Epilepsy Research 126 (2016) 45–52

Contents lists available at www.sciencedirect.com

Epilepsy Research

journal homepage: www.elsevier.com/locate/epilepsyres

# Altered expression of neuronal CCR6 during pilocarpine induced status epilepticus in mice

Jian-Xin Liu[a,1], Xia Cao[b,1], Yong Liu[a], Feng-Ru Tang[c,*]

[a] Institute of Neurobiology, School of Basic Medical Sciences, Xi'an Jiaotong University Health Science Center, 76 West Yanta Road, Xi'an 710061, China
[b] The Second Affiliated Hospital of Kunming Medical University, 374 Dianmian Road, Kunming 650101, China
[c] Radiobiology Research Laboratory, Singapore Nuclear Research and Safety Initiative, National University of Singapore, Singapore

ARTICLE INFO

*Article history:*
Received 17 February 2016
Received in revised form 2 June 2016
Accepted 25 June 2016
Available online 26 June 2016

*Keywords:*
Chemokine receptor
Epilepsy
Hippocampus
Status epilepticus

ABSTRACT

Chemokine and receptor systems play important roles in different animal models of status epileptics and epileptogenesis. Here, we identified protein and gene expression of chemokine receptor 6 in the hippocampus of Swiss mice with immunohistochemistry and RT-PCR respectively. Immunohistochemical study showed that CCR6 immunopositive product was localized in different subtypes of hippocampal interneurons, in apical dendrites of pyramidal neurons of CA1 area and other laminas of the hippocampus. Strongly stained CCR6 immunopositive product was found in calbindin, calretinin, parvalbumin immunopositive interneurons in the stratum oriens of CA1 area. During pilocarpine induced status epilepticus, a transient down-regulation of neuronal CCR6 in the stratum oriens of CA1 was demonstrated at 2 h during status epilepticus. The present study provides evidence that CCR6 may be involved in normal neuronal activity in the hippocampus and play an important role in maintenance of the status epilepticus.


## 1. Introduction

Chemokine ligand-receptor family is originally described as chemotactic cytokines which are involved in leukocyte trafficking. Based on the position of cysteine residues, chemokines are classified into four groups, i.e., CXC (α), CC (β), C (γ) and CX3C (δ), and the respective receptors are therefore named as CXCR, CCR, CR and CX3CR. While most chemokine ligand-receptors are only inductively expressed in neurons of central nervous system (CNS) in different animal models of neurological disorders, the constitutive expression of several chemokine ligand and/or receptors such as CCR1 (Meucci et al., 1998), CCL2/CCR2 (Foresti et al., 2009; Arisi et al., 2015), CCL3/CCR3 (Cowell and Silverstein, 2003; Xu et al., 2009), CXCR4 (for review, see Nash and Meucci 2014), CCR7, CCR8, CCR9, CCL28/CCR10 (Liu et al., 2007, 2012) have also been reported. The latter favors the view that the chemokine may serve as a signaling system in cellular communication within the mammalian CNS (for review, see Adler et al., 2006).

In the last decade, several studies suggest a role of the neuronal chemokines, especially CC chemokines including CCL2/CCR2, CCL3/CCR3 (Xu et al., 2009), CCR5 (for review, see Louboutin and Strayer 2013; Louboutin et al., 2011), CCR7, CCR8, CCR9 and CCL28/CCR10 (Liu et al., 2007, 2012) in the maintenance of status epilepticus (SE), and delayed cell loss of hippocampus following SE and epileptogenesis.

In the present study, we aimed to (1) identify if CCR6 was expressed in the normal hippocampus at both gene and protein levels, (2) examine the localization of CCR6 in different laminin and subtypes of neurons in the hippocampus, (3) show the progressive changes of CCR6 in the hippocampus in the mouse pilocarpine model of SE.

## 2. Methods

### 2.1. Housing and handling of animals

Female Swiss mice were group-housed in a temperature-controlled facility with a 12 h light/dark cycle (the lights were turn on at 6 am and turn off at 6 pm) and fed standard rodent diet and water ad libitum. Female mice were chosen in this study because of our experience that female mice had a lower death rate during and after pilocarpine induced SE compared with male ones. In addition, because the mice were not fully mature when the experiments were done, the potential influences of an ovarian cycle on the expression of CCR6 could be excluded. All experiments were approved by the National Neuroscience Institute of Singapore Animal Care and Use Committee. In the handling and care of all animals,

* Corresponding author.
*E-mail address:* tangfr@gmail.com (F.-R. Tang).
[1] These authors contribute equally to this work.

http://dx.doi.org/10.1016/j.eplepsyres.2016.06.011
0920-1211/

the guidelines of the NIH for animal research were strictly followed. Efforts were made throughout the study to minimize animal suffering and to use the minimum number of animals.

### 2.2. Pilocarpine treatment

Female Swiss mice weighing 25–30 g were used for the study. Mice were given a single subcutaneous injection of methylscopolamine nitrate (1 mg/kg) (Sigma, USA) 30 min before the injection of either saline in the control or pilocarpine in the experimental group. In the latter group, the mice received a single i.p. injection of 300 mg/kg pilocarpine (Sigma, USA) and experienced acute SE.

### 2.3. RNA extraction and RT-PCR

Following deep anesthesia with chloral hydrate (0.4 g/kg), the brain was taken out, and the whole hippocampus was separated quickly. Isolation of total RNA from mouse hippocampus was performed according to the protocol from RNeasya Mini kit handbook (QIAGEN, Germany). On-column DNaseI digestion was performed to eliminate any genomic DNA contamination. All RNA samples were stored at −80 °C. RT-PCR was performed using the OneStep RT-PCR Kit from QIAGEN (Germany). The primers for amplification of the CCR6 were: FW, 5′-GAGTTCATGCAGCATCCAGA-3′, RV, 5′-AGGCTCTCATCCACTGCTTC-3′. Each reaction was set up as described in the RNeasya Mini kit handbook using 50 ng total RNA as the starting template. The mixture was incubated at 50 °C for 30 min, 95 °C for 15 min, and followed by 35 cycles at 94 °C for 1 min, 60 °C for 50 s, 72 °C for 30 s, and a final cycle at 72 °C for 7 min. The PCR product was separated on a 1.8% agarose gel electrophoresis with TBS electrophoresis buffer and the bands (expected size is 110 bp for CCR6, 146 bp for β-actin) were then visualized under UV light after staining by ethidium bromide. For negative control, the RNA template was replaced by the same volume of miniQ water.

### 2.4. Cloning, sequencing and analysis of mouse CC6 in hippocampus

RT-PCR product of CC6 from mouse hippocampus was purified and ligated to the pGEM-T Easy vector according to the user's manual (Promega, USA). The vector was then transformed into *Escherichia coli* TOP10F' competent cells. Plasmids DNA was isolated from the transformed clones using the QIAprep Miniprep kit (QIAGEN, Germany). DNA sequencing was performed using ABI PRISM BigDye Terminator v3.1 Cycle Sequencing Kit and ABI 3100 Genetic Analyzer. The comparison between the cloned inserts and published sequence of CCR6 was done using the BLASTN program from the National Center for Biotechnology Information.

### 2.5. Quantitative real-time PCR

Following deep anesthesia with chloral hydrate (0.4 g/kg), the brain was taken out, and the whole hippocampus was separated quickly. Isolation of total RNA from mouse hippocampus was performed according to the protocol from RNeasya Mini kit handbook (QIAGEN, Germany). 2 μg total RNA from the mouse hippocampus was reversed transcribed using the Murine Leukemia virus reverse transcriptase (Promega, USA) for real-time PCR. PCR was carried out in a Gene-Amp 9700 PCR system (Applied Biosystems, ABI, USA).The SYBRGreen I double-stranded DNA binding dye (Roche Diagnostics, GmbH Mannheim, Germany) was used for the assay. Gene specific primers for CC6 were designed using primer 3: FW, 5′-CCTGCCTGGGGAATGAATTC-3′, RV, 5′-ACCTCTTCTAGGGAGCATGG-3′. Synaptophysin was chosen as the reference gene for the samples of mouse hippocampus, respectively, according to Chen et al. (2001). All reactions were performed in triplicate with 20 μl reaction volumes including 5 μl of cDNA, 2 μl of each primer and 4 μl of a LightCycler FastStart DNA Master SYBR Green I, 9 μl SYBR Green $H_2O$, and incubated in Light-Cycler under the following conditions: at 95 °C for 10 min, followed by 45 cycles of 94 °C for 1 s, 60 °C for 5 s, 72 °C for 10 s and finally cooled to 40 °C. The hot start PCR method was applied to prevent incomplete DNA denaturation. Following amplification, real-time PCR product was removed from the reaction capillaries and analyzed by 1.8% agarose gel electrophoresis to verify the product size. To quantify the results obtained by real-time RT-PCR, plasmid DNA concentration of CC6 was measured spectrophotometrically and 4 serial ten-fold dilutions of plasmid DNA were amplified to construct a standard curve which was used for extrapolation of expression level for CCR6 based on their threshold cycle (Ct) values.

### 2.6. Immunohistochemical study of the expression of CCR6

Six mice were sacrificed at each of the survival intervals, i.e. at 10 min, 30 min, 1 h, 2 h during pilocarpine-induced SE and 24 h after pilocarpine induced SE. Six mice with saline instead of pilocarpine injection were sacrificed for the control group. Following deep anesthesia with chloral hydrate (0.4 g/kg), the mice were perfused transcardially with 10 ml of saline initially, followed by 100 ml of 4% paraformaldehyde in 0.1 M phosphate buffer (PB) (pH 7.4) for 10 min. After perfusion, the brain of each mouse was removed, and kept overnight in 30% sucrose in 0.1 M PB. Coronal sections at 40 μm thickness were cut through the entire antero-posterior axis of the hippocampus in a cryostat (Slee Technik, Mainz, Germany). Serial sections of hippocampus were transferred to 5 different wells of a 24-well tissue culture dish for immunohistochemical reactions. Each well had 6–8 sections of dorsal hippocampus. For immunohistochemical study, free-floating sections were treated in 4% normal goat serum for 2 h at room temperature. All sections were then incubated with primary rabbit antibodies (1:1000) (Abcam, UK) in 0.1 M Tris-buffered saline (TBS) containing 0.1% Triton X-100 (TBSTX, pH 7.6) overnight. After incubation, sections were washed in TBS-TX and placed for 2 h in biotinylated anti-rabbit secondary antibodies (Chemicon International Inc., USA). After three washes in TBS-TX, the sections were placed in avidin—biotin complex (ABC) reagent (Chemicon International Inc., CA, USA) in TBS-TX for 2 h. They were then washed in 0.1 M Tris buffer (TB, pH 7.6), reacted in a solution of 0.012% H2O2 and 0.05% 3, 3′-diaminobenzidine in TB for 3 min, mounted, and covered. In negative control section, the primary antibodies were omitted.

### 2.7. Double labeling immunofluorescence microscopy

To identify the neuronal types expressing CC6 in the stratum oriens of CA1 area, double labeling of CCR6 with different subpopulations of calcium binding proteins (CBP) i.e. calbindin (CB), calretinin (CR) or parvalbumin (PV) was done. Tissue sections from six controls and 2 h during SE mice were used to co-localize CCR6 with CB, CR or PV. The tissue preparation procedure was the same asthe immunohistochemical study. The sections were washed in 0.1 M TBS–TX and placed overnight in mixed primary antibodies (CCR6 1:1000 with CB/1:2000, calretinin CR/1:1500, parvalbumin PV/1:1500 or GFAP/1:200 respectively) (mouse anti-calbindin and calretinin, parvalbumin were from Chemicon International Inc., CA, USA). After incubation, sections were washed in TBS–TX and placed for 4 h in Cy3-conjugated donkey anti-Rabbit IgG (1:200, Chemicon International Inc., CA, USA), followed by incubation with FITC-conjugated goat anti-mouse IgG (1:200, Chemicon International Inc., CA, USA) for another 4 h. The sections were then mounted, dried, and covered with FluorSaveTM Reagent to retard fading, and