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Neuronal preservation and reactive gliosis attenuation following neonatal sciatic nerve axotomy by a fluorinated cannabidiol derivative

Matheus Perez[a], Luciana Politti Cartarozzi[a], Gabriela Bortolança Chiarotto[a], Simone Alves de Oliveira[a], Francisco Silveira Guimarães[b], Alexandre Leite Rodrigues de Oliveira[a,*]

[a] University of Campinas, Department of Structural and Functional Biology, Campinas, SP, Brazil
[b] University of São Paulo, Department of Pharmacology, Ribeirão Preto, SP, Brazil

HIGHLIGHTS

- The fluorinated cannabidiol, HUF-101, displays enhanced neuroprotective properties.
- HUF-101 rescues spinal motoneurons following neonatal sciatic nerve axotomy.
- HUF-101 rescues dorsal root ganglia neurons following neonatal sciatic nerve axotomy.
- HUF-101 neuroprotection reduces glial reaction and has anti-apoptotic effects.

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ABSTRACT

Immature peripheral nervous system damage, such as the transection of a peripheral nerve, results in the extensive degeneration of motoneurons and dorsal root ganglia (DRG) sensory neurons, mostly due to apoptotic events. We have previously shown that cannabidiol (CBD), the most abundant non-psychotropic molecule present in the *Cannabis sativa* plant, exhibits neuroprotective action when administered daily at a dose of 15 mg/kg. This study shows that use of the fluorinated synthetic version of CBD (4′-fluoro-cannabidiol, HUF-101) significantly improves neuronal survival by 2-fold compared to that achieved with traditional CBD at one-third the dose. Furthermore, we show that HUF-101 administration significantly upregulates anti-apoptotic genes and blocks the expression of pro-apoptotic nuclear factors. Two-day-old Wistar rats were subjected to unilateral sectioning of the sciatic nerve and treated daily with HUF-101 (1, 2.5, 5 mg/kg/day, i.p.) or a vehicle solution for five days. The results were evaluated by Nissl staining, immunohistochemistry, and qRT-PCR. Neuronal counting revealed a 47% rescue of spinal motoneurons and a 79% rescue of DRG neurons (HUF-101, 5 mg/kg). Survival was associated with complete depletion of p53 and a 60-fold elevation in BCL2-like 1 gene expression. Additionally, peroxisome proliferator-activated receptor gamma (PPAR-gamma) gene expression was down-regulated by 80%. Neuronal preservation was coupled with a high preservation of synaptic coverage and a reduction in astroglial and microglial reactions that were evaluated in nearby spinal motoneurons present in the ventral horn of the lumbar intumescence. Overall, these data strongly indicate that HUF-101 exerts potent neuroprotective effects that are related to anti-apoptotic protection and the reduction of glial reactivity.

1. Introduction

Loss of motor and sensory neurons after traumatic injuries leads to lifelong disabilities (Carlstedt, 2009). One example of this type of injury is the brachial plexus injury, caused by motorcycle and other high energy accidents, which have drastically increased in recent years (Kachramanoglou et al., 2013). Such a proximal lesion at the interface of the central and peripheral nervous system results in a substantial loss of neurons, resulting in a poor recovery prognosis (Chew et al., 2011, 2013; Risling et al., 2011; Carlstedt et al, 1986, 1990; Koliatsos et al., 1994).

Research on avoiding neuronal degeneration following proximal lesions has shown positive results. The use of neurotrophic factors soon after axotomy can partially rescue lesioned neurons, although the

* Corresponding author.
E-mail address: alroliv@unicamp.br (A.L.R.d. Oliveira).

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delivery efficiency combined with side effects preclude clinical use (Araujo et al., 2017; Barbizan et al., 2013; Eggers et al., 2010; Barbizan and Oliveira, 2010; Rodrigues Hell et al., 2009; Chu and Wu, 2009; Chu et al., 2008, 2009; Schlegel et al., 2007; Zhou and Wu, 2006; Parsadanian et al., 2006; Hayashi et al., 2006; Watabe et al., 2005; Blits et al., 2004; Bergerot et al., 2004; Sakamoto et al., 2003). In turn, the search for alternative molecules that may confer neurotrophic activity in combination with easy delivery and reduced unwanted effects has been prioritized.

The screening of neuroprotective substances can be carried out in neonatal rats following a sciatic nerve axotomy (Kemp et al., 2015a). In fact, a peripheral lesion in newborns induces motor and sensory loss similar to that caused by a brachial plexus injury, making it a well-accepted experimental model for neurodegeneration studies (Kemp et al., 2015a); also, this model provides a means for studying the early non-neuronal response to injuries, including glial reactions and inflammation (Perez et al., 2013). We have already investigated drugs with neuroprotective and anti-inflammatory profiles, including cannabidiol (CBD), which is a major phytocannabinoid of *Cannabis sativa* (Perez et al., 2013). CBD has been shown to have important effects on both motor and sensory neuron survival, including the preservation of synapses in the spinal cord. One important finding was that astroglial and microglial reactions decreased by 30% following intraperitoneal treatment with CBD. While the mechanism of action of CBD is not entirely understood, CBD is known to facilitate the activation of CB1 and CB2 endocannabinoid receptors by anandamide and 2-arachidonoylglycerol (2-AG) (Thomas et al., 2007). In fact, CBD activates TRPV1 channels, thereby inhibiting the uptake and metabolization of anandamide and adenosine. CBD also interferes with intracellular Ca^{++} levels and reduces oxidative effects, consequently decreasing the apoptosis of interneurons following neonatal sciatic nerve section (Thomas et al., 2007).

It has recently been shown that the activity of synthetic drugs can be improved by the introduction of a fluorine atom, such as a fluorinated synthetic version of CBD 4′-fluoro-cannabidiol (HUF-101) (Silva et al., 2017; Breuer et al., 2016). Compared to CBD, the positive effects of HUF-101 on anxiety, depression, nociception and compulsive behavior are significantly improved, indicating that HUF-101 is considerably more potent (Silva et al., 2017; Breuer et al., 2016). Some of these effects were mediated by the facilitation of CB1 and CB2 neurotransmission. Based on these results, we aimed to evaluate the efficiency of HUF-101 on neuroprotection following neonatal sciatic nerve crushing. The results confirmed that the fluorinated version of CBD is a more potent neurotrophic agent, as 50% of the axotomized motoneurons in the spinal cord as well as 80% of the dorsal root ganglia (DRG) sensory neurons were rescued. Interestingly, the dose used herein was three times lower than that used in previous experiments with CBD (Perez et al., 2013). Survival was associated with complete depletion of p53 and a 60-fold elevation in BCL2-like 1 gene expression in the DRG. Additionally, peroxisome proliferator-activated receptor gamma (PPAR-gamma) gene expression was downregulated by 80%. Neuronal preservation was coupled with substantial reductions in astroglial and microglial reactions in the spinal cord, evaluated in nearby spinal motoneurons present in the ventral horn of the lumbar intumescence, and a high preservation of synaptic coverage. Overall, these data strongly indicate that HUF-101 has potent neuroprotective effects that are related to anti-apoptotic protection and the reduction of glial reactivity.

2. Materials and methods

2.1. Animals, surgical procedures and HUF-101

Forty neonatal Wistar rats (2 days old – P2) were obtained from the Multidisciplinary Center for Biological Investigation (CEMIB/UNICAMP) and housed under a 12/12 h light/dark cycle at a controlled temperature (23 °C). The litters were kept with their respective females. This study was approved by the Institutional Committee for Ethics in Animal Experimentation (CEUA/IB/UNICAMP, proc no. 3939–1), and the experiments were performed in accordance with the guidelines of the Brazilian College for Animal Experimentation. ARRIVE guidelines were used to improve the design, analysis and reporting of data obtained herein.

The animals were deeply anesthesized (hypothermia) and subjected to mid-thigh unilateral sciatic nerve transection. They were then ramdomly distributed into 4 groups: axotomy + vehicle treatment (saline phosphate buffer with 2% Tween), n = 10; axotomy + HUF-101 – 1 mg/kg treatment, n = 10; axotomy + HUF 101–2.5 mg/kg treatment, n = 10, and axotomy + HUF-101 – 5 mg/kg treatment, n = 10. Drug administration (i.p.) was repeated every 24 h for five days.

HUF-101 was synthesized by Professor Raphael Mechoulam's group at the Hebrew University of Jerusalem, Israel as previously described (Silva et al., 2017; Breuer et al., 2016). HUF-101 was diluted in saline phosphate buffer containing 2% Tween.

2.2. Tissue preparation

Animals were sacrificed five days post-lesion (P7). They were deeply anestesized (Ketamine, Fort Dodge, USA, 50 mg/kg and Xilasin, König, Argentina, 10 mg/kg; i.p.), transcardially perfused and fixed with 0.1 M phosphate buffer saline (PBS) followed by fixative (10% paraformaldehyde in 0.1 M PBS, pH 7.4). Their lumbar spinal cords (L5-L6) and L5 DRG were obtained and processed for neuronal survival counting and immunohistochemistry (n = 5 each group) and RT-qPCR (n = 5 each group) analyses.

Specimens were post-fixed for 12 h in the same fixative solution and then washed with phosphate buffer (PB) and stored in 10, 20 and 30% sucrose (12 h each) before being frozen in dichloroethane kept at − 25 °C in liquid nitrogen. Cryostat transverse sections (12 µm thick) of spinal cords and DRG were obtained and transferred onto gelatin-coated slides, dried at room temperature for 30 min and stored at - 20 °C until use.

2.3. Spinal motoneurons and DRG neuron survival counting

Cell counting was performed in 12 µm alternate sections obtained from the lumbar intumescences (n = 5 animals/group) and DRG (n = 5 animals/group). Sections were stained with cresyl violet (Nissl staining) for 50 s, dehydrated, diaphanized and mounted with Entellan (Merck, Darmstadt, Germany).

In spinal cord sections, cell counting was performed in approximately 20 sections on both ipsi and contralateral sides at the lamina IX of Rexed. In DRG sections, cell counting was performed in 5 adjacent sections on both ipsi and contralateral sides. Only cells with a visible nucleus and nucleolus were counted. To correct for the double counting of neurons, the following Abercrombie formula was used (Abercrombie, 1946):

$$N = n\,t/(t + d)$$

where N is the corrected number of counted neurons, n is the counted number of cells, t is the thickness between sampled sections (48 µm for spinal cord and 36 µm for DRG) and d is the average diameter of the cells. Since the neuronal size significantly affects cell counts, the value of d was calculated specifically for each experimental group. The diameter of neurons was measured in 15 randomly selected neurons per group using Image Tool software (version 3.0, The University of Texas Health Center, TX, USA), and the mean value was calculated.

2.4. Immunohistochemistry

For immunohistochemistry analysis, microscopic slides were