20: 223–227.

13. Fabene PF, Weiczner R, Marzola P, Nicolato E, Calderan L, et al. (2006) Structural and functional MRI following 4-aminopyridine-induced seizures: a comparative imaging and anatomical study. Neurobiol Dis 21: 80–89.

14. Yamada K, Wu O, Gonzalez RG, Bakker D, Ostergaard L, et al. (2002) Magnetic resonance perfusion-weighted imaging of acute cerebral infarction: effect of the calculation methods and underlying vasculopathy. Stroke 33: 97–94.

15. van den Pol AN, Gallay F (1990) Trauma-induced Golgi-like staining of neurons: a new approach to neuronal organization and response to injury. J Comp Neurol 296: 654–673.

16. Shabani A, Pietra C, Ballassarri AM, Guerrini U, Ziviani L, et al. (1996) The microvascular system in ischemic cortical lesions. Acta Neuropathol (Berl) 92: 56–63.

17. Meldrum BS, Brierley JB (1973) Prolonged epileptic seizures in primates. Ischemic cell change and its relation to ictal physiological events. Arch Neurol 28: 10–17.

18. Meldrum BS, Nilsson B (1976) Cerebral blood flow and metabolic rate early and late in prolonged epileptic seizures induced in rats by bicuculline. Brain 99: 532–547.

19. Nersesyan H, Herman P, Erdogan E, Hyder F, Blumenfeld H (2004) Relative changes in cerebral blood flow and neuronal activity in local microdomains during generalized seizures. J Cereb Blood Flow Metab 24: 1057–1064.

20. Hirase H, Crespo J, Buzsaki G (2004) Capillary level imaging of local cerebral blood flow in bicuculline-induced epileptic foci. Neuroscience 128: 209–216.

21. Barber AJ, Leth F (1997) Agrin accumulates in the brain microvascular basal lamina during development of the blood-brain barrier. Dev Dyn 208: 62–74.

22. Gallay F, Goldner FH, Zohay G, Wolff JR (1990) Golgi-like demonstration of “dark” neurons with an argyrophil III method for experimental neuropathology. Acta Neuropathol (Berl) 79: 620–628.

23. Anguelova E, Smirnova T (2000) Differential expression of small heat shock protein 10 in human neurons and glial cells in culture. J Neurosci Res 41: 805–813.

24. Valentim LM, Geyer AB, Tavares A, Cimarosti H, Worm PV, et al. (2001) Effects of global cerebral ischemia and preconditioning on heat shock protein 27 immunoreactivity and phosphorylation in rat hippocampus. Neuroscience 107: 43–49.

25. Lo EH, Dalkara T, Moskowitz MA (2000) Mechanisms, challenges and opportunities in stroke. Nat Rev Neurosci 4: 399–415.

26. Fan XM, Otzai ZN, Korsemeyer SJ (1994) BH1 and BH2 domains of Bcl-2 are required for inhibition of apoptosis and heterodimerization with Bax. Nature 360: 321–323.

27. Kane DJ, Ord T, Anton R, Bredesen DE (1995) Expression of bcl-2 inhibits necrotic neuronal cell death. J Neurosci Res 40: 269–275.

28. Otzai ZN, Milliman CL, Korsemeyer SJ (1993) Bcl-2 heterodimerizes in vivo with a conserved homolog, Bax, that accelerates programmed cell death. Cell 74: 609–619.

29. Korsemeyer SJ, Shutter JL, Veis DJ, Merry DE, Otzai ZN (1993) Bcl-2/Bax: a thread that regulates an anti-oxidant pathway and cell death. Semin Cancer Biol 4: 327–332.

30. Sofroniew MV, Hawe CL, Mohley WC (2001) Nerve growth factor signaling, neuroprotection, and neural repair. Annu Rev Neurosci 24: 1217–1261.

31. Sofroniew MV, Caplin R, De Koninck Y (2000) Differential progression of Dark Neuron and Fluoro-Jade labelling in the rat hippocampus following pilocarpine-induced status epilepticus. Brain Res 739: 244–250.

32. Anguelova E, Smirnova T (2000) Differential expression of small heat shock protein 10 in human neurons and glial cells in culture. J Neurosci Res 41: 805–813.

33. Valentim LM, Geyer AB, Tavares A, Cimarosti H, Worm PV, et al. (2001) Effects of global cerebral ischemia and preconditioning on heat shock protein 27 immunoreactivity and phosphorylation in rat hippocampus. Neuroscience 107: 43–49.

34. Anguelova E, Smirnova T (2000) Differential expression of small heat shock protein 27 in the rat hippocampus and septum after fimbria-fornix lesion. Neurosci Lett 280: 99–102.

35. Meldrum BS, Brierley JB (1973) Prolonged epileptic seizures in primates. Ischemic cell change and its relation to ictal physiological events. Arch Neurol 28: 10–17.

36. Meldrum BS, Nilsson B (1976) Cerebral blood flow and metabolic rate early and late in prolonged epileptic seizures induced in rats by bicuculline. Brain 99: 532–547.

37. Shabani A, Pietra C, Ballassarri AM, Guerrini U, Ziviani L, et al. (1996) The microvascular system in ischemic cortical lesions. Acta Neuropathol (Berl) 92: 56–63.

38. Meldrum BS, Brierley JB (1973) Prolonged epileptic seizures in primates. Ischemic cell change and its relation to ictal physiological events. Arch Neurol 28: 10–17.