

Mice Lacking Functional Fas Death Receptors Are Protected from Kainic Acid-Induced Apoptosis in the Hippocampus

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Abstract The Fas receptor (FasR)/Fas ligand (FasL) system plays a significant role in the process of neuronal loss in neurological disorders. Thus, in the present study, we used a real-time PCR array focused apoptosis (Mouse Apoptosis RT² PCR Array) to study the role of the Fas pathway in the apoptotic process that occurs in a kainic acid (KA) mice experimental model. In fact, significant changes in the transcriptional activity of a total of 23 genes were found in the hippocampus of wild-type C57BL/6 mice after 12 h of KA treatment compared to untreated mice. Among the up-regulated genes, we found key factors involved in the extrinsic apoptotic pathway, such as *tnf*, *fas* and *fasL*, and also in caspase genes (*caspase-4*, *caspase-8* and *caspase-3*). To discern the importance of the FasR/FasL pathway, mice lacking the functional Fas death receptor (*lpr*) were also treated with KA. After 24 h of neurotoxin treatment, *lpr* mice exhibited a reduced number of apoptotic positive cells, determined by the

terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) method in different regions of the hippocampus, when compared to wild-type mice. In addition, treatment of *lpr* mice with KA did not produce significant changes in the transcriptional activity of genes related to apoptosis in the hippocampus, either in the *fas* and *fas ligand* genes or in *caspase-4* and *caspase-8* and the executioner caspase-3 genes, as occurred in wild-type C57BL/6 mice. Thus, these data provide direct evidence that Fas signalling plays a key role in the induction of apoptosis in the hippocampus following KA treatment, making the inhibition of the death receptor pathway a potentially suitable target for excitotoxicity neuroprotection in neurological conditions such as epilepsy.

Keywords Kainic acid · Apoptosis · FAS · Neuroprotection · Microglia · Caspase-3

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Introduction

It is widely known that the excitotoxic process is a contributing factor to acute neuronal damage in most chronic neurodegenerative diseases, such as Alzheimer's disease, Parkinson's disease and Huntington's disease [1–3]. Previous reports have demonstrated that the administration of kainic acid (KA) in rodents, an analogue of the excitotoxin glutamate, produces epileptic seizures [4–9]. The greatest abundance of kainate receptors is present in the hippocampal CA3 region, and their activation results in neuronal loss of selective populations in the hippocampus and other brain structures. Moreover, numerous studies have demonstrated that neuronal loss observed in this excitotoxic model involves, at least in part, apoptotic cell death [10, 11]. Likewise, KA can stimulate CA3 neurons directly through stimulation of their own KA receptors and also indirectly by favouring glutamate release secondary to KA stimulation of mossy fibres [4].

One important point is the characterisation of the mechanism involved in KA-induced neurodegeneration. Thus, a large number of studies have been conducted to identify the potential biochemical pathways involved in KA-induced apoptosis, such as oxidative stress, cell cycle re-entry and calpain/cdk5 activation [8, 10–16]. Programmed cell death is primarily mediated by the extrinsic or death receptor pathway, and the intrinsic or mitochondrial pathway, which converge to activate mitochondria [17, 18]. This constitutes a key point because it favours the release of some proteins such as cytochrome c, Smac/Diablo and Omi/HrtA2 into the cytosol, which facilitates the activation of common executioner caspases (caspase-3/7) with the final cleavage of downstream targets that include DNA repair enzymes [19].

It has been previously shown that the extrinsic apoptotic pathway induces apoptosis in lymphoid cells and in neuronal cells deprived of critical survival factors and also in models of excitotoxicity [20]. The Fas receptor is known to induce apoptosis by binding to FasL. Thus, the Fas ligand (FasL) and TNF-related apoptosis-inducing ligand (TRAIL) are up-regulated following experimental KA administration to rodents [21–23]. In addition, previous studies have shown that the binding of the Fas ligand to FasR triggers the formation of a death-inducing signalling complex (DISC), which consists of FasL, FasR, the adapter protein FADD (Fas-associated death domain protein), and the TNFR1-associated death domain protein (TRADD) to induce the activation of caspase-8, a protease which then activates downstream effector caspase-3, which in turn is responsible for apoptosis. Thus, both extrinsic and intrinsic apoptotic pathways converge to activate common executioner caspases (caspase-3/7) with the final cleavage of downstream targets. Inhibition of Fas-mediated apoptosis is regulated by the FLICE-inhibitory protein (FLIP), the structural homologue of procaspase-8 [24–29].

lpr mice have alterations in the *Fas* receptor gene due to the insertion of a retrotransposon that causes disruption in its transcription [30]. Attenuation of the Fas signalling system has been related to neurological damage protection in various experimental models of pathophysiological diseases such as ischemia and multiple sclerosis [27, 29–33]. More precisely, *lpr* mice were protected from the neurotoxicity mediated by the neurotoxin MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine), which causes permanent symptoms of Parkinson's disease [34, 35]. Interestingly, in post-mortem brains of Parkinson's patients, the levels of Fas are increased in the nigrostriatal region [36]. In addition, deletions of the Fas receptor reduced lesion size and improved brain function after a traumatic brain injury, confirming the role of these signalling pathways in the pathogenesis of delayed brain damage [7, 25]. All these data raised the possibility that Fas signalling activated in different pathologies may render neurons vulnerable to dying [31, 37].

Furthermore, recent studies have specifically exemplified the role of Fas in Alzheimer's disease (AD) neurodegeneration [28, 38–40]. It has been suggested that β -amyloid can induce neuronal death via processes of apoptosis [40, 41]. Similarly, it has been demonstrated that neurons in the AD brain and neuronal cultures treated with beta-amyloid show a significant increase in Fas ligand expression and immunoreactivity [42]. Moreover, cortical cultures of *lpr* mice that carry mutations leading to a loss of function of Fas genes are protected from beta-amyloid toxicity. These results, therefore, lend support to the involvement of this pathway in AD apoptosis.

Given the lack of direct evidence linking FasR with the intrinsic apoptotic pathway, we sought to clarify the role of apoptosis mediated by Fas signalling after KA treatment. The present study examined the impact of KA administration in C57BL/6 Fas-deficient (*lpr*) mice, which exhibit low levels of Fas receptor expression [43, 44]. To investigate whether FasL signalling is also relevant to the neurotoxic process mediated by KA, we analysed the hippocampal apoptotic cell death after intraperitoneal KA administration in Fas receptor-deficient mice (*lpr* mice). Moreover, a study of apoptotic gene expression was performed in the hippocampus and here we demonstrated that loss of Fas receptor activity (*lpr* mice) is neuroprotective against excitotoxicity.

Material and Methods

Animals

Wild-type C57BL/6 mice were obtained from Harlan Laboratories or the Jackson Laboratory. Fas-deficient *lpr* mice (C57BL/6 background) were obtained from Jackson Laboratory (B6.MRL-FasLpr/J, stock number 000482). All mice

were housed at the Centro Biología Molecular *Severo Ochoa* in Madrid. Mice were housed four per cage with food and water available ad libitum and maintained in a temperature-controlled environment on a 12-h light/12-h dark cycle with light onset at 7 am. All experiments were performed under a protocol approved by the CSIC ethical committee.

Kainic Acid Treatment and Sample Preparation

A total of 40 mice were used for the present study. Six-week-old mice were treated with a single intraperitoneal (i.p.) dose (30 mg/kg) of KA (Sigma-Aldrich, St. Louis, MO, USA) or with saline solution for controls. For RNA analysis, 10 wild-type C57BL/6 mice and 10 *lpr* mice were used. Five mice from each group were used as controls and were treated with KA and killed after 12 h; the hippocampus was then excised and frozen. For immunohistochemistry and immunofluorescence experiments, we used 10 wild-type C57BL/6 mice and 10 *lpr* mice. Five from each group served as control and were injected with KA and killed after 24 h.

All mice were anaesthetised by injection (i.p.) of pentobarbital 80 mg/kg. After that, the mice were perfused with 40 g/L paraformaldehyde in 0.1 mol/L phosphate buffer (PB) and the brains were removed and post-fixed for 24 h. The brains were subsequently rinsed in the same solution with 300 g/L sucrose for 24 h and then frozen. Coronal sections of 30 μ m were obtained by a Cryostat (Leica Microsystems, Wetzlar, Germany).

RT² Profiler™ PCR Array

The Mouse Apoptosis RT² Profiler PCR Array (PAMM-012A, Biosciences, Frederick, MD, USA) profiles the expression of 84 genes involved in apoptosis together with five housekeeping genes. Controls for genomic DNA contamination and for the efficiency of the RT-PCR and PCR were also included in the array and were evaluated after each run. One microgram of total RNA was reverse transcribed in a final reaction mix of 20 μ L using the RT² First Strand Kit according to the manufacturer's instructions. For one 96-well plate of the PCR array, 1,100 μ L of PCR master mix containing 2 \times SuperArray RT² qPCR Master Mix and 102 μ L of diluted cDNA were prepared, and aliquots of 10 μ L were added to each well. The PCR was performed on the LightCycler 480 (Roche Applied Science, Mannheim, Germany), and universal cycling conditions (10 min at 95 °C, 15 s at 95 °C, 1 min at 60 °C for 40 cycles) were used. Expression of each gene was normalised to the mean Ct for five housekeeping genes in the PCR array (β -glucuronidase, hypoxanthine phosphoribosyltransferase, heat shock protein 90 α , glyceraldehyde-3-phosphate dehydrogenase and β -actin). Relative fold change in expression was calculated using the $\Delta\Delta$ Ct method, and the values were expressed as 2- $\Delta\Delta$ Ct.

The statistical calculation was based on the web-based program of RT² Profiler™ PCR Array Data Analysis. Significant changes in gene expression between KA-treated mice and untreated controls were assumed as a fold change ≥ 1.5 and ≤ 0.6 , with a *P* value < 0.05 . The experiments were repeated three times.

RNA Extraction and Real-Time Reverse Transcription-Polymerase Chain Reaction (qRT-PCR)

Total RNA was isolated from mouse hippocampus using TRIzol (Invitrogen, Carlsbad, CA, USA) followed by chloroform, according to the manufacturer's protocol. RNA concentration was measured using a NanoDrop™ 1000 Spectrophotometer (Thermo Scientific, MA, USA).

As a general procedure, 1 μ g of total RNA was reverse transcribed using a high-capacity cDNA reverse transcription kit (Applied Biosystems, Carlsbad, CA, USA). The same amounts of cDNA were subsequently used for quantitative real-time PCR using SYBR Green® PCR Master Mix and performed on the StepOnePlus™ Real-Time PCR System (Applied Biosystems, Carlsbad, CA, USA). All samples were run in triplicate, and expression values were normalised to the housekeeping β -actin in the same reaction. Relative normalised mRNA levels were calculated using the $\Delta\Delta$ Ct method. Primers used for quantitative real-time PCR are shown in Table 1. Gene-specific primers corresponding to the PCR targets on the PCR RT² array were designed using Primer Express® Software v2.0 (Applied Biosystems, Carlsbad, CA, USA).

Fluoro-Jade Staining

Neurodegeneration was assessed using Fluoro-Jade B (Chemicon Europe Ltd). Slides were rinsed with phosphate-buffered saline (PBS), followed by two washes in distilled water. Afterwards, slides were immersed in 0.6 g/L potassium permanganate (KMnO₄) for 15 min in the dark. Then, after two washes in distilled water, the slides were transferred to the staining solution containing 0.1 mL/L acetic acid and 0.004 mL/L of the fluorochrome Fluoro-Jade B for 30 min in the dark. Slides were rinsed in distilled water, dried, and then submerged directly into xylene and mounted in DPX medium. Slides were analysed using epifluorescence microscopy (Olympus BX61, Olympus, Barcelona, Spain). To determine the positive cell number for Fluoro-Jade B, we counted the cells in each area from serial sections of hippocampus from each mouse brain.

TUNEL Assay

DNA damage was assessed using terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) staining method. TUNEL staining was performed as described in the In situ

Table 1 Significant changes in transcriptional activity of genes included in the Mouse Apoptosis RT² Profiler PCR Array, PAMM-012A, and from the hippocampus of KA-treated C57/Bl6 wild-type mice, at 12 h. The administration of this neurotoxin caused significant changes in the expression of 23 genes: 17 genes resulted significantly up-regulated; the most significant up-regulation corresponded to the *tnf* gene that showed a

fold change of 15.95, and the lowest significant gene activation levels corresponded to *hells*, with a fold change of 1.5. Another six genes resulted down-regulated. Significant changes in gene expression between KA-treated mice and untreated controls were assumed as a fold change ≥ 1.5 and ≤ 0.6 , with a P value < 0.05 . The experiments were repeated three times

GenBank number	Gene symbol	Gene name	Fold change	P value
NM_013693	Tnfa	Tumour necrosis factor	15.95	0.0017
NM_013863	Bag3	Bcl2-associated athanogene 3	9.03	0.0002
NM_011609	Tnfr1	Tumour necrosis factor receptor superfamily, member 1a	6.51	0.0004
NM_007987	Fas	Fas (TNF receptor superfamily member 6)	3.53	0.0001
NM_008562	Mcl1	Myeloid cell leukaemia sequence 1	3.47	0.0001
NM_009805	Cflar	CASP8 and FADD-like apoptosis regulator	2.91	0.0003
NM_007609	Casp4	Caspase-4, apoptosis-related cysteine peptidase	2.21	0.0002
NM_010177	FasL	Fas ligand (TNF superfamily, member 6)	2.00	0.0017
NM_010736	LtBr	Lymphotoxin B receptor	1.99	0.0001
NM_009740	Bcl10	B cell leukaemia/lymphoma 10	1.89	0.0336
NM_009422	Traf2	Tnf receptor-associated factor 2	1.83	0.0001
NM_138606	Pim2	Proviral integration site 2	1.78	0.0002
NM_009810	Casp3	Caspase-3	1.78	0.0086
NM_030693	Atf5	Activating transcription factor 5	1.73	0.0001
NM_172858	Pak7	P21 protein (Cdc42/Rac)-activated kinase 7	1.62	0.0003
NM_009812	Casp8	Caspase-8	1.54	0.0002
NM_008234	Hells	Helicase, lymphoid specific	1.50	0.0214
NM_009950	Cradd	CASP2 and RIPK1 domain containing adaptor with death domain	-1.56	0.0001
NM_007702	Cidea	Cell death-inducing DNA fragmentation, α subunit-like effector A	-1.56	0.0089
NM_001163138	Card6	Caspase recruitment domain family, member 6	-1.61	0.0003
NM_173378	Trp53bp2	Transformation related protein 53 binding protein 2	-1.69	0.0008
NM_030152	Nol3	Nuclear protein 3 (apoptosis repressor with CARD domain)	-1.88	0.0007
NM_080637	Nme5	Non-metastatic cells 5	-2.34	0.0001

Cell Death Detection Kit, TMR red (Roche Molecular Biochemicals, IN, USA) with minor modifications. Sections were rinsed with PBS and immersed in a permeabilisation solution (0.1 % Triton X-100, 0.1 % sodium citrate) for 2 min at 4 °C. Then, slides were washed twice with PBS and incubated with TUNEL reaction mixture in a humidified atmosphere for 60 min at 37 °C in the dark. Afterwards, slides were rinsed three times with PBS and then incubated with 0.1 μ g/mL Hoechst 33258 (Sigma-Aldrich, St Louis, MO, USA) nuclear stain for 5 min in the dark. Following a final rinse with PBS, slides were mounted in Fluoromount medium (Sigma-Aldrich, St Louis, MO, USA) and analysed under a confocal fluorescence microscope (Leica TCS SP2 confocal microscope).

Immunohistochemistry

Free-floating coronal sections, 30 μ m thick, were rinsed in 0.1 mol/L PB pH 7.2 and treated with 5 mL/L H₂O₂ and 100 mL/L methanol in PBS. After that, they were pre-

incubated in a blocking solution (100 mL/L of foetal bovine serum (FBS), 2.5 g/L of bovine serum albumin and 0.2 mol/L of glycine in PBST, PBS with 5 mL/L of Triton X-100). Then, sections were incubated overnight (O/N) at 4 °C with different primary antibodies: rabbit anti-GFAP (1:2,000; Dako, Glostrup, Denmark) and rabbit anti-Iba1 (1:1,000; Wako, Osaka, Japan), and sequentially incubated for 2 h with Alexa Fluor 594 goat anti-rabbit antibody (1:500; Invitrogen, Eugene, OR, USA). Sections were counterstained with 0.1 μ g/mL Hoechst 33258 (Sigma-Aldrich, St Louis, MO, USA) nuclear stain for 5 min in the dark. Immediately, sections were rinsed with PBS and mounted onto gelatinised slides with Fluoromount medium (Sigma-Aldrich, St Louis, MO, USA). Stained sections were examined under a confocal fluorescence microscope (Leica TCS SP2 confocal microscope).

Statistical Analysis

All experiments were conducted in triplicate, and the results were expressed as mean values \pm SEM. Differences are

considered significant at $P < 0.05$. P values were determined by Student's t test.

Results

Identification of the Expression of Apoptotic Genes in the Hippocampus of Wild-Type Mice, After Treatment with Kainic Acid

The administration of KA at a dose of 30 mg/kg in mice favours the onset of seizures and a variety of behavioural changes that have been previously described by a number of laboratories [7–9, 45]. The *lpr* mice compared to the wild type did not show an improvement in the number of seizures.

To assess whether apoptosis-related genes showed changes in expression in KA-treated mice at 12 h, 84 genes were analysed using the real-time PCR array (Mouse Apoptosis RT² Profiler PCR Array). The results indicated that administration of this neurotoxin caused significant changes in the expression of 23 genes (Table 1). Among them, a total of 17 genes were significantly up-regulated, whereas the transcriptional activity of six genes was below normal levels. The most significant up-regulation corresponded to the *tnf*, which showed a fold change of 15, 95 with P value=0.0017 and the lowest significant gene activation levels corresponded to *Hells*, with a fold change of 1.5 with P value=0.0214. In relation to this observation, the exposure of the wild-type strain to KA toxicity significantly activated genes related to the Fas signalling pathway: *fas*, *fasl*, and *caspase-4*, *caspase-3* and *caspase-8*.

Evaluation of Kainic Acid-Induced Neurotoxicity in the Hippocampus of Fas-Deficient *lpr* Mice

The real-time PCR array results suggested an involvement of the Fas pathway in the apoptotic process occurring in the hippocampus of C57/Bl6 mice, so the next step was to evaluate the implication of this pathway in KA-induced neuronal death. Then, we decided to analyse the neurodegeneration induced in Fas-deficient *lpr* mice following KA injection. After 24 h of treatment with KA, the neuronal death in hippocampal areas was assessed using the Fluoro-Jade B technique. Interestingly, brain sections from *lpr* mice exhibited a significant stain reduction compared to the wild type, suggesting the existence of a neuroprotective effect, most probably due to the lack of a functional Fas/CD95 protein (Fig. 1). To clarify whether the identified dying cells were apoptotic, DNA integrity was assessed using TUNEL staining. A decrease in the cell apoptosis in *lpr* mice treated with KA compared to the wild type (WT) evidenced that the lack of

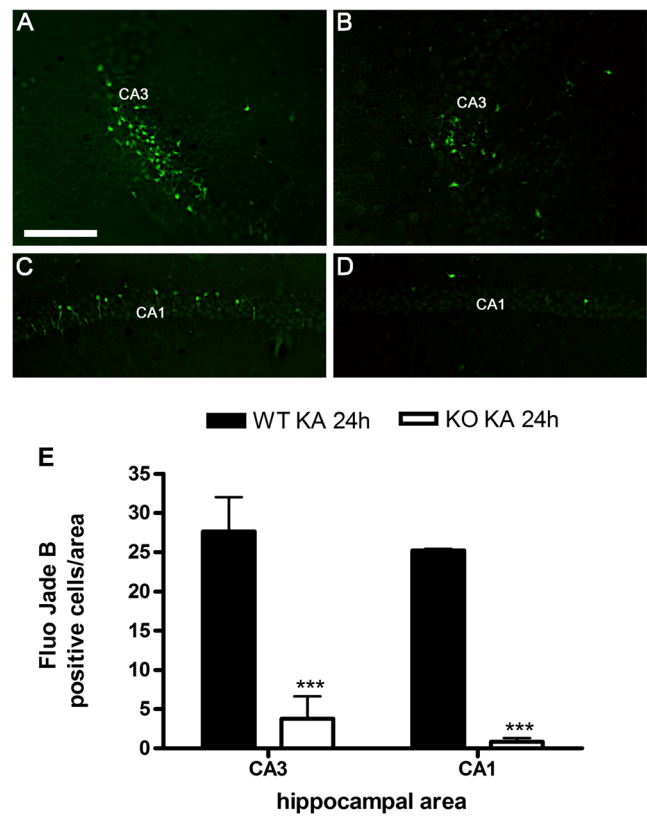


Fig. 1 Neurodegeneration observed by Fluoro-Jade B stain in the CA3 and CA1 regions of the hippocampus after 24 h of KA treatment. A decrease of labelled neurons is observed in the CA3 region of kainic acid (KA)-treated *lpr* mice versus wild-type (wt) mice (b vs a). A decrease of labelled neurons is observed in the CA1 region of kainic acid (KA)-treated *lpr* mice versus wild-type (wt) mice (d vs c). Scale bar 100 μ m. (e) A bar graph shows the quantification of positive neurons for Fluoro-Jade B labelling in the CA3 and CA1 areas of the hippocampus in wild-type and *lpr* mice. Each point is the mean \pm SEM of three independent experiments, determined in five pictures per experiment (***) $p < 0.001$; with respect to kainic acid-treated wild-type mice)

functionality of the Fas receptor plays an antiapoptotic role (Fig. 2).

Evaluation of Glial Activation in Fas-Deficient *lpr* Mice Hippocampus After Kainic Acid Treatment

In the literature, it is widely described that, in addition to neuronal death, the administration of KA produces a glial activation due to excitotoxicity [4, 5]. This glial reactivity is reflected in a change in the morphology of the astrocytes and microglia. In the case of astrocytes, these acquire a star shape because of the growth of their extensions. In the hippocampal brain areas of the KA-treated wild-type mice, a prominent astrogliosis was observed; thus, we detected an increase in GFAP immunoreactivity as well as shape alterations in astrocytes. The astrocyte activation mediated by KA was not abrogated in *lpr* mice because the pattern of GFAP immunoreactivity was similar to the wild type (Fig. 3). Microglia are a type

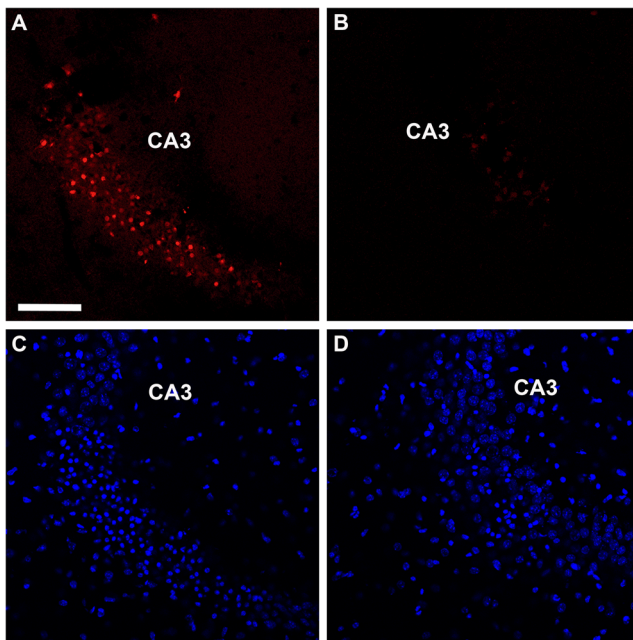


Fig. 2 TUNEL and Hoechst 33342 staining in the CA3 region of the hippocampus from kainic acid-treated wild-type mice (**a** and **c**) and kainic acid-treated *lpr* mice (**b** and **d**), after 24 h of treatment. Scale bar 100 μ m

of glial cell acting as the first and main form of active immune defence in the central nervous system, offering an extremely sensitive reaction when neural damage is induced. Thus, microglial activation was analysed in wild-type mice and *lpr* mice after KA treatment, with an Iba1 antibody that allows

measurement of the inflammatory response by means of changes in the cell shape of microglial cells, characterised by their amoeboid form. KA treatment evidenced microglial reactivity in the hippocampus, insofar as changes in the morphology of the microglial cells were detected in comparison to saline-treated mice (Fig. 3). Likewise, activated microglial morphology was visualised in *lpr* mice after KA injection, with the data suggesting that the neuroprotection detected in *lpr* mice after KA is not mediated by a prevention of glial activation.

Expression of Apoptotic Genes in *lpr* Mice After Treatment with Kainic Acid

We studied the transcriptional activity of genes involved in the apoptotic response to the exposure to KA in the *lpr* mice strain (Table 2). As a first approach, we determined the basal expression of genes in the hippocampus of both C57/Bl6 and *lpr* mice strains (Fig. 4). In fact, results show a clear down-regulation of the *fas* gene in *lpr* mice, together with *trp53bp2*, *traf2* and *nol3* genes. By contrast, a total of six genes were significantly up-regulated: *tnf*, *casp4*, *casp8*, *fasl*, *cflar* and *cidea*. Contrary to what was observed in the C57/Bl6 wild-type strain, treatment of *lpr* mice with kainic acid does not produce significant changes in the transcriptional activity of genes related to apoptosis (Table 2). In fact, no significant changes were observed either in the *fasl* gene or in *caspase-4* and *caspase-8* or the executioner *caspase-3* genes. In addition to the lack of

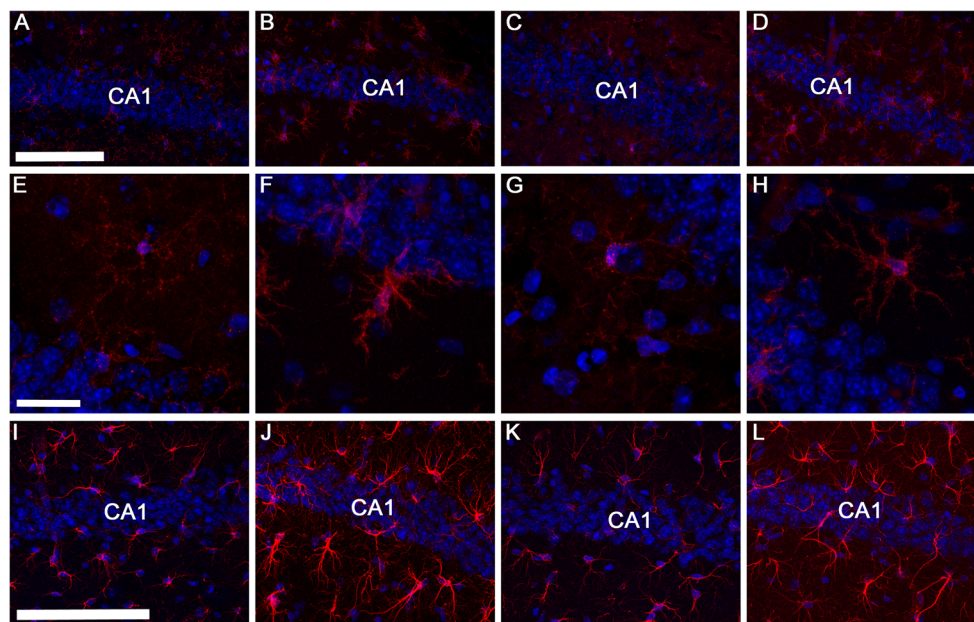


Fig. 3 Microglial and astroglial activation under kainic acid treatment. Iba-1 immunohistochemistry counterstained with Hoechst 33342 in the CA1 region of the hippocampus from saline control wild-type mice (**a** and **e**), kainic acid-treated wild-type mice (**b** and **f**), saline control *lpr* mice (**c** and **g**) and kainic acid-treated *lpr* mice (**d** and **h**). GFAP

immunohistochemistry counterstained with Hoechst 33342 in the CA1 region of the hippocampus from saline control wild-type mice (**i**), kainic acid-treated wild-type mice (**j**), saline control *lpr* mice (**k**) and kainic acid-treated *lpr* mice (**l**). CA1, CA1 field, hippocampus. Scale bars **a–d**, 100 μ m; **e–h**, 25 μ m; **i–l**, 100 μ m

Table 2 Significant changes in transcriptional activity of genes included in the Mouse Apoptosis RT² Profiler PCR Array, PAMM-012A, from the hippocampus of KA-treated *lpr* mice, at 12 h. Genes that did not prove to be related, directly or indirectly, to apoptotic processes were excluded from the analysis. By contrast to the observation in the C57/Bl6 wild-type strain, treatment of *lpr* mice with KA, at 12 h, did not produce significant changes in transcriptional activity of genes related to apoptosis, neither in *fas* ligand gene *fasl*, nor in caspase-4 and caspase-8 or the executioner caspase-3 genes. Any changes were also not observed in *atf5*

Gene symbol	<i>lpr</i> KA versus <i>lpr</i> CT
<i>tnf</i>	8.86**
<i>bag3</i>	5.00***
<i>tnfrsf1a</i>	5.05**
<i>fas</i>	3.14**
<i>mcl1</i>	2.69**
<i>cflar</i>	1.25*
<i>casp4</i>	0.76 ^{ns}
<i>fasl</i>	0.95 ^{ns}
<i>ltBr</i>	1.63**
<i>traf2</i>	1.47**
<i>atf5</i>	1.39 ^{ns}
<i>bcl10</i>	1.4**
<i>casp3</i>	1.11 ^{ns}
<i>casp8</i>	1.09 ^{ns}
<i>nol3</i>	0.48**
<i>cidea</i>	0.85 ^{ns}
<i>cradd</i>	0.43*
<i>trp53bp2</i>	0.56***

Asterisks different levels of significance

ns non significant (non statistical differences)

activity in cytoplasmic caspase genes, the cell death-inducing DNA fragmentation factor gene, *cidea*, showed no significant change in transcriptional activity. Nor were any changes observed in the *atf5* gene. Therefore, it can be concluded that Fas signalling is involved in kainic acid-induced apoptosis in hippocampal cells (Fig. 5).

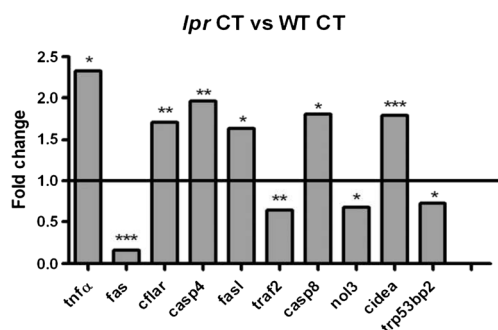


Fig. 4 Significant changes in the transcriptional activity of genes included in the Mouse Apoptosis RT² Profiler PCR Array, PAMM-012A, from the hippocampus of both C57/Bl6 wild-type and *lpr* mice. In *lpr* mice, a clear down-regulation of the *fas*, *trp53bp2*, *traf2* and *nol3* genes is observed when compared with C57/Bl6. By contrast, a total of six genes were significantly up-regulated: *tnf*, *casp4*, *casp8*, *fasl*, *cflar* and *cidea*

Discussion

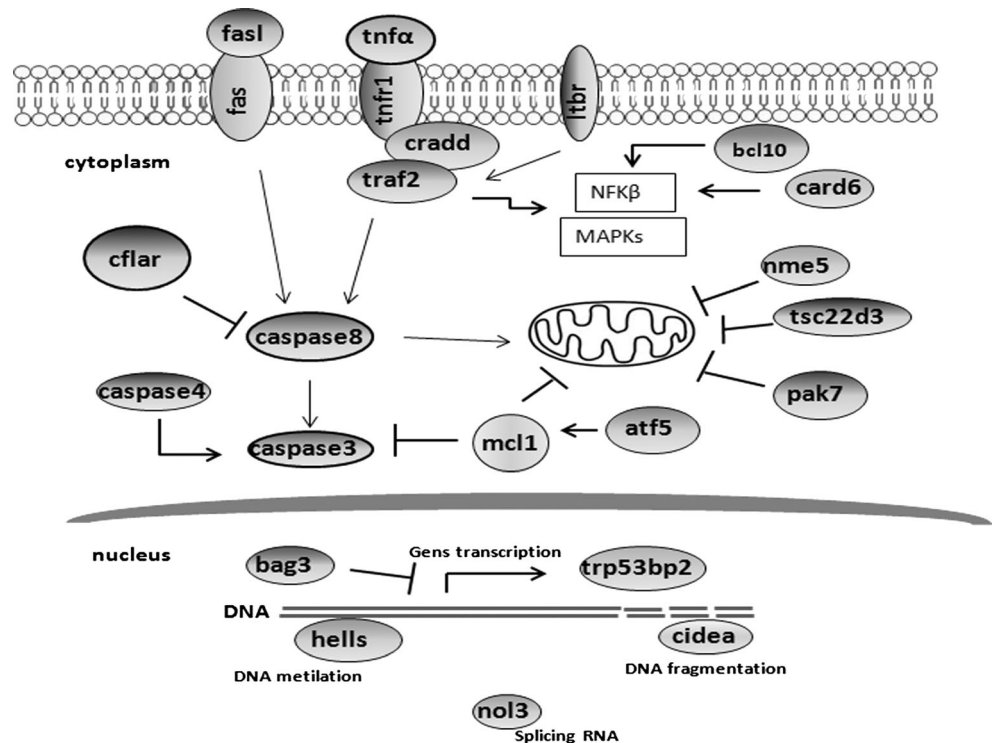
In the present paper, we report evidence of a novel role for Fas in the apoptosis of hippocampal neurons after treatment with the neurotoxin KA. To provide a mechanistic basis for these findings, we demonstrated that *lpr* mice treated with this neurotoxin exhibit a significant reduction in neuronal cell death and apoptosis compared with wild-type mice.

Real-time PCR array analysis showed that KA significantly regulated 23 apoptosis-related genes (fold change $\geq \pm 1.5$ and *P* value 0.05). It is well established that the extrinsic signalling pathway that initiates apoptosis involves death receptor members of the *Tnf receptor* gene superfamily [21, 23, 26, 42, 46, 47]. Our study demonstrated that *Fas*, *Tnfr1*, *FasL* and *Tnf* genes were highly expressed in the hippocampus following KA treatment in wild-type animals. In addition, our findings also prove an overexpression of the *Casp8* gene that encodes an initiator caspase and the *Casp3* gene that encodes an effector caspase. However, it is noteworthy that KA treatment at 12 h does not promote significant changes in the expression of key genes involved in the intrinsic apoptotic pathway such as *caspase-9* and *Apaf1*, among others. These data suggest that the cell death observed at 24 h of KA treatment may be due mainly to the activation of genes involved in the extrinsic pathway of apoptosis.

Several reports have demonstrated that the expression of the *Fas receptor* gene is induced after neuropathological lesions including those produced by KA [23–25, 38, 48]. With the aim of reporting more data in the involvement of the Fas signalling pathway in the mechanisms of neuronal death induced by excitotoxicity, we performed a treatment with KA in *lpr* mice. Previous studies have described that these mice exhibit neuroprotection against traumatic brain injury (TBI), in preclinical models of Parkinson's disease (MPTP) and amyotrophic lateral sclerosis (ALS), and also models of ischemia, demonstrating the involvement of the Fas pathway in neuronal death [24, 25, 29, 32, 41, 47, 49]. We observed a significant decrease in neurodegeneration in the hippocampus of the KA-treated *lpr* mice compared to the wild type, so the results strongly suggest that the lack of Fas receptor functionality plays a neuroprotective role following KA administration.

In the model of KA-induced excitotoxicity, it is widely demonstrated that there is clear astroglial and microglial activation [4, 5, 50]. Here, in the present study, we have also observed glial reactivity after KA treatment in the hippocampus of wild-type mice. In the case of the *lpr* mice, although there was a significant reduction in neuronal death, the glial reactivity induction was similar to that of wild-type mice, indicating that while the Fas pathway prevents neuronal death, it is not involved in the regulation of neuroinflammatory processes. Likewise, KA-induced glial activation may occur through mechanisms independent of Fas [27].

Fig. 5 Drawing of the 23 genes identified by the Mouse Apoptosis RT² Profiler PCR Array, PAMM-012A, with a significant differential expression (fold change $\geq \pm 1.5$ and P value 0.05) between KA wild-type treated and untreated mice, at 12 h. Fas/FasL interaction induces caspase-8 and caspase-3 activity. Caspase-3 is a key mediator of apoptosis in neuronal cells. This pathway is blocked in the *lpr* mice fold change $\geq \pm 1.5$



Because reduced neuronal death was observed in *lpr* mice following KA treatment, we analysed the differentially expressed apoptosis-related genes detected, comparing wild-type KA-treated mice to untreated mice. For this, first of all, we compared the gene expression levels between the two genotypes (wild type and *lpr*). The *lpr* mice showed higher expression levels of genes encoding the death receptor ligands (FasL and TNF): *caspase-8* that codifies an essential protease for the extrinsic apoptotic pathway, *caspase-4* encoding a caspase that plays a role in inflammation, *cflar* that codifies an apoptotic regulator protein that lacks caspase activity and appears to be itself cleaved into two peptides by Caspase-8 and the pro-apoptotic *cidea* gene [42, 51]. Furthermore, a down-regulation of the *nol3* (nucleolar protein 3) gene was detected, which encodes the antiapoptotic protein ARC (apoptosis repressor with a caspase recruitment domain) that interacts with Caspase-2 and Caspase-8 and inhibits receptor-mediated apoptosis. In addition, *nol3* does not play a key role in *lpr* neuroprotection, because we did not find an increase in gene expression after *lpr* mice KA treatment.

A down-regulation was also detected in *traf2* encoding TRAF2 protein required for TNF- α -mediated activation of MAPK8/JNK and NF- κ B and in *trp53bp2* that codifies a pro-apoptotic member of a family of p53 binding proteins [52]. All these data indicate that the *lpr* mice genotype does not respond to a neuroprotective profile.

However, after KA administration, we found that some genes implicated in the death pathway did not show significant changes in expression in *lpr* mice compared to WT, such

as *fasl*, decreasing the FasR/FasL interaction: *caspase-3* and *caspase-8*, the main caspases involved in the extrinsic apoptotic pathway; *caspase-4* involved in inflammatory processes; *cidea* that codifies a apoptotic protein activator and *pim2* that codifies an enzyme serine/threonine kinase that has roles in apoptosis and regulation of signal transduction cascades [18, 25, 52–58]. This fact suggests that the expression of these genes could be regulated by the expression of *fas* in the model of KA-induced neurotoxicity, indicating that they play an important role in the apoptotic cascade in the excitotoxic process.

In agreement with Torres-Peraza et al. [59], we also observed an increase of *atf5* gene expression mediated by KA-induced seizures; interestingly, a neuronal increase in *atf5* expression has a neuroprotective role [59]. It has been proposed that the neuroprotective effects of ATF5 are mediated by the transcriptional induction of some proteins with a neuroprotective effect, such as Hsp27, Bcl2 and Mcl1 [58–61]. Likewise, in our study, we found a significant up-regulation in *mcl1*, which was also up-regulated in other models of status epilepticus, such as pilocarpine administration in rodents [62].

Furthermore, in our study, after KA treatment in *lpr* mice compared to WT, we observed a non-significant increase in *atf5*, possibly resulting from the decrease in apoptotic neuronal death that occurs in *lpr* KA-treated mice. At the same time, other genes showed similar changes in gene expression profiles between the two genotypes, including *bag3*, *mcl1*, *traf2* and *bcl10*. Interestingly, these genes encode proteins that confer antiapoptotic neuroprotection, which could account

for the increase in expression in both WT and *lpr* mice after KA treatment [62]. Therefore, our results suggest neuroprotective effects in *lpr* mice after KA administration based on genes that are not activated in this model. Furthermore, these genes could be considered as potential therapeutic drug targets in neurodegenerative diseases. In this line, recent data have demonstrated that a mouse with neuronal *caspase-8* deficiency was protected against neuronal injury caused by KA-induced excitotoxicity [63]. The apoptotic prevention observed in *lpr* mice after KA administration may be at least partly attributable to a decrease in *caspase-8*, which is also involved in *caspase-3* activation. At the same time, the differential gene expression observed in some apoptosis-related genes between untreated genotypes should be intensively studied to determine their functional significance in the process of neuroprotection observed in mice deficient in the Fas receptor.

In conclusion, in the present work, we report evidence of the key role played by the Fas/FasL-mediated apoptosis of neurons in contributing to the process of KA-induced neurodegeneration and affecting neuronal function and survival (Fig. 5). Interestingly, FasR-deficient mice exhibit significantly reduced cell death, although inflammatory cells (microglia/macrophages) and the level of GFAP when compared with the wild type were unchanged. Thus, we propose that targeting the Fas receptor could provide a compelling rationale for therapeutic strategies against neurodegeneration but not against neuroinflammation.

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Conflict of Interest The authors declare no conflict of interest.

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