

CCAAT/enhancer binding protein β deficiency provides cerebral protection following excitotoxic injury

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Summary

The CCAAT/enhancer-binding protein β (C/EBP β , also known as CEBPB) was first identified as a regulator of differentiation and inflammatory processes in adipose tissue and liver. Although C/EBP β was initially implicated in synaptic plasticity, its function in the brain remains largely unknown. We have previously shown that C/EBP β regulates the expression of genes involved in inflammatory processes and brain injury. Here, we have demonstrated that the expression of C/EBP β is notably increased in the hippocampus in a murine model of excitotoxicity. Mice lacking C/EBP β showed a reduced inflammatory response after kainic acid injection, and exhibited a dramatic reduction in pyramidal cell loss in the CA1 and CA3

subfields of the hippocampus. These data reveal an essential function for C/EBP β in the pathways leading to excitotoxicity-mediated damage and suggest that inhibitors of this transcription factor should be evaluated as possible neuroprotective therapeutic agents.

Supplementary material available online at <http://jcs.biologists.org/cgi/content/full/121/8/1224/DC1>

Key words: C/EBP β , Excitotoxicity, Inflammation, Neurodegeneration

Introduction

Cell death resulting from excitotoxicity has been associated with different brain disorders (Coyle and Puttfarcken, 1993; Doble, 1999; Meldrum, 2000). In these diseases, accumulating evidence implicates an inflammatory response in affected regions. Glial cells, as mediators of the inflammatory response, also have an important role in the course of kainic acid (KA)-induced hippocampal neurodegeneration. Activated astrocytes and microglia cells proliferate and increase the expression of genes implicated in the production of nitric oxide and pro-inflammatory cytokines. These agents, when released from activated glial cells, can contribute noticeably to the expansion of brain injury and the increased loss of neurons (Oprica et al., 2003).

Excitotoxicity can be triggered by administration of KA, which results in behavioral changes and neuronal damage initiated by activation of KA-receptors in the CA3 region of the hippocampus (Krnjevic et al., 1980). This activation results in a depolarization of the cells, resulting in a significant increase in Ca²⁺ entry, which leads to neuronal death. Particularly sensitive are the hippocampal CA1 and CA3 regions, and the hilar neurons of the dentate gyrus (Coyle, 1983; Sperk et al., 1985). Injection of KA into rodents also results in the activation of glial cells and inflammatory responses typically found in neurodegenerative diseases. Hence, this model has been used extensively to analyze the cellular and molecular mechanisms that underlie central nervous system damage.

The CCAAT/enhancer binding protein β (C/EBP β , also known as CEBPB) is a member of the transcription factor family consisting

of six functionally and structurally related basic leucine-zipper DNA-binding proteins (Vinson et al., 1989). As a transcription factor, C/EBP β regulates many genes involved in different cell processes including metabolism, hematopoiesis, adipogenesis, the immune response, and morphogenesis (Poli, 1998; Ramji and Foka, 2002). Recently, it has been shown that C/EBP β mRNA is expressed in the central nervous system of adult mice (Nadeau et al., 2005; Sterneck and Johnson, 1998), as well as in mouse cortical astrocytes and in hippocampal neurons in vitro (Cardinaux and Magistretti, 1996; Yano et al., 1996; Yukawa et al., 1998). Several studies, including those from our laboratory, have suggested that this protein has important functions in the brain. It has been shown that C/EBP β has an important role in the consolidation of long-term memory, suggesting a very important role for this protein in the hippocampus (Alberini et al., 1994; Taubenfeld et al., 2001), and Menard et al. have defined the MAP kinase kinase (MEK)-C/EBP signaling pathway as being essential for the differentiation of cortical progenitor cells into postmitotic neurons (Menard et al., 2002). We have demonstrated that C/EBP β serves as a crucial factor in neuronal differentiation (Cortes-Canteli et al., 2002). In addition, using microarray analysis in neuronal cells overexpressing C/EBP β , we have found that this protein induces the expression of several genes involved in inflammatory processes and brain injury (Cortes-Canteli et al., 2004).

Given the limited understanding of C/EBP β involvement in brain injury and our prior speculation that this transcription factor has a role in this process, we sought to address the function of C/EBP β

in cells of the central nervous system in response to a brain insult in vitro and in vivo. Our results reveal an strong induction of C/EBPβ in the hippocampus of wild-type mice following KA injection. Notably, there was a pronounced reduction in glial activation and neuronal damage in C/EBPβ knockout mice. These data point to a key role for C/EBPβ in excitotoxic brain injury.

Results

C/EBPβ induction in glial primary cultures from C/EBPβ wild-type mice

We have previously demonstrated that cell lines stably transfected with C/EBPβ exhibit an increase in the expression of many genes involved in inflammation and brain injury (Cortes-Canteli et al., 2004). In addition, we have shown that, when these cells were subjected to injury using an in vitro ‘scratch-wound’ model, expression of C/EBPβ was also upregulated. These observations

have prompted us to investigate whether expression of the endogenous C/EBPβ gene in primary cultures of glial cells is regulated after neural injury. Therefore, we first investigated the expression of C/EBPβ in glial cultures from C/EBPβ wild-type (C/EBPβ^{+/+}) mice in response to different agents that are known to cause neural damage through different mechanisms. C/EBPβ protein levels in these experiments were significantly induced after treating the cells with lipopolysaccharide (LPS), the kinase inhibitor staurosporine, KA or glutamate. As shown in Fig. 1A, basal C/EBPβ levels measured by western blot analysis were low, both in microglial and astrocyte primary cultures prepared from wild-type mice. However, an increase in C/EBPβ was detected after treatment of microglial cells with either LPS or KA (2.3-fold or 1.5-fold, respectively, compared with non-treated cultures). Addition of LPS or staurosporine to astrocytes also potentially induced C/EBPβ expression (4.2-fold or 2.2-fold, respectively, compared with non-treated cultures); and a lesser increase was observed after treatment with KA (1.7-fold) or glutamate (1.3-fold). At the subcellular level (see confocal images shown in Fig. 1B,C) the increase in C/EBPβ protein expression was localized to the nucleus in both microglia and astrocytes. KA and glutamate-treated astrocyte cultures (Fig. 1B) also showed an increase in C/EBPβ protein levels in the nucleus, although the induction was lower than that observed following LPS or staurosporine treatment. No C/EBPβ signal was detected in astrocytes and microglial cultures from C/EBPβ^{-/-} mice after LPS treatment, confirming the specificity of the antibody used (Fig. 1D,E). A similar increase in C/EBPβ

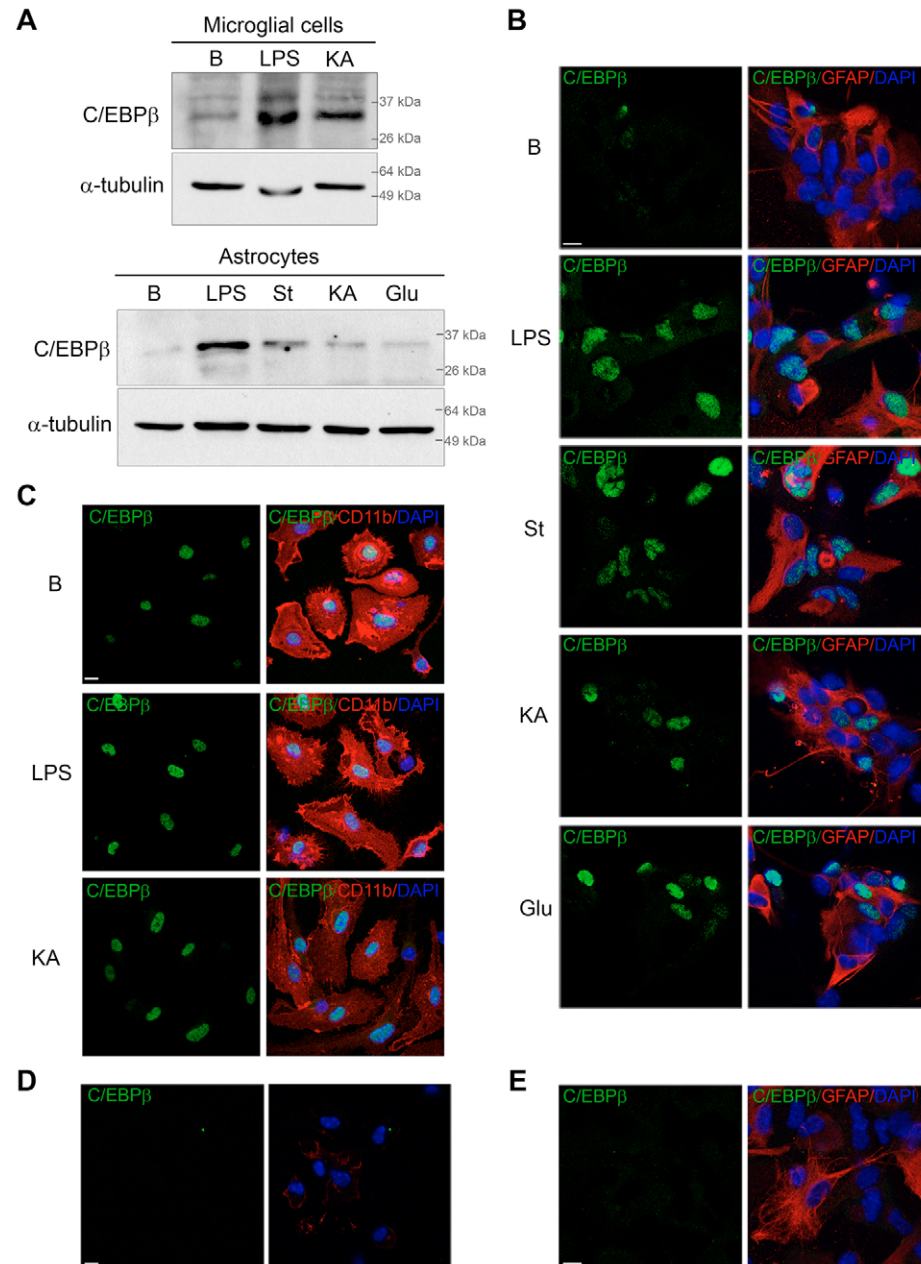


Fig. 1. Increased expression of C/EBPβ in mouse glial primary cultures after treatment with different stimuli. (A) Western blot showing an increase in C/EBPβ expression in microglial cells treated with LPS and KA and in astrocytes treated with LPS, staurosporine, KA or glutamate for 24 hours. Only a small induction was observed in astrocyte cultures after treatment with KA or glutamate. (B,C) Primary astroglial (B) and microglial (C) cultures prepared from C/EBPβ^{+/+} were treated as in A, and C/EBPβ expression was evaluated by immunofluorescence analysis and confocal microscopy using a specific anti-C/EBPβ antibody (clone A16), as described in the Materials and Methods. Cultures were also stained with anti-CD11b (clone M1/70.15) or anti-GFAP antibodies to detect microglia or astrocytes, respectively. (D,E) To confirm the specificity of the anti-C/EBPβ antibody, primary microglial (D) and astroglial (E) cultures from C/EBPβ^{-/-} mice were treated with LPS for 24 hours, and C/EBPβ expression was evaluated by immunofluorescence analysis and confocal microscopy. Representative results from three experiments are shown. Scale bar, 10 μm. Nuclei were counterstained by DAPI (blue). B, basal; LPS, lipopolysaccharide; St, staurosporine; KA, kainic acid; Glu, glutamate.

protein levels was also observed in rat primary cultures after treatment with LPS or staurosporine (see supplementary material Fig. S1).

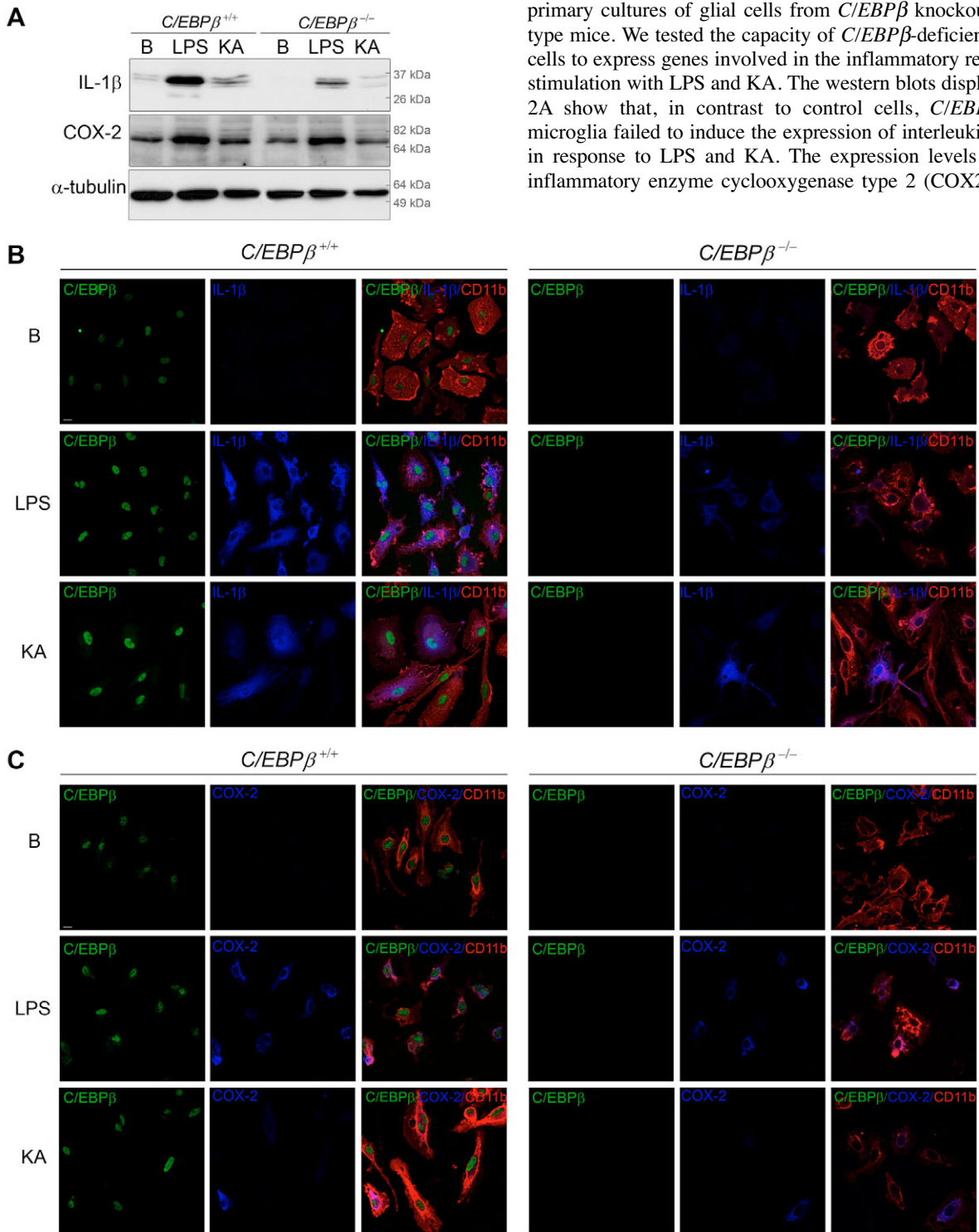


Fig. 2. Induction of pro-inflammatory mediators is attenuated in microglial primary cultures from *C/EBPβ*-deficient mice. (A) Western blots showing a reduction in the expression of IL1β and COX2 in *C/EBPβ*^{-/-} microglial cultures after treatment with LPS or KA for 24 hours, compared with cultures established from *C/EBPβ*^{+/+} mice. (B,C) Primary microglial cultures prepared from *C/EBPβ*^{+/+} or *C/EBPβ*^{-/-} mice were treated as in A, and the expression of *C/EBPβ* (green), IL1β (blue in B) and COX2 (blue in C) was evaluated using immunofluorescence techniques, and confocal microscopy using specific antibodies as described in the Materials and Methods. The last column of panels in each set shows colocalization of *C/EBPβ* with IL1β (B) or COX2 (C). The microglial marker CD11b is shown in red. Representative results from three experiments are shown. Scale bar, 10 μm. B, basal; KA, kainic acid; LPS, lipopolysaccharide.

Decreased induction of pro-inflammatory proteins in *C/EBPβ*^{-/-} microglial primary cultures

To further evaluate the possible role of *C/EBPβ* in the inflammatory response in neural tissues, we performed in vitro experiments using primary cultures of glial cells from *C/EBPβ* knockout and wild-type mice. We tested the capacity of *C/EBPβ*-deficient microglial cells to express genes involved in the inflammatory response after stimulation with LPS and KA. The western blots displayed in Fig. 2A show that, in contrast to control cells, *C/EBPβ*-deficient microglia failed to induce the expression of interleukin 1β (IL1β) in response to LPS and KA. The expression levels of the pro-inflammatory enzyme cyclooxygenase type 2 (COX2) were also

slightly reduced in protein lysates from *C/EBP β ^{-/-}* microglial cultures. These data were further confirmed by confocal analysis. As shown in Fig. 2, the number of microglial cells expressing IL1 β (Fig. 2B) or COX2 (Fig. 2C) was reduced in cultures established from *C/EBP β -deficient* animals. Quantification of both western blot and immunocytochemistry analysis revealed that IL1 β expression was significantly reduced (~50%) in *C/EBP β -deficient* microglial cultures after treatment with both LPS and KA. A decrease in COX2 levels (~15%) was also observed although, in this case, the reduction was not statistically significant. Together, these data suggested that C/EBP β helps to modulate the induction of proinflammatory mediators in microglial cells.

To ascertain a possible colocalization of IL1 β and COX2 with C/EBP β , multiple labeling was performed. As depicted in Fig. 2B and 2C, both IL1 β and COX2 colocalized with C/EBP β in the majority of microglial cells established from wild-type animals. These data provide evidence that the LPS- or KA-triggered upregulation of C/EBP β essentially takes place within the same cells in which induction of pro-inflammatory agents is observed.

Expression levels of C/EBP β increase in the hippocampus after excitotoxic injury

We next investigated the *in vivo* role of C/EBP β in a well-established model of excitotoxic brain injury. Adult mice received intra-hippocampal injections of either vehicle or KA; at different times post-injection, the animals were perfused and brain tissue was prepared for immunohistochemical analyses. These studies revealed that the injection of KA noticeably increased the expression of C/EBP β on the ipsilateral side of the hippocampus of wild-type mice when compared with vehicle-injected controls at 24 hours and 72 hours after surgery (Fig. 3A). Activation was found mainly in the dentate gyrus and, as anticipated, was completely absent in *C/EBP β -deficient* mice (Fig. 3C), a finding that underscores the appropriateness of the monoclonal anti-C/EBP β antibody (A16) for the *in vivo* immunohistochemical studies. In the KA-injected animals occasional C/EBP β -positive cells were also detected within the CA1 and CA3 subfields (data not shown), and in the stratum radiatum. To identify the cell type specificity of C/EBP β activation, we next performed double labeling using Neurotrace, anti-gial fibrillary acidic protein (GFAP) and tomato lectin to identify neurons, astrocytes and microglia, respectively. C/EBP β immunoreactivity was detected in the nuclei of granule neurons of the dentate gyrus as well as in glial cells 24 hours after KA injection (Fig. 3B). This increase was more pronounced 72 hours post-injection in granule neurons although, at this time, less C/EBP β -active cells in the hippocampus of KA-treated animals stained positive for GFAP or tomato lectin.

C/EBP β -deficient mice are less vulnerable to glial activation

One of the events that takes place in the hippocampus following excitotoxic injury is the sequential activation of microglia and astroglia. Astrocytes and microglial cells are considered to be key players in the induction of the neuronal damage following excitotoxic injury. Analysis of hippocampal sections revealed that *C/EBP β ^{-/-}* mice exhibited reduced astrocytic activation and astrogliosis (Fig. 4A). Twenty-four hours after KA injection, wild-type mice displayed the expected accumulation of GFAP-positive astrocytes (Fig. 4A) relative to vehicle-injected animals. At 72 hours, there was an increase in both the number and intensity of

GFAP-expressing cells. The increase in GFAP immunoreactivity was mainly detected in the hilus and the molecular layers surrounding the dentate gyrus of the hippocampus. By contrast, the *C/EBP β ^{-/-}* animals displayed very little staining for GFAP, indicating a reduced astroglial response to KA-induced injury. Quantification of the data revealed a 30% decrease in the number of GFAP-positive cells, both in the hilus and the molecular layer of the hippocampus of *C/EBP β ^{-/-}* mice, when compared with *C/EBP β ^{+/+}* animals 72 hours after KA injection (Fig. 4C). In addition, the evaluation of CD11b staining for microglial infiltration revealed a considerably larger response in *C/EBP β ^{+/+}* animals than in their *C/EBP β ^{-/-}* counterparts (Fig. 4B). Significantly lower levels of CD11b were detected in the hippocampus of *C/EBP β ^{-/-}* animals 24 hours after KA injection (Fig. 4D). Although the number of CD11b-positive cells was not statistically different between both groups of animals at 72 hours, *C/EBP β ^{+/+}* mice exhibited a strong microglial activation, as evidenced by morphological changes in these cells, which included swelling of the cell soma as well as a thickening and retraction of cell processes (see insets in Fig. 4B) – features characteristic of reactive microglia. By contrast, the number of activated microglial cells in *C/EBP β ^{-/-}* mice at this time was significantly lower than that found in their *C/EBP β ^{+/+}* littermates (data not shown). The CD11b⁺ cells observed in Fig. 4B can also represent infiltrated macrophages; it is well known that brain injury promotes the extravasation of these cells towards the sites of brain injury (Wang et al., 2007). In this regard, it has been shown that C/EBP β has an important role in macrophage activation, because their function is severely compromised in *C/EBP β ^{-/-}* mice (Screpanti et al., 1995).

Impaired pro-inflammatory protein induction in *C/EBP β -deficient* mice

We also evaluated the production of the cytokine IL1 β , which is secreted by glial cells in response to KA administration and known to enhance neuronal death. IL1 β was elevated in the hippocampus of *C/EBP β ^{+/+}* mice 72 hours after injection (Fig. 5A), although a slight increase was already detected at 24 hours (data not shown). In *C/EBP β ^{-/-}* mice, no upregulation of IL1 β was observed, consistent with a lack of glial activation. These results are in agreement with those obtained in primary cultures of *C/EBP β ^{-/-}* microglial cells (Fig. 2A,B), where induction of IL1 β was reduced in response to LPS and KA treatment. The IL1 β ⁺ cells also express the astroglial marker GFAP in both the hilus and the molecular layers of the dentate gyrus (Fig. 5B). We also found a small number of cells that expressed IL1 β and bound to the tomato lectin (Fig. 5B). We did not identify any cells that coexpressed IL1 β and the neuronal marker Neurotrace.

We also studied the accumulation of inducible COX2. Overexpression of COX2 in neural cells appears central to many neuroinflammatory conditions. As shown in Fig. 5C, protein levels of COX2 were clearly increased relative to vehicle-injected controls in the hippocampus 24 and 72 hours following KA injection of *C/EBP β ^{+/+}* animals. COX2 staining was mainly observed in the granule cells of the dentate gyrus, although some scattered cells were also detected in the stratum radiatum. A small increase in COX2 levels was also observed in some of the KA-treated *C/EBP β ^{-/-}* animals (mainly at 24 hours), albeit not as robustly or as persistently as that seen in *C/EBP β ^{+/+}* mice. Using double labeling, we found that all COX2-positive cells in wild-type animals were indeed neurons (Fig. 5D). In these cells, the intracellular distribution of the immunoreactivity was predominantly perinuclear,

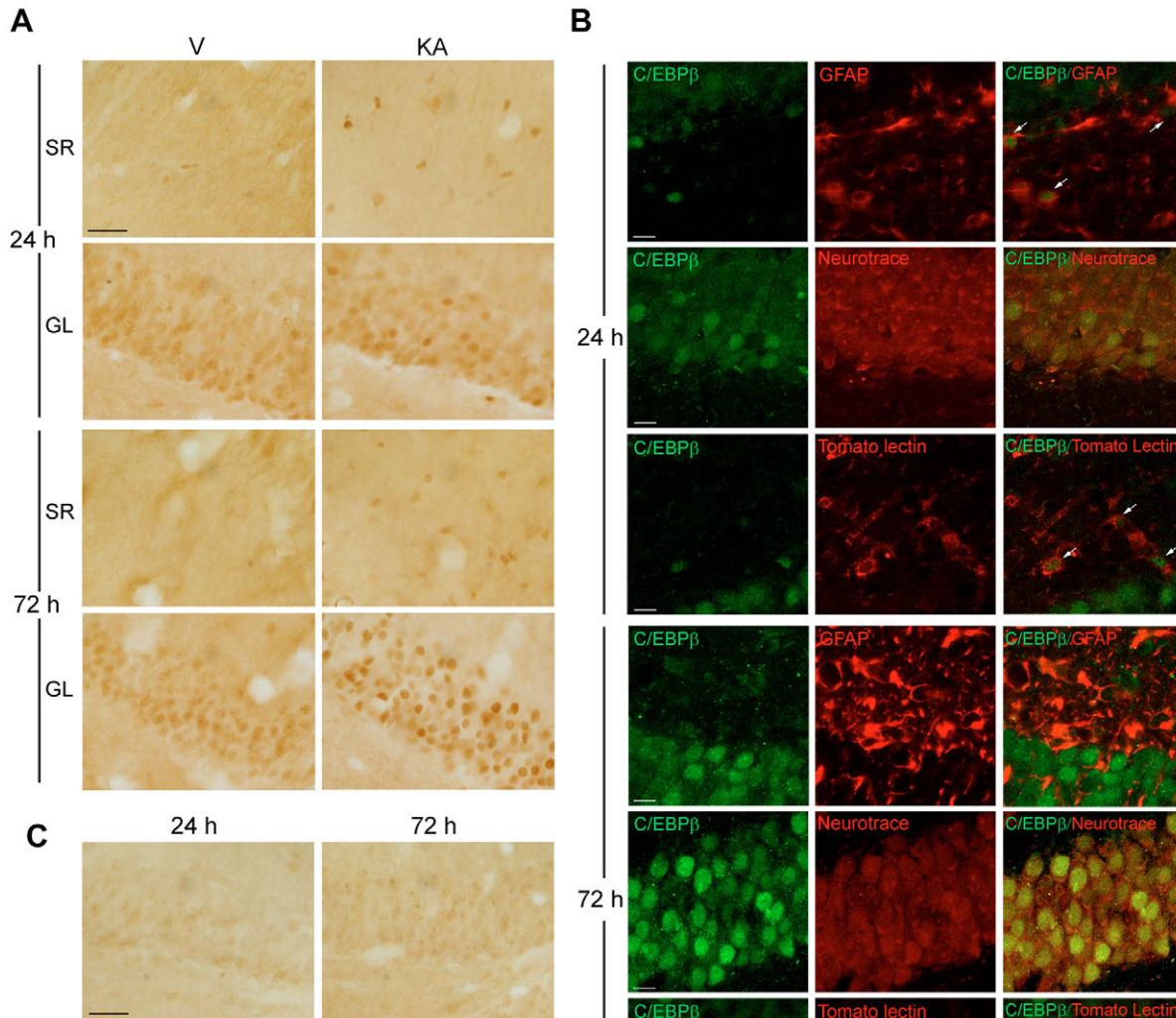


Fig. 3. Induction of *C/EBPβ* expression after KA injection in the adult mouse brain. (A) Immunohistochemical analyses of coronal brain sections prepared from *C/EBPβ*^{+/+} mice 24 and 72 hours after KA injection. *C/EBPβ* protein levels were substantially enhanced in the stratum radiatum (SR) and in the granular layer (GL) of the dentate gyrus of the hippocampus of *C/EBPβ*^{+/+} mice 24 hours and 72 hours post injection, compared with vehicle-injected controls. Images are representative for experiments from five animals. Scale bar, 25 μ m. (B) Double immunofluorescent labeling and confocal microscopic analyses of *C/EBPβ* after KA injection in *C/EBPβ*^{+/+} mice. Double-labeling studies were performed with an antibody recognizing *C/EBPβ* (green) and different reagents to detect astrocytes (anti-GFAP), microglia (tomato lectin), and neurons (Neurotrace) (red labeling). Complete colocalization (yellow) was found in neurons, although 24 hours and 72 hours after KA injection some astrocytes and microglia were found that express *C/EBPβ* (arrows). Scale bar, 10 μ m. (C) Immunohistochemical analyses of coronal brain sections prepared from *C/EBPβ*^{-/-} mice 24 hours and 72 hours after KA injection. As expected, *C/EBPβ*^{-/-} mice did not present any *C/EBPβ* staining. Images are representative for experiments from five animals. Scale bar, 25 μ m.

which is consistent with the known subcellular localization of this enzyme (Nogawa et al., 1997). COX2 did not appear to colocalize with the microglial marker tomato lectin or with the astrocytic marker GFAP (data not shown).

Next, we analyzed the protein expression of lipocalin 2 (LCN2) and histidine decarboxylase because their respective genes are upregulated by *C/EBPβ* (Cortes-Canteli et al., 2004) and probably implicated in inflammation and brain injury. Fig. 6 shows that protein levels of both LCN2 and histidine decarboxylase were increased 24 hours after treatment with KA, and that this increase – as previously observed for IL1 β and COX2 – were markedly reduced in the hippocampus of *C/EBPβ*^{-/-} animals.

C/EBPβ-deficient mice are resistant to excitotoxin-mediated neuronal degeneration

To better understand the role of *C/EBPβ* in vivo, we examined the sensitivity of *C/EBPβ*^{-/-} mice to KA-induced neurodegeneration. To determine whether *C/EBPβ* expression is associated with the death of CA1 and CA3 pyramidal cells after excitotoxicity, hippocampal sections were stained with the fluorescent dye Fluoro-Jade B (Schmued et al., 1997) to detect degenerating neurons, or with NeuN (neuronal nuclei) antibody to detect surviving neurons. The *C/EBPβ*^{-/-} mice exhibited significantly less damage in the CA1 and CA3 regions at both 24 and 72 hours post-injection, relative to their littermate controls. By contrast, *C/EBPβ*^{+/+} mice displayed

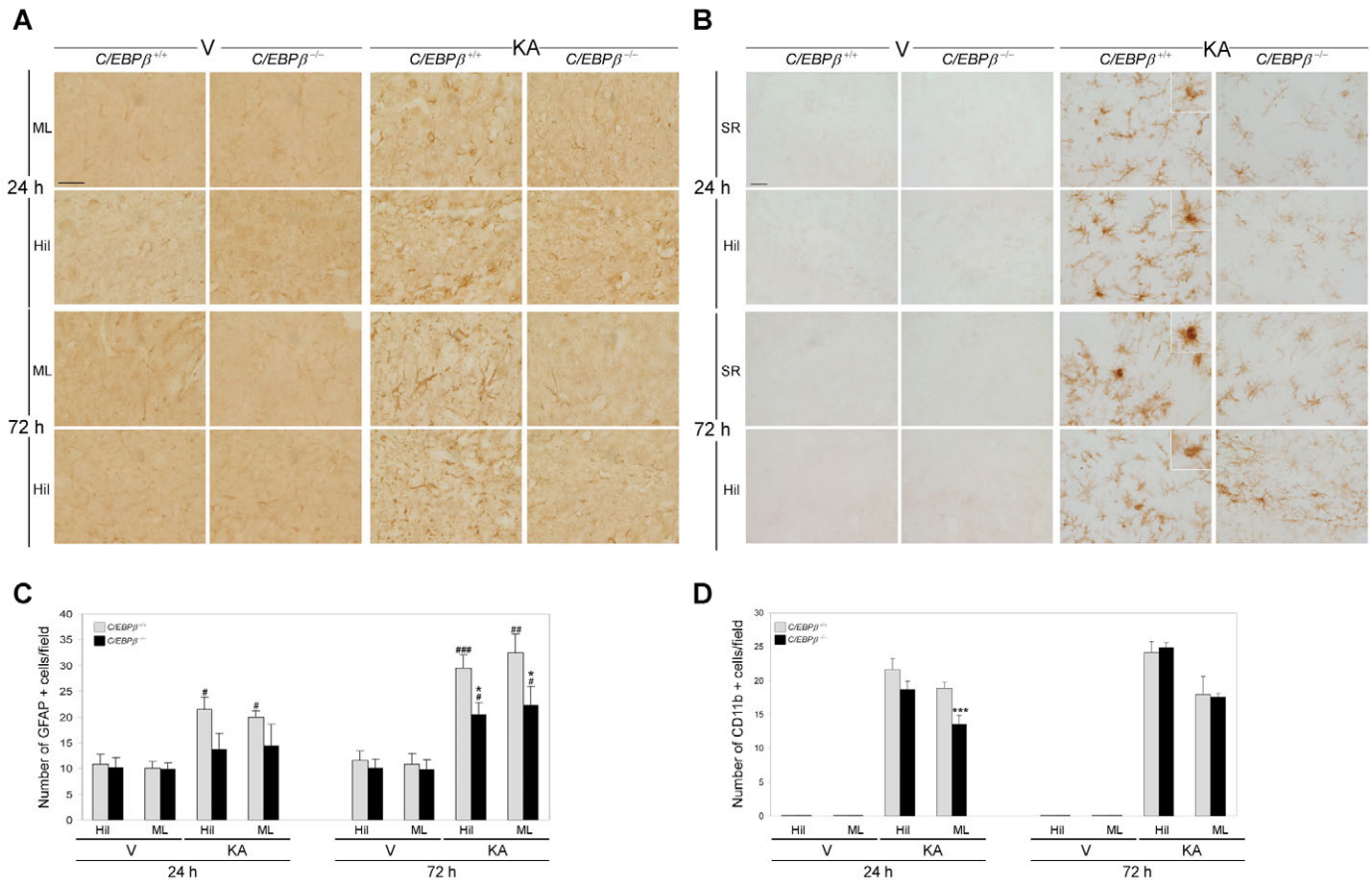


Fig. 4. Deficiency of C/EBP β ameliorates KA-induced gliosis. (A,B) C/EBP $\beta^{+/+}$ and C/EBP $\beta^{-/-}$ adult mice were injected with vehicle (V) or KA and sacrificed 24 hours and 72 hours post injection. Coronal sections (30 μ m) were stained with antibodies against (A) GFAP or (B) CD11b to detect astrocytes or microglia, respectively. A significant decrease in both astrogliosis and microgliosis is observed in C/EBP $\beta^{-/-}$ mice. Substantial gliosis is observed in C/EBP $\beta^{+/+}$ mice after KA-injection, which is prevented in C/EBP $\beta^{-/-}$ mice (A). Immunohistochemical detection using anti-CD11b antibodies reveals highly ramified microglia in the hippocampus of KA-treated C/EBP $\beta^{-/-}$ mice (B). By contrast, in C/EBP $\beta^{+/+}$ mice the activated microglial cells exhibit shorter and thicker processes, and larger cell bodies. Insets show higher magnification of the images corresponding to C/EBP $\beta^{+/+}$ mice. (C,D) Quantification of the number of reactive astrocytes (C) and microglial cells (D) analyzed in the hilus, stratum radiatum or molecular layer of the hippocampus. Values are the mean \pm s.e. from five different animals and two independent sections per animal. [#] $P \leq 0.05$, ^{##} $P \leq 0.01$, ^{###} $P \leq 0.001$; vehicle vs KA-injected animals. ^{*} $P \leq 0.05$, ^{***} $P \leq 0.001$; knockout vs wild-type KA-injected animals; SR, stratum radiatum; Hil, hilus; ML, molecular layer. Images representative for each group are shown. Scale bars, 25 μ m.

stronger Fluoro-Jade B fluorescence in the CA1 and CA3 subfields of the hippocampus 24 hours (4.6-fold and fivefold, respectively) and 72 hours (threefold and 4.5-fold, respectively) after KA injection, compared with the moderate damage observed in C/EBP $\beta^{-/-}$ animals (Fig. 7A,C). The apparent resistance to injury in C/EBP $\beta^{-/-}$ mice was also supported by the higher percentage of healthy cells shown by NeuN staining (Fig. 7B). Seventy-two hours after KA injection, quantitative studies showed 6.5-fold and threefold increases in the number of neurons in the CA1 and CA3, respectively, subfields of the hippocampus in C/EBP $\beta^{-/-}$ mice, compared with control C/EBP $\beta^{+/+}$ littermates (Fig. 7D).

Discussion

Here, we have shown that mice lacking C/EBP β are less susceptible to glial activation and neuronal damage following KA exposure. These results clearly establish C/EBP β as a factor required in the development of excitotoxic brain injury. Excitotoxic brain damage is considered one of the major mechanisms by which neurons of the adult central nervous system die; it contributes to the pathogenesis of many central nervous system disorders, including

neurodegenerative disease, brain ischemia, epilepsy, and trauma (Coyle and Puttfarcken, 1993; Guo et al., 1999; Hossmann, 1994; Lynch and Dawson, 1994). Therefore, our results suggest that C/EBP β should be considered as a therapeutic target in brain injury and neurodegenerative disorders where excitotoxic neuronal cell death and inflammation are involved.

The first clues that suggested a possible role for C/EBP β in brain injury stemmed from in vitro studies showing increased activity of this transcription factor during the induction of inflammation and damage in neural cells. The expression of C/EBP β has been previously shown to be induced by pro-inflammatory cytokines in primary cultures of murine astrocytes (Cardinaux et al., 2000) and by mechanical injury in neuroblastoma cells (Cortes-Canteli et al., 2004). In addition, we have demonstrated that overexpression of C/EBP β induces terminal differentiation and cell death in neuroblastoma N2A cells (Cortes-Canteli et al., 2002) and also upregulates the expression of genes suggested to be involved in brain injury and inflammatory processes (Cortes-Canteli et al., 2004). The in vitro studies presented here show that LPS, staurosporine, KA and glutamate are each capable of increasing the

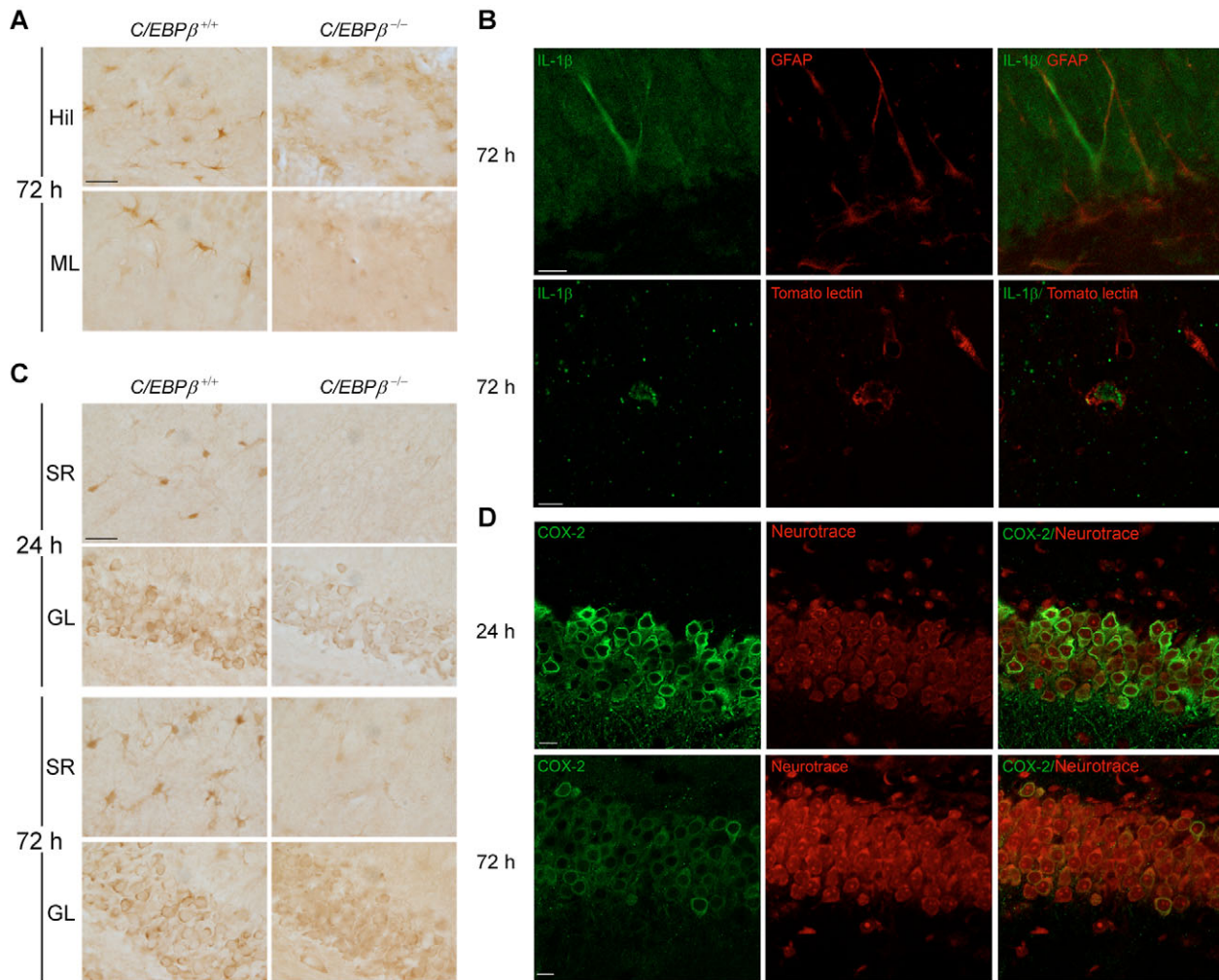


Fig. 5. *C/EBPβ* deficiency impairs KA induction of pro-inflammatory mediators. (A–D) *C/EBPβ*^{+/+} and *C/EBPβ*^{-/-} adult mice were injected with KA and sacrificed 24 and 72 hours post-injection. Coronal sections (30 μm) were stained with antibodies against (A,B) IL1β and (C,D) COX2. Neurotrace was used to identify neurons, and anti-GFAP and tomato lectin to detect astrocytes and microglia, respectively. IL1β expression was mainly localized in the hilus (Hil) and the molecular layers (ML) surrounding the dentate gyrus, and was completely absent in the *C/EBPβ*^{-/-} mice after KA injection, compared with their *C/EBPβ*^{+/+} littermates. COX2 immunoreactivity was mainly detected in the stratum radiatum (SR) and the granule cell layer of the dentate gyrus (GL). In contrast to controls, COX2 immunoreactivity was noticeably reduced in *C/EBPβ*^{-/-} mice. Confocal analysis of (B) IL1β and (D) COX2 subcellular localization indicates that IL1β is expressed primarily in glial cells, whereas COX2 immunostaining is restricted to neurons. Images are representative for experiments from five animals. Scale bars, 25 μm (A,C) and 10 μm (B,D).

expression of *C/EBPβ* in primary cultures of murine glial cells. These findings are consistent with previous studies indicating that *C/EBPβ* is induced in primary cultures of glial cells under inflammatory conditions (Cardinaux et al., 2000; Chen et al., 2004; Jana et al., 2005) or after glutamate treatment (Yano et al., 1996). Importantly, we have shown here that the activation of IL1β is markedly reduced in glial cultures from *C/EBPβ*^{-/-} mice, suggesting that IL1β gene expression is regulated by *C/EBPβ*. In this context, *C/EBPβ* has been previously shown to regulate the protein expression of inflammatory mediators and pro-inflammatory cytokines, including IL1β and COX2, in different tissues (Gorgoni et al., 2001; Yang et al., 2000); moreover, *C/EBPβ* response elements have been described in the *IL1β* and *COX2* genes (Wadleigh et al., 2000; Yang et al., 2000).

Excitotoxicity proceeds through a complex signaling pathway that includes participation of numerous signaling molecules. The identification of the genes that are activated or repressed in specific

responses to brain injury, and the understanding how such alterations in gene expression affect survival and neuronal function, is a central issue in the treatment of neurodegenerative diseases. We have demonstrated that the *in vivo* intra-hippocampal injection of KA results in a strong induction of *C/EBPβ*, and this was detected in the nuclei of granule neurons of the dentate gyrus as well as in a subset of hippocampal astrocytes and microglia. These observations are consistent with previous findings showing that *C/EBPβ* mRNA is expressed in neurons throughout the mature brain, and at particularly high levels in the hippocampus (Sterneck and Johnson, 1998). The observed induction is also consistent with previous studies showing that the expression of *C/EBPβ* mRNA is increased in facial motor neurons following axonal injury (Nadeau et al., 2005). There is considerable evidence that KA treatment is associated with a substantial activation of astrocytes and microglial cells and the increased expression of classic pro-inflammatory agents, probably through direct regulation by *C/EBPβ* (Wadleigh

et al., 2000; Yang et al., 2000). This hypothesis is supported by our data, which show that the morphological changes associated with the activation of astrocytes and microglial cells, as well as the expression of IL1 β and COX2, are markedly reduced in *C/EBP β* -null mice following KA injection.

The regulation of pro-inflammatory mediators is generally considered a key mechanism in neuronal cell death. The enzyme COX2 is involved in the pathogenesis of multiple neurological disorders associated with inflammation (Giovannini et al., 2003; Teismann et al., 2003) and local increases in COX2 expression in vivo have been associated with inflammation, seizures and ischemia (Nogawa et al., 1997; Strauss et al., 2000). In addition, an increase in IL1 β expression has been observed in several types of brain injury including excitotoxicity (Rothwell and Luheshi, 2000), where it has been shown that exogenous IL1 β enhances chemically induced seizures in rats (Vezzani et al., 2002). IL1 β has also been implicated in a number of neurodegenerative conditions and is generally believed to have neurotoxic actions (Rothwell and Luheshi, 2000). Therefore, the suppression of both COX2 and IL1 β expression in neurons and glial cells, respectively, in response to tissue injury in *C/EBP β* ^{-/-} mice may be directly responsible for the observed reduction in hippocampal neuronal loss. Consistent with this idea, it has been shown previously that the promoter of both genes is directly regulated by *C/EBP β* (Caivano et al., 2001; Shirakawa et al., 1993; Sirois and Richards, 1993; Wadleigh et al., 2000; Wu et al., 2005; Yang et al., 2000; Zhang and Rom, 1993). The *C/EBP β* -deficient animals in this study displayed a dramatic reduction in neuronal degeneration in the CA1 and CA3 subfields of the hippocampus (Fig. 7), as well as a less marked disruption of these hippocampal neuronal fields 24 hours and 72 hours post injection. In this regard, it has been suggested previously that *C/EBP β* is

involved in several models of neurodegenerative disease (Bonin et al., 2004; Colangelo et al., 2002; Giri et al., 2002; Obrietan and Hoyt, 2004). Taken together, there is accumulating evidence that *C/EBP β* has a key role in the response to the excitotoxic damage that occurs as a result of brain injury or neurodegeneration.

In support of this concept, we have demonstrated previously that other genes involved in brain injury and inflammatory processes are upregulated by *C/EBP β* (Cortes-Canteli et al., 2004) in neuronal cells. Here, we have analyzed two of these genes *LCN2* and histidine decarboxylase, and our results show that the induction of both proteins was reduced in *C/EBP β* ^{-/-} mice after KA injection. *LCN2*, which is most probably a direct target of *C/EBP β* (Cortes-Canteli et al., 2004), encodes lipocalin 2, a protein that modulates immune and inflammatory responses (Logdberg and Wester, 2000), and it is also involved in brain damage (Anwaar et al., 1998). Prior reports have shown that *LCN2* is upregulated after ischemia (Anwaar et al., 1998; MacManus et al., 2004) and it has been implicated in the apoptosis that follows an inflammatory response (Devireddy et al., 2001). Since this gene is mainly expressed in the granular layer of the dentate gyrus (Fig. 6A), the same place where the induction in *C/EBP β* takes place, it is tempting to speculate that this gene could also be a mediator of the effects of *C/EBP β* after an excitotoxic insult. The other gene analyzed, histidine decarboxylase, converts L-histidine in the neurotransmitter histamine and its expression has been linked to brain inflammation processes (Musio et al., 2006). Increased histidine decarboxylase activity has been found in the hypothalamus after intracerebroventricular administration of LPS (Niimi et al., 1993). Interestingly, histamine has been implicated in the pathogenesis of Parkinson disease, contributing to the loss of dopaminergic neurons in 6-hydroxydopamine-lesioned rats (Liu et al., 2007), and increased histamine innervation has been described

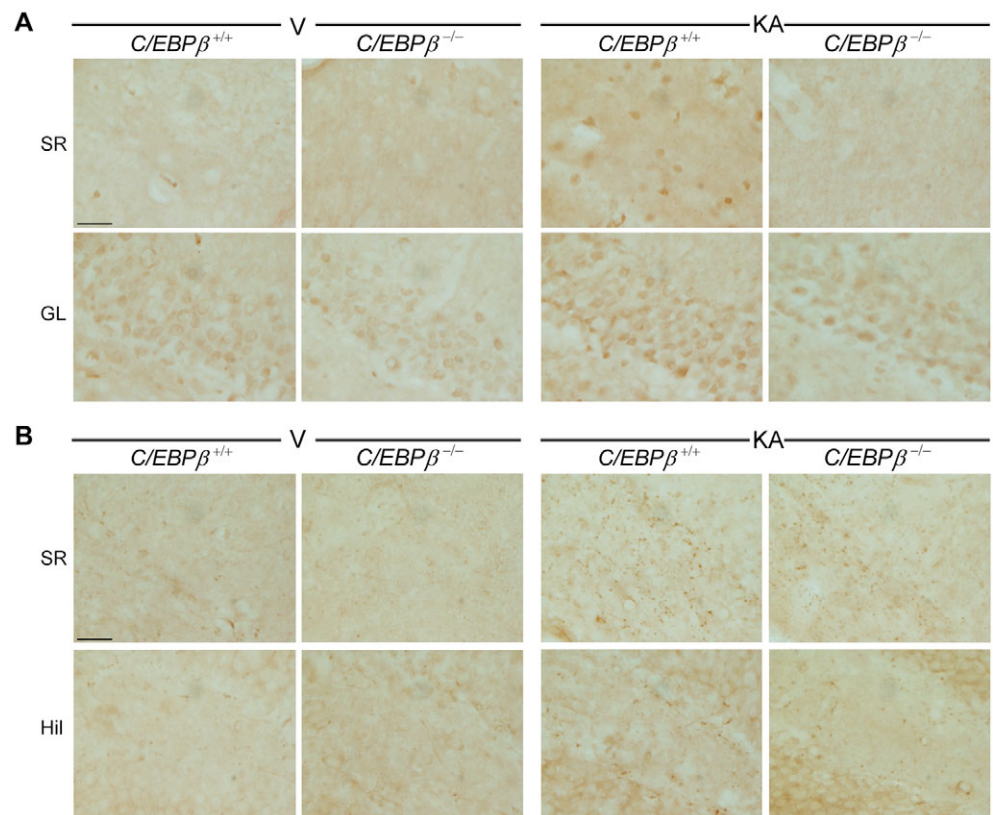


Fig. 6. Deficiency of *C/EBP β* decreases KA-induced expression of *LCN2* and histidine decarboxylase. (A,B) *C/EBP β* ^{+/+} and *C/EBP β* ^{-/-} adult mice were injected with vehicle (V) or KA and sacrificed 24 hours post-injection. Coronal sections (30 μ m) were stained with antibodies against (A) *LCN2* or (B) histidine decarboxylase. *C/EBP β* ^{-/-} mice exhibited lower levels of *LCN2* and histidine decarboxylase after KA injection, as compared with littermate controls. Representative results from three independent experiments are shown. Scale bars, 25 μ m. SR, stratum radiatum; GL, granular layer; Hil, hilus.

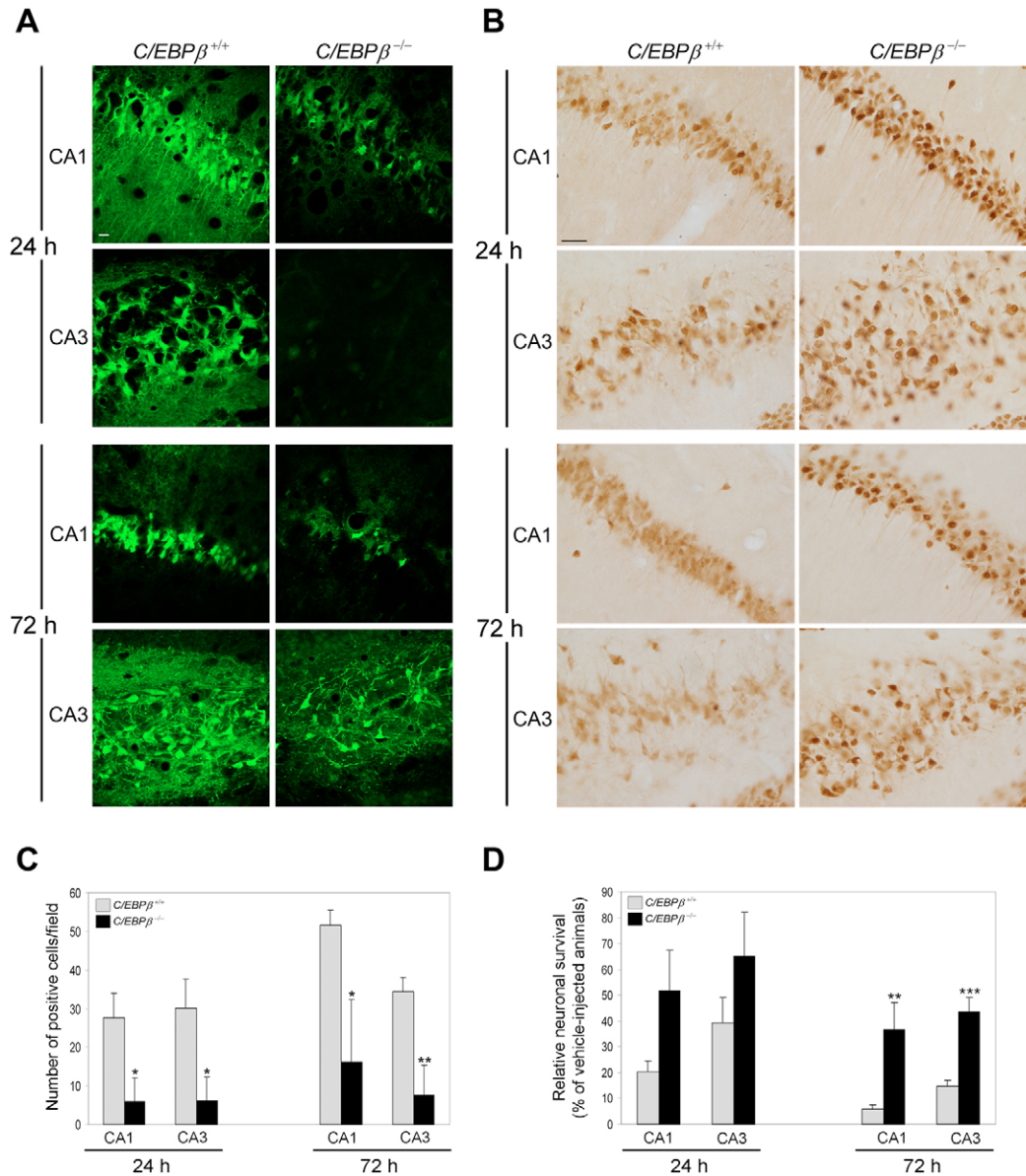


Fig. 7. Deficiency of *C/EBPβ* protects neurons from excitotoxic brain damage. (A,B) *C/EBPβ*^{+/+} and *C/EBPβ*^{-/-} adult mice were injected with KA and sacrificed 24 hours and 72 hours post-injection. Coronal sections (30 μm) were stained with (A) Fluoro-Jade B and (B) anti-NeuN antibody. *C/EBPβ*^{-/-} mice exhibited low levels of neuronal degeneration (as detected by Fluoro-Jade B staining) and a diminished loss of neurons in the CA1 and CA3 regions (as shown by NeuN staining) when compared with littermate controls. Scale bars, 10 μm (A) and 25 μm (B). (C) Quantification of degenerating neurons analyzed in the CA1 and CA3 areas of the hippocampus. Values represent the mean ± s.e. from five different animals and two independent sections per animal. (D) The extent of neuronal damage in the CA1 and CA3 areas of the hippocampus was quantified as described in Materials and Methods. Data were normalized against the mean values obtained from vehicle-injected mice. Values represent the mean ± s.e. from five different animals and two independent sections per animal. **P* ≤ 0.05, ***P* ≤ 0.01, ****P* ≤ 0.001, *C/EBPβ*^{-/-} mice versus *C/EBPβ*^{+/+} mice at each time point.

in the substantia nigra of Parkinson disease patients (Anichtchik et al., 2000). Another gene that we found to be upregulated by *C/EBPβ* in our microarray analysis was ornithine decarboxylase (Cortes-Canteli et al., 2004), which encodes an enzyme that has a key role in the polyamine biosynthetic pathway. Intriguingly, it has been shown that inhibition of polyamine synthesis abolishes neurodegeneration (Soulet and Rivest, 2003) and that increased polyamine metabolism is neurotoxic (Porcella et al., 1991). These observations collectively suggest that one of the mechanisms by which the loss of *C/EBPβ* in the CNS leads to the attenuation of neuronal injury after excitotoxic damage is by inhibiting the induction of *C/EBPβ*-dependent proinflammatory genes. In this regard, Kapadia et al. – by using a model of transient cerebral ischemia – have recently provided evidence for the upregulation of many of the genes previously identified by us to be involved in inflammatory processes and brain injury (Cortes-Canteli et al., 2004), including ornithine decarboxylase and LCN2 (Kapadia et al., 2006).

In summary, our findings suggest that *C/EBPβ* has a crucial role in the processes leading to the glial cell activation and resulting in neuronal damage that occurs in response to excitotoxic insults. Accordingly, this transcription factor merits serious consideration as a potential therapeutic target for the treatment of brain disorders.

Materials and Methods

Mice

C/EBPβ^{+/+} and *C/EBPβ*^{-/-} mice were generated from heterozygous breeding pairs, kindly provided by C. M. Croniger and R. W. Hanson (Case Western Reserve University, Cleveland, OH) (Screpanti et al., 1995). Genotypes were identified using genomic PCR, with DNA prepared from tail using the REDExtract-N-Amp™ tissue PCR kit (XNAT kit, Sigma, St Louis, MO). All procedures with animals were carried out in accordance with the European Communities Council, directive 86/609/EEC. Special care was taken to minimize animal suffering.

Primary cell culture and treatment

Rat primary astrocyte and microglial cultures were prepared as previously described (Luna-Medina et al., 2005). Mouse primary *C/EBPβ*^{+/+} and *C/EBPβ*^{-/-} astrocyte and microglial cultures were prepared as rat primary cells with some minor modifications. Each culture was generated from a single animal. Cultures were stimulated with

LPS (10 μ g/ml), staurosporine (50 nM), KA (100 μ M) or glutamate (100 μ M) and cells were harvested 24 hours later for evaluation of C/EBP β , IL1 β or COX2.

Western blot analysis

Cultured primary cells were collected in ice-cold RIPA buffer and equal quantities of total protein were separated by 10% SDS-PAGE. After electrophoresis, proteins were transferred to nitrocellulose membranes (Protran, Whatman, Dassel, Germany) and blots were probed with the indicated primary antibodies, as previously described (Cortes-Canteli et al., 2004). The following antibodies were used: DF77 polyclonal anti-rat C/EBP β (Cortes-Canteli et al., 2002), mouse monoclonal anti-mouse C/EBP β (clone A16, Abcam, Cambridge, UK), goat polyclonal anti-COX2 (Santa Cruz Biotechnologies, CA), monoclonal anti-IL1 β (clone SILK 6, Serotec, Düsseldorf, Germany), and anti- α -tubulin (Sigma). Secondary peroxidase-conjugated donkey anti-rabbit, rabbit anti-mouse and donkey anti-goat antibodies were from Amersham Biosciences (GE Healthcare, Buckinghamshire, England), Jackson ImmunoResearch (West Grove, PA) and Santa Cruz, respectively. Quantification analysis was performed using the Scion Image software. Values in the text are the mean of at least three experiments.

Immunocytochemistry

At the end of the treatment period cultures, grown on glass coverslips in 24-well cell culture plates, were washed with phosphate-buffered-saline (PBS) and processed for immunocytochemistry as previously described (Luna-Medina et al., 2005). Briefly, cells were fixed for 30 minutes with 4% paraformaldehyde at 25°C, and permeabilized with 0.5% Triton X-100 for 30 minutes at 37°C. After 1 hour incubation with the corresponding primary antibody, cells were washed with PBS and incubated with an AlexaFluor-labeled secondary antibody (AlexaFluor-488, AlexaFluor-546 or AlexaFluor-647; Molecular Probes, Leiden, The Netherlands) for 45 minutes at 37°C. Subcellular localization was determined using a Radiance 2100 confocal microscope (Carl Zeiss, Jena), with a 405-nm blue diode, a 488-nm Argon laser, a 543-Helion/Neon laser and a 633-nm red diode to excite 4',6-diamidino-2-phenylindole (DAPI), AlexaFluor-488, AlexaFluor-546 and AlexaFluor-647, respectively. Confocal microscope settings were adjusted to produce the optimum signal-to-noise ratio. Fluorescence analysis was performed using LaserPix software (Bio-Rad, Hercules, CA). To compare fluorescence signals from different preparations, settings were fixed for all samples within the same analysis. The following antibodies were used: DF77 polyclonal anti-rat C/EBP β (Cortes-Canteli et al., 2002), mouse monoclonal anti-mouse C/EBP β (clone A16, Abcam), polyclonal anti-COX2 (Cayman, Ann Arbor, MI), rabbit polyclonal anti-IL1 β (Abcam), rabbit polyclonal anti-GFAP (Dako, Glostrup, Denmark), mouse monoclonal anti-GFAP (Sigma), mouse monoclonal anti-rat CD11b (clone OX-42, Serotec), and rat monoclonal anti-mouse CD11b (clone M1/70.15, Serotec). Quantification of IL1 β -positive and COX2-positive cells was obtained using Image J program using a pixel size between 20 and ∞ .

Intrahippocampal injections

Adult male C/EBP β ^{+/+} and C/EBP β ^{-/-} mice ($n=5$ per group) were anaesthetized by intraperitoneal injection of ketamine (60 mg/kg) and medetomidine (0.125 mg/kg) and positioned in a stereotaxic apparatus (Kopf Instruments, CA). Kaicnic acid (0.25 μ g in 2.5 μ l PBS) was delivered unilaterally into the left hippocampus at a speed of 1 μ l/minute using the following coordinates from Bregma: posterior -2.0 mm; lateral -1.25 mm and a depth of 1.75 mm, according to the atlas of Paxinos and Franklin (Paxinos and Franklin, 2001). Control animals of the same age were injected with vehicle control. Mice were then housed individually to recover.

Immunohistochemistry

Twenty four or 72 hours after stereotaxic injection, animals were anaesthetized and perfused transcardially with 4% paraformaldehyde solution. Brains were removed, postfixed in the same solution at 4°C overnight, cryoprotected in the paraformaldehyde solution containing 30% sucrose, frozen, and 30 μ m coronal sections were obtained in a cryostat. Free-floating sections were processed for immunohistochemistry using the diaminobenzidine method or double-immunofluorescence analysis.

For the diaminobenzidine method, floating sections were immersed for 15 minutes in 3% H₂O₂ to inactivate endogenous peroxidase, and then blocked for 2 hours at room temperature (RT) in 5% normal goat serum (Vector Labs, Burlingame, CA) in PBS, containing 4% bovine serum albumin, 0.1 M lysine and 0.1% Triton X-100. Afterwards, the sections were incubated overnight at 4°C with the following primary antibodies: rat monoclonal anti-CD11b (Serotec) and rabbit polyclonal antibodies anti-GFAP (Dako), anti-COX2 (Cayman), anti-LCN2 (Davis et al., 1991) and anti-histidine decarboxylase (Eurodiagnostica, Arnhem, The Netherlands). After several rinses, sections were incubated for 1 hour with the correspondent biotinylated secondary antibody and processed following the avidin-biotin protocol (ImmunoPure Ultra-Sensitive ABC Peroxidase Staining kit, Pierce, Rockford, IL). Finally, the sections were washed, dehydrated, cleared in xylene and mounted with DePeX (Serva, Heidelberg, Germany). The slides were examined with a Zeiss Axiophot microscope equipped with an Olympus DP-50 digital camera. All the images shown correspond to the ipsilateral site of the hippocampus. The extent of microgliosis and

astrogliosis was quantified by counting the number of CD11b-positive cells in the hilus and the stratum radiatum and the number of GFAP-positive cells in the hilus and the molecular layer, respectively, of the hippocampus in twenty independent well-defined high-magnification ($\times 630$) fields per animal using a computer-assisted image analysis software (Soft Imaging System Corp.).

For COX2 double immunofluorescence, the protocol was similar to the one described above with some modifications. Briefly, the floating sections were blocked in PBS containing 0.25% Triton X-100 and 3% normal goat serum and incubated overnight with COX2 polyclonal antibody (Cayman). Then, AlexaFluor secondary antibody was added for 1 hour at RT together with Neurotrace fluorescent Nissl stain (Molecular Probes). Finally, the tissue was mounted with Vectashield (Vector Labs) mounting medium with DAPI (Vector Labs) to counterstain nuclei, and the sections were examined as described for immunocytochemistry. The sequential mode was used to acquire fluorescence images to avoid any interference from overlapping fluorescence.

For double immunofluorescence and for the diaminobenzidine method with mouse monoclonal antibodies, we used the Vector Mouse on Mouse immunodetection kit (Vector Labs) to eliminate background due to endogenous mouse immunoglobulins, following the manufacturer's instructions. The monoclonal antibodies used were anti-C/EBP β (clone A16, Abcam), anti-IL1 β (Serotec) and NeuN (Chemicon, Temecula, CA). For double-immunofluorescence analysis, we used rabbit polyclonal anti-GFAP antibody to detect astrocytes, Neurotrace fluorescent Nissl stain to identify neurons, and Texas-Red-labeled *Lycopersicon esculentum* (tomato) lectin (Vector Labs) to identify microglial cells. Neuronal integrity was assessed by counting the percentage of NeuN-positive cells in the CA1 and CA3 regions of the hippocampus in 20 independent well-defined high-magnification ($\times 630$) fields per animal, as described above.

Fluoro-Jade staining

To evaluate neuronal degeneration, Fluoro-Jade B staining was used (Schmued et al., 1997). Briefly, sections were mounted on gelatin-coated slides and then dried at RT. After that, the slides were immersed in 100% alcohol, followed by 70% alcohol and distilled water. Slides were then incubated while shaking gently for 15 minutes in 0.06% KMnO₄ solution, followed by 30 minutes in staining solution (0.001% Fluoro-Jade B dye, Chemicon) in the dark. After staining, sections were rinsed in distilled water, dried, immersed in xylene and mounted with DePeX (Serva). Images were analyzed using a confocal microscope, as detailed above. The degree of neuronal degeneration was quantified by counting the number of Fluoro-Jade B-stained neurons in the CA1 and CA3 regions of the hippocampus. As described above, at least 20 independent well-defined high-magnification ($\times 630$) fields per animal were analyzed.

Statistical determinations

Data are given as the mean \pm s.e. of at least five different animals per group. Comparisons of different groups of animals were performed using the Student's *t*-test, with $P \leq 0.05$ being considered significant.

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