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# A transient increase in CCK mRNA levels in hippocampus following audiogenic convulsions in audiogenic seizure-prone rats[1]

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**KEY WORDS** cholecystokinin; messenger RNA; *in situ* hybridization; hippocampus; convulsions; $P_{77}$PMC

## ABSTRACT

**AIM**: To examine the effects of a single convulsion and multiple convulsions on cholecystokinin (CCK) mRNA expression in hippocampus of audiogenic seizure-prone rats ($P_{77}$PMC). **METHODS**: Ringing (electric bell, 100 dB, 60 s) was used to induce convulsions, hippocampal CCK mRNA expression was exhibited by *in situ* hybridization. **RESULTS**: 1) The number of CCK mRNA-positive neurons in principal hippocampus of normal rats was 34 ± 5, which elevated markedly after a single (155 ± 7, $P < 0.01$) or multiple convulsions (95 ± 8, $P < 0.01$). 2) CCK mRNA levels in multiple consecutive convulsion rats were lower than that in a single convulsion rats ($P < 0.01$). **CONCLUSION**: The increased number of CCK mRNA-positive neurons in hippocampus may have important functional consequences in convulsion-associated processes.

## INTRODUCTION

Cholecystokinin sulphated-octapeptide (CCK-8S) is widely distributed in the neurons of the central nervous system, and is thought to act as a neurotransmitter or neuromodulator[1]. CCK-8S has been shown to exert anti-convulsant activity in several animal epilepsy models[2]. Many previous studies have demonstrated that epilepsy affects brain peptide content and synthesis. The changes in the cortical and hippocampal CCK immunoreactivity and preproCCK messenger RNA have been previously reported following pentylenetetrazole or electrical kindling and kainic acid-induced epilepsy[3]. The aim of this work was to investigate the expression of hippocampal CCK messenger RNA in response to convulsions by using a new animal epilepsy model — audiogenic seizure-prone rats ($P_{77}$PMC, supplied by Department of Experiment Animal, Beijing Medical University).

## MATERIAL AND METHODS

**Rats** Audiogenic seizure-prone adult rats ($n = 15$, 210－250 g, 237 ± 12 g, supplied by Department of Experiment Animal, Beijing Medical University, Grade 2, № 01-3056) were used. They were randomly divided into three groups (5/group)：1) Nonconvulsion group (control). No ringing stimulus was given to them, the other experimental conditions were the same as with convulsion groups; 2) Single-convulsion group. Convulsions were induced only once; 3) Multiple consecutive convulsions group. Convulsion was induced twice daily (morning and afternoon) for consecutive 2 d.

The rats were put in a special sound-insulated box, and an electric bell was fixed in the box to ring at 100 dB for 60 s. The detailed procedure of CHEN Yun-Cai[4] was followed to induce audiogenic convulsions by the stimuli of ringing. The rats that reached the fourth and fifth convulsion stage were used in our experiments[5].

**Purification and labeling of CCK cDNA** The plasmid PUC13 (containing 577 bp CCK cDNA) was presented by Prof JE Dixon (Department of Biological chemistry, University of Michigan, Ann Arbor, MI). The cDNA contains 345 nucleotides coding for a precursor to CCK, which has 115 amino acids. Recombinant rat CCK cDNA/pUC13 was digested with Hind Ⅲ and EcoR Ⅰ and CCK cDNA fragments were further purified by low-melting agarose electrophoresis. CCK cDNA was digoxin-labeled with random priming according to the procedure of ZHOU *et al*[6].

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**Tissue preparation for *in situ* hybridization** Rats after a single seizure ($n = 5$) or consecutive seizures ($n = 5$) and their controls ($n = 5$) were anesthetized with 0.4 % amylobarbitone and perfused through the ascending aorta with 50 mL phosphate buffered saline (PBS, 50 mmol·L$^{-1}$, pH 7.4) followed by 200 mL chilled 4 % paraformaldehyde in PBS. The brains were divided into three parts by coronal cuts. The tissues were postfixed in the same fixative for 90 min at 4 ℃, then transferred to 20 % sucrose in PBS and kept there for 24 h at 4 ℃. The brain was then immersed in −70 ℃ isopentane for 3 min and stored in tightly sealed vials at −70 ℃.

***In situ* hybridization** Rats were decapitated and their brains were frozen in isopentane at −70 ℃. Brain coronal sections (20 μm) were cut, thaw-mounted on gelatinized slides and kept dessicated at −20 ℃ until *in situ* hybridization was carried out.

The procedure of Vaudano[7] *et al* was followed. Frozen sections were rapidly immersed in 2 % paraformaldehyde, 10 mmol·L$^{-1}$ PBS for 10 min at room temperature (18 − 25 ℃), rinsed in 1 × PBS, immersed in 0.25 % acetic anhydride in triethylamine hydrochloride 0.1 mol·L$^{-1}$ for 10 min, dehydrated by ethanol series and delipidated with chloroform. They were hybridized with about 1 mg·L$^{-1}$ of CCK cDNA probe in 50 μL hybridization buffer at 42 ℃ for 24 h. The hybridization buffer consisted of 50 % formamide, 5 × SSC (1 × SSC is NaCl 150 mmol·L$^{-1}$, 15 mmol·L$^{-1}$ sodium citrate, pH 7.2), 500 mg·L$^{-1}$ salmon sperm DNA, 250 mg·L$^{-1}$ yeast tRNA, 1 × Denhardz' solution (0.02 % ficoll, 0.02 % polyvinylpyrrolidone, and 0.02 % bovine serum albumin), 10 % dextran sulfate, and dithiothreitol 20 mmol·L$^{-1}$ (all the chemicals were purchased from Sigma, St Louis). After hybridization and stringency washes, the detection procedure was carried out according to the method of ZHOU *et al*[6].

To test the sensitivity and specificity of the results, some sections were prepared by preincubating with excess unlabeled probe in the same hybridization buffer for 2 h.

Under the optical microscope, the *in situ* hybridization reaction product showed as blue-black, well-distributed bead materials located in cytoplasm of the neurons. The positive neurons could be divided into three types: strong, medium and weak positive neurons. The strong positive neurons are those whose reaction product was deeply dyed as thick blue-black; while the reaction product of weak positive neurons was dyed a light blue; the dyeing degree in medium positive neurons was between the above two neuron types.

**Statistical analysis** Five slices were chosen from each rat to count the individual labeled CCK-mRNA-expressing cell numbers in principal hippocampus, the accumulated number from the five slices of each rat is the total number of positive CCK-mRNA-expressing cells in principal hippocampus of the rat. The positive neuron number of each group was represented by $\bar{x} \pm s$. The above parameters were evaluated by a paired $t$ test.

## RESULTS

In non-convulsion rats, CCK mRNA positive neurons were scarce in principal hippocampus. The blue-black reaction product in cytoplasm was light. The number of positive neurons was about 34 ± 5, most of them were medium and weak positive neurons, only 21 % ± 2 % of all positive neurons were ones (Fig 1A).

The shape, size, and distribution of CCK mRNA positive neurons after a single or multiple consecutive convulsions was the same as with the control rats. The number of positive neurons significantly increased compared with the control (Fig 1). The positive neuron number after a single convulsion was 155 ± 7 ($P < 0.01$), 90 % ± 5 % of them were strongly dyed cells ($P < 0.01$). The positive number after multiple consecutive convulsions was 95 ± 8 ($P < 0.01$), 68 % ± 3 % of them were strongly dyed cells ($P < 0.01$).

An interesting finding is that CCK mRNA levels (the number of CCK mRNA positive neurons), was lower in consecutive convulsion rats than in single convulsion rats ($P < 0.01$).

## DISCUSSION

The present results demonstrate that the CCK mRNA expression in principal hippocampus is strongly increased after a single convulsion or multiple consecutive convulsions. The results clearly indicate that the increased CCK mRNA is associated with convulsion-associated processes.

A role for CCK as a potential endogenous anticonvulsant substance has been suggested by several studies. Systemic injections of CCK into rodents can attenuate or delay the appearance of generalized convulsions induced by picrotoxin, other convulsant drugs and electroshock[8]. Convulsion of audiogenic seizure-prone rats could be suppressed by intracerebroventricular (icv) or intraperitoneal (ip) injection of CCK-8S and even by icv

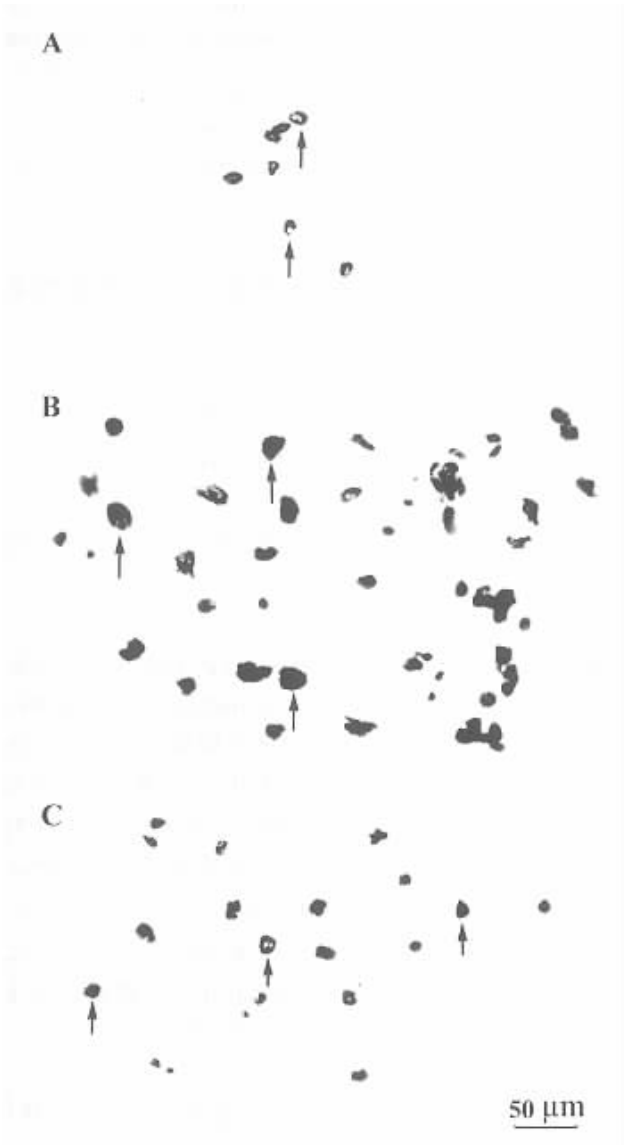


**Fig 1. CCK mRNA positive neurons in principal hippocampus (× 150, NBT stain). A) Nonconvulsion group. B) CCK mRNA positive neurons increased markedly after a single convulsion. C) Number of CCK mRNA positive neurons decreased after multiple consecutive convulsions *vs* a single convulsion group ($P < 0.01$), but are still strongly increased *vs* nonconvulsion rats ($P < 0.01$).**

injection of a CCK expression vector[9,10]. So our data may suggest that the increased CCK mRNA in principal hippocampus may have something to do with anti-convulsant processes.

Previous work has suggested that glutamate and/or enkephalin may be important for CCK regulation. Glutamate has been demonstrated to be necessary for CCK expression in kindled convulsion processes[11]; enkephalin can induce limbic convulsion when injected directly into the ventral hippocampus[12]. These studies lead one to speculate that CCK and enkephalin may exert antagonistic physiological effects during convulsion. Thus, the increased CCK mRNA expression in our experiment may reflect a compensatory anticonvulsant response to counteract glutamate or the putative proconvulsant effect of convulsion-induced increased enkephalin expression.

Of particular interest is the finding that CCK mRNA expression in principal hippocampus is lower in consecutive convulsions rats than that in single convulsion rats. An early *in situ* hybridization experiment investigated the changes in cholecystokinin mRNA expression after amygdala kindled convulsions, and found that a single kindled convulsion and multiple consecutive kindled convulsions have different effects on CCK mRNA expression in the amygdala, cerebral cortex, and hippocampus[2]. So our data provides new evidence for this idea hinting differential regulation of CCK expression by single *vs* multiple convulsions.

In conclusion, our *in situ* data indicates that the increased number of CCK mRNA positive neurons in hippocampus may have important functional consequences in convulsion-associated processes; the mechanisms that regulate CCK expression during a single *vs* multiple convulsions may be different.

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## 听源性惊厥致 $P_{77}$ PMC 大鼠海马内胆囊收缩素 mRNA增加[1]

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关键词 缩胆囊素；信使 RNA；原位杂交；海马；惊厥；$P_{77}$PMC

目的：研究听源性惊厥易感大鼠（$P_{77}$PMC）单次惊厥发作和多次发作对海马 CCK mRNA 表达的影响. 方法：电铃声（100 Db，60 s）诱发惊厥发作，原位杂交法显示海马 CCK mRNA 的表达. 结果：1）惊厥未发作大鼠海马本部 CCK mRNA 阳性神经元数目为 34 ± 5；单次和多次惊厥发作后海马本部 CCK mRNA 阳性神经元数目显著增加（155 ± 7 或 95 ± 8，$P < 0.01$）. 2）多次惊厥发作后 CCK mRNA 阳性神经元数目较单次发作明显下降（$P < 0.01$）. 结论：海马 CCK mRNA 参与惊厥发作的病理过程.

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