

## IL-12p35 deficiency alleviates kainic acid-induced hippocampal neurodegeneration in C57BL/6 mice

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The role of IL-12 in excitotoxic neurodegeneration of brain is largely unknown. To address this issue, we used the model of kainic acid (KA)-induced hippocampal injury in IL-12p35 knockout (KO) mice, a well-characterized model for human neurodegenerative diseases. After KA treatment, hippocampal neurodegeneration was significantly less severe in the IL-12p35 KO mice than in wild-type mice as demonstrated by reduced pathological changes and astrogliosis. One day after KA treatment, levels of F4/80 and CD86 expression on microglia were significantly lower in IL-12p35 KO mice than in wild-type mice analyzed by flow cytometry, indicating that IL-12p35 deficiency resulted in lower levels of microglial activation. Five days after KA treatment, CD86 expression on microglia of wild-type mice was still higher, whereas F4/80 expression in wild-type mice decreased and was similar to that in IL-12p35 KO mice. Because microglial activation is necessary for KA-induced neurodegeneration, the lower level of microglial activation in the absence of IL-12p35 may alleviate hippocampal injury in KO mice. In summary, this study indicates that IL-12 may play a critical role in excitotoxin-induced brain injury.

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### Introduction

Glutamate excitotoxicity plays a key role in inducing neuronal cell death in many neurological diseases. In mice, administration of kainic acid (KA), an analog of the excitotoxin glutamate, results in

hippocampal cell death and seizures. KA-induced seizures in mice provide a well-characterized model for studies of human neurodegenerative diseases (Chen et al., 2002). In the course of KA-induced hippocampal injury, neurons interact with the surrounding cells, including astrocytes and microglia. The modulation of the activity of these cells may influence the survival and repair of hippocampal neurons after injury.

IL-12 is a heterodimeric cytokine that consists of a heavy chain p40 and a light chain p35 (Kobayashi et al., 1989). The two genes encoding p40 and p35 are unrelated and located on different chromosomes (Ebner et al., 2001; Sieburth et al., 1992; Wolf et al., 1991). Expression of both genes in the same cell and secretion of the covalently linked heterodimer p70 are required for biological activity (D'Andrea et al., 1992). In the peripheral system, IL-12 is produced mainly by monocytes, macrophages, neutrophils, dendritic cells (DCs), and B cells in response to infectious agents (Trinchieri, 1995). The primary target cells of IL-12 activity are T cells and NK cells. Upon IL-12 stimulation, these cells produce pro-inflammatory cytokine such as IFN- $\gamma$  and IL-2 (Trinchieri, 1998; Wolf et al., 1991). In addition to T and NK cells, macrophages and DCs also respond to IL-12 (Jacobsen et al., 1993; Jelinek and Braaten, 1995). In the central nervous system (CNS), microglia are considered to be the main source of IL-12. Human CNS-derived microglia produce IL-12 in vitro after activation with LPS and IFN- $\gamma$  (Becher et al., 1996). Murine microglia can be induced to express mRNA encoding the IL-12 receptor (IL-12R) (Suzumura et al., 1998), indicating an autocrine regulation pathway of IL-12 in these cells. The IL-12 receptor comprises two chains, IL-12R $\beta$ 1 and  $\beta$ 2 (Gately et al., 1998). Expression of both chains is necessary for high affinity binding and signal transduction. Not only microglia but also astrocytes produce IL-12 p70 (Stalder et al., 1997).

IL-12 plays a critical role in autoimmune diseases and viral infections of the CNS, such as multiple sclerosis (MS) and Borna disease. The release of IL-12p40 subunit into CSF and serum is

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increased in MS patients (Fassbender et al., 1998). IL-12p40-deficient mice or mice administered with anti-IL-12p40 monoclonal antibodies are resistant to the development of experimental autoimmune encephalomyelitis (EAE), an animal model for MS (Brok et al., 2002; Gran et al., 2002). In contrast, IL-12p35-deficient mice are susceptible to EAE (Becher et al., 2002; Gran et al., 2002). Borna disease virus, which does not trigger disease in most strains, is however harmful after infecting the CNS of mice that overexpress IL-12, indicating an important role of IL-12 in viral infection of the CNS (Freude et al., 2002).

To determine whether IL-12 is an active participant in excitotoxic brain injury, we used the model of KA-induced hippocampal injury in IL-12 knockout mice. We observed that IL-12p35 deficiency increased hippocampal resistance to KA-induced excitotoxicity.

## Materials and methods

### *Animals*

In total, 84 male 5- to 6-week-old IL-12 p35-deficient mice (IL-12p35 KO mice), which carry a mutation in the p35 subunit gene of IL-12 and are unable to produce biologically active IL-12, and 80 male age-matched wild-type C57BL/6 mice were used in the present study. The mice were purchased from the Jackson Laboratory (Bar Harbor, Me) and bred at the animal facilities of the Microbiology and Tumor Biology Center, Karolinska Institute, Stockholm, Sweden. All mice were housed on a 12-h light–dark schedule with water and food available ad libitum.

### *KA administration and assessment of clinical signs*

Fourteen wild-type and 18 IL-12p35 KO mice were partially anesthetized with Isofluen (Abbott Laboratories Ltd, Kent, England) and held on their backs by hand. KA (Sigma-Aldrich, Stockholm, Sweden) dissolved in distilled water (10 mg/1.3 ml) was slowly and gently dropped by micropipette into the noses at a dose of 45 mg KA per kg body weight as described previously (Chen et al., 2002). Six age- and body weight-matched wild-type and KO mice received the same amount of distilled water intranasally as controls. Mice were monitored continuously for 5 h to register the onset and extent of seizure activity. Seizures were rated as follows: 0, normal; 1, immobilization; 2, rearing and falling; 3, seizure for less than 1 h; 4, seizure for 1–3 h; 5, seizure for more than 3 h; and 6, death.

### *Histopathological analysis*

Nine surviving KA-treated wild-type and 13 surviving KA-treated KO mice as well as water-treated wild-type and KO mice (3 mice for each group) were anesthetized with sodium pentobarbital and transcardially perfused with phosphate-buffered saline (PBS) 7 days after the administration of KA. The brains were excised, fixed in 4% buffered formaldehyde, and embedded in paraffin. Coronal sections (7- $\mu$ m slices) from  $-1.15$ ,  $-1.94$ , and  $-2.80$  mm, respectively, relative to the bregma were prepared according to the information in Franklin's brain atlas (Franklin and Paxinos, 1997). Sections were stained by Nissl's method to evaluate the morphology of neurons. Using a blinded protocol, two different examiners duplicated the counting. For assessment of severity and extent of

neurodegeneration in the hippocampus after Nissl's staining, sections were scored using a semiquantitative grading system: 0, normal; 1, slight shrinkage of neurons (1–4% pyknotic neurons in area CA3); 2, moderate shrinkage of neurons (5–15% pyknotic neurons in area CA3); 3, severe shrinkage of neurons (more than 15% pyknotic neurons in area CA3); 4, slight loss of neurons (5–10% neuron loss in area CA3); 5, moderate loss of neurons (11–40% neuron loss in area CA3); and 6, severe loss of neurons (more than 40% neuron loss in area CA3). Mean value of pathological changes in both sides of hippocampus was used for each mouse.

### *Immunohistochemistry of brain sections*

Three surviving mice 1 day after KA treatment and three water-treated control mice were perfused with PBS followed by 4% buffered formaldehyde and the brains were kept in 10% sucrose until being frozen and cryosectioned at the thickness of 12  $\mu$ m. Frozen sections were then incubated with 0.3% Triton X-100 in Tris buffer for 20 min at RT. Paraffin sections prepared as described for histopathological analysis were dewaxed and immersed in citric buffer (pH 6.0) and heated by microwave at 94°C for 9 min. After washes with water and Tris buffer, both the frozen and the paraffin-embedded sections were blocked by "protein block" (DAKO A/S, Glostrup, Denmark) at room temperature for 30 min. Subsequently, the paraffin-embedded sections were exposed to rabbit antibodies to FAS (1:50, Santa Cruz Biotechnology, CA, USA) and rabbit antibodies to glial fibrillary acidic protein (GFAP) (1:1600; DAKO), respectively, followed by staining with the avidin–biotin technique (Vectastain Elite Kit; Vector Labs, Burlingame, CA, USA). Peroxidase-substrate solution DAB (Sigma-Aldrich) was added until the desired intensity of color (yellow) developed. Frozen sections were exposed to rat anti mouse IL-12 antibody (Biosource, Camarillo, CA, USA) and DAB substrate, followed by incubation with rat anti mouse F4/80 (Serotec, Oxford, UK) and SG substrate (blue–gray color, Vector Labs). Omission of primary antibodies served as negative controls.

### *Western blotting*

Seven days after KA treatment, six KA-treated and six water-treated mice from each wild-type and IL-12p35 KO group in two separate experiments were perfused with PBS. The hippocampi were dissected out and put into 1.5 ml microcentrifuge tubes with 200  $\mu$ l IP buffer (1 mM PMSF, 0.1 M Tris–Cl (pH 8.0), 0.15 M NaCl, 5 mM EDTA, and 1% Triton X-100). After sonication for 1 s, 67  $\mu$ l Laemmli buffer [248 mM Tris–Cl (pH 6.8), 40% glycerol, and 8% SDS] was added. The samples were boiled at 100°C for 10 min and centrifuged at 12,000 rpm for 10 min. The supernatant was collected and stored at  $-70^{\circ}\text{C}$  until use. Protein concentrations were quantified by using a DC protein assay kit (Bio-Rad, Stockholm, Sweden). For protein separation, 100  $\mu$ g of each sample was electrophoresed on a 12% polyacrylamide gel and transferred to nitrocellulose membranes followed by staining with Ponceau S (Sigma-Aldrich) for loading control. After several washes, membranes were blocked in 5% nonfat dry milk in PBS-Tween 20 for 1 h at room temperature with gentle agitation. After blocking, membranes were incubated with rabbit anti-mouse FAS (1:600, Santa Cruz Biotechnology) or rabbit anti-cow/mouse GFAP antibody (1:5000, DAKO) overnight at 4°C with gentle agitation. After extensive washing in PBS-Tween 20, membranes were incubated with a secondary antibody, peroxidase-conjugated donkey

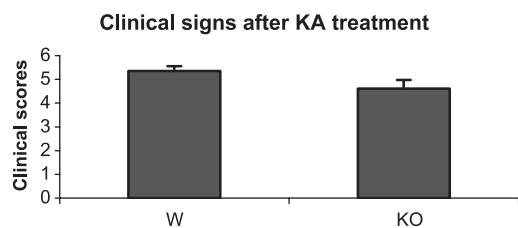


Fig. 1. Clinical signs after KA treatment. Wild-type ( $n = 14$ ) and IL-12p35 KO ( $n = 18$ ) mice, 5–6 weeks of age, were given KA at a dose of 45 mg per kg body weight by the intranasal route. Within 20–30 min, all recipients of KA displayed continuous seizures, which lasted less than 5 h. Some serious cases died in this period. Mice were monitored continuously for 5 h to register the onset and extent of seizure activity. Clinical signs were rated by a scoring system described in Materials and methods. Average clinical scores and SEM values are presented. No significant difference of clinical signs existed between the two groups.

anti-rabbit (1:50000, Jackson ImmunoResearch, West Grove, PA, USA) for 1 h at room temperature. Membranes were rinsed again in PBS-Tween 20. ECL Western blotting detection reagents (Bio-Rad) were used for exposure according to the manufacturer's instructions. Densitometric analysis was performed using the Scion Image program (NIH, USA). Results are presented as percentages relative to the expression level of the wild-type mice given only water.

#### Isolation and flow cytometry analysis of microglia

One (early activation) and five days after KA or water treatments, respectively, 24 KA-treated and 24 water-treated mice (12 mice per group) in two separate experiments were perfused

with PBS and sacrificed. After removal of meninges, the cerebral hemispheres were dissected and dissociated by pipetting. Next, after trypsinization at 37°C for 15 min, fetal bovine serum (10%, final concentration) was added to inactivate trypsin activity. The tissues were then dissociated with repeated pipetting in KRB solution [120 mM NaCl, 5 mM KCl, 1.2 mM  $\text{KH}_2\text{PO}_4$ , 25 mM  $\text{NaHCO}_3$ , 14 mM D-glucose, 2.5 mM  $\text{MgSO}_4$ , 0.3% bovine serum albumin (BSA)] containing DNase I (Sigma-Aldrich). Cell suspensions were passed through a 70- $\mu\text{m}$ -pore-size strainer and spun down. The cell pellets were resuspended in 30% Percoll in PBS and centrifuged at  $500 \times g$  for 20 min. These pellets were resuspended, passed through a 30- $\mu\text{m}$  pore-size strainer, collected, and stained for flow cytometry.

Microglia were washed with PBS containing 1% BSA (BSA/PBS) and incubated with the following antibodies: FITC-labeled rat anti-mouse CD11b (PharMingen, San Diego, CA, USA), APC-labeled rat anti-mouse CD11b (Caltag, Burlingame, CA, USA), RPE-labeled rat anti-mouse CD45 (Serotec), APC-labeled rat anti-mouse F4/80 (an activation marker for microglia, Caltag), RPE-labeled rat anti-mouse CD86 (Caltag). Cells were double stained with antibodies against CD11b and antibodies against either CD45, F4/80, or CD86. FITC-, RPE (Serotec)-, and APC (Caltag)-labeled rat IgGs were used as negative controls for flow cytometry staining. After washing, cells were analyzed by FACSCalibur flow cytometer and CellQuest software (both from Becton Dickinson, CA, USA). The mean fluorescence intensity (MFI) of each antibody on the microglia was analyzed. At each time point, microglia from all four groups (wild-type groups with or without KA treatment and KO groups with or without KA treatment) were collected and analyzed on the same day with the same cytometer settings.

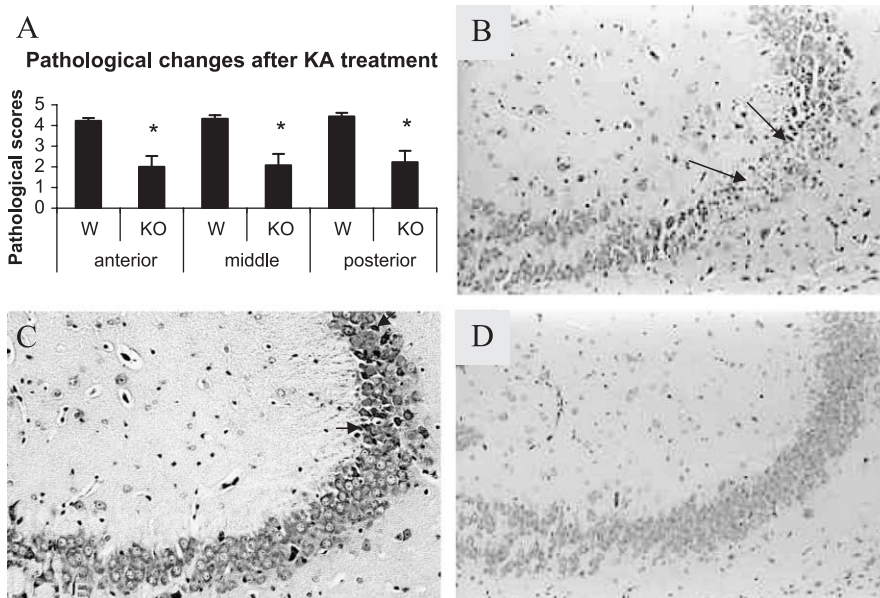


Fig. 2. Pathological changes after KA treatment. Coronal brain sections (7- $\mu\text{m}$  slices) from -1.15 (anterior hippocampus), -1.94 (middle hippocampus), and -2.80 mm (posterior hippocampus), respectively, relative to the bregma were prepared from mice sacrificed 7 days after the administration of KA. Sections were stained by Nissl's method to evaluate the morphology of neurons. For assessment of severity and extent of neurodegeneration, sections were scored using a semiquantitative grading system described in Materials and methods. Pathological scores and SEM values in each group are presented (A). At all three hippocampal levels, IL-12p35 KO mice ( $n = 13$ ) exhibited less neurodegeneration than wild-type mice ( $n = 9$ ). Representative sections at the mid-level for each group of mice are presented (B, KA-treated wild-type mice with pathological score 4; C, KA-treated IL-12p35 KO mice with pathological score 2; D, water-treated mice). Neither the wild-type nor the IL-12p35 KO mice treated with water had pathological change in the hippocampus (D).  $P$  values refer to comparisons between KA-treated IL-12p35 KO and KA-treated wild-type mice.  $*P < 0.05$ . The long arrows in B indicate the area of neuronal loss and the short arrows in C indicate pyknotic neurons.

### Statistics

Differences between more than two groups were tested by one-factor analysis of variance (ANOVA). Differences between two groups were tested by Student's *t* test. All tests were two-sided. Values are expressed as means  $\pm$  SEM, and the level of significance was set at  $P < 0.05$ .

### Ethics

The KA-induced excitotoxic model in mice was approved by the South Stockholm Research Animal Ethics Committee, Huddinge County Court, Stockholm, Sweden.

### Results

#### *IL-12p35 deficiency does not significantly affect KA-induced clinical signs*

Neither wild-type nor KO mice given water intranasally, as expected, showed any clinical change (data not shown). However, both groups of mice treated with KA displayed catatonic and staring behavior within 15 min, followed by myoclonic twitching and often frequent rearing and falling. Within 20–30 min after KA administration, all mice displayed continuous seizures, which lasted less than 5 h; sometimes seizures were fatal. After the seizure activity ceased, mice assumed a hunched posture and were immobile for some hours. The average clinical scores are presented in Fig. 1. Severity of seizure did not differ significantly between the two groups.

#### *IL-12p35 KO mice show reduced hippocampal neurodegeneration*

After KA administration, selective hippocampal neurodegeneration in region CA3 was observed. The average scores of histopathological changes at anterior (−1.15 mm to bregma), middle (−1.94 mm), and posterior (−2.80 mm) hippocampi of mice 7 days after KA treatment are presented in Fig. 2A. Continuous sectioning showed that samples at these three levels represented the overall changes in the hippocampus (data not shown). At all three hippocampal levels, wild-type mice exhibited more severe neurodegeneration (Fig. 2B) than IL-12p35 KO mice (Fig. 2C). No group treated with water had any pathological change in the hippocampi (Fig. 2D).

#### *KA treatment increases microglia-produced IL-12 in C57BL/6 wild-type mice*

Very few microglia were found positive for IL-12 expression in water-treated wild-type mice (Fig. 3A). However, significantly increased number of IL-12-positive microglia accumulated in the lesioned CA3 area 1 day after KA treatment (Fig. 3B).

#### *IL-12p35 deficiency diminishes the expression of FAS and GFAP in hippocampus*

FAS (CD95/APO-1) is a marker for apoptosis and GFAP is a marker for astrocytes. KA treatment resulted in an increase of FAS

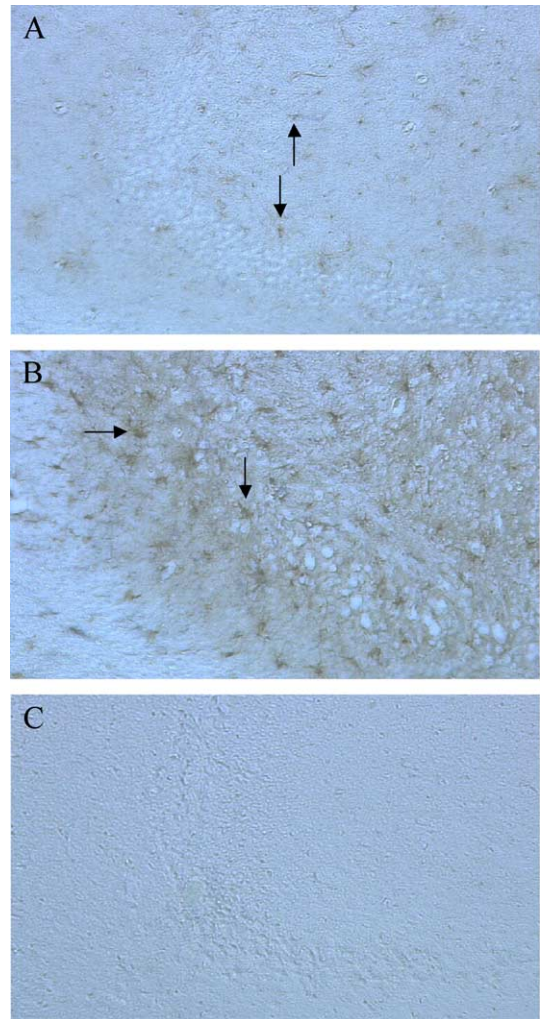


Fig. 3. KA treatment enhances IL-12 expression in microglia. Very few microglia were found positive for IL-12 expression in water-treated wild-type mice (A). However, 1 day after KA treatment, the number of IL-12-positive microglia significantly increased in the lesioned CA3 area (B). Negative control section with the primary antibodies omitted showed no positive staining (C). Arrows indicate IL-12-positive microglia.

and GFAP expression in hippocampus in both groups, as detected by immunohistochemistry and Western blotting 7 days after KA treatment (Figs. 4 and 5). However, the expression of FAS and GFAP in KA-treated wild-type mice (Figs. 4A1 and 5A,C for FAS; Figs. 4A2 and 5B,C for GFAP) was significantly higher than those in KA-treated IL-12p35 KO mice (Figs. 4B1 and 5A,C for FAS; Figs. 4B2 and 5B,C for GFAP) ( $P < 0.05$ ). No staining for FAS (Fig. 4C1) and very weak staining of GFAP (Fig. 4C2) were detected in water-treated mice. No difference in FAS and GFAP expression detected by Western blotting was observed between wild-type and KO mice treated with water (data not shown). Ponceau S staining showed no difference in the loadings of total proteins between samples (data not shown). Although the neuropathological change after KA treatment was not found in other areas of brain except hippocampus, higher expression of GFAP was found in cortex of both KA-treated groups. Furthermore, wild-type mice and KO mice after KA treatment clearly differed; wild-type mice showed more enhanced GFAP expression than KO mice (data not shown).

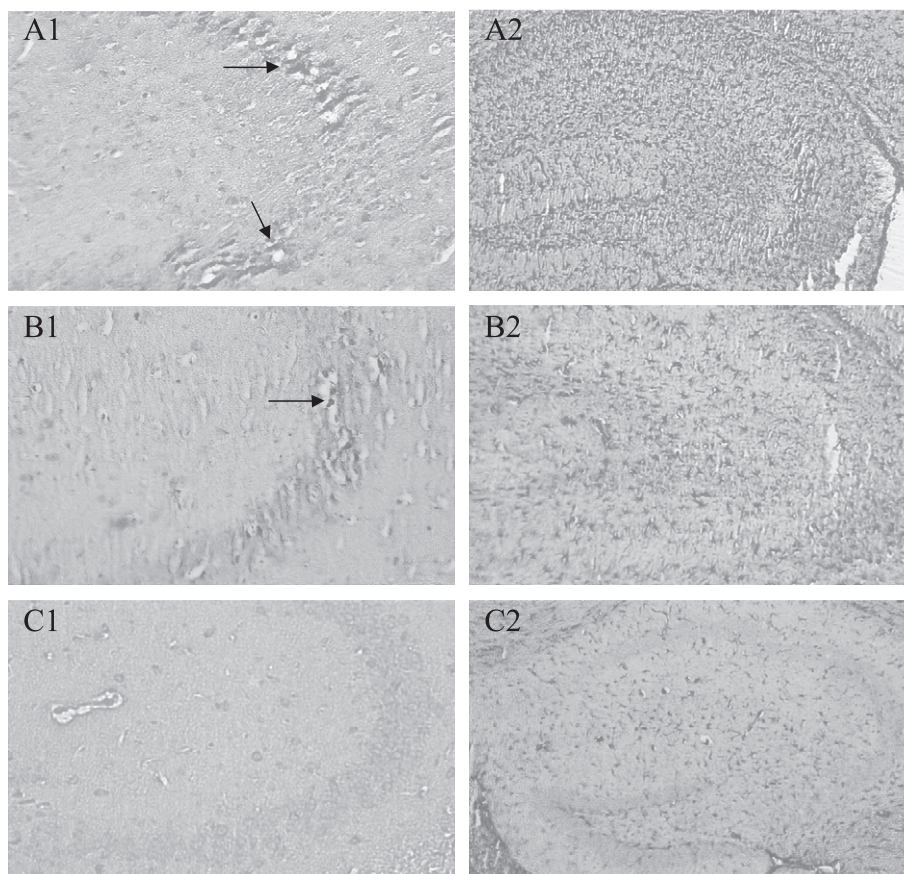


Fig. 4. FAS and GFAP expression 7 days after KA or water treatment as detected by immunohistochemistry. Representative sections at the middle level of hippocampus demonstrated that FAS expression was higher in KA-treated wild-type mice (A1) than in KA-treated IL-12p35 KO mice (B1). Sections from water-treated mice had no positive FAS staining (C1). GFAP expression at the hippocampus of the KA-treated wild-type mice (A2) was also higher than that of the KA-treated IL-12p35 KO mice (B2). Very weak GFAP staining was observed on sections from water-treated mice (C2). Arrows indicate the positive staining for FAS.

#### *IL-12p35 deficiency inhibits KA-induced microglia activation in brain*

To better understand mechanisms behind reduced neurodegeneration in IL-12-deficient mice, we examined their microglial activation 1 and 5 days after KA treatment using flow cytometry analysis.

Microglia of intracerebral parenchyma were enriched by Percoll gradient and identified via light scatter analysis as a homogeneous population of cells (Fig. 6). Table 1 presents the MFI of CD45, F4/80, and CD86 expressed on the microglia (CD11b<sup>+</sup> cells) 1 day after KA treatment. At that time, MFIs of all three molecules were distinctly enhanced by KA treatment in wild-type mice. However, only CD45 expression increased in IL-12p35 KO mice 1 day after KA treatment ( $P < 0.05$ ). Levels of F4/80 and CD86 were significantly lower in IL-12p35 KO mice than in wild-type mice after KA administration. Because such decreases indicate reduced activation of microglia, it is tempting to speculate that the IL-12p35 deficiency inhibited KA-induced microglial activation in brain.

Five days after KA treatment, the MFI profile of CD45 and CD86 expression remained the same as at 1 day post-treatment in all the groups, whereas the MFI of F4/80 in the KA-treated wild-type mice diminished to the levels of untreated mice. Still, no difference of F4/80 was observed between the KA-treated and

untreated IL-12p35 KO mice 5 days after KA treatment (Table 2). No significant difference in the three molecules was detected between the untreated wild-type mice and IL-12p35 KO mice.

#### **Discussion**

The present study shows that IL-12 was induced by KA treatment in C57BL/6 wild-type mice and that IL-12 deficiency reduced KA-induced excitotoxic injury of the hippocampus. Although the clinical signs of such injury were similar in both IL-12p35 KO and wild-type mice, we found significantly less neurodegeneration in the hippocampus and lower FAS expression in IL-12p35 KO mice compared with wild-type mice. Additionally, deficiency in IL-12 was also associated with a diminished astrocyte and microglial activation. Therefore, this study demonstrates that IL-12 may play an important role in KA-induced hippocampal injury.

Excitotoxic injury appears to be mediated predominantly by an excessive influx of calcium into neurons through ionic channels. The target receptors for KA can be divided into two major types, AMPA and kainite receptors, based on their selective agonists. During KA-induced excitotoxicity, the excessive influx of calcium into neurons can be mediated by voltage-dependent NMDA receptors and voltage-gated calcium channels (Mayer et al.,

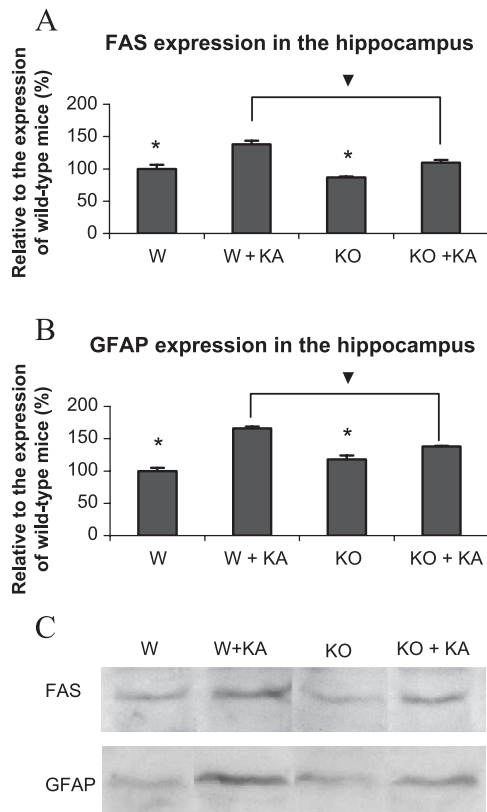


Fig. 5. FAS and GFAP expression before and 7 days after KA treatment as detected by Western blotting. Signal intensities were measured by densitometric analysis and compared by using ANOVA. After KA treatment, levels of FAS (A and C) and GFAP (B and C) expression increased in both IL-12p35 KO and wild-type mice. Both molecules were expressed at significantly lower levels in KO mice than wild-type mice after KA treatment. One representative experiment of three is shown.  $\nabla P < 0.05$ , compared between KA-treated wild-type and KO groups.  $* P < 0.05$ , comparison to the corresponding groups after KA treatment.

1984). Calcium entry into neurons plays a critical role in seizure genesis (McNamara, 1992; Meyer, 1989; Speckmann et al., 1989). In the present study, IL-12 deficiency seemed not to relate to the acute seizure activity because the clinical signs of IL-12p35 KO mice were similar to those of wild-type mice. Calcium entry into cells through L channels causes calcium overload and mitochondrial disruption that eventually leads to the release of mediators

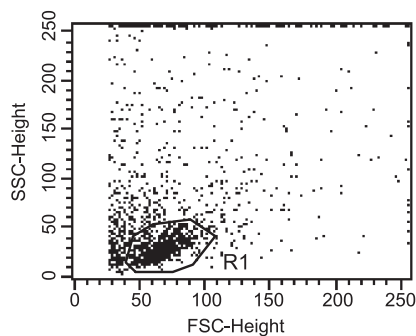


Fig. 6. Light scatter analysis of cells isolated from the brain. Cell preparation is described in Materials and methods. A homogeneous population of cells was identified and gated (R1) for subsequent analysis.

Table 1

MFI of indicated molecules on microglia (CD11b<sup>+</sup> cells) 1 day after KA treatment

	W	W + KA	KO	KO + KA
CD45	107 ± 1.45**	141 ± 5.21	105 ± 2.96**	145 ± 10.4
F4/80	92.0 ± 4.33**	136 ± 4.73*	100 ± 7.54	107 ± 7.54
CD86	9.21 ± 0.34**	14.8 ± 0.34*	10.4 ± 0.66	10.8 ± 1.33

MFI, mean fluorescence intensity; W, wild-type mice; W + KA, wild-type mice with KA treatment; KO, IL-12 knockout mice; KO + KA, IL-12 knockout mice with KA treatment.

\*  $P < 0.05$ , compared with KA-treated KO group.

\*\*  $P < 0.05$ , compared with the corresponding KA-treated groups.

responsible for activation of the apoptotic cascade and cell death (Cano-Abad et al., 2001). FAS, a transmembrane glycoprotein and receptor for the FAS ligand, plays an important role in apoptosis and FAS induction correlated with neuronal apoptosis after KA treatment (Tan et al., 2001). Although both necrosis and apoptosis occur in KA-induced hippocampal injury, the extent of apoptosis can partially represent the level of neurodegeneration. In KA-induced excitotoxic model, FAS induction is restricted in neurons but not in glial cells (Tan et al., 2001). FAS expression in the hippocampus detected by Western blotting accords with immunohistochemical staining of FAS on the brain sections (Tan et al., 2001). In the present study, enhancement of FAS expression was observed in both the wild-type and IL-12p35 KO groups after KA treatment but was significantly higher in the wild-type mice than in the KO mice. The pathological analysis using Nissl's staining gave similar results, that is, neurodegeneration was significantly less severe in IL-12p35 KO mice at three representative levels of the hippocampus.

We evaluated astrocyte reactivity by immunoblotting of GFAP, a marker of astrocytes. Astroglia is associated with neurotoxicity and strong neuronal activity (Norton et al., 1992; Steward et al., 1991; Torre et al., 1993). Excitotoxicity can lead to astroglia, a feature of KA-induced hippocampal injury (Chen et al., 2002) secondary to neuronal injury and believed to be mainly neuroprotective (Ding et al., 2000; Ridet et al., 1997). After KA treatment, a significant higher level of astrocyte activation was observed in wild-type mice than in IL-12p35 KO mice, suggesting that a deficiency in IL-12 may directly or indirectly influence astrocyte activity and that the level of astroglia may be associated with the extent of neuronal injury. However, because no IL-12 receptor has been detected on astrocytes, it seems impossible that IL-12 can interact with astrocytes directly (Suzumura et al., 1998). In addition, GFAP expression was similar in both wild-type and KO mice before KA treatment, indicating that an IL-12 deficiency may not influence astrocyte activity under normal conditions. Therefore, whether IL-12 deficiency indirectly

Table 2

MFI of indicated molecules on microglia (CD11b<sup>+</sup> cells) 5 days after KA treatment

	W	W + KA	KO	KO + KA
CD45	104 ± 2.91**	149 ± 6.11	110 ± 2.52**	149 ± 5.86
F4/80	94.0 ± 3.06	95.3 ± 2.03	92.7 ± 2.40	94.7 ± 3.53
CD86	9.24 ± 0.05**	13.2 ± 0.61*	10.6 ± 0.25	11.0 ± 0.39

MFI, mean fluorescence intensity; W, wild-type mice; W + KA, wild-type mice with KA treatment; KO, IL-12 knockout mice; KO + KA, IL-12 knockout mice with KA treatment.

\*  $P < 0.05$ , compared with KA-treated KO group.

\*\*  $P < 0.05$ , compared with the corresponding KA-treated groups.

influences astrocyte activity during KA-induced toxicity remains unknown.

Microglia are resident monocyte-lineage cells in the CNS that can react to injury and diseases of the brain, and become morphologically and functionally activated. In this study, we evaluated microglial reactivity by analyzing the expression of CD45, F4/80, and CD86. CD45 expression increased in both wild-type and KO mice 1 and 5 days after KA treatment. However, F4/80 and CD86 expression was enhanced only in wild-type mice 1 day after KA treatment. Five days post-treatment, F4/80 expression in wild-type mice dropped to the same level as in untreated mice whereas CD86 expression remained high in KA-treated wild-type mice. The intensities of CD45 staining are moderate (MFI about  $10^2$ ) in microglia and high (MFI about  $10^3$ ) in macrophages (Aloisi et al., 2000; Sedgwick et al., 1991). The MFIs of CD45 staining before and after KA treatment were about 100 and 150, respectively, indicating that no macrophages infiltrated into the CNS either before or after KA treatment. Some studies have shown that CD45 is a negative regulator of microglial activation because ligation to CD45 can markedly inhibit microglial activity via inhibition of p44/42 mitogen-activated protein kinase (MAPK) (Tan et al., 2000a,b). Enhancement of CD45 expression in the present study may reflect a mechanism for microglia to avoid overstimulation. F4/80, a glycoprotein with homology to the G-protein linked transmembrane 7 hormone receptor family, is considered a marker for microglia (Castano et al., 1996; Lawson et al., 1993; O'Donnell et al., 2002). CD86 is related to costimulatory signaling and can represent the antigen presenting function of microglia. The decreased levels of these two molecules in IL-12p35 KO mice after KA treatment indicate that IL-12 deficiency may have reduced the capacity of microglia for activation. The expression of both IL-12 and IL-12R on microglia indicates an autocrine regulation pathway of microglial activation and may account for the reduced activation in IL-12p35 KO mice observed in the present study. Microglial activation is necessary, but not sufficient for excitotoxin-induced neurodegeneration in the mouse hippocampus (Rogove and Tsirka, 1998; Tsirka et al., 1997). Activated microglia can potentiate excitotoxin-mediated neuronal death by secreting neurotoxic substances, such as glutamate, quinolinate, reactive oxygen species, and nitric oxide (Chao and Hu, 1994; Chao et al., 1992, 1995; Colton and Gilbert, 1987; Espey et al., 1997; Piani et al., 1991, 1992). In the present study, the diminished activation of microglia may account for the less neurodegeneration in IL-12p35 KO mice.

The new cytokine IL-23 also comprises the p40 subunit of IL-12 and another different p19 subunit. IL-23 can be produced by microglia and macrophages, and shows activity on macrophages and memory T cells through ligation to IL-23 receptor (IL-23R) (Cua et al., 2003). IL-23, not IL-12, has been found to be critical for the development of EAE (Cua et al., 2003). However, because macrophages, not microglia, have IL-23R (Cua et al., 2003) and there was no infiltrated macrophages detected by FACS, it seems unlikely that IL-23 could directly influence the activity of microglial cells in the current excitotoxic model. However, whether IL-23 can affect the functions of neurons or astrocytes or both is unclear.

The constitutive absence of IL-12 in p35KO mice may have unknown consequences on the developmental state of some hematopoietic cell types including IL-12 receptor-positive microglia, because IL-12 has been shown to play a dual stimulatory and inhibitory effect on hematopoiesis (Bellone and Trinchieri, 1994). Although there are no obvious differences after we examined brain

sections from both water-treated IL-12p35 KO and water-treated wild-type mice, this question needs to be further explored.

Taken together, the present study indicates that IL-12 may have an important role in excitotoxic injury in that KA-induced hippocampal neurodegeneration was alleviated in the IL-12p35 KO mice, evident from their less severe lesions, and reduction in FAS expression, astrogliosis, and microglial activation compared to animals with normal supply of IL-12.

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