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# A reduced susceptibility to chemoconvulsant stimulation in adenylyl cyclase 8 knockout mice

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## Abstract

**Objective—**Adenylyl cyclases (ACs) catalyze the synthesis of cAMP from ATP, and cAMP signaling affects a large number of neuronal processes. $Ca^{2+}$-stimualted adenylyl cyclase 8 (AC8) expressed in the CNS plays a role in synaptic plasticity, drug addiction and ethanol sensitivity, and chronic pain. This study was to aim at examining the contributions of AC8 to epileptogenesis.

**Methods—**In this study, we observed the seizure behavior induced by kainic acid (20mg/kg or 30mg/kg) or pilocarpine (350mg/kg) in AC8 KO and wild-type mice. Next we injected kainic acid or pilocarpine to induce status epilepticus (SE), and examined neuronal degeneration (by Fluoro-Jade B staining) and mossy fiber sprouting (by Timm staining) 24 hr and 2 weeks after SE termination in the hippocampus, respectively. Finally, 15min after intraperitoneal injection of kainic acid (30mg/kg), we examined phosphor-ERK1/2 in the hippocampus by western blot and immunochemistry staining.

**Results—**We first observed that AC8 KO mutants display reduced susceptibility (including seizure latency and episodes) to two chemoconvulsants, kainic acid and pilocarpine. Moreover, we found that degenerative neurons and mossy fiber sprouting induced by chemoconvulsants were significant decreased in the hippocampus. Further, western blot and immunochemistry analysis

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**Conflict of interests**

The authors claimed no conflict of interest

**Author contributions**

Dr. Xianju Zhou and Wenwei Yun (MD): conceived the idea and designed the experiments, explained and analyzed the data, wrote the manuscript and made significant contributions in the revision. Ms. Xia Chen and Ms. Guoying Dong conducted these experiments, acquired and analyzed the data. Dr. Changhong Zheng participated in western blot and immunohistochemistry experiments. Dr. Hongbing Wang analyzed and explained the data, and provided important intellectual content for the critical revision of the manuscript.

revealed that the MAPK signaling in the hippocampus was attenuated in kainic acid-injected AC8 KO mice.

**Conclusion—**AC8 is involved in epileptogenesis, and may serve as a potential target for the treatment of epilepsy.

### Keywords

Epilepsy; cAMP; Adenylyl cyclase; AC8 knockout; Calcium

## Introduction

Two ubiquitous messengers, $Ca^{2+}$ and cAMP (cyclic adenosine monophosphate), play an extremely important role in neuronal activity. Direct link between $Ca^{2+}$ and cAMP signaling is essential for neuronal activity, such as neurotransmitter release, learning memory, and synaptic development (Ferguson et al., 2004). Adenylyl cyclases (ACs) catalyze the synthesis of cAMP. It is known that there are nine membrane-bound mammalian ACs (AC1-9) in the brain(Defer et al., 2000). Four ACs (AC1, AC5, AC6 and AC8) are under regulation of physiological concentrations of $Ca^{2+}$, providing a crucial link between the $Ca^{2+}$ and cAMP-signaling pathways (Cooper et al., 1995). AC5 and AC6 are directly suppressed by $Ca^{2+}$, whereas AC1 and AC8 are stimulated by $Ca^{2+}$ in a calmodulin-dependent manner (Cooper et al., 1995).

There is increasing evidence that AC1 and AC8, the two major ACs in the CNS, are involved in activity-dependent synaptic plasticity, such as long-term potentiation and long-term depression (Wang and Zhang, 2012). Moreover, the two ACs are associated with addiction to several drugs of abuse and ethanol sensitivity (DiRocco et al., 2009; Maas et al., 2005; Zachariou et al., 2008), as well as chronic pain (Vadakkan et al., 2006; Wei et al., 2002). Interestingly, AC8 plays some specific roles, such as retrieval from adaptive presynaptic silencing (Moulder et al., 2008) and acquisition of new spatial information (Zhang et al., 2008). Although there is no report that AC1 and AC8 play a role in epileptogenesis, their coupled downstream signaling (cAMP-ERK1/2-CREB pathway), is associated with epileptogenesis. For example, cAMP and cAMP-dependent PKA are involved in epileptiform afterdischarge in rat hippocampal slices (Higashima et al., 2002). cAMP-dependent protein kinase A (PKA) is involved in epileptogenesis and maintenance of seizure activity (Vazquez-Lopez et al., 2005). Persistent ERK activation by genetic manipulation results in spontaneous seizures in transgenic mice(Nateri et al., 2007), whereas inhibition of ERK activation by pharmacological approach prevents rat autogenic seizure behavior (Glazova et al., 2015). Sustained CREB activity by expression of constituently active CREB causes spontaneous seizures (Lopez de Armentia et al., 2007). In contrast, decreased CREB activity by mutation of CREB gene suppresses epileptogenesis (Jancic et al., 2009). Moreover, increased or decreased expression of *Bdnf* gene (under the control of CREB signaling) enhanced or inhibited epileptogenesis, respectively (Barton and Shannon, 2005; Heinrich et al., 2011).

In this study, we observed a reduced sensitivity to chemoconvulsant stimulation in AC8 mutants, which is associated with the attenuation of MAPK signaling pathway. Our findings

suggest a role of adenylyl cyclase in epileptogenesis, and provide evidence on AC8 as a potential target for the treatment of epilepsy.

## Materials and methods

### Animals

The AC8$^{-/-}$ knockout (AC8 KO) mice were created by gene-specific recombination as described previously (Schaefer et al., 2000). The mutants were crossed into wildtype (WT) C57BL/6 background for at least 10 generation backcross. AC8 KO and WT mice were genotyped three weeks after birth. Male mice 2–3 months of age were used for all experiments. Animals were raised in the university laboratory animal research facility, and all the protocols were in compliance with the guidelines of Institutional Animal Care and Use Committee at Nanjing Medical University and Michigan State University. The mice had ad libitum access to water and food and were housed under a 12 h: 12h dark-light cycle. The researchers who conducted experiments were blinded to the genotypes of mice in this study.

### Seizure behaviors

Kainic acid (Sigma, St. Louis, USA) dissolved in 0.9% saline was administered intraperitoneally at a dose of 20 mg/kg to mice. The time of seizure onset was determined when animals first reached at least class 4 seizure. Mice were observed continuously by video monitoring for 3 h after kainic acid injection. The seizure intensity and classification were evaluated according to Racine's classification (Racine, 1972). Pilocarpine hydrochloride (Sigma) dissolved in 0.9% saline was administered intraperitoneally (i.p.) at a dose of 350 mg/kg to animals. Scopolamine methylbromide (2 mg/kg, i.p.; Sigma) was injected 30 min before pilocarpine to suppress peripheral muscarinic cholinergic effects. Diazepam (2 mg/kg, i.p.; Sigma) was administered 2 h after the onset of status epilepticus (SE), characterized by continual recurrent seizures (classes 3, 4, or 5), to terminate seizure and standardize duration of seizure activity.

### Timm staining

Timm staining was used to visualize mossy fiber sprouting in the inner molecular layer of dentate gyrus. According to previous work (Tan et al., 2011), mice were deeply anesthetized and perfused for 5 min with sulfide solution (1.2 % $Na_2S \cdot 9H_2O$ and 1.0 % $NaH_2PO4$), and 5 min with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) via the ascending aorta. Next, the brain was removed and post-fixed overnight, immersed in 30% sucrose at 4°C for 3 d. Then the brain sections (30μm) were prepared for further analyses. The sections were dehydrated using graded ethanol (100% for 15 min, 70% for 2 min, 50% for 2 min and distilled water for 2 min), and then immersed in a solution containing a 12:6:2:1 mixture of gum arabic (50% w/v), hydroquinone (5.67% w/v), citric acid–sodium citrate buffer (26% citric acid, w/v; 24% sodium citrate, w/v), and silver nitrate (17% w/v) (all above agents from Sigma) and developed for 45 min in dark at 25°C. Timm staining was quantified by Scion Image software (Scion Corp., Frederick, Maryland). Assessment of mossy fiber sprouting (Timm index) was obtained from the absolute value of the total area of Timm granules divided by total length of dentate gyrus (Watanabe, et al., 1996).

### Fluoro-Jade B staining

Degenerative neurons were detected with Fluoro-Jade B staining. Briefly, brain sections (30 μM) were immersed for 3 min in 100% ethanol, 1 min in 70% ethanol, 1 min in distilled water, and then transferred to a solution containing 0.0004% Fluoro-Jade B (Millipore,) and 0.1% acetic acid for 30 min. After three washes, the sections were cover-slipped. Finally, the sections were mounted with resinous medium and analyzed under a Nikon fluorescence microscope. Fluoro-Jade B-positive cells were counted (n=6 for each genotype, three sections for each animal) using Image Pro-plus 6.0 software, and the number in each section was divided by the respective area sampled. The staining and data analysis were conducted by an individual blinded to the experiment.

### Western Blotting

After cervical dislocation, brains were quickly removed and placed in ice-cold saline for about 3 min. Next, hippocampi were isolated and stored at 80°C until further use. Samples were homogenized by sonication in 200 μl homogenization buffer (10 mM Tris-HCl buffer, pH 6.8, 10% glycerol, 2% sodium dodecylsulfate, 0.01% bromophenol blue, and 5% β-mercaptoethanol) and boiled for 10 min. Protein concentration was measured using BCA protein assay (Pierce, Rockford, IL, USA) Samples were prepared by addition of 2× sample buffer followed by heating to 95°C for 5 min. Twenty μg were loaded onto 10% polyacrylamide gels. Proteins were transferred electrophoretically to nitro membranes (Millipore Co., Bedford, MA, USA). Blots were blocked 20 min in PBS + Tween 20 (PBS-T) with 5% dry milk. Milk was omitted for phospho-ERK1/2 blots. Blots were then incubated overnight at 4 °C in PBS-T ± milk and primary anti-body (phospho-p42/44 ERK1/2, 1: 1000; Total ERK1/2, 1: 1000; Cell Signaling Technology). Blots were washed 3 times and incubated in PBS-T with milk and horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (1:10,000; Cell Signaling). The blots were then thoroughly washed and subjected to ECL detection (SuperSignal West Pico; Pierce, Rockford, IL). The signal intensity was determined by Scion Image software (Scion Corp. Frederick, Maryland). The pERK1/2 level was normalized to total ERK1/2.

### Immunohistochemistry

30 μM brain slices were immersed in 1% $H_2O_2$ for 20 min and washed three times with 1× PBS, incubated with 5% NGS (normal goat serum, Invitrogen) for 1h at room temperature to reduce nonspecific binding and washed three times with 1× PBS. Next, the slices were incubated with primary antibody against phospho-ERK1/2 (1:200; Cell Signaling Technology) dissolved in 5% NGS overnight at 4°C. Then, the secondary antibody (Alexa Fluor 594-conjugated goat anti-rabbit IgG, 1:1000; Invitrogen) was incubated at room temperature for 1 h. Slices were imaged on a Nikon fluorescence microscope. Quantification (n=6, and three sections for each animal) was performed by analyzing the fluorescence intensity of phospho-ERK1/2 with Scion Image software (Scion Corp. Frederick, Maryland).

### Statistical analysis

All statistical analyses were performed using SPSS 17.0 software (SPSS Inc., Chicago, IL, USA). Values are expressed as mean ± SEM. Student's t-test was used to analyze data between two groups. Statistical significance was defined as $p < 0.05$.

## Results

### AC8 KO mice exhibit a reduced susceptibility to chemoconvulsants

To investigate whether AC8 KO mice exhibited some changes in seizure behavior, we first injected intraperitoneally (IP) the chemoconvulsant kainic acid (20 mg/kg) to induce seizures in AC8 KO and WT mice. Eleven of twelve (91.7%) WT mice developed seizures (at least at the stage of class 4) after kainic acid injection, and seizure behavior disappeared 3hr after injection in all mice. In contrast, 33.3% (4/12) of AC8 KO mice developed behavior seizures with kainic acid treatment, which also disappeared within 3hr after injection. As compared to WT mice, AC8 KO mice showed a delayed latency to first seizure (at least class 4) (Figure 1A) (34.3±3.6 min versus 24.6±4.7 min, p=0.032). In addition, seizure episodes within 3 hrs were significantly reduced in AC8 KO mice than in WT mice (Figure 1B) (6.1±2.1min versus 18.2±3.4 min, p=0.002). When increasing the dose of kainic acid to 30 mg/kg, all WT mice (n=14) developed seizures and 42.9% (6/14) mice developed behavioral status epilepticus as indicated by continuous seizure (at least at the stage of class 3). Moreover, a 28.6% mortality was observed. In contrast, only 66.6% (8/12) of AC8 KO mutants developed seizures with a 25% (3/12) occurrence of status epilepticus and a 16.7% of mortality. To next confirm that the phenotype of AC KO mice was non-specific to kainic acid, we used another chemoconvulsant pilocarpine (350mg/kg). As compared to WT mice (n=12), AC8 KO mutants (n=12) also displayed a delayed latency (Figure 1C) (40.3±4.2 min versus 31.6 ±3.5 min, p=0.028), a decreased SE occurrence (16.7 % vs 33.3%), as well as a reduced mortality. Taken together, these results suggest a reduced susceptibility to chemoconvulsants in AC8 KO mice.

### AC8 KO mice exhibit a reduced cell death and mossy fiber sprouting to chemoconvulsants

Neuronal cell death can be induced by seizures in human patients and animal models(Chang and Lowenstein, 2003). To determine the contribution of AC8 to SE-induced cell death, we examined cell death in the hippocampus from WT (n=6) and AC8 KO mice (n=6) 24 h after kainic acid-induced seizures. Extensive neuronal cell loss in the hippocampus, especially CA1 region, was shown by Fluoro-Jade B staining (Figure 2A) in WT mice. In contrast, AC8 KO mice displayed a remarkable reduced the severity of SE-induced cell death in CA1 region (85 ± 11 cells/0.2mm$^2$ versus 253 ± 15 cells/0.22mm$^2$, p=0.0004) (Figure 2A). Seizure-induced mossy fiber sprouting is a feature of human limbic epilepsy (Sutula et al., 1996), thus we next examined whether inhibition of epileptogenesis by AC8 knockout is reflected in seizure-induced mossy fiber sprouting. As shown in Figure 2B, we found extensive Timm-stained mossy fiber sprouting in the supragranular region of the dentate gyrus of the hippocampus 2 weeks after pilocarpine-induced seizure. The mean Timm index was lower in AC8 KO mice (n= as compared to WT mice (18.5±2.5 versus 41.3±4.2, p=0.0007). This result suggested that the elimination of AC8 contributes to seizure-induced long-term change in neural circuitry.

### A reduced MAPK signaling in response to chemoconvuslants in AC8 KO mutants

Previous studies showed that the ERK1/2 pathway plays an important role in epileptogenesis (Nateri et al., 2007). We thus investigated the contribution of AC8 in seizure-induced ERK1/2 activation. As illustrated in Figure 3A, KA induced a significant increase in phosphorylation of ERK1/2 15 min after injection with fold changes compared to saline injection in the hippocampus of WT mice. Although KA also induced a marked increase in phosphorylation of ERK1/2 15 min after injection, the relative change was reduced in the hippocampus of AC8 KO mice compared to WT mice (arbitrary value, 0.81±0.07 versus 1±0.1, p=0.03). Additionally, immunohistochemistry staining revealed that kainic acid induced a decreased phospho-ERK/12 signaling in the hippocampal slices of AC8 KO mice as compared to WT mice (arbitrary value, 0.48±0.14 versus 1±0.15, p=0.018) (Figure 3B). These findings suggested a reduced MAPK signaling in response to KA injection in AC8 KO mutants.

## Discussion

In this study, we found that AC8 KO mice exhibit a reduced seizure susceptibility, decreased cell death and mossy fiber sprouting to chemoconvulsants, which were associated with a reduced MAPK signaling. These results suggest that AC8 plays an important role in epileptogenesis and serves as a potential target for treatment of epilepsy.

cAMP, one of most ubiquitous second messengers, affects a large number of neuronal activity by initiating downstream signaling. The cAMP level is under the regulation of AC and PDE, the former catalyzing cAMP formation from ATP and the latter catalyzing the degradation of cAMP to AMP. Pharmacological studies showed that cAMP accumulation is associated with epilepsy. For instance, forskolin (an AC activator) and rolipram (a PDE inhibitor) promoted epileptiform afterdischarges in the CA1 region of rat hippocampal slices (Higashima et al., 2002). These effects were blocked by a cAMP antagonist Rp-cAMPS. Similarly, in rat neocortex slices, cyclic AMP analogues augmented epileptiform activity(Boulton et al., 1993). The data from in vivo studies also showed that repeated injection of cAMP into the rat amygdala leaded to chemical kindling in a dose-dependent way, and the cAMP PDE inhibitor EDTA facilitated the effect (Yokoyama et al., 1989). Moreover, the clinically used anticonvulsant carbamazepine inhibited AC activity (Chen et al., 1996). In this study, we first provide genetic evidence that knockdown of a specific AC, $Ca^{2+}$-stimulated AC8, inhibited epileptogenesis. And the downstream MAPK signaling was attenuated in epileptogenesis induced by chemoconvulsants, consistent with the notion that cyclic AMP (cAMP)-ERK1/2-cAMP-responsive element-binding protein (CREB) cascade is involved in regulation of neuronal excitability and epileptic activity.

AC8, one of two kinds of $Ca^{2+}$-stimulated membrane bound enzymes in the CNS, plays a critical role in a large number of neuronal processes (Wang and Zhang, 2012), such as activity-dependent plasticity, addiction, pain and anxiety. In this study, we showed a role of AC8 in epileptogenesis. However, we could not exclude indirect effects of AC8 on epileptogenesis due to the use of genetic mutants. Although there is no direct evidence that partial inhibition of calcium-stimulated AC activity in AC8 KO mice affects neurodevelopment and in turn neuronal excitability, the level of cAMP is very critical for

activity-dependent refinement of neuronal circuits (Suzuki et al., 2015). But we proposed that knockout of AC8 has little effect on neurodevelopment based on the following reports. First, AC1 is the major calcium-stimulated AC (Nielsen et al., 1996), thus it is possible that the existence of AC1 would be compensatory for deletion of AC8; Second, AC8 is most extensively expressed in adulthood, but not in embryonic life (Nicol et al., 2005). Interestingly, we observed an increased susceptibility to kainic acid and pilocarpine injection in AC1 KO mice and AC1/AC8 double KO mice (there was no significant difference between them, unpublished data). It is known that AC1 is more sensitive to $Ca^{2+}$ and contributes to cAMP production in a greater degree (Nielsen et al., 1996). Importantly, the peak of AC1 gene expression occurs in embryonic life (Nicol et al., 2005). Thus we speculated that AC1 deletion would substantially affect cAMP production induced by neuronal stimulation during development, leading to greater developmental alterations. In contrast, AC8 deletion might have little effect on cAMP generation and further on neurodevelopment. However, the conditioned expression of AC8 in the CNS after adulthood or the development of a highly specific AC8 inhibitor will help to address its precise roles in the epileptogenesis.

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## Highlights

- AC8 KO mutants display reduced susceptibility to chemoconvulsant stimulation
- Neuronal death in the hippocampus is reduced in kainic acid-injected AC8 KO mice
- Mossy fiber sprouting in the DG area is decreased in pilocarpine-injected AC8 KO mice
- MAPK signaling in the hippocampus is attenuated in kainic acid-injected AC8 KO mice

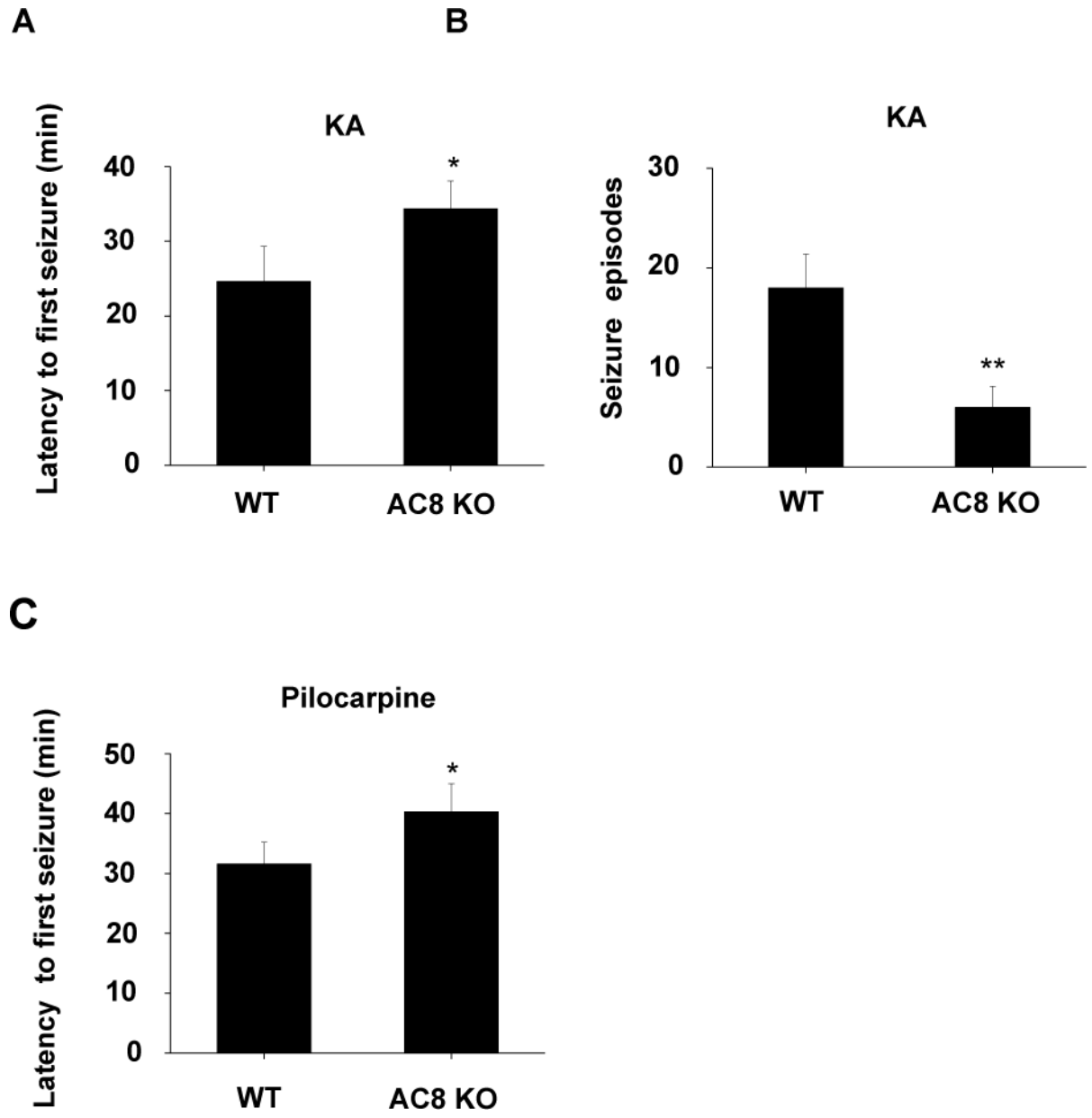


**Figure 1.**

AC8 KO mice display a reduced sensitivity to chemoconvulsants. Following kainic acid (KA, 20 mg/kg) ip injection, (A) the latency of the first seizure (at least class 4) and (B) seizure episodes within 3 hr were recorded in AC8 KO (n=12) and WT mice (n=12). (C) Following pilocarpine (350 mg/kg) i.p. injection, the latency of the first seizure (at least class 4) was recorded in AC8 KO (n=12) and WT mice (n=12). *P<0.05, **P<0.01, compared to WT mice, Student's t-test.

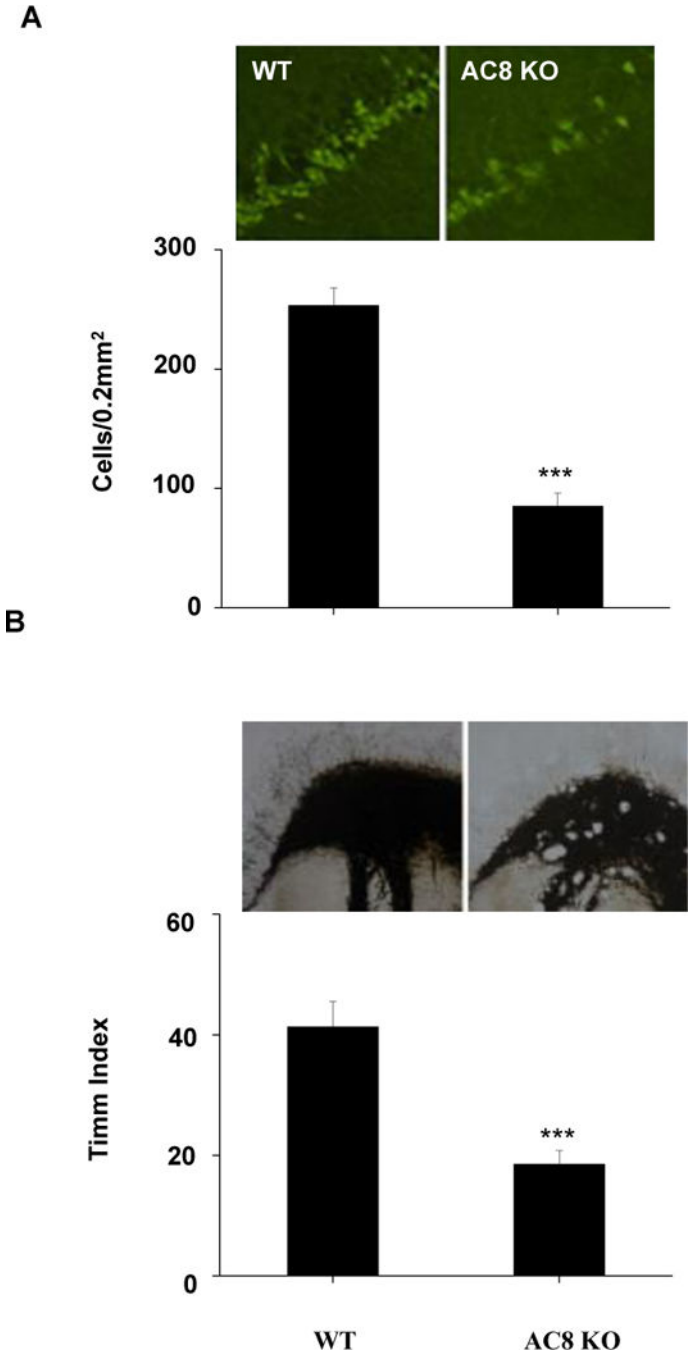


**Figure 2.**

AC8 KO mice exhibit reduced cell death and mossy fiber sprouting to the chemoconvulsant in the CA1 and DG area of the hippocampus, respectively. (A) 2hr after kainic acid (30 mg/kg)-induced SE, Diazepam (2mg/kg) was used to terminate SE. 24 later, degenerative neurons were examined by Fluoro-Jade B staining (n=6 for each genotype, three sections for each animal). (B) 2hr after pilocarpine-induced SE, diazepam (2mg/kg) was used to terminate SE; two weeks later, mossy fiber sprouting was examined by Timm staining (n=6

for each genotype, three sections for each animal); Representative images are shown; ***P<0.001, compared to WT mice, Student's t-test.

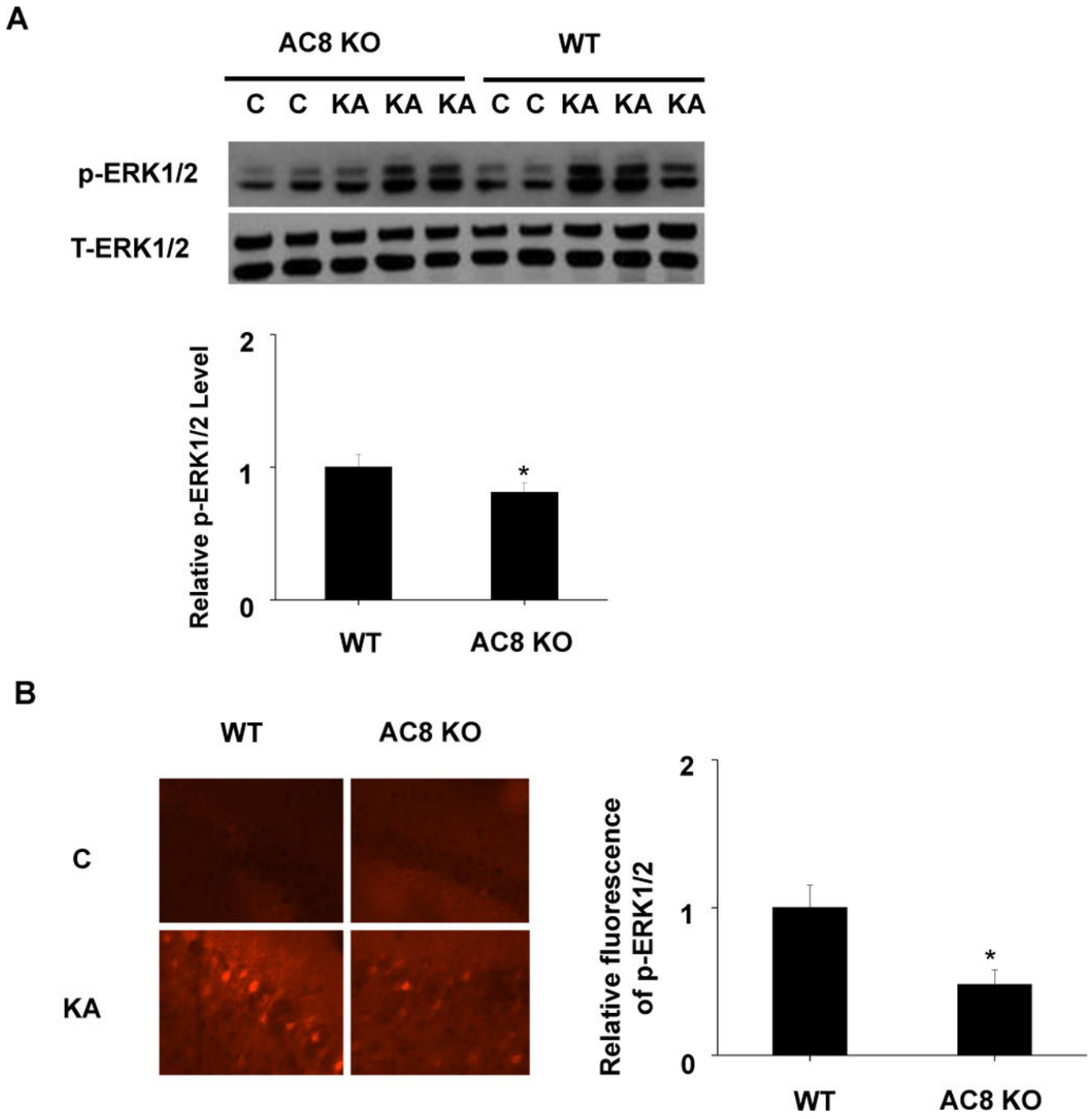


**Figure 3.**

The MAPK signaling is reduced in response to chemoconvulsant in AC8 KO mutants. 15 min after intraperitoneal injection of kainic acid (30mg/kg), the hippocampus was separated for detection of p-ERK1/2 by western blot (A) (n=12 for each genotype; 6 mice for saline injection and 6 mice for KA injection) and by immunochemistry staining (B) (n=12 for each genotype; 6 mice for saline injection or KA injection). In (A), the p-ERK1/2 level was normalized to total ERK1/2 and relative to saline injection, and presented as arbitrary values (the level in WT mice was set as 1). In (B), the relative intensity of p-ERK1/2 fluorescence

in the CA1 is expressed as arbitrary values relative to the saline injection (the intensity in WT was set as 1). C, saline injection; KA kainic acid; Representative images are shown; *P<0.05, compared to WT mice, Student's t-test.