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Status epilepticus evokes prolonged increase in the expression of CCL3 and CCL4 mRNA and protein in the rat brain

Anna Guzik-Kornacka, Anna Sliwa, Gabriela Plucinska and Katarzyna Lukasiuk*

The Nencki Institute of Experimental Biology, Warsaw, Poland; *Email: k.lukasiuk@nencki.gov.pl

CCL3 and CCL4 are proinflammatory chemokines belonging to the CC family. Increase in expression of mRNA coding for various chemokines including CCL3 and CCL4 has been often detected with global transcriptome profiling of brain tissue following epileptogenic stimuli as well as in epilepsy in experimental models and in human patients. Despite this, little is known about the expression of these proteins in epileptogenesis or epilepsy. In the present work CCL3 and CCL4 mRNA and protein expression were studied in the amygdala stimulation model of temporal lobe epilepsy using quantitiative PCR and immunohistochemistry. Expression of CCL3 and CCL4 mRNA in the block of tissue containing enthorinal and piriform cortices, amygdala and piriform nucleus was markedly up-regulated at 1, 4, 14 and 30 days following stimulation and in hippocampal CA1 was significantly increased at 1 and 4 days following stimulation. Expression of CCL3 and CCL4 proteins was elevated in astrocytes in the enthorinal and piriform cortices, amygdala, and hippocampus showing the largest increase at 4D after status epilepticus. Increase in mRNA and protein levels of CCL3 and CCL4 in the animal model of temporal lobe epilepsy suggests their role in disease development or recovery form epileptogenic insult. Existence of multiple targets for these chemokines in the damaged brain allows several possibilities of influencing neuronal and glial functions.

Key words: astrocyte, chemokine, epilepsy, Macrophage Inflammatory Protein 1-alpha, Macrophage Inflammatory Protein 1-beta, neurodegeneration

INTRODUCTION

Chemokines (chemotactic cytokines) constitute a large family of small (8-14 kDa) cytokines, first discovered as chemotactic agents. According to their protein structure chemokines are classified into 4 subfamilies: C, CC, CXC and CX3C depending on the number and spacing of conserved cysteine residues at the N-terminal end of the protein. There are at least 50 known chemokines (Zlotnik and Yoshie 2000, Murphy 2002, Miller et al. 2008). Chemokines exert their biological function through G_i protein-coupled receptors. Twenty two chemokine receptors have been characterized up to date (Bacon and Harrison 2000, Rossi and Zlotnik 2000). Chemokines have been shown to be involved in a variety of biological processes in the brain including development, neuroinflammation, migration, cell communication and synaptic transmission (Rostene et al. 2007, Biber, Vinet et al. 2008, Guyon et al. 2008, Melik-Parsadaniantz and Rostene 2008, Miller et al. 2008). The role of chemokines has also been suggested in various pathologies of the nervous system such as inflammation, trauma, ischemia and multiple sclerosis (Semple et al. 2009)

CCL3 (chemokine C-C motif ligand 3, also known as Macrophage Inflammatory Protein 1-alpha, MIP-1a, or small inducible cytokine A3) and CCL4 (chemokine C-C motif ligand 4, also known as Macrophage Inflammatory Protein 1-beta, MIP-1b or small inducible cytokine A4) are proinflammatory chemokines belonging to the CC family. They are mostly known as chemoattractants for monocytes and T cells. They have also been extensively studied in the context of HIV infection, since they compete with the HIV virus for binding to the same receptor (Cocchi et al. 1995).

Our interest in these chemokines was fueled by the observation that increase in expression of mRNA coding for various chemokines and chemokine receptors has been often detected in transcriptome profiling

Correspondence should be addressed to K. Lukasiuk
Email: k.lukasiuk@nencki.gov.pl

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studies of brain tissue following epileptogenic stimuli as well as in epilepsy in experimental models and in human patients (Tang et al. 2002, Lukasiuk et al. 2006, Lee et al. 2007, van Gassen et al. 2008, Cacheaux et al. 2009). Chemokines most consistently upregulated by epileptogenic stimuli are CCL2, CCL3 and CCL4 (Lukasiuk et al. 2006). Up to now, the most studied chemokine was CCL2 (MCP-1; Wu et al. 2008, Foresti et al. 2009, Xu et al. 2009), while little is known about the expression and distribution of CCL3 and CCL4 mRNA and protein in epileptogenesis or epilepsy.

In this study we aimed to investigate CCL3 and CCL4 mRNA expression in the brain at different time points following status epilepticus. We investigated also if alterations in gene expression were accompanied by changes in protein expression and characterized the spatiotemporal pattern of CCL3 and CCL4 protein expression during different stages of epileptogenesis in the amygdala stimulation model of the temporal lobe epilepsy in rat.

METHODS

Adult male Spraque-Dawley rats (290 - 360 g) were obtained from Medical Research Centre (Warsaw, Poland). Animals were housed in controlled environment (temperature 24°C, lights on 07:00 AM to 07:00 PM) with free access to food and water. Starting from the day of surgery, each animal was housed in a separate cage. All animal procedures were approved by the Ethical Committee on Animal Research of the Nencki Institute, and conducted in accordance with the guidelines set by the European Council Directives 86/609/ECC.

Status epilepticus (SE) was triggered by electrical stimulation of the amygdala as previously described by Nissinen and coauthors with some modifications (Nissinen et al. 2000). For electrode implantation animals were first injected with butorphanol (Butomidor, Richter Pharma AG, 0.5 mg/kg, i.p.) and than subjected to isoflurane anesthesia (2 - 2.5% in 100% O_2) Home made stimulating and recording bipolar wire electrode (wire: Franco Corradi, Milan, Italy, diameter 0.127 mm, #316) connected with two gold-plated female socket contacts (Plastic One Inc., #E363/0) was implanted into the left lateral nucleus of the amygdala (3.6 mm posterior and 5.0 mm lateral to bregma, 6.5 mm ventral to the surface of the brain; Paxinos and Watson 2007). A stainless steel screw electrode (Plastic One Inc., #E363/20) was implanted contralaterally into the skull over the right frontal cortex (3.0 mm anterior and 2.0 mm lateral to bregma) for a surface EEG recording. Two stainless steel screw electrodes were placed bilaterally over the cerebellum (10.0 mm posterior and 2.0 mm lateral to bregma) as a ground and reference. Socket contacts of all electrodes were placed in a multichannel electrode pedestal (Plastic One Inc., #MS363) which was attached to the skull with dental acrylate (Duracryl Plus). After two weeks of recovery animals were electrically stimulated via the intra-amygdala electrode to evoke status epilepticus. Stimulation consisted of a 100-ms train of 1-ms biphasic square-wave pulses (400 μA peak to peak) delivered at 60 Hz, every 0.5 s for 20 min. If the animal did not reveal SE behavior following 20 min of stimulation, stimulation was continued for the next 10 min. The SE has been stopped 1.5 - 2 h after stimulation by intraperitoneal injection of diazepam (20 mg/kg). If the first dose of diazepam did not suppress SE, animal received subsequent doses of 5 mg/kg. Time-matched control animals had electrodes implanted but did not receive electrical stimulation. Starting from stimulation, rats were monitored continuously with video-EEG (24 h/day) every second day to detect spontaneous epileptic seizures. Spontaneous seizures were identified from EEG recordings by browsing the EEG manually on the computer screen. Electrographic seizure was defined as a high frequency (>8 Hz), high amplitude (>2× baseline) discharge lasting for at least 5 s.

For mRNA isolation, rats were anaesthetized with carbon dioxide and decapitated with a guillotine 1 day (n=6), 4 days (n=6), 14 days (n=5 for temporal tissue; n=6 for CA1) and 1 month (n=6 for temporal tissue; n=5 for CA1) after SE. Control rats were sacrificed at the same time as corresponding stimulated groups. Separate, time matched control group was prepared for each time point (temporal tissue samples: n=6 for 1-d and 30-d, and n=5 for 4-d and 14-d; CA1 samples: n=6 for 1-d and 4-d, n=5 for 14-d, and n=4 for 30-d). Brains were quickly removed and cut to 1 mm thick coronal slices with acrylic brain matrix (WPI Inc., #RBMA-600C). Slices were preserved in RNAlater (Ambion, #AM7024) solution according to manufacturer's instructions and stored at -20°C for further processing. Temporal tissue (containing the piriform nucleus, amygdala, entorhinal and piriform cortices) and CA1 fields of the hippocampus were dissected and stored at -20°C until RNA isolation. Total RNA was

isolated using RNAeasy Mini Kit (Qiagen, #74104). Genomic DNA was digested on column during RNA isolation with DNase I (Qiagen, #79254) according to the manufacturer's protocol. The concentration and quality of RNA was measured by capillary electrophoresis with RNA 6000 Nano Chip (Agilent, #5067-1511) in 2100 Bioanalyzer (Agilent, #G2938B). Purified total RNA was stored at -70°C.

Reverse transcription was performed according to manufacturer's instructions in a final volume of 20 µl containing 0.5 µg of RNA, deoxynucleotide triphosphates (dNTPs, 1 mM each, Promega, #U1410), 0.5 µg random primers (Promega, #C1181) and 200 units of reverse transcriptase M-MLV (Sigma-Aldrich, #M1302-40KU) in M-MLV buffer (Sigma-Aldrich). After reaction the volume was adjusted to 40 µl and samples were stored at -20°C.

For qPCR, 18S rRNA gene was chosen as an internal control. Primers sequences for CCL3 (forward CATGGCGCTCTGGAACGAA, reverse TGCCGTCCATAGGAGAAGCA), CCL4 (forward TATGAGACCAGCAGCCTTTGC, reverse GCACAGATTTGCCTGCCTTT) and for 18S rRNA (forward AACGAACGAGACTCTGGCATG, reverse CGGACATCTAAGGGCATCACA) were designed in the Primer Express software (Applied Biosystems). PCR reactions were performed in duplicates in a total volume of 20 µl containing 1× ready to use SYBR Green Master Mix (Applied Biosystems, #4309155), 1 µl of cDNA and primers in appropriate concentration (for CCL3 or CCL4 - 500 nM each and 18S rRNA - 125 nM each). Reactions were performed in the ABI PRISM 7500 System (Applied Biosystems) using the following programme (1 cycle: 50°C - 2 min, 95°C - 10 min; 2 - 40 cycles: 95°C - 15 s, 60°C - 1 min). The relative level of CCL3 or CCL4 mRNA expression was quantified using a standard curve method. For the standard curves, series of 5-fold dilutions, spanning the concentrations in experimental samples, were prepared from a concentrated cDNA sample. Relative concentration values for each chemokine sample were normalized to the values of corresponding 18S rRNA samples. Data are presented as a fold change of mean of expression in the control group. Statistical significance was determined by the Mann-Whitney's *U*-test. Correlations between experimental groups were determined using the Spearman test.

For immunohistochemistry studies brains were collected at 6 hours (n=4), 1 day (n=4), 4 days (n=4) and 14 days (n=4) after stimulation. Two time matched control rats were prepared for each time point. For statistical analysis early time controls (6-h and 1-d) or late time controls (4-d and 14-d) were pooled. Control rats had implanted electrodes but were not stimulated. Rats were deeply anaesthetized by intraperitoneal injection of sodium pentobarbital (Vetbutal, Biowet, 120 mg/kg) and intracardially perfused, first for 2 min with saline and then for 20 min with cold 4% paraformaldehyde in 0.1 M sodium phosphate buffer, pH 7.4. After perfusion the brains were isolated, additionally fixed for 4 h in 4% paraformaldehyde, and cryoprotected with 30% sucrose in 0.02 M potassium phosphate buffer, pH 7.4 until complete saturation. Brains were frozen in dry ice, and stored at -70°C until cutting. Brains were cut to 30 µm thick coronal sections using a microtome at -20°C. Every sixth section was collected to the 2% formalin solution and used for Nissl staining. The remaining sections were collected to a crioprotectant tissue collecting solution TCS (30% ethylene glycol, 25% glycerol, 0.05 M sodium phosphate buffer pH 7.4) and stored at -20°C.

For double immunohistochemical staining brain sections were washed (3 × 10 min) in 0.02 M potassium phosphate buffered saline, pH 7.4 (KPBS: 0.02 M K_2HPO_4, 0.02 M KH_2PO_4, 0.15 M NaCl) with addition of 0.5% Triton X-100 (TX-100, Sigma-Aldrich). Blocking was performed in 5% normal horse serum (NHS, Vector Labs., #S-2000), 1% bovine serum albumin (BSA, Vector Labs., #SP-5050) in 0.02 M KPBS with 0.5% TX-100 for 4 hours at room temperature. Subsequently, sections were incubated overnight at 4°C in primary antibody solution containing goat polyclonal anti-CCL3 antibody (1:500, Santa Cruz Biotechnology, #SC-1383) or anti-CCL4 antibody (1:500, Santa Cruz, #SC-1387) and monoclonal anti-glial fibrillary acidic protein (GFAP)−Cy3 antibody (1:100, Sigma Aldrich, #C9205) in 0.02 M KPBS with 0.5% TX-100 and 1% NHS. After incubation sections were washed in 0.02 M KPBS with 0.5% TX-100, 3 × 10 minutes to remove unbound antibody and incubated for 6 hours at room temperature with secondary fluorescein conjugated anti-goat antibody (1:200, Vector Labs., #FI-5000) in 0.02 M KPBS with 0.5% TX-100. Subsequently sections were washed as previously, rinsed with 0.1 M sodium phosphate buffer and mounted on gelatine-coated glass slides, dried and coverslipped using a mounting medium with DAPI (Vector Labs., #H-1200).

The unspecific staining for CCL3 and CCL4 antibodies was determined by omission of primary antibody or by preincubation of primary antibody with the peptide used for antibody production (1:250, Santa Cruz, #sc-1383 P for CCL3 and #sc-1387 P for CCL4). Unspecific staining was present in fibers, mainly in corpus callosum, but was not detected in cell somata.

Microscopic analyses were performed using a Nikon Eclipse 80i epifluorescence microscope (lens 10×) equipped with automated tiling system. In epifluorescent microscope the fluorescein signal was excited by 465-495 nm wavelength light. For chemokine localization and expression study whole brain sections images were tiled using the Image Pro Plus 5.0 software (Media Cybernetics Inc.). CCL3 and CCL4 immunostainings were scored in hippocampal areas CA1, CA3 and dentate gyrus as well as in nuclei of the amygdala and in the enthorinal and piriform cortices according to the following scale: score 0 - no immunoreactivity; score 1 – low; score 2 – moderate; score 3 – high; score 4 - very high immunoreactivity. Statistical significance of the immunoreactivity scores for each structure was determined using non-parametric Mann-Whitney *U*-test. A *p*-value of less than 0.05 was considered significant. Since in control, un-stimulated brains collected at early time points, light to moderate chemokine immunoreactivity was observed, the data from 6-h and 1-d time-matched control rats (n=2 for each control group) were combined for comparison with 6-h and 1-d stimulated animals and the data from 4-d and 14-d time-matched control rats (n=2 for each control group) were combined for comparison with 4-d and 14-d stimulated animals.

Confocal microscopy (Leica TCS SP2 spectral confocal microscope, lens 40×) analysis was performed to visualize colocalization of CCL3 and CCL4-like immunohistochemistry with astroglial marker GFAP. Fluorescein was excited with argon laser (488 nm) and cyanine 3 with helium-neon laser (543 nm).

RESULTS

CCL3 and CCL4 mRNA expression following status epilpticus (SE)

Expression of mRNA coding for CCL3 and CCL4 was evaluated in the CA1 of the hippocampus and in the block of tissue containing the entorhinal and piriform cortices, piriform nucleus and the amygdala (we refer to this block of tissue as "temporal tissue" throughout the manuscript) at: 1-d, 4-d, 14-d, and 30-d following amygdala stimulation induced status epilepticus. Time-matched controls were prepared for each experimental group. CCL3 and CCL4 mRNA expression level is presented as a fold change of mean of the time-matched control level (Fig. 1 and Fig. 2, respectively).

Expression of CCL3 mRNA in the temporal lobe was markedly up-regulated throughout the observation

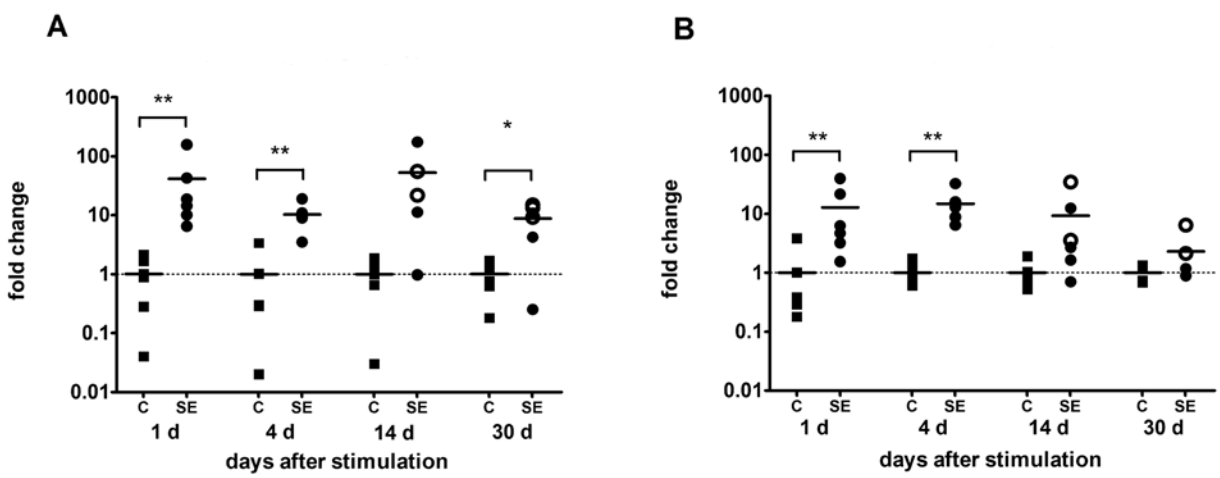

Fig. 1. Relative level of CCL3 mRNA expression in the temporal tissue (A) and the CA1 field of hippocampus (B) in individual rats at different time following stimulation induced status epilepticus. mRNA levels are presented as a fold change of mean control level (note the logarithmic scale). Individual control rats are marked by ■. Epileptic rats are marked by ○, while non-epileptic rats are marked by ● .**p<0.01; *p<0.05 as compared with control group (Mann-Whitney *U*-test).

period (Fig. 1A). At 1-d after stimulation the expression of CCL3 mRNA was 41.5±58.0 fold higher than mean expression in the time-matched control group (p<0.01). The increase in expression of CCL3 mRNA at this time point ranged from 6.5 to as much as 157.0 fold over the mean of the control level. Expression of CCL3 mRNA remained elevated at 4-d after SE and was 10.2±5.0 fold higher than in the control group (range: 3.5 to 18.9, p<0.01). At 14-d, the mean fold of increase from the control level was 52.4±70.5 (1.0 to 173.2, p=0.056). At 30 days after SE expression of CCL3 mRNA was still significantly increased to 8.8±5.6 (0.3 to 14.9, p<0.05).

In CA1 of the hippocampus CCL3 mRNA expression was significantly elevated at 1-d and 4-d following SE (12.8±15.0, range from 1.5 to 39.6, p<0.01, and 14.9±9.1, range from 6.4 to 32.3, p<0.01, respectively, Fig. 1B). At later time points differences in CCL3 mRNA expression following SE were not statistically significant in comparison with respective controls. However some animals still had high CCL3 mRNA expression, especially at 14 days after SE. The average level of expression at this time point exceeds the control level 9.2±13.1 times (range from 0.7 to 34.5, p>0.05). Thirty days after stimulation the mean CCL3 mRNA expression in CA1 was 2.3±2.3 fold of the control.

The levels of expression of CCL3 mRNA in CA1 and the temporal tissue were correlated (R=0.52, p<0.05).

Changes in expression of CCL4 mRNA in the temporal tissue following SE were similar to these observed for CCL3 mRNA (Fig. 2A). At 1-d after SE, CCL4 mRNA level was strongly elevated. At this time point the mean CCL4 mRNA expression was 203.7±291.3 fold higher then in controls (p<0.01). In one rat the expression was almost 800 times higher than in the control group (Fig. 2A). Four days following stimulation, CCL4 mRNA expression was 25.0±15.3 times higher than in controls (range from 2.0 to 43.7, p<0.01). At two weeks after SE, the average level of expression was not statistically different from that in time-matched controls, but it was nonetheless 17.2±22.5 times higher than in the control group (range from 1.0 to 52.4, p=0.056). Thirty days after stimulation, expression of CCL4 mRNA was 10.7±9.8 times higher than in controls and this difference was statistically significant (range from 0.9 to 28.2, p<0.05).

Analysis of the CCL4 mRNA expression in CA1 of the hippocampus revealed similar changes as in the case of CCL3 expression with significant increase at 1-d and 4-d after SE, a trend toward increased expression at 14-d and relative normalization of expression at 30-d after SE (Fig. 2B). The highest increase in CCL4 mRNA in CA1 was observed at 1-d following stimulation. At this time point the relative level of CCL4 mRNA expression in the CA1 field of the hippocampus was 101.1±152.7 fold of control (p<0.01). The expression ranged from 6.9 to as much as 401.0 times

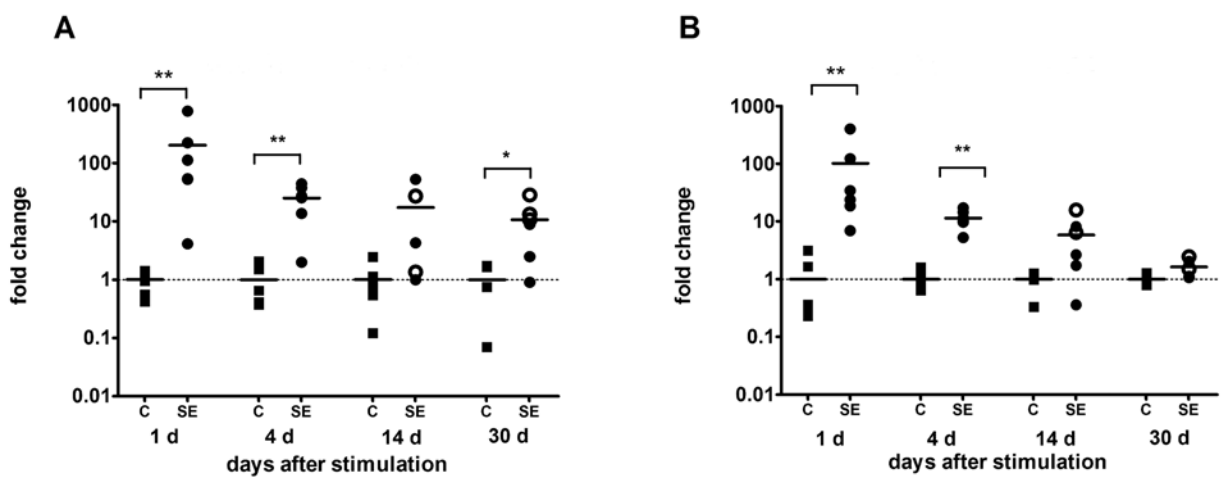

Fig. 2. Relative level of CCL4 mRNA expression in the temporal tissue (A) and the CA1 field of hippocampus (B) in individual rats at different time following stimulation induced status epilepticus. mRNA levels are presented as a fold change of mean control level (note the logarithmic scale). Individual control rats are marked by ■. Epileptic rats are marked by ○, while non-epileptic rats are marked by ● .**p<0.01; *p<0.05 as compared with control group (Mann-Whitney U-test).

higher than in the time-matched control group. Four days after SE, the average level of CCL4 mRNA expression was 11.4±4.1 fold of the control level (range from 5.2 to 17.2, p<0,01) and the difference was statistically significant. At 14-d following SE, the difference in CCL4 mRNA level decreased to 5.8±5.7 fold (range from 0.4 to 15.7, p=0.052) and ceased to be statistically significant. At 30-d after stimulation, CCL4 mRNA was only slightly elevated, on average 1.6±0.6 times (range from 1.1 to 2.5). There was no statistical difference between stimulated and control rats.

The levels of expression of CCL4 mRNA in CA1 and in the temporal tissue were correlated (R=0.52, p<0.05).

There was a correlation between the levels of CCL3 and CCL4 expression in both CA1 and the temporal tissue (R=0.77, p<0.001 and R=0.46, p<0.05, respectively).

Distribution and time course of CCL3-like and CCL4-like immunoreactivity following status epilepticus

To determine the spatial and temporal distribution of the CCL3 and CCL4 proteins following status epilepticus, immunohistochemical staining was performed. CCL3-like and CCL4-like immunoreactivities were analyzed in CA1, CA3 of the hippocampus, the dentate gyrus, the entorhinal and piriform cortices and in the lateral and basal nuclei of the amygdala. Since mRNA levels seem to stabilize at 30-d after SE, we omitted this time point in the immunohistochemical analysis. Instead, we introduced an earlier time point of 6 hours. Therefore we have analyzed the CCL3-like and CCL4-like immunoreactivity at 6-h, 1-d, 4-d, and 14-d after SE (n=4). Detailed analysis of the CCL3-like immunoreactivity is presented in Table I and that of the CCL4-like immunoreactivity in Table II.

Control brains had implanted electrodes but were not stimulated. Unexpectedly in unstimulated brains collected as controls for the 6-h and 1-d group, light to moderate CCL3-like immunoreactivity was observed around the tract of the implanted electrode and in the hippocampus (Table I). In the case of CCL4 immunohistochemistry, the staining could be also observed to some extent at later time points (Table II). We assume, that induction of chemokine immunoreactivity around the tract of electrode resulted from the electrode implantation and persisted for more than two weeks from the surgery, a time that is usually considered long enough for recovery.

The CCL3-like immunoreactivity in the control brain, apart from the above mentioned staining around the electrode, was very week. Faint immunoreactivity was observed in neurons of the entorhinal and perirhinal cortices or in CA1 of the hippocampus as well as in glial cells. Following status epilepticus a massive increase in extent and intensity of CCL3-like immunoreactivity in glial cells morphologically resembling astrocytes was observed (Table I, Fig. 3A1-B3). Double immunohistochemistry with anti-GFAP (glial fibrillary acidic protein, astrocytic marker) antibody revealed that following status epilepticus virtually all astrocytes display also CCL3-like immunoreactivity (Fig. 1C1-C3). Therefore we have further analyzed increase in CCL3-like immunoreactivity in astrocytes only (Table I).

At 6 h following status epilepticus, the CCL3-like immunoreactivity was present in all examined structures. The strongest signal was observed in the hippocampus and in the amygdala. The immunoreactivity observed in stratum lacunosum moleculare of CA1 was significantly higher than in controls (p<0.05). At 1-d after stimulation, the CCL3-like immunoreactivity was lower then at 6-h, and in one rat (#S54) there were only single CCL3-like positive cells. At the 4-d time point, only single, scattered CCL3-like positive cells were observed in controls (Fig. 3A). In stimulated brains the CCL3-like immunoreactivity was high (Fig. 3B), and in 2 rats (#S24 and #S25) it was very strong. The level of CCL3-like immunoreactivity in all analyzed structures was significantly higher in the stimulated group than in controls (p<0.05). In control brains at 14-d, the CCL3-like immunoreactivity in astrocytes was almost absent, whereas in the stimulated group two rats (#S30 and #S31) had a large number of CCL3-like immunopositive cells in the entorhinal and piriform cortices as well as in the amygdala. Rat #S32 had only week to moderate CCL3-like immunoreactivity. The CCL3-like immunoreactivity following stimulation was significantly higher than in control (p<0.05) in CA1 and in stratum radiatum of CA3, in the granular layer of the dentate gyrus and in layer I of the entorhinal and piriform cortices.

The CCL4-like immunoreactivity resembles in many respects the CCL3-like immunoreactivity. In control brains pale CCL4-like immunoreactivity was present in neurons of the amygdala, cortex and CA1 of the hip-

Table I

CCL3 immunoreactivity after Status Epilepticus evoked by electrical stimulation of the amygdala

	Rat group and code																							
	early time points												late time points											
	control				6 hours				1 day				control				4 days				14 days			
Structure	C59	C62	C55	C56	S57	S58	S60	S61	S35	S36	S53	S54	C1	C2	C3	C9	S21	S22	S24	S25	S23	S30	S31	S32
CA1																								
Oriens l.	2	3	1	2	3	3	4	2	2	2	1	0	0	0	0	0	2	2	4	4 [b]	2	1	1	2 [b]
Pyramidal l.	1	2	1	1	2	2	3	1	2	1	1	0	0	0	0	0	2	2	4	4 [b]	2	2	2	2 [b]
Radiatum l.	2	3	1	2	3	3	3	2	3	2	1	0	1	0	0	0	3	3	4	4 [b]	2	1	1	2
Lacunosum molec. l.	2	2	2	3	3	4	4	4 [a]	3	2	2	1	1	0	0	0	2	2	4	4 [b]	3	2	2	2 [b]
CA3																								
Oriens l.	2	2	1	2	3	3	2	1	1	0	1	0	0	0	0	0	2	2	4	3 [b]	1	0	1	1
Pyramidal l.	1	1	1	1	2	2	1	1	1	0	1	0	0	0	0	0	2	2	4	3 [b]	1	0	1	1
Radiatum l.	2	2	1	2	2	1	3	2	1	1	1	1	0	0	0	0	2	2	3	4 [b]	1	1	1	1 [b]
Dentate gyrus																								
Granular l.	1	1	1	1	2	2	2	1	1	1	1	0	0	0	0	0	1	2	3	3 [b]	2	1	1	1 [b]
Molecular l.	1	1	1	2	2	1	3	2	1	1	1	0	0	0	0	0	3	2	4	4 [b]	1	0	2	1
Hilus	2	2	2	2	3	1	3	2	3	1	2	0	1	1	0	0	2	2	4	4 [b]	4	1	2	1
Entorhinal cx.																								
I	1	1	1	2	1	1	2	1	2	1	1	NS	0	0	0	0	3	2	3	4 [b]	1	4	4	1 [b]
II	0	0	2	1	0	0	1	0	0	0	0	NS	0	0	0	0	2	1	2	3 [b]	0	3	3	0
III/VI	0	0	2	2	2	1	2	2	0	0	0	NS	0	0	0	0	3	2	3	4 [b]	2	4	4	0
Piriform cx.																								
I	1	1	2	2	1	1	2	1	2	1	1	0	0	0	0	0	3	2	3	4 [b]	1	4	4	1 [b]
II	0	0	1	1	0	0	1	0	0	0	0	NS	0	0	0	0	2	1	1	1 [b]	0	1	NS	0
III	0	0	2	2	2	0	2	2	0	0	0	NS	0	0	0	0	3	1	3	4 [b]	3	4	NS	0
Amygdala																								
lateral	1	0	2	1	2	3	3	2	0	0	0	NS	0	0	0	2	3	2	3	4 [b]	2	4	4	1
basal	0	0	2	2	2	2	3	2	1	0	1	NS	1	0	0	0	2	2	3	4 [b]	2	4	4	0

The intensity of immunoreactivity was scored as follows: 0 - no immunoreactivity, 1 – low, 2- moderate, 3 – high, 4 - very high, NS - not scored.

[a] $p<0.05$ as compared to controls from 6-h and 1-d groups (Mann-Whitney *U*-test).

[b] $p<0.05$ as compared to controls from 4-d and 14-d groups (Mann-Whitney *U*-test).

Table II

CCL4 immunoreactivity in astrocytes after SE evoked by electrical stimulation of the amygdala

	Rat group and code																							
	early time points												late time points											
	control				6 hours				1 day				control				4 days				14 days			
Structure	C59	C62	C55	C56	S57	S58	S60	S61	S35	S36	S53	S54	C1	C2	C3	C9	S21	S22	S24	S25	S23	S30	S31	S32
CA1																								
Oriens l.	2	2	2	2	2	2	3	3	2	2	2	1	1	1	0	0	4	4	4	3 [b]	3	1	1	3 [b]
Pyramidal l.	1	2	2	2	1	1	2	2	2	2	2	1	1	1	0	0	3	4	4	3 [b]	2	2	1	3
Radiatum l.	2	2	2	2	2	1	3	2	2	2	2	1	2	1	0	0	4	4	4	3 [b]	3	1	1	3
Lacunosum molec. l.	3	3	3	3	3	2	3	3	4	3	3	2	2	1	0	1	4	4	4	4 [b]	3	2	2	3
CA3																								
Oriens l.	2	2	1	2	2	2	2	2	1	1	1	1	1	1	0	0	4	4	4	3 [b]	2	0	0	2
Pyramidal l.	1	1	1	1	1	1	1	1	1	1	1	0	1	0	0	0	3	3	3	3 [b]	2	0	0	2
Radiatum l.	2	2	1	1	2	1	2	2	1	1	1	1	1	0	0	0	4	3	4	3 [b]	3	0	1	2
Dentate gyrus																								
Granular l.	1	1	2	1	1	1	2	2	1	2	1	1	1	0	0	0	2	4	3	4 [b]	2	2	1	3 [b]
Molecular l.	1	1	1	1	1	1	2	2	1	2	2	1	1	0	0	0	4	4	4	4 [b]	2	1	1	2
Hilus	2	1	2	2	2	2	3	3	3	3	2	2	2	0	0	0	4	4	4	4 [b]	4	2	2	3
Entorhinal cx.																								
I	1	0	1	1	0	0	1	1	1	1	2	2	1	1	1	1	3	3	3	3 [b]	1	3	3	3
II	0	0	0	0	0	0	0	1	0	0	1	0	0	1	0	0	2	1	1	2	1	2	1	2
III/VI	0	0	0	2	0	0	1	2	1	0	2	1	3	0	0	0	4	3	3	3	3	4	3	3
Piriform cx.																								
I	1	0	1	1	0	0	1	1	1	1	2	2	1	1	1	1	3	3	3	3 [b]	1	3	4	3
II	0	0	0	0	0	0	0	1	0	0	1	0	0	0	0	0	1	1	1	1 [b]	1	2	1	2 [b]
III	0	0	0	2	0	0	1	2	1	1	2	1	0	1	0	0	4	3	3	4 [b]	4	4	3	3 [b]
Amygdala																								
lateral	1	1	1	2	NS	1	2	2	NS	NS	1	NS	0	0	0	0	4	4	4	4 [b]	4	4	4	4 [b]
basal	0	0	0	2	1	2	2	1	NS	NS	2	NS	0	1	0	1	4	4	4	4 [b]	4	4	4	3

The intensity of immunoreactivity was scored as follows: 0 - no immunoreactivity, 1 – low, 2- moderate, 3 – high, 4 - very high, NS - not scored.
[b] $p<0.05$ as compared to controls from 4-d and 14-d groups (Mann-Whitney *U*-test).

pocampus. Light, but clear staining in glial cells could be also observed all over the brain (Fig. 4A1-A3). A massive increase in CCL4-like immunoreactivity following status epilepticus was evident in cells with astroglial morphology (Fig. 4B1-B3). The identity of cells expressing CCL4-like staining was confirmed by double immunostaining with anti-GFAP antibody (Fig. 4C1-C3).

At the 6-h time point, in control rats CCL4-positive astrocytes were present in the hippocampus and their number was especially high in stratum lacunosum moleculare of CA1 (Table II). At 6 hours following SE, the CCL4-like immunoreactivity was either moderate or strong in the hippocampus and weaker in the remaining analyzed structures. At 24-h in control rats the CCL4-like immunoreactivity was either moderate or strong in the CA1 field, light or moderate in CA3 and in the dentate gyrus; however one rat (#C56) had a low-to-moderate number of CCL4-like immunoposi-

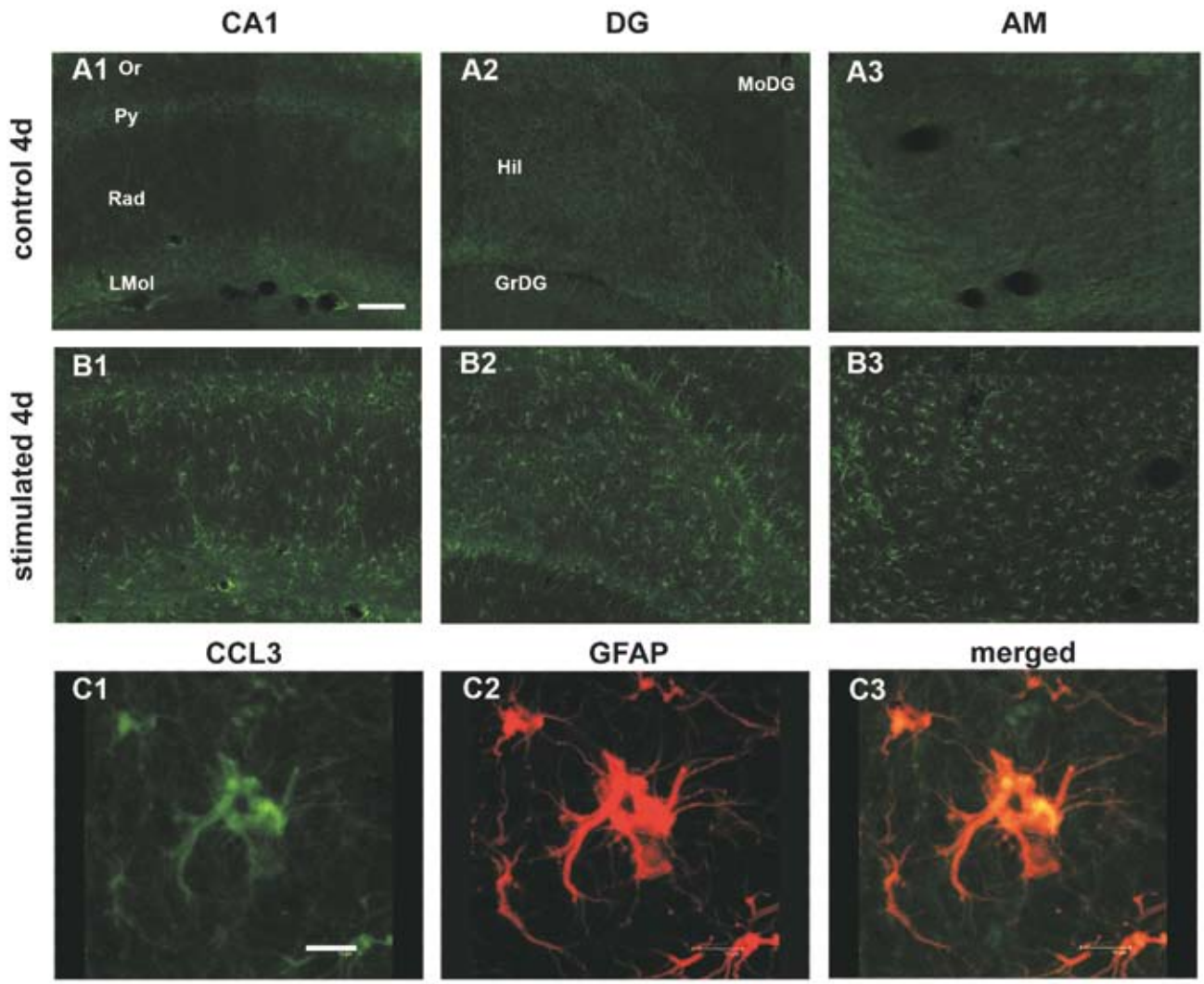

Fig. 3. CCL3-like immunoreactivity**.** Panels A-B present representative images of CCL3-like immunoreactivity in the control brain (A1-A3, rat #C1) and 4 days following status epilepticus (B1-B3, rat #S25) in the CA1 field of hippocampus (A1,B1), dentate gyrus (A2, B2) and in the basal nucleus of amygdala (A3, B3). Note the lack of CCL3 immunoreactivity in the control brain and widespread CCL3 immunoreactivity at 4 days following SE that is localized in cells morphologically resembling astrocytes. Panels C (1-3) present colocalization of CCL3 immunoreactivity (C1) with immunoreactivity for the astrocytic marker, GFAP (C2). As presented on the merged image (C3), most of CCL3-like immunoreactivity is localized in astrocytes and virtually all astrocytes express CCL3. Scale bars A and B - 100 μm, C - 10 μm. Abbreviations: Or - stratum oriens, Py - pyramidal layer of CA1, Rad - stratum radiatum, LMol - stratum lacunosum molecular, MoDG - molecular layer of the dentate gyrus, Hil – hilus, GrDG - granular layer of the dentate gyrus.

tive cells also in the entorhinal and piriform cortices as well as in the amygdala. In the stimulated group, the pattern of CCL4-like immunoreactivity was similar to that in controls, but rat #S35 had a very high level of CCL4 reactivity in stratum lacunosum moleculare. At 4-d, CCL4-like immunoreactivity was low or moderate in control rats (Fig. 4A), whereas in stimulated rats a robust increase in CCL4-like immunoreactivity occurred (Fig. 4B). In all rats the intensity of staining and the number of CCL4-like immunopositive cells was high or very high in the hippocampus, entorhinal and piriform cortices and in the amygdala. In all structures except for layers II-VI of the entorhinal cortex, the level of CCL4-like immunoreactivity was significantly higher ($p<0.05$) than in controls. At 14-d, control CCL4-like immunoreactivity was very low. In

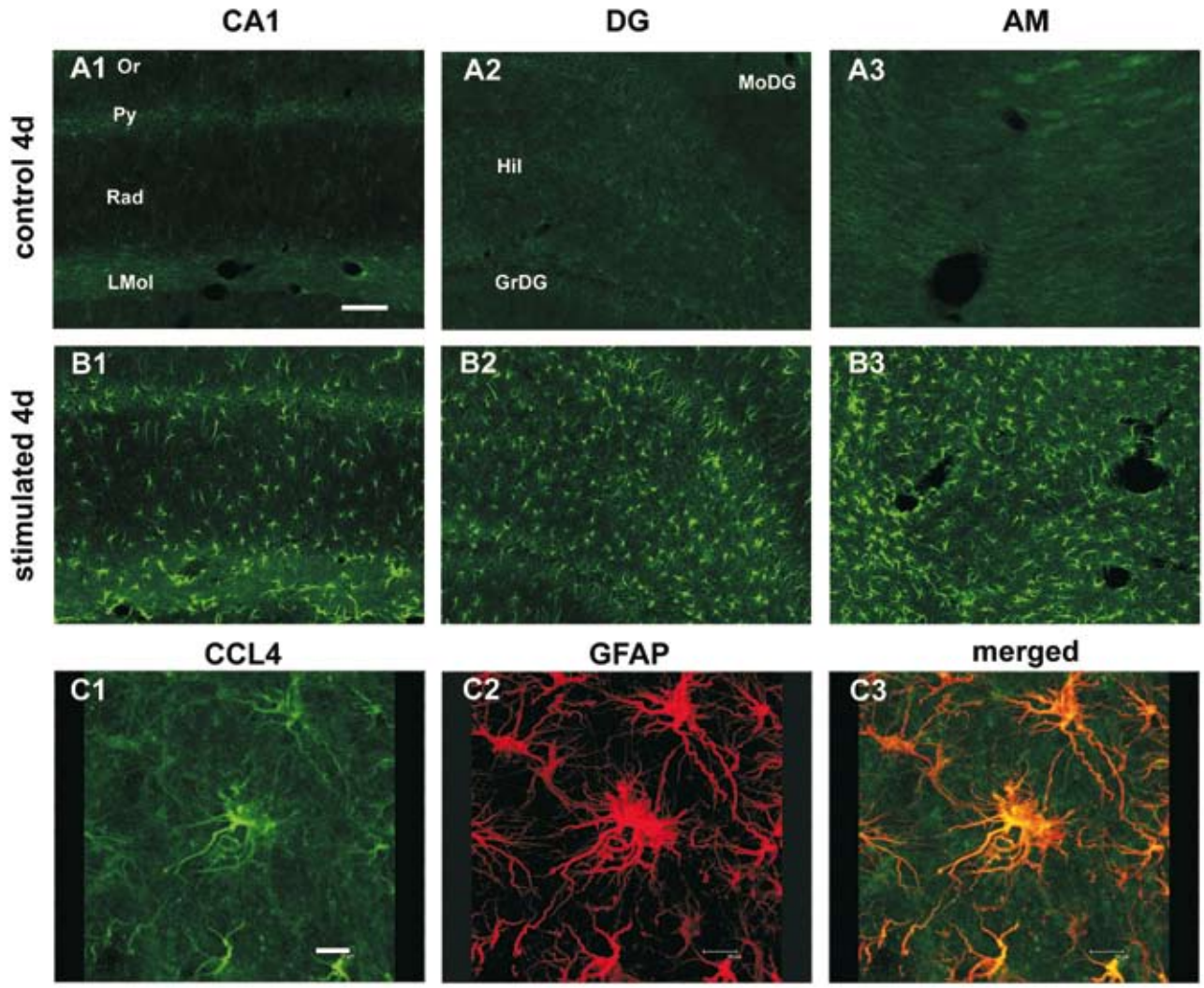

Fig. 4. CCL4-like immunoreactivity**.** Panels A-B present representative images of CCL4-like immunoreactivity in the control brain (A1-A3, rat #C1) and 4 days after status epilepticus (B1-B3, rat #S25) in the CA1 field of hippocampus (A1,B1), dentate gyrus (A2, B2) and in the basal nucleus of amygdala (A3, B3). In the time-matched control brain single CCL4-like positive cells are present (A1-A3); At 4 days following SE a large number of CCL4-like positive cells with glial morphology was observed (B1-B3); Panels C (1-3) present colocalization of CCL4-like immunoreactivity (C1) with immunoreactivity for the astrocytic marker, glial fibrillary acidic protein GFAP (C2). As presented on the merged image (C3), most of CCL4-ike immunoreactivity is localized in astrocytes and virtually all astrocytes express CCL4. Scale bars A and B - 100 µm, C - 10 µm. Abbreviations: Or - stratum oriens, Py - pyramidal layer of the CA1, Rad - stratum radiatum, LMol - stratum lacunosum molecular, MoDG - molecular layer of the dentate gyrus, Hil – hilus, GrDG - granular layer of the dentate gyrus.

stimulated rats CCL4-like immunoreactivity was strong or very strong in the entorhinal and piriform cortices and in the amygdala. In rats #S30 and #S31 no or only single CCL4-like immunopositive cells were present in the CA3 field of the hippocampus. The level of CCL4-like immunoreactivity in stratum oriens of CA1, the granule cell layer of the dentate gyrus, layer II and III of the piriform cortex and in the lateral nucleus of the amygdala was significantly higher ($p<0.05$) than in controls.

DISCUSSION

In this study we present data on long term increase in CCL3 and CCL4 mRNA expression and immunoreactivity following epileptogenic stimulus in a rat model of temporal lobe epilepsy. The previous data on increase in CCL3 and CCL4 mRNA levels in epileptogenesis or epilepsy come from microarray studies and most were not further validated with other methods. Here we confirm the increase in CCL3 and CCL4 mRNA expression with qPCR. To our best knowledge this is the first report on expression of the CCL4 protein and the second one on expression of the CCL3 protein following status epilepticus.

Our data show massive increase in CCL3 and CCL4 mRNA expression, especially at early time points, that is at 1-d and 4-d following status epilepticus. Later on the up-regulation of mRNAs expression is less striking and in CA1 it almost normalizes by 30-d. In the case of CCL3-like and CCL4-like immunoreactivity we observed the highest increase at 4-d after status epilepticus. These differences in mRNA and protein expression patterns could be explained by tight regulation of translation or protein stability, however there are no data supporting this thesis in the literature. We should also admit here, that mRNA levels and immunoreactivity for both chemokines show high within the group variability, particularly in stimulated animals.

There is little information about the regulation of CCL3 or CCL4 gene expression in epileptogenesis or epilepsy, but existing data indicate, that these genes are induced in the brain by other damaging stimuli that can lead to epilepsy development. Xu and coauthors used quantitative rt-PCR to study CCL3 mRNA in the mouse hippocampus at early time points during pilocarpine induced status epilepticus. The level of CCL3 mRNA increased at 1-h and remained elevated up to 2-h, that was the latest time point studied (Xu et al. 2009). The increase in CCL3 mRNA expression was found in the rat entorhinal cortex by means of microarrays at 1-d and 7-d, but not at 3-4-months following electrically induced status epilepticus (Gorter et al. 2006). In microarray studies of alterations of gene expression following traumatic brain injury on gene expression increase in CCL3 and CCL4 mRNA was detected within hours following controlled cortical impact in mouse (Kobori et al. 2002) and rat (Matzilevich et al. 2002, Raghavendra Rao et al. 2003) and fluid percussion injury in rat (Li et al. 2004). Closed head injury in mice induces CCL3 mRNA expression at 3-d following weight drop (Israelsson et al. 2009). In immature brain hypoxic-ischemic injury results in increase in CCL3 mRNA expression up to 72-h after the insult (Bona et al. 1999). Takami and others (1997) studied CCL3 mRNA expression in transient and permanent middle cerebral artery occlusion. The increase in expression was observed in both models in microglia/macrophages at 4-6 hours after occlusion. In human patients suffering from posttraumatic brain contusion, increase in CCL3 and CCL4 mRNA expression was observed early after trauma (median interval to biopsy 44 hours) in the tissue resected during surgical evacuation of brain contusions (Stefini et al. 2008). CCL3 and CCL4 mRNA was also induced in the hippocampus at 1-d after entorhinodentate lesion (Babcock et al. 2003). In mentioned studies mRNA expression was investigated only in selected time points, but together these reports indicate that CCL3 and CCL4 mRNA expression is increased in the brain from hours up to many days following the injury, in agreement with our data.

There are no data about the CCL4 protein expression in epilepsy and the only work about the CCL3 protein expression is the recent report by Xu and coworkers describing CCL3 immunoreactivity in pilocarpine induced status epilepticus in mice (Xu et al. 2009). Expression of CCL3 in the brain was studied by immunohistochemistry during (10 min, 30 min, 1-h and 2-h) and after (1-d, 7-d, 2-mo) status epilepticus. Immunopositive neurons were found in the control brain in the dentate gyrus and CA1-3 stratum pyramidale. Interestingly, expression of the CCL3 protein decreased in neurons during first two hours in the stratum pyramidale of CA1 and from the 2-h time point in the hilus. In astrocytes the CCL3 immunoreactivity was massively increased at 7 days after pilocarpine treatment and persisted up to 2 months but at

a much lower level (Xu et al. 2009). The time points evaluated in our study do not fully match those studied by Xu and colleagues, however the general picture remains similar with the highest increase in CCL3-like immunoreactivity occurring in astrocytes within days (in our case 4-d) following status epilepticus. We have not systematically evaluated the expression of CCL3 in neurons since it was in general very weak (not shown). Decrease in immunoreactivity could be occasionally observed in CA1 stratum pyramidale (not shown). However we have assumed that it is related to neurodegeneration.

Few other studies using brain damaging, potentially epileptogenic stimuli described an increase in CCL3 and CCL4 protein expression. Ghirnikar and coauthors proved that CCL3 and CCL4 expression is upregulated in reactive astrocytes following stab wound injury in the rat. CCL4 was also expressed in macrophages (Ghirnikar et al. 1996). CCL3 protein expression was studied with ELISA in homogenates of mice brain following weight-drop closed brain injury. The level of protein increased as early as 4-h after trauma and persisted up to 7-d. Due to the nature of the detection system the cellular localization of these changes remains unknown (Otto et al. 2001).

Jiang and others studied expression of the CCL3 protein in the middle cerebral occlusion model of stroke in rat (Jiang et al. 2008). The protein expression at 24 hours was increased in stroked hemispheres when compared to the contralateral ones. CCL3 was expressed by neurons, astrocytes and some microglial cells. Hypoxic-ischemic injury to the rat immature brain caused widespread increase in CCL3 protein expression from 8-h up to 5-d after insult. In this model CCL3 immunoreactivity was restricted to the monocyte/macrophage lineage (Cowell et al. 2002).

It could be then concluded, that CCL3 and CCL4 protein expression is increased following brain damaging insults in animal models and in humans. The localization of increased protein expression depends on the model and/or age, but usually increased expression occurs in astrocytes.

Whether there is a link between increased expression of CCL3 or CCL4 and development of epilepsy and what could be the role of this phenomenon following status epilepticus is not known. The increase in CCL3 and CCL4 expression could be associated with astrocyte activation and merely be a reaction to ongoing neurodegeneration or could have an active influence on repair and/or plasticity occurring in the brain following injury.

Although the precise biological function of CCL3 and CCL4 is not known, several possibilities emerge in the light of existing literature. One can hypothesize that the action of CCL3 and CCL4 in the diseased brain would depend on the availability and localization of their receptors. The understanding of the action of a particular chemokine is diffcult due to substantial promiscuity in chemokine binding to their receptors. In the case of CCL3 the predominant receptors are CCR5 and CCR1, while CCL4 binds with highest affinity to CCR5 (Bacon and Harrison 2000, Rossi and Zlotnik 2000, Rostene et al. 2007). Expression of CCR5 and CCR1 receptors in normal brain is low and has been shown in microglia, astrocytes and around blood vessels. Neuronal expression at the protein level is less consistent, but CCR5 seems to be present in neurons at least at the mRNA level (Meucci et al. 1998, Coughlan et al. 2000, McManus et al. 2000, van der Meer et al. 2000, Mennicken et al. 2002, Halks-Miller et al. 2003, Torres-Munoz et al. 2004). Increase in expression of these receptors was observed in brain pathology (Galasso et al. 1998, Spleiss et al. 1998, Vallat et al. 1998, Xia and Hyman 1999, Mennicken et al. 2002, Halks-Miller et al. 2003, Trebst et al. 2003, Cowell et al. 2006). In particular CCR5 mRNA and protein expression is elevated following kainic acid induced status epilepticus (Mennicken et al. 2002). Increase in protein expression occurs within hours following status epilepticus in blood vessels and within days in neurons and glia (Mennicken et al. 2002). If similar changes in expression of CCR5 occur also in our model of status epilepticus, it would imply possibility of a dynamic crosstalk between astrocytes, the main source of CCL3 and CCL4, and cells expressing the CCR5 receptor, that is neurons, microglia and blood vessels. The net effect of these interactions is difficult to predict, since CCR5 activation can be both protective and harmful to the brain. It has been proposed that CCL3 signaling through the CCR5 receptor can be detrimental to the brain. Takami and coauthors showed that CCL3 application increased the infarct volume following middle cerebral artery occlusion while CCR5 antagonist reduced infarct volume in a dose-dependent manner (Takami et al. 2001). On the other hand, it has been also suggested, that CCR5 ligands can evoke neuroprotection by suppressing microglial activation and release of inflammatory cytokines and

iNOS (Gamo et al. 2008). The other interesting possible function of chemokines is regulation of synaptic plasticity, however as yet there are no data that CCL3, CCL4 or their receptors are involved in such processes (Rostene et al. 2007). Altogether the picture of the function of CCL3 and CCL4 in the diseased brain and their potential role in development of epilepsy is unclear.

In the present report we describe increase in mRNA and protein levels of two proinflammatory chemokines CCL3 and CCL4 in the animal model of epileptogenesis. The current knowledge on the action of these chemokines in the diseased brain does not allow for definite conclusions about their involvement in epilepsy development. Existence of multiple targets for these chemokines in the damaged brain suggests several possibilities of influencing neuronal and glial functions so as the combinatorial effect is difficult to predict. Further studies are required to elucidate the role of CCL3, CCL4 and their receptors in epileptogenesis and epilepsy.

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