CCAAT Enhancer Binding Protein β deficiency provides cerebral protection following excitotoxic injury.

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Summary

The CCAAT/Enhancer-binding protein β (C/EBPβ) was first identified as a regulator of differentiation and inflammatory processes in adipose tissue and liver. Although C/EBPβ was initially implicated in synaptic plasticity, its function in the brain remains largely unknown. We have previously shown that C/EBPβ regulates the expression of genes involved in inflammatory processes and brain injury. Here, we have demonstrated that the expression of C/EBPβ is notably increased in the hippocampus in a murine model of excitotoxicity. Mice lacking *C/EBPβ* 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/EBPβ in the pathways leading to excitotoxicity-mediated damage and suggest that inhibitors of this transcription factor should be evaluated as possible neuroprotective therapeutic agents.

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 play 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 glia, can contribute noticeably to the expansion of brain injury and the increased loss of neurons (Oprica et al., 2003).

Excitotoxicity can be triggered by the 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 Ca^{2+} 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/EBPβ) is a member of the transcriptional factor family consisting of six functionally and structurally related basic leucine zipper DNA-binding proteins (Vinson et al., 1989). The C/EBPβ protein 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/EBPβ 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 may have important functions in the brain. It has been shown that C/EBPβ plays 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 a MEK-C/EBP pathway as being essential for the differentiation of cortical progenitor cells into postmitotic neurons (Menard et al., 2002). We have demonstrated that C/EBPβ serves as a critical factor in neuronal differentiation (Cortes-Canteli et al., 2002). In addition, using microarray analysis in neuronal cells overexpressing C/EBPβ, 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/EBPβ involvement in brain injury and our prior speculation that this transcription factor may play a role in this process, we sought to address the function of C/EBPβ 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* $^{+/+}$ mice. We have previously demonstrated that cell lines stably transfected with *C/EBPβ* exhibited 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, the expression of *C/EBPβ* was also up-regulated. These observations have prompted us to investigate whether the expression of the endogenous *C/EBPβ* gene in primary cultures of glia could be regulated after neural injury. In order to address this question we first investigated the expression of C/EBPβ in glial cultures from *C/EBPβ*$^{+/+}$ mice, in response to different agents known to cause neural damage through different mechanisms. The C/EBPβ signal in these experiments was significantly induced by lipopolysaccharide (LPS), the kinase inhibitor staurosporine, KA and glutamate. As shown in Fig. 1A, C/EBPβ basal expression 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- and 1.5-fold, respectively, compared to non-treated cultures). Addition of LPS or staurosporine to astrocytes also potently induced C/EBPβ expression (4.2- and 2.2-fold, respectively, compared to non-treated cultures), and a lower increase was observed 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 in the nucleus, although the induction was lower than the one 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 levels was also observed in rat primary cultures after treatment with LPS or staurosporine (see Supplementary Fig. S1).

Less pro-inflammatory protein induction in *C/EBPβ* $^{-/-}$ primary microglial cultures. To further evaluate the possible role of C/EBPβ in the inflammatory response in neural tissues, we next performed in vitro experiments with 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 (IL-1β) in response to LPS and KA. The expression levels of the pro-inflammatory enzyme cyclooxygenase type-2 (COX-2) were also slightly reduced in protein lysates from *C/EBPβ* $^{-/-}$ microglial cultures. These data were further confirmed by confocal analysis. As shown in Fig. 2, the number of microglial cells expressing IL-1β (Fig. 2B) or COX-2 (Fig. 2C) was reduced in cultures established from *C/EBPβ*-deficient animals. Quantification of both western blot and immunocytochemistry analysis revealed that IL-1β expression was significantly reduced (~50%) in *C/EBPβ*-deficient microglial cultures after treatment with both LPS and KA. A decrease in COX-2 levels (~15%) was also observed, although in this case the reduction was not statistically significant. Together, these data suggested that C/EBPβ helps to modulate the induction of proinflammatory mediators in microglial cells.

To ascertain a possible colocalization of IL-1β and COX-2 with C/EBPβ, a multiple labeling approach was performed. As depicted by Fig. 2B and C, both IL-1β and COX-2 colocalized with C/EBPβ in the majority of microglial cells established from wild type animals. These data provide evidence that the up-regulation of the gene encoding *C/EBPβ* triggered by LPS or KA takes place essentially within the same cells where induction of pro-inflammatory agents is observed.

The expression level of C/EBPβ protein increases in the hippocampus with excitotoxic injury. We next investigated the in vivo role of C/EBPβ in a well-established model of excitotoxic brain injury. Adult mice received intrahippocampal injections of either vehicle or KA and, 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/EBPβ on the ipsilateral side of the hippocampus of wild type mice when compared to vehicle-injected controls at 24 hours and 72 hours after surgery (Fig. 3A). Activation was noted mainly in the dentate gyrus and, as anticipated, was completely absent in *C/EBPβ*-deficient mice (Fig. 3C), a finding that underscores the utility of the A16 antibody for the in vivo immunohistochemical studies. In the KA-injected animals occasional C/EBPβ-positive cells were also detected within the CA1 and CA3 subfields (data not shown), and in the stratum radiatum. In order to identify the cell type-specificity of C/EBPβ activation, we next performed a double-labeling analysis using Neurotrace, anti-glial fibrillary acidic protein (GFAP), and tomato lectin to identify neurons, astrocytes, and microglia, respectively. C/EBPβ immunoreactivity was detected in the nuclei of granule neurons of the dentate gyrus as well as in glial cells 24 h after KA injection (Fig. 3B). This increase was more pronounced 72 hours post-injection in

granule neurons whilst at this time a lower percentage of C/EBPβ-active cells in the hippocampus of KA-treated animals were positive for GFAP or tomato lectin.

***C/EBPβ*-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/EBPβ* $^{-/-}$ mice exhibited reduced astrocytic activation and astrogliosis (Fig. 4A). Twenty-four hours following 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. In contrast, the *C/EBPβ* $^{-/-}$ 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 $GFAP^+$ cells, both the hilus and the molecular layer of the hippocampus of *C/EBPβ* $^{-/-}$ mice, compared with *C/EBPβ* $^{+/+}$ animals, seventy two hours after KA injection (Fig 4C). In addition, the evaluation of CD11b staining for microglial infiltration revealed a considerably larger response in *C/EBPβ* $^{+/+}$ animals than in their *C/EBPβ* $^{-/-}$ counterparts (Fig. 4B). Significantly lower levels of CD11b signal were detected in the hippocampus of *C/EBPβ* $^{-/-}$ animals 24 hours after KA injection (Fig 4D). Although the number of $CD11b^+$ cells was not statistically different between both groups of animals at 72 hours, *C/EBPβ* $^{+/+}$ mice exhibited a strong microglial activation as evidenced by morphological changes in these cells, which included swelling of the cell somas as well

as a thickening and retraction of the cell processes (see insets in Fig. 4B), features characteristic of reactive microglia. In contrast, the number of activated microglial cells in *C/EBPβ* $^{-/-}$ mice at this time was significantly lower than that found in their *C/EBPβ*$^{+/+}$ littermates (data not shown). The CD11b^{+} cells observed in Fig. 4B can also represent infiltrated macrophages, since it is well known that brain injury promotes the extravasation of these cells towards the sites of brain injury. In this regard, it has been shown that C/EBPβ plays an important role in macrophage activation, as their function is severely compromised in *C/EBPβ*$^{-/-}$ mice (Screpanti et al., 1995).

Impaired pro-inflammatory protein induction in *C/EBPβ*-deficient mice. We also evaluated the production of the cytokine IL-1β, which is secreted by glia in response to KA administration and known to enhance neuronal death. IL-1β was elevated in the hippocampus of *C/EBPβ* $^{+/+}$ mice 72 hours after injection (Fig. 5A), although a slight increase was also detected at 24 hours (data not shown). In the *C/EBPβ*$^{-/-}$ mice, no up-regulation of IL-1β was observed, consistent with a lack of glial activation. These results are in agreement with those obtained in primary cultures of *C/EBPβ*$^{-/-}$ microglial cells (Fig. 2A,B) where a reduced induction of IL-1β in response to LPS and KA treatment was observed. The IL-1β^{+} 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 IL-1β and also bound to the tomato lectin (Fig. 5B). We did not identify any cells that coexpressed IL-1β and the neuronal marker Neurotrace.

In addition, we also studied the accumulation of the inducible COX-2 enzyme. Overexpression of COX-2 in neural cells appears central to many neuroinflammatory conditions. As shown in Fig. 5C, the protein levels of COX-2 were clearly increased in the hippocampus at both 24 and 72 hours following KA injection of *C/EBPβ* $^{+/+}$ animals, relative to vehicle-injected controls (not shown). COX-2 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 COX-2 levels was also observed in some of the KA-treated *C/EBPβ* $^{-/-}$ animals (mainly at 24 hours), but not as robustly or as persistently as that seen in the *C/EBPβ* $^{+/+}$ mice. Using a double-labeling analysis we found that all COX-2-positive cells in wild-type animals were indeed neurons (Fig. 5D). In these cells, the intracellular distribution of the immunoreactivity was predominantly perinuclear, which is consistent with the known subcellular localization of this enzyme (Nogawa et al., 1997). COX-2 did not appear to colocalize with the microglial marker tomato lectin or with the astrocytic marker GFAP (data not shown).

Next, we analyzed the expression of 24p3/LCN2 and histidine decarboxylase, two genes up-regulated by C/EBPβ (Cortes-Canteli et al., 2004) and probably implicated in inflammation and brain injury. Fig. 6 shows that the protein levels of both 24p3 and histidine decarboxylase were increase 24 hours after treatment with KA and that this increase, as previously observed for IL-1β and COX-2, was markedly reduced in the hippocampus of *C/EBPβ* $^{-/-}$ animals.

***C/EBPβ*-deficient mice are resistant to excitotoxin-mediated neuronal degeneration.** In order to better understand the role of *C/EBPβ* in vivo, we examined

the sensitivity of mice lacking *C/EBPβ* to KA-induced neurodegeneration. To determine whether C/EBPβ expression was associated with CA1 and CA3 pyramidal cell death after excitotoxicity, hippocampal sections were stained with Fluoro-Jade B (Schmued et al., 1997) in order to detect degenerating neurons or NeuN 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. In contrast, *C/EBPβ* $^{+/+}$ mice displayed stronger Fluoro-Jade B fluorescence in both the CA1 and CA3 subfields of the hippocampus 24 (4.6- and 5-fold, respectively) and 72 (3- and 4.5-fold, respectively) hours after KA injection, compared with the moderate damage observed in *C/EBPβ* $^{-/-}$ animals (Fig. 7A,C). The apparent resistance to injury in *C/EBPβ* $^{-/-}$ mice was also supported by the higher percentage of healthy cells shown by NeuN staining (Fig. 7B). Quantitative studies showed an increase of 6.5- and 3.0-fold in the number of neurons in the CA1 and CA3, respectively, subfields of the hippocampus in *C/EBPβ* $^{-/-}$ mice, compared to control *C/EBPβ* $^{+/+}$ littermates, 72 hours after KA injection (Fig 7D).

Discussion

In the present study we have shown that mice lacking *C/EBPβ* are less susceptible to glial activation and neuronal damage following KA exposure. These results clearly establish C/EBPβ as a factor required for the development of excitotoxic brain injury. Excitotoxic brain damage is considered one of the major mechanisms by which neurons die in the adult central nervous system and 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/EBPβ should be considered as a possible therapeutic target in brain injury and neurodegenerative disorders where excitotoxic neuronal cell death and inflammation processes are involved.

The first clues suggesting a possible role for C/EBPβ 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/EBPβ 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/EBPβ induces terminal differentiation and cell death in neuroblastoma N2A cells (Cortes-Canteli et al., 2002) and also up-regulates the expression of genes previously 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

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 IL-1β is markedly reduced in glial cultures from *C/EBPβ* $^{-/-}$ mice, suggesting that the expression of this gene could be regulated by C/EBPβ. In this context, C/EBPβ has been previously shown to regulate expression of inflammatory mediators and pro-inflammatory cytokines, including IL-1β and COX-2, in different tissues (Gorgoni et al., 2001; Yang et al., 2000), and C/EBPβ response elements have been described in the *IL-1β* and *COX-2* genes (Wadleigh et al., 2000; Yang et al., 2000).

Excitotoxicity proceeds through a complex signaling pathway that includes the participation of numerous signaling molecules. The identification of the genes that are activated or repressed in specific responses to brain injury, and 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 intrahippocampal injection of KA resulted in a strong induction of C/EBPβ, which 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, with 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

significant activation of astrocytes and microglial cells and the increased expression of classic pro-inflammatory agents, likely through direct regulation by C/EBPβ (Wadleigh et al., 2000; Yang et al., 2000). This hypothesis is supported by our data showing that the morphological changes associated with the activation of astrocytes and microglial cells, as well as the induction of IL-1β and COX-2, are markedly reduced in *C/EBPβ* null mice following KA injection.

The regulation of pro-inflammatory mediators is generally considered a key mechanism in neuronal cell death. The enzyme, COX-2, is involved in the pathogenesis of multiple neurological disorders associated with inflammation (Giovannini et al., 2003; Teismann et al., 2003) and local increases in COX-2 expression in vivo have been associated with inflammation, seizures and ischemia (Nogawa et al., 1997; Strauss et al., 2000). In addition, an increase in IL-1β expression has been observed in several types of brain injury including excitotoxicity (Rothwell and Luheshi, 2000), where it has been shown that exogenous IL-1β enhances chemically induced seizures in rats (Vezzani et al., 2002). IL-1β 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 COX-2 and IL-1β expression in neurons and glial cells, respectively, in response to tissue injury in *C/EBPβ* $^{-/-}$ mice may be directly responsible for the observed reduction in hippocampal neuronal loss. Consistent with this idea, it has been previously shown that the promoter of both genes is directly regulated by C/EBPβ (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/EBPβ*-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 and 72 hours post-injection. In this regard, it has been previously suggested that C/EBPβ may be 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 an accumulating body of evidence that C/EBPβ may play 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 previously demonstrated that other genes involved in brain injury and inflammatory processes are up-regulated by C/EBPβ (Cortes-Canteli et al., 2004) in neuronal cells. In this work, we have analyzed two of these genes, *24p3/LCN2* and histidine decarboxilase, and the results obtained show that the induction of both proteins after KA injection was reduced in *C/EBPβ* $^{-/-}$ mice. The *24p3* gene, which is most likely a direct target of C/EBPβ (Cortes-Canteli et al., 2004), codes for a lipocalin that modulates the immune and inflammatory responses (Logdberg and Wester, 2000), and it is also involved in brain damage. Prior reports have shown that lipocalin 24p3 is up-regulated 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/EBPβ takes place, it is tempting to speculate that this gene could also be a mediator of the effects of C/EBPβ 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 in the substantia nigra of Parkinson Disease patients (Anichtchik et al., 2000). Another gene that we found to be up-regulated by C/EBPβ in our microarray analysis was ornithine decarboxilase (Cortes-Canteli et al., 2004), an enzyme that plays a key role in the polyamine biosynthetic pathway. Intriguingly, it has been shown that the inhibition of polyamine synthesis abolished 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, using a model of transient cerebral ischemia, have recently provided evidence for the up-regulation of many of the genes previously identified in our studies (Cortes-Canteli et al., 2004), including ornithine decarboxylase and *24p3* (Kapadia et al., 2006).

In summary, our findings have suggested that C/EBPβ plays a critical role in the processes leading to the glial activation and resulting neuronal damage that occurs in response to an excitotoxic insult. 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 Dr. C.M. Croniger and Dr. R.W. Hanson (Case Western Reserve University, Cleveland, Ohio) (Screpanti et al., 1995). Genotypes were identified based on genomic PCR with DNA prepared from tail using the REDExtract-N-Amp^{TM} 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 μg/ml), staurosporine (50 nM), KA (100 μM) or glutamate (100 μM) and cells were harvested 24 hours later for evaluation of C/EBPβ, IL-1β, and COX-2.

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 antibodies used were the following: DF77 polyclonal anti-rat C/EBPβ (Cortes-Canteli et al., 2002), mouse monoclonal anti-mouse C/EBPβ (clone A16, Abcam, Cambridge, UK), goat polyclonal anti-COX-2 (Santa Cruz Biotechnologies, CA), monoclonal anti-IL-1β

(clone SILK 6, Serotec, Dusseldorf, Germany), and anti-α-tubulin (Sigma) antibodies. 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 average of at least three experiments.

Immunocytochemistry. At the end of the treatment period the cultures, grown on glass cover-slips 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 37ºC. After 1 hour incubation with the corresponding primary antibody, cells were washed with PBS and incubated with an Alexa-labeled secondary antibody (Alexa 488, Alexa 546 or Alexa 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-blue diode, a 488-Argon laser, a 543-Helio/Neon laser and a 633-red diode to excite 4',6-diaminidine-2-phenylindole (DAPI), Alexa 488, 546 and 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 antibodies used were the following: DF77 polyclonal anti-rat C/EBPβ (Cortes-Canteli et al., 2002), mouse monoclonal anti-mouse C/EBPβ (clone A16, Abcam), polyclonal anti-COX-2 (Cayman, Ann Arbour, MI), rabbit polyclonal anti- IL-1β (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 IL-1β^+ and COX-2^+ cells was done using Image J program using a pixel size between 20 and ∞.

Intrahippocampal injections. Adult male *C/EBP*$\beta^{+/+}$ and *C/EBP*$\beta^{-/-}$ 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). Kainic acid (0.25 μg in 2.5 μl PBS) was delivered unilaterally into the left hippocampus at a speed of 1 μl/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. The mice were then housed individually to recover.

Immunohistochemistry. Twenty four or seventy two hours after stereotaxic injection, the animals were anaesthetized and perfused transcardially with 4% paraformaldehyde solution. The brains were removed, postfixed in the same solution at 4ºC overnight, cryoprotected in the paraformaldehyde solution containing 30% sucrose, frozen, and 30 μm 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% H_2O_2 to inactivate endogenous peroxidase, and then blocked for 2 hours at 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 4ºC with the following primary antibodies: rat monoclonal anti-CD11b (Serotec) and rabbit polyclonal antibodies anti-GFAP (Dako), anti-COX-2 (Cayman), anti-24p3 (Davis et al. 1991) and anti-histidine decarboylase (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 (x630) fields per animal using a computer-assisted image analySIS software (Soft Imaging System Corp.).

For the COX-2 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 COX-2 polyclonal antibody (Cayman). Then, Alexa 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-immunofluorescences and for the diaminobenzidine method with mouse monoclonal antibodies, we used the Mouse on Mouse immunodetection kit (Vector Labs) to eliminate background due to endogenous mouse immunoglobulins, following the manufacture's instructions. The monoclonal antibodies used were anti-C/EBPβ (clone A16, Abcam), anti-IL-1β (Serotec), and anti-NeuN (Chemicon, Temecula, CA) antibodies. For double-immunofluorescence analysis, we used rabbit polyclonal anti-GFAP antibody to detect astrocytes, Neurotrace Fluorescent Nissl stain (Molecular Probes) 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 twenty independent well-defined high-magnification (x630) fields per animal, as described above.

Fluoro-Jade staining. To evaluate neuronal degeneration, Fluoro-Jade B staining was used (Schmued et al., 1997). Briefly, the sections were mounted on gelatin-coated slides and then let them dried at RT. After that, the slides were immersed in 100% alcohol, followed by 70% alcohol and distilled water. Then the slides were incubated shaking gently for 15 minutes in 0.06% potassium permanganate solution followed by 30 minutes in dark in the staining solution (0.001% Fluoro-Jade B dye, Chemicon). After staining, the sections were rinsed in distilled water, let them dry, immersed in xylene and mounted with DePeX (Serva). Images were analyzed by 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 twenty independent well-defined high-magnification (x630) fields per animal were analyzed.

Statistical determinations. The data shown are the means±s.e. of at least five different animals/group. Statistical comparisons for significance between different groups of animals were performed using the Student's test, with $p \leq 0.05$.

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Figure legends

Figure 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. 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) and anti-GFAP antibodies to detect microglia or astrocytes, respectively. To confirm the specificity of the anti-C/EBPβ antibody used, 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 3 experiments are shown. Scale bar, 10 μm. Nuclei were counterstained by DAPI (blue). B, basal; LPS, lipopolysaccharide; St, staurosporine; KA, kainic acid; Glu, glutamate.

Figure 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 IL-1β and COX-2 expression in *C/EBPβ* $^{-/-}$ microglial culture after treatment with LPS and KA for 24 hours, compared to cultures established from *C/EBPβ* $^{+/+}$ mice. **B, C**, Primary microglial cultures prepared from *C/EBPβ* $^{+/+}$ and *C/EBPβ*$^{-/-}$ mice were treated as in (**A**), and the expression of C/EBPβ (green), IL-1β (**B**, blue) and COX-2 (**C**, blue) was evaluated using immunofluorescent detection and

confocal microscopy using specific antibodies, as described in the Materials and Methods. The third panels show colocalization of C/EBPβ with IL-1β (B) or COX-2 (C). Microglial marker CD11b is shown in red. Representative results from 3 experiments are shown. Scale bar, 10 μm. B, basal; KA, kainic acid; LPS, lipopolysaccharide.

Figure 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 kainic acid 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 and 72 hours post-injection, compared with vehicle-injected controls. Images are representative for experiments from 5 animals. Scale bar, 25 μm. **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 and 72 hours after KA injection some astrocytes and microglia can be found that express C/EBPβ (arrows). Scale bar, 10 μm **C**, Immunohistochemical analyses of coronal brain sections prepared from *C/EBPβ* $^{-/-}$ mice 24 and 72 hours after KA injection. As expected, *C/EBPβ* $^{-/-}$ mice did not present any C/EBPβ staining. Images are representative for experiments from 5 animals. Scale bar, 25 μm.

Figure 4. Deficiency of C/EBPβ ameliorates KA-induced gliosis. *C/EBPβ* $^{+/+}$ and *C/EBPβ*$^{-/-}$ adult mice were injected with vehicle (V) or KA and sacrificed 24 and 72 hours post-injection. Coronal sections (30 μm) were stained with antibodies against GFAP (**A**) and CD11b (**B**) to detect astrocytes and microglia, respectively. A significant decrease in both astrogliosis and microgliosis is observed in *C/EBPβ* $^{-/-}$ mice. **A**, A substantial gliosis is observed in *C/EBPβ* $^{+/+}$ mice after KA-injection, which is prevented in *C/EBPβ* $^{-/-}$ mice **B**, Immunohistochemical detection using anti-CD11b antibodies reveals highly ramified microglia in the hippocampus of KA-treated *C/EBPβ* $^{-/-}$ mice. In contrast, in *C/EBPβ* $^{+/+}$ mice the activated microglial cells exhibit shorter and thicker processes and larger cell bodies. Insets are shown at higher magnification in the upper right corner of the images corresponding to *C/EBPβ* $^{+/+}$ mice. 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 represent the mean±s.e. from five different animals and two independent sections/animal. #, $p \leq 0.05$, ##, $p \leq 0.01$ and ###, $p \leq 0.001$, vehicle *vs* KA-injected animals. *,$p \leq 0.05$ and ***,$p \leq 0.001$, 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 μm.

Figure 5. Deficiency of *C/EBPβ* impairs KA induction of pro-inflammatory mediators. *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 IL-1β (**A, B**) and COX-2 (**C, D**). Neurotrace was used to identify neurons and anti-GFAP and tomato lectin to detect astrocytes and microglia, respectively. IL-1β expression,

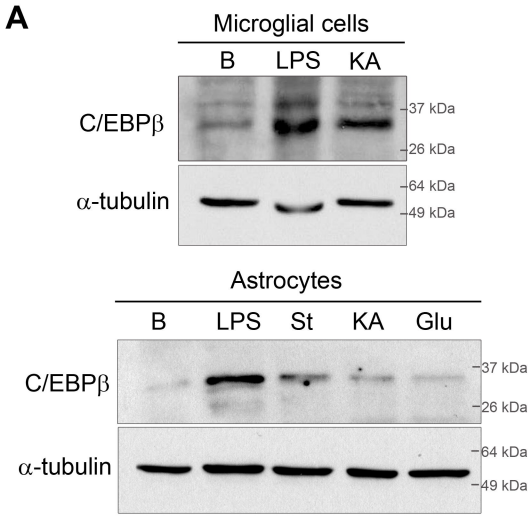
mainly localized in the hilus (Hil) and the molecular layers (ML) surrounding the dentate gyrus, was completely absent in the *C/EBPβ* $^{-/-}$ mice after KA injection, compared to their *C/EBPβ*$^{+/+}$ littermates. COX-2 immunoreactivity was mainly detected in the stratum radiatum (SR) and the granule cell layer of the dentate gyrus (GL). In contrast to controls, COX-2 immunoreactivity was noticeably reduced in the *C/EBPβ* $^{-/-}$ mice. Confocal analysis of IL-1β (**B**) and COX-2 (**D**) subcellular localization indicate that IL-1β is expressed primarily in glial cells whereas COX-2 immunostaining is restricted to neurons. Images are representative for experiments from 5 animals. Scale bars represent 25 μm (**A, C**) and 10 μm (**B, D**).

Figure 6. Deficiency of *C/EBPβ* decreases KA-induced expression of 24p3/LCN2 and histidine decarboxylase. *C/EBPβ* $^{+/+}$ and *C/EBPβ* $^{-/-}$ adult mice were injected with vehicle (V) or KA and sacrificed 24 hours post-injection. Coronal sections (30 μm) were stained with antibodies against 24p3 (**A**) and histidine decarboxylase (**B**). *C/EBPβ* $^{-/-}$ mice exhibited lower levels of 24p3 and histidine decarboxylase after KA injection, as compared to littermate controls. Representative results from 3 independent experiments are shown. Scale bars, 25 μm. SR, stratum radiatum; GL, granular layer; Hil, hilus.

Figure 7. Deficiency of *C/EBPβ* protects neurons from excitotoxic brain damage. *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 Fluoro-Jade B (**A**) and anti-NeuN antibody (**B**). *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 to littermate controls. Scale bars represent 10 μm (**A**) and 25 μm (**B**). **C**, Quantification of the number 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/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 given by vehicle-injected mice. Values represent the mean±s.e. from five different animals and two independent sections/animal. *,$p \leq 0.05$; **,$p \leq 0.01$; ***,$p \leq 0.001$, versus vehicle-injected animals at each time point.

Figure 1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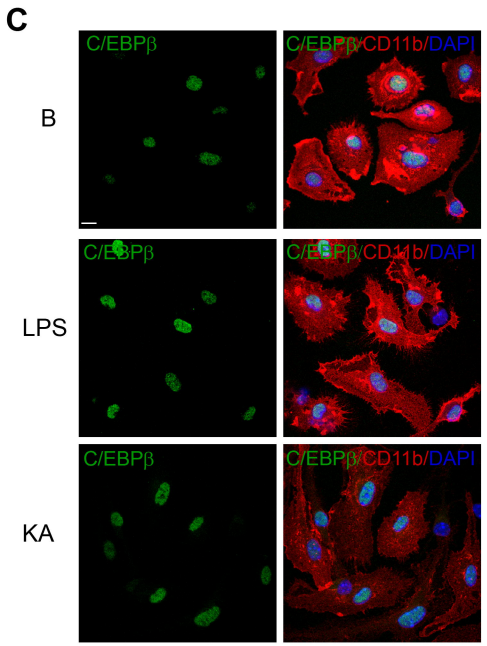
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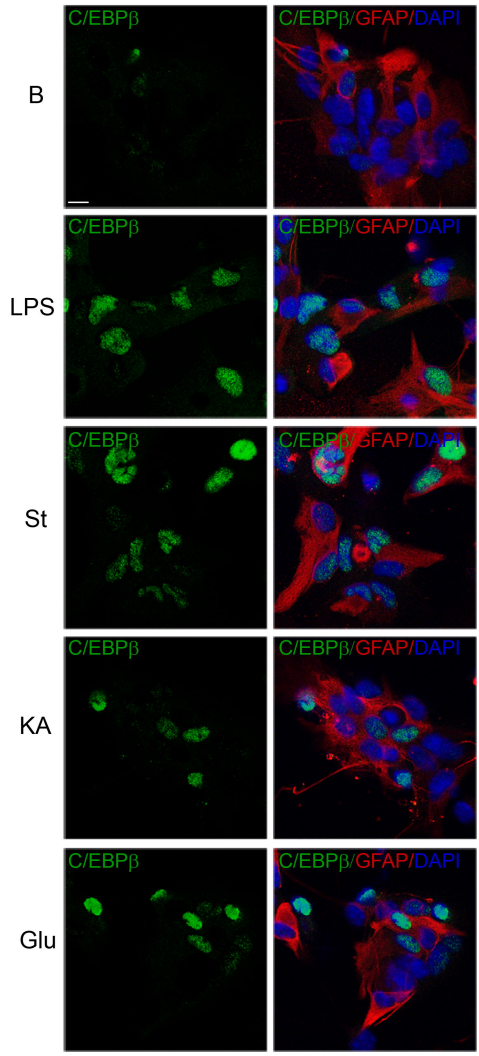
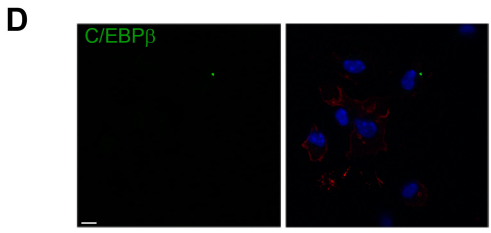
C/EBPβ
C/EBPβ/CD11b/DAPI


B
B
LPS
St
KA
Glu
C/EBPβ
C/EBPβ/GFAP/DAPI


D
C/EBPβ

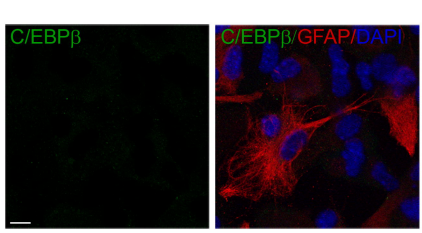

E
C/EBPβ
C/EBPβ/GFAP/DAPI

Figure 2

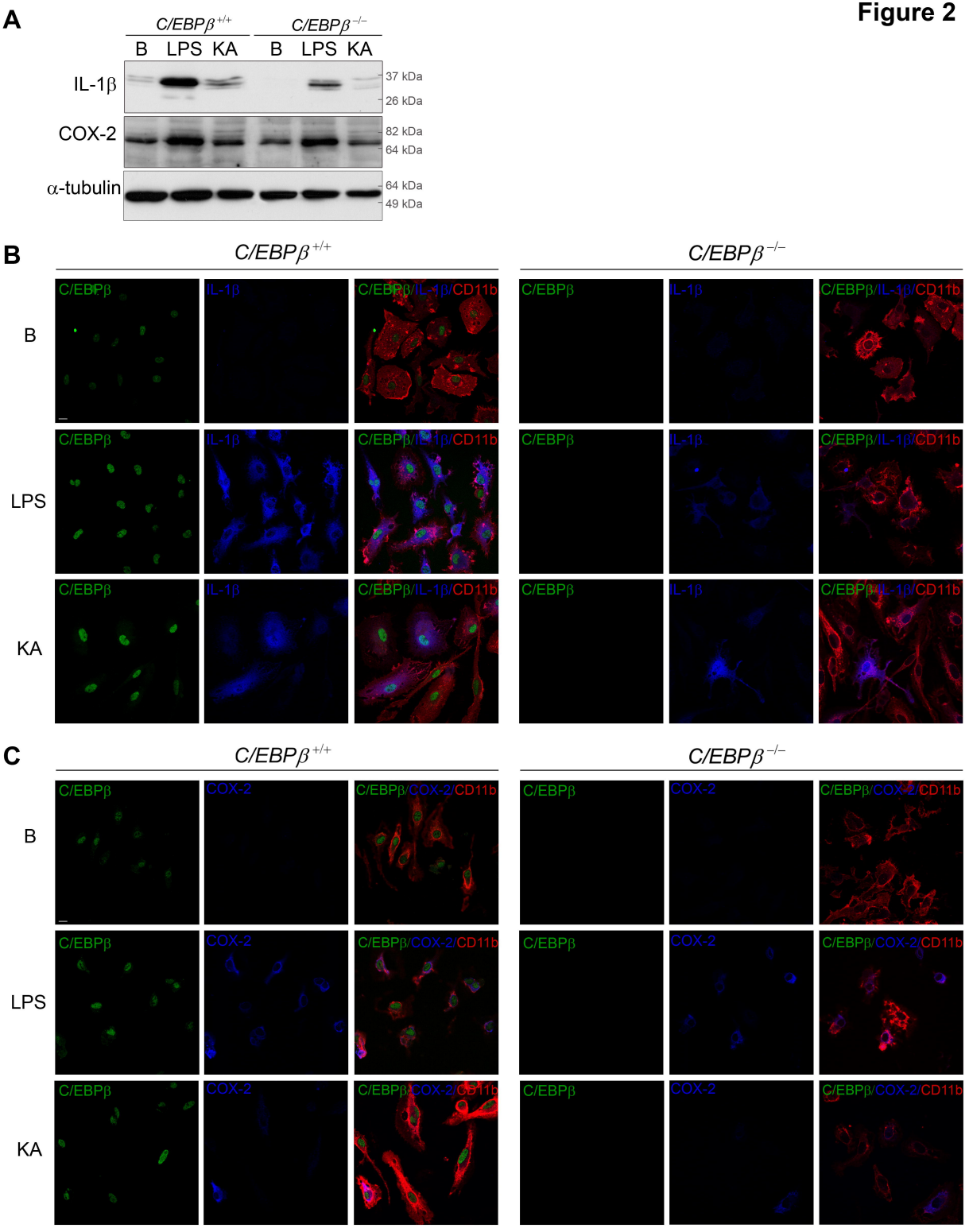

A
C/EBPβ+/+
C/EBPβ−/−
B LPS KA B LPS KA
IL-1β
COX-2
α-tubulin
37 kDa
26 kDa
82 kDa
64 kDa
64 kDa
49 kDa
B
C/EBPβ+/+
C/EBPβ−/−
B
LPS
KA
C/EBPβ
IL-1β
C/EBPβ/IL-1β/CD11b
C
C/EBPβ+/+
C/EBPβ−/−
B
LPS
KA
C/EBPβ
COX-2
C/EBPβ/COX-2/CD11b

Figure 3

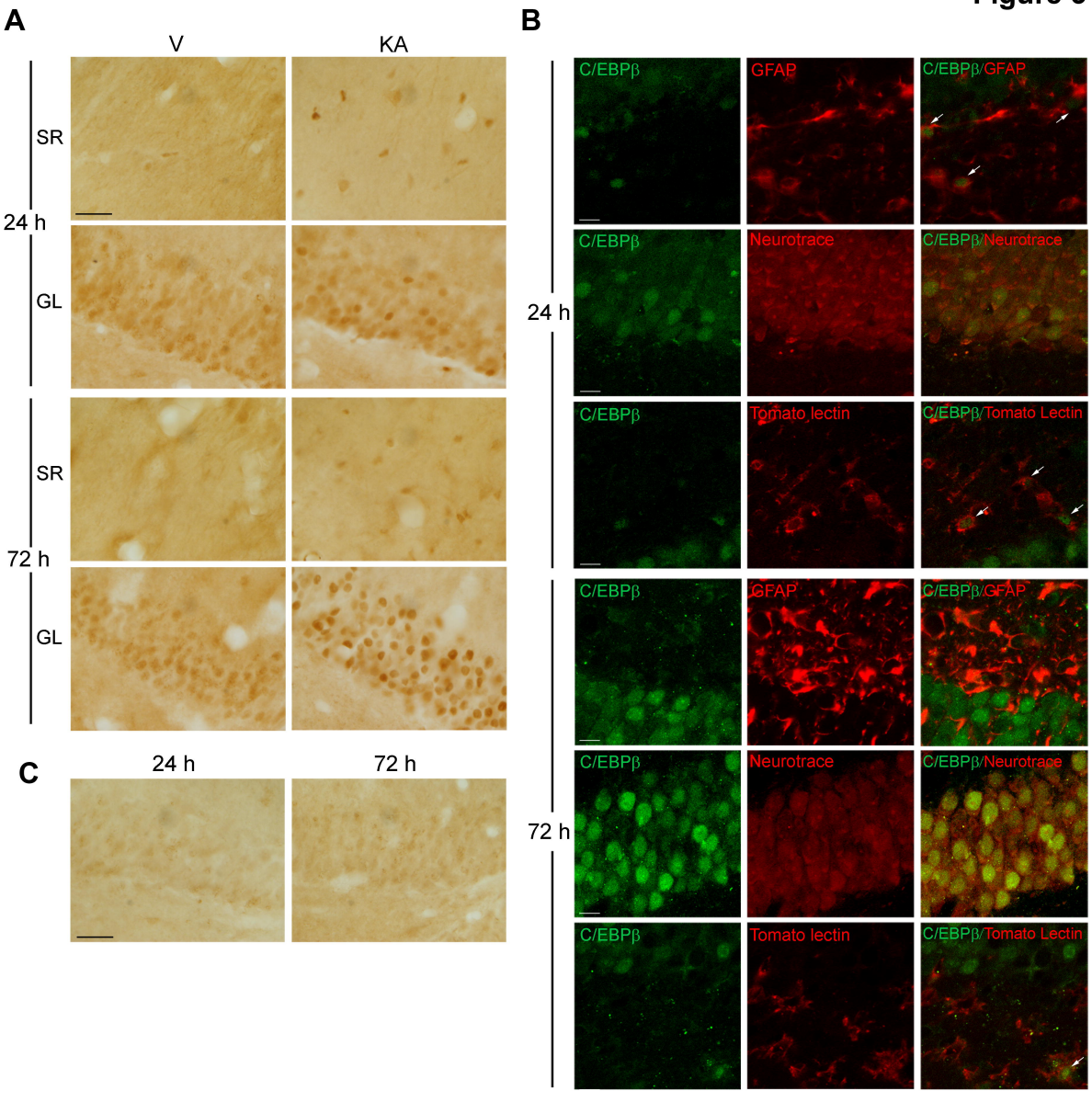

A
V
KA
SR
24 h
GL
SR
72 h
GL
B
24 h
C/EBPβ
GFAP
C/EBPβ/GFAP
C/EBPβ
Neurotrace
C/EBPβ/Neurotrace
C/EBPβ
Tomato lectin
C/EBPβ/Tomato Lectin
72 h
C/EBPβ
GFAP
C/EBPβ/GFAP
C/EBPβ
Neurotrace
C/EBPβ/Neurotrace
C/EBPβ
Tomato lectin
C/EBPβ/Tomato Lectin
C
24 h
72 h

Figure 4

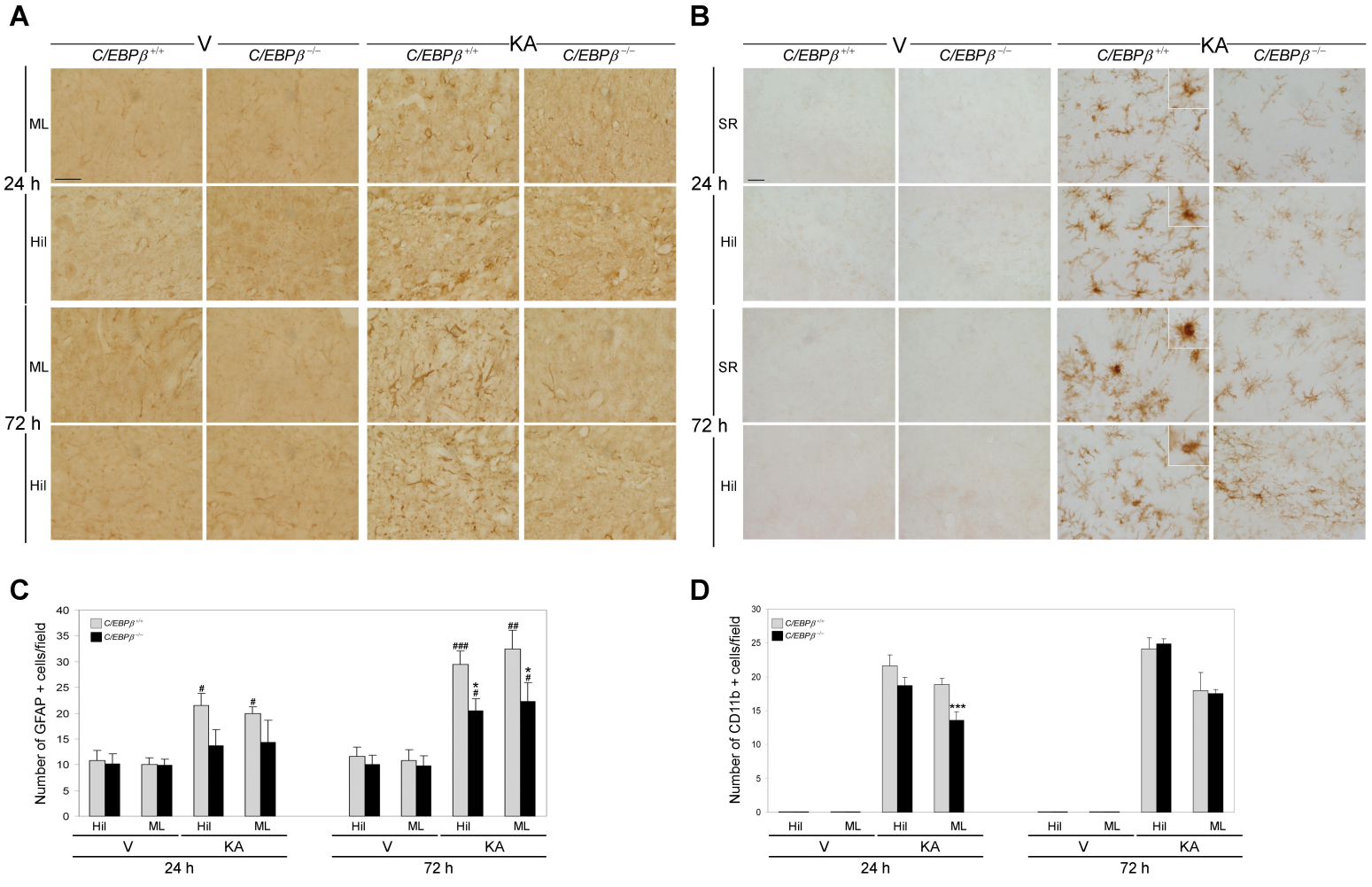

A
B
C
D
C/EBPβ+/+
C/EBPβ−/−
V
KA
24 h
72 h
ML
Hil
SR
Number of GFAP + cells/field
Number of CD11b + cells/field

Figure 5

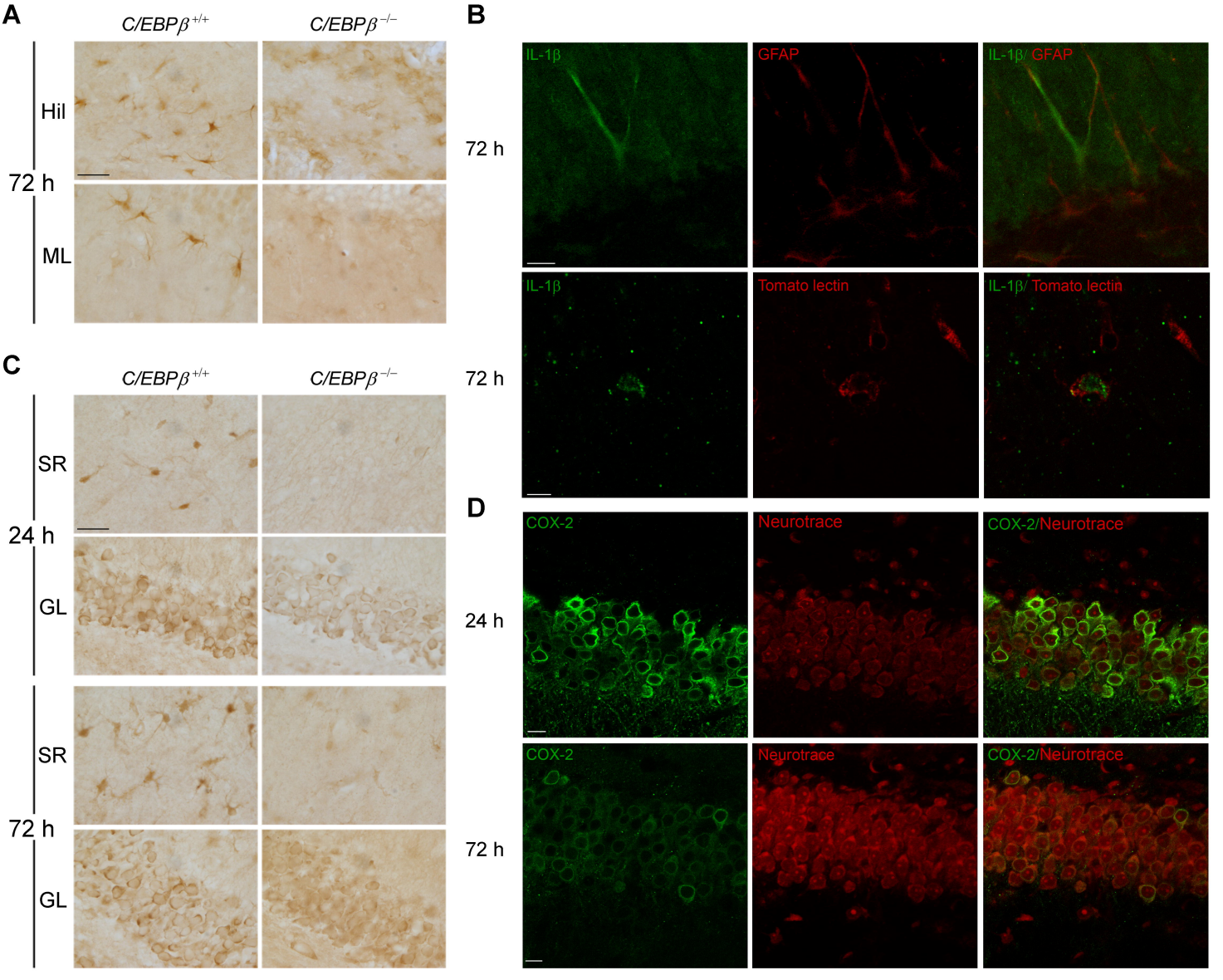

A
C/EBPβ+/+
C/EBPβ−/−
Hil
72 h
ML
B
IL-1β
GFAP
IL-1β/ GFAP
72 h
IL-1β
Tomato lectin
IL-1β/ Tomato lectin
72 h
C
C/EBPβ+/+
C/EBPβ−/−
SR
24 h
GL
SR
72 h
GL
D
COX-2
Neurotrace
COX-2/Neurotrace
24 h
COX-2
Neurotrace
COX-2/Neurotrace
72 h

Figure 6

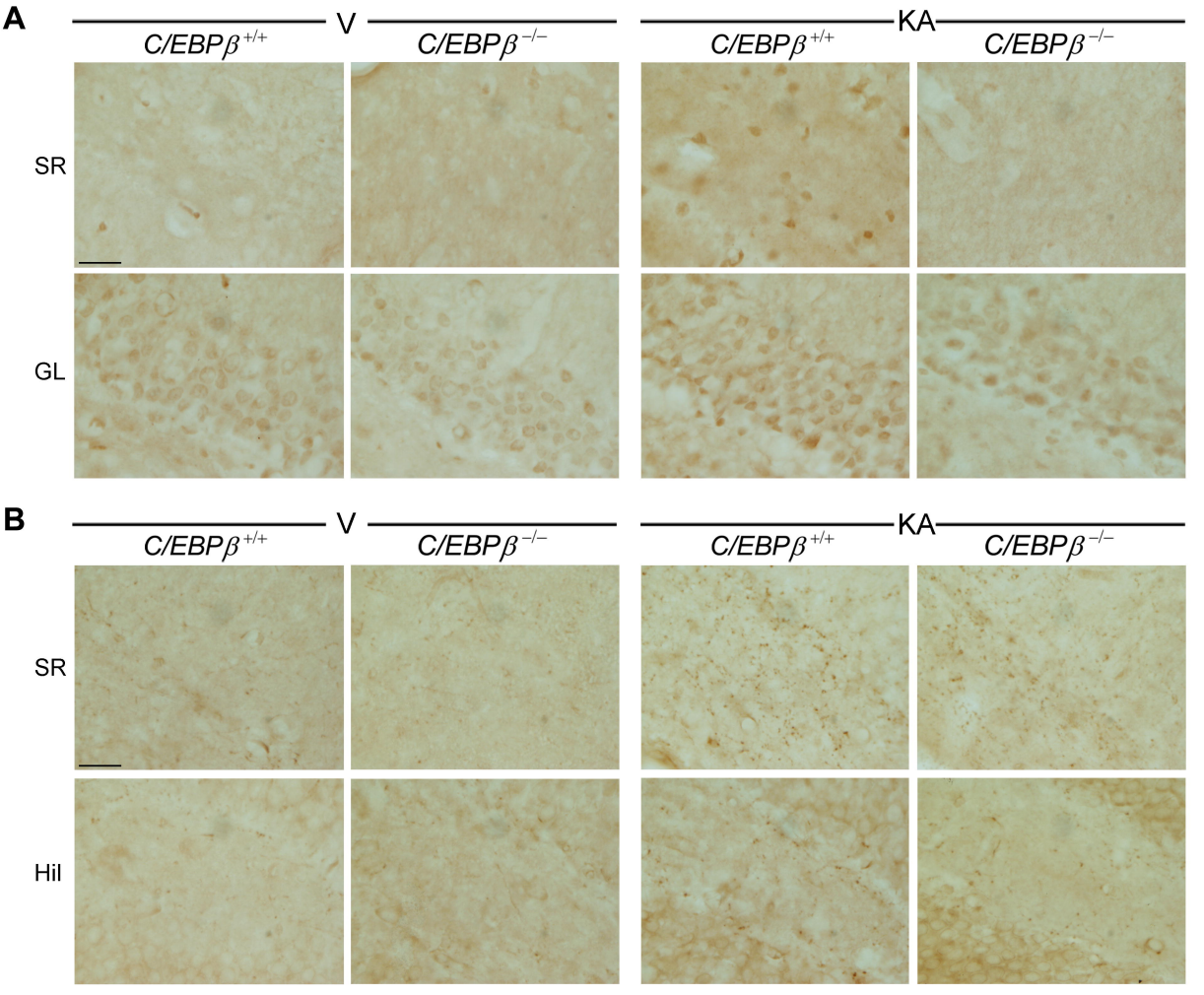
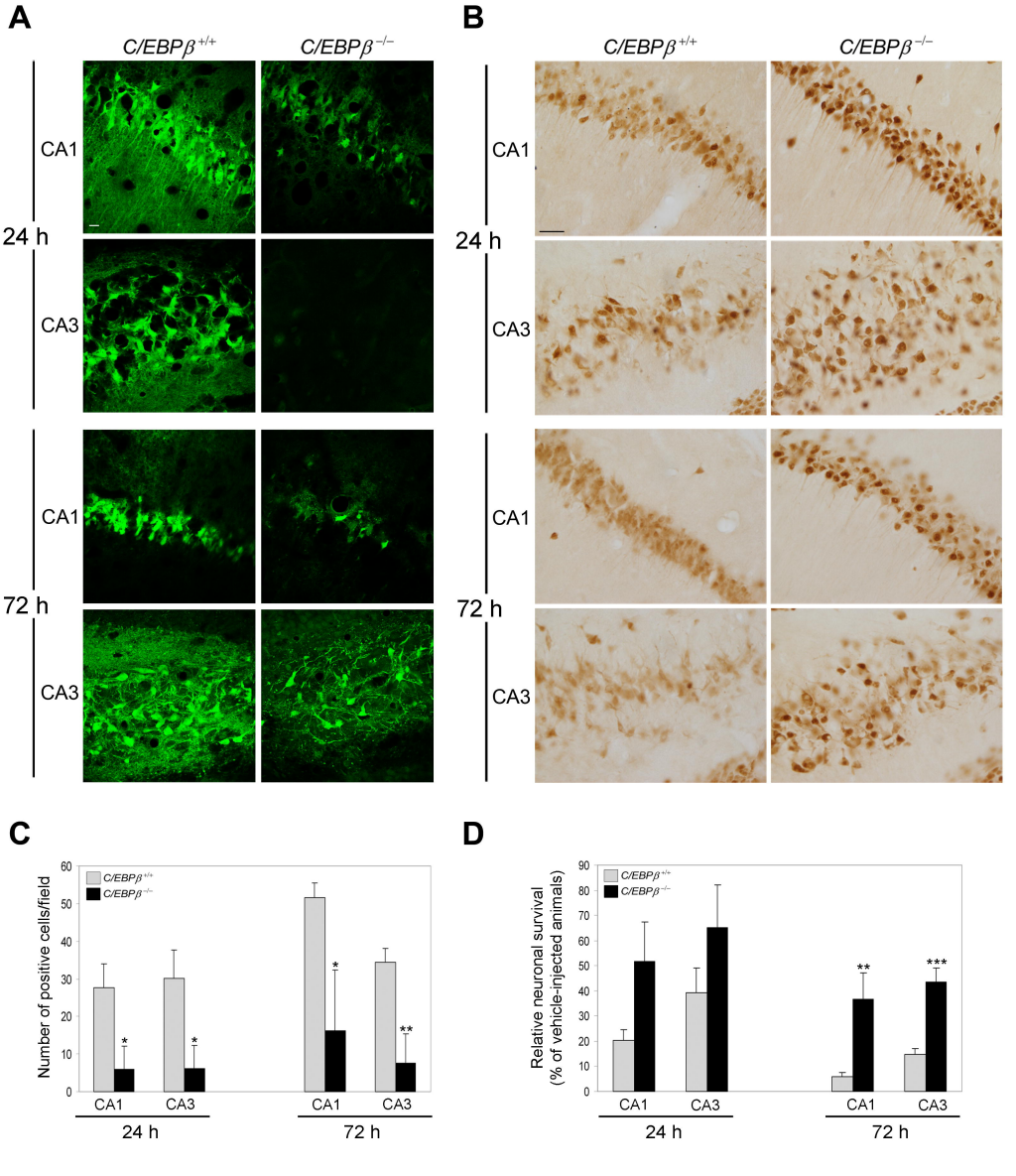

A
V
KA
C/EBPβ+/+
C/EBPβ−/−
C/EBPβ+/+
C/EBPβ−/−
SR
GL
B
V
KA
C/EBPβ+/+
C/EBPβ−/−
C/EBPβ+/+
C/EBPβ−/−
SR
Hil

Figure 7


A
C/EBPβ+/+
C/EBPβ−/−
CA1
CA3
24 h
72 h
B
C/EBPβ+/+
C/EBPβ−/−
CA1
CA3
24 h
72 h
C
Number of positive cells/field
C/EBPβ+/+
C/EBPβ−/−
CA1
CA3
24 h
72 h
D
Relative neuronal survival
(% of vehicle-injected animals)
C/EBPβ+/+
C/EBPβ−/−
CA1
CA3
24 h
72 h