Neuropharmacology 75 (2013) 337–346

Contents lists available at ScienceDirect

Neuropharmacology

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

CCL5-glutamate interaction in central nervous system: Early and acute presynaptic defects in EAE mice

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ARTICLE INFO

Article history:
Received 11 February 2013
Received in revised form
24 July 2013
Accepted 30 July 2013

Keywords:
Glutamate release
CCL5
EAE mice
cAMP
Spinal cord
Cortex

ABSTRACT

We investigated the CCL5-glutamate interaction in the cortex and in the spinal cord from mice with Experimental Autoimmune Encephalomyelitis (EAE) at 13 and 21/30 days post immunization (d.p.i.), representing the onset and the peak of the disease, respectively. An early reduction of the KCl-evoked glutamate release was observed in cortical terminals from EAE mice at 13 d.p.i., persisting until 21/30 d.p.i. A concomitant reduction of the depolarization-evoked cyclic adenosine monophosphate (cAMP), but not of the inositol 1,4,5-trisphosphate (IP_3) cortical production also occurred at 13 d.p.i, that still was detectable at the acute stage of disease (21 dp.i.). Inasmuch, the CCL5-mediated inhibition of glutamate exocytosis observed in control mice turned to facilitation in EAE mouse cortex at 13 d.p.i., then becoming undetectable at 21/30 d.p.i. Differently, glutamate exocytosis, as well as IP_3 and cAMP productions were unaltered in spinal cord synaptosomes from EAE mice at 13 d.p.i., but significantly increased at 21/30 d.p.i., while the presynaptic CCL5-mediated facilitation of glutamate exocytosis observed in control mice remained unchanged. In both CNS regions, the presynaptic defects were parallelled by increased CCL5 availability. Inasmuch, the presynaptic defects so far described in EAE mice were reminiscent of the effects acute CCL5 exerts in control conditions. Based on these observations we propose that increased CCL5 bioavailability could have a role in determining the abovedescribed impaired presynaptic impairments in both CNS regions. These presynaptic defects could be relevant to the onset of early cognitive impairments and acute neuroinflammation and demyelinating processes observed in multiple sclerosis patients.

1. Introduction

The idea that the immune and the central nervous systems (CNS) are independent has been challenged by the observations that immune cells infiltrate the CNS, and that endogenous molecules such as chemokines and classic neurotransmitters are commonly shared between the two systems (Kerschensteiner et al., 2010). Thus, studies aimed at defining the physiopathological consequences of the functional cross-talk between central neurotransmitters and proinflammatory agents in pathological conditions are particularly relevant to the comprehension of the mechanisms at the basis of neuroinflammatory diseases (Pitt et al., 2000; Groom et al., 2003; Hohlfeld et al., 2007; Rosténe et al., 2007; Centonze et al., 2009).

In recent years, evidence showing that proinflammatory cytokines can modulate glutamatergic transmission in CNS has been

Abbreviations: [^{3}H]D-ASP, [^{3}H]D-aspartate; AC, adenylyl cyclase; cAMP, cyclic adenosine monophosphate; CD45, cluster of differentiation 45; CNS, central nervous systems; CSF, cerebrospinal fluid; d.p.i., days post immunization; DAPI, 4′-6′-diaminidino-2-phenylindole; EAE, Experimental Autoimmune Encephalomyelitis; HE, haematoxylin eosin; IP_3, inositol-1,4,5-trisphosphate; f.c., final concentration; LFB, Luxol Fast Blue; MS, multiple sclerosis; MOG, myelin oligodendrocyte glycoprotein; n.s., not significant; PLC, phospholipase C; CCL5, Regulated upon activation normal T cells expressed and secreted, Tris-buffered saline; Tris, Tris-(hydroxymethyl)-amino methane.

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http://dx.doi.org/10.1016/j.neuropharm.2013.07.037

produced. In particular, the chemokine Regulated upon Activation Normal T cell Expressed and Secreted (RANTES, immunological designation CCL5) was shown to inhibit glutamate release from cultured rat hippocampal neurones (Meucci et al., 1998), from human and mouse cortical nerve endings terminals (Musante et al., 2008a), and from mouse spinal cord terminals (Di Prisco et al., 2012). Conversely, glutamate was reported to inhibit CCL5 production in cultured astrocytes (Besong et al., 2002), suggesting that the two systems are functionally connected.

Endogenous CCL5 bioavailability in CNS dramatically increases in pathological conditions associated to central neuroinflammation. In particular, abnormal CCL5 expression was detected in the cerebrospinal fluid (CSF) of patients suffering from multiple sclerosis (MS) and in the CNS of mice with Experimental Autoimmune Encephalomyelitis (EAE), one of the most used animal models for this human pathology (Sørensen et al., 1999; Furlan et al., 2005; Ubogu et al., 2006; Szczucinski and Losy, 2007); this implicates this chemokine in the onset of the demyelinating disease(s). Accordingly, altered expression of chemokine receptors selectively targeted by CCL5 in immunocompetent cells was found to correlate with the severity of EAE (Navratilova, 2006; Eltayeb et al., 2007), while modified CCL5 ligands with antagonist profile were efficacious in controlling EAE symptoms (Proudfoot et al., 2008). Impaired central glutamate transmission also has a role in MS etiopathogenesis (Stover et al., 1997; Ohgoh et al., 2002; Sarchielli et al., 2003) as suggested by the abnormal glutamate level(s) detected in the CSF of MS patients, as well as by the altered glutamate release capability observed in both mouse and rats suffering demyelinating diseases. Thus, the possibility that impaired CCL5-glutamate cross-talk could be relevant to MS etiopathogenesis deserves attention.

The present study aimed at ascertaining whether the CCL5-glutamate interaction in selected CNS regions (namely the cortex and the spinal cord) could undergo pathologically relevant modifications in EAE mice suffering a non-remitting form of demyelinating disease [i.e. the myelin oligodendrocyte glycoprotein $(MOG)_{35-55}$-induced form of disease]. Our findings suggest that CCL5-glutamate interaction undergo severe area-dependent modifications that became evident in EAE mice at different stages of disease and possibly depend on impaired second messenger productions. Notably, presynaptic defects did not necessarily correlate with diffuse inflammatory and demyelinating histological hallmarks. These results provide novel information on the molecular events involved in the etiopathogenesis of MS.

2. Materials and methods

2.1. Animals and EAE induction

Female mice (C57BL/6J; 18–20 g, 6–8 weeks) were obtained from Charles River (Calco, Italy) and were housed in the animal facility of DIFAR, Section of Pharmacology and Toxicology (authorization n° 484 of 2004, June, 8th). Female mice were immunized according to a standard protocol previously described (Zappia et al., 2005), with minor modifications. Briefly, animals were subcutaneously injected with incomplete Freund's adjuvant containing 4 mg/ml *Mycobacterium tuberculosis* (strain H37Ra) and 200 μg of the MOG_{35-55} peptide. Immunization with MOG_{35-55} was followed by i.p. administration of 250 ng of pertussis toxin on day 0 and after 48 h. Clinical scores (0 = healthy; 1 = limp tail; 2 = ataxia and/or paresis of hindlimbs; 3 = paralysis of hindlimbs and/or paresis of forelimbs; 4 = tetraparalysis; 5 = moribund or death) were recorded daily. Control mice received the same treatment in the absence of the MOG_{35-55} peptide. All efforts were made to minimize animal suffering and to use the minimal number of animals necessary to produce reliable results. We refer to naive mice when considering animals that were not administered with MOG and incomplete Freund's adjuvant containing 4 mg/ml *M. tuberculosis*.

At different stages of disease [13, 21 and 30 days post immunization (d.p.i.)], MOG-immunized EAE and control mice were killed by decapitation. When indicated, naive (untreated) and control animals were concomitantly sacrificed to analyse whether administration of incomplete Freund's adjuvant containing 4 mg/ml *M. tuberculosis* and PTx toxification could affect the functional parameters under study. The spinal cords and the cortices were rapidly removed and purified isolated nerve endings (synaptosomes) prepared within minutes. All the experimental procedures described here were in accordance with the European legislation (European Communities Council Directive of 24 November 1986, 86/609/EEC) and the ARRIVE guidelines and they were approved by the Italian Ministry of Health (protocol number n° 50/2011-B). Experiments were performed following the Guidelines for Animal Care and Use of the National Institutes of Health.

2.2. Preparation of synaptosomes

Purified synaptosomes were prepared according to Dunkley et al. (1986), with some modifications (Musante et al., 2008b). Briefly, the tissue was homogenized in 10 volumes of 0.32 M sucrose, buffered to pH 7.4 with Tris-(hydroxymethyl)-amino methane [Tris, final concentration (f.c.) 0.01 M] using a glass/Teflon tissue grinder (clearance 0.25 mm); the homogenate was centrifuged at 1000 × *g* for 5 min to remove nuclei and debris and the supernatant was gently stratified on a discontinuous Percoll gradient (2%, 6%, 10% and 20% v/v in Tris-buffered sucrose) and centrifuged at 33,500 × *g* for 5 min. The layer between 10% and 20% Percoll (synaptosomal fraction) was collected and washed by centrifugation. The synaptosomal pellets were resuspended in a physiological solution (standard medium) with the following composition (mM): NaCl, 140; KCl, 3; $MgSO_4$, 1.2; $CaCl_2$, 1.2; NaH_2PO_4, 1.2; $NaHCO_3$, 5; HEPES, 10; glucose, 10; pH 7.2–7.4.

2.3. Experiments of release

Synaptosomes were incubated for 15 min a 37 °C in a rotary water bath in the presence of [^{3}H]D-aspartate ([^{3}H]D-ASP, spinal cord synaptosomes, f.c.: 20 nM; cortical synaptosomes, f.c.: 50 nM, Musante et al., 2010). After the labelling period, identical portions of the synaptosomal suspensions were layered on microporous filters at the bottom of parallel chambers in a Superfusion System (Raiteri et al., 1974; Ugo Basile, Comerio, Varese, Italy), and maintained at 37 °C. The apparatus consists of 24 identical units of superfusion where synaptosomes are plated under moderate vacuum on a microporous filters that are located on a filter holder of porous glass and then superfused at 0.5 ml/min with standard physiological solution for a total period of 48 min of superfusion. Each experimental condition was run in triplicate to mitigate variability.

When studying the effect of high KCl or forskolin on the release of preloaded [^{3}H]D-ASP, synaptosomes were transiently (90 s) exposed, at t = 39 min, to high KCl-containing medium (12 or 15 mM, NaCl substituting for an equimolar concentration of KCl), or to the adenylyl cyclase (AC) activator in the absence or in the presence of CCL5. Dialysed 0.1% Polypep was present to avoid sticking of peptides to glass walls and tubing. Fractions were collected according to the following scheme: two 3-min fractions (basal release), one before (t = 36–39 min) and one after (t = 45–48 min) a 6-min fraction (t = 39–45 min; evoked release). Fractions collected and superfused synaptosomes were measured for radioactivity. Synaptosomal protein contents were determined according to the BCA (Thermoscientific, Rockford, IL, USA) protein assay.

The amount of radioactivity released into each superfusate fraction was expressed as percentage of the total radioactivity in the synaptosomal fraction. The K^+ induced overflow was expressed as "induced overflow (%)" and it was estimated by subtracting the neurotransmitter content into the first and the third fractions collected (basal release, b1 and b3) from that in the 6-min fraction collected during and after the depolarization pulse (evoked release, b2).

2.4. Preparation of slices and histopathology

The cortices and the spinal cords from the control and EAE mice at day 13 and 21 days after immunization were dissected and routinely embedded in paraffin wax. 8 μm sagittal paraffin embedded slices of the cortex and of the spinal cord (the lumbar part) were prepared; haematoxylin and eosin (HE), and Luxol Fast Blue (LFB) staining were used to reveal perivascular inflammatory infiltrates and demyelination, respectively. CD45 positive cells were detected using an anti CD45 antibody (1:100) and revealed using a secondary antibody anti mouse conjugated with Alexa594. Cells were considered to be positive for CD45 if specific fluorescence was observed. Nuclei were after stained by 4′-6′-diaminidino-2-phenylindole (DAPI) to show total cells. In immuno fluorescent experiment all primary antibodies were revealed using FITC or rhodamine-labelled secondary antibodies. CNS pathologic score was calculated on an average of 6 CNS tissue sections per mouse (at 40 × magnification) and expressed as mean ± S.E.M. In each section, we determined the total number of positive elements and reported it as number of positive elements/mm^2 LFB^+ cells were quantified as already described (Morando et al., 2012). LFB^+ and $CCL5^+$ cells were quantified as number of positive cells per mm^2.

2.5. Quantification of endogenous CCL5

Serum, cortical, and spinal cord tissue samples were obtained from control and EAE mice at 13, 21 and 30 d.p.i. Blood samples were collected immediately before animal sacrifice. The cortices and the spinal cord (about 100 mg wet tissue) were collected, rapidly homogenized at 4 °C in 150 μl of the assay diluent RD buffered