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LETTER TO THE EDITOR

Decreased post-synaptic density-95 protein expression on dendrites of newborn neurons following CX3CR1 modulation in the epileptogenic adult rodent brain

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Temporal lobe seizures generate a large pool of new hippocampal neurons in the adult brain. Their role within the hyperexcitable neuronal network and how they are regulated by the immune response is unclear. Fractalkine and its receptor CX3CR1 constitute a chemokine pathway known to modulate the immune response in temporal lobe epilepsy. Here, 6 weeks of intracerebroventricular infusion of anti-CX3CR1 antibody, starting 1 week after electrically induced prolonged seizures in the temporal lobe (status epilepticus (SE)), reduced microglial activation within the hippocampus. This was associated with a several-fold decrease in expression of post-synaptic density-95 (PSD-95), a scaffolding protein specific for excitatory synapses on individual retroviral vector-mediated GFP-labeled hippocampal neurons at 6 weeks of age. In contrast, the scaffolding protein at inhibitory synapses, gephyrin, and related adhesion molecules were unaltered. These results indicate that expression of synaptic proteins on adult-born neurons in an epileptogenic environment is sensitive to the inflammatory milieu and may in turn affect excitation/inhibition balance.

Depending on the precipitating injury that precedes epilepsy development, the new neurons may either migrate abnormally into the hilar region of the dentate gyrus and contribute to the pathophysiology of epilepsy or correctly integrate within the granule cell layer (GCL) and have possible dampening effects.[1] Previous studies have shown spatial and temporal changes in expression of synaptic proteins, known to regulate the excitatory/inhibitory synaptic balance,[2,3] on newborn neurons in animal models of brain inflammation and epileptogenesis.[4,5] Modulating the fractalkine-CX3CR1 chemokine pathway has led to reduced cell proliferation and immature neurons in the hippocampus one week after SE.[6] In this study, we aimed to evaluate changes in the expression of synaptic scaffolding proteins and adhesion molecules on individual new integrated neurons born and differentiated into the GCL of the hippocampus following temporal SE, with and without immune modulation.

We used adult male 200–250 g Sprague–Dawley rats (Charles River, Germany). Experimental procedures were performed in accordance with guidelines set by Malmö-Lund Ethical Committee. Rats were subjected to electrically induced temporal lobe SE; $n=7$ rats as vehicle-treated SE (SE-Veh) and $n=7$ SE rats received anti-CX3CR1 antibody (Ab) treatment (SE-CX3CR1). Rats were implanted with a bipolar electrode in the right ventral hippocampus and a brain infusion cannula in the lateral ventricle ipsilaterally for CX3CR1 Ab administration, as previously described.[6] Following a week of recovery, rats were subjected to SE[4] and only rats that displayed temporal lobe seizure semiology (including automatisms and chewing) were included in the study. Seven days after SE induction, rats were injected intrahippocampally with a retroviral vector (1.0–1.1 transducing units/ml) expressing the GFP gene under the CAG promoter.[4] Immediately after the RV-GFP injection, the brain infusion cannula was connected with an osmotic pump (2006, Alzet), carrying either rabbit anti-CX3CR1 Ab (20 μg/ml (Abcam, UK) or vehicle (phosphate buffer saline). Infusion rate was 0.15 μl/h and continued for 6 weeks.

Animals were transcardially perfused 6-weeks post-RV-GFP injections and brains were prepared for immunohistochemical assessments of microglia/macrophage marker Iba1, phagocytic marker CD-68/ectodermal dysplasia 1 (ED1), synaptic scaffolding proteins (gephyrin and PSD-95), and adhesion

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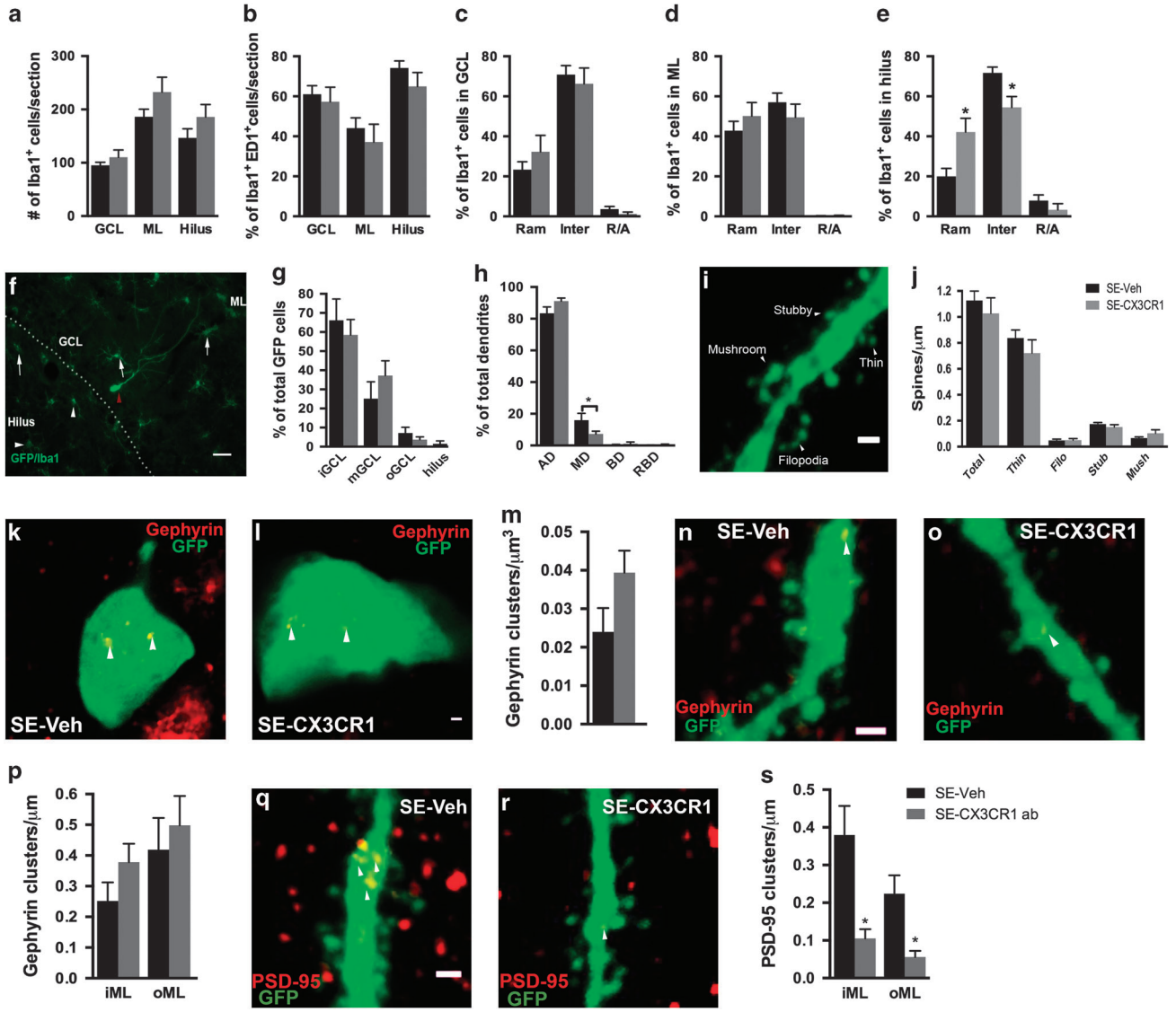

Figure 1 Microglial activation and morphology as well as synaptic protein expression of 6-week old GFP-labeled newborn neurons, 7 weeks after temporal lobe status epilepticus (SE). (**a**) Quantification of Iba1$^+$ and (**b**) percentage of Iba1$^+$ED1$^+$ cells in granule cell layer (GCL), molecular layer (ML) and dentate hilus of the ipsilateral hippocampus in SE-Veh and SE-CX3CR1 rats. Relative percentage of microglia with different morphologies including ramified (Ram), intermediate (Inter) and round/ameboid (R/A) in the ipsilateral GCL (**c**), ML (**d**) and dentate hilus (**e**). (**f**) Representative image of a GFP-labeled neuron (red arrowhead) in the middle GCL (mGCL), surrounded by round/ameboid activated microglia (arrows) and ramified microglia (arrowheads). (**g**) Location of new neurons within the dentate gyrus region, including inner (iGCL), mGCL, outer GCL (oGCL), and dentate hilus. (**h**) Percentage of new neurons that displayed apical (AD), medial (MD), basal (BD) or recurrent basal dendrites (RBD). (**i**) Representative image showing different spine types on a dendritic shaft of a GFP-labeled neuron. (**j**) Density of each spine phenotypes. Representative images showing gephyrin clusters (red) on cell soma of newly formed neurons (green) in SE-Veh (**k**) and SE-CX3CR1 (**l**) rat. (**m**) Density of gephyrin clusters on cell soma. Representative image showing gephyrin clusters on dendrites in SE-Veh (**n**) and SE-CX3CR1 rats (**o**). (**p**) Density of gephyrin clusters on the dendritic shaft in inner (iML) and outer molecular layer (oML). PSD-95 clusters (red) on dendrites of newly formed neurons in SE-Veh (**q**) and SE-CX3CR1 (**r**) rat. (**s**) Density of PSD-95 clusters on dendrites in iML and oML. Graphs represent Mean ± SEM, $n = 6$–7 in each group and $^*P \leqslant 0.05$. Scale bar is 10 μm for (**f**) and 1 μm for all other images.

molecules (neuroligin-1 (NL-1) and neuroligin-2 (NL-2)).[5] Quantification of Iba1/ED1 staining was performed in 3–4 brain sections.[6] Gross morphology of GFP$^+$ neurons, and expression of synaptic proteins on newborn neurons were analyzed in images from confocal laser-scanning microscope (Zeiss, Germany), 63× oil immersion objective and 5× digital zoom.[5] Unpaired Student's *t*-test was used when comparing two groups, while two-way ANOVA and Bonferroni *post hoc* test was used for microglial and GFP$^+$ cell morphology (Graphpad).

Seizure-induced microglial activation is associated with an increased number of microglial cells as well as increased percentage of intermediate and amoeboid/round microglial morphology.[4] Here, following 6 weeks of CX3CR1 Ab treatment, total number of Iba1$^+$ cells

and percentage of Iba1$^+$ cells expressing ED1 in the GCL, molecular layer (ML) and the dentate hilus of the hippocampus were unaltered when compared to SE-Veh animals 7 week after SE (Figures 1a and b). Instead, percentage of different morphological profiles of microglia, including ramified, intermediate and round/ameboid, changed with treatment (Figures 1c–e). In the dentate hilus ipsilateral to the epileptic focus, increased numbers of ramified/surveying and reduced intermediate phenotype of Iba1$^+$ cells were evident. We observed a similar trend in the contralateral hilus, but it did not reach significance ($P=0.053$) (not shown). There were no differences between groups in microglial morphology within the ML or GCL of ipsilateral hippocampus (Figures 1c and d). However, although the CX3CR1 Ab-induced alteration in microglial activation was restricted to the dentate hilus within the hippocampal epileptic focus, additional functional changes and altered release of immune mediators in ramified/surveying microglia is likely.[7] The entire pool of modulated microglia and the affected brain regions may be more extensive.

Epileptic seizures *per se* could lead to ectopic migration and structural abnormalities of the newly formed neurons, such as enhanced numbers of basal dendrites and altered spine density. Here, the CX3CR1 Ab treatment for 6 weeks after SE induced no difference in the location of the 6 weeks old GFP$^+$ cells, (Figures 1f and g) and only subtle change in the origin of their dendrites from the cell soma (Figure 1h). The number and morphology of dendritic spines, the main sites of excitatory input onto neurons, were evaluated, as previously described.[4] The overall spine density or spine morphology did also not change (Figures 1i and j). This is despite previous reports showing how fractalkine-CX3CR1 pathway could play an important role in neuron-glia interactions via synaptic pruning during postnatal neuronal development.[8]

Finally we evaluated the expression of synaptic protein clusters on GFP-labeled neurons. The cluster density of gephyrin, a scaffolding protein for $GABA_A$ and glycine receptors at the postsynaptic terminals, were unaltered on individual GFP$^+$ cell soma between SE-veh and SE-CX3CR1 groups ($P=0.09$) (Figures 1k–m). Also, no changes were observed either on proximal or on distal dendritic processes within the outer and inner ML (Figures 1n–p). In addition, no differences were observed between groups regarding cluster density of NL-2 on the soma ($P=0.4$) or dendrites in inner ($P=0.4$) or outer ML ($P=0.9$) between the SE-veh and SE-CX3CR1 rats (data not shown). Instead, PSD-95, a scaffolding protein that mediates the clustering of glutamatergic receptors at the excitatory synapses[3] was reduced in both proximal and distal dendritic processes in the SE-CX3CR1 group compared to SE-Veh group (Figures 1q–s). The reduction occurred without alterations in cluster density of the excitatory synapse-associated adhesion molecule NL-1 (proximal ($P=0.9$) and distal dendritic processes (0.9), data not shown). Alterations in the expression of PSD-95 have previously been reported on new neurons during pathological conditions such as after SE and bacterial endotoxin LPS infection.[4,5] Changes in the expression of these proteins may impart excitation/inhibition balance and contribute to epileptogenesis.[2,3] We have previously shown changes in the expression levels of these synaptic proteins just before the development of epileptic seizures in a genetic mouse model of epilepsy.[9] To our knowledge our present study is the first to determine that such changes in PSD-95 expression on individual newly formed hippocampal neurons can be altered by immune modulation.

The reason for a specific immune-induced altered expression of PSD-95 and not gephyrin, NL-1 or NL-2 is not clear. Previous studies from our group have shown that newly formed neurons that migrate correctly within the GCL following SE show reduced afferent excitatory connections, with higher gephyrin and lower PSD-95 expression.[4] These functional and molecular changes may represent a homeostatic mechanism of an epileptic brain to control strong excitatory afferent inputs and thereby to prevent seizures. Conversely, we have also previously reported reduced gephyrin and enhanced PSD-95 expression on new neurons during a strong pro-inflammatory LPS infection, which may act to trigger epileptogenesis.[5] In the current experimental set up, it can be hypothesized that lowering inflammation by interfering with fractalkine-CX3CR1 pathway further inhibits the excitatory input on the newly formed GCL neurons. Determining the electrophysiological functional properties of these neurons in future studies are needed to confirm the hypothesis. Moreover, we evaluated the expression of synaptic proteins only on individual correctly migrated neurons within the GCL after SE. Following temporal SE, these are the majority of newborn neurons.[10] However, even though the percentage of aberrantly migrated new neurons are relatively low, they may still have a significant impact on the hippocampal network and so studying how immune modulation may affect the synaptic properties of both groups of new neurons are of interest for understanding the overall impact of these cells after SE. Here we provide some initial novel insights on the role of the fractalkine-CX3CR1-mediated immune pathway, in particular, for the synaptic function of newly formed hippocampal neurons in an epileptogenic environment.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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