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Upstream open reading frames regulate cannabinoid receptor 1 expression under baseline conditions and during cellular stress

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ABSTRACT

The cannabinoid receptor subtype 1 gene *CNR1* is not only associated with phenotypes such as cognitive performance, addiction and anxiety, but is also known to be crucially involved in responses to acute and chronic psychological and cellular stress conditions. Functional analysis of the 5' untranslated regions of the five known mRNA variants of the human *CNR1* gene revealed that two of these variants contain upstream open reading frames that are able to modulate gene expression both under baseline condition and conditions of cellular stress including hypoxia, glucose restriction and hyperthermia. The upstream open reading frames might provide a mechanism that enables the cannabinoid 1 receptor to escape the general repression of protein synthesis that is typical for conditions of cellular stress.

1. Introduction

The endocannabinoid system (ECS) plays a crucial role in the regulation of a variety of physiological functions, such as learning and memory processing, vegetative control, energy homeostasis, immunity and stress response (Bermudez-Silva et al., 2012; Hillard, 2014; Puighermanal et al., 2012; Strewe et al., 2012). It acts through different endocannabinoids (ECs) which are able to bind to the cannabinoid receptor subtypes 1 and 2 (CB1 and CB2 receptor) (Strewe et al., 2012). The CB1 receptor is mainly located in the central and peripheral nervous system, but also e.g. in the skeletal and gastrointestinal system, whereas the CB2 receptor can be predominantly found in organs and cells involved in immune response but also in the brain (Onaivi et al., 2012). Both subtypes are members of the G-protein coupled receptor family and act mainly through the activation of an associated guanine nucleotide-binding protein, but other, non-G protein mediated effects of endocannabinoids such as extracellular signal-regulated kinase (ERK) 1/2 activation via beta-arrestin have also been described (Franklin et al., 2013). In neurons of the brain, endocannabinoids function as retrograde synaptic messengers. They are synthesized and released postsynaptically and travel backwards across the synapses, stimulating CB1 receptors on presynaptic axons and suppressing the release of several excitatory neurotransmitters (McLaughlin et al., 2014).

Particularly the CB1 receptor has been intensively studied as this receptor subtype is known to be linked to several neurological and endocrinological pathways (Vlachou and Panagis, 2014). A growing body of literature indicates a crucial involvement of the CB1 receptor in acute and chronic conditions of global (psychological) and cellular stress. Cellular stress is the consequence of disturbed homeostasis, and the cannabinoid receptor plays an important role within the functional network that helps the cell to survive such critical situations (Feuerecker et al., 2012; Sanchez and Garcia-Merino, 2012; Zogopoulos et al., 2013b).

Yet the important function of the endocannabinoid system as a regulator of homeostasis is not limited to global stress but is also seen on a cellular level. Endocannabinoid signaling through the CB1 receptor has been shown to facilitate the survival of stressed neurons as a result of acute brain injury, neuroinflammation and neurodegenerative diseases. Furthermore, the endocannabinoid system plays an important role in neurogenesis and in repair mechanisms after neuronal injury (Zogopoulos et al., 2013a). The molecular mechanisms leading to altered cannabinoid receptor 1 gene (*CNR1*) expression under acute or chronic cellular stress exposure are not completely understood. It is known that the 5'- and 3'untranslated regions (UTRs) of genes can harbor regulatory elements that are capable of influencing the expression pattern of the main protein coding region. Multiple regulatory elements, like hairpins, protein-binding sites, internal ribosomal entry sites (IRESs), polyadenylation signals, microRNA binding sites and iron-responsive elements may be found in these regions (Chatterjee and Pal, 2009; Wethmar et al.,

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2010). Furthermore, many genes carry one or more upstream open reading frames (uORFs) in their 5'UTR (Calvo et al., 2009; Iacono et al., 2005; Matsui et al., 2007). Certain uORFs are able to govern protein expression, for example by establishing barrier functions to scanning ribosomes, altering the rate of ribosomal re-initiation, or increasing mRNA instability. In the present study we performed a systematic search for functionally relevant uORFs in the 5'UTRs of the five known *CNR1* gene variants. Our experiments revealed that two of these variants possess uORFs that are functional under baseline and/or cellular stress conditions.

2. Material and methods

2.1. In silico *analysis of the 5'UTR of* CNR1 *variants*

CNR1 variants 1 (NM_016083.4), 2 (NM_033181.3), 3 (NM_001160226.1), 4 (NM_001160258.1) and 5 (NM_001160259.1) were screened for in-frame start and stop codons upstream of the main start codon using the StarORF Finder (http://star.mit.edu/orf/runapp.html) (Fig. 1). The putative uORFs contained no validated SNPs (NCBI gene view). Furthermore, none of the SNPs present in *CNR1* 5'UTRs created or deleted a putative uORF.

2.2. Plasmid construction

Firefly luciferase vector pGL4.10 and renilla luciferase vector pGL4.74 were purchased from Promega (Mannheim, Germany). The TK-Promoter sequence was cut out of pGL4.74 with *Kpn*I and *Xho*I (Fermentas, St. Leon-Rot, Germany) after generating a *Xho*I restriction site at position bp 783 using GeneArt site-directed mutagenesis system (Invitrogen, Karlsruhe, Germany). After *Kpn*I and *Xho*I digestion of pGL4.10, the TK-Promoter sequence was ligated into the multiple cloning site of pGL4.10 upstream of the firefly luciferase coding sequence. The 5'UTR inserts of four different variants of the *CNR1* gene were synthesized (MWG Eurofins, Ebersberg, Germany) and cloned into pGL4.10 with *Xho*I and *Nco*I directly between the TK-Promoter and the firefly luciferase coding sequence. After cloning the inserts were confirmed by sequencing. To create constructs lacking a certain uORF, the ATG of the uORF was mutated to TTG. For *CNR1* variant 1 (NM_016083.4), harboring one putative uORF, two constructs were created: one with the wild type mRNA (*CNR1*-1.1) and one with the mutated uORF start codon (*CNR1*-1.2).

For *CNR1* variant 3 (NM_001160226.1), possessing two putative uORFs, four constructs were created: one with the wild type mRNA (*CNR1*-3.1), one with the first uORF switched off (*CNR1*-3.2), one with the second uORF switched off (*CNR1*-3.3), and one construct with both uORFs switched off (*CNR1*-3.4).

CNR1 variant 4 (NM_001160258.1) harbors three putative uORFs. As the third uORF is identical in sequence with the second uORF of variant 3 (and has been analyzed there), constructs were created only for the first two uORFs: one with the wild type mRNA (*CNR1*-4.1), one with the first uORF switched off (*CNR1*-4.2), one with the second uORF switched off (*CNR1*-4.3), and one construct with both uORFs switched off (*CNR1*-4.4).

For the additional experiments, three more constructs were created, harboring an in-frame stop codon (tga) three bases downstream of the uORF start codon: one with a premature stop codon within the first uORF (*CNR1*-4.5), one with a premature stop codon within the second uORF (*CNR*1-4.6), and one construct, in which the putative protein coding sequences of both uORFs were truncated by premature stop codons (*CNR*1-4.7).

For *CNR1* variant 5 (NM_001160259.1), harboring one putative uORF, two constructs were created: one with the wild type mRNA (*CNR1*-5.1) and one with the mutated uORF start codon (*CNR1*-5.2).

An overview of the constructs used in our experiments is shown in the Supplementary Table S1 and S2.

2.3. Cell culture

The human embryonic kidney cells (HEK) 293 were purchased from Cell Lines Service (Eppelheim, Germany). The HEK cell line was chosen because, unlike neuronal cell lines, it has the advantage of not expressing endogenous *CNR1* (information provided by the company 'Cell Lines').

Culturing of the cells was performed in T25 flasks in monolayer with DMEM (Sigma Aldrich, Hamburg, Germany) containing 4.5 g/l glucose, 1% L-glutamine, 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (Invitrogen, Karlsruhe, Germany). The cells were maintained in a humidified atmosphere at 37 °C and 5% CO_2. For all experiments HEK293 cells were seeded with 3×10^5 cells in 24 well plates (Greiner Bio-One, Frickenhausen, Germany). Co-transfection was performed with the reporter plasmid pGl4.10 + TK containing the 5'UTR of *CNR1* variant 1, 3, 4, and 5, respectively, and the control plasmid pGL4.74 at a ratio 20:1 using TransIT®-LT1

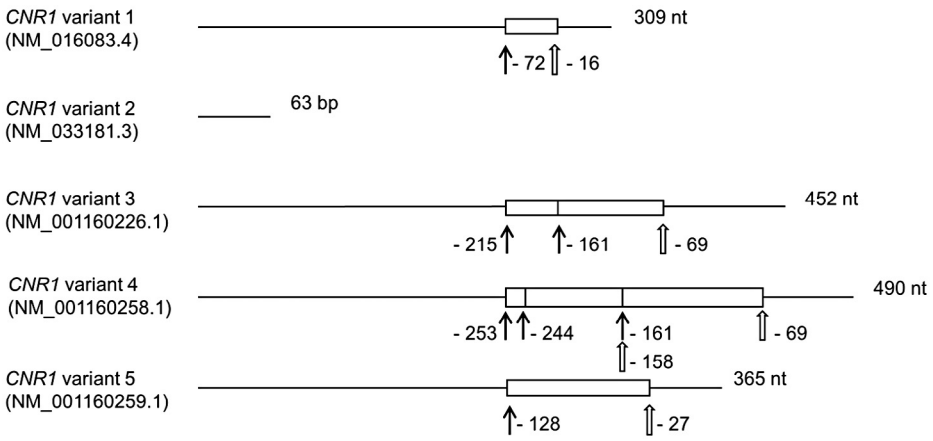

Fig. 1. 5'UTRs of the five cannabinoid 1 receptor (*CNR1*) variants with putative uORFs. The bp position for each uORF (start/stop) is depicted starting from the main start codon in 5'- direction.