Neurochemistry International xxx (2015) 1–13

Contents lists available at ScienceDirect

Neurochemistry International

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

Lack of presynaptic interaction between glucocorticoid and CB_1 cannabinoid receptors in GABA- and glutamatergic terminals in the frontal cortex of laboratory rodents

Rafael M. Bitencourt [a, b, 1], Alán Alpár [c], Valentina Cinquina [d, e], Samira G. Ferreira [a, f], Bárbara S. Pinheiro [a, 2], Cristina Lemos [a], Catherine Ledent [g], Reinaldo N. Takahashi [b], Fernando J. Sialana [h, i], Gert Lubec [h], Rodrigo A. Cunha [a, f], Tibor Harkany [c, d], Attila Köfalvi [a, j, *]

[a] CNC, Center for Neuroscience and Cell Biology of Coimbra, University of Coimbra, 3004-504 Coimbra, Portugal
[b] Laboratory of Psychopharmacology, Dept. Pharmacology, Universidade Federal de Santa Catarina, Florianopolis 88049-900, Brazil
[c] Division of Molecular Neurobiology, Department of Medical Biochemistry and Biophysics, Karolinska Institutet, S-17177 Stockholm, Sweden
[d] Department of Molecular Neurosciences, Center for Brain Research, Medical University of Vienna, A-1090 Vienna, Austria
[e] University of Insubria, Via Ravasi, 2, 21100 Varese, Italy
[f] FMUC, Faculty of Medicine, University of Coimbra, 3004-504 Coimbra, Portugal
[g] IRIBHM, Université Libre de Bruxelles, Brussels B-1070, Belgium
[h] Department of Pediatrics, Medical University of Vienna, Währinger Gürtel 18, A-1090 Vienna, Austria
[i] CeMM Research Center for Molecular Medicine of the Austrian Academy of Science, Lazarettgasse 14, AKH BT 25.3, A-1090 Vienna, Austria
[j] Institute for Interdisciplinary Research, University of Coimbra, 3030-789 Coimbra, Portugal

ARTICLE INFO

Article history:
Received 11 October 2014
Received in revised form
13 July 2015
Accepted 17 July 2015
Available online xxx

Keywords:
CB_1 cannabinoid receptor
Frontal cortex
GABA
Glucocorticoid receptor
Glutamate
Presynaptic

ABSTRACT

Corticosteroid and endocannabinoid actions converge on prefrontocortical circuits associated with neuropsychiatric illnesses. Corticosteroids can also modulate forebrain synapses by using endocannabinoid effector systems. Here, we determined whether corticosteroids can modulate transmitter release directly in the frontal cortex and, in doing so, whether they affect presynaptic CB_1 cannabinoid receptor-(CB_1R) mediated neuromodulation. By Western blotting of purified subcellular fractions of the rat frontal cortex, we found glucocorticoid receptors (GcRs) and CB_1Rs enriched in isolated frontocortical nerve terminals (synaptosomes). CB_1Rs were predominantly presynaptically located while GcRs showed preference for the post-synaptic fraction. Additional confocal microscopy analysis of cortical and hippocampal regions revealed vesicular GABA transporter-positive and vesicular glutamate transporter 1-positive nerve terminals endowed with CB_1R immunoreactivity, apposing GcR-positive post-synaptic compartments. In functional transmitter release assay, corticosteroids, corticosterone (0.1−10 microM) and dexamethasone (0.1−10 microM) did not significantly affect the evoked release of [^{3}H]GABA and [^{14}C]glutamate in superfused synaptosomes, isolated from both rats and mice. In contrast, the synthetic cannabinoid, WIN55212-2 (1 microM) diminished the release of both [^{3}H]GABA and [^{14}C]glutamate, evoked with various depolarization paradigms. This effect of WIN55212-2 was abolished by the CB_1R neutral antagonist, O-2050 (1 microM), and was absent in the CB_1R KO mice. CB_2R-selective agonists did not affect the release of either neurotransmitter. The lack of robust presynaptic neuromodulation by corticosteroids was unchanged upon either CB_1R activation or genetic inactivation. Altogether,

Abbreviations: 3Rs, Replacement, Refinement and Reduction of Animals in Research; 4-AP, 4-aminopyridine; ARRIVE, Animals in Research: Reporting *In Vivo* Experiments; $CB_1R(s)$, $CB_2R(s)$, cannabinoid CB_1 and CB_2 receptor(s); corti, corticosterone; DAGLα, diacylglycerol lipase α; dexa, dexamethasone; DMSO, dimethylsulfoxide; DPM, disintegration per minute; FELASA, Federation for Laboratory Animal Science Associations; FR%, fractional release expressed as percentage; GPCR, G protein-coupled receptor; GcR(s), glucocorticoid receptor(s); HPA, hypothalamo-pituitary-adrenal; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; [^{3}H]GABA, tritiated γ-aminobutyric acid; McR(s), mineralocorticoid receptor(s); mife, mifepristone; PFC, prefrontal cortex; SDS-PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis; VGAT, vesicular GABA transporter; VGCC(s), voltage-gated calcium channel(s); VGLUT1, vesicular glutamate transporter 1; WB, Western blotting; WIN, WIN55212-2.

* Corresponding author. CNC, Center for Neuroscience and Cell Biology of Coimbra, University of Coimbra, 3004-504 Coimbra, Portugal.
E-mail address: akofalvi@uc.pt (A. Köfalvi).
[1] Present address: Dept. Pharmacy, University of Western Santa Catarina − UNOESC, Campus Videira, Rua Paese, N 198, Bairro Universitário, 89560-000 Videira, SC, Brazil.
[2] Present address: Experimental Psychiatry Unit, Center for Psychiatry and Psychotherapy, Medical University Innsbruck, Austria.

http://dx.doi.org/10.1016/j.neuint.2015.07.014

corticosteroids are unlikely to exert direct non-genomic presynaptic neuromodulation in the frontal cortex, but they may do so indirectly, via the stimulation of trans-synaptic endocannabinoid signaling.

1. Introduction

Corticosteroids produce diverse responses in various classes of neurons and astrocytes in the brain (Chaouloff and Groc, 2011; Joëls et al., 2012; Maggio and Segal, 2012; Yu et al., 2011). Alternative splicing and post-translation modifications yield pharmacologically and structurally distinct subtypes of corticosteroid-sensing mineralocorticoid and glucocorticoid receptors (McR and GcR). These receptors can either mediate slow genomic responses when located intracellularly or, they can directly control other receptors, membrane conductance, release probability and synaptic plasticity when inserted in the plasmalemma (Chaouloff and Groc, 2011; Chen et al., 2012; Joëls et al., 2012; Maggio and Segal, 2012; Tse et al., 2011). Only a few of these responses were associated with presynaptic loci, probably because non-genomic corticosteroid modulation of ionic currents at the second to minute scale is less typical (Joëls et al., 2012; Zaki and Barrett-Jolley, 2002). The particular mechanism(s) leading to rapid presynaptic neuromodulation by corticosteroids has not been fully elucidated, and the underlying receptor has been ascribed either as McR (Joëls et al., 2012; Karst et al., 2005; Maggio and Segal, 2012) or as GcR (Wang and Wang, 2009).

Corticosteroids alone are often insufficient to increase presynaptic neurotransmitter release: acute stress rapidly raises the readily releasable pool of glutamate in the rat (pre)frontal cortex, and augments its depolarization-induced *ex vivo* release (Popoli et al., 2011). However, corticosterone applied *in vitro* onto isolated nerve terminals (synaptosomes) does not affect glutamate release *per se* (Treccani et al., 2014).

In the prefrontal cortex (PFC), acute stimulation of glutamate release by corticosteroids under stressful stimuli is among the first steps to terminate the stress response via a feed-back loop feeding into the hypothalamo-pituitary-adrenal (HPA) axis (Herman et al., 1996; Joëls et al., 2012). Chronic stress, however, can impair the delicate context of neuromodulation in the PFC, which can elicit numerous neuropsychiatric illnesses (Opris and Casanova, 2014), including impaired fear extinction (Bitencourt et al., 2013). We recently found that GcRs rather than McRs facilitate prefrontal cortex-dependent conditioned fear extinction by promoting endocannabinoid signaling at CB_1 cannabinoid receptors (CB_1Rs) (Bitencourt et al., 2014). $G_{i/o}$ protein-coupled metabotropic CB_1Rs are expressed at high density in subsets of GABA-ergic interneurons, while their expression at lower density is typical for many pyramidal cells throughout the neocortex, with predominant axonal (presynaptic) localization (Egertová and Elphick, 2000; Hill et al., 2007; Marsicano and Lutz, 1999; Tsou et al., 1998). An additional CB_1R paralogue, the CB_2 cannabinoid receptor (CB_2R) is largely expressed peripherally with circumstantial evidence inferring central roles (Zhang et al., 2015).

CB_1Rs serve as downstream mediators of glucocorticoid action in frontocortical and hippocampal areas. CB_1Rs control the feedback loop along the HPA axis to terminate stress responses (Hill et al., 2011; Hill and Tasker, 2012), as well as to facilitate the extinction and impair the retrieval of aversive memories (Atsak et al., 2012; Bitencourt et al., 2013, 2014; Hill et al., 2011; Marsicano et al., 2002). Due to these important roles, endocannabinoids (endogenous CB_1R agonists) have been implicated in the pathogenesis of chronic stress-related psychiatric illnesses (Bitencourt et al., 2013; Köfalvi and Fritzsche, 2008; Trezza and Campolongo, 2013).

Prompted by the lack of conclusive evidence on a signaling interplay between GcRs and CB_1Rs, we aimed at mapping the acute presynaptic effects of corticosteroids on the resting and depolarization-evoked release of radiolabeled GABA and glutamate in superfused synaptosomes of the frontal cortex of laboratory rodents, which is a model free from polysynaptic and glial influences (Popoli et al., 2011). We also asked whether corticosteroids could modulate the presynaptic CB_1R activity in relation to the evoked release of GABA and glutamate.

2. Materials and methods

2.1. Ethics statement and animals

All studies were conducted in accordance with the principles and procedures outlined as "3Rs" in the guidelines of EU (86/609/EEC), FELASA, and the National Centre for the 3Rs (the ARRIVE; Kilkenny et al., 2010), and were approved by the Animal Care Committee of the Center for Neuroscience and Cell Biology of the University of Coimbra, Portugal. We also applied the ARRIVE guideline for the design and execution of *in vitro* pharmacological experiments (see below), as well as for data management and interpretation (McGrath et al., 2010).

In specific detail, 90 male Wistar rats (180–240 g, 8–10-week old) were purchased from Charles-River (Barcelona, Spain). Six CB_1 receptor null-mutant (knockout, KO) male mice with a CD-1 background (Ledent et al., 1999) and six of their wild-type littermates (35–45 g, 8–12-week old) were also used in pharmacological experiments. Animals were housed with 12 h light on/off cycles under controlled temperature (23 ± 2 °C), and *ad libitum* access to food and water. All efforts were made to minimize the number of animals used and to minimize their stress and discomfort. Animals used *in vitro* studies were deeply anesthetized with halothane before (5%, 1 L/min flow rate) before cervical dislocation.

2.2. Subcellular fractionation and Western blotting

After decapitation, rat brains were rapidly collected in ice-cold Krebs-HEPES assay solution (in mM: NaCl 113, KCl 3, KH_2PO_4 1.2, $MgSO_4$ 1.2, $CaCl_2$ 2.5, $NaHCO_3$ 25, glucose 5.5, HEPES 10), and frontal cortices were dissected (Paxinos and Watson, 1998). Frontal cortices were used instead of the much smaller prefrontal cortex to obtain sufficient amount of protein. To test the presence of GcR in frontocortical nerve terminals, we prepared a purified synaptosome (nerve terminal) fraction from three rats, following Dunkley et al. (2008). As Fig. 1A and E demonstrate, brain membrane fractions contain high densities of PSD95 - a membrane-bound marker of post-synapses (Ehlers et al., 1996), whereas this marker is largely diluted in crude (total) brain homogenate by the copious amounts of intracellular proteins, and was largely absent in the purified synaptosomes. To support the specificity of the anti-GcR antibody available to us (Abcam), we tested for GcR translocation from cytoplasmic to nuclear fractions upon its stimulation with dexamethasone (Sarabdjitsingh et al., 2010) (Fig. 1A–A_1). In brief, frontal

Please cite this article in press as: Bitencourt, R.M., et al., Lack of presynaptic interaction between glucocorticoid and CB_1 cannabinoid receptors in GABA- and glutamatergic terminals in the frontal cortex of laboratory rodents, Neurochemistry International (2015), http://dx.doi.org/10.1016/j.neuint.2015.07.014