Neuroscience Letters 508 (2012) 67–72

Contents lists available at SciVerse ScienceDirect

Neuroscience Letters

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

Identification of receptors and enzymes for endocannabinoids in NSC-34 cells: Relevance for *in vitro* studies with cannabinoids in motor neuron diseases

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ARTICLE INFO

Article history:
Received 13 October 2011
Received in revised form
17 November 2011
Accepted 14 December 2011

Keywords:
Cannabinoids
CB_1 and CB_2 receptors
Endocannabinoid enzymes
Cannabinoid signaling system
NSC-34 cells
Motor neuron disease

ABSTRACT

NSC-34 cells, a hybridoma cell line derived from the fusion of neuroblastoma cells with mice spinal cord cells, have been widely used as an *in vitro* model for the study of motor neuron diseases [*i.e.* amyotrophic lateral sclerosis (ALS)]. In the present study, they were used to characterize different elements of the cannabinoid signaling system, which have been reported to serve as targets for the neuroprotective action of different natural and synthetic cannabinoid compounds. Using RT-PCR, Western blotting and immunocytochemistry, we first identified the presence of the cannabinoid CB_1 receptor in these cells. As expected, CB_2 receptor is not expressed in this neuronal cell line, a result that is concordant with the idea that this receptor type is preferentially expressed in glial elements. Diacylglycerol-lipase (DAGL) and N-arachidonoylphosphatidylethanolamine-phospholipase D (NAPE-PLD), the enzymes that synthesize endocannabinoids, have also been detected in these cells using RT-PCR, and the same happened with the endocannabinoid-degrading enzymes fatty acid amide hydrolase (FAAH) and monoacylglycerol-lipase (MAGL). The presence of the CB_1 receptor in these cells supports the idea that this receptor may play a role in the regulation of cellular survival face to excitotoxic injury. Interestingly, the expression of CB_1 receptor (and also the FAAH enzyme) was strongly up-regulated after differentiation of these cells, as previously reported with glutamate receptors. No changes were found for NAPE-PLD, DAGL and MAGL. Assuming that glutamate toxicity is one of the major causes of neuronal damage in ALS and other motor neurons diseases, the differentiated NSC-34 cells might serve as a useful model for studying neuroprotection with cannabinoids in conditions of excitotoxic injury, mitochondrial malfunctioning and oxidative stress.

1. Introduction

Amyotrophic lateral sclerosis (ALS) is a fatal neurodegenerative disorder characterized by the selective loss of motor neurons in the spinal cord, brainstem, and motor cortex [19]. ALS exists in two forms, familial ALS (only 5% of cases) and sporadic ALS (most of cases) [4]. Complete pathogenic causes of ALS are presently unknown but several mechanisms have been suggested and these include excitotoxicity, chronic inflammation, oxidative damage and protein aggregation [4,9,17]. For example, several studies have identified changes in the function of glutamate transporters that have been associated with the initiation of the disease (reviewed in [9]). High amounts of activated microglia have been found in those brain regions that are affected in ALS patients [21]. Lastly, genetic studies have identified several mutations in the copper-zinc superoxide dismutase (SOD-1), a key antioxidant enzyme, in approximately 20% cases of familial ALS [16], being pathological through a gain-of-neurotoxic function. However, other mutated genes, *i.e.* TDP-43 and FUS, have also been recently identified and related to the disease and to mechanisms other than oxidative injury, leading to a novel molecular exclusive classification of ALS cases (reviewed in [13]).

Despite the intensive research conducted in the last years, an effective treatment for this disease remains elusive, with Rilutek® as the only licenced medicine [11]. Recent evidence points that

Abbreviations: ALS, amyotrophic lateral sclerosis; CB1 receptor, cannabinoid receptor type 1; CB2 receptor, cannabinoid receptor type 2; DAGL, diacylglycerol-lipase; FAAH, fatty acid amide hydrolase; MAGL, monoacylglycerol-lipase; NAPE-PLD, N-arachidonoylphosphatidylethanolamine-phospholipase D; SOD-1, superoxide dismutase-1.

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doi:10.1016/j.neulet.2011.12.020

cannabinoids may be beneficial as neuroprotectant agents in ALS (reviewed in [3]). Cannabinoids include some active ingredients present in *Cannabis sativa* (termed phytocannabinoids), various intercellular signaling lipids (so-called endocannabinoids) and different synthetic molecules. Their neuroprotective properties have been studied in different neurodegenerative disorders and would be based on their ability to decrease excitotoxicity, microglial activation, neuroinflammation and oxidative stress (see [8] for a recent review), then becoming an interesting therapeutic option in ALS too. In fact, the administration of Δ^9-tetrahydrocannabinol was effective in delaying motor impairment and prolonging survival in the SOD-1 (G93A transgenic mice) mouse model of ALS [14]. Similar results were reported with cannabinol, a less psychotrophic plant-derived cannabinoid [22], with the synthetic cannabinoid WIN55,212-2 [2], or with the selective CB_2 agonist AM1241 [12,18]. In parallel, genetic ablation of fatty acid amide hydrolase (FAAH) enzyme leading to elevated levels of endocannabinoids also prevented the appearance of disease signs in SOD1 mutant mice [2], whereas genetic ablation of the CB_1 receptor had no effect on the onset of the disease in this model [2]. Taken together, these results show that cannabinoids might have neuroprotective effects in ALS mediated by the combination of different mechanisms. In part, these mechanisms might be related to the cannabinoid receptor-independent antioxidant properties of certain cannabinoids, but the data obtained with WIN55,212-2 [2], which is not antioxidant, as well as using FAAH deficient mice [2] or treatments with selective CB_2 receptor agonists [12,18], suggest an additional contribution of this cannabinoid receptor type associated with the important role of glial elements in this disease [17,21]. An important aspect of these previous pharmacological studies is that they were conducted in absence of data on the changes that the development of ALS causes in the receptors and enzymes for endocannabinoids, which may be an important factor to determine the efficacy of potential cannabinoid treatments. Only a couple of studies have explored this issue and described elevated levels of CB_2 receptors in microglia from post-mortem human spinal cords of ALS patients [25] or elevated levels of endocannabinoids in the spinal cord of SOD-1 mutant mice [23].

In the present study, we used NSC-34 cells, a hybridoma cell line derived from the fusion of neuroblastoma cells with mice spinal cord cells [5]. These cells have been widely used as an *in vitro* model for the study of ALS and other disorders affecting motor neurons, in particular after they become differentiated by serum depletion [7]. Both non-differentiated and differentiated cells have been used for the evaluation of the effects of potential neuroprotective compounds against different insults (*i.e.* excitotoxins, mitochondrial toxins, oxidants, *etc.*) affecting cell survival [7,10,15,20,24]. We plan to use these cells for evaluating the effects of various types of cannabinoid compounds that have shown neuroprotective effects in other disorders. However, before these studies, we wanted to identify and analyze, using RT-PCR, Western blotting and immunocytochemistry, the presence in these cells of those elements of the cannabinoid signaling system (receptors and enzymes) that have been reported to serve as targets for the neuroprotective action of different natural and synthetic cannabinoid compounds.

2. Materials and methods

2.1. Cell culture

NSC-34 cells (purchased from Cedarlane Laboratories Ltd., Ontario, Canada) were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 2 mM ultra-glutamine and 1% penicillin/streptomycin (LONZA, Verviers, Belgium), and under a humidified 5% CO_2 atmosphere at 37 °C. To slow the proliferation of these cells and enhance their maturation towards a differentiated state, they were grown in a medium containing 1:1 DMEM plus Ham's F12, 1% fetal bovine serum, 1% penicillin/streptomycin, and 1% modified Eagle's medium with non-essential amino acids (Sigma–Aldrich, St. Louis, MO, USA), as previously described [7]. For each experiment, cells were seeded at 2×10^4 cells/mm^2.

2.2. Immunocytochemistry

Cells were grown in 24-well laminin-coated plates (200,000 cells per well) overnight, then fixed in cold 4% paraformaldehyde for 30 min at 4 °C and permeabilized in 1:1 methanol–acetone. Cells were incubated overnight at 4 °C with the primary antibodies anti-CB_1 (1:400, Pierce Biotechnology, Rockford, IL, USA) or anti-SMI-312 (1:1000, Covance, Emerville, CA, USA). The secondary antibody was added for 2 h at 37 °C (Alexa Fluor® 488 donkey anti-rabbit IgG; Invitrogen Corp., Life Technologies, Madrid, Spain). Hoechst staining was also used for the identification of cell nuclei. A Leica DM-IL microscope and a Leica DFC-300FX camera were used for well observation and photography.

2.3. Western blotting

Cells were lysed in ice-cold RIPA buffer and subjected to centrifugation at $40{,}000 \times g$ for 30 min at 4 °C. Proteins for each extract were electrophoresed in SDS-PAGE. Precision Plus Protein™ Standards (Bio-Rad Laboratories, Madrid, Spain) were included on each gel. Proteins were then electroblotted onto PVDF membranes (GE Healthcare UK Ltd., Buckinghamshire, UK). The resulting blot was incubated with an antibody anti-CB_1 (1:400, Pierce Biotechnology, Rockford, IL, USA) during 2 h at room temperature in blocking buffer. Finally, blots were incubated with monoclonal anti-rabbit Ig peroxidase conjugate (1:2500; GE Healthcare UK Ltd., Buckinghamshire, UK) for 1 h at room temperature and revealed with Amersham ECL™ Western Blotting Detection Reagents (GE Healthcare UK Ltd., Buckinghamshire, UK).

2.4. Reverse transcription (RT) and real-time polymerase chain reaction (PCR)

mRNA (1 μg) was reverse transcribed into cDNA using the QuantiTect® reverse transcription kit (Qiagen) with poly-dT primers. For the genes used in this study (CB_1, CB_2, DAGL, NAPE, FAAH) we used specific TaqMan gene expression assays (Cnr2, NM_009924.3; Cnr1, NM_007726.3; DAGL,

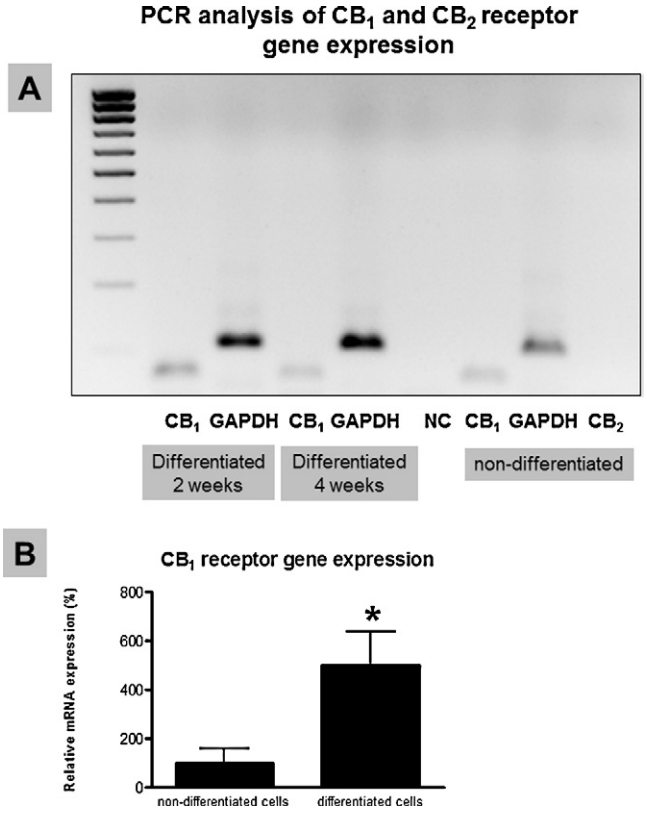

Fig. 1. PCR analysis of CB_1 and CB_2 receptor gene expression in non-differentiated and differentiated (after 2 or 4 weeks) NSC-34 cells (panel A; NC = negative control). Quantitative RT-PCR analysis of gene expression for the CB_1 receptor in non-differentiated *versus* differentiated (after 4 weeks) (panel B). Values represent means ± SEM of more than 5 different groups of cells and are expressed as % over the level of expression in non-differentiated cells. Data were analyzed by the Student's *t*-test (*$p < 0.05$).